Probing Calmodulin in Living Neurones

Jacobus Martinus Milikan

A thesis submitted for the degree of
Doctor of Philosophy
in the
University of London

Department of Physiology
University College London
2000
ABSTRACT

In this thesis I explored the role of the calcium binding protein calmodulin in neuronal signalling in detail. Nerve cells have a high calmodulin content and calmodulin is involved in neuronal processes such as the memory models Long Term Potentiation and Long Term Depression, and neurite outgrowth.

Previous studies mainly relied on biochemical and pharmacological techniques and immunocytochemistry to study calmodulin function. I investigated the suitability of the potent calmodulin antagonist calmidazolium for studying the calmodulin-mediated processes in living pheochroma cytoma 12 (PC12) cells.

I used two fluorescently labelled calmodulins to study calmodulin function in living neurones: FL-CaM, which does not change its fluorescence when it binds calcium, and TA-CaM, which increases its fluorescence upon calcium binding. In addition, I measured the intracellular calcium concentration using low-affinity calcium indicator dyes.

I used FL-CaM as a concentration and localisation marker for fluorescent calmodulin and the fluorescence of TA-CaM as an indicator of calmodulin activation. I introduced fluorescent calmodulins into rat dorsal root ganglion neurones by micro-injection or by loading via a patch-pipette under whole-cell voltage-clamp. Fluorescence was collected using confocal microscopy.

I found that fluorescently labelled calmodulins locate to the cell nucleus of resting cells. Calmodulin immunofluorescence, however, had a different subcellular distribution and would only accumulate in the nucleus after depolarisation of the cells.

In response to a brief depolarisation, TA-CaM was activated to the same extent in nucleus and cytosol of dorsal root ganglion neurones. When calcium levels fell again, TA-CaM fluorescence showed a residual plateau when the calcium level had recovered back to baseline. Since calmodulin activity is retained after the calcium signal, calmodulin can serve as part of a system in which repetitive stimulation leads to longer term changes in nerve activity. I investigated this phenomenon and binding of calmodulin to target proteins appeared responsible.
## CONTENTS:

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>1</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>2</td>
</tr>
<tr>
<td>CONTENTS</td>
<td>3</td>
</tr>
<tr>
<td>LIST OF FIGURES AND TABLES</td>
<td>10</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>12</td>
</tr>
<tr>
<td>QUOTE</td>
<td>14</td>
</tr>
<tr>
<td>PREFACE</td>
<td>15</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>15</td>
</tr>
</tbody>
</table>

## CHAPTER 1: INTRODUCTION

### 1.1. GENERAL

### 1.2 INTRACELLULAR SIGNALLING

#### 1.2.1 Why?

#### 1.2.2. Parameters of signalling molecules

#### 1.2.3. Signal/noise ratio

#### 1.2.4. Specificity

#### 1.2.5. Change in activity

### 1.3. SECOND MESSENGERS

### 1.4. INTRACELLULAR CALCIUM

#### 1.4.1. Gradient

#### 1.4.2. Calcium changes

### 1.5. SOURCES OF CALCIUM IN CELLS

#### 1.5.1. Calcium influx

##### 1.5.1.1. Voltage operated calcium channels

##### 1.5.1.2. Receptor operated calcium channels

##### 1.5.1.3. Sodium calcium exchanger

##### 1.5.1.4. Capacitative calcium influx

#### 1.5.2. Calcium release

##### 1.5.2.1. The IP₃ pathway
1.5.2.2. The cADPr pathway 26
1.5.2.3. other organelles 26

1.6. CALCIUM CLEARANCE 26
   1.6.1. Calcium pumps 26
      1.6.1.1. The plasma membrane calcium/magnesium-ATPase 26
      1.6.1.2. Smooth endoplasmic reticulum calcium ATPase 27
   1.6.2. Calcium exchanger 27
   1.6.3. Mitochondria 27
   1.6.4. Contribution to calcium clearance of individual pathways in neurones 28

1.7. CALCIUM SIGNALS CONTAIN INFORMATION 28

1.8. CALCIUM BINDING PROTEINS 30
   1.8.1. EF-hand calcium binding domains 32

1.9. CALMODULIN 34
   1.9.1. The discovery of calmodulin 34
   1.9.2. Genes & evolution 34
   1.9.3. expression 35
   1.9.4. Structure 36
   1.9.5. Calcium binding 38
   1.9.6. Selection of calcium over magnesium 39
   1.9.7. Co-operativity in calcium binding 39

1.10. TARGET BINDING 41
   1.10.1. Binding to calmodulin 41
   1.10.2. Calmodulin binding site 41
   1.10.3. Binding modes 42
   1.10.4. Calcium independent binding 42
   1.10.5. Calcium dependent binding 44
      1.10.5.1. cAMP system 45
      1.10.5.2. Calcium:calmodulin activated kinases and phosphatases 45
         1.10.5.2a Myosin light chain kinase 45
1.10.5.2. Calmodulin dependent protein kinases (CaMKs) 46
1.10.5.2.b. Calmodulin kinase II 46
1.10.5.2.b.2. CaMKI 47
1.10.5.2.b.3. CaMKIII 48
1.10.5.2.b.4. CaMKIV 48
1.10.5.2.c. Calmodulin kinase kinase 49
1.10.5.2.d. Phosphatidylinositol 3-kinase 49
1.10.5.2.e. Other calmodulin activated kinases 50
1.10.5.2.f. Calmodulin activated phosphatases 50
1.10.5.3. Ion channels 51
1.10.5.4. Cytoskeletal interactions 52
1.10.5.5. Other targets 52
1.10.5.5.a. Nitric oxide synthase 52
1.10.5.5.b. pmCa\(^{2+}\)-ATPase 53
1.10.5.5.c. Other calmodulin binding proteins 53
1.10.6. Kinetics of calmodulin binding 54
1.10.7. Layers of complexity 55
1.10.7.1. How are calmodulin activated processes co-ordinated in response to a calcium rise? 55
1.10.7.2. Spatial considerations 58
1.10.7.3. Subcellular distribution of calmodulin 58
1.10.7.4. Bound/free calmodulin 58
1.10.7.5. Subcellular distribution of calmodulin in neurones 60
1.10.7.6. Dynamic subcellular redistribution of calmodulin 60
1.10.7.6.a. Membrane to cytosol 61
1.10.7.6.b. Cytosol to nucleus 61
1.10.7.7. Temporal coding 63

1.11. AIM OF THIS THESIS: 66
CHAPTER 2: MATERIALS AND METHODS

2.1. CELLS:
   2.1.1. PC12 cells
   2.1.2. PC12 cells methods
   2.1.3. Dorsal root ganglion neurones
   2.1.4. Dorsal root ganglion neurones methods

2.2. FLUORESCENCE:

2.3. FLUORESCENTLY LABELED PROTEINS, DEXTRANS AND CALMODULIN BINDING PEPTIDES: TOOLS FOR STUDYING CALMODULIN IN LIVING CELLS.
   2.3.1. Fluorescent calmodulins
   2.3.2. Fluorescently labelled calmodulin studies
   2.3.3. Calmodulin purification
   2.3.4. Labelling

2.4. PEPTIDES

2.5. EPIFIUORESCENCE IMAGING

2.6. MICRO-INJECTION
   2.6.1. Injection of cells
   2.6.2. Data analysis for micro-injected cells of chapter 5

2.7. CONFOCAL LASER SCANNING MICROSCOPY:
   2.7.1. Confocal Imaging
   2.7.2. Confocality of the Zeiss LSM510 system
   2.7.3. Image analysis confocal
   2.7.4. Calculation of the intracellular calcium concentration

2.8. IMMUNOCYTOCHEMISTRY

2.9. WESTERN BLOTTING:

2.10. PATCH-CLAMP EXPERIMENTS
   2.10.1. Patch-clamp experiments at UCL:
      2.10.1.1. Protocol using TA-CaM, FL-CaM and Fura Red
      2.10.1.2. Protocol using TA-CaM & Oregon Green Bapta 5N
2.10.2. Patch-clamp experiments at NIMR-Mill Hill (collaboration with David Ogden's group; Chapter 6) 94
2.10.3. Image analysis of patch-clamp data 95

2.11. GENERAL 96

CHAPTER 3: CALMIDAZOLIUM INDUCES CALCIUM TRANSIENTS IN PC12 CELLS. 97

3.1. INTRODUCTION:
   3.1.1. Pharmacological calmodulin antagonists 97
   3.1.2. Binding mechanism of antagonists 98
   3.1.3. Calcium management in neurones 99

3.2. RESULTS:
   3.2.1. Calmidazolium induces an increase in the [Ca^{2+}]_{i} 99
   3.2.2. Source of calcium 103
      3.2.2.1. Calcium release from mitochondria 103
      3.2.2.2. Calcium release from endoplasmic reticulum calcium stores 103
      3.2.2.3. Calcium influx 105
         3.2.2.3a Influx via voltage operated calcium channels 105
         3.2.2.3b Contribution from sodium channels 105
         3.2.2.3c Contribution of potassium channels 107

3.3. DISCUSSION 108
   3.3.1. Calmidazolium causes calcium rises 108
   3.3.2. Effect of high concentrations of calmidazolium on the cell membrane 108
   3.3.3. Calcium rises are due to influx via L-type VOCC 109
   3.3.4. Mechanism of calmidazolium induced depolarisation 110
   3.3.5. Sodium and potassium channel mediated depolarisation 110
   3.3.6. Contribution from intracellular calcium stores 112
   3.3.7. Contribution from mitochondria? 112
   3.3.8. Inhibition of plasma membrane Ca^{2+}-ATPase activity? 113

3.4. CONCLUSION 113
CHAPTER 4: LOCALISATION OF MICRO-INJECTED FLUORESCENTLY LABELLED CALMODULIN.

4.1 INTRODUCTION 116

4.2 RESULTS:

4.2.1. Localisation of micro-injected fluorescently labelled calmodulin 119
4.2.2. Localisation of calmodulin immunoreactivity 122
4.2.3. Localisation of patch-loaded fluorescently labelled calmodulin 129

4.3. DISCUSSION & CONCLUSIONS 131

CHAPTER 5: STEADY STATE CALCIUM ACTIVATION OF CALMODULIN.

5.1. INTRODUCTION 135

5.2. RESULTS 137

5.3. DISCUSSION & CONCLUSIONS 140

CHAPTER 6. INTEGRATION OF CALCIUM SIGNALS BY CALMODULIN IN RAT SENSORY NEURONES.

6.1 INTRODUCTION 143

6.1.1 Target activation by calmodulin 143
6.1.2. Parameters of target activation 143
6.1.3. Calmodulin-target interaction 144
6.1.3.1. Steady-state interaction 144
6.1.3.2. Dynamic interaction 145
6.1.4. Hysteresis by calmodulin 147
6.1.5. The study of calmodulin activation 147

6.2. RESULTS 148

6.2.1. Calmodulin activation in rat dorsal root ganglion neurones. 148
6.2.2. Kinetics of Calmodulin activation. 149
6.2.3. Subcellular calmodulin activation. 156
6.2.4. Extra calmodulin binding sites do not affect the plateau. 159
6.2.5. TA-CaM response partially similar to high affinity calcium indicator 163
6.3. DISCUSSION & CONCLUSIONS

6.3.1. Kinetics of the calcium response. 163
6.3.2. The calmodulin response 165
6.3.3. Integration of calcium transients by calmodulin 165
6.3.4. Are there any localised hotspots in calmodulin activity during the plateau? 169
6.3.5. Calmodulin as a memory molecule 170

CHAPTER 7: GENERAL DISCUSSION & CONCLUSIONS 171

7.1. Using pharmacological calmodulin inhibitors 171
7.2. Subcellular calmodulin localisation 172
7.3. Subcellular calmodulin activation 175

8. BIBLIOGRAPHY 178

9. APPENDIX 189
LIST OF FIGURES AND TABLES

CHAPTER 1:

Figure 1.1. Bacterial chemotaxis: a signal transduction cascade. 18
Figure 1.2. Calcium sinks and sources in cells. 22
Figure 1.3. EF-hand calcium binding domain. 33
Figure 1.4. Sequence of vertebrate calmodulin. 35
Figure 1.5. Calmodulin has a dumbbell structure. 37
Figure 1.6. Calcium occupation of individual binding sites. 40
Figure 1.7. GAP-43 sequesters calmodulin at membranes. 43
Figure 1.8. Calmodulin signalling network. 56
Figure 1.9. Autophosphorylation of CaMKII. 65
Table 1.1. Properties of voltage operated calcium channels (VOCCs). 23
Table 1.2. Calcium binding proteins in cells. 31
Table 1.3. Sequence of calcium binding sites adapted from (Levine, B.A., Williams, R.J.P., 1988). 32
Table 1.4. Affinity for calcium binding by calmodulin. 38
Table 1.5. Affinities of target proteins for calcium:calmodulin binding. 57
Table 1.6. Subcellular distribution of calmodulin binding proteins. 59

CHAPTER 2:

Figure 2.1. Distribution of DRGN cell sizes used. 70
Figure 2.2. Excitation and emission spectrum of TA-CaM. 78
Figure 2.3. The concentration of injected FL-CaM in cells. 82
Figure 2.4. Schematic of the Zeiss LSM 510 confocal microscope. 86
Figure 2.5. Confocality of the microscope set-up. 88
Table 2.1. Binding constants for TA-CaM target interactions. 75

CHAPTER 3:

Figure 3.1. Responses to 5 microM calmidazolium. 100
Figure 3.2. Dose-response curves calmidazolium & W13. 102
Figure 3.3. Thapsigargin does not abolish the calmidazolium effect. 104
Figure 3.4. No responses in calcium-free buffer. 106
Figure 3.5. Dissection of the calcium source. 107
Figure 3.6. Structure of calmidazolium & W13. 107
Table 3.1. Affinities of calmodulin antagonists for calmodulin. 98
Table 3.2. Concentrations of calmidazolium used in other studies. 114

CHAPTER 4:
Figure 4.1. Subcellular localisation of injected fluorescently labelled calmodulins. 120
Figure 4.2. Discrepancy between the localisation of immunofluorescence and FL-CaM. 123
Figure 4.3. Histone H1 immunoreactivity. 125
Figure 4.4. Micro-injection causes an increase in [Ca^{2+}]. 125
Figure 4.5. Western blot of labelled and unlabelled CaM. 127
Figure 4.6. Effect of co-injecting FL-CaM with Trp-peptide or unlabelled calmodulin. 129
Figure 4.7. TA-CaM activation in nucleus and cytosol. 130

CHAPTER 5:
Figure 5.1. Nucleus over cytosol fluorescence distribution. 138
Figure 5.2. Injected cells. 138
Figure 5.3. Subcellular calmodulin activation. 139
Table 5.1. Relative subcellular distribution of calmodulin binding proteins. 136

CHAPTER 6:
Figure 6.1. Response to high K^+ of TA-CaM injected DRGN. 150
Figure 6.2. Response to a single depolarisation. 151
Figure 6.3. TA-plateau recovers in +/- 1 minute. 153
Figure 6.4. Furaptra response. 154
Figure 6.5. Calcium response in the absence of TA-CaM. 156
Figure 6.6. Geography of TA-CaM activation. 158
Figure 6.7. Calmodulin binding peptides. 160
Figure 6.8. Peak fluorescence increase. 161
Figure 6.9. Amplitude slow component of fit. 162
Figure 6.10. Time constant of slow component of fit. 162
Figure 6.11. Single depolarisation in excess unlabelled calmodulin. 164
Figure 6.12. Calcium indicators of different affinity. 164

CHAPTER 7:
Figure 7.1. Calmodulin redistribution in response to a calcium rise. 175
Figure 7.2. Recovery of TA-CaM activation after depolarisation. 177

ABBREVIATIONS

ADP adenosine 5'-diphosphate
AKAP A kinase anchoring protein
ANOVA analysis of variance
AOTF acousto optical tuning filter
4-AP 4-aminopyridine
ATP adenosine 5'-triphosphate
BAPTA 1, 2-bis(2-aminophenoxo)ethane-N,N,N',N'-tetra acetic acid
cADPr cyclic adenine dinucleotide ribose
CaM calmodulin
CaMBP calmodulin binding protein
CaMK calmodulin kinase
CaMKK calmodulin kinase kinase
cAMP cyclic adenosine 5'-monophosphate
cGMP cyclic guanosine 5'-monophosphate
CRE cAMP response element
CREB CRE binding protein
DMEM Dubecco's modified Eagles' medium
DMSO ditmethylsulphoxide
DNA deoxyribonucleic acid
DRGN dorsal root ganglion neurone
DTAF 5-(4, 6-dichlorotriazinyl)-amino-fluorescein
EDTA ethylene glycol-\textit{bis}(beta-aminoethyl ether)-N, N, N', N'-tetra acetic acid

EGTA ethylene glycol \textit{bis}(beta-aminoethyl ether)-N, N, N', N'-tetraacetic acid

FL-CaM DTAF-calmodulin

FL-dextran fluorescein-dextran

GAP-43 growth cone associated protein-43

GFP green fluorescent protein

GTP guanosine 5'-triphosphate

HBS HEPES buffered saline

HEPES N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid

hnRNP heterogeneous nuclear ribonucleoprotein particle

IAF iodoacetamido-fluorescein

IP$_3$ inositol 1,4,5 \textit{tris}phosphate

MARKS myristoylated alanine rich C-kinase substrate

Mero tetramethinemerocyanine

MLCK myosin light chain kinase

mRNA messenger ribonucleic acid

NIMR National Institute for Medical Research

NMDA N-methyl-D-Aspartate

PB phosphate buffer

PC12 pheochromocytoma 12

Pl$_3$K phosphatidylinositol 3-kinase

NA numerical aperture

NFAT nuclear factor of activated T-cells

NGF nerve growth factor

nNOS neuronal nitric oxide synthase

OGB5N Oregon Green 488 Bapta-5N

PBS phosphate buffered saline

PDE cyclic nucleotide phosphodiesterase

pmCa$^{2+}$-ATPase plasma membrane calcium/magnesium-ATPase

PMT photomultiplier tube

RITC rhodamine isothiocyanate

SEM standard error of the mean

SRE serum response element

TA-CaM TA-calmodulin

TA-Cl 2-chloro-(e-amino-Lys$_{75}$)-(6-(4-N,N-diethylamino-phenyl)-1,4,5-triazin-4-y1)-Cl

TEA tetra-ethylammonium

TTX tetrodotoxin

UV ultra violet

UCL University College London

VOCC voltage operated calcium channel
'Our deepest fear is not that we are inadequate
Our deepest fear is that we are powerful beyond measure.
It is our light, not our darkness that most frightens us.
We ask ourselves, who am I to be brilliant, gorgeous,
talented and fabulous?
Actually, who are you not to be?
You are a child of God-You playing small does not serve the world.
There is nothing enlightened about shrinking so other people will not feel insecure around you.
We were born to make manifest the glory of God that is within us.
It is not just in some of us; it is in everyone.
And as we let our own light shine, we unconsciously give people permission to do the same.
As we are liberated from our own fear, our presence automatically liberates others.'

-Nelson Mandela, inaugural speech, 1994-
PREFACE

Some of the experiments described in this thesis involved the operation of complex equipment that could not be performed alone. All the work described including initiating, performing and analysing experiments and writing the thesis were performed by me under normal supervision, with the exception of the experiments described in chapter 6. These experiments involved patching cells, operating the confocal microscope, and operating the patch-clamp computer and required the help of an additional person. Steve Bolsover, my supervisor and I took turns in operating these machines, resulting in about one third of the experiments being performed by him.

The experiments involving the use of Furapatra described in chapter 6 were performed in collaboration with the lab. of David Ogden at the National Institute for Medical Research in Mill Hill. One third of the data resulting from this collaboration including all the data in the figures of chapter 6 were acquired by me. All the analysis for chapter 6 as well as all the writing have been performed by me.

ACKNOWLEDGEMENTS

Soon after I started the work on my thesis project I also met a girl. During the progression of my research, my romantic life, too, progressed; both on winding roads with peaks and troughs, but always progressing uphill. Both trajectories have culminated now, above the clouds: I married Nabila on April 7, 2000 and now my thesis is ready. Both events provide stepping stones for a brighter future.

I would like to thank Nabila Milikan for her patience and support and for ensuring that I never forgot about other things in life. I would like to thank my parents for unintentionally evoking my interest in all what lives and how it works, they are the reason I am here. I would like to thank my family and friends for their patience.
I am grateful to Steve Bolsover, my supervisor for the support, guidance and help and the whole Bolsover laboratory for helpful discussions and a nice working atmosphere. I thank everyone in the department, past and present, for an enjoyable social atmosphere. I would like to thank the Physiology Department for awarding me a Bayliss-Starling studentship to support me for the first one-and-a-half year and the European community for the Marie-Curie Fellowship that supported me during the rest of my thesis work.
CHAPTER 1: INTRODUCTION.

1.1. GENERAL

One of the biggest and most fundamental steps in evolution (still responsible for the creation of a lot of jobs world-wide), was the formation of membrane compartments, forming primitive cells in the prebiotic soup that covered the earth about 3.5 billion years ago. Over time this resulted in a composition of the solution inside cells that was different from the outside world and with it the possibility of signalling from outside to inside arose.

1.2. INTRACELLULAR SIGNALLING

1.2.1. why?

To ensure survival and thus for evolutionary advantage, cells, from the most primitive to the most complicated, face the enduring task to respond to pressing changes in their environment.

The simplest example is found in prokaryotes. Bacteria are capable of chemotaxis: movement in response to a chemical stimulus. An example is the case of *Escherichia coli* that will automatically move in the direction of the highest concentration of glucose, unless this concentration is too high, then it will move away. The mechanism underlying the attraction to glucose in *E. coli* is simple (Figure 1.1). *E. coli* has a flagellar motor that can either turn clockwise, causing a random tumbling movement of the bacterium, or counter clockwise causing smooth swimming. If there is a glucose gradient however, swimming in the direction of the highest concentration is promoted because the flagellum twists counter clockwise. Glucose causes this chemotactic behaviour by binding to a membrane receptor. Binding of an attractant, like glucose, to the receptor reduces its activity. Consequently, the kinase CheA, that in turn phosphorylates the ‘messenger’ molecule CheY, is less active and therefore less phosphorylated CheY will be available. CheY modulates the motor proteins that make the flagellum twist. Phosphorylated CheY
induces clockwise rotation and thus tumbling, so when phosphorylation of CheY is reduced as a result of glucose binding to the receptor, smooth directional swimming is the result (Alberts, B., Bray, D., Lewis, L., Raff, M., Roberts, K. and Watson, J., 1994).

![Bacterial chemotaxis: a signal transduction cascade.](image)

Figure 1.1: Bacterial chemotaxis: a signal transduction cascade.

Bacteria move towards a source of sugars. Sugar binding to receptors reduces the activity of CheA, that phosphorylates the messenger protein CheY. CheY diffuses towards the motor protein that drives bacterial locomotion using a flagellum. Upon CheY-P binding to the motor proteins, rotation in clockwise direction will become dominant resulting in random motion of the bacterium. When less CheY-P is available counter clockwise rotation will cause directional motion.

Similar systems are in operation in the cells in the body of a complex vertebrate like ourselves. During embryonic development cells migrate to their correct position in the body, divide and become more and more specialised to perform their specific function. All these processes are governed by extracellular signals influencing intracellular processes.

### 1.2.2. Parameters of signalling molecules

For a molecule to be a signalling or messenger molecule, it has to have a certain number of properties to be effective: it has to be able to provide a sufficient signal to noise ratio, have a high affinity, show specificity and it should have an effect on another process.
1.2.3. Signal/noise ratio
First of all, a signal can only have significance when its amplitude is different from noise, i.e. random changes in the signal. Usually this is achieved by increasing the concentration of the signalling molecule several fold.

1.2.4. Specificity
For a signalling molecule to be effective, not only does the signal need to be higher than the noise level, there also needs to be a receptor to pick up the signal. Receptors have to be selective for the signal to ensure that the effect is a result from this signal only and not influenced by irrelevant other signals. This is usually achieved by the effector molecule having a high affinity for the signal molecule.

1.2.5. Change in activity
For a signal to be a signal, it needs to have an effect. Most intracellular signals work by changing the activity of a target protein or process, either increasing or decreasing it and these changes nearly always are accompanied by a change in the conformation of the target molecule.

1.3. SECOND MESSENGERS

The process by which an extracellular signal influences an intracellular process often involves a number of intermediate steps involving multiple molecules. In many cases, a signal transduction cascade converts the signal from the first messenger, the molecule or process which instigates the change (usually by interacting with a component of the cell membrane) to a second messenger, another molecule that changes its concentration or properties in response to the first messenger. The second messenger in turn activates another cascade that culminates at the target protein or proteins. In eukaryotic cells the most important second messengers are inositol 1,4,5 triphosphate (IP₃) and diacylglycerol (both generated by the action of phospholipase Cβ or Cγ), cyclic adenosine monophosphate (cAMP), generated by
adenylate cyclase, and calcium. Calcium in turn binds to and affects a number of proteins including calmodulin, some adenylate cyclases, protein kinase C and annexins (Berridge, M.J., 1993)(listed in 1.2.).

In the rest of this introduction I will concentrate on the second messenger calcium and its effectors.

1.4. INTRACELLULAR CALCIUM

Evolution has selected calcium as one of the most versatile and effective second messengers in the cell. The origin for this lies probably in the fact that calcium readily binds to inorganic molecules like phosphates and negatively charged proteins inside the membrane enclosed space and thus the concentration of unbound calcium inside primitive cells is likely to have been lower than the concentration outside (Toescu, E.C., Verkhratsky, A., 1998). Under such circumstances it is easy to imagine that some primitive proteins might have been more sensitive to calcium binding than others, simply by different distributions of negative charge over the protein. It is therefore also conceivable that some proteins could have responded to a change in the intracellular calcium concentration with a change in conformation, a capacity which over a the course of evolution would have given an selective advantage in that proteins inside the cell became responsive to changes in the calcium concentration, for example when it varied outside the cell. Thus a calcium signalling system was born.

1.4.1. Gradient

Nowadays, a similar calcium signalling system is highly conserved in all eukaryotic cells where the concentration of calcium inside is about 10,000 fold less than that outside the cell and typically in the order of 50-150 nM. Furthermore, ionic gradients across eukaryotic cell membranes generate a membrane voltage that is usually negative inside with respect to the extracellular space. The concentration and voltage gradient add to give an electrochemical gradient that thus exists provides a strong driving force for calcium to enter the cell.
1.4.2. Calcium changes

As I mentioned before, a molecule or ion will only be effective as a signal if its stimulus-induced concentration change is significantly greater than the amplitude of the noise level. The intracellular calcium concentration can rise typically to 1-5 microM, 10 times the baseline, and with a noise amplitude no higher than 50 nM, this means that the signal to noise ratio is at least 20 in the worst case and usually better. In localised compartments and close to the source of calcium, as for example beside the endoplasmic reticulum and directly underneath the plasma membrane, local calcium concentrations of 10-100 microM can be achieved increasing the signal to noise ratio dramatically.

Continuous high intracellular calcium concentrations are lethal for cells, especially for neurones, therefore cells have developed not only a sophisticated range of calcium influx pathways, but also calcium stores and extrusion systems to generate in concert functional calcium signals of limited duration (Sattler, R., Tymianski, M., 1998) In the following section I will outline the most important pathways in cells that shape calcium signals by raising and lowering the intracellular calcium concentration (Fig 1.2).

1.5. SOURCES OF CALCIUM IN CELLS

The calcium that increases the cytosolic concentration can come from a number of sources. Calcium either originates extracellularly and enters via influx pathways or it originates intracellularly and is released into the cytosol. Both calcium influx and release can have several possible mediators, the importance of which differs between cell types. I will concentrate here on the players that are important for neuronal calcium changes (figure 1.2).
Figure 1.2 Calcium sinks and sources in cells.
The cytosolic [calcium] in neurones rises due to influx via receptor operated calcium channels (ROC), voltage operated calcium channels (VOCC), capacitative calcium influx, reverse operation of the sodium/calcium exchanger or release from the endoplasmic reticulum (ER) via IP$_3$ receptors (IP$_3$R) or ryanodine receptors (RyR). Mitochondria can also contribute to the removal of cytosolic calcium.

1.5.1. Calcium influx

Calcium influx can occur via voltage operated calcium channels, receptor operated calcium channels, the reverse mode of the sodium calcium exchanger and the capacitative influx pathway.

1.5.1.1. Voltage operated calcium channels
Voltage operated calcium channels reside in the plasma membrane and contain a voltage sensor that opens up a pore inside the protein complex that is more or less selectively permeable to calcium ions. Several subtypes of these channels are known and they can be classified pharmacologically and by their activation voltage; they consist of L-type, N-type, R-type, T-type and P/Q-type voltage operated calcium channels (Birnbaumer, L. et al., 1994; Smith, S.M. et al., 1999).
Table 1.1. Properties of voltage operated calcium channels (VOCCs).

<table>
<thead>
<tr>
<th>Calcium channel type</th>
<th>inhibitors</th>
<th>activation voltage (mV)</th>
<th>inactivation voltage (mV)</th>
<th>conductance (pS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-type</td>
<td>nifedipine, nimodipine,</td>
<td>above -10</td>
<td>-50 to -10</td>
<td>25</td>
</tr>
<tr>
<td>T-type</td>
<td>flunarizine</td>
<td>&gt;-70</td>
<td>-100 to -60</td>
<td>8</td>
</tr>
<tr>
<td>N-type</td>
<td>omega-conotoxin, GIVA, Cd²⁺</td>
<td>&gt;-20</td>
<td>-120 to -30</td>
<td>13</td>
</tr>
<tr>
<td>P/Q-type</td>
<td>omega-agatoxin</td>
<td></td>
<td></td>
<td>9, 14, 19</td>
</tr>
<tr>
<td>R-type</td>
<td>not sensitive to any of the above</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Different subtypes of voltage operated calcium channels have different subcellular distributions and perform different tasks in cells. For example, the N- and P-type channels are found on nerve terminals and are specifically linked to exocytosis of synaptic vesicles (Takahashi, T. and Momiyama, A., 1993). L-type channels are found on the axons of dorsal root ganglion neurones, whereas N-type voltage operated calcium channels are found on the cell body and provide a strong calcium signal that can regulate gene expression and release large dense core vesicles (Bading, H. et al., 1993; George, E.B., Chen, W.J. and Deng, L.P., 1999). L-type channels have also been reported to be concentrated at the base of apical dendrites of hippocampal pyramidal neurones and at the growth cones of mouse neuroblastoma cells (Sculptoreanu, A. et al., 1993).

1.5.1.2. Receptor operated calcium channels

Receptor operated calcium channels are calcium channels that are activated by the binding of a ligand, usually a neurotransmitter for neurones. Binding of the ligand induces opening of a channel that usually is permeable to sodium, potassium or chloride, but for a number of channels also has considerable calcium permeability. Channels of this type that are calcium permeable have very different functions and are listed below.
*N-methyl-D-Aspartate (NMDA) receptors are involved in the induction of the memory models long term potentiation and long term depression (Ghosh, A. and Greenberg, M.E., 1995). They are activated physiologically by binding of glutamate to the receptor and the simultaneous depolarisation of the plasma membrane. This depolarisation is needed to relieve the block of the channel pore by magnesium at depolarised membrane potentials (Sharma, G. and Stevens, C.F., 1996). Opening of NMDA receptors can be greatly facilitated by the binding of the co-agonist glycine (Mayer, M.L. et al., 1989).

*Nicotinic acetylcholine receptors are found on synapses at neuromuscular junctions, in the peripheral and central nervous system. Nicotinic acetylcholine receptor opening is normally triggered by acetylcholine binding. Acetylcholine receptors are responsible for the activation of muscle contraction and have been implicated in memory formation (Mayer, M.L. et al., 1989).

*ATP receptors are found in the central as well as the peripheral nervous system. They need extracellular ATP for activation, and have been implicated in pain transduction (McCleskey, E.W. and Gold, M.S., 1999).

*Neuronal 5-hydroxytryptamine receptors open in response to 5-hydroxytryptamine binding and are responsible for modulating depressive states (Grant, K.A., 1995).

1.5.1.3. Sodium calcium exchanger
The sodium/calcium exchanger is a protein that shuttles three sodium ions out of the cell in exchange for a calcium ion. In this mode it does not belong here with the calcium influx pathways. The action of this exchanger, however, is driven by the sodium electrochemical gradient across the membrane. When this gradient is reduced by cell depolarisation, the exchanger can operate in reverse and contribute significantly to the calcium influx caused by depolarisation. Any cytosolic sodium increase that accompanies depolarisation will increase the tendency of the exchanger to work in reverse mode (Stys, P.K. et al., 1991).

1.5.1.4. Capacitative calcium influx
When intracellular calcium stores are emptied, which can be achieved experimentally by using an inhibitor of the endoplasmic reticulum calcium ATPase like thapsigargin or
cyclopiazic acid, many cell types exhibit a calcium influx that is not associated with any of the previously described calcium influx pathways (Putney, J.W., Jr., 1986). This store activated calcium influx is thought to depend on the degree of filling of the intracellular stores with calcium and has been proposed to either operate via a 'calcium influx factor', the exact mechanism of which has to date not been identified, or by a direct coupling between IP₃ receptors and calcium influx (Randriamampita, C. and Tsien, R.Y., 1993). There is still considerable debate as to which of these mechanisms is responsible. The existence of capacitative calcium influx in neurones is debatable. Only a few studies have been able to demonstrate its existence in nerve cells and there it does not seem to play a significant role (Garaschuk, O. et al., 1997).

1.5.2. Calcium release

Apart from the pathways that cause calcium influx from the extracellular space, there are other pathways that use a second messenger to release calcium from intracellular stores. Two such second messengers that promote calcium release exist: IP₃ and cyclic adenosine phosphate ribose (cADPr) (Berridge, M.J. and Irvine, R.F., 1984).

1.5.2.1. The IP₃ pathway

IP₃ is generated at the plasma membrane by hydrolysis of phosphatidylinositol-4,5-bisphosphate by Phospholipase Cγ or Cβ to IP₃ and diacyl glycerol. IP₃ is a quickly diffusing messenger that binds to IP₃ receptors on the endoplasmic reticulum. IP₃ facilitates opening of IP₃ receptor calcium channels that causes release of calcium from the endoplasmic reticulum into the cytosol. The IP₃ receptor has a bell shaped sensitivity to the calcium concentration: at low intracellular calcium concentrations, calcium works as a co-agonist, helping to open the channel, at higher calcium concentrations, calcium inhibits channel opening (Berridge, M.J., 1993). Inhibition by calcium of calcium release triggered by IP₃ has been proposed to be mediated by the calcium binding protein calmodulin that binds to the IP₃ receptor (Patel, S. et al., 1997).
1.5.2.2. The cADPr pathway
The putative second messenger cADPr is synthesised from nicotinamide adenine dinucleotide and in analogy to IP$_3$ works as a co-agonist together with calcium to release calcium from intracellular stores (Lee, H.C., 1993). cADPr acts on the ryanodine receptor and its effect has been proposed to be mediated by calmodulin (Lee, H.C. et al., 1994).

1.5.2.3. Other organelles
In some cell types calcium release from non-mitochondrial, non-endoplasmic reticulum stores has been reported (Korkotian, E. and Segal, M., 1997; Volpe, P. et al., 1988). However, the contributions to changes in intracellular calcium in neurones by these stores remain to be investigated in detail.

1.6. CALCIUM CLEARANCE

Calcium clearance pathways in neurones consist of plasma membrane and endoplasmic reticulum calcium pumps, the sodium-calcium exchanger, mitochondria and possibly other organelles.

1.6.1. Calcium pumps
Two types of calcium pump work to reduce intracellular calcium concentrations. Both pump calcium against its concentration gradient and hydrolyse ATP to ADP to provide the energy for this process.

1.6.1.1. The plasma membrane calcium/magnesium-ATPase
The plasma membrane calcium/magnesium-ATPase (pmCa$^{2+}$-ATPase) removes calcium from the cytosol and pumps it back into the extracellular space. The pump is regulated by calmodulin: the Km for calcium decreases from ~30 microM to 0.25 microM when calmodulin binds and calmodulin binding speeds up the rate of ATP dependent transport by up to ten-fold (Jeffery, D.A. et al., 1981; Muallem, S. and Karlish, S.J., 1981).
1.6.1.2. Smooth endoplasmic reticulum calcium ATPase
The smooth endoplasmic reticulum calcium ATPase (SERCA) is a calcium pump that is located on the endoplasmic reticulum. The SERCA pumps calcium from the cytosol into the endoplasmic reticulum and hydrolyses ATP to fuel this process. SERCA-pumps can be inhibited by the tumor promotor thapsigargin and by 2,5-Di(tertbutyl)1,4-benzohydroquinone (Jackson, T.R. et al., 1988; Kass, G.E. et al., 1989)

1.6.2. Calcium exchanger

The sodium/calcium exchangers (described in section 1.5.1.3) in its normal mode exchanges intracellular calcium for extracellular sodium, using the electrochemical driving force for sodium to energise the process of removing calcium against its concentration gradient.

1.6.3. Mitochondria

Mitochondria possess a low affinity calcium uptake system that couples mitochondrial ATP production to cellular activity by action of calcium on mitochondrial enzymes. It has been demonstrated that mitochondrial calcium increases are more profound when triggered by a localised high calcium domain than by global calcium elevation. These data stem from work on permeabilised cells where IP3 caused large peak rises in mitochondrial calcium that were not observed when mitochondria of permeabilised cells were directly exposed to micromolar calcium. The built up calcium gradient between mitochondria and cytosol then slowly dissipates (Rizzuto, R. et al., 1998). Overwhelming the calcium buffering capacity of mitochondria will lead to the irreversible opening of the permeability transition pore, which incapacitates mitochondrial function (Huser, J. et al., 1998).
1.6.4. Contribution to calcium clearance of individual pathways in neurones

Not all the calcium clearance pathways described above contribute to the termination of calcium signals to the same extent. In hippocampal pyramidal neurones, both in dendrites and cell body alike, it was found that clearance of calcium during recovery from a KCl induced calcium rise was mainly due to activity of the pmCa\(^{2+}\)ATPase and the SERCA, whereas mitochondria and the sodium/calcium exchanger played a minor role (Mironov, S.L., 1995).

Similarly, the clearance of dendritic calcium rises caused by back-propagating action potentials in neocortical pyramidal neurones was mainly dependent on SERCAs, not affected by mitochondrial uncouplers or inhibitors of mitrochondrial calcium uptake and was only slightly reduced by inhibitors of the sodium/calcium exchanger or calmodulin (pmCa\(^{2+}\)ATPase (Markram, H. et al., 1995)). In cultured rat dorsal root ganglion neurones, analysis of calcium transients evoked by voltage clamp depolarisation (similar to calcium signals induced by trains of action potentials) indicated a minor role in calcium clearance for the sodium/calcium exchanger and SERCA. The strongest slowing of clearance was obtained by inclusion in the patch-pipette of a peptide inhibitor of the pmCa\(^{2+}\)ATPase (Werth, J.L. et al., 1996).

1.7. CALCIUM SIGNALS CONTAIN INFORMATION

In the B-cells within the immune system, the amplitude of a calcium signal is a determining factor in the decision of which gene to switch on (Dolmetsch, R.E. et al., 1997).

In addition it is becoming increasingly clear that repetitive calcium signals have specific functions in cells. The frequency of calcium signals is important, for example for fluid secretion in salivary glands and glycogen metabolism in liver cells (Woods, N.M. et al., 1986; Zimmermann, B. and Walz, B., 1997). In T-cells a differential dependence of the expression levels of the transcription factors NFAT, Oct/OAP and NFkappaB depending on the frequency of calcium oscillations has been determined using reporter gene
constructs. In contrast the actual calcium concentration had similar (non-linear) effects on gene expression of all three reporter constructs. Taken together, these results suggested that in these cells the amplitude of the calcium signal is less important for selective gene expression than its frequency. These differences in the expression of immediate early genes are translated into differential expression of the Interleukin 2 (NFAT and Oct/OAP dependent) and 8 (NFκB dependent) late genes and thus have real significance (Dolmetsch, R.E. et al., 1998).

In neurones transient elevations of intracellular calcium are important for the induction of long term changes in cellular parameters during induction of the memory models of long term potentiation or long term depression, neurite outgrowth and neuronal differentiation (Ghosh, A. and Greenberg, M.E., 1995). The responsiveness of chick ciliary ganglion neurones to basic fibroblast growth factor, for example, can be induced by a depolarisation induced transient calcium rise and will last for up to five hours (Schmidt, M.F. et al., 1996). Depolarisation of neurones causes the activation of gene expression, the mechanism of which is particularly well studied for the induction of the prototypical proto-oncogene \( c-fos \) (Sheng, M. et al., 1990). The \( c-fos \) gene product is an immediate early gene that serves as a transcription factor binding to promoters containing an AP-1 site. Induction of \( c-fos \) expression is mediated by either the serum response element (SRE) or cAMP response element (CRE) on the \( c-fos \) promoter. Activation of \( c-fos \) expression via the SRE occurs in response to growth factors and cytosolic calcium signals, whereas CRE induced \( c-fos \) expression requires nuclear calcium and phosphorylation of the CRE binding protein (CREB) as well as phosphorylation of the CREB binding protein (Chawla, S., Bading, H., 1998).

Repetitive bursts of depolarising action potentials, induced in cultured dorsal root ganglion neurones by extracellular stimulation, were able to induce \( c-fos \) expression. Expression of \( c-fos \) was inversely related to the interburst interval and correlated with CREB phosphorylation and microtubule associated protein kinase activity, but not with the integral of the accompanying calcium signal. This indicates that the frequency of the calcium signals is the determining factor in depolarisation induced gene expression. \( c-fos \)
expression could be induced from a threshold frequency of 0.1 Hz (Fields, R.D. et al., 1997). Different immediate early genes have a different frequency response, so the frequency of stimulation can dictate different patterns of gene expression (Fields, R.D. and Nelson, P.G., 1994).

Patterned calcium signals occur spontaneously and are dependent on extracellular calcium. In developing spinal Xenopus neurones, in culture as well as in situ, both calcium spikes (rise to ~400% of baseline in <1s and decay with double exponential time constants of 10s and 2min) and calcium waves (rise to ~200% of baseline; rise and decay time variable, but many seconds to minutes) (Spitzer, N.C. et al., 1995). Spikes usually are global whereas waves are often compartmentalised within the cell and differ between soma and growth cone. When these naturally occurring patterns were imposed on cultured neurones, it emerged that maturation of a potassium current (I_Kv) as well as synthesis of the neurotransmitter γ-amino-butyric acid was regulated by spikes at frequencies of >2-3/hr, whereas frequencies <8-9/hr were most effective in affecting neurite outgrowth (Gu, X. and Spitzer, N.C., 1995; Gu, X. and Spitzer, N.C., 1997).

1.8. CALCIUM BINDING PROTEINS

Calcium has been chosen by evolution to become the most important and versatile messenger inside cells. This selection process was based on the ability of constituents of the cell to bind calcium.

Nowadays many cellular calcium binding proteins with specific functions are known and for many more the function has yet to be discovered.
Table 1.2. calcium binding proteins in cells.

<table>
<thead>
<tr>
<th>calcium binding protein</th>
<th>Kd calcium (microM)</th>
<th>localisation</th>
<th>function</th>
</tr>
</thead>
<tbody>
<tr>
<td>calmodulin</td>
<td>~1 (table1.4.)</td>
<td>throughout cell</td>
<td>buffer, confers calcium sensitivity onto other proteins</td>
</tr>
<tr>
<td>calbindin D28K</td>
<td>0.3-0.5</td>
<td>nucleus/cytosol (3)</td>
<td>buffer?</td>
</tr>
<tr>
<td>parvalbumin</td>
<td></td>
<td>cytosol</td>
<td>buffer?</td>
</tr>
<tr>
<td>annexins I and II</td>
<td>&gt;1 to &gt;100 (2)</td>
<td></td>
<td>not clear</td>
</tr>
<tr>
<td>troponin-C</td>
<td>1.8 (N-term)(1)</td>
<td></td>
<td>calcium sensor in muscle contraction</td>
</tr>
<tr>
<td>S-100P</td>
<td>1.6 site 1</td>
<td></td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>800 site 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>calgizzarin (S100C)</td>
<td>520 (EC50)</td>
<td></td>
<td>?</td>
</tr>
<tr>
<td>synaptotagmin</td>
<td>4-6</td>
<td>membrane/vesicles</td>
<td>calcium sensor in exocytosis</td>
</tr>
<tr>
<td>calretinin</td>
<td>0.3-0.5</td>
<td></td>
<td>buffer?</td>
</tr>
<tr>
<td>calpain</td>
<td>~1 calpain I</td>
<td></td>
<td>protease</td>
</tr>
<tr>
<td></td>
<td>~1000 calpain II</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Calcium binding proteins can be classified into three different categories, based on a common theme in their calcium binding domains.

The first group consists of proteins that contain a calcium binding domain characterised by a ~30 amino acid helix-loop-helix repeat, called an EF-hand. EF-hand regions show a high affinity for calcium and consist of a 12 amino acid pocket with α helices on either side (England, P.J., 1986). EF-hand domains bind calcium complexed with seven oxygenated ligands in a structure which can be described as pentagonal bipyramid. Six of the oxygenated groups are contributed by the amino acids of calmodulin and one comes from a contributing water molecule (Fig 1.3). Calmodulin, parvalbumin, troponin C, myosin light chains, S-100 proteins and the β subunit of calcineurin are calcium binding proteins of this type. A second group of calcium binding proteins is characterised by conserved repeat units that bind calcium and interact with phospholipids in a calcium
dependent manner. These are the annexins. The last group is formed by proteins that bind calcium in a similar manner to protein kinase C, using the C2 domain. Proteins like this include protein kinase C and synaptotagmin.

1.8.1. EF-hand calcium binding domains
I will now describe EF-hand domains in more detail. The requirements for a protein to be able to bind calcium become clear if we look at what some known EF-hands have in common (table 1.3). First of all they contain a high number of aspartate and glutamate residues. These amino acids are negatively charged and can therefore help to complex the positively charged calcium ion at the binding site. Having a binding site with a strong negative charge, however, will also attract other positive ions like sodium, potassium and magnesium, all of which are present in millimolar concentrations, and hydrogen, which is present at similar concentrations to calcium in a resting cell.

Table 1.3. sequence of calcium binding sites adapted (Levine, B.A., Williams, R.J.P., 1988).

<table>
<thead>
<tr>
<th>Calcium binding protein</th>
<th>amino acids involved in calcium binding (for each site the amino acids and their position are shown)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asp-56. Asn-60. Thr-60. Thr-62. Asp-64. Glu-67</td>
</tr>
</tbody>
</table>
The intracellular calcium concentration on the other hand, is less than a microM. So how do calcium binding sites obtain selectivity for calcium over the other cations? The ionic radius of the calcium ion is 0.099 nm and that for the magnesium ion 0.066 nm, hydrogen, potassium and sodium are even smaller (Vogel, H.J., 1988). It is found that other polar amino acids such as Ser, Thr, Tyr, Asn, Gln, Arg, Lys, contribute to the calcium binding site, helping to shape the binding site to give an ideal fit for the calcium ion. Other cations, like magnesium, are therefore not able to interact as strongly as calcium with the charges in the binding site and hence show a much lower affinity (Malmendal, A. et al., 1998). This 'fit' for calcium is illustrated in figure 1.3. In addition, the binding of magnesium is about $10^3$ times slower than calcium binding as measured by the rate of H$_2$O displacement on oxygenated ligands (Levine, B.A., Williams, R.J.P., 1988).

**Figure 1.3 EF-hand calcium binding domain.**
Amino acids that participate in calcium binding are indicated with their single letter amino acid code and number in the calmodulin sequence. Amino acid side chains are shown as green sticks, the main protein backbone as a grey ribbon and hydrogen bonds as dashed lines. Calcium is represented by a red sphere (Figure adapted from Nelson-MR and Chazin-WJ (1998) in Calmodulin and signal transduction (Van Eldik-LJ and Waterson-DM eds) Academic Press, NY, pp 17-64; Fig 1).

EF-hand calcium binding domains usually occur in facing pairs, where the presence of one domain stabilises the other and increases the relative affinity of the binding site pair for calcium compared with single EF-hands (Nelson, M.R., Chazin, W.J., 1998).
1.9. CALMODULIN

The most abundant calcium binding protein is calmodulin. Calmodulin content is high in many tissues: in brain, testis, gizzard, uterus its concentration is 20-100 microM, in aorta, lung, prostrate, adrenal gland, kidney medulla, erythrocytes it is 10-20 microM while its concentration is less than 10 microM in liver, kidney cortex, spleen, heart and skeletal (Ebashi, S., Ogawa, Y., 1988; Kakiuchi, S. et al., 1982). Calmodulin is involved in numerous cellular processes and in my studies I will concentrate on this versatile calcium binding protein.

1.9.1. The discovery of calmodulin

When working in the 1960s to elucidate the effect of calcium on the brain, Kakiuchi discovered the stimulating effect of calcium on phosphodiesterase and isolated the responsible modulating factor. Cheung isolated an effector of cyclic nucleotide phosphodiesterase (PDE) activation and demonstrated that it was the same as Kakiuchi's (Cheung, N.Y., 1970; Kakiuchi, S., Yamazki, R. and Nakajima, H., 1969; Kakiuchi, S., Yamazki, R. and Nakajima, H., 1970). It was the work by Teo and Wang that linked Cheung's factor to calcium and thus greatly speeded up the identification of this factor as a calcium binding protein that was renamed calmodulin (Teo, T.S. and Wang, J.H., 1973).

1.9.2. Genes & evolution

The total amino acid sequence of calmodulin (Figure 1.4) became first known in 1980 (Watterson, D.M. et al., 1980). This triggered the search for calmodulin in many species and it became increasingly clear that calmodulin was highly conserved, from unicellular algae to higher plants and from yeast to humans. The calmodulin sequence is extremely highly conserved among eukaryotes with a generic consensus sequence of 148 amino
acids that is characterised by a pattern of hydrophobic and charged residues (Fig 1.4) (Klee, C.B. and Vanaman, T.C., 1982).

Figure 1.4. Sequence of vertebrate calmodulin.
The consensus amino acid sequence for vertebrate calmodulin is shown here in the single amino acid code. Charged amino acids are indicated by colour, as are hydrophobic amino acids. Amino acids participating in calcium binding are boxed. Adapted from (VanEldik, L.J., Waterson, D.M., 1998).

Calmodulin is either coded by one or by multiple genes, depending on the species, and genes vary in the number and position of introns, but even in the case of mammals, where three genes for calmodulin are found, all have a very similar intron/exon organisation. The main differences between these genes occur in the 3' and 5' non-coding sequences. The DNA-sequence in the promotor regions of these genes are essentially distinct, implying some differences in the regulation of expression (Nojima, H., 1989; Nojima, H. and Sokabe, H., 1987).

1.9.3. Expression
The three mammalian calmodulin genes (CaM I, II and III) code for different mRNAs. CaM I codes for a 1.7 and a 1.4kb transcript, CaM II for just one 1.4 kb transcript and
CaM III for transcripts of 1.0 and 2.3 kb (Sola, C. et al., 1999). The protein products of all these mRNAs however are identical (VanEldik, L.J., Watterson, D.M., 1998). The extreme degree of conservation of calmodulin between species is illustrated by a sequence homology of 78% between the coding regions of calmodulin mRNA for chicken and potato (Jena, P.K. et al., 1989).

1.9.4. Structure

Calmodulin, a 16,790 Da protein, has a structure which is interesting at all levels. Calmodulin is quite an acidic protein (pI 4.2) (England, P.J., 1986). This is because about one third of the 148 amino acids are either aspartate or glutamate and this helps calmodulin to bind calcium and to interact with other proteins (Fig. 1.4). In addition, calmodulin is unusual in that its sequence is completely void of cysteines, which would normally be involved in the formation of disulphide bonds. There are also no hydroxyprolines that are normally involved in stabilising turns. The absence of disulphide bridges and stabilised turns make calmodulin a very flexible molecule.

Calmodulin has four EF-hand domains. Two EF-hand domains are located in the C-terminal half of the protein and two are located near the N-terminus (Fig. 1.5A). Each calcium binding site is flanked by \( \alpha \) helices and the two EF hands are separated by a \( \beta \) sheet (England, P.J., 1986; Nelson, M.R., Chazin, W.J., 1998). Each pair of EF-hands forms a globular structure, both of which are connected by a very flexible linker region (28 amino acids long) giving an overall dumbbell appearance (Fig 1.5A). The globular domains forming the 'weights' of the dumbbell measure approximately 25 x 25 x 20 Angstrom and the total structure is ~65 Angstrom long (Nelson, M.R., Chazin, W.J., 1998).

The four EF-hands in calmodulin each have an affinity for calcium of about 1 microM. Measurements of the overall affinity for calcium binding of calmodulin have yielded differing results with some studies reporting different affinities for the four binding sites and other studies finding all four sites have similar affinities (Table 1.4).
Figure 1.5. Calmodulin has a dumbbell structure.

When calcium binds to the binding sites located at calmodulin’s N- and C-terminus, calmodulin undergoes a structural change that allows it to wrap around a target protein, with its globular domains rather resembling two hands ‘grasping’ the target.
1.9.5. Calcium binding

### Table 1.4. Affinity for calcium binding by calmodulin.

<table>
<thead>
<tr>
<th>binding site</th>
<th>calcium affinity (microM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>equal for all 4</td>
<td>4 (England, P.J., 1986)</td>
</tr>
<tr>
<td>overall</td>
<td>2 (Stull, J.T., Sanford, C.F., Manning, D.R., Blumenthal, D.K. and High, C.W., 1981)</td>
</tr>
<tr>
<td>overall</td>
<td>2.4 (Dedman, J.R. et al., 1977)</td>
</tr>
<tr>
<td>successive binding to all four sites</td>
<td>15.0+/−2.0, 13.0+/−0.9, 82.4+/−8.4 and 102.0+/−9.2 (Mirzoeva, S. et al., 1999)</td>
</tr>
<tr>
<td>N-terminal sites</td>
<td>2.6 (5*10⁻¹⁰ with MLCK) (Davis, J.P. et al., 1999)</td>
</tr>
<tr>
<td>C-terminal sites</td>
<td>1 (Davis, J.P. et al., 1999)</td>
</tr>
</tbody>
</table>

This diversity in the determined affinity of calmodulin for calcium stems from the fact that the calmodulin molecule undergoes extensive structural changes upon calcium binding. Upon calcium binding there is an increase of 10-20% in α helical content and crystal models revealed that in calcium bound calmodulin the two globular domains approach each other, flexing around the linker region that connects them (fig. 1.5b)(England, P.J., 1986). Different experimental methods (equilibrium dialysis, fluorescence anisotropy, nuclear magnetic resonance spectroscopy) detect this conformational change with different sensitivities and therefore variations in the determination of kinetic parameters can arise (reviewed in (Forsén, S., Vogel, H.J. and Drakenberg, T., 1986)). In addition the calcium binding of a highly charged protein like calmodulin is sensitive to the ionic strength, pH and the concentration of the protein in solution, parameters that easily differ between studies (Nelson, M.R., Chazin, W.J., 1998).

The overall binding constant for calcium binding (K_b) is about ~5 x 10⁵ and offrates are 3-10 s⁻¹ for the C-terminus and 250-400 s⁻¹ for the N-terminus (both offrates decrease ~10-fold when calmodulin is complexed with drugs (Levine, B.A., Williams, R.J.P.,
1988). These parameters are in line with onrates for the N- and C-terminal half of calmodulin (C-2.3 and N-0.016 microM·s⁻¹ (Davis, J.P. et al., 1999)).

1.9.6. Selection of calcium over magnesium

As mentioned before, the ionic radii of the calcium ion (0.099 nm) and magnesium ion (0.066 nm) differ enough to explain why in an environment where the magnesium concentration is ~10⁴ times the resting calcium concentration and is about 10³-10² times higher in activated cells, it is calcium, and not magnesium that binds to calmodulin. The competition of magnesium for calcium binding is in particular strong at the N-terminal calcium binding sites, because these have the lowest affinity for calcium. It is thought that magnesium could be bound to this site at resting calcium concentrations and that this site might be fully populated with calcium when calmodulin binds to a target protein (Malmendal, A. et al., 1999). For further discussion of the calcium affinity of these binding sites see section 1.10.6. The conformation of calmodulin that is partially magnesium bound is closer to the conformation of calcium-free calmodulin and magnesium seems to function as an allosteric effector that does not change the cooperativity of calcium binding. A real effect of the high intracellular magnesium concentration seems to be that there is negative correlation between the magnesium concentration and the affinity of calmodulin for target binding (Ohki, S. et al., 1997).

Cadmium (ionic radius 0.097 nm) occupies calmodulins EF-hands in the same order as calcium and induces a similar biphasic conformational change. Sodium (ionic radius 0.097 nm) binds in a different order to the binding sites and does not induce a large scale conformational change like calcium or cadmium do (Vogel, H.J., 1988).

1.9.7. Co-operativity in calcium binding

The question whether there is co-operativity in the binding of calcium to the four binding sites on calmodulin has long been controversial. Equilibrium binding studies with ⁴⁵Ca yielded no co-operativity, whereas other approaches, (⁴³Ca NMR and spectroscopic:
ultra violet (UV), circular dichroism, fluorescence) gave a biphasic relation (reviewed by (Burger, D., Cox, J.A., Comte, M. and Stein, E.A., 1984; Forsén, S., Vogel, H.J. and Drakenberg, T., 1986)). Both camps were finally united when Wang et al. communicated that the data from equilibrium binding studies could be explained equally well by assuming co-operative binding to 2 x 2 calcium-binding sites as well as four independent ones and thus the equilibrium method cannot discriminate between these options (Wang, C.L., 1985). There is now reasonable agreement that calmodulin binding is by biphasic co-operative binding to 2 x 2 binding sites. Binding of calcium to these binding sites occurs in the order III, IV and then to the N-terminal sites I and II (Vogel, H.J., 1988). The predictions are that at resting calcium concentrations, hardly any calmodulin will have calcium bound (and might carry magnesium on some of its binding sites) and progressively, with increasing calcium concentrations, more sites will be occupied by calcium ((Ebashi, S., Ogawa, Y., 1988) Fig 1.6). The co-operativity in calcium binding, linked to a conformational change, allows calmodulin to act as a calcium 'trigger' with an all or none response to calcium changes.

![Figure 1.6. Calcium occupation of individual binding sites.](image)

The probability of occupancy of calmodulin's four calcium binding sites is plotted against the calcium concentration. The broken line indicates calcium binding to calmodulin. The figure is adapted from (Ebashi, S., Ogawa, Y., 1988), figure 3. The probabilities are calculated assuming intrinsic affinities of 1.2 10^-5 M^-1 of the binding sites for calcium in the presence of 1 mM magnesium.
1.10. TARGET BINDING

Calmodulin does not have any intrinsic enzymatic activity and would in the absence of any other protein, just function as a calcium buffer. The conformational change calmodulin undergoes in response to calcium binding reveals hydrophobic patches on both globular domains. 46% of the exposed hydrophobic surface consist of methionine side chains. The presence of methionines is higher in EF-hand calcium binding sites (>5%) than in average database sequences (<2%) and the long and flexible side chains of this hydrophobic amino acid are thought to help stabilise the calcium bound conformation of calmodulin and allow calmodulin to bind a variety of target proteins (Nelson, M.R., Chazin, W.J., 1998). Binding of calmodulin will confer calcium sensitivity to proteins normally unresponsive to changes in the intracellular calcium concentration.

1.10.1. Binding to calmodulin

In the following section I will discuss the mechanism of target binding by calmodulin and I will review some of calmodulin’s targets and their properties to illustrate the widespread physiological consequences of calmodulin activation in cells.

1.10.2. Calmodulin binding site

Although calmodulin has many specific cellular targets, no great sequence homology is found among calmodulin binding domains. Rather, the recognition of a binding site by calmodulin depends upon several parameters: calmodulin binding sites generally span approximately 20 amino acids, have a net positive charge, a small amount of hydrophilicity and a reasonably high amount of helical hydrophobic moment (reviewed in (O’Neil, K.T. and DeGrado, W.F., 1990)).
1.10.3. Binding modes

Calmodulin binds to a plethora of target proteins and these can be divided into proteins that are not dependent on (a rise in intracellular) calcium for calmodulin binding and those that need (an elevation in intracellular) calcium to bind calmodulin.

1.10.4. Calcium independent binding

Targets that bind calmodulin in the absence of calcium, can be divided into those that have calmodulin permanently bound and those that transiently associate with calmodulin when intracellular calcium is low. Phosphorylase kinase is an example of a multi-subunit protein that permanently binds calmodulin. The subunit composition of phosphorylase kinase is $\alpha_4\beta_4\gamma_4\delta_4$ and calmodulin forms the $\delta$ subunit. As part of phosphorylase kinase, calmodulin helps to couple cell activity to cell metabolism. Another example in this category is the $\alpha_{1c}$ subunit of the L-type voltage operated calcium channel. The L-type channel undergoes dual regulation by calmodulin: calmodulin supports both inactivation and facilitation of L-type calcium channels (Zuhlke, R.D. et al., 1999). Proteins that bind calmodulin only at low calcium concentrations are the presynaptic membrane bound protein GAP-43 (also known as neuromodulin or B50), postsynaptic membrane bound neurogranin and unconventional myosins. These proteins have as a consensus calmodulin binding IQ motif:


Where x stands for any amino acid. The best studied example of this group is GAP-43. GAP-43 is a membrane bound protein that sequesters calmodulin to sites where it might be needed at high local concentrations, such as the neuronal growth cone. Although, other targets have been suggested, calmodulin appears to be the only protein that binds to GAP-43 as assessed with precipitation with fusion protein, yeast two-hybrid system and chemical cross linking (VanDalen, J.J.W., deWit, M., Edgar, M.A.N., Huigsloot, M.,
The same is true for neurogranin, a post-synaptic GAP-43 equivalent (Prichard, L. et al., 1999). GAP-43 and neurogranin as well as myristoylated alanine rich C-kinase substrate (MARKS), are phosphorylated in the calmodulin binding domain by protein kinase C, phosphorylation and calmodulin binding being mutually exclusive (Fig. 1.7).

On the left in figure 1.7, the situation at resting calcium is depicted: calmodulin is sequestered at the plasma membrane by GAP-43. When calmodulin binds calcium, it leaves GAP-43 and can activate targets like calcineurin. Protein kinase C can phosphorylate GAP-43 and when this happens (at the right of figure 1.7), this inhibits calmodulin binding. GAP-43 can be dephosphorylated, for example by calcineurin, which will allow calmodulin to bind again. This mechanism can function as a means of redistribution of calmodulin, increasing the local calmodulin concentration, so calmodulin will activate lower affinity targets (Chakravarthy, B. et al., 1999). This sort of redistribution of calmodulin is thought to be important in neuronal growth cones where

![Figure 1.7. GAP-43 sequesters calmodulin at the membrane.](image-url)

See text for explanation. CaN is calcineurin, PKC is protein kinase C, CaM is calmodulin. The P attached to GAP-43 indicates phosphorylation.
Another example of calcium independent binding of calmodulin is the 68 kDa DNA helicase that is regulated by calmodulin and protein kinases and links calmodulin directly to gene expression (Bachs, O. et al., 1994; Buelt, M.K. et al., 1994).

1.10.5. Calcium dependent binding

The majority of target proteins bind calmodulin in a calcium dependent manner. Two consensus motifs have been proposed for calcium dependent binding:

the 1-8-14 motif

**type A** = Phe-Ile-Leu-Val-Trp-xxx-Phe-Ala-Ile-Leu-Val-Trp-xx-Phe-Ala-Ile-Leu-Val-Val-Trp-xxxx-Phe-Ile-Leu-Val-Trp

(eg calcineurin, nNOS, skMLCK)

**type B** = Phe-Ile-Leu-Val-Trp-xxxxxx-Phe-Ala-Ile-Leu-Val-Trp-xxxxx-Phe-Ile-Leu-Val-Trp

(e.g. α-fodrin, caldesmon, smMLCK)

the 1-5-10 motif

xxx-Phe-Ile-Leu-Val-Trp-xxx-Phe-Ile-Leu-Val-xxxx-Phe-Ile-Leu-Val-Trp.

(e.g. CaMKI, CaMKII, MARKS, HSP90)

Although some targets that bind calmodulin in a calcium dependent manner have divergent motifs, most calmodulin binding motifs fit these general patterns of hydrophobic amino acids with an overall net positive charge (Rhoads, A.R. and Friedberg, F., 1997).

Calcium dependent calmodulin binding affects target proteins that are part of important cellular systems and in the section below I outline the binding of these targets and give a brief description of their function to illustrate the importance and diversity of calmodulins influence on cellular processes.
1.10.5.1. cAMP system

The cAMP system is a second messenger cascade that has significant cross-talk with calcium signals. cAMP is generated by adenylate cyclases, which is are regulated by G-protein coupled receptors, for example, cAMP is produced in response to dopamine in brain. A number of adenylate cyclase isoforms is also activated by calcium loaded calmodulin (adenylate cyclases I, III and VIII)(Xia, Z. and Storm, D.R., 1997).

Activation of adenylate cyclases by calmodulin raises the cellular cAMP concentration. cAMP activates protein kinase A, which in turn has many cellular targets, including the phosphorylation of CREB that functions as a transcription factor for the expression of immediate early genes (Sheng, M. and Greenberg, M.E., 1990).

The cAMP signal is terminated via hydrolysis by a PDE that is also stimulated by calmodulin. The $V_{max}$, the maximal reaction rate, is 6-10 fold increased by calcium loaded calmodulin without any change in the $K_m$, the concentration at which half of $V_{max}$ is achieved. In short, calmodulin has profound effects on the cAMP signal transduction pathway (England, P.J., 1986).

1.10.5.2. Calcium:calmodulin activated kinases and phosphatases

1.10.5.2a Myosin light chain kinase

Myosin light chain kinase (MLCK) is the prototypical calmodulin binding protein. Two different forms, coded by separate genes, are known in vertebrates: smooth muscle MLCK (smMLCK) and skeletal muscle MLCK (skMLCK).

MLCK phosphorylates a specific serine in the N-terminal of myosin II. In smooth muscle cells this phosphorylation step leads to stronger interactions of actin and myosin, resulting in muscle contraction. Proposed functions of MLCK in non-muscle cell types include cell motility, receptor capping (basically actin mediated clustering of receptors), tension development and neuronal growth cone motility. MLCKs have a functional structure that is typical for many calmodulin regulated enzymes: they consist of a catalytic domain, the function of which is blocked by an inhibitory domain. When calmodulin binds to the calmodulin binding domain, inhibition is relieved and the kinase is activated (reviewed by (Lukas, T.J., Mirzoeva, S., Watterson, D.M., 1998)).
calmodulin binding domain of skMLCK consists of the extreme C-terminal 27 amino acids and a peptide corresponding to this sequence, named the M13 peptide, is sufficient to bind calmodulin (Blumenthal, D.K. et al., 1985). Similarly a 20 amino acid peptide corresponding to the calmodulin binding region of smMLCK has been identified (Lukas, T.J. et al., 1986). Both peptides bind calmodulin with high affinity and are powerful tools to study conformational changes in the calmodulin molecule in response to target binding (Mirzoeva, S. et al., 1999; Török, K. and Trentham, D.R., 1994). In addition they have been used to inhibit calmodulin function by preventing binding to other targets in in vitro essays and in cells (Török, K., Wilding, M., Groigno, L., Patel, R. and Whitaker, M., 1998; Török, K. et al., 1998; Wang, J. et al., 1996; Wilding, M. et al., 1995).

1.10.5.2.b. Calmodulin dependent protein kinases (CaMKs)

CaMKs are important effectors of calmodulin activity. They are responsible for some of calmodulin's effects on cellular metabolism, neurotransmitter synthesis and release, gene expression, regulation of calcium homeostasis, and modulation of the cytoskeleton by phosphorylation of over 40 potential (Hanson, P.I. and Schulman, H., 1992). This family consist of four members, Calmodulin kinases I, II, III and IV.

1.10.5.2.b.1 Calmodulin kinase II

The prototypical and best studied example of this family is calmodulin kinase II (CaMKII). CaMKII is a multimeric calmodulin binding protein with Ser/Thr kinase activity. The CaMKII multimere can be a homomeric or heteromeric assembly of 6-12 closely related subunits, α through to δ, of 54-64 kDa. The α and β subunits are exclusively neuronal, whereas the γ and δ subunits are also found in other tissues. In fact CaMKII makes up 0.25-2.0% of total protein in brain and 1-2% of total protein in the hippocampus (Sola, C. et al., 1999). Interestingly, although most CaMKII subunits are cytosolic, the αb and δb isoforms contain nuclear localisation signals that target them to the cell nucleus where they could phosphorylate the transcription factor CREB (Brocke, L. et al., 1995; Srinivasan, M. et al., 1994).
Each subunit has the same basic functional structure, consisting of catalytic, regulatory and (structural) association domains. The catalytic domain alone is constitutively active and its function is normally blocked by an auto-inhibitory domain. Upon calmodulin binding the auto-inhibitory domain is displaced and kinase activity enabled. With calmodulin bound, in the presence of calcium, kinase activity is maximal. However, when the CaMKII subunits are phosphorylated on Thr286, the offrate of calmodulin is reduced 1000-fold, from 0.4 to several 100 seconds, essentially 'trapping' calmodulin on the molecule and prolonging maximal activity (Meyer, T. et al., 1992). The onrate is not affected and thus the apparent affinity for calmodulin binding increases from 45 nM to 60 pM (Schulman, H., 1998). Calmodulin binding allows for CaMKII to autophosphorylate, in this process one subunit that has calmodulin bound can phosphorylate an adjacent subunit if this also has calmodulin bound to reveal the phosphorylation site. Autophosphorylated CaMKII retains about 60% of its activity, even when calmodulin is no longer bound and thus has attracted a lot of interest as a potential integrator of calcium signals arising from neuronal activity (Hanson, P.I. et al., 1994). Indeed calmodulin-independent CaMKII activity has been observed in several neuronal cell types in response to calcium influx via voltage operated calcium channels, receptor operated channels or store release and induction of the memory model long term potentiation is accompanied by an increase of autophosphorylated CaMKII (Schulman, H., 1998). In agreement with the hypothesis that autophosphorylation of CaMKII is important for learning, mutation of the autophosphorylation site Thr286Ala in mice, impairs their spatial learning (Giese, K.P. et al., 1998). Because at least two calmodulins need to bind to adjacent subunits for autophosphorylation to occur, the local calmodulin concentration needs to be significant to increase the chance of autophosphorylation.

1.10.5.2.b.2. CaMKI

In contrast to CaMKII, CaMKI is a monomeric kinase. CaMKI has three isoforms (α-γ). CaMKI α and γ show a high expression level only in brain, but are also found in other tissues. CaMKI has activation requirements distinct from CaMKII: it needs calcium loaded calmodulin as well as phosphorylation by a calmodulin kinase kinase on a
threonine residue for full activation. Mutation of this residue T177A prevents phosphorylation by calmodulin kinase (Haribabu, B. et al., 1995). There is some overlap in the targets of CaMKI with both CaMKII and PKA phosphorylation sites; CaMKI phosphorylates the transcription factor CREB, activating transcription factor-1, but CaMKI specifically phosphorylates synapsins I and II (Lukas, T.J., Mirzoeva, S., Watterson, D.M., 1998).

1.10.5.2.b.3. CaMKIII
Uniquely in the calmodulin activated kinase family, CaMKIII has only one known target which is the small guanosine 5'-triphosphate (GTP)-binding protein elongation factor that is responsible for translocation of the growing protein chain during protein synthesis on eukaryotic ribosomes (Mitsui, K. et al., 1993). Phosphorylated elongation factor 2 has also been detected in situ in response to a rise in the intracellular calcium concentration (Redpath, N.T. and Proud, C.G., 1994). Once phosphorylated, elongation factor 2 loses its ability to induce translocation, the overall physiological significance of this is currently unclear. CaMKIII has low nanomolar affinity for and displays autonomous activity after autophosphorylation analogous to CaMKII (Mitsui, K. et al., 1993; Redpath, N.T. and Proud, C.G., 1994).

1.10.5.2.b.4. CaMKIV
The youngest member of the calmodulin kinase family is CaMKIV and in depth studies of this kinase are only just taking off. CaMKIV displays a high expression in the forebrain and cerebellum, but is generally abundant in brain as well as in thymus; an expression pattern different from that of CaMKI and CaMKII. CaMKIV is associated with nuclei and, contrary to CaMKII, is not found in post-synaptic densities and growth cones (Ohmstede, C.A. et al., 1989). CaMKIV is capable of phosphorylating several transcription factors (CREB, activating transcription factor-1 and CREMt (Lukas, T.J., Mirzoeva, S., Watterson, D.M., 1998)) and the CREB binding protein, that functions as a switch in transcription activation (Chawla, S., Bading, H., 1998). In addition CaMKIV can potentially regulate adenylate cyclase I activity (Wayman (1996)). Adenylate cyclase
I (as well as adenylate cyclase III) immunoreactivity has recently been demonstrated in the nucleus of dorsal root ganglion neurones in culture and in brain slices (Parkinson, N.A., Bolsover, S.R., 2000). The activity of CaMKIV, like that of CaMKI is regulated by calmodulin kinase kinase and calmodulin kinase kinase has been shown to enhance CaMKIV mediated gene expression (Park, I.K. and Soderling, T.R., 1995). Not much is yet known about the exact interactions of calmodulin with CaMKIV.

1.10.5.2.c. Calmodulin kinase kinase

Two isoforms of calmodulin kinase kinase exist (CaMKKa and β) and both can phosphorylate CaMKI and CaMKIV, with preference for CaMKI. CaMKKs are activated by calcium loaded calmodulin and can undergo autophosphorylation (Edelman, A.M. et al., 1996). The calmodulin binding site has not been exactly identified and the parameters of calmodulin binding remain unknown, however it was recently established that although CaMKK activity increased with the intracellular calcium concentration, there was substantial activity of CaMKK at resting calcium concentrations (Anderson, K.A. et al., 1998).

1.10.5.2.d. Phosphatidyl inositol 3-kinase (PI3K)

Cross-talk between calmodulin and another signalling pathway has been reported. PI3K regulates events downstream of insulin and growth factor receptors by phosphorylating the membrane lipid phosphatidyl inositol on the three position. PI3K contains a high affinity (Kd 5 nM) calmodulin binding domain that is highly conserved among PI3K isoforms and binds calmodulin in a calcium dependent way in vitro (Fischer, R. et al., 1998). Furthermore, it has been demonstrated that calmodulin immunoprecipitates with PI3K and that a calmodulin antagonist inhibits both basal and calcium stimulated phosphatidyl inositol phosphorylation in intact cells (Joyal, J.L. et al., 1997).
1.10.5.2.e. Other calmodulin activated kinases

More calmodulin activated kinases are known, such as the 'death-associated protein kinase' and probably more will be discovered, but I will limit my description to the most well studied calmodulin activated kinases known at present (Deiss, L.P. et al., 1995).

1.10.5.2.f. Calmodulin activated phosphatases

Calmodulin is involved in the activation of the two major protein phosphatases: calcineurin (or protein phosphatase 2B) and protein phosphatase 1. Calcineurin is a Ser/Thr phosphatase that is particularly abundant in brain where it constitutes 1% of total protein in the hippocampus (Sola, C. et al., 1999). In total brain its concentration has been estimated to be 10 microM (England, P.J., 1986). Calcineurin is absolutely dependent on intracellular calcium for calmodulin binding and in the presence of calcium, calmodulin and calcineurin bind tightly with a Kd of 0.1-1.0 nM (Klee, C.B. et al., 1988). This makes calcineurin potentially an important calmodulin buffer. The Kact for calcium of this process is 0.3-1 microM (Stemmer, P.M. and Klee, C.B., 1994). The memory models long term depression and long term potentiation are activated by low and high frequency stimulation and activation of calcineurin at lower intracellular calcium concentrations as opposed to activation of CaMKII at higher intracellular calcium concentrations, fits with the finding that the switch between long term depression and long term potentiation depends on the relative activation of phosphatases and kinases (Coussens, C.M. and Teyler, T.J., 1996). Calcineurin consists of a catalytic α subunit containing the calmodulin binding domain and a regulatory calcium binding β subunit. Although the β subunit itself binds calcium, both calmodulin and the β subunit are needed for full activation of phosphatase activity and calmodulin and the β subunit cannot substitute for each other.

In neurones, calcineurin is equally distributed between the plasma membrane and the cytosol and is also found in the cell nucleus (Pujol, M.J. et al., 1993). Calcineurin can regulate several membrane proteins, including the AMPA receptor, involved in long term potentiation, and the protein dynamin, part of the endocytotic machinery. It has been proposed that calcineurin can associate with membranes in a calcium dependent way,
thus increasing its effective concentration up to 1000-fold by limiting itself to a two-
dimensional diffusion surface. Other calcineurin substrates include microtubule associated
protein 2, τ protein, γ-amino-butyric acid A receptors and sodium channels. Calcineurin
increases gene transcription in T-cells by dephosphorylation of the transcriptional
regulator Nuclear Factor of Activated T-cells (NFAT). Dephosphorylation of NFAT
induces nuclear translocation and induction of the interleukine-2 gene. Calcineurin also
has role in CREB induced transcription (Perrino, B.A., Soderling, T.R., 1998). In
addition to its own phosphatase activity, calcineurin can recruit the activity of a second
phosphatase that has different substrate specificity. This phosphatase is protein
phosphatase-1. Protein phosphatase-1 activity is normally inhibited by Inhibitor-1.
Inhibitor-1 can be dephosphorylated by calcineurin thus relieving inhibition of protein
phosphatase-1.
Calcineurin is thus an important player in counteracting the effect of phosphorylation by
kinases like protein kinase A and C and the calmodulin activated kinases.

1.10.5.3. Ion channels
Many ion channels have been shown to be regulated by intracellular calcium, either
enhancing activation or inactivation. At least part of the modulation of ion channels by
calcium is increasingly being demonstrated as due to modulatory actions of calmodulin
(Saimi, Y. and Kung, C., 1994). Calmodulin is responsible for inactivation of the NMDA
receptor upon calcium, Calmodulin activates SK potassium channels and modulates L-
insensitive calmodulin mutant ablates the calcium dependent inactivation of L-VOCCs
(Peterson, B.Z. et al., 1999).

Calmodulin binds to the ryanodine receptor and reduces channel activity by more than 2-
fold, but in addition to inhibition at high intracellular calcium concentrations, calmodulin
has also been shown to potentiate the ryanodine receptor at low calcium (Ikemoto, T. et
al., 1998; Smith, J.S. et al., 1989). Calmodulin modulates IP$_3$ receptors in a way
functionally similar to the modulation of the ryanodine receptor (Yamada, M. et al.,
By regulating calcium release channels in a calcium dependent way, calmodulin provides direct feedback on these channels.

1.10.5.4. Cytoskeletal interactions
Several proteins that associate with the cytoskeleton are calmodulin targets. They include microtubule associated protein-2, fodrin (non-erythrocyte spectrin), unconventional myosins, kinesin light chains, MARKS, tubulin, adducin, the post synaptic density protein NAP-22, a neuronal synaptic and microtubule associated heat labile protein of 80kDa and heat shock proteins 90 and 100 (Gnegy, M.E., 1995; Wood, J.G. et al., 1980). Calmodulin has also been shown to regulate the calcium binding site of actin (Vandenkerckhove, J., 1990). Calmodulin inhibits microfilament re-polymerisation by preventing interaction between actin and microtubule associated protein-2 and τ proteins (Kotani, S. et al., 1985). In the presence of microtubule associated protein-2 calmodulin also modulates the formation of microtubuli (Lee, Y.C. and Wolff, J., 1984). Together, by modulating tubulin and actin polymerisation, calmodulin contributes to cytoskeletal remodelling during axon guidance and neurite outgrowth (Archer, F., Ashworth, R. and Bolsover, S.R., 1998). Indeed, targeted disruption of calmodulin in the growth cones of extending neuronal processes in Drosophila leads to increased stalling of the extending axons and errors in pathfinding (VanBerkum, M.F. and Goodman, C.S., 1995).

1.10.5.5. Other targets

1.10.5.5.a. Nitric oxide synthase
Nitric oxide is an important enzyme that coverts L-Arginine into L-Citrulline and the very volatile, diffusible second messenger nitric oxide. Nitric oxide regulates smooth muscle relaxation in the blood vessel wall and is a candidate for the retrograde messenger that helps induce long term potentiation by signalling between pre and postsynaptic neurones, activating cyclic guanoside monophosphate synthesis by guanylate cyclase.
Three isoforms are known: nNOS, eNOS and iNOS. I will concentrate here on the neuronal isoform (nNOS). Calmodulin binds nNOS with a Kd of ~3.5 nM and
inactivation of nNOS is about ten-fold faster than the unbinding of calcium from the calmodulin C-terminal binding sites and also faster than MLCK enzyme inactivation (Schmidt, H.H. and Murad, F., 1991). The EC50 for calcium activation of calmodulin:nNOS is ~100-400 nM (Hu, J. and el Fakahany, E.E., 1996). nNOS activity is decreased by phosphorylation by CaMKII and increased by dephosphorylation by calcineurin. This means that nNOS will be activated at relatively low calmodulin and calcium concentrations and with the rapid inactivation, nNOS activity will track changes in the intracellular calcium concentration.

1.10.5.5.b. pmCa$^{2+}$-ATPase
The interactions of calmodulin with the pmCa$^{2+}$-ATPase have been described in section 1.6.1.1.

1.10.5.5.c. Other calmodulin binding proteins
Calmodulin targets are numerous and more are still being discovered. Rather than trying to give a comprehensive review of every single one, I will here just list calmodulin binding proteins that I have not discussed above:

* histone H1
* the calcium activated potassium channel
* caldesmon (Bachs, O. et al., 1994),
* synapsins (Rhoads, A.R. and Friedberg, F., 1997)
* basic helix loop helix (bHLH) transcription (Bachs, O. et al., 1994).
* heterogeneous nuclear ribonucleoprotein particle (hnRNP) (Bachs, O. et al., 1994)
* p62 (Bachs, O. et al., 1994)
* GAP junction connexons (Török, K. et al., 1997)
* ribosomal proteins L3 & S7 (Agell, N. et al., 1998)
* NAP-22 (Iino, S. and Maekawa, S., 1999)

* Spc 110 (Agell, N. et al., 1998)
* τ proteins (Bachs, O. et al., 1992)
* TRPL (Rhoads, A.R. and Friedberg, F., 1997)
* the nuclear auto-antigen La/SSB (Agell, N. et al., 1998)
* the small G-protein Rin (Lee, C.H.J. et al., 1996)
* calmodulin also binds to the metabotropic glutamate receptor (mGluR) and the competition of calmodulin binding with the G-protein subunit Gβγ regulates the amount of Gβγ available for modulating m-currents (O'Connor, V. et al., 1999)
* estrogen receptor: calmodulin forms an integral component of the estrogen receptor complex that binds to the estrogen response element and is required for activation of the 17 β-estradiol promoter in mammary epithelial cells (Biswas, D.K. et al., 1998).

1.10.6. Kinetics of calmodulin binding

A simple scheme of equilibrium binding of calcium:calmodulin to a target protein would be the following (where CaMBP represents a calmodulin binding protein):

\[
[\text{CaM}:(\text{Ca}^{2+})_4] + [\text{CaMBP}] \leftrightarrow [\text{CaM}:(\text{Ca}^{2+})_4]:\text{CaMBP} \quad \text{equation 1.1}
\]

This scheme assumes calmodulin to be fully calcium liganded before binding occurs, in practise this is probably not the case and the following scheme would be closer to reality (equation 1.2, based on (Lukas, T.J., Mirzoeva, S., Watterson, D.M., 1998)):
Where $K_{1-4}$ are association constants for binding of calcium by calmodulin, $K_{ae}$ are association constants for binding of calmodulin to enzyme (target) with 1-4 calcium ions bound and $K'_{1-4}$ are association constants for calcium binding by the calmodulin:enzyme complex. Equation 1.2 illustrates the difficulty in examining the mechanism underlying the overall rate constants for calcium:calmodulin binding and binding of calcium:calmodulin to target proteins. The rate constants of calcium binding to different sites are impossible to determine on the intact molecule, because of the co-operative binding to the different sites. For the inactivation route of the calcium-bound calmodulin:target complex it is not currently clear at which stage the complex dissociates on loosing calcium. The scheme clarifies however, why dissociation constants and on and offrates are not easily estimated. $K_1$ to $K_4$ for calcium binding of calmodulin have been estimated though, as 15.0$\pm$2.0, 13.0$\pm$0.9, 82.4$\pm$8.4 and 102.0$\pm$9.2 microM respectively. In the presence of the RS20 peptide $K_1-K_4$ have also been determined, as 0.4$\pm$0.1, 2.5$\pm$0.2, 0.8$\pm$0.2 and 1.1$\pm$0.1 microM (Mirzoeva, S. et al., 1999). The offrate for calcium-loaded calmodulin bound to target is significantly slower than the onrate for most targets and extremely slow when autophosphorylated CaMKII 'traps' calmodulin (>100s (Meyer, T. et al., 1992). This effectively causes the reaction in equation 1.1 to be one way, resulting in a dramatic apparent increase of the Kd up to 200-fold (Cox, J.A., 1984). This increased apparent affinity creates the possibility that some calmodulin will be calcium-bound and active when bound to target at resting intracellular calcium concentrations. Competition of other ions for calcium binding is discussed in section 1.9.6.

1.10.7. Layers of complexity
1.10.7.1. How are calmodulin activated processes co-ordinated in response to a calcium rise?

Are all the processes calmodulin is involved in activated at the same time? How is specificity achieved? Basically, not all calmodulin targets and the affected processes will be active at the same time, although some will coincide. For example, activation of phosphorylation processes will cost energy, so it makes sense to activate phosphorylase
kinase at the same time as kinases to keep up with energy demand. Also, when a neurone is depolarised and calcium floods in, then it is good to inactivate the voltage operated calcium channels and activate repolarising potassium channels to stop calcium influx and activate the pmCa\(^{2+}\)-ATPase to pump calcium out of the cell. On the other hand, activating calmodulin activated kinases and phosphatases to the same extent would only be useful if a truly short-lived response is desired. And also, some cell activity like induction of long term potentiation might require gene expression, but it would be less appropriate for other processes such as neurotransmitter release. So how are all these responses co-ordinated? Separation of responses can occur on three levels: by spatial separation, temporal separation (frequency modulation) and by different affinities for calcium:calmodulin binding. All of these occur.

The complex interactions of signalling pathways that are activated in concert by calcium:calmodulin to evoke a physiological response have been clarified by simulation studies (Fig 1.8 (Bhalla, U.S. and Iyengar, R., 1999)).

**Figure 1.8. Calmodulin signalling network.**
See text for explanation. AC= adenylate cyclase, PP1= protein phosphatase 1, CaN= calcineurin, CaM= calmodulin, PKA= protein kinase A, PDE= phosphodiesterase, CaMKII= calmodulin activated kinase II, PMCA= pm Ca\(^{2+}\)-ATPase, ROC= receptor operated channel, ER= endoplasmic reticulum, cAMP= cyclic adenosine monophosphate, VOCC= voltage operated calcium channel, Na\(^+\)/Ca\(^{2+}\)= sodium/calcium exchanger (Adapted from: Bhalla-US and Ilyengar-R (1999) Science 283: 381-387).
The model used contains the NMDA receptor, calcium and calmodulin. Calmodulin activates adenylate cyclase (AC), PDE, CaMKII and calcineurin (CaN). This in turn can lead to activation of cAMP and PKA and prolonged activity of CaMKII, a scenario that promotes gene transcription. Alternatively, calcineurin can dephosphorylate inhibitor protein 1, that leads to activity of protein phosphatase 1 (PP1). PP1 dephosphorylates CaMKII, leading to reduced gene transcription. By assuming Michaelis-Menten kinetics for the reactions and using rate constants obtained from experimental data, it was possible to mathematically model the activity of the adenylate cyclase pathway and the activity of the calmodulin-CaMKII/calcineurin pathways simultaneously. In this way the relative importance of each pathway for the induction of gene expression could be clarified. Simulations revealed the importance of the cAMP-PKA pathway to amplify CaMKII activity and thus increase gene expression. These simulations indicate an important role of the affinity of calmodulin targets and compartmentalisation for the physiological response. First of all, calmodulin’s targets have different affinities for calmodulin binding (Table 1.5), therefore the local availability of calcium:calmodulin is an important determinant in whether or not a specific target enzyme will be active.

Table 1.5. Affinities of target proteins for calcium:calmodulin binding.

<table>
<thead>
<tr>
<th>target protein</th>
<th>Kd (nM)</th>
<th>Km (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>calcineurin</td>
<td>0.1-1 (4)</td>
<td>0.09 (1)</td>
</tr>
<tr>
<td>CaMKII</td>
<td>0.06 (3)</td>
<td></td>
</tr>
<tr>
<td>Thr286-P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaMKII</td>
<td>45 (3)</td>
<td>375 (1)</td>
</tr>
<tr>
<td>MLCK</td>
<td>0.10-0.22 (2)</td>
<td>3.1 (1)</td>
</tr>
<tr>
<td>nNOS</td>
<td>~3.5 (5)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>PDE</td>
<td></td>
<td>45 (1)</td>
</tr>
<tr>
<td>pmCa(^{2+})ATPase</td>
<td>nM range (7)</td>
<td>10 (1)</td>
</tr>
<tr>
<td>MAP-2</td>
<td>700 (8)</td>
<td></td>
</tr>
<tr>
<td>PI3K</td>
<td>5 (6)</td>
<td></td>
</tr>
</tbody>
</table>

Both the dissociation constant Kd, as an indicator for the affinity in a steady state reaction and the Km, the concentration at which half maximal reaction speed (Vmax) is reached, as an indicator of how tightly a substrate is bound (the higher Km, the weaker the binding), are given. 1. (Quadroni, M. et al., 1998), 2. (Török, K. and Trentham, D.R., 1994), 3. (Schulman, H., 1998), 4. (Klee, C.B. et al., 1988), 5. (Schmidt, H.H. and Murad, F., 1991), 6. (Fischer, R. et al., 1998), 7. (Brandt, P.C., Vanaman,T.C., 1998), 8. (Bonafe, N.M., Sellers, J.R., 1998).
1.10.7.2. Spatial considerations
Spatially, three considerations are important: the local availability of calmodulin, the local calcium concentration and the quantity and affinity of local targets. Targets like GAP-43 increase local availability by sequestering calmodulin on the membrane, resulting in a high availability of both calcium and calmodulin when calcium concentrations rise. Co-localisation of targets is a way to solve the concentration problem that is used for example by the NMDA receptor that has CaMKII bound to it, ready to phosphorylate when calcium:calmodulin concentrations rise (Leonard, A.S. et al., 1999). CaMKII, as well as calmodulin, is enriched in the post synaptic density and translocates there upon NMDA receptor (Shen, K. and Meyer, T., 1999). Post synaptic density proteins have been proposed to concentrate NMDA receptors and AMPA receptors as well as potassium channels and signalling units thus formed would be capable of both generating a calcium signal in response to glutamate and allowing calmodulin to modulate the response and repolarise the membrane by acting on potassium channels. With a similar rationale, calmodulin activated adenylate cyclases are membrane bound as are calmodulin activated phosphodiesterases. The A-kinase anchoring protein (AKAP) could potentially also bring protein kinase A within reach by sequestering protein kinase at the membrane. In a broader context, there exists a subcellular distribution of calmodulin targets that is non-uniform (table 1.6) allowing differential activation of cellular targets.

1.10.7.3. Subcellular distribution of calmodulin
In analogy to the specificity lent to the response to calcium:calmodulin by the subcellular distribution of target proteins, the distribution of calmodulin itself can also be dynamically regulated to provide localised increases in the concentration of calmodulin in response to particular stimuli.

1.10.7.4. Bound/free calmodulin
Although the total concentration of calmodulin in brain has been estimated to be between 10 and 100 microM, given the presence of targets that bind calmodulin in the absence of calcium and some high affinity targets it is conceivable that only a proportion of cellular
calmodulin will be freely diffusible, but this is hard to investigate (Kakiuchi, S. et al., 1982). By studying the mobility of fluorescently labelled calmodulin in smooth muscle cells, it was estimated that about 5% of total calmodulin was mobile (Luby Phelps, K. et al., 1995). Another study of human embryonic kidney 293 cells estimated that free calmodulin comprised about 0.1% of the total cellular amount (Romoser, V.A. et al., 1997). Little is known about whether bound calmodulin activates these targets and about the calcium loading of calmodulin in (resting) cells.

Table 1.6. Subcellular distribution of calmodulin binding proteins.

<table>
<thead>
<tr>
<th>membrane</th>
<th>cytosol</th>
<th>nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaMKII</td>
<td>CaMKII</td>
<td>CaMKII (1,2)</td>
</tr>
<tr>
<td>ACI, III, VIII</td>
<td>CaMKI, CaMKIII</td>
<td>CaMIV (1,2)</td>
</tr>
<tr>
<td>PDE</td>
<td>calcineurin</td>
<td>calcineurin (1,2)</td>
</tr>
<tr>
<td>unconventional myosins (?)</td>
<td>nNOS</td>
<td>α-spectrin</td>
</tr>
<tr>
<td>pmCa^{2+}-ATPase</td>
<td>RyR</td>
<td>histone 1</td>
</tr>
<tr>
<td>NMDAR</td>
<td>IP3R</td>
<td>p68 DNA replitase (1)</td>
</tr>
<tr>
<td>K_{Ca^{2+}}</td>
<td></td>
<td>AC7</td>
</tr>
<tr>
<td>L-VOCC</td>
<td>MLCK</td>
<td>MLCK (1)</td>
</tr>
<tr>
<td>MARKS</td>
<td>caldesmon</td>
<td>caldesmon (1)</td>
</tr>
<tr>
<td>synapsins (3)</td>
<td>phosphorylase kinase</td>
<td>bHLH transcription factors (1,2,7)</td>
</tr>
<tr>
<td>neurogranin (3)</td>
<td></td>
<td>CaMKK</td>
</tr>
<tr>
<td>GAP-43 (3)</td>
<td></td>
<td>hnRNP (1,2)</td>
</tr>
<tr>
<td>α spectrin (2)</td>
<td></td>
<td>p62 (1,4)</td>
</tr>
<tr>
<td>GAP junction connexons</td>
<td></td>
<td>ribosomal proteins L3</td>
</tr>
<tr>
<td>NAP-22 (6)</td>
<td></td>
<td>&amp; S7 (2)</td>
</tr>
<tr>
<td>TRPL (3)</td>
<td></td>
<td>HSP90 (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spc 110 (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>τ proteins (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>myosin I (5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>La/SSB (2)</td>
</tr>
</tbody>
</table>

1.10.7.5. Subcellular distribution of calmodulin in neurones

In neurones, the distribution of calmodulin immunoreactivity at the light microscopic level appears to be quite homogeneous throughout the cytosol with the amount of immunoreactivity in the nucleus sometimes reported as slightly higher than in the cytosol, sometimes as lower (Sola, C. et al., 1999). An explanation for these discrepancies, apart from differences in methodology, could be found in the fact that the distribution of calmodulin immunoreactivity has been found to change during differentiation and development (Natsukari, N. et al., 1995). On an electronmicroscopic level (Caceres, A. et al., 1983), calmodulin immunoreactivity in neurones was associated with the:

* nuclear chromatin, leaving the nucleolus unstained,
* nuclear, reticular and mitochondrial membranes,
* axonal microtubules (but not actin), increasing with development
* apical growth cones of developing neurones.
* postsynaptic densities of spine and shaft synapses

1.10.7.6. Dynamic subcellular redistribution of calmodulin

In addition to the differential distribution of calmodulin's targets, calmodulin itself can redistribute between subcellular compartments in response to cellular activity and the calmodulin levels in the cell can be regulated both by the expression level of calmodulin binding proteins and by hormone action (Gnegy, M.E., 1993).

The effect of the expression of calmodulin binding proteins is illustrated by knockout models that lack a calmodulin binding protein. Knock-out of phosphorylase kinase in rabbit skeletal muscle or unconventional myosin in Drosophila photoreceptors in both cases resulted in a decrease in the amount of calmodulin detected in these cells (Cohen, P. et al., 1978). The down-regulation of calmodulin in phosphorylase deficient skeletal muscle is accompanied by a 54% decrease in all calmodulin mRNA so regulation is likely to occur at the transcriptional level (Bender, P.K. et al., 1988). On the other hand various drugs and hormones (e.g. nerve growth factor, melatonin, amphetamine,
oestrogens) can cause an overall increase in calmodulin content after prolonged (days) treatment (Gnegy, M.E., 1993).

Movement of calmodulin between membrane, cytosol and nucleus has mainly been studied biochemically, using cell fractionation methods and assessing the calmodulin content of the different cellular fractions.

1.10.7.6.a. Membrane to cytosol

Stimulation induced translocation of calmodulin from the membrane to the cytosolic fraction of neurones has been observed in response to dopamine, apomorphine, amphetamine, morphine, Leu-enkephalin, carbachol and bradykinin. The drugs cocaine and high doses of amphetamine have the opposite effect and induce translocation of calmodulin from the cytosol to the membrane. These translocations take place over a timescale in the order of tens of minutes (Gnegy, M.E., 1993). The increase of cytosolic calmodulin in response to muscarinic receptor stimulation has been studied in more detail in human neuroblastoma cells. Muscarinic stimulation leads to an increase in both IP3, causing calcium release from intracellular stores, and protein kinase C activation, which work in concert to liberate calmodulin from calmodulin sequestering proteins like GAP-43 (Mangels, L.A. and Gnegy, M.E., 1990).

1.10.7.6.b. Cytosol to nucleus

The nucleus is separated from the cytosol by a double membrane layer, the nuclear envelope, that forms an extension of the endoplasmic reticulum. The nuclear envelope contains a nuclear pore complex, a large (~125MDa) multi-subunit protein that regulates nuclear import and export of proteins. The nuclear pore complex forms a barrier for diffusion of proteins larger than ~60kDa (Doye, V. and Hurt, E., 1997). Bigger proteins are imported in an active, energy dependent process and usually require a nuclear localisation signal to gain access to the nucleus. Nuclear import depending on a nuclear localisation signal can be inhibited by chilling to 0-4 degrees C, addition of wheat germ agglutinin or the non-hydrolysable guanosine triphosphate analogue GTPγS.
Translocation of calmodulin from the cytosol to the cell nucleus has been demonstrated using immunocytochemistry as well as imaging of fluorescently labelled calmodulin. Use of fluorescently labelled proteins is an increasingly popular way to study dynamics of subcellular localisation on a sub-second time-scale, which is hard to achieve with traditional biochemical or immunohistochemical techniques.

An increase of nuclear calmodulin content is observed during cell division and inhibition of nuclear calmodulin with the M13 peptide, tagged with a nuclear localisation signal arrests dividing cells at the start of the S-phase (Wang, J. et al., 1996). Calmodulin activated enzymes like MLCK, CaMKII, CaMKIV and calcineurin are found in the nucleus, and translocation of calmodulin immunoreactivity to the nucleus after depolarisation of hippocampal neurones correlates with the phosphorylation of CREB and thus with the induction mechanism for gene expression (Deisseroth, K. et al., 1998).

Nuclear translocation of fluorescently labelled calmodulin in response to a transient increase in intracellular calcium has been shown in smooth muscle cells and recently in pancreatic acinar cells (Craske, M., Takeo, T., Török, K., Gerasimenko, O., Peterson, O.H. and Tepikin, A.V., 1998; Luby Phelps, K. et al., 1995). Calmodulin translocation in pancreatic acinar cells was confirmed by immunocytochemistry and, interestingly, in these cells the nuclear concentration of fluorescent calmodulin oscillates with the intracellular calcium changes in a way that allows integration of calcium signals by nuclear calmodulin accumulation. From a recent study investigating the mechanism of nuclear translocation of fluorescently labelled calmodulin in permeabilised A7R5 cells, it was concluded that calmodulin enters the nucleus via a calcium dependent passive process and is retained in the nucleus by calmodulin binding proteins. It was suggested that calmodulin's distribution over nucleus and cytosol reflects the pattern of affinities of the available calmodulin binding sites (Liao, B. et al., 1999). These studies contrast with recent findings by Hardingham, who, using fluorescently labelled calmodulin and calmodulin antibodies, could not find any calcium induced nuclear translocation in hippocampal neurones and found nuclear accumulation in resting cells, that was mediated by an active process (Hardingham, personal communication and Hardingham et al, (1999) SFN Miami
A study of nuclear calmodulin import by Pruschy et al. shows data of a permeabilised cell essay somewhere in-between the previously described studies, with ATP-independent nuclear calmodulin accumulation that could be inhibited with wheat germ agglutinin or chilling in both the presence and absence of calcium (Pruschy, M. et al., 1994). I think the first study I described is the most thorough and probably the most accurate (Liao, B. et al., 1999). These authors report that if a wash step after each translocation assay is omitted, the results are a lot more variable. It could be this difference that leads the other interpretations (Pruschy, M. et al., 1994) (Hardingham, personal communication and Hardingham et al, (1999) SFN Miami Beach; see also chapter 4 for discussion).

1.10.7.7. Temporal coding

The range of affinities of calmodulin binding proteins provides a diverse spectrum of targets. At a given calcium and calmodulin concentration, some targets will be activated, whereas others are not. Stronger or longer lasting signals will liberate more calcium:calmodulin and thus increase the likelihood that a low affinity calmodulin target will be activated. If calcium:calmodulin signals are transient and occur intermittently, then calcium:calmodulin might not fully activate a given target if the signal is not strong enough (Hanson, P.I. et al., 1994). However, because offrates vary between targets it is possible that, if a signal is repeated faster than the offrate for target binding, a proportion of target will have calmodulin bound continuously during stimulation. By repeating the signal, a situation can be created where subsequent stimulations summate to provide the parameters for target activation. This can occur by a staggered local increase in the calcium:calmodulin concentration or by increasing amounts of calcium:calmodulin staying bound to the target between stimuli, if the offrates are slower than the repetition frequency. These scenarios allow for the integration of a repetitive signal. CaMKII has been proposed to act as an integrator of calcium signals and the underlying mechanism has been investigated.
CaMKII is a suitable candidate to be a detector of the frequency of calcium signals. It is for example activated during frequency dependent stimulation protocols as needed for the induction of long term potentiation. Autophosphorylation of the kinase, as discussed in section 1.5.2.b.1, results in prolonged autonomous activity of the kinase after the termination of the calcium signal. Autophosphorylation requires binding of calmodulin to adjacent subunits and once it has occurred, the offrate for calmodulin is dramatically decreased, effectively trapping calmodulin on CaMKII (Hanson, P.I. et al., 1994). The fact that more than one calmodulin needs to bind before autophosphorylation can occur and the slow offrate for calmodulin imply that CaMKII will be sensitive to the interval of calcium transients: if the interval is long, calmodulin would drop off and the chances of autophosphorylation would be reduced, so the prediction is that only at higher frequencies CaMKII would bind enough calcium:calmodulin to achieve the autonomous state. Simulations of this process showed that when calcium transients occur at a 20s interval, no increase in auto-phosphorylation would occur, whereas at 2 s intervals autophosphorylation would reach ~70% of maximal (Fig 1.9 (Hanson, P.I. et al., 1994)).

This theory was investigated in vitro using rapid perfusion of calcium and calmodulin at different frequencies and assessing the extent of CaMKII auto-phosphorylation by measuring kinase activity after stimulation (De Koninck, P. and Schulman, H., 1998). Stimulation protocols delivering a total of 6 s of fixed length calcium:calmodulin spikes at different frequencies revealed that under these circumstances the level of CaMKII autonomy was dictated by the stimulation frequency. There was no increase in autonomy at 1 Hz, intermediate autonomy at 2.5 Hz and near maximal autonomy at over 4Hz stimulation. In cells however, the picture is likely to be more complicated, first of all because CaMKII will have to compete for calcium:calmodulin with other calmodulin binding proteins and secondly because phosphatases might counteract kinase activity. Although CaMKII probably represents an extreme in calmodulin binding and trapping, other calmodulin binding proteins might be sensitive to the frequency of the calcium:calmodulin response and the real picture will have to emerge from work on living cells.
Figure 1.9. Autophosphorylation of CaMKII.
A. Low frequency calcium signals fail to induce CaMKII autophosphorylation. At lower frequency calcium:calmodulin binds CaMKII, but because the concentration of free calcium:calmodulin remains relatively low, the chance of calcium calmodulin binding to two adjacent CaMKII subunits is low and calcium:calmodulin dissociates between transients. Therefore autophosphorylation does not easily occur, meaning that CaMKII is inactivated as soon as the calcium concentration drops. B. CaMKII activity outlasts calcium signals of high frequency. At higher frequencies, the increases in local [calcium:calmodulin] increases the chance of adjacent binding to subunits of CaMKII with each subsequent spike, because calcium:calmodulin is slow to dissociate. When this happens autophosphorylation occurs. When [calcium] drops, calmodulin will dissociate from autophosphorylated CaMKII at a reduced rate, but even without calmodulin bound, CaMKII will retain some autonomous activity for many minutes, until dephosphorylation occurs.
1.11. AIM OF THIS THESIS

The lack of data from living cells is a recurring theme: regarding calmodulins role in calcium metabolism, in the questions regarding the dynamic redistribution of calmodulin, the proportion of calmodulin that is bound to targets in resting living cells and the extent to which calmodulin contributes to integration of calcium signals in neurones. Neurones have very interesting calcium signalling properties, a very high calmodulin content and high concentrations of calmodulin binding proteins that have been shown to be involved in mechanisms that rely on repetitive calcium signals causing functional changes like calcium dependent gene expression, neurite outgrowth and differentiation, long term potentiation and long term depression. I therefore expect to find that calmodulin signals in neurones have interesting properties.

The questions I set out to investigate are:

1. Can the effect of pharmacological calmodulin inhibitors on changes in the intracellular calcium concentrations be used to investigate the concerted action of calmodulin on calcium homeostasis?

Calmodulin modulates many component\textit{s} that shape neuronal calcium signals, ranging from voltage operated calcium channels, NMDA receptors, potassium channels ryanodine and IP\textsubscript{3} receptors, to the p\textit{m}Ca\textsuperscript{2+}-ATPase. How calmodulins actions affect these processes in a concerted way is however not clear. I set out to investigate this in the first part of my thesis, using a classical approach with fluorescent calcium indicators to monitor intracellular calcium concentrations and pharmacological calmodulin inhibitors to influence the calcium handling parameters of the cell. The cell type I choose to use for this study are rat PC12 cells. PC12 cells can be made to differentiate into a model of neuronal cells by exposure to nerve growth factor. Calcium influx, release and
pump systems, as well as the calmodulin content have been well characterised in this cell
type rendering them suitable for this study (Chapter 3).

2. What is the subcellular distribution of calmodulin in neurones?
Can fluorescently labelled calmodulins be used to study subcellular distribution
and calmodulin activation in living neurones?

Calmodulin localisation and its dynamics in neurones have only very recently become a
topic of wider interest, with the availability of fluorescent labels for proteins increasing.
Until recently the only data available about the subcellular localisation of calmodulin
came from studies using either cell fractionation or immunocytochemistry. Little is
known about calmodulin localisation in living neurones. The advent of good fluorescently
labelled calmodulins allows the study of both the subcellular distribution and the
activation of calmodulin in detail (Chapter 4).

3. Are there subcellular differences in the state of activation of calmodulin?

The fractions of free and bound calmodulin in living cells are largely, and for neurones
totally, unknown. Using fluorescently labelled calmodulins the extent of calmodulin
binding can be studied at the subcellular level (chapter 5).

4. Does calmodulin have role in the integration of calcium signals in neurones in
response to repetitive stimulation?

Several neuronal processes are induced by calcium signals of different frequencies. This
strongly suggests the presence of a frequency detection system in the calcium signalling
pathway in neurones. There is evidence from in vitro work that CaMKII is part of the
this frequency detection system. Given the wealth of calmodulin binding proteins in
neurones, CaMKII is not necessarily the only component of this system and its
mechanism has not been investigated in living cells. I investigated the activation of
fluorescent calmodulin during repetitive stimulation in living neurones (Chapter 6).
CHAPTER 2: MATERIALS AND METHODS.

2.1. CELLS:

The cells used for this study are neuronal cells of two types, nerve growth factor differentiated PC12 cells and dorsal root ganglion neurones (DRGNs).

2.1.1. PC12 cells

The rat adrenal cytoma cell line PC12 can be differentiated into a neuronal cell type by exposure to nerve growth factor (reviewed by (Tischler, A.S. and Greene, L.A., 1975)). After 4-6 days in culture cells display a neuronal morphology with an enlarged cell body and dendrites. Neuronal PC12 cells express a full calcium: calmodulin signalling system, including L-type VOCCs, CaMKI, CaMKK and display depolarisation induced c-fos expression (Aletta, J.M. et al., 1996). Calcium influx and pump systems, as well as the calmodulin content and subcellular distribution, have been well characterised in this cell type (Koizumi, S. et al., 1999). PC12 cells therefore form a suitable cell system to study the suitability of calmodulin inhibitors as tools to interfere with calmodulin function in living neurones.

2.1.2. PC12 cells methods:

PC12 cells at passage 7 were a kind gift of Dr. Wiedemann at University College London, UK. PC12 cells were seeded at 300,000 cells per flask in 25 ml Falcon polystyrene tissue culture flasks (Becton Dickinson, Le Pont de Claix, France) in Dubecco's modified Eagles' medium (DMEM, with l-glutamine and 4.5 g/l glucose; ICN-Biomedicals, Aurora, Ohio, USA) supplemented with 10% fetal calf serum (Advanced Protein Products, Brierly Hill, UK) and 5% horse serum (Life Technologies, Paisley, UK), further referred to as culture medium. Cells were passaged every 7 days and fed every 2 to 3 days. Passaging was done by rinsing once with Ca\(^{2+}\)/Mg\(^{2+}\)-free Hank's
balanced salt solution (Life Technologies, Paisley, UK) and then with a 1x Trypsin (0.5 g/l) / ethylene glycol-bis(beta-aminoethyl ether)-N, N', N'-tetra acetic acid (EDTA: 0.2 g/l) solution and subsequent incubation for 3 min at 37°C and 5% CO₂. For experiments described in chapter 3, passage numbers 10-16 were used. Flasks were then washed with culture medium and the cell suspension was spun down for 3 min at 1000 rpm (126.6G) and resuspended in 1 ml culture medium and counted with a hemocytometer (Improved Neubauer, Weber, UK). PC12 cells were then diluted to a density of ~40,000 cells per ml in differentiating medium consisting of DMEM with 5% horse serum and 100 ng/ml nerve growth factor (7S fraction; Life Technologies, Paisley, UK) The PC12 cell suspension was plated in 0.5 ml drops onto rectangular glass coverslips (22 x 40 mm, number 1 glass, BDH, UK) that were coated with poly-l-lysine (0.1 mg/ml) for one hour and then washed with phosphate buffered saline (PBS; in mM: NaCl 137, KCl 2.7, 10 phosphate buffer) and left to dry. Coverslips were kept in 60 mm plastic tissue culture dishes (Becton Dickinson, Le Pont de Claix, France) and topped up with 1.5 ml differentiating medium. Plated PC12 cells were fed every two days with differentiating medium and used at day four or five after plating.

2.1.3. Dorsal root ganglion neurones

DRGNs are a well characterised cell type. DRGNs are sensory neurones, involved in the sensory processing of for example pain (Vyklicky, L. and Knotkova Urbancova, H., 1996). DRGNs are a good model to study neurite outgrowth and growth cone dynamics and activity dependent gene expression mediated by calcium and CREB (Archer, F., Ashworth, R. and Bolsover, S.R., 1998).

In culture DRGNs form a heterogeneous population with cell types of different sizes present. Some efforts have been made to characterise the different DRGN types, but there seems to be overlap in expression of certain markers between cells of different sizes. Small and medium sized DRGNs, for example are sensitive to capsaicin and ATP whereas medium size DRGNs are only sensitive to ATP and large DRNs are insensitive
to both (Li, C. et al., 1999). In this study, mainly DRGNs of intermediate size were used (Figure 2.1), with an approximate capacitance of 80 pF.

Figure 2.1. Distribution of DRGN cell sizes used.

Spherical

Volumes were calculated using the measured cell radius and assuming a \( \frac{4}{3} \pi r^3 \) cell body; volumes are expressed as '20k' meaning 20,000 \( \mu l \) (see text for method).

2.1.4. Dorsal root ganglion neurones methods

DRGNs were obtained from adult Sprague Dawley rats (obtained from Biological Sciences, UCL, UK) weighing between 150 to 200 g. Rats of either sex were killed by asphyxiation with \( \text{CO}_2 \) and death was confirmed by testing ptosis and tail and paw pinching. The spine was removed and cut in half sagittally. Ganglia were gently taken from their pockets and the ventral roots that run along their sides were removed. Ganglia were then transferred to Ham's F14 solution (with L-glutamine, Imperial, UK) enriched with 2% Ultroser G (Life Technologies, Paisley, UK) and 1% of 100 i.u. penicillin/100g/l-streptomycin, further referred to as F14. Ganglia were incubated for 3 hours in 2.5 mg/ml Collagenase XI in F14 at 37\(^\circ\)C and 5% \( \text{CO}_2 \). The ganglia were then spun down for 3 min at 1000 rpm (126.6G) and washed once in F14. Cells were triturated
through flame narrowed Pasteur pipettes (Volac, John Poulton, Barking, UK) of
decreasing diameter and plated in 0.5 ml drops onto 10 poly-ornithine (5 mg/ml;
1h)/laminin (0.5 mg/ml; 1h, Life Technologies, Paisley, UK) coated Petriperm dishes
(Heraeus, UK). Cultures were maintained under a humidified atmosphere at 37°C and
5% CO₂ and used 1 to 2 days after plating. Nerve cells were identified morphologically
by their big, rounded phase bright cell bodies and axon growth.

2.2. FLUORESCENCE

Luminescence occurs when a system that is emitting light loses energy; in order to keep
emitting light, energy must be supplied in some form from somewhere else. In the case of
photoluminescence this energy is supplied by the absorption of light. Fluorescence is a
form of photoluminescence where the delay between absorption and emission of light is
10⁻¹⁰ s or less. Emitted light is called delayed fluorescence when delays are 10⁻⁸ to 10⁻⁶ s
and when delays are longer one speaks of phosphorescence (Ploem, J.S., 1999).

Fluorescence occurs after a molecule absorbs a photon and is temporarily put in an
unstable state of higher energy, a photon of a slightly lower energy is then released. The
energy of the emitted photon is Ephoton = E₂-E₁ = h·c/λ, where E₂ and E₁ are
the energy states of the excited molecule, h is Planck's constant, c is the speed of light
and λ is the wavelength of the emitted light. The energy difference occurs because
a molecule resonates when it absorbs a photon and the resonance generates heat, a loss
of energy, which in turn means that the emitted light is of a longer wavelength than the
excitation light. This phenomenon is called the Stokes shift and is exploited by scientists
who use fluorescent molecules as measurement tools. Higher frequency, shorter
wavelength excitation light can be separated from the lower frequency, longer
wavelength light by using a dichroic mirror; this reflects excitation light onto the
preparation, but allows the returning emitted light to pass through, for example onto a
detector.

Fluorescent molecules can be used as measurement tools when they respond to a change
in their environment with a change in either their absorbance or emission properties.
A number of dyes respond to changes in the local intracellular calcium concentration, others respond to changes in pH, membrane voltage or the concentrations of other ions. The following discussion applies to calcium sensitive dyes, the only indicator dyes used in this work.

The simplest behaviour is shown by single wavelength excitation dyes. When excited at the wavelength where absorption is maximal, the intensity of emitted light will change depending on whether or not calcium has bound to the molecule, the spectral properties of the emitted light remaining unchanged. When interpreting the emitted light signal from single wavelength indicators one should be careful, because an increase in the intensity of emitted fluorescence can arise both from binding of calcium or from an increase in the concentration of the fluorescent dye. The second type of indicator dye, termed ratiometric, does not have this problem. Ratiometric dyes rely on a change in the absorption and/or emission spectrum of an indicator when it binds calcium. The most widely used ratiometric dye is Fura-2. Fura-2 has an emission maximum at 510 nm. When Fura-2 has bound calcium, the emission will be high when excited at 340 nm, but low when excited at 380 nm. When no calcium is bound this relationship reverses. These properties allow the use the of Fura-2 ratio as an indication of calcium binding and because the ratio is used, the measurements become independent of the dye concentration.

The relationship between the calcium-loading of an indicator dye and its fluorescence can be established, allowing for the conversion of fluorescence values to calcium concentration. This is done by using simple equations. First of all the emitted fluorescence is a reflection of the proportion of indicator dye that has calcium bound to it and thus different fluorescent properties. This is expressed as:

\[
K_d = \frac{[Ca^{2+}][dye]}{[Ca^{2+}:dye]}
\]

Using this relation for ratiometric calcium indicators, the \([Ca^{2+}]\) can be calculated using the Grynkiewicz equation (Grynkiewicz, G. et al., 1985):
\[
[Ca^{2+}] = K_d \cdot S \cdot \frac{(R-R_{\text{min}})}{(R_{\text{max}}-R)} \quad \text{(equation 2.2)}
\]

where \(K_d\) is the dissociation constant (see Eq 1), \(S\) is the ratio of the fluorescence at the denominator wavelength of a fixed dye concentration in the absence of calcium to its fluorescence in the presence of saturating amounts of calcium, \(R\) is the observed fluorescence ratio and \(R_{\text{max}}\) and \(R_{\text{min}}\) are the maximal and minimal ratio values measured at saturating and zero calcium respectively.

Fluorescent calcium indicators used in this study are Fura Red, Furaptra, Oregon Green 488 BaptaSN (OGB5N) and Fura-2.

Fluorescent dyes can also be linked to proteins or other molecules to signal their location or even activation.

2.3. FLUORESCENTLY LABELLED PROTEINS, DEXTRANS AND CALMODULIN BINDING PEPTIDES: TOOLS FOR STUDYING CALMODULIN IN LIVING CELLS.

For this study I have used fluorescently labelled calmodulin, bovine serum albumin and dextran of 10,000 molecular weight.

2.3.1. Fluorescent calmodulins

Fluorescently labelled calmodulins have been used in several studies. Their fluorescent labels are of two kinds: either they consist of a fluorescent chemical that is covalently attached to calmodulin or they can be produced by the cell itself by inserting code for the sequence of fluorescent proteins like the green fluorescent protein (GFP) of the jellyfish \textit{Aequorea victoria} that will be expressed as part of a chimaeric molecule. Different fluorescent labels can be used. Apart from GFP, one can use blue, yellow or red
fluorescent protein, although up till now for the study of calmodulin itself, only GFP has been used.

The use of different fluorescent chemicals for calmodulin labelling has been reported. Most calmodulin studies use a fluorescein based labelling agent like 5-(4.6-dichlorotriazinyl)-amino-fluorescein (DTAF), iodoacetamido-fluorescein (IAF), rhodamine isothiocyanate (RITC), 2-chloro-(ε-amino-Lys75)-(6-(4-N,N-diethylamino-phenyl)-1,4,5-triazin-4-yl-Cl (TA-Cl) or tetramethinemerocyaninem (Mero).

2.3.2. Fluorescently labelled calmodulin studies

Fluorescently labelled calmodulins have been used in several studies. They have been used to study the localisation of calmodulin:
* during the cell cycle in yeast and Hela cells (GFP-CaM (Moser, M.J. et al., 1997))
* sea urchin embryos (TA-calmodulin and DTAF-calmodulin (Wilding, M. et al., 1995))
* Swiss 3T3 fibroblasts ((Luby Phelps, K. et al., 1985)IAF-calmodulin (Luby Phelps, K. et al., 1995))
* pancreatic acinar cells (DTAF-calmodulin and TA-CaM (Craske, M., Takeo, T., Török, K., Gerasimenko, O., Peterson, O.H. and Tepikin, A.V., 1998))
* A7R5 cells (IAF-calmodulin (Liao, B. et al., 1999))
* rat kangaroo PtK cells, BS-C-1 monkey kidney cells human Hela cells, mouse 3T3 cells (RITC-calmodulin (Zavortink, M. et al., 1983))
* dorsal root ganglion neurones (TA-calmodulin (Zimprich, F. et al., 1995))

Fluorescent calmodulins are mostly used to give information about the local calmodulin concentration within a cell, but two fluorescently labelled calmodulins exist that change their fluorescence in response to calcium binding. Tayler and Török both managed to produce such indicators named Merocam and TA-CaM (Hahn, K.M. et al., 1990). Merocam and TA-CaM are labelled with an environmentally sensitive fluorophore that increases its emission when calcium binds (Merocam 3.4-fold and TA-CaM ~5-fold). When calcium-loaded TA-CaM binds to a calmodulin binding protein this will result in an
additional increase in fluorescence, depending on the nature of the target, of a further 0–100% (Török, K. and Trentham, D.R., 1994).

TA-CaM and DTAF-CaM were kindly provided for my studies by Dr. Katalin Török of St. Georges Medical School London, department of Pharmacology. The covalent attachment of a fluorescent label provides a good tool to study the localisation of proteins in living cells. They work best when:

* The properties of the protein are not disturbed by the fluorophore

The interactions of TA-CaM with a peptide corresponding to the MLCK calmodulin binding domain as well as with MLCK, PDE and peptides corresponding to the calmodulin binding domains of rat GAP junction connexins have been characterised (Table 2.1).

### Table 2.1. Binding constants for TA-CaM target interactions.

<table>
<thead>
<tr>
<th>Target</th>
<th>TA-CaM (all K values are nM)</th>
<th>unlabelled calmodulin (all K values are nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp-peptide</td>
<td>Kd = 0.011</td>
<td>Kd = 0.006</td>
</tr>
<tr>
<td>MLCK</td>
<td>Ki = 0.23–0.51</td>
<td>Kd = 0.10–0.22</td>
</tr>
<tr>
<td></td>
<td>K_i = 12.6</td>
<td>K_m = 3.5</td>
</tr>
<tr>
<td>PDE</td>
<td>K_m = 0.45</td>
<td>V_max = 0.13</td>
</tr>
<tr>
<td></td>
<td>V_max = 0.13</td>
<td>V_max = 0.13</td>
</tr>
<tr>
<td>RS 20 peptide</td>
<td>NA</td>
<td>Kd = 0.09–0.64</td>
</tr>
<tr>
<td>Connexin 32: GAP 10</td>
<td>Kd = 27</td>
<td>NA</td>
</tr>
<tr>
<td>GAP 8M</td>
<td>Kd = 1200</td>
<td></td>
</tr>
</tbody>
</table>


Although the Kd of calmodulin is typically 0.4 fold that of TA-CaM, the Kds for TA-CaM are generally of the same order of magnitude and the behaviour of TA-CaM and unlabelled calmodulin is largely similar (Török, K. et al., 1998). The $V_{\text{max}}$ for
phosphodiesterase activation by TA-CaM and unlabelled calmodulin is for example the same. On the other hand, TA-CaM inhibits, whereas unlabelled calmodulin activates smMLCK, but this appears to be specific to interaction of TA-CaM with smMLCK (Török, K. et al., 1998). The subcellular distribution of FL-CaM in sea urchin eggs is the same as for TA-CaM and there is no reason to believe that both dyes would bind to targets in a dissimilar way (Török, K., Wilding, M., Groigno, L., Patel, R. and Whitaker, M., 1998).

*The protein that is to be labelled is expected to have a specific distribution within the cell so the fluorophore can be expected to show a non-homogeneous distribution in the cell.

Immunohistochemical data have established that calmodulin shows a non-homogeneous distribution throughout cells (see section 1.10.7.5) and the distribution of FL-CaM and TA-CaM follows the expected distribution pattern in developing sea urchin oocytes (Török, K., Wilding, M., Groigno, L., Patel, R. and Whitaker, M., 1998). Overall the combination of FL-CaM and TA-CaM seems to provide a promising tool to study the activation and localisation of calmodulin in living cells.

### 2.3.3. Calmodulin purification:

FL-CaM and TA-CaM were prepared by Dr. Török of St.George's Medical School in London from homogenised pig brain calmodulin, as described before for calmodulin purified from bovine brain (Török, K. et al., 1992). Briefly, calmodulin obtained from pig brain was homogenised and purified by diethyl-amino-ethyl-cellulose, eluted using a 0-1 M NaCl gradient and fractions were assayed for activation of phosphodiesterase to determine the calmodulin elution peak (typically 0.3-0.5 M NaCl). Active fractions were pooled and loaded with calcium, mixed with calcium-saturated phenyl-Sepharose and, after washing, calmodulin was eluted with EDTA and ethylene glycol bis(beta-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA), both at 2 mM. Calmodulin was further purified, to separate it from S100 protein, using fast
pressure liquid chromatography on a Mono-Q column in Tris-buffer. Tris-buffer (20 mM, pH 7.5, EGTA 1 mM, EDTA 1 mM) was used throughout the purification and manipulations were done at 4°C where possible. Purified calmodulin was gel filtered to rid it of remaining salts and lyophilised for storage and further use.

2.3.4. Labelling:

TA-CaM
Calmodulin has a reactive lysine group at position 75 and to create TA-CaM, purified CaM was labelled with TA-Cl at lysine 75 as described by Török and Trentham (Török, K. and Trentham, D.R., 1994): 15 mg of calmodulin diluted to 1 mg/ml was treated with 200 μM TA-Cl from a 2.5 mM stock in dimethylformamide in 20 mM CaCl₂, 100 mM Tris pH adjusted to 8.5 with HCl. The progress of the reaction was monitored spectrophotometrically by observing a slow change in absorption maximum from 403 to 365 nm during incubation of the reaction mixture at 22°C. The reaction mixture was stirred occasionally. The reaction was terminated by gel-filtration to remove soluble and insoluble excess reagent and further purified by fast pressure liquid chromatography and high pressure liquid chromatography to homogeneity, as judged by absorption at 215 nm and fluorescence emitted at 455 nm when excited at 360 nm.

TA-CaM fluorescence increases up to five-fold in the presence of a saturating concentration of CaCl₂. In figure 2.2 the excitation spectrum and the emission spectrum of TA-CaM in the presence of either 1 mM EGTA or 1 mM CaCl₂ is shown. TA-CaM has its excitation maximum around 360 nm and emits maximally around 410 nm.

In addition, TA-CaM increases its fluorescence by up to a further 0-100% when it binds to certain target peptides or proteins in the presence of CaCl₂ (Török, K. and Trentham, D.R., 1994).
Figure 2.2. Excitation and emission spectrum of TA-CaM

Excitation and emission spectra were acquired in a fluorometer set-up in absence of calcium and in a buffer containing a saturating calcium concentration. The excitation spectrum of TA-CaM (in blue) is shown together with the emission spectrum (green lines, exciting at 360 nm) in zero and saturating calcium. The vertical line indicates the wavelength of the laser line used to excite TA-CaM for experiments on the Zeiss confocal microscope. The grey vertical bar on the right shows the dynamic range.

Binding of TA-CaM to MLCK or the Trp-peptide (see below), can be interpreted as a two step process: one association step and a subsequent isomerisation step.

FL-CaM

Calmodulin was labelled with DTAF-fluorescein (Molecular Probes, Oregon, USA) as described for TA-CaM. FL-CaM was purified using reverse phase HPLC from the reaction mixture and the first peak eluted by an acetonitrile gradient was isolated as FL-CaM (as in Török et al, 1998). FL-CaM as used here was labelled in a ratio of fluorescein:calmodulin, 1:1 (Török, personal communication). FL-CaM shows a negligible fluorescence increase upon binding to the Trp-peptide (for peptide see below) in the presence of calcium at both pH 8.5 and pH 7.0 (Török, K., Wilding, M., Groigno,
FL-CaM and TA-CaM were dissolved in filtered (Millipore 0.22 microm) internal solution comprising 130 mM KCl (Fisons, Loughborough, UK)/10 mM HEPES (BDH, UK) pH (NaOH) 7.20 to obtain millimolar stock solutions and stored at -20°C.

Fl-albumin and FL-dextran were obtained from Molecular Probes (Leiden, Netherlands).

2.4. PEPTIDES

Two peptides were used that bind tightly to calmodulin, the Trp-peptide and the α1c-peptide

Trp-peptide


This peptide is derived from the sequence of the CaM binding domain of smMLCK and has nanomolar affinity for calcium:calmodulin (Török, K. and Trentham, D.R., 1994).

Binding of TA-CaM to this peptide is detected as an initial increase in the fluorescence of TA-CaM.

α1c-peptide:

The α1c-peptide, Y-A-T-F-L-I-Q-E-Y-F-R-K-F-K-K-R-K-E-Q-G (Alta Bioscience, Birmingham, UK), corresponds to the IQ CaM-binding domain of the L-type voltage operated Calcium-channel (L-VOCC; (Zuhlke, R.D. et al., 1999)). The α1c-peptide binds calmodulin maximally at a calcium concentrations of 100 nM and higher and since the resting intracellular calcium concentration is around 100 nM, calmodulin is likely to be bound to the α1c peptide at all physiological intracellular calcium concentrations.
2.5. EPIFLUORESCENCE IMAGING

Non-confocal imaging was performed on the stage of a Zeiss Axiovert 100TV inverted microscope using a 20 times Zeiss Fluar objective of 0.75 numerical aperture (NA) or a 40 times Zeiss Fluar/1.30NA-oil objective. Images were acquired using a cooled CCD camera (Theta systems cooled CCD) and Till Photonics (Germany) software.

TA-CaM was excited for 200 ms at 364 nm and emitted light >410 nm was collected as TA-CaM fluorescence. Images were acquired once every second.

In experiments with Fura-2 on this set-up the preparation was excited in turn by 340 and 380 nm light. Exposure to UV excitation light ranged from 200-1000 ms and images were taken once every 5 s.

Cells grown on coverslips (see PC 12 cell culture) were exposed to 5 microM Fura-2 in its esterified membrane permeable form (Fura-2-axetoxymethyl ester, from 2 mM stock dissolved in dimethylsulphoxide (DMSO), 2.5% w/w Pluronic acid) for 30 minutes at room temperature to allow uptake and de-esterification of the dye.

A diamond shaped plastic perfusion chamber (Warner, Hamden, Connecticut, USA) was mounted onto the coverslip using silicon grease (Dow Corning, Wiesbaden, Germany). Coverslips were washed with HBS after dye loading and briefly rinsed when transferred to the microscope stage to wash off the remaining extracellular Fura-2. Fura-2 fluorescence was then imaged before, during and after the application of either of the calmodulin inhibitors calmidazolium or W13 (Calbiochem-Novabiochem, UK) Other pharmacological inhibitors were added to the test solutions as described in the results.

Kinetic data files were processed in Excel (Microsoft) and printed from Sigma plot (Jandel Scientific) software.

Average calcium concentrations in the cell body of PC 12 cells were calculated from the average background subtracted fluorescence ratio of the cell bodies, using equation 2. A response was defined as a 50% increase in the intracellular calcium concentration and accepted if this value was higher than three times the standard deviation of the averaged baseline before application of any agonist or antagonist. The total number of cells in the

80
field of vision (about 80) was counted and the number of responding cells was expressed as a proportion of the total number of cells in the corresponding field of vision.

2.6. MICRO-INJECTION

2.6.1. Injection of cells

Cells were injected on the stage of a Zeiss Axiovert 100TV inverted microscope using a 20 times Zeiss Fluar objective of 0.75 NA or a 40 times Zeiss Fluar/1.30 NA-oil objective. An Eppendorf injection system (5171/5246, set to 641 hPa pressure) was used to inject 2 mM stock solutions of labelled CaM. DRGNs were injected with pipettes pulled from borosilicate glass of 1.0 microm outer diameter, 0.58 microm inner diameter (Clark 100F-10, Reading, UK), with an electrical resistance of 7 to 10 MOhm (when filled with 130 mM KCl), using an upright, one-stage electrode puller (David Kopf Instruments 720, CA, USA). Cells were injected in culture medium to the same brightness, judged by eye. To estimate the injected amount, droplets of injection solution (130 mM KCl/10 mM HEPES pH (NaOH) 7.20) containing a known fixed concentration of FL-CaM were imaged on the confocal microscope. By imaging a droplet at one setting of the photomultiplier tube (PMT) voltage and subsequent droplets at increasingly higher PMT voltages, a voltage intensity relation can be plotted for each of the FL-CaM concentrations used (Figure 2.3). When the measured fluorescence intensity of cells injected with FL-CaM was plotted in the same graph against the PMT voltage at which the images were acquired, the corresponding concentration could be estimated. The estimated final concentration of injected FL-CaM was therefore ~0.1-2.0 microM.

Injected DRGNs were placed back into a humidified incubator and kept at 37 degreesC and 5% CO\textsubscript{2} for a number of hours (on average the same amount for all experiments) to recover from the injection process. DRGNs were then either imaged live in growth medium, with CO\textsubscript{2}-gas supplied above the medium, or fixed for 30 min (cooling down from 37°C to room temperature, 22°C in 4% paraformaldehyde in phosphate buffer (PB,
consisting of 0.1M Na$_2$HPO$_4$ and 0.1M NaH$_2$PO$_4$) before imaging; no differences in calmodulin distribution were observed between fixed and live cells.

![Amount of FL-CaM injected](image)

**Figure 2.3. The concentration of injected FL-CaM in cells**
The fluorescence intensity of a confocal slice through droplets containing a known concentration of FL-CaM is shown as a function of the photomultiplier tube (PMT) voltage. The best fit is shown for each concentration 0.1 (diamonds, blue line), 0.2 (squares, scarlet line) and 1 microM (triangles, yellow line). The fluorescence intensity of individual cells is plotted at the PMT voltage at which the image was acquired. By comparing the fluorescence intensity of injected cells with the line standards, an estimate of the final concentration of injected FL-CaM can be obtained.

### 2.6.2. Data-analysis for micro-injected cells of chapter 5

Images were analysed using Zeiss LSM software. Regions of interest were chosen covering the nucleus (identified from transmitted light and fluorescence images) and cytosol of injected dorsal root ganglion neurones. Autofluorescence of these regions of uninjected cells was collected as background. The average fluorescence intensity of the
chosen regions was calculated by the software and numerical data were exported to Excel for further analysis. Background fluorescence at the respective channels and locations was subtracted from the intensity values for TA-CaM and FL-CaM of injected cells. To improve the fidelity of the analysis, only cells that had fluorescence levels after background subtraction of >30 arbitrary units (where 4095 represents saturation) for both the TA-CaM and FL-CaM channels were used. This method rejected cells with the lowest amounts of injected calmodulin.

Calculation of concentration of injected calmodulin:

The concentration of FL-CaM and TA-CaM in injected cells was calculated by comparing the background subtracted fluorescein fluorescence of injected cells ($I_{cell}$) with the fluorescence of a droplet containing 3.33 microM FL-CaM and 6.67 microM TA-CaM in injection buffer (composition: 130 mM KCl/10 mM HEPES pH (NaOH) 7.20) containing a high concentration of calcium (4.5 mM), imaged at the same microscope settings ($I_{droplet}$). The concentrations of FL-CaM and TA-CaM in cellular compartment were then calculated as follows:

$$[\text{FL-CaM}_{\text{cell}}] = \frac{I_{cell}}{I_{droplet}} \times 3.33 \text{ microM} \quad (\text{equation 2.3})$$

$$[\text{TA-CaM}_{\text{cell}}] = \frac{I_{cell}}{I_{droplet}} \times 6.67 \text{ microM} \quad (\text{equation 2.4})$$

$$\text{total } [\text{CaM}_{\text{injected}}] = \frac{I_{cell}}{I_{droplet}} \times 10 \text{ microM} \quad (\text{equation 2.5})$$

Fluorescein fluorescence, rather than TA-fluorescence, was used to estimate the injection amount because TA-fluorescence in the cell will be a function of calmodulin activation as well as concentration.
Calculation of nuclear and cytosolic volume:

Assuming the nucleus and the cytosol are in approximation spheres, the volume of both compartments can be calculated using the formula:

\[ V = \frac{4}{3} \pi r^3 \]  
(equation 2.6)

Rather than estimate \( r \) from a single line, I measured the cross section of nuclei and cells in the confocal slice, both when focused at the level where the widest diameter of the nucleus could be observed.
\( r \) can be calculated from the area using the formula:

\[ r = \sqrt{\frac{\text{area}}{\pi}} \]  
(equation 2.7)

This gave an \( r \) for nucleus and cell of 7.86 microm and 24.48 microm respectively and volumes of 2.2 \( \text{pl} \) for the nucleus and 54.7 \( \text{pl} \) for the whole cell.

Knowing the injected calmodulin concentration in the nucleus and in the cytosol and having an estimate of the two volumes, the amount present can now be calculated as 0.17 pg in the nucleus and 2.32 pg in the cytosol (subtracting the nuclear volume from the whole cell volume before calculation). Although the calculated volumes are approximations, they give a good idea about the relative volumes and calmodulin contents of both compartments.

2.7. CONFOCAL LASER SCANNING MICROSCOPY

Confocal microscopy has been developed to prevent contamination of the in focus signal by fluorescence of out-of-focus structures. This is achieved by illuminating the preparation with a small light spot, generated from the parallel, high intensity light beam of a laser. This spot is then scanned across the preparation to compose a complete image
of the preparation on a computer. The emitted light is passed through a pinhole, so that out of focus light, which does not converge in a point at the plane of the pinhole, is rejected. This means that effectively fluorescence can be collected from a thin slice of a living cell, creating the possibility of resolving subcellular events.

Figure 2.4 illustrates how this basic concept is achieved on one of the systems I used, the Zeiss LSM 510. Laser lines of an UV and Argon laser are selected with an acousto optical tuning filter (AOTF). The laser light is focussed onto mirrors by the collimator lenses. Laser beams are then guided to an x/y-scanner box using mirrors. The X/Y-scanner box contains a galvano mirror system that scans the laser light spot across the preparation in the x and y direction. The laser spot is focussed onto the preparation via a scanning lens that focusses the light spot onto a mirror and light is reflected onto the preparation through the microscope objective. Light emitted from the preparation travels through dichroic mirror two, to be separated into the desired wavelength bands for specific dyes by dichroic mirrors three, four and five. Light that does not concentrate in a point at the plane of the pinholes (A, B, C, D) in each of the wavelength bands now separated is rejected and the remaining light in each of the wavelength bands is send to individual photomultiplier tubes, where the light intensity is converted into an electrical signal. The values of the electrical signals for each pixel are composed to form an x/y-image and stored on a computer for offline analysis.

2.7.1. Confocal Imaging

Confocal images were acquired using two systems. A Leica CLSM employed a Leitz Fluotar 40 times/1.30 NA-oil objective and the 488nm line of an argon laser (Melles Griot. Carlsbad. CA, USA). Light was reflected onto the preparation by a 510 nm dichroic and emitted fluorescence was collected through a 515 longpass filter. The readout of the photomultiplier tube was converted into a 512 times 512 pixel image on a VME computer with Motarola 68020 processor and 8 Mb RAM and saved to magneto-optical disc
Figure 2.4. Schematic of the Zeiss LSM 510 confocal microscope
Light emitted from either a UV or Argon laser is split into different laser lines by an acoustic optical tuning filter. The laser lines are focused onto a mirror (M 3,4) by a collimator lens and then passed to a scanning box via dichroic D1. The laser light is scanned across the preparation via the objective lens after reflection off mirror M 2. Light passing through the preparation can be picked up by a separate PMT to form a transmitted light picture. The light that is reflected back from the preparation passes through dichroic D1 and, after reflection off mirror 5, is split further by dichroics 2-4 to separate the emitted light in the appropriate wavelength bands. The intensity of the emitted light for each individual wavelength band is measured at PMT 1-4. A mercury lamp allows previewing of fluorescent specimens. Mirror M 1 is moved out of the light path when confocal images are acquired.

The gray values in the acquired pictures were converted into a pseudo-colour scale using a look-up table with the lowest intensities appearing in black and increasingly warmer colours representing increasingly higher intensities from red, through orange, yellow and white for the highest intensities. Average intensities of selected regions of interest were calculated using the CLSM software.

Images requiring a UV-laser were acquired on a Zeiss LSM 510 system employing a Zeiss Axiovert 100 M microscope, a Zeiss Fluar 40 times/1.30 NA oil objective and the
488nm line of a 15 mW 80% powered argon laser (Zeiss, Jena, Germany) plus the 364 line of a 80 mW 60% powered argon UV-laser (Enterprise, Coherent, Santa Clara, CA, USA). Laser light was reflected onto the preparation using a dual band dichroic (HFT488, Zeiss Oberkochen, Germany) and light emitted from the preparation was separated into bands corresponding to emission of TA-CaM (385nm<\lambda<470nm) and fluorescein or OGB5N (\lambda>505nm), except where Fura Red was also present, where fluorescein emission was taken as 505nm<\lambda<570nm and Fura Red as \lambda>650nm. When excited at 488nm, Fura Red emission falls upon binding calcium. Images were collected using Zeiss LSM510 software on a Dell computer with Intel 5233 processor and 200 Mb random access memory, temporarily stored on hard disc and archived on recordable compact disc media.

2.7.2. Confocality of the Zeiss LSM510 system

The confocality of the Zeiss CLSM microscope using the 40 times oil immersion objective, was tested using microbeads that can be excited at several wavelengths (Tetraspec, Molecular Probes, Leiden, Netherlands). Although a high magnification, z-corrected high quality objective (63 times water immersion) was available, I found that the UV-transmission of the 40 times oil immersion objective was about 10 times better and this objective has been used for all the experiments on the Zeiss confocal microscope. I used beads of 4 \mu m or 1 \mu m diameter. The picture in figure 2.5A shows that a 1 \mu m bead can be resolved by the microscope when the pinholes are set for an optical slice thickness of 1 \mu m at a resolution of 512 x 512 pixels, using dual directional scanning. However, focussing up and down, it was found that there was a ~6.7 \mu m difference in focal plane between the 364 nm-excited fluorescence and the 488 nm-excited fluorescence. This creates a serious potential problem: the emitted fluorescence, for these two wavelengths, is collected from slices at different depths in the cell. To avoid potential problems I used a larger pinhole diameter to give a slice thickness of 2 \mu m. To collect fluorescence from both the cell nucleus (average radius 7.86+/-.136 microm (n=31)) and the cytosol in my experiments, I chose the focal plane such that it visibly transected the
nucleus and cytosol in all channels. Images 2.5 B, C, and D are collected at these settings, at a spatial resolution of 64x64 pixels, as is used for the experiments in chapter 6. Note the spatial separation in the z-direction in figure 2.5 D(z).

**Figure 2.5. Confocality of the microscope set-up**

Multi-coloured fluorescent beads of 1 and 4 microm diameter were used to assess the confocality of the microscope lens that was used for the experiments using TA-CaM.

A. A 1 micron fluorescent bead at a 512x512 pixel resolution in a two-directional scan. B. A 1 micron bead at the 64x64 resolution used for the experiments of chapter 6. C. A 4 micron bead at 64x64 pixel resolution. D. A 4 micron bead is shown in a reconstruction in the xy, xz and yz direction. The yellow line indicates that there is shift in the centre of the image for the UV (blue) and the fluorescein (green) channel.
2.7.3. Image analysis confocal

Using the Zeiss LSM or Leica CLSM software, regions of interest were chosen. The average fluorescence intensity of regions of interest of dye loaded cells or fixed cells was determined. In order to estimate the background signal, the fluorescence intensity of the corresponding regions of uninjected cells or the same cell before dye loading were also determined and later subtracted from the fluorescence values of the loaded cell. Time series of fluorescence values were normalised to the average baseline fluorescence intensity before the stimulus.

2.7.4. Calculation of the intracellular calcium concentration

For the single wavelength calcium indicator dyes used in chapter 6 it is difficult to get a reliable estimate of the intracellular calcium concentration. I calculated the intracellular calcium concentration assuming a basal level \( (Cr) \) of 50 nM before stimulation by using the standard equation:

\[
C_t = \frac{K_d \cdot (F_t - N \cdot F_o)}{(S \cdot N \cdot F_o - F_t)} \quad \text{(equation 2.8)}
\]

with

\[
N = \frac{F_{\text{min}}}{F_o} = \frac{(K_d + Cr)}{(Cr \cdot S + K_d)} \quad \text{(equation 2.9)}
\]

where \( C_t \) is the calcium concentration at a given time, \( K_d \) is the dissociation constant for the fluorophore, \( F_t \) is the fluorescence value at a given time, \( F_o \) is the fluorescence value at basal calcium, \( S \) is the ratio of the fluorescence of a fixed dye concentration in the absence of calcium to its fluorescence in the presence of saturating amounts of calcium. \( I_{\text{min}} \) is the lowest fluorescence value of the fluorophore.

The parameters used were:

- **Fura Red**: \( K_d \) is 140 nM, \( S \) is 0.44, \( I_o \) is 1 for normalised data.
- **OGB5N**: \( K_d \) is 20 microM, \( S \) is 41, \( I_o \) is 1 for normalised data.
For Furaptra, the following equation

\[ Ct = \left( K_d \times \frac{F_{\text{zero calcium}}}{F_t} \right) - K_d \]  

(equation 2.10)

was used with the parameters \( K_d \) as 48 microM (Ogden, D. et al., 1995), assuming a resting calcium concentration (\( Ct \)) of 50 nM and \( I_{\text{zero calcium}} \) was calculated as:

\[ F_{\text{zero calcium}} = (Cr + K_d) \times F_0 / K_d \]  

(equation 2.11)

with \( F_0 \) as 1 for normalised data. Because OGB5N and Furaptra are low affinity dyes, the calculated calcium concentration can be scaled, meaning that an increase in fluorescence equivalent to a calcium change from 50 to 100 nM would be equal in magnitude to the fluorescence change from an increase in calcium from 150 to 300 nM (for derivation see appendix).

2.8. IMMUNOCYTOCHEMISTRY

Cells were rinsed three times with HBS, composition (mM) NaCl 120, glucose 25, KCl 5.5, CaCl\(_2\) 1.8 (Aldrich, Milwaukee, USA), MgCl\(_2\) 1 (BDH), HEPES 20, pH (NaOH) 7.2) at 37°C. Cells were then either bathed in HBS or were depolarized with High K\(^+\) HBS (68 mM KCl, replacing NaCl in HBS) and then fixed for 30 min (cooling down from 37°C to room temperature, 22°C) in 4% paraformaldehyde in PB with 1mM EGTA; to ensure both calcium-free and Calcium-loaded calmodulin would be recognized equally well (Deisseroth, K. et al., 1998). Cells were rinsed three times with PB at room temperature, permeabilised with 0.4% saponin in PB for 10 min and unspecific sites were blocked with 10% goat serum in PB for 30 min. Cells were then exposed to the primary monoclonal calmodulin antibody raised to 20 amino acids at the C-terminal of bovine brain calmodulin (UBI, (Sacks, D.B. et al., 1991)) in phosphate buffered saline plus 0.8% Triton and 1% goat serum at a 1:400 dilution and left overnight at 4°C. Cells were rinsed three times with PB plus 0.8% Triton and exposed to the secondary
Cy5 linked goat-anti-mouse antibody (Amersham, Little Chalfont, UK) at 1:1000 in PB plus 2% goat serum for 30-60 min, washed three times with PB and either mounted for later use or used on the confocal microscope immediately. Background values were obtained from cells incubated with the primary antibody in the presence of 50 microM calmodulin. This background fluorescence value was the same as for cells for which the primary antibody was omitted as an antibody control.

2.9. WESTERN BLOTTING

Two filtered buffers were used for Western blotting; buffer A contained (mM) KCl 130, HEPES 10, EGTA 1; buffer B KCl 130, HEPES 10, CaCl₂ 0.1. Both buffers contained 0.02% Tween and were adjusted to pH (NaOH) 7.2. The whole experiment was done in parallel with either buffer A or B throughout. Blotting paper (Sigma, St. Louis, USA) was washed with 100% methanol (BDH) and with buffer and then 17 and 8.5 microg 'spots' of either pure unlabeled calmodulin (>99% pure, Calbiochem-Novabiochem, UK), FL-CaM or TA-CaM were blotted before the paper dried up for condition A, low Calcium, and B, high calcium. The paper was kept wet all the time to provide the most optimal conditions for the proteins to maintain a natural conformation. The blots were then washed (with continuous motion) for 5 min at room temperature and blocked with 5% goat serum for 1h, washed twice at room temperature, for 5 min each time, and left with 0.75 microg/ml calmodulin antibody (UBI, USA) for 2 hrs. The blots were washed 4 times for 5 min and incubated for 45 min with the secondary, rabbit anti mouse antibody conjugated to horse radish peroxidase (HRP, Sigma, St. Louis, USA) at 1:10,000 dilution. Blots were washed 4 times for 5 min and incubated with ECL (enhanced chemiluminescence) mixture (ECL-plus kit, Amersham, Little Chalfont, UK) for a few minutes. The blots were exposed to photographic film in a dark room for increasing lengths of time, starting at 5s. The film was developed and fixed and the blot washed with Indian ink 1:1000 2 times for 2 min and then washed to control for the amount of protein present in the dots. This procedure used an exorbitantly large amount of TA-CaM and FL-CaM and was therefore performed only once.
2.10. PATCH-CLAMP EXPERIMENTS

The whole cell voltage clamp technique, developed by Neher and Sakmann, was used in these studies for two reasons (Hamill, O.P. et al., 1981). First of all the control of the membrane potential offered by this technique allows for calcium transients to be imposed onto the cell by opening voltage operated calcium channels in the membrane and secondly, in the whole-cell mode the contents of the patch-pipette slowly equilibrates with cell interior and this provides a means to introduce fluorescently labelled calmodulin to the cell interior.

Two protocols for patch clamp experiments were followed at UCL and one at the institute of medical research at Mill Hill, in collaboration with David Ogden's group.

2.10.1. Patch-clamp experiments at UCL

2.10.1.1. Protocol using TA-CaM, FL-CaM and Fura Red (Chapter 4)

DRGns were patched using pipettes pulled from Clark GC-150-TF-10 borosilicate glass, 1.5 mm outer diameter, 1.17 mm inner diameter, using an upright two-stage pipette puller (Narashige pp-830, Japan). Pipettes tips (resistance 2-4 MOhm) were dipped into filtered intracellular solution (in mM; CsCl 135, MgCl₂,6H₂O 1, TEA 25, HEPES 7.5, Mg²⁺-ATP 5, pH (KOH) 7.2; to which sucrose (BDH, UK) was added to achieve an osmolarity of 300-310 mOsm (Roebling osmometer Camlab, UK), then back-filled with ~0.1microl of internal solution containing one or more of 33 microM FL-CaM, 66 microM TA-CaM, 400 microM Fura Red and topped up with protein-free internal solution. Cells were washed with external solution containing (in mM NaCl 120, MgCl₂,6H₂O 1, KCl 5.5, TEA 1.8, HEPES 8, CaCl₂ 5, D-glucose 25, tetrodotoxin (TTX) 0.001; osmolarity adjusted to 310-320 mOsm) in which recordings were also performed. Cells were patched on the stage of the Zeiss confocal microscope and voltage was controlled using a Axoclamp 2A amplifier (Axon instruments Foster City,
USA) in continuous voltage clamp mode and pClamp6 software (Axon instruments Foster City, USA).

Patch pipettes were mounted on an Axon HS-2 headstage (Axon instruments Foster City, USA) and voltage was recorded using an Ag/AgCl recording electrode and an Ag/AgCl bath electrode. The headstage ground was earthed to the microscope body. The headstage was mounted onto a x/y/z piezo-electric micromanipulator (Burleigh Instruments, NY, USA). Pipettes were brought within 5 μm of the top of the DRGN, the cell nucleus was then brought into focus and the microscope sliders were moved to their scanning positions. The electrode was then lowered until a sudden increase in seal resistance was observed. Gentle suction was then applied to achieve a seal of GOhm resistance in the cell-attached mode. A holding voltage of -50 mV was imposed and when a giga-seal had formed, a series of 100 background pictures of the cells' autofluorescence immediately before dye-loading was taken and averaged in time offline. The average intensity value of the nucleus and cytosol region was then subtracted as background from the average intensity of the corresponding regions of the same cell after loading with the fluorescently labeled proteins. The whole-cell configuration was then achieved by sharp and abrupt suction and diffusion of dye and protein into the cell was monitored by taking snap-shot images at regular intervals. When the loading rate seemed to have plateaued, which could take as little as 5 or as long as 30 minutes, an experiment was started.

Patch-loaded DRGNs were held at -50 mV and depolarized to +10 mV for 200 ms preceded by a 100 ms step to -70 mV to remove channel inactivation. At the start of the recording the voltage clamp software was set to trigger the start of the acquisition of images by the LSM510 software by giving a 200 ms pulse on a digital output port of the digital-analogue-converter (Digidata 1200) and to depolarise the cell after a delay of a few seconds. Confocal imaging was performed as a dual directional scan at a spatial resolution of 64 x 64 pixels to achieve an acquisition time of 51.2 ms per image. Whole cell currents were low pass filtered, digitised and stored on hard disc for off-line analysis using a Dell computer with a 486/50 Intell processor and 8 Mb random access memory. Current traces were leak subtracted in pClamp6 software.
2.10.1.2. Protocol using TA-CaM & Oregon Green Bapta 5N (Chapter 6)

Image acquisition and patching were essentially the same as for the protocol using TA-CaM, FL-CaM and Fura Red described above, apart from: DRGNs were patched using as intracellular solution (in mM) TA-CaM 0.1, OGB5N 0.05, CsCl2 125, Na2ATP 4, HEPES 10 creatine phospho-4-kinase 50 units/ml, di-tris phosphocreatine 14, pH (KOH) 7.4 and as extracellular solution (in mM) NaCl 120, KCl 5.5, CsCl 10, MgCl2 1.2, CaCl2 5, TEA 1.8, HEPES 8, D-glucose 25, TTX 0.001, patch pipettes had a resistance of 1.6 MOhm and were not dipped, but just back filled with dye containing solution and then filled with dye-free solution. Patch-loaded DRGNs were held at -70 mV and depolarised to 0 mV for 200 ms.

2.10.2. Patch-clamp experiments at NIMR-Mill Hill (collaboration with David Ogden's group; Chapter 6)

DRGs were harvested as described before and cultured on poly-l-lysine/laminin on 30 mm round coverslips in DMEM containing 10% horse serum. DRGNs were patched using 0.5-2 MOhm pipettes back-filled first with 50 microM TA-CaM and 250 microM Furaptra in an internal solution of the composition (in mM): CsCl 125, MgCl2 5, HEPES 10, Na2ATP 4, creatine phospho-4-kinase 50 units/ml, di-tris phosphocreatine 14, pH (NaOH) 7.4 and topped up with protein-free internal solution. Cells were washed with external solution containing (in mM) Na-Gluconate 125, MgSO4·7H2O 1.2, K-Gluconate 5.6, TEA·HCl 20, HEPES 8, CaSO4·2H2O 5, D-glucose 10, TTX 0.005, pH (NaOH) 7.4, in which recordings were also performed. Coverslips were mounted in a circular metal recording chamber on the stage of a Nikon Diaphot microscope and viewed using a Nikon 40x/oil 1.3NA objective. Cells were visualised on a monitor and fluorescence emitted from the cell body of a single cell was isolated with a rectangular diaphragm. After establishing the cell attached mode, the whole cell mode was established by applying a sharp negative pressure and the time course of the dye loading was followed by monitoring the fluorescence emitted by both dyes during excitation at 360 nm. Excitation light was reflected by a DM 380 dichroic towards the preparation and emitted light >380 nm passed through the first dichroic to a second dichroic that sent light <450
nm, corresponding to TA-CaM fluorescence to a photomultiplier tube (PMT; Photon Technologies Ltd), giving a time resolution of 10 ms (100 s recording) to 20 ms (200 s recording). The remaining light <650 nm was reflected by a second dichroic through a 490 nm long pass filter to the second PMT to monitor the Furaptra fluorescence, while the light >650 nm was sent to a camera to form an image of the cell, using infra red light from the microscope transluminator.

Patched cells were held in the voltage clamp mode at -70 mV by an Axopatch 1B patch clamp amplifier. Series resistance and whole cell capacitance were compensated and monitored intermittently during the course of the experiment. Signals were filtered at 10 kHz. A series of depolarisations from -70 mV to 0 mV was delivered, the spacing of the depolarisations being varied from 2.5 to 20 s. Signals were analysed using the in-house and PTI software. Fluorescence traces were smoothed by a 4-point running average and exported as ASCII files for processing with Microsoft Excel software.

2.10.3. Image analysis of fluorescence data acquired from patch-clamped cells.

Time series of TA-CaM and OGB5N fluorescence values were imported into Excel to analyse the kinetics of the offrates. First a straight line was subtracted to correct for drift. This line was chosen to be as close as possible to the trend in the baseline fluorescence before depolarisation. Fluorescence values, taken from the time point at which the fluorescence starts to decline back to baseline, were selected and the parameters for a two-exponential fit were roughly determined by adjusting the parameters c, d, J and K in a model of the equation \( z = c \exp\left(-\frac{x}{d}\right) + j \exp\left(-\frac{x}{k}\right) \) (equation 3) in an approximation of the data. The fluorescence values as well as the corresponding time values were then exported to Sigma plot. In Sigma plot the data was fit with equation 3, priming the iterative protocol with the estimated parameters c, d, j, and k. The parameters of the best fit reported by Sigma plot were then plugged back into the model in Excel and if a good fit was obtained, accepted as amplitudes c, j and time constants d, k for the offrate of an individual response.
2.11. GENERAL

All handling and experiments were performed at room temperature (22-24°C) and were performed in HBS, unless indicated otherwise. All chemicals were from Sigma (St. Louis, USA) and fluorescent dyes from Molecular Probes (Leiden, Netherlands), unless indicated otherwise.

Statistical analysis of data:
Data are expressed as mean +/-standard error of the mean (SEM), with n = number of cells, throughout the paper.
Group means were compared using a two tailed unpaired (unless otherwise stated) tailed Student's t-test, Bonferroni corrected t-test after ANOVA or Mann-Whitney non-parametric test, where appropriate.
CHAPTER 3: CALMIDAZOLIUM INDUCES CALCIUM TRANSIENTS IN PC12 CELLS.

3.1. INTRODUCTION

3.1.1. Pharmacological calmodulin antagonists.

A whole range of drugs is now available that have been found to bind to and antagonise calmodulin function. The classic examples of these drugs are the anti-psychotic compounds chlorpromazine and trifluoroperazine (Levin, R.M. and Weiss, B., 1976). These compounds bind to the hydrophobic moieties that calmodulin exposes upon calcium-binding (LaPorte, D.C. et al., 1980). However, these classical calmodulin antagonists have later been shown to have considerable effects that are not related to calmodulin function (England, P.J., 1986) and are therefore only suitable to study interactions of calmodulin and target proteins in isolation in a test tube.

In the eighties more selective calmodulin antagonist became available. The class of the naphthalenesulfonamides shows better calmodulin selectivity (Hidaka, H. and Tanaka, T., 1983). W7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide) is a widely used example of this family that binds calmodulin with a Kd of 25 microM and penetrates cell membranes, making it a very suitable antagonist for use on tissue and cultured cells. Unfortunately W7 also binds to troponin-c and S-100 protein to a limited extent (England, P.J., 1986). Other calmodulin antagonists of this family are W5, with a far lower affinity than W7, and usually employed as a structure specific control, and W13.

A more selective and more potent calmodulin antagonist is calmidazolium (1-[bis(pchlorophenyl)methyl]-3-[2.4-dichloro-beta-(2,4-dichlorobenzyl oxy)phenethyl]imidazolium chloride: R24571 (VanBelle, H., 1981). Calmidazolium has a Kd of 10 nM (Gietzen, K. et al., 1982; VanBelle, H., 1981) and is generally used as the most potent and selective pharmacological calmodulin inhibitor.
3.1.2. Binding mechanism of antagonists

Binding of antagonists to calmodulin is due to interaction with the hydrophobic pockets revealed upon calcium binding and by a positive charge found in many calmodulin antagonists (Trifluoperazine, W7, calmidazolium). Specificity of binding is relative as aspecific effects occur for all antagonists at higher concentrations. I therefore choose to use the calmodulin antagonist with the highest affinity for calmodulin (see table 3.1), calmidazolium, to limit aspecific interactions to the minimum.

Table 3.1. Affinities of calmodulin antagonists for calmodulin.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Kd binding (microM)</th>
<th>reference</th>
<th>IC50 (microM)</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpromazine</td>
<td>2.4-22 (K0.5)</td>
<td>(Ebashi, S., 1988)</td>
<td>19 (PDE)</td>
<td>(Gietzen, K. et al., 1983; Marshak, D.R. et al., 1985)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Ogawa, Y., 1988)</td>
<td>22 (PMCA)</td>
<td></td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>1.5-5 (K0.5)</td>
<td>(Ebashi, S., 1988)</td>
<td>7 (PDE)</td>
<td>(Gietzen, K. et al., 1983; Itoh, H. and Hidaka, H., 1984)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Ogawa, Y., 1988)</td>
<td>9 (PMCA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Ogawa, Y., 1988)</td>
<td>51 (PMCA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>58 (MLCK)</td>
<td></td>
</tr>
<tr>
<td>Calmidazolium</td>
<td>0.002-0.003</td>
<td>(Johnson, J.D. and Wittenauer, L.A., 1983)</td>
<td>0.01 (PDE)</td>
<td>(Gietzen, K. et al., 1982; VanBelle, H., 1981)</td>
</tr>
</tbody>
</table>

Calmidazolium is the most suitable pharmacological tool to try to elucidate calmodulin function in living cells. Many studies have used calmidazolium to show calmodulin dependence of intracellular processes (see table 3.2). In neurones, using calmidazolium, calmodulin has been shown to be involved in the memory model long term potentiation,
activity dependent gene expression and neurite outgrowth (Neveu, D. and Zucker, R.S., 1996).

### 3.1.3. Calcium management in neurones

Neurones show complex calcium dynamics. Calcium can enter the cell either through voltage operated calcium channels or via receptor operated channels. Calcium can also be released from intracellular calcium stores via either IP$_3$ receptors or ryanodine sensitive receptors (Berridge, M.J., 1993). The intracellular calcium concentration will then be lowered by the action of removal mechanisms, mainly via the plasma membrane and smooth endoplasmic reticulum calcium-ATPases. Although the sodium/calcium-exchanger and mitochondria play a role in calcium removal in neurones, the contribution of these has been found to be minor (Mironov, S.L., 1995).

When cultured with nerve growth factor, PC12 cells differentiate into a neuronal phenotype (reviewed (Tischler, A.S. and Greene, L.A., 1975)). Nerve growth factor differentiated PC12 cells have complex calcium dynamics and have therefore recently been chosen for studies of the subcellular aspects of calcium signals (Koizumi, S. et al., 1999). PC12 cells form a versatile cell system to study the suitability of calmodulin inhibitors as tools to interfere with calmodulin function in living neurones.

### 3.2. RESULTS

#### 3.2.1. Calmidazolium induces an increase in the intracellular calcium concentration

When nerve growth factor differentiated PC12 cells were exposed to 5 microM calmidazolium a proportion of cells (19+/−3%; n=23; fig 3.5) showed transient...
Figure 3.1 Responses to 5 μM calmidazolium.

A-D Increases in $[\text{Ca}^{2+}]_{i}$ of individual cells upon application of calmidazolium (black bar).

E These increases in $[\text{Ca}^{2+}]_{i}$ do not occur in the absence of calmidazolium. Every trace in the figures A-E represents the fluorescence measured from a single cell.
elevations in the intracellular calcium concentration (Fig 3.1A-D). Spontaneous calcium elevations were very rare in the absence of calmidazolium (Fig 3.1E).

To study the dose dependency of these calcium rises, the number of cells in the field of vision of which the intracellular calcium concentration passed a set threshold was determined. The threshold was defined as 50% above baseline and cells were accepted when this value was greater than three times the standard deviation of the averaged baseline. I will further refer to these cells as 'passing threshold'.

The triggering of calcium rises by calmidazolium was a dose-dependent effect, as the proportion of cells in which calcium passed threshold, increased with the dose (Fig3.2A).

At physiological pH (7.2) calmidazolium concentrations of 25μM and higher precipitated out of solution when dissolved in HBS. This was apparent from a white cloudiness that was not observed when the vehicle (DMSO) alone was dissolved in HBS at the same concentration. Therefore the concentrations of '25' and '50' microM in the dose response curve (rightmost points in Figure 3.2A) should be taken as 'maximal'. At these concentrations there was still an increase in the proportion of cells passing threshold, but note that at 10 microM initially the proportion of responding cells went down. These calcium transients were not a response to the DMSO vehicle (Figure 3.2B).

The calcium rises in cells that passed threshold in response to calmidazolium were of two distinct types: most cells exhibited a series of calcium transients (Figure 3.1A, B) but occasionally I observed a more maintained increase (Figure 3.1C).

The lower affinity (table 3.1) antagonist W13 only very rarely evoked responses at concentrations below 100 microM (Figure 3.2a). Transient responses similar to calmidazolium responses are rare even at 100 microM (Figure 3.2c) and rises in intracellular calcium mainly take the form of delayed slow rises that occur in nearly all cells at a concentration of 300 microM (86+/-14% (n=3); Figure 3.2A), implying that this is a distinct phenomenon, not directly related to the calmidazolium-induced calcium rises.
Figure 3.2. Dose response curves calmidazolium & W13.
A. The proportion of cells that showed an increase in [Ca\(^{2+}\)] that passed the set threshold are plotted as function of the calmidazolium and W13 concentration. Each point represents the average of at least five dishes and ~80 cells per dish. B. Vehicle alone does not induce a calcium rise in individual cells. C, D. Calcium rises induced by W13 are distinct from those caused by calmidazolium and appear as a delayed rising plateau (black arrows). Only rarely is a transient calcium rise observed using W13 (white arrow). Each coloured trace represents the fluorescence measured from a single cell.
3.2.2. Source of calcium

So where does calmidazolium act in the cell to cause these calcium transients?

3.2.2.1. Calcium release from mitochondria

A possible action of calmidazolium is inhibition of the oligomycin sensitive ATP-synthase in mitochondria (Phenothiazines disrupt mitochondrial energy production by inhibition of the oligomycin sensitive ATPase (Ruben, L. and Rasmussen, H., 1981)). Mitochondria can take up and release calcium, causing brief calcium transients in the cytosol (Verkhratsky, A.J. and Petersen, O.H., 1998). If calmidazolium causes calcium transients by inhibiting the mitochondrial ATP-synthase, then oligomycin should mimic the calcium transients caused by calmidazolium. However, applying oligomycin at 2.5 microg/ml did not reliably trigger calcium elevations (cells passing threshold 4+/−1% ; n=2; Figure 3.5).

3.2.2.2. Calcium release from endoplasmic reticulum calcium stores

Calmodulin can modulate IP₃ receptor and or ryanodine receptor mediated calcium release (Patel, S. et al., 1997). To investigate whether calmidazolium causes calcium transients by releasing calcium from the endoplasmic reticulum calcium stores, I used thapsigargin to inhibit the SERCA, which empties the stores. When I applied thapsigargin alone, a gradual increase in the intracellular calcium concentration indicated that the stores were emptying (Figure 3.3A). These thapsigargin-induced calcium rises are indicated with a filled arrow throughout figure 3.3. When calmidazolium was added immediately after the thapsigargin induced calcium rise, I saw calcium rises similar to the types observed when calmidazolium was applied alone (Figure 3.3B, indicated with open arrows). The proportion of cells that passed threshold in response to calmidazolium application was reduced to 10+/−3% (n=17; p<0.05; Figure 3.5), indicating that some of the calcium increases were due to release from intracellular stores.
Figure 3.3. Thapsigargin does not abolish the calmidazolium effect.
A. When cells were treated with thapsigargin, a slow increase in $[\text{Ca}^{2+}]_i$, due to emptying of intracellular stores could be observed in all individual cells (black arrows). B. Co-application of calmidazolium together with thapsigargin to cells pretreated with thapsigargin (open bar) caused transient rises in $[\text{Ca}^{2+}]_i$ (white arrow). C. Calmidazolium-induced rises in $[\text{Ca}^{2+}]_i$ did not occur in calcium-free buffer. ‘B’ is HBS.
In the absence of extracellular calcium no calmidazolium-induced calcium rises can be observed and the thapsigargin-induced plateau-phase observed in the presence of extracellular calcium is also lacking. This shows extracellular calcium is involved in the calmidazolium induced calcium rises.

3.2.2.3. Calcium influx

Indeed, in the absence of extracellular calcium (Figure 3.4A) the fraction of cells passing threshold upon calmidazolium application is reduced to nearly zero (3+/-1%; n=25; p <0.001; Fig3.5). Calmidazolium does still exert its effect in the absence of extracellular calcium, because when calcium is added back after application of calmidazolium in a calcium free solution, pronounced calcium elevations were seen (Figure 3.4B).

3.2.2.3a Influx via voltage operated calcium channels

How does calcium enter the cell? It has been suggested that calmidazolium can affect the calcium permeability of the plasma membrane (Grosman, N., 1986) and this could cause, or be caused by, depolarisation. Depolarisation would open voltage sensitive calcium channels to allow calcium influx.

When I applied calmidazolium in the presence of the L-type voltage operated calcium channel blocker nifedipine (10 microM), the calmidazolium effect is reduced to the same level as in the absence of extracellular calcium (2.4+/-.0.8%; n=3; p>0.05 fig3.5), indicating that calcium influx occurs through L-type calcium channels.

3.2.2.3b Contribution from sodium channels

Normal depolarisation in neurones, as occurs during firing of action potentials, involves both sodium and potassium channels. Depolarisation could occur through opening of sodium channels or closing of potassium channels. If sodium channel opening was involved in the calmidazolium response, then blocking sodium channels with TTX should prevent depolarisation. In the presence of TTX, the proportion of responding cells is reduced to 8.9+/-.0.5% (n=3), significantly lower than caused by calmidazolium.
Figure 3.4. No responses in calcium-free buffer.
A. When calmidazolium was applied in a calcium-free buffer, no increase in $[\text{Ca}^{2+}]_{i}$ was observed and often a decrease in the resting calcium concentration occurred. B. The mechanism that causes the calmidazolium-induced rise in $[\text{Ca}^{2+}]_{i}$ did operate in calcium-free buffer as restoring the extracellular calcium concentration to normal immediately evoked rises in $[\text{Ca}^{2+}]_{i}$. 'CMZ' is calmidazolium.
3.2.2.3c Contribution from potassium channels

Block of all potassium channels would enhance depolarisation and might therefore be expected to enhance the proportion of cells that respond to calmidazolium. When calmidazolium was applied in the presence of 4-aminopyridine (4-AP; 5 mM) and tetraethylammonium (TEA; 20 mM), however, the proportion of responding cells was significantly lower than when calmidazolium is applied alone (1.0±0.6%; n=3; p<0.05 fig5) and was not different from the response in zero calcium (p>0.05).
3.3. DISCUSSION

3.3.1. Calmidazolium causes calcium rises

I have shown here that calmidazolium, widely used as an inhibitor of calmodulin function, causes transient increases in the intracellular calcium concentration, in a dose dependent manner, in nerve growth factor differentiated PC12 cells. Only a limited dose response curve for this effect of calmidazolium could be constructed because the solubility limit of calmidazolium at a physiological pH in HBS, is reached between 10 and 25 microM. The manufacturer claims a solubility limit for calmidazolium of 12 mg/ml in aqueous solutions at pH 4, equivalent to 17 mM (Calbiochem, personal communication) and 10 mg/ml in DMSO or ethanol. However, precipitation of calmidazolium out of solution at concentrations of 25 microM and higher has been confirmed by other users (Bregestovski, personal communication). Surprisingly, in the literature there is mention of concentrations as high as 100 microM being used, but most studies use concentrations of 10 micromolar or less (Wyskovsky, W. et al., 1988)(see table 3.2). The fact that the proportion of responding cells still increases at these ‘maximal’ concentrations of calmidazolium indicates that the precipitating drug somehow has an additional effect on the intracellular calcium concentration. This is also reflected by the change in the dose response curve, that reveals a more linear process above 10 microM (Fig3.2A).

3.3.2. Effect of high concentrations of calmidazolium on the cell membrane

This additional effect of calmidazolium might be due to an aspecific effect on membrane permeability. It has been shown that calmidazolium interacts with serum components and phospholipids, probably because of the hydrophobic properties that also allow it to bind to calmodulin (Grosman, N., 1986). At higher concentrations calmidazolium causes an increase in the spontaneous outflow of radioactively labelled noradrenaline (Grosman, N., 1986) and at 20 microM calmidazolium increases the permeability to ethydium bromide of Trypanosoma membranes (Grosman, N., 1986).
3.3.3. Calcium rises are due to influx via L-type voltage operated calcium channels

The calcium rises induced by calmidazolium are due to influx of extracellular calcium, because they disappear when extracellular calcium is omitted. Calcium influx must occur through L-type voltage operated calcium channels, because nifedipine, a blocker of L-channels, reduces the number of cells that passed threshold, to the level found in the absence of extracellular calcium.

Calmidazolium has been reported to inhibit the barium current through voltage operated calcium channels by about 30% in undifferentiated PC12 (Ito, K. et al., 1996). Although it was recently confirmed that calmodulin mediates calcium dependent modulation of L-type calcium currents (Zuhlke et al, 1999), other studies using calmidazolium have not found any effect on these channels (Zuhlke, R.D. et al., 1999). In addition, calmidazolium treated synaptosomes would still show an increase in calcium uptake when they were depolarised, confirming that calmidazolium leaves the function of voltage operated calcium channels intact (Snelling, R. and Nicholls, D., 1984) and it has been suggested that ethidiumbromide permeability in Trypanosoma in response to calmidazolium could be due to influx via calcium channels (Vercesi, A.E. et al., 1991). It seems therefore unlikely that calmidazolium acts on L-type calcium channels in a mechanism that involves calmodulin.

In addition, the calcium rises induced by calmidazolium are different from those caused by a structurally different (Figure 3.6) calmodulin antagonist W13. For an effect of a pharmacological inhibitor to be interpreted as specifically acting on calmodulin, both inhibitors should have the same effect, with a potency order that is the same as for the effect on isolated enzymes.

Therefore it seems rather more likely that the calmidazolium induced calcium rises I observed are due to an effect of calmidazolium on the membrane potential, unrelated to calmodulin function, which in turn leads to the opening of L-type voltage operated
calcium channels. This means results of studies using calmidazolium to inhibit calmodulin function need to be treated with caution.

3.3.4. Mechanism of calmidazolium induced depolarisation

The main effector of calmidazolium induced calcium rises is the depolarisation of the plasma membrane. This could be either achieved directly by induction of pore formation or indirectly, by activating existing permeation pathways. The fact that a calcium channel blocker can inhibit the calcium rises suggest calmidazolium does not induce formation of calcium permeable pores, nor does it work as a calcium-ionophore.

It has been shown that calmidazolium depolarises the cell plasma membrane at concentrations of 0.5 microM and higher (Snelling, R. and Nicholls, D., 1984). If depolarisation does not occur through pore formation or a direct effect on calcium channels, this effect of calmidazolium must either be on potassium or sodium conductances.

3.3.5. Sodium and potassium channel mediated depolarisation

Both sodium and potassium channels are normally involved in the depolarisation of neurones. To depolarise cells, calmidazolium should either open up sodium channels or close down potassium channels.

In the presence of the sodium channel blocker TTX, the calmidazolium response is reduced, but not to the level it reaches in zero calcium. This indicates that the calmidazolium response is in part due to opening of sodium channels during the firing of action potentials. The only report in the literature, to my knowledge, that has investigated sodium currents in relation to calmodulin, identifies calmodulin as a facilitator of opening of sodium channels in Paramecium, if calmidazolium interfered with this calmodulin
effect however, the result should be the opposite: calmidazolium should keep sodium channels closed, thus preventing depolarisation (Saimi, Y. and Ling, K.Y., 1990).

Most likely is that opening of voltage sensitive sodium channels could be contributing to and be caused by general depolarisation of the cell.

Besides via sodium channels, depolarisation of the cell could be caused by opening of sodium permeable receptor channels in response to depolarisation or by the closing of potassium channels. There are some reports on calmidazolium causing an increase in transmitter release. Stimulation of release by calmidazolium has been reported for insulin release in pancreatic Beta cells and histamine release, but transmitter release and secretion can be an indirect effect of membrane depolarisation and these effects of calmidazolium are hard to dissect out (Kindmark, H. et al., 1995). The potassium channels inhibitors 4-Aminopyridine (4-AP) and tetra-ethyl ammonium would block between them delayed rectifier, $K_A$, $K_M$, $K_S$, calcium activated Maxi-K channels and sodium activated potassium channels (Nichols, J.G., Martin, A.R. and Wallace, B.G., 1992). If these would constitute the complete make-up of potassium conductances in the cell the membrane, depolarisation of the cell membrane would be enhanced. As a result of this I would expect to have seen an increase in the proportion of cells showing calcium rises when I applied calmidazolium together with 4-AP and TEA. However, the opposite is true: when 4-AP, TEA and calmidazolium were applied together, there was a decrease in the proportion of responding cells to the level of responses in zero calcium. This could mean two things: either the effect of calmidazolium is inhibited by 4-AP and TEA, in which case calmidazolium might somehow open potassium channels, or, not all potassium channels are blocked by 4-AP and TEA and enough potassium conductances remain to retain a resting membrane potential below the activation threshold for L-type calcium channels. The effect of 4-AP and TEA under these conditions would be to prevent depolarisation and consequently, 4-AP and TEA would reduce the number of cells that pass threshold.

So, although the exact mechanism is unknown, it is clear that calmidazolium causes calcium influx through L-type voltage operated calcium channels, most likely through
depolarisation of the cell membrane and possibly a route that involves potassium channels.

3.3.6. Contribution from intracellular calcium stores

If influx is the source for the calcium rises caused by calmidazolium, why does thapsigargin also reduce the number of responding cells?

Both IP₃ receptor and ryanodine receptor mediated calcium release show a bell-shaped dependence on the intracellular calcium concentration, with slightly elevated calcium levels facilitating release and higher calcium levels inhibiting calcium release. If a proportion of the calmidazolium induced calcium rises therefore is caused by calcium influx triggering store release, thapsigargin treatment would obliterate this part of the response and thus reduce the total fraction of responding cells. Similarly, in Dictyostelium other inhibitors of the SERCA only partially eliminated calmidazolium induced calcium transients (Schlatterer, C. and Schaloske, R., 1996). In the absence of extracellular calcium, no calmidazolium induced calcium rises are observed in thapsigargin treated cells. This means that external calcium is needed for the calmidazolium induced calcium rises that are seen in the presence of thapsigargin.

3.3.7. Contribution from mitochondria?

Calmidazolium has an effect on mitochondria illustrated by the finding that glucose can reduce the effect of calmidazolium on histamine release from mast cells (Grosman, N., 1986). Calmidazolium has been reported to depolarise mitochondria, which would result in any stored free calcium being released, in Trypanosoma and guinea pig cortical synaptosomes (Vercesi, A.E. et al., 1991). Furthermore, there is evidence that calmodulin inhibitors disrupt mitochondrial energy production by inhibition of the oligomycin sensitive ATP-(Ruben, L. and Rasmussen, H., 1981). However, in my experiments oligomycin, at a concentration that is known to inhibit ATP-synthase in hippocampal neurones (2.5 microg/ml; J. Keelan personal communication), fails to induce an increase in the
proportion of cells displaying calcium rises, making mitochondrial ATP-synthase an unlikely player in the calmidazolium induced calcium rise.

3.3.8. Inhibition of plasma membrane Ca\(^{2+}\)-ATPase activity?

Calmodulin stimulates the activity of the pmCa\(^{2+}\)-ATPase (Brandt, P.C., Vanaman, T.C., 1998) and potentially, reduced clearance of calcium could appear as calcium rises. The extent to which the pmCa\(^{2+}\)-ATPase contributes to calcium clearance in hippocampal neurones, is limited and in addition, calcium efflux has been reported to not be greatly affected by calmidazolium (Snelling, R. and Nicholls, D., 1984). The pmCa\(^{2+}\)-ATPase should still work in the presence of nifedipine and since these agents inhibit the effect of calmidazolium, a major role for the pmCa\(^{2+}\)-ATPase in the calmidazolium response is excluded.

I have not further investigated the exact site of action for calmidazolium for causing these calcium rises, as it has become clear that use of calmidazolium to study subcellular processes that are modulated by calmodulin is greatly impaired by effects on membrane potential and calcium influx.

Since the same calcium rises are not observed when I use calmodulin antagonist W13, which has a different chemical structure (Fig 3.8), it seems unlikely that they are caused by a direct inhibition of calmodulin, but are due to an indirect unrelated effect of calmidazolium on the membrane potential that leads to opening of L-type voltage operated calcium channels.

3.4. CONCLUSION

In this study I showed that calmidazolium at concentration widely used to inhibit calmodulin function in cells (Table 2, italic entries), causes transient rises in intracellular calcium by causing influx of extracellular calcium through L-type voltage operated calcium channels, most likely caused by depolarisation of the cell membrane. This
appears to be an action on a target other than calmodulin, possibly transmitter release or potassium conductances.

Table 3.2. Concentrations of calmidazolium used in other studies.
Where authors claim calmidazolium acted specifically on calmodulin, entries in the table are italic. Where authors raised doubt about the specificity of calmidazolium entries are in plain text. '-' indicates an inhibitory effect and '+' a stimulated effect.

<table>
<thead>
<tr>
<th>calmidazolium</th>
<th>cell type</th>
<th>effect</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(microM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>N1E-115</td>
<td>-nitric oxide release (5microM-60%; 10 microM-95%)</td>
<td>(Forstermann, U. et al., 1990)</td>
</tr>
<tr>
<td>10</td>
<td>SH-SH5Y</td>
<td>-metylcholine receptor mediated PLC activation</td>
<td>(Larsson, C. and Alling, C., 1995)</td>
</tr>
<tr>
<td>10</td>
<td>thoracic aortae</td>
<td>-Et3 and ACh induced nitric oxide mediated relaxation</td>
<td>(Moritoki, H. et al., 1993)</td>
</tr>
<tr>
<td>1, 3, 10, 30</td>
<td>C6-glioma</td>
<td>+ ATP induced PI turnover (Calmodulin specific)</td>
<td>(Lin, W.W., 1995)</td>
</tr>
<tr>
<td>100</td>
<td>skel muscle sarcopl reticulum</td>
<td>+ Ca2+ release</td>
<td>(Wyskovsky, W. et al., 1988)</td>
</tr>
<tr>
<td>0.01-1</td>
<td>PC12</td>
<td>- I L-VOCC</td>
<td>(Ito, K. et al., 1996)</td>
</tr>
<tr>
<td>20</td>
<td>dictyostelium</td>
<td>+ [Ca2+] stores + influx</td>
<td>(Malchow, D. et al., 1996)</td>
</tr>
<tr>
<td>9</td>
<td>Dictyostelium</td>
<td>+ [Ca2+] stores + influx</td>
<td>(Schlatterer, C. and Schaloske, R., 1996)</td>
</tr>
<tr>
<td>20</td>
<td>Trypanosoma Cruzi</td>
<td>+ Ca release from mitos (mito depol) effect at 20μM (perm membr for EtBr)</td>
<td>(Vercesi, A.E. et al., 1991)</td>
</tr>
<tr>
<td>&gt;0.5 (15)</td>
<td>synaptosomes</td>
<td>+ depol V mito</td>
<td>(Snelling, R. and Nicholls, D., 1984)</td>
</tr>
<tr>
<td>10</td>
<td>cholera toxin treated chief cells</td>
<td>-phrobol ester + A23187 induced calmodulinP rise (no effects on its own)</td>
<td>(Raufman, J.P., 1992)</td>
</tr>
<tr>
<td>10</td>
<td>synaptosomes</td>
<td>-noradrenaline release, but increased spontaneous outflow</td>
<td>(Barrington, M. and Majewski, H., 1994)</td>
</tr>
<tr>
<td>5</td>
<td>rat mast cells</td>
<td>+max histamine release</td>
<td>(Grosman, N., 1986)</td>
</tr>
</tbody>
</table>

In general care should be taken to interpret effect of calmodulin inhibitors as arising from interaction with calmodulin alone, when using these on cells or tissue, because aspecific actions are common (table 3.2, non-italic entries).
To ascertain that the observed effect of an inhibitor indeed results from specific action, the effect should: a. be similar to that observed in response to structurally different calmodulin antagonists. b. have a sensitivity profile to antagonists that corresponds to the order of sensitivity of calmodulin in a known system of isolated enzymes to these antagonists. Because calmodulin function changes with changes in the intracellular calcium concentration, so should the effect of antagonists be influenced by changes in intracellular calcium concentration. This could be tested, for example, by using calcium chelators or ionophores.

Pharmacological calmodulin antagonists are therefore not suitable for the study of an isolated aspect of modulation of cellular function by calmodulin if this action is not easily isolated from other consequences of calmodulin inhibition, or the parameter that is being measured. The risk of aspecific effects of calmodulin inhibitors on the parameter that is being measured reduces the potential use of these tools to specific cell systems and interactions of isolated proteins.
CHAPTER 4: LOCALISATION OF MICRO-INJECTED FLUORESCENTLY LABELLED CALMODULIN.

4.1. INTRODUCTION

Calmodulin and its function have mainly been studied biochemically, using fractionated cells (e.g. (Gnegy, M.E., 1993)) or using immunocytochemistry on fixed tissue (e.g. (Caceres, A. et al., 1983)). In living cells calmodulin's function has been mostly studied using pharmacological inhibitors of limited specificity. A few studies have been published in which calmodulin was labelled with a fluorescent group in order to monitor its localisation and activation during cell activity (Hahn, K.M. et al., 1990). Two of these fluorescently labelled calmodulins were available to me: TA-CaM, that changes its fluorescence up to five-fold in vitro upon the binding of calcium (Török, K. et al., 1998) and FL-CaM, which does not change its fluorescence when calcium binds (Török, K., Wilding, M., Groigno, L., Patel, R. and Whitaker, M., 1998). In each case, the fluorophore is attached to lysine 75 of pig calmodulin, which is identical to the rat protein.

The addition of the fluorescent group is a prerequisite for visualising the behaviour of calmodulin-probes in the intracellular environment in living cells. However, the fluorophores that are attached to calmodulin should not disturb binding to normal downstream targets. In resting cells most cellular calmodulin is believed to be bound to CaMBPs and the free calmodulin concentration can be a thousand times lower than the total calmodulin concentration (Gnegy, M.E., 1995). This means that calmodulin could be limiting for many reactions unless the intracellular calcium concentration is raised. The fluorescent calmodulin analogues I use in this study have been characterised in vitro (Török, K. and Trentham, D.R., 1994) see materials and methods). TA-CaM binds to and activates the calmodulin-activated PDE with a $K_m$ that is 3.5 times greater than the $K_m$ for PDE of native calmodulin and TA-CaM binds to smooth muscle myosin light chain kinase (smMLCk) and to smMLCK-derived peptide with a $K_d$ of 0.23–0.51 nM.
(compared to 0.10-0.22 nM for unlabelled calmodulin), but inhibits this kinase in an 
TA-CaM has also been shown to bind to connexin of rat liver gap junctions (Török, K. et 
al., 1997). Wilding et al. micro-injected FL-CaM into *Lytechinus pictus* embryos and 
studied the distribution of this fluorescent calmodulin analogue during the first cell cycle 
(Wilding, M. et al., 1995). The distribution they observed very much resembled that 
found using calmodulin antibodies in similar systems, with FL-CaM locating to the 
kinetochore complex and mitotic spindle. Other groups deploying fluorescently labelled 
calmodulin-analogues showed that micro-injected calmodulin labelled with rhodamine 
iodo-thiocyanate located to the mitotic spindle of PtK1, BS-C-1, HeLa and mouse 3T3 cells 
and found that this distribution mimicked that of calmodulin antibodies or known 
calmodulin binding targets (Zavortink, M. et al., 1983). TA-CaM, as used in this study, 
has been successfully used to report calmodulin activation in pancreatic acinar cells and 
Hela cells (Craske, M., Takeo, T., Török, K., Gerasimenko, O., Peterson, O.H. and 
Tepikin, A.V., 1998; Li, C.J. et al., 1999). These studies all demonstrate that 
fluorescently labelled calmodulins are valuable and valid tools to study the physiology of 
calmodulin in living cells.

Brain tissue has a high calmodulin content (10-100 microM; (Kakiuchi, S. et al., 1982)). 
Fluorescent calmodulin analogues however, have not been extensively used to study 
calmodulin behaviour and distribution in nerve cells. To my knowledge the only 
published result showing the distribution of fluorescently labelled calmodulin in neurones 
stems from previous experiments in the Bolsover laboratory by Zimprich et al. (Zimprich, 
F. et al., 1995). Although Deisseroth et al. mention the use in neurones of MeroCaM, a 
labelled calmodulin generated by Taylor's group, they do not show any data using it 
(Deisseroth, K. et al., 1998).

The normal subcellular distribution of endogenous calmodulin in neurones is non-uniform 
as is illustrated by the following examples. Electronmicroscopical analysis of calmodulin 
antibodies in cerebellar cells showed intense labelling in the nucleus and dendrites, with
less labelling in the cytosol of the cell body and in axons. Calmodulin immunoreactivity was found on axonal and dendritic microtubules and in postsynaptic densities (Caceres, A. et al., 1983). In PC12 cells there was a diffuse calmodulin immunoreactivity and a weak nuclear signal in undifferentiated cells. However, when PC12 cells were made to differentiate to a neuronal phenotype, cells with a high level of differentiation showed a high nuclear calmodulin immunoreactivity (Natsukari, N. et al., 1995). When neuronal cells are stimulated with agonists the relative calmodulin content of the membrane compartment, cytosolic compartment and nuclear compartments of the cell change; dopamine, apomorphine, Leu-enkephalin, carbachol and bradykinin all stimulate release of calmodulin bound to the isolated membranes of neuronal cells into the cytosolic fraction over a time-course of 1-90 minutes and oestrogens and norepinephrine can induce calmodulin-translocation from cytosol to nucleus in liver cells (Gnegy, M.E., 1993).

A study by Deisseroth and co-workers demonstrated that depolarisation of rat hippocampal neurones in culture, either electrically or with high potassium induced an increase in the nuclear calmodulin immunoreactivity. This was interpreted as a translocation of calmodulin to the cell nucleus and translocation was maximal within a few minutes after depolarisation (Deisseroth, K. et al., 1998). Experiments in the Bolsover laboratory reported in 1995 that nuclear calmodulin was rapidly activated (that is, became calcium loaded) after even brief depolarisation of rat sensory neurones. This conclusion was based on their recording of a rapid increase of TA-CaM fluorescence following depolarisation (Zimprich, F. et al., 1995). However, the report by Deisseroth raised the possibility that the fluorescence changes they recorded were not the result of calcium binding, but instead simply reflected relocation of cytosolic TA-CaM to the nucleus. Indeed, Deisseroth and co-workers have suggested that calcium influx activates calmodulin dependent processes in the nucleus not by activating calmodulin already in the nucleus but by causing the movement of cytosolic calmodulin to the nucleus (Deisseroth, K. et al., 1996; Deisseroth, K. et al., 1998). In this chapter I evaluate the suitability of FL-CaM as a calcium-insensitive control to correct for possible
relocation of TA-CaM and test the previous conclusion that brief depolarisation of rat sensory neurones causes calcium loading of nuclear calmodulin.

A prerequisite for this approach is that both FL-CaM and TA-CaM assume the same subcellular distribution.

To investigate this I micro-injected FL-CaM and TA-CaM into rat DRGs.

4.2. RESULTS

4.2.1. Localisation of micro-injected fluorescently labelled calmodulin:

TA-CaM has been used previously in the Bolsover laboratory (Zimprich, F. et al., 1995) to compare activation of nuclear and cytosolic calmodulin in dorsal root ganglion neurones. However, there was no correction in that study for possible stimulus induced translocation of TA-CaM. One approach of controlling for localised fluorescence increases that result from local accumulation of fluorescently labelled calmodulin is to coinject FL-CaM and TA-CaM. Using this technique, a stimulus that causes a local change of TA-CaM fluorescence without a corresponding change of FL-CaM fluorescence can be taken to result from a change in the degree of calcium loading of TA-CaM. If the FL-CaM signal does change, indicating calmodulin translocation, the degree of calcium:calmodulin activation can still be estimated by calculating the ratio of TA-CaM fluorescence to FL-CaM fluorescence. This method has been used successfully
Figure 4.1. Subcellular localisation of fluorescently labelled calmodulins.

Confocal sections through dorsal root ganglion cells micro-injected with fluorescently labeled calmodulins. Cells were injected to a final concentration of 1 to 6μM while bathed in growth medium and maintained at 37°C, 5% CO₂ for several hours after injection. The images are presented using the pseudocolor scale illustrated in A in which increasing intensities are represented by increasingly warmer colours. A: FL-CaM fluorescence preferentially localizes to the cell nucleus. Living cell bathed in growth medium imaged 1 hour after injection of FL-CaM. Open arrow: nucleus. Filled arrow: growth cone. B: TA-CaM shows a distribution similar to FL-CaM. Cell fixed about 4 hours after injection. Open arrow: nucleus. Filled arrow: growth cone. C: A FL-dextran injected cell is only slightly brighter in the nucleus. Living cell bathed in growth medium imaged 6 hours after injection. Open arrow: nucleus. D: FL-Albumin (FL-Alb) injected into the cell nucleus stays there. Living cell bathed in growth medium imaged 2 hours after injection. E: When FL-Albumin is injected into the cytosol the nucleus remains dark. Living cell bathed in growth medium imaged 2 hours after injection. F: Average nucleus/cytosol fluorescence intensity ratio ± SEM for FL-Dextran (n=14), FL-CaM (n=14) and TA-CaM (n=4); **: significantly different from FL-Dextran value, P<0.01. Scale bars in A through E correspond to 10μm.
in sea urchin eggs (Török, K., Wilding, M., Groigno, L., Patel, R. and Whitaker, M., 1998) and during the secretory response in pancreatic acinar cells (Craske, M., Takeo, T., Török, K., Gerasimenko, O., Peterson, O.H. and Tepikin, A.V., 1998). For the method to be valid in neurones, FL-CaM and TA-CaM need to have a similar intracellular distribution. To investigate this point, I micro-injected FL-CaM and TA-CaM into rat dorsal root ganglion neurones. Micro-injection has the added advantage over the patch-loading technique used earlier in the Bolsover laboratory, that the cell contents are not dialyzed by the solution in the patch pipette and there is no continued loading of fluorescent protein from the patch-pipette. When either FL-CaM or TA-CaM were injected into dorsal root ganglion neurones they localised to the cell nucleus (figure 4.1A, B, F). The ratio of nuclear over cytosolic fluorescence of micro-injected calmodulin was 1.92±0.14 (n=14) for FL-CaM and 1.31±0.06 (n=4) for TA-CaM. The nuclear localisation of fluorescent calmodulin was not a result of damage to the nuclear envelope, because fluorescein-albumin (66KDa), that should not easily cross the nuclear pore complex, remained nuclear when it was injected into the nucleus (figure 4.1D) and cytosolic when it was injected into the cytosol (figure 4.1E).

The observed distribution of FL-CaM could be a result of dye exclusion by cytosolic organelles that are absent from the nucleus, that is, FL-CaM might have the same concentration in nucleoplasm and cytosol, but cytosol occupies only a fraction of the cytoplasmic volume. To correct for this effect, I injected fluorescein conjugated to a 10 kDa dextran (FL-dextran). FL-dextran has been used as a control for the space-water distribution of fluorescently labelled molecules (Luby Phelps, K. et al., 1985). A molecule of this size is expected to pass through the nuclear pores without the aid of a nuclear localisation signal. The distribution of FL-dextran (nucleus/cytosol=1.29±0.16 (n=14); figure 4.1C) was significantly different from that of FL-CaM (p<0.01; Bonferroni corrected Students t-test; figure 4.1F) therefore the localisation of FL-CaM to the nucleus cannot simply be a result of exclusion by organelles in the cytosol nor can it be an effect of the nuclear environment on the fluorescein fluorophore.

This was a curious finding because Deisseroth's work showed increased nuclear
calmodulin immunofluorescence after depolarisation, whereas my experiments used resting cells that were allowed to recover after the injection.

To investigate the discrepancy between injected fluorescent calmodulin and the immunoreactivity result of Deisseroth, I looked at the subcellular distribution of calmodulin immunoreactivity in fixed DRGNs.

4.2.2. Localisation of calmodulin immunoreactivity:

I used a monoclonal calmodulin antibody, raised against the C-terminus of calmodulin, on DRGNs that were fixed in the presence of 1 mM EGTA to ensure that the antibody-labelling reflected the true calmodulin distribution of all calmodulin. In this way a preference of the antibody for calcium-free calmodulin or calcium-calmodulin would not distort the data.

In direct contrast to the results obtained using injected FL-CaM, the cell’s own calmodulin, as revealed by immunofluorescence of fixed unstimulated cells, was concentrated in the cytoplasm (Fig. 4.2A). Deisseroth and co-workers described a similar finding for rat hippocampal neurones and further reported that electrical or high potassium stimulation caused a relocation of the immunofluorescence to the nucleus (Deisseroth, K. et al., 1998). In agreement with their finding, calmodulin immunofluorescence in sensory neurones also relocated to the nucleus upon high potassium treatment (figure 4.2B, C). However, the mismatch between FL-CaM distribution and immunofluorescence persisted when the injected FL-CaM and calmodulin immunoreactivity were imaged in the same cells (Fig. 4.2D).

One explanation of the measured mismatch between the location of injected FL-CaM (always concentrated in the nucleus) and calmodulin immunofluorescence (in cytosol until cells are depolarised) is that the anti-calmodulin antibodies (>144 KDa) used to reveal endogenous calmodulin could not gain entry into the nuclei of these mildly permeabilised cells (0.4% saponin for 10 minutes) until the cells were depolarised. To test for this possible artifact, I examined whether the access of an antibody of similar
Figure 4.2. Discrepancy between the localisation of immunofluorescence and Fl-CaM.
Nuclear translocation of calmodulin immunofluorescence upon depolarization with high K⁺ saline. A. Confocal section of calmodulin immunofluorescence in resting dorsal root ganglion cells. Cells were rinsed with HBS followed by HBS with formalin and then stained for calmodulin as described in the Methods. The nucleus is indicated by open arrow. B. Calmodulin immunofluorescence in a stimulated dorsal root ganglion cell. Cells were rinsed with HBS followed by high K⁺ saline for 180s then fixed in normal HBS and stained for calmodulin. C. Average nucleus/cytosol immunofluorescence ratio after background subtraction (n=34 and 32 respectively). **: different at P<0.0001, Mann-Whitney non-parametric test. D. Spatial mismatch of calmodulin immunofluorescence and fluorescence of injected FL-CaM in a resting cell. Cells were injected with FL-CaM to a final concentration of 1 to 6µM while bathed in growth medium and maintained at 37°C, 5% CO₂ for several hours after injection. They were then rinsed with HBS followed by HBS with formalin and stained for calmodulin as described in the Methods. Scalebars correspond to 10µm.

size to that for calmodulin, but with a target that is always entirely nuclear would label the nuclei of resting cells. A histone H1 antibody did indeed label the nucleus in resting
cells (figure 4.3), implying that the calmodulin antibody will also have access to the nucleus of unstimulated cells that have been permeabilised and fixed.

Another possible explanation was that micro-injection of DRGNs would allow calcium to enter the cells. This could be sufficient to induce the nuclear localisation of FL-CaM as seen in the immunocytochemistry data.

To test this hypothesis, I injected DRGNs, that had been acetoxymethyl-loaded with Fura-2, with vehicle (the injection buffer without any calmodulin). Cells thus injected showed an increase in intracellular calcium (figure 4.4A) that declined back to baseline over a time period of 20 minutes (figure 4.4B). As a control the fluorescence of uninjected cells near the injected cells was monitored. These control cells did not show a change in either the 340 nm or 380 nm excited fluorescence, showing that neither movement of the dish nor a calcium increase unrelated to the injection event could be the basis of the calcium rise seen in injected cells. Although this injection induced calcium rise could cause calmodulin translocation, it is, unlikely that this would explain the distribution of fluorescent calmodulin I observed here. In experiments by Deisseroth and experiments by Alexei Tepikin, translocated calmodulin moves out of the nucleus over a time course of tens of minutes, whereas my cells injected with fluorescent calmodulin were left to recover for up to 6 hours and still showed a high nuclear fluorescence intensity (Deisseroth, K. et al., 1998). In addition, the mismatch persisted when the injected FL-CaM and calmodulin immunoreactivity were imaged in the same cells (Fig. 2D).

Two models could explain the mismatch of calmodulin immunoreactivity and FL-CaM fluorescence:

1. The different distribution of FL-CaM and immunofluorescence is due to an artifact in antibody binding.
2. The distributions of endogenous and labelled calmodulin are actually different.
Figure 4.3. Histone H1 immunoreactivity.
An antibody against histone H1, of similar size to the calmodulin antibody used in figure 4.2., can penetrate into the nucleus of resting cells to label its nuclear target. Confocal image of a DRGN fixed with paraformaldehyde Scalebar corresponds to 20 microm.

Figure 4.4. Micro-injection causes an increase in \([\text{Ca}^{2+}]_i\).
A. Injection of the vehicle used for the injection of FL-CaM causes an increase in \([\text{Ca}^{2+}]_i\) in the Fura-2 fluorescence ratio of Fura-2 loaded cells. The average of five cells is shown. Neighbouring uninjected cells were imaged to control for movement of the dish. B. Time course of Fura-2 ratio change of an injected and three uninjected cells over 30 minutes.
For the first explanation two possible scenarios are:
a. The calmodulin antibody did not recognise calmodulin in the cell nucleus unless the cells were depolarised
or b. the antibody did not recognise labelled calmodulin.

In scenario a, the antibody would label neither nuclear endogenous calmodulin nor nuclear TA-CaM and FL-CaM in resting cells. Shielding of the antibody binding epitope by nuclear calmodulin binding proteins could cause this and upon depolarisation of the cell membrane, calcium binding could reveal the antibody epitope. The shielding of antibody epitopes by CaBPs can not be excluded, since the exact nature of the CaMBPs in question is unknown, however it is unlikely, because the same antibody has been successfully used by others to immunoprecipitate CaMKII and CaMKIII bound to calmodulin (Mitsui, K. et al., 1993).

In scenario b, fluorescently labelled calmodulin would not be recognised by the antibody. Therefore, immunocytochemistry on injected cells would only reflect the distribution of unlabelled calmodulin.

On a Western Blot, it appeared that the calmodulin antibody bound poorly to TA-CaM and FL-CaM when 1 mM EGTA was included in the buffers (fig 4.5B). These conditions were the same as used for fixing cells, to ensure the antibody would recognise calcium:calmodulin and calcium-free calmodulin equally well (Deisseroth, K. et al., 1998). The fact that immunofluorescence and FL-CaM distribution show a different nucleus/cytosol fluorescence ratio either means that in resting cells some shielding of the antibody epitope on FL-CaM by the fluorophore occurs, mainly in the cytosol or that the distribution of FL-CaM is different from that of endogenous calmodulin. In the later case model 2 would apply.

2-In the second model, the distributions of endogenous and fluorescently labelled calmodulin are indeed different.
Figure 4.5. Western blot of labelled and unlabelled calmodulin. Purified unlabelled calmodulin (CaM), FL-CaM and TA-CaM were spotted onto blotting paper that was kept wet during the whole blotting and labelling procedure with a calcium containing or a calcium-free buffer, to encourage a natural conformation of the proteins. Immunofluorescence was visualised using an enhanced chemiluminescence protocol and exposure to photographic film. Staining of the blots afterwards with indian ink revealed similar amount of protein in all spots.

In this model the cytosolic antibody signal was simply much stronger than the nuclear signal because it reflected the much higher amount of endogenous calmodulin present in
the cytosol. Endogenous calmodulin would occupy most of the high affinity binding sites, so additional injected FL-CaM would be left with lower affinity binding sites, more of which might be present in the cell nucleus. This phenomenon could be enhanced by the slightly reduced affinity for targets of TA-CaM that has been reported (Török, K. and Trentham, D.R., 1994). Alternatively, labelled calmodulin might have a slightly elevated affinity for some nuclear targets, although there is no evidence for this.

In an attempt to confirm that the FL-CaM distribution was due to binding to CaBPs, I used the peptide Ac-R-R-K-W-Q-K-T-G-H-A-V-R-A-I-G-R-L-COH₂ (Trp-peptide). The Trp-peptide has nanomolar affinity for calmodulin and inhibits binding of calmodulin to smMLCK with a Kᵢ of 8.6 x 10⁻¹² in vitro (Török, K. and Trentham, D.R., 1994). Because of its high affinity the Trp-peptide would be expected to compete for calmodulin binding with other CaBPs and effectively work as a calmodulin inhibitor. Co-injection of Trp-peptide at 2.5 times the FL-CaM concentration did however not change the nucleus/cytosol distribution (Figure 4.6).

Other evidence supports the scenario of competition between already bound unlabelled calmodulin and injected calmodulin.

When I injected FL-CaM together with a 15-fold excess of unlabelled calmodulin, the nucleus over cytosol ratio for FL-CaM fluorescence was significantly higher (3.30±0.44; n=5; p<0.001), compared to that for FL-CaM injected alone, but when a 30 fold excess of unlabelled calmodulin was injected, the ratio was 1.70±0.22 (n=3), not different from FL-CaM alone (Figure 4.6).

This indicates that increasing the binding competition for a set of binding sites with high affinity, by including unlabelled calmodulin, would drive the labelled calmodulin to a set of binding sites with lower affinity more of which, apparently, were located in the nucleus. When the proportion of unlabelled calmodulin that was injected was higher, the
Figure 4.6. Effect of co-injecting FL-CaM with Trp-peptide or unlabelled calmodulin. FL-CaM was injected together with either of Trp-peptide or unlabelled purified calmodulin at 2.5, 15 or 30 times the concentration of the injected FL-CaM respectively. After recovery from injection for several ours at 37°C, 5% CO₂, the average nucleus/cytosol fluorescence ratio of confocal sections transecting the cell nucleus was determined for n cells. *** significantly different from FL-CaM ratio in figure 4.1.C. p<0.001.

competition for these lower affinity binding sites increased and less labelled calmodulin was retained in the nucleus.

4.2.3. Localisation of patch-loaded fluorescently labelled calmodulin:

When dorsal root ganglion neurones were loaded via a patch-pipette, circumventing the calcium transient associated with the pressure-injection process, both FL-CaM and TA-CaM distributed over nucleus and cytosol in a ratio not different from each other (figure 4.7A; nucleus/cytosol for FL-CaM and TA-CaM 1.84±/−0.42 and 1.52+/-0.24 respectively, n=7; p>0.05) or from the distribution of micro-injected Fl-CaM. Depolarisation for 200 ms in voltage clamp mode caused an average peak inward current of 2.9±0.9 nA (figure 4.7B insert) and increased calcium concentration equally in nucleus and cytosol (Fura-Red traces, Figure 4.7B). TA-CaM fluorescence also rose rapidly and to the same extent in nucleus and cytosol (Figure 4.7B). There was no depolarisation-induced increase in nuclear FL-CaM fluorescence over the five second recording period indicating that calmodulin translocation was minimal over this brief recording period.
Figure 4.7. TA-CaM activation in nucleus and cytosol.
Distribution and activation of FL-CaM and TA-CaM in patch-loaded dorsal root ganglion cells. A: Average nucleus/cytosol fluorescence intensity ratio of cells patch-loaded with FL-CaM (n=6) and TA-CaM (n=5). Measurements were made between 15 and 30 minutes after attaining whole cell configuration with a pipette containing 33μM FL-CaM, 66μM TA-CaM and 400μM Fura Red. B: Fluorescence of TA-CaM, FL-CaM and Fura Red measured simultaneously during voltage clamp depolarisation, normalised to the average fluorescence level before depolarisation, the average ± SEM of 7 cells is shown. A 200ms depolarisation (open square) increased the fluorescence of TA-CaM in both nucleus (nu) and cytosol (cy) (top traces) while FL-CaM fluorescence (middle traces) remained steady or fell slightly. The bottom traces show a decrease in the fluorescence of Fura Red, corresponding to an increase in the intracellular calcium concentration. The insert shows the averaged current with scale bars corresponding to 1nA vertical and 100ms horizontal.
4.3. DISCUSSION & CONCLUSIONS

My aim was to establish the intracellular localisation of FL-CaM and TA-CaM and to assess the suitability of FL-CaM as a control for changes in the local concentration of TA-CaM. The data presented in this chapter show that both FL-CaM and TA-CaM show significant localisation to the cell nucleus after they have been micro-injected or patch-loaded into the cytosol of resting cells. The same phenomenon has been reported for FL-CaM, micro-injected into sea urchin eggs or patch-loaded into mouse pancreatic acinar cells (Wilding, M. et al., 1995). Localisation of calmodulin to the cell nucleus seems not unreasonable, considering the fact that endogenous calmodulin in neurones is also found at elevated levels in the cell nucleus (Caceres, A. et al., 1983).

In contrast a well-characterised antibody to calmodulin recognises sites that are mainly cytoplasmic in resting cells, although in depolarised cells the immunofluorescence relocates to the nucleus. The nuclear localisation of FL-CaM is not a result of a different water space distribution or chemical environment, because the distribution of a 10 kDa fluorescein-dextran over nucleus and cytosol is significantly more uniform. The observed distribution of FL-CaM is also not a consequence of damage to the nuclear membrane.

What is the explanation of the spatial mismatch between endogenous calmodulin, as recognised by antibody, and injected calmodulin? Models to explain this mismatch are of two types. In the first, endogenous calmodulin and injected calmodulin have the same distribution; the mismatch reflects location-specific variation in the accessibility of the antibody epitope on calmodulin. In the second type of model, endogenous calmodulin is indeed primarily cytoplasmic until cells are depolarised.

The first type of model states that endogenous calmodulin and injected calmodulin have the same distribution; the mismatch between calmodulin immunofluorescence and injected calmodulin reflects location-specific variation in the accessibility of the antibody epitope on calmodulin. My results with histone H1 show that antibody is not excluded from the nucleus after fixation and permeabilisation. However, it remains possible that in
resting cells nuclear calmodulin is not recognised by the antibody because the epitope (the C terminal 20 amino acids) is screened by an attached CaMBP. In this model an increase of intracellular calcium concentration causes a dissociation from the CaMBP, allowing the antibody to bind. The antibody used has been reported to bind calmodulin even when complexed with the known CaMBPs CaMKII and CaMKIII (Mitsui, K. et al., 1993), rendering this hypothesis unlikely. However, since the nature of the relevant CaMBP is unknown, I cannot rule out that such a phenomenon underlies my results and similar findings in hippocampal neurones (Deisseroth, K. et al., 1998). Further study of this model with fluorescently labelled calmodulins is prohibited by the reduced accessibility of the antibody epitope of FL-CaM and TA-CaM in the presence of EGTA, needed to provide equal binding to all calmodulin regardless of calcium loading. The paper by Deisseroth et al. provided additional evidence that an actual translocation of calmodulin took place. By isolating nuclei of resting and depolarised cells and assessing the calmodulin content by Western blot they showed that depolarisation increased the nuclear calmodulin content 2.4-fold (Deisseroth, K. et al., 1998).

The second type of model states that endogenous calmodulin is indeed primarily cytoplasmic until cells are depolarised. Introduced fluorescent calmodulin, on the other hand, is at a higher level in the nucleus of even resting cells. Two factors may act to cause this phenomenon. First, it may be that endogenous calmodulin already occupies all the high affinity CaMBPs, and that these are located predominantly in the cytosol. Introduced calmodulin therefore cannot bind these cytosolic sites, and distributes according to the location of lower affinity sites, many of which would be nuclear. A recent paper by Liao et al. elegantly demonstrated, using a permeabilised cell assay, that FL-CaM entered the nucleus in a calcium dependent way (Liao, B. et al., 1999). Some translocation of FL-CaM occurred at calcium concentrations equivalent to resting intracellular calcium (100 nM) and translocation increased with the calcium concentration in a dose dependent way. The mechanism of FL-CaM translocation was not affected by chilling, wheat germ agglutinin or GTPyS and nuclear accumulation was blocked by the skMLCK M13 peptide, so FL-CaM translocation was not an active process and involved
binding to CaMBPs. Nuclear FL-CaM recovered from photobleaching with the same kinetics as a 20 kDa FL-dextran indicating that translocation was a first-order process. The rate of efflux of FL-CaM from the nucleus was reduced at high calcium concentrations but could be increased by adding cytosolic smMLCK. This could be taken as evidence that nuclear accumulation of calmodulin is determined by the subcellular distribution of CaBPs of different affinity.

My results injecting FL-CaM with excess unlabeled calmodulin support this idea: injection of a 15-fold excess of unlabelled calmodulin drove more FL-CaM of cytosolic (presumably high affinity) binding sites and increased the nucleus/cytosol ratio. When a 30-fold excess of unlabelled calmodulin was co-injected, competition for target binding for the (presumably lower affinity) nuclear sites increased and the nucleus/cytosol ratio is reduced.

If the distribution of FL-CaM over nucleus and cytosol is regulated by binding to CaBPs, than injection of FL-CaM with the Trp-peptide in my experiments, should have altered this distribution. Assuming that the intracellular concentration of FL-CaM after injection is 0.1-2.0 μM (see methods), the intracellular concentration of the Trp-peptide is calculated to have been in the range of 0.25-5.0 μM. It might be that this concentration was too low for the peptide to be an effective inhibitor in living neurones. The concentration that was used to inhibit nuclear envelope breakdown in sea urchin eggs was 100 microM and sea urchin eggs reportedly have a lower calmodulin content (20-50 microM (Török, K., Wilding, M., Groigno, L., Patel, R. and Whitaker, M., 1998)) than neurones have (10-100 microM (Kakiuchi, S. et al., 1982)). The rate constant for dissociation of the Trp-TA-CaM complex in vitro is 0.0082 s⁻¹, therefore, at too low a concentration of Trp, FL-CaM may also bind to other CaMBPs (Török, K. and Trentham, D.R., 1994). The binding of the Trp-peptide to calmodulin is calcium-dependent and it is possible that at resting intracellular calcium concentrations, FL-CaM would still bind to CaMBPs that bind calmodulin at low calcium concentration or in a calcium-independent way.
A second possible factor contributing to the mismatch between FL-CaM distribution and calmodulin immunoreactivity is that fluorescently labelled calmodulin may show a spectrum of affinity for the various CaMBPs that is different from that of native calmodulin, having a lower affinity for cytosolic CaMBPs but a higher affinity for nuclear CaMBPs. That addition of a fluorophore can alter the properties of calmodulin is demonstrated by the finding that TA-CaM has a $K_m$ for activation of phosphodiesterase that is 3.5 times that of unmodified calmodulin (Török, K. and Trentham, D.R., 1994) and in general the $K_d$ of unlabelled calmodulin is 0.4 times that of TA-CaM (Török, K. et al., 1998).

I therefore conclude that although injected calmodulins can give very useful information on stimulus-induced activation and translocation they should not be regarded as accurately indicating the spatial distribution of the cell's own calmodulin (Zimprich, F. et al., 1995).

However, although the intracellular localisations of FL-CaM and TA-CaM do not match that of endogenous calmodulin as reported by antibody, the distributions of the two fluorescent calmodulins are similar to each other (e.g. in patch-clamped cells nucleus/cytosol ratio = 1.84±0.42 and 1.52±0.24 respectively, difference not statistically significant). This indicates that the two fluorescent calmodulin analogues have a similar relative affinity for nuclear and cytosolic binding sites and therefore that FL-CaM can be used as a control to test for possible relocation artifacts in the TA-CaM signal. The absence of an increase in FL-CaM fluorescence in Fig.47b demonstrates that no significant relocation occurs over the 5 seconds following a brief voltage clamp depolarisation. This therefore gives me confidence that the marked increase in TA-CaM signal reflects a true activation of nuclear calmodulin by a 200 ms depolarisation. I therefore conclude that the conclusion of the 1995 paper on earlier research in the Bolsover laboratory 'Nuclear calmodulin responds rapidly to calcium influx at the plasmalemma' (Zimprich, F. et al., 1995), is correct.
CHAPTER 5: STEADY STATE CALCIUM ACTIVATION OF CALMODULIN.

5.1. INTRODUCTION

Although binding of calcium to calmodulin and of calcium:calmodulin to isolated target proteins has been widely investigated, little is known about the calcium loading of calmodulin and the extent of target binding inside living cells (Török, K. and Trentham, D.R., 1994; Van Eldik, L.J., Waterson, D.M., 1998).

Calmodulin binding proteins have been suggested to outnumber calmodulin by a factor of about two, implying that a lot of calmodulin will be bound to targets in a resting cell (Persechini, A. and Cronk, B., 1999). In fact, it has been previously estimated that the free calmodulin concentration in human embryonic kidney cells was 1/1000 of the total cellular content and less than 1/20 of the total calmodulin content of smooth muscle (Romoser, V.A. et al., 1997). It was estimated that in human embryonic kidney cells the maximum free calmodulin concentration is ~45 nM and is achieved at a calcium concentration of ~3 microM (Persechini, A. and Cronk, B., 1999).

There is remarkable range of calmodulin binding sites of different affinities available inside cells. Calmodulin binding proteins can roughly be divided into high (Kd<10 nM), intermediate (10<Kd<100 nM) and low affinity (Kd>100 nM) groups. High affinity calmodulin binding proteins include calcineurin, myosin light chain kinase, neuronal nitric oxide synthase (nNOS), phosphodiesterase and cyclic guanosine 5'-monophosphate (cGMP)-gated ion channels. Examples of intermediate affinity targets are CaMKIV, G-protein coupled receptor kinase 5, AKAP and synapsin. Low affinity targets include calmodulin dependent adenylate cyclases, G-protein coupled receptor kinase 2, spectrin, Gβγ and caldesmon (Persechini, A. and Cronk, B., 1999).

If subcellular differences in the distribution of calmodulin targets of different affinity existed, this would have implications for the local activation state of calmodulin. There
are indications that target proteins have specific subcellular distributions but for many quantitative studies into subcellular distribution have yet to be done. Table 5.1 gives an overview of the subcellular distribution of calmodulin binding proteins.

### Table 5.1. Relative subcellular distribution of calmodulin binding proteins.
Names in brackets indicate the location where the relative concentration of a protein is the lowest.

<table>
<thead>
<tr>
<th></th>
<th>membrane and synapses</th>
<th>cytosol and organelles</th>
<th>nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High affinity targets Kd &lt; 10 nM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI3K (16)</td>
<td>PI3K (16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>{MLCK (10,17)}</td>
<td>MLCK (11,17)</td>
<td>{MLCK (1, 10,17)}</td>
<td></td>
</tr>
<tr>
<td>nNOS (8,17)</td>
<td>nNOS (8,17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>phosphorylase kinase (9)</td>
<td>phosphorylase kinase (9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-VOCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAP-43 (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MARKS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>neurogranin (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>{PDE (13,17)}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cGMP gated ion channels (18)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Medium/Low affinity targets Kd &gt; 10 nM</strong></td>
<td>CaMKII (15)</td>
<td>CaMKIV (1,2,17)</td>
<td></td>
</tr>
<tr>
<td>{CaMKII}</td>
<td>CaMKII (15)</td>
<td>CaMKIV (1,2,17)</td>
<td></td>
</tr>
<tr>
<td>G-prot RK 5</td>
<td>CaMKII, CaMKIII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>synapsins (3,17)</td>
<td>synapsins (3,17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AKAP (17)</td>
<td>AKAP (17)</td>
<td>AKAP (17, 18)</td>
<td></td>
</tr>
<tr>
<td>AC1, III, VIII (17, 19)</td>
<td></td>
<td>AC1, III (17, 19)</td>
<td></td>
</tr>
<tr>
<td>G-prot RK 2 (17)</td>
<td></td>
<td>CaMKK</td>
<td></td>
</tr>
<tr>
<td>Gβγ</td>
<td>caldesmon</td>
<td>caldesmon (1,17)</td>
<td></td>
</tr>
<tr>
<td>alfa spectrin (2,20)</td>
<td>alfa-spectrin (20)</td>
<td>alfa-spectrin (20)</td>
<td></td>
</tr>
<tr>
<td>TRPL (3)</td>
<td>Ryanodine receptor</td>
<td>SPC 110 (2)</td>
<td></td>
</tr>
<tr>
<td>NAP-22 (6)</td>
<td>IP3 receptor</td>
<td>τ proteins (4)</td>
<td></td>
</tr>
<tr>
<td>GAP junction connexons</td>
<td></td>
<td>myosin I (5)</td>
<td></td>
</tr>
<tr>
<td>unconventional myosins</td>
<td></td>
<td>La/SSB (2)</td>
<td></td>
</tr>
<tr>
<td>pmCa$^{2+}$-ATPase</td>
<td>{HSP90 (2, 12)}</td>
<td>HSP90 (2, 12)</td>
<td></td>
</tr>
<tr>
<td>NMDA receptor</td>
<td></td>
<td>bHLH transcription factors</td>
<td></td>
</tr>
<tr>
<td>K$_{\text{Ca}^2+}$</td>
<td></td>
<td>(1,2,7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>hnRNP (1,2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p62 (1,4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>histone 1 (14)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p68 DNA replitase (1)</td>
<td></td>
</tr>
</tbody>
</table>

Although the exact contribution to the total of calmodulin binding sites of these proteins is not known, it is likely that there will be differences in the affinity of available binding sites in different subcellular locations. In this chapter I will use the activity of calmodulin as reported by TA-CaM as an indicator for steady state calmodulin binding at different subcellular locations in resting living cells.

5.2. RESULTS

To establish whether different degrees of calmodulin activation existed in compartments of living neurones, I micro-injected FL-CaM and TA-CaM together into rat dorsal root ganglion neurones. The injection mixture contained 0.33 mM FL-CaM and 0.67 mM TA-CaM. On average the total concentration of injected calmodulin was 4.67 +/- 1.01 microM (n=24) in the nucleus and 2.60 +/- 0.59 microM (n=24; p<0.001; paired t-test) in the cytosol. The nuclear volume comprises +/-4% of the total cell volume (2.2 microl versus 54.7 microl for the whole cell) and contains +/-7% of the total amount of injected calmodulin present in the cell.

Micro-injection of FL-CaM and TA-CaM, as I have shown in chapter 4, results in a similar distribution over nucleus and cytosol of both FL-CaM and TA-CaM. In these doubly injected cells, as in the cells injected with FL-CaM or TA-CaM, the nucleus was significantly brighter than the cytosol, this excess brightness being more noticeable with TA-CaM than with FL-CaM. However, the nucleus over cytosol fluorescence ratio is not significantly different between FL-CaM and TA-CaM (figure 5.1 FL-CaM 1.99 +/- 0.13 (n=24) versus 1.66 +/- 0.18 (n=24); p>0.05, paired Student's t-test).
Figure 5.1. Nucleus over cytosol fluorescence distribution.
Nucleus over cytosol fluorescence distribution of cells injected with a combination of FL-CaM 33 microM ($n=24$, open box) and TA-CaM 67 microM ($n=24$ striped box). The average fluorescence intensity was measured from the nuclear and cytosolic area of a confocal slice transsecting the nucleus of the cell.

Figure 5.2. Injected cells
A confocal slice through nucleus and cytosol of DRGNs injected with TA-CaM (blue) and FL-CaM (green). A transmitted light picture is shown at the bottom left. The bottom right picture shows the ratio of TA-CaM/FL-CaM after a pixel by pixel mathematical division of the TA-CaM and FL-CaM images. Image intensities were enhanced to the same extent, to visualise the fluorescence distribution.
Another way of presenting this phenomenon is to calculate the ratio of TA-CaM fluorescence to FL-CaM fluorescence in each cell region. The nuclear ratio of TA-CaM and FL-CaM fluorescence was 0.71 +/- 0.08 (n=24) whereas the cytosolic ratio was 0.99 +/- 0.13 (n=24). These values are significantly different (p<0.01; paired t-test; Figure 5.3).

![TA-CaM activation](image)

**Figure 5.3. Subcellular calmodulin activation**
The ratio of background corrected TA-CaM/FL-CaM fluorescence for the nuclear (open box) and cytosolic region (striped box).
I attempted to calibrate the TA-CaM/FL-CaM ratio with respect to the calcium concentration. I imaged drops of the injection solution containing 6.67 microM TA-CaM and 3.33 microM FL-CaM in injection buffer containing either 1 mM Bapta or 5 mM calcium. The ratio measured was 0.082+/-0.0019 (n=6) for calcium-free and 0.313+/-0.014 (n=6) in saturated calcium, significantly different with p<0.0001. This gives a dynamic range of 3.8.

5.3. DISCUSSION & CONCLUSIONS

FL-CaM and TA-CaM are labelled at the same amino acid (Lysine 75). It is therefore likely that the nuclear/cytosol equilibration of each is the same and the lower relative TA-CaM fluorescence in the nucleus is caused by a lower activation of TA-CaM.

FL-CaM and TA-CaM distribute throughout the cell depending on the pattern of calmodulin binding sites available. TA-CaM fluorescence reports the activation state (fluorescence of TA-CaM due to calcium and/or target binding) of calmodulin and FL-CaM just reports the distribution of labelled calmodulin. The activation state of calmodulin in different regions of the cell can be compared by determining the fluorescence ratio of TA-CaM / FL-CaM fluorescence. The significantly lower TA-CaM/FL-CaM ratio in the nucleus as compared to the cytosol therefore indicates that the degree of calmodulin activation is less in the nucleus.

The finding that the activation of fluorescent calmodulin in the nucleus of the cell is lower than in the cytosol is consistent with the view that at resting intracellular calcium concentrations, calmodulin will only be bound to high affinity targets. Resting intracellular calcium concentrations are 90.85+/-14.26 nM (n=38) in these cells in normal HBS.

High affinity targets (Kd<10 nM) include calcineurin, myosin light chain kinase, nNOS, phosphodiesterase and cGMP-gated ion (Persechini, A. and Cronk, B., 1999). Most of these high affinity targets are more abundant in the cytosol.

The nucleus contains more intermediate and low affinity calmodulin binding sites and consequently, less calmodulin will be bound to these at resting intracellular calcium
concentrations (CaMKIV [Bachs, 1994 #91(Agell, N. et al., 1998), AKAP (Coghlan, V.M. et al., 1994), adenylate cyclase (Parkinson, N.A., Bolsover, S.R., 2000) and τ-proteins (Uberti, D. et al., 1997). This view is consistent with my explanation of the discrepancy between the distribution of fluorescently labelled calmodulin and calmodulin immunoreactivity described in chapter 4. Here fluorescent calmodulin, that has a slightly lower affinity for target binding than endogenous calmodulin (Torok, K. and Trentham, D.R., 1994), is competed away from the high affinity binding sites by endogenous calmodulin and preferentially partitions into the nucleus, presumably binding to lower affinity binding sites.

Experiments on nuclear calmodulin accumulation by Liao et al., using permeabilised cells are consistent with this explanation (Liao, B. et al., 1999). Liao et al. found that the efflux rate of fluorescently labelled calmodulin from the nucleus decreased when calcium levels were elevated to 600 nM, indicating the existence of a significant pool of low affinity targets in the nucleus. In addition they found that increasing the amount of high affinity calmodulin binding sites outside the nucleus increased the nuclear efflux rate of fluorescent calmodulin, thus demonstrating that nuclear retention of calmodulin and the subcellular distribution of calmodulin in a resting cell are a consequence of the distribution of target proteins of different affinity. The distribution of calmodulin is therefore shifted when calcium levels change.

The calcium calibration data give a maximal TA-CaM/FL-CaM ratio of around 0.3, the ratios found in cells range from 0.7 to 1.0. It is possible that this is caused by the further fluorescence increase that is observed when TA-CaM is bound to targets. If this were the case, that would provide an extra argument to presume that the distribution of the TA-CaM/FL-CaM ratio in resting cells gives an indication of the distribution of calmodulin binding sites of different affinity.

My data using the TA-CaM/FL-CaM fluorescence ratio to assess the activation state of calmodulin in different cellular compartments shows good agreement with predictions of
the distribution of calmodulin targets of different affinity. Therefore, at resting intracellular calcium levels, the TA-CaM/FL-CaM can be interpreted as mainly reflecting the distribution of calmodulin binding sites of high affinity, more of which appear to be present in the cytosol.
CHAPTER 6: INTEGRATION OF CALCIUM SIGNALS BY CALMODULIN IN RAT SENSORY NEURONES.

6.1 INTRODUCTION

6.1.1 Target activation by calmodulin

Calmodulin confers calcium sensitivity to a wide range of target proteins. Targets, whose function is changed by calmodulin binding are diverse and involved in a wide array of cellular functions. Targets are summarised in table 5.1 and in neurones they include:

- glycogen phosphorylase kinase
- CaM kinases
- Calcineurin
- NMDA receptor
- metabotropic glutamate receptor
- ryanodine receptor
- inositoltrisphosphate receptor
- L-type and P/Q-type voltage operated calcium channels
- nNOS
- phosphodiesterase and adenylate cyclases
- pmCa$^{2+}$-ATPase.

To activate a specific target, several conditions need to be met: the local calcium concentration must be right to provide the appropriate calcium loading of calmodulin, free calmodulin must be present in a high enough concentration to bind to the target in an environment of competing CaMBPs and calmodulin must stay bound to the target for long enough to exert its activating effect.

6.1.2 Parameters of target activation

One of the parameters for activation of a specific set of targets is the local calmodulin concentration. In chapter 4, I discussed the mobility of calmodulin in neuronal cells and demonstrated that depolarisation of sensory neurones causes calmodulin to translocate to
the cell nucleus. Other work explored the translocation of calmodulin from the plasma membrane to the cytosol (Gnegy, M.E., 1993). The occurrence and extent of these translocations of calmodulin and the known differences in affinity of calmodulin targets can account for activation of different calmodulin targets in different sub-cellular compartments. However, this does not make the story complete, for the binding of calmodulin to a target and effective activation of a cellular process are also dependent on the duration of the permitting circumstances. Calmodulin is a mediator in longer term processes like the putative memory models long term potentiation and long term depression and several of the CaMBPs mentioned above (CaMKII, nNOS, calcineurin) are involved in these models (Ghosh, A. and Greenberg, M.E., 1995).

For some processes, a short transient activation of calmodulin and targets can be enough to have a long term effect, for example in the dephosphorylation of integrin β3 by calcineurin. When dephosphorylated, integrin β3 is not protected against proteolysis and is degraded (Pomies, P. et al., 1995). In other cases, like in the example of CaMKII described in chapter 1, a transient calcium:calmodulin signal is translated into long lasting kinase activity. A high local concentration of calcium:calmodulin is needed for the autophosphorylation and full activation of CaMKII. Autophosphorylation of this multimeric kinase is initiated by the binding of calcium:calmodulin to two adjacent subunits. The chance that this happens increases with the local concentration of calcium:calmodulin. Upon autophosphorylation the off-rate for calmodulin is dramatically reduced, effectively trapping calmodulin on the kinase and even when the calcium concentration has returned to baseline levels, the autophosphorylated kinase can retain ~60% of its activity for many minutes afterwards.

6.1.3. Calmodulin-target interaction

6.1.3.1. Steady-state interaction

Under steady state conditions at saturating calcium concentrations, calmodulin will be calcium-bound and bind to targets when they are present. This means that, due to the law
of mass action, the effective Kd of the calmodulin:target complex for calcium will decrease.

Binding of calcium to the reporter probe TA-CaM, as mentioned in chapter 2 and 4, leads to an up to five-fold increase in TA-CaM fluorescence. When calcium:TA-CaM binds to a target a further fluorescence increase of up to 100% can be observed, depending on the nature of target (Török, K. and Trentham, D.R., 1994).

6.1.3.2. Dynamic interaction

When the calcium concentration rises

Binding of calmodulin to target proteins is extremely rapid. Binding of calcium:TA-CaM to the Trp-peptide has a rate constant of \( \sim 8 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \) and is thought to be diffusion limited (Török, K. and Whitaker, M., 1994).

When calcium:calmodulin binds to some targets, as for example the N-acetylated Trp-peptide (corresponding to residues 797-813, the calmodulin binding domain of smooth muscle myosin light chain kinase), a slow isomerisation step takes place after the initial binding to the target (Török, K. and Trentham, D.R., 1994). This slow isomerisation could be responsible for the 200-400 ms lag phase between the rise in calcium concentration and contraction observed in smooth muscle (Török, K. and Whitaker, M., 1994).

When the calcium concentration falls

When the intracellular calcium concentration drops calcium:calmodulin loses its calcium. In stopped flow experiments this process can be monitored by mixing a fluorescent calcium indicator with calcium:calmodulin. The indicator will compete for calcium with calmodulin and the rate of fluorescence change of the indicator reflects the rate at which calmodulin loses its calcium. Calmodulin looses its calcium in a dual exponential process. The off rates for calmodulin have been determined as 400·s\(^{-1}\) for the low affinity N-terminal and 2.4·s\(^{-1}\) for the C-terminal calcium binding sites (Johnson, J.D. and Snyder, C.H., 1995). In the presence of the calmodulin binding peptides M13 and
RS-20 (corresponding to the calmodulin binding domain of skeletal muscle and smooth muscle MLCK, respectively) the off-rates for calcium are reduced to \( \sim 1.8 \cdot s^{-1} \) for calmodulin's N-terminus and \( \sim 0.1 \cdot s^{-1} \) for the C-terminus. This implies that target binding has an effect on the structure of the hydrophobic calcium binding pockets that results in large increases in the affinity for calcium. This is reflected in the reductions in the calcium dissociation rate.

By monitoring the Trp-fluorescence of the RS-20 peptide in the same experiments, the kinetics of the unbinding of the peptide from calmodulin could also be determined (Davis, J.P. et al., 1999). The rate of dissociation of the peptide tracked the dissociation of calcium from the N- and C-terminus at \( 2 \cdot s^{-1} \) and \( 0.1 \cdot s^{-1} \).

The off-rates for protein-calmodulin interactions depend not only on the rate at which calcium dissociates, but also on the presence of other CaBPs. In the presence of EGTA, the offrate for MLCK interaction with a reporter probe for target binding, MIANS-CaM, was \( 3.5 \cdot s^{-1} \). However, when caldesmon and calponin were also present, to reflect the competition encountered in a smooth muscle cell, the off rate was reduced to \( 0.031 \cdot s^{-1} \) (Davis, J.P. et al., 1999). When unlabelled calmodulin was used to compete the Trp-peptide off TA-CaM in the continuous presence of calcium, the off rate was lower at \( 0.0075 \cdot s^{-1} \) (Török, K. and Trentham, D.R., 1994).

When a brief calcium transient (0.6 ms half width) was applied to a mixture of MIANS-CaM and MLCK and the low affinity calcium indicator Furaptra was used to monitor changes in the calcium concentration, peak Furaptra fluorescence was reached 25 ms before the peak in the MIANS-CaM fluorescence. Furaptra fluorescence was already falling back to baseline before the MIANS-CaM peak was reached. It was estimated that \(-60\%\) of MIANS-CaM had associated with MLCK. MIANS-CaM fluorescence then slowly recovered back to baseline at a rate of \( \sim 1 \cdot s^{-1} \) (Davis, J.P. et al., 1999).

About 90% of the RS-20 Trp-fluorescence change after a brief (1.1 ms halfwidth) calcium transient accounted for dissociation from the N-terminal and the remaining 10% for dissociation from the C-terminal of calmodulin.
Since the affinity for calcium binding and the dissociation constant for calcium are increased when calmodulin binds a target and thus the inactivation of calmodulin is reduced, it is likely that, in a situation where there is competition with other CaMBPs, calmodulin:target complexes can outlast even very rapid calcium transients and calmodulin could function as an integrator of calcium signals.

6.1.4. Hysteresis by calmodulin

An indication that calmodulin activity outlasts a calcium signal comes from an in vitro study of the pmCa\(^{2+}\)-ATPase, where after full activation of the pmCa\(^{2+}\)-ATPase by calmodulin, activity was retained at calcium levels that did not show activity before; this phenomenon then went away at an intracellular calcium concentration below 1 microM (Scharff, O. and Foder, B., 1982). This is consistent with the calcium dynamics in red blood cells and shows that hysteresis occurs (Scharff, O. et al., 1983).

6.1.5. The study of calmodulin activation

The consequences of calmodulin activation have been the subject of many studies, however, little is known about the activation of calmodulin itself and what is known stems mainly from in vitro studies (see chapter two). Only a few tools are currently suitable to study calmodulin activation directly in living cells, all fluorescently labelled calmodulins. One, Merocam, has been used in fibroblasts and hippocampal neurones (Hahn, K. et al., 1992). An other, TA-CaM, has been used to study calmodulin activation in sea urchin oocytes, pancreatic acinar cells and Hela cells (Craske, M., Takeo, T., Török, K., Gerasimenko, O., Peterson, O.H. and Tepikin, A.V., 1998). However, none of these studies provides detailed insight in the kinetics of calmodulin activation in living cells and currently little is known about the mechanism of calmodulin activation on the cellular and sub-cellular level.
To identify the mechanisms by which this occurs, I have used TA-CaM as a tool to investigate subcellular activation of calmodulin and its relation to the intracellular calcium concentration and target binding.

Previous studies on calmodulin translocation used strong stimuli, depolarisation trains, high potassium depolarisations and continuous hormone treatment. However, in neurones, action potentials have a duration of only a few milliseconds. In the chapter 4, I have shown that a short depolarisation in neurones is sufficient to activate TA-CaM in the nucleus as well as cytosol of dorsal root ganglion neurones. Work by others has demonstrated that repetitive action potentials could lead to long term changes in these cells when repeated at critical intervals (Fields, R.D. et al., 1997). This suggest an integration process takes place under such circumstances and here I will investigate the possibility that calmodulin functions as one of the integrators.

6.2. RESULTS

6.2.1. Calmodulin activation in rat dorsal root ganglion neurones.

The use of neurones permits the induction of global calcium signals of a fixed duration by depolarising them for a limited period, allowing calcium influx via voltage operated calcium channels. DRGNs have L-, N-, P, Q, R- and T-types of voltage operated calcium channels (Acosta, C.G. and Lopez, H.S., 1999; Nakashima, Y.M. et al., 1998; Todorovic, S.M. and Lingle, C.J., 1998). When DRGNs are injected with TA-CaM and depolarised for 10 seconds with 68 mM potassium containing HBS, an increase in TA-CaM fluorescence can be observed (figure 6.1), that gradually declines to a plateau value, 26.62±4.83% (n=8) of the peak value. This plateau phase is maintained for tens of seconds even though the extracellular potassium concentration has returned to normal, suggesting that calmodulin may show hysteresis in intact neurones.
6.2.2. Kinetics of Calmodulin activation.

To investigate this delay in the recovery of the TA-CaM signal in more detail, I used the whole-cell patch-clamp technique. Under whole cell voltage-clamp a good control of the membrane voltage can be obtained and controlled depolarisation of a short duration can be imposed on the neurone. To enable me to study activation of TA-CaM with good spatial as well as temporal resolution, these experiments were performed on the stage of a confocal microscope. As a calcium indicator I used OGB5N, a low affinity calcium indicator (quoted kD 20 microM, Molecular Probes) that should track changes in the intracellular calcium concentration up into the high micromolar range in a linear fashion and not saturate easily in the physiological calcium range. In addition, OGB5N has a high quantum yield, so a limited concentration is sufficient to monitor the intracellular calcium concentration.

In response to a single short (200 ms) depolarisation from -70 to 0 mV, the fluorescence of OGB5N increases quickly and begins to fall immediately when the depolarisation terminates and calcium is removed from the cytosol (figure 6.2A).
Figure 6.1. Response to high $K^+$ of TA-CaM injected DRGN.
High $K^+$ was superfused during the period indicated by the black bar. Inset shows a transmitted light and a fluorescence image of the same cell on an epifluorescence microscope set-up. Intensity in arbitrary units.
Figure 6.2. Response to a single depolarisation.

A. A DRGN patch-loaded with TA-CaM and OGB5N was depolarised for 200 ms under whole-cell voltage clamp from -70 to 0 mV. The time course of the average fluorescence of TA-CaM and OGB5N of the whole confocal slice, normalised to the average baseline before depolarisation, is shown for a single cell. The recovery was well fit with a double exponential equation, although the recording is not long enough to allow an accurate determination of whether the second component is actually exponential. The inset shows the onset of the response in more detail.

B. The amplitude of the fitted slow component is expressed as a percentage of the total of the amplitude of the slow and fast component. The average of these slow amplitudes of independent depolarisations of n different cells is plotted for OGB5N and TA-CaM. * significantly different from OGB5N, p<0.05. Purple bar indicates depolarisation.
On average OGB5N fluorescence increased by 1.79 +/-0.13 fold (n=9; measured from the whole confocal slice), corresponding to an increase in intracellular calcium from an estimated 50 nM to 597.0 +/-38.4 nM. The majority of the recovery of the intracellular calcium concentration back to baseline can be fitted with a single exponential of $\tau = 1.12\pm0.13$ seconds (n=9). A small second component was observed with an amplitude of 13.81 +/-5.36% of the fluorescence peak (n=9) and a $\tau$ of 14.5 +/-5.89 s (n=9).

The kinetics of TA-CaM showed some interesting differences compared with those of OGB5N. Although OGB5N fluorescence began to fall as soon as the calcium influx terminated, TA-CaM fluorescence kept on increasing when OGB5N fluorescence had already begun to fall and peaked 0.74 +/-0.08 seconds (n=9) after the onset of depolarisation. TA-CaM fluorescence then fell exponentially with an initial $\tau$ that is not different from that of OGB5N (1.30 +/-0.17 seconds, n=9; paired t-test).

The second component of the recovery of the TA-CaM signal is slower than that of OGB5N (64.7 +/-12.93 versus 14.5 +/-5.89, n=9; paired t-test) and shows a plateau phase in the recovery that has an amplitude of 30.28 +/-6.64% of the peak fluorescence, significantly different from OGB5N (figure 6.2B; paired t-test). This is reminiscent of the amplitude in micro-injected cells and indeed not significantly different.

This plateau in the TA-CaM signal decays back to baseline eventually over a time course of minutes (Figure 6.3A). The slow plateau phase in the recovery of the TA-CaM signal indicates that indeed some hysteresis occurs. This being the case, then adequately spaced subsequent depolarisations should keep a fraction of TA-CaM continuously active. When DRGNs are depolarised five times at 2.5 second intervals, OGB5N fluorescence tracks the calcium peaks and almost completely recovers between depolarisations (Figure 6.3B). The TA-CaM trace, however, shows a smoothing of the signal: each subsequent depolarisation starts at an elevated level, on average 46.3 +/-8.3% (n=5) of the subsequent peak.
Figure 6.3. TA-plateau recovers in +/- 1 minute.
A. Long time-course of normalised average TA-CaM and OGB5N fluorescence of a whole confocal slice after a single voltage-clamp depolarisation (purple bar). B. Smoothing of the TA-CaM signal occurs during multiple depolarisations (purple bars) delivered at an interval of 2.5 s, whereas the transients in the OGB5N signal are largely independent.
**Figure 6.4. Furaptra response.**

**A.** Long time-course of normalised average TA-CaM and Furaptra fluorescence of an isolated cell on a photomultiplier set-up after a single voltage-clamp depolarisation (purple bar). Note that Furaptra fluorescence decreases when calcium levels increase. **B.** Smoothing of the TA-CaM signal occurs during multiple depolarisations (purple bars) delivered at an interval of 2.5 s, whereas the transients in the Furaptra signal are largely independent.
To make sure that the channel for collection of TA-CaM fluorescence was properly separated from the OGB5N channel and there was no bleedthrough from OGB5N fluorescence to the TA-CaM signal, I used a different low affinity calcium indicator, Furaptra, in combination with TA-CaM on an epifluorescence photomultiplier based set-up. I excited Furaptra at 360 nm and collected fluorescence 490<\lambda<650 \text{ nm}. Under these circumstances, Furaptra fluorescence falls upon calcium binding, so an increase in TA-CaM fluorescence is accompanied by a decrease in Furaptra fluorescence, thus ruling out possible cross contamination of two rising signals.

Using this system, depolarisation induces an increase of TA-CaM fluorescence and a decrease in Furaptra fluorescence (figure 6.4A). Like with OGB5N, TA-CaM fluorescence continued to rise when Furaptra fluorescence was already recovering back to baseline and also, like in the experiments with OGB5N before, depolarisations at a 2.5 second interval showed a smoothing of the TA-CaM response (figure 6.4B).

Autofluorescence excited in the range of TA-CaM excitation is mainly caused by mitochondrial NADH and it is possible that this autofluorescence changes with depolarisation and is picked up with the TA-CaM fluorescence (Schafer, B. et al., 1999). I therefore repeated the depolarisation experiment in the absence of TA-CaM, with only OGB5N in the pipette (figure 6.5). Under these circumstances, no detectable increase in fluorescence in the TA-CaM channel accompanied the fluorescence increase of OGB5N, so autofluorescence does not affect the TA-CaM response (n=4 cells).

In addition, the fast $\tau$, slow $\tau$ and relative amplitudes of the OGB5N signal were not significantly different from those of OGB5N in the presence of TA-CaM, so the amount of TA-CaM that diffuses into the cell does not dramatically alter the overall calcium buffering properties of these cells.
Calcium signal in absence of TA-CaM

Figure 6.5. Calcium response in the absence of TA-CaM.
Average fluorescence of a whole confocal slice during a patch-clamp depolarisation (purple bar) of a DRGN loaded with OGB5N in the absence of TA-CaM. The TA-CaM channel reports changes in the cells autofluorescence.

6.2.3. Sub-cellular calmodulin activation.

Calmodulin binding proteins show a specific cellular distribution with preferences for cell membrane, cytosol or nucleus (Gnegy, M.E., 1993). Membrane bound proteins include GAP43, neuromodulin and MARKS that bind calmodulin in the absence of calcium (Chakravarthy, B. et al., 1999). The pmCa\textsuperscript{2+}-ATPase, phosphodiesterase, nNOS and most adenylate cyclases are cytosolic proteins. Proteins like CaMKII and calcineurin can be nuclear, but are largely cytosolic, but CaMBPs like CaMKIV, a 68 kDa CaMBP associated with DNA polymerase alpha and alpha-spectrin are mainly nuclear (see tables 1.6 and 5.1). These CaMBPs have different affinities for calcium:calmodulin and thus can affect the activation level of calmodulin differently. Previously, I did not observe any differences in the peak activation of TA-CaM between nucleus and cytosol (Figure 4.7B),
however, there could be local heterogeneity in the TA-CaM activation, which would show up as 'hotspots' of TA-CaM activity in the confocal images.

To investigate whether any 'hotspots' of calmodulin activation occurred during the TA-CaM response, I compared single images of the plateau phase, normalised pixel by pixel by a pre depolarisation image (figure 6.6A). The first image in both the series for OGB5N and TA-CaM shows the rim of high calcium and calmodulin activation that occurs immediately upon depolarisation. Two subsequent images, taken during the plateau phase, are averages of two subsequent sets of ten images each covering 0.4 s, and show an irregular fluorescence pattern. Some of these 'activity spots' in the TA-CaM signal were consistent between subsequent images, others were not. Overall there appeared to be no consistency of location of these 'activity spots' during a plateau phase nor between plateau phases and they most likely arose from measurement noise. One interpretation of the plateau phase is that it represents TA-CaM within the calcium:calmodulin:target protein complexes that dissociate slowly, even when the intracellular calcium concentration is low.

The distribution of calmodulin target protein is likely to be different in the submembrane space and in the nucleus as compared to the bulk cytosol. I therefore examined whether the amplitude of the plateau differed in these three regions. I found no significant difference: in all three regions the plateau had an amplitude about 30% of the total peak TA-CaM fluorescence change (Figure 6.6B).

Figure 6.6. Geography of TA-CaM activation (figure on next page).
A. Confocal images of OGB5N and TA-CaM fluorescence during the peak, and two subsequent time points during the plateau-phase of a depolarisation response (blue trace at the top). The coloured bars at the top of the each image column correspond to the time points indicated by similarly coloured bars on the trace. Images were normalised pixel by pixel to a time averaged image of the fluorescence before depolarisation. The top row represents OGB5N fluorescence and the bottom row TA-CaM fluorescence. A false colour scale was applied to reveal localised hotspots of TA-CaM activity. The arrows indicate two pixels, one of which the fluorescence value changes during the plateau phase, and one that maintains the same fluorescence value. B. The amplitude of the slow component of a dual exponential fit to the data was expressed as a percentage of the sum of both the amplitudes of the fast and the slow phase. The average amplitudes of the areas representing the cell nucleus, the cytosol and a 2 micron area directly underneath the plasma membrane were compared.
Figure 6.6. Geography of TA-CaM activation (legend on previous page).
6.2.4. Extra calmodulin binding sites do not affect the plateau.

To test whether the plateau phase in the TA-CaM signal represents binding of TA-CaM to target proteins, I added calmodulin binding peptides to the pipette solution. The peptides I used were synthetic peptides with the sequence of the calmodulin binding domains of known CaMBPs.

The first peptide, the α1C peptide, corresponds to 20 amino acids in the IQ region of the L-type calcium channel α1c subunit (figure 6.7A) and binds calmodulin at all physiological calcium concentrations (Zuhlke, R.D. et al., 1999).

The second peptide corresponded to residues 797-813 of myosin light chain kinase (Trp-peptide, figure 6.7B) and was acetylated at its N-terminus. This peptide has picomolar affinity for calcium:calmodulin, so will bind very tightly to calmodulin when intracellular calcium levels are raised, but does not bind calmodulin in the absence of calcium (Torok, K. and Trentham, D.R., 1994).

When I included the α1c peptide at a pipette concentration twice that of TA-CaM, the amplitude and time course of the TA-CaM signal were not affected. In particular, the peak amplitude (Figure 6.8A; F/F0 = 2.71+/−0.28; n=3) and the relative amplitude of the plateau (Figure 6.9; 22.25+/−8.08%; n=5) were not significantly different from those for TA-CaM alone. The increase in the time constant of the plateau phase was not significant (Figure 6.10; τ = 151+/−55s; n=5).

When 112 microM of the Trp-peptide was included in the patch pipette with 100 microM TA-CaM, the peak fluorescence increase was significantly greater compared to TA-CaM alone (Fig 6.8A; F/F0 = 5.29+/−1.03; n=8). The amplitude of the slow phase was not different (Figure 6.9; 24.29+/−5.34%; n=8), however the τ of the slow phase was significantly higher (Figure 6.10; τ = 150+/−38s; n=8).
Figure 6.7. Calmodulin binding peptides.
An illustration of the amino acid sequence (single letter code) and working mechanism of two peptides corresponding to the calmodulin binding domain of target proteins were included in the patch-pipette: the Trp-peptide (MLCK calmodulin binding domain) that binds calmodulin at elevated calcium concentrations and the alfa1C peptide (L-type VOCC calmodulin binding domain) that binds calmodulin at all physiological calcium concentrations.
Figure 6.8. Peak fluorescence increase.
Peak fluorescence of n whole confocal slices (fold-increase over the average baseline), during a 200 ms voltage clamp depolarisation, +/-SEM. Peaks for OGB5N and TA-CaM were measured in the same cells, in the absence or presence of calmodulin binding peptides and compared using paired t-test. * significantly different, p<0.05.
Figure 6.9. Amplitude of slow component of fit.
Effects of adding calmodulin binding peptides on the average amplitude of the slow component of the fit expressed as a percentage of the total amplitude +/- SEM for n cells. * significantly different, p<0.05. No TA means no TA-CaM was present.

Figure 6.10. Time constant of slow component of fit.
Average time constant of the slow component of the fit in the absence or presence of calmodulin binding peptides. Significant differences * p<0.05; ** p<0.01.

The increase in peak amplitude and the tau of the slow component in the presence of the Trp-peptide are not a result of differences in the intracellular calcium concentration, because the average peak increase in OGB5N fluorescence is the same in the presence and absence of the peptides (Figure 6.8B; F/F0 = TA-alone 1.79+/-.013 {n=7}; TA + Trp 1.96+/-.025 {n=5}; TA + alpha1C 1.69+/-.019 {n=4}) and is also not different from experiments where TA-CaM was absent from the pipette solution (1.98+/-.026; n=4). Neither is the amplitude of the slow phase in the OGB5N signal in the presence of the Trp-peptide (8.5+/-.8%; n=8), different from the amplitude of OGB5N in the presence of TA-CaM alone (13.81+/-.5.36%; n=9) or in the absence (11.53+/-.4.8%; n=4) of TA-CaM.

In an attempt to directly compete TA-CaM off targets, I included excess unlabelled calmodulin in the patch pipette. Patching under these conditions had a very low success-
rate because such high amounts of protein in the pipette made it difficult to get good seals and often when the whole-cell configuration was achieved, the cell failed to load with calmodulin. Only in one case did I manage to get a reasonable loading in a well clamped cell. In this experiment the TA-CaM response recovers at the same rate as the OGB5N signal (figure 6.11), consistent with the hypothesis that indeed target binding is responsible for the plateau in the recovery phase.

6.2.5. TA-CaM response partially similar to high affinity calcium indicator.

The affinity of calmodulin:target protein complexes for calcium is usually higher than the calcium affinity of free calmodulin. For this reason TA-CaM bound to target would be expected to show calcium dependent fluorescence changes similar to those of a high affinity calcium indicator. To illustrate this phenomenon, I repeated the patch experiment, but using 400 microM Fura Red, in place of 100 microM TA-CaM, thus maintaining the same calcium buffering capability. At the wavelength used, Fura Red fluorescence falls when the calcium concentration rises. In response to a single depolarisation, Fura Red fluorescence decreased, reaching its minimum after the end of the depolarisation (Figure 6.12). The OGB5N fluorescence increase stopped as soon as the depolarisation terminated, indicating an accurate report of the calcium concentration in the confocal slice through the cell. Also note that when Fura Red fluorescence begins to recover, it only slowly recovers back towards baseline.

6.3. DISCUSSION & CONCLUSIONS


The OGB5N signal shows that this low-affinity indicator is indeed good at following the calcium transient: fluorescence increases rapidly upon depolarisation and starts to drop as soon as repolarisation occurs. The decline in calcium concentration after influx terminates has two components: a fast component that reduces calcium levels within seconds from a
~10-fold increase from baseline values, to about twice the baseline (1.08+/−0.05 times the baseline fluorescence value, corresponding to 94 nM calcium when assuming a resting level of 50 nM) and a slower phase during which the calcium concentration declines back to baseline in less than a minute.

**Figure 6.11.** Single depolarisation in excess unlabelled calmodulin.
The slow plateau in the recovery phase of the TA-CaM signal in response to a 200 ms depolarisation, is greatly reduced when 3 mM unlabelled calmodulin is included in the patch pipette (containing 100 microM TA-CaM).

**Figure 6.12.** Calcium indicators of different affinity.
The low affinity calcium indicator dye OGB5N is imaged together with the high affinity indicator Fura Red (note Fura Red fluorescence falls when calcium levels rise). When the depolarisation ends, the OGB5N signal starts to fall immediately, whereas the Fura Red signal continues to decrease and reaches its lowest level when the intracellular calcium concentration is actually falling. The inset shows the calcium peak of same experiment in more detail.

The rapid clearance of calcium during the fast component of the recovery is probably due to buffering by calcium binding proteins, uptake by mitochondria and extrusion via the
sodium/calcium-exchanger, whereas the slow component is probably mainly due to action of the pmCa\textsuperscript{2+}-ATPase (Werth, J.L. et al., 1996).

The calcium signal travels as a wave, from a high ‘rim’ of calcium directly underneath the membrane upon depolarisation, further into the cell interior and when influx is terminated, the high calcium ‘rim’ collapses into the cytosol and enters the nucleus of the cell.

6.3.2. The calmodulin response

Subcellular mobility of calmodulin has been suggested as a means to create localised calmodulin activity where needed, as is the case with the translocation of calmodulin to the cell nucleus as observed by Deisseroth (Deisseroth, K. et al., 1998). In chapter 4, however, I have shown that this translocation does not occur over short time periods after a brief depolarisation. This is consistent with a timecourse of minutes reported for nuclear calmodulin translocation by Deisseroth et al. and Craske et al. (Deisseroth, K. et al., 1998). The spatial geometry of the TA-CaM signal resembles the OGB5N signal in that a rim of high activity spreads into the cytosol and reaches 50% of peak level in the centre of the cell after 0.37±0.08 seconds (n=8), not different from OGB5N (0.30±0.11 seconds (n=8). These similarly fast propagation rates indicate, that calcium, rather than calmodulin itself, diffuses into the cell interior and into the cell nucleus, to then activate nuclear calmodulin directly.

6.3.3. Integration of calcium transients by calmodulin

TA-CaM reports calcium binding with an up to five fold increase in fluorescence. Subsequent binding of target proteins can result in a further fluorescence increase of up to 100%, depending on the nature of the target. In my experiments, I cannot distinguish the relative contributions of these two phenomena to the observed increase in
fluorescence and I will therefore refer to an increase in TA-CaM fluorescence as indicating calmodulin activation.

The calmodulin response shows some striking differences to the OGB5N response, indicating that TA-CaM does not just track the calcium response.

First of all, the TA-CaM signal reaches its peak 0.30 +/- 0.07 seconds (n=9) after the peak in the calcium signal. This phenomenon is similar to what happens when the high affinity calcium indicator Fura Red is used: immediately upon depolarisation, indicator directly under the membrane is saturated by the high local concentration of calcium. Even though calcium influx stops after 200 ms, calcium from this high concentration region diffuses into the cytosol and more indicator dye reports calcium binding. At this time, although submembrane calcium levels are falling, the submembrane indicator is still calcium saturated, therefore the global indicator signal will report an increasing calcium concentration until global calcium reaches a level at which the saturated indicator start losing calcium, then the global signal will drop.

Low affinity indicators like OGB5N and Furaptra do not show this phenomenon, because they are not saturated at any location and will accurately report a (submembrane) fluorescence decrease as soon as the calcium concentration starts to fall. This phenomenon ensures increasing global TA-CaM activation when calcium levels are actually dropping and TA-CaM activity thus can act as an integrator of calcium signals.

Secondly, the TA-CaM signal shows a slowly declining plateau phase during the recovery, when the intracellular calcium concentration has returned back to baseline levels. Because the decline in the TA-CaM signal during this plateau phase is slow (τ is 64.7 ± 12.9 s; n=9) and the endpoint is not known, an accurate determination of whether the slow phase is exponential is not possible. However, I can test the null hypothesis that the plateau in the TA-CaM signal has the same τ as the OGB5N signal (14.5 ± 0.2 s; n=9) by fitting an exponential to the slow phase. These time constants are indeed different at high significance (p<0.01) and calmodulin activity can thus outlast the calcium signal that caused it. This phenomenon shows that calmodulin functions as a real
integrator of calcium transients: as long as the interval between calcium transients is less than the minute or so it takes for TA-CaM activity to return to baseline, TA-CaM will show some continuous activity. This is illustrated by the smoothening of the TA-CaM signal when depolarisations are delivered at a 2.5 second interval.

My hypothesis for explaining this slow recovery of calmodulin activity is that calmodulin has bound to target proteins during the calcium transient. From test tube experiments it is known that this causes a decrease in the off rate of calcium from calmodulin:target protein complexes (Johnson, J.D. and Snyder, C.H., 1995). In fact, the dissociation of the whole complex is slow even when the calcium concentration has returned to baseline levels (Scharff, O. and Foder, B., 1982).

In stop-flow experiments it was shown that including only two more target proteins could reduce the off rate of the calmodulin:MLCK complex 10-fold (Davis, J.P. et al., 1999). In cells, where, in contrast to the test tube environment, a whole array of calmodulin binding proteins is present, I would expect this phenomenon to be more prominent.

When I included the Trp-peptide in the patch-pipette the recovery during the plateau phase was significantly slower, with a $\tau$ of $150+/-38$ s (n=8) compared to $64.7+/-12.9$ s (n=9) in the absence of the Trp-peptide. The $\tau$ value in the presence of the Trp-petide agrees with the $\tau$ for the unbinding of the Trp-peptide:TA-CaM complex in vitro ($\tau = 1$/rate constant $=121$s (Török, K. and Trentham, D.R., 1994)). In addition, the peak increase in TA-CaM fluorescence was significantly increased by the presence of the Trp-peptide. This is a phenomenon that also occurs when calcium:TA-CaM and the Trp-peptide are mixed in stopped flow experiments and confirms that the peptide binds to TA-CaM (Török, K. and Trentham, D.R., 1994).

The L-channel α1C peptide did not change any of the fit parameters compared with experiments in the absence of peptide. First of all, the extent of a further increase in peak TA-CaM fluorescence when a peptide binds calcium:TA-CaM depends on the identity of the peptide and varies from less than 10 to 100% (Török, K. and Trentham, D.R., 1994).
The binding mode of calmodulin to the α1C calcium channel subunit, being bound to a target at resting intracellular calcium concentrations and showing a further interaction when the concentration rises is not well understood. During the plateau phase there was too much variability between responses to accurately determine whether the τ of the slow phase was indeed different from τ in the absence of peptide. This variability might be caused by an effect of the peptide on the calcium channel itself since the variability in the amplitude of the slow component was also increased.

That the slow plateau phase was caused by interaction of calmodulin with target proteins was further confirmed by the absence of the plateau when an excess of unlabelled calmodulin was included in the patch-pipette to compete with TA-CaM for target binding.

Two scenarios are possible for the unbinding of the calcium:calmodulin:target protein complex (Brown, S.E. et al., 1997).

In scenario one, the complex first loses calcium rapidly before calmodulin and the target protein slowly dissociate.

In scenario two, calcium:calmodulin would first slowly dissociate from the target protein and then the calcium:calmodulin complex would rapidly unbind.

Experiments with calmodulin binding peptides have demonstrated that in vitro the first scenario is most prominent. My experiments are consistent with the same route being the most important one in neurones.

During the fast component of the recovery phase both calcium:TA-CaM, bound and unbound, would lose its calcium when calcium levels drop. Because the calcium concentration in cells drops more slowly than in a stop-flow experiment in a test tube, where EGTA can rapidly pull the calcium of the complex, any effects of peptides on this phase in living cells would be masked by the rate-limiting decrease of the calcium. However, during the slower plateau phase, when the calcium concentration has fallen significantly the slowing of the unbinding of the TA-CaM:target protein complex would
be expected to be more prominent, which is what I observed when I added extra target in the form of the Trp-peptide.

Since TA-CaM appears to be a particularly good reporter for interactions with the high affinity calcium binding site, effects of target binding on the off rate from calcium would be expected during the slow phase of the recovery.

If scenario two would be predominant, I would expect the slow unbinding of the calcium:TA-CaM complex to increase the amplitude of the second component. The fast phase would reflect free TA-CaM losing its calcium. When extra target was provided in the form of the Trp-or α1C peptide, however, this increase in amplitude of the slow component was absent and this scenario seems not to play a major role in neurones.

6.3.4. Are there any localised hotspots in calmodulin activity during the plateau?

As mentioned before, calmodulin binding proteins show some differences in subcellular localisation, therefore, the global plateau phase could be caused by some localised calmodulin activity, possibly associated with the membrane, nucleus or organelles. Analysis of individual pictures during the plateau phase showed a patchy pattern of TA-CaM activity. I could, however, not detect any consistency in the location of these 'hotspots', either during or between depolarisations; it was just as easy to find a pixel that would change as its was to find one that would remain high. Its is therefore most likely that either hotspots did not occur or they where below the detection level of this system and disappeared in the measurement noise. To reduce the contribution of noise, I compared the averaged fluorescence in the first 2 microm directly underneath the membrane, an area in the cytosol and the nucleus of the cell, but this, too, failed to reveal any differences in the plateau between these regions. Considering the wealth of calmodulin binding proteins and their diffuse distribution, it is not surprising than I could not find any consistent ‘hotspots’. If they would at all be detectable in a background of calmodulin, then a system with a higher spatial resolution (without loss in the time resolution) should be used.
6.3.5. Calmodulin as a memory molecule

Calcium signals in nerve cells are mostly associated with action potential activity of millisecond duration, still they have been implicated in many cellular processes that involve long term changes and gene expression and it has been shown that repetitive calcium transients can bring about these changes (Fields, R.D. et al., 1997). Calmodulin is one of the players that team up to integrate calcium signals and does so by binding to downstream target proteins. The effect of this binding is an increase in the affinity of calmodulin for calcium and this change in affinity seems a likely mechanism for the integrative properties of calmodulin. Calmodulin can thus remain active after calcium signals disappear and, when calcium transients are repeated at a sufficiently high frequency, maintain an increased level of activity, ultimately resulting in a 'memory of activity', converting a brief calcium signal into a lasting biochemical change.
CHAPTER 7. GENERAL DISCUSSION & CONCLUSIONS

The study of calmodulin function has classically been performed either as protein-protein interactions in cell-free systems or using immunocytochemistry on fixed tissue. Both methods have the disadvantage that dynamic subcellular events can only be studied with a limited time resolution.

In recent years it has become increasingly clear that inside cells, as in inter-human relations, it matters very much to be in the right place at the right time and to know the right people. In cells this concept applies to proteins like calmodulin that are concentrated in specific subcellular locations and can change location when the intracellular calcium concentration changes. Calmodulin interacts with a limited number of target proteins and the individual activation of these depends on local availability of calcium and calmodulin. These interactions can be very fast and to understand their dynamics high resolution techniques are needed that enable the study of calmodulin in living cells.

7.1. USING PHARMACOLOGICAL CALMODULIN INHIBITORS

Calmodulin inhibitors are an obvious choice when one wants to investigate the dynamic modulation by calmodulin of cellular processes. My work in this thesis emphasises that great care should be taken when employing these tools. Even inhibitors that are considered specific and potent can affect the cells in unanticipated ways. Calmidazolium at commonly used concentrations causes increases of the intracellular calcium concentration that appear to be unrelated to inhibition of calmodulin and are caused by indirectly opening nifedipine sensitive voltage operated calcium channels. A probable cause for this is the depolarisation of the cell membrane by calmidazolium, possibly by acting on potassium channels. When cellular parameters, like for example the membrane potential, are not monitored during experiments with calmodulin inhibitors, there is a risk of misinterpreting the obtained data.
7.2. SUBCELLULAR CALMODULIN LOCALISATION

Fluorescently labelled calmodulins have the advantage over pharmacological tools that they allow the specific study of calmodulin function in living cells.

Using micro-injection to introduce fluorescently labelled calmodulins into rat sensory neurones, I found that fluorescently labelled calmodulins preferentially locate to the cell nucleus.

This finding is in line with other reports on the localisation of micro-injected fluorescently labelled calmodulin in rat hippocampal neurones (Giles Hardingham, Hilmar Bading lab, Cambridge, personal communication), electroporation of fluorescent calmodulin into rat dorsal root ganglion neurones (Jack Horne, David Ogden lab, Mill Hill, personal communication) and pancreatic acinar cells patch-loaded with FL-CaM or TA-CaM (Craske, M., Takeo,T., Török, K., Gerasimenko, O., Peterson, O.H. and Tepikin, A.V., 1998). In sea urchin embryos and permeabilised A7R5 cells fluorescence of labelled calmodulin was also highest in the nucleus (Liao, B. et al., 1999; Wilding, M. et al., 1995).

Craske et al. and Deisseroth et al. reported translocation of calmodulin immunoreactivity to the cell nucleus of pancreatic acinar cells and hippocampal neurones, after an increase in the intracellular calcium concentration, confirming my finding in dorsal root ganglion cells (Craske, M., Takeo,T., Török, K., Gerasimenko, O., Peterson, O.H. and Tepikin, A.V., 1998; Deisseroth, K. et al., 1998).

Both Deisseroth and Craske mention that endogenous calmodulin and FL-CaM leave the nucleus again, minutes after stimulation. However, stimulus-induced translocation of FL-CaM was not observed after micro-injection in hippocampal neurones (Hardingham) or electroporation into DRGNs (Jack Horne), in their experiments FL-CaM seemed to translocate and stay in the nucleus regardless whether or not a stimulus was applied.
In permeabilised cell systems as used by Liao et al., the distribution of FL-CaM depended on the distribution of high affinity CaM binding sites (Liao, B. et al., 1999). My results on steady state activation of calmodulin show that activation of calmodulin in resting cells is lowest in the nucleus. This suggests that the subcellular distribution of targets with a different affinity for calmodulin could be an important determinant of subcellular CaM activation and thus could dictate where CaM sensitive processes are activated in a cell at a given calcium concentration.

It proved to be important in Liao’s experiments with permeabilised cells to wash away excess, unbound FL-CaM in order to monitor FL-CaM translocation. This provides a possible explanation why activity induced FL-CaM translocation was not observed by Hardingham or Horne. Maybe this reflected a population of unbound FL-CaM that was in dynamic equilibrium with the bound FL-CaM and thus mimicked the distribution of the high and low affinity binding sites. This would be in line with my finding that co-injection of a 15 fold excess of unlabelled CaM causes an increased nuclear translocation of FL-CaM due to increased competition for cytosolic binding sites, whereas a 30 fold excess of unlabelled calmodulin resulted in a distribution similar to that of injected FL-CaM alone.

On the other hand Craske et al. could see translocation even when concentrations of calmodulin were added that reached the level of the endogenous calmodulin concentration (Craske, M., Takeo,T., Torok, K., Gerasimenko, O., Peterson, O.H. and Tepikin, A.V., 1998). The explanation here could well lie in differences between the cell types used. Liver cells, for example contain a high concentration of calmodulin because of its role in their high metabolic activity and continuous cell division. Unknown differences in the identity and quantity of target proteins in subcellular locations between cells mean that one cell type might well show a more pronounced translocation than another and direct conclusions cannot be easily drawn from this.

To understand the subcellular distribution of calmodulin, the relative subcellular concentrations of different target proteins needs to be known and calmodulin localisation should be studied with improved spatial and temporal resolution.
To summarise, a model for the activity dependent subcellular redistribution of calmodulin is shown in figure 7.1. At resting intracellular calcium concentrations, most of the cellular calmodulin is bound to high affinity targets, more of which are possibly found in the cytosol. When the intracellular calcium concentration increases, calcium:calmodulin binds to lower affinity targets, more of which could be found in the nucleus. This redistribution of calmodulin would position calmodulin in a favourable position to enhance gene expression and translocation of calmodulin could work as an integrating step in signal transduction when calcium transients are frequent enough to cause a continuous increase in the nuclear calmodulin concentration during intermittent stimulation (Craske, M., Takeo, T., Török, K., Gerasimenko, O., Peterson, O.H. and Tepikin, A.V., 1998).

In general, when using fluorescently labelled calmodulins and proteins, great care must be taken with the interpretation of the data. When the endogenous protein is present in the cell, the behaviour of the fluorescent probes might not be the same as that of the endogenous equivalent, because the fluorescent label might interfere with target binding and change the parameters of calmodulin: target interaction. This is for example the case for the interaction of TA-CaM and MLCK, where TA-CaM inhibits MLCK, whereas unlabelled calmodulin stimulates it (Török, K. and Trentham, D.R., 1994). In addition the behaviour of the labelled protein will be affected by calmodulin already present. This is especially important when the affinity of labelled calmodulin for various targets is different for that of endogenous calmodulin. This could lead to a representation of a only subset of binding sites by the labelled calmodulin. It is for example possible that the nuclear translocation of endogenous calmodulin increases the rate at which FL-CaM leaves the nucleus by increasing the competition for nuclear binding sites.
It seems crucial when investigating the subcellular localisation of calmodulin in living cells, to also monitor the localisation of endogenous calmodulin. Ideally one would replace the existing cellular calmodulin with fluorescent calmodulin, for example by expressing GFP-calmodulin chimeras. GFP-calmodulin can functionally substitute for endogenous calmodulin in yeast (Moser, M.J. et al., 1997) indicating that the labelling with GFP might not prove as intrusive as chemical labelling. The main advantage would be to be able to study the dynamic regulation of the concentration as well as the dynamic translocation of calmodulin, without damaging the cells in any way or altering the total calcium buffering capacity of the cell.

7.3. SUBCELLULAR CALMODULIN ACTIVATION

When TA-CaM is used together with FL-CaM, FL-CaM reports localised concentration differences that should also apply to the TA-CaM fluorescence distribution. By interpreting the ratio of the TA-CaM and FL-CaM fluorescence signals, the extent of
subcellular calmodulin activation can be determined. In resting cells this would represent
the degree to which calmodulin is bound to target proteins. It appears that in the cell
nucleus this ratio is lower than in the cytosol and therefore that less calmodulin is bound
in the cell’s nucleus. This finding agrees with the idea that less high affinity calmodulin
binding sites are present in the cell nucleus.

When patch-loaded dorsal root ganglion neurones are depolarised, no FL-CaM
translocation can be observed in the first 40 s after a 200 ms depolarisation. This means
that the TA-CaM signal during this time can be interpreted as representing calmodulin
activation. This makes it possible to study calmodulin activation in a living neurone, in
the presence of a plethora of different calmodulin binding proteins.

Following a 200 ms depolarisation, TA-CaM activation keeps on increasing when the
actual intracellular calcium concentration has already started to fall. In addition, while the
intracellular calcium concentration declines quickly to baseline, the TA-CaM signal
recovers to a slowly declining plateau that outlasts the calcium signal. This slowly
decaying plateau in the TA-CaM signal is likely to be caused by the binding of TA-CaM
to target proteins. When extra target, in the form of calmodulin binding peptide is
included in the patch-pipette, the τ of this slowly declining plateau is increased and when
excess unlabelled calmodulin is included, the plateau is reduced. The slow phase in the
recovery of the calmodulin signal is due to the slow dissociation of calcium from
calmodulin’s high affinity C-terminal calcium binding sites. From stop-flow experiments it
is known that dissociation of calcium from calmodulin’s C-terminal calcium binding sites
is significantly slower when calmodulin has bound to target protein. In a model best
describing the events underlying these kinetics (figure 7.2), calcium would dissociate
from the low affinity calcium binding sites on calmodulins N-terminus. The N-terminus
would subsequently dissociate from the target protein. This is represented by the fast
component of the decay in TA-CaM fluorescence. Binding to target protein by
calcium:calmodulin slows this reaction down, but this cannot be distinguished in my
experiments because the intracellular calcium concentration falls slowly in cells. During
the slower second phase of the decay in TA-CaM fluorescence, calcium now dissociates
from calmodulin’s high affinity C-terminal calcium binding sites and subsequently also dissociates from the target protein. Because this process is slower than the N-terminal dissociation, a slowing of the decline in fluorescence due to target binding can be observed when extra target is provided in the form of Trp-peptide.

The finding that calmodulin activity itself can outlast calcium signals, means that calmodulin can function as an integrator of calcium signals and thus forms component of the cellular mechanism that provides a memory of cellular activity.

**Figure 7.2. Recovery of TA-CaM activation after depolarisation.** Calmodulin’s globular domains are represented in green, a target protein as a red rod, and calcium as blue spheres. The blue trace is TA-CaM fluorescence, the green trace OGB5N fluorescence. For explanation see text.
8. BIBLIOGRAPHY


Barrington, M., et al. (1994) Trifluoperazine and calmidazolium have multiple actions on the release of noradrenaline from sympathetic nerves of mouse atri. Naunyn-Schmiedebergs-Arch-Pharmacol 349, 133-139.


Ehlers, M. D., et al. (1996) Inactivation of NMDA receptors by direct interaction...
of calmodulin with the NR1 subunit. Cell 84, 745-755.


Luby Phelps, K., et al. (1985) Behavior of a fluorescent analogue of calmodulin in


Shen, K., et al. (1999) Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. Science 284, 162-166.


Smith, J. S., et al. (1989) Calmodulin modulation of single sarcoplasmic
reticulum Ca2+-release channels from cardiac and skeletal muscle. Circ-Res 64, 352-359.


Török, K., et al. (1994) Taking a long, hard look at calmodulin's warm embrace
[see comments]. Bioessays 16, 221-224.


9. APPENDIX

The equations for the calculation of the intracellular calcium concentration using Furaptra (2.7.4. page 90) were derived from the standard formula for the Kd, assuming the resting calcium concentration to be 50 nM.

\[ K_d = \frac{Ca^{2+} \times B}{Ca^{2+} \times B + Ca^{2+}} \]

(\( T = \text{total furaptra} \))

\[ K_d(T-B) = Ca^{2+} \times B \]

\[ B = \frac{K_d(T-B)}{[Ca^{2+}]} \]

\[ B \times Ca^{2+} = K_d \times T - K_d \times B \]

\[ B \times Ca^{2+} + K_d \times B = K_d \times T \]

\[ B (Ca^{2+} + K_d) = K_d \times T \]

\[ B = \frac{K_d \times T}{Ca^{2+} + K_d} \]

Furaptra fluorescence is proportional to the concentration of unbound Furaptra. Thus:

\[ Ft = \frac{K_d}{Ct + K_d} \times F_{\text{zero calcium}} \]
When the calcium concentration in a resting cell is assumed to be at a level of 50 nM then the fluorescence value measured in resting cells \((F_{\text{resting Ca}^2+})\) can be used to calculate the fluorescence at zero calcium \((F_{\text{zero calcium}})\).

\[
F_{\text{resting calcium}} = \frac{K_d}{K_d + Cr} \times F_{\text{zero calcium}}
\]

From this it follows that:

\[
F_{\text{zero calcium}} = \frac{Cr + K_d}{K_d} \times F_{\text{resting calcium}}
\]

2. With \(F_{\text{zero calcium}}\) known, the calcium concentration at a given time can now be calculated as:

\[
F_t = \frac{K_d}{C_t + K_d} \times F_{\text{zero calcium}}
\]

\[
F_t (C_t + K_d) = K_d \times F_{\text{zero calcium}}
\]

\[
C_t + K_d = \frac{K_d \times F_{\text{zero calcium}}}{F_t}
\]

\[
C_t = \frac{K_d \times F_{\text{zero calcium}}}{F_t} - K_d
\]

Where:

\(K_d\) = dissociation constant
\(Ca^{2+}\) = calcium concentration
\(B\) = the free Furaptra concentration
\[ T = \text{the total furaptra concentration} \]
\[ F_{\text{zero calcium}} = \text{the fluorescence of the indicator in the absence of calcium} \]
\[ F_{\text{resting calcium}} = \text{the fluorescence of the indicator at resting calcium} \]
\[ F_t = \text{the fluorescence at a given time} \]
\[ C_t = \text{the calcium concentration at a given time} \]
\[ C_r = \text{the calcium concentration in resting cells} \]