THE CHARACTERIZATION AND PHARMACOLOGY OF THE
SLOW AFTERHYPERPOLARIZATION IN CULTURED RAT
HIPPOCAMPAL PYRAMIDAL CELLS

MALA M. SHAH

A thesis submitted for the degree of Doctor of Philosophy

Department of Pharmacology,
University College London,
Gower Street,
London,
WC1E 6BT.
ABSTRACT

Following a burst of action potentials in hippocampal pyramidal cells, a slow afterhyperpolarization (sAHP) is observed which is insensitive to the bee-venom toxin apamin (Sah, 1996). Most of the work published on this response has employed brain slices, the sAHP proving difficult to reproduce in cultured neurones (Alger et al., 1994). In this work, the use of appropriate culture conditions and perforated patch recording has allowed the sAHP to be successfully recorded in cultured neurones. The current underlying the sAHP (sIAHP) in the isolated pyramidal cells was pharmacologically characterized and proved to be similar to that recorded using slices. The sIAHP in these cells was partially inhibited by both L- and N-type calcium channel inhibitors as well as ryanodine, an inhibitor of calcium-induced calcium release. These results suggested that calcium entry via multiple pathways can contribute to the generation of the sIAHP in these cells.

The pharmacology of the sIAHP in these cells, as expected, differed greatly from the pharmacology of the apamin-sensitive afterhyperpolarization. The sIAHP could be inhibited by clotrimazole, an antifungal agent which blocks intermediate conductance calcium-activated potassium channels in red blood cells. Two derivatives of clotrimazole, UCL 2027 and UCL 2077, were the most selective blockers of the sIAHP, with very little block of calcium channels.

One of the cloned small conductance calcium-activated potassium channel (SK1 channel) has been suggested underlie the sIAHP. However, when SK1 cDNA was expressed in mammalian cell lines, the channels formed were apamin-sensitive. UCL 2027 and UCL 2077 also had little effect on the expressed SK1 channels. These results therefore, question whether SK1 channels underlie the sIAHP.
In conclusion, two novel blockers of the $sI_{AHP}$ have been identified which may prove useful in establishing the physiological role and in the identification of the molecular correlate of the current.
ACKNOWLEDGEMENTS

I am extremely grateful to my supervisor, Dr. Dennis Haylett for his continuous support, encouragement and advice. I would also like to thank Prof. D. H. Jenkinson, Dr. D. C. H. Benton and Dr. T. G. J. Allen for all their advice and help; Dr. P. M. Dunn for help in setting up the hybrid-clamp protocol; Dr. S. J. Marsh for the photography of the cells; Dr. G. W. J. Moss and Dr. R. Hosseini for help with the molecular biology experiments; and Prof. C. R. Ganellin and members in his team for the synthesis of the novel compounds and their advice.

I would like to dedicate this thesis to my parents, without whose support and encouragement this would not have been possible.
PUBLICATIONS ARISING FROM THIS THESIS

Papers


Abstracts


## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>4</td>
</tr>
<tr>
<td>Publications arising from this thesis</td>
<td>5</td>
</tr>
<tr>
<td>List of Figures</td>
<td>14</td>
</tr>
<tr>
<td>List of Tables</td>
<td>18</td>
</tr>
<tr>
<td><strong>Chapter 1: Introduction</strong></td>
<td>19</td>
</tr>
<tr>
<td>1.1 K⁺ channels</td>
<td>19</td>
</tr>
<tr>
<td>1.1.1 Classification of K⁺ channels</td>
<td>21</td>
</tr>
<tr>
<td>1.1.2 Structure of K⁺ channels</td>
<td>21</td>
</tr>
<tr>
<td>1.1.3 The importance of K⁺ channels</td>
<td>22</td>
</tr>
<tr>
<td>1.2 Afterhyperpolarizations</td>
<td>23</td>
</tr>
<tr>
<td>1.3 Ca²⁺-activated K⁺ channels</td>
<td>24</td>
</tr>
<tr>
<td>1.3.1 BK channels</td>
<td>24</td>
</tr>
<tr>
<td>1.3.2 IK channels</td>
<td>25</td>
</tr>
<tr>
<td>1.3.3 SK channels</td>
<td>26</td>
</tr>
<tr>
<td>1.3.3.1 Biophysical properties</td>
<td>26</td>
</tr>
<tr>
<td>1.3.3.2 Pharmacology of SK channels</td>
<td>26</td>
</tr>
<tr>
<td>1.3.3.3 Evidence for subtypes of SK channels</td>
<td>27</td>
</tr>
<tr>
<td>1.3.3.4 Cloning of SK channels</td>
<td>28</td>
</tr>
<tr>
<td>1.3.3.5 Distribution and physiological role of SK channels</td>
<td>31</td>
</tr>
<tr>
<td>1.3.3.5a Central Nervous System (CNS)</td>
<td>31</td>
</tr>
<tr>
<td>1.3.3.5b Peripheral tissues</td>
<td>31</td>
</tr>
<tr>
<td>1.4 The mAHP and sAHP</td>
<td>33</td>
</tr>
<tr>
<td>1.5 The sAHP in hippocampal neurones</td>
<td>34</td>
</tr>
<tr>
<td>1.5.1 General properties</td>
<td>34</td>
</tr>
<tr>
<td>1.5.2 Pharmacological characteristics</td>
<td>34</td>
</tr>
<tr>
<td>1.5.3 Do SK1 channels underlie the sAHP?</td>
<td>35</td>
</tr>
<tr>
<td>1.5.4 Ca²⁺ channels in hippocampal neurones</td>
<td>36</td>
</tr>
<tr>
<td>1.5.5 Possible reasons for the slow time course of the sI&lt;sub&gt;AHP&lt;/sub&gt;</td>
<td>37</td>
</tr>
<tr>
<td>1.5.6 The physiological role of the sAHP</td>
<td>40</td>
</tr>
</tbody>
</table>
1.6 Aims of the project 41

Chapter 2: Methods 43

2.1 Studies using hippocampal cells 43
2.1.1 Cell culture methods 43
2.1.1.1 Method 1 43
2.1.1.2 Method 2 44
2.1.1.3 Method 3 45
2.1.2 Electrophysiological studies using cultured hippocampal pyramidal neurones 46
   2.1.2.1 External solutions 46
   2.1.2.2 Patch pipettes 47
   2.1.2.3 Equipment used for patch-clamp studies 47
   2.1.2.4 General procedure for patch clamp recordings 49
   2.1.2.5 Identification of hippocampal neurones 49
   2.1.2.6 Recording of the sIahp 50
   2.1.2.7 Estimation of the reversal potential of the sIahp 51
   2.1.2.8 Recording of outward currents evoked using NMDA 51
   2.1.2.9 Recording of outward currents produced by caffeine 52
   2.1.2.10 Data Analysis 53
      2.1.2.10a Measurement of effects of drugs on the sIahp 53
      2.1.2.10b Measurement of the reversal potential of the sIahp 55
      2.1.2.10c Measurement of the currents evoked by application of NMDA 55
      2.1.2.10d Measurement of the outward current evoked by caffeine 56
   2.1.3 Recording of Ca^{2+} currents 56
   2.1.3.1 Cell preparation 56
   2.1.3.2 Electrophysiological recordings 56
   2.1.3.3 Data analysis 58
2.2 Studies using Superior Cervical Ganglion(SCG) cells 59
   2.2.1 Cell culture methods 59
   2.2.2 Electrophysiological studies 59
   2.2.2.1 Data analysis 60
2.3 Studies using the cloned rSK1 and hSK1 cDNAs 60
### 2.3.1 Maintenance of cell lines

- **2.3.1.1 HEK293 cells**
- **2.3.1.2 Cos-7 cells**

### 2.3.2 Modification of rSKI cDNA

- **2.3.2.1 Experimental design**
- **2.3.2.2 Step 1; Digestion reaction**
- **2.3.2.3 Step 2: PGR reaction**
- **2.3.2.4 Step 3; A-tailing reaction and sub-cloning into the pGEM-T-Easy vector**
- **2.3.2.5 Step 4; Identification of the pGEM-T-Easy vector containing the insert**

### 2.3.3 The calcium phosphate transfection method

### 2.3.4 Electrophysiological studies with transfected cells

- **2.3.4.1 Data analysis**

### 2.4 Materials

- **2.4.1 Materials used for tissue culture methods**
- **2.4.2 Materials used for electrophysiological studies**
- **2.4.3 Materials used for molecular biology studies and transfection purposes**

### Chapter 3: Characterization of the sI_AHP in cultured hippocampal pyramidal neurones

- **3.1 Introduction**
- **3.2 Results**
- **3.2.1 Properties of cells isolated using different methods**
- **3.2.2 Comparison of sAHPs recorded using different conditions**
- **3.2.3 General characterization of the sI_AHP**
- **3.2.4 Channels underlying the sI_AHP**
- **3.2.4.1 Reversal potential of the sI_AHP**
- **3.2.4.2 Ba^{2+} sensitivity**
- **3.2.4.3 Dependence of the sI_AHP on Ca^{2+}**
- **3.2.5 Pharmacological characterization of the sI_AHP**
- **3.2.5.1 Effects of apamin and TEA**
- **3.2.5.2 Effects of alternative blockers of the apamin-sensitive AHP on the sI_AHP**
5.2.3 Effects of clotrimazole, UCL 1851 and UCL 1880 on the outward holding current at -50mV
5.2.4 Effects of clotrimazole, UCL 1851 and UCL 1880 on Ca\(^{2+}\) currents in freshly dissociated cells
5.2.5 Effects of clotrimazole, UCL 1851 and UCL 1880 on the mI\(_{AHP}\) in hippocampal pyramidal neurones
5.2.6 Effects of clotrimazole, UCL 1851 and UCL 1880 on the mI\(_{AHP}\) in SCG neurones
5.2.7 Effects of analogues of UCL 1851
5.2.8 Effects of analogues of UCL 1880
5.2.9 Effects of UCL 2027 and UCL 2077 on the sI\(_{AHP}\)
5.2.10 Effects of UCL 2027 and UCL 2077 on action potentials
5.2.11 Effects of UCL 2027 and UCL 2077 on the outward holding current in freshly dissociated hippocampal neurones
5.2.12 Effects of UCL 2027 and UCL 2077 on the HVA Ca\(^{2+}\) current in freshly dissociated hippocampal neurones
5.2.13 Effects of UCL 2027 and UCL 2077 on the mI\(_{AHP}\) in cultured hippocampal neurones
5.2.14 Effects of analogues of UCL 2077
5.3 Discussion
5.3.1 Effects of clotrimazole, UCL 1851 and UCL 1880 on the sI\(_{AHP}\) and the Ca\(^{2+}\) current in hippocampal neurones
5.3.2 Selectivity profile of clotrimazole, UCL 1851 and UCL 1880
5.3.3 Effects of analogues of UCL 1851
5.3.4 Effects of analogues of UCL 1880
5.3.5 Pharmacological profile of UCL 2027 and UCL 2077
5.3.6 Exploring the structural-activity relationship of UCL 2077
5.3.7 Conclusions

Chapter 6: The pharmacology of SK1 channels in mammalian cell lines
6.1 Introduction
6.2 Results
6.2.1 Transfection of rSK1 cDNA in HEK293 cells
6.2.2 Transient transfection of hSK1 cDNA into HEK293 cells
6.2.3 Pharmacological characterization of hSK1 current in HEK293 cells

6.2.4 hSK1 expression in cos-7 cells

6.2.5 rSK2 expression in HEK293 cells

6.3 Discussion

6.3.1 rSK1 expression in HEK293 cells

6.3.2 Characterization of hSK1 currents in mammalian cell lines

6.3.3 Effect of 1-EBIO on the hSK1 current

6.3.4 Effects of the inhibitors of the sIAHP on the hSK1 current

6.3.5 Possible reasons for the apamin-sensitivity of hSK1 channels in mammalian cell lines

6.3.6 Role of SK1 channels in hippocampal pyramidal neurones

Chapter 7: Characterization of outward currents produced by raising [Ca\(^{2+}\)]\(_i\) via alternative methods

7.1 Introduction

7.2 Results

7.2.1 Characterization of the currents evoked by NMDA receptor activation

7.2.1.1 Effects of pressure application of 100\(\mu\)M NMDA

7.2.1.2 Effects of pressure application of 1mM NMDA

7.2.1.3 Effects of 7-chlorokynurenic acid on currents produced by application of 1mM NMDA

7.2.1.4 Pharmacological characterization of the outward current produced by application of 1mM NMDA

7.2.1.5 Effects of ouabain on the currents produced by pressure application of 1mM NMDA

7.2.1.6 Do cells that demonstrate a sIAHP generate a UCL 1848-insensitive outward current when 1mM NMDA is applied?

7.2.2 Characterization of the currents evoked by caffeine

7.3 Discussion

7.3.1 Currents evoked by pressure application of NMDA

7.3.1.1 Characterization of inward and outward currents evoked by NMDA pressure application
7.3.1.2 Dependence of the generation of the outward current evoked by NMDA application on Ca\(^{2+}\)
7.3.1.3 Effects of UCL 1848 and apamin on the currents evoked by pressure application of NMDA
7.3.1.4 Effects of muscarine and UCL 1880 on the currents evoked by pressure application of NMDA
7.3.1.5 Kinetics of the outward currents sensitive to UCL 1848, apamin, muscarine and UCL 1880
7.3.1.6 Correlation between the presence of a \(I_{\text{AHP}}\) and a UCL 1848-insensitive outward current in response to pressure application of NMDA
7.3.1.7 Could other Ca\(^{2+}\)-activated conductances underlie the outward current evoked by NMDA pressure application?
7.3.1.8 Effects of ouabain on the NMDA induced outward currents
7.3.1.9 Effects of ouabain on the outward holding current present at \(-50\text{mV}\)
7.3.1.10 Could changes in intracellular pH contribute to the generation of the outward current evoked by NMDA?
7.3.1.11 Summary
7.3.2 Currents evoked by application of caffeine
7.3.2.1 Pharmacology of the outward current evoked by caffeine
7.3.2.2 Pharmacology of the inward current caused by application of caffeine
7.3.3 Conclusions

Chapter 8: Discussion
8.1 The pharmacology of the \(I_{\text{AHP}}\)
8.2 Do SK1 channels underlie the \(I_{\text{AHP}}\)?
8.2.1 Comparison of the pharmacology of the \(I_{\text{AHP}}\) and expressed SK1 channels
8.2.2 Could the apamin-sensitivity of SK1 channels be dependent on the association of auxiliary subunits?
8.2.3 Comparison of the distribution of SK1 mRNA and \(I_{\text{AHP}}\) currents
8.2.4 Comparison of the kinetics of the \(I_{\text{AHP}}\) and the expressed SK channels
8.3 The role of Ca\(^{2+}\) channels in the generation of the \(I_{\text{AHP}}\)
8.3.1 Dependence of the generation of the sI_{AHP} on particular Ca^{2+} channel subtypes

8.3.2 Dependence of the time course of the sI_{AHP} on \([Ca^{2+}]_i\)

8.4 Future Directions

References
LIST OF FIGURES

Fig 1.1 Schematic representation of the three principal K⁺ channel groups
Fig 1.2 An alignment of the sequences of hSK1, rSK1, rSK2 and rSK3
Fig 1.3 The sIAHP and the associated somatic and dendritic Ca²⁺ transients
Fig 2.1 An illustration of a fit of the sIAHP to the exponential equation described in Methods Section 2.1.2.10a
Fig 2.2 Ca²⁺ currents recorded from cultured hippocampal pyramidal cells
Fig 2.3 The unmodified sequence of rSK1
Fig 2.4 Amino acid sequence of the primers designed for the PCR reaction
Fig 2.5 The first 270 base pairs of the modified rSK1 sequence
Fig 2.6 Gel electrophoresis of restriction enzyme products of the modified and unmodified rSK1
Fig 3.1 M-like, Q-currents and the sAHP recorded from cells cultured using either Method 2 or Method 3
Fig 3.2 Morphology of a hippocampal pyramidal cell approximately 3h after isolation and after 8 days in culture
Fig 3.3 Three different types of IAHP can be detected from cells that have the morphology of a typical pyramidal cell
Fig 3.4 The dependence of the sIAHP on the holding potential and the number of action potentials
Fig 3.5 An illustration of the protocol used to measure the reversal potential of the sIAHP and the I-V curve obtained
Fig 3.6 Effects of 2min bath applications of 1mM Ba²⁺ and 200μM Cd²⁺
Fig 3.7 Effects on the sIAHP of 2min bath applications of 100nM apamin, 1mM TEA, 100μM tubocurarine, 100nM UCL 1848 and 100nM charybdotoxin
Fig 3.8 Effects of 2min bath applications of 1μM noradrenaline and 3μM muscarine
Fig 3.9 Effect of a 2min bath application of 300μM 1-EBIO on the sIAHP and the mlAHP.
Fig 4.1 Records of the sIAHP (when evoked using the hybrid-clamp protocol and a depolarizing step) and the last action potential in the train in the presence of 10μM nifedipine
Fig 4.2 Effects of nimodipine on the sI_AHP
Fig 4.3 Effects of 10μM nimodipine on action potentials
Fig 4.4 Effects of 10μM nifedipine, 3μM nimodipine and 10μM nimodipine on Ca^{2+} currents recorded from freshly dissociated hippocampal neurones
Fig 4.5 Effects of 100nM ω-conotoxin GVIA on the sI_AHP and the last action potential in the train
Fig 4.6 Records of the sI_AHP in the absence and presence of 10μM ryanodine
Fig 4.7 Effects of 10μM clotrimazole, UCL 1851 and UCL 1880 on the sI_AHP recorded in cultured rat hippocampal pyramidal neurones
Fig 4.8 Concentration-inhibition curves for the sI_AHP and HVA Ca^{2+} currents
Fig 4.9 Effects of 3μM UCL 1880 and 3μM UCL 1851 on action potentials
Fig 4.10 Effects of clotrimazole on the outward holding current present at -50mV
Fig 4.11 Effects of clotrimazole on the sI_AHP and the last action potential in the train
Fig 4.12 Effects of clotrimazole on the mI_AHP and the sI_AHP recorded in freshly dissociated hippocampal neurones
Fig 5.1 Effects of clotrimazole on the sI_AHP in the absence and presence of 10μM ryanodine
Fig 5.2 Effects of clotrimazole, UCL 1851 and UCL 1880 on the sI_AHP recorded in cultured rat hippocampal pyramidal neurones
Fig 5.3 Concentration-inhibition curves for the sI_AHP and HVA Ca^{2+} currents
Fig 5.4 Effects of 3μM UCL 1880 and 3μM UCL 1851 on action potentials
Fig 5.5 Effects of clotrimazole on the outward holding current present at -50mV
Fig 5.6 Effects of UCL 1880 on the outward holding current present at -50mV
Fig 5.7 Effects of 3μM and 10μM clotrimazole on the Ca^{2+} current in freshly dissociated hippocampal neurones
Fig 5.8 Effects of 10μM clotrimazole on the mI_AHP and the sI_AHP
Fig 5.9 Effects of 10μM UCL 1851 and 10μM UCL 1880 on the mI_AHP and the sI_AHP
Fig 5.10 Effects of 10nM UCL 1848, 10μM UCL 1851, 10μM clotrimazole and 10μM UCL 1880 on the mI_AHP in SCG neurones
Fig 5.11 The general structure of compounds related to UCL 1851 that were tested on the sI_AHP in cultured rat hippocampal pyramidal neurones
Fig 5.12 General structure of compounds related to UCL 1880 that were tested on cultured hippocampal pyramidal neurones
Fig 5.13 Effects of 10μM UCL 2027 and 3μM UCL 2077 on the sI_AHP recorded in cultured hippocampal pyramidal neurones
Fig 5.14 Concentration-inhibition curves for UCL 2027 and UCL 2077 on the sI_AHP in cultured hippocampal pyramidal neurones
Fig 5.15 Effects of 10μM UCL 2027 and 3μM UCL 2077 on action potentials
Fig 5.16 Effects of 10μM UCL 2027 and 3μM UCL 2077 on the Ca\(^{2+}\) current recorded from freshly dissociated hippocampal neurones

Fig 5.17 Effects of 10μM UCL 2027 and 1μM UCL 2077 on the mI\(_{AHP}\) and the sI\(_{AHP}\)

Fig 5.18 General structure of the compounds related to UCL 2077

Fig 5.19 Chemical structures of UCL 2077, UCL 1495 and UCL 2339

Fig 6.1 Currents recorded from HEK293 cells transfected with rSK1 and hSK1 cDNA

Fig 6.2 An example of the block by apamin of the current produced by hSK1 transfected in HEK293 cells

Fig 6.3 Concentration-inhibition curves for apamin, UCL1848, tubocurarine and dequalinium.

Fig 6.4 I-V curves in the presence of dequalinium, tubocurarine, UCL 1848 and 1-EBIO

Fig 6.5 I-V curves in the presence of UCL 1880, UCL 2027 and UCL 2077

Fig 6.6 Whole cell currents from untransfected cos-7 cells and cos-7 cells transfected with hSK1

Fig 6.7 Effect of apamin on I-V curves obtained from cos-7 cells transfected with hSK1 and HEK293 cells transfected with rSK2

Fig 7.1 300ms and 10s pressure application of 100μM NMDA onto cultured hippocampal pyramidal neurones

Fig 7.2 300ms 1mM NMDA pressure applications onto 2 different hippocampal cells

Fig 7.3 Effects of 10μM 7-chlorokynurenic acid (7-CK) on the inward and outward currents produced by 300ms NMDA pressure applications

Fig 7.4 Effects of 100nM UCL 1848 on the currents produced by 300ms pressure applications of 1mM NMDA

Fig 7.5 Effects of 100nM apamin, 3μM muscarine and 3μM UCL 1880 on cells where the outward current was totally inhibited by UCL 1848

Fig 7.6 A comparison of the peaks of the subtracted UCL 1848-sensitive current and the control current generated by NMDA receptor activation
Fig 7.7 Effects of 3μM muscarine and 3μM UCL 1880 on the outward currents, generated by NMDA receptor activation, which were not inhibited by 100nM UCL1848.

Fig 7.8 To show that the peak of the muscarine-sensitive current occurs earlier than the peak of the outward current generated by NMDA receptor activation.

Fig 7.9 Effects of 100μM ouabain on the inward and outward currents generated by 300ms pressure applications of NMDA.

Fig 7.10 Currents generated by 10s bath applications of 10mM caffeine in the absence, presence and after washout of 1mM Ba^{2+}.

Fig 7.11 Records showing currents generated by 10s bath applications of 10mM caffeine in the absence and presence of 3μM muscarine.

Fig 8.1 An illustration of the possible mechanism that may be responsible for the generation of the sI_{AHP}.
LIST OF TABLES

Table 5.1 Effects of clotrimazole, UCL 1851 and UCL 1880 on the decay time constant ($\tau_3$) and the time to peak (TP) of the $sI_{\text{AHP}}$ recorded in cultured hippocampal pyramidal neurones  
124

Table 5.2 Effects of clotrimazole, UCL 1851 and UCL 1880 on the width of the last action potential in the train used to evoke the $sI_{\text{AHP}}$  
125

Table 5.3 Summary of effects of compounds structurally related to UCL 1851 on the $sI_{\text{AHP}}$ and the width of the last action potential (APW) in the train used to evoke the $sI_{\text{AHP}}$  
136

Table 5.4 Summary of effects of compounds structurally related to UCL 1880 on the $sI_{\text{AHP}}$ and the width of the last action potential (APW) in the train used to evoke the $sI_{\text{AHP}}$  
138

Table 5.5 Decay time constants ($\tau_3$) and times to peak (TP) of the $sI_{\text{AHP}}$ in the presence of UCL 2027 and UCL 2077  
143

Table 5.6 Effects of UCL 2027 and UCL 2077 on the last action potential width (APW) in the train used to evoke the $sI_{\text{AHP}}$  
145

Table 5.7 Summary of effects of compounds structurally related to UCL 2077 on the $sI_{\text{AHP}}$ and the width of the last action potential (APW) in the train used to evoke the $sI_{\text{AHP}}$  
150

Table 5.8 Summary of effects of UCL 2077 and the related compounds, UCL 1495 and UCL 2339, on the $sI_{\text{AHP}}$ and the width of the last action potential (APW) in the train used to evoke the $sI_{\text{AHP}}$  
152
CHAPTER 1

INTRODUCTION

Ion channels are macromolecular pores in cell membranes that allow passive diffusion of ions across the cell membrane. Although ion channels are most often selective for a particular ion such as potassium ($K^+$), sodium ($Na^+$) or calcium ($Ca^{2+}$), they can also allow the flow of more than one type of ion (e.g. the hyperpolarization-activated cation channels, HAC; Ludwig et al., 1998). Ion channels are commonly gated by a stimulus such as a chemical change (e.g. in $Ca^{2+}$ concentration), a change in membrane potential or a mechanical deformation.

1.1 $K^+$ Channels

Hodgkin and Huxley (1952) first described $K^+$ channels in the squid giant axon. Since then multiple $K^+$ channels have been described. Cloning and expression of $K^+$ channel cDNAs has allowed progress to be made into determining the mechanisms of ionic selectivity, voltage- and $Ca^{2+}$-dependent gating, inactivation and pharmacology of these channels. Recently, a bacterial $K^+$ channel, KcsA, has been crystallised and a high-resolution structural analysis of it has been made (Doyle et al., 1998). The KcsA $K^+$ channel exhibits homology of sequence and structure to all known eukaryotic $K^+$ channels. This data has provided evidence in favour of a tetrameric $K^+$ structure and has also provided an insight into the mechanism of selective $K^+$ transport.

19
Fig 1.1 Schematic representation of the three principal K⁺ channel groups. Each group is divided into discrete families on the basis of sequence similarity. Each family can be further subdivided into several subfamilies which often contain several closely related subfamily members (for example, SK channels can be further subdivided into SK1, SK2 and SK3; adapted from Coetzee et al., 1999).
1.1.1 Classification of $K^+$ channels

Based on structural properties, $K^+$ channels can be divided into three groups (see Fig 1.1; Coetzee et al., 1999). The first group consists of proteins that have six transmembrane domains (6TMD) (S1-S6) and a conserved pore (P or H5) domain. When expressed in heterologous expression systems, they form voltage- and/or Ca$^{2+}$-activated $K^+$ channels. The S4 domain is conserved among the voltage-gated $K^+$ channels and is responsible for the voltage-dependence of the channels (Coetzee et al., 1999).

The second group of $K^+$ channels has two transmembrane domains (2TMD) (M1-M2) and a pore domain that is analogous to the S5-P-S6 region of the 6TM $K^+$ channel family. These proteins all exhibit some degree of inward rectification and are therefore also known as inward rectifiers.

The third class of $K^+$ channels form proteins that have four putative transmembrane domains (4TMD) and two P domains. The 4TMD subunits form $K^+$ selective channels that are not voltage-or Ca$^{2+}$-gated nor do they possess conserved properties such as inward rectification. There have been reports that substances such as arachidonic acid can modulate these channels. They are also referred to as ‘leak’ $K^+$ channels (for example see Duprat et al., 1997; Millar et al., 2000; Telley et al., 2000).

1.1.2 Structure of $K^+$ channels

In order to form functional channels, the 6TMD (MacKinnon, 1991) and 2TMD subunits are thought to assemble as tetrameric proteins. In contrast, the 4TMD subunits are thought to dimerize, thereby retaining the fourfold symmetry around the central pore (Lesage et al., 1996). All the channels formed have the
conserved amino acid sequence, GYG, which is thought to be an essential component of the K⁺ selective filter (Coetzee et al., 1999).

Heteromultimeric assembly can occur within each subfamily in the 6TMD and 2TMD class of K⁺ channels. There are also examples of heteromultimerization between different subfamilies of the 2TMD subunits. For example, Kir5.1 significantly increases expression levels and single channel conductance of Kir4.1 (Pessia et al., 1996). It has not yet been determined whether heteromultimeric assembly can occur within the 4TMD family of K⁺ channels. There is also increasing evidence for the existence of auxiliary subunits (such as β subunits) that regulate the expression levels and functional properties of K⁺ channel subunits (see Coetzee et al., 1999). Heteromultimerization, the presence of auxiliary subunits, alternative splicing, RNA editing by adenosine deamination, post-translational modification particularly by phosphorylation and dephosphorylation and the ion channel interactions with the cytoskeleton can all increase the diversity of functional K⁺ channels. Hence, although a large number of principle subunits have been cloned and expressed, the properties of the channels formed by these subunits often differ from native channels.

1.1.3 The importance of K⁺ channels

In excitable tissues such as neurones, cardiac and smooth muscle cells and pancreatic β cells, the major function of K⁺ channels is likely to be the maintenance of a negative membrane potential and in shaping action potentials and controlling action potential firing rates. In non-excitable tissues (e.g. glandular tissue), K⁺ channels are often involved in processes such as secretion.
In neurones, K⁺ channels are involved in the repolarization of action potentials and also in the afterhyperpolarization that follows an action potential (Vergara et al., 1998; Castle 1999). This project has been concerned with the K⁺ channels underlying the afterhyperpolarization in hippocampal neurones and therefore, afterhyperpolarizations and the K⁺ channels underlying them will be discussed in more detail below.

1.2 Afterhyperpolarizations

Following the firing of action potentials in neurones, a hyperpolarization of the membrane commonly occurs. This afterhyperpolarization (AHP) can have several phases (Sah, 1996). Immediately following the action potential there is a fast AHP (fAHP) that typically lasts between 5ms and 10ms. This is usually followed by a more prolonged phase. The kinetics of this prolonged AHP vary between cells. In some cells (e.g. superior cervical ganglionic cells (SCGs); Dunn, 1994), the AHP has a fast onset (<50ms) and lasts several hundred milliseconds. In this thesis, this type of AHP will be referred to as the mAHP (medium duration AHP). In a few cell types (e.g. hippocampal pyramidal neurones (Lancaster and Adams, 1986), cortical neurones (Constanti and Sim, 1987; Pineda et al., 1998) and vagal motor neurones (Sah and McLachlan, 1991)), the prolonged AHP has a slower rising phase and can last for several seconds. For this reason, this type of AHP will be referred to as the slow AHP (sAHP). In some cell types (e.g. hippocampal pyramidal neurones; Storm 1987, 1989; Williamson and Alger, 1990; Stocker et al., 1999), both a mAHP and sAHP are present.
The AHP is dependent upon a rise in $[\text{Ca}^{2+}]_i$ for activation. As $K^+$ channels underlie the AHP, it is likely that $\text{Ca}^{2+}$-activated $K^+$ channels ($K_{\text{Ca}}$ channels) are responsible for the generation of the current (see Sah, 1996; Vergara et al., 1998). Therefore, this class of $K^+$ channels will be discussed in greater detail, with most emphasis placed on the small conductance $K_{\text{Ca}}$ channels (SK channels).

### 1.3 $\text{Ca}^{2+}$-activated $K^+$ channels

In the late 1950s, G. Gardos presented the first evidence for $K_{\text{Ca}}$ channels in red blood cells. The main observation was that there was a large increase in $K^+$ efflux when red blood cells were ATP-depleted. This rise in $K^+$ efflux, however, was dependent on the presence of $\text{Ca}^{2+}$ in the external solution (Gardos, 1958). Since then evidence for $K_{\text{Ca}}$ channels have been found in both excitable and non-excitable cells such as hepatocytes.

Based on their biophysical and pharmacological properties, $K_{\text{Ca}}$ channels can be further subdivided into three distinct families: large conductance $K_{\text{Ca}}$ or BK channels, intermediate conductance $K_{\text{Ca}}$ or IK channels and small conductance $K_{\text{Ca}}$ or SK channels.

#### 1.3.1 BK channels

BK channels or maxi $K$ channels have single channel conductances in excess of 100pS in symmetrical, high (146mM) $K^+$ solutions (for a review, see Siemer and Grissmer, 1999). BK channels are voltage-dependent as well as $\text{Ca}^{2+}$-activated. Two cDNAs (known as slo) which encode for the $\alpha$ subunit of the BK channels have been cloned. The primary amino acid sequence of BK channels shares a high degree of
homology with the sequences of the S1-S6 regions of the voltage-gated $K^+$ ($K_v$) channels (Vergara et al., 1998) but has little homology to the amino acid sequences of the other $K_{Ca}$ channels (Coetzee et al., 1999). BK channels can also be distinguished pharmacologically from IK and SK channels in that they are blocked by the toxins, charybdotoxin and iberiotoxin as well as TEA (Castle, 1999). Recently, Olesen et al., (1994a, b) have identified a novel class of compounds, the benzimidazolones, as direct activators of BK channels, with the most widely studied being 5-trifluoromethyl-1-(5-chloro-2-hydroxyphenyl)-1,3-dihydro-2H-benzimidazole-2-one (NS-004) and 1-(2'-hydroxy-5'-trifluoromethylphenyl)-5-trifluoromethyl-2[H] benzimidazolone (NS-1619). BK channels are found in a variety of tissues and they underlie the fast afterhypolarization that follows an action potential in neurones (Sah, 1996). In hippocampal neurones, BK channels are also involved in the repolarization phase of the action potential (Lancaster and Nicoll, 1987; Yoshida et al., 1991).

1.3.2 IK channels

IK channels have unitary conductance values of 20-80pS in symmetrical, high $K^+$ solutions (Vergara et al., 1998). They are usually voltage-insensitive, though voltage-sensitive IK channels have been reported in molluscan nerve cells (Meech, 1974). The IK channel mostly widely studied is that present in red blood cells and is responsible for the Gardos effect. IK channels can also be found in lymphocytes. Charybdotoxin (Wolff et al., 1988), clotrimazole (Brugnara et al., 1993, 1995) and cetiedil (Dunn, 1998; Benton et al., 1999a) have all been shown to block these channels. 1-ethyl-2-benzimidazolinone (1-EBIO) and the related compounds, chlorzoxazone and zoxazolamine, have been found to increase the open probability
of IK channels (Devor et al., 1996; Jensen et al., 1998; Pedersen et al., 1999; Warth et al., 1999; Syme et al., 2000). Recently, a cloned cDNA (termed hIK1/hSK4), which has approximately 50% homology to SK channels has been shown to represent an IK channel (Ishii et al., 1997a; Joiner et al., 1997). So, far hIK1 mRNA has only been found in peripheral tissues (Ishii et al., 1997a; Joiner et al., 1997).

1.3.3 SK channels

1.3.3.1 Biophysical properties

SK channels have single channel conductances between 5-20pS in symmetrical, high K⁺ solutions. Native SK channels are half-maximally activated by 400-800nM Ca²⁺ (see e.g. Blatz and Magleby, 1986; Grissmer et al., 1992; Park, 1994; Selyanko et al., 1998; Hirschberg et al., 1999). The single channel conductance and Ca²⁺ sensitivity of these channels were first confirmed by Blatz and Magleby (1986) in skeletal muscle and then by Capiod and Ogden (1987, 1989) in guinea-pig hepatocytes. They are voltage-insensitive.

1.3.3.2 Pharmacology of SK channels

SK channels are insensitive to block by low concentrations of TEA (1-5mM) and all the known toxins that block BK and IK channels (Castle, 1999). The bee venom toxin, apamin (Burgess et al., 1981) and the scorpion toxin, scyllatoxin (Chicchi et al., 1988), selectively block SK channels (at least SK2 and SK3 channels, see Section 1.3.3.4). Apamin has, in fact been a useful tool for identifying the presence of these channels. Radiolabelled apamin has been used to define tissue distribution of the apamin-sensitive SK channels (e.g. see Mourre et al., 1986) and also to identify other pharmacological agents that interact with these potassium ion channels.
channels (e.g. see Cook and Haylett, 1985). The IK channel openers, 1-EBIO and the related compounds, chlorzoxazone and zoxazolamine, have also recently been described as SK channel openers (Syme et al., 2000; Oliver et al., 2000; see Chapter 3 and Chapter 6).

Structure-activity studies have shown that two arginine residues at positions 13 and 14 are important for the biological activity of apamin (Vincent et al., 1975). Bisquaternary and some trisquaternary neuromuscular blocking agents also have two positively charged residues and some of these (e.g. tubocurarine and gallamine) have been shown to displace $^{125}\text{I}$ apamin from guinea pig hepatocytes (Cook and Haylett, 1985). Tubocurarine and gallamine, like apamin, have also been shown to block the afterhyperpolarization in neurones (Nohmi and Kuba, 1984; Bourque and Brown, 1987; Goh and Pennefather, 1987; Tanaka and Kuba, 1987; Dunn et al., 1996). The antiseptic agent, dequalinium has also been shown to be a selective and potent inhibitor of apamin-sensitive SK channels (Castle et al., 1993; Dunn, 1994). Using dequalinium as a lead compound, Professor Ganellin and colleagues (Department of Chemistry, UCL) have developed a series of non-peptidic compounds that are inhibitors of the apamin-sensitive SK channels. Two of the compounds, UCL 1684 and UCL 1848, have similar potency to apamin, with the added advantage that the block produced by these compounds is more reversible than that produced by apamin (Campos Rosa et al., 1998; Benton et al., 1999b).

1.3.3.3 Evidence for subtypes of SK channels

Even before SK channels had been cloned, subtypes of the SK channels had been postulated. Dequalinium, gallamine and UCL 1530, a dequalinium analogue, have been shown to block the SK channels in hepatocytes and SCG neurones with
different rank orders of potency, thereby implying that the SK channel types differ in hepatocytes and SCG neurones (Dunn et al., 1996). Wadsworth et al., (1997) have also shown that polypeptides of different sizes but which can all be labelled by apamin exist in rat brain and liver plasma membranes, thus providing biochemical evidence for the presence of subtypes of SK channels. However, the cloning of SK channels, initially from rat and human brain libraries, have provided the most definitive proof for the existence of SK channel subtypes (Köhler et al., 1996).

1.3.3.4 Cloning of SK channels

Three cDNAs (SK1-3) that express functional proteins and have the properties of SK channels have been cloned (Köhler et al., 1996). The amino acid sequences for SK1-3 show 90% homology (see Fig 1.2). Injection of the mRNA into Xenopus oocytes produced Ca\(^{2+}\)-dependent, voltage-independent, mildly inwardly rectifying K\(^+\) channels. The channels exhibit unitary conductances of about 10pS in symmetrical, high K\(^+\) solutions (Köhler et al., 1996). Hydropathy analysis indicates that the SK channels belong to the 6TM family (Vergara et al., 1998).

The cloned SK \(\alpha\) subunits form channels that have a steep dependence (Hill coefficients of approximately 4; Xia et al., 1998; Bond et al., 1999) on \([Ca^{2+}]_i\); with the half-maximal activation by Ca\(^{2+}\) being 400-600nM, similar to the values reported for native SK channels (Blatz and Magleby, 1986; Grissmer et al., 1992; Park, 1994; Selyanko et al., 1998; Hirschberg et al., 1999). Ca\(^{2+}\) ions, however, do not interact directly with the cloned SK channel \(\alpha\) subunits. Cloned SK channels have been shown to interact with calmodulin. Opening of the SK channels is a consequence of the conformational alterations that occur due to Ca\(^{2+}\) binding to calmodulin (Xia et al., 1998).
Fig 1.2 An alignment of the sequences of hSK1, rSK1, rSK2 and rSK3. The transmembrane regions (blue), the pore region (purple) and the amino acid residues that have identified to bind to apamin (green) have been highlighted.
Three members of the SK channel family, SK1-3, have so far been identified. Both the human (denoted by an h) and the rat (denoted by an r) homologues have been cloned. The channels formed by these clones show similar biophysical properties and only differ in their pharmacology (Bond et al., 1999). In the Xenopus oocyte expression system, both rSK2 and rSK3 have been shown to be apamin-sensitive, with IC₅₀s of 60pM and 1nM respectively (Köhler et al., 1996; Ishii et al., 1997b). rSK2 also displayed relatively high affinity for d-tubocurarine (IC₅₀ = 5.4μM; Köhler et al., 1996). hSK1, however, was found to be insensitive to 100nM apamin and also relatively insensitive to d-tubocurarine (IC₅₀ =350μM; Ishii et al., 1997b). The high affinity of SK2 for apamin and d-tubocurarine was shown to be due to two particular residues, an aspartic acid (D) and an asparagine (N), which reside on opposite sites of the pore (Fig 1.2; Ishii et al., 1997b). Mutations of the residues in SK1 to the equivalent residues in the SK2 sequence resulted in apamin-sensitive SK1 channels being formed (Ishii et al., 1997b). SK3 channels, which have intermediate sensitivity to apamin, have only one of the residues that determine apamin binding, consistent with the mutagenesis studies (Fig 1.2; Ishii et al., 1997b; Bond et al., 1999). Recently, however, it has been reported that injection of hSK1 mRNA into Xenopus oocytes results in the formation of channels with at least two different sensitivities to apamin (Grunnet et al., 1999). Some of the work presented in this thesis and recently published data also show that hSK1 cDNA can form apamin-sensitive channels when expressed in mammalian cell lines (Shah and Haylett, 2000a; Strobaek et al., 2000). Hence, it is very likely that there are also other residues that can determine apamin binding.
1.3.3.5 Distribution and physiological role of SK channels

1.3.3.5a Central Nervous System (CNS)

SK channels are present in neurones and are usually activated by a rise in intracellular Ca\(^{2+}\) that occurs during an action potential. Intracellular Ca\(^{2+}\) decay is over a period of several hundred milliseconds, allowing SK channel activation to generate a prolonged afterhyperpolarization (Castle, 1999). The AHP, and therefore, SK channels are important for spike frequency adaptation and are necessary for normal neurotransmission (Vergara et al., 1998). Apamin-sensitive SK channels may have a role in epilepsy (McCown and Breese, 1990), in the circadian cycle and sleep patterns (Gandolfo et al., 1996) and memory and learning (Messier et al., 1991).

As mentioned above, there are 3 subtypes of SK channels. Within the central nervous system (CNS), \textit{in situ} hybridization studies have revealed that the different SK subtypes have different distribution patterns (e.g. the SK3 gene alone is expressed in the dopaminergic neurones of the substantia nigra; Köhler et al., 1996; Stocker and Pedarzani, 2000). Therefore, it is possible that one subtype of SK channels may be more important than others in particular disorders of the CNS; e.g. the SK3 gene has been linked to schizophrenia (Chandy et al., 1998).

1.3.3.5b Peripheral tissues

In the periphery, apamin-evoked changes in smooth muscle contractility as well as apamin binding sites have been found throughout the alimentary canal (e.g. see Gater et al., 1985; Hugues et al., 1982b, Pacha et al., 1992). Apamin-sensitive SK channels are also present in vascular smooth muscle where they have been shown to be possibly involved in relaxation (Simonsen et al., 1997).
identified in vascular endothelial cells, too and may be involved in the regulation of nitric oxide production (Muraki et al., 1997).

Although SK channels are not present in innervated adult skeletal muscle, there is an increase in apamin binding sites and the presence of an AHP in skeletal muscle fibres following denervation (Hugues et al., 1982a; Schmid-Antomarchi et al., 1985; Blatz and Magleby, 1986). Exposure of the sciatic nerve to agents such as colchicine that inhibit axonal transport also results in upregulation of apamin binding sites in rat skeletal muscle and these findings have been used to argue that neurotrophic factors released from nerve endings may exert an inhibitory control of SK channel expression in skeletal muscle (Ramirez et al., 1996). Skeletal muscle of patients with myotonic muscular dystrophy also express apamin-binding sites (Renaud et al., 1986). A specific role for SK channels in this disorder is supported by the findings that the injection of apamin into the muscle of myotonic dystrophy patients results in myotonic discharges after muscle percussion being more difficult to trigger and of smaller intensity and duration (Behrens et al., 1994). The SK3 gene has been shown to be present in denervated skeletal muscle (Pribnow et al., 1999).

Apamin-sensitive SK channels are also present in adrenal glands where they are thought to provide a negative feedback control for adrenal catecholamine secretion (Park, 1994; Montiel et al., 1995).

Although there is little biophysical or functional evidence for the presence of SK channels in cardiac muscle, apamin-binding sites have been found in rabbit cardiac muscle membrane preparations (Schetz and Anderson, 1995). In situ hybridisation studies have also identified the presence of SK1 mRNA in rat heart (Köhler et al., 1996). However, any physiological role of these channels in the heart still remains to be elucidated.
Apamin-sensitive K⁺ conductances were identified in hepatocytes of some species more than 15 years ago (Burgess et al., 1981). However, as with cardiac muscle, the functional significance of these channels in hepatocytes still remains to be found.

1.4 The mAHP and sAHP

These two AHPs can generally be distinguished by their apamin-sensitivity. The mAHP usually has an apamin-sensitive component whereas the sAHP is usually insensitive to apamin. It is, therefore likely that either SK2 or SK3 homomers or a combination of the two mediates the apamin-sensitive AHP (but see Chapter 6). Heteromultimers between SK channels can also form (Ishii et al., 1997b) and hence depending on the expression of the SK channel in the particular cell type, a combination of all the SKs could potentially form the channels underlying the apamin-sensitive AHP.

As mentioned above, in hippocampal pyramidal neurones both a mAHP and a sAHP can be generated following action potentials. In these cells, it is possible that a number of different types of K⁺ conductances, including apamin-sensitive SK channels (Stocker et al., 1999), contribute to the mAHP (Storm, 1989, 1990; Alger et al., 1994). The sAHP is apamin-insensitive and since this current in hippocampal pyramidal neurones has been the main object of study, it is discussed below in greater detail.
1.5 The sAHP in hippocampal neurones

1.5.1 General properties

The sAHP is usually detected following a depolarising step or a train of action potentials (Lancaster and Nicoll, 1987). The current underlying the sAHP (sI_{AHP}) shows a distinct rising phase, peaks between 400 and 700ms after a train of action potentials and decays with a time constant of approximately 1.5s at 30°C (Lancaster and Adams, 1986). It has been established from reversal potential experiments that K⁺ channels underlie the sI_{AHP} (Lancaster and Adams, 1986). Noise analysis studies estimate the single channel conductance of these K⁺ channels to be approximately 10pS in symmetrical K⁺ solutions (Sah and Isaacson, 1995; Valiante et al., 1998). The generation of the sI_{AHP} has also been shown to be dependent upon Ca²⁺ (Lancaster and Adams, 1986). Taken together, these findings might suggest that SK channels underlie the sI_{AHP}.

1.5.2 Pharmacological characteristics

The sI_{AHP} is insensitive to apamin at concentrations up to 100nM (Lancaster and Nicoll, 1987). The sI_{AHP} has also been reported to be relatively insensitive to the common K⁺ channel blockers tetraethylammonium (TEA; 1mM) and 4-aminopyridine (4AP; 1mM) (Lancaster and Nicoll, 1987; Storm, 1990).

As yet, no specific blocker of the sI_{AHP} channel has been identified. It is possible that BAPTA and some of its derivatives may be potential 'openers' of the channels underlying the sI_{AHP} (Lancaster and Batchelor, 2000). Low concentrations of BAPTA and its derivatives have also been shown to potentiate the sI_{AHP} (Schwindt et al, 1992; Zhang et al, 1995; Velumian and Carlen, 1999). However, the sI_{AHP} can be inhibited by neurotransmitters such as acetylcholine, noradrenaline,
dopamine and histamine and potentiated by adenosine (Storm, 1990). The effects of these neurotransmitters occur via the activation of second messenger systems and do not involve inhibition of Ca\(^{2+}\) entry via voltage-gated Ca\(^{2+}\) channels (Knopfel et al., 1990; Sah and Clements, 1999). It is clear that ligands that interact with receptors leading to activation of cyclic adenosine-monophosphate (cAMP) and protein kinase A (PKA) will inhibit the sI\(_{AHP}\) (Pedarzani and Storm, 1993, 1995; Pedarzani et al., 1998). Similarly, the sI\(_{AHP}\) can be enhanced by ligands such as adenosine (Haas and Greene, 1984) that produce their effect by inhibiting cAMP production via activation of the G\(_i\) protein (Gerber and Gahwiler, 1994). Phosphorylation of the channels underlying the sI\(_{AHP}\) by PKA has been suggested to occur (Pedarzani and Storm, 1995), though there is no direct evidence for this. There is some evidence that a balance between the basal activities of PKA and a protein phosphatase (protein phosphatase 1 or 2A) regulates the amplitude of the sI\(_{AHP}\) (Pedarzani et al., 1998). It is still not clear as to how muscarine exerts its effects except that the effect of muscarine does not involve protein kinase C (PKC; Sim et al., 1992; Krause and Pedarzani, 2000).

1.5.3 Does SK1 underlie the sAHP?

As SK1 mRNA injected in *Xenopus* oocytes can form channels that are relatively insensitive to apamin (Köhler et al., 1996; Ishii et al., 1997b; Grunnet et al., 1999), it is possible that SK1 channels underlie the sI\(_{AHP}\). The cloned SK1 channel, however, does not appear to be modulated by kinases or phosphatases (Xia et al., 1999). There are also no specific blockers of SK1 channels to test the possibility that they may underlie the sI\(_{AHP}\) (but see Chapter 6). SK1 knockout mice have also not yet been generated.
The sI_AHP also has very slow activation and deactivation rates. SK1 channels, on the other hand, are activated very rapidly following exposure to Ca^{2+} (Köhler et al., 1996; Xia et al., 1999). To meet this difficulty, Bond et al., (1999) have suggested that the kinetics of the sI_AHP may depend on the kinetics of the Ca^{2+} channels involved in the generation of the sI_AHP rather than the K^+ channels themselves. Various studies have investigated the time course of Ca^{2+} transients following action potentials in neurones and this will be discussed after a consideration of the Ca^{2+} channels in pyramidal cells.

1.5.4 Ca^{2+} channels in hippocampal neurones

Hippocampal pyramidal neurones have both the high voltage-activated (HVA; L-, N-, P-, Q-, and R- type) Ca^{2+} channels and the low voltage-activated (LVA; T-type) Ca^{2+} channels (Christie et al., 1996). Although, the overall Ca^{2+} channel density appears to be uniform throughout hippocampal pyramidal neurones (Magee et al., 1998), the relative proportions of the HVA Ca^{2+} channels varies on the surface of the pyramidal neurones. The L-type Ca^{2+} channel has been shown to be localized preferentially to the cell body and clustered at the base of major dendrites (Westenbroek et al., 1990). The N-, P- and Q-type Ca^{2+} channels are predominantly found on the dendrites (Westenbroek et al., 1992; Westenbroek et al., 1995), though currents from these channels can also be recorded from the soma of hippocampal pyramidal neurones (e.g. see Potier and Rovira, 1999).

The L-type Ca^{2+} channels are unique in showing 'delayed facilitation' following a long depolarizing step or a train of action potentials (Cloues et al., 1997). Under these circumstances, the open probability of single L-type Ca^{2+} channels increases with time after the step(s), reaching a peak approximately 600ms
later and decaying with a time constant of approximately 1.3s (Cloues et al., 1997).
Significantly, the voltage gating of the channels is altered, so that the channels are
open at potentials as negative as −50mV (Cloues et al., 1997). The kinetics of the
opening and closings of the L-type channels then mirror the kinetics of the sIAHP. If
Ca\(^{2+}\)-entry via L-type Ca\(^{2+}\) channels contributed exclusively to the generation of the
sIAHP, then the time course of the sIAHP might be explained by this delayed
facilitation process. However, studies of the source of Ca\(^{2+}\) required to activate the
sIAHP in hippocampal cells have provided conflicting results, with some studies
reporting that the sIAHP is only partially reduced by L-type Ca\(^{2+}\) channel inhibitors
(Rascol et al., 1991; Moyer et al., 1992) and others suggesting that the sIAHP is
generated entirely by the activation of L-type Ca\(^{2+}\) ion channels (Tanabe et al., 1998;
Borde et al., 2000). Hence, further work might usefully be done to determine the
source of Ca\(^{2+}\) for the generation of the sIAHP and whether the time-course of the
sIAHP can be explained by the kinetics of Ca\(^{2+}\) channels rather than the kinetics of the
K\(^+\) channels involved.

1.5.5 Possible reasons for the slow time course of the sIAHP

In addition to the suggestion that the time course of the sIAHP is due to
unusual L-type Ca\(^{2+}\) channel kinetics (Theory 3 below), several other hypotheses
have also been put forward to explain the slow activation and deactivation kinetics of
the sIAHP. These are discussed below:

1) The slow activation of the sIAHP may be due to a delay in the diffusion of Ca\(^{2+}\)
to a site distant from the influx of Ca\(^{2+}\) (Lancaster and Zucker, 1994; Zhang et
al., 1995). The slow deactivation of the sIAHP, in this model, is due to slow
removal of Ca\(^{2+}\) from the cells. This theory would depend on the location of
the channels underlying the sI\textsubscript{AHP}. It has been suggested that most of the sI\textsubscript{AHP} channels are localized to the basal dendrites (Bekkers, 2000). If these channels are in the dendrites and they are dependent upon a rise in Ca\textsuperscript{2+} in the soma for activation, then a Ca\textsuperscript{2+} diffusional delay may explain the slow activation of the sI\textsubscript{AHP}. However, upon depolarization of the cell, Ca\textsuperscript{2+} entry occurs in dendrites and [Ca\textsuperscript{2+}]\textsubscript{i} rises and falls quickly in all parts of the cell (see Fig 1.3; Lasser-Ross \textit{et al.}, 1997; Jahromi \textit{et al.}, 1999; Sah and Clements, 1999; Lancaster and Batchelor, 2000). Therefore, it is unlikely that the reason for the slow kinetics of the sI\textsubscript{AHP} is the time course of the Ca\textsuperscript{2+} transients.

2) The slow time course might alternatively be due to Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) which would introduce a delay in the rise in [Ca\textsubscript{i}] (see Sah, 1996). However, in hippocampal pyramidal neurones, it is not clear as to whether CICR contributes to the generation of the sI\textsubscript{AHP}. There is evidence both for (Torres \textit{et al.}, 1996; Tanabe \textit{et al.}, 1998; Borde \textit{et al.}, 2000) and against (Lancaster and Zucker, 1994; Zhang \textit{et al.}, 1995) a role for CICR. It should also be noted that CICR in hippocampal neurones occurs within milliseconds following action potentials (Sandler and Barbara, 1999).

3) The time course of the sI\textsubscript{AHP} is due to the time course of the delayed facilitation of L-type Ca\textsuperscript{2+} channels (Cloues \textit{et al.}, 1997; Marrion and Tavalin, 1998). It has been shown that L-type Ca\textsuperscript{2+} channels co-localize with SK channels in freshly dissociated hippocampal neurones (Marrion and Tavalin, 1998). Moreover, isoprenaline, a β-adrenoreceptor agonist inhibits the delayed facilitation mechanism (Cloues \textit{et al.}, 1997). It was, therefore, proposed that Ca\textsuperscript{2+} entry via L-type Ca\textsuperscript{2+} channels exclusively triggered the sAHP. However, as mentioned above, several studies have shown that application of L-type
Fig 1.3 Traces of the \( s_{AP} \) (A), evoked using 100ms voltage step from -50 to +10 mV, and the associated \( Ca^{2+} \) transients in the cell soma (B) and in the dendrite (66\( \mu \)m from the cell body, (C)) obtained in control Ringer's solution and after addition of 10\( \mu \)M isoprenaline are shown. Records obtained in the presence of isoprenaline are indicated with an asterisk (Adapted from Sah and Clements, 1999).
channel blockers do not abolish the sI\textsubscript{AHP} (Rascol \textit{et al.}, 1991; Moyer \textit{et al.}, 1992).

4) The channels underlying the sI\textsubscript{AHP} may not be directly gated by Ca\textsuperscript{2+} (Schwindt \textit{et al.}, 1992; Lasser-Ross \textit{et al.}, 1997). Although it has been established that the sI\textsubscript{AHP} is dependent upon Ca\textsuperscript{2+}, the relationship between activation of the channels underlying the sI\textsubscript{AHP} and [Ca\textsuperscript{2+}]\textsubscript{i} is still not certain. It should also be noted that overall changes in intracellular Ca\textsuperscript{2+} concentration do not parallel the time course of the sI\textsubscript{AHP} (see Fig 1.3; Lasser-Ross \textit{et al.}, 1997; Jahromi \textit{et al.}, 1999; Sah and Clements, 1999; Lancaster and Batchelor, 2000). Hence, it has been suggested a secondary synergistic factor activated by Ca\textsuperscript{2+} may control the time course of the sI\textsubscript{AHP}.

5) It has also been suggested that the channels underlying the sI\textsubscript{AHP} may themselves respond slowly to a rise in Ca\textsuperscript{2+} (Sah and Clements, 1999). This contradicts the idea that SK1 channels may underlie the sI\textsubscript{AHP}. Therefore, establishing the molecular correlate of the sI\textsubscript{AHP} is an essential requirement in understanding the reasons behind the slow kinetics of the sI\textsubscript{AHP}.

1.5.6 The physiological role of the sAHP

The sAHP has been suggested to have a role in learning and memory (Moyer \textit{et al.}, 1992; Disterhoft \textit{et al.}, 1996; Giese \textit{et al.}, 1998; Weiss \textit{et al.}, 2000). The amplitude of the sAHP has been shown to increase with age in animals and this has been correlated with a deficit in learning (Disterhoft \textit{et al.}, 1996). Indirect block of the sAHP by the Ca\textsuperscript{2+} channel inhibitor, nimodipine (Moyer \textit{et al.}, 1992) and the M1 muscarinic receptor agonist, CI-1017 (Weiss \textit{et al.}, 2000) enhance learning in ageing animals. Additional evidence for the role of the sAHP in learning and memory
comes from the Kvβ1.1 knockout mice. They have a smaller sAHP and have deficits in learning (Giese et al., 1998). However, specific blockers of the sAHP would clearly help in showing whether the sAHP is indeed important for learning and memory.

The sAHP may also have a role in epilepsy. Genetically prone epileptic rats have been shown to have a reduced sAHP in comparison to normal rats (Verma-Ahuja et al., 1995; 1998). In this case openers of the channels underlying the sAHP would be beneficial.

The sAHP is important for spike frequency adaptation and thereby prevents Ca\(^{2+}\) entry into cells. Massive increases in Ca\(^{2+}\) within the cell can lead to cell death. It has been shown that in scrapie-infected mice, the sAHP is absent and it may be one of the primary pathological mechanisms triggering neurodegeneration in scrapie and other related diseases (Johnston et al., 1998). Therefore, it is important to also understand the transcriptional regulation of the channels underlying the sAHP.

1.6 Aims of the project

As mentioned above, no blockers of the apamin-insensitive sAHP have yet been described. Also the channel underlying the sAHP has not yet been identified and knockout mice in which the sAHP is absent have not yet been generated. Hence, it has not been possible to determine the physiological role of the sAHP. On the other hand, a selective and potent blocker of the sAHP would not only be useful for \textit{in vivo} studies to determine the role of the sAHP, but could also be beneficial in the identification of the K\(^{+}\) channel underlying the sAHP. The major aim of this project was therefore to extend the pharmacology of the sAHP and to hopefully identify
most extensively studied and therefore, they would be the most appropriate cell type for the study of the pharmacology of the current.

Pharmacological studies using slices face some problems in that drug access to patched cells can be poor. Therefore, it is potentially better to do pharmacological studies using isolated cells. Previous studies, however, have reported difficulty in recording the sAHP in isolated cells (Alger et al., 1994; Sah, 1996). Therefore, the first aim was to attempt to develop a preparation that would allow the sAHP to be recorded from single isolated cells. The $I_{\text{AHP}}$ in these cells would have to be characterized to ensure that its properties are similar to those in slices and presumably to those in vivo. In view of the discrepant reports in the literature, it would also be of interest to revisit the question of the source of $Ca^{2+}$ for the $I_{\text{AHP}}$.

The laboratory, through Professor Ganellin (Department of Chemistry, University College London), has access to a number of compounds that are potent inhibitors of $K_{Ca}$ channels. A few of these key compounds were tested to see whether they would reduce the sAHP. If any of these compounds inhibited the $I_{\text{AHP}}$, others of similar structure would also be tested.

Generation of the sAHP is dependent upon $Ca^{2+}$ entry via voltage-gated $Ca^{2+}$ channels. It would therefore also be important to assess the block of $Ca^{2+}$ channels by any compound that might block the sAHP.

Finally, as SK1 channels have been hypothesised to underlie the sAHP (Vergara et al., 1998; Bond et al., 1999), it would be sensible to test any of compounds that appear to block the sAHP current on the SK1 current as well.
CHAPTER 2

METHODS

2.1 Studies using hippocampal cells

2.1.1 Cell culture methods:

A variety of different methods have been described for the isolation and culture of hippocampal pyramidal cells. Three different methods were tried before hippocampal pyramidal cells that were suitable for electrophysiological studies could be obtained. Each of the three methods that were attempted are described below.

2.1.1.1 Method 1:

The procedure followed was that described by Mynlieff (1997). A 4-day-old Sprague-Dawley rat was decapitated and both hippocampi were dissected into cold oxygenated Ringer solution of the following composition: NaCl 146mM; KCl 5mM; MgCl₂ 1mM; CaCl₂ 2mM; N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES free acid) 10mM; glucose 11mM; pH adjusted to 7.4 with NaOH. The tissue was cut into approximately 1mm³ pieces and rinsed in piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffered solution of the following composition: NaCl 120mM; KCl 5mM; CaCl₂ 1mM; MgCl₂ 1mM; glucose 25mM; PIPES 20mM; pH adjusted to 7.2 with NaOH. The tissue was then incubated at room temperature for 30min in continuously oxygenated PIPES buffered solution containing 0.5%w/v trypsin and 0.01%w/v DNase I followed by incubation for a further 60min at 35°C with gentle shaking. The tissue was then rinsed in Ringer solution containing 1mg/ml bovine serum albumin and then transferred to the growth medium.
medium supplemented with B27 serum free supplement, 0.02mg/ml gentamicin, 0.25mM L-glutamine). Trituration was carried out in the growth medium using fire-polished Pasteur pipettes to release individual cells and the cell suspension was centrifuged at 27g to remove cell debris. The cells were resuspended in the growth medium and plated onto Falcon plastic tissue culture dishes pre-coated with Poly-L-Lysine (Mol. wt. > 300,000) by exposure to 1mg/ml of the substrate for 4hr. The cells were then maintained in culture at 37°C in incubators continuously gassed with 95%O2/ 5%CO2.

2.1.1.2 Method 2:

The procedure followed was adapted from the method for hippocampal cell isolation described by Sodickson and Bean (1996). A 4-day-old Sprague-Dawley rat was decapitated and the whole brain removed and placed into cold (4°C) solution 1 (NaCl 126mM; KCl 3.5mM; NaH2PO4 1.2mM; MgCl2 1.3mM; CaCl2 2mM; glucose 11mM; NaHCO3 25mM). The brain was hemisected in the cold solution. Coronal slices (500μM thick) were obtained using a MacIlwain tissue chopper and collected in the cold isolation solution. The CA1 and CA3 regions were then dissected out and incubated in continuously gassed solution 1 at room temperature for 1hr. The CA1 and CA3 tissue was then transferred to and incubated for 1 hr at room temperature in solution 2 (Na2SO4 82mM; MgCl2 5mM; K2SO4 30mM; HEPES free acid 5mM; NaHCO3 1mM; glucose 10mM; pH adjusted to 7.2 with NaOH) containing 1mg/ml Pronase E. The tissue was rinsed in solution 2 and triturated with fire-polished sterilized Pasteur pipettes (1mm-0.2mm) to release individual pyramidal cells. The cells were centrifuged at 27g for 5min and resuspended in Neurobasal™ medium supplemented with B27 serum free supplement, 0.02mg/ml gentamicin, 0.25mM L-
glutamine, 10% heat-inactivated fetal calf serum (FCS) and 0.6mg/ml ethylene glycol-bis(b-aminoethyl ether)- N,N,N',N'-tetraacetic acid (EGTA). The amount of EGTA added to the Neurobasal™ medium was calculated to reduce the free Ca\(^{2+}\) concentration from 1.8mM to 1.37\(\mu\)M. The free Ca\(^{2+}\) concentration was calculated using the program ‘React’, written by G. L. Smith (Department of Physiology, University of Glasgow, U.K.). The cells were then plated onto 35mm tissue culture dishes (Nunc) coated with Poly-D-Lysine (PDL) (Mol.wt. > 300,000) at a concentration of 0.05mg/ml and placed in an incubator maintained at 37°C and continuously gassed with 95%\(\text{O}_2/\text{CO}_2\). After 2hrs in culture the cells were re-fed with Neurobasal™ medium supplemented with B27 serum free supplement, 0.02mg/ml gentamicin, 0.25mM L-glutamine and 10% FCS. The cells were then maintained in this culture medium. After 2 days in culture the medium was supplemented with the antimitotic Ara C at a concentration of 0.1mM for approximately 6 hr, to reduce glial cell growth.

2.1.1.3 Method 3:

4-day-old Sprague-Dawley rats were decapitated and the whole brain removed and placed in cold (4°C) Gey's Balanced Salt Solution (GBSS) supplemented with 0.6% w/v glucose and 8mM MgCl\(_2\) as described by Allen et al. (1993). The brain was hemisected and 500\(\mu\)m thick coronal slices were obtained using a MacIlwain tissue chopper. The CA1 and CA3 regions were dissected out. These regions were then incubated in Ca\(^{2+}\)- and Mg\(^{2+}\)-free Hanks Balanced Salt solutions (HBSS) containing 0.125%w/v trypsin (Sigma, T1005) and 1mM HEPES buffer (pH 7.3) at 37°C for 1hr. Following the 1hr enzyme treatment, the slices were repeatedly washed with Ca\(^{2+}\) and Mg\(^{2+}\)-free HBSS supplemented with 8mM MgCl\(_2\).
1 mg/ml bovine serum albumin and 10% FCS. Trituration was then carried out in this solution with 3 fire-polished sterilized Pasteur pipettes of decreasing bore diameters (1mm - 0.2mm) to release individual pyramidal cells. The cell suspension was centrifuged at 27g and the cells resuspended in Neurobasal™ medium supplemented with 2% B27 serum free supplement, 0.02mg/ml gentamicin, 0.25mM L-glutamine and 10% FCS. 200μl of the cell suspension was plated in 35mm tissue culture dishes (Nunc) coated with Poly-D-Lysine. The cells were then maintained in culture in incubators continuously gassed with 95%O₂/ 5%CO₂ at 37°C. After 24h in culture the cells were re-fed with the culture medium, but without FCS, and retained in this medium until use. As the cells were maintained in media not containing FCS, there was no need to treat the cells with the antimitotic Ara C. B27 serum free supplement also inhibits glial cell growth (Brewer et al., 1993).

2.1.2 Electrophysiological studies using cultured hippocampal pyramidal neurones

2.1.2.1 External solutions

The hippocampal pyramidal cell cultures were used after a period of 4 days if cultured using Method 2 or 8 days if cultured using Method 3. The cell cultures were superfused at a flow rate of 5ml min⁻¹ with an external solution of the following composition: NaCl 130mM; KCl 3mM; CaCl₂ 2.5mM; MgCl₂ 1.2mM; HEPES free acid 5mM; glucose 10mM; NaHCO₃ 26mM; pH maintained at 7.2 by continuously gassing with 95% O₂/ 5%CO₂. The final osmolarity of this external solution was 300mosmol l⁻¹. 5μM DNQX (6,7-dinitroquinoxaline-2,3-dione) was added to the external solution to reduce the spontaneous activity of the cells caused by release of glutamate. The solution flowed under gravity into the 35mm culture dishes via a stainless steel inflow tube. The tube was positioned such that the flow was directed
onto the patched cell. The level of the solution in the culture dish was maintained by another stainless tube connected to a suction pump. The solution was preheated by passage through a warming coil to give a temperature of approximately 28°C in the culture dish. Drugs were dissolved in the external solution and stored in separate reservoirs. Drugs were applied by switching to a superfusion fluid containing the drug using a multiway tap. The time taken for the new drug to reach the patched cell was between 2s and 3s.

2.1.2.2 Patch pipettes

Patch electrodes with resistances of 3-8MΩ were pulled from thin borosilicate glass containing an inner filament (i.d. 1.17mm; Clark Electromedical Instruments) using a vertical pipette puller (List Medical L-MP-30) and fire-polished using a microforge (Narashige, MF-9). In order to reduce the capacitance, the tips of the pipettes were coated with Sylgard (Dow Corning, USA). The tips of the electrodes were dipped into a filling solution (KMeSO₄ 126mM; KCl 14mM; HEPES 10mM; MgCl₂ 3mM; pH adjusted to 7.25 with 1M KOH; Zhang et al., 1994) and then backfilled with the same solution containing amphotericin B at a concentration of 0.12mg/ml. In some initial experiments, KMeSO₄ in the filling solution was replaced by 126mM potassium gluconate, 126mM KCl or 70mM K₂SO₄. Occasionally, the filling solution included Na₂ATP and Na₂GTP at concentrations of 2mM and 0.3mM respectively. The final osmolarity of the pipette filling solution was 280mosmol l⁻¹.

2.1.2.3 Equipment used for patch-clamp studies

The culture dishes were mounted onto the stage of a Nikon TMS inverted
microscope equipped with phase contrast optics. The microscope was placed on steel and concrete slabs supported on a Micro-g vibration isolation table (Technical Manufacturing Corporation) to minimize external vibrations. A Faraday cage was used to screen any electrical interference. A Narashige hydraulic micromanipulator system controlled the fine movement of the patch pipette.

With all the studies described below, either a List EPC-7 or an Axoclamp 2A (Axon Instruments) amplifier was used. When using the List EPC-7 amplifier, pulse protocols were generated using pClamp6 software (Axon Instruments). When using the Axoclamp 2A amplifier pulse protocols were generated either using the pClamp6 software or using a Master 8 pulse generator (Intracel). Voltage and current signals were filtered at 10kHz using the List EPC-7 low pass filter or at either 3kHz (for voltage signals) or 0.3kHz (for current signals) using the Axoclamp 2A low pass filter. The signals were amplified 5-fold (using an in-house amplifier) and digitized at 48kHz using a VR-10 digital data recorder (Instrutech Corporation). Signals were displayed on an oscilloscope (Gould 20MHz digital storage type 1424) and a computer monitor. Signals were recorded on a video recorder (Panasonic NV-L20 HQ) and on chart recorder paper using the Gould Easy Graf TA240 pen recorder. Data was also acquired and stored on a computer (Acer) using the Axon TL1 Labmaster digitizer and pClamp6 software.

The patch pipettes were held in perspex holders (Clark Electromedical Instruments). The perspex holders were directly attached to the headstage of the amplifier being used. The headstage being used was mounted on the micromanipulator.
2.1.2.4 General procedure for patch recordings

When using the List EPC-7 amplifier, the pipette and seal resistances were estimated using constant voltage pulses (usually -10mV and lasting 10ms) by the 'test-seal' program provided by pClamp6 software. With the Axoclamp 2A amplifier, the pipette resistance was estimated using the bridge balance setting on the Axoclamp 2A amplifier. The pipette was then lowered onto the cell and gentle suction was applied to obtain giga-ohm seals. The seal resistance was estimated by eye from the traces recorded on the oscilloscope in response to a constant current pulse. Perforated patches were obtained about 5-10min after the seal. Only cells that had membrane potentials greater than -50mV were used. When using the list EPC-7 amplifier, no series resistance compensation was carried out. The Axoclamp 2A amplifier was used in the discontinuous current-clamp (DCC) and discontinuous single electrode voltage-clamp (DSEVC) mode as appropriate. The sampling rate was usually between 2kHz and 6kHz. The design of experiments and pulse protocols used are described below.

2.1.2.5 Identification of hippocampal pyramidal neurones

Hippocampal pyramidal neurones were identified by their morphology. The soma of the neurones was pyramidal shaped with a width of 12-20μm and had one or more thick processes extending from it (see Fig 3.2B, Chapter 3). Pyramidal neurones also have the M current and the Q current (Halliwell and Adams, 1982). In initial experiments, these currents were recorded using conventional whole-cell voltage-clamp and the List EPC-7 patch amplifier. Cells with these currents and the appropriate morphology were positively identified as pyramidal cells. Cells with resting membrane potentials of -50mV or above, were held at -30mV and a 2s,
30mV hyperpolarising step was applied to measure the M current. To record the Q current, cells were held at -60mV and a 2s, 30mV hyperpolarising step was applied. Data was acquired at a sampling rate of 2.5kHz on the computer using pClamp6 software.

2.1.2.6 Recording of the sI_{AHP}

In a few early experiments, the sAHP, rather than the sI_{AHP}, was recorded under current clamp conditions using the List EPC-7 amplifier. 6-9 10ms current pulses were passed to elicit action potentials and so generate the sAHP. The command pulses for the List were generated using an isolated stimulator (Digitimer, DS2). The data was acquired at a sampling frequency of 2.5kHz on a computer using the Axon TL1 Labmaster interface and pClamp6 software and stored for later analysis.

It is, however, difficult to measure the true inhibition produced by a drug on an AHP. The magnitude of the AHP depends on the driving force of the K^+ current (E_M - E_K) and accordingly there is a non-linear relationship between the AHP and the K^+ conductance change. Therefore, the change in the AHP amplitude produced by application of drugs will not be directly proportional to the change in the K^+ conductance. Also, under current clamp conditions, changes in voltage can cause voltage-gated ion channels to open or close and, thereby, change the shape of the AHP recorded. It is preferable to record the current underlying the AHP (sI_{AHP}) at a fixed voltage which, in the absence of complicating factors, will increase linearly with K^+ conductance. To mimic the normal generation of the sI_{AHP} by action potential firing, the sI_{AHP} was recorded under hybrid clamp conditions using the Axoclamp 2A amplifier (Axon Instruments). A train of 13 action potentials was
evoked by passing 5ms current pulses (at a frequency of 76.5Hz) under discontinuous current clamp conditions and the cell was then voltage-clamped at approximately -50mV to record the \( sI_{AHP} \) (Axoclamp sampling rate 1.5-5kHz). The \( sI_{AHP} \) was evoked every 10s. \( sI_{AHP}s \) were also stored on a computer using pClamp6 for later analysis. The first 2s of the \( sI_{AHP} \) was acquired at a sampling frequency of 2kHz and the remainder at a sampling frequency of 0.5kHz. Action potentials were acquired separately at a sampling frequency of 20kHz.

2.1.2.7 Estimation of the reversal potential of the \( sI_{AHP} \)

The cells were voltage-clamped at -50mV using the DSEVC mode on the Axoclamp 2A amplifier. 1\( \mu \)M tetrodotoxin (TTX) was added to the external solution to prevent the activation of Na\(^+\) channels. The cells were depolarized from the holding potential of -50mV to nominally '+20mV' to evoke the \( sI_{AHP} \). To determine the reversal potential of the \( sI_{AHP} \), a ramp protocol was applied before applying the depolarizing step and then during the \( sI_{AHP} \). During the ramp, the cells were depolarized from -120mV to -40mV at a speed of 650mV s\(^{-1}\). The ramp protocol was generated using pClamp6. The \( sI_{AHP} \) and ramps were evoked every 10s. \( sI_{AHP}s \) and current evoked during the ramps were digitized at 2.0kHz using the Axon TLI Labmaster interface and pClamp6 software and stored on a computer for later analysis.

2.1.2.8 Recording of outward currents evoked using NMDA

When recording currents evoked as a result of the application of NMDA, the external solution was supplemented with glycine (1\( \mu \)M), TTX (1\( \mu \)M) and DNQX (5\( \mu \)M). Addition of glycine is required for the activation of NMDA receptors and
TTX and DNQX were added to block activation of Na\(^+\) channels and AMPA and kainate receptors respectively. Mg\(^{2+}\) ions block NMDA receptors in a voltage-dependent manner (Ozawa et al., 1998) and therefore, MgCl\(_2\) was removed from the external solution. Cells were voltage-clamped using the DSEVC mode on the Axoclamp 2A amplifier. 100\(\mu\)M or 1mM NMDA was pressure-applied (5psi) for 300ms every 30s onto cells using a puffer pipette (tip diameter 1-2\(\mu\)m). The pressure was applied via a solenoid-operated valve that was triggered using the Master8 stimulator. The puffer pipette was positioned less than 5\(\mu\)m from the patched cell. The signals evoked by application of NMDA were acquired at a sampling frequency of 667Hz on the computer with the aid of the Axon TL1 Labmaster digitizer and pClamp6 software.

In a number of experiments, \(I_{\text{AHPs}}\) were activated every 10s by applying a 60mV depolarising step from a holding potential of -50mV before pressure application of NMDA. The \(I_{\text{AHPs}}\) were acquired and stored as described above.

2.1.2.9 Recording of outward currents produced by caffeine

The cells were voltage-clamped at -50mV using the DSEVC mode on the Axoclamp 2A amplifier. 1\(\mu\)M TTX and 5\(\mu\)M DNQX were added to the external solution. 10mM caffeine was dissolved in the external solution and 10s bath applications of this solution were made every 5min. The data was acquired and stored on videocassettes and on a computer using the Axon TL1 Labmaster digitizer and pClamp6 for later analysis.
2.1.2.10 Data Analysis

2.1.2.10a Measurement of effects of drugs on the sI\text{AHP}

Data were analyzed using pClamp6 software. The average amplitude of 3 successive records of the sI\text{AHP} in the presence of the drug was expressed as a percentage of the amplitude of the average of 6 successive records before application of the drug. The effects of drugs on action potential duration were measured at a potential of -20mV. The effects of drugs on the time course of the I\text{AHP} were measured by fitting the I\text{AHP} to the following empirical equation in Microcal Origin 6.0:

\[ y = y_0 + A_1e^{-t/\tau_1} + A_2e^{-t/\tau_2} + A_3e^{-t/\tau_3} \]

The three exponentials adequately described the decay phase of the mI\text{AHP} and the rising and decay phases of the sI\text{AHP} (see Fig 2.1). On no occasion was it possible to discern a rising phase for the mI\text{AHP} following the action potential train. \( \tau_1, \tau_2 \) and \( \tau_3 \) are the decay time constant of the mI\text{AHP}, the growth time constant of the sI\text{AHP} and the decay time constant of the sI\text{AHP} respectively. \( t \) is the time from switching to voltage-clamp and \( y_0 \) represents the holding current at the voltage-clamp potential. Note that the value of \( A_2 \) in the above expression is negative. The value for \( A_1 \) was used to estimate the amplitude of the mI\text{AHP}. The time to peak of the sI\text{AHP} was estimated from the fitted curve by differentiation. Results are expressed as mean ± S.E.M. Statistical analysis was carried out using the Student’s t-test (paired or unpaired as appropriate). Concentration-inhibition curves were fitted with the Hill equation:

\[ y = y_{\text{max}} [I]^n / (IC_{50}^n + [I]^n) \]
Fig 2.1 To illustrate that the exponential decay equation described in section 2.1.2.10a fits well to the shape of the $I_{\text{AHP}}$ evoked using a train of 13 action potentials. The original trace is shown in black with the fitted line drawn in red. In this case $\tau_1$, $\tau_2$ and $\tau_3$ were 0.1s, 0.8s and 1.9s respectively. The corresponding values of $A_1$, $A_2$ and $A_3$ were 0.6nA, -0.49nA and 0.48nA respectively.
where \( y \) is the percentage inhibition, \([I]\) is the drug concentration, \( y_{max} \) is the maximum inhibition and \( n \) is the Hill coefficient. The IC\(_{50}\) value is the concentration of drug causing 50% inhibition.

2.1.2.10b Measurement of the reversal potential of the sI\(_{AHP}\)

The current obtained during the ramp in the presence of sI\(_{AHP}\) was subtracted from the current obtained during the ramp in the absence of the sI\(_{AHP}\). Microcal Origin 6.0 was used to do the subtraction. The difference current was assumed to be the current due to the sI\(_{AHP}\) and this was plotted against voltage.

2.1.2.10c Measurement of the currents evoked by application of NMDA

Data were analyzed using pClamp6. The peak amplitudes of the inward and outward currents produced by NMDA application in the presence of the drug were expressed as a percentage of the peak amplitudes of 2 successive records in the absence of the drug. The time course of the outward current was estimated by fitting the outward current alone to the following exponential equation in Microcal Origin 6.0:

\[
y = y_0 + A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}
\]

where \( \tau_1 \) and \( \tau_2 \) are the respective growth and decay time constants of the outward current. \( t \) represents the time from the termination of the inward NMDA current and \( y_0 \) is the holding current at a potential of -50mV. The fitted curve was differentiated to obtain an estimation of the time to peak.

Because of considerable overlap between the inward NMDA current and the outward current evoked due to activation of NMDA receptors (see Chapter 7), the
measurement of inhibitory effects on the outward current was not straightforward. In order to discern the shape of the current inhibited by the application of drugs, the current in the presence of drugs was subtracted from the current in the absence of drugs. The difference current was also fitted with the above equation. Again the fitted curve was differentiated to obtain an estimation of the time to peak.

2.1.2.10d Measurement of the outward current evoked by caffeine

The maximum amplitude of the outward current in the presence of the drug was expressed as a percentage of the mean amplitudes of the outward current in the absence and after washout of the drug.

2.1.3 Recording of Ca^{2+} currents

2.1.3.1 Cell preparation

Initially Ca^{2+} currents were evoked in hippocampal pyramidal cells that were cultured using Method 3 employing the ramp protocol described by Deak et al. (1998). However, from the current traces recorded (for example see Fig 2.2), it was clear that the voltage-clamp was inadequate. Due to the difficulty of adequately voltage-clamping cultured hippocampal pyramidal neurones, the effects of most drugs were studied on Ca^{2+} currents in freshly dissociated cells. The pyramidal cells were isolated and plated as described for Method 3. Recordings were made from cells between 3.5h and 8h after isolation.

2.1.3.2 Electrophysiological recordings

Pyramidal cells could again be identified by their morphology (see Fig 3.2A, Chapter 3). The Ca^{2+} current was produced using a protocol similar to that described
Fig 2.2 (A) The Ca\(^{2+}\) current recorded from cultured hippocampal pyramidal neurones in the absence and presence of the non-specific voltage-gated Ca\(^{2+}\) channel blocker, Cd\(^{2+}\) when the voltage protocol shown in (B) is applied. The dotted line shows the maximal recovery after washout of the Cd\(^{2+}\). As the cells were not voltage-clamped properly, an escape current was observed. The escape current could have been due to a propagating Ca\(^{2+}\) spike or a Ca\(^{2+}\)-activated current.
by Deak et al. (1998). Briefly, the cell cultures were superfused with a solution of
the following composition: NaCl 115mM; KCl 2mM; CaCl₂ 2mM; MgCl₂ 0.5mM;
glucose 11mM; HEPES 10mM; pH adjusted to 7.4 with NaOH. To block K⁺ currents
and Na⁺ currents, 25mM TEA and 0.3μM TTX were added to the external Krebs
solution and CsCl was incorporated in the pipette filling solution. The cells were
patch-clamped with a List EPC-7 patch amplifier using 7-10MΩ pipettes filled with
the following solution: CsCl 135mM; CaCl₂ 0.5mM; MgCl₂ 2mM; HEPES 10mM;
EGTA 3mM; Na₂ATP 2mM; pH adjusted to 7.3 with NaOH. The free Ca²⁺
concentration was calculated to be 30nM using REACT. The cells were voltage-
clamped at −80mV and then depolarized with a ramp from −100mV to +40mV, at a
speed of 1400mV s⁻¹, every 10s. The leak current was determined using Cd²⁺
(200μM) to block Ca²⁺ currents. Since the maximum voltage error was calculated to
be less than 5mV, series resistance compensation was not carried out. The signals
were filtered at a frequency of 1kHz (in-house 8 pole bessel filter) and acquired at a
sampling frequency of 3.33kHz using the Axon TL1 Labmaster digitizer and
pClamp6 software.

2.1.3.3 Data analysis

When analyzing the effects of drugs on the Ca²⁺ current, the average peak
current of the last 2 records in the presence of the drug (2min bath application) was
expressed as a percentage of the average peak current of 2 successive records
obtained both before application of the drug and after its washout.
2.2 Studies using Superior Cervical Ganglion (SCG) cells:

2.2.1 Cell culture methods

17-day old rat pups were killed using a rising concentration of CO\textsubscript{2} and then decapitated. Both SCG were dissected out into L-15 medium and subsequently desheathed in order to produce a 'clean' preparation. The SCG were cut into small pieces and then washed twice with HBSS supplemented with 10mM HEPES buffer. The SCG were then incubated for 15min at 37°C in HBSS containing 10mM HEPES buffer, 400iu/ml collagenase class 2 (Sigma, C9891) and 6mg/ml bovine serum albumin. The SCG were washed twice again with HBSS supplemented with 10mM HEPES buffer and re-incubated with this solution containing 1mg/ml trypsin (Sigma, T2271) and 6mg/ml bovine serum albumin for 30min. The ganglia were then triturated to release individual neurones using fire-polished Pasteur pipettes. The disaggregation procedure was carried out in L-15 growth medium supplemented with 10% FCS, 0.2mM L-glutamine, 0.6%w/v D-glucose, 0.19%w/v NaHCO\textsubscript{3}, 100u/ml penicillin, 100u/ml streptomycin and 50ng/ml nerve growth factor (mouse submaxillliary gland). The cell suspension was plated onto 35mm cell culture dishes (Nunc) coated with 10\mu g/ml laminin. The cells were then maintained in the L-15 growth medium at 37°C for up to 10days.

2.2.2 Electrophysiological studies

Cells that had been in culture for at least 4 days were used to record the current underlying the mAHP (mAHP). The cells were superfused with an external solution of an identical composition as that used for hippocampal pyramidal neurones. 1\mu M TTX was added to the solution to prevent activation of Na\textsuperscript{+} channels. Patch electrodes with resistances of 3-8M\Omega were pulled using the List vertical puller.
from thin borosilicate glass with an inner filament (i.d. 1.17mm) The tips of the patch electrodes were coated with Sylgard and fire-polished. Perforated patches were obtained with the internal pipette filling solution used for patching hippocampal pyramidal neurones. Recordings were made from cells that had membrane potentials greater than -50mV. The cells were voltage-clamped at -50mV using the List EPC-7 amplifier. As these cells are almost spherical in shape, up to 70% series resistance compensation could be carried out. I_{AHP} were generated by applying 50ms depolarizing steps from -50mV to +40mV were applied every 10s. The signals were filtered at a frequency of 1kHz (in-house 8 pole bessel filter) and were then acquired on computer at a sampling frequency of 3.33kHz with the aid of the Axon TL1 Labmaster digitizer and pClamp6 software. Signals were displayed on the oscilloscope and computer monitor Data was analyzed later using pClamp6 software.

2.2.2.1 Data analysis

The average of the amplitude of 3 successive records of the mI_{AHP} in the presence of the drug was expressed as a percentage of the amplitude of the average of 6 successive records before application of the drug.

2.3 Studies using the cloned rSK1 and hSK1 cDNAs

2.3.1 Maintenance of cell lines

2.3.1.1 HEK293 cells:

HEK293 cells were grown in 25ml Nunc sterile tissue culture flasks and maintained using Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 2mM L-glutamine, 50μg ml⁻¹ Penicillin-Streptomycin and 10% FCS. When the cells
were more than 70% confluent, the cells were lifted by first washing with HBSS and then adding 1ml of a commercially available trypsin/ethylenediaminetetraacetic acid (EDTA) solution in HBSS (Life Technologies, U.K.). The lifted cells were resuspended in the culture media and centrifuged for 5min at 27g. The pellet of cells formed was then resuspended in the culture media and re-plated at a lower density in sterile culture flasks.

2.3.1.2 Cos-7 cells:

The cos-7 cells were maintained in the same way as the HEK293 cells but were grown in culture media containing 10% new born calf serum (NBCS) instead of 10% FCS.

2.3.2 Modification of rSK1 cDNA

Dr. W. Joiner (Yale University, USA) kindly provided the rSK1 cDNA. However, when this cDNA was transiently transfected into HEK293 cells, no SK currents could be recorded (data presented in this thesis; W. Joiner, personal communication). Upon examination of the nucleotide sequence of rSK1 (shown in Fig 2.3), it was noticed that there was potentially a false start sequence (weak Kozak consensus site followed by a methionine) in a different frame upstream from the desired methionine start sequence (G.W.J. Moss, personal communication). One possible explanation for the lack of rSK1 current could be that this false start sequence caused an incorrect translation by the cell ribosomes and hence, prevented SK1 protein being made. Therefore, it was thought that removal of the methionine upstream and an improvement of the Kozak sequence in front of the correct methionine might aid the expression of rSK1.

61
Fig 2.3 The unmodified sequence of rSK1. The three frames as well as the cDNA sequence are shown. The specific EcoR1 restriction enzyme site (green), the possible false start sequence with the false methionine (blue) and the correct start sequence together with the correct methionine (purple) have all been highlighted.
2.3.2.1 Experimental design

At the 5' end, the incorrect initiation codon was preceded by the unique restriction site for the enzyme, EcoRI (Fig 2.3 and 2.4). Within the rSK1 sequence (3' to the correct initiation codon), there is a unique restriction site for the enzyme, BsrGI (Fig 2.4). To modify rSK1, the sequence between the two restriction sites could be removed by digestion with the respective restriction enzymes and replaced with a re-designed fragment. The oligos shown in Fig 2.4 were designed and used to perform a polymerase chain reaction (PCR) to generate a product that contained only the correct methionine, an EcoRI site and a perfect Kozak consensus sequence prior to this methionine at the 5'end of the sequence. The PCR product would then be ligated into the cut rSK1 sequence to create the full-length rSK1 cDNA without the incorrect methionine.

2.3.2.2 Step 1: Digestion reaction

The restriction enzymes EcoRI (12u/µl final concentration; Promega, U.K.) and BsrGI (10u/µl final concentration; New England Bio Labs, U.K.) were mixed together with 0.89µg rSK1 cDNA in a 1X EcoRI buffer (Buffer H; Promega, U.K.) solution. This mixture was incubated at 37°C for 1.5hr. The sample was run on a 1% low melting point (LMP) agarose gel (Life Technologies, U.K.) for approximately 30min along with cDNA markers (100base-pair ladder; Promega, U.K.). This resulted in the separation of a 380base-pair band (the 5' end of rSK1) from a high molecular weight band (the rest of rSK1 and the plasmid). The high molecular weight band was excised from the gel and recovered by purification using the Geneclean III kit (Anachem., U.K.).
Fig 2.4 The primers (oligos) that were designed for the PCR reaction to generate a product in which the incorrect initiation sequence (Fig 2.1) would be eliminated. The EcoR1 site, the BsrG1 site and the Kozak consensus sequence prior to the correct methionine have been highlighted.
2.3.2.3 Step 2: PCR reaction

A 50μl PCR reaction was set up containing 0.89μg rSK1 cDNA and final concentrations of the 2 oligos (Fig 2.4; 1μM), de-oxynucleotide triphosphates (dNTP; 2.5mM), pfu polymerase (2.5u/μl) in pfu reaction buffer (1X; Promega, U.K.). The reaction mixture was heated to a temperature of 95°C and then maintained at this temperature for 30s to separate the DNA strands. It was then cooled to a temperature of 55°C for oligo annealing and maintained at this temperature for 30s. The reaction mixture was finally heated to 70°C for polymerase extension and maintained at this temperature for 1min. The above cycle of events were repeated 30 times.

The PCR product was run on a 2%w/v LMP agarose gel. A band of approximately 350base-pairs was observed and this band was excised and purified using the Geneclean III kit.

2.3.2.4 Step 3: A-tailing reaction and sub-cloning into the pGEM-T-Easy vector

The oligos were designed such that the PCR product would contain restriction sites for EcoRI and BsrGI at the ends (Fig 2.4). Therefore, following digestion with these enzymes, the PCR product can be ligated with the digested rSK1 sequence (obtained from Step 1). However, as the enzyme restriction sites are very close to the ends of the PCR product, it may result in inadequate digestion of the product. To avoid this, the PCR product was sub-cloned into the vector pGEM-T-Easy and then digested. The pGEM-T-Easy vector has an overhanging thymidine (T) base at each end. Therefore, addition of an overhanging adenosine (A) base (A tailing) onto the PCR product would make it suitable for sub-cloning into the pGEM-T-Easy vector.
The A tailing reaction was carried out using Taq polymerase. To the purified PCR product, 0.6mM dATP and 0.1μl of Taq polymerase (Promega, U.K.) were added. The mixture was incubated at 70°C for 15min and then placed on ice.

The mixture was then diluted 1:1 with the Ligase buffer (Promega, U.K.). To this mixture, 5ng/μl pGEM-T-Easy vector and 10μl T4 DNA ligase enzyme (Promega, U.K.) were added. The mixture was incubated for 48hr at 16°C.

2.3.2.5 Step 4: Identification of the pGEM-T-Easy vector containing the insert

JM109 cells (Promega, U.K.) were transformed with the ligation product as follows: 30% of the ligation mixture was added to 50μl JM109 competent cells and incubated on ice for 30min. The cells were heatshocked for 45s at 42°C and then returned to ice for 2min before 1ml LB media was added and the cells incubated for 1hr with shaking at 37°C. The cells were centrifuged at 12000g for 30s and resuspended in 0.1ml LB media. This mixture was then spread onto LB agar plates that were coated with 4μl of 20%w/v IPTG and 20μl of 50mg/ml XGal to allow blue/white screening. The plates were incubated for 24hr at 37°C.

This above procedure resulted in a mixture of blue and white bacterial colonies. The white colonies would be those that contained the pGEM-T-Easy vector with the insert. Several white colonies were selected and grown in LB media containing 50μg/ml ampicillin. Plasmids were harvested using the alkaline lysis 'miniprep' method (as described by Sambrook, Fritsch and Maniatis, 1989). The pGEM-T-Easy vector containing the insert was sequenced using the BigDye Terminator Cycle sequencing kit (PE Applied Biosystems, UK) run on a ABI377 fluorescent sequencer. The sequence of the insert (Fig 2.5) no longer contained the false initiation codon (see Fig 2.3) that was present in the original sequence. The
Fig 2.5 The first 270 base pairs of the modified rSK1. The three frames together with nucleotide base sequence are shown. The specific EcoR1 site (green) and the correct kozak consensus sequence together with the methionine (blue) have been highlighted.
Fig 2.6 Gel electrophoresis of restriction enzyme products of the modified and unmodified rSK1. Both plasmids have been digested with the specific restriction enzymes, EcoRI and BsrGI.
vector was then digested using the restriction enzymes EcoRI and BsrGI as described in Step 1 above to release the insert. The digestion mixture was run on a 2% w/v LMP agarose gel. This allowed the separation of a band of approximately 350 base-pairs long. The band was excised and purified using the Geneclean III kit as described above.

The insert was then ligated into the cut rSK1 sequence (from Step 1) using a similar ligation reaction to that described above. Once again the JM109 cells were transformed with the ligation product (as described above). The cells were plated onto agar plates containing 50μg/μl ampicillin. Several colonies were selected and these were then grown in LB media containing 50μg/μl ampicillin. The rSK1 plasmids were harvested from the cells using the alkaline lysis ‘miniprep’ method together with the phenol-chloroform extraction procedure. The modified rSK1 cDNA together with the original rSK1 cDNA were digested with EcoRI and BsrGI as described in Step 1. The bands produced with the modified rSK1 were of a lower molecular weight than those produced by digestion of the original rSK1 sequence (Fig 2.6), suggesting that the ligation was successful.

2.3.3 The calcium phosphate transfection method

6μg of DNA was added to 250mM CaCl₂ to obtain a total volume of 75μl. An equal volume of 2X HEPES buffered saline (HBS) solution (280mM NaCl, 50mM HEPES, 2.8mM Na₂HPO₄, pH adjusted to 7.2 using 1M NaOH) was added dropwise to the CaCl₂ solution. After 20min incubation at room temperature, half of this solution was added dropwise to a dish of cells. The cells were then incubated for 3hr at 37°C. The medium was then removed from each dish and replaced with 15% glycerol in HBSS and left for 1min at room temperature. The cells were then washed
once with HBSS before addition of the growth medium. Cells were transfected with either rSK1 (both modified and unmodified constructs) or hSK1 (a generous gift from Dr. J. P. Adelman, Vollum Institute, USA) and CD8 DNAs. As a matter of routine, the pore region of hSK1 was re-sequenced using the BigDye Terminator Cycle sequencing kit (PE Applied Biosystems, UK) run on a ABI377 DNA sequencer. The sequence was identical to that of hSK1 deposited in Genbank (Accession no. HSU69883).

A few experiments were performed with rSK2 transfected stably in HEK293 cells (a gift from Dr. W. Joiner, Yale University, USA).

2.3.3.1 Identification of transiently transfected cells

HEK293 cells were used a minimum of 24hrs after transfection whereas cos-7 cells were used at least 48hrs after transfection since expression was slower in these cells. Transiently transfected cells were identified by adding CD8 antibody coated microspheres (Dynabeads M-450 CD8, Dynal, U.K.) to the cells 30min before use as described by Jurman et al. (1994). Briefly the beads were diluted 1:50 in water and vortexed. The suspension of beads was then placed on a magnetic stand (Dynal, U.K.) to draw the beads to the bottom of the container. The water was removed and the beads resuspended in one-fifth of the volume of water. 100μls of suspension was added to each dish of cells.

2.3.4 Electrophysiological studies with transfected cells

Cells were superfused in the culture dishes at 5ml min⁻¹ with the following solution: 150mM NaCl, 5mM KCl, 1mM MgCl₂, 2mM CaCl₂, 10mM glucose, 10mM HEPES, pH adjusted to 7.4 with 1M NaOH. Drugs were applied by switching
to bathing fluid containing the drug using a multiway tap. The inlet tube was positioned such that the flow was directed onto the patched cell. Whole cell recordings from isolated cells were made using glass micropipettes (Clark Electromedical Instruments; GC15OTF-15) that were fire-polished and coated with Sylgard. They had resistances of 3-5MΩ when filled with the following solution: 130mM KCl, 5mM HEDTA, 10mM HEPES, 2mM Na₂ATP, 3mM MgCl₂, 0.67mM CaCl₂; pH adjusted to 7.2 using 1M KOH. The Ca²⁺ concentration was calculated to give 1μM free Ca²⁺ using the program 'REACT' (G. L. Smith, Dept. of Physiology, University of Glasgow, UK). Cells were voltage-clamped at -80mV using a List EPC-7 amplifier. 100ms voltage steps from -140mV to +40mV in 20mV increments (see Fig 6.1A, Chapter 6) were applied every minute. Signals were filtered at 3kHz and stored on a computer using pClamp6 (sampling frequency of 5kHz, Axon Instruments) for further analysis.

2.3.4.1 Data analysis

Data were analyzed using pClamp6 software. To minimize series resistance errors and the contribution of a significant amount of delayed rectifier current, the amplitude of the SK current was measured at a potential of -40mV. The amplitude of the SK current in the presence of the drug was expressed as a percentage of the average amplitude before and after recovery from the drug. Results are expressed as mean ± S.E.M. The concentration-inhibition curves were fitted with the Hill equation:

\[ y = y_{\text{max}} [I]^n / (IC_{50}^n + [I]^n) \]
where \( y \) is the percentage inhibition, \([I]\) is the drug concentration, \( y_{\text{max}} \) is the maximum inhibition and \( n \) is the Hill coefficient.

### 2.4 Materials

#### 2.4.1 Materials for tissue culture methods

With the exception of bovine serum albumin, all tissue culture reagents including the culture media were obtained from Life Technologies, U.K. Bovine serum albumin was purchased from ICN Biomedicals Inc., U.K. The chemicals used for the solutions in hippocampal culture Methods 1 and 2 (Sections 2.1.1.1 and 2.1.1.2) were obtained from Sigma, U.K.

#### 2.4.2 Materials used for electrophysiological studies

All chemicals were obtained from Sigma, U.K. apart from tetrodotoxin, N-methyl-D-aspartate and glycine, which were purchased from Tocris, U.K. Charybdotoxin was obtained from Peptide Institute, Inc. The UCL compounds were synthesized in Professor Ganellin’s laboratory at the Department of Chemistry, UCL.

#### 2.4.3 Materials used for molecular biology studies and transfection purposes

All reagents were purchased from Promega, U.K. apart from the gel materials and the Geneclean III kit which were obtained from Life Technologies, U.K. and Anachem., U.K. respectively. The reagents used for transfection purposes were purchased from Sigma, U.K. The Dynabeads (CD8 antibody coated microspheres) were obtained from Dynal, U.K.
CHAPTER 3

CHARACTERIZATION OF THE $sI_{AHP}$ IN CULTURED
HIPPOCAMPAL PYRAMIDAL NEURONES

3.1 Introduction

In hippocampal pyramidal neurones, a train of action potentials is followed by
an AHP with fast (fAHP), medium (mAHP) and slow components (sAHP; for
reviews see Storm, 1990; Sah, 1996). Whereas it has been possible to record the fast
and medium AHPs in isolated cells, the sAHP has proven difficult to reproduce using
whole cell voltage-clamp conditions (Alger et al., 1994). Most studies of the sAHP
have, therefore, employed brain slices in which it is readily recorded. In general, drug
access is improved when using isolated cells and, accordingly there is some advantage
in doing pharmacological studies with isolated cells. The first part of the project,
therefore, involved establishing a method suitable for isolating hippocampal pyramidal
cells from postnatal rats and maintaining them in culture. Various cell isolation and
culture conditions, patch electrode filling solutions as well as whole cell and
perforated patch conditions have been explored to find conditions under which a $sI_{AHP}$
can be recorded.

Hippocampal pyramidal neurones have a number of K⁺ conductances
including the M current ($I_m$, Halliwell and Adams, 1982; Storm, 1990). They also
possess the cation-mediated hyperpolarizing current, $I_Q$ (Halliwell and Adams, 1982).
In initial experiments, the identification of neurones as pyramidal was, in addition to
their morphology and the presence of the sAHP, substantiated by confirming the presence of $I_M$ and $I_Q$.

In this chapter, results will be presented which show that the sAHP can be recorded from pyramidal cells in culture. As literature contains no records of the properties of the sAHP in isolated hippocampal neurones, it was necessary to characterize the sAHP recorded from these cells and to ensure that it has similar characteristics to the sAHP recorded from brain slices. Experiments were also carried out to determine whether the pharmacology of the sI_{AHP} might resemble the pharmacology of the known cloned K_{Ca} channels.

3.2 Results

3.2.1 Properties of cells isolated using different methods

Hippocampal pyramidal cells were first isolated using Method 1 (see Chapter 2, Section 2.1.1.1). The dissociated cells initially appeared to be phase bright and did stick to the plastic dishes coated with poly-L-lysine. The freshly dissociated cells, however, were mostly round and free of processes and, therefore, could not be identified morphologically. Hence, these cells were left in culture to allow the cells to develop structurally. Unfortunately, the cells did not survive for more than 3 days. At this stage of development, the pyramidal cells were unidentifiable morphologically. The cells that were patched onto had resting membrane potentials of approximately -40mV and did not exhibit either M-like or Q currents.

Method 2 yielded cells that were initially structurally identifiable and were phase-bright. The advantage of using this method was that recordings could be made from the freshly dissociated cells as they could be identified morphologically. The
freshly dissociated cells had membrane potentials of at least $-50\text{mV}$ but no sAHP could be recorded. Also, the cells did not adhere strongly to the poly-D-lysine coated dishes and this made it difficult to patch onto them. Changing the type of plastic dishes did not make the cells adhere any better nor did changing the concentration of the poly-D-lysine used. Cells that did adhere to the plastic dishes remained healthy for a period of 5 days and maintained their morphology over this period. After 3 days in culture, M-like and Q currents could be recorded as shown in Fig 3.1A and 3.1C. Using perforated patches with KMeSO$_4$ as the major salt in the pipette filling solution, the sAHP could also be detected in these cells under current clamp conditions (Fig 3.1E). The sAHP lasted for several seconds and was also insensitive to 30nM apamin (data not shown). However, due to the difficulty of maintaining the cells for a long period of time in culture, Method 3 was tried.

With Method 3, the cell yield was good and the cells adhered well to the plastic dishes. The cells remained healthy for a period of over 2 weeks. The cells retained their basic morphology after dissociation (Fig 3.2A). The sAHP could be recorded from these cells within a few hours (data not shown) but not on a regular basis. The sAHP could be recorded more stably and robustly only after the cells had been in culture for more than 7 days (Fig 3.1F). By this time, the soma had a width of 12-20$\mu\text{m}$ and an extensive dendritic tree also emerged from it (Fig 3.2B). Glial cell growth was minimal due to the use of the B27 serum free supplement (Brewer et al., 1993). After 7 days in culture, M-like and Q currents could be recorded as well (Fig 3.1B and 3.1D). Hence, recordings of the sAHP were routinely obtained from cells that had been cultured for at least 8 days using Method 3.
Fig 3.1 The M-like current, Q current and the sAHP could all be detected in cells cultured using either Method 2 (A, C and E respectively) or Method 3 (B, D and F respectively). To detect the M- (panels A and B) and the Q current (panels C and D), the cells were voltage-clamped at -30mV and -60mV respectively and a 30mV, 2s long hyperpolarising step applied in each case. To detect the sAHP (panels E and F), a train of 13 action potentials were evoked under current clamp conditions. The time scale shown in A also applies to B, C and D.
Fig 3.2 The morphology of a dissociated hippocampal pyramidal cell approximately 3h after isolation (A) and after 8 days in culture (B). Both cells can be identified by as pyramidal from their morphology. sIAHPs were routinely recorded from cells such as the one shown in (B). Horizontal bar on each picture represents 20μm.
3.2.2 Comparison of sAHPs recorded using different conditions

Initial experiments involved recording the sAHP under current-clamp conditions. Under these conditions, the sAHP is recorded as a change in membrane potential (Fig 3.1F). Changes in membrane potential can lead to the activation or inactivation of other voltage-dependent ion channels and these may affect the shape of the sAHP. The amplitude of the sAHP under current-clamp conditions is also dependent on the difference between the resting membrane potential of the cell and the reversal potential for K⁺, E_K. The relationship between current flow through the open K⁺ channels and voltage-change is also non-linear. Hence, effects of drugs on the AHP would not be proportional to changes in the K⁺ conductance.

Application of a voltage step from a holding potential of -50mV to a potential of +20mV under DSEVC conditions could evoke the sI_AHP. However, under physiological conditions the channels underlying the sI_AHP are activated by Ca²⁺ influx that occurs during the firing of action potentials. It was therefore decided to use a hybrid current/voltage-clamp protocol to evoke the sI_AHP. The sI_AHP recorded using the hybrid-clamp protocol in these cells (Fig 3.3) had similar kinetics to the sAHP recorded under current clamp conditions (Fig 3.1F). The hybrid-clamp protocol involved evoking a train of action potentials using the discontinuous current-clamp mode and then automatically switching to DSEVC conditions to record the sI_AHP.

To obtain stable recordings of the sI_AHP for up to 60min, perforated patches had to be employed. Under whole cell conditions, even when Na₂ATP and Na₂GTP were included in the filling solution, a rapid rundown of the sI_AHP occurred making it much more difficult to characterize and study the current. The sI_AHP could be most reliably recorded when KMeSO₄ was present as the major salt in the filling solution. With potassium gluconate or K₂SO₄ as the major salt in the filling solution, it was
Fig 3.3 Three different types of $I_{AHP}$ can be detected from cells that have the morphology of a typical pyramidal cell. The typical shape of the $sI_{AHP}$ is shown in (A). The $sI_{AHP}$ has a distinctive growth phase, peaks approximately 600ms after the action potentials and can last up to 8s. In some cells, a $mI_{AHP}$ can also be detected together with the $sI_{AHP}$ (B). 30% of the cells have no $sI_{AHP}$ but only a $mI_{AHP}$ as shown in (C).
more difficult to evoke the $I_{AHP}$, especially under whole cell conditions.

3.2.3 General characterization of the $I_{AHP}$

The shape of $I_{AHP}$ recorded using the hybrid-clamp protocol varied (Fig 3.3). In some cells, the $I_{AHP}$ was dominant (Fig 3.3A) whereas in others (40%) both the $I_{AHP}$ and the $mI_{AHP}$ could be clearly identified (Fig 3.3B). Approximately 30% of cells had just the $mI_{AHP}$ (Fig 3.3C). These cells, however, could not be distinguished on the basis of their morphology. Approximately 55% of the cells displayed a $I_{AHP}$ that had an amplitude in excess of 20pA. The effect of drugs on the $I_{AHP}$ was investigated in cells exhibiting a $I_{AHP}$ in excess of 50pA.

The amplitude of the $I_{AHP}$ was dependent upon the holding potential of the cell. As shown in Fig 3.4A, the amplitude of the $I_{AHP}$ increased at more positive potentials as expected for a selective change in $K^+$ permeability. To record the $I_{AHP}$, cells were routinely held at -50mV. The amplitude of the $I_{AHP}$ also increased with the number of action potentials (Fig 3.4B and 3.4C) as has been reported in slices (Lancaster and Adams, 1986). The amplitude of the $I_{AHP}$ was maximal with a train of 13 or more action potentials (Fig 3.4C). Based on this observation, a train of 13 action potentials was used to generate the $I_{AHP}$ in subsequent experiments. The time to peak and the decay time constant of the $I_{AHP}$ did not change with the action potential number.

Using the recording conditions described above, the mean amplitude of the $I_{AHP}$ (for currents in excess of 20pA) was $173 \pm 15$pA at 28°C (n=78). The $I_{AHP}$ peaked approximately 600ms after the last action potential and had a decay time constant of $1.30 \pm 0.06s$ (n=32), when fitted with the exponential equation described in METHODS (Section 2.1.2.10a). This value is similar to that reported for the $I_{AHP}$.
Fig 3.4 (A) The sI_{AHP} recorded using the hybrid-clamp protocol at (i) -65mV and (ii) -50mV. The extra holding current at -50mV has been offset in order to superimpose the traces. (B) sI_{AHP}s activated using (i) 12, (ii) 10, (iii) 8 and (iv) 6 action potentials. (C) The dependence of the sI_{AHP} on the number of action potentials in a train. The amplitude of the sI_{AHP} has been expressed as a percentage of the amplitude following 13 action potentials. The values plotted are the means of the number of observations shown in brackets. Where n > 2, the standard error is also shown.
(1.5s at 30°C) in hippocampal slices (Storm, 1990).

The amplitude or the time to peak of the mI_AHP could generally not be determined directly in these experiments as often the rising phase of the mI_AHP occurred during the action potential train (Fig 3.3). The amplitude and the decay time constant (τ₁) of the mI_AHP could be estimated by fitting the recorded trace with the exponential equation described in METHODS (Section 2.1.2.10a). On average, τ₁ was 45ms, which is comparable to the values reported in the literature (Storm, 1987; Alger et al., 1994).

3.2.4 Channels underlying the sI_AHP

3.2.4.1 Reversal potential of the sI_AHP

To measure the reversal potential of the sI_AHP, the sI_AHP was evoked in the presence of 1μM tetrodotoxin (TTX) by applying a 70mV, 170ms depolarising step from a holding potential of -50mV under DSEVC conditions using the Axoclamp 2A amplifier. A ramp (from -120mV to -40mV) was applied before and after the depolarising step as shown in Fig 3.5A. Above potentials of -40mV, opening of Ca²⁺ channels and the voltage-gated K⁺ channels would occur and these would distort the current obtained during the ramp. Hence, the I-V curve for the sI_AHP current was plotted for potentials from -120mV to -40mV (Fig 3.5). This was adequate to determine the reversal potential of the sI_AHP which was -90.5 ± 5.6mV (n=4; Fig 3.5B). The junction potential was not corrected for. E_K was calculated to be -98.7mV and this suggests that it is likely that K⁺ channels underlie the sI_AHP. The I-V plot of the sI_AHP showed outward rectification as would be expected from the Goldman Hodgkin Katz equation for
Fig 3.5 (A) An example of the protocol used to measure the reversal potential of the $I_{\text{AHP}}$. Ramps (from -120mV - -40mV) were applied before and after the depolarising step. The increased outward current after the ramps may represent $I_{\text{AHP}}$ current activated by Ca$^{2+}$ entry through voltage-gated Ca$^{2+}$ channels opened during the ramp. (B) The difference in currents observed in the 2 ramps shown in (A), was plotted against voltage. The current-voltage relationship (I-V curve) shown for this particular cell has a reversal potential of -88.8mV and is outwardly rectifying.
current produced from non voltage-gated ion channels.

3.2.4.2 Ba$^{2+}$ sensitivity

Ba$^{2+}$ is a non-selective inhibitor of K$^+$ channels (Hille, 1992). More importantly, Ba$^{2+}$ ions do not inhibit Ca$^{2+}$ channels, Na$^+$ channels, Cl$^-$ channels or other non-selective ion channels (Hille, 1992). Ba$^{2+}$ can, therefore, be used to distinguish K$^+$ channels from the other ion channels. A 2min bath application of 1mM Ba$^{2+}$ (barium chloride) abolished the $s_{\text{AHP}}$ (% inhibition = 95 ± 6.1%; n=3; Fig 3.6A), also indicating that K$^+$ channels very likely underlie the $s_{\text{AHP}}$. The effect of Ba$^{2+}$ was rapidly reversible, often within 2min of washout.

A number of K$^+$ channels are activated during the repolarization phase of action potentials and therefore, not surprisingly, the width of the action potentials was also increased significantly (% increase = 114.3 ± 26%; n=3; p < 0.01) in the presence of Ba$^{2+}$.

At a holding potential of -50mV, a variable outward current can also be detected (the exact magnitude depending on how much more positive this potential is than the resting membrane potential of the cell). Ba$^{2+}$ caused a significant reduction of this holding current (% inhibition = 72 ± 24%). In agreement with this, current clamp traces showed that 1mM Ba$^{2+}$ reduced the resting membrane potential from -62.3 ± 0.3mV to -55.7 ± 0.7mV (n=3; p < 0.01). It has been suggested that two-pore K$^+$ channels may be partially responsible for maintaining the membrane potential in a number of central neurones (for example see Duprat et al., 1997; Millar et al., 2000; Telley et al., 2000). It is possible that Ba$^{2+}$ would have blocked these K$^+$ channels (for example see Millar et al., 2000).
Fig 3.6 Effects of 2min bath applications of (A) 1mM Ba\(^{2+}\) and (B) 200\(\mu\)M Cd\(^{2+}\). The traces in the presence and absence of the ions have been superimposed. Calibration bars shown in B also apply to A.
3.2.4.3 Dependence of the sI_{AHP} on Ca\(^{2+}\)

Activation of the sI_{AHP} by action potentials follows the opening of voltage-gated Ca\(^{2+}\) channels which results in Ca\(^{2+}\) influx into the cells. A simple test of the importance of voltage-gated Ca\(^{2+}\) entry was provided by a 2min bath application of a non-selective inhibitor of voltage-gated Ca\(^{2+}\) channels, Cd\(^{2+}\) (cadmium chloride 200μM). This markedly reduced the sI_{AHP} (% inhibition = 87.3 ± 1.9%; n=4; Fig 3.6B). This provides support for the notion that the generation of the sI_{AHP} is dependent upon Ca\(^{2+}\) entry via voltage-gated Ca\(^{2+}\) channels.

In the presence of Cd\(^{2+}\), the action potential width significantly increased by 25.0 ± 4.8% (n=4; p < 0.05). This is most easily explained as a reduction in BK channel opening since in hippocampal pyramidal neurones, BK channels have been shown to be activated during the repolarization phase of the action potential (Lancaster and Adams, 1986; Yoshida et al., 1991).

In cells that had both a mI_{AHP} and a sI_{AHP}, 200μM Cd\(^{2+}\) abolished the mI_{AHP} (n=3; data not shown) as well as the sI_{AHP} indicating that the generation of mI_{AHP} is also dependent upon Ca\(^{2+}\) entry.

3.2.5 Pharmacological characterization of the sI_{AHP}

3.2.5.1 Effects of apamin and TEA

The sI_{AHP} was observed to be insensitive to 100nM apamin (inhibition = -8.9 ± 7.6%, n=6; Fig 3.7A) and 1mM TEA (2.0 ± 6.4%, n=6; Fig 3.7B), as previously reported (Lancaster and Adams, 1986; Lancaster and Nicoll, 1987). Although TEA appeared to have no effect on the sI_{AHP}, there was a widening of the action potentials, as previously reported (Lancaster and Adams, 1986). The action potential width

86
Fig 3.7 Effects on the $s\text{I}_{\text{AHP}}$ of 2 min bath applications of (A) 100nM apamin, (B) 1mM TEA, (C) 100μM tubocurarine, (D) 100nM UCL 1848 and (E) 100nM charybdotoxin. All control traces were recorded just before the drugs were applied and are shown superimposed on the traces in the presence of the drug just before washout. Calibration bars shown in (B) apply for (A) - (E). (F) shows the effects of 100nM charybdotoxin on the last action potential from the train of action potentials in the same cell as in (E). The last action potential in the control train is indicated by the dotted line.
increased from 1.3 ± 0.2 ms to 2.0 ± 0.5 (p = 0.057) in the presence of TEA. Apamin did not have any affect on the action potentials (p > 0.05). In cells that had both a \( m_{\text{AHP}} \) and a \( s_{\text{AHP}} \), 2 min bath applications of 100 nM apamin (Fig 3.7A) completely inhibited the \( m_{\text{AHP}} \) suggesting that apamin-sensitive SK channels mediate the \( m_{\text{AHP}} \) in these cultured neurons (see also Stocker et al., 1999). This effect of apamin was irreversible at least for 15 min after washout.

3.2.5.2 Effects of alternative blockers of the apamin-sensitive AHP on the \( s_{\text{AHP}} \).

Apamin-sensitive SK channels are sensitive to the plant alkaloid, d-tubocurarine (Cook and Haylett, 1985; Köhler et al., 1996) and AHPs in various neurones can also be blocked by tubocurarine (IC_{50} < 100 \mu M; Dun et al., 1986; Bourque and Brown, 1987). In chromaffin cells, which also express apamin-sensitive SK channels, the IC_{50} for tubocurarine is 20 \mu M (Park, 1994). In cultured hippocampal pyramidal cells, tubocurarine at a concentration of 50 \mu M had little effect on the \( s_{\text{AHP}} \) (potentiation of 15.4 ± 18.5%, n=4). Increasing the concentration to 100 \mu M reduced the amplitude of the \( s_{\text{AHP}} \) by 25.9 ± 8.3% (n=6; Fig 3.7C), which was completely reversed within a 5 min washout period. The time to peak and decay time constants of the \( s_{\text{AHP}} \) was unaffected by tubocurarine. Tubocurarine also had no effect (p > 0.05) on action potential duration.

It was observed that both 50 \mu M and 100 \mu M tubocurarine completely abolished the \( m_{\text{AHP}} \), providing further evidence that apamin-sensitive SK channels underlie the \( m_{\text{AHP}} \) in these cultured neurons (Stocker et al., 1999).

UCL 1848 is a novel analogue of dequalinium and is a more potent and selective blocker than dequalinium of the apamin-sensitive AHP in SCG neurones
A 2min bath application of a supramaximal concentration of UCL 1848 (100nM) had little effect on the sI_{AHP} (% inhibition = -3.6 ± 5.0%; n=6) but abolished the mI_{AHP} (Fig 3.7D), providing further evidence that apamin-sensitive SK channels underlie the mI_{AHP}. The effects of UCL 1848 on the mI_{AHP} reversed within 5min of washout. UCL 1848 did not modify action potential duration (p > 0.05).

**3.2.5.3 Effects of neurotransmitters on the sI_{AHP}**

The sI_{AHP} was sensitive to muscarinic receptor and β-adrenoreceptor agonists, as previously reported (see Storm, 1990, for references). 3μM muscarine (n=4) and 1μM noradrenaline (n=3) abolished the sI_{AHP} (Fig 3.8 A and B). The effects of both reversed in less than 5min. Both agonists also caused a reduction of the outward holding current (Fig 3.8C), an effect which has also been demonstrated in previous studies (Storm, 1990). In keeping with this, the resting membrane potential values in the presence of muscarine and noradrenaline were reduced to -50.2 ± 6.0mV (n=4; p < 0.01; control = -69.9 ± 6.7mV) and -62.0 ± 3.8mV (n=3; p > 0.05; control = -64.4 ± 1.7mV) respectively. Neither agonist appeared to modify the action potentials or the mI_{AHP}.

**3.2.5.4 Effects of charybdotoxin**

Charybdotoxin is a potent blocker of both BK and IK channels (McManus, 1991). As magnocellular neurons of the rat supraoptic nucleus have been demonstrated to have a charybdotoxin-sensitive sAHP (Greffrath et al., 1998), it was decided to test the effects of charybdotoxin on the sI_{AHP} in cultured hippocampal
Fig 3.8 The effects of 2min bath applications of (A) 1μM noradrenaline and (B) 3μM muscarine. The sI_{AHP} traces in the presence and absence of the two agents have been superimposed. (C) The effects of muscarine on the outward holding current in the same cell as in (B). The action potentials have been removed to clarify the effects of muscarine on the mI_{AHP} and sI_{AHP}. Note that although noradrenaline and muscarine have abolished the sI_{AHP}, the mI_{AHP} (as indicated in (B) and (C)) has remained largely unaffected.
pyramidal cells. A 2min application of charybdotoxin (100nM) significantly increased the amplitude of the $s_{\text{I}_{\text{AHP}}}$ by $21.7 \pm 5.4\%$ ($n=7$; $p < 0.01$; Fig 3.7E) and also significantly broadened ($\%$ increase = $22.9 \pm 4.6\%$; $p < 0.01$) the action potentials (Fig 3.7F). This broadening of the action potentials can be explained by block of BK channels, which are involved in action potential repolarization in hippocampal pyramidal cells (Yoshida et al., 1991). The increase in amplitude of the $s_{\text{I}_{\text{AHP}}}$ may then be due to increased influx of calcium through the voltage-gated $Ca^{2+}$ channels which remain open longer.

3.2.5.5 Effects of 1-ethyl-2-benzimidazolinone (1-EBIO)

1-EBIO has been described as an opener of the cloned IK/SK4 channel (Jensen et al., 1998; Pedersen et al., 1999; Warth et al., 1999) and the cloned SK2 channels (Syme et al., 2000). Although the effect of 1-EBIO on SK1 channels is unknown, 1-EBIO may also be expected to be an opener of SK1 channels since SK1 and SK2 sequences show 90% homology (see Chapter 6). As SK1 channels have been hypothesized to underlie the $s_{\text{I}_{\text{AHP}}}$ (Bond et al., 1999), the effect of 1-EBIO on the $s_{\text{I}_{\text{AHP}}}$ was studied. Application of a near-maximal concentration of 1-EBIO ($300\mu$M; Jensen et al., 1999; Syme et al., 2000), had little effect on the amplitude or duration of the $s_{\text{I}_{\text{AHP}}}$ ($\%$ inhibition = $9.0 \pm 6.1\%$; $n = 3$; Fig 3.9A) as well as on the action potential and outward holding current. This concentration of 1-EBIO, however, did increase the amplitude of the $m_{\text{I}_{\text{AHP}}}$ from $0.10 \pm 0.03nA$ (control conditions) to $0.20 \pm 0.04nA$ ($n=3$; $p < 0.05$; Fig 3.9B). The effect was reversible within 2min of washout. The decay time constant of the $m_{\text{I}_{\text{AHP}}}$ ($\tau_1$) also increased significantly by $21.1 \pm 6.0\%$ ($n=3$; $p < 0.05$) in the presence of 1-EBIO.
Fig 3.9 (A) Effect of a 2min bath application of 300μM 1-EBIO on the \( s_{AHP} \) and the \( m_{AHP} \). The trace in (A) is shown in (B) on an expanded time course. The traces in the absence and presence of 1-EBIO have been superimposed. The vertical scale bar in panel (A) also applies to panel (B).
3.3 Discussion

3 different methods that have been used to culture hippocampal pyramidal cells in previous studies were tried. Method 3 (Section 2.1.1.3) was found to be the best for yielding pyramidal cells that were suitable for long term culture. Pyramidal cells obtained using this method were identifiable morphologically both when freshly dissociated and after 7 days in culture. The M-like and Q currents, which are observed in pyramidal cells in slices, could also be recorded in these cells. Although previous studies had suggested that the $I_{AHP}$ was undetectable in cultured hippocampal pyramidal cells (Alger et al., 1994), the $I_{AHP}$ could be recorded from cells cultured using either Method 2 or Method 3. The $I_{AHP}$ was prone to rundown if perforated patches were not used. As shown in previous studies, the $I_{AHP}$ could be most reliably recorded using KMeSO$_4$ in the filling solution (Zhang et al., 1994). The difficulties encountered by Alger et al. (1994) may have been due to the use of a high concentration of Ca$^{2+}$ chelating agent, BAPTA in the pipette filling solution and the use of conventional whole cell recordings.

3.3.1 General characteristics of the $I_{AHP}$

The time-course of the $I_{AHP}$ in cultured hippocampal pyramidal cells was observed to be similar to that reported using hippocampal slices (Storm, 1990). The amplitude of the $I_{AHP}$ was dependent on both the holding potential of the cell and the number of action potentials that were used to evoke the $I_{AHP}$ as has been reported previously using hippocampal slices (Lancaster & Adams, 1986). As the $I_{AHP}$ had a reversal potential of $-90$ mV (similar to $E_k$) and could be abolished by Ba$^{2+}$, K$^+$ channels are likely to underlie the $I_{AHP}$. The $I_{AHP}$ was dependent on Ca$^{2+}$ entry as it was suppressed by the application of 200 µM Cd$^{2+}$. Hence, as suggested by previous
studies (Lancaster and Adams, 1986), it can be concluded that K⁺ channels that are
dependent upon a rise in \([\text{Ca}^{2+}]_i\) for activation, probably mediate the sI_{AHP}. The
general characteristics of the sI_{AHP} in these cultured neurones are, therefore, very
similar to those of the sI_{AHP} recorded in brain slices.

3.3.2 Pharmacology of the sI_{AHP}

The sI_{AHP} was shown to be insensitive to 100nM apamin and 1mM TEA but
was suppressed by application of 1µM noradrenaline and 3µM muscarine, as reported
in the literature (see Storm, 1990; Sah, 1996, Vergara et al., 1998). The inhibition of
the sI_{AHP} by noradrenaline occurs via β-adrenoreceptors and is due to activation of
The effect of muscarine involves the activation of second messengers including
Ca²⁺/calmodulin-dependent protein kinase (Pedarzani and Storm, 1996b) and protein
kinase G (Krause and Pedarzani, 2000). The pharmacological characteristics of the
sI_{AHP} in the cultured pyramidal cells, thus, appear to be similar to those reported for
pyramidal cells in slices.

Inhibition of the sI_{AHP} by muscarine has been reported to reveal an inward
current (Knopfel et al., 1990). The inward current has been shown to be due to a
Ca²⁺-activated cation conductance (Caeser et al., 1993). In these cells, however, this
current was not observed following inhibition of sI_{AHP} by muscarine, suggesting that
perhaps these channels are not expressed.

UCL 1848 has been discovered to be the most potent non-peptidic compound
to block the apamin-sensitive AHP in SCG neurones (Benton et al., 1999b). It also
displaces apamin from its binding site in guinea-pig liver hepatocytes (Benton et al.,
1999b). It is assumed that UCL 1848 binds to the same binding site as apamin. It is,
therefore, not surprising that UCL 1848 does not affect the sI_{AHP} in these cultured hippocampal pyramidal cells.

The same amino acid residues determine the tubocurarine- and apamin-sensitivity of SK channels (Ishii et al., 1997b) and suggest a common binding site. SK1 channels expressed in *Xenopus* oocytes are relatively insensitive to tubocurarine (IC_{50} = 354μM for hSK1; Ishii et al., 1997b) in comparison to apamin-sensitive channels (IC_{50} = 5.4μM for rSK2; Ishii et al., 1997b). Tubocurarine at a concentration of 50μM had no effect on the sI_{AHP} (n=4) but at a concentration of 100μM, tubocurarine reduced the sI_{AHP} by 25.9 ± 8.3% (n=6). These results would be consistent with the effects of tubocurarine on the hSK1 channels formed in *Xenopus* oocytes (but see Chapter 6).

The amplitude of the sI_{AHP} was significantly increased in the presence of 100nM charybdotoxin. This is probably a consequence of the widening of action potentials (Fig 3.6). The lack of effect of charybdotoxin on the sI_{AHP} suggests that IK channels do not underlie this current.

Interestingly, although TEA (1mM) also caused broadening of the action potentials, there was no significant increase in the amplitude of the sI_{AHP}. This suggests that TEA at a concentration of 1mM may inhibit the sI_{AHP} but the effect may have been masked by a rise in [Ca^{2+}].

1-EBIO, which has been described as an opener of the IK and SK2 channels (Jensen et al., 1998; Pedersen et al., 1999; Warth et al., 1999; Syme et al., 2000), had little effect on the sI_{AHP}. The effect of 1-EBIO on SK1 channels is unknown (but see Chapter 6). As the SK1 sequence shares 90% homology with the SK2 sequence, 1-EBIO may be expected to be an opener of SK1 channels (see Chapter 6). This
result, therefore, raises the possibility that SK1 channels may not underlie the sI\textsubscript{AHP} in these cultured hippocampal pyramidal neurones.

### 3.3.3 Pharmacology of the mI\textsubscript{AHP}

45% of the cultured hippocampal pyramidal neurones displayed both a sI\textsubscript{AHP} and a mI\textsubscript{AHP}. In these cells, 100nM apamin, 100nM UCL 1848 and 50μM tubocurarine were all observed to block a significant proportion of the mI\textsubscript{AHP} (see Fig 3.5). Application of 200μM Cd\textsuperscript{2+} also abolished the mI\textsubscript{AHP} suggesting that a Ca\textsuperscript{2+} dependent current underlies the mI\textsubscript{AHP}. These results are consistent with previous reports (Stocker et al., 1999) and suggest that apamin-sensitive SK channels predominantly underlie the mI\textsubscript{AHP} in these cells. In situ hybridization studies have shown the presence of SK2 and SK1 mRNA in hippocampal CA regions of the rat brain, with SK2 channels being hypothesized to underlie the mI\textsubscript{AHP} in rat brain slices (Stocker et al., 1999). In the current study, 1-EBIO, an opener of SK2 channels (Syme et al., 2000), also significantly enhanced the amplitude and duration of the mI\textsubscript{AHP}. However, it is possible that 1-EBIO can activate other SK channels (see Chapter 6). Also, the concentrations of apamin and tubocurarine employed in this study can block SK3 channels (Köhler et al., 1996; Ishii et al., 1997b). Hence, it cannot be confirmed that homomeric SK2 channels underlie the mI\textsubscript{AHP} in these cultured cells.

When the AHP is recorded under current clamp conditions, other K\textsuperscript{+} currents such as I\textsubscript{M} have also been suggested to contribute to the generation of the mAHP (Storm, 1987). In these cells, muscarine had very little effect on the mI\textsubscript{AHP} recorded at a potential of −50mV, suggesting that K\textsuperscript{+} channels underlying I\textsubscript{M} at least do not contribute to the generation of the mI\textsubscript{AHP}. 

96
3.3.4 Conclusion

The results presented in this chapter show that hippocampal pyramidal cells cultured using Method 3 display similar currents to those recorded in pyramidal cells in brain slices. In particular, using perforated patches the $s_{AHP}$ can be recorded in these cells. The $s_{AHP}$ appears to have identical properties to those recorded from brain slices. As reported for the $s_{AHP}$ in brain slices, the $s_{AHP}$ in these cultured hippocampal pyramidal cells has a unique pharmacological profile. The $s_{AHP}$ is insensitive to blockers and openers of the $m_{AHP}$ as well as the BK and IK channel blocker, charybdotoxin. These results confirm that neither the apamin-sensitive SK channels nor IK channels underlie the $s_{AHP}$. It is possible that SK1 channels which are relatively apamin-insensitive when expressed in *Xenopus* oocytes (Köhler et al., 1996; Ishii et al., 1997b) may underlie the $s_{AHP}$. 
CHAPTER 4

Ca\textsuperscript{2+} CHANNELS INVOLVED IN THE GENERATION OF THE sI\textsubscript{AHP} IN CULTURED RAT HIPPOCAMPAL PYRAMIDAL NEURONES

4.1 Introduction

In recent years, there has been much speculation as to which Ca\textsuperscript{2+} channels are involved in the generation of the sI\textsubscript{AHP} in hippocampal neurones. Conflicting results have been obtained in previous studies. Some studies have shown that only a partial inhibition of the sI\textsubscript{AHP} occurs with L-type Ca\textsuperscript{2+} channel blockers (Rascol \textit{et al.}, 1991; Moyer \textit{et al.}, 1992). Others, however, have reported that Ca\textsuperscript{2+} entry via L-type Ca\textsuperscript{2+} channels is the main contributor to the activation of the sI\textsubscript{AHP} (Tanabe \textit{et al.}, 1998; Borde \textit{et al.}, 2000).

The time course of the sAHP has been suggested to be due to the kinetics of the underlying Ca\textsuperscript{2+} channels rather than the underlying K\textsuperscript{+} channels (Bond \textit{et al.}, 1999). When a long depolarizing pulse or a series of short pulses are applied under voltage-clamp conditions to hippocampal pyramidal cells, the L-type Ca\textsuperscript{2+} channels undergo a change in their gating properties, termed 'delayed facilitation' (Cloues \textit{et al.}, 1997). This delayed facilitation mechanism has therefore, been highlighted as a possible cause of the slow kinetics of the sAHP in hippocampal pyramidal neurones and would then imply a dominant role for L-type Ca\textsuperscript{2+} channels in the generation of the sAHP (Cloues \textit{et al.}, 1997; Marrion and Tavalin, 1998; see INTRODUCTION, Section 1.5.4 for details).
It should be noted, however, that the sAHP in other neurones, for example cortical pyramidal (Pineda et al., 1998), is predominantly generated via Ca\(^{2+}\) entry through N-type Ca\(^{2+}\) channels. The properties and time course of the sAHP in these other types of neurones is very similar to the sAHP recorded from hippocampal pyramidal neurones (see Sah, 1996). Like cortical pyramidal neurones, hippocampal pyramidal neurones express L-, N-, P-, Q-, R- and T-type Ca\(^{2+}\) channels (Christie et al., 1996). Assuming there are no Ca\(^{2+}\) diffusion barriers, no microdomains or no compartmentalization, it might be expected that Ca\(^{2+}\) entry via Ca\(^{2+}\) channels other than the L-type might also contribute to the generation of the sAHP in hippocampal pyramidal neurones.

In some cell types, such as vagal motoneurones, the slow time course of the sAHP has been attributed to Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR; Sah and McLachlan, 1995). Again, in hippocampal pyramidal neurones, there is evidence both for (Torres et al., 1996; Tanabe et al., 1998; Borde et al., 2000) and against (Lancaster and Zucker, 1994; Zhang et al., 1995) a role for CICR in the generation of the sAHP.

The aim of this part of the study was thus, to identify the sources of Ca\(^{2+}\) for the generation of the sAHP in cultured hippocampal pyramidal neurones. Specifically, the roles of L- and N-type Ca\(^{2+}\) channels and CICR for the activation of the sI\(_{AHP}\) were explored.

**4.2 Results**

**4.2.1 Effects of L-type Ca\(^{2+}\) channel inhibitors on the sI\(_{AHP}\) evoked by a train of action potentials**

2min bath applications of the L-type Ca\(^{2+}\) channel inhibitor, nifedipine
(1 mM), inhibited the sI_AHP by 29.1 ± 4.0% (n=10). This effect was reversible within 5 min of washout. Increasing the concentration of nifedipine to 10 μM did not inhibit the sI_AHP any further (28.3 ± 5.1%, n=14; Fig 4.1A), suggesting that L-type Ca^{2+} channels had been maximally inhibited at both concentrations. In neurones, L-type Ca^{2+} channels are inhibited by nifedipine with an IC_{50} between 75 nM - 368 nM (Trombley and Westbrook, 1991; Stengel et al., 1998). Nifedipine had no effects on the time to peak or the decay time constant (τ₃) of the sI_AHP (control value for time to peak = 0.49 ± 0.04 s and τ₃ = 0.68 ± 0.06 s; in the presence of nifedipine, time to peak = 0.49 ± 0.04 s and τ₃ = 0.66 ± 0.05 s; n=14; p > 0.05; Fig 4.1A). Nifedipine, at concentrations of both 1 μM and 10 μM, had no effect on the action potentials used to evoke the sI_AHP (Fig 4.1B). In cells that exhibited both the mI_AHP and the sI_AHP, 10 μM nifedipine had very little effect on the mI_AHP (% inhibition = 3.5 ± 1.7%, n=8; data not shown).

As some studies have suggested that nifedipine may be less effective on neuronal L-type Ca^{2+} channels than other L-type channel blockers (Tanabe et al., 1998), the effects of a second L-type Ca^{2+} channel antagonist, nimodipine, were also studied. Nimodipine, at concentrations up to 3 μM, caused only a partial inhibition of the sI_AHP (Fig 4.2A and 4.2B). The block by 3 μM nimodipine was not significantly (p > 0.05) different from that with 1 μM. The block by both concentrations of nimodipine were not significantly different (p > 0.05) from that produced by nifedipine. The IC_{50} for inhibition of L-type Ca^{2+} channels by nimodipine is approximately 50 nM in neurones (Marchetti et al., 1995) so it is likely that almost maximal inhibition of L-type channels was achieved by nimodipine at the concentrations used in the present work (0.3-10 μM; see also results of direct measurements of inhibition of Ca^{2+} currents). There was no effect of nimodipine (at
Fig 4.1: (A) Record of the sIAHP in the presence of a supramaximal concentration of nifedipine applied for 2 min. The sIAHP was evoked using hybrid clamp conditions. (B) Effects of 10μM nifedipine on the last action potential in the train of 13. The dotted line represents the control trace. (C) The effects of nifedipine on the sIAHP evoked using a 170ms depolarizing step from -50mV to +25mV in the presence of 1μM TTX.
Fig 4.2: (A) A histogram to show the effects of different concentrations of nimodipine on the amplitude of the $s_{I_{AHP}}$. The means and the standard errors have been plotted. The number of observations are shown in brackets. (B) and (C) are illustrations of the effects of 3μM and 10μM nimodipine on the $s_{I_{AHP}}$ in two different cells. The tails of the currents have been superimposed. The calibration bars in (C) apply to (B) as well.
either 1μM or 3μM) on the time to peak or $\tau_3$ of the sI_{AHP} (control values for time to peak = 0.51 ± 0.01s and $\tau_3 = 0.67 \pm 0.15$s; in the presence of nimodipine time to peak = 0.54 ± 0.03s and $\tau_3 = 0.64 \pm 0.11$s, n=9; p > 0.05; Fig 4.2B), nor was there any change in the action potential width. Nimodipine, at concentrations of 1μM or 3μM, also had little effect on the mI_{AHP} (% inhibition of pooled data = 19.8 ± 7.6%, n=6; p < 0.05; data not shown).

Increasing the nimodipine concentration to 10μM resulted in complete abolition of the sI_{AHP} (Fig 4.2C) within 2min of bath application. This can be at least partially, explained by the ability of 10μM nimodipine to reduce the action potential amplitude during a train of 13 action potentials in a use-dependent fashion (Fig 4.3). The threshold of firing of action potentials was also raised (see Fig 4.3B and 4.3D) and the last current pulse during the train of 13 often failed to trigger an action potential (Fig 4.3B). The use-dependent effect on the action potentials reversed during the 10s intervals between trains. These findings suggest that the complete block of the sI_{AHP} by nimodipine at high concentrations cannot be solely attributed to block of L-type Ca$^{2+}$ channels. The effects of nimodipine on the sI_{AHP} as well as the action potentials were reversible with 5min of washout at all concentrations.

**4.2.2 Effects of L-type channel inhibitors on the sI_{AHP} evoked using depolarizing steps**

The studies that showed that Ca$^{2+}$ entry via L-type Ca$^{2+}$ channels predominantly contributes to the generation of the sI_{AHP} (Tanabe et al., 1998; Borde et al., 2000), evoked the sI_{AHP} using single depolarizing steps that were at least 200ms long. To generate a sI_{AHP} under similar conditions, the current was activated by Ca$^{2+}$ entry occurring during a single 170ms long depolarizing step from −50mV
Fig 4.3: (A) A train of action potentials before application of 10μM nimodipine. (B) In the same cell, a train of 13 action potentials in the presence of 10μM nimodipine applied 2min beforehand. The calibration bar in (B) also applies to (A). (C) and (D) The first and second last action potentials in the train of 13 in the absence and presence of 10μM nimodipine. The traces have been superimposed. The dotted lines represent the control action potentials. Calibration bars in (D) also apply to (C).
to +20mV in the presence of 1μM tetrodotoxin (TTX). A sIAHP with a similar time course to the sIAHP evoked using action potentials could be recorded (Fig 4.1C). Interestingly, under these conditions, nifedipine produced a greater inhibition of the sIAHP: a 52.5 ± 2.0% reduction (n=3; Fig 4.1C) occurred by a 2min bath application of 10μM nifedipine. This inhibition was significantly (p < 0.01) different from the inhibition obtained in the presence of 10μM nifedipine when the sIAHP was evoked using a train of 13 action potentials. Again, \( \tau_3 \) and the time to peak of the sIAHP were not altered significantly in the presence of nifedipine. The amplitude of the mIAHP elicited in this was also unchanged by nifedipine (% inhibition = 1.3 ± 1.3%, n=3; Fig 4.1C).

### 4.2.3 Effects of L-type Ca\(^{2+}\) channel inhibitors on Ca\(^{2+}\) currents

The effects of both nifedipine and nimodipine on the Ca\(^{2+}\) current were examined directly in freshly dissociated cells. Using the protocol described in METHODS (Section 2.1.3) low voltage-activated (LVA) and high voltage-activated (HVA) Ca\(^{2+}\) currents were observed in all cells (Fig 4.4). It should be noted that rundown of the Ca\(^{2+}\) currents was quite common and hence the inhibition of drugs was calculated as a percentage of the average of the control and recovery after washout. Data were only collected from cells in which the Ca\(^{2+}\) current recovered to within 70% of the control after washout.

10μM nifedipine, 3μM nimodipine and 10μM nimodipine reduced the HVA Ca\(^{2+}\) current by 18.8 ± 3.5% (n=7), 20.8 ± 4.3% (n=4) and 23 ± 1.7% (n=5) respectively (Fig 4.4). There were no significant differences between these values (p > 0.05). These results are consistent with those published in previous studies (Deak et al., 1998; Potier et al., 1999) and it can therefore, be concluded that both
Fig 4.4: Records in the absence and presence of (A) 10μM nifedipine, (B) 3μM nimodipine and (C) 10μM nimodipine. Ca^{2+} currents were evoked from a holding potential of −80mV using a voltage ramp (shown in (D)). The bathing solution contained 1μM TTX and 25mM TEA to inhibit Na^+ and K^+ channels, respectively. The patch pipette filling solution contained CsCl to provide additional block of K^+ currents. In each record, the leak current was determined in the presence of 200μM Cd^{2+}, which completely blocked the HVA current but only part of the LVA current. The records are from 3 different cells. The calibration bar shown in (C) applies to (A) and (B) as well.
nifedipine and nimodipine at these concentrations cause a complete inhibition of the current carried by L-type Ca\(^{2+}\) channels in these cells. The L-type current is, therefore, of the order of 20% of the total HVA Ca\(^{2+}\) current.

3\(\mu\)M nimodipine caused a small reduction of the (LVA) calcium ion current (Fig 4.4B). 10\(\mu\)M nimodipine inhibited a larger component of the LVA current (Fig 4.4C). In earlier studies, 10\(\mu\)M nimodipine has also been observed to inhibit an LVA current component (Avery et al., 1996). Since the \(s_I_{AHP}\) was recorded at a holding potential of -50mV, it is unlikely that LVA Ca\(^{2+}\) channels contribute to the generation of the \(s_I_{AHP}\).

4.2.4 Effects of an N-type Ca\(^{2+}\) channel inhibitor on the \(s_I_{AHP}\)

A 20min bath application of a supramaximal concentration of \(\omega\)-conotoxin GVIA (100nM; \(IC_{50} = 60\mu\)M, Wagner et al., 1988), a selective blocker of N-type Ca\(^{2+}\) channels (Tsien et al., 1988; Zhang et al., 1993), irreversibly reduced the \(s_I_{AHP}\) by 35.8 ± 3.4% (n=8; Fig 4.5A). This finding suggests that Ca\(^{2+}\) entry via N-type Ca\(^{2+}\) channels also plays a role in the activation of the \(s_I_{AHP}\). Because of the irreversibility of the effects of \(\omega\)-conotoxin, it was essential to use only those cells that demonstrated stable \(s_I_{AHP}\)s for at least 5min before drug application (see Fig 4.5C). It should also be noted that after washout of the \(\omega\)-conotoxin, the \(s_I_{AHP}\)s remained at their reduced amplitude for at least 15min and occasionally up to 30min (see Fig 4.5C). Application of \(\omega\)-conotoxin had no effects on \(\tau_3\) or the time to peak of the \(s_I_{AHP}\) (control values for time to peak = 0.50 ± 0.06s and \(\tau_3 = 1.15 \pm 0.12\)s; after 20min application of \(\omega\)-conotoxin, time to peak = 0.50 ± 0.03s and \(\tau_3 = 1.15 \pm 0.18\)s, n=8; p < 0.05; Fig 4.5A). \(\omega\)-conotoxin had no effects on the action potential duration (Fig 4.5B). The effects of \(\omega\)-conotoxin on the \(m_I_{AHP}\) could not be assessed.
Fig 4.5 (A) Effects of a supramaximal concentration of \( \omega \)-conotoxin (100nM) applied alone for 20min and then together with nifedipine (10\( \mu \)M). The traces in the absence and presence of the drugs have been superimposed. The effect of \( \omega \)-conotoxin was irreversible. (B) Effects of 100nM \( \omega \)-conotoxin on the last action potential in a train of 13. The dotted line represents the control trace. (C) For the cell shown in (A), the amplitude of the sI_{AHP} before, during and after application of \( \omega \)-conotoxin GVIA alone and together with nifedipine has been plotted against time.

Location of records shown in (A) are indicated in (C) as (i), (ii) and (iii).
as the cells used to study the effects of α-conotoxin did not exhibit mI_{AHP}.

α-conotoxin GVIA has been suggested to block L-type Ca^{2+} channels in hippocampal neurones (Takahashi et al., 1989). To re-examine this possibility, after application of 100nM α-conotoxin alone, both nifedipine (10µM) and α-conotoxin (100nM) were applied together. The co-application of nifedipine and α-conotoxin resulted in the sI_{AHP} being reduced further by 40.0 ± 1.6% (i.e. total % inhibition in the presence of both nifedipine and α-conotoxin = 69.6 ± 2.6%, n=4; Fig 4.5A and 4.5C). The action potential width also increased from 0.98 ± 0.10ms (under control conditions) to 1.70 ± 0.31ms (p = 0.056) in the presence of both nifedipine and α-conotoxin.

4.2.5 Effects of ryanodine

Ryanodine was applied at a maximal concentration of 10µM (Tanabe et al., 1998). A steady block of 29.4 ± 6.1% (n=5; Fig 4.6A and 4.6B) of the sI_{AHP} was achieved within 20min of application (Fig 4.6B). This block was partially reversible after 20mins of washout (Fig 4.6B). Because of the slow onset of action, particular care was taken to select cells that showed very little rundown of the sI_{AHP} (as shown in Fig 4.6B). This result indicates CICR plays a role in activation of the sI_{AHP} in cultured neurones. There were no obvious effects of ryanodine on the duration or amplitude of the action potential, confirming previous reports (Sandler and Barbara, 1999) or on the mI_{AHP}.
Fig 4.6: (A) Record of the sI_{AHP} in the absence (i) and presence (ii) of a maximal concentration (10\mu M) of ryanodine. Ryanodine was applied for 20min. The traces have been superimposed. (B) The amplitude of the sI_{AHP} before, during and after application of ryanodine has been plotted against time. The location of the records shown in (A) are included in (B) as (i) and (ii).
4.3 Discussion

4.3.1 Ca\(^{2+}\) channels involved in the generation of the sI\(_{AHP}\)

The work presented in this chapter has explored the role of Ca\(^{2+}\) channels in the generation of the sI\(_{AHP}\) in cultured hippocampal pyramidal neurones. Nifedipine, at both 1\(\mu\)M and 10\(\mu\)M, inhibited the sI\(_{AHP}\) by only 30% when the sI\(_{AHP}\) was evoked using action potentials. Another L-type Ca\(^{2+}\) channel inhibitor, nimodipine, at concentrations that specifically inhibits Ca\(^{2+}\) channels, also only partially reduced the sI\(_{AHP}\). This suggests that since L-type channels were maximally inhibited at these concentrations, L-type Ca\(^{2+}\) channels are not exclusively concerned in the activation of the sI\(_{AHP}\) in cultured hippocampal pyramidal cells. This conclusion is in keeping with studies employing hippocampal slices (Rascol et al., 1990; Moyer et al., 1992) in which inhibition of L-type Ca\(^{2+}\) channels produced only partial inhibition of the sAHP.

In a more recent study also employing hippocampal pyramidal slices (Borde et al., 2000), Ca\(^{2+}\) entry via L-type Ca\(^{2+}\) channels appeared to be the main contributor to the generation of the sI\(_{AHP}\) when evoked using a depolarizing step. When the sAHP was evoked using action potentials under current-clamp conditions, potentiation of the sAHP occurred with repeated activation. Under these conditions, nifedipine only reduced the potentiation of the sAHP and a significant amount of the sAHP could still be recorded in the presence of nifedipine. Therefore, the source of Ca\(^{2+}\) for the generation of the sI\(_{AHP}\) may differ when evoked using a long depolarizing step or action potentials. This possibility has been explored and, when a 170ms long depolarizing step was used to evoke the sI\(_{AHP}\), the inhibition by nifedipine was significantly (\(p < 0.01\)) increased to 53%. This finding is perhaps not surprising as N-type Ca\(^{2+}\) channels have been reported to inactivate within 50-100ms.
at a potential of $-10\text{mV}$ (Takahashi et al., 1989) whereas L-type $\text{Ca}^{2+}$ channels do not inactivate. More importantly, the inactivation rate of N-type $\text{Ca}^{2+}$ channels increases at more positive potentials. Hence, application of depolarizing steps to $+20\text{mV}$ or so may result in a significant portion of N-type channels becoming inactivated and therefore, $\text{Ca}^{2+}$ entry via $\text{Ca}^{2+}$ channels that inactivate more slowly such as the L-type may contribute to a greater degree to the generation of the $\text{sIAHP}$. In addition, the presence of TTX in these experiments would prevent action potential invasion of the dendrites and thus, somatic $\text{Ca}^{2+}$ channels may be preferentially activated. L-type $\text{Ca}^{2+}$ channels are mainly located on the soma (Westenbroek et al., 1990) and, hence, $\text{Ca}^{2+}$ entry via these channels may contribute to a greater extent in the generation of the $\text{sIAHP}$. However, when the $\text{sIAHP}$ is triggered via action potentials, $\text{Ca}^{2+}$ entry via $\text{Ca}^{2+}$ channels located on dendrites may also contribute to the $\text{sIAHP}$ activation.

In organotypic slices, the $\text{sIAHP}$ was abolished in the presence of the L-type $\text{Ca}^{2+}$ channel inhibitor, isradipine (Tanabe et al., 1998). In the same study, however, application of a maximal concentration of nifedipine resulted in only partial block of the $\text{sIAHP}$. This discrepancy was explained by the suggestion that nifedipine is less effective at blocking L-type channels than isradipine. Dihydropyridines have been found to inhibit $\text{K}^+$ channels in various preparations (for example the IK channel in red blood cells (Ellory et al., 1992) and A-type $\text{K}^+$ channels in bovine adrenal cells (Mlinar and Enyeart, 1994)). Thus the possibility that isradipine at supramaximal concentrations may also have other actions that could lead to the inhibition of the $\text{sIAHP}$ cannot be ruled out. In this particular study, a supramaximal concentration ($10\mu\text{M}$) of nimodipine abolished the $\text{sIAHP}$. It appeared to block HVA $\text{Ca}^{2+}$ channels to the same extent as $10\mu\text{M}$ nifedipine and $3\mu\text{M}$ nimodipine (Fig 4.4). However, the
Supramaximal concentration of nimodipine also had inhibitory actions on action potentials (Fig 4.3) which might be expected to lead to a further reduction of Ca\(^{2+}\) entry into the neurones. Nimodipine thus appears to have multiple actions that are not shared by nifedipine.

Although in previous studies N-type channel inhibitors have been shown to have minimal effects on the sIAHP in rat hippocampal slices (Rascol et al., 1990; Borde et al., 2000) or in rat hippocampal organotypic slice cultures (Tanabe et al., 1998), in our cultured hippocampal pyramidal cells o-conotoxin GVIA (100nM) irreversibly inhibited the sIAHP by 35% (Fig 4.5B). It has also been recently confirmed that in hippocampal slices when the sIAHP is evoked using action potentials, Ca\(^{2+}\)-entry via N-type Ca\(^{2+}\) channels contributes to the generation of the sIAHP (H. Hua and J.F. Storm, personal communication). This suggests that N-type Ca\(^{2+}\) channels also have a role in activation of the sIAHP. This observation coupled with the finding that L-type Ca\(^{2+}\) channel blockers only partially reduced the sIAHP in these cells, supports the conclusion that Ca\(^{2+}\) entry via L-type Ca\(^{2+}\) channels is not solely responsible for the activation of the sIAHP. Application of nifedipine and o-conotoxin together reduced the sIAHP by 70% (Fig 4.5B), significantly less than 100%. As Cd\(^{2+}\) abolished the sIAHP, this indicates that Ca\(^{2+}\) entry via other types of HVA Ca\(^{2+}\) channels may also play a role in activation of the sIAHP.

It should be noted that in rat neocortical pyramidal cells (Pineda et al., 1998), and in guinea-pig nucleus basalis neurones (Williams et al., 1997), an apamin-insensitive sIAHP is activated predominantly by Ca\(^{2+}\) entry via N-type Ca\(^{2+}\) channels. Thus, it is clear that the types of Ca\(^{2+}\) channels involved in the activation of the apamin-insensitive sIAHP vary between neurones. It is, therefore, likely that the source of Ca\(^{2+}\) for the generation of the sIAHP depends on the Ca\(^{2+}\) channels in the
vicinity of the K⁺ channels underlying the sI_{AHP} but not necessarily coupled to them. The K⁺ channels underlying the sI_{AHP} in hippocampal pyramidal neurones have been suggested to be principally located on dendrites (Sah and Bekkers, 1996; Bekkers, 2000). L-type Ca²⁺ channels, however, are predominantly found at the base of major dendrites and the cell soma (Westenbroek et al., 1990). N-type Ca²⁺ channels are mainly distributed on dendrites (Westenbroek et al., 1992). Therefore, if the channels underlying the sI_{AHP} are indeed located on dendrites (Sah and Bekkers, 1996; Bekkers, 2000), then Ca²⁺ entry via N-type channels should also be able to contribute to the generation of the sI_{AHP}.

The slow kinetics of the sI_{AHP} have been suggested to be due to the delayed facilitation of L-type Ca²⁺ channels (Cloues et al., 1997; Marrion and Tavalin, 1998). Unlike L-type Ca²⁺ channels, facilitation of N-type Ca²⁺ channels does not seem to occur in hippocampal pyramidal neurones (Fisher et al., 1990). However, the results presented in this chapter suggest that Ca²⁺ entry via N-type Ca²⁺ channels contributes significantly to the generation of the sI_{AHP}. Hence, the time course of the sI_{AHP} may not necessarily reflect the kinetics of Ca²⁺ channels (this is discussed in greater detail in DISCUSSION, Section 8.3).

Application of 10μM ryanodine reduced the sI_{AHP} in cultured hippocampal pyramidal neurones by about 30%, suggesting that CICR plays a role in the activation of the sI_{AHP}. This finding is in agreement with the results of Tanabe et al. (1998) and Borde et al. (2000) but differs from those of Zhang et al. (1995) and Torres et al. (1996), neither of whom detected any effect of ryanodine at a concentration of 20μM. However, Torres et al. (1996) as well as Tanabe et al. (1998) observed a reduction of the sI_{AHP} with thapsigargin. Ca²⁺ release from stores
is a very rapid event occurring within milliseconds (Sandler and Barbara, 1999), and therefore, is unlikely to be responsible for the slow onset of the $s_{IAHP}$.

4.3.2 $Ca^{2+}$ channels involved in the generation of the $m_{IAHP}$

Although the role of the different $Ca^{2+}$ channels and CICR involved in the generation of the $m_{IAHP}$ was not specifically studied, it was noted that L-type $Ca^{2+}$ channel inhibitors as well as ryanodine had little effect on the amplitude of the $m_{IAHP}$. Furthermore, nifedipine did not affect the amplitude of the $m_{IAHP}$ when evoked using a depolarizing step. This is in keeping with the recent findings of Aoki and Baraban (2000), who also did not detect any effect of nifedipine on the $m_{IAHP}$. Therefore, $Ca^{2+}$ entry via $Ca^{2+}$ channels other than the L-type is likely to contribute to the activation of the channels underlying the $m_{IAHP}$. Recent findings suggest that $Ca^{2+}$ entry via N-type $Ca^{2+}$ channels contributes largely to the generation of the $m_{IAHP}$ in hippocampal neurones (P. Pedarzani, personal communication). It should be noted that in other cell types which demonstrate an apamin-sensitive $m_{IAHP}$, $Ca^{2+}$ entry via N-type $Ca^{2+}$ channels is mostly responsible for the activation of the $m_{IAHP}$ (see for example Davies et al., 1996; Pineda et al., 1998). It, therefore, seems likely that there is a close association between N-type $Ca^{2+}$ channels and the SK channels that underlie the $m_{IAHP}$. It also suggests that there is compartmentalization of $Ca^{2+}$ such that $Ca^{2+}$ entry via N-type $Ca^{2+}$ channels only can generate a $m_{IAHP}$.

4.3.3 Conclusions

From the results presented in this chapter, it can be concluded that $Ca^{2+}$ entry via $Ca^{2+}$ channels other than the L-type can also contribute to the generation of the $s_{IAHP}$. The type of $Ca^{2+}$ channels that may contribute to the generation of the $s_{IAHP}$
may depend on the stimulus used to evoke the $sI_{AHP}$ and the cell preparation used. These factors need to be examined in more detail. Nevertheless, these results suggest that a particular coupling between L-type Ca$^{2+}$ channels and the channels underlying the $sI_{AHP}$ is probably not crucial for the generation of the $sI_{AHP}$. 
CHAPTER 5

THE PHARMACOLOGY OF THE $sI_{AHP}$ IN CULTURED RAT HIPPOCAMPAL NEURONES

5.1 Introduction

Previous studies (see Storm, 1990 and Sah, 1996 for reviews) as well the results presented in this thesis so far have shown that the pharmacology of the $sI_{AHP}$ is unique. The $sI_{AHP}$ is insensitive to apamin, the dequalinium analogue, UCL 1848, as well as the common $K^+$ channel blockers, TEA and 4-AP (see Chapter 3; Lancaster and Adams, 1986; Lancaster and Nicoll, 1987). The $sI_{AHP}$ is, however, inhibited by relatively high concentrations of tubocurarine (see Chapter 3). As yet, no known specific openers or blockers of the channel underlying the $sI_{AHP}$ have been described. The $sI_{AHP}$ is important for spike frequency adaptation and therefore, might serve to limit neuronal damage due to excessive rises in $Ca^{2+}$ concentration within the cells (Vergara et al., 1998). By limiting the firing of action potentials, the $sI_{AHP}$ may also play a role in reducing epilepsy (Verma-Ahuja et al., 1995; 1998). A combination of behavioural and electrophysiological studies have shown that the $sI_{AHP}$ may be important in learning and memory (Disterhoft et al., 1996; Giese et al., 1998). Therefore, specific openers or blockers of the $sI_{AHP}$ have a number of possible clinical uses. They may also be beneficial in determining the physiological role of the $sI_{AHP}$ and the identification of the channel underlying the $sI_{AHP}$.

So, far the $sI_{AHP}$ has proved to be resistant to most inhibitors of $K^+$ channels (see Chapter 3). There are also no potent inhibitors of the apamin-insensitive SK1 channels formed in Xenopus oocytes (Ishii et al., 1997b; Khawaled et al., 1999).
Fig 5.1 The chemical structures of (A) clotrimazole, (B) UCL 1851 and (C) UCL 1880.
Clotrimazole (Fig 5.1A), an antifungal agent, is a potent, though rather unselective, blocker of the intermediate conductance (IK) channel found in red blood cells and lymphocytes (Alvarez et al., 1992; Brugnara et al., 1993; Rittenhouse et al., 1997a; Logsdon et al., 1997; Dunn, 1998; Jensen et al., 1999). Clotrimazole has also been reported to block BK channels (for example see Rittenhouse et al., 1997b and Wu et al., 1999) and voltage-gated K⁺ channels (for example see Kim and Gregor, 1999). Given that it is capable of inhibiting most types of K⁺ channels, it was decided to test the effects of clotrimazole on the sIAHP.

The major in vivo metabolite of clotrimazole, 2-chlorophenyl-bisphenyl-methanol (referred to here by the code number for the UCL synthesized compounds, UCL 1851; Fig 5.1B), has also been shown to be a potent inhibitor of the IK channels in red blood cells (Rittenhouse et al., 1997a). Both clotrimazole and UCL 1851 are also effective blockers of the recombinant IK channels (Ishii et al., 1997a; Logsdon et al., 1997; Vandorpe et al., 1998; Jensen et al., 1998). As IK channels have about 55% identity to SK channels (Castle, 1999), blockers of the IK channel could also be effective at blocking the K⁺ channel underlying the sIAHP. It was, therefore, decided to also investigate the effects of UCL 1851 and other analogues of clotrimazole on the sIAHP in these cultured hippocampal pyramidal neurones.

Since action potentials were used to evoke the sIAHP and a change in the action potential width can affect the amplitude of the sIAHP by altering Ca²⁺ entry (see Chapter 3), it was important to establish whether any of the drugs modified the action potential width. To take into account any use-dependence of their action, the effects of the compounds on the width of the last action potential in the train of action potentials were noted.
L-type Ca\(^{2+}\) channels in cardiac myocytes can be inhibited by the application of high concentrations of clotrimazole (Thomas et al., 1999). Clotrimazole is also a potent blocker of currents due to the expressed human cardiac L-type Ca\(^{2+}\) \(\alpha_{1c}\) subunits (Fearon et al., 2000). As the generation of the sI\(_{AHP}\) is dependent upon Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels, it was also essential to test the effects on clotrimazole and the related compounds on the Ca\(^{2+}\) currents in hippocampal pyramidal neurones.

Finally, to determine the selectivity profile of clotrimazole and the more selective analogues of it, the effects of these compounds were studied on the mI\(_{AHP}\) in hippocampal pyramidal neurones and the mI\(_{AHP}\) in superior cervical ganglion (SCG) cells.

5.2 Results

5.2.1 Effects of clotrimazole and the related compounds, UCL 1851 and UCL 1880 on the sI\(_{AHP}\)

Clotrimazole was first tested at a concentration of 3\(\mu\)M which within 2min of application reduced the amplitude of the sI\(_{AHP}\) by 69.1 ± 3.7\% (n=6; Fig 5.2A). This effect was reversible within 5min of washout. In view of the blocking effect of clotrimazole, the related compounds UCL 1851 (Fig 5.1B) and UCL 1880 (Fig 5.1C) were tested at a concentration of 3\(\mu\)M. UCL 1851 and UCL 1880 inhibited the sI\(_{AHP}\) at this concentration by 61.7 ± 5.8\% (n=7; Fig 5.2B) and 71.8 ± 7.0\% (n=8; Fig 5.2C) respectively. With both compounds, maximal block of the sI\(_{AHP}\) occurred within 2-4min and the effect was reversible within 10min (see Fig 5.6 for an example). The effects of clotrimazole and the related compounds were found to be
Fig 5.2 Effects of clotrimazole (A), UCL 1851 (B) and UCL 1880 (C) on the sI_{AHP} recorded in cultured rat hippocampal pyramidal neurones. The traces under control conditions and in the presence of the drug have been superimposed in each panel. The calibration bars in (B) also apply to (A).
Fig 5.3 Concentration-inhibition curves for the sI_{AHP} (■) and HVA Ca^{2+} currents (●). Each point is the mean of 3-7 observations. The lines show least square fits to the Hill equation with $y_{max}$ constrained to 100%.
concentration dependent (Fig 5.3). The IC$_{50}$ values for clotrimazole, UCL 1851 and UCL 1880 were $1.7 \pm 0.04 \mu M$, $2.2 \pm 0.5 \mu M$ and $1.2 \pm 0.1 \mu M$ respectively with Hill coefficients of $1.3 \pm 0.04$, $1.3 \pm 0.3$ and $1.4 \pm 0.1$ respectively. The $sI_{AHP}$ could be abolished at the maximal concentrations tested and there was some suggestion that a small inward current could be revealed, though this was not investigated further.

The $I_{AHP}$ were also fitted with the exponential equation described in METHODS (Section 2.1.2.10a) and the time to peak and the decay time constant ($\tau_3$) were estimated from the fit. In the presence of $3\mu M$ and $10\mu M$ concentrations of the compounds, the amplitude of the $sI_{AHP}$ was often reduced to below $20pA$. In these cases, $\tau_3$ and the time to peak could no longer be measured reliably, and the data is, therefore, not presented. For clotrimazole and UCL 1880 at concentrations lower than or equal to $3\mu M$, there were no significant differences of the times to peak and the $\tau_3$ of the $sI_{AHP}$ in the absence of the drug from that in the presence of the drug (Table 5.1). Although it is not apparent from the traces (Fig 5.2B), $\tau_3$ and time to peak were significantly altered in the presence of $3\mu M$ UCL 1851.

5.2.2 Effects of clotrimazole, UCL 1851 and UCL 1880 on action potentials

At concentrations up to $1\mu M$, clotrimazole and UCL 1880 had little effect on the width of the action potentials (see Table 5.2 for values; Fig 5.4A). In the presence of clotrimazole and UCL 1880 at both $3\mu M$ and $10\mu M$, the action potential width tended to widen though the increase was not significant (Table 5.2). The action potentials became broader in the presence of UCL 1851 at concentrations of $3\mu M$ and $10\mu M$ (Table 5.2; Fig 5.4B), though the effects were significant only at $3\mu M$. The amplitude of the action potentials appeared to be little affected in the presence of all drugs tested (see Fig 5.4C and 5.4D for an example).
<table>
<thead>
<tr>
<th>Drug</th>
<th>Control $\tau_3$ (s)</th>
<th>$\tau_3$ in the presence of 1µM drug (s)</th>
<th>Control $\tau_3$ (s)</th>
<th>$\tau_3$ in the presence of 3µM drug (s)</th>
<th>Control TP (s)</th>
<th>TP in the presence of 1µM drug (s)</th>
<th>Control TP (s)</th>
<th>TP in the presence of 3µM drug (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>clotrimazole</td>
<td>1.38 ± 0.22</td>
<td>1.09 ± 0.13</td>
<td>0.91 ± 0.08</td>
<td>0.79 ± 0.09</td>
<td>0.50 ± 0.06</td>
<td>0.50 ± 0.04</td>
<td>0.46 ± 0.07</td>
<td>0.50 ± 0.06</td>
</tr>
<tr>
<td>UCL 1851</td>
<td>0.99 ± 0.09</td>
<td>0.81 ± 0.10</td>
<td>1.11 ± 0.15</td>
<td>0.77 ± 0.07*</td>
<td>0.38 ± 0.03</td>
<td>0.39 ± 0.06</td>
<td>0.49 ± 0.06</td>
<td>0.58 ± 0.06*</td>
</tr>
<tr>
<td>UCL 1880</td>
<td>0.96 ± 0.09</td>
<td>0.92 ± 0.09</td>
<td>1.00 ± 0.07</td>
<td>1.07 ± 0.15</td>
<td>0.45 ± 0.03</td>
<td>0.45 ± 0.01</td>
<td>0.48 ± 0.03</td>
<td>0.51 ± 0.05</td>
</tr>
</tbody>
</table>

Table 5.1: Effect of clotrimazole, UCL 1851 and UCL 1880 on the decay time constant ($\tau_3$) and the time to peak (TP) of the sI$_{AHP}$ recorded in cultured hippocampal pyramidal neurones. * indicates significance at the 5% level.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Control APW (ms)</th>
<th>APW in the presence of 1μM drug (ms)</th>
<th>Control APW (ms)</th>
<th>APW in the presence of 3μM drug (ms)</th>
<th>Control APW (ms)</th>
<th>APW in the presence of 10μM drug (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotrimazole</td>
<td>0.95 ± 0.12</td>
<td>1.07 ± 0.14</td>
<td>1.07 ± 0.17</td>
<td>1.18 ± 0.22</td>
<td>0.66 ± 0.11</td>
<td>1.84 ± 0.52</td>
</tr>
<tr>
<td>UCL 1851</td>
<td>1.14 ± 0.20</td>
<td>1.18 ± 0.20</td>
<td>1.00 ± 0.13</td>
<td>1.33 ± 0.16**</td>
<td>1.13 ± 0.24</td>
<td>1.70 ± 0.35</td>
</tr>
<tr>
<td>UCL 1880</td>
<td>1.38 ± 0.38</td>
<td>1.43 ± 0.42</td>
<td>1.35 ± 0.17</td>
<td>1.66 ± 0.22</td>
<td>1.25 ± 0.24</td>
<td>2.05 ± 0.40</td>
</tr>
</tbody>
</table>

Table 5.2: Effects of clotrimazole, UCL 1851 and UCL 1880 on the width of the last action potential in the train used to evoke the \( sI_{AHP} \). Action potential width (APW) was measured at a potential of -20mV. ** indicates significance at the 1% level (i.e. \( p < 0.01 \)).
Fig 5.4 Effects of (A) 3μM UCL 1880 and (B) 3μM UCL 1851 on the last action potential in a train. The grey lines represent the last action potential in a train before application of the agents. The records are superimposed to align the upstroke. The negligible effect of UCL 1851 on the amplitude of the action potentials is seen by comparing (C) (in the absence) with (D) (in the presence) of the drug. The action potentials shown in (B) are from the records in (C) and (D). The calibration bars in (B) and (D) also apply to (A) and (C).
5.2.3 Effects of clotrimazole, UCL 1851 and UCL 1880 on the outward holding current at -50mV

Clotrimazole was effective at inhibiting the outward holding current (see Fig 5.5A), the effect being concentration-dependent. The concentration-inhibition curve could be fitted with a Hill equation having a Hill coefficient of 1.2 ± 0.1 and an IC<sub>50</sub> value of 2.8 ± 0.1µM (Fig 5.5B). In keeping with this, the resting membrane potential was also significantly decreased in the presence of 3µM and 10µM clotrimazole by 5.4 ± 1.1mV (n=7; p < 0.05) and 8.7 ± 2.4mV (n=4; p < 0.05) respectively. 1µM clotrimazole had no significant effect on resting membrane potential.

At concentrations of 10µM, both UCL 1851 and UCL 1880 caused a slight inhibition of the outward holding current (see Fig 5.6 for an example). This inhibition, however, was very variable and the values ranged from 5 - 40%. The resting membrane potential values were not significantly altered.

5.2.4 Effects of clotrimazole, UCL 1851 and UCL 1880 on Ca<sup>2+</sup> currents in freshly dissociated cells

In freshly dissociated neurones, clotrimazole reduced the HVA Ca<sup>2+</sup> current in a concentration-dependent manner (see Fig 5.3A and 5.7A). Maximal inhibition and recovery of the HVA Ca<sup>2+</sup> current occurred within 2min of application and washout. As the generation of the sI<sub>AHP</sub> is largely dependent upon Ca<sup>2+</sup> entry via HVA Ca<sup>2+</sup> channels, only the effects of drugs on the HVA current were quantified (see Chapter 4). The concentration-response curve could be fitted with the Hill equation having a Hill coefficient of 1.7 ± 0.2 and an IC<sub>50</sub> value of 4.7 ± 0.4µM (Fig
Fig 5.5 (A) Effects of clotrimazole (10μM) on the outward current present at the holding potential of -50mV. The action potentials have been removed to make the effects on the outward holding current, the $sI_{AHP}$ and the $mI_{AHP}$ easier to see. The axis break represents 150s. (B) The concentration-inhibition curve for clotrimazole on the outward holding current. The curve was obtained by fitting the Hill equation to the points with $y_{max}$ constrained to 100%. Each point is the mean and S.E. of 3-7 observations.
Fig 5.6 Effects of UCL 1880 on the outward holding current present at -50mV. Again, the action potentials have been removed so that the effects of UCL 1880 on the outward holding current, the mI_{AHP} and the sI_{AHP} can be clearly seen. The axis break represents 650s.
Fig 5.7 Effects of (A) 3μM and (B) 10μM clotrimazole on the Ca^{2+} current in freshly dissociated hippocampal pyramidal neurones. The voltage protocol shown in (C) was applied to evoke both the low voltage-activated (LVA) and high voltage-activated (HVA) Ca^{2+} currents. The calibration bar shown in (B) also applies to (A).
5.3A). At concentrations of 3μM and 10μM, a significant effect on the LVA Ca^{2+} current was also observed (see Fig 5.7A and 5.7B).

UCL 1851 also inhibited the HVA Ca^{2+} current in a concentration dependent manner (Fig 5.3B). The concentration-inhibition curve yielded an IC_{50} value of 2.4 ± 1.0μM with a Hill coefficient of 2.7 ± 0.4. UCL 1880 was a less potent blocker of the Ca^{2+} current. The block of the HVA Ca^{2+} current by UCL 1880 was dose-dependent (Fig 5.3C) with an IC_{50} value of approximately 10μM. The low solubility of the compound in the external solution did not allow concentrations greater than 10μM to be tested. Like clotrimazole, maximal effects of both UCL 1851 and UCL 1880 on the Ca^{2+} current occurred within 2min of application and were reversible within 1min of washout.

5.2.5 Effects of clotrimazole, UCL 1851 and UCL 1880 on the mlAHP in hippocampal pyramidal neurones

3μM clotrimazole had very little effect on the mlAHP (% inhibition estimated to be 2.1 ± 4.9%, n=3) whereas at 10μM, the mlAHP was clearly reduced (see Fig 5.5 and 5.8A). This effect of clotrimazole can be clearly observed when the current underlying the AHP (I_{AHP}) in the presence of clotrimazole is subtracted from the current in the absence (see Fig 5.8B). The inhibition of the mlAHP by 10μM clotrimazole was estimated by fitting the I_{AHP} to the exponential equation described in METHODS (Section 2.1.2.10a) to be 56.8 ± 3.9% (n=4). In contrast, UCL 1851 and UCL 1880 at concentrations of 10μM had no effect on the mlAHP (see Fig 5.9). On average, they increased it by estimated values of 13.8 ± 8.7% (n=3; Fig 5.9A) and 5.1 ± 1.9% (n=3; Fig 5.9C) respectively. In support of this data, when the I_{AHP} in the presence of UCL 1851 and UCL 1880 is subtracted from the I_{AHP} present under
Fig 5.8: (A) Effects of 10μM clotrimazole on the $\text{ml}_{\text{AHP}}$ and the $\text{sI}_{\text{AHP}}$. (B) The current sensitive to clotrimazole (current in the absence of the drug subtracted from the current in its presence). In both A and B, the current trace is presented on an expanded time base in the inset. The horizontal time bar represents 1s (full record) and 200ms (inset) while the vertical bar represents 100pA (full record) and 200pA (inset). The calibration bars apply to both (A) and (B).
Fig 5.9 Effects of (A) 10μM UCL 1851 and (C) 10μM UCL 1880 on the mI_{AHP} and sI_{AHP}. The traces in the presence and absence of the compounds have been superimposed. Clotrimazole and UCL 2027 sensitive currents were obtained by subtraction and are shown in (B) and (D). The calibration bars in (D) apply also to (A), (B) and (C) respectively.
control conditions, no current resembling the mI_{AHP} is observed (Fig 5.9B and 5.9D). These results show that at concentrations above 3μM, clotrimazole inhibits the apamin-sensitive mI_{AHP} present in these cells. On the other hand, UCL 1851 and UCL 1880 appear to be selective for the sI_{AHP}.

5.2.6 Effects of clotrimazole, UCL 1851 and UCL 1880 on the mI_{AHP} in SCG neurones

In SCG neurones, using perforated patches and with the application of a 50ms depolarizing step, a mI_{AHP} could be recorded stably for up to 60min. The mI_{AHP} was reduced by 76.2 ± 7.2% (n=5) within 2min of bath application of 10nM UCL 1848 (Fig 5.10A). The effect of 10nM UCL 1848 was almost totally reversible, too. These effects are consistent with those reported previously (Benton et al., 1999b).

In the same cells, 2min applications of 10μM clotrimazole, UCL 1851 and UCL 1880 reduced the mI_{AHP} by 5.7 ± 6.6% (n=5; Fig 5.10B), 3.3 ± 2.0% (n=4; Fig 5.10C) and -15.7 ± 13.5% (n=4; Fig 5.10D) respectively. It, therefore, appears that the effects of all three compounds are selective for hippocampal pyramidal neurones.

5.2.7 Effects of analogues of UCL 1851

From the results presented above, the effects of UCL 1851 on the sI_{AHP} can possibly be attributed to its effects on the HVA Ca^{2+} channels. However, as UCL 1851 is known to directly inhibit the IK channel (Rittenhouse et al., 1997a; Vandorpe et al., 1998), it was decided to test a few other analogues of the compound. The analogues were tested at a concentration of 3μM on the sI_{AHP} in hippocampal pyramidal neurones and the results together with the structures of the
Fig 5.10 Effects of (A) 10nM UCL1848, (B) 10μM UCL 1851, (C) 10μM clotrimazole and (D) 10μM UCL 1880 on the mAHP in the same SCG neurone. Currents were activated by a 50ms depolarizing pulse to +20mV from a holding potential of -50mV. All records are from the same cell and have been superimposed. The dark grey records represent the control mAHP before application of the agents. Although it appears as though UCL 1851 and UCL 1880 may have inhibited a fast component, this effect was not reproducible. The calibration bars in (D) also apply to (A), (B) and (C).
Table 5.3: Summary of effects of compounds structurally related to UCL 1851 on the sI_{AHP} and the width of the last action potential (APW) in the train used to evoke the sI_{AHP}. R₁, R₂, R₃ and R₄ denote the chemical substituents present on the general structure shown in Fig 5.11. ** indicates significance at the 1% level (i.e. p < 0.01).

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>% inhibition of sI_{AHP} at 3µM</th>
<th>IC₅₀ value</th>
<th>% increase in APW at 3µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCL 1851</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>61.7 ± 5.8 (n=7)</td>
<td>2.2 ± 0.5µM</td>
<td>34.4 ± 5.1**</td>
</tr>
<tr>
<td>UCL 1864</td>
<td>Cl</td>
<td>H</td>
<td>Cl</td>
<td>Cl</td>
<td>14.3 ± 9.3 (n=6)</td>
<td>&gt;3µM</td>
<td>6.0 ± 7.8</td>
</tr>
<tr>
<td>UCL 1887</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>17.5 ± 3.6 (n=4)</td>
<td>&gt;3µM</td>
<td>16.3 ± 7.2</td>
</tr>
<tr>
<td>UCL 1893</td>
<td>H</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
<td>62.5 ± 2.6 (n=4)</td>
<td>&lt;3µM</td>
<td>52.7 ± 13.9</td>
</tr>
</tbody>
</table>

Fig 5.11: The general structure of compounds related to UCL 1851 that were tested on the sI_{AHP} in cultured rat hippocampal pyramidal neurones.
compounds are summarized in Table 5.3 and Fig 5.11. The effects of the analogues on the last action potential width in a train are also presented in Table 5.3. Maximal effects occurred within 2min of bath application and were reversible within 5min of washout. At 3μM, these analogues had very little effect either on the outward holding current at -50mV or on the resting membrane potential of the cells. Unlike UCL 1851, the decay time constants and time to peak of the $I_{AHP}$ in the presence of the analogues were unchanged. From the results presented in Table 5.3, it is clear that none of the analogues of UCL 1851 are likely to be more potent or selective than the parent compound. Therefore, a full pharmacological profile of these compounds in hippocampal pyramidal neurones was not established.

5.2.8 Effects of analogues of UCL 1880

UCL 1880 blocks the $Ca^{2+}$ current less effectively than the $I_{AHP}$ raising the possibility that some of its effects may possibly be due to selective block of the $K^+$ channel underlying the $I_{AHP}$. Therefore, the effects of a number of structurally related compounds were studied on the $I_{AHP}$ and the action potentials used to evoke the $I_{AHP}$ in cultured hippocampal pyramidal cells. The general structure of the compounds is shown in Fig 5.12. The effects of the compounds applied at a concentration of 3μM on the $I_{AHP}$ and the last action potential width in a train are summarized in Table 5.4. Maximal inhibition of the $I_{AHP}$ occurred within 2min of bath application of the compounds and, with the exception of UCL 1892, was reversible within 5min of washout (see Fig 5.13C for an example). The reversal of the block of the $I_{AHP}$ by UCL 1892 was very slow and could take up to 20min after washout. Again, $\tau_3$ and the times to peak were only estimated for $I_{AHP}$s having
<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>% inhibition of sI\textsubscript{AHP} at 3\mu M</th>
<th>IC₅₀ value</th>
<th>% increase of APW at 3\mu M</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCL 1880</td>
<td>-</td>
<td>Cl</td>
<td>H</td>
<td>71.8 ± 7.0 (n=7)</td>
<td>1.3 ± 0.2\mu M</td>
<td>32.1 ± 17.6%</td>
</tr>
<tr>
<td>UCL 1892</td>
<td>-</td>
<td>H</td>
<td>Cl</td>
<td>68.9 ± 9.9 (n=4)</td>
<td>&lt;3\mu M</td>
<td>68.2 ± 11.1%*</td>
</tr>
<tr>
<td>UCL 2019</td>
<td>-</td>
<td>H</td>
<td>H</td>
<td>51.3 ± 10.5 (n=4)</td>
<td>~3\mu M</td>
<td>19.5 ± 9.5%</td>
</tr>
<tr>
<td>UCL 2026</td>
<td>-</td>
<td>H</td>
<td>H</td>
<td>55.4 ± 5.0 (n=4)</td>
<td>~3\mu M</td>
<td>6.5 ± 14.1%</td>
</tr>
<tr>
<td>UCL 2027</td>
<td>-</td>
<td>H</td>
<td>H</td>
<td>77.6 ± 3.9 (n=5)</td>
<td>1.2 ± 0.1\mu M</td>
<td>70.1 ± 16.9%*</td>
</tr>
<tr>
<td>UCL 2049</td>
<td>-</td>
<td>H</td>
<td>H</td>
<td>63.3 ± 3.6 (n=7)</td>
<td>&lt;3\mu M</td>
<td>39.0 ± 11.0%*</td>
</tr>
<tr>
<td>UCL 2110</td>
<td>-Cl</td>
<td>H</td>
<td>H</td>
<td>-12.9 ± 10.8 (n=3)</td>
<td>&gt;3\mu M</td>
<td>16.7 ± 9.6%</td>
</tr>
<tr>
<td>UCL 2077</td>
<td>-</td>
<td>H</td>
<td>H</td>
<td>98.2 ± 8.5 (n=3)</td>
<td>0.5 ± 0.07\mu M</td>
<td>45.4 ± 3.2%*</td>
</tr>
</tbody>
</table>

Table 5.4: Summary of effects of compounds structurally related to UCL 1880 on the sI\textsubscript{AHP} and the width of the last action potential (APW) in the train used to evoke the sI\textsubscript{AHP}. R₁, R₂ and R₃ denote the chemical substituents present in the general structure shown in Fig 5.12. * indicates significance at the 5% level.
Fig 5.12 General structure of the compounds related to UCL 1880 that were tested on cultured hippocampal pyramidal neurones.
Fig 5.13 Effects of (A) 10µM UCL 2027 and (B) 3µM UCL 2077 on the sI<sub>AHP</sub> recorded in cultured hippocampal pyramidal neurones. The traces in both (A) and (B) are superimposed records with and without drug. Note that in (B) the presence of a small inward current was revealed when the sI<sub>AHP</sub> was abolished by application of UCL 2077. The calibration bars in (B) also apply to (A). (C) Effects of UCL 2027 on the outward holding current present at -50mV. The action potentials have been removed from the trace so that the effects on the outward holding current, the sI<sub>AHP</sub> and the mI<sub>AHP</sub> can be clearly seen. The traces shown in (A) are from the time points at (a) and (b) in (C). Note that the mI<sub>AHP</sub> is not affected in the presence of UCL 2027.
amplitudes greater than 20pA in the presence of the compounds. Neither \( \tau_3 \) nor the
time to peak were significantly altered in the presence of the compounds.

The effects of the compounds on the outward holding current at \(-50\text{mV}\) were
very variable and inconsistent. In general, compounds that produced less than 60%
block of the \( \text{sI}_{\text{AHP}} \) at 3\( \mu \text{M} \) had no effect on the outward holding current. The
remainder of the compounds inhibited the outward holding current by 10-30%. None
of the compounds tested had any significant effect on the resting membrane
potential.

From the results shown, two of the compounds tested, UCL 2027 (Fig 5.13A)
and UCL 2077 (Fig 5.13B), appeared to be more potent than UCL 1880 at inhibiting
the \( \text{sI}_{\text{AHP}} \) when tested at the concentration of 3\( \mu \text{M} \). Therefore, the effects of these
compounds at different concentrations on the \( \text{sI}_{\text{AHP}} \), action potentials and the \( \text{Ca}^{2+}\)
current were analysed in greater detail.

5.2.9 Effects of UCL 2027 and UCL 2077 on the \( \text{sI}_{\text{AHP}} \)

The effects of both UCL 2027 and UCL 20277 were concentration-dependent
(Fig 5.14). Maximal inhibition of the \( \text{sI}_{\text{AHP}} \) was achieved within 2min (for an
example, see Fig 5.13C). The effects were also reversible within 5min of washout
(Fig 5.13C). The \( \text{IC}_{50} \) values obtained for UCL 2027 and UCL 2077 were 1.1 \( \pm \)
0.2\( \mu \text{M} \) and 0.5 \( \pm \) 0.1\( \mu \text{M} \) respectively with Hill coefficients of 1.5 \( \pm \) 0.2 and 1.7 \( \pm \) 0.4
respectively (Fig 5.14). Like UCL 1880, the decay time constant (\( \tau_3 \)) and the time to
peak of the \( \text{sI}_{\text{AHP}} \) in the presence of 3\( \mu \text{M} \) UCL 2027 and 1\( \mu \text{M} \) UCL 2077 were
unchanged in comparison to the controls (Table 5.5).
Fig 5.14 Concentration-inhibition curves for the $s_{IAHP}$ in cultured hippocampal pyramidal neurones. The lines show least squares fits of the Hill equation with $y_{max}$ constrained to 100%.
Table 5.5 Decay time constants ($\tau_3$) and times to peak (TP) of the sI_{AHP} in the presence of UCL 2027 and UCL 2077.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Control $\tau_3$ (s)</th>
<th>$\tau_3$ in the presence of drug (s)</th>
<th>Control TP (s)</th>
<th>TP in the presence of drug (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCL 2027 (3µM)</td>
<td>0.84 ± 0.11</td>
<td>1.11 ± 0.29</td>
<td>0.47 ± 0.04</td>
<td>0.52 ± 0.05</td>
</tr>
<tr>
<td>UCL 2077 (1µM)</td>
<td>1.15 ± 0.36</td>
<td>0.93 ± 0.37</td>
<td>0.50 ± 0.02</td>
<td>0.53 ± 0.02</td>
</tr>
</tbody>
</table>
Fig 5.15 Effects of (A) 10μM UCL 2027 and (B) 3μM UCL 2077 on the last action potential in a train. The grey lines represent the last action potential in the train before application of the agents. The records are superimposed so as to align the base of the records. The negligible effect of UCL 2077 on the amplitude of the action potentials in a train is seen by comparing (C) (in the absence) with (D) (in the presence) of the drug. The action potentials shown in (B) are from the records in (C) and (D). The calibration bars in (B) and (D) also apply to (A) and (C) respectively.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Control APW (ms)</th>
<th>APW in the presence of 1μM drug (ms)</th>
<th>Control APW (ms)</th>
<th>APW in the presence of 3μM drug (ms)</th>
<th>Control APW (ms)</th>
<th>APW in the presence of 10μM drug (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCL 2027</td>
<td>0.90 ± 0.15</td>
<td>1.17 ± 0.37</td>
<td>1.36 ± 0.25</td>
<td>2.08 ± 0.38*</td>
<td>1.80 ± 0.17</td>
<td>2.26 ± 0.35</td>
</tr>
<tr>
<td>UCL 2077</td>
<td>1.24 ± 0.20</td>
<td>1.42 ± 0.24</td>
<td>1.90 ± 0.47</td>
<td>2.73 ± 0.62*</td>
<td>Not tested</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

Table 5.6: Effects of UCL 2027 and UCL 2077 on the last action potential width (APW) in the train used to evoke the $sI_{AHP}$. All measurements were made at a potential of -20mV. * indicates significance at the 5% level (i.e. $p < 0.05$).
5.2.10 Effects of UCL 2027 and UCL 2077 on action potentials

In the presence of 3μM concentrations of UCL 2027 and UCL 2077, the width of the last action potential in the train was significantly broader than in the absence of the drugs (see Table 5.6 for values; Fig 5.15A and Fig 5.15B). At lower concentrations, there was no significant effect (Table 5.6). The broadening of action potentials by 10μM UCL 2027 was not statistically significantly (Table 5.6). Neither compound appeared to have effects on the action potential amplitude (Fig 5.15C and Fig 5.15D).

5.2.11 Effects of UCL 2027 and UCL 2077 on the outward holding current

10μM UCL 2027 and 3μM UCL 2077 reduced the outward current present at a holding potential of -50mV (see Fig 5.13C). However, as with UCL 1880, the percentage inhibition was variable and ranged from 5 - 40%. The resting membrane potential was not significantly altered in the presence of the compounds.

5.2.12 Effects of UCL 2027 and UCL 2077 on the HVA current in freshly dissociated hippocampal neurones

10μM UCL 2027 and 3μM UCL 2077 reduced the HVA Ca^{2+} currents by 3.4 ± 4.6% (n=4; Fig 5.16A) and 1.1 ± 1.2% (n=7; Fig 5.16B). The LVA current was also not affected by these compounds. It is, therefore, very unlikely that the effects of these compounds on the sI_{AHP} are due to a reduction of Ca^{2+} entry.

5.2.13 Effects of UCL 2027 and UCL 2077 on the ml_{AHP} in cultured hippocampal neurones

UCL 2027 and UCL 2077 at concentrations of 10μM and 1μM respectively,
Fig 5.16 Effects of (A) 10μM UCL 2027 and (B) 3μM UCL 2077 on the Ca\(^{2+}\) current recorded from freshly dissociated hippocampal neurones. The voltage protocol shown in (C) was applied to evoke both the low voltage-activated (LVA) and the high voltage-activated (HVA) Ca\(^{2+}\) current. In each case, 200μM Cd\(^{2+}\) was also applied to determine the magnitude of the HVA Ca\(^{2+}\) current. The calibration bar shown in (B) also applies to (A).
Fig 5.17 Effects of (A) 10µM UCL 2027 and (C) 1µM UCL 2077 on the mI_{AHP} and sI_{AHP}. The traces in the presence and absence of the compounds have been superimposed. UCL 2027 and UCL 2077 sensitive currents were obtained by subtraction and are shown in (B) and (D). The calibration bars in (D) apply also to (A), (B) and (C) respectively.
reduced the mI_{AHP} by estimated values of -11.6 ± 12% (n=3; Fig 5.17A) and -18.9 ± 10.0% (n=3; Fig 5.17B) respectively. When the I_{AHP} in the presence of UCL 2027 or UCL 2077 is subtracted from the I_{AHP} present under control conditions, only a current with similar kinetics to the sI_{AHP} is observed (Fig 5.17C and 5.17D). The mI_{AHP} also appeared to be unaffected in the presence of 3μM UCL 2077. However, with this concentration, the I_{AHP} was often completely inhibited and a small inward current revealed (see Fig 5.13B), making it difficult to fit the I_{AHP} with the exponential equation described in METHODS (Section 2.1.2.10a). It was, therefore, difficult to estimate the percentage inhibition. The effects of both UCL 2027 and UCL 2077 would thus seem to be quite selective for the sI_{AHP} in hippocampal pyramidal neurones.

5.2.14 Effects of analogues of UCL 2077

From the results presented above, it is clear that both UCL 2027 and UCL 2077 are selective blockers of the sI_{AHP} in hippocampal pyramidal neurones. UCL 2077 is more potent and selective than UCL 2027 and, therefore, compounds that were structurally related to UCL 2077 (Fig 5.19A) were tested on the sI_{AHP} at concentrations of 3μM. The general structure of the compounds is shown in Fig 5.18 and the results are summarized in Table 5.7. As can be seen, none of the analogues were more potent blockers of the sI_{AHP} than UCL 2077. All of the compounds tested produced their maximal effects within 2min of bath application. Reversal of block of the sI_{AHP} occurred within 5min of washout as with UCL 2077. Neither the action potential width nor the time course of the sI_{AHP} were modified in the presence of these compounds.
<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>% inhibition of sI\textsubscript{AHP} at 3\mu M</th>
<th>IC\textsubscript{50} value</th>
<th>% increase in APW at 3\mu M</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCL 2077</td>
<td>\chem{-}</td>
<td>H</td>
<td>98.2 ± 8.5 (n=3)</td>
<td>0.5 ± 0.07\mu M</td>
<td>45.4 ± 3.2%*</td>
</tr>
<tr>
<td>UCL 2323</td>
<td>\chem{\text{N}}</td>
<td>H</td>
<td>60.7 ± 9.8 (n=3)</td>
<td>&lt;3\mu M</td>
<td>18.5 ± 7.4%</td>
</tr>
<tr>
<td>UCL 2328</td>
<td>\chem{-}</td>
<td>H</td>
<td>16.7 ± 15.4 (n=3)</td>
<td>&gt;3\mu M</td>
<td>11.4 ± 7.3%</td>
</tr>
<tr>
<td>UCL 2332-B</td>
<td>\chem{-}</td>
<td>CH\textsubscript{2}CH\textsubscript{3}</td>
<td>-27.3 ± 18.1 (n=3)</td>
<td>&gt;&gt;3\mu M</td>
<td>7.9 ± 4.0%</td>
</tr>
<tr>
<td>UCL 2336-A</td>
<td>\chem{\text{N}}</td>
<td>H</td>
<td>63.1 ± 5.1 (n=3)</td>
<td>&lt;3\mu M</td>
<td>-11.4 ± 11.6%</td>
</tr>
<tr>
<td>UCL 2337</td>
<td>\chem{-}</td>
<td>H</td>
<td>59.7 ± 12.3 (n=4)</td>
<td>~3\mu M</td>
<td>14.3 ± 8.8%</td>
</tr>
<tr>
<td>UCL 2338</td>
<td>\chem{-}</td>
<td>H</td>
<td>12.1 ± 13.7 (n=3)</td>
<td>&gt;3\mu M</td>
<td>21.1 ± 10.6%</td>
</tr>
</tbody>
</table>

Table 5.7: Summary of effects of compounds structurally related to UCL 2077 on the sI\textsubscript{AHP} and the width of the last action potential (APW) in the train used to evoke the sI\textsubscript{AHP}. R\textsubscript{1} and R\textsubscript{2} denote the chemical substituents present on the general structure shown in Fig 5.18. * indicates significance at the 5% level.
Fig 5.18 General structure of the compounds related to UCL 2077.
<table>
<thead>
<tr>
<th>Compound</th>
<th>% inhibition of sIₐHₚ at 3μM</th>
<th>IC₅₀ value</th>
<th>% increase of APW at 3μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCL 2077</td>
<td>98.2 ± 8.5 (n=3)</td>
<td>0.5 ± 0.07μM</td>
<td>45.4 ± 3.2%*</td>
</tr>
<tr>
<td>UCL 1495</td>
<td>28.5 ± 5.2 (n=5)</td>
<td>&gt;3μM</td>
<td>17.2 ± 7.6%</td>
</tr>
<tr>
<td>UCL 2339</td>
<td>9.3 ± 14.7 (n=4)</td>
<td>&gt;3μM</td>
<td>7.2 ± 10.1%</td>
</tr>
</tbody>
</table>

Table 5.8: Summary of the effects of UCL 2077 and the related compounds, UCL 1495 and UCL 2339, on the sIₐHₚ and the width of the last action potential (APW) in the train. * indicates significance at the 5% level.
Fig 5.19 Chemical structures of (A) UCL 2077, (B) UCL 1495 and (C) UCL 2339
Two other compounds that were also related structurally to UCL 2077 (Fig 5.19A), UCL 1495 (Fig 5.19B) and UCL 2329 (Fig 5.19C) were also tested. These compounds had very little effect on the amplitude (Table 5.8) or the kinetics of the sIAHP, action potential width (see Table 5.8), the outward holding current and resting membrane potential of these hippocampal neurones. Like the other compounds tested, the onset of block was very rapid with maximum inhibition occurring within 2min of application. The effects were also reversible within 5min of washout.

5.3 Discussion

5.3.1 Effects of clotrimazole, UCL 1851 and UCL 1880 on the sIAHP and the Ca²⁺ current in hippocampal neurones

In this study, the pharmacology of the sIAHP in hippocampal pyramidal neurones has been explored further. In seeking to identify compounds that may block the sIAHP, we selected for investigation clotrimazole and some simple analogues. Clotrimazole proved to be an effective blocker, inhibiting the sIAHP with an IC₅₀ of 1.7μM. The structurally related compounds UCL 1851 (Fig 5.1B) and UCL 1880 (Fig 5.1C) were also effective with similar IC₅₀s. The imidazole ring present in the structure of clotrimazole is unlikely to contribute to the suppression of the sIAHP as UCL 1851, an in vivo metabolite of clotrimazole lacking the imidazole ring, was an effective inhibitor. The activity of UCL 1880 provides further evidence that the imidazole ring is not crucial. These results also, coincidentally, indicate that the cytochrome P450 inhibitory action of clotrimazole is not involved in the reduction of the sIAHP as this effect depends on the presence of the imidazole ring (Rittenhouse et al., 1997a).
The time to peak and $\tau_3$ as well as the amplitude of the $sI_{AHP}$ were significantly different in the presence of 3$\mu$M UCL 1851 in comparison to control values. With clotrimazole and UCL 1880 only the amplitude of the $sI_{AHP}$ was significantly reduced. These results suggest that UCL 1851 may have different actions to clotrimazole and UCL 1880, though this possibility was not further pursued.

UCL 1851 also differs from clotrimazole, UCL 1880 and other K$^+$ channel blockers such as TEA and 4-AP in that it does not ionise as a base (see Fig 5.1). This may indicate that it may bind to a site distinct from that of clotrimazole and UCL 1880 to exert its actions.

All three compounds also inhibited the Ca$^{2+}$ current in freshly dissociated neurones, with UCL 1851 being the most potent inhibitor ($IC_{50} = 2.7\mu$M). Although there may not be a linear relationship between Ca$^{2+}$ entry and activation of the channels underlying the $sI_{AHP}$, these results make it possible that most of the effects of UCL 1851 are likely to be due to inhibition of Ca$^{2+}$ entry rather than direct block of the $sI_{AHP}$ channels. On the other hand, clotrimazole and UCL 1880 are 5 and 10 times respectively less effective at blocking Ca$^{2+}$ channels than the channels underlying the $sI_{AHP}$ and therefore, the inhibition of the $sI_{AHP}$ by these compounds is less likely to be due to Ca$^{2+}$ channel block. Since clotrimazole is a known K$^+$ channel blocker (see Introduction, Section 5.1), it is quite possible that inhibition of the $sI_{AHP}$ is caused by direct interaction with the K$^+$ channel.

Interestingly, all three compounds inhibit the IK channel present in red blood cells with $IC_{50}$ values between 1$\mu$M and 2$\mu$M (M. Malik-Hall and D.H. Jenkinson, unpublished observations) when the K$^+$ sensitive electrode method (described by Malik-Hall et al. (2000)) is used. However, it should be noted that previous studies
have reported the half-maximal block of IK channels by clotrimazole and UCL 1851 to be in the low nanomolar range (Brugnara et al., 1993; Ishii et al., 1997a; Jensen et al., 1997; Vandorpe et al., 1998). Therefore, it is possible that under the conditions of other studies, UCL 1880 may also be a very potent inhibitor of the IK channel.

5.3.2 Selectivity profile of clotrimazole, UCL 1851 and UCL 1880

Since Ca$^{2+}$ entry through HVA Ca$^{2+}$ channels will depend on action potential width, the effects of the agents on this parameter were also determined. At 10μM, all three compounds broadened action potentials. This effect was also observed at the lower concentration of 3μM for UCL 1851. An increase in action potential width is not surprising for clotrimazole which has been shown to block BK channels (Rittenhouse et al., 1997b; Wu et al., 1999) and voltage-gated K$^+$ channels (Wu et al., 1999; Kim & Gregor, 1999; Hatton & Peers, 1996). Since the compounds tested are structurally related (Fig 5.1), it is possible that UCL 1851 and UCL 1880 (especially at high concentrations) may also inhibit voltage-gated K$^+$ channels and BK channels, thereby causing action potential widening. All three compounds also inhibit Ca$^{2+}$ channels (Fig 5.3). Any Ca$^{2+}$ channel block by the compounds would lead to a reduction in Ca$^{2+}$ entry and reduced activation of BK channels, thereby broadening action potentials. A complicating factor is that action potential broadening, however caused, would tend to increase Ca$^{2+}$ entry and so potentially enhance the sI_AHP; this is indeed seen with charybdotoxin (see Chapter 3).

Significant inhibition of Ca$^{2+}$ channels would also be expected to cause reduction of the mI_AHP in these cells (as described for Cd$^{2+}$; see Chapter 3). The finding that clotrimazole reduced the mI_AHP by approximately 50% at 10μM is consistent with its Ca$^{2+}$ channel blocking activity but might also be at least partly due
to blocking the SK channels that underlie the mI\textsubscript{AHP}. At 10\mu M, both UCL 1851 and UCL 1880 also block a large fraction of the HVA Ca\textsuperscript{2+} current and might, therefore, be expected to also inhibit the mI\textsubscript{AHP}. However, neither UCL 1851 nor UCL 1880 had any significant effect on the mI\textsubscript{AHP} at 10\mu M. From this result, it would appear that SK channel activation is not linearly related to Ca\textsuperscript{2+} entry though HVA channels. It is also possible that both UCL 1880 and UCL 1851 may be less effective at inhibiting Ca\textsuperscript{2+} entry via N-type Ca\textsuperscript{2+} channels, which has been suggested to be the main contributor to the generation of the mI\textsubscript{AHP} (P. Pedarzani, personal communication). These results suggest that both UCL 1851 and UCL 1880 do not block the SK channels underlying the mI\textsubscript{AHP} and, more importantly that there is sufficient Ca\textsuperscript{2+} entry in the presence of these compounds to activate the mI\textsubscript{AHP}, reinforcing the suggestion that the relationship between Ca\textsuperscript{2+} entry and the activation of the mI\textsubscript{AHP} is not linear.

Homomeric SK2 or heteromeric SK1/SK2 channels have been suggested to underlie the mI\textsubscript{AHP} (Stocker \textit{et al.}, 1999). \textit{In situ} hybridization studies show that there is very little SK3 mRNA present in hippocampal pyramidal cells (Stocker \textit{et al.}, 1999; Stocker and Pedarzani, 2000). SK3 mRNA, however, is present abundantly in SCG neurones (Hosseini \textit{et al.}, 1999). Moreover, the pharmacology of the cloned rSK3 channels matches the pharmacology of the mAHP in SCG neurones (Hosseini \textit{et al.}, 1999) and therefore, it is likely that homomeric SK3 channels underlie the mAHP in these neurones. In light of recent results showing that SK1 mRNA is also present in SCG neurones (R. Hosseini, personal communication), it is possible that SK1/SK3 heteromeric channels or a combination of SK1 and SK3 homomeric channels might also underlie the mI\textsubscript{AHP}. To obtain a selectivity profile of clotrimazole and the analogues, these compounds were tested on the mI\textsubscript{AHP} in SCG
neurones. Neither clotrimazole nor the analogues UCL 1851 or UCL 1880 were effective at inhibiting the mIAHP in these neurones at concentrations up to 10μM suggesting that these compounds are not likely to inhibit SK3 channels or the Ca\textsuperscript{2+} channels that are involved in the generation of the mIAHP. Ca\textsuperscript{2+} entry via N-type Ca\textsuperscript{2+} channels selectively activates the mIAHP in SCG neurones (Davies et al., 1996). SCG neurones have been reported to express a different splice variant of the N-type Ca\textsuperscript{2+} channel to that present in the brain (Lin et al., 1997). In view of the large inhibition of the Ca\textsuperscript{2+} current in hippocampal neurones by clotrimazole and UCL 1851, it is possible that these compounds may be able to distinguish between the two N-type channel splice variants described so far.

In this study clotrimazole was found to inhibit the outward current present at the holding potential of -50mV in a concentration dependent manner (IC\textsubscript{50} = 3μM; Fig 5.5B). UCL 1851 and UCL 1880 were less effective inhibitors of this outward current. The amount of outward current present at a given potential is determined by the resting membrane potential of the cell. In keeping with this, clotrimazole at concentrations greater than 3μM, significantly depolarized the neurones. Two pore K\textsuperscript{+} channels are likely to significantly contribute to the resting membrane potential of many cells including neurones (for example see Duprat et al., 1997; Millar et al., 2000; Talley et al., 2000) and hence, it is a possibility that clotrimazole may inhibit these channels.

5.3.3 Effects of analogues of UCL 1851

As UCL 1851 has been described to be a potent blocker of the IK channel (Rittenhouse et al., 1997a; Vandorpe et al., 1998), the structurally related analogues, UCL 1893, UCL 1864 and UCL 1887 (Table 5.3) were tested. Only UCL 1893
(where the chlorine substituent is in the para instead of the ortho position) was as effective as UCL 1851 at blocking the sI_{AHP}. The other compounds had very little effect. In contrast to UCL 1851, t_3 was not altered in the presence of UCL 1893, indicating that the mechanism of block for UCL 1893 may be different to that of UCL 1851. As UCL 1893 was not more potent than UCL 1851, its selectivity for the sI_{AHP} was not further explored.

Interestingly, when these compounds were tested for their blocking action of the IK channel in red blood cells using the K⁺ sensitive electrode method, UCL 1864 was the most potent blocker with an IC₅₀ of 0.5μM (M. Malik-Hall and D.H. Jenkinson, unpublished observations). UCL 1851, UCL 1887 and UCL 1893 had IC₅₀ values of 1μM on the red cell IK channel. These results therefore also indicate that the channel underlying the sI_{AHP} is not likely to be the conventional IK channel.

### 5.3.4 Effects of analogues of UCL 1880

The effects of several compounds that were structurally related to UCL 1880 (Table 5.4) were also studied on the sI_{AHP}. For all compounds tested, apart from UCL 2110, the reduction of the sI_{AHP} at 3μM was comparable to that produced by UCL 1880. The results suggest that altering the position of the nitrogen atom in the pyridine ring (UCL 2049) or changing the ring structure to a pyrimidine (UCL 2019) or a pyrazine (UCL 2026) may not be crucial for activity. Increasing the distance between the amino nitrogen atom and the pyridine ring (UCL 1880 to UCL 2077; Table 5.4 and Fig 5.19A) improved the activity. However, the importance of the pyridine ring is difficult to comment upon as UCL 2027 (Table 5.4) which contains a thiazole ring still has very high activity. The low potency of UCL 2110 suggests that the addition of a chlorine atom to the aminopyridine ring has detrimental effects.
With the exception of UCL 1892, where recovery after washout was very slow, the onset and offset rates were quite fast and very similar for all compounds. The difference in structure between UCL 1892 and UCL 1880 is in the position of the chlorine atom on the triphenyl ring structure (Table 5.4 and Fig 5.12). This indicates that moving the chlorine atom to the para position on the phenyl ring may reduce the rate of dissociation of UCL 1892 from its target.

UCL 1892, UCL 2027, UCL 2049 and UCL 2077 all increased the action potential width at concentrations of 3μM, suggesting that these compounds may block the K⁺ channels involved in the repolarization phase of action potentials either directly or indirectly via Ca²⁺ channel block (but this is not likely for UCL 2027 or UCL 2077). Unlike clotrimazole, all of the compounds tested had little effect on the outward holding current or on the resting membrane potential.

5.3.5 Pharmacological profile of UCL 2027 and UCL 2077

Construction of concentration-inhibition curves for UCL 2027 and UCL 2077 revealed IC₅₀ values of 1.1μM and 0.5μM respectively. These compounds also had negligible Ca²⁺ channel blocking effects at the doses that abolished the sIₐHₚ, indicating that the effects on the sIₐHₚ were independent of inhibition of Ca²⁺ entry. At concentrations of 3μM, both the compounds broadened the action potentials. These effects are likely to be due to direct block of the K⁺ channels involved in the repolarization phase rather than inhibition of Ca²⁺ entry. At the maximum doses used, both compounds had a small effect on the outward holding current present at the potential of -50mV but no effect on the resting membrane potential was detectable. There was also no effect of either of the two compounds on the mIₐHₚ. When tested on the IK channel present in red blood cells using the K⁺ sensitive
electrode method, the IC$_{50}$ value for UCL 2077 was approximately 5µM whereas UCL 2027 had an IC$_{50}$ value greater than 10µM (M. Malik-Hall and D.H. Jenkinson, unpublished observations). Hence, both compounds show useful selectivity for the sI$_{AHP}$.

The sI$_{AHP}$ is regulated by a number of intracellular kinases and phosphatases (Pedrazani and Storm, 1993, 1995; Pedrazani et al., 1998; Krause and Pedrazani, 2000). The possibility that these compounds may exert their effects by activating or inhibiting these enzymes cannot be ruled out and will be returned to in DISCUSSION (Chapter 8).

5.3.6 Exploring the structure-activity relationship of UCL 2077

As UCL 2077 was the most selective compound for inhibition of the sI$_{AHP}$, compounds related to UCL 2077 were synthesized and tested for inhibition of the sI$_{AHP}$. From the results obtained, it was very clear that the presence of two nitrogen atoms enhances activity as UCL 2328, which has a phenyl ring instead of the pyridine ring as in UCL 2077, and UCL 2338, which has a thiophene instead of the pyridine ring, are less effective. It is difficult to assess the importance of the pyridine ring present in UCL 2077. Replacing this ring with a benzimidazole ring (UCL 2336-A) or a morpholine ring (UCL 2337) reduced activity slightly. However, it should be noted that with UCL 2337 an ethylene chain separated the morpholine ring from the nitrogen attached to the triphenylmethyl moiety instead of a methylene group as in UCL 2077. The presence of an ethylene chain instead of methylene (UCL 2323) does reduce activity. It should be noted that UCL 2323 has a 2-pyridyl group and not the 3-pyridyl group present in UCL 2077.
Another essential feature of blockers of the sI_{AHP} is the presence of the secondary amino group attached to the triphenylmethyl moiety. A tertiary amine as in UCL 2332 and UCL 2339 has lower activity, suggesting that the presence of the hydrogen atom at R_2 (Fig 5.18) may be important for hydrogen bonding to the target. Replacing the secondary amino group with an ester group (UCL 1495) appears to reduce activity, though it should be noted that UCL 1495 differs structurally from UCL 2077 in other respects, too (Fig 5.19). UCL 1495 is also an effective blocker of the IK channel in red blood cells (Benton et al., 1994) and therefore, can be used as a tool to distinguish between IK channels and the sI_{AHP} channels.

From the data presented, it is not known whether increasing the distance of the amino nitrogen from the triphenylmethyl moiety may improve activity. Future experiments should also assess the importance of the triphenylmethyl structure.

5.3.7 Conclusions

In this section, the pharmacology of the sI_{AHP} has been explored further, with the discovery of at least two novel, selective inhibitors of the sI_{AHP}, UCL 2027 and UCL 2077. The mechanism of their action and their target still remain to be identified.
CHAPTER 6

THE PHARMACOLOGY OF SK1 CHANNELS IN MAMMALIAN CELL LINES

6.1 Introduction

Noise analysis studies have suggested that the channel underlying the sAHP has a conductance of 10pS in symmetrical K⁺ (Sah and Isaacson, 1995; Valiante et al., 1998). This finding taken together with the fact that the generation of the sAHP is dependent upon Ca²⁺ has led to the suggestion that small conductance Ca²⁺-activated K⁺ channels (SK channels) underlie the sAHP (for reviews see Sah, 1996 and Vergara et al., 1998). In recent years, three SK channels (SK1-3) have been cloned (Köhler et al., 1996). Of these 3 SK channels, SK2 and SK3 homomeric channels have high affinities for apamin (IC₅₀s of 60pM and ~1nM respectively; Köhler et al., 1996; Ishii et al., 1997b; Vergara et al., 1998). Only SK1 formed homomeric channels that were insensitive to 100nM apamin when expressed in Xenopus oocytes (Köhler et al., 1996; Ishii et al., 1997b). In addition, the distribution of SK1 mRNA appeared to match that of the sIAHP (Köhler et al., 1996). These channels, therefore, have been suggested to underlie the apamin-insensitive sIAHP in neurones (Vergara et al., 1998; Bond et al., 1999; Castle, 1999).

In the previous chapter, the pharmacology of the sIAHP was studied, with the identification of two novel selective inhibitors of the current, UCL 2027 and UCL 2077. However, it is unknown as to whether these compounds inhibit the channel underlying the sIAHP directly or via an intracellular mechanism. As it has been hypothesized that SK1 homomeric channels may underlie the sIAHP (Vergara et al.,
1998; Bond et al., 1999), it was decided to test the compounds, UCL 1880, UCL 2027 and UCL 2077 on the current formed by SK1 homomorphic channels. Both rSK1 and hSK1 cDNA were transiently transfected in HEK293 cells. In addition, hSK1 cDNA was also expressed in cos-7 cells. As neither hSK1 nor rSK1 cDNA has been expressed previously in mammalian cell lines, it was necessary to characterize the currents recorded from the transfected cells.

6.2 Results

6.2.1 Transfection of rSK1 cDNA in HEK293 cells

rSK1 cDNA together with GFP cDNA or CD8 cDNA was transiently transfected into HEK293 cells. Whole cell recordings with 1µM free Ca\(^{2+}\) in the patch pipette were made from cells that were fluorescent (indicative of GFP expression) or from cells that had at least 5 Dynal beads on the surface (indicative of CD8 expression) at least 24hrs after transfection. The input resistance of the transfected cells was at least 800M\(\Omega\) and was similar to the input resistance of untransfected cells (at least 1G\(\Omega\)). Only delayed rectifier type currents (Fig 6.1B) could be recorded from rSK1 transfected HEK293 cells (n=8) when patched with pipettes containing 1µM free Ca\(^{2+}\) and a step voltage protocol (shown in Fig 6.1A) was applied from a holding potential of −80mV. Similar currents could be recorded from untransfected cells when they were patched onto with pipettes containing 1µM free Ca\(^{2+}\) and the step voltage protocol applied (Fig 6.1A). This agrees with the findings of Yu and Kerchner (1998) for native HEK293 cells. The resting membrane potentials of transfected cells under these conditions were between −20mV and 0mV.
Fig 6.1 (A) Current recorded from untransfected HEK293 cells dialyzed with 1\(\mu\)M free Ca\(^{2+}\) and subjected to voltage steps from -140mV to +40mV (shown in inset). (B) and (C) Current obtained from HEK293 cells transiently transfected with rSK1 and hSK1 respectively and dialyzed with 1\(\mu\)M free Ca\(^{2+}\) (same voltage protocol as in A). (D) The I-V curve for the cell in (C). The calibration bars in (A) also apply to (B) and (C).
(n=8). Again, these values were similar to those recorded from untransfected cells. These results suggested that functional rSK1 channels were not being expressed.

Examination of the nucleotide sequence of rSK1 (see Fig 2.1, METHODS) revealed a potentially false start sequence (weak Kozak consensus site followed by a methionine) in a different frame upstream from the desired methionine start sequence. Hence, a possible explanation for the lack of rSK1 current could be that an incorrect protein was being formed. As described in METHODS (Section 2.3.2), the potentially false start sequence was removed from the sequence and the modified rSK1 cDNA was then transfected together with CD8 cDNA in HEK293 cells. The transfected cells were identified by the presence of more than 5 Dynal beads on their surface. Whole cell recordings with patch pipettes containing 1µM Ca²⁺ were made at least 24hrs after transfection. The input resistance of these cells was still at least 800MΩ and the membrane potential of the cells was between −30mV and 0mV. Application of the step voltage protocol shown in Fig 6.1A from a holding potential of −80mV resulted in only delayed rectifier type currents, not dissimilar to those of untransfected cells (data not shown). These results suggest that the modified rSK1 cDNA was also incapable of forming functional SK1 channels.

6.2.2 Transient transfection of hSK1 cDNA into HEK293 cells

As hSK1 mRNA when injected into *Xenopus* oocytes does form functional channels (Köhler *et al.*, 1996; Ishii *et al.*, 1997b), it was decided to transfect hSK1 cDNA into HEK293 cells. Transfected cells usually had 5 or more CD8 antibody coated microspheres attached to them. Transfected HEK293 cells when patched with pipettes containing 1µM free Ca²⁺ gave currents up to 5nA (at +40mV), generally showing outward rectification at negative potentials (Fig 6.1C and 1D). The SK1
currents ran down initially but often stabilized within 5 min. These cells had membrane potentials of \(-80\) mV and the current also reversed at approximately \(-80\) mV (Fig 6.1D). \(E_K\) was set to be \(-80\) mV and this suggests that the current was due to \(K^+\) ions. In contrast to untransfected HEK293 cells, the input resistance was usually less than 100 M\(\Omega\). Therefore, unlike \(\text{rSK1 cDNA}\), transfection of \(\text{hSK1 cDNA}\) results in the formation of channels in mammalian cell lines as well as in \textit{Xenopus} oocytes.

6.2.3 Pharmacological characterization of \(\text{hSK1 current in HEK293 cells}\)

To ensure that the pharmacological properties of the \(\text{hSK1 current}\) in HEK293 cells were similar to those in \textit{Xenopus} oocytes, 30 nM apamin was applied. This concentration of apamin blocked the \(\text{hSK1 current}\) rapidly (within 2 min) by \(75.6 \pm 5.0\%\) (\(n=4\); Fig 6.2 and 6.3). As block by apamin in many types of neurones is slow to reverse (for example see Kawai and Watanabe, 1986 and Chapter 3), it was expected that the effect of apamin may take a long while to recover. Surprisingly, the effect reversed within 5 min of washout (Fig 6.2). The \(I_{C50}\) value was \(7.7 \pm 1.7\) nM (Hill coefficient of unity, Fig 6.3). It should be noted, however, that in 15\% (3/20) of the cells tested the current was found to be only partially inhibited by 30 nM apamin (37.2 \(\pm\) 2.0\%, \(n=3\)). The results from these cells were excluded from the data presented above.

As apamin significantly inhibits the \(\text{hSK1 current}\) in HEK293 cells, other blockers of the apamin-sensitive current were also tested. Tubocurarine and dequalinium also reduced the \(\text{hSK1 current}\) significantly (Fig 6.3, 6.4A and 6.4B). The \(I_{C50}\) values for the block by tubocurarine and dequalinium were found to be 23.5 \(\pm\) 5.3 \(\mu\)M (Fig 6.3) and 0.48 \(\pm\) 0.1 \(\mu\)M (Fig 6.3) respectively. UCL 1848 potently
Fig 6.2 An example of the block by apamin of the current produced by hSK1 transfected in HEK293 cells (A). The trace in the presence of 30nM apamin (B) was obtained 2 min after bath-application of the drug. The recovery trace (C) was obtained 4 min after washout. The I-V curves for (A) - (C) are shown in (D). The dotted line represents the I-V curve for the recovery trace. The calibration bars in (C) also apply to (A) and (B).
Fig 6.3 Concentration-inhibition curves for apamin, UCL 1848, tubocurarine and dequalinium. All the curves were fitted using the Hill equation with $y_{\text{max}}$ constrained to 100%. For the dose-response curves for apamin, tubocurarine and dequalinium, the Hill slopes have been constrained to one. Each data point is the mean and standard error of 3-7 observations.
Fig 6.4 I-V curves in the presence of (A) dequalinium, (B) tubocurarine, (C) UCL 1848 and (D) 1-EBIO. HEK293 cells were transiently transfected with hSK1 cDNA and patched onto with pipettes containing 1µM free Ca^{2+}. The I-V curves were obtained by applying the voltage protocol shown in Fig 6.1A. The dotted lines represent the I-V curves for the recovery traces.
blocked the hSK1 current ($IC_{50} = 1.1 \pm 0.4\text{nM}$; Fig 6.3 and 6.4C). The concentration-response curve produced by UCL 1848, however, had a shallow slope of $0.4 \pm 0.1$ when fitted with the Hill equation. Like apamin, the block produced by all these compounds was rapid, with recovery occurring within 5min of washout.

1-EBIO (300μM), an activator of the hIK1 current (Jensen et al., 1998; Pedersen et al., 1999; Warth et al., 1999) and rSK2 channels (Syme et al., 2000), increased the magnitude of the hSK1 current by 41.8 ± 16.2% at -40mV (n=3; Fig 6.4D) in cells patched onto with pipettes containing 1μM free Ca$^{2+}$. This finding indicates that 1-EBIO is an ‘opener’ of SK1 channels, also.

The effects of the sI_{AH}P inhibitors, UCL 1880, UCL 2027 and UCL 2077 were also studied on the hSK1 current. 10μM UCL 1880 and 3μM UCL 2077 inhibited the hSK1 current by 44.2 ± 3.0% (n=4; Fig 6.5A) and 27.2 ± 3.8% (n=4; Fig 6.5C) respectively. In contrast, 10μM UCL 2027 had very little effect on the hSK1 current (% inhibition = 1.6 ± 5.6%, (n=5); Fig 6.5B). It should be noted that in all these cells application of 10nM UCL 1848 significantly inhibited the hSK1 current by 61.5 ± 4.3% (n=7), a value comparable to the previous results (see Fig 6.3).

6.2.4 hSK1 expression in cos-7 cells

As the pharmacology of hSK1 expressed in HEK293 cells is very different from that in Xenopus oocytes, it was decided to express hSK1 cDNA in cos-7 cells to see whether the above findings can be confirmed in a second mammalian cell line. Untransfected cos-7 cells possess only a very small delayed rectifier type current when patched with pipettes containing 1μM free Ca$^{2+}$ (n=5; Fig 6.6A). The membrane potential of these cells was around 0mV and the input resistance was
Fig 6.5 I-V curves in the presence of (A) UCL 1880, (B) UCL 2027 and (C) UCL 2077. The I-V curves were obtained by applying the voltage protocol shown in Fig 6.1A to HEK293 cells transiently transfected with hSK1 and patched with pipettes containing 1μM free Ca^{2+}. The dotted lines represent the traces after drug washout.
Fig 6.6 (A) and (B) represent the whole cell currents in an untransfected cos-7 cell and a transfected cos-7 cell respectively when the cells were dialyzed with a pipette solution containing 1μM free Ca^{2+} and the voltage protocol shown in (A) applied. (C) is the I-V curve for the current shown in (B). The calibration bars shown in (B) also apply to (A).
Fig 6.7 Effect of apamin on I-V curves obtained from (A) cos-7 cells transfected with hSK1 and (B) HEK293 cells transfected with rSK2. The cells were patched with pipettes containing 1μM free Ca$^{2+}$. The dotted lines represent the traces after 2min of washout for (A) and 10min of washout for (B).
more than 1GΩ. In contrast, transfected cos-7 cells had an input resistance of less than 100MΩ and membrane potentials between -70mV and -80mV. Currents of up to 4nA (at +40mV) could be recorded (Fig 6.6B). These currents were generally stable, showing little rundown. The current reversed at -80mV (Fig 6.6C), suggesting that it was carried by K⁺.

Apamin also rapidly blocked the hSK1 current in cos-7 cells (Fig 6.7A) with an IC₅₀ value of 12.2 ± 1.3nM (Hill coefficient constrained to 1; Fig 6.3). This IC₅₀ value is comparable to that observed in HEK293 cells (IC₅₀ of 8nM). The effect was reversible within 5min (Fig 6.7A).

6.2.5 rSK2 expression in HEK293 cells

To determine whether the properties of other SK channels also vary when expressed in mammalian cell lines, we tested the effects of apamin on HEK293 cells stably transfected with the rSK2 gene. In HEK293 cells expression of rSK2 generated currents of up to 5nA (at +40mV) when patched onto with 1μM free Ca²⁺ in the patch pipette (Fig 6.7B). As found in Xenopus oocytes, apamin was an extremely potent blocker, with 100pM apamin giving 71 ± 8% inhibition within 5min (n=4; Fig 6.7B). The effects were reversible within 10min of washout.

6.3 Discussion

6.3.1 rSK1 expression in HEK293 cells

The sIₐHP previously had been recorded from rat hippocampal pyramidal neurones. A proper study of the role of SK1 channels in hippocampal neurones would have ideally compared the pharmacology of the sIₐHP with currents obtained
from the rat SK1 channels. Attempts were accordingly made to express the rat clone. However, currents recorded from transfected cells did not differ from untransfected cells when patched onto with 1μM free Ca$^{2+}$ in the pipette (Fig 6.1). The input resistance and the membrane potentials of the cells were indistinguishable from those of untransfected cells. These results indicated that no functional SK1 channels were being formed.

A potentially false start sequence was identified in a different frame in the rSK1 nucleotide sequence and was successfully removed (see METHODS, Section 2.3.2). However, transfection of the modified rSK1 sequence into HEK293 cells also failed to generate currents attributable to SK1 expression. Again, no difference in the input resistance or membrane potentials of the cells could be detected. One possibility could be that the rSK1 mRNA is not being translated. However, results from in vitro translation assays show that the cell ribosomes are capable of translating the rSK1 mRNA (W. J. Joiner, personal communication). With the modified rSK1 sequence, there is only one start sequence and, hence, the correct protein should be formed. These findings, therefore, suggest that the protein being formed is either not being directed to the cell surface or may require additional subunits to form functional channels. Although the rSK1 pore sequence is almost identical to the hSK1 pore sequence, the C and N termini sequences do differ between the two (Köhler et al., 1996). Hence, it is possible that rSK1 protein may require additional subunits to form functional channels. To detect whether the SK1 protein formed is being directed within the cell, antibodies that specifically recognize the SK1 protein are required. As yet, no antibodies are commercially available that recognize the expressed SK1 proteins.
Both *in situ* hybridization studies (Stocker *et al.*, 1999) and northern blot analysis (R. Hosseini, personal communication) have shown that SK1 mRNA is present in rat hippocampal tissue. However, again antibodies would be required to determine the cell surface expression pattern of SK1 in native hippocampal pyramidal neurones. To determine whether rSK1 requires additional subunits to form functional channels in native cells, biochemical and molecular biological studies would have to be done.

### 6.3.2 Characterization of hSK1 currents in mammalian cell lines

In contrast to rSK1, hSK1 cDNA transfection into both HEK293 cells and cos-7 cells resulted in the generation of Ca$^{2+}$-activated K$^+$ currents. The surprising and important finding of this study is the current due to hSK1 channels is predominantly apamin-sensitive (IC$_{50}$ of 8nM; Fig 6.2 and 6.3). The effect of apamin is very fast in onset, with the maximum effect occurring within 2 min. Moreover, the effect of apamin was also readily reversible (Fig 6.2). In neurones, the effect of apamin on the AHP is usually very slowly reversible (for example see Kawai and Watanabe, 1986 and Goh and Pennefather, 1987; also see Chapter 3). This finding suggests that although apamin does bind to hSK1 channels expressed in mammalian cell lines, the association may not be so ‘tight’ as with the endogenous neuronal SK channels. These results, however, are very different from those obtained when hSK1 is expressed in *Xenopus* oocytes (Köhler *et al.*, 1996; Ishii *et al.*, 1997b).

To find out whether this effect was unique to apamin, other blockers of the apamin-sensitive channels were also tested. The hSK1 current in HEK293 cells was also moderately sensitive to tubocurarine (IC$_{50}$ of 23μM; Fig 6.3 and 6.4) whereas the current obtained when hSK1 is expressed in *Xenopus* oocytes is relatively
Insensitive (IC\textsubscript{50} of 350\textmu M; Ishii \textit{et al.}, 1997). In addition, hSK1 channels in mammalian cell lines were also blocked by dequalinium and UCL 1848 (Fig 6.3 and 6.4), which are known inhibitors of apamin-sensitive K\textsuperscript{+} channels (Castle \textit{et al.}, 1993; Dunn, 1994; Benton \textit{et al.}, 1999b). UCL 1848 was particularly potent (Fig 6.3). Interestingly, the dose-response curve observed for this compound was shallow (Hill slope = 0.4), suggesting that binding sites with different affinities for UCL 1848 may be present.

In contrast to the findings with SK1, the pharmacology of SK2 and SK3 expressed in mammalian cell lines does not seem to differ from that in \textit{Xenopus} oocytes. With rSK2, the results presented in this chapter give an IC\textsubscript{50} for apamin just below 100pM, which is comparable to the IC\textsubscript{50} of 60pM in oocytes (Kähler \textit{et al.}, 1996) and 83pM in mammalian cell lines (Strobaek \textit{et al.}, 2000). For rSK3 expressed in HEK293 cells, the IC\textsubscript{50} for apamin is approximately 2nM (Hosseini \textit{et al.}, 1999), close to the value of 1nM observed in oocytes (Ishii \textit{et al.}, 1997b; Vergara \textit{et al.}, 1998). Therefore, of the SK channels so far cloned, only the pharmacology of hSK1 significantly differs in different expression systems.

\textbf{6.3.3 Effect of 1-EBIO on the hSK1 current}

1-EBIO, an opener of IK1 (Jensen \textit{et al.}, 1998; Pedersen \textit{et al.}, 1999; Warth \textit{et al.}, 1999) and rSK2 channels (Syme \textit{et al.}, 2000), could also activate hSK1 channels in the presence of Ca\textsuperscript{2+} (Fig 6.4). Interestingly, although 300\textmu M 1-EBIO increased the magnitude and duration of the mI\textsubscript{AHP} in hippocampal pyramidal neurones, it had no effect on the sI\textsubscript{AHP} (see Chapter 3). These results provide further evidence that SK1 channels, at least in this conformation, do not underlie the sI\textsubscript{AHP}. 

178
1-EBIO is only effective as an opener of IK and SK channels in the presence of Ca\(^{2+}\) (Pedersen et al., 1999; Syme et al., 2000; Oliver et al., 2000). However, conflicting evidence has been presented on the possible interaction of 1-EBIO with SK channels in *Xenopus* oocytes. Syme et al. (2000) have shown that an increase in the open probability of the channels occurs in the presence of 1-EBIO with no change in the Ca\(^{2+}\) sensitivity of the channels, resulting in an enhancement of the current amplitude. On the other hand, Oliver et al. (2000) have reported a shift in the Ca\(^{2+}\) sensitivity of SK channels towards lower concentrations in the presence of 1-EBIO, without affecting activation kinetics or current amplitude. In the present work, the magnitude of the SK1 current was increased in the presence of Ca\(^{2+}\). Assuming that the Ca\(^{2+}\) sensitivity of SK1 channels in mammalian cell lines is similar to that reported for oocytes, i.e. that 1\(\mu\)M Ca\(^{2+}\) is maximal (Xia et al., 1998), these results suggest that 1-EBIO may increase the open probability of SK1 channels without affecting the Ca\(^{2+}\) sensitivity of the channels. However, further experiments would have to be carried out to determine whether Ca\(^{2+}\) is required for the effect of 1-EBIO to occur and whether the Ca\(^{2+}\) sensitivity of the channels is altered in mammalian cell lines.

### 6.3.4 Effects of inhibitors of the sI\_AHP on the hSK1 current

In the previous chapter, novel selective inhibitors of the sI\_AHP were described. Their mechanism of action, however, is unknown. As SK1 channels have been suggested to underlie the sAHP (Vergara et al., 1998; Bond et al., 1999), it was clearly of interest to test the effects of these compounds on the hSK1 current. One of the most selective inhibitors of the sI\_AHP, UCL 2027 (10\(\mu\)M) had little effect on this current in HEK293 cells. Application of 10\(\mu\)M UCL 1880 and 3\(\mu\)M UCL 2077 did
inhibit the apamin-sensitive hSK1 current by 44% and 23%. In contrast to these results, UCL 1880, UCL 2027 and UCL 2077 all inhibit the $i_{\text{AHP}}$ with IC$_{50}$s of 1.3μM, 1.2μM and 0.5μM respectively (see Chapter 5). However, it should be noted that UCL 1848 (10nM) significantly inhibited the current in the cells that the compounds were tested on. These compounds have very little effect on apamin-sensitive currents (see Chapter 5) and, therefore, it is perhaps not surprising that they have small effects on the apamin-sensitive conformation of the hSK1 channels. It is possible that these compounds may have different effects on the rat SK1 channels or on the apamin-insensitive hSK1 channels formed in *Xenopus* oocytes.

6.3.5 Possible reasons for the apamin-sensitivity of hSK1 channels in mammalian cell lines

These results show that the hSK1 protein can form an apamin-sensitive channel when expressed in mammalian cell lines. One possible explanation is that there is a change in the folding or assembly of the channels as, for example, occurs with nicotinic receptors (Lewis *et al.*, 1997). Interestingly, in this case, a small proportion of the channels in the mammalian cell line behaved as in oocytes whilst the properties of the majority of the channels corresponded to the native channel phenotype. We have also found that in 3/20 of the cells tested, the current was only partially blocked by apamin, suggesting that some apamin-insensitive hSK1 channels, possibly similar to those in oocytes, may assemble in the mammalian cell lines we have examined.

An alternative explanation for these findings is that additional accessory subunits expressed in either some mammalian cell lines or *Xenopus* oocytes may be able to combine with hSK1 and change the properties of the channel that is formed.
Indeed, Wadsworth et al. (1997) have purified a number of polypeptides with different molecular weights, which can be photolabelled by apamin. These may represent SK (α) subunits and possible β subunits' alone or in combination. The presence or absence of these additional subunits may determine the apamin-sensitivity of hSK1.

It is worth noting that SK1 channels in these mammalian cell lines are still less sensitive to apamin than SK2 and SK3 channels. Therefore, the amino acid residues that Ishii et al. (1997b) suggested were important for apamin sensitivity in oocytes, are probably also important for apamin binding in mammalian cell lines. However, these residues may also influence the folding or the binding of auxiliary subunits to SK1 subunits as suggested by the result that mutated SK1 subunits form channels that are less susceptible to rundown in oocytes (Khawaled et al., 1999).

6.3.6 Role of SK1 channels in hippocampal pyramidal neurones

The present work shows that hSK1 in mammalian cell lines mainly forms apamin-sensitive channels. As the pore region and transmembrane regions of rSK1 and hSK1 are almost identical, it is likely that rSK1 may also be able to form apamin-sensitive channels. This suggests that in hippocampal pyramidal neurones, SK1 channels, under certain conditions, could exist in a form that could bind to apamin. This raises the possibility that homomeric SK1 or SK2 channels or heteromeric SK1/SK2 channels underlie the apamin-sensitive mlAHP. The IC₅₀ for inhibition by apamin of the mlAHP has been reported to be approximately 0.5nM (Stocker et al., 1999). As apamin is a more potent blocker of homomeric rSK2 channels in both Xenopus oocytes and mammalian cell lines (Köhler et al., 1996; Ishii et al., 1997b; Strobaek et al., 2000), it suggests that either a combination of
homomeric SK1 and SK2 channels or heteromeric SK1/SK2 channels could underlie
the mI_AHP. It should be noted that though both UCL 1880 and UCL 2077 did inhibit
the apamin-sensitive hSK1 channels, neither had any detectable effects on the mI_AHP
in rat hippocampal pyramidal neurones. As rSK1 cannot be expressed, the affinity of
UCL 1880 and UCL 2077 for rSK1 channels is uncertain.

Because hSK1 cDNA can form channels that are inhibited by apamin and can
be activated by 1-EBIO in the presence of Ca^{2+}, it is doubtful as to whether SK1
subunits can be solely responsible for the apamin-insensitive sAHP. Therefore,
the sAHP.
CHAPTER 7

CHARACTERIZATION OF OUTWARD CURRENTS PRODUCED BY RAISING [Ca\(^{2+}\)] \textit{VIA ALTERNATIVE METHODS}

\subsection*{7.1 Introduction}

The most common methods of activation of the \(i_{\text{AHP}}\) in hippocampal pyramidal neurones are by the use of a train of action potentials or a depolarizing step. These methods of activation, however, involve Ca\(^{2+}\) entry via voltage-gated Ca\(^{2+}\) channels. Experiments involving caged Ca\(^{2+}\) compounds have suggested that a rise in [Ca\(^{2+}\)], without the activation of voltage-gated Ca\(^{2+}\) channels can also activate a current with the properties of the \(i_{\text{AHP}}\) (Sah and Clements, 1999). Whether a current resembling the \(i_{\text{AHP}}\) can be activated by Ca\(^{2+}\) entry via ligand gated ion channels or by Ca\(^{2+}\) released from intracellular stores by caffeine is still unclear (Nicoll and Alger, 1981; Zorumski \textit{et al.}, 1989; Mistry \textit{et al.}, 1990; Uneyama \textit{et al.}, 1993; Shirasaki \textit{et al.}, 1994; Harata \textit{et al.}, 1996; Yu \textit{et al.}, 1999).

Previous studies have demonstrated that under voltage-clamp conditions, glutamate receptor activation can induce an outward K\(^+\) current (Nicoll and Alger, 1981; Zorumski \textit{et al.}, 1989; Mistry \textit{et al.}, 1990; Shirasaki \textit{et al.}, 1994; Harata \textit{et al.}, 1996; Yu \textit{et al.}, 1999). The outward K\(^+\) current has very slow kinetics and requires a rise in [Ca\(^{2+}\)] for activation. Glutamate receptors have been divided into two categories: the ion channel forming or ionotropic receptors and the metabotropic receptors which are coupled to G-proteins and linked to various second messenger systems (Ozawa \textit{et al.}, 1998). The ionotropic receptors can be further subdivided
into those that are activated by N-methyl-D-aspartic acid (NMDA), which are Ca\(^{2+}\) permeant, and two others which respond to kainic acid or to \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), which are relatively Ca\(^{2+}\) impermeant. Native NMDA receptors are heteromers, containing the NMDA receptor subunit, NMDAR1 (NR1) and one or more of four other subunits, NMDAR2A (NR2A) – NMDAR2D (NR2D; Ozawa et al., 1998). Hippocampal neurones mainly express NR2A and NR2B subunits together with the NR1 subunit (Monyer et al., 1994; Petralia et al., 1994).

NMDA receptor activation but not AMPA or kainate receptor activation results in the generation of an outward current following an inward current in both hippocampal pyramidal neurones (Zorumski et al., 1989) and in cortical pyramidal neurones (Mistry et al., 1990; Yu et al., 1999). The inward current is carried by both Na\(^+\) and Ca\(^{2+}\), with P\(_{Ca}\)/P\(_{Na}\) calculated to be 10.6 (Mayer and Westbrook, 1987). The outward current in both hippocampal and cortical neurones has been suggested to be due to a Ca\(^{2+}\)-activated K\(^+\) conductance (Zorumski et al., 1989; Mistry et al., 1990; Yu et al., 1999) and has very slow kinetics, similar to those of the sI\(_{AHP}\). In hippocampal pyramidal neurones, it is reported to be insensitive to apamin but can be partially inhibited by 10mM TEA and 500\(\mu\)M d-tubocurarine (Zorumski et al., 1989). The outward current generated by NMDA receptor activation is less well characterized in cortical neurones (Mistry et al., 1990; Yu et al., 1999). Recent experiments have shown that excitatory post-synaptic potentials generated by synaptic release of glutamate and the activation of NMDA receptors are broadened in the presence of isoprenaline (Lancaster et al., 1999). As the sI\(_{AHP}\) is inhibited by isoprenaline, it has been suggested that the K\(^+\) channels underlying the sI\(_{AHP}\) may be activated by Ca\(^{2+}\) influx through NMDA receptor channels (Lancaster et al., 1999).
Metabotropic glutamate receptor (mGluR) activation can also result in the activation of a K$^+$ current in hippocampal pyramidal neurones (Shirasaki et al., 1994; Harata et al., 1996). The activation of this K$^+$ current is due to Ca$^{2+}$ released from the IP$_3$-sensitive Ca$^{2+}$ pool and the ryanodine sensitive Ca$^{2+}$ pool (Shirasaki et al., 1994; Harata et al., 1996). The Ca$^{2+}$-activated K$^+$ current recorded at holding potentials of $-45$ mV to $-40$ mV was insensitive to apamin and iberiotoxin but abolished by charybdotoxin and TEA (Shirasaki et al., 1994; Harata et al., 1996). The current was voltage-independent and therefore, it has been suggested that the K$^+$ channels activated by mGluR stimulation may be of the IK channel type (Shirasaki et al., 1994; Harata et al., 1996). However, northern blot studies have shown that no mRNA of the cloned DCl channel is present in brain tissue (Joiner et al., 1997; Ishii et al., 1997a; Jensen et al., 1998; Vergara et al., 1998).

Release of Ca$^{2+}$ from ryanodine-sensitive pools by caffeine also results in the activation of an outward current in hippocampal pyramidal neurones that is carried by K$^+$ (Uneyama et al., 1993). The outward K$^+$ current at a holding potential of $-40$ mV is suppressed by charybdotoxin, TEA, quinine and Ba$^{2+}$ and is, thus, probably mostly due to activation of BK channels. However, the results presented in Chapter 4 show that in cultured hippocampal pyramidal neurones, CICR can contribute to the activation of the sI$_{AHP}$ (also see Tanabe et al., 1998; Borde et al., 2000), which suggest that the sAHP channels can be activated by Ca$^{2+}$ from this source. Indeed, previous studies have also shown that application of low concentrations of caffeine can enhance the sI$_{AHP}$ (Torres et al., 1996). An outward K$^+$ current evoked by caffeine or theophylline (a second methylxanthine that also causes Ca$^{2+}$ release from ryanodine-sensitive Ca$^{2+}$ stores) at a holding potential of approximately $-40$ mV has also been described in cortical (Munakata and Akaike, 1993) and in bullfrog...
sympathetic neurones (Akaike and Sadoshima, 1989; Marrion and Adams, 1992). In both these cell types Ca\(^{2+}\)-activated conductances including BK channels were found to be involved in the generation of the outward current.

The aim of this study was to characterize further the outward currents evoked by both NMDA receptor activation and ryanodine receptor activation in hippocampal pyramidal neurones. A second goal of this study was to determine whether raising [Ca\(^{2+}\)]\(_i\) other than by activation of voltage-gated Ca\(^{2+}\) channels can evoke a current with the pharmacological properties of the \(\text{sI}_{\text{AHP}}\).

### 7.2 Results

#### 7.2.1 Characterization of the currents evoked by NMDA receptor activation

### 7.2.1.1 Effects of pressure application of 100µM NMDA

300ms pressure applications of 100µM NMDA at a holding potential of −50mV produced inward currents of 0.55 ± 0.17nA (n=9; Fig 7.1A) which developed rapidly and decayed quickly after the NMDA pulse. However, with this concentration of NMDA, no outward current followed the inward current. Increasing the holding potential to −20mV to increase the K\(^+\) driving force also failed to reveal an outward current. Extending the pressure application to 10s (n=4) produced a sharp inward current that showed considerable desensitization to leave a smaller steady state depolarisation (Fig 7.1B). However, there was still no outward current following the inward NMDA response. This suggested that the rise in [Ca\(^{2+}\)]\(_i\) produced by application of 100µM NMDA was not sufficient to activate any Ca\(^{2+}\)-activated channels. In 3 cells a depolarising step from −50mV to +10mV was also applied and in each case a \(\text{mI}_{\text{AHP}}\) as well as a \(\text{sI}_{\text{AHP}}\) were produced. This suggested
Fig 7.1 (A) 300ms and (B) 10s pressure applications of 100μM NMDA onto cultured hippocampal pyramidal neurones. The external solution contained 1μM glycine, 1μM TTX, 5μM DNQX and no Mg²⁺. The horizontal bar on top of the inward NMDA response in (A) shows the duration of application of NMDA. $V_H = -50\text{mV}$. 
SK channels, at least, were present in these cells and that given a sufficient rise in [Ca^{2+}], an outward K$^+$ current might be expected.

7.2.1.2 Effects of pressure application of 1mM NMDA

At a holding potential of -50mV, pressure application of 1mM NMDA produced an inward current of significantly greater magnitude to that produced by 100μM NMDA (0.73 ± 0.06nA, p < 0.05, n=103). The inactivation rate of the NMDA inward current varied between cells, with some cells showing a very fast rate of inactivation (Fig 7.2A) whereas others demonstrated a slower rate or a biphasic offset (Fig 7.2B). In 80% of the cells there was no clear rundown of the inward current during repeated NMDA applications.

47% of the cells (48/103) displayed an outward current following the inward current produced by NMDA receptor activation (Fig 7.2A). The outward current activated by NMDA application increased with successive exposure to NMDA (at 30s intervals). It usually appeared after 2-3 successive applications and reached a peak after 5min. In a few cells, rundown of the outward current was observed. The outward current had a slow rising phase. It peaked 2.5 ± 0.2s (n=48) from the initiation of the inward NMDA current. The outward current produced also had a slow decay and the decay time constant was estimated to be 1.4 ± 0.16s (n=48) by fitting the outward current to the exponential equation described in METHODS (Section 2.1.2.10c). The magnitude of the outward current varied (on average 0.062 ± 0.01nA, n=48). The magnitude of the inward current induced by NMDA receptor activation in cells that had no outward current was not different (0.68 ± 0.07nA, n=55) to cells that had an outward current following the inward current (0.77 ± 0.10nA; n=48; p > 0.05). The presence of the outward current was not dependent on
Fig 7.2 300ms 1mM NMDA pressure applications onto 2 different cells. The cells used in (A) and (B) were of comparable size. The calibration bars shown in (B) also apply to (A). $V_h = -50$mV.
7.2.1.3 Effects of 7-chlorokynurenic acid on currents produced by application of 1mM NMDA

At a concentration of 1mM, NMDA might be expected to have effects on other glutamate receptors. However, application of 1mM NMDA in a glycine-free solution containing the glycine site antagonist 7-chlorokynurenic acid (7-CK, 10μM; Moroni et al., 1991), which will prevent activation of NMDA receptors but leave AMPA, kainate and metabotropic receptors functional, failed to produce both inward and outward currents (inhibition = 98.6 ± 1.4% and 106.2 ± 16.7% respectively, n=5; Fig 7.3). This effect was reversible within 2min. This strongly suggests that the inward current was produced by NMDA receptor-gated channels and the outward current was a consequence of this activation.

7.2.1.4 Pharmacological characterization of the outward current produced by application of 1mM NMDA

As part of the further characterization of the NMDA-induced outward K⁺ current, the effect of 100nM UCL 1848 was studied. UCL 1848 abolished the outward current in 52% (14/27) of the cells tested. In these cells, the inward current decayed less rapidly in the presence of UCL 1848 (Fig 7.4A) and as a result, the method used to calculate the inhibition gave a value in excess of 100% (% inhibition = 177.1 ± 36.8%, n=24; Fig 7.4). The effects of UCL 1848 occurred within 2min of bath application and were reversible within 5min of washout (Fig 7.4). Not surprisingly, in these cells apamin also completely inhibited the outward current.
Fig 7.3 Effects of 10μM 7-chlorokynurenic acid (7-CK) on the inward and outward currents produced by 300ms NMDA pressure applications. 7-CK was dissolved in a glycine-free solution. Both the control and recovery traces were recorded in the presence of 10μM glycine. The inset shows the effect of 7-CK on the outward current on an expanded time scale. The traces have all been superimposed. The records shown in the inset have been smoothed in Microcal Origin 6.0.
Fig 7.4 (A) Effect of 100nM UCL 1848 on the outward current produced by 300ms pressure application of 1mM NMDA. All three traces have been superimposed. (B) Recording of the inward and outward currents in the absence, presence and after washout of UCL 1848. The location of the traces shown in (A) are indicated in (B) as (a), (b) and (c). (NB the apparent reduction in the outward holding current by UCL 1848 in (B) is probably artificial since it was not observed in other cells). $V_H = -50\text{mV}$. 
Fig 7.5 Effects of (A) 100nM apamin, (B) 3μM muscarine and (C) 3μM UCL 1880 on the cells where the outward current was totally inhibited by UCL 1848. The traces have been superimposed. The dotted lines represent the maximum recoveries after washout of the agents. The calibration bars shown in (C) also apply to (A) and (B).
produced, with the inward current also decaying less rapidly (% inhibition = 131.7 ± 16.3%, n=6; Fig 7.5A). The effect of apamin did not, however, reverse after 15min of washout. In all cells tested, both UCL 1848 and apamin had negligible effect on the inward current produced by NMDA (-0.19 ± 3.8% and 4.3 ± 6.3% inhibition respectively; for example see Fig 7.4). In these cells, where the outward current was completely inhibited by UCL 1848, application of 3μM muscarine and 3μM UCL 1880 had little effect (% inhibition = 9.9 ± 8.9% (n=3; Fig 7.5B) and −9.5 ± 20.3% (n=5; Fig 7.5C) respectively).

Since in the presence of both UCL 1848 and apamin a prolonged inward current was detected, it indicated that there was an overlap between the UCL 1848/apamin-sensitive outward currents and the NMDA-induced inward currents (for example see Fig 7.4). Indeed when the currents in the presence of UCL 1848 or apamin were subtracted from the currents in the absence of the drugs, the difference outward current peaked significantly earlier (1.1 ± 0.2s, n=14; p < 0.05; Fig 7.6) than the absolute peak (2.5 ± 0.2s, n=14; Fig 7.6). The magnitude of the subtracted outward current was also greater than that of the control. The decay time constants (1.3 ± 0.1s, n=14) of the subtracted UCL 1848- and apamin-sensitive were not significantly different from the controls (1.1 ± 0.1s, n=14; Fig 7.6).

In 30% (8/27) of the cells on which UCL 1848 was tested, 100nM caused only partial inhibition of the outward current; by 44.2 ± 5.0% (n=8). In the remainder of the cells (5/27), UCL 1848 had very little effect on the outward current (% inhibition = -15.3 ± 4.4%, n=5). In cells in which the outward current was either partially inhibited or insensitive to UCL 1848, both 3μM muscarine and 3μM UCL
peak of UCL 1848-sensitive current

peak of outward current following NMDA receptor activation in the absence of UCL 1848

Fig 7.6 To show that the peak of the UCL 1848-sensitive current (difference of the currents obtained by NMDA pressure application in the absence and presence of 100nM UCL 1848; solid line) occurs earlier than the peak of the net outward current generated by NMDA receptor activation (dotted line). The traces have been superimposed and smoothed using Microcal Origin 6.0.
Fig 7.7 Effects of (A) 3μM muscarine and (B) 3μM UCL 1880 on the outward currents, generated by NMDA receptor activation, which were not inhibited by 100nM UCL 1848. The data has been obtained from two different cells. The traces have been superimposed.
Fig 7.8 To show that the peak of the muscarine-sensitive current occurs earlier than the peak of the outward current generated by NMDA receptor activation (dotted line). Traces have been superimposed. NB the muscarine-sensitive current has been obtained by subtraction of the currents shown in Fig 7.7.
1880 partially reduced the outward current by 42.6 ± 8.9% (n=6; Fig 7.7A) and 33.5 ± 9.7% (n=4; Fig 7.7B) respectively. Both 3μM muscarine and UCL 1880 had small effects on the peak of the inward current due to NMDA receptor activation (% inhibition = -14.2 ± 11.2% (n=9, p > 0.05) and 18.7 ± 5.5% (n=9, p = 0.09) respectively). Muscarine also significantly reduced the outward holding current present at -50mV as described in Chapter 3.

When the currents in the presence of muscarine or UCL 1880 were subtracted from the currents in the absence of the agents, only outward currents were revealed (see Fig 7.8). The kinetics of the outward currents produced by subtraction of the currents in the presence of muscarine or UCL 1880 from the controls did not differ significantly (p > 0.05) from each other. It was, therefore, supposed that both muscarine and UCL 1880 were inhibiting the same channels and hence the data was pooled together. The average decay time constant of the subtracted muscarine- and UCL 1880-sensitive currents (1.6 ± 0.6s, n=9; Fig 7.8) was not significantly different from the average decay time constant of the UCL 1848-sensitive current (1.4 ± 0.2s; n=8) and the control current (1.1 ± 0.1s, n=14). As with the UCL 1848-sensitive current, the peak of the muscarine- and UCL 1880-sensitive current occurred significantly earlier (1.1 ± 0.2s, n=9; p < 0.05; Fig 7.8) than the absolute peak of the control currents (2.7 ± 0.3s, n=9).

7.2.1.5 Effects of ouabain on the currents produced by pressure application of 1mM NMDA

As both muscarine and UCL 1880 only partially inhibited the UCL 1848-insensitive outward current, it suggested that there is probably a third type of conductance that contributes to the generation of the current. In some cell types
in the presence of 100µM ouabain

Fig 7.9 Effects of 100µM ouabain on the inward and outward currents generated by 300ms pressure applications of NMDA. Each trace is the average of 3 consecutive recordings. To clearly show the effects of ouabain, the traces have been superimposed.
(Johnson et al., 1992; Mercuri et al., 1996), an outward current that is due to the activity of the Na\(^+/\)K\(^+/\)-ATPase and which can be reduced by ouabain is generated following NMDA receptor activation. A maximal concentration of ouabain (100\(\mu\)M; Munakata et al., 1998) was, therefore, applied to cells that exhibited a UCL 1848-insensitive current. This concentration of ouabain, rather than reducing, increased the outward current generated by NMDA receptor activation by 67.6 ± 26.1\% (n=5; Fig 7.9). The inward NMDA current was little affected by ouabain (% inhibition = 3.9 ± 5.3\%, n=5).

100\(\mu\)M ouabain also affected the holding current at -50mV. In 3/5 cells, this concentration of ouabain reduced the outward holding current by 23.1 ± 1.8\%, indicating that the Na\(^+/\)K\(^+/\)-ATPase contributes to the maintenance of the membrane potential. In the remainder of the cells tested, however, ouabain caused an increase (% inhibition = -25.9 ± 8.4\%, n=2) in the outward holding current.

7.2.1.6 Do cells that demonstrate a sI\(_{AHP}\) generate a UCL 1848-insensitive outward current when 1mM NMDA is applied?

Whether cells that displayed a UCL 1848-insensitive outward current when NMDA was pressure applied, also exhibited the sI\(_{AHP}\) and vice versa was also investigated. The sI\(_{AHP}\) was evoked using a 170ms depolarizing step from -50mV to + 20mV. 11/26 cells tested showed an outward current as well as both the mI\(_{AHP}\) and the sI\(_{AHP}\), of which 8/11 cells exhibited an NMDA-induced outward current that was totally inhibited by 100nM UCL 1848. Only 3/11 cells displayed a current that was partially or completely insensitive to UCL 1848 and a sI\(_{AHP}\). A sI\(_{AHP}\) but no outward current could be recorded in 13/26 cells. In the remainder of the cells (2/26), an
outward current insensitive to UCL 1848 was generated by NMDA receptor activation but no sIAHP could be detected.

7.2.2 Characterization of the currents evoked by caffeine

The cultured cells were voltage-clamped at -50mV and 10mM caffeine was bath applied for approximately 10s every 5min. Caffeine was only applied to cells that demonstrated a sIAHP following the application of a 170ms, depolarizing step from -50mV to +20mV. Application of caffeine resulted in an outward current followed by an inward current (Fig 7.10). The average amplitude of the outward current was 66.8 ± 12.6pA (n=11) and the outward current lasted for approximately 20s. Rundown of both the outward and inward current occurred in some cells. Initial experiments suggested that the outward currents produced by application of caffeine were not sensitive to 100nM UCL 1848. However, in order to investigate the possibility that caffeine could activate the sIAHP, a maximal concentration of UCL 1848 (100nM) was included to inhibit the apamin-sensitive SK channels. 1mM Ba^{2+} inhibited the outward current by 71.2 ± 5.4% (n=3, Fig 7.10). The outward current, however, was insensitive to 3μM muscarine (% inhibition = 1.3 ± 14.7%, n=5, Fig 7.11). Neither Ba^{2+} nor muscarine significantly affected the inward current that resulted from the caffeine application (% inhibition = 18.8 ± 9.7, n=3, Fig 7.10 and 11.4 ± 7.3, n=5, Fig 7.11 respectively) Both Ba^{2+} and muscarine inhibited the outward holding current present at -50mV, as described previously (see Chapter 3).
Fig 7.10 Currents generated by 10s bath application of 10mM caffeine. (A), (B) and (C) are records in the absence, in the presence and after washout of 1mM Ba\(^{2+}\) respectively. The three traces were obtained consecutively at 5min intervals. The calibration bars shown in (A) also apply to (B) and (C). 100nM UCL 1848 was included throughout the experiment.
Fig 7.11 Records showing currents generated by 10s bath application of 10mM caffeine in the absence (A) and in the presence (B) of 3μM muscarine. Record (B) was obtained 5min after record (A). The outward holding current present at -50mV was greater than 100pA and thus, the 50pA current level is shown. The calibration bars shown in (A) also apply to (B).
7.3 Discussion

7.3.1 Currents evoked by pressure application of NMDA

7.3.1.1 Characterization of inward and outward currents evoked by NMDA pressure application

Pressure applications of both 100μM and 1mM NMDA resulted in an inward current, which is carried by both Na\(^+\) and Ca\(^{2+}\). The offset decay time constants of the inward currents differed between cells (Fig 7.2). This could be due to a difference in the bath flow rate. However, the offset decay time constants are also dependent upon the NR2 subunits present in the NMDA receptors (Monyer et al., 1994). Assuming that the flow rate into the bath remained constant and the tip diameters of the pressure pipettes were similar, this might suggest that the subunit composition of the NMDA receptors may differ between cells. The NMDA subunit composition may change with time in culture, depending on the stage of development of the pyramidal cells.

Pressure application of 1mM NMDA, but not 100μM NMDA, resulted in an outward current (which followed the inward current) in approximately 47% of the cells at a holding potential of −50mV. This is in agreement with previous studies which reported that NMDA concentrations of 300μM and greater, could more reliably evoke outward currents following the inward NMDA response (Zorumski et al., 1989). In the present study, it was noted that application of 100μM NMDA produced a smaller inward current than application of 1mM NMDA, presumably reflecting the activation of fewer NMDA receptors. Accordingly, the rise in \([\text{Ca}^{2+}]\), is likely to be greater with 1mM NMDA applications. Reichling and MacDermott (1993) have, in fact, reported that Ca\(^{2+}\) entry through NMDA receptors is maximal
when 300µM NMDA is applied, the rise in \([\text{Ca}^{2+}]_i\) declining with higher concentrations.

The outward current evoked by pressure application of 1mM NMDA was shown to be due to the activation of NMDA receptors since it was abolished (along with the inward current) by the use of 7-CK in conjunction with a glycine-free solution to prevent NMDA receptor activation (Fig 7.3). Activation of other glutamate receptors by 1mM NMDA was, therefore, not implicated.

The ion permeability and selectivity of NMDA receptors containing different NR2 subunits is similar (Monyer et al., 1994) and, therefore, the generation of the outward current is also not likely to be dependent upon the decay of the inward NMDA current (Fig 7.2). An additional complication is that if the decay of the inward current is slower, \(\text{Ca}^{2+}\) entry would be expected to be greater and any outward current larger in magnitude. It is, however, clear that there is a significant amount of overlap between the inward and outward currents and it is, therefore, possible, that if there is a prolonged inward current present (as in the example shown in Fig 7.2B), an outward current may be masked.

7.3.1.2 Dependence of the generation of the outward current evoked by NMDA application on \(\text{Ca}^{2+}\)

The outward current following NMDA receptor activation in hippocampal neurones is most likely dependent upon \(\text{Ca}^{2+}\) for generation (Zorumski et al., 1989). In approximately 50% of the cells tested, the outward current was totally inhibited by UCL 1848 and apamin, suggesting that SK channels were activated and that \(\text{Ca}^{2+}\) entry through the NMDA receptors was most likely responsible for the generation of the outward current. Application of \(\text{Ca}^{2+}\)-free solutions onto cells that demonstrated a
UCL 1848-insensitive current was attempted. However, each time this experiment was tried, the recordings tended to become unstable and eventually, the cell would die. The NMDA receptor-gated channel is a cation channel, and allows permeation of both Na⁺ and Ca²⁺. The permeability ratio, $P_\text{Ca}/P_\text{Na}$ has been calculated to be 10.6 (Mayer and Westbrook, 1987), suggesting that significant amounts of Ca²⁺ will enter the cell during the NMDA application and lead to a significant rise in [Ca²⁺]. Therefore, the outward current is likely to be Ca²⁺-dependent. However, the possibility that Na⁺ ions may activate Na⁺-activated K⁺ currents or the Na⁺/K⁺ ATPase (see Section 7.2.1.5 and 7.3.1.8) and lead to the generation of an outward current cannot be excluded. Na⁺-activated K⁺ channels have been described in embryonic hippocampal neurones (for example see Liu et al., 1998).

7.3.1.3 Effects of UCL 1848 and apamin on the currents evoked by pressure application of NMDA

In 52% of the pyramidal cells, UCL 1848 abolished the outward current following the inward NMDA response. The outward current in these cells was also sensitive to apamin, indicating that Ca²⁺ entry via NMDA receptors activated apamin-sensitive SK channels. Complete inhibition of the outward current by UCL 1848 or apamin often revealed the presence of a slower decaying phase of inward current (see Fig 7.4). This inward current may be attributed to a slower phase of NMDA receptor current. However, an alternative explanation follows the report that hippocampal neurones possess a Ca²⁺-activated cation conductance, activation of which results in an afterdepolarization (Caeser et al., 1993). Hence, it is possible that Ca²⁺ entry via NMDA receptors can activate this current, resulting in a prolonged inward current (see for example Fig 7.2B and 7.4).
These results show that there is an overlap between the generation of the outward current and the inward current. Indeed, when the current in the presence of UCL 1848 or apamin is subtracted from the controls, the peak of the resultant outward current occurs significantly earlier than the peak of the control outward current (Fig 7.6). In addition, the magnitude of the subtracted outward current is significantly greater than the magnitude of the control. Clearly, the SK channels are activating before the NMDA receptor current is terminated. This overlap may make the measurement of any effects on the activated current difficult.

In the remainder of the cells tested (48%), the outward current generated was either partially or totally insensitive to UCL 1848, indicating that the expression of apamin-insensitive SK channels may vary between pyramidal cells and may depend on the stage of development of the cells in culture. On the other hand, if there is Ca$^{2+}$ compartmentalization in these neurones, then the generation of an apamin-sensitive outward current may depend on the location of the apamin-sensitive SK channels.

7.3.1.4 Effects of muscarine and UCL 1880 on the currents evoked by pressure application of NMDA

In cells that demonstrated a partially insensitive or a totally UCL 1848-insensitive current, both muscarine and UCL 1880 partially inhibited the outward current generated by NMDA receptor activation, suggesting that a current with the properties of the SI_{AHP} might be activated in this manner. Subtraction of the outward currents in the presence of muscarine and UCL 1880 from the controls also revealed an outward current that peaked during the inward current due to NMDA receptors. This suggested that the channels that were inhibited by UCL 1880 and muscarine were also activated during the inward NMDA current.
UCL 1880 can inhibit $\text{Ca}^{2+}$ channels (see Chapter 5) so it is necessary to consider whether this action could contribute to the inhibition of the NMDA-induced outward current. Note that initially these experiments were done in the presence of TTX to prevent the opening of $\text{Na}^+$ channels and, thereby, inhibit any action potential generation and propagation into the dendrites where the voltage-clamp would be inadequate. However, it is possible that depolarization caused by the opening of NMDA receptors may itself have activated voltage-gated $\text{Ca}^{2+}$ channels in the vicinity of the receptors. This possibility could not be easily tested by the use of divalent cation blockers of $\text{Ca}^{2+}$ channels such as $\text{Cd}^{2+}$, since they are also effective at blocking NMDA receptors (Ascher and Nowak, 1988). Hence, it is possible that at least a part of the effect of UCL 1880 on the outward current evoked by NMDA is a consequence of $\text{Ca}^{2+}$ channel block. However, as UCL 1880 did not inhibit the UCL 1848-sensitive current, it suggests that the effects of UCL 1880 on the outward current may be independent of $\text{Ca}^{2+}$ channel block. The inhibition of the NMDA-induced outward current by muscarine is also unlikely to be due to the inhibition of $\text{Ca}^{2+}$ channels since its inhibition of the sAHP in hippocampal pyramidal neurones has been shown to be independent of $\text{Ca}^{2+}$ channel block (Knopfel et al., 1990). However, it should be noted that muscarine can inhibit $\text{Ca}^{2+}$ channels in these neurones via G-protein and second messenger activation (see for example Toselli and Taglietti, 1995).

7.3.1.5 Kinetics of the outward currents sensitive to UCL 1848, apamin, muscarine and UCL 1880

The surprising finding of this study was that when the currents in the presence of UCL 1848, apamin, muscarine and UCL 1880 were subtracted from the
controls, the outward currents revealed had very similar kinetics. When AHPs are evoked using a train of action potentials or a depolarizing step, the apamin-sensitive AHP usually lasts for a period less than 1s whereas the apamin-insensitive, muscarine and UCL 1880 sensitive sAHP has a slow rising phase and lasts for several seconds. NMDA induced outward currents, irrespective of the underlying channels, all displayed a rising phase and lasted for several seconds. The cloned SK channels (Xia et al., 1998) as well as native hippocampal SK channels (Selyanko et al., 1998; Hirschberg et al., 1999) respond rapidly to Ca\(^{2+}\). The slow kinetics of the outward current, therefore, probably reflect the time course of the Ca\(^{2+}\) transient in the cells, though it would be necessary to perform Ca\(^{2+}\) imaging studies to confirm this. In this context, it should also be noted that the time-course of the apamin-sensitive mIAHP corresponds to the time-course of the Ca\(^{2+}\) transients that follow action potential generation (Sah and Clements, 1999). These results, therefore, also indicate that Ca\(^{2+}\) entry is likely to have occurred over a period of several seconds. In keeping with this, it was observed that the inward current could last for up to 1s, although the NMDA was only pressure applied for 300ms, indicating that Ca\(^{2+}\) entry is likely to have occurred for at least 1s. Ca\(^{2+}\) entry via NMDA receptors can also induce Ca\(^{2+}\) release from stores (for example see Emptage et al., 1999) and this may also delay the peak of the Ca\(^{2+}\) transient and contribute to the generation of the outward current.

7.3.1.6 Correlation between the presence of a sIAHP and a UCL 1848-insensitive outward current in response to pressure application of NMDA

A sIAHP can only be activated in approximately 60% of these cultured cells. It was of interest, therefore, to investigate whether it was only these cells which
exhibited a UCL 1848-insensitive outward current on pressure application of NMDA. No such correlation was, however, discovered. Pressure application of NMDA onto cells that had both the sI\textsubscript{AHP} and mI\textsubscript{AHP}, usually produced an outward current that was totally sensitive to UCL 1848. The presence of both the sI\textsubscript{AHP} and a UCL 1848-insensitive NMDA activated outward current could only be demonstrated in 3/26 cells. Also, in half the cells, though the sI\textsubscript{AHP} could be detected, NMDA receptor activation did not generate an outward current. These results suggest that there is compartmentalization of Ca\textsuperscript{2+} in these neurones and that the channels underlying the sI\textsubscript{AHP} are more likely to be activated by Ca\textsuperscript{2+} entry through voltage-gated Ca\textsuperscript{2+} channels than through NMDA receptor channels.

7.3.1.7 Could other Ca\textsuperscript{2+}-activated conductances underlie the outward current evoked by NMDA pressure application?

In the present study, both muscarine and UCL 1880 only partially inhibited the totally UCL 1848-insensitive current. Previous studies have shown that in hippocampal pyramidal neurones the outward current generated by NMDA receptor activation is totally Ca\textsuperscript{2+}-dependent (Zorumski et al., 1989). If this is also the case in these cells, it raises the possibility that Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels other than SK channels and the channels underlying the sI\textsubscript{AHP} are activated. A single application of 100nM charybdotoxin did not affect the UCL 1848-insensitive current (data not shown), suggesting that BK channels or IK-like channels (Shirasaki et al., 1994; Harata et al., 1996) were not activated. It should, however, be noted that neurones possess auxiliary subunits that can drastically change the both the pharmacology and the biophysical properties of BK channels (see for example Behrens et al., 2000 and Meera et al., 2000). This possibility, therefore, has to be further explored.
There have also been reports that hippocampal neurones possess Ca\(^{2+}\)-activated Cl\(^{-}\) conductances (Owen et al., 1988, Brown et al., 1990). Assuming E\(_{\text{Cl}}\) to be approximately \(-70\text{mV}\) (Brown et al., 1990), the possibility that the outward current generated by NMDA receptor activation is due to Cl\(^{-}\) ions cannot be ruled out.

7.3.1.8 Effects of ouabain on the NMDA induced outward current

It should be noted, though, that in other cell types such as dopaminergic neurones (Johnson et al., 1992; Mercuri et al., 1996), the outward current generated by NMDA receptor activation is inhibited by the Na\(^+/\)K\(^+\) ATPase inhibitor, ouabain. In the present study, ouabain tended to increase the outward current generated by NMDA receptor activation rather than to inhibit it. Low concentrations of ouabain have been reported to stimulate the Na\(^+/\)K\(^+\) ATPase (Cohen et al., 1976). This is an unlikely explanation as the concentration of ouabain used in the present study was near maximal (Munakata et al., 1998). In hippocampal neurones, ouabain has also been reported to cause a sustained rise in [Ca\(^{2+}\)]\(_{i}\) (Zhan et al., 1998). This would lead to an increased uptake of Ca\(^{2+}\) into intracellular stores. It is highly probable that a larger amount of Ca\(^{2+}\) will then be released from Ca\(^{2+}\) stores following Ca\(^{2+}\) entry through the NMDA receptor-gated channels (CICR), and hence the magnitude of the outward current generated by NMDA receptors could be greater in the presence of ouabain.

7.3.1.9 Effects of ouabain on the outward holding current present at -50mV

The Na\(^+/\)K\(^+\) ATPase contributes to the resting membrane potential in a number of cell types (Munakata et al., 1998). In 3/5 of these cultured cells, the
outward holding current present at −50mV was inhibited by ouabain, suggesting the Na⁺/K⁺ ATPase is involved in maintaining the resting membrane potential in these cells, too. In 2/5 cells, however, the outward current present at −50mV was increased. Previous studies have also shown that ouabain causes a sustained decrease in intracellular pH in hippocampal neurones (Zhan et al., 1998). Significantly, acidosis has been reported to activate K⁺ channels such TREK-1, a member of the two pore K⁺ channel family (Maingret et al., 1999). TREK-1 mRNA is found abundantly in the hippocampus (Fink et al., 1996) and since two pore K⁺ channels may be involved in maintaining the resting membrane potential (for example see Duprat et al., 1997; Millar et al., 2000; Talley et al., 2000), it is likely that activation of TREK-1 could cause an increase in the outward holding current in the presence of ouabain.

7.3.1.10 Could changes in intracellular pH contribute to the generation of the outward current evoked by NMDA?

Interestingly, activation of NMDA receptors in a number of cell types including hippocampal neurones induces transient reversible, Ca²⁺-dependent intracellular acidosis (Irwin et al., 1994; Zhan et al., 1997; Zhan et al., 1998; Wu et al., 1999). This also raises the possibility that the UCL 1848- and muscarine insensitive current generated by NMDA receptor activation may be due to proton-gated channels such as TREK-1.

7.3.1.11 Summary

Application of 1mM NMDA can generate an outward current following an inward current with a variable overlap between the decline of the inward and onset
of the outward currents. A variety of K⁺ channels, including SK channels, could potentially underlie this outward current evoked by NMDA in pyramidal cells and the relative contribution of each is likely to depend on the stage of development of the cells in culture. It is also possible that Ca²⁺-activated cation and Cl⁻ conductances may also be activated following NMDA receptor activation. Further experiments are required to determine the molecular correlate of the UCL 1848- and muscarine-insensitive current generated by NMDA receptor activation.

7.3.2 Currents evoked by application of caffeine

7.3.2.1 Pharmacology of the outward current evoked by caffeine

Application of 10mM caffeine resulted in an outward current followed by an inward current, as reported in previous studies (for example see Akaike and Sadoshima, 1989; Marrion and Adams, 1992; Uneyama et al., 1993). 1mM Ba²⁺ inhibited the outward current by approximately 70%. Both the outward and the inward currents remained unaffected by muscarine. Previous studies have also shown that the outward current generated by application of 10mM caffeine in dissociated neurones is insensitive to noradrenaline (Uneyama et al., 1993). In the case of the sI_{AHP}, the effects of these neurotransmitters are likely to be independent of inhibition of Ca²⁺ entry (Knopfel et al., 1990). These results, therefore, suggest that the channels underlying the sI_{AHP} may not be activated by the release of Ca²⁺ from stores by caffeine. Hence, the analogues of clotrimazole were not tested on the currents activated by caffeine.

A potential complication is that the concentration of caffeine used in this study could inhibit phosphodiesterases and, thereby increase cAMP and PKA levels within the cell (Nehlig et al., 1992). Activation of PKA leads to inhibition of the
slAHP (Pedarzani and Storm, 1993, 1995; Pedarzani et al., 1998). This might mask any activation of the channels underlying the slAHP by CICR. It would obviously be preferable to use a Ca^{2+} releasing agent that does not inhibit phosphodiesterases. At present, suitable agents are unavailable.

Previous studies have reported that the channels underlying the outward current generated by caffeine in hippocampal pyramidal neurones are likely to be BK channels (Uneyama et al., 1993). A single application of 100nM charybdotoxin had little effect on the outward and inward current generated by caffeine (data not shown). This suggests that BK channels or IK-like channels do not underlie the outward current generated by caffeine at the holding potential of -50mV. However, as mentioned above, the possibility that BK channels were activated by application of caffeine cannot be excluded as there are auxiliary subunits in neurones that can change the biophysical and pharmacological properties of BK channels (Behrens et al., 2000; Meera et al., 2000). Hence, further work has to be done to establish which channels underlie the outward current generated by application of caffeine in these hippocampal neurones.

7.3.2.2 Pharmacology of the inward current caused by application of caffeine

Application of caffeine resulted in the production of an inward current following the outward current (Fig 7.10 and 7.11). Neither Ba^{2+} nor muscarine had any effect on the inward current. Previous studies have suggested that inhibition of the M-current causes the inward current (Akaike and Sadoshima, 1989; Marrion and Adams, 1992; Munakata and Akaike, 1993; Uneyama et al., 1993). However, as the inward current persisted in the presence of muscarine in these cells, this seems unlikely. Depletion of intracellular Ca^{2+} stores leads to capacitative Ca^{2+} entry via
Ca\(^{2+}\)-release activated Ca\(^{2+}\) (CRAC) channels (for a review see Parekh and Penner, 1997). This then is an alternative mechanism that may underlie the inward current produced by caffeine in these neurones.

### 7.3.3 Conclusions

The results of this study have shown that the Ca\(^{2+}\) entry via voltage-gated Ca\(^{2+}\) channels is more likely to activate sI\(_{AHP}\) channels than Ca\(^{2+}\) entry via ligand-gated ion channels, suggesting that there is Ca\(^{2+}\) compartmentalization. In order to activate a current with the properties of the sI\(_{AHP}\) without activation of voltage-gated Ca\(^{2+}\) channels, it may be more appropriate to cause a rise in [Ca\(^{2+}\)]\(_i\) by flash photolysis of caged Ca\(^{2+}\) compounds.

The results presented in this chapter have also hinted that there may be unidentified, novel Ca\(^{2+}\)-activated channels present in hippocampal neurones as activation of both NMDA receptors and ryanodine receptors resulted in the generation of outward currents with novel pharmacological properties.
CHAPTER 8

DISCUSSION

The major aim of this project was to extend the pharmacology of the sI_{AHP} in hippocampal pyramidal neurones. In order to achieve this aim, a method was developed that allowed the recording of the sI_{AHP} from isolated hippocampal neurones. This method involved isolating hippocampal pyramidal neurones using a technique similar to that described by Allen et al. (1992) and culturing these neurones for up to 15 days using Neurobasal medium and the serum-free supplement, B27 (Brewer et al., 1993). The sI_{AHP} could be reliably and robustly recorded from cells that had been in culture for more than 7 days. The time course and other characteristics of this current were very similar to those that have been described previously for the sI_{AHP} in brain slices (see Chapter 3 and for reviews see Storm, 1990 and Sah, 1996). In addition to the sI_{AHP}, a predominantly apamin-sensitive mI_{AHP} could also be recorded from a large proportion of these cells. The presence of an apamin-sensitive mI_{AHP} has also recently been shown in brain slices (Stocker et al., 1999). When it was clear that the characteristics of the currents recorded from the cultured neurones were similar to those recorded from brain slices, the pharmacology of the sI_{AHP} was further explored. In addition to this, issues such as the source of Ca^{2+} for the generation of the sI_{AHP} and the role of SK1 channels in the generation of the current have been addressed. The implications of these results are discussed below in detail, starting with the pharmacology of the sI_{AHP}. 
8.1 The pharmacology of the sI\textsubscript{AHP}

The sAHP in hippocampal neurones was first described independently by two separate groups (Alger and Nicoll, 1980 and Hotson and Prince, 1980). The sAHP has been established to be a Ca\textsuperscript{2+}-activated K\textsuperscript{+} conductance (Alger and Nicoll, 1980; Lancaster and Adams, 1986) that is insensitive to the common K\textsuperscript{+} channel blockers, TEA and 4-AP as well as the SK channel blocker, apamin (Lancaster and Adams, 1986; Lancaster and Nicoll, 1987). However, no selective blockers or openers of the sAHP channel have, as yet, been described. The Ca\textsuperscript{2+} chelating agent, BAPTA and some of its derivatives, under appropriate conditions, enhance the sI\textsubscript{AHP} (Schwindt \textit{et al}, 1992; Zhang \textit{et al}, 1995; Velumian and Carlen, 1999). However, the mechanism of this action is uncertain. It is possible that these may be openers of the channels underlying the sI\textsubscript{AHP} (Lancaster and Batchelor, 2000). The sAHP is inhibited by second messengers activated by neurotransmitters and is one of the best-studied examples of neuromodulation (for reviews see Brown \textit{et al}, 1990; Storm, 1990; and Sah, 1996).

In this study, the effects of analogues of dequalinium and clotrimazole have been explored on both the sI\textsubscript{AHP} and the apamin-sensitive mI\textsubscript{AHP}. The most potent SK channel blocking analogue of dequalinium, UCL 1848, had little effect on the sI\textsubscript{AHP} but was a selective blocker of the mI\textsubscript{AHP}. In contrast, clotrimazole and its analogues were found to inhibit the sI\textsubscript{AHP}. Clotrimazole is also a potent inhibitor of the IK channel (Alvarez \textit{et al}, 1992; Brugnara \textit{et al}, 1993; Rittenhouse \textit{et al}, 1997a; Logsdon \textit{et al}, 1997; Dunn, 1998; Jensen \textit{et al}, 1999). Most of the clotrimazole analogues tested that were effective blockers of the sI\textsubscript{AHP} also inhibit the IK channel in red blood cells with similar potency (M. Malik-Hall and D.H. Jenkinson, unpublished data) suggesting that the channel underlying the sI\textsubscript{AHP} may be related to
the IK channel. However, UCL 1495 and UCL 1864, which are potent inhibitors of the IK channel in red blood cells (Benton et al., 1994; M. Malik-Hall and D.H. Jenkinson, unpublished data), were ineffective inhibitors of the sI_{AHP}, suggesting that the channel underlying the sI_{AHP} is not the conventional IK channel.

The clotrimazole derivatives, UCL 2027 and UCL 2077 were found to be relatively selective inhibitors of the sI_{AHP}, with IC_{50}s of 1\mu M and 0.5\mu M respectively. These compounds had little effect on Ca^{2+} currents, the mI_{AHP} and the outward holding current in hippocampal pyramidal neurones and are also relatively less potent at inhibiting the IK channel in red blood cells (M. Malik-Hall and D.H. Jenkinson, unpublished data). Both compounds, however, caused widening of action potentials indicating that they may inhibit one or more of the Kv or BK channels, which are involved in the repolarization of action potentials. The ability of clotrimazole analogues to block a range of K^+ channels suggests that these compounds might be direct inhibitors of the K^+ channel underlying the sI_{AHP}. As mentioned above the sI_{AHP} is regulated by neurotransmitters and therefore, it is also possible that the compounds may alternatively inhibit the sI_{AHP} by activating second messenger systems. It is possible that a consideration of the effects of the drugs on the time course of the sI_{AHP} may help in distinguishing between these possibilities. Thus, the partial inhibition of the sI_{AHP} by second messenger activation usually results in the sI_{AHP} peaking earlier and decaying faster (see for example Malenka and Nicoll, 1986 and Krause and Pedarzani, 2000). Partial inhibition of the sI_{AHP} by either UCL 2027 or UCL 2077, however, did not cause a change in the shape of the current, suggesting that these agents act differently to neuromodulators and may be direct K^+ channel inhibitors. These blockers could therefore, be useful in the identification of the molecular correlate of the sI_{AHP}. 

218
As mentioned in the INTRODUCTION (Section 1.5.6), the physiological role of the sI_{AHP} has not yet been firmly established. The sI_{AHP} has been suggested to have an important role in learning and memory (Moyer et al., 1992; Disterhoft et al., 1996; Giese et al., 1998; Weiss et al., 2000). Selective blockers of the sI_{AHP} could thus be tested in experimental models of learning and memory to provide further information on this possible role. The inhibitors that have been described in this study, however, are probably not ideal for these experiments, as potentially large doses of these agents would have to be administered. Large doses are more likely to be toxic. The selective inhibitors described in this study could, however, be useful in studying the role of the sI_{AHP} using in vitro assays (for example the role of the sI_{AHP} during long term potentiation (LTP) induced in brain slices). A potential drawback, however, for the clinical use of sI_{AHP} inhibitors is that they are likely to increase cell excitability leading to epileptiform activity (Verma-Ahuja et al., 1995; 1998).

8.2 Do SK1 channels underlie the sI_{AHP}?

8.2.1 Comparison of the pharmacology of the sI_{AHP} and expressed SK1 channels

Noise analysis studies have indicated that the channels underlying the sI_{AHP} have a small conductance (Sah and Isaacson, 1995; Valiante et al., 1998). As activation of the current is dependent upon Ca^{2+} entry and K^+ channels underlie the current (Lancaster and Adams, 1986), it has been suggested that SK channels may underlie the sI_{AHP} (Sah, 1996; Vergara et al., 1998; Bond et al., 1999). Of the three SK channels that have been cloned (Köhler et al., 1996), only SK1 cDNA formed apamin-insensitive channels when expressed in Xenopus oocytes. SK1 mRNA was also present in tissues that expressed the sI_{AHP}, thereby providing additional support
for a possible role of SK1 subunits in this current. Hence, it was suggested that SK1 channels may underlie the sI_AHP (Vergara et al., 1998; Bond et al., 1999). However, results presented in this study have shown that hSK1 cDNA can form apamin-sensitive channels when expressed in mammalian cell lines (Shah and Haylett, 2000a). These results have been independently confirmed by another laboratory (Strobaek et al., 2000). Moreover, UCL 1848 and UCL 1684 (Strobaek et al., 2000) which are the most potent inhibitors of SK1 channels expressed in mammalian cell lines have little effect on the sI_AHP, while the sI_AHP inhibitors, UCL 2027 and UCL 2077 are relatively ineffective blockers of SK1 channels. These results indicate that SK1 channels, at least in the conformation present in mammalian cell lines, are unlikely to underlie the sI_AHP.

An additional complication is that although the sI_AHP can be recorded from both rat and human (Lorenzon and Foehring, 1992) pyramidal neurones, as yet the rSK1 clone cannot be expressed in oocytes or mammalian cell lines (also see Hirschberg et al., 1999). On the other hand, the human version (hSK1) can readily be expressed. There are also no commercially available SK1 antibodies to determine whether rSK1 is being directed to the cell plasma membrane in the expression systems or indeed on hippocampal pyramidal cell membranes. Also, although SK channels have been recorded from hippocampal pyramidal neurones (Marrion and Tavalin, 1998; Selyanko et al., 1998; Hirschberg et al., 1999), it is not known whether these are SK1 or SK2. Hence, the role of SK1 in native tissues still has to be established.

The rSK1 and hSK1 sequences are 98% identical (see Fig 1.2). Since the amino-acids within the pore region are likely to influence the apamin-sensitivity of the channels (Ishii et al., 1997b), it is unlikely that the apamin binding ability of rat
and human SK1 channels would differ substantially. In hippocampal pyramidal cells, the mI_{AHP} can be inhibited by apamin (see Chapter 3) with an IC_{50} of approximately 0.5nM (Stocker et al., 1999). This value does not correlate with the IC_{50} values for apamin on either expressed SK2 or SK1 channels (Köhler et al., 1996; Ishii et al., 1997b; Strobaek et al., 2000), raising the possibility that a combination of SK1 and SK2 homomeric channels or heteromeric SK1/SK2 channels may underlie the mI_{AHP} (Stocker et al., 1999).

8.2.2 Could the apamin-sensitivity of SK1 channels be dependent on the association of auxiliary subunits?

Recently, it has been suggested that injection of SK1 mRNA in Xenopus oocytes can result in the formation of both apamin-sensitive and apamin-insensitive channels (Grunnet et al., 1999). This indicates that, in addition to the amino acid residues in the pore region (Ishii et al., 1997b), the presence or absence of a secondary factor may determine the apamin-sensitivity of SK1 channels. Although SK1 is 90% homologous to SK2 and SK3, the N and C terminal sequences are quite different from SK2 and SK3 (Köhler et al., 1996) and may interact differently with putative auxiliary subunits. Some evidence for auxiliary subunits was provided by Wadsworth et al. (1997) who purified a number of polypeptides of different molecular weights from brain tissue, which could all be photolabelled by apamin. These could potentially represent SK ‘α’ subunits together with auxiliary subunits. Hence, it is possible that binding of auxiliary subunits could change the apamin-sensitivity of SK1. The possibility of such modulation is supported by the recent demonstration that binding of a novel β subunit to BK channels can alter their
charybdotoxin sensitivity as well as their Ca$^{2+}$-sensitivity and voltage-gating (Behrens et al., 2000; Meera et al., 2000).

8.2.3 Comparison of the distribution of SK1 mRNA and sI_{AHP} currents

Although initial reports suggested that the distribution pattern of SK1 matched that of the sI_{AHP} (Köhler et al., 1996), detailed in situ hybridization studies have revealed that there is a mismatch between the distribution of SK1 and the existence of the sI_{AHP} (Stocker and Pedarzani, 2000). For example, although an apamin-insensitive sI_{AHP} can be recorded from rat locus coeruleus neurones (Osmanovic et al., 1990; Osmanovic and Shefner, 1993), no rSK1 mRNA was detectable in these neurones (Stocker and Pedarzani, 2000).

8.2.4 Comparison of the kinetics of the sI_{AHP} and the expressed SK channels

A rise in [Ca$^{2+}$]$_i$ results in a rapid opening (within milliseconds) of both expressed and native SK channels (Xia et al., 1998; Selyanko et al., 1998; Hirschberg et al., 1999). The sI_{AHP}, however, has very slow kinetics, the peak of the current occurring 400-500ms after the peak of the rapidly rising Ca$^{2+}$ transient (Lasser-Ross et al., 1997; Jahromi et al., 1999; Sah and Clements, 1999). If SK channels do underlie the sI_{AHP}, a possible explanation for the slow rate of opening of the channels could be the presence of auxiliary subunits in hippocampal neurones, which modify the Ca$^{2+}$ sensitivity and, perhaps, also the gating properties of the channels. This possibility is supported by the recent finding that apamin-sensitive SK channels can contribute to both the mI_{AHP} and sI_{AHP} activated by action potentials in guinea-pig sympathetic neurones (Martinez-Pinna et al., 2000), though it must be emphasized that the shape of the Ca$^{2+}$ transients in these cells are unknown.
Also, the UCL 1848-sensitive outward current evoked by 300ms pressure applications of NMDA has similar kinetics to the $s_{AHP}$ (see Chapter 7). Pressure-applications of NMDA onto cultured hippocampal neurones might be expected to cause a rapid increase in $[Ca^{2+}]_i$ throughout the cells as occurs during a train of action potentials or a depolarizing step (Jahromi et al., 1999; Sah and Clements, 1999). UCL 1848 has no effect on the $s_{AHP}$ (see Chapter 3); rather its actions seem to be confined to SK channels (see Chapter 6; Shah and Haylett, 2000a; D.C.H. Benton, unpublished observations). This then implies that under certain circumstances SK channel activation can lag behind the rise in $[Ca^{2+}]_i$ in the cell, perhaps by the binding of auxiliary subunits. Alternatively, as suggested in Chapter 7, Ca$^{2+}$ compartmentalization occurs within neurones and the slow kinetics of the UCL 1848-sensitive outward current induced by NMDA receptor activation is due to the submembrane Ca$^{2+}$ diffusional rate. Similarly, the slow activation of the $s_{AHP}$ may not be governed by the kinetic properties of the K$^+$ channels involved but by submembrane Ca$^{2+}$ levels.

8.3 The role of Ca$^{2+}$ channels in generation of the $s_{AHP}$

8.3.1 Dependence of the generation of the $s_{AHP}$ on particular Ca$^{2+}$ channel subtypes

It has been suggested that L-type Ca$^{2+}$ channels co-localize with SK channels (Marrion and Tavalin, 1998) and that the slow time-course of the $s_{AHP}$ is due to the delayed facilitation of L-type Ca$^{2+}$ channels that occurs following a depolarizing step(s) (Cloues et al., 1997). If this were the case, then L-type Ca$^{2+}$ channel inhibitors should abolish the $s_{AHP}$. However, in the hippocampal pyramidal neurones used in this study, Ca$^{2+}$ entry via N-type Ca$^{2+}$ channels contributed as much
to the generation of the sI_{AHP} as L-type Ca^{2+} channels (see Chapter 4; Shah and Haylett, 2000b). This has also recently been confirmed in hippocampal slices (H. Hua and J.F. Storm, personal communication). Also, the sI_{AHP} in neocortical neurones, which has similar properties to the sI_{AHP} in hippocampal pyramidal neurones, is activated by Ca^{2+} entry predominantly via N-type Ca^{2+} channels (Pineda et al., 1997). These observations make it less likely that the slow kinetics of the sI_{AHP} are due to the delayed facilitation of L-type Ca^{2+} channels.

In contrast to the sI_{AHP}, the time course of the mI_{AHP} can be more easily explained by changes in intracellular Ca^{2+} (Sah and Clements, 1999). Interestingly, the apamin-sensitive SK channels that underlie the mI_{AHP} in hippocampal pyramidal neurones (P. Pedarzani, personal communication) and in other neurones such as SCG (Davies et al., 1996), are dependent mainly on Ca^{2+} entry via N-type Ca^{2+} channels for activation. This indicates that apamin-sensitive SK channels are, perhaps, preferentially located adjacent to N-type Ca^{2+} channels. The dependence of the mI_{AHP} specifically on N-type Ca^{2+} channels also suggests that there is Ca^{2+} compartmentalization within neurones.

8.3.2 Dependence of the time course of the sI_{AHP} on [Ca^{2+}]i

A number of possible theories to explain the kinetics of the sI_{AHP} have been outlined in INTRODUCTION (Section 1.5.5). From the results presented in this thesis, it is quite clear that neither CICR nor delayed facilitation of L-type Ca^{2+} channels can in themselves explain the time course of the sI_{AHP}. Although the generation of the sI_{AHP} may not be dependent on Ca^{2+} entry through specific subtypes of Ca^{2+} channels, the results presented in Chapter 7 suggest that Ca^{2+} entry via voltage-gated Ca^{2+} channels is more likely to activate the K^+ channels involved
in the sI_{AHP} than Ca^{2+} entry via NMDA receptor channels, which is more likely to produce an apamin-sensitive current. This indicates that although the K^{+} channels underlying the sI_{AHP} may not be coupled to particular Ca^{2+} channel subtypes, they may be preferentially located in the vicinity of voltage-gated Ca^{2+} channels. These results also suggest that compartmentalization of Ca^{2+} occurs such that high submembrane Ca^{2+} levels may be limited to the immediate area of Ca^{2+} entry and the Ca^{2+} that activates the sI_{AHP} is in a distinct compartment. It is, therefore, possible that submembrane Ca^{2+} levels may determine the time course of the sI_{AHP}. With large submembrane Ca^{2+} transients that occur during, for example, the rising phase of the AHP, novel states of the sAHP channels with slow opening kinetics may occur (see Jahromi et al., 1999). This mechanism could occur if the Ca^{2+} signal associated with the sAHP not only directly activated the sAHP channels but also enhanced their sensitivity to Ca^{2+}. This could lead to increased opening of the channels, despite a decline in the Ca^{2+} transient and may explain the rising phase of the sI_{AHP}. However, as yet, none of the Ca^{2+}-activated K^{+} channels that have been cloned display these characteristics.

It must also be emphasized that the Ca^{2+} affinity of the channels underlying the sI_{AHP} is unknown. It is not even established that Ca^{2+} directly gates the K^{+} channels involved. Simultaneous recordings of Ca^{2+} transients and the sI_{AHP} have shown that the peak of the sI_{AHP} occurs several hundred milliseconds after the peak of the Ca^{2+} transients (Lasser-Ross et al., 1997; Jahromi et al., 1999; Sah and Clements, 1999; Lancaster and Batchelor, 2000). It should be noted that the sAHP channels have been suggested to be located on dendrites (Sah and Bekkers, 1996; Bekkers, 2000) and in dendrites the Ca^{2+} transients have much faster kinetics compared with somatic Ca^{2+} transients (Jahromi et al., 1999; Sah and Clements,
This would suggest an even bigger discrepancy between the time course of the Ca\(^{2+}\) transients and the shape of the sI\(_{AHP}\), thereby reinforcing the idea that the time course of the sI\(_{AHP}\) is not determined by that of [Ca\(^{2+}\)]. It has thus, been proposed that a secondary factor activated by Ca\(^{2+}\) may control the time course of the sI\(_{AHP}\). Large concentrations of Ca\(^{2+}\) are likely to occur just inside the cell membrane where the Ca\(^{2+}\) channels are located and these may be able to activate a second messenger cascade. Since the sI\(_{AHP}\) can be inhibited by activation of protein kinases and enhanced via activation of phosphatases (Pedarzani and Storm, 1993, 1995; Pedarzani et al., 1998; Krause and Pedarzani, 2000), it could be possible that changes in [Ca\(^{2+}\)] alters the relative activity of these enzymes (see e.g. Soderling, 1999) and thereby leads to activation of the sI\(_{AHP}\) channels.

**8.4 Future Directions**

The sI\(_{AHP}\) channels appear to be activated by Ca\(^{2+}\) entry via a number of different Ca\(^{2+}\) channels as well as by Ca\(^{2+}\) released from Ca\(^{2+}\) stores by CICR (see Chapter 4; Fig 8.1). From previous studies and the results presented in this thesis, it is clear that the properties of the currently identified SK (α) channels cannot explain the properties of the sI\(_{AHP}\). It is, therefore very likely that auxiliary subunits (which could be enzymes) are involved (see Fig 8.1).

SK1 channels may be involved in the generation of the sI\(_{AHP}\). However, more evidence is require to support this hypothesis. The generation of inducible SK1 'knock-out' mice (like the recently described SK3 ‘knock-out’ mice by Bond et al. (2000)) might be particularly useful. Cultured hippocampal pyramidal cells which
Fig 8.1 An illustration of the possible mechanism that may be responsible for the generation of the $s_{I_{AHP}}$. As it is not clear whether SK channels underlie the $s_{I_{AHP}}$, the channels underlying the $s_{I_{AHP}}$ have been represented as sAHP channels.
display the sIAHP current (like the ones used in this study) would also be useful for anti-sense experiments and transfecting dominant negatives. Such studies would be useful in the positive identification of the molecular correlate of the sIAHP.

It is possible that if SK1 channels are involved in the generation of the sIAHP, they may be coupled to a secondary factor present in hippocampal neurones other than calmodulin. The coupling of this factor to the channels may influence the pharmacology as well as the time course and modulation by second messengers. Molecular and biochemical approaches are required to identify the involvement of any such molecule.

Previous work as well as the work presented in this thesis suggests that the relationship between Ca$$^{2+}$$ and the sIAHP may be far more complex than originally thought (Jahromi et al., 1999; Sah and Clements, 1999). It is also possible that submembrane Ca$$^{2+}$$ levels are more important than total cell Ca$$^{2+}$$ levels. Experiments that involve measurements of submembrane Ca$$^{2+}$$ levels would be of great value. These experiments, however, are technically very demanding and require advanced microscopy.

Work from other laboratories has also indicated that the channels involved in the sIAHP may not be directly gated by Ca$$^{2+}$$ (Schwindt et al., 1992; Lasser-Ross et al., 1997; Jahromi et al., 1999; Sah and Clements, 1999; Lancaster and Batchelor, 2000). Selective, potent inhibitors and openers would, therefore, be very useful in not only identifying the channels underlying the sIAHP but also in biochemical approaches used to extract the channels and thereby, clone them. The work done in this project has led to the identification of two novel, selective inhibitors of the sIAHP. However, more potent agents would be required to allow biochemical experiments to
be done. These agents could also prove to be useful in establishing the physiological role of the $sI_{AHP}$ using both in vitro and in vivo assays.
REFERENCES


56) Gerber, U. & Gahwiler, B. H. (1994). GABA(B) and adenosine receptors mediate enhancement of the K\(^+\) current, I\(_{AHP}\) by reducing adenylyl -cyclase activity in rat CA3 hippocampal neurons. *Journal of Neurophysiology* 72, 2360-2367.


