The electrophysiological properties of freshly isolated and cultured human and guinea-pig detrusor smooth muscle cells

GUIPING SUI

A thesis presented to the University of London
in fulfilment of the regulations for the degree of
Doctor of Philosophy

Institute of Urology and Nephrology
University College London Medical School
1999
Abstract

Uncontrolled contraction of the detrusor is a major factor in bladder instability, but the cellular mechanisms remain unknown and clinical treatment still leaves much to be desired. The objectives of this study were to characterise the electrophysiological properties of freshly isolated adult and cultured detrusor smooth muscle cells from human biopsy samples and guinea-pig bladder in order to develop a model for study of the causes of bladder instability; and to facilitate the development of functional materials suitable for bladder implants.

Single detrusor myocytes were obtained from either adult guinea-pig or human urinary bladders by enzymatic dissociation after removal of the mucosa and epithelium. A suitable culture medium was developed which consisted of D-valine substituted minimal essential medium, supplemented with 10% foetal bovine serum. Cultures were maintained in a humidified incubator at 37°C with 5% CO₂, grown to confluence and passaged up to three times. Electrophysiological properties of freshly isolated, primary cultured and passaged cells were investigated using patch-type electrodes.

Cultured detrusor smooth muscle cells presented a "hills and valleys" appearance after growing to confluence; smooth muscle specific α-actin staining was positive. The electrophysiological properties of the freshly isolated cells were retained in the cultured cells, but with some modifications. Specific membrane resistance was decreased, but membrane capacitance was larger; the resting membrane potential and action potential threshold were shifted to more depolarised values; action potential upstroke velocity was slower, attributed to smaller inward currents and action potential duration was longer. Similar results were obtained for both guinea-pig and human detrusor smooth muscle cells.

Inward and steady-state outward currents were maintained in cultured cells, the current density was lower in primary cultures but outward currents increased again during subsequent passages. Activation and inactivation curves of the inward currents were shifted to more positive potentials after culture. The spontaneous transient outward current, which was recorded in freshly isolated cells, was not recorded in cultured cells. Both inward and outward currents were further characterised in guinea-pig cells. This revealed the existence of a T-type Ca²⁺ current in addition to the L-type currents found in freshly isolated cells, but in cultured cells only the L-type Ca²⁺ current was found. In freshly-isolated cells K⁺ currents showed transient and steady-
state components; the transient current and part of the steady-state current were \( \text{Ca}^{2+} \)-dependent, the transient current was blocked by iberiotoxin but not apamin. The results show that myocytes are less excitable in culture, this could be due to the reduced density and altered steady-state kinetics of \( \text{Ca}^{2+} \) currents, as well as a proportionately smaller reduction of \( \text{K}^+ \) currents.
Acknowledgement

I am very grateful to the many people who have help me throughout this research. In particular I would like to thank the following:

Professor Chris Fry, my supervisor for encouraging and guiding me to pursue this challenging project. I am greatly indebted to him for his enthusiasm and advice, for his friendship and support, leading to the completion of this thesis.

Dr John Masters for his friendship and help. I am very grateful to him for the freedom he gave me to use cell culture facilities.

Drs Changhao Wu, Mark Bayliss, Jo Hockey, Xianghong Wang, David Hudson, Mr Neal Appleton, Miss Valerie Procter, Mrs Betty Swan-Taylor, Mrs Bernadett Daly-Burns for their technical support, advice and help.

My colleagues in the research laboratories, Institute of Urology who have made research life enjoyable.

For financial support from the St. Peter's Trust, which allowed this work to proceed.

My family, in particular my twin sons for their support and understanding.
Chapter 1. Introduction ........................................................................................................... 27

I. Clinical problem ................................................................................................................ 27
II. Experimental considerations .......................................................................................... 28
III. Electrical activity of excitable tissues ........................................................................... 28
   III.1. Classic description of the ionic basis of axon membrane excitability .................. 28
   III.2. Membrane capacitance and subthreshold depolarisation ................................... 29
   III.3. Non-propagating action potentials under current clamp ..................................... 31
   III.4. Ion channels ........................................................................................................... 31
      III.4.1. The concept of ion channels ........................................................................... 31
      III.4.2. Channel gating model .................................................................................... 32
      III.4.3. Ionic conductance ......................................................................................... 34
IV. Contractile activation of detrusor muscle ..................................................................... 34
   IV.1. Neurotransmission and Pharmacomechanical coupling ....................................... 35
      IV.1.1. Neurotransmission .......................................................................................... 35
      IV.1.2. Pharmacomechanical coupling ...................................................................... 37
         IV.1.2.1. Receptor-operated cation channels ......................................................... 37
         IV.1.2.2. Pharmacomechanical coupling ............................................................... 37
   IV.2. Electrophysiological activity and Electromechanical coupling .............................. 39
      IV.2.1. Electrophysiological activity .......................................................................... 39
         IV.2.1.1. Resting membrane potentials .................................................................. 39
         IV.2.1.2. Action potentials ..................................................................................... 39
         IV.2.1.3. Ion channels in detrusor muscle ............................................................... 40
            IV.2.1.3.1. Voltage-dependent Ca^{2+} channels .................................................... 40
               IV.2.1.3.1.1. Voltage-dependent Ca^{2+} channels in detrusor muscle .......... 42
IV.2.1.3.2. Voltage-dependent transient outward K⁺ channels ......43
   IV.2.1.3.2.1. Voltage-dependent transient outward K⁺ channels
       in detrusor muscle .....................................................45
IV.2.1.3.3. Steady-state outward K⁺ channels: iK<sub>ATP</sub> and iK ..........46
   IV.2.1.3.3.1. K<sub>ATP</sub> channels in detrusor ........................................46
   IV.2.1.3.3.2. Delayed rectifier K⁺ channels (iK) in detrusor ......47
IV.2.1.3.4. Other currents: iCl<sub>Ca</sub> and stretch-activated
cation channels ............................................................48
IV.2.2. Electromechanical coupling ................................................49
   IV.2.2.1. Electromechanical coupling in detrusor muscle .................49
IV.3. Interaction between different pathways ...........................................51
V. Smooth muscle cell culture ..........................................................52
VI. Hypothesis ....................................................................................52
VII. Aims and objectives of the study ..................................................53

Chapter 2. Principles of experimental techniques .........................55

I. Principle of patch-clamp technique ...............................................55
   I.1. Voltage-clamp ................................................................................55
   I.2. Current-clamp ................................................................................56
   I.3. Patch-clamp ....................................................................................57
   I.4. Whole-cell patch clamp ..................................................................58
II. Primary monolayer culture ............................................................62
III. Principle of immunocytochemistry ...............................................64
   III.1. Antibodies ...................................................................................64
   III.2. Requirements for immunocytochemistry .....................................65
   III.3. Immunoenzyme methods .........................................................67

Chapter 3. Methods .......................................................................69

I. General protocol ..........................................................................69
II. Solutions and chemicals ..............................................................69
   II.1. Extracellular solution .................................................................69
       II.1.1. Normal Tyrode’s solution .....................................................70
       II.1.2. Ca²⁺-free Tyrode’s solution ...............................................70
       II.1.2.1. Determination of [Ca²⁺] using a Ca²⁺-selective electrode ......71
II. 1.2.1. Data collection .................................................................71
II. 1.2.1.2. Data handling ...............................................................73
II. 1.2.1.3. Determination of [Ca\(^{2+}\)] in Ca\(^{2+}\)-free Tyrode's solution ..76
II. 1.2.2. Composition of the very low Ca\(^{2+}\) Tyrode's solution ..........77
II. 1.3. HEPES buffered-Tyrode's solutions .........................................78
II. 2. Intracellular solutions ......................................................................79
II. 3. Enzyme solution ...............................................................................81

III. Cell isolation and culture .................................................................82
III. 1. Cell isolation .................................................................................82
III. 2. Cell culture .....................................................................................83
   III. 2.1. Primary culture ...................................................................83
   III. 2.2. Subculture ...........................................................................84
III. 3. Cell viability and Cell Counting ....................................................84
   III. 3.1. Cell viability .........................................................................84
   III. 3.2. Cell Counting .....................................................................84
III. 4. Immunocytochemistry .................................................................86
   III. 4.1. The Avidin-biotin complex (ABC) method ...............................87
   III. 4.2. The procedure of the ABC method ........................................88
   III. 4.3. Results ................................................................................92

IV. Whole-cell patch clamp experiments ..............................................93
IV. 1. Equipment setup ........................................................................93
IV. 2. Patch-electrodes ..........................................................................95
IV. 3. Whole-cell configuration ............................................................95
IV. 4. Cells ..........................................................................................97
IV. 5. Current-clamp protocols ............................................................97
   IV. 5.1. Measurement of cell membrane passive properties ...............98
      IV. 5.1.1. Cell capacitance \(c_m\) .........................................................98
      IV. 5.1.2. Measurement of passive membrane properties ................99
      IV. 5.2. Measurement of action potentials, APs ..............................101
IV. 6. Voltage-clamp protocols ............................................................102
   IV. 6.1. Measurement of inward and outward currents .....................102
      IV. 6.1.1. Inward and outward current activation .............................102
         IV. 6.1.1.1. Voltage protocol .....................................................102
         IV. 6.1.1.2. Current-voltage (i-v) relationships .............................103
         IV. 6.1.1.3. Determination of inward current activation kinetics ....104
         IV. 6.1.1.4. Steady-state outward current activation kinetics ..........104
      IV. 6.1.2. Inward current inactivation ...........................................105
         IV. 6.1.2.1. Voltage protocol .....................................................105
         IV. 6.1.2.2. Inactivation kinetics ................................................105
Chapter 4. results

I. Characterisation of cultured smooth muscle cells

I.1. The growth characteristics and morphology of cultured detrusor smooth muscle cells

I.2. Immunocytochemistry with monoclonal smooth muscle specific a-actin antibody

I.3. Functional response to cholinergic stimulation

II. Membrane passive properties

II.1. Comparison of freshly isolated guinea-pig (fGP) and human (fH) detrusor smooth muscle cells

II.2. Comparison of cultured guinea-pig (cGP) and human (cH) detrusor
II.3. Comparison of freshly isolated and cultured human cells ........................................ 126

II.4. Comparison of freshly isolated and cultured guinea-pig cells .............................. 127

III. Membrane Action potentials .................................................................................. 127

III.1. Measurement of action potentials in freshly isolated guinea-pig (fGP) and human (fH) cells .......................................................... 128

III.1.1. Spontaneous action potentials .............................................................. 128

III.1.2. Action potentials elicited by a current stimulus ........................................ 129

III.1.2.1. Action potentials elicited by a short-duration of step current .......... 129

III.1.2.2. Action potentials elicited by a long-duration of step current ... 131

III.1.2.3. The effect of Ca²⁺ channel blockers on action potentials ...... 133

III.1.2.4. Comparison of action potentials in cells from freshly isolated guinea-pig and human cells ................................. 133

III.2. Measurement of action potentials in cultured guinea-pig (cGP) and human (cH) cells ............................................................................................. 135

III.2.1. Action potentials elicited from cultured guinea-pig and human detrusor cells by short and long pulses ............................................. 135

III.2.2. The effect of Ca²⁺ channel blockers on action potentials ............... 137

III.2.3. Comparison of action potentials from cultured guinea-pig and human cells .............................................................. 139

III.3. Comparison of action potentials in freshly isolated (fH) and cultured (cH) human cells ............................................................................. 140

III.4. Comparison of action potentials in freshly isolated (fGP) and primary cultured (cGP) guinea-pig cells ....................................................... 141

III.5. Comparison of action potentials in freshly isolated (fGP) and passaged (cGP1-3) guinea-pig cells ............................................................... 142

III.6. Comparison of action potentials in cells from primary cultured (cGP) and passaged (cGP1-3) guinea-pig cells .......................................... 143

IV. Action potential and membrane currents .......................................................... 144

IV.1. The relationship between action potential $V_{\text{max}}$ and inward Ca²⁺ currents .......................................................... 144

IV.2. Comparison of action potentials in freshly isolated guinea-pig cells with KCl+5mM EGTA and KCl+0.1mM EGTA filled electrodes ........... 145

IV.3. The action potential and underlying ionic currents ..................................... 146

V. Measurement of inward and outward currents ................................................. 153

V.1. Comparison of currents from freshly isolated guinea-pig and human cells .................................................................................. 153

V.1.1.1. Inward and outward current-voltage relationships ............. 155
V.1.1.2. Net currents .................................................................155
V.1.2. Inward current steady-state activation and inactivation ..........157
  V.1.2.1. Inward current activation ............................................157
  V.1.2.2. Inward current inactivation ...........................................158
V.1.3. Steady-state outward current activation and inactivation ..........159
  V.1.3.1. Steady-state outward current activation .........................159
  V.1.3.2. Steady-state outward current inactivation ......................160
V.2. Ionic currents in primary cultured guinea-pig and human cells ....162
  V.2.1. Currents in primary cultured cells ........................................162
    V.2.1.1. The i-v relationships for inward and outward currents ......163
    V.2.1.2. Net inward current ....................................................165
  V.2.2. Inward current activation and inactivation ..........................165
    V.2.2.1. Inward current activation .............................................165
    V.2.2.2. Inward current inactivation ..........................................165
  V.2.3. Steady-state outward current activation and inactivation ........167
    V.2.3.1. Steady-state outward current activation ..........................167
    V.2.3.2. Steady state outward current inactivation .......................168
V.3. Comparison of currents from freshly isolated and cultured human cells .................................................................168
  V.3.1. Current-voltage (i-v) relationships ....................................169
  V.3.2. Inward current activation and inactivation ..........................171
  V.3.3. Steady-state outward current activation and inactivation ........171
V.4. Summary of membrane currents from freshly isolated and cultured human cells .................................................................173
V.5. Membrane currents recorded from passaged cells ......................173
  V.5.1. Comparison of current i-v relationships of freshly isolated, primary cultured and passaged guinea-pig cells ......................175
    V.5.1.1. Inward and steady-state outward current-voltage relationships .................................................................175
    V.5.1.2. Net currents .................................................................178
  V.5.2. Steady-state inward current activation and inactivation of freshly isolated, primary cultured and passaged guinea-pig cells .........179
    V.5.2.1. Steady-state inward current activation ..............................179
    V.5.2.2. Steady-state inward current inactivation ...........................180
  V.5.3. Steady-state outward current activation and inactivation of freshly isolated, primary cultured and passaged guinea-pig cells ..........181
    V.5.3.1. Steady-state outward current activation .............................181
    V.5.3.2. Steady-state outward current inactivation ..........................183
VI. Characterisation of peak inward and steady-state outward currents ....184
VI.1. $i_{Ca}$ from freshly isolated, primary cultured and passaged
guinea-pig cells ...............................................................185
VI.2. $i_{Ca}$ activation curves, $d_{oo}$, of freshly isolated, primary cultured
and passaged guinea-pig cells ........................................188
VI.3. $i_{Ca}$ inactivation curves, $f_{oo}$, of freshly isolated, primary cultured
and passaged guinea-pig cells ........................................190
VI.4. Steady-state outward current, $i_K$, in guinea-pig cells .................192
VI.4.1. Steady-state $i_K$ in freshly isolated guinea-pig cells .................192
VI.4.1.1. ATP-sensitive $K^+$ channels ....................................193
VI.4.1.2. CsCl-sensitive outward currents ..............................196
VI.4.1.3. $Ca^{2+}$-dependent steady-state outward currents .............199
VI.4.1.4. Large conductance $Ca^{2+}$-dependant $K^+$ channel (BKCa)
in steady-state outward currents ....................................200
VI.4.1.5. TEA-insensitive components .....................................201
VI.4.2. Steady-state $i_K$ in cultured guinea-pig detrusor cells ............202
VI.4.2.1. CsCl-sensitive outward currents .................................202
VI.4.2.2. $Ca^{2+}$-dependent component ..................................202
VI.4.2.3. TEA-insensitive components .....................................204
VII. Spontaneous transient outward current, STOC .................................205
VII.1. STOC is $Ca^{2+}$ dependent ..............................................205
VII.2. STOC is a $K^+$ current ....................................................211
VII.3. STOC is a BKCa current ..................................................214
VII.4. STOC and intracellular $Ca^{2+}$ ..........................................216
VII.4.1. STOC and membrane depolarisation ................................216
VII.4.2. STOC and intracellular store release ..............................221
VIII. T-type $Ca^{2+}$ current ................................................................223
VIII.1. Low (negative) voltage activated $i_{Ca}$ in freshly isolated
guinea-pig cells ............................................................226
VIII.2. Sensitivity to L-type $Ca^{2+}$ channel blockers .......................227
VIII.3. Sensitivity to low concentrations of NiCl$_2$ ............................229
VIII.4. $Ca^{2+}$ as a Ni$^{2+}$-sensitive current carrier ...............................230
VIII.5. The $i$-$v$ relationships of L-type ($i_{LCa}$) and T-type ($i_{TCa}$) currents
in freshly isolated guinea-pig cells .....................................231
VIII.6. Steady-state activation and inactivation variables of $i_{TCa}$ and $i_{LCa}$
from freshly isolated guinea-pig cells .................................233
VIII.7. $i_{TCa}$ and $i_{LCa}$ window currents in freshly isolated guinea-pig cells .......235
VIII.8. Comparison of $i_{Ca}$ activation and inactivation curves
for freshly isolated and culture guinea-pig cells .......................236
VIII.8.1. $i_{Ca}$ activation curves for freshly isolated (fGP)
and cultured (cGP) guinea-pig cells .................................................237

VIII.8.2. \( \text{iCa} \) inactivation curves for freshly isolated (fGP) and
cultured (cGP) guinea-pig cells .........................................................238

Chapter 5. discussion .................................................................240

I. Methods .........................................................................................240

I.1. Voltage clamp technique for whole-cell recording ..................240

I.1.1. Series resistance and capacitive artefacts considerations .....240

I.1.2. Space-clamp considerations .................................................242

I.1.3. Diffusional exchange between pipette solution and cytosol 243

I.2. Single cells as a model system for electrophysiological studies .244

I.3. Cell culture .................................................................................245

I.4. Measurement of intracellular \( \text{Ca}^{2+} \) with fluorescent indicators 248

II. Results ............................................................................................248

II.1. The electrical activities of freshly isolated and cultured detrusor cells 248

II.1.2. Excitability of freshly isolated and cultured detrusor cells ....250

II.1.3. Ionic currents in freshly isolated and cultured detrusor cells ..253

II.2. Two distinct types of \( \text{iCa} \) in freshly isolated detrusor

smooth muscle cells ...........................................................................256

II.2.1. \( \text{iTCA} \) and \( \text{iLCA} \) in freshly isolated detrusor muscle cells .256

II.2.2. Only \( \text{iLCA} \) is present in cultured detrusor muscle cells ...258

II.2.3. Physiological roles of \( \text{iTCA} \) in detrusor .........................258

II.3. \( \text{K}^{+} \) currents in detrusor smooth muscle .............................260

II.3.1. STOC in freshly isolated detrusor muscle cells ...............260

II.3.2. Steady-state outward rectifier, \( \text{iK} \) ..................................261

III. Conclusions ..................................................................................263

IV. Further experiments ...............................................................265

IV.1. Studies of steady-state \( \text{iK} \) .......................................................265

IV.2. Studies of the spread of excitation in detrusor muscle ...........265

IV.3. Studies of bladder instability ...................................................266

IV.4. Relationship between electrophysiological activity and

second messenger pathways ..........................................................267

IV.5. Mechanical properties of cultured cells ...............................267

References .....................................................................................268
List of published and presented work from this thesis .......................286
List of Figures

Figure 2.1. Equivalent circuit of whole-cell recording .............................................59
Figure 3.1. A Scatchard plot of calcium bound versus the ratio of calcium bound/calcium free in an EGTA containing calibration solution ......75
Figure 3.2. Plot of the linear part of the Scatchard plot of Figure 3.1 ..............75
Figure 3.3. Calibration curve of a Ca^{2+}-selective electrode ...............................76
Figure 3.4. A schematic diagram of the counting chamber ......................................86
Figure 3.5. Patch-clamp system setup ........................................................................94
Figure 3.6. Parallel resistance and capacitance circuit used to represent the cell membrane impedance .................................................................98
Figure 3.7. Protocol for calculation of cell capacitance, membrane input resistance and corresponding membrane voltage change .........................100
Figure 3.8. Long duration current protocols for determination of action potential threshold, V_{th} ..................................................................................101
Figure 3.9. Current activation protocol for both inward and outward currents ...102
Figure 3.10. Whole-cell membrane currents recorded under the voltage-clamp mode using a "KCl + 5 mM EGTA" filled electrode .....................103
Figure 3.11. Inward current inactivation voltage protocol ..................................105
Figure 3.12. Steady-state outward current inactivation voltage protocol ............106
Figure 3.13. Voltage protocol for measurement of STOCs as well as for steady-state i_k ..................................................................................107
Figure 3.14. Spontaneous transient outward currents, STOCs .........................108
Figure 3.15. Voltage protocol A to elicit T-type and L-type Ca^{2+} currents ....109
Figure 3.16. Voltage protocol B for activation of i_{TCa} and i_{LCa} ....................109
Figure 3.17. Voltage protocol for i_{LCa} inactivation ..........................................110
Figure 3.18. Voltage protocol for i_{TCa} inactivation .........................................110
Figure 3.19. A schematic diagram of the experimental setup for combined epifluorescence microscopy and patch electrode recording for the measurement of [Ca^{2+}]_i with fura-2 and membrane currents ......112
Figure 3.20. A voltage ramp to determine intracellular Ca^{2+} accumulation and outward current oscillations .......................................................115
Figure 4.1. Guinea-pig detrusor smooth muscle cells in culture (top) ..............119
Figure 4.2. Human detrusor smooth muscle cells in culture (bottom) .............119
Figure 4.3. Positive immunocytochemical staining for guinea pig smooth muscle α- actin (top) .........................................................................120
Figure 4.4. Serum control showing negative staining to antibody for smooth muscle α-actin (bottom) .................................................................120

Figure 4.5. Intracellular Ca\(^{2+}\) rise in response to 100μM carbachol in a cultured guinea-pig detrusor smooth muscle cell .........................122

Figure 4.6. Intracellular Ca\(^{2+}\) rise in response to 100μM carbachol in a cultured human detrusor smooth muscle cell ............................122

Figure 4.7. Membrane potential response to current steps under current clamp mode .................................................................123

Figure 4.8. Histogram of the resting membrane potential for freshly isolated guinea-pig detrusor smooth muscle cells ..........124

Figure 4.9. A: A train of spontaneous action potentials (APs) recorded from a freshly isolated guinea-pig detrusor cell under I= 0 mode; B: Spontaneous APs recorded from a freshly isolated human detrusor cell .................................................................129

Figure 4.10. An action potential recorded from a freshly isolated guinea-pig cell ..................................................................................130

Figure 4.11. An action potential recorded from a freshly isolated human cell ....130

Figure 4.12. Action potentials in a freshly isolated guinea-pig cell .......................131

Figure 4.13. Action potentials in a freshly isolated human cell ....................132

Figure 4.14. The effect of verapamil on the action potential in a freshly isolated guinea-pig cell .................................................................132

Figure 4.15. An action potential from a cultured guinea-pig cell elicited by a 50ms 60pA step current .................................................................135

Figure 4.16. An action potential from a cultured human cell ....................136

Figure 4.17. Action potentials from a cultured guinea-pig cell .......................136

Figure 4.18. An action potential from a cultured human cell ....................137

Figure 4.19. Action potentials produced by 600ms step currents in a cultured guinea-pig cell .................................................................138

Figure 4.20. The effect of nifedipine on action potentials in a cultured guinea-pig cell .................................................................138

Figure 4.21. The correlation of action potential maximum upstroke velocity and peak iCa current density from freshly isolated guinea-pig cells ......144

Figure 4.22. Action potentials in freshly isolated guinea-pig cells using a "KCl + 5mM EGTA" filled electrode and a "KCl + 0.1mM EGTA" filled electrode .................................................................146

Figure 4.23. The relationship between APD50 and pure transient outward current magnitude .................................................................148

Figure 4.24. Variations in voltage and net ionic current during a membrane action potential from a freshly isolated guinea-pig cell initiated
by a brief square pulse current ..............................................................150

Figure 4.25. The relationship between action potential and ionic current $i_1$
in a freshly isolated guinea-pig cell .....................................................151

Figure 4.26. Part A: the action potential and ionic currents in a freshly isolated
guinea-pig cell with a CsCl filled electrode.
Part B shows the detail of the inward current $i_1$ and maximum
dV/dt of Part A .......................................................................................152

Figure 4.27. Inward and outward currents in a freshly isolated guinea-pig cell ...154
Figure 4.28. Inward and outward currents in a freshly isolated human cell .......154
Figure 4.29. Mean current-voltage relationships for inward and
outward currents in freshly isolated guinea-pig and human cells .........155

Figure 4.30. The mean current-voltage relationships of net current densities
from freshly isolated guinea-pig and human cells ..............................156

Figure 4.31. Inward current steady-state activation curves from
freshly isolated guinea-pig cells and human cells .........................157

Figure 4.32. Membrane currents recorded in a freshly isolated guinea-pig cell ...158

Figure 4.33. Inward current inactivation curves from freshly isolated
guinea-pig cells and human cells .........................................................159

Figure 4.34. Steady-state outward current activation curves from
freshly isolated guinea-pig and human cells ........................................160

Figure 4.35. Steady-state outward current inactivation curves from
freshly isolated guinea-pig and human cells ........................................161

Figure 4.36. Inward and outward currents in a primary cultured guinea-pig cell ...162
Figure 4.37. Inward and outward currents in a primary cultured human cell ....163

Figure 4.38. The mean current-voltage relationships of inward and
outward currents from cultured guinea-pig and human cells ..........164

Figure 4.39. The mean current-voltage relationships of net inward current
densities in cultured guinea-pig and human cells ..............................164

Figure 4.40. Inward current activation curves for cultured guinea-pig
and human cells ......................................................................................166

Figure 4.41. Inward current inactivation curves in cultured guinea-pig
and human cells ......................................................................................166

Figure 4.42. The mean steady-state outward current activation curves
in cultured guinea-pig and human cells ................................................167

Figure 4.43. Steady-state outward current inactivation curves
in cultured guinea-pig and human cells ................................................168

Figure 4.44. Inward current densities in freshly isolated and
cultured human cells ..............................................................................169

Figure 4.45. Comparison of steady-state outward current i-v relationships
from freshly isolated and cultured human cells ..............................................170

Figure 4.46. Comparison of net currents in cultured and freshly isolated human cells .................................................................170

Figure 4.47. Inward current activation curves in freshly isolated and cultured human cells .............................................................................171

Figure 4.48. Inward current inactivation curves for freshly isolated and cultured human cells .............................................................................172

Figure 4.49. Steady-state outward current activation curves for freshly isolated and cultured human cells .................................................................172

Figure 4.50. Steady-state outward current inactivation curves for freshly isolated and cultured human cells .................................................................173

Figure 4.51. Currents recorded in a cultured human cell after first passage .................................................................176

Figure 4.52. Currents recorded in a cultured guinea-pig cell after second passage ..............................................................................176

Figure 4.53. Inward current i-v relationships for freshly isolated, primary cultured and passaged guinea-pig cells .................................177

Figure 4.54. The steady-state outward current i-v relationships in freshly isolated, primary cultured and passaged guinea-pig cells ..........177

Figure 4.55. Net currents from freshly isolated, primary cultured and passaged guinea-pig cells ..............................................................178

Figure 4.56. Comparison of steady-state inward current activation from freshly isolated, primary cultured and passaged guinea-pig cells ..................179

Figure 4.57. A comparison of inward current steady-state inactivation in freshly isolated, primary cultured and passaged guinea-pig cells ..........181

Figure 4.58. Steady-state outward current activation from freshly isolated, primary cultured and passaged guinea-pig cells ..................182

Figure 4.59. Steady-state outward current inactivation curves for freshly isolated, primary cultured and passaged guinea-pig cells ..........183

Figure 4.60. The effect of 5|xM nifedipine on peak inward current in a cultured guinea-pig cell .................................................................186

Figure 4.61. Peak iCa i-v relationships in freshly isolated and primary cultured guinea-pig cells with CsCl filled electrodes ..........187

Figure 4.62. Peak iCa i-v relationships in freshly isolated and passaged guinea-pig cells with CsCl filled electrodes ..........187

Figure 4.63. Comparison of iCa i-v relationships in freshly isolated, primary cultured and passaged guinea-pig cells with CsCl filled electrodes .188

Figure 4.64. iCa activation curves in freshly isolated, primary cultured and passaged guinea-pig cells with CsCl filled electrodes ..........189
Figure 4.65. iCa inactivation curves in freshly isolated, primary cultured and 
passaged guinea-pig cells with CsCl filled electrodes ......................191
Figure 4.66. iCa inactivation time constants for freshly isolated and 
primary cultured or passaged guinea-pig cells .................................192
Figure 4.67. Steady-state outward current in freshly isolated guinea pig cells 
using different filling solutions ..........................................................193
Figure 4.68. Membrane currents recorded from a freshly isolated guinea-pig 
detrusor cells using ATP-containing "KCl+5mM EGTA" 
filling solution ......................................................................................194
Figure 4.69. The effect of 30μM BRL38227 on steady-state outward current 
in freshly isolated guinea-pig cells .........................................................194
Figure 4.70. Membrane current recorded from a freshly isolated guinea-pig cells 
using ATP-free "KCl+5mM EGTA" filling solution ..............................195
Figure 4.71. The i-v relationships in freshly isolated guinea-pig cells 
using an ATP-free "KCl+5mM EGTA" filling solution .......................195
Figure 4.72. The effect of CsCl on the outward current 
in a freshly isolated guinea-pig cell .........................................................196
Figure 4.73. Effects of CsCl, verapamil and NiCl2 on the currents 
in a freshly isolated guinea-pig cell .........................................................197
Figure 4.74. Steady-state outward currents in freshly isolated guinea-pig cells 
with KCl or CsCl-filled electrodes ..........................................................198
Figure 4.75. The effect of verapamil on the steady-state outward current 
in freshly isolated guinea-pig cells .........................................................199
Figure 4.76. The verapamil-sensitive component of steady-state outward current 
in freshly isolated guinea-pig cells ..........................................................200
Figure 4.77. The effect of iberiotoxin (100nM) and apamin (1μM) on the steady-state outward current in freshly isolated guinea-pig cells .....201
Figure 4.78. The TEA-resistant components in steady-state outward current 
in freshly isolated guinea-pig cells ..........................................................202
Figure 4.79. Comparison of steady-state outward currents recorded with KCl 
or CsCl filled electrodes in cultured guinea-pig cells .........................203
Figure 4.80. Verapamil-sensitive component of steady-state outward current 
in cultured guinea-pig cells .................................................................203
Figure 4.81. TEA-insensitive steady-state outward current in cultured 
guinea-pig cells .....................................................................................204
Figure 4.82. Spontaneous transient outward current (STOC) in freshly isolated 
and cultured guinea-pig cells .............................................................205
Figure 4.83. The Ca2+ dependence of STOC in a freshly isolated 
guinea-pig cell .....................................................................................206
Figure 4.84. The effect of BaCl$_2$ on the STOC .........................................................207
Figure 4.85. The i-v relationship of STOC in freshly isolated guinea-pig cells 
in the presence and absence of Ca$^{2+}$ .................................................................208
Figure 4.86. The effect of extracellular [Ca$^{2+}$] on STOC amplitude recorded 
at +10mV ........................................................................................................208
Figure 4.87. The effect of verapamil on the STOC in a freshly isolated 
guinea-pig cell ..................................................................................................209
Figure 4.88. The effect of verapamil and NiCl$_2$ on the STOC in a freshly isolated 
guinea-pig cell ...............................................................................................210
Figure 4.89. Averaged data for the effect of verapamil on the STOC amplitude 
in freshly isolated guinea-pig cells ...................................................................211
Figure 4.90. The effect of 3mM [TEA]$_0$ on STOC in a freshly isolated 
guinea-pig cell .................................................................................................212
Figure 4.91. The effect of 30mM [TEA]$_0$ on the STOC ........................................213
Figure 4.92. The effect of a low concentration (3mM) of TEA on STOC 
(i-v relationship) in freshly isolated guinea-pig cells ....................................213
Figure 4.93. The effect of iberiotoxin on the STOC ..............................................215
Figure 4.94. Averaged data for the effect of iberiotoxin on STOC 
in freshly isolated guinea-pig cells ..................................................................215
Figure 4.95. The relationship between inward current, the first peak of STOC 
and intracellular bulk Ca$^{2+}$ simultaneously recorded 
in a freshly isolated guinea-pig cell .................................................................216
Figure 4.96. Simultaneous [Ca$^{2+}$] and current recording 
in a freshly isolated guinea-pig cell ..............................................................218
Figure 4.97. The relationship between [Ca$^{2+}$] and pure STOC .......................218
Figure 4.98. Correlation between the pure STOC amplitude and 
the mean [Ca$^{2+}$] ........................................................................................................219
Figure 4.99. [Ca$^{2+}$] accumulation and outward current 
in response to membrane depolarisation ......................................................220
Figure 4.100. The voltage dependence of STOC ..................................................221
Figure 4.101. STOC and caffeine-induced intracellular Ca$^{2+}$ release ..................223
Figure 4.102. Inward i$_{Ca}$ characteristics in cells from freshly isolated (fGP) and 
cultured (cGP) guinea-pig cells with "CsCl+5mM EGTA" filled 
electrodes ...........................................................................................................224
Figure 4.103. The correlation between action potential threshold,$V_{th}$ and holding 
potential in freshly isolated guinea-pig cells using KCl+0.1EGTA 
solution filled electrodes .................................................................................225
Figure 4.104. Low voltage activated i$_{Ca}$ in a freshly isolated guinea-pig cell ......226
Figure 4.105. The nifedipine-resistant component of i$_{Ca}$
in a freshly isolated guinea-pig cell .....................................................227

Figure 4.106. Cd$^{2+}$-insensitive component of i$\text{Ca}$ .....................................................228

Figure 4.107. A Ni$^{2+}$-sensitive component of i$\text{Ca}$ .....................................................229

Figure 4.108. Ca$^{2+}$ is the charge carrier of the Ni$^{2+}$-sensitive inward current ......230

Figure 4.109. The current-voltage relationships of i$\text{L\text{Ca}}$ and i$\text{T\text{Ca}}$ .................231

Figure 4.110. The "total Ca$^{2+}$ current" i-$v$ relationship in freshly isolated
guinea-pig cells .....................................................................................232

Figure 4.111. i$\text{Ca}$ inactivation time constants, $\tau_{\text{inact}}$, as a function of voltage
following a preconditioning voltage step to -100mV
for 2 seconds ..........................................................................................232

Figure 4.112. i$\text{T\text{Ca}}$ and i$\text{L\text{Ca}}$ steady-state activation curves in freshly isolated
guinea-pig cells from a 2s preconditioning voltage at -100mV ...........234

Figure 4.113. i$\text{T\text{Ca}}$ and i$\text{L\text{Ca}}$ steady-state inactivation curves
from freshly isolated guinea-pig cells ..................................................234

Figure 4.114. The window current of i$\text{L\text{Ca}}$ in freshly isolated guinea-pig cells ....235

Figure 4.115. The window current of i$\text{T\text{Ca}}$ in freshly isolated guinea-pig cells ....236

Figure 4.116. Comparison of i$\text{Ca}$ (cGP), i$\text{T\text{Ca}}$ and i$\text{L\text{Ca}}$ (fGP) activation curves
(preconditioning potential -100mV, CsCl+5mM EGTA
filling solution) ......................................................................................237

Figure 4.117. Comparison of i$\text{Ca}$ (cGP), i$\text{T\text{Ca}}$ and i$\text{L\text{Ca}}$ (fGP)
inactivation curves .................................................................................239

Figure 5.1. A summary of the voltage-dependent membrane currents
in freshly isolated guinea-pig detrusor muscle cells ..............................263
List of Tables

Table 3.1. Composition of normal Tyrode's solution ............................................70
Table 3.2. Results from an experiment to determine the constant $K_{Ca^{2+}}$ ....72
Table 3.3. Data from Table 2.2 for calculation of $K_{Ca^{2+}}$ ....................................74
Table 3.4. Results of a determination of the $[Ca^{2+}]$ in a nominally Ca-free Tyrode's solution ................................................................................................................77
Table 3.5. Composition of the pCa 8.0 Tyrode's solution
("EGTA-Ca^{2+}-free" solution) ........................................................................78
Table 3.6. Composition of Ca^{2+}-free HEPES-Tyrode's solution ....................78
Table 3.7. Composition of Ca^{2+}-containing HEPES-Tyrode's solution ..........79
Table 3.8. Composition of basic "KCl+5mM EGTA" filling solution .............80
Table 3.9. Composition of "KCl+0.1mM EGTA" filling solution ....................80
Table 3.10. Composition of "CsCl+0.1mM EGTA" filling solution ..............80
Table 3.11. Composition of "CsCl+5 mM EGTA" filling solution ................81
Table 3.12. Composition of "high Ca^{2+}" filling solution ............................81
Table 3.13. Composition of enzyme solution .........................................................82
Table 4.1. The passive electrical properties of isolated detrusor smooth muscle cells from freshly isolated guinea-pig (fGP) and human (fH) cells ..124
Table 4.2. The passive electrical properties of isolated detrusor smooth muscle cells from cultured guinea-pig (cGP) and human (cH) cells ........125
Table 4.3. Comparison of the passive electrical properties of freshly isolated and cultured human detrusor cells .........................126
Table 4.4. Comparison of the passive electrical properties of freshly isolated and cultured guinea-pig detrusor cells ....................127
Table 4.5. Comparison of action potential threshold currents from short pulses in freshly isolated guinea-pig and human cells ..........129
Table 4.6. Comparison of action potentials (APs) from freshly isolated guinea-pig and human cells ........................................134
Table 4.7. Comparison of action potential parameters in cultured cells from guinea-pig and human detrusor ........................................139
Table 4.8. Comparison of action potential properties in cultured and freshly isolated human cells ........................................140
Table 4.9. Comparison of action potential properties in freshly isolated and primary cultured guinea-pig cells ........................141
Table 4.10. AP parameters in freshly isolated and passaged (cGP1-3) guinea-pig cells .................................................................142
Table 4.11. AP properties in primary cultured and passaged guinea-pig cells ....143
Table 4.12. Comparison of AP properties in freshly isolated guinea-pig cells with K+ containing filling solution using a high or low EGTA concentration .................................................................147
Table 4.13. Steady-state outward current inactivation variables for freshly isolated guinea-pig and human cells ..................162
Table 4.14A. Summary of comparisons of current densities from freshly isolated guinea-pig and human; cultured guinea-pig and human cells in section V.1-V.3 ..............174
Table 4.14B. Summary of current activation and inactivation kinetics in section V.1-V.3 from freshly isolated guinea-pig and human; cultured guinea-pig and human cells..........................174
Table 4.15. Steady-state inward current activation parameters, freshly isolated vs primary cultured cells ..................179
Table 4.16. Steady-state inward current activation, freshly isolated vs passaged cells .............................................180
Table 4.17. Steady-state inward current activation, primary cultured vs passaged cells .............................................180
Table 4.18. Steady-state inward current activation parameters for freshly isolated and primary cultured guinea-pig cells ..........180
Table 4.19. Steady-state inward current inactivation variables for freshly isolated and passaged guinea-pig cells .............180
Table 4.20. Steady-state inward current inactivation variables for primary cultured and passaged cells .....................181
Table 4.21. Steady-state outward current activation parameters for freshly isolated and primary cultured cells .............182
Table 4.22. Steady-state outward current activation parameters for freshly isolated and passaged cells .....................182
Table 4.23. Steady-state outward current activation parameters from primary cultured and passaged cells ...............183
Table 4.24. Steady-state outward current inactivation parameters for freshly isolated and primary cultured cells ..........184
Table 4.25. Steady-state outward current inactivation parameters for freshly isolated and passaged guinea-pig cells ..........184
Table 4.26. Steady-state outward current inactivation parameters for primary cultured and passaged cells ...............184
Table 4.27. Peak iCa in freshly isolated, primary cultured and passaged cells with CsCl filled electrodes ......................188
Table 4.28. iCa activation properties (CsCl filled) in freshly isolated
Table 4.29. $i_{Ca}$ activation properties (CsCl filled) in freshly isolated and passaged guinea-pig cells ..............................................................189

Table 4.30. $i_{Ca}$ activation properties (CsCl filled) in primary cultured and passaged guinea-pig cells ..............................................................190

Table 4.31. $i_{Ca}$ inactivation properties (CsCl filled) in freshly isolated and primary cultured guinea-pig cells ..............................................................190

Table 4.32. $i_{Ca}$ inactivation properties (CsCl filled) in freshly isolated and passaged guinea-pig cells ..............................................................190

Table 4.33. $i_{Ca}$ inactivation properties (CsCl filled) in passaged and primary cultured guinea-pig cells ..............................................................191

Table 4.34. Steady-state activation parameters for $i_{TCa}$ & $i_{LCa}$ in freshly isolated guinea-pig cells ..............................................................233

Table 4.35. Steady-state inactivation parameters for $i_{TCa}$ and $i_{LCa}$ in freshly isolated guinea-pig cells ..............................................................235

Table 4.36. Activation parameters for $i_{TCa}$ (fGP) vs $i_{Ca}$ (cGP) ..............................................................237

Table 4.37. Activation parameters for $i_{LCa}$ (fGP) vs $i_{Ca}$ (cGP) ..............................................................238

Table 4.38. Inactivation parameters for $i_{TCa}$ (fGP) vs $i_{Ca}$ (cGP) ..............................................................238

Table 4.39. Inactivation variables for $i_{LCa}$ (fGP) vs $i_{Ca}$ (cGP) ..............................................................239

Table 5.1. Alterations of membrane passive properties after culture ..............................................................250

Table 5.2. Alterations to membrane action potential properties in cultured detrusor cells compared to freshly isolated counterparts ..............................................................252

Table 5.3. Alterations of membrane currents in cultured detrusor cells ..............................................................254

Table 5.4. Summary of L-type and T-type Ca$^{2+}$ currents in freshly isolated detrusor muscle cells ..............................................................257
List of Abbreviation

a radius of muscle cell (cm)
ABC method avidin-biotin complex, an indirect immunoenzyme binding assay
AP action potential
AP dV/dt rate of action potential upstroke (in thesis AP dV/dt = AP V_{max})
AP V_{max} maximum rate of AP upstroke
APD_{50} action potential duration at 50% repolarisation
ATP adenosine 5'-triphosphate
BK_{Ca} a large conductance Ca^{2+}-activated K+ channel
BRL38227 Levromakalim, K_{ATP} channel opener
c offset values of K+ current activation and inactivation.
CaEGTA Ca^{2+} binds to EGTA
cGP primary cultured guinea-pig detrusor cells
cGP_{1-3} subcultured guinea-pig detrusor cells up to three times
cH primary cultured human detrusor cells
cH_{1-3} subcultured human detrusor cells up to three times
C_{m} specific membrane capacitance (µF/cm²)
c_{m} membrane capacitance(pF)
DAB diaminobenzidine
d_{∞} activation variable of Ca^{2+} current
E_{Ca} potential difference measured by the Ca^{2+}-selective electrode, reference electrode pair
E_{Ca, corr} the E_{Ca} value offset to zero at the highest [Ca^{2+}]
EGTA ethylene glycol-bis-(β-aminoethyl ether) N,N,N',N'-tetra acetic acid
fGP freshly isolated guinea-pig detrusor cells
fH freshly isolated human detrusor cells
f_{∞} inactivation variable of Ca^{2+} current
g gram
g membrane conductance (= l/r)
g_{max} maximum membrane conductance
GTP guanosine 5'-triphosphate
H_{2}EGTA EGTA without bound Ca^{2+}
HEPES N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]
I current density (pA/pF)
i current
i_{c} capacitive current
I_{Ca} Ca^{2+} current density (pA/pF)
iCa \quad \text{Ca}^{2+} \text{ current}

iClic \quad \text{Ca}^{2+}-\text{activated Cl}^- \text{ current}

i_i \quad \text{ionic current}

I_K \quad \text{K}^+ \text{ current density (pA/pF)}

i_K \quad \text{K}^+ \text{ current}

i_{kCa} \quad \text{Ca}^{2+} \text{ dependent K}^+ \text{ current}

i_{LCa} \quad \text{L-type Ca}^{2+} \text{ current}

i_m \quad \text{membrane current}

i_{max} \quad \text{maximum membrane ionic current}

i_p \quad \text{pipette current}

IP_3 \quad 1,4,5\text{-triphasphate}

i_{rCa} \quad \text{T-type Ca}^{2+} \text{ current}

i-v \quad \text{current-voltage (relationship)}

K_{ATP} \quad \text{ATP-dependent K}^+ \text{ channel}

K_{Ca} \quad \text{the equilibrium constant of the reaction between Ca}^{2+} \text{ and EGTA}

K_{Ca}^{app} \quad \text{apparent K}_{Ca}

k_d \quad \text{Ca}^{2+} \text{ current activation slope factor}

k_f \quad \text{Ca}^{2+} \text{ current inactivation slope factor}

k_n \quad \text{steady-state outward K}^+ \text{ current activation slope factor}

k_p \quad \text{steady-state outward K}^+ \text{ current inactivation slope factor}

\lambda \quad \text{space constant (cm)}

M \quad \text{molar}

ml \quad \text{millilitre}

mM \quad \text{millimolar}

ms \quad \text{millisecond}

mV \quad \text{millivolt}

ns \quad \text{not significant}

n_\infty \quad \text{activation variable of steady-state outward K}^+ \text{ current}

p.d. \quad \text{potential difference}

pA \quad \text{picoamp}

PBS \quad \text{phosphate buffered saline}

pCa \quad -\log[\text{Ca}^{2+}]

pF \quad \text{picofarad}

pK_{Ca} \quad -\log K_{Ca}

pK_{Ca}^{app} \quad \text{apparent pK}_{Ca}

p_\infty \quad \text{inactivation variable of steady-state outward K}^+ \text{ current}

Q \quad \text{charge}

R \quad \text{ratio of fluorescent output}
\( r \) correlation coefficient
\( R_i \) internal specific resistance (\( \Omega \text{cm} \))
\( \text{Rinp} \) cell input resistance
\( \text{Rm} \) specific membrane resistance (\( \Omega \text{cm}^2 \))
\( \text{rm} \) membrane resistance
\( \text{RMP} \) resting membrane potential
\( \text{r_s} \) series resistance
\( \text{SD} \) standard deviation
\( \text{SKCa} \) a small conductance Ca\(^{2+}\)-activated K\(^+\) channel
\( \text{SR} \) sarcoplasmic reticulum
\( \text{STOC} \) spontaneous transient outward current
\( \text{TEA} \) tetraethylammonium chloride
\( \tau_{\text{inact}} \) \( i_{\text{LCa}} \) and \( i_{\text{TCa}} \) inactivation time constants
\( \tau_{\text{m}} \) membrane time constant
\( \text{TTP} \) action potential time to peak
\( \text{Tyr} \) Tyrode's solution
\( \text{UDC} \) uncontrolled detrusor contraction
\( \text{V} \) volt
\( V_{0.5} \) half maximum voltage of activation or inactivation variables
\( V_h \) holding potential
\( V_m \) membrane potential
\( V_{\text{max}} \) steady-state value of voltage
\( V_{\text{rev}} \) reversal potential of current
\( \text{vs} \) versus
\( V_{\text{th}} \) action potential threshold voltage
\([ \quad ]_i \) intracellular concentration
\([ \quad ]_o \) extracellular concentration
\( \mu l \) microlitre
\( ^{\circ} \text{C} \) centigrade
Chapter 1  Introduction

I. Clinical problem

The normal functions of bladder filling (storage) and voiding depend on a low intravesical pressure during filling which allows free drainage of urine from the upper urinary tract, and a fall of bladder outflow resistance in conjunction with a sufficient rise in intravesical pressure when voiding. Therefore disorders of these processes will result in bladder dysfunction. Urinary incontinence, urgency and frequency of micturition, for example, are common problems especially in older age. These conditions may arise from involuntary contractions of the detrusor smooth muscle (uncontrolled detrusor contraction, UDC). The two main forms are detrusor instability and detrusor hyperreflexia. Detrusor instability occurs when the detrusor contracts spontaneously or on provocation (e.g. in clinical urodynamic evaluation), during the filling of the bladder when the patient is trying to inhibit micturition. This type of bladder dysfunction is often found in patients with partial outflow obstruction (e.g. due to benign prostatic hyperplasia) and secondary bladder hypertrophy, but is also seen in patients with no underlying pathology (idiopathic form). Detrusor hyperreflexia is defined as detrusor overactivity due to an obvious perturbation of the nervous control mechanism, particularly in patients with spinal lesions, diabetes and multiple sclerosis.

Pharmacological or surgical intervention in the management of UDC has been aimed at the reduction or abolition of detrusor contraction. It cannot yet be targeted at the underlying pathophysiological abnormality, since normal detrusor smooth muscle physiology and the fundamental pathophysiological processes that initiate and underlie unstable detrusor contractions are not well understood (Mundy 1988, Sibley 1997). Due to the lack of bladder specificity, the clinically available pharmacological therapies therefore have unsatisfactory results of inefficiency, unsuitability and side-effects. Surgical management is reserved for those resistant to conservative therapy. Augmentation cystoplasty, for example, denervates the bladder by the interposition of a bowel segment thus rendering the bladder unable to function in a coordinated fashion so as to reduce the symptoms of incontinence. However this procedure is associated with complications such as electrolyte disorders, stones and even bladder carcinoma (Stone et al 1987).

This leaves a challenging subject for basic research, as the clinical treatment of the unstable bladder still leaves much to be desired.
II. Experimental considerations

To elucidate the cellular mechanisms underlying functional disorders of the bladder, human samples provide the best source of tissue. However there is a limitation to such samples so that other model systems are required. Cell cultures have become a very useful supplementary model in many research fields, such as molecular biology, and is beginning to play a role in the physiological study of the human bladder (Baskin et al 1993; Harriss 1995; Chambers et al 1996). However most research using cultured cells, especially from human bladder has been restricted to areas of morphology, molecular biology, biochemistry and more recently intracellular \( \text{Ca}^{2+} \) signalling. The electrical properties of cultured detrusor smooth muscle cells have not been investigated. Moreover even the electrical properties of normal adult detrusor cells have not been systematically studied. Whether or not cells after culture still represent the normal tissue remains a question to answer. If cultured cells could express normal physiological properties, it would be an ideal model for basic research to approach the clinic problem; i.e., it would be possible to manipulate their cellular function and lead to the development of new pharmaceutical therapies and other clinical treatments, such as the bladder implant.

Freshly isolated adult mammalian detrusor, as another supplementary experimental system, has been in widespread use in urological research. The advantage is to provide a well controlled experimental model and most research has been performed on this from animal sources. It is important to assess how relevant the physiological properties of cells from animal models are to those from humans in terms of revealing the physiological mechanisms and pathophysiological changes of human detrusor. It is practical and economical to make full use of tissue sources including human samples, cultured cells and animal models in laboratory research if these models shed light on abnormal detrusor function.

III. Electrical activity of excitable tissues

III.1. Classic description of the ionic basis of axon membrane excitability

The electrical excitability of nerve and muscle has attracted scientists for several centuries. Hodgkin and Huxley (the HH model) were the first to give a quantitative description of ion fluxes and permeability changes that would explain membrane excitability and the action potential of the squid giant axon. Their model was the first
to describe the ionic basis of excitation correctly and revolutionised electrophysiology.

In nerves, action potentials are rapidly propagated electrical messages along the axon. They are brief, travel at constant velocity, and maintain a constant amplitude. For determining the laws which govern movements of ions during electrical activity, Hodgkin and Huxley developed an experimental method to measure the flow of current through a definite area of the membrane of a giant axon from *Loligo*, while the membrane potential was kept uniform over this area. This was based on the theoretical assumption that membrane current density $I_m$, may be divided into a capacity current, $(I_c = C_m \frac{dV}{dt}, C_m$: membrane capacitance) which involves a change in ion density at the outer and inner surfaces of the membrane, and an ionic current density $I_i$ which depends on the movement of charged particles through the membrane (i.e., $I_m = C_m \frac{dV}{dt} + I_i$). In their experiments $\frac{dV}{dt} = 0$, so that the ionic current was obtained directly from the experimental recording. Hodgkin and Huxley then identified three components to the ionic current flow that generates the action potential in the squid axon. An inward current carried by $\text{Na}^+$, termed $I_{\text{Na}}$, enters the nerve fibre during the rising phase of the action potential; an outward current carried by $\text{K}^+$, named $I_{\text{K}}$ leaves during the falling phase, and a small "leak current" ($I_L$) made up by chloride and other ions. They determined the "instantaneous current-voltage relation of $I_{\text{Na}}$ and $I_{\text{K}}"$ on the basis of voltage-clamp experiments in the giant axon and provided the complete description of the kinetics of conductance changes (Hodgkin 1951; Hodgkin and Huxley 1952a, b, c; Hodgkin et al 1952).

However, the way in which ions actually crossed the membrane still remained a question in the HH model until ion channels were discovered.

III.2. Membrane capacitance and subthreshold depolarisation

A description of membrane structure is now clear. The lipid bilayer of the membrane, embedded with conducting ionic channels (and ion transporters etc.), separates two highly conductive media which comprise the internal and external environments of the cell. This structure of cells is the foundation of the electrophysiological properties of all excitable cells. Electrical current may flow therefore either within the cells as intracellular current, i.e., along the axon, or it may flow between the intracellular and extracellular fluids by entering or leaving the cells as membrane current. The impedance to current flow within cells is referred to as intracellular impedance, even though in some cases this may involve cell-to-cell impedance in addition to that of the
intracellular medium. The impedance to current entering or leaving cells is referred to as membrane impedance. Provided the diameter of cells is relatively small and the resistance of the cell membrane much higher than the resistance to current flow through the cell cytoplasm, the equivalent electrical circuit representing the membrane impedance of such a cell is a simple one, consisting of a resistance $r_m$ (ionic channels) and capacitance $c_m$ in parallel. This follows because the injected current spreads out and passes evenly through all parts of the cell membrane.

The membrane capacitance, determined by the thickness and dielectric constant of the bilayer membrane, is a measure of how much charge separation, $\Delta Q$, must occur to generate a given change of potential, $\Delta V$ (i.e., $\Delta V=\Delta Q/c_m$). The direction of potential change is determined by the change in charge with time, i.e., a deposition of positive charge on the inner face of the membrane will depolarise it. The voltage established across the membrane is therefore proportional to the amount of charge separation which has occurred. In the resting state, it is assumed that the cell membrane conductance, which is the reciprocal of membrane resistance, remains constant; the current-voltage relation is linear. Since the cell membrane behaves as capacitor, when a sudden step change of subthreshold current is uniformly applied to the membrane (the space-clamp condition), an instantaneous membrane voltage change in response to a step current injection does not occur. Instead, there is a slow rise of voltage due to the time taken to charge or discharge the membrane capacity and is given by the equation

$$\Delta V = i_m r_m \left(1 - \text{exp}\left(-t/\tau_m\right)\right)$$  \hspace{1cm} \text{(1.1)}

where $i_m r_m$ is the steady-state value of $\Delta V$; $\tau_m$ is the membrane time constant, the time taken for voltage to reach 63% (i.e., $1-e^{-1}$) of the steady-state voltage value. The time constant is equivalent to the product of the resistance and capacitance of the cell membrane ($\tau_m = r_m c_m$).

Electrophysiologists have used this theory to determine the passive properties of excitable cells, since this information is required to assess the changes in membrane properties that occur, for example, with some neurotransmitters, hormones or other biologically active substances. Membrane capacitance per unit area of membrane is considered to be a biological constant, approximately $1\mu F/cm^2$ in all biological membranes. Knowledge of the passive electrical membrane properties of smooth muscle cells, including the coupling between cells, is essential for our understanding of the propagation of action potentials and other active responses arising from voltage-dependent changes in membrane conductance, and remains a central problem.

III.3. Non-propagating action potentials under current clamp

In excitable cells, transient alterations to their membrane properties result in membrane potential changes. The action potential is such a transient change to the transmembrane electrical potential and depends on nonlinear impedance properties displayed by the membrane. When a cell is isolated and small enough for the membrane to be excited uniformly, the cable complications are eliminated. In this condition, the electrical properties of the cell will be determined entirely by the specific membrane properties.

An action potential can be initiated when a depolarising current sufficient to exceed threshold is uniformly applied to the intracellular fluid from an external circuit via an electrode. In this case, the membrane conductance is no longer linear but changes as a function of time and voltage. Initially the applied current will simply add charge to the inside of the cell and so depolarise it. When the potential difference exceeds threshold, inward ionic current will flow across the cell membrane (via Ca$^{2+}$ channels in most smooth muscles and Na$^{+}$ channels in an axon) to add more positive charge to the inside of the cell and so further depolarise it. The inward ionic current is used to change the charge on the local membrane capacity; outward current flow then repolarises the cell. It should be emphasised that the action potential evoked from an isolated single cell under current-clamp would follow a different time course from one evoked in a bundle of muscle fibres, since the muscle fibres are electrically connected in a syncytium, so acting more like a cable. The condition for excitation of a uniformly polarised membrane is that the voltage should exceed a critical value at which the net ionic current becomes inward. The corresponding condition in a cable is that the voltage should exceed a critical value at which the net ionic current generated by the cable as a whole becomes inward (Jack et al. 1975; Tomita 1975).

III.4. Ion channels

III.4.1. The concept of ion channels

Ionic channels as elementary excitable elements in the cell membrane produce and transduce electrical signals in living cells. They are membrane proteins that span the
lipid bilayer and act as macromolecular pores in the cell membrane. It has been found that the membranes of all living cells contain ion channels. Excitable channels respond to appropriate stimuli; for example, voltage-dependent (or voltage-gated) channels sense membrane potential changes; ligand-gated channels respond to the binding of a neurotransmitter or other chemical stimulus with its receptor, involving G-protein (ligand-gated G-dependent channels) or without involving G-protein (ligand-gated G-independent channels); stretch-activated channels are sensitive to direct mechanical stretch (mechanoreceptors). The response of the channels is called "gating", representing the pore of a channel opening and closing.

Since the 1990s, many channel proteins have been isolated, cloned and their amino acid sequences determined. For example, the voltage-dependent L-type Ca^{2+} channel, the dihydropyridine receptor in muscle, is composed of two distinct high molecular weight subunits $\alpha_1$ and $\alpha_2$, plus three smaller subunits $\beta$, $\gamma$ and $\delta$. cDNAs for all of these subunits have been cloned and sequenced. The $\alpha_1$ subunit of the L-type Ca^{2+} channel contains strong binding sites for the dihydropyridines, verapamil and diltiazem, acts as the voltage sensor and the ion-selective pore which is highly selective for Ca^{2+}. The $\alpha_1$ subunit of all known Ca^{2+} channels bears a characteristic structure which has a strong resemblance to other voltage-dependent channels, particularly the Na^{+} channel. That is it has a single polypeptide chain with four repeated units of homology named I-IV respectively, each containing six $\alpha$-helical membrane spanning segments, named S1- S6 respectively. The fourth segment S4 in each repeat contains positively charged residues at every third or fourth position, and it is thought to form part of the voltage-sensor. These structures allow ions across the membrane through the open pore at high rate (>10^6 ions/s) as ions passively flow down their electrochemical gradients. This transfer is both independent of cellular energetics and is stoichiometrically uncoupled, distinguishing the process from other ion transport devices such as the Ca^{2+} pump (Tsien et al 1991).

III.4.2. Channel gating model

Excitable channels have sensors. Voltage-gated channels have a voltage sensor (so voltage-gated channels are also called voltage-sensitive channels): a collection of gating charges that move under the influence of the membrane electric field. Work done to move these gating charges in the field is the free-energy source for gating. The movement of the charges can be measured as tiny gating currents. The ligand-gated channels have receptors; binding sites for the chemical message. The free energy of binding is the free-energy source for opening the channel. The gating of
many channels is modulated through signalling pathways involving phosphorylation and dephosphorylation of certain sites on the cytoplasmic face. Some channels also interact with G-proteins.

Single channels contributing to current flow open abruptly in a step from a nonconducting to a highly conducting form, although the whole cell macroscopic current is graded and continuous. The simplest gating kinetics are described by a state diagram:

\[
\text{closed} \quad \xrightarrow{\text{activation}} \quad \text{open} \quad \xrightarrow{\text{deactivation}} \quad \text{inactivation}
\]

The channel gates switch the channel between activated, deactivated and inactivated states, only the first one is a conductive state when ions pass through the channel pore. In the case of voltage-dependent channels, the gating is controlled by membrane potential. During membrane depolarisation, e.g. positive to -30mV in the case of the L-type Ca\(^{2+}\) channel, the activation gate opens, and Ca\(^{2+}\) flow through the channel as an inward current \(i_{\text{Ca}}\). As membrane depolarisation continues, the inactivation gate operates and current flow is turned off, although the activation gate is still open. When membrane repolarisation occurs, the activation gate is closed and inactivation slowly recovers until the channel can open again. The movements of ions through the channels depend on the value of membrane depolarisation and time after the onset of depolarisation, i.e., gating is not only voltage- but also time-dependent. However actual channel gating is much more complicated. Rather, the open state is the last of a sequence of several gating states or substates, varying from a fully closed state to a fully open configuration (Colquhoun & Hawkes 1982; Katz 1993). The signal has to be passed along each of these state of configuration until eventually the open state is reached and the channel opens.

Many theories have been proposed for the nature of gates. A typical model is the ball-and-chain model developed by Armstrong and colleagues depicting the inactivation of Na\(^{+}\) and K\(^{+}\) channels (Armstrong and Bezanilla 1977; Hille 1992). In this model, it is believed that at the inner mouth of voltage-dependent Na\(^{+}\) and K\(^{+}\) channels, there is
an inactivation gate composed of an amino-acid sequence, which changes after the activation gate opens and blocks the channel. This model has been well tested by molecular biological methods. Deletion of the appropriate cytoplasmic part of a channel, i.e., amino acids 6-46 of the cytoplasmic N-terminal of the A-type K+ channel eliminated fast inactivation. However inactivation was restored in the deletion mutant by perfusing the cytoplasmic face of an outside-out patch with a peptide containing the first 20 residues of the N-terminal of the A-type K+ channel (Hoshi et al 1990; Zagotta and Aldrich 1990; Zagotta et al 1990).

III.4.3. Ionic conductance

Ionic currents are passive fluxes of current down their electrochemical potential gradients. Current flow is zero when the electrochemical potential gradient is zero. The relation between ionic current \( i_i \) and transmembrane potential \( V_m \) is described as

\[
\begin{align*}
i_i &= g_i (V_m - V_i) \\
g &= i_i/(V_m - V_i)
\end{align*}
\]

Where \( V_i \) is the equilibrium potential of one given ion "i" and defined by the Nernst equation; \((V_m - V_i)\) is the driving force on ion "i" (Hille 1992). The conductance \( g \) in equation 1.3, known as chord conductance, is given by \( i_i/(V_m - V_i) \). Differentiating the current-voltage relationship (i-v curve) from equation 1.2, yields values of the slope conductance, \( g \), given by \( di_i/dV_m \). When the i-v curve is linear, the conductance is independent of voltage (or current) and the chord conductance and slope conductance are equal.

In detrusor the predominant ion channels are selective towards either Ca\(^{2+}\) or K\(^+\). Section IV of this chapter describes in more detail different forms of Ca\(^{2+}\) or K\(^+\) channels which may have a role in detrusor smooth muscle.

IV. Contractile activation of detrusor muscle

Initiation of contraction in detrusor smooth muscle is primarily controlled by postganglionic autonomic motor nerves embedded in the muscle mass. In human detrusor from normally functioning bladders these motor nerves are predominantly cholinergic derived from the parasympathetic system. In smooth muscle, the link between membrane activation and mechanical events is a rise of the sarcoplasmic free
Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) which could be achieved by electromechanical coupling and/or pharmacomechanical coupling mechanisms.

A pharmacomechanical coupling mechanism is characteristic of smooth muscle and operates through multiple cellular signalling mechanisms that can change the level of force without necessarily changing the membrane potential (Somlyo and Somlyo, 1968). The major mechanisms of this coupling are: (i) Ca$^{2+}$ release from intracellular stores by 1,4,5-inositoltrisphosphate (IP$_3$) (Berridge and Irvine 1989), following neurotransmitter or agonist binding to specific receptors on the cell membrane, and (ii) modulation of the Ca$^{2+}$ sensitivity of myosin phosphorylation to alter smooth muscle contractility (Somlyo and Somlyo 1994).

Electromechanical coupling operates through changes of membrane potential and their subsequent effects on [Ca$^{2+}$]$_i$. The characteristic of this coupling is that depolarisation of the cell membrane opens voltage-dependent Ca$^{2+}$ channels leading to Ca$^{2+}$ influx and therefore an increase in [Ca$^{2+}$]$_i$. Ca$^{2+}$ influx through voltage-dependent Ca$^{2+}$ channels may also trigger Ca$^{2+}$ induced Ca$^{2+}$ release (CICR) from intracellular stores (Iino 1989).

Investigations show that both the electromechanical and pharmacomechanical coupling processes can be evoked in detrusor muscle. The existence of a dual coupling mechanism adds complexity to the contractile activation in detrusor muscle and implies the importance of a fine balance between the two systems. Modulation of individual components in each of these systems may influence the contractility under both physiological and pathophysiological conditions.

IV.1. Neurotransmission and Pharmacomechanical coupling

IV.1.1. Neurotransmission

Detrusor smooth muscle is innervated by unmyelinated, postganglionic efferent, parasympathetic nerve fibres. The axons form varicosities along their length which contain synaptic vesicles and are possibly the release sites for neurotransmitters (Brading 1987). These motor nerves are primarily cholinergic, particularly in detrusor from human and large mammals (Sibley 1984). However, there is increasing evidence that other active substances also co-exist in these nerve endings (Burnstock 1972, 1981) and may be released as transmitters or cotransmitters. Neurotransmitter binding
to receptors on the cell membrane of the detrusor muscle cells results in initiation of the activation process.

(i). Cholinergic activation

The intrinsic motor nerves in isolated detrusor muscle strips can be stimulated by tetanic pulse trains with short pulse durations (<0.1ms). These selectively excite the nerves since their membrane time constant is much less than that of the smooth muscle, thus depolarising the nerve membrane more rapidly than the detrusor cell membrane. Excitation of these nerves releases a transmitter which will then activate the muscle cell. In detrusor muscle from normally functioning human bladders, the contraction can be effectively abolished by addition of atropine, a muscarinic antagonist (Sibley 1984). Thus the motor nerves are predominantly cholinergic and derived from the parasympathetic system.

Several subtypes of muscarinic receptors exist in detrusor muscle. Although M2 is the most abundant, the functional postsynaptic muscarinic receptor mediating muscle contraction is probably M3 (Wang et al 1995; Harriss et al 1995). It has been shown in bladder smooth muscle that the M3 receptor is coupled with G-proteins, which are probably Gq/11α or related subtypes (Wang et al 1995).

(ii). Non-cholinergic mechanisms

In isolated preparations from many small animals, considerable atropine-resistant contractions are observed, suggesting the existence of other non-cholinergic neurotransmitters (Sibley 1984). Atropine-insensitive contractions have also been observed in some isolated human preparations from abnormally functioning bladders (Palea et al 1993). The atropine-resistance cannot be an artefact due to direct muscle stimulation since the atropine-insensitive component is also TTX-sensitive (Luheshi and Zar 1990). As nerves, but not detrusor smooth muscle, possess fast Na+ channels, blockade of any contraction with TTX implies the involvement of an excitatory nerve.

The non-cholinergic neurotransmitters have not yet been unequivocally identified. The observation that the non-cholinergic nerve-mediated bladder contraction can be abolished by α, β-methylene-ATP (Palea et al 1993) suggests that ATP is an important candidate and that the action is probably mediated by P2x receptors.
IV.1.2. Pharmacomechanical coupling

IV.1.2.1. Receptor-operated cation channels

ATP-gated purinergic P2X receptors found in both human and animal detrusor muscle, are cation channels permeable to both Na\(^+\) and Ca\(^{2+}\) (Schneider et al 1991). Activation of this channel by ATP or its analogue α, β-methylene ATP induces inward current, Ca\(^{2+}\) entry and membrane depolarisation (Inoue and Brading 1990; Schneider et al 1991). In human detrusor muscle cells, ATP is capable of inducing \([\text{Ca}^{2+}]_i\) transients of comparable magnitude and time course to those by carbachol; and the dose-response relationship shows similar half-maximal effective concentrations for both agonists, suggesting a functional role in detrusor muscle cells (Wu et al 1995).

In contrast to the carbachol-induced rise of intracellular Ca\(^{2+}\), ATP-evoked \([\text{Ca}^{2+}]_i\) transients could be attenuated by Ca\(^{2+}\) channel antagonists (Wu et al 1995). This indicates that the ATP-induced rise of intracellular Ca\(^{2+}\) consists of two components, one attributable to Ca\(^{2+}\) entry through the ATP-receptor-gated cation channel, the other attributable to secondary stimulation of the L-type Ca\(^{2+}\) channel, which is due to membrane depolarisation caused by inward cation flow. Whether there is further release of Ca\(^{2+}\) from the SR via a Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism in this ATP-evoked \([\text{Ca}^{2+}]_i\) transients remains to be seen.

IV.1.2.2. Pharmacomechanical coupling

In many smooth muscle types, activation of receptors by excitatory transmitters results in G-protein-coupled stimulation of phospholipase C (PLC) as a primary response. This is followed by hydrolysis of membrane-associated phosphatidyl inositol by the phosphodiesterase action of the enzyme and results in the formation of two second messengers, diacyl glycerol (DAG) and IP\(_3\). The generated IP\(_3\) acts as a receptor agonist on intracellular store sites resulting in Ca\(^{2+}\) release and a transient rise of \([\text{Ca}^{2+}]_i\). Thus, there are two pathways for increasing the cytosolic \([\text{Ca}^{2+}]_i\): IP\(_3\)-induced release from intracellular store sites and stimulated Ca\(^{2+}\) entry from the extracellular space.

The mechanisms underlying the pharmacomechanical coupling process in detrusor muscle has been partly clarified following the discovery of the role of the second
messenger IP3 in vascular smooth muscle (Berridge and Irvine 1984, 1989). Application of acetylcholine, or its analogue carbachol, to isolated detrusor muscle can elicit a contraction or a transient rise of the intracellular Ca\(^{2+}\); the half-maximal effective concentration of carbachol is about 1\(\mu\)M (Kishii et al 1992; Wu et al 1995). Both the contraction and the rise of intracellular Ca\(^{2+}\) by application of cholinergic agonists can be blocked by atropine, suggesting an action mediated via muscarinic receptors. Carbachol can also induce the rise of intracellular Ca\(^{2+}\) in Ca\(^{2+}\)-free superfusates (Wu and Fry 1998), suggesting the source of Ca\(^{2+}\) comes from intracellular sites. The \([\text{Ca}^{2+}]_i\) transients exhibited an undershoot following relaxation, similar to that evoked by caffeine (Wu et al 1998), which was explained by a release and re-uptake of Ca\(^{2+}\) by the SR (Baro et al 1993). Of the functional muscarinic receptor subtypes identified in smooth muscle, the M3 subtype is of physiological importance in detrusor muscle, and is coupled to the hydrolysis of phosphoinositides in the surface membrane and inositol phosphate turnover (Yang et al 1991a).

The evidence for an IP3-mediated pathway in detrusor smooth muscle has been found from experiments which measured tension and inositol phosphate production upon application of carbachol (Iacovou et al 1990). Both tension generation and inositol phosphate production occurred over a similar range of carbachol concentrations providing strong evidence for a role of IP3 in carbachol-mediated force generation. In addition it was shown that muscarinic antagonists inhibited both phosphoinositide (PI) turnover and detrusor muscle contraction and the decrease in contraction was significantly correlated to PI reduction (Noronha Blob et al 1989). More recent work has determined that carbachol produced a half-maximal stimulation of inositol phosphates at 8.3\(\mu\)M in cultured human detrusor myocytes (Harriss et al 1995). This potency is similar to those found in other smooth muscles where IP3 pathways have been more established (Chilvers et al 1994). Furthermore, production of inositol phosphates in this preparation is linked to stimulation of the muscarinic M3 receptor, as M3 receptor antagonists significantly inhibited the response generated by submaximal carbachol concentrations (Harriss et al 1995). A study has also shown that \(^{45}\text{Ca}^{2+}\) efflux from permeabilized cultured detrusor cells is stimulated by incubation with IP3 which suggests a role for IP3 as a messenger for triggering intracellular Ca\(^{2+}\) release (Chambers et al 1996). Direct evidence for triggering of Ca\(^{2+}\) release from the SR by IP3 and the blocking effect by heparin in carbachol-induced Ca\(^{2+}\) release in adult detrusor cells awaits further investigation.

An agonist-induced increase in the Ca\(^{2+}\)-sensitivity of the contractile proteins has been observed as a separate mechanism for pharmacomechanical coupling in some
smooth muscles (Sato et al 1994). The evidence for such a mechanism in detrusor is lacking at present.

IV.2. Electrophysiological activity and Electromechanical coupling

IV.2.1. Electrophysiological activity

IV.2.1.1. Resting membrane potentials

Most of the electrophysiological information on detrusor muscle has been obtained from several animal species, such as rabbits and guinea-pigs (Creed et al 1983; Mostwin 1986; Brading and Mostwin 1989). The resting potential measured from detrusor muscle strips using microelectrodes is around -40mV (Creed 1971; Mostwin 1986). Direct measurement of membrane potential in isolated guinea-pig detrusor smooth muscle cells shows that these cells have a more negative resting potential of -52±2mV (Klockner and Isenberg 1985a). The first intracellular recording on isolated human detrusor myocytes yielded a resting potential of -47±17 mV (Montgomery and Fry 1992).

IV.2.1.2. Action potentials

Detrusor muscle can generate action potentials when electrically stimulated (Creed 1971; Mostwin 1986). In guinea-pig muscle strips superfused in isotonic or hypertonic Krebs solutions containing 2.5mM CaCl₂, microelectrode recording showed that the electrically-elicited action potential had an upstroke and a downstroke, each lasting 10 ms (Mostwin 1986). The rate of rise was 2-4V/s and there was no plateau phase as seen in cardiac and ureteral smooth muscle. Most action potentials exhibited an overshoot of 15-20mV. In isolated single guinea-pig cells superfused with a physiological solution containing 3.6mM CaCl₂, using patch-type electrodes, single action potentials, generated by stimuli, rise from a threshold of about -38mV, with a maximum depolarisation rate of 6.5V/s to an overshoot of 22±3mV. The action potentials lasted for 36±4ms, measured between threshold and repolarisation to -40mV. Continuous depolarising current produced repetitive activity, a pacemaker depolarisation followed the action potential and preceded the next upstroke (Klockner and Isenberg 1985a).
Action potentials were less easy to elicit using K+-filled electrodes compared to Cs+-filled electrodes, because a large or rapidly activating outward current would attenuate any inward or depolarising current. Using Cs+-filled electrodes, the threshold shifted to less depolarised values and the repolarisation phase was significantly prolonged.
It is also possible to elicit electrically action potentials in human detrusor smooth muscle cells (Montgomery and Fry, 1992). Using patch-type electrodes, filled with a high K\textsuperscript+ intracellular medium, data recorded from two cells gave a threshold potential between -22 and -30mV, an upstroke velocity of 2.1-3.2V/s, and an overshoot potential of 0-20mV. Action potentials were not easy to elicit using K\textsuperscript+-filled electrodes probably because a large or rapidly activating outward current would damp any inward or depolarising current. Action potentials could be more easily recorded using Cs\textsuperscript+-filled electrodes. Under this condition, the threshold shifted to less depolarised values and the repolarisation phase was significantly prolonged. Action potentials could not be recorded in the presence of 5mM NiCl\textsubscript{2}, which blocked the L-type Ca\textsuperscript{2+} channel.

Spontaneous action potentials can also be recorded in human detrusor muscle cells (Wu et al 1997). Whether the generation of spontaneous action potentials is related to spontaneous contractile activity in human bladder (Kinder and Mundy 1987) remains to be answered.

IV.2.1.3. Ion channels in detrusor muscle

IV.2.1.3.1. Voltage-dependent Ca\textsuperscript{2+} channels

Voltage-dependent Ca\textsuperscript{2+} channels regulate Ca\textsuperscript{2+} entry and thereby contribute to Ca\textsuperscript{2+} signalling in many cells. The critical role of Ca\textsuperscript{2+} in the contractile process has been established in skeletal, cardiac and smooth muscle. Ca\textsuperscript{2+} also plays a crucial role in secretory responses and is a widespread second messenger in cells (Hille 1992). Functional studies have uncovered several types of Ca\textsuperscript{2+} channels, such as high-voltage-activated (HVA) L-, N-, P-, Q-, R-type and low-voltage-activated(LVA) T-type channels. They are distinguished by their activation and inactivation kinetics, single channel conductance, their sensitivity to drugs and toxins, and their cellular distribution (Tsien et al 1991; Spedding and Paoletti 1992).

It is clear that a primary subunit α1 is an invariable subunit for both low-voltage-activated and high-voltage-activated Ca\textsuperscript{2+} channels; and auxiliary β, α2, δ and/or γ subunits for high-voltage-activated Ca\textsuperscript{2+} channels (Jones 1998; Walker and Waard 1998; Perez-Reyes 1998). The α1 subunit is responsible for pore-formation, voltage-sensing and drug/toxin binding (see section III.4.1, this chapter). The auxiliary subunits dramatically influence the properties and surface expression of Ca\textsuperscript{2+} channels (Walker and Waard 1998). Upon co-expression with the α1 subunit, for
example, β subunits regulate Ca²⁺ channel properties including facilitation of activation and deactivation in response to voltage and modulation of inactivation by voltage. L-type and non-L-type channels have different responses to voltage which are affected by β subunits, therefore the effects of β subunits vary somewhat between these two type channels (Singer et al 1991; Birnbaumer et al 1998).

(i). L-type Ca²⁺ channels
L-type (long lasting) Ca²⁺ channels are activated at relatively depolarised voltages and inactivate slowly. They are distributed in all excitable cells, particularly in heart and smooth muscle, and also many non-excitable cells. L-type Ca²⁺ channels occur in different isoforms of L1, 2, 3, 4, respectively, from heart, brain, skeletal muscle and smooth muscle. The channels have a large unitary conductance of 25pS, and are very sensitive to Cd²⁺, but less sensitive to Ni²⁺. They are a major pathway for Ca²⁺ entry in heart and smooth muscle, and help control transmitter release from endocrine cells and some neuronal preparations.

(ii). T-type Ca²⁺ channels
T-type (transient) Ca²⁺ channels have very different electrophysiological characteristics from L-type channels. T-type Ca²⁺ channels require a smaller depolarisation (~70mV, Hille 1992) for activation (LVA, low-voltage-activated) and are rapidly inactivated. In contrast to L-type channels, T-type Ca²⁺ channels have a small single channel conductance (8pS). They are DHP and Cd²⁺ resistant, but Ni²⁺ sensitive. They are located in a wide variety of excitable and non-excitable cells, especially concentrated in the sinoatrial node, atrioventricular node and specialised conducting tissue of the heart, smooth muscle cells and neurones, where they may be important for pacemaker activity, responsible for burst firing or Ca²⁺ entry at negative membrane potentials (Carbone and Lux 1984; Bean 1985; Nilius et al 1985; Tseng and Boyden 1989; Loirand et al 1989; White et al 1989).

(iii). N-type Ca²⁺ channels
N-type (neuronal) channels are also "high-voltage-activated" (> -20mV) but the inactivation rate is variable. They differ pharmacologically from L-type channels in being resistant to dihydropyridines and largely blocked by ω-conotoxin (ω-CTx-GVIA); the single channel conductance is 13pS. They are largely restricted to neurons and play a role in mediating transmitter release.
It has been shown... Nakayama and Brading (1993) demonstrated that L-type Ca\textsuperscript{2+} channels in guinea-pig detrusor have two available open states, since two distinct nifedipine-sensitive inward currents were recorded. One, which rapidly deactivated and was activated by simple step depolarisation, showed a similar voltage dependence to that reported by Klockner & Isenberg (1985 b); another deactivated more slowly and was recorded as tail currents when repolarizing from positive potentials, with an amplitude related to the size of the depolarising step.
(iv). P-type Ca\(^{2+}\) channels

P-type (Purkinje) channels are high-voltage-activated (> -50mV) with very slow inactivation and particularly located in cerebellar Purkinje cells and some peripheral neurons. P-type channels can be potently blocked by FTX (funnel-web spider toxin, Llinas et al 1992) and ω-agatoxin-IVA (ω-Aga-IVA). These channels may be responsible for neurotransmitter release in certain circumstances (Llinas et al 1992).

(v). Q- and R-type Ca\(^{2+}\) channels

Q- and R-type channels are high-voltage-activated and located in cerebellar granule neurons, brain, hippocampal CA3 and CA1 neurons. They are resistant to blockade by ω-CTx-GVIA, nimodipine, and ω-Aga-IVA at concentrations sufficient to eliminate N-, L-, or P-type channels, respectively. The Q-type channels are completely blocked by 1.5 µM ω-CTx-MVIIC and largely suppressed by ω-Aga-IVA at 1µM, a concentration 100 to 1000 times that needed to block P-type channels. In contrast, R-type channels are little affected by either of these treatments. Q- and R-type Ca\(^{2+}\) channels may cooperate with other type Ca\(^{2+}\) channels to trigger neurotransmitter release under physiological conditions (Wheeler et al 1994).

IV.2.1.3.1.1. Voltage-dependent Ca\(^{2+}\) channels in detrusor muscle

Voltage-clamp experiments in single cells from detrusor smooth muscle have revealed a large inward Ca\(^{2+}\) current similar to L-type channels in other tissue. Inward current was activated at a membrane potential about -40mV, with a rapid time-dependent inactivation and sensitive to nifedipine and verapamil. Whole-cell current measured with Cs\(^{+}\)-filled patch electrode was about 500pA in guinea-pig detrusor cells, with a current density of 10pA/pF with extracellular CaCl\(_2\) 1.8mM (Wu and Fry 1998). Corresponding values of about 200pA (100-300pA) and 4pA/pF have been found in human preparations (Montgomery and Fry 1992; Gallegos and Fry 1994). It has been shown that the L-type Ca\(^{2+}\) channels in detrusor have two open states, one which rapidly deactivates at normal membrane potentials; and one which deactivates much slowly at very positive potentials (Nakayama and Brading 1993a, 1995).
IV.2.1.3.2. Voltage-dependent transient outward K+ channels

Outward currents, recorded under whole-cell voltage-clamp, usually exhibit two distinct components: a transient and a steady-state component. The transient outward current has very fast activation and inactivation time courses with Ca\(^{2+}\)-independent and Ca\(^{2+}\)-dependent fractions, such as A-current (\(i_{\text{KA}}\)) and Ca\(^{2+}\)-dependent K\(^{+}\) current (\(i_{\text{KCa}}\)).

(i). A-current (\(i_{\text{KA}}\))

The A-current is a fast transient outward K\(^{+}\) current, potently blocked by 4-aminopyridine (4-AP) but less sensitive to TEA. This \(i_{\text{KA}}\) channel can be activated when a cell is depolarised after a period of hyperpolarisation. The \(i_{\text{KA}}\) component can be separated from the total outward current during a voltage clamp step by manipulating the holding potential. The voltage dependence of inactivation is steep, with a \(V_{1/2}\) near -70mV and falling almost to zero at -40mV. The activation is also steep and occurs at potentials more positive than -65mV. Therefore \(i_{\text{KA}}\) conducts only within a narrow window of negative potentials between -65 and -40mV. The inactivation of \(i_{\text{KA}}\) is gradually removed as the cell is hyperpolarised. \(i_{\text{KA}}\) is distributed in a wide variety of tissues, including neurons, myocardium and smooth muscle cells. It is involved in maintaining the resting membrane potential and modulates the onset of the action potential (Hille 1992; Smirnov et al 1992; Akbarali et al 1995).

(ii). Ca\(^{2+}\) dependent K\(^{+}\) current (\(i_{\text{KCa}}\))

\(i_{\text{KCa}}\) is a K\(^{+}\) current activated when the cytoplasmic [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_{i}\)) increases. The channels are distributed in a wide variety of both electrically excitable and non-excitable cells. There are several distinct subtypes comprising this class of K\(^{+}\)-channel, and they differ in their single channel conductance, Ca\(^{2+}\) sensitivity, voltage-dependence and pharmacological properties. The most common distinguishing feature among this group is the unitary conductance of the channel.

The physiological kinetics of \(i_{\text{KCa}}\) channels in different cells is determined by a complex blend of the kinetics of Ca\(^{2+}\) entry; Ca\(^{2+}\) buffering and diffusion; Ca\(^{2+}\) extrusion and uptake into intracellular organelles; Ca\(^{2+}\) binding to channels; and voltage-dependent transitions. The response would always be a repolarisation or a hyperpolarisation of the cell when the internal free Ca\(^{2+}\) rises above a certain level (Garcia and Kaczorowski 1992; Hille 1992).
BKCa channels

BKCa channels have a large conductance (unitary conductance, 100-250pS), can be dissected from SKCa by specific toxins: charybdotoxin (10-100nM range in guinea-pig bladder and coli), and iberiotoxin (10-100nM range in guinea-pig bladder) block BKCa, as do low concentrations of external TEA (0.3-3.0mM in guinea-pig bladder, Garcia and Kaczorowski 1992).

BKCa acquires its voltage-dependence from the voltage-dependence of Ca2+ entry, but it also has a voltage sensitivity of its own, since at a fixed level of [Ca2+]i, BKCa is activated upon depolarisation. The gating kinetics of BKCa channels are remarkably complex. Binding of Ca2+ on the inner surface of the channel shifts the voltage dependency of gating and allows the channel to open at more negative membrane potentials. The shut-open transition of the channel gates requires numerous kinetics states, including some with two and three Ca2+ ions bound to achieve a high probability of opening. For optimal opening, the channel must have three bound Ca2+.

BKCa channels are found in neurons, striated muscle, kidney tubules, the choroid plexus, blood cells such as platelets and B-lymphocytes, in endocrine and exocrine glands and particularly are abundant in many types of smooth muscle. Therefore BKCa functions to repolarise cells following membrane depolarisation and elevation of intracellular Ca2+, resulting in regulation of neuroendocrine secretion, in control of muscle contractility and in a variety of other diverse cellular processes. In quiescent tissue such as aorta, BKCa helps to maintain the cellular resting potential. Thus, BKCa can affect excitation-contraction (e-c) coupling responses in smooth muscle in a tissue-specific fashion (Marty 1989; Garcia and Kaczorowski 1992; Haylett and Jenkinson 1990).

BKCa channels are targets of pharmaceutical therapy for muscle relaxation. BKCa channel openers include: NS004 (5-trifluoromethyl-(5-chloro-2-hydroxyphenyl)-1,3-dihydro-2H-benzimidazole-2-one) and its analog, NS1608 (N-(3-(trifluoromethyl)phenyl)-N'-(2-hydroxy-5-chlorophenyl)urea) (Hu and Kim 1996).

(iib). SKCa channels

SKCa channels have a small unitary conductance (4-14pS). Apamin is a specific and potent blocker of the SKCa, and therefore it has become a valuable tool in identifying the presence of SKCa. SKCa is resistant to external TEA. The opening of SKCa has little voltage dependence, and it controls the action potential firing rate leading to afterhyperpolarisation (AHP). In intestinal smooth muscle its activation underlies the
inhibitory action of α1-adrenoceptors, and of the receptors for neurotensin and ATP (P2-subtype) (Haylett and Jenkinson 1990).

(iic). Intermediate K\textsubscript{Ca}

The intermediate K\textsubscript{Ca} has a unitary conductance of 18-50pS, with no specific blocker. The intermediate K\textsubscript{Ca} is found in red blood cells and Aplysia neurones (unitary conductance of 40 and 35 pS respectively), and can be blocked by charybdotoxin but not by apamin (Garcia and Kaczorowski 1992). Similar channels may be present in lymphocytes and macrophages.

The channels found in red blood cells are voltage insensitive. The prime stimulus for channel opening is an increase of [Ca\textsuperscript{2+}]\textsubscript{i}. In addition to Ca\textsuperscript{2+} the channels are also activated by Sr\textsuperscript{2+} and Pb\textsuperscript{2+}, but Mg\textsuperscript{2+} inhibits channel opening. Open channels do not show inactivation. The channel opening is modulated by the extracellular [K\textsuperscript{+}] and intracellular pH affects their permeability. Their function includes volume regulation in red blood cells.

The channels found in Aplysia neurones are voltage sensitive, that is the proportion of channels that are open at a given Ca\textsuperscript{2+} concentration increases with depolarisation. Their physiological role is thought to lie in the control of the bursts of action potentials that characterise the activity of these neurones. The channel permeability sequence for monovalent cations is Tl\textsuperscript{+}>K\textsuperscript{+}>Rb\textsuperscript{+}>>NH\textsubscript{4}\textsuperscript{+}>Cs\textsuperscript{+}>Li\textsuperscript{+}, Na\textsuperscript{+}. This is very similar to that of the BK\textsubscript{Ca} channel, and also of the delayed rectifier in various tissues (Haylett and Jenkinson 1990).

IV.2.1.3.2.1. Voltage-dependent transient outward K\textsuperscript{+} channels in detrusor muscle

Voltage-clamp has also demonstrated that macroscopic outward currents in detrusor muscle have a transient outward current following Ca\textsuperscript{2+} entry through Ca\textsuperscript{2+}-channels on the cell membrane, and a steady-state outward-rectifier, activated by membrane depolarisation (Klockner and Isenberg 1985a; Montgomery and Fry 1992).

(i). Ca\textsuperscript{2+}-dependent outward K\textsuperscript{+} current.

There is evidence that detrusor smooth muscle may have Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels: whole cell currents from guinea-pig bladder myocytes exhibit a large transient outward current after I\textsubscript{Ca} activation. The i-v curves of the peak outward current followed an N-shaped relation (Klockner and Isenberg 1985a). The open channel
probability with inside-out patches was dependent on $[\text{Ca}^{2+}]_i$ (Markwardt and Isenberg, 1992); and the BK$_{\text{Ca}}$ opener NS004 relaxed guinea-pig bladder strips precontracted with 15mM KCl, an effect which was inhibited by iberiotoxin (Trivedi et al 1995), suggesting that there may be a Ca$^{2+}$-activated BK$_{\text{Ca}}$. There may also exist apamin-sensitive SK$_{\text{Ca}}$ since after-hyperpolarisations, probably attributed to activation of Ca$^{2+}$-dependent K$^+$ channels, can be abolished by the specific blocker apamin (Creed et al 1983).

However, their presence has not demonstrated in human detrusor, nor the relative importance of the different channels in any detrusor specimens.

ik$_{\text{Ca}}$ is of physiological interest as a membrane depolarisation results in Ca$^{2+}$ influx through the L-type Ca$^{2+}$ channels, which then would activate ik$_{\text{Ca}}$. This would repolarise the cell membrane, and therefore reduce the voltage-dependent Ca$^{2+}$ entry. This would prevent excessive rises of intracellular Ca$^{2+}$ and presumably could diminish contractile activation. Therefore outward K$^+$ currents in detrusor muscle play an important role in maintaining the resting potentials, and K$^+$ channels are promising targets for pharmaceutical therapy in the clinical treatment of UDC.

IV.2.1.3.3. Steady-state outward K$^+$ channels: ik$_{\text{ATP}}$ and iK

IV.2.1.3.3.1. K$_{\text{ATP}}$ channels in detrusor

K$_{\text{ATP}}$ channels were first described in cardiac muscle (Noma 1983), then in skeletal muscle, pancreatic β-cells, central neurons and smooth muscles (Spruce et al 1985; Cook and Hales 1984; Ashford et al 1988, 1989; Standen et al 1989). They form a family of channels with differences to some extent from tissue to tissue in properties such as ATP sensitivity. In addition, the channel density of K$_{\text{ATP}}$ varies in different tissues. A characteristic of K$_{\text{ATP}}$ channels is that their open-state probability is reduced by an increase in intracellular concentration of adenosine-5'-triphosphate ([ATP]$_i$) to millimolar levels. The channel activity is affected by ATP, GTP, etc., and their sensitivity to [ATP]$_i$ is also modulated by intracellular pH. The sulfonylurea anti-diabetic compounds tolbutamide and glibenclamide block K$_{\text{ATP}}$ channels and they are opened by cromakalim.

Both human and guinea-pig detrusor possess K$_{\text{ATP}}$ channels (Bonev and Nelson 1993; Wammack et al 1994), and an ATP-dependent K$^+$ current (ik$_{\text{ATP}}$) has been
dissected from steady-state outward current. The channels were fully opened after cells from guinea-pig detrusor muscle were dialysed with an electrode filling solution with ATP omitted. Using intracellular filling solution containing 5mM ATP, it has been demonstrated that these channels could be opened by levromakalim (BRL38227), a $K_{ATP}$ channel opener, and blocked by glibenclamide (Bonev and Nelson 1993). In muscle strips from both guinea-pig and human bladders levromakalim reduced the contraction in a dose-dependent manner (Sui et al 1995).

This repolarising current will make the muscle less excitable, limiting $Ca^{2+}$ influx, contractile activation and excessive consumption of ATP. Therefore these channels together with $Ca^{2+}$-dependent outward $K^+$ currents are the targets of $K^+$ channel openers aiming at providing a therapeutic avenue of controlling abnormal detrusor contractility such as occurs in bladder instability (Nurse et al 1991). However their use is limited so far as they also reduce blood pressure by dilating arterial resistance vessels.

**IV.2.1.3.3.2. Delayed rectifier $K^+$ channels ($i_K$) in detrusor**

Delayed rectifier $K^+$ current, $i_K$, is an important $K^+$ current in many cells. It is activated by membrane depolarisation, not activated by a rise in the internal $[Ca^{2+}]$ and sufficiently distinct from the transient outward A-current. It is widely distributed, with diverse kinetics, voltage-dependence and pharmacology; and may or may not inactivate with time (Rudy 1988; Bolton and Beech 1992).

A $K^+$ current recorded from both human and guinea-pig detrusor smooth muscle has the characteristics of a delayed rectifier (Klockner and Isenberg 1985a; Montgomery and Fry 1992). Using K-filled electrodes, the current recorded using K-filled electrodes is activated as the membrane is depolarised over -30mV, with a slow inactivation (Montgomery and Fry 1992). Extracellular TEA (20mM) largely reduced but did not block it. However, the use of Cs-filled electrodes blocked $i_K$ within 2 minutes (Klockner and Isenberg 1985a,b). Further characterisation of this current is required under the condition of blockade of ATP-dependent and $Ca^{2+}$-dependent $K^+$ channels.
IV.2.1.3.4. Other currents: iClCa and stretch-activated cation channels

(i) Ca2+-activated Cl− current (iClCa)

iClCa is a Ca2+-activated Cl− current which has been demonstrated in a variety of tissue types, such as exocrine and secretory cells, neuronal tissue, as well as skeletal, cardiac and smooth muscle cells. The rise of [Ca2+]i due to Ca2+ release from the sarcoplasmic reticulum (SR) or Ca2+ entry through voltage-dependent Ca2+ channels, is the essential trigger for activation of iClCa in both smooth muscle and non-smooth muscle cells. The unitary conductance of iClCa is small (<10pS) and may be less sensitive than iKCa to [Ca2+]i. In general, there is a lack of selectivity of Cl− channel blockers. All compounds which act as inhibitors of several different types of Cl− current are active. The agents that have been most used in smooth muscle are: anthracene-9-carboxylic acid (9-A-C); the stilbene derivatives 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS), 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), and niflumic acid (Large and Wang 1996).

When using depolarising pulses to produce Ca2+ entry, activated iClCa, induced in this manner is usually referred to as a "tail current". In experiments on the rat anococcygeus muscle in K+-free conditions, depolarising voltage steps from the holding potential of -50mV to more positive test potentials during activation of iClCa produced an instantaneous current that was followed by relaxation to an equilibrium current level (Large and Wang 1996). The current relaxation is outward when the test potential is positive to the reversal potential of iClCa (Cl− in). When the test potential is negative to the reversal potential of iClCa, the iClCa relaxation is inward (the spontaneous transient inward current, STIC). iClCa then leads to membrane depolarisation and opens voltage-dependent Ca2+ channels, resulting in an influx of Ca2+ to produce contraction and secretory processes.

There has been no reports of iClCa in detrusor smooth muscle.

(ii) Stretch-activated cation channels (SACs)

In guinea-pig detrusor muscle there may exist also stretch-activated cation channels which open by physical stretch of the cell membrane. Non-selective cation currents were recorded when the detrusor cell was longitudinally stretched. Ca2+ influx through stretch-activated non-selective cation channels results in Ca2+ activation of
BK channels. Activity of SACs is modulated by cAMP (Wellner and Isenberg 1994; Wellner and Isenberg 1995).

IV.2.2. Electromechanical coupling

In cardiac and skeletal muscles, electrical excitation of the cell membrane induces depolarisation of the triad at the junctional area between the transverse tubular structure (T-system) and SR in the case of skeletal muscle, or activates the L-type Ca$^{2+}$ channel in the case of cardiac muscle. Both trigger release of Ca$^{2+}$ from the SR, and the subsequent rise of cytoplasmic Ca$^{2+}$ activates the contractile proteins following the formation of a Ca$^{2+}$-troponin C complex and hydrolysis of ATP by myosin ATPase. These processes finally cause cyclic conformational changes in the actin-myosin interaction and lead to force generation. Thus excitation-contraction crucially involves the mobilisation of Ca$^{2+}$ from the SR.

In smooth muscles such as detrusor muscle, there are differences in the triggering mechanisms of contraction compared with striated muscles. The T-system found in cardiac and skeletal muscle is not present in smooth muscle and troponin is also absent. Ca$^{2+}$ required for activation of the contractile proteins acts as a repressor of the actin-myosin interaction in striated muscle, but in smooth muscle Ca$^{2+}$ binds to calmodulin and this Ca$^{2+}$-calmodulin binding triggers activation of calmodulin-dependent myosin light chain kinase. This catalyses the phosphorylation of the myosin light chain and leads to the interaction between actin and myosin, and force generation. This means that although the final goal of the excitation-contraction coupling is the same, i.e. Ca$^{2+}$ mobilisation and muscle contraction, the coupling process between Ca$^{2+}$ and the contractile proteins are different in striated and in smooth muscles.

IV.2.2.1. Electromechanical coupling in detrusor muscle

Electromechanical coupling operates in detrusor muscle through changes to the surface membrane potential resulting in Ca$^{2+}$ entry and their effects on intracellular Ca$^{2+}$, including Ca$^{2+}$-induced Ca$^{2+}$ release.
(i). Role of the L-type Ca$^{2+}$ channel

Contractile activity can be accompanied by action potentials in detrusor muscle (Brading and Mostwin 1989) where the L-type Ca$^{2+}$ channel plays a crucial role. Inotropic interventions which manipulate the L-type Ca$^{2+}$ current cause a proportional increase or decrease of tension and a close linear dependence of these two variables was demonstrated (Fry et al 1995).

The relationship between the Ca$^{2+}$ current (i$_{Ca}$) and [Ca$^{2+}]_{i}$ has been studied in detrusor muscle cells by simultaneous recording of ionic current and [Ca$^{2+}]_{i}$ using fluorescent Ca$^{2+}$ indicators under voltage-clamp (Ganitkevich and Isenberg 1991; Wu and Fry 1998). The rise of intracellular Ca$^{2+}$ followed the inward Ca$^{2+}$ current. The magnitude of the [Ca$^{2+}]_{i}$ transient was correlated to that of peak i$_{Ca}$. Both the [Ca$^{2+}]_{i}$ transient and peak i$_{Ca}$ were voltage-dependent and bell-shaped. These mirror images suggested that Ca$^{2+}$ current flow through voltage-dependent L-type Ca$^{2+}$ channels caused the intracellular Ca$^{2+}$ to rise. The Ca$^{2+}$ channel antagonist verapamil completely abolished both the Ca$^{2+}$ current and the [Ca$^{2+}]_{i}$ transient. These observations support the idea that the rise of [Ca$^{2+}]_{i}$ is controlled by Ca$^{2+}$ influx through the L-type Ca$^{2+}$ channels.

(ii). Ca$^{2+}$-induced Ca$^{2+}$ release

The Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) mechanism involves Ca$^{2+}$ release from the SR through the Ca$^{2+}$ release channel, the ryanodine receptors, triggered by a rise of cytoplasmic Ca$^{2+}$ (Fabiato 1985a,b,c; Callewaert 1992). In detrusor muscle, Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels may also trigger further release of Ca$^{2+}$ from the SR (Ganitkevich and Isenberg 1992; Wu and Fry 1998). The magnitude of the depolarisation-induced transient [Ca$^{2+}]_{i}$ was attenuated by ryanodine applied via the pipette, or extracellular application of high concentrations of ryanodine, to block the ryanodine receptor. Manipulation of the SR Ca$^{2+}$ store by using thapsigargin, an inhibitor of Ca$^{2+}$-ATPase on the SR membrane, or caffeine to the superfusate, also attenuated the [Ca$^{2+}]_{i}$ transient.
IV.3. Interaction between different pathways

The interaction between different activation pathways in the signal transduction process forms the basis for the fine tuning of the regulation of cell functions as described in other smooth muscle types (Somlyo and Somlyo 1994). Possible interactions between second messenger systems and electrophysiological pathways are an area of interest for future research.

The voltage-gated ion channels may be modulated by activation of second messenger systems following transmitter-receptor activation. For example, the Ca$^{2+}$ current activated by depolarisation under voltage-clamp can be attenuated by muscarinic agonists, due to channel inactivation by raised [Ca$^{2+}$], constituting a negative feedback mechanism in detrusor cell function. However, the influence of second messenger mobilisation on the voltage-dependent membrane events under normal physiological conditions is less clear. Many types of smooth muscle have non-specific cation channels as well as iCl$_{Ca}$ and iK$_{Ca}$, which are activated by a rise of intracellular Ca$^{2+}$ and result in a change to the membrane potential, and hence regulation of other voltage-gated ion channels. In intestinal smooth muscle cells, activation of muscarinic receptors is able to induce inward current through non-specific cation channels, mediated by a raised [Ca$^{2+}$]. This depolarises the cell membrane and further activates the L-type Ca$^{2+}$ channels (Benham et al 1985; Cousins et al 1993; Ito et al 1993). A consequence of such interaction is hyperactivity of the cell, due to the transient inward current in response to a rise of intracellular Ca$^{2+}$. Preliminary observations have shown that membrane depolarisation could occur following a carbachol-induced rise of intracellular Ca$^{2+}$ in a proportion of detrusor cells (Wu and Fry 1997), but the presence of such a positive feedback in detrusor muscle needs to be confirmed.

The possible role of electrophysiological events in second messenger pathways is another aspect of interaction. The role of the L-type Ca$^{2+}$ channel in the cholinergic activation process itself is not clear. There is evidence that the L-type Ca$^{2+}$ channel may have an important role in the refilling of the SR Ca$^{2+}$ store, since the Ca$^{2+}$ antagonist verapamil significantly depresses the refilling of Ca$^{2+}$ stores which had been depleted by carbachol (Wu and Fry 1997); and refilling is related to the value of the membrane potential (Wu et al 1998).
It is well known that cultured smooth muscle cells undergo spontaneous modulations in cell phenotype and ultrastructure, including their morphological, immunobiochemical characteristics, receptor density and ion channels (Campbell, Campbell and Ross, 1979; Groschel-Stewart et al 1975; Gown et al 1988; Yang et al 1991b; Richard et al 1992 and Snetkov, Hirst and Ward 1996). It is now becoming clear that in many cell types the phenotypic state influences the properties of ion currents. For example, Richard, Neveu, Carnac, Bodin, Travo and Nargeot (1992) have reported differential expression of two Ca^{2+}-channels associated with different proliferative phenotypes in cultured rat aortic smooth muscle cells. Snetkov, Hirst and Ward (1996) have displayed differential distribution of K^{+} channels in freshly isolated, proliferating cultured and growth-arrested human bronchial smooth muscle cells.
V. Smooth muscle cell culture

(i) Smooth muscle cell in culture
Descriptive reports appeared in the literature on cultured smooth muscle which can be traced back to the early years of this century. Studies on cultured smooth muscle cells have involved in several different tissues. Chamley-Campbell, Campbell and Ross (1979) summarized the then current knowledge of both vascular and visceral smooth muscle (vas deferens, taenia coli, etc.) culture: such as the methods of culture; morphology of contractile smooth muscle cells in culture and distinction from fibroblasts and endothelial cells; factors influencing smooth muscle phenotype and proliferation; synthesis of connective tissue; lipid uptake, synthesis and metabolism; innervation of smooth muscle in culture; functional neuromuscular junctions and electrical activity in cultured cells.

Smooth muscle culture has been widely used for different experimental purposes. In particular, vascular smooth muscle cell culture has become an experimental model for normal vascular development and atherosclerotic lesions (Ross 1971; Owens 1995); and human airway smooth muscle in cell culture provides a novel preparation for investigation of the physiology of the human airway and respiratory disease (Twort and Breemen 1988).

(ii) Detrusor smooth muscle cell in culture
Detrusor smooth muscle culture has been developed from both human and mammal in recent years (Harriss 1995; Lau and Chacko 1996). The effect of muscarinic receptor agonists and antagonists on the accumulation of inositol phosphates indicates that human detrusor smooth muscle cells in culture express M3 muscarinic receptors which are linked to phosphoinositide hydrolysis (Harriss et al 1995). Carbachol which induced a transient rise of intracellular Ca^{2+} in cultured human detrusor smooth muscle cells gave further evidence that functional muscarinic receptors are expressed in cultured detrusor muscle cells (Chambers et al 1996). However, whether or not the membrane electrophysiological properties are retained after culture has not been characterised.

VI. Hypothesis

Uncontrolled contraction of the detrusor is a major factor in bladder instability, but the cellular mechanisms remain unknown. To investigate the cellular causes of the
unstable contraction it is necessary to study the cell physiology of detrusor smooth muscle and how it may change with disease.

Although some studies have been performed on mammalian detrusor muscle, there have been no systematic investigations of detrusor smooth muscle electrophysiological properties. Furthermore, elucidation of the mechanisms underlying bladder instability using animal models relies on how similar animal models are to human detrusor, about which there is also little information. Several questions are raised:

1. There is evidence that both pharmacomechanical and electromechanical coupling co-exist in bladder smooth muscle. Spontaneous action potentials occur in unstable bladder significantly more than in normal human bladder (Wu et al 1997), suggesting that electromechanical coupling might be deranged in the unstable bladder. It is therefore important to determine the electrophysiological properties in normal bladder in order to reveal the mechanism underlying detrusor instability.

2. What are the electrophysiological passive and active properties of detrusor smooth muscle and the ionic channels which confer these properties? Are there any differences between human and animal tissue?

3. Are cultured cells excitable? What are the membrane electrical properties after long term culture?

4. Are the electrophysiological properties of passaged cells the same as primary cultured cells?

5. Are cultured cells comparable with fresh tissue? Is there any change following culture? If so, what causes it?

VII. Aims and objectives of the study

This study aims to characterise the electrophysiological properties of freshly isolated adult and cultured detrusor smooth muscle cells from human and guinea-pig bladder in order to (1) develop a model to study the causes of bladder instability; and (2) to facilitate the development of functional materials suitable for bladder implants.
The study is in the first instance an attempt to culture and subculture detrusor smooth muscle both from human and guinea-pig, and to characterise the cultured cells by morphology, immunocytochemistry and functional response to receptor stimulation. To address the question: are cultured cells comparable to fresh tissue, the passive and active electrical properties and ionic currents of the cell membrane from freshly isolated and cultured human and guinea-pig detrusor smooth muscle were investigated using the patch-clamp technique.

The following experimental plan and objectives are to be pursued in this study:

1. For all cell preparations the same standard procedures were used to dissociate cells from biopsy samples of detrusor and from cultures of cells.
   (1) For freshly isolated cells: a fraction was used immediately for experiment; the remainder was used for culture.
   (2) For primary cultured cells: a fraction was used for experimentation, and the remainder was used for subculture.

2. Measurement and comparison of the passive and active membrane electrical properties of the following groups: (1) freshly isolated human and guinea-pig detrusor muscle cells; (2) human detrusor muscle cells before and after culture; (3) guinea-pig detrusor muscle cells before and after culture; (4) cultured human and guinea-pig detrusor muscles; (5) freshly isolated and subcultured cell; (6) primary and subcultured cells.

3. Determination and comparison of the ionic currents in the above groups.
Chapter 2 Principles of experimental techniques

I. Principle of patch-clamp technique

The development of the patch-clamp technique by Neher and Sakmann (Neher and Sakmann 1976) has greatly improved our understanding of ion channels. The patch-clamp recording was originally developed for high-resolution current recording from single ion channels. The key step in this process is the formation of a gigaohm seal between the electrode glass and the membrane. This tight seal electrically isolates the several square microns of membrane "patch" covered by the pipette, reducing the leak current to a level where picoampere currents flowing through single channels can be resolved. A tight-seal is obtained by positioning a blunt-ended, low-resistance micropipette against a membrane and applying gentle suction to the pipette interior. If successful, a gigaohm seal will form between pipette and membrane and isolate a patch. By rupturing the patch, whole-cell access is established; this allows recording of current flowing across the entire cell membrane - whole cell recording. This is similar to conventional intracellular recording but offers advantages of high resolution, low noise, excellent stability, and control over intracellular constituents, due to the low electrode resistance and its tight glass-membrane seal. The relevant experimental considerations are discussed below section I.1.3.

I.1. Voltage-clamp

Voltage-clamp means that the membrane potential is held constant (i.e. clamped) while transmembrane current is measured.

The principle underlying a voltage-clamp can be understood by considering a simplified equivalent circuit for a cell membrane: a membrane resistance \( r_m \) (ion channel conductance) in parallel with a membrane capacity \( c_m \) (see section III.2, chapter 1); current flows through \( r_m \) (i\(_i\)) and across \( c_m \) (i\(_c\)). The net current i\(_m\) flowing through the circuit is the sum of i\(_i\) and i\(_c\):

\[
i_m = i_i + i_c = i_i + c_m \cdot \frac{dV}{dt}
\]

In a voltage-clamp experiment the membrane potential is generally clamped at a constant level as rapidly as possible after a brief spike of capacitive current i\(_c\) which flows at the edges of the voltage pulse. When the voltage is steady, \( \frac{dV}{dt} \) is zero and
so \( i_c \) is zero. The characteristics of the voltage-clamp are that: (1) clamping the voltage eliminates the capacitive current, except for a brief time when establishing a step to a new voltage; (2) except for the brief charging time, the currents that flow are proportional only to the membrane conductance, i.e., to the number of open channels; (3) if channel gating is determined by the transmembrane voltage, voltage-clamp offers control over the key variables that determine the opening and closing of ion channels.

A variety of different voltage-clamp methods have been used; the choice of methods depends mainly on the size and the shape of the preparation investigated. In essence, all the methods have a feedback amplifier which receives a signal from a voltage follower via an intracellular electrode and compares this with a command potential. The difference between these signals is amplified and a proportional current passed to the cell via a second electrode; this current can be recorded as that required to maintain a given membrane potential. The patch-clamp circuit operates rather differently; it is essentially a current-to-voltage converter. In traditional micropipette voltage-clamp techniques for whole-cell current measurement, potential measurement and current passing are performed by two different intracellular micropipettes. This is done using the conventional two-electrode voltage-clamp technique, or it is simulated by time-sharing using the discontinuous single electrode voltage-clamp technique. The whole-cell voltage-clamp is a technique in which only one micropipette is used full-time for both voltage recording and current passing.

### I.2. Current-clamp

In a current-clamp experiment, a known constant or time-varying current is applied to the cell and the change in membrane potential caused by the applied current is measured (this type of experiment mimics the current produced by, for example, a synaptic input).

The usual method for recording the transmembrane potential during the current-clamp technique uses a "bridge recording" mode. The essence of the technique is the connection of a micropipette to a buffer amplifier (often unity gain) that has an input resistance many orders of magnitude greater than that of micropipette and the input resistance of the cell. The output of the buffer amplifier follows the voltage at the tip of the electrode. The ideal buffer amplifier draws no input bias current, therefore the current through the micropipette is "clamped" at zero. When a current is injected down the micropipette in a variable waveform, it causes a corresponding voltage drop
across the micropipette which is difficult to be distinguished from the intracellular potential. Special compensation circuitry in modern recording system can be used to minimise the micropipette voltage drop from the cellular recording. The essence of this technique is to generate a signal that is proportional to the product of the micropipette current and the microelectrode resistance. This signal is then subtracted from the buffer amplifier output. This subtraction technique is commonly known as "bridge balance".

I.3. Patch-clamp

The patch-clamp technique is a special voltage- (or current-) clamp. This technique allows the experiment to isolate electrically a patch of membrane from the external solution and record membrane current as described above.

The configurations of the patch-clamp techniques include cell-attached; whole-cell; perforated-patch and inside-out, outside-out isolated patch configurations. The technique relies on the formation of a giga-seal and usually uses a single patch electrode to apply the command voltage and to record the current. By definition, the patch-clamp command voltage is positive if it increases the potential inside the patch pipette. Whether it is hyperpolarising or depolarising depends on whether the patch is "cell attached", "inside out" or "outside out".

After formation of a cell-attached patch, the whole-cell configuration can be achieved by rupturing the patch of membrane isolated by the patch pipette. This brings the cell interior into contact with the pipette interior. In practice, this is done by a short pulse of suction after a giga-seal has been formed (Hamill et al 1981). This method is called tight-seal "whole-cell recording", and will be used in present project. This is in contrast to various other patch-clamp techniques and it allows recording from the whole-cell membrane rather than from small patches of membrane. In this configuration, the potential on the outside surface is 0mV (bath potential), and the command potential controls the voltage at the tip of the intracellular (voltage-recording) pipette. The transmembrane potential is thus equal to the command potential. This configuration records the current from ion channels on the whole-cell membrane. The intracellular environment can be altered by changing the compositions of the pipette solution. This is useful for investigating the effects of intracellular ions or specific mediators on the behaviour of the ion channels of interest.
In the perforated-patch configuration, the membrane patch is still attached to the cell. However the patch has not been ruptured but perforated by antibiotics such as nystatin in the pipette. This configuration, as in the whole-cell configuration, can record whole-cell currents but does not dialyse the cell, thus the intracellular contents have not been washed out. This method has certain advantages: whole-cell recording can be done without dialysing important substances from the cytoplasm of the cell; current run-down is significantly slower; and physiologically relevant second messenger cascades and mechanisms important to cell signalling and channel regulation remain operative. But it does not allow replacement of the intracellular content with compounds other than small ions included in the pipette filling solution, as is possible with conventional methods.

The characteristics of patch-clamp are: (1) The currents measured are very small, in the order of picoamperes in single-channel recording and usually up to several nanoamperes in whole-cell recording. Due to the small currents, particularly in single-channel recording, the electrode polarisations and nonlinearities are negligible and the Ag/AgCl electrode can record voltage accurately even while passing current. (2) The electronic ammeter must be carefully designed to avoid adding appreciable noise to the currents it measures.

I.4. Whole-cell patch-clamp

The whole-cell configuration can be used to record the current when membrane potential is clamped (V-clamp), or used to record the membrane potential when a constant current is passed (I-clamp). This can be achieved using two electrodes or a single electrode.

In whole-cell voltage-clamp experiments, the membrane potential of the cell is controlled by applied command potentials: the command potential controls the voltage at the tip of the intracellular recording micropipette, and the whole-cell current response to the applied potential are recorded. The transmembrane potential is thus equal to the command potential ($V_m$=inside-outside).

The equivalent circuit used to represent the whole-cell recording condition is as follows.
Figure 2.1. Equivalent circuit of whole-cell recording. a. Current $i_m$ flows through the cell resistance $r_c$ and $i_c$ through the capacitance $c_m$. Pipette current, $i_p = i_m + i_c$, flows through the series resistance $r_s$ between pipette and cell and produces a voltage error $V_p - V_c = i_p r_s$. b. Time course of changes of $V_c$ and $i_p$ following a step of $V_p$ (Ogden and Stanfield 1994).

(i). resistance
In patch-clamp experiments, a tight seal between the pipette electrode and the membrane is required for successful patch recording. Seal resistance is the resistance due to the seal formed between the membrane and the pipette tip. This seal resistance can be estimated from the amplitude of the current deflection on the oscilloscope under a known voltage step, according to the relation: $r = V/i$.

Series resistance is the resistance between the cell and recording system. Series resistance is mainly from the pipette resistance plus other any access resistance to the patch or cell, such as the residual resistance of the ruptured patch.

Membrane resistance is the electrical resistance from the cell membrane.

(ii). Series resistance errors in whole-cell recording
The series resistance is the major source of error in whole-cell recording.

For whole-cell recording, current in the pipette ($i_p$) is defined as positive when flowing from pipette to the cell; and flows first through a resistance ($r_s$) in series with the cell membrane originating at the pipette tip. This $r_s$ results in a potential error
(i_p r_s) between pipette and cell which need to be compensated to produce the membrane potential of the cell.

The current flowing through the pipette contains capacitive transient and ionic current. The latter is what one is interested in but the former needs to be compensated. The time constant \( \tau \) (usually approximated well by \( \tau = c_m r_s \), since the cell resistance \( r_c >> r_s \), (Ogden and Stanfield 1994)) characterises the response of the voltage-clamped cell to applied voltage steps and also to currents originating in the cell membrane. The initial current flowing into the cell discharges the cell membrane capacitance and then the pipette current declines exponentially with \( \tau = c_m r_s \) to a steady state (Figure 2.1b). Signals are therefore low pass filtered with a bandwidth: \( f_c = 1/(2\pi \tau) \). These errors can be electronically minimised by using a series resistance compensation and capacitance compensation. The series resistance compensation adds a fraction of the voltage drop over \( r_s \) (i.e. \( i_p r_s \)) to the command voltage. This works well if \( r_s \) is small and should be used with caution because of frequent fluctuations of \( r_s \) during recording. Moreover, for best results, the cell input resistance should be many times the pipette resistance, but this is normally the case for cells at rest. However, during voltage activation, the cell input resistance could fall dramatically with two possible consequences: 1), there will be a significant error due to the voltage drop across the pipette; 2), the setting of the Series Resistance and Whole-Cell Capacitance Compensation controls will become erroneous because they are set based on the time constant of discharging the membrane capacitance before the change in the membrane resistance. The better solution is to use low resistance pipettes and small cells. The magnitude of the current investigated should also be considered in assessing the suitability of the technique for recording. In addition, care must be taken that the resistance of the pathway between the cell interior and pipette does not increase with time during the experiment due to the formation of an obstruction in the pipette tip. This is indicated by an increase in the time constant of the capacitive current transient. In some cases, the obstruction can be removed by applying another rapid suction. If the obstruction causes the access resistance to increase so that the voltage drop across the electrode is excessive, significant errors in measurements will occur and the experiments should be discontinued.

Therefore reducing \( r_s \) will minimise the size and time constant of the error voltage. The currents associated with charging the cell capacitance should be compensated, using the "slow" capacity compensation controls of Axopatch-1D amplifier by injecting the current into the pipette to cancel the capacitive currents.
(iii). Background noise
The main source of background noise in a whole-cell recording is likely to be the membrane capacitance \( (C_m) \) in series with the access resistance \( (r_s) \), in addition to the noise of the membrane conductance. Over most of the frequency range of interest, the noise from the series combination \( r_sC_m \) is much greater than the conductance noise. Therefore one should reduce the membrane capacitance and access resistance as much as possible. Thus low-resistance pipettes and small cells are preferred to be selected for whole-cell configuration.

To evaluate this resistance \( r_s \) of the connective pathway between the patch and cell, it is essential to examine the cell capacitive current. The value of \( r_s \) and of the cell capacitance \( C_m \) can be simply derived from the total charge \( \Delta Q \) displaced during a step command of amplitude \( \Delta V \) and the time constant \( \tau \) of the capacitive current according to \( C_m = \Delta Q / \Delta V \) and \( r_s = \tau / C_m \). Alternatively, one can simply read from the dial setting of the capacitance cancellation network after capacitance compensation has been performed (NB: \( C_m \) is membrane capacitance read from Whole-cell CAP, \( r_s \) is access resistance read from the Series Resistance after adjusting as the whole-cell recording established. Pipette resistance \( r_p \) represents the resistance before touching the cell).

(iv). Usage
The whole-cell recording of patch-clamp has extensive applications to study ion channels. Its popularity comes from the ease of use of the technique compared with other voltage-clamp techniques. It is particularly useful to small cells for which conventional voltage-clamp techniques are not feasible. Much information on whole-cell ion channel function and how ion channels are influenced by receptor activation can be obtained using this configuration. By combining the whole-cell clamping procedure with experimental protocols that aid in the isolation of individual currents, channels which carry the same ion can be separated; kinetics of channel activation and inactivation can be measured; and modulation of channel activity by different factors can be investigated. The factors of interest include both physical forces (e.g. membrane potential) and small molecules (e.g. ions, agents produced by intracellular regulatory pathways). The whole-cell recording configuration of patch clamp is especially suitable to the latter study, since this technique allows manipulation of the intracellular environment (e.g. small molecules can be introduced directly via the patch electrode, cytoplasmic \( \text{Ca}^{2+} \) can be buffered, intracellular ionic composition can be effectively controlled). This approach is particularly useful for investigating effects of different second messengers on ion channel activity. Membrane currents as well as
membrane potential can be recorded using this configuration. In recent years, use of this technique has been made in many cell types such as cardiac cells, smooth muscle cells, certain secretory cells, epithelial cells, some small nerve cells, etc.

If small cells are used, their input resistance is very large compared to the access resistance of the pipette tip, so that meaningful electrical measurements may be performed even though it is the whole-cell recording in which the signal originates from a much larger membrane area than that of initial patch (Sakmann and Neher 1983).

II. Primary monolayer culture

(i). Tissue culture

Tissue culture is a general term and includes organ culture and cell culture.

Primary culture means the culturing of freshly isolated single cells or fragments (explants) and are recorded as P0. The tissue can be chopped into tiny pieces (1mm³) and attached to a dish; the cells will then grow out from the fragment. Primary cultures can also be made by enzyme dissociation of the fragment and the cell suspension allowed to settle on to culture dishes. This type of culture gives a higher yield of cells and it can be more selective as only certain cells will survive dissociation. If the culture proliferates, then any non-dividing or slow-growing cells will be diluted out. Hence it may be necessary at this stage to select specific cell types by cloning, using selective media or physical cell separation.

Subculture is the further culture of primary cultured cells, a process known as passaging (recorded as P1, 2, etc.). The subcultures have a lower growth rate, but are more representative of the cell types in the tissue from which they are derived and in the expression of tissue specific properties.

Cell cultures include 2 dimensional (2D) monolayer cultures and suspension cultures. Monolayer culture is a method used for all cell types, in which cells need to attach to the substrate surface first, they then dedifferentiate and proliferate, so it is a stationary culture. This will be used for the detrusor cells used in the project. Suspension culture is the preferred method for some cells, it requires gentle agitation of the bottle during culture to prevent cell attachment to the surface of the growth bottle.
To successfully culture cells in vitro, in vivo conditions must be mimicked as closely as possible. These conditions consist of optimal temperature, pH, osmotic pressure, growth factors and hormones, cell growth matrix, essential metabolites and supplementary metabolites (Freshney 1989) which will differ for various cell types.

(ii). Requirements for primary monolayer culture:

(iia). The source of tissue
This could be embryonic or adult tissue. In general, cultures derived from embryonic tissue will survive and grow better than adult tissue. Embryonic or foetal tissue has many practical advantages, but in some instances the cells will be different from adult cells and it cannot be assumed that they will mature into adult-type cells unless this can be confirmed by appropriate characterisation.

Cultured cells can be from normal or neoplastic tissue. Normal tissue usually gives rise to cultures with a finite lifespan while cultures from tumours usually give continuous cell lines. During proliferation, normal cells often dedifferentiate until growth is terminated when redifferentiation occurs. These cells may then be used for experimentation or allowed to proliferate again (passage).

(iib). Culture medium
The culture medium contains 13 essential amino acids, 8 vitamins, salts and glucose, and omission of any single component can cause cell death. The medium requires supplementation with 5-10% serum (e.g. foetal calf serum) to provide hormones, growth factors, attachment and spreading factors and transport proteins, minerals and lipids. Media also require antibiotics and anti-fungal agents, such as penicillin, streptomycin and fungizone.

There are many types of culture medium available. The most commonly used medium for smooth muscle culture is DMEM (Dulbecco's Modified Eagle's Medium). For inhibition of fibroblast proliferation, a selective medium should be used, in which the amino acid L-valine is replaced by D-valine.

(iic). Substrate
Disposable plastic is usually used as a substrate for tissue culture. Choosing a suitable type and volume of substrate mainly depends on cell type, the number of cells required, the number of culture wells and the time of sampling. A 12-24 well plate is good for a large number of separate cultures for simultaneous sampling, but
individual dishes or bottles are preferable where sampling is carried out at different time. A flask is easy to control contamination of fungi, etc.

(iid). pH
Ideally the pH should be near 7.4 at the initiation of culture 37°C and should not fall below a value of 7.0 during the culture. A pH below 6.8 is usually inhibitory to cell growth. Factors affecting the pH stability of the medium are buffer capacity, type of buffer and glucose concentration. The normal buffer in tissue culture media is a CO₂/HCO₃⁻ buffer system, therefore culture is usually carried out in a 5% CO₂ atmosphere with 23-25mM NaHCO₃ in the medium.

(iie). Feeding
Complete culture media (i.e. added 10% foetal calf serum, antibiotics and antifungi) should be changed under sterile condition, usually 2-3 times per week if cells grow fast.

(ii) Preventing contamination.
All of the apparatus and materials used in culture require sterilisation and the procedures should be operated under sterile conditions.

III. Principle of immunocytochemistry

Immunocytochemistry is the use of labelled antibodies as specific reagents for the location of tissue antigens in situ (Polak and Noorden 1992).

III.1. Antibodies

Antibodies used in immunocytochemistry, mainly γ-globlins (IgG's), are raised by immunising animals such as rabbits with antigen. This antigen should be as pure as possible, or preferably synthetic, to ensure as specific an antibody as possible. For immunisation, the small antigen molecule is usually chemically coupled to a larger carrier protein molecule (e.g. limpet haemocyanin, thyroglobin, or bovine serum albumin) since the larger complex is a better stimulant for antibody formation. The antibodies produced will then be directed to various parts of antigen molecule and to the carrier protein. The donor animal serum will also contain many natural antibodies, which also react with the tissue component. However the antibody to the carrier molecule will either not react with the tissue to be stained or can be absorbed, for
example, by addition of the carrier protein, say. bovine albumin, to the antiserum prior to use which increases specificity.

Monoclonal antibodies are more advanced in their purity and specificity. Monoclonal antibodies are produced in mice, and lymphocytes from spleen, the source of the antibodies, are fused with mouse myeloma cells in culture. The fusion allows the hybrid cells to continue to grow and divide in culture and also produce antibody. One cell produces only one type of antibody and cultured hybrid myeloma cells are gradually cloned into cell lines producing that antibody only. Monoclonal anti-α smooth muscle actin, for example, is derived from the hybridoma produced by the fusion of mouse myeloma cells and splenocytes from an immunised mouse. The NH$_2$ terminal synthetic decapeptide of α-smooth muscle actin coupled to a carrier molecule was used as immunogen. Therefore monoclonal anti-α smooth muscle actin is used as a marker specific for smooth muscle in distinguishing smooth muscle cells from fibroblasts in a mixed culture.

The main requirements for a good antibody are: (1) the high avidity (stickiness) for its specific antigen, so that they are not washed off the section, and its lack of avidity to other antigens so that they are easily washed off the section during the staining process; (2) high specificity for its expected antigen; (3) high stability for easy storage and (4) rapid reaction rate, which mainly depends on the tissue fixation and antibody concentration.

III.2. Requirements for immunocytochemistry

The essential conditions for immunocytochemistry include: ease of preservation of the antigen; specific staining; a well-characterised antibody of high avidity and easy visualisation.

(i). Fixation

An essential part of all immunocytochemistry is the preservation of cells and tissue by immersing the sections in a fixative fluid. The fixatives employed prevent the autolysis by inactivating lysosomal enzymes and inhibit the growth of bacteria and moulds; they also stabilise the cells and tissues to protect them from the rigours of subsequent processing and staining techniques. Successful immunostaining requires that tissue antigen sites must be available to the applied antibody without great alteration of their tertiary structure and that the cellular structure is preserved so that
the immunoreactive cell may be identified. Fixation can break down the membrane of viable cells, which allows relatively large molecules to penetrate or escape.

(ii). Specificity problems and essential controls
In immunocytochemistry, there is a risk of non-specific reactions which must be eliminated before the result can be accepted. Therefore essential controls have to be performed during the staining procedure.

(iia). Method controls
For achieving method controls, two procedures are used: (1) the known positive control tissue is included each time; (2) a negative control is performed, that is, test tissue is stained with non-immune serum (or an inappropriate antisera) as a first layer, or the first layer is omitted.

(iib). Antiserum specificity controls
Antibody at the maximum dilution which gives a good positive staining and pre-absorbed with the specific antigen should give a negative stain; but when pre-absorbed with an inappropriate antigen should give a positive stain.

(iic). Non-specificity of antisera
This is due to: (1) the heterogeneous population of antibodies in the donor serum which may react with tissue components additional to the required specific antigen; (2) shared amino-acid sequences between related substances particularly peptides, which may mean that a given antibody will react with several substances instead of just one.

Non-specific staining will be reduced by: (1) diluting the primary or secondary antibody: high dilution of the antiserum results in diminution of the amount of unwanted antibody compared to the amount of specific antibody in the serum. The amount of unwanted binding may be reduced to be negligible; (2) more effective blockade of other antigens with a more concentrated normal serum from the donor species (if the indirect method is used, the blockade of other antigens with the normal serum is performed before staining with primary antibody; between the first and second layers as well); (3) blocking endogenous peroxidase with a stronger, or different peroxidase or for a longer time if peroxidase is used to visualise the antibody label; (4) absorbing the primary antibody with albumin.
(iii). Visualisation
The end-product of the reaction is visualised by labelling the antibody with fluorescein (immunofluorescence method); or an enzyme (peroxidase or alkaline phosphatase; immunoenzyme method). The advantage of the immunoenzyme method is that a permanent stain is obtained which can be seen by light microscopy.

III.3. Immunoenzyme methods

Immunoenzymatic staining methods utilise enzyme-substrate reactions to convert colourless chromogens into coloured end products. There are many immunoenzymatic staining methods which can be used to localise antigens (Naish 1989).

(i). Direct method
An enzyme-labelled primary antibody reacts with the antigen in the tissue. Subsequent use of substrate and chromagen concludes the reaction sequence. Because only one antibody is used and therefore completed quickly, non-specific reactions are limited. The disadvantage is that little signal amplification is achieved since staining involves only one labelled antibody.

(ii). Two-step indirect method
In this method, an unconjugated primary antibody binds to the antigen. An enzyme-labelled secondary antibody directed against the primary antibody is then applied, followed by a substrate-chromogen solution. The advantage is that it is more sensitive than the direct method because several secondary antibodies are likely to react with different epitopes on the primary antibody. Undesired cross-reaction may occur if the secondary antibody cross-reacts with endogenous immunoglobulin in the specimen. This can be eliminated by using a preabsorbed antiserum as described above.

(iii). Three-step indirect method
In this method, the first layer is an unconjugated primary antibody which binds to the antigen; the second layer is an enzyme-labelled secondary antibody directed against the primary antibody; and the third layer is another enzyme-conjugated antibody specific to the secondary antibodies. Both secondary and tertiary antibody must conjugate to the same enzyme. The three layers are applied sequentially. The advantage of this method is to further amplify the signal, since more antibodies are capable of binding to the previously-bound secondary antibody reagent. This places additional enzyme at the site of the antigen and therefore produces greater colour
intensity. The avidin-biotin coupled (ABC) method belongs to this three layer indirect method and will be used in this thesis.
Chapter 3. Methods

I. General protocol

Experiments were designed to characterise the electrophysiological properties of the cell membrane of freshly isolated and cultured detrusor smooth muscle cells. To this end, guinea-pig and human detrusor smooth muscle were used as models in the experiments. Cells were isolated enzymatically; a fraction was used for experiments and the remainder used for culture. Comparison was made between freshly isolated and cultured detrusor smooth muscle cells, as well as between the two species. Initial experiments established the possibility of achieving successful primary and subculture of detrusor smooth muscle and determined whether the cultured cells were smooth muscle by characterising their morphological growth pattern, immunocytochemical markers and functional responses to cholinergic stimulation. Then in the second stage, cellular electrophysiological approaches were used to characterise their passive and active properties using current-clamp; the resting potential and spontaneous action potentials under voltage follower mode (I=0), and measurement of membrane currents using voltage-clamp technique. The final investigations involved the determination of the ionic channels possessed by freshly isolated cells and their disappearance during culture using various protocols including a combination of patch-clamp with fluorescence imaging. The extracellular solution in all experiments was gassed with 95% O₂+5%CO₂, pH at 7.40±0.03 and superfused at 36.5 ±0.5 °C.

II. Solutions and chemicals

II.1. Extracellular solutions

Extracellular solutions used in the present studies included normal Tyrode’s solution (Tyr), Ca²⁺-free solution, HEPES-buffered Tyrode’s solution and various intervention solutions, some of which were made in stock. The enzyme solution will be described in the section: cell isolation.
II.1.1. Normal Tyrode’s solution

Normal Tyrode’s solution was the basic solution used in experiments and its composition is given in Table 3.1. The chemicals were Analar grade agents from BDH Chemicals Ltd. (Poole, Dorset, UK). The solution was made up in deionised water from Milli-RO 10 plus (Millipore, Croxley Green, Watford, UK).

Table 3.1. Composition of normal Tyrode’s solution

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>118.0</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>24.0</td>
</tr>
<tr>
<td>KCl</td>
<td>4.0</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>1.0</td>
</tr>
<tr>
<td>NaH₂PO₄·2H₂O</td>
<td>0.4</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>1.8</td>
</tr>
<tr>
<td>Glucose</td>
<td>6.1</td>
</tr>
<tr>
<td>Na pyruvate</td>
<td>5.0</td>
</tr>
</tbody>
</table>

II.1.2. Ca²⁺-free Tyrode’s solution

Ca²⁺-free solution (see Table 3.5) was made up in deionised water from Milli-RO 10 plus and used to determine the Ca²⁺-dependence of membrane ion channels. Because of contaminant quantities of Ca²⁺ in the water and commercially available preparations of other salts required for preparation of the final solution, it was important to regulate the concentration of Ca²⁺ in the solution with the Ca²⁺ chelator EGTA.

The equation for EGTA and free Ca²⁺ binding at physiological pH values can be approximately described as,

\[ \text{H}_2\text{EGTA} + \text{Ca}^{2+} \iff \text{CaEGTA} + 2\text{H}^+ \]

The calculated value of the [Ca²⁺] is given from the Law of Mass Action as,

\[ K_Ca = \frac{[\text{H}_2\text{EGTA}][\text{Ca}^{2+}]}{[\text{CaEGTA}][\text{H}^+]^2} \]
\[
\log K_{Ca} = \log \left( \frac{[H_2EGTA]}{[CaEGTA]} \right) + \log([Ca^{2+}]) - 2\log[H^+]
\]
\[
-\log([Ca^{2+}]) = \log \left( \frac{[H_2EGTA]}{[CaEGTA]} \right) - \log K_{Ca} - 2\log[H^+]
\]
\[
pCa = \log([H_2EGTA]) - [CaEGTA]) + pK_{Ca} + 2pH
\]

where \( pCa = -\log([Ca^{2+}]) \) and \( pK_{Ca} = -\log K_{Ca} \). \( K_{Ca} \) is the equilibrium constant of the reaction in equation 3.1. Thus the \( pK_{Ca} \) will be a function of pH and must be carried out at a stable value. The correct value will only be achieved when the pH is titrated back to the desired value of pH7.0. Thereafter pCa can be calculated according to equation 3.2 as soon as \( pK_{Ca}^{app} \) and total [EGTA] in the solution has been determined:

\[
pCa = \log([H_2EGTA]) - [CaEGTA]) + pK_{Ca}^{app}
\]

where \([H_2EGTA])=[EGTA]_{total} - [CaEGTA]. Note that the value of \( K_{Ca}^{app} \) here is only an apparent equilibrium constant. In addition, the solution may not only contain EGTA and CaCl₂ but also other molecules which could bind to \( Ca^{2+} \) or EGTA.

II.1.2.1. Determination of \([Ca^{2+}]\) using a \( Ca^{2+} \)-selective electrode

II.1.2.1.1. Data collection

\( Ca^{2+} \) and \( H^{+} \)-selective electrodes were used to determine the pCa in solutions according to the standard procedure set up in this laboratory (Fry, 1990; Langley and Fry 1995).

The experiment was performed at 37°C and pH7.0. The bath solution was a basic EGTA-containing calibration solution, which contained (mM) KCl 120, NaCl 10, MgCl₂ 1.0, HEPES 20 and EGTA 5.0; the pH was adjusted to 7.0 with KOH.

A dip-cast \( Ca^{2+} \) selective electrode was used with ETH1001 as the neutral ligand (Fluka Chemicals, Gillingham, Dorset, UK). The \( Ca^{2+} \)-selective electrode (filled with 150mM NaCl plus 1mM CaCl₂) and a reference electrode filled with 3M KCl, were placed into a well-stirred 5ml chamber containing the EGTA calibration solution. Samples from the 1M CaCl₂ stock solution were added to the solution with a Hamilton syringe, which resulted in the reaction of \( Ca^{2+} \) and EGTA following equation 3.1. The pH was titrated back to 7.0 after the addition of each CaCl₂ sample, monitored by a \( H^{+} \)-selective electrode built inside the chamber. The pH electrode was
calibrated separately before the experiment in standard pH7.0 and pH4.0 calibration solutions (BDH, Poole, UK). CaCl2 additions were repeated until excess CaCl2 has been added (usually 7mmoles/l CaCl2 to yield a nominal excess of 2mmoles/l Ca2+). The results were recorded as shown in Table 3.2 and used to determine the constant $K_{Ca^{app}}$ for the EGTA-containing calibration solution.

Note that it was very important that the pH was monitored throughout this procedure. The addition of CaCl2 to the EGTA-containing solution results in the release of H+ (see equation 3.1) when Ca2+ binds to EGTA, which itself would reduce the stoichiometric binding of Ca2+.

### Table 3.2. Results from an experiment to determine the constant $K_{Ca^{app}}$ for an EGTA-containing solution at pH 7.0, 37°C; $E_{Ca}$ is the p.d. measured by the Ca2+-selective electrode, reference electrode pair; $E_{Ca, corr}$ is the $E_{Ca}$ value offset to zero at the highest [Ca2+].

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vol (ml)</td>
<td>KOH added (μl)</td>
<td>Ca added (μmol)</td>
<td>Ca added (μmol/ml)</td>
<td>$E_{Ca}$ (mV)</td>
<td>$E_{Ca, corr}$ (mV)</td>
</tr>
<tr>
<td>1</td>
<td>5.0000</td>
<td>5.008</td>
<td>7</td>
<td>1.9968</td>
<td>-12.5</td>
</tr>
<tr>
<td>2</td>
<td>5.0055</td>
<td>5.008</td>
<td>7</td>
<td>1.9968</td>
<td>-12.5</td>
</tr>
<tr>
<td>3</td>
<td>5.0145</td>
<td>5.0145</td>
<td>10.5</td>
<td>0.79769</td>
<td>-9</td>
</tr>
<tr>
<td>4</td>
<td>5.022</td>
<td>5.022</td>
<td>15</td>
<td>1.39387</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>5.028</td>
<td>5.028</td>
<td>18</td>
<td>1.98886</td>
<td>10.5</td>
</tr>
<tr>
<td>6</td>
<td>5.035</td>
<td>5.035</td>
<td>22</td>
<td>2.58193</td>
<td>16.5</td>
</tr>
<tr>
<td>7</td>
<td>5.0415</td>
<td>5.0415</td>
<td>25.5</td>
<td>3.17366</td>
<td>24.5</td>
</tr>
<tr>
<td>8</td>
<td>5.048</td>
<td>5.048</td>
<td>29</td>
<td>3.76387</td>
<td>36.5</td>
</tr>
<tr>
<td>9</td>
<td>5.054</td>
<td>5.054</td>
<td>32</td>
<td>4.35299</td>
<td>60.5</td>
</tr>
<tr>
<td>10</td>
<td>5.056</td>
<td>5.056</td>
<td>33</td>
<td>4.54905</td>
<td>83</td>
</tr>
<tr>
<td>11</td>
<td>5.057</td>
<td>5.057</td>
<td>33</td>
<td>4.74590</td>
<td>94</td>
</tr>
<tr>
<td>12</td>
<td>5.058</td>
<td>5.058</td>
<td>33</td>
<td>4.94267</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>5.059</td>
<td>5.059</td>
<td>33</td>
<td>5.13936</td>
<td>103</td>
</tr>
<tr>
<td>14</td>
<td>5.061</td>
<td>5.061</td>
<td>33</td>
<td>5.53250</td>
<td>109</td>
</tr>
<tr>
<td>15</td>
<td>5.063</td>
<td>5.063</td>
<td>33</td>
<td>5.92534</td>
<td>113</td>
</tr>
<tr>
<td>16</td>
<td>5.0665</td>
<td>5.0665</td>
<td>33.5</td>
<td>6.51337</td>
<td>116.5</td>
</tr>
<tr>
<td>17</td>
<td>5.069</td>
<td>5.069</td>
<td>34</td>
<td>6.90471</td>
<td>122</td>
</tr>
</tbody>
</table>

(1). Column A is total volume in chamber.
(2). Column B and C are accumulated quantities of added KOH and CaCl2 respectively.
(3). Column D=Column C / Column A
II.1.2.1.2. Data handling

Table 3.3 lists values obtained from the data in Table 3.2 to calculate $K_{Ca^{app}}$. It is initially assumed that the electrode is perfectly Nernstian, i.e.:

$$E_{Ca} = E^0 + \frac{2.303RT}{zF} \log_{10} ([Ca^{2+}])$$

where $2.303RT/zF = 29.5$ mV per 10x change of $[Ca^{2+}]$ at 22°C. It was also initially assumed the total $[EGTA]$ in the calibration solution was 5.0 mM and that 6.905 mmol/l Ca was added to the final volume (= 5.069 ml, line 18, Table 3.2) to yield a free $[Ca^{2+}]$ of $1.905 \times 10^{-3}$ M $Ca^{2+}$, or a $pCa = 2.720$ (line 18, Table 3.3). This $pCa$ value was added to column A values in Table 3.3 to yield $pCa$ values (column B), which then yield $[Ca^{2+}]$ values (column C), at each increment of added CaCl$_2$. The free $[Ca^{2+}]$ was subtracted from the total added Ca (Table 3.2, column D) to yield the values of bound Ca, then the ratio of Ca bound / $Ca^{2+}$ free was calculated (Table 3.3, column D and E).

A Scatchard plot of Ca bound (x-axis) versus the Ca bound / $Ca^{2+}$ free ratio (y-axis) is shown in Figure 3.1. The linear region of this plot was fitted by a least-squares regression as shown in figure 3.2. The $K_{Ca^{app}}$ was calculated from the slope ($=1/K_{Ca^{app}}$) and the intercept with the x-axis gave the total $[EGTA]$ in the solution. The data in Figure 3.2 yielded values for $K_{Ca^{app}} = 3.46 \times 10^{-7}$ ($pK_{Ca^{app}} = 6.46$) and an $[EGTA] = 4.44$ mM. Similar values were obtained in three other determinations.

At $pCa > 4.5$ the added Ca did not exceed the total $[EGTA]$ and so $pCa$ values were calculated using equation 3.2 and derived values of $K_{Ca^{app}}$ and total $[EGTA]$. At $pCa < 4.5$ the added Ca exceeded the total $[EGTA]$ and was calculated using equation 3.4:

$$pCa = -\log_{10}([Ca added - 4.44 \text{ mM}]/1000)$$

A calibration curve of a $Ca^{2+}$-selective electrode using calculated $pCa$ values is shown in Figure 3.3.
### Table 3.3. Data from Table 3.2 for calculation of $K_{Ca^{app}}$

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECa, corr</td>
<td>pCa</td>
<td>[Ca^{2+}]</td>
<td>Ca bound</td>
<td>Ca</td>
<td>bound/free</td>
</tr>
<tr>
<td>1</td>
<td>29.5</td>
<td>4.76271</td>
<td>7.48288</td>
<td>3.289E-05</td>
<td>3799.3862</td>
</tr>
<tr>
<td>2</td>
<td>4.55932</td>
<td>7.27949</td>
<td>5.254E-05</td>
<td>0.19963</td>
<td>11551.616</td>
</tr>
<tr>
<td>3</td>
<td>4.44068</td>
<td>7.16085</td>
<td>6.905E-05</td>
<td>0.79762</td>
<td>8553.3685</td>
</tr>
<tr>
<td>4</td>
<td>4.06780</td>
<td>6.78797</td>
<td>0.0001629</td>
<td>1.39370</td>
<td>5108.5785</td>
</tr>
<tr>
<td>5</td>
<td>3.77966</td>
<td>6.49983</td>
<td>0.0003164</td>
<td>1.98855</td>
<td>3362.6823</td>
</tr>
<tr>
<td>6</td>
<td>3.57627</td>
<td>6.29644</td>
<td>0.0005053</td>
<td>2.5814</td>
<td>1562.5413</td>
</tr>
<tr>
<td>7</td>
<td>3.30508</td>
<td>6.02525</td>
<td>0.0009435</td>
<td>3.17272</td>
<td>10.889032</td>
</tr>
<tr>
<td>8</td>
<td>2.89831</td>
<td>5.61848</td>
<td>0.0024073</td>
<td>3.76146</td>
<td>7.0125301</td>
</tr>
<tr>
<td>9</td>
<td>2.08475</td>
<td>4.80492</td>
<td>0.0156706</td>
<td>4.33732</td>
<td>13.450867</td>
</tr>
<tr>
<td>10</td>
<td>1.32203</td>
<td>4.04220</td>
<td>0.0907394</td>
<td>4.45831</td>
<td>21.163628</td>
</tr>
<tr>
<td>11</td>
<td>0.94915</td>
<td>3.66932</td>
<td>0.2141300</td>
<td>4.53177</td>
<td>13.450867</td>
</tr>
<tr>
<td>12</td>
<td>0.74576</td>
<td>3.46593</td>
<td>0.3420324</td>
<td>4.60663</td>
<td>21.163628</td>
</tr>
<tr>
<td>13</td>
<td>0.64407</td>
<td>3.36424</td>
<td>0.4322771</td>
<td>4.70708</td>
<td>10.889032</td>
</tr>
<tr>
<td>14</td>
<td>0.44068</td>
<td>3.16085</td>
<td>0.6904815</td>
<td>4.84202</td>
<td>5.2801221</td>
</tr>
<tr>
<td>15</td>
<td>0.30508</td>
<td>3.02525</td>
<td>0.9435073</td>
<td>4.98183</td>
<td>4.2531111</td>
</tr>
<tr>
<td>16</td>
<td>0.18644</td>
<td>2.90661</td>
<td>1.2399076</td>
<td>5.27346</td>
<td>2.6250647</td>
</tr>
</tbody>
</table>

(1). Column A is calculated from column F values (Table 3.2)/29.5mV.

(2). The pCa value in Column B line 18 was obtained from the molar [Ca^{2+}] (see text), other values in column B are equal to Column A+2.72017.

(3). Column C is the calculated free [Ca^{2+}] from column B.

(4). Column D=Column D, Table 3.2 - Column C, Table 3.3.

(5). Column E=Column D/Column C.
Figure 3.1. A Scatchard plot of calcium bound (column D, Table 3.3) versus the ratio of calcium bound/calcium free (column E, Table 3.3) in an EGTA containing calibration solution.

Figure 3.2. Plot of the linear part of the Scatchard plot of Figure 3.1. The line was fitted by a least squares regression, the slope and x-intercept were equivalent to $-1/K_{Ca^{2+}}$ and total [EGTA] respectively.
Figure 3.3. Calibration curve of a Ca$^{2+}$-selective electrode. The pCa values were calculated using equation 3.2 (pCa > 4.5) or equation 3.4 (pCa < 4.5) with pK$_{Ca^{2+}}$=6.46 and [EGTA]=4.44. The y-axis is the E$_{Ca,corr}$ of the Ca$^{2+}$-selective electrode with a maximum value offset to zero at the maximum [Ca$^{2+}$] used. The straight line has a slope of 28.0mV per pCa unit, data obtained at 37°C, pH 7.0.

II.1.2.1.3. Determination of [Ca$^{2+}$] in Ca$^{2+}$-free Tyrode's solution

The previous calibration procedure showed the Nernstian behaviour of the Ca$^{2+}$-selective electrode at [Ca$^{2+}$] ≥ 0.1μM in a KCl-based medium. This implied that K$^+$ had a negligible interferent effect on the electrode over this range of [Ca$^{2+}$]. The electrode shows a larger interference from Na$^+$ (Fry, 1990) so this effect was quantified before it was used to measure the [Ca$^{2+}$] in a nominally Ca-free Tyrode's solution. The response of the Ca$^{2+}$-selective electrode to the primary ion, Ca$^{2+}$, and a single interferent ion, Na$^+$, is given by the Nikolsky equation:

$$E_{Ca} = E^0 + (2.303RT/zF) \log_{10} \left( [Ca^{2+}] + k_{pot}[Na^+]^2 \right)$$

3.5

$k_{pot}$ is the potentiometric selectivity coefficient to be determined and $E^0$ is a constant.

Table 3.4 shows the results of a typical determination of the [Ca$^{2+}$] of a nominally Ca-free solution. The E$_{Ca}$ value was recorded in four solutions:

1) the high EGTA-calibrating solution containing 120mM KCl + 10mM NaCl;
(2) the high EGTA solution with 130 mM NaCl;
(3) the high Ca\(^{2+}\) solution at the end of the previous calibration (i.e. 6.90471-4.44=2.46471 mM Ca\(^{2+}\), Table 3.2, line 18);
(4) the test solution - the nominally Ca-free solution.

**Table 3.4. Results of a determination of the [Ca\(^{2+}\)] in a nominally Ca-free Tyrode’s solution.**

<table>
<thead>
<tr>
<th>Solution no.</th>
<th>Solution composition</th>
<th>E(_{Ca}), mV</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EGTA / KCl</td>
<td>-149.0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>EGTA / NaCl</td>
<td>-134.0</td>
<td>(k_{pot}[Na^+]^2 \gg [Ca^{2+}])</td>
</tr>
<tr>
<td>3</td>
<td>2.46471 mM Ca</td>
<td>0</td>
<td>([Ca^{2+}] \gg k_{pot}[Na^+]^2)</td>
</tr>
<tr>
<td>4</td>
<td>Ca-free Tyrode’s</td>
<td>-60.0</td>
<td>Test solution</td>
</tr>
<tr>
<td></td>
<td>(147 mM Na)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Substitution of \(E_{Ca}\) values for solutions 2 and 3 into equation 3.5 was used to calculate the value of \(k_{pot}\). Using a value of the slope factor \((2.303RT/zF)\) derived from the previous calibration (= 27.9 mV/10x \(\Delta[Ca^{2+}]\)) the \(k_{pot}\) was \(2.297 \times 10^{-9}\) mM\(^{-2}\). Substitution of \(E_{Ca}\) values for solutions 3 and 4 into equation 3.5 was used to calculate the value of [Ca\(^{2+}\)] in the Ca-free Tyrode’s. This yielded a value of 16.9 \(\mu M\).

**II.1.2.2. Composition of the very low Ca\(^{2+}\) Tyrode’s solution**

A Tyrode’s solution with a [Ca\(^{2+}\)] of 10 nM (pCa = 8.0) was made using the known contaminant [Ca\(^{2+}\)] in the solution, the nominal [EGTA] and the p\(K_{Ca^{app}}\) value. The calculation also took into account that the solution pH was 7.4 not 7.0 which necessitated changing the p\(K_{Ca^{app}}\) from 6.46 to 7.26 (6.46 + 2\(\Delta p\)H).

From equation 3.2: \(pCa - pK_{Ca^{app}} = 0.74 = \log10([H_2EGTA]/[CaEGTA])\)

Thus: \(\frac{[H_2EGTA]}{[CaEGTA]} = 5.495\)

If [CaEGTA] = 16.9 \(\mu M\) from the contaminant in solution, the [H\(_2\)EGTA] = 92.9 \(\mu M\) and the total [EGTA] required is 110 \(\mu M\). The purity of the EGTA was \((4.44 / 5.00)\times100\% = 88.8\%\) so the total nominal EGTA required was 124 \(\mu M\) (=0.12 mM). The composition of the pCa 8.0 Tyrode’s solution is given in table 3.5, note that the MgCl\(_2\) was increased by 1.8 mM to keep the total divalent cation concentration approximately constant.
Table 3.5. Composition of the pCa 8.0 Tyrode's solution
("EGTA-Ca²⁺-free" solution)*.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>118.0</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>24.0</td>
</tr>
<tr>
<td>KCl</td>
<td>4.0</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>2.8</td>
</tr>
<tr>
<td>NaH₂PO₄·2H₂O</td>
<td>0.4</td>
</tr>
<tr>
<td>Glucose</td>
<td>6.1</td>
</tr>
<tr>
<td>Na pyruvate</td>
<td>5.0</td>
</tr>
<tr>
<td>EGTA</td>
<td>0.12</td>
</tr>
</tbody>
</table>

* Made from deionised water and final [Ca²⁺] about 10 nM used for experiments, as precise [Ca²⁺] required.

II.1.3. HEPES buffered-Tyrode's solutions

HEPES-buffered Tyrode's solutions were made up with ultra pure Analar water (BDH) and used to make enzyme solutions for cell isolation (Ca²⁺-free HEPES-Tyrode's solution), or with the 1.8mM CaCl₂ for storage of isolated single cells (Ca²⁺-containing HEPES-Tyrode's solution).

Table 3.6. Composition of Ca²⁺-free HEPES-Tyrode's solution*

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>105.4</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>22.3</td>
</tr>
<tr>
<td>KCl</td>
<td>3.6</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>0.9</td>
</tr>
<tr>
<td>NaH₂PO₄·2H₂O</td>
<td>0.4</td>
</tr>
<tr>
<td>HEPES</td>
<td>19.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.4</td>
</tr>
<tr>
<td>Na pyruvate</td>
<td>4.5</td>
</tr>
</tbody>
</table>

*pH7.1 adjusted with 1M NaOH for cell isolation
Table 3.7. Composition of Ca$^{2+}$-containing HEPES-Tyrode’s solution*

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>105.4</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>22.3</td>
</tr>
<tr>
<td>KCl</td>
<td>3.6</td>
</tr>
<tr>
<td>MgCl$_2.6$H$_2$O</td>
<td>0.9</td>
</tr>
<tr>
<td>NaH$_2$PO$_4.2$H$_2$O</td>
<td>0.4</td>
</tr>
<tr>
<td>HEPES</td>
<td>19.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.4</td>
</tr>
<tr>
<td>Na pyruvate</td>
<td>4.5</td>
</tr>
<tr>
<td>CaCl$_2.2$H$_2$O</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* Based on the solution in Table 3.6 plus 1.8mM CaCl$_2$, then pH 7.4 adjusted with 1M NaOH for cell storage.

II.2. Intracellular solutions

Experiments involving the measurement of the passive and active membrane properties under physiological condition as well as measurement of K$^+$ currents required mimicking the intracellular ionic concentrations. Therefore a basic patch pipette filling solution used in present study contained KCl+5mM EGTA. For measurement of other currents different filling solutions were required, such as: KCl+0.1mM EGTA for Ca$^{2+}$-dependent K$^+$ currents; and CsCl+0.1mM EGTA or +5mM EGTA (to reduce Ca$^{2+}$-dependant Ca$^{2+}$ inactivation) to determine L-type and T-type Ca$^{2+}$ currents.

All solutions were made up using ultra pure Analar water. The pH of these solutions was carefully adjusted to pH7.2. GTP and ATP (except for an "ATP-free" filling solution) were added to all solutions to attempt to prevent current run down. Filling solutions were made up in batches, filtered through a 0.2μm filter and stored at -20 °C for later use.

For measurement of voltage dependence of Ca$^{2+}$-dependent K$^+$ current, the Ca$^{2+}$-containing pipette filling solution was made using high concentration of EGTA (Table 3.12). It gave a constant intracellular free Ca$^{2+}$ of 0.25μM.
**Table 3.8.** Composition of basic "KCl+5mM EGTA" filling solution*

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>20</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>110</td>
</tr>
<tr>
<td>MgCl$_2.6H_2O$</td>
<td>5.45</td>
</tr>
<tr>
<td>Na$_2$ATP***</td>
<td>5.0</td>
</tr>
<tr>
<td>Na$_4$GTP 2H$_2$O</td>
<td>0.1</td>
</tr>
<tr>
<td>EGTA**</td>
<td>5.0</td>
</tr>
<tr>
<td>HEPES</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* pH 7.2 adjusted with 1 M KOH; ** dissolved in KOH first.

*** replaced by Na$_2$ Aspartate in "ATP-free" filling solution.

**Table 3.9.** Composition of "KCl+0.1mM EGTA" filling solution*

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>20</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>110</td>
</tr>
<tr>
<td>MgCl$_2.6H_2O$</td>
<td>5.45</td>
</tr>
<tr>
<td>Na$_2$ATP</td>
<td>5.0</td>
</tr>
<tr>
<td>Na$_4$GTP 2H$_2$O</td>
<td>0.1</td>
</tr>
<tr>
<td>EGTA**</td>
<td>0.1</td>
</tr>
<tr>
<td>HEPES</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* pH 7.2 adjusted with 1 M KOH; ** dissolved in KOH first.

**Table 3.10.** Composition of "CsCl+0.1mM EGTA" filling solution*

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsCl</td>
<td>20</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>110</td>
</tr>
<tr>
<td>MgCl$_2.6H_2O$</td>
<td>5.45</td>
</tr>
<tr>
<td>Na$_2$ATP</td>
<td>5.0</td>
</tr>
<tr>
<td>Na$_4$GTP 2H$_2$O</td>
<td>0.1</td>
</tr>
<tr>
<td>EGTA**</td>
<td>0.1</td>
</tr>
<tr>
<td>HEPES</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* pH 7.2 adjusted with 1 M CsOH; ** dissolved in CsOH first.
Table 3.11. Composition of “CsCl+5 mM EGTA” filling solution*

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsCl</td>
<td>20</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>110</td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O</td>
<td>5.45</td>
</tr>
<tr>
<td>Na$_2$ATP</td>
<td>5.0</td>
</tr>
<tr>
<td>Na$_4$GTP 2H$_2$O</td>
<td>0.1</td>
</tr>
<tr>
<td>EGTA**</td>
<td>5.0</td>
</tr>
<tr>
<td>HEPES</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* pH 7.2 adjusted with 1 M CsOH; ** dissolved in CsOH first.

Table 3.12. Composition of “high Ca$^{2+}$” filling solution*

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$</td>
<td>11.6</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>114</td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O</td>
<td>5.45</td>
</tr>
<tr>
<td>Na$_2$ATP</td>
<td>5.0</td>
</tr>
<tr>
<td>Na$_4$GTP 2H$_2$O</td>
<td>0.1</td>
</tr>
<tr>
<td>EGTA**</td>
<td>20.0</td>
</tr>
<tr>
<td>HEPES</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* pH 7.15 adjusted with 1 M KOH; ** dissolved in KOH first.

II.3. Enzyme solution

Enzyme-containing solutions were used for cell dissociation, which mainly contained collagenase (Type I, Sigma Chemicals Co. Ltd., Poole Dorset, UK), papain (1.7u/mg solid, Sigma) as well as bovine albumin (essential fatty acid-free; Sigma). The enzymes were dissolved in Ca$^{2+}$-free HEPES-Tyrode’s (Table 3.6) aliquots and stored at -20°C for weekly use.
Table 3.13. Composition of enzyme solution.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase type I</td>
<td>0.7*</td>
</tr>
<tr>
<td>Papain</td>
<td>0.7*</td>
</tr>
<tr>
<td>Albumin</td>
<td>1</td>
</tr>
</tbody>
</table>

* variable amount for human and guinea-pig detrusor.

III. Cell isolation and culture

Next morning, the centrifuge tube containing the detrusor muscle strips was put in a heat-block and warmed up to 36.5°C for variable times from 15 to 30 minutes and the supernatant was discarded. Some digested cells were stored in Ca²⁺-containing HEPES-Tyrode’s (Table 3.7.) for subsequent experiments, others were used for cell culture.

Cultured cells were digested with the same enzyme solution. Briefly, the petri dish was rinsed with Ca²⁺-free Mg²⁺-free phosphate buffered saline (PBS, GIBCOBRL) three times, then was soaked in the enzyme solution for about 1 minute. The wet petri dish was covered with a lid after the solution was discarded and put in an incubator for about 2 minutes. As soon as the cells were dissociated, they were suspended in the culture medium by a gentle trituration using a blunt glass pipette, transferred into a centrifuge tube and stored at 4°C for experimental measurements.
III.2. Cell culture

III.2.1. Primary culture

Cell culture was performed under aseptic conditions - that is, all instruments and materials were sterilised and manoeuvres performed in a flow cabinet according to the following procedure:

Digested muscle strips were transferred to a 1ml sterile centrifuge tube with PBS (phosphate buffered saline (Dulbecco's formula), GIBCOBRL Life Technologies Ltd., Paisley, UK) with penicillin/streptomycin 100units/ml +fungizone 2.5μg/ml (GIBCOBRL) and centrifuged at a speed of 1000-1500 rpm (110-250 g) for 1 minute (BS400 Bench Centrifuge. Denley Instruments Ltd, West Sussex). The supernatant was discarded and centrifugation repeated in the above solution for three times to get rid of possible contamination with bacteria and remaining enzyme.

The tissue was then cut into pieces as small as possible with sterile scissors which had been soaked in 70% ethanol for more than 30 minutes. The pieces were transferred to another 1ml sterile centrifuge tube with a selective D-valine culture medium (Minimum Essential Medium D-Val, GIBCOBRL) supplemented with 10% FBS (Foetal Bovine Serum, GIBCOBRL), 2mM L-glutamine (GIBCOBRL).and penicillin/streptomycin 100units/ml +fungizone 2.5μg/ml (GIBCOBRL).

The tissue pieces were triturated gently using an autoclave sterile glass pipette with a blunt tip and then transferred into a 35mm petri dish (GIBCOBRL) and cultured in an incubator at 37°C and 5% CO₂ atmosphere.

The cultured cells were not disturbed for a week; subsequently the culture medium was changed twice a week for another 2-3 weeks to confluence when they formed a characteristic “hills and valleys” growth pattern. At this time the cells were harvested using the same enzyme solution as that used for fresh cell dissociation. The procedure for cultured cell digestion with enzyme solution is described in the section III.1, cell isolation.
III.2.2. Subculture

The primary cultured cells which were going on to subculture were digested with sterile enzyme solution of 0.25% trypsin / versene (100ml of 2.5% (1:250) trypsin + 900ml of 1:5000 versene; GIBCOBRL). The cultured cells were quickly rinsed in the enzyme solution (<1 minute), then the solution discarded. The wet petri dish was covered with a lid and then put in an incubator for about 2 minutes. As soon as the cells were dissociated, as observed under the microscope, they were quickly supplied with the same culture medium as used in primary culture to stop the enzyme reaction. The cultured cells were then gently triturated with a pipette and the cell suspension was aliquoted into two dishes for subculture, following the same procedure as primary culture.

III.3. Cell viability and Cell Counting

III.3.1. Cell viability

How long the enzyme solution was used for in dissociation, especially with cultured cells, was very critical for cell survival and function.

Cell viability was determined by means of 0.1% trypan blue (GIBCOBRL) exclusion. This is a viability stain and is only taken up by dead cells, enabling them to be excluded from the cell count. Under a phase contrast microscope, the cells which were positively stained by trypan blue were blue and the cells which were not stained (live cells) possessed a shiny membrane. Under the microscope of the patch clamp system, the live cells were very dark, by contrast with the clear dead cells. After digestion, the live cell was also spindle shaped. The dissociation procedure used allowed a high percentage (more than 80%) of cells to survive after enzyme digestion.

III.3.2. Cell Counting

1. Preparation of haemocytometer:
The haemocytometer was kept clean and free of grease. The surface of the chamber and the coverslip was gently cleaned using a 70% alcohol-soaked tissue. The clean, dry chamber was held in both hands, with one finger of each hand extending underneath the central area to support the chamber at its weakest point. After
breathing on the chamber the coverslip was gently pushed from the edge of the chamber into the central position. When the coverslip was correctly attached, a rainbow effect, ("Newton's rings") could be seen on either side of the central chamber.

2. Preparation of single cell suspension:
The single cell suspension was mixed with 0.1% trypan blue in a ratio of 1:1 (v/v) using a Pasteur pipette.

A small volume of suspension was taken into the tip of the Pasteur pipette and transferred to the edge of the coverslip. By application of gentle pressure, the suspension ran out of the pipette and was drawn under the coverslip by capillarity. It was important not to overfill or underfill the chamber as this would lead to inaccuracies in cell distribution.

Excess fluid was blotted off (without drawing it from under the coverslip) and the chamber placed onto the stage of microscope. The objective was focused on the grid lines of chamber.

3. Counting cells:
Figure 3.4 shows the counting chamber. Each large square consists of 16 small squares and the four large squares are marked A, B, C and D on the diagram. The area is 1 mm² per large square. The number of viable cells (i.e., those which have not taken up the stain) in each of the four large squares was counted, using the 16 small squares as an aid to counting. Cells were included which lay across the top and left-hand lines of each large square, but were excluded which lay across the bottom and right-hand lines of the large square.

The number of cells present in each large square was recorded in the order A to D.

The procedure was repeated for a second chamber and an absolute minimum of 100 cells was counted.
4. Calculation of cell numbers:
The area of each large square is 1 mm² and the depth of the counting chamber is 0.1 mm, therefore the volume of each large square is 0.1 mm³ (0.0001 cm³), i.e. 10⁻⁴ ml. The cell suspension was diluted 1+1 in trypan blue. Therefore the mean number of cells per large square was multiplied by 2; then multiplied by 10⁴ (the volume of each large square=10⁻⁴ ml) to get the number of cells per ml cell suspension.

Thus, in 1 ml of single cell suspension,

\[ \text{cells} = 2 \times 10^4 \times \frac{\text{total cells}}{\text{number of large squares counted}} \]

III.4. Immunocytochemistry

To identify the cultured cells as smooth muscle, an indirect immunoenzyme binding assay, the avidin-biotin complex (ABC) method, was used to determine α-smooth muscle actin in the cultured cells. In this method, peroxidase was used as an antibody label in the immunocytochemical reaction and was visualised by the diaminobenzidine (DAB) reaction for peroxidase. Peroxidase-labelled antibodies have the advantage that an ordinary transmission-light microscope can be used and a permanently stained preparation can be prepared which does not fade, as will a fluorescence-labelled preparation. The development of the enzyme reaction is
progressive, it can be monitored and stopped when the signal-to-noise ratio is adequate, and the method has been well-established in this Institute.

III.4.1. The Avidin-biotin complex (ABC) method

The avidin-biotin complex method is a three-layer indirect method, which utilises the high affinity of avidin with four binding sites for biotin. It consists of:

(1) The first layer is a primary antibody: mouse monoclonal antibody to the N-terminal of α-smooth muscle actin A2547 (Sigma). This monoclonal antibody, also called anti-α-Sm-1, recognises only smooth muscle α-actin, as it was produced using the NH₂ terminal synthetic decapeptide of α-smooth muscle actin as immunogen. It is therefore specific for the single isoform of α-smooth muscle actin, not for fibroblasts or any other non-muscle actin.

(2) The second layer is an enzyme-conjugated antibody, a biotinylated secondary anti-mouse antibody.

(3) The third layer is a complex of avidin and biotinylated peroxidase. Some biotin-binding sites on the avidin molecule of this third layer are not filled by biotinylated peroxidase (open sites), and bind to biotin on the second layer antibody. The peroxidase on the third layer is then developed by the diaminobenzidine (DAB). It is thus obvious that a very large amount of peroxidase will be attached to each primary antigen site.

Therefore the sequence of reagent application is primary antibody, biotinylated secondary antibody, avidin-biotin-peroxidase complex (ABC) and DAB/peroxide solution.

The peroxidase used in the ABC method has an iron-containing heme group (hematin) as its active site and in solution is coloured brown. Peroxidase activity in the presence of an electron donor first results in the formation of a complex of hematin and hydrogen peroxide, an enzyme-substrate complex, and then the oxidation of the electron donor (e.g., the chromogenic substance, DAB). Oxidation of DAB produces a brown end product which is highly insoluble in alcohol and other organic solvents. The electron donor provides the 'driving force' in continuing the catalysis of H₂O₂, while its absence effectively stops the reaction.

Similar to many other enzymes, peroxidase can be inhibited by excess substrate. The enzyme-substrate complex formed between peroxidase and excess hydrogen peroxide is catalytically inactive and in the absence of an electron donor, is reversibly
inhibited. The excess hydrogen peroxide and absence of an electron donor (e.g., DAB) brings about quenching of endogenous peroxidase activity, which is a necessary procedure used in the method, although cyanide and azide (sodium azide, an antibacterial agent present in buffers) are two other strong (reversible) inhibitors of peroxidase, which prevent binding of the peroxidase enzyme to its substrate and inhibit colour development. Some tissues such as liver, mammary gland, adipose tissue and kidney are relatively rich in endogenous biotin. Since free avidin or avidin conjugates employed in biotin-avidin systems will bind endogenous biotin, the endogenous biotin must be blocked to avoid non-specific staining. Therefore a negative control, in which the replacement of primary specific antibody by normal serum results in negative staining of the section, is necessary in the application of this ABC method and provides an evidence that endogenous avidin-binding activity is absent.

III.4.2. The procedure of the ABC method

The procedure of the ABC method is as follows:

1. Cells are cultured on 8-chamber slides (Nunc Inc. Naperville, Illinois U.S.), or on coverslips in a petri dish.

2. Remove excess medium:
   Gently rinse the slides with normal PBS (pH7.0-7.2, GIBCOBRL) three times to remove excess medium (by placing the slides in a PBS bath three times, each for 5 minutes).

3. Remove excess liquid from around the 8-chamber slides:
   Put the slides upside down on tissue paper and tap gently with fingers to drain off excess liquid. Keep the slides in a large box with water or wet tissue paper in the bottom to provide a humid atmosphere.

4. Fixation:
   The slides are immersed in cold 100% methanol and kept at -20°C for 6 minutes if the slides are plastic. Note that the 100% methanol is kept at -20°C as long as possible before use. If cells are cultured on coverslips in a petri dish (i.e., glass material is used) the dishes are filled with 50% acetone & 50% methanol and kept at -20°C for 6 minutes. Note that the mixture of 50% acetone & 50% methanol is made the day before use and stored at -20°C.
5. Allow to air-dry:
Remove the methanol (and acetone) and let the solvents evaporate completely from the sections for about 5 minutes by repeating step 3 of the procedure. If coverslips are used then encircle the cell area of coverslips with a Dako pen (Dako Ltd, Cambridge), which provides a circular barrier to the sections. This helps to keep the antibody solution within the circle and makes the drying after rinsing easier.

6. Rinse in PBS three times for 5 minutes each wash (i.e., repeat step 2 of the procedure).

7. Block endogenous peroxidase:
Block endogenous peroxidase by exposure for 30 minutes to a hydrogen peroxide solution. The solution contains: 0.3ml of 30% hydrogen peroxide; 70 ml 100% methanol and 30 ml PBS.

8. Rinse in PBS three times for 5 minutes each wash (by repeating step 2 of the procedure).

9. Background blocking:
Repeat step 3 to get rid of excess PBS and provide a humid atmosphere to prevent evaporation of antibody solutions.

Then block the background:
Add 1 drop of diluted normal non-immune horse serum (Vector Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) in which the biotinylated secondary antibody is made, to each chamber of 8-chamber slides. The slides are kept in the humidified box at all times. Incubate at room temperature for 20 minutes.

The normal serum is diluted according to instructions in the kit: add 1 drop of stock normal serum to 3.33ml of PBS (approximately 1ml=20 drops). Keep some of this diluted normal serum for later controls.

10. Tap off serum and wipe away excess by repeating step 3 of the procedure. Do not rinse the sections at this stage.
11. Apply primary antibody:
Add 1 drop of diluted primary antibody to each test chamber of the 8-chamber slides and add 1 drop of diluted normal serum (the same as that used in step 9) to each control chamber as a negative control.

Then put the slides back into the humidified box, replace the lid and leave overnight at 4°C in a cold room. Make sure the sections are fully covered with solution and each is marked (e.g., test or controls: culture medium, percentage of serum, antibody and dilution ratios).

The primary antibody, mouse monoclonal antibody to the N-terminal of α-smooth muscle actin, is diluted in a diluent of PBS containing 0.1% BSA (bovine serum albumin) and 0.1% NaN₃ (sodium azide), at ratios of 1:100; 1:200; and 1:400, respectively.

12. Repeat step 2 and 3. Do not let the section dry as PBS will precipitate on them.

13. Dilute the biotinylated secondary (anti-mouse) antibody: add 3 drops of normal serum stock in the kit to 10ml of PBS and add 1 drop biotinylated antibody stock. Then add 1 drop of this diluted secondary antibody to each chamber of the 8-chamber slides. Incubate at room temperature for 30 minutes.

Be particularly careful not to let the slides dry, so keep them in the covered humidified box. Immediately prepare step 14 after application of the secondary antibody.

14. Prepare a dilution of the ABC complex (Vector Elite ABC kit, Vector Laboratories) as the third layer reagent: add 1 drop of reagent A to 2.5ml PBS followed by 1 drop of reagent B. This has to be prepared 30 minutes before use. Reagents A and B are parts of the Vector Elite ABC kit.

15. After 30 minutes in the second layer antibody, repeat steps 2 and 3.

16. Add 1 drop of ABC complex to each chamber of the 8-chamber slides. Replace the slides in the covered humidified box, incubate at room temperature for 30 minutes.

17. Repeat step 2.
18. Wear gloves and make up (0.05%) DAB / (0.01%) peroxide solution by placing one DAB tablet (diaminobenzidine tetrahydrochloride, Sigma) and one peroxide tablet (Sigma) in 5ml (as instructed) distilled water in a universal bottle. Spin with a Whirlmixer to dissolve completely. Freshly make it just before use.

19. Development of peroxidase by DAB and hydrogen peroxide:
Develop peroxidase with the DAB/peroxide solution for 2-10 minutes by filling the chambers of the slides.

An optimal colour occurs after about 5 minutes. At two minutes intervals, take out the slides and wipe the back with tissue paper to check the reaction on the sections under a microscope. Do not let the colour become too intense: optimal staining is dark brown against a light background. If the reaction is not sufficient, replace the slides in DAB and check the reaction again after a few more minutes.

Note that the DAB development should be performed in a fume cupboard and gloves should be worn when handling DAB solutions as DAB may be carcinogenic. When throwing away DAB/peroxide solution, mix the solution as well as glassware and instruments with a few drops of bleach which contains sodium hypochloride to oxidise DAB. Pour the black oxidised DAB solution down a sink in the fume cupboard followed by plenty of water and put the DAB container in a bin near the fume cupboard.

20. When the colour is adequate (usually 5-10 minutes), transfer the slides to another container or slide rack and rinse them in tap water, i.e., tap water is run into the side of the container, and not directly run onto the slides, for 2-3 minutes.

21. Nuclear counterstain:
Counterstain nuclei lightly for 10-15 seconds with filtered Harris Haematoxylin (BDH) by putting the slides in a jar containing the dye as following procedure:
(A) filter the dye through filter paper.
(B) take off the upper chamber structure of the 8-chamber slides and put the slides in the jar containing dye for 10-15 seconds.
(C) rinse in running tap water by the method in step 20.
(D) dip rapidly in acid alcohol (about 1 second) to remove cytoplasmic staining by haematoxylin. The acid alcohol contains 1% HCl in 95% ethanol.
(E) rinse in running tap water.
(F) examine under a microscope. (If the staining is inadequate, repeat all the steps). If it is satisfactory then;
(G) Dehydrate the sections by passing them through graded alcohols: twice in 70% alcohol each for 1 minute, then twice in 100% alcohol each for 1 min, then

(H) Clear in solvent by twice dipping into xylene (BDH), each for 30 seconds. Note that if a plastic dish is used then ignore this last step.

22. Coverslip mounting:
After the second xylene dip, mount the section in an aqueous mountant DPX (DPX mountant, BDH, Poole Dorset), taking care not to let the solvent evaporate from the section before the mountant and coverslip are applied

Mounting follows the following procedure:

(A) Place 1 small drop of the mountant, which is kept in hot tap water when using it, on a coverslip. If cells are grown on a coverslip, place a drop of mountant on a slide and put the coverslip onto it.

(B) Lower the coverslip slowly onto the slide so that the drops meet and spread. This should avoid air bubbles over the section. Gently press out excess mountant with a mounting needle.

(C) Leave overnight at room temperature to dry. Put a heavy object on the slides to avoid warping when dry.

23. Count cells and take photos.

III.4.3. Results

The α-smooth muscle actin should be stained dark brown; nuclei should be blue. There should be no immunostaining on the negative control section which was incubated with normal serum.
IV. Whole-cell patch clamp experiments

The aim of this project was to characterise the electrical properties of the detrusor smooth muscle cell membrane using the patch-clamp technique.

IV.1. Equipment setup

The patch-clamp system included: (1) a headstage (CV-4; Axon Instruments, Inc., Burlingame, California, USA) with an electrode holder (Axon Instruments), (2) a CV-4 boost box (Axon Instruments), (3) Axopatch-1D clamp box (Axon Instruments), (4) an interface (TL-1 DMA Interface, Axon Instruments), (5) an oscilloscope (Gould 20 MHz Digital Storage, Type1421, UK), (6) a micromanipulator (Narishige Co. Ltd., Setagaya-Ku, Tokyo, Japan), (7) a pen recorder (EasyGraf TA240S; Gould Electronics Ltd, Hainault, Essex, U.K), and (8) a computer (Dell UltraScan VC5EN; Dell Computer Corp., Texas, U.S.A).

The headstage was connected to an electrode holder, which was used to hold the pipette. Inside the holder was a length of silver wire coated with AgCl. The headstage served as current-to-voltage (CV) converter and a model CV-4 1/100 was used for all experiments. For whole-cell recording, the feedback resistor in the CV-4 1/100 is 500 MΩ (gain b=1).

The boost box had a high frequency boost circuit with a gain proportional to frequency. This box was connected between the headstage probe and the Axopatch-1D clamp box and was plugged into the rear panel of the clamp box. The function of the boost box was to recover the output signal bandwidth that had been lost because of the presence of stray capacitance associated with feedback resistors in the headstage probe.

The Axopatch-1D was the central instrument for the patch-clamp experiments. It could be used for single-channel and whole-cell recording, with various function controls and in both the voltage-clamp and current-clamp modes.

The TL-1DMA interface, A-D converter was connected between the Axopatch-1D output and a computer. The analogue voltage and current output were converted to digital data (A to D) which were stored on-line by an IBM-compatible microcomputer. These data could be retrieved for subsequent analysis.
protocols designed using the pClamp software in the computer were delivered to the cell membrane via D-A conversion.

An oscilloscope was used to display the analogue outputs of membrane current and membrane potential. This was necessary to assist in obtaining a giga seal between the patch electrode and cell membrane.

A fine micromanipulator, on which the headstage was mounted, was carried on a second coarse manipulator (Prior Instruments Ltd). The use of two manipulators allowed coarse and fine positioning of the micropipette tip. A chart recorder was connected to the output of the Axopatch-1D. Permanent analogue data such as spontaneous membrane potentials under I=0 mode were recorded onto chart paper. An IBM compatible microcomputer was used 1) to acquire and store digital data for on-line as well as off-line analysis; 2) to design protocols and apply them for experiments. pClamp software was used to carry out these functions.

The headstage and a water-jacketed Perspex perfusion bath were placed on an inverted microscope stage, which in turn was placed on an anti-vibration air-table (TMC micro-g Vibration isolation system, Technical Manufacturing Corp. Massachusetts USA), and surrounded by a grounded Faraday cage to minimise electrical pickup. The base of the perfusion bath was formed by a thin glass microscope coverslip, the bath being illuminated from above, and observed using a inverted stage light microscope (Nikon TMS, Nikon Corporation, Tokyo, Japan). This system was arranged as following (see Figure 3.5).

**Figure 3.5.** Patch-clamp system setup.
The glass patch electrode was inserted into the pipette holder with the non-polarisable Ag/AgCl electrode wire immersed in the pipette filling solution. The reference electrode was made from a length of translucent PVC tubing (Portex Ltd, Hythe, UK) with a porous ceramic plug inserted into one end, filled with 3 M KCl solution and a Ag/AgCl electrode inserted in from another end. This reference electrode was placed in the superfusate at the downstream end of the bath. The non-polarisable Ag/AgCl electrodes connect the filling solution with the headstage to form a circuit.

IV.2. Patch electrodes

Patch electrodes (GC 150F; Clark Electromedical, Pangbourne, U.K) were pulled on the Brown-Flaming microelectrode puller (Model P-87; Sutter Instrument Co., San Francisco, California, USA) and polished over a customised glass-coated nickel/chrome heating element.

Electrodes were filled with intracellular solutions shown in Tables 3.8 to Table 3.12. (see Method II.2.). Filled electrodes usually had an average resistance of 5 MΩ, measured by applying a 20mV square pulse from the Rseal test control of the Axopatch 1D.

IV.3. Whole-cell configuration

Whole-cell recording was performed using the Axopatch 1D system. Glass patch pipettes were made and 1/3 filled with filling solution. The pipette was then inserted into a pipette holder which was connected to the headstage.

Under voltage-clamp mode, square voltage steps of +20mV at a frequency of 100Hz were imposed on the patch pipette so that the amplitude of the voltage and resulting current pulses could be monitored on the oscilloscope. The patch electrode was immersed in the bath solution, and the Rseal Test control was turned off. The junction potential between the pipette filling solution and the bath solution was nulled by adjusting the Manual Junction Null control to about zero and then switching the Auto Junction Null control to the Auto Track position in order to keep the pipette current equal to zero.

The patch pipette was advanced to approach the cell with care, and suction gently applied through the suction tube when the electrode was pressed against the
membrane. The indication for seal formation was that on the oscilloscope, the amplitude of the current response to the 20mV Rseal Test pulse disappeared, as the resistance of the system increased. After the giga-seal was formed, the Track control and Rseal Test control were turned off in order to set the holding potential at -60mV, using a manual dial, since the holding potential on the computer program was set to zero. The Rseal Test control was turned on again in order to adjust the Fast and Slow capacitance compensation controls when necessary. Gentle suction resulted in rupture of a membrane patch, and larger capacitive currents occurred in response to the 20mV Rseal Test step-command. The Series Resistance and Whole-cell CAP (i.e. whole cell capacitance) controls were adjusted to eliminate the large slow capacitance transients and the value recorded from the control of Whole-Cell Capacitance. The Rseal Test control was turned off to establish whole-cell recording.

In order to record the resting potential and spontaneous action potentials, the system was switched to the I=0 mode and all command and holding potential controls as well as capacitance compensation control turned off.

For membrane potential recordings including action potentials and passive membrane depolarisations, the system was switched to I-CLAMP mode after establishing whole-cell recording in V-CLAMP mode. In this I-CLAMP mode, the voltage commands became current commands of amplitude (10/β) pA/mV. Thus at β=1 usually used, 1mV voltage command corresponds to 10pA.

If whole-cell recording was established, special care was taken when switching between current-clamp and voltage-clamp modes. This is because in voltage-clamp the holding potential must be set to a negative value to hold the cell at rest. In current-clamp mode, the holding potential which acts as a DC current command must be equal to zero for the cell to be at rest. The intermediate "I=0" mode is provided to allow the system to be switched between current and voltage clamp (Axopatch-1D patch clamp theory and operation, Axon Instruments, Inc. California, USA). Therefore, to switch from voltage-clamp to current-clamp, I=0 mode was first selected to turn off the holding potential and any other commands (i.e., Rseal Test and capacitance compensation); then the I-CLAMP mode was selected. To switch from current-clamp to voltage-clamp, the system was first switched back to I=0 mode to adjust the holding potential back to -60mV, all other current commands were turned off and, then switched to voltage-clamp mode. The series resistance and whole cell capacitance settings were restored to their original values for compensation when necessary.
The headstage gain $\beta = 1$, output gain $\alpha = 1$ were set and signals were transferred at 0-2KHz bandwidth for all experiments.

IV.4. Cells

A small volume of cell suspension was placed in the perfusion bath. The cells were allowed to settle before superfusion was commenced with Tyrode's solution gassed with 95% $O_2$, 5% $CO_2$ at pH at 7.40±0.03 and at 36.5 ±0.5 °C. Solution flow was maintained at a rate of 2 ml/min by gravity feed from water jacketted glass reservoirs, passed to the bath via heated plastic tubing and removed by suction using a pump (HYFLO, Medcalf Bros Ltd, Herts, UK) allowing a constant bath level to be maintained.

The reference electrode was filled with 3 M KCl and put at the downstream end of the bath as described in section IV.1. of this chapter. Patch pipettes were filled with different intracellular filling solutions according to experimental need. Once the electrode was patched on the cell, it was lifted off the floor of the bath using the fine micromanipulator to allow the solution to flow evenly around the cell. After whole-cell recording was obtained, the system then switched between $I=0$, current-clamp and voltage-clamp modes to record resting membrane potential, passive and active properties and membrane currents. The microscope light was turned off before recording to minimise electrical pick-up.

IV.5. Current-clamp protocols

Protocols were designed and generated using the pClamp software package, pClamp / clampex version 5.5.1 (Axon Instruments Inc, 1984-90) on a Dell UltraScan VC5EN personal computer (Dell Computer Corp. Texas USA) and delivered to the amplifier via the TL-1 DMA interface. The computer was fitted with a Labmaster DMA mother board set (Labmaster TM100) for operation with pClamp. Voltage recordings produced by cells were temporally stored in computer RAM and written to hard disk for future analysis using pClamp software package (pClamp/clampan version 5.5.1;
IV.5.1. Measurement of cell membrane passive properties

IV.5.1.1. Cell capacitance $c_m$

The electrophysiological phenomena were explained on the basis that the cell interior behaves electrically as a simple electrolyte separated from an electrolytic extracellular solution by a thin polarisable bilayer cell membrane. This cell membrane then behaves as a capacitor in parallel with a resistor which is very sparingly permeable to ions in the resting state. Therefore the cell membrane has a high impedance compared to the intracellular electrolyte fluid. The membrane impedance may then be represented by an equivalent electrical circuit as shown in Figure 3.6.

![Figure 3.6. Parallel resistance and capacitance circuit used to represent the cell membrane impedance. $c_m$ stands for membrane capacitance, $r_m$ for membrane resistance, $i_i$ for ionic current, $i_c$ for capacitive current and $i_m$ for membrane currents. $i_m = i_i + i_c$.](image)

In this equivalent circuit, the cell membrane is considered as a capacitance $c_m$ and resistance $r_m$ in parallel. The membrane current $i_m$ is the sum of two components: the current $i_i$ which flows through the resistance $r_m$ and the current $i_c$ which is required to charge and discharge the capacitance $c_m$. Thus:

$$i_m = i_i + i_c \quad 3.6$$

$i_i$ is defined as the ratio $V/r_m$. The voltage established across the membrane is proportional to the amount of charge separation which has occurred, so that the capacitive current determines the rate at which the voltage changes. Therefore $i_c$ is given by $c_m dV/dt$, and equation 3.6 can be rewritten as,
Because of the membrane capacitance an instantaneous step of $i_m$ will ensure that the membrane potential $V$ will not achieve a steady-state value ($=i_m r_m$) instantaneously, but will achieve the limit exponentially with a time constant, $\tau_m$, given by

$$\tau_m = r_m c_m.$$ 

Therefore if a sudden step current $i_m$ is applied uniformly to a cell membrane under current-clamp mode, the determination of $\tau_m$ from the time course of the resultant voltage response will give the value for $r_m c_m$. Since the value of $r_m$ can be calculated from the steady-state voltage reached and $\tau_m$ can be obtained from exponential fit of the time course of voltage change, the value of $c_m$ be determined.

### IV.5.1.2. Measurement of passive membrane properties

Experiments were designed to obtain the value of the specific membrane resistance, $R_m$. Specific membrane values refer to that for 1 cm$^2$ of cell membrane and have units: $R_m$ (\(\Omega\cdot\text{cm}^2\)), $C_m$ (F/cm$^2$). Cell values of $r_m$ and $c_m$ have units \(\Omega\) and \(\text{F}\) respectively. However, note that:

$$\tau_m = r_m c_m = R_m C_m.$$

If it is assumed that $C_m=1$ \(\mu\text{F/cm}^2\) (Hille, 1992), $R_m$ can be calculated from $\tau_m$. Whole-cell recording was first achieved under voltage-clamp mode then switched to current-clamp. The protocol is shown in Figure 3.7. A 600 ms hyperpolarising current step (-30 to -20 pA) was applied to cells from all experimental groups: fresh and cultured cells from human and guinea-pig detrusor, and the corresponding membrane voltage change recorded.
Figure 3.7. Protocol for calculation of cell capacitance, membrane input resistance and corresponding membrane voltage change. The bottom trace is the step current change. The top trace is the corresponding membrane voltage change recording from a fresh isolated guinea-pig detrusor cell, resting potential -50 mV. The trace was fitted to equation 3.9.

The membrane potential change was fitted by a single exponential equation,

\[ V = V_{\text{max}} \exp(-t/\tau_m) + c \]  

where \( V_{\text{max}} \) is the steady-state value of voltage (mV), \( t \) is time in ms, \( c \) is a offset. This equation gave the value for the time constant \( \tau_m \) in ms. This process was performed using pClamp6/clampfit program.

The value of \( V_{\text{max}} \) can also be used to calculate the input resistance, \( r_{\text{inp}} \), of the cell \( = (V_{\text{max}}/i_m) \). Assuming the resistance offered by the cytoplasmic space is negligible this \( r_{\text{inp}} \) will be equivalent to the membrane resistance (\( r_m \)) of the cell. The value of \( r_m \) could then be used to give an independent estimate of \( c_m \) from the relation \( \tau_m = r_m c_m \). Assuming a constant specific membrane capacitance (\( C_m \)) of 1\( \mu \)F/cm\(^2\), the cell surface area, \( s \), will be given by \( c_m/C_m \).
IV.5.2. Measurement of action potentials, APs

The long duration pulse protocol for action potentials is shown in Figure 3.8. A train of 600ms depolarising steps from 5 to 45pA with 5pA increments were applied. An action potential was elicited when threshold current was applied to the cell. The action potential fired from such a resting state, (i.e., it was not spontaneous see Chapter 4 results) was measured from a threshold voltage $V_{th}$. $V_{th}$ was defined as the voltage at which the action potential trajectory deviated by 10% from the curve of a single exponential fit to a subthreshold depolarisation.

![Figure 3.8](image)

**Figure 3.8.** Long duration current protocols for determination of action potential threshold, $V_{th}$. An action potential was elicited from a depolarising step current (25pA). $V_{th}$ was defined as a 10% deviation from a single exponential fit to a subthreshold depolarisation using equation 3.10. The trace was recorded from a cultured cell of guinea-pig detrusor.

As shown in the top traces of Figure 3.8, the subthreshold depolarisation before action potential firing was fitted by a single exponential equation 3.10,

$$V = V_{\text{max}} [1 - \exp(-t/\tau_m)] + c$$  \hspace{1cm} 3.10

The short duration (50ms) pulse protocol was the same as that using long pulses. The action potential elicited by short pulses was suitable for determination of the relation
IV.6. Voltage-clamp protocols

Membrane currents were evoked under voltage-clamp mode using a voltage protocol designed as follows. The cell was held at -60 mV which was close to its resting potential. Protocols were also generated using pClamp software with the same procedure as that in current-clamp. Recorded currents produced by cells were also written to hard disk for future analysis using the pClamp software package.

IV.6.1. Measurement of inward and outward currents

For determination of inward and outward currents under physiological conditions electrodes were filled with KCl+5mM EGTA (see Table 3.8).

IV.6.1.1. Inward and outward current activation

IV.6.1.1.1. Voltage protocol

A "current activation protocol" for both inward and outward currents was used. 500 ms steps were used from a holding potential of -60 mV between -100 and +40 mV in 10 mV increments and are shown in Figure 3.9.

This protocol was also used to further elicit $i_{Ca}$, which was recorded using CsCl filled electrodes (see Tables 3.10 and 3.11).

![Current activation protocol](image)

**Figure 3.9.** Current activation protocol for both inward and outward currents.
IV.6.1.1.2. Current-voltage (i-v) relationships

Figure 3.10 shows a whole-cell current recording produced by a step from -60 to 0 mV using a KCl-filled electrode. The peak inward current was defined as the current difference between the peak of inward and the end of steady-state outward (i.e. point 3 referred to point 2 in Figure 3.10). The steady-state outward current was defined as the current difference between point 1 and point 2. The "net inward" current was the difference between point 3 and point 1.

![Current-voltage relationship diagram](image)

**Figure 3.10.** Whole-cell membrane currents recorded under the voltage-clamp mode using a "KCl + 5 mM EGTA" filled electrode.

The current-voltage (i-v) relationships of inward, outward and net inward currents were presented by plotting the currents versus the test voltages.

Note that the terms 'inward current', 'net inward current' are used throughout. Even when the two have the same value as when CsCl-filled electrodes are used. See also STOC protocol (section IV.6.2.2.).
IV.6.1.1.3. Determination of inward current activation kinetics

The steady-state current activation kinetics were determined from their i-v relationships. The membrane conductance \( g \) (1/r) was determined from the i-v relationship for each cell individually, according to

\[
g = \frac{i}{(V_m - V_{rev})} \tag{3.11}
\]

where, \( i \) is inward current at a given membrane potential \( V_m \), \( V_m - V_{rev} \) is the driving force acting on the inward current. \( V_{rev} \) is the reversal potential of the inward current, which was determined for each individual i-v relationship by extrapolation of a regression line, fitted by a least-squares fit, to the data points between 4-10 to 4-40mV.

The maximum whole cell conductance for inward currents, \( g_{max} \), was determined from the slope conductance, of the i-v relationship between +10 to +40mV, assuming that maximum activation was achieved over this range.

For more negative voltages the slope of the chord conductance, \( g \), was expressed as a ratio of \( g_{max} \). Values of \( g/g_{max} \) were fitted to the Boltzmann equation,

\[
d_{\infty} = \frac{1}{1+\exp(-(V_m-V_{0.5})/k_d)} \tag{3.12}
\]

where \( V_m \) is the membrane potential, \( V_{0.5} \) is a membrane potential at which conductance \( g \) is half \( g_{max} \) and \( k_d \) is a slope factor.

IV.6.1.1.4. Steady-state outward current activation kinetics

\( g_{max} \) was determined by the positive maximum chord conductance of the outward rectifier parts of steady-state i-v relationships between 0 to +40mV. This was obtained from the slope of the regression line, fitted by a least-squares fit to these data. The \( V_{rev} \) was determined for individual i-v relationship using steady-state outward currents. The \( g/g_{max} \) was plotted against the membrane voltages between -40 to +40mV where there was outward current, and fitted with the equation,

\[
g/g_{max} = \frac{(1-c)}{(1+\exp(-(V_m-V_{0.5})/k_n))} + c \tag{3.13}
\]
where $V_{0.5}$ is the membrane potential at which conductance $g$ is half maximum, $k_n$ is a slope factor and $c$ is offset.

**IV.6.1.2. Inward current inactivation**

**IV.6.1.2.1. Voltage protocol**

The effect of steady-state membrane potential $V_m$ on the proportional availability of inward current was investigated using an "inward current inactivation protocol", shown in Figure 3.11. The cell was preconditioned for 2 seconds over a range of voltages between -60 to +10mV in 10mV increments. Each of the preconditioning voltages was returned to -60mV (for 1ms) to allow the cell to be activated with the same driving force: from -60 to a test potential of +10mV for 500ms. Therefore the current response was only related to the different preconditioning voltages.

\[ f_\infty = \frac{1}{1 + \exp((V_m - V_{0.5})/k_f)} \]  

Where $V_{0.5}$ is the membrane potential at half maximal availability of inward current and $k_f$ is a slope factor.
IV.6.1.3. Steady-state outward current inactivation

IV.6.1.3.1. Voltage protocol

The inactivation kinetics of steady-state outward currents were studied by using the "outward current inactivation protocol" shown in Figure 3.12. This is similar to that in Figure 3.11 except that the range of preconditioning pulses was from -110 to +40mV and activated to +40mV.

![Figure 3.12. Steady-state outward current inactivation voltage protocol.](image)

IV.6.1.3.2. Outward current inactivation kinetics

Steady-state outward current was measured during the second test pulse and the current value obtained at the end of this test step, i, referred to holding potential $V_h$ -60mV. The maximum steady-state outward current $i_{\text{max}}$ was determined from the first few most negative preconditioning steps. The relationship between the ratio of $i/i_{\text{max}}$ and preconditioning voltage, $V_m$ was described by

$$i/i_{\text{max}} = (1-c)/(1+\exp((V_m-V_{0.5})/k_p))+c$$  \hspace{1cm} 3.15

where $V_{0.5}$ is the membrane potential at half maximum availability of steady state outward current, $k_p$ is a slope factor and $c$ is offset.
IV.6.2. Spontaneous transient outward currents, STOCs

V.6.2.1. Voltage protocol

For determination of the components of outward currents: spontaneous transient outward currents, STOCs, as well as steady-state $i_K$ components, patch electrodes were filled with KCl + 0.1mM EGTA (Table 3.9). The voltage protocol was similar to that in Figure 3.9, but with a 2-second duration test step, "STOC protocol", shown in Figure 3.13.

![Voltage protocol for measurement of STOCs as well as for steady-state $i_K$.](image)

**Figure 3.13.** Voltage protocol for measurement of STOCs as well as for steady-state $i_K$.

IV.6.2.2. Determination of pure STOCs

Pure STOCs were measured as the current difference between peak STOC"+" and early-state outward current "□" (Figure 3.14). The peak STOC was the difference between "+" and "X", automatically measured by the computer program (pClamp software package, pClamp/claamp version 5.5.1. Axon Instruments). The early-state (i.e. early steady-state) outward current was the current difference between "X" and "□". The steady-state (i.e. late steady-state) outward $i_K$ was measured at the end of pulses (i.e., "O"."X").
IV.6.3. Determination of low-voltage activated $i_{Ca}$, $i_{TCa}$

Experiments were designed to measure inward currents at membrane potentials more negative than the activation threshold for the L-type Ca$^{2+}$ current $i_{L_Ca}$. Patch electrodes were filled with CsCl+5mM EGTA (Table 3.11) in these experiments. Occasionally KCl+0.1mM EGTA filling (Table 3.9) was used to observe STOCs to corroborate the fact that the low-voltage activated inward current was a Ca$^{2+}$ current.

The holding potential for all experiments was set at -60mV but followed by different preconditioning pulses. The test pulses all commenced after a short (1ms) return to -60mV to ensure equal driving forces for the subsequent inward currents. The protocol is shown in Figure 3.15 (see below).
IV.6.3.1. $i_{TCa}$ and $i_{LCa}$ activation

IV.6.3.1.1. Voltage protocols

A simplified "voltage protocol A" shown in Figure 3.15 was used to dissect a low (negative) voltage activated $i_{Ca}$ from a high voltage activated $i_{Ca}$ by their different voltage dependence. The cell was activated from a 1 ms pulse -60mV to -30, -20, -10 and +10mV, after a 2-second preconditioning pulse of either -80 or -30mV. Preconditioning at -80mV was expected to activate L- and T-type Ca$^{2+}$ channels whilst preconditioning of -30mV would activate L-type Ca$^{2+}$ channels only.

![Figure 3.15. Voltage protocol A to elicit T-type and L-type Ca$^{2+}$ currents.](image)

IV.6.3.1.2. The i-v relationship and activation kinetics

$i_{TCa}$ was characterised pharmacologically using protocol A (Figure 3.15) and voltage protocol B in Figure 3.16. In voltage protocol B, activation pulses from between -100 to +50mV after a brief 1ms -60mV pulse, with 10mV increments, was preceded by a 2-second precondition voltage of -100mV.

![Figure 3.16. Voltage protocol B for activation of $i_{TCa}$ and $i_{LCa}$.](image)
The i-v relationships for $i_{TCa}$ and $i_{LCa}$ were obtained using protocol B and in the presence of a specific L-type $Ca^{2+}$ channel blocker, nifedipine, or a low concentration (0.1-0.2mM) of NiCl$_2$ to block T-type $Ca^{2+}$ channel.

IV.6.3.2. $i_{TCa}$ and $i_{LCa}$ inactivation

IV.6.3.2.1. Voltage protocol for $i_{LCa}$ inactivation

The voltage protocol for $i_{LCa}$ inactivation is shown in Figure 3.17. The protocol was applied to the cell while the solution contained 0.2mM NiCl$_2$. An activation step to +10mV was applied after a 1ms -60mV pulse and preceded with 2-second preconditioning pulses from -120mV to +30mV with 10mV increments.

Figure 3.17. Voltage protocol for $i_{LCa}$ inactivation.

Figure 3.18. Voltage protocol for $i_{TCa}$ inactivation.
IV.6.3.2.2. Voltage protocol for $i_{TCa}$ inactivation

The voltage protocol for $i_{TCa}$ inactivation is shown in Figure 3.18. The only difference between the two protocols for $i_{LCa}$ and $i_{TCa}$ was that the activation step was from -60 to -10mV (instead of +10mV), the maximum activation voltage for $i_{TCa}$.

V. Simultaneous measurement of intracellular Ca$^{2+}$ and STOCs

STOCs were assumed to result from a rise of the intracellular [Ca$^{2+}$] ([Ca$^{2+}$]$_i$), as Ca$^{2+}$ were released from the SR or entered via Ca$^{2+}$ channels. Experiments were designed to measure simultaneously intracellular Ca$^{2+}$ and STOCs by combining epifluorescence microscopy and patch-clamp recordings.

V.1. Intracellular loading of fluorescent indicators

The fluorescent indicator Fura-2 was used as index of [Ca$^{2+}$]$_i$. Fura-2 has a high affinity and selectivity for Ca$^{2+}$. The fluorescence excitation spectrum of Fura-2 shifts progressively to shorter wavelengths as [Ca$^{2+}$] increases; as a result, the emission intensity at 340nm excitation is increased and at 380nm excitation decreased. The emission spectrum is unaltered by Ca$^{2+}$ binding and is maximal at about 510nm (Grynkiewicz, et. al. 1985). Fura-2 was excited with light at 340 and 380nm and the emitted light was collected at 400-510nm. The ratio of fluorescence outputs at 340/380nm was used as an index of [Ca$^{2+}$]$_i$.

Fura-2 pentapotassium salt (K$_5$-Fura-2, Sigma Co.) was used in dual recordings of [Ca$^{2+}$] and membrane currents. It was dissolved in deionised water at a concentration of 10mM and stored frozen at -20°C in aliquots of 20μl. Intracellular loading was achieved via dialysis from the patch pipette. K$_5$-Fura-2 was mixed in the electrode filling solution of KCl+0.1mM EGTA (Table 3.9) at a concentration of 100μM. Such a concentration of K$_5$-Fura-2 was required to achieve a sufficient signal-to-noise ratio while not significantly attenuating the Ca$^{2+}$ rise (Wu and Fry 1998).
V.2. The experiment setup

A schematic diagram of the experimental setup for combined epifluorescence microscopy and patch-clamp is shown in Figure 3.19.

Figure 3.19. A schematic diagram of the experimental setup for combined epifluorescence microscopy and patch electrode recording for the measurement of $[\text{Ca}^{2+}]_i$ with fura-2 and membrane currents.
V.2.1. The microscope

Cells were placed in a heated (36.5±0.5°C) Perspex superfusion bath similar to the one used for the patch clamp experiments. The bath and patch-clamp amplifier were placed on an inverted stage microscope (Diaphot-TMD, Nikon Corporation, Tokyo, Japan), all were mounted on an air table (Ealing Optics Ltd., Watford, U.K.) and enclosed in a Faraday cage covered with a black blanket. Solutions flowed into the bath at a rate of 2ml/min through a system similar to that used with whole-cell patch clamp experiments (see section IV in this chapter), maintained at 36.5±0.5°C by a heated water jacket tubing system connected to a thermostatic pump. The solution in the bath was drawn to waste by suction to ensure a constant level of fluid.

V.2.2. Light source, transmission and collection

The epifluorescence system was obtained from Cairn Research Ltd. (Sittingbourne, Kent, U.K.). A xenon short arc light (75W XBO; Osram Ltd., Berlin, Germany) was used to provide a focused, high intensity and broad bandwidth light source, which was filtered prior to transmission to the microscope stage via a quartz fibre optic cable. Filtering of the light was achieved using a rotating wheel containing several radially distributed filters of different wavelengths (340, 360, 380, 400, 420 and 500nm). Two desired filters (340 and 380nm) were used for Fura-2, the others being masked with black tape. The wheel was rotated at 50rps. A dichroic mirror (400nm for Fura-2), situated in the substage of the microscope, directed the filtered excitation light beam through the objective of the microscope to be focused on the cell in the perfusion bath. Emitted light passed back through the objective and straight through the 400nm dichroic mirror. This emitted light was reflected by the microscope mirror and focused in the light tube prior to a variable rectangular diaphragm which could be adjusted to eliminate the extracellular light from around the test cell. A red light produced by filtering the microscope's own light source (>580nm) was used to position the cell and adjust the diaphragm. This independent light source was turned off during data acquisition. A second dichroic mirror (510nm) acted as a beam splitter. The higher wavelengths were directed to a CCD camera (Heimann CCD; Alrad Instruments Ltd., Newbury, Berks, U.K.) and the image displayed on a monitor to facilitate adjustment of the rectangular diaphragm. The lower wavelengths were transmitted to the photomultiplier (PMT) for signal collection. Light at 400-510nm was collected for Fura-2 fluorescence recordings.
V.2.3. Signal recording

The intensity of the emitted light at the two exciting wavelengths (340, 380nm) were recorded by two "sample and hold" amplifiers incorporated into the spectrophotometer system. The switch frequency between these two amplifiers was synchronised to the rotating filter wheel using an internal high frequency time clock so that sampling occurred only during the passage of the specific filter. The magnitude of the output could be adjusted by changing the voltage applied to the PMT. An analogue division circuit was also incorporated into the spectrophotometer system to produce a simultaneous ratio of the two signals which was displayed on an oscilloscope (Model DSO 1604; Gould Inc., Essex, U.K.). The emitted light intensities and ratio from two exciting wavelengths were recorded onto a pen recorder (Model E2007; Gould, France) or displayed and printed out on a storage oscilloscope (Model DSO 420; Gould). The currents as well as ratio of fluorescence signal were recorded in computer and written in hard disc for subsequent analysis.

V.2.4. The experimental protocol

The cell suspension was placed in the superfusate bath and allowed to settle on the bottom of the trough before the superfusion was turned on. Electrodes were filled with KCl+0.1mM EGTA+K5-fura-2 solution. The cell was lifted up before whole cell recordings were made, then the rectangular diaphragm was adjusted close to the cell and the microscope light being turned off. Under voltage-clamp mode, intracellular Ca^{2+} transients and membrane currents were measured simultaneously when voltage protocols or 20mM caffeine were applied to cells. The Ca^{2+} transients and membrane currents were recorded in computer for further analysis.

V.2.4.1. Voltage protocols

The voltage protocol used in simultaneous recordings was the same for STOC recordings in Figure 3.13.

A voltage ramp -90 to +60mV at 15mV/second was also applied to display intracellular Ca^{2+} accumulation and simultaneous outward current oscillations (see Figure 3.20).
Figure 3.20. A voltage ramp to determine intracellular Ca$^{2+}$ accumulation and outward current oscillations

V.2.4.2. Ca$^{2+}$ release from SR

The cell was held at different membrane potentials, and 20mM caffeine added to induce Ca$^{2+}$ release from SR. Membrane currents and Ca$^{2+}$ transients were simultaneously recorded to investigate the voltage dependence of STOC.

VI. General data handling and statistics

VI.1. Data storage and analysis

Cultured cells and the cells treated by immunocytochemistry were photographed.

All data acquisition from voltage-, and current-clamp recordings and simultaneous intracellular Ca$^{2+}$ recording, were made using the Clampex program (Axon Instruments). These analogue data were digitised through the TL1 A/D converter and written to hard disc (DAT files). Traces were analysed using the pClamp software package, (pClamp/clampan version 5.5.1; pClamp6/clampfit version 6.0.3.; Axon Instruments). Individual derived data were then stored in an ASCII file format for further analysis on an Apple Macintosh computer (Power Macintosh 6100/60, Apple computer Inc., California, U.S.A.). The stored original DAT files were also could directly read by an AxoGraph program (Axograph 3.0, Axon Instruments) to apply single exponential fits to subthreshold membrane depolarisations and the differentiations of the action potential waveform. Microsoft Excel (version 5.0 1985-1994, Microsoft Corporation, Soft-Art, Inc. USA) was used to spread the data for mathematical calculations and statistical for F-tests and corresponding t-tests.
Mathematical functions, graphical presentation and curve fitting were performed using Kaleidagraph (version 3.0.4, Synergy Software (PCs Inc.), Reading, UK).

Ratio signals were used as a qualitative index of $[\text{Ca}^{2+}]_i$ and therefore changes of $[\text{Ca}^{2+}]_i$. When the cell was stimulated, a transient increase in the ratio was apparent and this was assumed to correspond to the transient rise in the sarcoplasmic $[\text{Ca}^{2+}]$. The $[\text{Ca}^{2+}]_i$ rise and corresponding STOC were simultaneously recorded in computer and analysed using the same program as used in patch-clamp data.

VI.2. Statistics

The $F$-test was performed to test the change of variance before Student's $t$-test for the mean values. The Student's $t$-test was performed using the StatWorks™ program on the computer (Macintosh) or Statistics in Excel program when their variances were equal. When the variances were not equal, their means were tested using a Wilcoxon signed rank test or unequal variance Student's $t$-test (Statistics in Excel program).

Two tail tests were performed for all tested data sets and $p<0.05$ was accepted as a significance level. All data were expressed as mean±SD (one standard deviation) and presented in graphs as mean±SD; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. 
Chapter 4 Results

I. Characterisation of cultured smooth muscle cells

One objective of the present study was to characterise the electrophysiological properties of cultured detrusor smooth muscle cells. A prerequisite for this was to obtain smooth muscle cells in culture. Freshly isolated cells from muscle strips of guinea-pig detrusor wall mainly consist of three types of cells: epithelium, fibroblast and smooth muscle; while muscle strips of human detrusor are usually taken from large lumps of tissue after cystectomy and are less contaminated with epithelium. A pool of cells in a petri dish therefore may contain other cells in addition to smooth muscle cells. Thus standard protocols were used to minimise the contamination of other cells instead of smooth muscle.

First of all, the mucosa and epithelium of detrusor specimens were rigorously peeled away under the microscope before enzymatic dissociation in order to minimise the contamination of epithelial cells in culture.

Then to optimise the culture conditions for smooth muscle cell growth, D-valine minimum essential medium was used and supplemented with 10% fetal bovine serum. The cells from detrusor muscle strips, without mucosa and epithelium, were incubated in this selective medium. Cells settled without disturbance in the first week then grew for another 1-2 weeks to reach confluence.

In the D-valine medium, D-valine is substituted for L-valine which is an essential amino acid required for cell growth (Eagle, 1955; Litwin, 1974). D-amino acid oxidase, which catalyses the oxidative deamination of several D-amino acids, converts D-valine to 2-ketoisovaleric acid. This keto acid can then be converted to L-valine by the ubiquitous enzyme: branched-chain amino acid/2-oxoglutarate aminotransferase. Cells without D-amino acid oxidase lack a pathway for this transformation and should not be able to proliferate in a nutrient medium where D-valine is substituted for L-valine. Only those cells containing D-amino acid oxidase can convert the D-amino acid into its essential L-enantiomer and can grow in such nutrient medium.
This D-amino acid oxidase is not found in fibroblasts. Therefore the proliferation of fibroblasts could be inhibited by using this selective medium, whilst the detrusor smooth muscle cells survived (Gilbert and Migeon 1975).

Although these standard protocols were used to minimise the epithelium cell contamination and inhibit fibroblast growth, it was necessary to characterise further the cultured cells to ensure that these were smooth muscle cells with little contamination from other cell types.

I.1. The growth characteristics and morphology of cultured detrusor smooth muscle cells

The cultured cells started to proliferate after they attached on a petri dish; then they grew to confluence as a monolayer. Both human and guinea-pig detrusor smooth muscle cells became elongated during culture, and displayed a characteristic spindle-shaped morphology. The cells did not exhibit contact inhibition, instead they proliferated at a steady rate and formed multilayered arrays adopting a characteristic "hills and valleys" pattern of growth (see Figure 4.1 and 4.2). These cells grew vigorously in culture and were maintained in subculture for up to three passages in the present study.

The cultured cells kept their spindle shape like freshly isolated detrusor smooth muscle cells in HEPES-buffered Tyrode's solution as a short time of collagenase digestion was used (see Chapter 3 Methods).

I.2. Immunocytochemistry with monoclonal smooth muscle specific \(\alpha\)-actin antibody

The antibody, monoclonal anti-\(\alpha\)-smooth muscle actin A2547 (anti-\(\alpha\)-Sm-1) which recognises only smooth muscle \(\alpha\)-actin, was used to identify the cells as smooth muscle. This monoclonal antibody was produced using the NH\(_2\) terminal synthetic decapeptide of \(\alpha\)-smooth muscle actin as an immunogen. It is specific for the single isoform of \(\alpha\)-smooth muscle actin, and not for fibroblasts or any other non-muscle actin. The product shows wide reactivity with human and animal smooth muscle by indirect immunofluorescent labelling of blood vessels and human bladder (Chambers et al, 1996; Chamley et al 1977; Campbell et al 1979; Gown et al 1985; Omar et al
Figure 4.1. Guinea-pig detrusor smooth muscle cells in culture (top).
Figure 4.2. Human detrusor smooth muscle cells in culture (bottom).
Phase contrast microscopic appearance of cultured detrusor smooth muscle cells at confluence, showing a lack of contact inhibition by forming multilayered arrays adopting a "hills and valleys" pattern of growth.
Figure 4.3. Positive immunocytochemical staining for guinea-pig smooth muscle α-actin (top).

Figure 4.4. Serum control showing negative staining to antibody for smooth muscle α-actin (bottom).
However, two types of cells (short cells and long cells) could be observed under microscope during culture, which is consistent with the result reported by Lau and Chacko (1996, rabbit urinary bladder smooth muscle cells). In addition, human detrusor cell cultures needed a longer incubation time to reach confluence compared to guinea-pig detrusor cells. This could explain some difference between cultured human and guinea pig cells (see page 125), as the cell phenotype would be different when the culture period was different (see page 52).
1986; Tung and Fritz 1990). In the present study, the indirect immunoperoxidase labelling method which has been established in this Institute was used to determine smooth muscle actin (see Chapter 3 methods). Under the phase contrast microscope, the α-actin of the smooth muscle cells was then stained as dark brown (black colour in a black-and-white photo), contrasting it to blue stained nuclei. There was no immunostaining on the negative control section when it was incubated with normal serum instead of specific antibody.

Figure 4.3 shows positive immunocytochemical staining with a working dilution of 1:400 for guinea-pig smooth muscle α-actin as a black colour. Figure 4.4 is a negative control in which the same staining procedure was performed but serum instead of α-smooth muscle actin was used which excluded the possibility of any non-specific reaction in the assay. These results suggested that the cultured cells contained this characteristic α-actin and therefore were from smooth muscle.

I.3. Functional response to cholinergic stimulation

It is well established that detrusor contraction is under cholinergic nerve control. Activation of muscarinic M3 receptors on the detrusor smooth muscle cell membrane in response to acetylcholine or carbachol stimulation causes intracellular Ca\(^{2+}\) to rise via IP\(_3\)-induced Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) and results in muscle contraction.

The cultured cells from both guinea-pig and human detrusor responded to high concentrations of carbachol. As shown in Figures 4.5 and 4.6, brief application of 100μM carbachol caused transient rises of intracellular Ca\(^{2+}\) in cultured guinea-pig and human detrusor smooth muscle cells.

II. Membrane passive properties

Resting membrane potentials were recorded under I=0 (voltage follower) mode, the other parameters, membrane capacitance and resistance, were measured under current clamp mode by injection of a subthreshold current. Freshly isolated and cultured smooth muscle cells from guinea-pig and human detrusor were used. In this section, all experiments were performed under basic conditions, i.e., cells were superfused with normal Tyrode's solution at 36.5±0.5°C gassed with 5% CO\(_2\) + 95% O\(_2\) giving a
Figure 4.5. Intracellular Ca$^{2+}$ rise in response to 100μM carbachol in a cultured guinea-pig detrusor smooth muscle cell. The cell was exposed to carbachol during the time indicated by the bar above the trace.

Figure 4.6. Intracellular Ca$^{2+}$ rise in response to 100μM carbachol in a cultured human detrusor smooth muscle cell. The cell was exposed to carbachol during the time indicated by the bar above the trace.
pH 7.40±0.03; patch electrodes were filled with the basic filling solution of 
"KCl+5mM EGTA" (see Chapter 3 Methods). All data were expressed as mean±SD.

Figure 4.7 is an example of the voltage response obtained with a hyperpolarising 
current injection of 25 and 30pA. The solid lines are single exponential fits to the data 
for determination of the membrane time constant, $\tau_m$ (see Methods, section IV.5.1.2).

![Voltage response](image)

Figure 4.7. Membrane potential response to current steps under current-clamp mode.

II.1. Comparison of freshly isolated guinea-pig (fGP) and human (fH) 
detrusor smooth muscle cells

Resting membrane potential (RMP), specific membrane resistance ($R_m$), whole cell 
capacitance ($C_m$) and cell input resistance ($r_{inp}$) were recorded from both freshly 
isolated guinea-pig (fGP) and human (fH) detrusor smooth muscle cells. The RMP of 
cells from fGP was -58.6±11.3mV (n=146). The values were not significantly 
different from a normal distribution (Normality test, d=0.074, p=0.19) and are shown 
in Figure 4.8. The RMP of cells from fH was -53.1±14.5mV (n=30). The two mean 
values of RMP from fGP and fH were not significantly different. The $R_m$, $C_m$ and $r_{inp}$ 
were 138.2±29.7kΩ.cm², 50.1±17.1pF and 3.0±1.1GΩ in fGP (n=34), and 
132.7±36.6 kΩ.cm², 51.9±17.6pF and 2.9±1.3GΩ in fH (n=24). These parameters 
were also not statistically different in the human and guinea-pig cells. Data are shown 
in Table 4.1.
Figure 4.8. Histogram of the resting membrane potential (RMP) for freshly isolated guinea-pig detrusor smooth muscle cells, the values were not significantly different from a normal distribution (Normality test, d=0.074, p=0.19, n=146).

Table 4.1. The passive electrical properties of isolated detrusor smooth muscle cells from freshly isolated guinea-pig (fGP) and human (fH) cells.

<table>
<thead>
<tr>
<th></th>
<th>fGP</th>
<th>fH</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting membrane potential, RMP (mV)</td>
<td>-58.6±11.3</td>
<td>-53.1±14.5</td>
<td>ns</td>
</tr>
<tr>
<td>Specific membrane resistance*</td>
<td>138.2±29.7</td>
<td>132.7±36.6</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>Rm (kΩ.cm²)</td>
<td>138.2±29.7</td>
<td>132.7±36.6</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>Whole cell capacitance, Cm (pF)</td>
<td>50.1±17.1</td>
<td>51.9±17.6</td>
<td>ns</td>
</tr>
<tr>
<td>Cell input resistance, rinp (GΩ)</td>
<td>3.0±1.1</td>
<td>2.9±1.3</td>
<td>ns</td>
</tr>
<tr>
<td>Time constant, τm (ms)</td>
<td>138.2±29.7</td>
<td>132.7±36.6</td>
<td>ns</td>
</tr>
</tbody>
</table>

(1) *Calculated assuming a specific membrane capacitance of 1.0μF.cm⁻². (2) n=number of cells. (3) For all data, F-tests were first performed on the two data sets followed by two-tailed, unpaired t-tests for sets of equal or unequal variance.
II.2. Comparison of cultured guinea-pig (cGP) and human (cH) detrusor smooth muscle cells

Human and guinea-pig detrusor smooth muscle cells were cultured in the selective medium until they were confluent. Cells with a characteristic "hills and valleys" growth pattern were chosen for experiments. Cultured cells were detached from the petri dish by a short incubation with the same enzyme solution as used in fresh cell dissociation and then stored in HEPES-buffered Tyrode's solution for later use (for detail see Chapter 3 Methods).

The cells used for measurement of the passive electrical properties were primary cultured cells from guinea-pig (called cGP) and from human (called cH) detrusor smooth muscle. The same protocols were used as in experiments for the fGP and fH.

Data are shown in Table 4.2. The values of RMP and $c_m$ were similar in the two groups of cGP and cH. The specific resistance $R_m$ and cell input resistance $r_{imp}$ in cH were significantly lower than those in cGP. The different mean $R_m$ values in cH and cGP suggests that the number of ionic channels per cm² area of membrane opened at

<table>
<thead>
<tr>
<th></th>
<th>cGP</th>
<th>cH</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting membrane potential</td>
<td>$-42.1 \pm 15.4$ (86)</td>
<td>$-43.4 \pm 17.9$ (59)</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>RMP (mV)</td>
<td>(86)</td>
<td>(59)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>Specific membrane resistance*</td>
<td>$124.1 \pm 26.0$ (46)</td>
<td>$103.6 \pm 34.8$ (21)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>$R_m$ (kΩ.cm²)</td>
<td>(46)</td>
<td>(21)</td>
<td></td>
</tr>
<tr>
<td>Whole cell capacitance,</td>
<td>$59.5 \pm 13.4$ (46)</td>
<td>$73.0 \pm 30.6$ (21)</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>$c_m$ (pF)</td>
<td>(46)</td>
<td>(21)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>Cell input resistance,</td>
<td>$2.2 \pm 0.8$ (46)</td>
<td>$1.6 \pm 0.9$ (21)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>$r_{imp}$ (GΩ)</td>
<td>(46)</td>
<td>(21)</td>
<td></td>
</tr>
<tr>
<td>Time constant,</td>
<td>$124.1 \pm 26.0$ (46)</td>
<td>$103.6 \pm 34.8$ (21)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>$\tau_m$ (ms)</td>
<td>(46)</td>
<td>(21)</td>
<td></td>
</tr>
</tbody>
</table>

(1) * Calculated assuming a specific membrane capacitance of $1.0 \text{µF.cm}^{-2}$. (2) n=number of cells. (3) For all data, F-tests were first performed on the two data sets followed by two-tailed, unpaired t-tests for sets of equal or unequal variance.
rest were different at this stage of culture. This difference was mirrored by a similar inequality of the $r_{\text{imp}}$ values in cH and cGP. By contrast, there were no significant differences between human and guinea-pig cells before culture.

II.3. Comparison of freshly isolated and cultured human cells

Comparison of the electrical properties of freshly isolated (fH) and cultured (cH) human detrusor cells showed that RMP, $R_m$, $c_m$ and $r_{\text{imp}}$ were all significantly changed. The $r_{\text{imp}}$ and $R_m$ of cH were decreased and $c_m$ increased compared to fH as shown in Table 4.3. This suggested that the size of the cultured cell was larger, and that there were more ionic channels open at rest or the same number of channels had a larger conductance resulting in the larger specific membrane conductance ($1/R_m$).

The RMP in cH was more depolarised than that in fH. These results also suggested that the relative resting membrane conductance, and possibly the permeability to K$^+$ or Cl$^-$ were modified during culture.

<table>
<thead>
<tr>
<th>Table 4.3. Comparison of the passive electrical properties of freshly isolated (fH) and cultured (cH) human detrusor cells.</th>
</tr>
</thead>
<tbody>
<tr>
<td>tv</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Resting membrane potential</td>
</tr>
<tr>
<td>RMP (mV)</td>
</tr>
<tr>
<td>Specific membrane resistance*</td>
</tr>
<tr>
<td>$R_m$ (k$\Omega$.cm$^2$)</td>
</tr>
<tr>
<td>Whole cell capacitance,</td>
</tr>
<tr>
<td>$c_m$ (pF)</td>
</tr>
<tr>
<td>Cell input resistance,</td>
</tr>
<tr>
<td>$r_{\text{imp}}$ (G$\Omega$)</td>
</tr>
<tr>
<td>Time constant,</td>
</tr>
<tr>
<td>$\tau_m$ (ms)</td>
</tr>
</tbody>
</table>

(1) * Calculated assuming a specific membrane capacitance of 1.0$\mu$F.cm$^{-2}$. (2) n=number of cells. (3) For all data, F-tests were first performed on the two data sets followed by two-tailed, unpaired t-tests for sets of equal or unequal variance.
II.4. Comparison of freshly isolated and cultured guinea-pig cells

The trend of modification of passive electrical properties during culture also existed with guinea-pig cells. Comparison of data from freshly isolated (fGP) and cultured (cGP) guinea-pig detrusor cells are listed in Table 4.4 and show highly significant differences for all parameters.

**Table 4.4.** Comparison of the passive electrical properties of freshly isolated (fGP) and cultured (cGP) guinea-pig detrusor cells.

<table>
<thead>
<tr>
<th></th>
<th>fGP</th>
<th>cGP</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n)</td>
<td>(n)</td>
<td></td>
</tr>
<tr>
<td>Resting membrane potential</td>
<td>-58.6±11.3</td>
<td>-42.1±15.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RMP (mV)</td>
<td>(146)</td>
<td>(86)</td>
<td></td>
</tr>
<tr>
<td>Specific membrane resistance*</td>
<td>138.2±29.7</td>
<td>124.1±26.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>R_m (kΩ.cm²)</td>
<td>(34)</td>
<td>(46)</td>
<td></td>
</tr>
</tbody>
</table>

III. Membrane Action potentials

Action potentials (APs) were elicited and measured under current-clamp mode in cells at a membrane potential between -50 to -60mV for all experimental groups. Short-(40-60ms) and long-duration (600ms) stimulating step currents were used to excite cells. However, the data of AP parameters in Tables 4.5 to 4.12 were collected from APs induced by long-duration pulses, as these allowed the most accurate determination of action potential threshold (V_th).
All experiments were carried out under the basic conditions, i.e., normal Tyrode's solution at 36.5±0.5°C, gassed with 5% CO$_2$ + 95% O$_2$ giving a pH of 7.40±0.03; patch electrodes were filled with the basic filling solution of "KCl+5mM EGTA". All data were expressed as mean±SD.

III.1. Measurement of action potentials in freshly isolated guinea-pig (fGP) and human (fH) cells

III.1.1. Spontaneous action potentials

Action potentials (APs) occurred spontaneously in some freshly isolated cells from both guinea-pig and human detrusor smooth muscle, but not from cultured cells, using KCl-filled and Cs-filled patch electrodes under the I=0 (voltage follower) mode. Spontaneous APs recorded with the KCl filling solution, most often occurred from membrane potentials of -40 to -25mV with a variable AP duration at 50% repolarisation (APD$_{50}$). Occasionally spontaneous APs were recorded at more negative potentials of about -50mV. At membrane potentials depolarised over -25mV only slow waves of membrane potential, without spontaneous APs, were observed. This may be due to inactivation of inward currents responsible for generating the action potential. Traces were recorded on a pen-recorder as shown in Figure 4.9.

A:
Figure 4.9. A: A train of spontaneous APs recorded from a freshly isolated guinea-pig detrusor cell under I= 0 mode. B: Spontaneous APs recorded from a freshly isolated human detrusor cell.

III.1.2. Action potentials elicited by a current stimulus

III.1.2.1. Action potentials elicited by a short-duration of step current

Cells from both freshly isolated guinea-pig (fGP) and human (fH) cells could be stimulated to fire action potentials (APs) by injecting a short-duration step current under current-clamp mode. The APs exhibited a slow rising phase with a fast repolarisation as seen in Figure 4.10. and Figure 4.11. Table 4.5 shows the excitation currents to initiate APs from both groups of cells.

Table 4.5. Comparison of action potential threshold currents from short pulses in freshly isolated guinea-pig (fGP) and human (fH) cells.

<table>
<thead>
<tr>
<th>Action potential stimulus</th>
<th>fGP Mean±SD</th>
<th>fH Mean±SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration (ms)</td>
<td>53.5±4.8</td>
<td>54.4±5.1</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(63)</td>
<td>(16)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>Strength (pA)</td>
<td>43.7±18.5</td>
<td>51.3±12.6</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(63)</td>
<td>(16)</td>
<td>(&gt;0.05)</td>
</tr>
</tbody>
</table>

(1) n is number of cells. (2) For all data, F-tests were first performed on the two data sets followed by two-tailed, unpaired t-tests for sets of equal or unequal variance.
Figure 4.10. An action potential recorded from a freshly isolated guinea-pig cell. A 10pA, 50ms of square current was used to initiate the AP under current clamp mode.

Figure 4.11. An action potential recorded from a freshly isolated human cell. The cell was excited by a 40pA, 50ms square current.
III.1.2.2. Action potentials elicited by a long-duration of step current

In practice, it was sometimes hard to determine the voltage threshold of action potentials (APs) elicited by a short-duration current since the time course was too short to separate AP initiation from the stimulus signal. Therefore it was not suitable to use exponential fitting to the voltage waveform to distinguish the subthreshold depolarisation phase from the upstroke of the AP. This was however more easily achieved by applying long-duration step currents.

The strength of stimulus current required to initiate an AP was reduced as the pulse duration was increased. A 600ms series of step currents triggered a group of APs in cells from freshly isolated guinea-pig and human cells and is illustrated in Figures 4.12 and 4.13. A subthreshold change of membrane potential or a single AP developed when a smaller stimulus strength was used, but faster firing APs or a family of repetitive APs occurred when more intensive currents were injected. Repetitive APs were more often seen in guinea pig than in human cells.

![Graph showing action potentials](image)

**Figure 4.12.** Action potentials (APs) in a freshly isolated guinea-pig cell. The APs were elicited by 600ms duration step currents from 15 to 45pA with 5pA increments. A subthreshold depolarisation occurred when a 15pA current was supplied, a single AP occurred at 20pA and repetitive APs occurred as 25pA or more.
Figure 4.13. Action potentials (APs) in a freshly isolated human cell. The APs were elicited by 600 ms step currents of strength 5 to 25 pA. A 5 pA stimulus current initiated the longest latency AP in this cell. There were no repetitive APs even at a stimulus current of 25 pA, but the latency decreased with increasing current strength.

Figure 4.14. The effect of verapamil on the AP in a freshly isolated guinea-pig cell. The APs were elicited by a 600 ms duration, 25 pA step current. 20 μM verapamil reversibly inhibited the AP and inward current (shown in the inset). Both AP and current were recorded from the same cell.
III.1.2.3. The effect of Ca\textsuperscript{2+} channel blockers on action potentials

The action potential (AP) upstroke was blocked by the specific L-type Ca\textsuperscript{2+} channel antagonists, 20\textmu M verapamil or 5\textmu M nifedipine. The inward currents recorded from the same cell were also blocked, suggesting that the AP upstrokes were supported by Ca\textsuperscript{2+} current entry through L-type Ca\textsuperscript{2+} channels (see also section VIII. T-type Ca\textsuperscript{2+} current).

Figure 4.14 illustrates the reversible inhibitory effect of 20\textmu M verapamil on the AP upstroke recorded from a freshly isolated guinea-pig cell under current-clamp mode. The inset shows that inward current from the same cell recorded under voltage-clamp mode was also inhibited by 20\textmu M verapamil.

III.1.2.4. Comparison of action potentials in cells from freshly isolated guinea-pig and human cells

For a more objective comparison of action potentials (APs) from human and guinea-pig cells, the APs were elicited, under current-clamp mode, from membrane potentials between -50 to -60mV. Using this potential range, a single AP or the first AP in a train in response to one step current were chosen for further analysis. This was done as these APs, called resting-state APs, would be obtained in response to a brief voltage change applied to the resting cell.

To estimate the voltage threshold for AP initiation, the passive membrane potential change in response to a depolarising current was fitted by an equation:

\[ V = V_{\text{max}} [1-\exp(-t/\tau_m)]+c \]

where, \( V_{\text{max}} \) is an extrapolated steady-state value of voltage in mV, \( t \) is time in ms, \( \tau_m \) is time constant in ms, \( c \) is offset. The point at which the real membrane voltage trajectory deviated by 10% from this subthreshold fitting was defined as AP threshold (\( V_{\text{th}} \)) (see Methods, Figure 3.8). Then all the measurements, such as AP amplitude, AP overshoot, AP duration at 50% repolarisation (APD\textsubscript{50}) and AP time to peak (TTP), were calculated from \( V_{\text{th}} \).

The data are shown in Table 4.6. The mean stimulus strength for AP initiation was not significantly different in human and guinea-pig cells. The AP threshold, APD\textsubscript{50}
and AP repolarisation time (the time from peak of overshoot to 50% repolarisation were not significantly different. Moreover AP overshoot, amplitude and upstroke velocity were significantly smaller, and time to peak (TTP) significantly longer in freshly isolated human (fH) than that in guinea-pig (fGP) cells. This suggests that the net inward current in human cells is smaller than in guinea-pig cells (see section V.1.1.2).

Table 4.6. Comparison of APs from freshly isolated guinea-pig (fGP) and human (fH) cells.

<table>
<thead>
<tr>
<th></th>
<th>fGP</th>
<th>fH</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n)</td>
<td>(n)</td>
<td></td>
</tr>
<tr>
<td>Stimulus strength (pA)#</td>
<td>37.9±10.4</td>
<td>41.3±8.9</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(37)</td>
<td>(16)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP threshold, Vth (mV)</td>
<td>-24.8±5.3</td>
<td>-26.4±5.1</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(37)</td>
<td>(16)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP overshoot (mV)</td>
<td>25.2±9.7</td>
<td>13.6±7.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(37)</td>
<td>(16)</td>
<td></td>
</tr>
<tr>
<td>AP amplitude (mV)*</td>
<td>49.9±11.6</td>
<td>40.0±8.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>(37)</td>
<td>(16)</td>
<td></td>
</tr>
<tr>
<td>AP duration 50, APD50 (ms)**</td>
<td>63.9±28.5</td>
<td>67.1±37.9</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(36)</td>
<td>(16)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP time to peak, TTP (ms)*</td>
<td>31.4±14.2</td>
<td>44.6±15.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>(37)</td>
<td>(16)</td>
<td></td>
</tr>
<tr>
<td>AP repolarisation (ms)***</td>
<td>32.1±19.7</td>
<td>22.5±22.9</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(36)</td>
<td>(16)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP upstroke velocity (V/s)</td>
<td>3.6±1.9</td>
<td>1.9±0.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>(37)</td>
<td>(16)</td>
<td></td>
</tr>
<tr>
<td>Peak inward current (pA/pF)+</td>
<td>-5.8±2.1</td>
<td>-4.8±2.3</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(37)</td>
<td>(16)</td>
<td>(&gt;0.05)</td>
</tr>
</tbody>
</table>

(1) # Cells excited by 600ms step currents with the intensity shown here.
(2) * measured from Vth to AP peak.
(3)** measured from Vth to 50% AP repolarisation.
(4) *** measured from AP peak to 50% AP repolarisation.
(5) + inward current at voltage of AP peak.
(6) For all data, F-tests were first performed on the two data sets followed by two-tailed, unpaired t-tests for sets of equal or unequal variance.
Page135:line3 of paragraph2, "All APs were elicited by a current stimulation." replaced by "APs could be induced by current stimulation in some cultured cells when the electrodes were filled with high K⁺."
III.2. Measurement of action potentials in cultured guinea-pig (cGP) and human (cH) cells

Primary cultured cells from guinea-pig (cGP) and human (cH) detrusor smooth muscle were used, as in section II.2. In later section experiments with passaged cultured cells are also described.

No spontaneous action potentials (APs) were found in any cultured cells in the present study including primary cultured and passaged cells from either human or guinea-pig detrusor. All APs were elicited by a current stimulation. Repetitive APs seen in freshly isolated cells were also not recorded from cultured cells.

III.2.1. Action potentials elicited from cultured guinea-pig and human detrusor cells by short and long pulses

Action potentials (APs) were elicited by short step currents in cultured cells from guinea-pig and human detrusor as shown in Figures 4.15 and 4.16; and by long step currents are shown in Figures 4.17 and 4.18. The APs in cultured cells whether from guinea-pig or human tissue had a prolonged APD_{50} because of the much slower repolarisation phase, compared to APs recorded from freshly isolated cells.

![Figure 4.15. An action potential (AP) from a cultured guinea-pig cell elicited by a 50ms 60pA step current.](image-url)
Figure 4.16. An action potential (AP) from a cultured human cell. The AP was elicited by a 20ms 150pA step current.

Figure 4.17. Action potentials (APs) from a cultured guinea-pig cell. The APs were elicited by 600ms step currents recorded in the same cell as in Figure 4.15.
Figure 4.18. An action potential (AP) from a cultured human cell. The recordings were elicited by 500ms step currents recorded in the same cell as in Figure 4.16.

III.2.2. The effect of Ca$^{2+}$ channel blockers on action potentials

In freshly isolated cells from guinea-pig and human detrusor smooth muscle, it has been shown that the AP upstroke was caused by Ca$^{2+}$ entry, since both the AP and inward current recorded in the same cell were abolished by superfusion with specific L-type Ca$^{2+}$ channel blockers. This was also confirmed in cultured detrusor smooth cells as shown in Figures 4.19 and 4.20. When the cell was superfused by 5μM nifedipine, the AP was blocked and left passive membrane responses only. The insets show inward currents obtained from the same cells under voltage-clamp which were also abolished by 5μM nifedipine. It was supposed that there was L-type Ca$^{2+}$ current only when the cell was held at -30, but both L- and T-type Ca$^{2+}$ currents were activated if the cell was held at -80mV (see section VIII, T-type Ca$^{2+}$ current). However both currents activated at either -30 (the smaller current in inset, Figure 4.19) or -80mV (the larger current in inset, Figure 4.19) were sensitive to 5μM nifedipine. These experiments suggest that the AP upstroke was caused by Ca$^{2+}$ flow through the L-type Ca$^{2+}$ channel in cultured cells.
**Figure 4.19.** Action potentials (APs) produced by 600ms step currents in a cultured guinea-pig cell. The inset shows inward currents recorded from the same cell. The patch electrode was filled with the CsCl+5mM EGTA solution in this example to block outward current.

**Figure 4.20.** The effect of nifedipine on action potentials (APs) in a cultured guinea-pig cell. The traces was recorded from the same cell as in Figure 4.19. The APs shown in Figure 4.19 were abolished after superfusion with 5μM nifedipine for 7 minutes and the inward current recorded from the same cell was also blocked as shown in the inset.
III.2.3. Comparison of action potentials from cultured guinea-pig and human cells

Resting-state action potentials (APs) from cultured cells of both guinea-pig and human detrusor were compared; all APs were elicited by 600ms step currents.

APs from both cultured cells exhibited a much slower repolarisation phase than from freshly isolated cells. In particular the repolarisation phase of the AP in some cells from human was very slow. One possibility is that AP duration could be artificially prolonged by longer stimulation pulses. Thus APD₅₀ values are not quoted in the data comparing cultured human and guinea-pig cells (Table 4.7 below).

Table 4.7. shows the AP variables measured from both group cells. There were no significant differences in the mean value of the parameters between the two groups.

<table>
<thead>
<tr>
<th></th>
<th>cGP Mean±SD</th>
<th>cH Mean±SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n)</td>
<td></td>
<td>(n)</td>
<td></td>
</tr>
<tr>
<td>Threshold stimulus strength (pA)</td>
<td>39.1±11.0</td>
<td>38.6±14.9</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(27)</td>
<td>(7)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP threshold, Vₚₘₜ (mV)</td>
<td>-15.6±6.0</td>
<td>-14.4±3.8</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(26)</td>
<td>(7)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP overshoot (mV)</td>
<td>19.5±13.5</td>
<td>18.7±13.0</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(27)</td>
<td>(7)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP amplitude (mV)</td>
<td>35.0±16.7</td>
<td>33.1±11.4</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(26)</td>
<td>(7)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP time to peak, TTP (ms)</td>
<td>68.5±35.3</td>
<td>83.7±57.2</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(26)</td>
<td>(7)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP upstroke velocity (V/s)</td>
<td>1.3±0.8</td>
<td>1.3±0.5</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(27)</td>
<td>(7)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>Peak inward current (pA/pF)</td>
<td>-2.3±1.3</td>
<td>-2.1±0.8</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(27)</td>
<td>(7)</td>
<td>(&gt;0.05)</td>
</tr>
</tbody>
</table>
Comparison of action potentials in freshly isolated (fH) and cultured (cH) human cells

Comparison of AP parameters was also made in cells from human detrusor smooth muscle before and after cell culture and are shown in Table 4.8. As described in section III.2.3., the AP repolarising phase in the cells from cH was much longer than that in fH, suggesting that the outward current in cH would be much smaller. The AP overshoot, amplitude, time to peak and maximum rate of AP upstroke velocity were not different after culture compared to fresh cells. This suggests that net inward current in cultured cells did not differ from that in the fresh cells. However AP threshold, Vth, and peak inward current were significantly less in cultured cells. The disparity between the lack of reduction of upstroke velocity and the significant reduction of the peak inward current will be considered in section IV, V and the Discussion.

Table 4.8. Comparison of action potential (AP) properties in cultured and freshly isolated human cells. For all data, F-tests were first performed on the two data sets followed by two-tailed, unpaired t-tests for sets of equal or unequal variance.

<table>
<thead>
<tr>
<th></th>
<th>fH Mean±SD</th>
<th>cH Mean±SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n)</td>
<td>(n)</td>
<td></td>
</tr>
<tr>
<td>Threshold stimulus strength (pA)</td>
<td>41.3±8.9</td>
<td>38.6±14.9</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>(7)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP threshold, Vth (mV)</td>
<td>-26.4±5.1</td>
<td>-14.4±3.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>(7)</td>
<td></td>
</tr>
<tr>
<td>AP overshoot (mV)</td>
<td>13.6±7.3</td>
<td>18.7±13.0</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>(7)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP amplitude (mV)</td>
<td>40.0±8.4</td>
<td>33.1±11.4</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>(7)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP duration 50, APD50 (ms)</td>
<td>67.1±37.9</td>
<td>270.9±210.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>(7)</td>
<td></td>
</tr>
<tr>
<td>AP time to peak, TTP (ms)</td>
<td>44.6±15.7</td>
<td>83.7±57.2</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>(7)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP upstroke velocity (V/s)</td>
<td>1.9±0.7</td>
<td>1.3±0.5</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>(7)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>Peak inward current (pA/pF)</td>
<td>-4.8±2.3</td>
<td>-2.1±0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>(7)</td>
<td></td>
</tr>
</tbody>
</table>
III.4. Comparison of action potentials in freshly isolated (fGP) and primary cultured (cGP) guinea-pig cells

The same comparisons were made between freshly isolated and cultured guinea-pig cells to help understand what electrophysiological alterations had occurred during cell culture. As with human cells, $V_{th}$ was significantly shifted to less negative potentials after culture, suggesting that some properties manifested in fresh cells were not maintained at this stage of culture. Table 4.9 also shows that not only the AP depolarising phase (the maximum rate of AP upstroke and TTP), but also the repolarising phase (AP repolarisation) were altered significantly after culture. This suggested that both net inward current, responsible for AP upstroke, and outward current responsible for repolarisation were significantly changed during culture (see section V, Net currents).

**Table 4.9.** Comparison of action potential (AP) properties in freshly isolated (fGP) and primary cultured (cGP) guinea-pig cells. For all data, F-tests were first performed on the two data sets followed by two-tailed, unpaired t-tests for sets of equal or unequal variance.

<table>
<thead>
<tr>
<th></th>
<th>fGP Mean±SD</th>
<th>cGP Mean±SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threshold stimulus strength (pA)</td>
<td>37.9±10.4 (37)</td>
<td>39.1±11.0 (27)</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>AP threshold, $V_{th}$ (mV)</td>
<td>-24.8±5.3 (37)</td>
<td>-15.6±6.0 (26)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AP overshoot (mV)</td>
<td>25.2±9.7 (37)</td>
<td>19.5±13.5 (27)</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>AP amplitude (mV)</td>
<td>49.9±11.6 (37)</td>
<td>35.0±16.7 (26)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AP duration 50, $APD_{50}$ (ms)</td>
<td>63.9±28.5 (36)</td>
<td>139.2±69.5 (15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AP time to peak, TTP (ms)</td>
<td>31.4±14.2 (37)</td>
<td>68.5±35.3 (26)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AP repolarisation (ms)</td>
<td>32.1±19.7 (36)</td>
<td>81.5±43.1 (15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AP upstroke velocity (V/s)</td>
<td>3.6±1.9 (37)</td>
<td>1.3±0.8 (27)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Peak inward current (pA/pF)</td>
<td>-5.8±2.1 (37)</td>
<td>-2.3±1.3 (27)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
III.5. Comparison of action potentials in freshly isolated (fGP) and passaged (cGP\textsubscript{1-3}) guinea-pig cells

Primary cultured guinea-pig cells were subcultured (passaged) up to three times. At each stage sample cells were prepared for electrophysiological experiments by collagenase dissociation. The passaged cells are denoted cGP\textsubscript{1-3}, the subscript depending on the number of passages. Data for cells from the three passages have been collected into one data set, cGP\textsubscript{1-3} and are shown in Table 4.10.

### Table 4.10. AP parameters in freshly isolated (fGP) and passaged (cGP\textsubscript{1-3}) guinea-pig cells

<table>
<thead>
<tr>
<th></th>
<th>fGP</th>
<th>cGP\textsubscript{1-3}</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n)</td>
<td>(n)</td>
<td></td>
</tr>
<tr>
<td>Threshold stimulus strength (pA)</td>
<td>37.9±10.4</td>
<td>45.0±0.0</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(37)</td>
<td>(8)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP threshold, V\textsubscript{th} (mV)</td>
<td>-24.8±5.3</td>
<td>-15.5±3.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(37)</td>
<td>(8)</td>
<td></td>
</tr>
<tr>
<td>AP overshoot (mV)</td>
<td>25.2±9.7</td>
<td>17.8±11.4</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(37)</td>
<td>(8)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP amplitude (mV)</td>
<td>49.9±11.6</td>
<td>33.3±12.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(37)</td>
<td>(8)</td>
<td></td>
</tr>
<tr>
<td>AP duration 50, APD\textsubscript{50} (ms)</td>
<td>63.9±28.5</td>
<td>91.7±49.2</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(36)</td>
<td>(6)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP time to peak, TTP (ms)</td>
<td>31.4±14.2</td>
<td>61.5±25.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>(37)</td>
<td>(8)</td>
<td></td>
</tr>
<tr>
<td>AP repolarisation (ms)</td>
<td>32.1±19.7</td>
<td>40.0±30.6</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(36)</td>
<td>(6)</td>
<td>(&gt;0.05)</td>
</tr>
<tr>
<td>AP upstroke velocity (V/s)</td>
<td>3.6±1.9</td>
<td>1.3±0.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>(37)</td>
<td>(8)</td>
<td></td>
</tr>
<tr>
<td>peak inward current (pA/pF)</td>
<td>-5.8±2.1</td>
<td>-3.2±2.1</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

(1) For all data, F-tests were first performed on the two data sets followed by two-tailed, unpaired t-tests for sets of equal or unequal variance.

(2) APs in cGP\textsubscript{1-3} was also elicited by long-duration (600ms) step currents. Parameters measured from V\textsubscript{th} as for other cells. Electrodes were filled with the same solution as other cells.
Compared to freshly isolated cells (fGP) the $V_{\text{th}}$ in passaged cells (cGP$_{1-3}$) was also shifted to less negative values. The slower maximum rate of AP upstroke velocity and TTP in cGP$_{1-3}$ suggest that the net inward current in cGP$_{1-3}$ was smaller. However the AP repolarisation time in cGP$_{1-3}$ was not significantly different from that in freshly isolated cells which suggests that the outward currents recovered compared to primary cultured cells (cGP, see Table 4.11).

### III.6. Comparison of action potentials in cells from primary cultured (cGP) and passaged (cGP$_{1-3}$) guinea-pig cells

Table 4.11 shows more clearly that there are no significant differences between cGP and cGP$_{1-3}$ except for a significant decrease of AP repolarisation time.

#### Table 4.11. AP properties in primary cultured (cGP) and passaged (cGP$_{1-3}$) guinea-pig cells.

<table>
<thead>
<tr>
<th></th>
<th>cGP Mean±SD</th>
<th>cGP$_{1-3}$ Mean±SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n)</td>
<td>(n)</td>
<td></td>
</tr>
<tr>
<td>Threshold stimulus strength (pA)</td>
<td>39.1±11.0 (27)</td>
<td>45.0±0.0 (8)</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>AP threshold, $V_{\text{th}}$ (mV)</td>
<td>-15.6±6.0 (26)</td>
<td>-15.5±3.6 (8)</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>AP overshoot (mV)</td>
<td>19.5±13.5 (27)</td>
<td>17.8±11.4 (8)</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>AP amplitude (mV)</td>
<td>35.0±16.7 (26)</td>
<td>33.3±12.5 (8)</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>AP duration 50, APD$_{50}$ (ms)</td>
<td>139.2±69.5 (15)</td>
<td>91.7±49.2 (6)</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>AP time to peak, TTP (ms)</td>
<td>68.5±35.3 (26)</td>
<td>61.5±25.3 (8)</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>AP repolarisation (ms)</td>
<td>81.5±43.1 (15)</td>
<td>40.0±30.6 (6)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>AP upstroke velocity (V/s)</td>
<td>1.3±0.8 (27)</td>
<td>1.3±0.6 (8)</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>peak inward current (pA/pF)</td>
<td>-2.3±1.3 (27)</td>
<td>-3.2±2.1 (8)</td>
<td>ns (&gt;0.05)</td>
</tr>
</tbody>
</table>

(1) For all data, F-tests were first performed on the two data sets followed by two-tailed, unpaired $t$-tests for sets of equal or unequal variance.
IV. Action potential and membrane currents

IV.1. The relationship between action potential $V_{\text{max}}$ and inward Ca$^{2+}$ currents

In smooth muscle the rising phase of an action potential is generally due to an influx of extracellular Ca$^{2+}$. This was verified in detrusor smooth muscle by the experiments in the present study. Action potentials were abolished in the presence of L-type Ca$^{2+}$ channel blockers in the superfusate and recovered after their removal (see section III.1.2. Figure 4.14 and section III.2.2 Figures 4.19 and 4.20).

To demonstrate the membrane electrophysiological behaviour under physiological situations, experiments carried out in previous sections used a high K$^+$ based intracellular medium to mimic the intracellular environment. However the net inward currents recorded with KCl-filled electrodes were interfered by outward K$^+$ currents. To clarify further the role of the L-type Ca$^{2+}$ channel in action potential generation, experiments were performed in which K$^+$ in the filling solution was replaced by Cs$^+$ to inhibit the outward K$^+$ current and to unmask the inward Ca$^{2+}$ current. These experiments used a "CsCl+0.1mM EGTA" filling solution (Table 3.10, in Methods),

\[ \text{AP } \frac{dV}{dt} \max (V/s) \]

\[ \begin{array}{c|c|c|c|c}
\text{Peak } \text{i}_{\text{Ca}}(-\text{pA/\text{pF}}) & 0 & 2 & 4 & 6 \\
\text{AP } \frac{dV}{dt} \max (V/s) & 0 & 2 & 4 & 6 \\
\end{array} \]

\[ \text{Figure 4.21. The correlation of AP maximum upstroke velocity and peak } \text{i}_{\text{Ca}} \text{ current density from freshly isolated guinea-pig cells. Electrodes were filled with a "CsCl+0.1mM EGTA" solution. The line was fitted using a least squares fit to the data points with slope} = 0.89 \ (r=0.99, \ n=8, \ p<0.001). \]
instead of the basic "KCl+5mM EGTA" filling solution and further confirmed the relationship between maximum rate of upstroke velocity \((V_{\text{max}})\) of APs and inward currents. Figure 4.21 shows the relationship between AP maximum upstroke velocity and peak inward \(i_{\text{Ca}}\) current, both recorded from 8 cells, under current-clamp and voltage-clamp modes respectively. The plot shows a highly significant linear correlation between \(V_{\text{max}}\) and peak \(i_{\text{Ca}}\), passing through the origin.

IV.2. Comparison of action potentials in freshly isolated guinea-pig cells with KCl+5mM EGTA and KCl+0.1mM EGTA filled electrodes

APs in freshly isolated cells exhibited a faster depolarisation rate than those in cultured cells. One possibility is a difference in net inward current density between freshly isolated and cultured cells. A difference of outward current density would shorten AP duration. Experiments were performed with freshly isolated guinea-pig cells with a "KCl+0.1mM EGTA" filling solution instead of the basic "KCl+5mM EGTA". This would accentuate the increase of the intracellular free \(\text{Ca}^{2+}\) concentration and activate any \(\text{Ca}^{2+}\) dependent \(K^+\) current.

APs elicited by short-duration pulses using the two filling solutions are shown in Figure 4.22. APs parameters were compared using the two filling solutions, elicited with long (600ms) current pulses and the data are shown in Table 4.12. APs recorded
Figure 4.22. APs in freshly isolated guinea-pig cells using a "KCl+ 5mM EGTA" filled electrode (part A) and a "KCl+ 0.1mM EGTA" filled electrode (part B). Note that the same time scales are used in both parts A and B.

with the "KCl+0.1mM EGTA" filling solution exhibited a significantly quicker repolarisation and therefore a shorter AP duration (p=0.001).

Under voltage-clamp mode, large outward transient currents were recorded when electrodes were filled with "KCl+0.1mM EGTA". (see Results, section VII STOC). Figure 4.23 shows the relationship between APD50 and the magnitude of the largest transient outward current (STOC) recorded during a clamp to the voltage of the AP overshoot. Each data point represents measurements from the same cell when switching between current- and voltage-clamp modes. The APD50 shows a significant negative correlation with pure outward transient current magnitudes (for measurement of "pure STOC", see section IV.6.2.2, in Methods) (n=19, r=0.58, p<0.05).

IV.3. The action potential and underlying ionic currents

Figure 4.21 has shown that there is a significant relationship between the maximum rate of AP upstroke velocity and icA current density using CsCl-filled electrodes to remove the interference from K+ currents. There was also a close correlation between
AP duration and outward current density as shown in Figure 4.23. However all these data were based on two different recording systems: current-clamp and voltage-clamp, in spite of the fact that both recordings were made from the same cell. Since there is large degree of variation in the rates of channel gating reactions, it is difficult to match both AP production and ionic current data from different recording systems to display their real relationship.

An alternative approach was to demonstrate the relation between AP and net ionic currents which produced the AP based on a single recording.

As described in the introduction and methods, the cell membrane impedance was represented by a simple circuit of a membrane capacitance ($c_m$) in parallel with a

| Table 4.12. Comparison of AP properties in freshly isolated guinea-pig cells with K$^+$ containing filling solution using a high or low EGTA concentration. |
|-------------------------------|-----------------|-----------------|-----------------|
| Filling solution: Filling solution: | KCl+5mM EGTA | KCl+0.1mM EGTA | p value |
| Threshold stimulus strength (pA) | 37.9±10.4 | 34.2±10.0 | ns |
| (n) | (37) | (19) | (>0.05) |
| AP threshold, $V_{th}$ (mV) | -24.8±5.3 | -26.0±5.5 | ns |
| (n) | (37) | (19) | (>0.05) |
| AP overshoot (mV) | 25.2±9.7 | 23.8±9.4 | ns |
| (n) | (37) | (19) | (>0.05) |
| AP amplitude (mV) | 49.9±11.6 | 49.8±10.5 | ns |
| (n) | (37) | (19) | (>0.05) |
| AP duration 50, APD$_{50}$ (ms) | 63.9±28.5 | 36.4±14.3 | <0.001 |
| (n) | (36) | (19) | |
| AP time to peak, TTP (ms) | 31.4±14.2 | 28.3±11.8 | ns |
| (n) | (37) | (19) | (>0.05) |
| AP repolarisation (ms) | 32.1±19.7 | 8.1±4.8 | <0.001 |
| (n) | (36) | (19) | |
| AP upstroke velocity (V/s) | 3.6±1.9 | 5.0±2.9 | ns |
| (n) | (37) | (19) | (>0.05) |

(1) Data were presented as mean±SD.

(2) For all data, F-tests were first performed on the two data sets followed by two-tailed, unpaired t-tests for sets of equal or unequal variance.
Figure 4.23. The relationship between APD$_{50}$ and pure transient outward current magnitude (pure STOC) recorded at a clamp step to the voltage of the AP overshoot. The APs and pure STOC were measured in freshly isolated guinea-pig cells with "KCl+0.1mM EGTA" filled electrodes under current-clamp and voltage-clamp modes, respectively (for pure STOC, see section IV.6.2.2 in Methods and section VII STOC, Results). The regression line was fitted using a least squares fit to the data points ($r=0.58$, $n=19$, $p<0.05$).

Membrane resistance $r_m$. Thus the membrane current $i_m$ will be given by the sum of two components,

$$i_m = i_c + i_i$$

Where $i_i$ is the ionic current which is carried by the flow of ions across the membrane, $i_c$ is the capacitive current flow which results in a change in the amount of charge separated by the membrane, while the membrane potential is a measure of the net difference of charge between the inside and outside of the dielectric layer of cell membrane. How much charge separation must occur to generate a particular potential is therefore determined by,

$$\Delta V = \Delta Q/c_m$$
The direction of potential change is determined by the change in $Q$ with time, that is, the capacity current $I_c$:

\[
\frac{dV}{dt} = \frac{i}{c_m} \quad 4.2
\]

\[
i_c = c_m \frac{dV}{dt} \quad 4.3
\]

Thus the capacitive current flow determines the rate at which the voltage changes, i.e., $\frac{dV}{dt}$.

When a brief positive step current is applied under current-clamp mode to the intracellular compartment of a cell from an external source, initially this current will simply add charge to the inside of the cell and depolarise it. As the depolarisation increases, ionic currents will also flow across the cell membrane resistance. In a uniformly polarised cell, all the ionic current is used to charge the local cell membrane capacity and none flows as local circuit (cable) current flow (i.e. $i_m = 0$). Then equation 3.6 and 4.3 can be combined:

\[
i_m = c_m \frac{dV}{dt} + i_i \quad (i_i = V/r_m(t,V)) \quad 4.4
\]

where $i_m$ is the stimulus current. When the current pulse is terminated $i_m = 0$ so that

\[
i_i = -c_m \frac{dV}{dt} \quad 4.5
\]

The ionic current $i_i$ can now be calculated from equation 4.5.

Figures 4.24 to 4.26 show membrane (non-propagated) APs initiated by passing short-duration depolarising current to exceed threshold. $i_m$ returned to zero before the APs were initiated. The ionic current $i_i$ and capacitive current $i_c$ were then always equal and opposite as $i_m = 0$ (Jack, et al 1975). The capacitive current $i_c$ was obtained by differentiating the action potential waveform as in equation 4.2, $i_c, i_i$ and the AP were plotted as a function of time as shown in Figures 4.24-4.26.

It was initially presumed that the inward current responsible for the AP upstroke was through an L-type $\text{Ca}^{2+}$ channel and the outward currents were through $\text{K}^+$ channels. In the AP of Figure 4.24 the outward $i_i$ was larger than the inward value and so resulted in a fast repolarisation. Thus it was presumed that $\text{K}^+$ current density is greater than $\text{Ca}^{2+}$ current density. To examine further the ionic dependencies the same procedure was carried out using different electrode filling solutions to manipulate selectively $\text{Ca}^{2+}$ and $\text{K}^+$ currents.
Figure 4.24. Variations in voltage and net ionic current during a membrane action potential from a freshly isolated guinea-pig cell initiated by a brief square pulse current. The patch electrode was filled with “KCl+0.1mM EGTA” solution. $i_m$ was equal to zero apart from a 50ms step. Part A shows the capacitive current given by $c_m dV/dt$. The initial wave is the applied current, most of which flows as capacitive current when the brief pulse was used, the later wave was due to ionic currents. Part B shows the membrane potential change as a function of time. Part C is the total ionic current, obtained as the inverse of the waveform in Part A. The three vertical dashed lines indicate the positions in which $dV/dt$
is maximum (also at peak inward current), zero and when \(-dV/dt\) is maximum (peak net outward current). Part D shows a subthreshold depolarisation to a smaller applied step current (40pA, 50ms) and the consequent capacitive current. There is no AP when there is no inward ionic current. The suprathreshold response (Part B) is also superimposed.

Figure 4.25 shows a record from a freshly isolated guinea pig cell obtained with an electrode filled with "KCl+5mM EGTA" solution. This solution would enhance inward Ca\(^{2+}\) flux by suppressing the rise of intracellular free Ca\(^{2+}\) and hence maintain the inward during force on Ca\(^{2+}\). In this cell, by contrast to Figure 4.24, the outward component of \(i_i\) was smaller than the inward component, so that the AP repolarising phase was longer. This was well confirmed by a recording from a CsCl-filled electrode as shown in Figure 4.26.

With the CsCl filled electrode, the potassium channels were blocked and the membrane resistance became very large so that little outward current would flow.

Therefore AP upstroke was caused by net inward ionic current and repolarisation depended on the outward K\(^+\) current.

![Figure 4.25](image)

**Figure 4.25.** The relationship between action potential (AP) and ionic current \(i_i\) in a freshly isolated guinea-pig cell. The electrode was filled with "KCl+5mM EGTA" solution.
Figure 4.26. Part A: the action potential (AP) and ionic currents in a freshly isolated guinea-pig cell with a CsCl-filled electrode. Part B shows the detail of the inward current $i_i$ and maximum $dV/dt$ of Part A. The outward current is virtually zero and the AP repolarisation very prolonged.
V. Measurement of inward and outward currents

Passive and active membrane properties have been compared between freshly isolated and cultured cells from human and guinea-pig detrusor smooth muscle. It has been shown that similar electrical properties exist in freshly isolated cells from the two species and are maintained after culture with some modifications.

Membrane currents, which underlie these membrane properties, were also investigated under the same experimental conditions; i.e., normal Tyrode's solution at 36.5±0.5°C, gassed with 5% CO₂ + 95% O₂ and using a basic "KCl+5mM EGTA" filling solution.

Under voltage-clamp mode, the cell membrane was held at a resting value of -60mV. A "current activation protocol" for both inward and outward currents was used to determine the channel activation properties; i.e., 500ms steps from -60mV over the range -100 to +40mV with increments of 10mV. The steady-state membrane potential inactivation kinetics for both inward and outward currents were studied by using two protocols: (1) an "inward current inactivation protocol": that is, 2-second preconditioning steps between -60 and +10mV with 10mV increments; and a 500ms activating step from -60mV (1ms) to +10mV, the maximal inward current activation voltage and (2) an "outward current inactivation protocol": that is, 2-second preconditioning steps between -110 and +40mV with 10mV increments; and a 500ms activating step from -60mV (1ms) to +40mV.

V.1. Comparison of currents from freshly isolated guinea-pig and human cells

V.1.1. Currents from freshly isolated guinea-pig and human cells

Currents were evoked using the "current activation protocol" as described above. Sample traces are shown in Figure 4.27 from a guinea-pig cell and Figure 4.28 from a human cell. Peak inward currents were measured as the difference between the maximum inward current and the current value obtained at the end of the each clamp step (see Methods, section IV.6.1.1.2, Figure 3.10). The steady-state outward current was measured as the current difference between that at the end of the voltage step and that at the holding potential (see Methods, Figure 3.10).
Figure 4.27. Inward and outward currents in a freshly isolated guinea-pig cell. Currents were evoked from a holding potential of -60mV and activated by steps between -20 to +10mV, with 10mV increments.

Figure 4.28. Inward and outward currents in a freshly isolated human cell. Currents were evoked from a holding potential of -60mV and activated by steps between -30 and 0mV, with 10mV increments.
V.1.1.1. Inward and outward current-voltage relationships

Membrane capacitance was measured in each cell to normalise the current magnitude. It was therefore possible to express currents as current density (pA/pF) to make comparison between data sets. Both inward and outward current densities were plotted against the activation voltages as shown in Figure 4.29.

There was no significant difference in outward current between freshly isolated guinea-pig and human cells. The peak inward current densities at +10mV were -4.5±2.2 pA/pF (mean±SD, n=17) from human cells, and -5.5±1.9 pA/pF (mean±SD, n=37) from guinea-pig cells. These values were also not significantly different from each other.

![Figure 4.29](image)

Figure 4.29. Mean current-voltage relationships for inward (filled symbols) and outward currents (open symbols) in freshly isolated guinea-pig and human cells. Data are expressed as mean±SD. The circles stand for human cells (n=17), and triangles for guinea-pig cells (n=37). There was no significant difference between guinea-pig and human for both inward and outward currents.

V.1.1.2. Net currents

Inward current, the difference between the peak inward current and the current value obtained at the end of the clamp step, is interfered by outward current when an electrode is filled with KCl solution. Net current was measured as the peak inward
current, referred with reference to the current at holding potential \((V_h-60mV)\) (see Methods, section IV.6.1.1.2, Figure 3.10). Therefore the net inward current is more closely related to AP upstroke, amplitude and time to peak amplitude.

Figure 4.30 shows that the current-voltage \((i-v)\) relationship for data from freshly isolated guinea-pig and human cells. Over the voltage range -20 to +20mV, the net inward current was significantly larger in guinea-pig than in human cells: peak net inward current at 0mV for guinea pig cells \(-2.9\pm1.9\ pA/pF\) (mean±SD, n=37) and human cells \(-1.1\pm1.3\) (mean±SD, n=17). This was consistent with the fact that the APs in cells from guinea-pigs has a faster rate of upstroke and a shorter time to peak amplitude, compared to human cells (see also Table 4.6 section III.1.2.4. in this chapter).

Figure 4.30. The mean current-voltage relationships of net current densities (referred to current at \(V_h-60mV\)) from freshly isolated guinea-pig and human cells. The filled triangles are guinea-pig, and the filled circles human cells. Data are expressed as mean±SD (guinea-pig n=37; human n=17). Unpaired, two-tailed \(t\)-test (* \(p<0.05\); ** \(p<0.01\)).
V.1.2. Inward current steady-state activation and inactivation

V.1.2.1. Inward current activation

The activation kinetics of inward currents for both freshly isolated guinea-pig and human cells were measured. The membrane conductance $g_{1/r}$ to inward current was determined from the current-voltage (i-v) relationships for each cell individually, according to equation 3.11 (see Methods, section IV.6.1.1.3.).

The relationships between relative conductance ($g/g_{\text{max}}$) and membrane potential are shown in Figure 4.31 for freshly isolated guinea-pig and human cells. The curves were constructed using the averaged data determined from several individual activation curves. The fitted curves in Figure 4.31 were constructed using the averaged $V_{0.5}$ and $kd$ determined from fitting each individual curve and the Boltzmann equation, equation 3.12 (Method, section IV.6.1.1.3), using a least-squares fit.

![Figure 4.31](image)

**Figure 4.31.** Inward current steady-state activation curves from freshly isolated guinea-pig cells (open circles, n=36) and human cells (filled circles n=17). The curves were fitted from mean $V_{0.5}$ and $kd$ values using the Boltzmann equation (equation 3.12).

The mean values for human cells were $V_{0.5} = -7.3\pm4.8 \text{ mV}$ and $kd = 7.3\pm1.3 \text{ mV}$ (n=17); and for guinea-pig cells were $V_{0.5}=-8.1\pm5.5 \text{ mV}$ and $kd=7.4\pm1.6 \text{ mV}$ (n=36).
There were no significant differences in $V_{0.5}$ and in slope $k_d$ values between human and guinea-pig cells.

V.1.2.2. Inward current inactivation

The effect of the steady-state membrane potential $V_m$ on the proportional availability of the inward current was investigated using the "inward current inactivation protocol". A typical set of recordings is shown in Figure 4.32. The proportion of inward current available following any particular preconditioning potential was measured as the ratio of the peak current and the maximum peak inward current recorded at negative preconditioning potentials ($i/i_{\text{max}}$). The relationship between the $i/i_{\text{max}}$ ratio and the preconditioning membrane potential $V_m$ was described by equation 3.14 and gave the $V_{0.5}$ and $k_f$ values (Methods, section IV.6.1.2.2.).

The relationships between the $i/i_{\text{max}}$ ratio and the preconditioning membrane potential is shown in Figure 4.33 for freshly isolated guinea-pig and human cells, and were constructed using the averaged data from individual curves. The fitted curves in Figure 4.33 were constructed using the averaged $V_{0.5}$ and $k_f$ values.

![Figure 4.32. Membrane currents recorded in a freshly isolated guinea-pig cell. The currents evoked by the "inactivation protocol" with three preconditioning voltages from -50 to -30mV and an activating step from -60 to +10mV are shown here as an example.](image-url)
Figure 4.33. Inward current inactivation curves from freshly isolated guinea-pig cells (open circles n=36) and human cells (closed circles n=17). The curves were fitted from mean $V_{0.5}$ and $k_f$ values using the Boltzmann equation (equation 3.14).

The mean values for $V_{0.5}$ and $k_f$, for human cells were $V_{0.5} = -25.8 \pm 4.3$ mV and $k_f = 9.7 \pm 2.6$ mV (mean±SD, n=17). For guinea-pig cells $V_{0.5} = -27.9 \pm 4.7$ mV, $k_f = 8.7 \pm 3.1$ mV (mean±SD, n=36). Both $V_{0.5}$ and $k_f$ were not significantly different in guinea-pig and human cells.

V.1.3. Steady-state outward current activation and inactivation

V.1.3.1. Steady-state outward current activation

Changes in the activation kinetics of outward current for both freshly isolated guinea-pig and human cells were also investigated. Steady-state outward current conductance, $g$, was determined from the data of individual current-voltage (i-v) relationships using equation 3.11 (Methods, section IV.6.1.1.3), expressed as the ratio, $g/g_{\text{max}}$ and plotted against the membrane potential between -40 to +40 mV. The curve was fitted to the equation 3.13 to give values of $V_{0.5}$, the membrane potential at which conductance $g$ is half maximum, a slope factor $k_n$ and an offset $c$ (Methods, section IV.6.1.1.4).
Page 160 add the following figure as Figure 4.34B to show outward current activation in freshly isolated guinea-pig and human cells.
The relationships are shown in Figure 4.34 for freshly isolated guinea-pig and human cells, using averaged data from individual cells. The fitted curves were constructed using the averaged $V_{0.5}$, $k_n$ and $c$ values determined from individual curves. Mean values were: guinea-pig, $V_{0.5} 1.3\pm10.1\text{mV}$, slope $k_n 11.7\pm4.4\text{mV}$ and offset $0.11\pm0.22$ (mean$\pm$SD, $n=36$); human, $V_{0.5} 0.3\pm9.8\text{mV}$, slope $k_n 11.4\pm3.2\text{mV}$ and offset $0.03\pm0.09$ (mean$\pm$SD, $n=17$). There were no significant differences between guinea-pig and human cells. There was no significant difference from zero in the offset value for human cells, but the value was significantly greater than zero for guinea-pig cells ($p<0.05$).

### Figure 4.34

**Steady-state outward current activation curves from freshly isolated guinea-pig and human cells.** The filled circles were human cells ($n=17$) and open circles from guinea-pig ($n=36$).

**V.1.3.2. Steady-state outward current inactivation**

The effect of membrane potential $V_m$ on the proportional availability of the steady-state outward current was investigated using the "outward current inactivation protocol".

The proportion of steady-state outward current available following any particular preconditioning voltage was measured as the ratio of the steady-state outward current elicited following a given potential to the maximum steady-state outward current.
Page 161 add the following figure as Figure 4.35B to show outward current inactivation in freshly isolated guinea-pig and human cells.

IK (fGP) and IK (fH)
$i/i_{\text{max}}$. The maximum steady-state outward current was that from the first few most negative preconditioning voltages.

The relationship between the ratio of steady-state outward current to maximum steady-state current, $i/i_{\text{max}}$ and preconditioning voltage, $V_m$, was described by equation 3.15 to give $V_{0.5}$, $k_p$ and $c$ values (Method, section IV.6.1.3.2.).

The relationships between $i/i_{\text{max}}$ and membrane potential are shown in Figure 4.35 for freshly isolated guinea-pig and human cells. The averaged data were obtained from individual curves. The fitted curves were constructed using the averaged $V_{0.5}$, $k_p$ and $c$ values derived from fitting individual curves. For human cells: $V_{0.5} -41.4\pm11.4\text{mV}$, slope $k_p 12.2\pm3.4\text{mV}$ and offset $c 0.56\pm0.12$ (mean±SD, n=7); for guinea-pig cells: $V_{0.5} -63.4\pm4.3\text{mV}$, slope $k_p 9.4\pm1.9\text{mV}$ and offset $c 0.32\pm0.06$ (mean±SD, n=14). Unpaired two-tailed $t$-test revealed a significant difference ($p<0.01$) for $V_{0.5}$ and $c$ values in guinea-pig and human cells. There was no significant difference between the $k_p$ values. Using the offset to calculate the non-inactivating current density, values of $4.5\pm0.7$ (mean±SD, n=7) pA/pF for human cells and $3.7\pm2.7$ (mean±SD, n=14) for guinea-pig cells were obtained; there was no significant difference in the current density of these non-inactivating components. The data are also shown in Table 4.13.

![Figure 4.35](image-url)

**Figure 4.35.** Steady-state outward current inactivation curves from freshly isolated guinea-pig and human cells. The filled circles were from human cells (n=7) and open circles from guinea-pig cells (n=14).
Table 4.13. Steady-state outward current inactivation variables for freshly isolated guinea-pig (fGP) and human (fH) cells.

<table>
<thead>
<tr>
<th></th>
<th>fGP Mean±SD (n)</th>
<th>fH Mean±SD (n)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{0.5}$ (mV)</td>
<td>-63.4±4.3 (14)</td>
<td>-41.4±11.4 (7)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Slope factor, $k_p$ (mV)</td>
<td>9.4±1.9 (14)</td>
<td>12.2±3.4 (7)</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>Offset, $c$</td>
<td>0.32±0.06 (14)</td>
<td>0.56±0.12 (7)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Non-inactivating current, (pA/pF)</td>
<td>3.7±2.7 (14)</td>
<td>4.5±0.7 (7)</td>
<td>ns (&gt;0.05)</td>
</tr>
</tbody>
</table>

V.2. Ionic currents in primary cultured guinea-pig and human cells

V.2.1. Currents in primary cultured cells

![Figure 4.36. Inward and outward currents in a primary cultured guinea-pig cell. Currents were evoked from a $V_h$ of -60mV to voltages between -20 to +20mV.](image_url)
Page 163, line 2 of paragraph 2: after "a fraction of cells" add: "(75% of cells in which AP could be recorded)."
Figure 4.37. Inward and outward currents in a primary cultured human cell. Currents were evoked from a $V_h$ of -60mV, to voltages between -30 to +20mV.

V.2.1.1. The i-v relationships for inward and outward currents

Inward and outward currents were measured in cultured smooth muscle cells from guinea-pig and human detrusor cells using the protocols described in section V.1. Examples of original current recordings from cultured guinea-pig and human cells are shown in Figures 4.36 and 4.37.

Currents could be recorded from all cultured cells, but action potentials (APs) could be generated in only a fraction of cells. This was different from freshly isolated cells when action potentials could always be evoked. The currents in cells from which APs were recorded were further analysed.

Current densities were plotted against the activation voltages as shown in Figure 4.38. Peak inward current at $+10\text{mV}$ was $-2.2\pm1.2 \text{pA/pF (n=27)}$ for guinea-pig cells and $-2.0\pm0.9 \text{pA/pF (n=8)}$ for human cells. There were no significant differences in peak inward current and steady-state outward current densities between cultured cells from guinea-pigs and humans.
**Figure 4.38.** The mean current-voltage relationships of inward and outward currents from cultured guinea-pig and human cells. The circles are human cells (n=8) and triangles guinea-pig cells (n=27). Currents were recorded from the cells in which membrane APs were elicited, expressed as mean±SD.

**Figure 4.39.** The mean current-voltage relationships of net inward current densities (referred to $V_h$ -60mV) in cultured guinea-pig and human cells. Triangles are for guinea-pigs (n=27) and circles for human cells (n=8). Data were expressed as mean±SD. There was no significant difference in peak net inward currents from guinea-pigs and humans.
V.2.1.2. Net inward current

Figure 4.39 shows the net inward current measured as the peak inward current referred to the holding potential ($V_{h}$-60mV) and plotted against the clamp step voltage.

The net inward current from both guinea-pig and human detrusor myocytes had a similar current density and voltage dependence. Peak net inward current was $-0.7 \pm 1.3$ pA/pF ($n=27$) at 0mV for guinea-pig cells and $-0.9 \pm 0.7$ pA/pF ($n=8$) at -10mV for human cells. Statistical analysis showed no significant difference in the net inward current between these two species.

V.2.2. Inward current activation and inactivation

V.2.2.1. Inward current activation

The inward current conductance $g$ and $g_{\text{max}}$ in cultured guinea-pig and human cells were determined as in section V.1. The relationships between relative conductance ($g/g_{\text{max}}$) and membrane potential are shown in Figure 4.40 using averaged data from individual curves as in section V.1. The fitted curves were constructed using averaged $V_{0.5}$ and $k_d$ values and the Boltzmann equation 3.12 using a least-squares fit. Mean values for cultured guinea-pig cells were: $V_{0.5}$ -3.2±7.5mV and slope $k_d$ 6.8±1.9mV (mean±SD, $n=36$); for cultured human cells: $V_{0.5}$ -5.9±5.6mV and slope $k_d$ 8.4±2.1mV (mean±SD, $n=8$). There was no significant difference in $V_{0.5}$ values for cultured guinea-pig and human cells, but a significant difference was found in slope $k_d$ values ($p < 0.05$).

V.2.2.2. Inward current inactivation

The effect of membrane potential on the proportional availability of inward current was investigated using the same protocol as described in section V.1. Peak inward current was taken as the difference between the maximum inward current and at the end of this test step (Methods, section IV.6.1.1.2.).

The relationships between $i/i_{\text{max}}$ and membrane potential are shown in Figure 4.41, constructed using the averaged data determined from individual curves. The fitted
Figure 4.40. Inward current activation for cultured guinea-pig and human cells. Open circles are for cultured guinea-pig cells (n=36) and filled circles for human cells (n=8).

Figure 4.41. Inward current inactivation in cultured guinea-pig and human cells. Open circles are guinea-pig (n=36) and filled circles human (n=8) data.
curves used averaged $V_{0.5}$ and $k_f$ values, for cultured guinea-pig cells: $V_{0.5}$ -23.1±10.2mV and slope $k_f$ 7.2±3.6mV (mean±SD, n=36); for cH: $V_{0.5}$ -19.0±14.0mV and slope $k_f$ 8.4±4.8mV (mean±SD, n=8). There were no significant differences in $V_{0.5}$ and slope $k_f$ values between guinea-pig and human cells.

V.2.3. Steady-state outward current activation and inactivation

V.2.3.1. Steady-state outward current activation

The mean steady-state outward current activation curves were constructed as in section V.1. The curves are shown in Figure 4.42, using mean values for $V_{0.5}$, slope factor $k_n$ and offset for cultured guinea-pig cells: $V_{0.5}$ 4.6±11.8mV, slope $k_n$ 8.1±3.8mV and offset 0.32±0.41 (mean±SD, n=36). for cultured human: $V_{0.5}$ 3.3±16.8mV, slope $k_n$ 9.3±8.1mV and offset 0.15±0.39 (mean±SD, n=8). There were no statistical differences in $V_{0.5}$, slope $k_n$ and offset between guinea-pig and human cells. There was no significant difference from zero in the offset value for human cells, but the value was significant for guinea-pig cells (p<0.05).

![Figure 4.42](image-url)  

**Figure 4.42.** The mean steady-state outward current activation curves in cultured guinea-pig and human cells. Open circles are guinea-pig (n=36) and filled circles human (n=8) data.
V.3.2.3.2. Steady state outward current inactivation

Steady-state outward current inactivation curves (Figure 4.43) were constructed as in section V.1. For cultured guinea-pig cells: $V_{0.5} = -26.7 \pm 17.9\text{mV}$, slope $k_p = 14.6 \pm 7.4\text{mV}$ and offset $0.46 \pm 0.03$ (mean±SD, n=6); for cultured human cells: $V_{0.5} = -55.7 \pm 14.7\text{mV}$, slope $k_p = 13.6 \pm 8.3\text{mV}$ and offset $0.42 \pm 0.11$ (mean±SD, n=5). There were significant differences in $V_{0.5}$ values for guinea-pig and human cells ($p<0.05$), but no significant differences in $k_p$ and offset values.

![Graph](image)

**Figure 4.43.** Steady-state outward current inactivation curves in cultured guinea-pig and human cells. Open circles are guinea-pig (n=6) and filled circles human (n=5) data.

V.3. Comparison of currents from freshly isolated and cultured human cells

The magnitude and kinetics of the inward and outward currents were compared in freshly isolated and cultured human cells to document any changes in culture. Data are shown in Figures 4.44 to 4.50.
V.3.1. Current-voltage (i-v) relationships

The mean peak inward current and steady-state outward current i-v relationships for freshly isolated and cultured human cells are shown in Figures 4.44 and 4.45. Figure 4.44 shows that the inward current density from cultured cells were significantly smaller at voltages between -30 and +40mV, and the inward current activation threshold potential was depolarised compared to freshly isolated cells. The steady-state outward current density in cultured cells was also significantly smaller than that freshly isolated cells at voltages from -20 to +40mV. These data suggest that both inward and outward current densities reduced during culture; more positive potentials were required to activate the inward current, which was consistent with the finding that the action potential threshold in cultured human cells were at more depolarised voltages, compared to freshly isolated cells.

Net currents from freshly isolated and cultured human cells are shown in Figure 4.46. The peak net inward currents from the two groups were not significantly different. Net inward current determines action potential parameters, such as overshoot, amplitude, TTP and maximum upstroke velocity, and explains why there was no significant differences in these parameters between freshly isolated and cultured cells.

![Graph showing inward current densities in freshly isolated and cultured human cells](image_url)

**Figure 4.44.** Inward current densities in freshly isolated and cultured human cells. Filled circles are cultured human cells (n=8) and open circles are freshly isolated human cells (n=17) data. Note that the inward current activation threshold potential for cultured cells was depolarised. (* p<0.05, ** p<0.01).
**Figure 4.45.** Comparison of steady-state outward current i-v relationships from freshly isolated and cultured human cells. Filled circles are cultured (n=8) and open circles freshly isolated human (n=17) data. (* p<0.05, ** <0.01).

**Figure 4.46.** Comparison of net currents in cultured and freshly isolated human cells. Filled circles are cultured (n=8) and open circles freshly isolated human cells (n=17). There was no significant difference between two peak net inward current values.
V.3.2. Inward current activation and inactivation

Inward current activation curves from freshly isolated and cultured human cells are shown in Figure 4.47 and inactivation curves in Figure 4.48. Both the activation and inactivation curves were not significantly different by comparing the mean $V_{0.5}$ and slope values.

V.3.3. Steady-state outward current activation and inactivation

Figures 4.49 and 4.50 show the comparisons of steady-state outward current activation and inactivation curves from freshly isolated and cultured human cells. Again there were no significant differences either in two activation or inactivation curves between freshly isolated and cultured human cells by comparing the $V_{0.5}$, slope and offset values.

Figure 4.47. Inward current activation curves in freshly isolated and cultured human cells. Open circles are freshly isolated (n=17) and filled circles cultured cells (n=8).
**Figure 4.48.** Inward current inactivation curves for freshly isolated and cultured human cells. Open circles are freshly isolated (n=17) and filled circles cultured cells (n=8).

**Figure 4.49.** Steady-state outward current activation curves for freshly isolated and cultured human cells. Open circles are freshly isolated (n=17) and filled circles cultured cells (n=8).
Figure 4.50. Steady-state outward current inactivation curves for freshly isolated and cultured human cells. Open circles are freshly isolated (n=7) and filled circles cultured cells (n=5).

V.4. Summary of membrane currents from freshly isolated and cultured human cells

Tables 4.14A and 4.14B summarise the comparisons of current kinetics from data obtained in section V.1-V.3. between freshly isolated, cultured human and guinea-pig cells. After culture inward and outward current densities were significantly smaller and inward current activation threshold potential was shifted to a more depolarised voltage. Net inward current from freshly isolated guinea-pig cells was significantly larger, compared to freshly isolated human, but not significantly different between cultured human and guinea pig cells, and between freshly isolated and cultured human cells. These data were consistent with their action potential properties (see Results, Tables 4.6-4.8)

V.5. Membrane currents recorded from passaged cells

Primary cultured cells from guinea-pig and human samples grew to confluence after 2-3 weeks. Cells were then dissociated with 0.25% trypsin/versene as described in Methods and subcultured up to three times (cGP<sub>1.3</sub> is for guinea-pig cells passaged up
**Table 4.14A.** Summary of comparisons of current densities from freshly isolated guinea-pig (fGP) and human (fH); cultured guinea-pig (cGP) and human (cH) cells in section V.1-V.3.

<table>
<thead>
<tr>
<th></th>
<th>fH/fGP</th>
<th>cH/cGP</th>
<th>cH/fH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inward current</td>
<td>fH/fGP:</td>
<td>cH/cGP:</td>
<td>fH: ≤ -40mV</td>
</tr>
<tr>
<td>activation threshold</td>
<td>≤ -40mV</td>
<td>≥ -30mV</td>
<td>cH: ≥ -30mV</td>
</tr>
<tr>
<td>Peak inward current</td>
<td>fH:-4.5±2.2 (17)</td>
<td>cH:-2.0±0.9(8)</td>
<td>cH &lt; fH</td>
</tr>
<tr>
<td>(pA/pF)</td>
<td>fGP:-5.5±1.9 (37)</td>
<td>cGP:-2.2±1.2(27)</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>(mean±SD, n)</td>
<td>fH/fGP: ns</td>
<td>cH/cGP: ns</td>
<td></td>
</tr>
<tr>
<td>Steady-state</td>
<td>ns</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>outward current</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pA/pF)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Net inward currents</td>
<td>-20 to +20 mV:</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>(pA/pF)</td>
<td>fH &lt; fGP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mean±SD, n)</td>
<td>p&lt;0.05 to p&lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ns:</strong> not significant (p&gt;0.05).</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.14B.** Summary of current activation and inactivation kinetics in section V.1-V.3 from freshly isolated guinea-pig (fGP) and human (fH); cultured guinea-pig (cGP) and human (cH) cells.

<table>
<thead>
<tr>
<th></th>
<th>fH/fGP</th>
<th>cH/cGP</th>
<th>cH/fH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inward current</td>
<td>V_{0.5}</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>activation</td>
<td>k_d</td>
<td>ns</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Inward current</td>
<td>V_{0.5}</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>inactivation</td>
<td>k_f</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Steady-state</td>
<td>V_{0.5}</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>outward current</td>
<td>k_n</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>activation</td>
<td>offset</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Steady-state</td>
<td>V_{0.5}</td>
<td>p&lt;0.01</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>outward current</td>
<td>k_p</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>inactivation</td>
<td>offset</td>
<td>p&lt;0.01</td>
<td>ns</td>
</tr>
<tr>
<td>ns: not significant (p&gt;0.05).</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
to 3 times; cH1-3: for equivalent human cells. Inward currents and steady-state outward currents were recorded in all subcultured cells, but action potentials could be recorded only in proportion of cells. Figures 4.51 and 4.52 show currents recorded from passaged human detrusor smooth muscle cells (cH1) and guinea-pig cells (cGP2), respectively, under the same conditions as in sections V.1 to V.3. These results provide the first evidence that excitability was retained in detrusor smooth muscle cells undergoing passage.

V.5.1. Comparison of current i-v relationships of freshly isolated, primary cultured and passaged guinea-pig cells

It has been shown that passaged guinea-pig detrusor smooth muscle cells could generate action potentials when electrically stimulated and ionic currents could also recorded. Here, fGP stands for freshly isolated and cGP for primary cultured cell as designated in previous sections; cGP1-3 for passaged guinea-pig cells.

V.5.1.1. Inward and steady-state outward current-voltage relationships

A comparison of the inward and steady-state outward current i-v relationships of guinea-pig detrusor myocytes is made in Figures 4.53 and 4.54.

As shown in Figure 4.53 the maximum inward currents in the i-v relationships from freshly isolated(fGP), primary cultured (cGP) and passaged(cGP1-3) guinea-pig cells are at +10mV. The peak inward currents were: -5.5±1.9pA/pF for fGP (n=37), -2.2±1.2pA/pF for cGP (n=27) and -2.8±2.0pA/pF for cGP1-3 (n=9) (mean±SD for all data sets). There are significant differences between fGP and cGP, and between fGP and cGP1-3 cells at voltages from -30 to +40mV. But no difference was found between cGP and cGP1-3 cells. The inward current activation threshold voltages for both cGP and cGP1-3 cells was shifted to more positive potentials compared to fGP.

There were no significant changes in steady-state outward currents between fGP and cGP1-3 cells or between cGP and cGP1-3 cells. However there were significant differences between fGP and cGP cells at voltages from -10 to +40mV as shown in Figure 4.54.
Figure 4.51. Currents recorded in a cultured human cell after first passage (cH1).

Figure 4.52. Currents recorded in a cultured guinea-pig cell after second passage (cGP2).
Figure 4.53. Inward current i-v relationships for freshly isolated (fGP, open circles n=37), primary cultured (cGP, triangles n=27) and passaged (cGP\textsubscript{1-3}, filled circles n=9) guinea-pig cells. Data expressed as mean±SD. The inset shows that there are significant differences between fGP and cGP and between fGP and cGP\textsubscript{1-3} at voltages from -30 to +40mV, but no difference between cGP and cGP\textsubscript{1-3} ( ** p<0.01 and *** p <0.001).

Figure 4.54. The steady-state outward current i-v relationships in freshly isolated (fGP), primary cultured (cGP) and passaged (cGP\textsubscript{1-3}) guinea-pig cells. The open circles are fGP (n=37), triangles
cGP (n=27) and filled circles cGP\textsubscript{1.3} (n=9). There were no significant changes in steady-state outward currents between fGP and cGP\textsubscript{1.3}, or between cGP and cGP\textsubscript{1.3}, but a significant difference between fGP and cGP from -10 to +40 mV as shown in the inset (\(*\, p<0.01\) and \(*\, *\, p<0.001\)).

V.5.1.2. Net currents

Peak net inward currents (referred to current at the holding potential \(V_{h}=-60\, mV\)) for passaged (cGP\textsubscript{1.3}) cells were determined using the same method as in section V.1 and V.2. A comparison of net currents is shown in Figure 4.55. The mean peak net inward currents were: fGP \(-2.9\pm1.9\, pA/pF\) (\(\pm\, SD\), \(n=37\)); cGP \(-0.7\pm1.3\, pA/pF\) (\(\pm\, SD\), \(n=27\)) and cGP\textsubscript{1.3} \(-0.8\pm0.9\, pA/pF\) (\(\pm\, SD\), \(n=9\)). There were significant differences in peak net currents between fGP and cGP\textsubscript{1.3}, and between fGP and cGP as shown in the inset in Figure 4.55., but no difference between cGP and cGP\textsubscript{1.3} cells.

These data, the same as the human cells, show that the current densities were reduced during culture.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4_55}
\caption{Net currents from freshly isolated (fGP), primary cultured (cGP) and passaged (cGP\textsubscript{1.3}) guinea-pig cells. The open circles are fGP (n=37), triangles cGP (n=27) and filled circles cGP\textsubscript{1.3} (n=9). There were statistical differences between cGP\textsubscript{1.3} and fGP; cGP and fGP as shown in the inset graph, but no difference between cGP and cGP\textsubscript{1.3} (* \(p<0.05\), ** \(p<0.01\) and **\, *\, p<0.001).}
\end{figure}
V.5.2. Steady-state inward current activation and inactivation of freshly isolated, primary cultured and passaged guinea-pig cells

V.5.2.1. Steady-state inward current activation

Steady-state inward current activation curves for freshly isolated (fGP), primary cultured (cGP) and passaged (cGP$_{1-3}$) guinea-pig cells are shown in Figure 4.56 and Tables 4.15 to 4.17. The curves from cultured cells were shifted to less negative potentials. There was a significant change in $V_{0.5}$ values between fGP and cGP ($p<0.01$), but not significant change either between fGP and cGP$_{1-3}$, or between cGP and cGP$_{1-3}$ cells. There was a significant difference in $k_d$ values between fGP and cGP$_{1-3}$, but not others.

\[
\frac{d_{\infty}}{g_{\max}}
\]

Figure 4.56. Comparison of steady-state inward current activation from freshly isolated (fGP, open circles $n=36$), primary cultured (cGP, triangles $n=36$) and passaged (cGP$_{1-3}$, filled circles $n=9$) guinea-pig cells. Statistical comparisons are shown in Tables 4.15 to 4.17.

Table 4.15. Steady-state inward current activation parameters, freshly isolated (fGP) vs primary cultured (cGP) cells.

<table>
<thead>
<tr>
<th></th>
<th>fGP (mean±SD, n=36)</th>
<th>cGP (mean±SD, n=36)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{0.5}$ (mV)</td>
<td>-8.1±5.5</td>
<td>-3.2±7.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>$k_d$ (mV)</td>
<td>7.4±1.6</td>
<td>6.8±1.9</td>
<td>ns (&gt;0.05)</td>
</tr>
</tbody>
</table>
Table 4.16. Steady-state inward current activation, freshly isolated (fGP) vs passaged (cGP1-3) cells.

<table>
<thead>
<tr>
<th></th>
<th>fGP</th>
<th>cGP1-3</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mean±SD, n=36)</td>
<td>(mean±SD, n=9)</td>
<td></td>
</tr>
<tr>
<td>( V_{0.5} ) (mV)</td>
<td>-8.1±5.5</td>
<td>-4.1±7.3</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>( k_d ) (mV)</td>
<td>7.4±1.6</td>
<td>6.0±1.9</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Table 4.17. Steady-state inward current activation, primary cultured (cGP) vs passaged (cGP1-3) cells.

<table>
<thead>
<tr>
<th></th>
<th>cGP</th>
<th>cGP1-3</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mean±SD, n=36)</td>
<td>(mean±SD, n=9)</td>
<td></td>
</tr>
<tr>
<td>( V_{0.5} ) (mV)</td>
<td>-3.2±7.5</td>
<td>-4.1±7.3</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>( k_d ) (mV)</td>
<td>6.8±1.9</td>
<td>6.0±1.9</td>
<td>ns (&gt;0.05)</td>
</tr>
</tbody>
</table>

V.5.2.2. Steady-state inward current inactivation

A comparison of steady-state inward current inactivation curves for freshly isolated (fGP), primary cultured (cGP) and passaged (cGP1-3) guinea-pig cells is shown in Figure 4.57. There was no significant difference between cGP1-3 and cGP (Table 4.20), but the curves were significantly shifted to less negative potentials in both the culture groups compared to freshly isolated cells (Table 4.18 and Table 4.19). This suggests that the inactivation of the inward current occurred at less negative potentials after culture, and remained less negative undergoing further passages.

Table 4.18. Steady-state inward current inactivation parameters for freshly isolated (fGP) and primary cultured (cGP) guinea-pig cells.

<table>
<thead>
<tr>
<th></th>
<th>fGP</th>
<th>cGP</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mean±SD, n=36)</td>
<td>(mean±SD, n=36)</td>
<td></td>
</tr>
<tr>
<td>( V_{0.5} ) (mV)</td>
<td>-27.9±4.7</td>
<td>-23.1±10.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>( k_f ) (mV)</td>
<td>8.7±3.1</td>
<td>7.2±3.6</td>
<td>ns (&gt;0.05)</td>
</tr>
</tbody>
</table>

Table 4.19. Steady-state inward current inactivation variables for freshly isolated (fGP) and passaged (cGP1-3) guinea-pig cells.

<table>
<thead>
<tr>
<th></th>
<th>fGP</th>
<th>cGP1-3</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mean±SD, n=36)</td>
<td>(mean±SD, n=9)</td>
<td></td>
</tr>
<tr>
<td>( V_{0.5} ) (mV)</td>
<td>-27.9±4.7</td>
<td>-20.7±9.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>( k_f ) (mV)</td>
<td>8.7±3.1</td>
<td>7.8±1.9</td>
<td>ns (&gt;0.05)</td>
</tr>
</tbody>
</table>
Table 4.20. Steady-state inward current inactivation variables for primary cultured (cGP) and passaged (cGP1-3) cells.

<table>
<thead>
<tr>
<th></th>
<th>cGP (mean±SD, n=36)</th>
<th>cGP1-3 (mean±SD, n=9)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{0.5}$ (mV)</td>
<td>-23.1±10.2</td>
<td>-20.7±9.3</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>$k_f$ (mV)</td>
<td>7.2±3.6</td>
<td>7.8±1.9</td>
<td>ns (&gt;0.05)</td>
</tr>
</tbody>
</table>

Figure 4.57. A comparison of inward current steady-state inactivation in freshly isolated (fGP), primary cultured (cGP) and passaged (cGP1-3) guinea-pig cells. The open circles are fGP (n=36), triangles cGP (n=36), and filled circles cGP1-3 (n=9). Statistical comparisons are shown in Tables 4.18 to 4.20.

V.5.3. Steady-state outward current activation and inactivation of freshly isolated, primary cultured and passaged guinea-pig cells

V.5.3.1. Steady-state outward current activation

Figure 4.58 and Tables 4.21 to 4.23 show comparisons between freshly isolated (fGP), primary cultured (cGP) and passaged (cGP1-3) guinea-pig cells. Although the slope of the steady-state outward current activation curves altered after primary culture compared with freshly isolated cells (Table 4.21), there were no significant
differences in the activation variables between primary and passaged cultured cells (Table 4.23) or between freshly isolated and passaged cells (Table 4.22).

![Graph](image)

**Figure 4.58.** Steady-state outward current activation from freshly isolated (fGP), primary cultured (cGP) and passaged (cGP\(_{1.3}\)) guinea-pig cells. The open circles are fGP (n=36), triangles cGP (n=36) and filled circles cGP\(_{1.3}\) (n=9).

<table>
<thead>
<tr>
<th>Table 4.21. Steady-state outward current activation parameters for freshly isolated (fGP) and primary cultured (cGP) cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>fGP (\text{(mean±SD, n=36)})</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>(V_{0.5}) (mV)</td>
</tr>
<tr>
<td>(k_n) (mV)</td>
</tr>
<tr>
<td>offset</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 4.22. Steady-state outward current activation parameters for freshly isolated (fGP) and passaged (cGP(_{1.3})) cells.</th>
</tr>
</thead>
<tbody>
<tr>
<td>fGP (\text{(mean±SD, n=36)})</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>(V_{0.5}) (mV)</td>
</tr>
<tr>
<td>(k_n) (mV)</td>
</tr>
<tr>
<td>offset</td>
</tr>
</tbody>
</table>
Table 4.23. Steady-state outward current activation parameters from primary cultured (cGP) and passaged (cGP<sub>1-3</sub>) cells.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>cGP (mean±SD, n=36)</th>
<th>cGP&lt;sub&gt;1-3&lt;/sub&gt; (mean±SD, n=9)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>V&lt;sub&gt;0.5&lt;/sub&gt; (mV)</td>
<td>4.6±11.8</td>
<td>6.4±14.5</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>k&lt;sub&gt;n&lt;/sub&gt; (mV)</td>
<td>8.1±3.8</td>
<td>8.6±4.2</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>Offset</td>
<td>0.32±0.41</td>
<td>0.24±0.25</td>
<td>ns (&gt;0.05)</td>
</tr>
</tbody>
</table>

V.5.3.2. Steady-state outward current inactivation

The steady-state outward current inactivation curves for freshly isolated (fGP), primary cultured (cGP) and passaged (cGP<sub>1-3</sub>) guinea-pig cells are shown in Figure 4.59 and Tables 4.24 to 4.26. There were changes to the steady-state outward current inactivation curves after culture, including primary and subcultured cells. In particular the V<sub>0.5</sub> and offset values were significantly changed.

Figure 4.59. Steady-state outward current inactivation curves for freshly isolated (fGP, open circles), primary cultured (cGP, triangles) and passaged (cGP<sub>1-3</sub>, filled circles) guinea-pig cells.
Table 4.24. Steady-state outward current inactivation parameters for freshly isolated (fGP) and primary cultured (cGP) cells.

<table>
<thead>
<tr>
<th></th>
<th>fGP</th>
<th>cGP</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>V_{0.5} (mV)</strong></td>
<td>-63.4±4.3</td>
<td>-26.7±17.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>k_{p} (mV)</strong></td>
<td>9.4±1.9</td>
<td>14.6±7.4</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td><strong>offset</strong></td>
<td>0.32±0.06</td>
<td>0.46±0.03</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 4.25. Steady-state outward current inactivation parameters for freshly isolated (fGP) and passaged (cGP1-3) guinea-pig cells.

<table>
<thead>
<tr>
<th></th>
<th>fGP</th>
<th>cGP1-3</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>V_{0.5} (mV)</strong></td>
<td>-63.4±4.3</td>
<td>-38.8±17.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td><strong>k_{p} (mV)</strong></td>
<td>9.4±1.9</td>
<td>17.4±8.0</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td><strong>offset</strong></td>
<td>0.32±0.06</td>
<td>0.55±0.07</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 4.26. Steady-state outward current inactivation parameters for primary cultured (cGP) and passaged (cGP1-3) cells.

<table>
<thead>
<tr>
<th></th>
<th>cGP</th>
<th>cGP1-3</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>V_{0.5} (mV)</strong></td>
<td>-26.7±17.9</td>
<td>-38.8±17.9</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td><strong>k_{p} (mV)</strong></td>
<td>14.6±7.4</td>
<td>17.4±8.0</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td><strong>offset</strong></td>
<td>0.46±0.03</td>
<td>0.55±0.07</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

VI. Characterisation of peak inward and steady-state outward currents

KCl-filled patch electrodes were used in the present study to investigate electrical membrane properties, under more intracellular physiological conditions. Although the peak inward current was likely to be mainly i_{Ca} and the steady-state outward current i_{K}, their precise characteristics could be distorted when both currents were present together.

To characterise more precisely the kinetics of the membrane ionic channels, "CsCl+0.1mM EGTA" filled electrodes were used in this sections VI.1 to VI.3 to construct i_{Ca} i-v relationships and activation and inactivation curves to understand
better the basis for alteration to action potential characteristics after cell culturing and to demonstrate more precisely the relationship between the action potential and ionic currents.

To characterise the steady-state outward current components varied patch-electrode filling solutions and pharmacological tools were used in section VI.4.

All other conditions were as described above, including the voltage-clamp protocols.

VI.1. \( i_{Ca} \) from freshly isolated, primary cultured and passaged guinea-pig cells

Inward currents in freshly isolated and cultured cells had been identified as \( i_{Ca} \) by using specific L-type Ca\(^{2+}\) channel blockers, 10-20 \( \mu \)M verapamil or 5 \( \mu \)M nifedipine. Figure 4.60 shows an example from a cultured guinea-pig cell where 5\( \mu \)M nifedipine blocked the inward current. Additional evidence has been shown in section III.2.2 (Figures 4.19 and 4.20). The effect of verapamil on the inward currents in a freshly isolated guinea-pig is shown in Figure 4.73. and reference can also be made to section III.1.2.3 (Figure 4.14) and section VIII. Therefore the peak inward currents were \( i_{Ca} \) via L-type Ca\(^{2+}\) channels, in both freshly isolated and cultured cells.

The i-v relationships of \( i_{Ca} \) from freshly isolated, primary cultured and passaged guinea-pig cells are shown in Figures 4.61 to 4.63. The peak \( i_{Ca} \) from the three cell types are listed in Table 4.27. There was a significant difference in the peak \( i_{Ca} \) magnitude between freshly isolated and primary cultured guinea-pig cells as well as between freshly isolated and passaged cells, but with no significant difference between primary cultured and passaged cells. Also note that the \( i_{Ca} \) activation threshold was shifted to less negative potentials in cells from both primary cultured and passaged cells. The appearance of a smaller \( i_{Ca} \) density and rightward shift of the activation threshold after culture could underlie the changes to action potential properties shown in section III.4, III.5 and III.6 (Tables 4.9, 4.10 and 4.11). The similarity of peak \( i_{Ca} \) and its activation voltage threshold between primary cultured and passaged cells suggests that alterations to the Ca\(^{2+}\) channels after culture remained constant following further passage.
Figure 4.60. The effect of 5µM nifedipine on peak inward current in a cultured guinea-pig cell. Traces A and B were recorded from the same cell. In part A currents was recorded in Tyrode's with CsCl-filled electrode and in part B after superfusion with 5µM nifedipine for 2.5 minutes.
Figure 4.61. Peak $i_{Ca}$ i-v relationships in freshly isolated (fGP, filled circles n=10) and primary cultured (cGP, open circles n=6) guinea-pig cells with CsCl-filled electrodes. There were significant differences between voltages -40 and +30mV (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

Figure 4.62. Peak $i_{Ca}$ i-v relationships in freshly isolated (fGP, filled circles, n=10) and passaged (cGP1-3, triangles, n=6) guinea-pig cells with CsCl-filled electrodes. There were significant differences between -40 and +20mV (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).
**Figure 4.63.** Comparison of $i_{\text{Ca}}$ i-v relationships in freshly isolated (fGP), primary cultured (cGP) and passaged (cGP$_{1.3}$) guinea-pig cells with CsCl-filled electrodes. There was no significant difference between cGP (open circles) and cGP$_{1.3}$ (triangles) cells. Note the $i_{\text{Ca}}$ activation threshold voltages shifted to right in both primary cultured and passaged cells.

**Table 4.27.** Peak $i_{\text{Ca}}$ in freshly isolated (fGP), primary cultured (cGP) and passaged (cGP$_{1.3}$) cells with CsCl-filled electrodes

<table>
<thead>
<tr>
<th></th>
<th>Peak $I_{\text{Ca}}$, pA/pF (mean±SD)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>fGP: at 0 mV</td>
<td>-3.6±1.4</td>
<td>10</td>
</tr>
<tr>
<td>cGP: at +10 mV</td>
<td>-1.7±0.9</td>
<td>6</td>
</tr>
<tr>
<td>cGP$_{1.3}$: at +20 mV</td>
<td>-1.6±0.3</td>
<td>6</td>
</tr>
</tbody>
</table>

VI.2. $i_{\text{Ca}}$ activation curves, $d_{\infty}$, of freshly isolated, primary cultured and passaged guinea-pig cells

The $i_{\text{Ca}}$ activation curves for freshly isolated, primary cultured and passaged cells were constructed as shown previously and illustrated in Figure 4.64. Comparison between groups is shown in Tables 4.28 to 4.30. Ca$^{2+}$ channel activation was shifted to more positive potentials after primary culture and subculture and the changes were highly significant ($p<0.001$); the slope factor $k_d$ was not significantly different. There
was no significant change in activation kinetics between primary (cGP) and subculture (cGP1-3), suggesting a similar expression of Ca\(^{2+}\) channel activation function in both conditions.

The rightward shift in \(i_{\text{Ca}}\) activation curve may contribute to a positive shift in the action potential threshold voltage, \(V_{\text{th}}\), in cultured cells.

![Graph showing \(i_{\text{Ca}}\) activation curves in freshly isolated (fGP), primary cultured (cGP) and passaged (cGP1-3) guinea-pig cells with CsCl-filled electrodes. The filled circles are fGP (n=10), open circles cGP (n=6) and triangles cGP1-3 (n=6).](image)

**Figure 4.64.** \(i_{\text{Ca}}\) activation curves in freshly isolated (fGP), primary cultured (cGP) and passaged (cGP1-3) guinea-pig cells with CsCl-filled electrodes. The filled circles are fGP (n=10), open circles cGP (n=6) and triangles cGP1-3 (n=6).

<table>
<thead>
<tr>
<th>(V_{0.5}) (mV)</th>
<th>fGP (mean±SD, n=10)</th>
<th>cGP (mean±SD, n=6)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-18.1±3.8</td>
<td>-7.0±1.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(k_d) (mV)</td>
<td>6.2±1.9</td>
<td>6.4±0.9</td>
<td>ns (&gt;0.05)</td>
</tr>
</tbody>
</table>

**Table 4.28.** \(i_{\text{Ca}}\) activation properties (CsCl-filled) in freshly isolated (fGP) and primary cultured (cGP) guinea-pig cells.
Table 4.29. $i_{Ca}$ activation properties (CsCl-filled) in freshly isolated (fGP) and passaged (cGP1-3) guinea-pig cells.

<table>
<thead>
<tr>
<th></th>
<th>fGP</th>
<th>cGP1-3</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{0.5}$ (mV)</td>
<td>-18.1±3.8</td>
<td>-2.0±7.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$k_d$ (mV)</td>
<td>6.2±1.9</td>
<td>6.7±1.4</td>
<td>ns (&gt;0.05)</td>
</tr>
</tbody>
</table>

Table 4.30. $i_{Ca}$ activation properties (CsCl-filled) in primary cultured (cGP) and passaged (cGP1-3) guinea-pig cells.

<table>
<thead>
<tr>
<th></th>
<th>cGP</th>
<th>cGP1-3</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{0.5}$ (mV)</td>
<td>-7.0±1.7</td>
<td>-2.0±7.1</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>$k_d$ (mV)</td>
<td>6.4±0.9</td>
<td>6.7±1.4</td>
<td>ns (&gt;0.05)</td>
</tr>
</tbody>
</table>

VI.3. $i_{Ca}$ inactivation curves, $f_{\infty}$, of freshly isolated, primary cultured and passaged guinea-pig cells

The Ca$^{2+}$ channel availability in response to steady-state preconditioning voltages was investigated and mean inactivation curves constructed as shown previously and illustrated in Figure 4.65. and Tables 4.31 to 4.33. There was no significant changes to Ca$^{2+}$ channel inactivation kinetics after cell culture.

Table 4.31. $i_{Ca}$ inactivation properties (CsCl-filled) in freshly isolated (fGP) and primary cultured (cGP) guinea-pig cells.

<table>
<thead>
<tr>
<th></th>
<th>fGP</th>
<th>cGP</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{0.5}$ (mV)</td>
<td>-32.0±5.0</td>
<td>-29.3±5.4</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>$k_f$ (mV)</td>
<td>9.1±1.5</td>
<td>7.1±3.8</td>
<td>ns (&gt;0.05)</td>
</tr>
</tbody>
</table>

Table 4.32. $i_{Ca}$ inactivation properties (CsCl-filled) in freshly isolated (fGP) and passaged (cGP1-3) guinea-pig cells.

<table>
<thead>
<tr>
<th></th>
<th>fGP</th>
<th>cGP1-3</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{0.5}$ (mV)</td>
<td>-32.0±5.0</td>
<td>-26.7±4.8</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>$k_f$ (mV)</td>
<td>9.1±1.5</td>
<td>8.8±2.5</td>
<td>ns (&gt;0.05)</td>
</tr>
</tbody>
</table>
Table 4.33. $i_{\text{Ca}}$ inactivation properties (CsCl-filled) in passaged (cGP$_{1.3}$) and primary cultured (cGP) guinea-pig cells.

<table>
<thead>
<tr>
<th></th>
<th>cGP$_{1.3}$ (mean±SD, n=5)</th>
<th>cGP (mean±SD, n=6)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{0.5}$ (mV)</td>
<td>-26.7±4.8</td>
<td>-29.3±5.4</td>
<td>ns (&gt;0.05)</td>
</tr>
<tr>
<td>$k_f$ (mV)</td>
<td>8.8±2.5</td>
<td>7.1±3.8</td>
<td>ns (&gt;0.05)</td>
</tr>
</tbody>
</table>

Figure 4.65. $i_{\text{Ca}}$ inactivation curves in freshly isolated (fGP), primary cultured (cGP) and passaged (cGP$_{1.3}$) guinea-pig cells with CsCl-filled electrodes. The filled circles are fGP (n=8), open circles cGP (n=6) and triangles cGP$_{1.3}$ (n=5).

$i_{\text{Ca}}$ was rapidly inactivated even when the membrane was still depolarised, as shown in Figure 4.60. The declining (inactivating) phase of $i_{\text{Ca}}$ was fitted to a single exponential function using current traces evoked over voltage range from -20 to +20mV for both freshly isolated (fGP) and cultured (including passaged cGP$_{1.3}$) cells. The inactivation time constant of $i_{\text{Ca}}$ is plotted in Figure 4.66. The time constant for peak $i_{\text{Ca}}$ inactivation was 22.1±10.3ms (±SD) at 0mV for freshly isolated guinea-pig cells and 31.5±8.0ms (±SD) at +10mV for primary cultured and passaged cells. These voltages are those at which $i_{\text{Ca}}$ was largest in the three cell groups. The difference was statistically significant.
VI.4. Steady-state outward current, \( i_K \), in guinea-pig cells

VI.4.1. Steady-state \( i_K \) in freshly isolated guinea-pig cells

Characterising the electrophysiological properties of detrusor smooth muscle was a major objective of this project. Therefore patch electrodes were filled with a high concentration of \( K^+ \) to mimic the cytosolic ionic environment. Under these conditions there was a steady-state outward current (i.e. the current at the end of step pulses, see Methods, Figure 3.10) apart from the early inward current \( i_Ca \) and some spontaneous outward currents (STOC, see section VII).

Currents were activated from a holding potential of -60mV to voltages between -100 and +50mV for 2 seconds, and then steady-state outward current was measured at the end of pulse steps as described in Methods (Figure 3.10).
VI.4.1.1. ATP-sensitive K+ channels

To determine ATP-sensitive K+ channels (K<sub>ATP</sub>) in detrusor smooth muscle two patch-electrode filling solutions were used: one was the basic "KCl+5mM EGTA" filling solution which contained 5mM Na<sub>2</sub>ATP (here called "ATP-containing" solution); another was an "ATP-free" filling solution, the same as the "ATP-containing" solution except that Na<sub>2</sub>ATP was replaced by Na<sub>2</sub>Aspartate (see Methods, Table 3.8). Data were collected from freshly isolated guinea-pig cells and are illustrated in Figures 4.67 to 4.71.

K<sub>ATP</sub> channels do not open if the patch electrode contains 5mM ATP, but they open when the electrode filling solution is ATP-free (Figure 4.67). Figures 4.68 and 4.69 show that the K<sub>ATP</sub> channel was activated by a specific K<sub>ATP</sub> channel opener BRL38227 (levcromakalim, gift from Smithkline Beecham) when electrodes contained ATP. However there was no effect of BRL38227 on the K<sub>ATP</sub> channel once the channel opened under intracellular ATP-free conditions (Figures 4.70 to 4.71). This suggested that the steady-state outward current in freshly isolated guinea-pig cells contains an ATP-sensitive K+ current component.

**Figure 4.67.** Steady-state outward current in freshly isolated guinea pig cells using different filling solutions. Open circles are currents using an ATP-containing "KCl+5mMEGTA" filling solution (n=19), filled circles are currents using an ATP-free "KCl+5mMEGTA" filling solution (n=19). There were significant differences between -50 and 0mV (* p<0.05 and ** p<0.01).
Figure 4.68. Membrane currents recorded from a freshly isolated guinea-pig detrusor cells using ATP-containing "KCl+5mM EGTA" filling solution. 30μM BRL38227, a K$_{ATP}$ channel opener, was preceded and followed by a control solution.

Figure 4.69. The effect of 30μM BRL38227 on steady-state outward current in freshly isolated guinea-pig cells. Patch electrodes were filled with "KCl+5mMEGTA" filling solution containing ATP. Open circles are currents in Tyrode's solution and filled circles current in Tyrode's+30μM BRL38227 (n=19pairs). There were significant difference between -50 and +30mV.
Figure 4.70. Membrane current recorded from a freshly isolated guinea-pig cells using ATP-free "KCl+5mM EGTA" filling solution, in which ATP was replaced by Na₂ Aspartate. 30μM BRL38227 was added after the control trace was obtained.

Figure 4.71. The i-v relationships in freshly isolated guinea-pig cells using an ATP-free "KCl+5mM EGTA" filling solution. Filled circles are Tyrode's solution +30μM BRL38227 and open circles Tyrode's solution only (paired data, n=8). There was no significant difference between two curves.
VI.4.1.2. CsCl-sensitive outward currents

All filling solutions used in the present study, except for the "ATP-free" solution which was only used in section VI.4.1.1 to determine ATP-sensitive K⁺ current, contained 5mM ATP to block the ATP-sensitive K⁺ current. The nature of the remaining steady-state current which was likely to contain the K⁺ current component was then investigated by pharmacological means.

The most effective way to block the outward \( I_{\text{k}} \) is by applying intracellular TEA or CsCl. To determine the steady-state outward current, patch electrodes were filled with nystatin (80μg/ml) and CsCl. Continuous recordings of currents under voltage-clamp were made as soon as a whole-cell configuration was achieved. An example is shown in Figure 4.72, where a large transient outward and a steady-state outward current were recorded as soon as whole-cell mode was established. The transient and steady-state outward currents were dramatically reduced 4 minutes after a membrane seal was established due to gradual dialysis of Cs⁺ from the patch electrode into the interior of the cell. There were no transient outward and steady-state outward currents left after another 30 seconds. This observation suggests that the outward currents were through K⁺ channels.

![Graph showing the effect of CsCl on the outward current in a freshly isolated guinea-pig cell. A perforated patch was achieved by nystatin-containing CsCl-filling solution to keep the cell intracellular environment relatively intact. Current traces 1-3 were continuous recordings from the same cell.](image-url)

**Figure 4.72.** The effect of CsCl on the outward current in a freshly isolated guinea-pig cell. A perforated patch was achieved by nystatin-containing CsCl-filling solution to keep the cell intracellular environment relatively intact. Current traces 1-3 were continuous recordings from the same cell.
Figure 4.73. Effects of CsCl, verapamil and NiCl$_2$ on the currents in a freshly isolated guinea-pig cell. Parts A to E were continuous recordings from one cell.

Figure 4.73 is also a continuous recording from a freshly isolated guinea-pig cell. The electrode was filled with CsCl. Two holding potentials were used (-80 and -30mV). The larger currents were elicited from the more negative holding potential. Part A
shows transient and steady-state outward currents recorded as soon as the electrode broke through the cell membrane. Outward currents then disappeared and left only inward currents (part B). This inward current was blocked by 0.1 mM NiCl$_2$+20 μM verapamil and no currents were left at this time (part C and D). There was some inward current recovery after washing out of the Ca$^{2+}$ channel blockers (part E). It thus seems that the inward current was a Ca$^{2+}$ current and the outward currents were K$^+$ currents.

Figure 4.74 gives a summary of the steady-state outward currents in freshly isolated guinea-pig cells using either CsCl or KCl-filling solutions (both n=11) in the patch electrodes. There was little steady-state outward current in CsCl-filled electrodes in contrast to the large current in KCl-filled electrodes, which suggests that the steady-state outward current was a K$^+$ current sensitive to intracellular CsCl.

**Figure 4.74.** Steady-state outward currents in freshly isolated guinea-pig cells with KCl (circles, n=11) or CsCl-filled (squares, n=11) electrodes. Currents were activated from -60 mV to voltages between -100 and +50 mV for 2 seconds and measured at the end of pulse steps. The currents recorded from these two conditions were statistically different from each other at voltages between -30 and +50 mV (* p<0.05, ** p<0.01 and *** p<0.001).
VI.4.1.3. $\text{Ca}^{2+}$-dependent steady-state outward currents

The components of the CsCl-sensitive steady-state outward current were further determined and the results are presented in this section.

Figure 4.75 shows that the steady-state outward current was reduced in a freshly isolated guinea-pig cell after extracellular perfusion with 20$\mu$M verapamil. This effect was more pronounced when the electrode filling solution contained 0.1mM EGTA (part A), rather than 5.0mM EGTA (part B). In the former situation the intracellular $\text{Ca}^{2+}$ was less buffered by the EGTA and therefore there was a higher free $\text{Ca}^{2+}$ concentration. Figure 4.76 summarises the effect of verapamil on the steady-state outward current in freshly isolated guinea-pig cells using "KCl+0.1mM EGTA" filled electrodes. 20$\mu$M verapamil significantly reduced the steady-state outward currents by between 50 and 83% control over the range -20 to +50mV. This suggests that the steady-state outward current contains a $\text{Ca}^{2+}$-dependent component.

Figure 4.75. The effect of verapamil on the steady-state outward current in freshly isolated guinea-pig cells. Part A and B were recorded from different cells using the same protocol. Part A: the electrode was filled with KCl+0.1mM EGTA. Part B: KCl+5mM EGTA filling solution was used.
Figure 4.76. The verapamil-sensitive component of steady-state outward current in freshly isolated guinea-pig cells. The filled circles were currents in Tyrode's and the squares in Tyrode's plus 20μM verapamil (n=11 pairs). Electrodes were filled with "KCl+0.1mM EGTA" and cells were activated by 2-second steps from -60mV. Data are mean±SD. Wilcoxon signed rank test (* p<0.05, **p<0.01)

VI.4.1.4. Large conductance Ca^{2+}-dependant K^{+} channel (BK_{Ca}) in steady-state outward currents

The above data suggest the existence of Ca^{2+}-dependent or Ca^{2+}-activated K^{+} channels. One type of Ca^{2+}-dependent K^{+} channel is the large conductance Ca^{2+}-dependant K^{+} channel (BK_{Ca}). Further experiments were performed to test this. Iberiotoxin, a specific BK_{Ca} channel blocker, significantly reduced the steady-state outward current over a voltage range from -20 to +50mV (except at -10 and 10 mV) by between 12 and 35% of control in freshly isolated guinea-pig cells, when electrodes were filled with "KCl+0.1mM EGTA". There was no significant effect of apamin (a blocker of small conductance Ca^{2+}-dependant K^{+} channels see next section) on the steady-state outward currents. A CsCl-filling solution blocked the current completely. These results are shown in Figure 4.77 and suggest that steady-state outward currents contained a component of BK_{Ca}. 
Figure 4.77. The effect of iberiotoxin (100nM) and apamin (1μM) on the steady-state outward current in freshly isolated guinea-pig cells. Electrodes were filled with "KCl+0.1mM EGTA". Filled circles are Tyrode's solution (n=7), open squares 100nM iberiotoxin (n=7), open diamonds 100nM iberiotoxin + 1μM apamin (n=5) and filled triangles were data with CsCl-filled electrodes (n=11).

VI.4.1.5. TEA-insensitive components

The K+ channel blocker, TEA, was used to determine further the nature of the verapamil-insensitive components in the steady-state outward current, recorded with "KCl+0.1mM EGTA" filled electrodes. Figure 4.78 shows that 20μM verapamil (filled diamonds, n=11) significantly reduced steady-state outward current compared to control (filled circle, n=11), see also Figure 4.76. Addition of 30mM TEA did not significantly reduce the current further (open circles, n=7), and was still greater than the residual current using CsCl-filled electrodes (filled triangles, n=11).
VI.4.2. Steady-state $i_K$ in cultured guinea-pig detrusor cells

VI.4.2.1. CsCl-sensitive outward currents

The steady-state outward current in cultured guinea-pig detrusor cells was also examined. Figure 4.79 shows that a large steady-state outward current was recorded with KCl filled electrodes, but there was little steady-state outward current when electrodes were filled with CsCl.

VI.4.2.2. Ca$^{2+}$-dependent component

Figure 4.80 shows the mean verapamil-sensitive currents recorded from 3 cultured guinea-pig cells. It shows that 20μM verapamil reduced the steady-state outward current, which suggests the presence of Ca$^{2+}$-activated or Ca$^{2+}$-dependent component in the steady-state outward current.
Figure 4.79. Comparison of steady-state outward currents recorded with KCl or CsCl filled electrodes in cultured guinea-pig cells. The open circles are with KCl-filled electrodes (n=9) and the filled circles CsCl-filled electrodes (n=9). Cells were activated from -60mV by 2-second steps. Data were expressed as mean±SD. From -20mV upwards there was significant difference between the two sets of data (t-test).

Figure 4.80. Verapamil-sensitive component of steady-state outward current in cultured guinea-pig cells. The filled circles are steady-state outward currents in Tyrode's and open circles with 20μM...
verapamil (n=3 pairs). Electrodes were filled with KCl+0.1mM EGTA. Cell were activated by 2-second steps from a holding potential of -60mV.

VI.4.2.3. TEA-insensitive components

TEA-sensitive steady-state outward current in cultured guinea-pig cells was also investigated. 20µM verapamil reduced the steady-state outward current (open circles) compared to control (filled circles) as shown in Figure 4.80. Figure 4.81 shows that further addition of 30mM TEA and 5mM BaCl2 further reduced the current (open triangle). However there was still a significant component compared to current with CsCl-filled electrodes (filled circles).

Figure 4.81. TEA-insensitive steady-state outward current in cultured guinea-pig cells. The open circles are steady-state outward current in 20µM verapamil (n=5), triangles in 20µM verapamil +30mM TEA+5mM BaCl2 (n=5). Electrodes were filled with KCl+0.1mM EGTA and cells were activated by 2-second steps from -60mV. Data were expressed as mean±SD. The filled circles represent steady-state outward currents recorded with CsCl filled electrodes (n=9).
VII. Spontaneous transient outward current, STOC

When electrodes were filled with the basic "KCl+5mM EGTA" solution, spontaneous transient outward currents (STOC) could be recorded in freshly isolated but not cultured guinea-pig and human cells. The STOC's occurred in 44.5% (53 out of 119 cells) of guinea-pig cells. When a "KCl+0.1mM EGTA" solution was used STOC's occurred in every cell. Therefore further investigations used guinea-pig cells and electrodes filled with the "KCl+0.1mM EGTA" solution. Currents were activated from a holding potential of -60 mV by 2-second clamp steps. The measurement of STOC magnitude is described in the Methods (section IV.6.2.2, Figure 3.14).

Figure 4.82 shows the voltage dependence of pure STOC in cells from freshly isolated guinea-pig cells, compared to cultured cells in which no STOC was recorded.

![Pure STOC](image)

**Figure 4.82.** Spontaneous transient outward current (STOC) in freshly isolated (open circles, n=8) and cultured (filled circles n=9) guinea-pig cells.

VII.1. STOC is Ca$^{2+}$ dependent

Figure 4.83 shows the Ca$^{2+}$-dependence of the STOC in a freshly isolated guinea-pig cell. The large STOC was induced by a 2-second step pulse from a holding potential of -60 to 0mV. It was abolished when the cell was superfused with a Ca$^{2+}$-free
Tyrode's solution containing 0.12 mM EGTA and extra 1.8mM MgCl₂. The STOC recovered as the Ca^{2+}-free was restored to Tyrode's, which suggests that the STOC is Ca^{2+}-dependent.

**Figure 4.83.** The Ca^{2+} dependence of STOC in a freshly isolated guinea-pig cell. Part A shows a control trace in Tyrode's solution. Part B shows the current recorded in a Ca^{2+}-free solution (0.12mM EGTA+2.8mM MgCl₂). Part C shows the post-control. Parts A to C were from a continuous recording in the same cell.
The Ca\(^{2+}\) dependence of the STOC was further confirmed by using extracellular BaCl\(_2\) instead of CaCl\(_2\) in superfusion solution. Figure 4.84 shows that there were large inward currents and there was no STOC.

**Figure 4.84.** The effect of BaCl\(_2\) on the STOC. The currents were recorded in a freshly isolated guinea-pig cell with an electrode filled with "KCl+0.1mM EGTA" solution. Part A shows the currents in Tyrode's solution. Part B was obtained in a Ca\(^{2+}\)-free solution plus 10mM BaCl\(_2\). Part A and B were continuous recording in the same cell.
Pure STOC
at 10mV
(pA/pF)

pCa
**Figure 4.85.** The i-v relationship of STOC in freshly isolated guinea-pig cells in the presence (open circles) and absence (filled circles) of Ca\(^{2+}\). The paired data, mean±SD, were obtained from 14 cells. **p<0.01** and ***p<0.001 (Wilcoxon signed rank test). The pure STOC in Tyrode's solution was significantly larger at voltage from -30 mV onwards. The open triangles overlapped with filled circles formed a baseline of current measured with CsCl filled electrodes (n=6).

**Figure 4.86.** The effect of extracellular [Ca\(^{2+}\)] on STOC amplitude recorded at +10mV.
Figure 4.87. The effect of verapamil on the STOC in a freshly isolated guinea-pig cell. The STOC was evoked by a step pulse from a holding potential of -60 to +40mV for 2s. Part A shows a control trace; part B the effect of 20μM verapamil for 5 minutes and part C the action of 20μM verapamil +30mM TEA. Part D is the current recorded 5 minutes after a return to control condition.
Figure 4.88. The effect of verapamil and NiCl₂ on the STOC in a freshly isolated guinea-pig cell. The STOC was evoked from a holding potential of -60mV to between 0 and -30mV for 2 seconds. Part A shows control traces; part B in the presence of 20μM verapamil and 1mM NiCl₂ for 5 minutes. Part C is after a return to control for 5 minutes. Parts A to C were a continuous recording from one cell.

Figure 4.85 summarises data demonstrating the Ca²⁺-dependence of pure STOC. There was no STOC when a Ca²⁺-free solution was used. Data were acquired from paired experiments in 14 cells. It shows the N-shaped i-v relationship of pure STOC, which significantly differs from the Ca²⁺-free current over -30 to +50mV.

The Ca-dependence of STOC was also studied in further experiments, in which a change of the extracellular Ca²⁺ concentration altered the STOC magnitude. The pure STOC measured at a clamp step to +10mV was increased as the extracellular Ca²⁺ concentration ([Ca²⁺]₀) was raised between 10⁻⁸M (Ca²⁺-free +0.12mM EGTA) and 5.4mM. Figure 4.86 shows STOC density as a function of nCa. Data were obtained.

The close linear correlation between STOC amplitude and [Ca²⁺]₀ raised the question as to the site of action of Ca²⁺ on the STOC channels. Figures 4.87 and 4.88 show that the STOC was greatly reduced by extracellular application of 20μM verapamil, and to greater extent by verapamil and 1mM NiCl₂, these are both Ca²⁺
Figure 4.89. Averaged data for the effect of verapamil on the STOC amplitude in freshly isolated guinea-pig cells. The filled triangles are STOC in Tyrode's and open squares with 20μM verapamil (n=11 pairs). The filled circles are verapamil plus 30mM TEA (n=7) which overlap the baseline current measured with CsCl-filled electrodes (crosses, n=6). 20μM verapamil blocked STOC by 78-90% of control between -20 and +50mV (Paired t-test).

channel blockers. Ca\(^{2+}\) entry into the cell therefore appears to be essential which strongly suggests that the action of Ca\(^{2+}\) on the STOC channel took place at intracellular side. The Ca\(^{2+}\) could be from an extracellular source but entry through the Ca\(^{2+}\) channel is a major route. A summary of the effect of verapamil on the STOC shown in Figure 4.89.

VII.2. STOC is a K\(^{+}\) current

The STOC recorded above could be either a K\(^{+}\) or Cl\(^{-}\) current. In the present experiments, the intracellular [Cl\(^{-}\)] was 30.9mM and extracellular [Cl\(^{-}\)] in Tyrode's was 127.6 mM, with experiments performed at 36.5°C. From the Nernst equation, the Cl\(^{-}\) equilibrium potential, E_{Cl}, is -37.6 mV when superfused with Tyrode's and -43.2 mV with Tyrode's plus 30mM TEACl. Thus when the membrane was depolarised positive to -30mV, any Cl\(^{-}\) current would be outward going.
Page 212, Figure 4.90 replaced by the following:

![Graph showing electrophysiological responses to Tyr, 3 mM TEA, and combined 30 mM TEA and 20 µM verapamil.]

Page 212, Figure 4.90 legend, line 3: cancel the sentence: "further application of 30 mM TEA and 20 µM verapamil completely blocked the STOC. In part D:"

Page 212, line 2 of paragraph 2: cancel the sentence: "and the remainder was blocked by addition of 20 µM verapamil and 30 mM TEA."
Figure 4.90. The effect of 3mM [TEA]₀ on STOC in a freshly isolated guinea-pig cell. The electrode was filled with KCl+0.1mM EGTA. Part A: A large STOC was evoked by a pulse from -60 to +20mV. Part B: 3mM TEA application for 3 minutes greatly attenuated the current. Part C: further application of 30mM TEA and 20μM verapamil completely blocked the STOC. In part D: return to Tyrode's partially restored the current. Traces A to D are a continuous recording from a single cell.

Evidence for STOC being a K⁺ current came from the fact that the remaining STOC after verapamil application was abolished by applying 30mM TEA Cl. In addition intracellularly applied CsCl progressively inhibited the transient outward current as shown in section VI.4. Figures 4.72 and 4.73. Further evidence is shown in Figure 4.90 and 4.91.

Figure 4.90. shows the STOC in a freshly isolated guinea-pig cell. A low concentration of extracellular TEA inhibited most of the current and the remainder was blocked by addition of 20μM verapamil and 30mM TEA. Figure 4.91 shows a second cell in which the STOC was evoked. The figure shows that after holding the cell at -80mV or -30mV for 2s a subsequent step to -20mV elicited the STOC. Addition of 30mM TEA blocked the STOC at all potentials.

Figure 4.92 shows the effects of 3mM extracellular TEA on the STOC i-v relationship (n=4). Pure STOC was measured as the difference between peak STOC
Figure 4.91. The effect of 30mM [TEA]₀ on the STOC. The cell was held at -60mV and activated from -60mV(1ms) to -20mV after 2 second preconditioning pulses to -80 or -30mV. Upper traces were in Tyrode’s solution; the lower traces: in Tyrode’s + 30mM TEA.

Figure 4.92. The effect of a low concentration (3 mM) of TEA on STOC in freshly isolated guinea-pig cells (n=4). Pure peak STOC (open circles) was measured as the difference between the peak STOC and early steady-state current. The filled diamonds are residual current using CsCl-filled electrodes (n=6). The crosses are current in 3 mM TEA (n=4).
and early steady-state component as defined in Methods (IV.6.2.2. Figure 3.14) The non-STOC baseline current was taken as the residual current measured with CsCl-filled electrodes (n=6) in separate experiments and shown as the diamonds in Figure 4.92. The residual current after TEA addition using KCl-filled electrodes is shown as the crosses. 3mM TEA reduced STOC by 70-91% over the voltage range -30 to 50 mV and 30 mM TEA blocked the STOC totally (not shown in Figure 4.92). The data suggest that the STOC was a K+ current and not a Ca2+-dependant Cl- current.

VII.3. STOC is a BKCa current

Iberiotoxin, a specific BKCa blocker, was used to determine the STOC components. In Figure 4.93, parts A to D were continuous recordings from one cell. 100nM iberiotoxin was superfused for 8 minutes and markedly reduced the STOC (part B); further exposure to 100nM iberiotoxin and 1μM apamin for 4 minutes did not show a further effect. The STOC recovered well after washing out the toxins for 9 minutes, suggesting that the STOC was mostly a BKCa current.

Figure 4.94 summarises the mean iberiotoxin effect on STOC from freshly isolated guinea-pig cells. The STOC was significantly reduced by 100 iberiotoxin by up to 73-91% of Tyrode's control at voltages -10 to +50mV, (Wilcoxon signed (paired) rank test p<0.05, n=7).
Figure 4.93. The effect of iberiotoxin on the STOC. Traces A to D were continuous recordings from one cell. Part A: control Tyrode's solution; part B: Tyrode's and 100nM iberiotoxin; part C: Tyrode's + 100nM iberiotoxin + 1μM apamin; part D: post intervention control in Tyrode's solution.

Figure 4.94. Averaged data for the effect of iberiotoxin on STOC in freshly isolated guinea-pig cells. The filled circles are STOC in Tyrode's solution (n=7), open triangles 100nM iberiotoxin (n=7), filled triangles 100nM iberiotoxin+1μM apamin (n=5) and crosses baseline current measured in CsCl-filled electrodes (n=6). Wilcoxon signed (paired) rank test * p<0.05: Tyrode's vs iberiotoxin.
VII.4. STOC and intracellular Ca$^{2+}$

VII.4.1. STOC and membrane depolarisation

STOC was inhibited by Ca$^{2+}$ channel blockers which suggested an action of intracellular Ca$^{2+}$ on BK$_{Ca}$ channels. Figure 4.95 shows a simultaneous recording of membrane current and intracellular [Ca$^{2+}$]. A large outward current precedes the rise of [Ca$^{2+}$]$\text{i}$. Therefore the Ca$^{2+}$-dependent outward current may be a response to a local rise of [Ca$^{2+}$] beneath the membrane before the bulk [Ca$^{2+}$]$\text{i}$ had increased. There are reports that STOC in some smooth muscles is more sensitive to a local intracellular Ca$^{2+}$ rise and STOC has been used in some studies as a more sensitive indicator of an intracellular Ca$^{2+}$ rise near the cell membrane compared with the whole cell [Ca$^{2+}$]$\text{i}$ (Bolton and Imaizumi 1996; Ganitkevich and Isenberg 1996). Thus Ca$^{2+}$ entry through Ca$^{2+}$ channels caused a high local concentration which would cause the first peak of STOC; while subsequent STOC oscillations (frequency) are more likely related to the bulk intracellular Ca$^{2+}$ rise.

\[
\frac{[\text{Ca}^{2+}]}{[\text{Ca}^{2+}]_{\text{bulk}}} (\text{Ratio 340/380})
\]

Figure 4.95. The relationship between inward current, the first peak of STOC and intracellular bulk Ca$^{2+}$ simultaneously recorded in a freshly isolated guinea-pig cell. The cell was depolarised by a step pulse from -60 to +10mV.
Figure 4.96. Simultaneous $[Ca^{2+}]_i$ and current recording in a freshly isolated guinea-pig cell. In parts A-C depolarisation increased from -20 to +50mV.

Figure 4.97. The relationship between $[Ca^{2+}]_i$ and pure STOC. The filled circles are pure STOC and open circles the mean $[Ca^{2+}]_i$ during depolarising pulses. Data are the mean values from 7 simultaneous recordings.
Figure 4.98. Correlation between the pure STOC amplitude and the mean [Ca^{2+}]_i. Data from the same source as in Figure 4.97. The regression line was fitted using a least-squares fit (r=0.862 p<0.05 n=7 in each data point).

Figure 4.96 shows the voltage dependence of STOC in relation to the rise of [Ca^{2+}]_i. In part A a depolarisation to -20mV caused only a modest rise of [Ca^{2+}]_i and no STOC. In part B depolarisation to +20mV caused a larger rise of [Ca^{2+}]_i and several STOCs. Depolarisation to +50mV(part C) caused a smaller rise of [Ca^{2+}]_i and only one large STOC.

Figure 4.97 shows the voltage dependence of pure STOC amplitude and magnitude of the intracellular Ca^{2+} transient for data recorded from seven cells. The [Ca^{2+}]_i is the mean value during the clamp step. The averaged correlation of STOC amplitude to mean intracellular [Ca^{2+}] is shown in Figure 4.98 where there is a significant correlation between the two variables.

An alternative way to generate the relationship between STOC amplitude and [Ca^{2+}]_i was to apply a slow ramp depolarisation (15mV.s^{-1}). Figure 4.99 shows such an experiment in which the [Ca^{2+}]_i and the outward current originated from approximately the same potential. Both STOC and steady-state outward currents increased as the [Ca^{2+}]_i rose.
The voltage-dependence of STOC was further investigated at a constant intracellular free Ca$^{2+}$ of 0.25μM using a filling solution containing Ca$^{2+}$ and high concentration of EGTA (Table 3.12). In the presence of 10μM nifedipine, membrane depolarisation caused outward current without the rise of intracellular Ca$^{2+}$. This outward current was blocked by 100nM iberiotoxin and exhibited voltage-dependence at membrane potentials beyond 0mV (Figure 4.100).
Figure 4.100. The voltage dependence of STOC. iberotoxin-sensitive (100nM) outward current was recorded under the condition of constant intracellular free $\text{Ca}^{2+}$ and presence of 10μM nifedipine. Data are the mean values (±SD) from 5 cells.

VII.4.2. STOC and intracellular store release

STOC could also be caused by intracellular $\text{Ca}^{2+}$ release from the sarcoplasmic reticulum (SR) as shown in Figure 4.101. Parts A-C were simultaneous recordings from one cell. From parts A to C, the cell was held at -60mV, 0 and +20mV respectively. 20mM caffeine induced $\text{Ca}^{2+}$ release from SR and caused intracellular $\text{Ca}^{2+}$ to rise. Although the intracellular $\text{Ca}^{2+}$ transients were similar, the STOC amplitude became progressively larger as the holding potential was more positive. These data further reinforce the dual-dependence of STOC activation on $[\text{Ca}^{2+}]_i$ and membrane potential.
A:

\[ [\text{Ca}^{2+}]_i \]

Ratio 340/380

0.7

300

0.3

0

pA

50 s

B:

\[ [\text{Ca}^{2+}]_i \]

Ratio 340/380

0.8

300

0.4

0

pA

50 s
Figure 4.101. STOC and caffeine-induced intracellular Ca\(^{2+}\) release. Parts A-C were simultaneous recordings from one cell of STOC and caffeine-induced intracellular Ca\(^{2+}\) release from SR. Part A at a holding potential of -60mV, B at 0mV and C at +20mV. Top traces in each graph are [Ca\(^{2+}\)]\(_i\) rise caused by 20mM caffeine; the bottom traces are STOC.

VIII. T-type Ca\(^{2+}\) current

A few questions have been raised by comparison of data from freshly isolated and cultured cells:

(1). Inward currents which was defined as i\(_{Ca}\) in freshly isolated and cultured cells exhibited a difference in activation properties (section V, Figures 4.44 and 4.53; section VI Figure 4.63), i.e., a "shoulder" current at negative voltages existed in freshly isolated cells from both human and guinea pig, but not in cultured cells from either species. Figure 4.102 is a summary of this difference between freshly isolated and cultured cells from guinea-pigs.

a). The filled circles are currents from cultured guinea-pig (cGP) (n=4) and the open circles from freshly isolated guinea-pig cells (fGP) (n=10). Both were recorded from a holding potential (V\(_h\)) of -100mV and their i\(_{Ca}\) thresholds, shown in the right inset, exhibited a significant difference (fGP: -51±10mV, n=10; cGP: -15±5mV, n=4 (mean±SD) unequal variance t-test p<0.001 ).
Figure 4.102. Inward $i_{Ca}$ characteristics in cells from freshly isolated (fGP) and cultured (cGP) guinea-pig cells with "CsCl+5mM EGTA" filled electrodes. The filled circles are cultured cells (n=4) with a holding potential (Vh) at -100mV, the open circles are freshly isolated guinea-pig cells (n=10) with a holding potential at -100mV and the triangles are freshly isolated guinea-pig cells (n=10) with a holding potential at -30mV. The right inset shows $i_{Ca}$ thresholds for fGP (Vh-100mV) and cGP (Vh -100mV). The left inset shows i-v relationships from a cultured guinea-pig cell with holding potentials of -100 or -30mV. The characteristics of $i_{Ca}$ from fGP and cGP are described in detail in the text.

b). In freshly isolated guinea-pig cells, currents from different holding potentials (-30mV, open triangles ; -100mV, open circles ) had different $i_{Ca}$ thresholds (arrows in Figure 4.102). In contrast to this, the left inset shows i-v relationship recorded from a cultured guinea-pig cell with holding potentials of -30mV or -100mV. The $i_{Ca}$ thresholds were at the same voltage as that of freshly isolated guinea-pig cells with a holding potential of -30mV. In other words, cultured guinea-pig cells lacked a low voltage activation component which was present in freshly isolated cells, when cells were held at a holding potential of -100mV.

(2) Action potentials in freshly isolated cells shown in section III.1.2 (Figure 4.14.) could not be blocked completely by L-type Ca$^{2+}$ channel blockers, but could be abolished totally by these agent in cultured cells, shown in section III.2.2 (Figure 4.19 and 4.20). This suggested that $i_{Ca}$ in freshly isolated cells contained a current component which is resistant to L-type Ca$^{2+}$ channel blockers.
(3) The action potential thresholds, $V_{\text{th}}$, from freshly isolated and cultured cells were different. The $V_{\text{th}}$ in cultured cells was more depolarised (see section III). The $V_{\text{th}}$ values were lower than that expected from the L-type Ca$^{2+}$ channel activation threshold when cells were held at a negative potential, i.e., about -100mV. The $V_{\text{th}}$ and holding potential are well correlated as shown in Figure 4.103.

![Graph](image)

**Figure 4.103.** The correlation between action potential threshold, $V_{\text{th}}$, and holding potential in freshly isolated guinea-pig cells using KCl+0.1EGTA solution filled electrodes. The regression line was fitted using a least-squares fit ($r=0.72$, $p<0.001$, $n=33$).

All these data are inconsistent with the hypothesis and suggest that freshly isolated detrusor cells contain non-L-type Ca$^{2+}$ channels, which have not persisted during culture.

Therefore further investigation was undertaken to determine if there existed low voltage activated Ca$^{2+}$ channels in detrusor myocytes freshly isolated from guinea-pigs. The electrodes were filled with "CsCl+5mMEGTA" or "KCl+0.1mM EGTA" solution in these experiments. Cells were held at -60mV but 2-second preconditioning pulses, at more variable potentials such as -100, -80 and -30 mV, were used. Other conditions such as temperature, pH of superfusion solution etc. remained the same.
VIII.1. Low (negative) voltage activated $i_{\text{Ca}}$ in freshly isolated guinea-pig cells

Transient Ca$^{2+}$-activated K$^+$ current (STOC) was activated as intracellular Ca$^{2+}$ rose either by Ca$^{2+}$ release from the SR or as inward Ca$^{2+}$ current. In the latter case, STOC can be a useful indicator of Ca$^{2+}$ entry through membrane channels. By recording this STOC and the inward current at various activation voltages, information on the activation voltage for Ca$^{2+}$ channel opening can be obtained.

Figure 4.104 shows that with K-filled electrodes, inward currents were recorded when cells were held at -100mV and activated to -70, -60 or -50mV, which would not be expected to activate L-type Ca$^{2+}$ channels. When the cell was depolarised to -70mV, a small inward current was recorded; when depolarised to -60mV, a small outward transient current appeared following an inward current. A large inward current was followed by a large transient outward current when the cell was depolarised to -50mV. The data suggest that these inward currents were Ca$^{2+}$ currents activated at negative voltage, which then activated the Ca$^{2+}$-dependent $i_K$.

Figure 4.104. Low voltage activated $i_{\text{Ca}}$ in a freshly isolated guinea-pig cell. The electrode was filled with a "KCl+0.1mM EGTA" solution, holding potential -100 mV. The inset shows the inward currents on a faster time-base.
VIII.2. Sensitivity to L-type Ca\(^{2+}\) channel blockers

To identify the non-L-type Ca\(^{2+}\) channels, further investigations were carried out. The experiments were carried in freshly isolated guinea-pig cells with electrodes filled with "CsCl+5mM EGTA" solution.

Figure 4.105. The nifedipine-resistant component of \(i_{\text{Ca}}\) in a freshly isolated guinea-pig cell. The cell was pre-conditioned to -70mV for 2s and then stepped to +10mV, after a 1ms step to -60mV. A second trace is superimposed after a 10 minute exposure to 5\mu M nifedipine. The inset shows the i-v relationship from another cell of the peak inward current before (closed circles) and during (open circles) exposure to 5\mu M nifedipine.

Figure 4.105 shows that there was a nifedipine-resistant component of \(i_{\text{Ca}}\). The cell was held at a holding potential of -60mV, with a pre-conditioning pulse to -70mV for 2 seconds and then stepped to 10mV. Superfusion with 5\mu M nifedipine for 10 minutes did not block \(i_{\text{Ca}}\) entirely. The inset is the i-v relationship recorded from another cell with a 2-second preconditioning step of -100mV; 5\mu M nifedipine blocked most of the inward current but was less effective at more negative holding potentials.

20-50 \mu M Cd\(^{2+}\), which is expected to block completely L-type Ca\(^{2+}\) channels, did not totally block \(i_{\text{Ca}}\), suggesting a Cd\(^{2+}\)-insensitive components of \(i_{\text{Ca}}\). Parts A to C in
Figure 4.106. Cd$^{2+}$-insensitive component of $i_{\text{Ca}}$. Part A and B: the effect of 20μM CdCl$_2$ on inward currents evoked from a preconditioning potential of -100mV to -30mV or -10mV. Part C: the i-v relationship of the inward current before (filled circles) and during (open circles) exposure to 20μM CdCl$_2$. Parts A to C are recordings from the same cell.
Figure 4.106 are recordings from the same cell. Part A and B show inward currents evoked at -30mV and -10mV after a 2-second preconditioning pulse at -100mV. The superimposed second trace was obtained after 11 minutes superfusion with a 20μM CdCl₂ solution. These traces were used to construct the i-v relationship of inward current in the absence (filled circles) and presence (open circles) of 20μM CdCl₂. Note that 20μM CdCl₂ blocked completely the sustained component of the inward current but only partially blocked the transient component.

VIII.3. Sensitivity to low concentrations of NiCl₂

More specific experiments were undertaken to characterise further the nature of the current. Figure 4.107 shows that continuous superfusion with 20μM verapamil for more than 9 minutes did not block totally the iCa. However, the verapamil or nifedipine-insensitive component was inhibited by exposure to 0.1mM NiCl₂ for 1-1.5 minutes. This suggested that the verapamil-resistant component of iCa was Ni²⁺-sensitive.

![Graph showing sensitivity to NiCl₂](image.png)

**Figure 4.107.** A Ni²⁺-sensitive component of iCa. Currents were evoked from a 2s preconditioning pulse of -80mV to -20mV in control condition; 20μM verapamil; and 20μM verapamil + 0.1mM NiCl₂.
VIII.4. Ca\(^{2+}\) as a Ni\(^{2+}\)-sensitive current carrier

To ascertain the ionic basis of the Ni\(^{2+}\)-sensitive current, the effect of Ca\(^{2+}\) removal on this current was tested. Parts A and B in Figure 4.108 are continuous recordings from the same cell. The inward current vanished when the cell was superfused with a Ca\(^{2+}\)-free solution (0.12mM EGTA) and recovered when Tyrode's was restored. Part B shows that this inward Ca\(^{2+}\) current could not be blocked by 20 µM verapamil but was blocked completely when 0.1 mM NiCl\(_2\) was included for 1 minute. Therefore the Ni\(^{2+}\)-sensitive inward current was carried by Ca\(^{2+}\).

Figure 4.108. Ca\(^{2+}\) is the charge carrier of the Ni\(^{2+}\)-sensitive inward current. Parts A and B were continuous recordings from the same cell. Part A shows traces before, during and after superfusion with a Ca\(^{2+}\)-free solution (Ca\(^{2+}\)-free+0.12mM EGTA). Part B shows the effect of 20µM verapamil and 20µM verapamil+0.1mM NiCl\(_2\) on the inward current. All currents were evoked from preconditioning 2s pulses of -80mV to -20mV.

These experiments therefore suggest that a low-voltage activated current is present in freshly isolated guinea-pig cells which is verapamil or nifedipine resistant, but blocked by NiCl\(_2\). This suggests the current is a T-type Ca\(^{2+}\) current, (\(i_{T_{Ca}}\)).
VIII.5. The i-v relationships of L-type ($i_{\text{L}Ca}$) and T-type ($i_{\text{T}Ca}$) currents in freshly isolated guinea-pig cells

To gain more precise information on the voltage characteristics of $i_{\text{L}Ca}$ and $i_{\text{T}Ca}$, these currents were studied in a quantitative way. $i_{\text{L}Ca}$ was quantified in the presence of 0.2mM NiCl$_2$ to block $i_{\text{T}Ca}$. $i_{\text{T}Ca}$ was quantified when 5µM nifedipine was used to block the L-type Ca$^{2+}$ current. The currents shown in Figure 4.109 were induced at a holding potential of -60mV and a 2s pre-conditioning pulses at -100 mV. As Figure 4.109 shows, the currents at more negative voltages were significantly different, which proved that the "shoulder currents" recorded in freshly isolated guinea-pig and human detrusor were $i_{\text{T}Ca}$. The peak $i_{\text{T}Ca}$ was at -10mV: -1.4±0.8pA/pF (mean±SD, n=11) and the peak $i_{\text{L}Ca}$ was at +10mV: -4.0±2.0pA/pF (n=14); unpaired t-test: p<0.001.

Figure 4.109 also shows the threshold voltages for $i_{\text{T}Ca}$ and $i_{\text{L}Ca}$. These were -56±10.6mV (±SD, n=24), and -24±6.3mV (±SD, n=14) respectively (p<0.001). The threshold was calculated by recording the most negative clamp step in each cell at which the current was measured.

![Figure 4.109](image-url)

**Figure 4.109.** The current-voltage relationships of $i_{\text{L}Ca}$ and $i_{\text{T}Ca}$. The filled circles are NiCl$_2$-sensitive current $i_{\text{T}Ca}$ (n=11) and the open circles the nifedipine-sensitive current $i_{\text{L}Ca}$ (n=14). Currents recorded from a 2-second pre-conditioning pulse at -100mV. The inset shows threshold for $i_{\text{T}Ca}$ and $i_{\text{L}Ca}$ (** p<0.01, *** p<0.001).
Figure 4.110. The "total Ca\(^{2+}\) current" i-v relationship in freshly isolated guinea-pig cells. The triangles are the "total Ca\(^{2+}\) current" \((= i_{\text{LCa}} + i_{\text{TCa}})\), closed circles \(i_{\text{TCa}}\) and open circles \(i_{\text{LCa}}\).

\(\tau\) (ms)

\(n=13\) and open circles are \(i_{\text{LCa}}\) (in 0.2mM NiCl\(_2\), \(n=10\)). Electrodes were filled with the CsCl+5mM EGTA solution; Tyrode's solution containing 1.8mM CaCl\(_2\) at 36.5±0.5 °C. Mean±SD, \(***\) \(p<0.001\) \(\tau_{\text{inact}}\) for \(i_{\text{TCa}}\) at -10mV versus \(i_{\text{LCa}}\) at +10mV.
Figure 4.110 plots the sum of $i_{kCa}$ and $i_{TCa}$ as the "total Ca$^{2+}$ current" in freshly isolated guinea-pig detrusor myocytes.

Figure 4.111 shows the inactivation time constants, $\tau_{inact}$, for $i_{kCa}$ and $i_{TCa}$ as a function of membrane potential with 2s preconditioning voltage of -100mV and in 1.8mM $[Ca^{2+}]_0$; the CsCl+5mM EGTA electrode filling solution was used. At -10mV $\tau_{inact} = 22.0\pm3.6$ms for $i_{TCa}$ (n=13, in 5µM nifedipine), and at +10mV $\tau_{inact} = 37.9\pm9.2$ms for $i_{kCa}$ (n=10, in the presence of 0.2mM NiCl$_2$). These values of $\tau_{inact}$ were smallest value for each current at the voltage at which peak current was largest. The values were significantly different (p<0.001, unequal variance t-test).

VIII.6. Steady-state activation and inactivation variables of $i_{TCa}$ and $i_{kCa}$ from freshly isolated guinea-pig cells

For comparison of the activation kinetics of $i_{TCa}$ and $i_{kCa}$, $i_{TCa}$ was recorded in the presence of 5µM nifedipine (n=11), and $i_{kCa}$ in the presence of 0.2mM NiCl$_2$ (n=14). Currents were evoked from a 2s preconditioning pulse of -100mV, switched to -60mV for 1ms and then to voltages between -100 to +40mV. The mean activation curves were constructed in the same way as described in section V.1.2. and shown in Figure 4.112. Comparison of the activation parameters is given in Table 4.34.

To compare $i_{TCa}$ and $i_{kCa}$ inactivation kinetics, $i_{TCa}$ was again recorded in the presence of 5µM nifedipine (n=11), and $i_{kCa}$ in the presence of 0.2mM NiCl$_2$ (n=15). Currents were evoked from 2s preconditioning voltages over a range of -120 to +30mV, pulsed to -60mV for 1ms and then to +10mV for $i_{kCa}$ and -10mV for $i_{TCa}$ for 500ms. The mean inactivation curves were constructed as described in section V.1.2. and are shown in Figure 4.113. Comparison of the inactivation parameters is given in Table 4.35.

Table 4.34. Steady-state activation parameters for $i_{TCa}$ & $i_{kCa}$ in freshly isolated guinea-pig cells.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$i_{TCa}$</th>
<th>$i_{kCa}$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{0.5}$ (mV)</td>
<td>-28.1±8.0</td>
<td>-9.3±6.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>slope factor (mV)</td>
<td>8.1±3.8</td>
<td>5.2±1.5</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

(1) Electrodes were filled with the "CsCl+5mM EGTA"solution, preconditioning voltage -100mV for 2 seconds. (2) p values from unpaired two-tailed t-tests after F-tests.
Figure 4.112. $i_{TCa}$ and $i_{ILCa}$ steady-state activation curves in freshly isolated guinea-pig cells from a 2s preconditioning voltage at -100mV. The filled circles are $i_{TCa}$ (n=11), the open circles are $i_{ILCa}$ (n=14). The mean activation curves were constructed in the same way as described in section V.1.2.

Figure 4.113. $i_{TCa}$ and $i_{ILCa}$ steady-state inactivation curves from freshly isolated guinea-pig cells. The filled circles are $i_{TCa}$ (n=11) and open circles $i_{ILCa}$ (n=15). The mean inactivation curves were constructed in the same way as described in section V.1.2.
Table 4.35. Steady-state inactivation parameters for $i_{T Ca}$ and $i_{L Ca}$ in freshly isolated guinea-pig cells.

<table>
<thead>
<tr>
<th></th>
<th>$i_{T Ca}$</th>
<th>$i_{L Ca}$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{0.5}$ (mV)</td>
<td>-61.5±8.2 (mean±SD, n=11)</td>
<td>-34.4±5.3 (mean±SD, n=15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>slope factor (mV)</td>
<td>9.9±3.1</td>
<td>12.9±3.3</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

(1) Electrodes were filled with the “CsCl+5mM EGTA” solution. Preconditioning voltages between -120 to +30mV for 2 seconds, activated from a lms of -60mV to +10mV for $i_{L Ca}$ and -10mV for $i_{T Ca}$.

(2) $p$ values from unpaired two-tailed $t$-tests after F-tests.

VIII.7. $i_{T Ca}$ and $i_{L Ca}$ window currents in freshly isolated guinea-pig cells

The activation and inactivation curves of $i_{L Ca}$ are plotted in Figure 4.114 to illustrate the window current as the overlap of the two curves. In 12 cells where both curves were obtained maximum overlap occurred at -17.7±4.6mV (SD) where 23.8±6.7% (SD) of channels were available. The maximum current in the 12 cells was 1.1±0.6pA/pF (SD) so that the window current at the peak was 0.3±0.18pA/pF (SD).

Figure 4.114. The window current of $i_{L Ca}$ in freshly isolated guinea-pig cells.
The window current of $i_{TCA}$ is shown in Figure 4.115. The peak window current was at -43.4±5.1mV (SD, n=11), the channels available at peak window were 16.6±4.6% (SD). The available current was 0.3±0.13pA/pF (SD), so that activated at peak window: 0.05±0.02pA/pF (SD). The whole cell capacitance, $C_m$, of freshly isolated guinea-pig myocytes is 50pF and the cell input resistance 3 GΩ, so the cell membrane would be depolarised by a mean voltage of 8mV. Thus if the resting potential was about -40mV activation of $i_{TCA}$ could depolarise the cell to about -32mV sufficient to activated L-type Ca$^{2+}$ current and generate an action potential. This could underlie the spontaneous action potentials in freshly isolated guinea-pig cells.

![Graph showing window current of $i_{TCA}$ in freshly isolated guinea-pig cells.](image)

**Figure 4.115.** The window current of $i_{TCA}$ in freshly isolated guinea-pig cells.

**VIII.8. Comparison of $i_{Ca}$ activation and inactivation curves for freshly isolated and culture guinea-pig cells**

Data presented in earlier sections on activation and inactivation curves of $i_{Ca}$ were obtained using standard voltage protocols, with no pharmacological interventions. To assess the contribution of $i_{TCA}$ to the difference between the total $i_{Ca}$ in freshly isolated and cultured cells, a more quantitative approach was used to compare the activation and inactivation curves of the two cell types.
VIII.8.1. $i_{Ca}$ activation curves for freshly isolated (fGP) and cultured (cGP) guinea-pig cells

Figure 4.116 and Tables 4.36 and 4.37 compare $i_{Ca}$ activation curves from cultured cells, without nifedipine or NiCl$_2$ (n=4), to $i_{TCa}$ in the presence of nifedipine (n=11) and $i_{LCA}$ in the presence of NiCl$_2$ (n=14) in freshly isolated cells. All currents were recorded from a 2-second preconditioning pulse of -100mV. The $i_{Ca}$ curve of cultured cells was significantly shifted to the right compared with $i_{TCa}$, but not different from $i_{LCA}$. These data suggest that the difference in the activation variables of total $i_{Ca}$ in freshly isolated cells and $i_{Ca}$ in cultured cells could be attributed to the existence of $i_{TCa}$ in the freshly isolated cells.

![Graph showing activation curves](image)

**Figure 4.116.** Comparison of $i_{Ca}$ (cGP), $i_{TCa}$ and $i_{LCA}$ (fGP) activation curves (preconditioning potential -100mV, CsCl+5mM EGTA filling solution). The filled circles are $i_{TCa}$ (n=11), open circles $i_{LCA}$ (n=14) and open triangles are cGP in Tyrode's (n=4).

**Table 4.36.** Activation parameters for $i_{TCa}$ (fGP) vs $i_{Ca}$ (cGP)

<table>
<thead>
<tr>
<th></th>
<th>fGP, $i_{TCa}$ (mean±SD, n=11)</th>
<th>cGP, $i_{Ca}$ (mean±SD, n=4)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{0.5}$ (mV)</td>
<td>-28.1±8.0</td>
<td>-3.9±2.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>slope factor (mV)</td>
<td>8.1±3.8</td>
<td>6.1±0.7</td>
<td>ns</td>
</tr>
</tbody>
</table>
Table 4.37. Activation parameters for \( i_{L, Ca} \) (fGP) vs \( i_{Ca} \) (cGP)

<table>
<thead>
<tr>
<th></th>
<th>( f_{GP} ), ( i_{L, Ca} ) (mean±SD, n=14)</th>
<th>( c_{GP} ), ( i_{Ca} ) (mean±SD, n=4)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_{0.5} ) (mV)</td>
<td>-9.3±6.2</td>
<td>-3.9±2.8</td>
<td>ns</td>
</tr>
<tr>
<td>slope factor (mV)</td>
<td>5.2±1.5</td>
<td>6.1±0.7</td>
<td>ns</td>
</tr>
</tbody>
</table>

(1) Electrodes were filled with the "CsCl+5mM EGTA" solution, preconditioning voltage -100mV for 2 seconds.

(2) \( i_{TCa} \) was determined in the presence of 5\( \mu \)M nifedipine and \( i_{L, Ca} \) in the presence of 0.2mM NiCl\(_2\). \( i_{Ca} \) was determined in Tyrode's solution.

(3) Unpaired two-tailed t-tests after F-tests.

VIII.8.2. \( i_{Ca} \) inactivation curves for freshly isolated (fGP) and cultured (cGP) guinea-pig cells

A comparison of \( i_{Ca} \) inactivation curves from cultured cells using CsCl-filled electrodes with \( i_{TCa} \) and \( i_{L, Ca} \) from freshly isolated cells is shown in Figure 4.117, Tables 4.38 and 4.39.

\( i_{TCa} \) (n=11) was recorded in the presence of 5 \( \mu \)M nifedipine, \( i_{L, Ca} \) (n=15) in the presence of 0.2mM NiCl\(_2\) and \( i_{Ca} \) (n=9) in Tyrode's solution. Currents were evoked from 2 second preconditioning voltages between -120 to +30mV for \( i_{TCa} \) and \( i_{L, Ca} \) and -100 to +30mV for \( i_{Ca} \). Activation voltages were -10mV for \( i_{TCa} \) and +10mV for \( i_{L, Ca} \) and \( i_{Ca} \). The \( i_{Ca} \) inactivation curve from cultured cells was significantly shifted to the right compared to \( i_{TCa} \), but was not significantly different compared to \( i_{L, Ca} \). The data are consistent with the contribution of \( i_{TCa} \) to the difference in inactivation kinetics between the gross \( i_{Ca} \) from freshly isolated and cultured guinea-pig cells.

These results strongly suggest that \( i_{TCa} \) exists in freshly isolated cells but did not persist in cultured cells.

Table 4.38. Inactivation parameters for \( i_{TCa} \) (fGP) vs \( i_{Ca} \) (cGP)

<table>
<thead>
<tr>
<th></th>
<th>( f_{GP} ), ( i_{TCa} ) (mean±SD, n=11)</th>
<th>( c_{GP} ), ( i_{Ca} ) (mean±SD, n=9)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_{0.5} ) (mV)</td>
<td>-61.5±8.2</td>
<td>-31.2±7.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>slope factor (mV)</td>
<td>9.9±3.1</td>
<td>11.5±4.9</td>
<td>ns</td>
</tr>
</tbody>
</table>
Table 4.39. Inactivation variables for \( i_{L,Ca} \) (fGP) vs \( i_{Ca} \) (cGP)

<table>
<thead>
<tr>
<th></th>
<th>fGP, ( i_{L,Ca} ) (mean±SD, n=15)</th>
<th>cGP, ( i_{Ca} ) (mean±SD, n=9)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_{0.5} ) (mV)</td>
<td>-34.4±5.3</td>
<td>-31.2±7.3</td>
<td>ns</td>
</tr>
<tr>
<td>slope factor (mV)</td>
<td>12.9±3.3</td>
<td>11.5±4.9</td>
<td>ns</td>
</tr>
</tbody>
</table>

(1) Electrodes were filled with the “CsCl+5mM EGTA” solution, preconditioning voltages between -120 to +30mV for 2 seconds and activated from -60mV (1ms) to voltages of +10mV for \( i_{L,Ca} \) and \( i_{Ca} \), -10mV for \( i_{TCa} \).

(2) \( i_{TCa} \) was determined in the presence of 5\( \mu \)M nifedipine, \( i_{L,Ca} \) in the presence of 0.2mM NiCl\(_2\), \( i_{Ca} \) in Tyrode’s solution.

(3) unpaired two-tailed t-tests after F-tests.

\[
\frac{f_{\infty}}{f_{\max}}
\]

Figure 4.117. Comparison of \( i_{Ca} \) (cGP), \( i_{TCa} \) and \( i_{L,Ca} \) (fGP) inactivation curves. The filled circles are \( i_{TCa} \) (n=11), open circles \( i_{L,Ca} \) (n=15) and open triangle \( i_{Ca} \) from cultured cell (n=9).
Chapter 5. Discussion

I. Methods

I.1. Voltage-clamp technique for whole-cell recording

The whole-cell recording of patch-clamp has extensive applications to study ion channel properties and is particularly useful with small cells where conventional voltage-clamp techniques are not feasible. Patch-clamp systems switch readily between current-clamp and voltage-clamp modes to obtain information on membrane potential and ion channel activities from the same cell. It is possible to separate ionic currents from the membrane capacitive current and by combining whole-cell recording with experimental protocols which isolate individual currents, channel conductance and kinetics can be measured. Under whole-cell configuration, macroscopic currents and membrane potential can be measured and the magnitude and the relative contribution of individual ionic currents to potential changes can be quantified. The patch electrode whole-cell recording also allows manipulation of the intracellular environment, e.g. cytoplasmic Ca$^{2+}$ can be buffered and intracellular ionic composition can be effectively controlled.

I.1.1. Series resistance and capacitive artefacts considerations

Despite many advantages of the patch-clamp technique, consideration must be made of its limitations. The patch electrode is used both to record voltage and as a path for current flow into the cell. This means that the access resistance of the electrode, usually in the order of a few MΩ, contributes to the series resistance $r_s$. This series resistance is the main source of error of whole-cell clamping experiments (Halliwell et al 1994). It mainly resides in the pipette resistance, plus any other access resistances to the patch or cell. The accuracy with which the membrane voltage is controlled depends on having sufficiently high gain in the clamping amplifier set by a feedback resistor. The membrane potential $V_m$ is measured and the clamping amplifier compares $V_m$ with a command potential and passes current to control $V_m$. In whole-cell recording, the current ($i_p$) flows from the pipette to the cell, through this series resistance and generates a potential error ($i_p r_s$) between pipette and cell. This leads to a discrepancy between the measured $V_m$ and the true potential difference across the membrane. The problem is most likely to be serious when large membrane
currents are flowing. Thus with membrane currents of several nanoamps or more, compensation should correct for the error due to the voltage drop across the electrode resistance. The series resistance compensation control carries out this function by adding a fraction of $ip_r$ to the command to partly correct the error. When the cell input resistance becomes comparable to or less than the pipette resistance, the whole-cell patch technique will be ineffective.

Another possible error when using whole-cell voltage-clamp recording arrives from capacitive artefacts. These capacitive artefacts come from electrode and/or membrane capacitances when a repetitive voltage step applied. They can mostly be cancelled by adjusting the representative compensation controls on the patch-clamp equipment, in addition to coating patch pipettes with an insulator, minimising the depth of bath solution in contact with the pipette wall, reducing the volume of the filling solution in the pipette and keeping the pipette holder clean and dry. Therefore to a very good approximation, the pipette-cell system may be represented by the simple equivalent circuit of a resistance $r_s$ in series with the parallel combination of membrane conductance $g_m (1/r_m)$ and membrane capacitance $c_m$.

Both series resistance and capacitive current errors can be electronically minimised by using series resistance compensation. This works well if $r_s$ is small and for best results, the cell input resistance should be many times the pipette resistance. This is normally the case for cells at rest. However, during voltage activation, the cell input resistance could fall dramatically with two possible consequences: 1), there will be a significant error due to the voltage drop across the pipette; 2), the setting of the Series Resistance and Whole-Cell Capacitance Compensation controls will become erroneous because they are set based on the time constant of charging the membrane capacitance before the change in the membrane resistance. Thus a better solution is to use low resistance pipettes and cell types of small size. The magnitude of the current investigated should also be considered in assessing the suitability of the technique for recording.

Bladder detrusor smooth muscle is suitable for whole-cell patch clamp experiments. (1) the cell is small and has a large input resistance. When patch electrodes were filled with a KCl-filling solution containing 5mM EGTA, the whole cell capacitance $c_m$ recorded from freshly isolated guinea-pig detrusor muscle cells was $50.1 \pm 17.1 \mu F$ (mean±SD, n=34). The calculated cell surface area therefore had a mean value of $50.1 \times 10^{-6} \text{ cm}^2$, if the specific membrane capacitance was assumed to be $1 \mu F/\text{cm}^2$ (Hille 1992). This surface area is about one quarter the size of cardiac cells in which whole-cell patch clamp recording has been widely used. From the same recordings, the cell
input resistance $r_{in} 3.0\pm1.1 \, (\text{M}\Omega)$ (mean±SD, n=34). The electrode resistance was 3-5 M\(\Omega\) so that total input resistance was approximately equal to cell resistance.

(2) Low resistance patch electrodes were used in these experiments to reduce the series resistance. The typical values of the series resistance were 3-20 M\(\Omega\) if low resistance (1-5M\(\Omega\)) pipettes were used (Ogden and Stanfield 1994). According to the Axon patch-clamp instruction hand book (Axopatch-1D theory and operation), it is possible to obtain the absolute value of series resistance shown on the Series Resistance dial after the whole-cell current transient had been eliminated. This gave a value of the series resistance for freshly isolated guinea-pig detrusor smooth muscle of $4.7\pm3.9\,\text{M} \Omega$ (mean±SD, n=46).

(3) In addition, whole cell currents are much smaller compared to cardiac myocytes; the largest currents were usually less than 0.5nA, e.g for the mean steady-state $I_K$ measured at $+40\text{mV}$ depolarisation voltage and less than 1.5nA for the mean STOC current at a maximum depolarisation of $+40\text{mV}$. These value are far less than the limitation values set by Axopatch-1D: the CV-4 1/100 headstage of Axopatch-1D has 500 M\(\Omega\) ($\beta=1$) feedback resistor with maximum current 20nA and series resistance compensation range of 100M\(\Omega\). The error voltage caused by series resistance is only a problem in whole-cell current clamping where the current is several nA or more (Axopatch-1D patch clamp theory and operation, 1990). Thus whole-cell clamping of the detrusor cell is accurate and for the small series resistance in detrusor muscle compensation was generally not required.

I.1.2. Space-clamp considerations

Isopotentiality (space-clamp) is also required when using whole-cell recording in single cell preparations. Membrane current should be recorded from an area of uniform potential, so that current comes from a population of channels that all experience the same voltage. How fully this criterion is met depends on the geometry of the preparation and the recording system used. In giant axons and giant muscle fibres local circuits were eliminated and uniformity of membrane potential was achieved (the space-clamp condition) by inserting a highly conductive axial wire inside the fibre. In other cells, uniformity is achieved by using a small membrane area or small cells (Halliwell et al 1994).

The enzymatically dissociated detrusor muscle cell varied in length and shape, and partially contracted, as it had a spindle shape. The human detrusor muscle cell was on average 110±59\(\mu\text{m}\) in length and 11±4\(\mu\text{m}\) (±SD) at the greatest diameter (Montgomery and Fry 1992). Guinea-pig detrusor muscle cells had about the same
size as human detrusor and also partially contracted: about 100-200\(\mu\)m long and 5-10\(\mu\)m wide at greatest part. If an average length of 160\(\mu\)m was taken (Brading 1987), the space constant, \(\lambda\), of guinea-pig detrusor smooth muscle could be estimated using the ordinary cable equation (Abe and Tomita 1968):

\[
\lambda = \sqrt{\frac{aR_m}{2R_i}}
\]

5.1

Where \(a\) is the radius of muscle cell, \(R_m\) is the specific membrane resistance, and \(R_i\) is the internal specific resistivity. The specific membrane resistance from freshly isolated guinea-pig detrusor muscle cells was 138±30 k\(\Omega\)cm\(^2\) (mean±SD, \(n=34\)), \(R_i\) is approximately 600\(\Omega\)cm in guinea-pig detrusor (Fry et al 1997). Assuming a fully relaxed detrusor smooth muscle cell with a uniform diameter of 5\(\mu\)m, the space constant is then about 0.17cm, i.e. 1.7 mm, which is approximately 10 times larger than the mean length of detrusor cells from guinea-pig. If the cell of length, \(l\), is modelled as a short-cable with an infinite impedance at its ends the voltage distribution is given by

\[
V(x) = V_{x=0} \frac{\cosh \left(\frac{(l-x)}{\lambda}\right)}{\cosh \left(l/\lambda\right)}
\]

5.2

At one space constant, \(\lambda\), the voltage would have declined to 66% of that at \(V(x=0)\) (compared to 37% for an infinite cable). If \(l=0.1\lambda\) which is more appropriate for the cells used here \(V(x)\) would be 99.5% of \(V(x=0)\). This result forms the basis of voltage-clamp techniques in this preparation in which fairly uniform polarisation of a large area of membrane is achieved by using the short-cable equation (Abe and Tomita 1968; Jack et al 1975). If the patch electrode was routinely positioned near the midportion of cells the short cable representing these cells predicts that the cell membrane would be virtually isopotential in the resting state and even during membrane depolarisation (the membrane input resistance allowed a 10-fold decrement), the space clamp is still likely maintained.

So a reliable space clamp can be obtained in detrusor muscle cells for whole-cell studies.

I.1.3. Diffusional exchange between pipette solution and cytosol

Dialysis of the sarcoplasma by the patch electrode filling solution allows control of intracellular and extracellular environments and ensures analysis of membrane
phenomena under reasonably controlled ionic conditions. The contents of the interior of the cell is in diffusional equilibrium with the solution in the patch pipette. It is important to realise that dialysis of the cell is not instantaneous. An equilibrium is achieved more rapidly for ions than for large intracellular molecules. The time course of exchange depends on the size of the molecules, the series (access) resistance between the pipette tip and the cell, the cell volume, as well as the degree of buffering of the molecules or ions by cytoplasmic constituents. Junction potentials due to small ion fluxes will also exist between the pipette and the cytoplasm. These sources of error are not constant but will change towards zero as equilibrium proceeds. It should be born in mind that both properties of the cell membrane and intracellular regulatory systems may be modified following the changes in composition of the intracellular space. Therefore measurements were usually made five minutes after rupture of the patch of membrane to ensure an equilibrium was largely achieved.

With the basic filling solution, the concentration of the major ions was similar to the intracellular concentration of these ions in intact cell. Particular consideration was given to the $[\text{Cl}^-]_i$. For convenience of solution preparation, many studies simply add KCl to the filling solution to achieve a physiological $[\text{K}^+]_i$, but results in higher than normal $[\text{Cl}^-]_i$. The present study used a normal $[\text{Cl}^-]_i$ with the remaining anion as aspartate. This provided an intracellular environment closer to physiological conditions and also allowed more reliable measurement of resting membrane potentials.

I.2. Single cells as a model system for electrophysiological studies

This project aimed to characterise the electrophysiological properties of detrusor smooth muscle. Single detrusor smooth muscle cells offer several advantages over multicellular smooth muscle preparations. 

(1) single cells are an ideal model for which adequate and uniform control of the membrane potential can be achieved in combination with recording via low resistance electrodes - the whole-cell patch-clamp technique. The high frequency response permitted by the low electrode impedance allows accurate quantification of the kinetics of voltage-gated ion channels.

(2) The interpretation of experimental results in multicellular strips is complicated by the existence of intercellular clefts, where the ionic composition may change due to accumulation or depletion phenomena, which restrict free diffusion (Johnson and Lieberman 1971; Bolton et al 1981). Moreover cells may be electrically coupled and
embedded in connective tissue (Brading 1987), so that accurate measurement of membrane voltage and current is difficult.

(3) The presence of terminal neurones throughout the tissue strip may contribute to the release of transmitters during extracellular current stimulation or ion replacement (Dixon and Gosling 1987).

(4) Application of the patch-clamp technique requires an identifiable cell with a particularly clean surface (Hamill et al. 1981).

(5) Single cells provide a unique model in which membrane current or potential can be simultaneously measured with intracellular Ca^{2+}, or pH, the kinetics of the interaction between the membrane electrophysiological activities and intracellular ions can be studied on a real-time basis.

I.3. Cell culture

The electrophysiological properties of cultured detrusor smooth muscle were also carried out in this study. The cultured cell model had to be initially developed and characterised for this purpose. A problem in the culture detrusor smooth muscle is the risk of contamination by other cell types, which results in difficulties in evaluating the properties of smooth muscle itself.

There are three types of the cells in the wall of the detrusor, i.e., fibroblasts, epithelial cells and smooth muscle cells. Fibroblasts share a similar growth pattern to smooth muscle cells, and it is not possible to distinguish them under light microscopy. However fibroblasts cannot survive in a D-valine selective medium, where L-valine is replaced by D-valine. Fibroblasts have no D-amino acid oxidase, they therefore cannot make use of this amino acid and will not survive in culture (Gilbert and Migeon 1975).

Epithelial cells can grow in such a medium if the culture dish is contaminated by a large number of epithelial cells. However they have an entirely different characteristic growth pattern and morphology from smooth muscle cells. Furthermore muscle strips were very rigorously dissected free from any overlying epithelium even though this necessitated losses of the smooth muscle layer to achieve a culture which was predominantly smooth muscle cells. On the other hand, it is well known that long-term cultured smooth muscle cells undergo spontaneous modulations in cell phenotype and ultrastructure, cells dedifferentiate before proliferation and redifferentiate after that. It is hard to distinguish a smooth muscle cell from a fibroblast purely by their morphology (Ross 1971; Campbell et al. 1974; Chamley and
Campbel 1974; Chamley et al 1974; Groschel-Stewart et al 1975; Campbell et al 1979; Absher et al 1989; Baskin et al 1993). Therefore, it was important to know the characteristics of the cells under study, in order to be able to evaluate how the electrophysiological properties of cultured cells resemble those of smooth muscle cells in situ. So the first principle to be followed during culture was to eliminate the fibroblasts and reduce the contamination of epithelial cells as much as possible.

For this purpose, the culture procedure was designed to be suited to smooth muscle growth: firstly epithelium and mucosa were rigorously peeled off under the dissection microscope, as described above. Contamination by bacteria was avoided by performing operation in an aseptic environment with sterilised vessels. Muscle strips were washed by centrifugation before seeding in a petri dish, to remove soluble contaminants (Burtis and Ashwood 1994). Antibiotics were added to the culture medium to prevent further any infection. Thus the muscle strips mainly contained smooth muscle cells and fibroblasts. At this stage the selective D-valine medium was used to inhibit fibroblast growth.

This selective medium inhibited fibroblast growth, and furthermore as the culture proliferated, any non-dividing or slow growing cells, would be diluted out (Freshney 1989) so that a few contaminating epithelial cells would not last. Contaminating epithelium in a smooth muscle-enriched culture nearly always become contact inhibited in small plaques and stop proliferating (Moore et al 1984; Devore-Carter et al 1988). In addition, epithelial cells and smooth muscle cells have different growth patterns and are easily distinguished under microscope: epithelial cells are polygonal and have a characteristic flat sheet growth pattern. Smooth muscle cells are spindle shaped and exhibit a characteristic "hills and valleys" growth pattern (i.e., formation of ridges and dense smooth muscle aggregates after monolayer of confluent growth). The preponderance of smooth muscle cells was confirmed by specific α-smooth actin antibody staining (see below).

In this study, cultured cells in petri dishes exhibited a characteristic "hills and valleys" growth pattern before being harvested to undergo further electrophysiological examination. However epithelial cells have been reported to exhibit electrical activity. An "epithelial action potential" and electrical coupling of excitable epithelial cells have been reported from the exumbrellar epithelium of the hydromedusa, *Euphysa japonica*, as well as from hydrozoan coelenterates, larval amphibians, tunicates, molluscs, ctenophores and anthozoan coelenterates (Josephson and Schwab 1979), although there is no report from mammalian epithelium yet. Therefore it is important
to characterise the cultured cells of smooth muscle origin before the electrode was struggled to patch on the cell.

Immunochemical and functional characterisation of the cell was undertaken before electrophysiological measurements were made to prove that the cultured cells were smooth muscle. An antibody against α-smooth muscle actin is widely used as a smooth muscle specific differentiation marker. This α-smooth actin stains positively in both differentiated and dedifferentiated smooth muscle, but negatively in fibroblasts and endothelial cells (Chamley et al 1977; Campbell et al 1979; Gown et al 1985; Omar et al 1986; Tung and Fritz 1990). In the present study, both culture cells from human and guinea-pig detrusor were stained positively by monoclonal anti-α-smooth muscle actin (Figure 4.3). Therefore they were considered to be smooth muscle rather than fibroblasts or epithelial cells.

Epithelial and smooth muscle cells of the urinary bladder are functionally different as well. Muscarinic receptor-activation induced Ca^{2+} release via IP_3 is considered as specific to detrusor smooth muscle rather than bladder epithelial cells; although in some secretory epithelial cells muscarinic receptors have been described, i.e., bile duct epithelium (Nathanson et al 1996). It has been reported that cultured smooth muscle cells from detrusor and other organs can express characteristic muscarinic receptors (Twort and Breemen 1988; Yang 1990; Yang et al 1991b; Harriss et al 1995). Amedee et al (1986) applied 500μM acetylcholine in long-term primary cultured myometrial cells and induced a maximal contraction which was inhibited by atropine. Carbachol-induced Ca^{2+} release from intracellular stores in cultured cells both from guinea-pig and human detrusor (Figure 4.5 and Figure 4.6) gave further evidence that cultured cells in the present study were smooth muscle.

Thus a cultured muscle cell model was successfully developed. In experiments only the confluent cell cultures which had formed characteristic "hills and valleys" growth pattern were used. The cells were dissociated just before experiments with the same enzyme as that used in fresh cell isolation and their viability was tested using trypan blue exclusios. A shiny appearance under the microscope, a reasonable resting potential (i.e., about -55 to -60mV) and large density i_Ca were also good criteria (Klockner and Isenberg 1985a, b).
I.4. Measurement of intracellular Ca\(^{2+}\) with fluorescent indicators

Measurement of intracellular [Ca\(^{2+}\)], using epifluorescence microscopy has been well established in many mammalian cells, including cultured cells. (Gryniewicz et al 1985; Morgan et al 1993; Chambers et al 1996). The fluorescent indicator method has many advantages: good specificity for measured ions, high sensitivity to small changes in their concentrations, dissociation constant values within the range of intracellular levels and ease of use. Use of the ratio method overcomes changes to the absolute magnitude of the signals due to change of dye concentration, bleach of fluorescence or variations of the cell dimensions. One disadvantage of using fluorescent dyes is that they buffer the ion, which may alter the kinetic changes of the ion and its transient response. To minimise the effect of dye buffering on the kinetics of [Ca\(^{2+}\)]\(_i\) changes, the indicator loading was kept to a minimum level while still giving a good signal-to-noise ratio. Although a limitation of non-imaging epifluorescence microscopy is that it only gives bulk [Ca\(^{2+}\)]\(_i\), not local [Ca\(^{2+}\)]\(_i\) which is more related to Ca\(^{2+}\) activated \(i_C\) in the present study, it does not affect its role in demonstrating the qualitative relationship between the rise of [Ca\(^{2+}\)]\(_i\) and STOC amplitude.

In the present study, 0.1 mM acid form of fura-2 was used in the patch electrode filling solution. This concentration of fura-2 has also been used in other studies on smooth muscle cells (Ohta et al 1993) and in detrusor cells has been found to give sufficient a signal-to-noise ratio (Wu and Fry 1998). Qualitative changes in [Ca\(^{2+}\)]\(_i\) are represented by changes in the ratio of the light emitted by the two excitation wavelengths of 340 and 380 nm to avoid the uncertainties in using a calibration curve required for determination of absolute value. These uncertainties come from intracellular viscosity, compartmentation of the dye (in the case of fura-2 AM) and binding of the indicator to cellular constituents.

II. Results

II.1. The electrical activities of freshly isolated and cultured detrusor cells

Characterisation of the electrophysiological properties of freshly isolated and cultured detrusor muscle cells included measurement of the membrane passive properties, action potential characteristics as well as membrane ionic currents (Chapter 4, sections II-VI, summaries in Tables 5.1-5.3). Electrodes were filled with a solution
containing 5mM EGTA, a high [K+] and low [Cl−] to mimic the physiological environment (Brading 1987) when recording action potential and membrane currents, since under physiological condition the action potential relies on the balance between $i_K$ and $i_{Ca}$. Membrane ionic currents are time-dependent, therefore inward current in this case was treated as $i_{Ca}$ and steady-state outward current as $i_K$. Results demonstrated that primary and subcultured cells both from human and guinea-pig detrusors retained most of their electrical properties with some modifications.

II.1.1. Passive properties

Before culture, freshly isolated cells from human and guinea-pig detrusor showed no difference in their passive electrical properties (see Table 4.1).

During culture, the cells were obviously larger under the light microscope. This was consistent with their membrane capacitance, $C_m$, measurement which increased in cultured cells. The resting membrane potential, RMP, in cultured cells was shifted to more positive potentials. In human cells it was depolarised by an average of 9.7 mV, compared to freshly isolated human detrusor cells; the resting potential in cultured guinea-pig depolarised by 16.5 mV, compared to freshly isolated guinea-pig detrusor cells. Table 5.1 gives a summary of the changes to passive properties shown in Tables 4.3 and 4.4. It is interesting that the trends of alterations in both human and guinea-pig detrusor after culture are the same. The resting potential of the cultured cells shifted in the depolarising direction, which indicated either a $K^+$ conductance was down-regulated or failed to recover during culture or a conductance with a more positive equilibrium potential emerged.
Table 5.1. Alterations of membrane passive properties after culture. fH and cH are freshly isolated and cultured human detrusor cells; fGP and cGP are freshly isolated and cultured guinea-pig cells.

<table>
<thead>
<tr>
<th></th>
<th>Human cells</th>
<th>Guinea-pig cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cH vs fH</td>
<td>cGP vs fGP</td>
</tr>
<tr>
<td>Resting membrane potential, RMP</td>
<td>depolarised</td>
<td>depolarised</td>
</tr>
<tr>
<td></td>
<td>(by 9.7mV)</td>
<td>(by 16.5mV)</td>
</tr>
<tr>
<td>Specific membrane resistance, R_m</td>
<td>reduced</td>
<td>reduced</td>
</tr>
<tr>
<td></td>
<td>(by 29.1kΩ.cm^2, 21.9%)</td>
<td>(by 14.1kΩ.cm^2, 10.2%)</td>
</tr>
<tr>
<td>Whole cell capacitance, C_m</td>
<td>increased</td>
<td>increased</td>
</tr>
<tr>
<td></td>
<td>(by 21.1pF, 28.9%)</td>
<td>(by 9.4pF, 15.8%)</td>
</tr>
<tr>
<td>Cell input resistance, r_inp</td>
<td>reduced</td>
<td>reduced</td>
</tr>
<tr>
<td></td>
<td>(by 1.3GΩ, 44.8%)</td>
<td>(by 0.8GΩ, 26.7%)</td>
</tr>
</tbody>
</table>

II.1.2. Excitability of freshly isolated and cultured detrusor cells

With freshly isolated cells, both human and guinea-pig detrusor muscle are excitable. They can generate action potentials spontaneously and also in response to a current stimulus. The action potential threshold, V_th, and duration were similar, the peak net inward current from freshly isolated human cells was significantly smaller than freshly isolated guinea-pig cells, but both i_Ca activation threshold potentials were the same (Figure 4.30, 4.29). These characteristics would underlie the smaller action potential amplitude, maximum upstroke velocity and slower time to peak potential in freshly isolated human cells (Table 4.6).

As with freshly isolated detrusor smooth muscle cells, cultured cells from both human and guinea-pig were also excitable, i.e., responded to electrical stimulation by generating action potentials (Tables 4.8, 4.9). These properties were retained in passaged cells from both human (not shown in this thesis) and guinea-pig detrusor (Tables 4.10, 4.11).

However, there were changes to action potential morphology. Cultured cells did not generate action potentials spontaneously but were elicited only in response to
electrical stimulation. The most pronounced change to the action potential from cultured cells was their near resemblance to a cardiac action potential with a slow repolarisation phase; while the action potential from freshly isolated detrusor cells had a short duration and a faster repolarisation phase. Such changes were very constant; for example, the action potential threshold ($V_{th}$) of all cultured cells was depolarised by approximately 10mV as shown in Table 5.2. Table 5.2 summarises the changes to action potential morphology in cultured cells as shown previously in Tables 4.8 to 4.11. The combination of changes to action potential threshold and their passive electrical properties suggests that some conductances involved in regulating the normal resting potential and subthreshold depolarisation were not evident in cultured cells. One observation was that specific L-type $\text{Ca}^{2+}$ channel blockers completely inhibited the action potential and inward currents in cultured cells (Figures 4.19 and 4.20), but not so in freshly isolated cells (Figure 4.14). A second observation was the shift in a depolarising direction of the inward current activation threshold voltage after culture. These and the preceding observations suggest that an inward current in freshly isolated cells is not retained in cultured cells. These changes to inward current would have consequent influences on the activation of $\text{Ca}^{2+}$-dependent outward currents.
Table 5.2. Alterations to membrane action potential (AP) properties in cultured detrusor cells compared to freshly isolated counterparts. fH and cH are freshly isolated and cultured human detrusor cells; fGP and cGP are freshly isolated and cultured guinea-pig cells; cGP\textsubscript{1,3} is passaged guinea-pig cells.

<table>
<thead>
<tr>
<th></th>
<th>cH vs fH</th>
<th>cGP vs fGP</th>
<th>cGP\textsubscript{1,3} vs fGP</th>
<th>cGP\textsubscript{1,3} vs cGP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. AP threshold</td>
<td>depolarised</td>
<td>depolarised</td>
<td>depolarised</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(by 12mV)</td>
<td>(by 9.2mV)</td>
<td>(by 9.3mV)</td>
<td></td>
</tr>
<tr>
<td>2. (1). AP amplitude</td>
<td>ns</td>
<td>reduced</td>
<td>reduced</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(by 14.9mV, 29.9%)</td>
<td>(by 16.6mV, 33.3%)</td>
<td></td>
</tr>
<tr>
<td>3. (2). AP time to peak</td>
<td>ns</td>
<td>slower</td>
<td>slower</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(by 37.1ms, 54.2%)</td>
<td>(by 30.1ms, 48.9%)</td>
<td></td>
</tr>
<tr>
<td>(3). AP upstroke velocity</td>
<td>ns</td>
<td>reduced</td>
<td>reduced</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(by 2.3V/s, 63.9%)</td>
<td>(by 2.3V/s, 63.9%)</td>
<td></td>
</tr>
<tr>
<td>4. AP repolarisation</td>
<td>slower</td>
<td>slower</td>
<td>ns</td>
<td>faster</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(by 41.5ms, 50.9%)</td>
</tr>
<tr>
<td></td>
<td>(by 49.4ms, 60.6%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: "*" data not shown in thesis; "ns": not significant.
II.1.3. Ionic currents in freshly isolated and cultured detrusor cells

An inward $i_{\text{Ca}}$ and an outward $i_{\text{K}}$ were recorded in cultured cells from which an action potential was recorded, but with lower densities than their equivalents in freshly isolated cells (Figures 4.44, 4.45, 4.53, 4.54, 4.61, 4.62). Table 5.3 is a summary of i-v relationships listed in Chapter 4 sections V.3 and V.5, which were responsible for the action potential changes described above (Table 5.2). The activation threshold of inward currents from cultured cells, including primary and passaged cells, was shifted to more positive potentials (see also Figures 4.44, 4.53, 4.61, 4.62). This could be explained by the presence of a low voltage-activated inward $i_{\text{Ca}}$ components ("shoulder" current) in i-v curves only in freshly isolated cells. Using Cs-filled electrodes, this difference was more obvious (see Figures 4.61-4.63). When the holding potential was at very negative potentials (-100mV), this low-voltage activated $i_{\text{Ca}}$ existed in freshly isolated detrusor muscle cells, but not in cultured cells. The cultured cells only contained the high voltage ($\geq -30\text{mV}$) activated component (see Figure 4.102). The same phenomenon also existed in human detrusor (not shown in thesis).

The peak net inward $i_{\text{Ca}}$ determines the action potential amplitude, time to peak and maximum upstroke velocity, and there were consistent correlations between these variables and $i_{\text{Ca}}$ kinetics and magnitude (see Tables 5.2 and 5.3).

Outward currents developing in the first tens of milliseconds are responsible for action potential repolarisation. In the first instance, the aim was to demonstrate the relationship between action potential morphology and net currents in freshly isolated and cultured cells under physiological conditions. Specific channel blockers were not used to block $i_{\text{Ca}}$ to isolated outward $K^+$ currents, as the magnitude of the outward current was dependent on the magnitude of $i_{\text{Ca}}$. As $i_{\text{Ca}}$ and $i_{\text{K}}$ have different gating kinetics and time-dependence, the steady-state outward current was used as an index of $i_{\text{K}}$. Cultured cells exhibited a large decrease of this current (see Figures 4.45 and 4.54 and Table 5.3) which would explain the prolongation of the action potential. Moreover, a spontaneous outward current recorded after $i_{\text{Ca}}$ activation could be recorded from freshly isolated cells but never in cultured cells, even using a low-EGTA filling solution.
Table 5.3. Alterations of membrane currents in cultured detrusor cells.

<table>
<thead>
<tr>
<th></th>
<th>fH/cH</th>
<th>fGP/cGP</th>
<th>fGP/cGP$_{i-3}$</th>
<th>cGP/cGP$_{i-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. i$_{\text{Ca}}$ activation threshold (mV)</td>
<td>fH: ≤-40</td>
<td>fGP: ≤-40</td>
<td>fGP: ≤-40</td>
<td>cGP: ≥ -30</td>
</tr>
<tr>
<td></td>
<td>cH: ≥ -30</td>
<td>cGP: ≥ -30</td>
<td>cGP$_{i-3}$ ≥ -30</td>
<td>cGP$_{i-3}$ ≥ -30</td>
</tr>
<tr>
<td>2. Peak net inward current</td>
<td>ns</td>
<td>cGP: reduced</td>
<td>cGP$_{i-3}$: ns</td>
<td>reduced</td>
</tr>
<tr>
<td>3. Steady-state outward current</td>
<td>cH: reduced</td>
<td>cGP: reduced</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

Note: "≤" means negative to or equal to; "≥" means positive to or equal to; "ns" is not significant.

This correlation between current magnitude and action potential morphology was then investigated more closely in freshly isolated cells from guinea-pig detrusor by using different filling solutions (Chapter 4, Section IV). When electrodes were filled with Cs+, peak i$_{\text{Ca}}$ and action potential maximum upstroke velocity, $V_{\text{max}}$, exhibited a close linear relation (Figure 4.21). When electrodes were filled with a solution (KCl) containing 0.1 mM EGTA instead of 5mM EGTA, action potential repolarisation was increased to an average of 8ms from 32ms, but other parameters (action potential threshold, amplitude, time to peak, maximum upstroke velocity) were not changed (Table 4.12). This indicated that the lower intracellular [EGTA] with a smaller buffer capacity for intracellular Ca$^{2+}$ allowed more Ca$^{2+}$-dependent $i_\text{K}$ to develop and thereby shorten the action potential (Figure 4.23).

A more direct correlation between the action potential waveform and the underlying membrane currents was generated by differentiation of the action potential, elicited with short current pulses. For a uniformly polarised cell the first derivative of the action potential is proportional to net membrane current when capacitative current from the stimulus pulse has declined. When the electrode was filled with KCl containing 0.1mM EGTA, the ionic current $i_\text{i}$ had two components: a large outward and a smaller inward current and the action potential exhibited a rapid repolarisation (Figure 4.24). When the electrode was filled with KCl containing 5mM EGTA, the outward current was smaller, and the inward current larger and the action potential repolarised more slowly (Figure 4.25). When the electrode was filled with Cs+,
Page255:line7 of paragraph3, add the sentence:"The modification of membrane ionic currents during cell culture has also reported in other smooth muscle types (Richard et al 1992, cultured rat aortic smooth muscle cells; Snetkov, Hirst and Ward 1996, freshly isolated, proliferating cultured and growth-arrested human bronchial smooth muscle cells).
instead of $K^+$, there was no outward component to net membrane current and the action potential was prolonged until the $i_{Ca}$ inactivated (Figure 4.26).

There are no published data on the electrophysiological properties of cultured detrusor smooth muscle cells. In freshly isolated cells, Klockner and Isenberg (1985a) measured the action potential from guinea-pig cells using patch-clamp techniques. They used 3.6mM CaCl$_2$ in the superfusate and the patch electrodes (3-4MΩ) contained either zero or 20μM EGTA. Their values of resting potential (-52mV), and cell capacitance (50pF), are consistent with the results in the present study: -58.6mV for resting potential and 50.1pF for cell capacitance. The action potential morphology was also similar in their experiments to those recorded in this study. Their values of action potential overshoot (22mV), duration (36ms with zero or 20μM EGTA in filling solution) and maximum upstroke velocity (6.5V/s with 3.6mM CaCl$_2$ in superfusate) were similar to those recorded here: the action potential overshoot 24mV, duration 36ms (with 100μM EGTA in the filling solution) and maximum upstroke velocity 5.0V/s (with 1.8mM CaCl$_2$ in superfusate). In cultured cells from other smooth muscle types, the current densities of voltage-dependent inward and outward currents were also lower than the corresponding acutely dissociated cells (Sasoshima et al 1988, aortic vascular smooth muscle; Hall and Kotlikoff 1995, bronchial smooth muscle).

In summary, muscle cells both from human and guinea-pig detrusor have similar electrophysiological properties. After culturing, both strains of cells from human and guinea-pig detrusor smooth muscle retained many electrical properties, although there are some modifications with the result that they were less excitable. This could result from lower densities of both $i_{Ca}$ and $i_K$, and loss of a low-voltage-activated $i_{Ca}$ component and spontaneous outward currents, which exist in freshly isolated detrusor smooth cells. What causes these changes is not clear and could be very complex (Simoncini and Moody 1991; Owens 1995). Hall and Kotlikoff (1995) considered that the decrease in current density in cultured cell is likely (1) to be due to a decrease in the expression of ion-channel proteins associated with a general shift in cellular protein synthesis, although altered regulation of existing channel proteins may occur. (2) The resting behaviour of membrane ion channels in synthetic or proliferative cells may reflect differences in the cytosolic environment of cultured cells, since the ion channel open probability can be modified by phosphorylation-dependent and -independent mechanisms. (3) Expression of a key transducer molecule in the channel functional coupling pathway decreased. (4) Some ion channels constitute individual members of a large and diverse family of proteins. Because differences in the electrical properties of cells arise substantially from the differential expression of
individual members within such a family, it is possible that the alteration of phenotype that occurs in cultured cells results from alterations to the structural properties of membrane ion channels (Hall and Kotlikoff, 1995). Although currents or action potentials have been reported from some cultured smooth muscle cells or cell lines (Sinback and Shain, 1979; Harder and Sperelakis 1979; Marvin et al 1979; Mclean et al 1979; Toro et al 1986; Toro and Stefani 1987; Yuan et al 1993), none have been from cultured detrusor smooth muscle. There are no reports evaluating the electrophysiological properties of cultured cells by comparison with their freshly isolated counterparts.

II.2. Two distinct types of $i_{Ca}$ in freshly isolated detrusor smooth muscle cells

II.2.1. $i_{TCa}$ and $i_{LCa}$ in freshly isolated detrusor muscle cells

Comparison of $i_{Ca}$ current-voltage relationships from freshly isolated and cultured cells using both guinea-pig and human biopsies demonstrated that a low-voltage-activated $i_{Ca}$ component was absent during cell culture. Further characterisation of this low-voltage-activated $i_{Ca}$ was performed on freshly isolated guinea-pig cells. The results revealed two distinct $Ca^{2+}$ channels. One was an L-type $Ca^{2+}$ channel which has been described previously (Klockner and Isenberg 1985b; Montgomery and Fry 1992; Nakayama and Brading 1993b; Wu and Fry 1998). The second was a T-type $Ca^{2+}$ channel, which has been described for the first time in detrusor smooth muscle, although it has been reported in some other smooth muscle types (Ganitkevich and Isenberg 1991, coronary artery; Xiong et al 1993, colonic smooth muscle; Janssen 1997, bronchial smooth muscle) and cardiac tissue (DiFrancesco 1991; Balke et al 1992; Sipido et al 1998).

In freshly isolated detrusor muscle cells, a small shoulder (or "small hump", Kitamura et al., 1991) can be observed on the current-voltage relationship when the holding potential is more negative than -60mV. The more negative the holding potential (down to -100mV), the more obvious was the shoulder. This shoulder disappeared when the holding potential was -30mV. When cells were held at -100mV, an inward current followed by an outward transient current (possibly $Ca^{2+}$-dependent $i_K$) was activated at -70mV when L-type channels would not yet open (Figure 4.104). This low-voltage-activated current was identified as a T-type $Ca^{2+}$ current, by its distinct voltage-gating characteristics and its pharmacological protocols. This current was insensitive to 5µM nifedipine, 20µM verapamil, and 20-50µM Cd^{2+}, which are
sufficient to block L-type Ca\(^{2+}\) channels; but was very sensitive to low concentrations of NiCl\(_2\) (Figures 4.105-4.108). This Ni\(^{2+}\)-sensitive inward current was abolished in Ca\(^{2+}\)-free solution (Figure 4.108). Table 5.4 is a summary of the characteristics of the two currents.

The L-type Ca\(^{2+}\) current demonstrated a faster decay time constant than in other studies (Hille, 1992). This could be attributed to different tissue types, ionic conditions, filling solutions (especially the inclusion of EGTA, ATP and GTP), holding potential and temperature as well as different cell dissociation procedure and enzyme solution components. Moreover, only a single exponential was fitted to the waveform when other authors have used two exponentials. The method of cell isolation in the present study was adopted from that Montgomery and Fry (1992). They reported the mean \(\tau\) for human detrusor at peak \(i_{Ca}\) was about 20ms and close to the value of 22ms for guinea-pig cells here under comparable conditions.

<table>
<thead>
<tr>
<th>Table 5.4. Summary of L-type and T-type Ca(^{2+}) currents in freshly isolated detrusor muscle cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-type (i_{Ca}) (mean±SD)</td>
</tr>
<tr>
<td>i(_{Ca}) threshold (mV)</td>
</tr>
<tr>
<td>Peak (i_{Ca}) (pA/pF)</td>
</tr>
<tr>
<td>(i_{Ca}) inactivation (\tau) (ms)</td>
</tr>
<tr>
<td>(i_{Ca}) activation:</td>
</tr>
<tr>
<td>(V_{0.5}) (mV)</td>
</tr>
<tr>
<td>slope factor (mV)</td>
</tr>
<tr>
<td>(i_{Ca}) inactivation:</td>
</tr>
<tr>
<td>(V_{0.5}) (mV)</td>
</tr>
<tr>
<td>slope factor (mV)</td>
</tr>
</tbody>
</table>
II.2.2. Only iLCA is present in cultured detrusor muscle cells

To dissect the T-type and L-type Ca\(^{2+}\) currents under whole-cell clamp a negative holding potential is required. When preconditioning voltages from -60 to +10 mV were used, the most available current was L-type iCa, and the T-type iCa could not be distinguished (see Figure 4.65). An experimental and analytical protocol was designed to verify that cultured cells expressed only an L-type iCa:

1. the same voltage-protocols of iCa activation (2 seconds preconditioning at -100 mV) and inactivation (2 seconds preconditioning at -120 mV to +30) were used in both freshly isolated and cultured cells.
2. KCl was replaced by CsCl in the patch pipette filling solutions to block iK.
3. activation and inactivation curves of iCa in cultured cells were compared to equivalent curves of L-type and T-type iCa in freshly isolated cells. In the latter cells the two current components were isolated by their differential sensitivity to channel blockers. The cultured cell curves were inseparable from the equivalent L-type curves in freshly isolated cells but highly significantly different from the T-type curves.

These results are consistent with the reports using long-term primary cultured and subcultured cells from some other smooth muscle cells (Simard 1991, basilar artery; Kent et al 1992, basilar artery).

II.2.3. Physiological roles of iTCA in detrusor

The physiological roles of iTCA in detrusor smooth muscle are not clear. In heart, T-type Ca\(^{2+}\) channels were shown to contribute to the pacemaker depolarisation in cardiac sino-atrial cells (Hagiwara et al 1988). The possible physiological roles of iTCA in detrusor smooth muscle are that it functions together with Ca\(^{2+}\)-dependent K\(^{+}\) current:

1. to regulate the resting membrane potential;
2. to make the action potential threshold more negative;
3. to contribute to spontaneous activity;
4. to refill intracellular stores.

Regulation of the resting potential

In freshly isolated detrusor muscle cells the Ca\(^{2+}\)-dependent iK is predominant and can be activated at -60 mV (see Figure 4.104). Occasionally this current was recorded at a constant holding potential of -60 mV without the requirement of a step
depolarising voltage. This suggests that resting Ca$^{2+}$ influx could only be via $i_{\text{TCa}}$, since $i_{\text{LCa}}$ activation at this voltage is unlikely. A $i_{\text{TCa}}$ window current is in the range -70 to -20mV, with a peak at about -43mV (Figure 4.115) and channel availability at this voltage is about 17%. This is equivalent to an inward current of 0.05-0.1 pA/pF (2.5 to 5.0pA per cell). In cultured cells, which have lost $i_{\text{TCa}}$, the resting potential was 10-17mV more depolarised in human and guinea-pig cells and presumes that any inward Ca$^{2+}$ current must be more than offset by an activation of Ca$^{2+}$-dependent outward currents. Furthermore, Knot et al (1998) reported 100nM iberiotoxin depolarised the membrane potential of cerebral arteries by 10mV further indicating an involvement of Ca$^{2+}$-dependent outward currents.

Modulation of action potential threshold
In detrusor smooth muscle $i_{\text{TCa}}$ seems to make action potential threshold more negative. The evidence for this is that the action potential threshold was a linear function of the holding potential (Figure 4.103). Since $i_{\text{TCa}}$ activates from about -60mV and peaks at -10mV, the initial activation of $i_{\text{TCa}}$ could depolarise the cell to open L-type Ca$^{2+}$ channels and cause an action potential with overshoot. In freshly isolated cells, specific L-type Ca$^{2+}$ channel blockers abolished the overshoot but left a residual component (Figure 4.14). In cultured cells, L-type blockers abolished the AP totally (see Figure 4.19, 4.20) since only $i_{\text{LCa}}$ was available. Therefore action potential threshold was at a more depolarised voltage in cultured cells than that in freshly isolated cells.

Contribution of spontaneous activity
$i_{\text{TCa}}$ in fresh detrusor muscle could contribute to spontaneous activity. Spontaneous action potentials were only recorded from freshly isolated detrusor cells, mostly in a range of membrane potentials between -40 to -25mV which was within the $i_{\text{TCa}}$ window current range. An inward $i_{\text{Ca}}$ of 5pA could depolarise a cell of 3 GΩ input resistance by 15mV. $i_{\text{TCa}}$ provides a slow depolarising current that drives the membrane potential into the $i_{\text{LCa}}$ threshold to generate action potential.

Refilling of intracellular stores
It may play a role to refill intracellular Ca$^{2+}$ store when the cell is in the resting state, particularly at negative resting potentials.
II.3. K+ currents in detrusor smooth muscle

II.3.1. STOC in freshly isolated detrusor muscle cells

Both freshly isolated guinea-pig and human detrusor muscle cells contain large spontaneous transient outward currents (STOC), which were not present in cultured cells (Figure 4.82). In freshly isolated cells from guinea-pig detrusor, this STOC disappeared when superfusing with Ca2+-free solution (Figure 4.83; 4.85). Replacement of extracellular Ca2+ by Ba2+ also eliminated STOC, although there was a large inward iBa (Figure 4.84). A change of the extracellular [Ca2+]o altered the amplitude of STOC with a close linear correlation between these two variables (Figure 4.86). Extracellular Ca2+ entry through the Ca2+ channels is required to activate outward current, as it was blocked by verapamil and NiCl2 (Figure 4.88, also see Figure 4.89). The current was carried by K+ as it was blocked by extracellular TEA (Figure 4.91, 4.92) or intracellular Cs+ (Figure 4.72 and 4.73 A, B). Caffeine-induced Ca2+ release from the sarcoplasmic reticulum also produced a voltage-dependent transient outward current (Figure 4.101). The specific BKCa blocker, iberiotoxin effectively abolished the current, but it was not affected by the SKCa blocker, apamin (Figure 4.93, 4.94). All these properties are characteristic of Ca2+-dependent large conductance K+ channels (BKCa). The present study also showed that the amplitude of the Ca2+-dependent K+ current is more closely related to the local [Ca2+]i instead of bulk [Ca2+]i rise by using a combination of patch clamp and epifluorescence techniques (Figure 4.95), similar to findings from other smooth muscle cells (Ganitkevich and Isenberg 1996; ZhuGe et al 1998). However, the frequency of the STOC may be more dependent on bulk [Ca2+]i rise (Figure 4.96). Therefore the Ca2+ source which caused the spontaneous outward current could be extracellular influx or intracellular release.

Ca2+-dependent K+ currents have been recorded in a wide variety of cells. The physiological role of BKCa in arterial smooth muscle is to regulate membrane potential and myogenic tone (Nelson and Quayle 1995). Ca2+-dependent ion channels also contribute to shaping action potential morphology and the firing pattern of excitable cells (Marty, 1989). Its functional role in detrusor muscle may be to contribute to determining the resting potential and be partially responsible for action potential repolarisation as described above. Ca2+-dependent K+ currents could modulate membrane potential oscillations, as well as spontaneous and repetitive action potentials (Hong et al 1997). Recently Heppner et al (1997) reported that 100nM iberiotoxin significantly increased the duration and frequency of action...
The iberiotoxin-insensitive component of Ca\(^{2+}\)-activated K\(^+\) currents in detrusor possibly indicates that there is an intermediate type of Ca\(^{2+}\)-activated K\(^+\) channel, since a maximal concentration (100 nM) of iberiotoxin was used. It has been reported that iberiotoxin is a selective BK\(_{Ca}\) inhibitor, acts exclusively at the outer face of the channel (IC\(_{50}\)=250pM) and does not block other types of voltage-dependent ion channels, especially other types of K\(^+\) channels that are sensitive to inhibition by charybdotoxin (Galvez, et al 1990). However the present study cannot exclude the possibility of an iberiotoxin-insensitive BK\(_{Ca}\) subtype in detrusor. A specific blocker for intermediate KC\(_{a}\) current is lacking, although charybdotoxin can block not only BK\(_{Ca}\), but also intermediate KC\(_{a}\) and other Ca\(^{2+}\)-independent K\(^+\) conductances in some tissue types (a charybdotoxin-sensitive Kv-type channel in human T-lymphocytes, Brewster and Strong 1992). Therefore further characterisation is necessary by using single channel recording in conjunction with pharmacological tools.
potentials elicited from bladder muscle strip, suggesting an involvement of $\text{BK}_{\text{Ca}}$ in modulating action potentials in detrusor muscle.

II.3.2. Steady-state outward rectifier, $i_K$

The present study demonstrated that freshly isolated detrusor muscle cells contain two voltage-dependent inward currents and a variety of voltage-dependent outward currents. The latter in most cases, especially when low intracellular [EGTA] was used, fall into two broad groups: one was STOC which as described above, was mediated mainly by $\text{BK}_{\text{Ca}}$; another was a steady-state component which resembles the "delayed rectifier" in other smooth muscle preparations. Cultured cells had only one inward, L-type current and only one steady-state outward current component, and lacked the inward T-type $i_{\text{Ca}}$ and outward STOC.

In freshly isolated guinea-pig detrusor cells, when all $\text{BK}_{\text{Ca}}$ was blocked, there was a remaining voltage-dependent outward current (see Figure 4.93). This apparent outward rectifier activated from about -20mV (Figures 4.74, 4.76, 4.77). This remaining steady-state outward current was an $i_K$ as it was absent when cells were dialysed with Cs+, instead of K+ (Figures 4.72-4.74). This steady-state $i_K$ contained no ATP-gated $i_K$ ($i_{\text{KATP}}$) since electrodes were filled with 5mM ATP to block ATP-gated $i_K$ ($i_{\text{KATP}}$). However, there was a considerable $\text{Ca}^{2+}$-dependent component, especially at depolarised voltages, because most of the current was reduced by extracellular verapamil (Figures 4.75, 4.76). These results were consistent with the finding that membrane depolarisation always induced a rise of [$\text{Ca}^{2+}$]; and then activated $\text{Ca}^{2+}$-dependent $i_K$ (Figures 4.97, 4.99). The U-shaped $i_{\text{Ca}}$ inactivation curve seen in detrusor cells suggests that the available channels were increased as voltage increased beyond +20mV (Nakayama and Brading 1993). The non-inactivating component of outward currents at potentials positive to +10mV, demonstrated in steady-state $i_K$ inactivation curves, could represent a $\text{Ca}^{2+}$-dependent $i_K$ (see Figure 4.59). This $\text{Ca}^{2+}$-dependent component of steady-state outward current was partially iberiotoxin-sensitive, but apamin-resistant (Figure 4.77). The $\text{Ca}^{2+}$-independent fraction of steady-state $i_K$ was mostly insensitive to extracellular TEA (Figure 4.78).

Under these present experimental conditions, small conductance $\text{Ca}^{2+}$-dependent $i_K$ ($SK_{\text{Ca}}$) was not evident in detrusor muscle cells. 1μM apamin, which is sufficient to block $SK_{\text{Ca}}$ channels (Blatz and Magleby 1986), produced no significant effect on the
STOC or on the steady-state $i_K$ (Figures 4.93, 4.94 and 4.77). This is in contrast to findings by Creed et al (1983) that 0.5μM apamin suppressed hyperpolarisations produced by field stimulation, suggesting that it could be attributed to activation of $SK_{Ca}$. One of the possible reasons for this contradiction is that the $SK_{Ca}$ in bladder was not large enough to be recorded easily in whole-cell recordings, but physiologically it could still have an effect on action potential after-hyperpolarisation, since at the resting state the detrusor smooth cell had a high input resistance (about 3 GΩ) a current as small as 5pA could change the membrane potential by 15mV. Also it should be noticed that the above effect of apamin was performed on muscle strips which contain nerve endings and thus could be involved in neurotransmitter responses in addition. To confirm further the presence of $SK_{Ca}$ in detrusor smooth muscle cells single channel recording would be a better tool to distinguish them by their unitary conductance, in conjunction with the pharmacological tools.

In cultured cells from guinea-pig detrusor, under the same experimental conditions as that in freshly isolated cells, the STOC could never be recorded but only the steady-state outward current component. This steady-state component was sensitive to intracellular Cs⁺ (Figure 4.79), only partially Ca²⁺-dependent (Figure 4.80) and partially sensitive to extracellular [TEA]₀ (Figure 4.81). Since TEA more effectively inhibits outward-going $i_K$ from the intracellular side (Colatsky, 1992), one cannot rule out the possibility that [TEA]₀-insensitive components, both in freshly isolated and cultured cells, could represent the same channel population as the [TEA]₀-sensitive component.

In summary, changes to membrane voltage-dependent ionic currents occurred during cell culture. In cultured cells, the inward $i_{TCa}$ and outward STOC were not functional; $i_{LCa}$ and steady-state $i_K$ occurred with smaller densities. Figure 5.1 summarises the voltage-dependent membrane currents in freshly isolated guinea-pig detrusor muscle cells.
**III. Conclusions**

A comparison of freshly isolated and cultured cells from human and guinea-pig detrusor uncovered a new \( \text{Ca}^{2+} \) current in freshly isolated detrusor muscle cells, a T-type \( \text{Ca}^{2+} \) current (\( i_{\text{TCA}} \)). Voltage-dependent currents in freshly isolated guinea-pig cells were comprised of two inward currents, \( i_{\text{TCA}} \) and \( i_{\text{LCA}} \), and a variety of outward currents including STOC (mainly \( i_{\text{BKCa}} \)), \( \text{Ca}^{2+} \)-dependent steady-state \( i_{\text{K}} \), \( \text{Ca}^{2+} \)-independent \( i_{\text{K}} \) and ATP-dependent \( i_{\text{K}} \) (\( i_{\text{KATP}} \)).

Freshly isolated muscle cells from guinea-pig and human detrusor bear a remarkable similarity in their membrane electrophysiological properties:

- there are no differences in their passive properties;
- they are excitable, either spontaneously or in response to electric stimuli to generate action potentials;
- their action potential threshold voltage and repolarisation time were not different, as both contained low-voltage activated \( i_{\text{TCA}} \);
- STOC and steady-state \( i_{\text{K}} \) density were not different;
the action potential amplitude was smaller, action potential maximum upstroke velocity and time to peak slower in human cells as a result of its smaller peak net inward current densities.

After culture, both human and guinea-pig detrusor muscle cells retained most of their electrical properties but with less excitability. They could not spontaneously fire action potentials but still responded to electrical stimuli to generate action potentials. Both cultured cells retained L-type Ca²⁺ current (\(i_{LCa}\)) and steady-state \(i_K\) with lower current densities, but lost the spontaneous outward transient current (STOC) and T-type Ca²⁺ current (\(i_{TCa}\)), which resulted in corresponding membrane potential changes:
resting membrane potential and action potential threshold shifted to more positive potentials;
action potential repolarisation was slower and therefore duration was longer;
the changes in action potential amplitude, time to peak and maximum upstroke velocity of both cultured cells were consistent with changes to their reduced peak net inward current densities.

The upstroke of the action potential is attributed to L-type \(i_{Ca}\) (\(i_{LCa}\)) and the T-type current (\(i_{TCa}\)) has a role in determining the action potential threshold. Both the steady-state outward rectifier \(i_K\) and STOC are involved in action potential repolarisation.

Ca²⁺-dependent \(i_K\), including STOC and steady-state outward current, is predominant in freshly isolated detrusor muscle. It plays an important role in action potential repolarisation, and may conjugate with \(i_{TCa}\) to regulate the resting potential, as well as the spontaneous and repetitive firing of action potentials.

Acutely dissociated detrusor muscle cells and cultured cells have advantages and disadvantages. Acutely dissociated cells represent the native, differentiated phenotype of the tissue and as such most accurately reflect the membrane ionic properties associated with muscle function in vivo. The process of enzymatic disaggregation, however, results in cells of variable cell quality and could potentially result in proteolytic damage to membrane proteins. Conversely, cultured cells provide a stable and reproducible cellular preparation which has an important advantage of allowing the growth of small amounts of human tissue for study. However the phenotypic alterations that occur in cultured cells present substantial difficulties. The current density of voltage-dependent inward and outward currents in cultured detrusor smooth muscle was substantially lower than in freshly dissociated cells, and some ion
channels were not preserved in cultured cells. These difference could result from alteration of the expression of membrane ion channels; differences in the cytosolic environment of cultured cells, or reflect decreased expression of key regulating or transducer molecules in cultured detrusor smooth muscle cells. Therefore studies on the relative contribution of specific electromechanical coupling mechanism of cultured cells may not accurately reflect the native phenotype.

However cultured detrusor muscle cells do provide the ability to study the regulation of expression of membrane ion channels. The differences in current densities between freshly isolated and cultured detrusor muscle cells suggested that synthesis of these proteins is actively regulated. The mechanism underlying this regulation is of considerable importance for the understanding of regulation of excitability of smooth muscle cells. A major potential use for cultured cells is to study those factors controlling the expression of ion channels with the availability of suitable molecular probes for a number of relevant ion channels.

IV. Further experiments

IV.1. Studies of steady-state $i_K$

Although the activation kinetics of macroscopic steady-state $i_K$ from both cultured and freshly isolated cells of guinea-pig and human detrusor showed no significant changes, the steady-state inactivation kinetics of $i_K$ in freshly isolated human and guinea-pig detrusor, were significantly different (see Figure 4.35). This could result from a difference of the components which contribute to steady-state $i_K$ in two species. During culture, steady-state $i_K$ inactivation kinetics also altered (see Figure 4.59). For further analysis of the changes, it will be helpful to use pharmacological tools to dissect the individual components of the current.

IV.2. Studies of the spread of excitation in detrusor muscle

Detrusor muscle cells, as with other smooth muscle cells, are very small compared to skeletal and cardiac muscle myocytes. Therefore synchronisation of excitation is particularly important for efficient force generation in detrusor. For a better understanding of the electrical properties of detrusor muscle, it is necessary to analyse the mechanism of the spread of excitation in whole tissue.
Spread of excitation can be explained by conduction of the action potential. The space constant is large enough to permit a reasonable volume of muscle to contract synchronously. When an action potential propagates a local circuit current flows in front of the waveform. It is assumed that the gap junction is the structure responsible for cell-to-cell coupling with a low electrical resistance. However adequate cell-to-cell coupling has not proved unequivocally in detrusor. Thus the structural basis for the electrophysiological observations has not yet been established and further investigations are necessary concerning this problem. Identification of connexin subtypes and determination of the cable properties of human detrusor muscle would be very valuable.

IV.3. Studies of bladder instability

The present study suggested that the STOC and iTCa might be related to spontaneous action potentials which might induce similar contractions. These uncontrolled contractions could result in detrusor instability. It would be interesting to compare the electrophysiological properties of unstable and normal detrusor to see if there was any difference between them. It would also be interesting to see whether there is any change to cell-to-cell coupling in unstable bladders to determine whether any electrical activity could propagate more or less effectively.

The physiological role of the L-type Ca\(^{2+}\) channel in normal bladder function is not clear. It has been found that an atropine-resistant component of detrusor muscle contraction, induced by electrical field stimulation of the intrinsic nerves, was increased in unstable bladders. This component has been found to be mediated by the neurotransmitter ATP (Palea et al 1993). Purinergic activation of a non-selective cation conductance would depolarise the cell and subsequently open voltage-dependent L-type Ca\(^{2+}\) channels. The resultant influx of Ca\(^{2+}\) would bring about contraction of the detrusor smooth muscle. In small mammals which have purinergic innervation to their bladders, it has been shown that activation of purinergic nerve produces excitatory junction potentials and subsequent activation of the L-type Ca\(^{2+}\) channels. Similar phenomena in human detrusor remains to be demonstrated; in particular in abnormally functioning bladders. The process of neurotransmitter release in prejunctional nerves also needs to be studied using modulators of synaptic release, such as \(\omega\)-conotoxin GVIA and \(\omega\)-agatoxin IVA.
IV.4. Relationship between electrophysiological activity and second messenger pathways

In detrusor muscle electromechanical and pharmacological coupling co-exist. Contractile function is a result of a fine balance between these two systems. Up to now, only limited studies have been performed on the interaction between them. It is of interest to reveal the roles of individual currents in IP$_3$-mediated Ca$^{2+}$ signalling. For example, could the i$_{L\text{Ca}}$, i$_{T\text{Ca}}$ and Ca$^{2+}$-dependent K$^+$ current modulate the IP$_3$-induced Ca$^{2+}$ release? and in what way? Are there any positive or negative feed back mechanisms existing between the inositol phosphate cascade due to cholinergic activation and membrane electrophysiological events? The roles of the i$_{L\text{Ca}}$ and i$_{T\text{Ca}}$ in the replenishing of the IP$_3$-sensitive intracellular Ca$^{2+}$ pools also needs to be addressed in detail.

IV.5. Mechanical properties of cultured cells

Cultured cells retained the electrophysiological properties and cholinergic receptor response although some alterations occurred. It is also interesting to investigate whether the contraction recovers after culture and is there any difference between freshly isolated and cultured cells.
References


Chamley J H, Groschel-Stewart U, Campbell G R and Burnstock G (1977). Distinction between smooth muscle, fibroblasts and endothelial cells in culture by the


List of published and presented work from this thesis


