REGULATION OF CALCIUM
AND POTASSIUM CHANNELS
IN VISCERAL SMOOTH
MUSCLES

By

Gregory Raymond Wade

Graduate Programme
In
Physiology

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University Of Western Ontario
London, Ontario
April 1999

© GREGORY R. WADE 1999
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.
ABSTRACT

These studies were undertaken to investigate the roles of intracellular Ca\textsuperscript{2+} stores in the regulation of membrane currents, and the participation of currents in excitation contraction coupling in visceral smooth muscles. Macroscopic currents were recorded using the nystatin perforated-patch technique in single cells freshly isolated from gastric (guinea-pig), tracheal (guinea-pig) and esophageal (human) smooth muscles. Single channels were recorded in the cell-attached patch configuration.

Macroscopic L-type Ca\textsuperscript{2+} channels were characterized by their voltage-dependence of activation and inactivation and pharmacological sensitivity in gastric smooth muscle. Depolarization of cells caused inward Ca\textsuperscript{2+} currents and contraction consistent with Ca\textsuperscript{2+} entry activating the contractile apparatus of these cells. Acetylcholine (ACh) caused contraction of cells both in the presence and absence of extracellular Ca\textsuperscript{2+} and when the membrane potential of cells was clamped at negative potentials consistent with ACh-mediated contraction due to release of Ca\textsuperscript{2+} from intracellular stores. ACh acutely inhibited Ca\textsuperscript{2+} currents and single Ca\textsuperscript{2+} channels with depletion of intracellular Ca\textsuperscript{2+} stores abolishing this effect, consistent with a requirement for Ca\textsuperscript{2+} stores to mediate the effects of ACh.

Large conductance Ca\textsuperscript{2+}-dependent K\textsuperscript{+} (\textit{K}\textsubscript{Ca}) channels were identified by their voltage-dependence, large unitary conductance, dependence of the reversal potential on the [K\textsuperscript{+}] gradient, and opening of channels following elevation of [Ca\textsuperscript{2+}]\textsubscript{i} in dog and guinea-pig tracheal smooth muscles. ACh and caffeine caused contraction of cells and activation \textit{K}\textsubscript{Ca} channels. The activation of \textit{K}\textsubscript{Ca} channels may serve to limit excitation and contribute to recovery of smooth muscle.
Depolarization of human esophageal cells activated a transient delayed rectifier (K\textsubscript{V}) and a non-inactivating Ca\textsuperscript{2+}-dependent K\textsuperscript{+} (K\textsubscript{Ca}) current. K\textsubscript{V} current exhibited voltage-dependent activation and inactivation and was blocked by 4-aminopyridine. K\textsubscript{Ca} current was active at voltages more positive than 0 mV, blocked by tetraethylammonium, iberiotoxin and charybdotoxin. Messenger RNA encoding the gene products of Kv1.2 and Kv1.5 were identified by RT-PCR. 4-AP increased resting tension of intact muscle strips, while TEA, augmented the amplitude and duration of electrically evoked contraction. Thus, K\textsuperscript{+} channels serve distinct roles regulating contraction of esophageal smooth muscle.

KEYWORDS

Esophagus, trachea and gastric, calcium-dependent K\textsuperscript{+}, L-type Ca\textsuperscript{2+} channels, delayed rectifier K\textsuperscript{+} channels, intracellular Ca\textsuperscript{2+} stores, acetylcholine, caffeine, contraction
Sections from Chapter 1 have been adapted from a review co-written by Stephen Sims and Greg Wade; Sims, S. M. & Wade, G.R. (In Press). Inward Currents in Smooth Muscle. In Smooth Muscle in Health and Disease. Edited by Lloyd Barr & C. Ladd Prosser. Published by JAI Press, Greenwich, Connecticut.

Chapter 2 is adapted from; Wade, G. R., Barbera, J., & Sims, S. M. (1996). Cholinergic inhibition of Ca\(^{2+}\) channels in guinea-pig gastric and tracheal smooth muscle. Journal of Physiology 491, 307-319. Reproduced here with permission (see APPENDIX I). G. Wade designed experiments, recorded and analyzed whole-cell currents and cell length and prepared the figures. Based upon G. Wade's findings, a summer student, J. Barbera recorded and analyzed single Ca\(^{2+}\) channels. All experiments were carried out within S. Sims' laboratory. S. Sims co-wrote the publication with G. Wade.

Chapter 3 is adapted from; Wade, G.R. & Sims, S.M. (1993). Muscarinic stimulation of tracheal smooth muscle cells activates the large conductance Ca\(^{2+}\)-dependent K\(^+\) channel. American Journal of Physiology 265, C658-C665. Reproduced here with permission (see APPENDIX 2). Experimental design, current recordings and data analysis and preparation of figures were carried out by G. Wade in the lab of S. Sims. G. Wade and S. Sims co-wrote the publication.

Chapter 4 has been adapted from a manuscript submitted for publication; Wade, G.R., Laurier, L.G., Preiksaitis, H.G. & Sims, S.M. (Submitted). Roles Of Delayed Rectifier And Ca\(^{2+}\)-Dependent K\(^+\) Currents In Regulating Contraction Of Human Esophageal Smooth Muscle. G. Wade recorded and analyzed all electrophysiological studies, prepared
the figures and co-wrote the manuscript with S. Sims with comments from H. Preiksaitis and L. Laurier. L. Laurier undertook molecular biological studies of the expression of delayed rectifier channels in human esophageal muscle cells. Muscle strip studies were carried out in the lab of H. Preiksaitis by T. Chrones. These experiments were planned and analyzed by G. Wade.
ACKNOWLEDGEMENTS

This work was carried out in the laboratories of Stephen Sims and Harold Preiksaitis. H. G. Preiksaitis was supported by an Ontario Ministry of Health Career Scientist award and S. M. Sims by a Medical Research Council of Canada Scientist award. G. Wade was supported for three years by a block-term-training grant from The Ontario Thoracic Society.

I am grateful to Dr. Zhu Gang Zheng and Yang Jiao for help in preparation of cells, Tom Chrones for advice and assistance with human esophageal muscle strips and Drs. R.I. Inculet, R.A. Malthaner, C. Rajgopal and S.E. Carroll for providing the esophagectomy specimens. I am also indebted to the co-authors whose contributions to the research are detailed above.

Helix Investments Canada Inc. and Procyon BioPharma Inc.’s patience and support during the completion of these studies is sincerely appreciated.

Stephen Sims provided much guidance, encouragement and critical oversight of these studies as they developed and were published. I am very grateful to have had the privilege to work with such an esteemed and supportive supervisor.
# TABLE OF CONTENTS

CERTIFICATE OF EXAMINATION .................................................................................. ii  
ABSTRACT .................................................................................................................. iii  
CO-AUTHORSHIP ....................................................................................................... v  
ACKNOWLEDGEMENTS .............................................................................................. vii  
TABLE OF CONTENTS ............................................................................................... viii  
LIST OF TABLES .......................................................................................................... xi  
LIST OF FIGURES ........................................................................................................ xii  
LIST OF APPENDICES ............................................................................................... xiv  
ABBREVIATIONS ........................................................................................................ xv  

Chapter 1 .................................................................................................................... 17  
1 Introduction .............................................................................................................. 17  
1.1 Background - Smooth Muscle ............................................................................. 18  
1.1.1 Structure of Smooth Muscle .......................................................................... 18  
1.2 Contraction Of Smooth Muscle .......................................................................... 20  
1.3 Excitation-contraction coupling ........................................................................ 20  
1.4 Muscarinic Signalling in Visceral Smooth Muscles ............................................ 23  
1.4.1 M3-Mediated Signalling ................................................................................. 24  
1.4.2 M2-Mediated Signalling .................................................................................. 24  
1.5 Inward Currents In Smooth Muscle .................................................................. 26  
1.5.1 Voltage-dependent Ca\(^{2+}\) Current .............................................................. 26  
1.5.1.1 Inhibition Of Ca\(^{2+}\) Current ................................................................ 28  
1.5.1.2 Enhancement of Ca\(^{2+}\) Current ............................................................ 30  
1.5.1.3 Ca\(^{2+}\)-induced Ca\(^{2+}\) Release (CICR) ................................................. 32  
1.5.1.4 Physiological Roles of Ca\(^{2+}\) Current .................................................. 32  
1.5.2 Cl\(^{-}\) Current .................................................................................................. 33  
1.5.2.1 Ca\(^{2+}\)-activation of Cl\(^{-}\) current .......................................................... 34  
1.5.2.2 Physiological Roles of Cl\(^{-}\) Current ....................................................... 35  
1.5.3 Nonselective Cation Currents ........................................................................ 36  
1.5.3.1 Muscarinic nonselective cation current .................................................. 37  
1.5.3.2 Protein kinase regulation of nonselective cation current ......................... 42  
1.5.4 Other nonselective cation channels ................................................................ 43  
1.5.4.1 Physiological Roles of Nonselective Cation Channels ............................ 44  
1.5.5 Future Directions of Inward Current Research ............................................ 45  
1.6 Outward Currents In Smooth Muscle ................................................................. 45  
1.6.1 Molecular Basis of K\(^{+}\) Channels ................................................................. 46
Chapter 1

1.6.2 Ca²⁺-Dependent K⁺ Channels .......................................................... 47
  1.6.2.1 Regulation of KCa ................................................................. 48
  1.6.2.2 Muscarinic Regulation of KCa ............................................... 50
  1.6.2.3 Regulation of KCa by Intracellular Ca²⁺ Stores ....................... 50
  1.6.2.4 Physiological Roles of KCa .................................................. 51
  1.6.3 Delayed Rectifier Currents (Kv) ................................................. 52
  1.6.4 Inward Rectifier K⁺ Currents .................................................... 52
  1.6.5 ATP-Sensitive K⁺ Currents .......................................................... 53

1.7 Rational And Objectives of the Research ........................................... 53

1.8 References ....................................................................................... 56

Chapter 2

2 Cholinergic Inhibition of Ca²⁺ Current in Guinea-pig Gastric and Tracheal
  Smooth Muscle Cells ........................................................................... 69
  2.1 Introduction ................................................................................ 70
  2.2 Methods .................................................................................... 71
    2.2.1 Isolation of single smooth muscle cells ................................... 71
    2.2.2 Patch-clamp recording .......................................................... 72
    2.2.3 Solutions ............................................................................. 73
  2.3 Results ....................................................................................... 74
    2.3.1 Action potentials elicit contraction of gastric smooth muscle cells.. 74
    2.3.2 L-type calcium channel current in gastric smooth muscle .......... 74
    2.3.3 Acetylcholine causes a reduction in Ca²⁺ current ..................... 77
    2.3.4 Mechanisms Underlying Cholinergic Regulation of Ca²⁺ Current... 79
    2.3.5 ACh repeatedly inhibits Ca²⁺ current in the presence of 1 mM Ca²⁺. 82
    2.3.6 Cyclopiazonic acid reversibly suppresses ACh inhibition of Ca²⁺
        currents. .................................................................................. 84
    2.3.7 Time course of ACh-mediated inhibition of Ica .......................... 84
    2.3.8 ACh decreases Ca²⁺ channel open probability ........................... 85
    2.3.9 Inhibition of Ca²⁺ current in tracheal smooth muscle cells ......... 87
  2.4 Discussion ................................................................................ 90
  2.5 References ................................................................................. 94

Chapter 3

3 Muscarinic Stimulation Of Tracheal Smooth Muscle Cells Activates The
  Large Conductance Ca²⁺-Dependent K⁺ Channel ...................................... 98
  3.1 Introduction ............................................................................... 99
  3.2 Methods .................................................................................. 101
    3.2.1 Preparation of isolated tracheal smooth muscle cells ............... 101
    3.2.2 Solutions ............................................................................ 101
    3.2.3 Patch-clamp methods ............................................................ 102
  3.3 Results ..................................................................................... 104
    3.3.1 Acetylcholine causes reversible cell contraction ....................... 104
    3.3.2 Identification of large-conductance KCa channels .................... 104
    3.3.3 ACh causes a transient increase in KCa channel activity ........... 105
3.3.4 ACh causes increased $K_{Ca}$ channel activity independent of Ca$^{2+}$ entry.

3.3.5 Caffeine causes activation of $K_{Ca}$ channels.

3.4 Discussion

3.5 References

Chapter 4

4 Roles Of Delayed Rectifier And Ca$^{2+}$-Dependent K$^+$ Currents In Regulating Contraction Of Human Esophageal Smooth Muscle

4.1 Introduction

4.2 Methods

4.2.1 Tissue retrieval and isolation of cells

4.2.2 Electrophysiology

4.2.3 Solutions

4.2.4 Reverse Transcription-PCR (RT-PCR)

4.2.5 Muscle contraction

4.2.6 Chemicals

4.2.7 Statistics

4.3 Results

4.3.1 Outward currents in freshly isolated human esophageal smooth muscle cells

4.3.2 Delayed rectifier and Ca$^{2+}$-dependent K$^+$ currents from longitudinal and circular muscle cells

4.3.3 Pharmacological isolation of $K_V$ and $K_{Ca}$ currents

4.3.4 Pharmacological sensitivity of $K_{Ca}$ and insensitivity of $K_V$ currents to ChTX and IbTX.

4.3.5 STOCS in human esophageal smooth muscle cells

4.3.6 RT-PCR identification of $K_V$ channels

4.3.7 Functional effects of K$^+$ channel blockers

4.4 Discussion

4.5 References

Chapter 5

5 Discussion

5.1 Summary And Conclusions

5.2 Physiological Roles For Ca$^{2+}$ and K$^+$ Channels In Smooth Muscle

5.3 New literature supporting the findings in the published studies

5.4 Limitations of the Studies and Suggestions For Future Experiments

5.4 Reference

5.5 s

APPENDIX 1

APPENDIX 2

CURRICULUM VITAE
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Number</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**CHAPTER 4**

1. Summary of the effects of $K^+$ channel blockers on contraction of human esophageal muscle........................................................................................................................................153
LIST OF FIGURES

Number   Page

CHAPTER 1

1. Action potentials elicit contraction of a single gastric smooth muscle cell. 27
2. Schematic diagram showing some of the participants involved in regulating excitation and recovery in smooth muscle. 54

CHAPTER 2

1. L-Type calcium channel currents in gastric smooth muscle cells. 76
2. Acetylcholine inhibits Ca²⁺ current in gastric smooth muscle. 78
3. Acetylcholine inhibits I_Ca over all potentials. 80
4. Ca²⁺ currents and contraction of smooth muscle cells in 1 mM Ca²⁺ and 0 mM Ca²⁺ solutions. 81
5. Ca²⁺ stores participate in ACh-mediated inhibition of Ca²⁺ channel current. 83
6. Single Ca²⁺ channels in gastric smooth muscle. 86
7. Cholinergic regulation of single Ca²⁺ channels in gastric cells. 88
8. Acetylcholine inhibits I_Ca in guinea-pig tracheal muscle cells. 89

CHAPTER 3

1. Identification of the large conductance Ca²⁺-dependent K⁺ (K_Ca) channels in canine tracheal smooth muscle cells. 107
2. ACh causes a transient increase in channel activity. 109
3. Amplitude distribution of the ACh-elicited current shown in figure 2. 111
4. Quantification of the ACh-induced K_Ca current. 112
5. ACh shifts the potential at which K_Ca channels are open to less positive potentials. 113
6. ACh acts on muscarinic receptors to elicit K_Ca channel current. 116
7. ACh causes increased K_Ca activity independent of Ca²⁺ entry into the cell. 117
8. Release of Ca²⁺ from intracellular stores is sufficient to increase unitary K_Ca currents. 119

CHAPTER 4

1. Outward currents in human esophageal smooth muscle cells. 138
2. Delayed rectifier and Ca²⁺-dependent K⁺ currents in longitudinal and circular esophageal smooth muscle layers. 140
3. Pharmacological separation of K_V and K_Ca currents in human esophagus. 143
4. Charybdotoxin (ChTX) and iberiotoxin (IbTX) block $K_{Ca}$ current. .................144
5. Spontaneous transient outward currents (STOCs) in esophageal muscle. ......147
7. Effects of $K^+$ channel blockers on nerve-mediated contraction of human esophagus. ........................................................................................................150
8. Myogenic contraction of human esophageal smooth muscle. ......................152
9. Examples of $K^+$ channel blockers causing oscillatory contraction of circular esophageal muscle.................................................................154

CHAPTER 5

1. Schematic diagram showing some of the molecular participants involved in regulating excitation and recovery of smooth muscle. .........................167
LIST OF APPENDICES

APPENDIX 1 (Permission from The Journal of Physiology) ............................................173

APPENDIX 2 (Permission from the American Physiological Society) ......................174
ABBREVIATIONS

Concentration
Extracellular concentration
Intracellular concentration
Free cytosolic concentration of Ca$^{2+}$
4-aminopyridine
20-hydroxyeicosatetraenoic acid
Acetylcholine
Analysis of variance
Adenosine triphosphate
Ionic calcium
Adenosine 3', 5'-cyclic monophosphate
Cholecystokinin
Calcitonin gene related protein
Guanosine cyclic 3', 5'-monophosphate
Charybdotoxin
Cyclopiazonic acid
Diacylglycerol
Dimethylsulfoxide
Deoxynucleic Acid
N', N' -1-2-Ethanediylbis[N-carboxymethyl]glycine] trisodium salt
ethylene glycol-bis(β-aminoethyl ether)-N, N, N', N'-tetraacetic acid
guanine nucleotide binding protein
5'guanylyl-imidodiphosphate
Guanidine tris-phosphate
Guanosine 5' O-(3-thiotriphosphate)
4-(2-Hydroxyethyl)-1-piperazineethane-sulfonic acid
Iberitoxin
Calcium current
IP3  1,4,5-trisphosphate
IP3R  Inositol 1,4,5-trisphosphate Receptor
IV  Current voltage
KCa  Large conductance Ca^{2+} dependent K^+
KV  Delayed rectifier
M2AChR  M2 muscarinic acetylcholine receptor
M3AChR  M3 muscarinic acetylcholine receptor
Na^+  Ionic sodium
NO'  The free radical nitric oxide
PCR  Polymerase Chain Reaction
PdBV  Phorbol 12, 13-dibutyrate
PKA  Protein Kinase A
PKC  Protein Kinase C
RNA  Ribonucleic Acid
RT-PCR  Reverse Transcription Polymerase Chain Reaction
S.E.M.  Standard Error of the Mean
SITS  4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid, disodium salt
Sparks  Localized elevation of Ca^{2+} due to release of Ca^{2+} from intracellular stores
SR  Sarcoplasmic reticulum
STICs  Spontaneous transient inward currents
STOCs  Spontaneous transient outward currents
Stores  Intracellular Ca^{2+} stores found in the sarcoplasmic reticulum of smooth muscle
TEA  Tetraethylammonium
TTX  Tetrodotoxin
VIP  Vasoactive intestinal polypeptide
CHAPTER 1

1 INTRODUCTION

Sections from this chapter have been adapted from a review co-written by Stephen Sims and Greg Wade;


Figure 1 was prepared from current-clamp experiments performed and analyzed by G. Wade in S. Sims laboratory.
1.1 Background - Smooth Muscle

While the coordinated contraction of smooth muscles serves to control the diameter and shape of hollow organs, there is much complexity and variation involved in the regulation of this activity. Variation occurs within regions of tissues, organs, organ systems and species. The source of this variation and complexity comes about via differences in innervation, expression of receptors, ion channels and enzymes, the characteristics of intracellular Ca\(^{2+}\) stores (sarcoplasmic reticulum in smooth muscle, SR), and the structure and orientation of cells. Isolation of the participants involved in excitation contraction coupling, and comparison between tissue regions, organs and species will provide an improved understanding of the physiology of smooth muscle in health and disease.

1.1.1 Structure of Smooth Muscle

Smooth muscle cells share similarities and differences with skeletal and cardiac muscles (Vander, Sherman & Luciano, 1994). Contraction of smooth muscle is subject to both electromechanical and pharmacomechanical regulation (Somlyo & Somlyo, 1994). Under electromechanical regulation of muscle, depolarization leads to contraction while hyperpolarization is associated with relaxation, as the level of Ca\(^{2+}\) entry into cells is determined by voltage-dependent L-type Ca\(^{2+}\) channels which are open at more positive potentials. Regulation of membrane potential may come about via neurotransmitter activation of inward currents, inhibition of outward currents, or, in the absence of neurotransmitters, via electrical coupling of smooth muscle cells. Several neurotransmitters cause pharmacomechanical excitation of muscle by activating signalling pathways that converge at release of Ca\(^{2+}\) from intracellular stores. However, such regulation often leads to electromechanical effects as excitatory agents and elevation of
[Ca\(^{2+}\)], often activate inward currents, leading to depolarization of cells and Ca\(^{2+}\) entry (Fig. 1.) While the primary roles of Ca\(^{2+}\) stores in contraction of cardiac and skeletal muscle are well known, the relative contributions of Ca\(^{2+}\) entry, and stores in various smooth muscles remain the subject of continuing research and debate.

The membrane of most smooth muscles has numerous invaginations or calveolae leading to increased surface area and permitting movement of Ca\(^{2+}\) into and out of the cell from deeper regions within the cytosol and less proximal to the cell’s surface. Unlike skeletal muscle cells, smooth muscle cells have a single nucleus. Smooth muscles have thick myosin containing filaments and thin actin containing filaments. The actin filaments are anchored to the plasma membrane on dense bodies, the functional equivalent of Z-lines in skeletal muscle. The contractile filaments in smooth muscle are not organized into myofibrils. Smooth muscle cells contain about 1/3 the concentration of myosin and up to twice as much actin as compared to skeletal muscle; however, smooth muscles develop similar tensile forces per unit of cross-sectional area. Smooth muscles may contain significant intracellular Ca\(^{2+}\) stores within their sarcoplasmic reticulum, although these are not coupled directly to ryanodine receptors as in skeletal muscle and despite the presence of ryanodine receptors, Ca\(^{2+}\)-induced Ca\(^{2+}\) release has not been widely observed in smooth muscles (see below).

Coordinated changes in diameter lead to regulation of blood pressure and perfusion, organ volume, as in the stomach or bladder, or cause mechanical effects on luminal contents as in peristalsis in the esophagus and intestinal tract. To effect such activity, smooth muscles are generally oriented with their long axis around the hollow organ (inner layer of circular smooth muscle) and along the organ’s length (outer layer of longitudinal muscles). While
contraction of circular smooth muscles would clearly lead to a reduction in lumenal diameter, the role of longitudinal excitation is less clear.

1.2 Contraction Of Smooth Muscle

Contraction of smooth muscle is regulated by the level of free cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) (Somlyo & Somlyo, 1994). Smooth muscle cells have a resting membrane potential of −40 to −70 mV. A variety of neurotransmitters elicit contraction by causing Ca\(^{2+}\) entry through the activation of inward currents or inhibition of outward currents, thus leading to depolarization of cells and opening of voltage-dependent Ca\(^{2+}\) channels. Conversely, agents or intracellular signals that cause increased outward currents cause relaxation of muscle by inhibition of action potentials or by hyperpolarizing cells, which leads to decreased Ca\(^{2+}\) channel open probability. In many cases receptor signalling also involves the activation of guanine nucleotide binding proteins (G proteins), leading to the generation of second messengers that can cause release of Ca\(^{2+}\) from intracellular stores. Many of the channels in smooth muscle are sensitive to intracellular Ca\(^{2+}\) (Carl, Lee & Sanders, 1996; Large & Wang, 1996). The role of Ca\(^{2+}\) stores in the regulation of channels, and evidence that Ca\(^{2+}\) entry is influenced by Ca\(^{2+}\) released from intracellular stores, is discussed below.

1.3 Excitation-contraction coupling

[Ca\(^{2+}\)]\(_i\) is of central importance in controlling contraction of smooth muscles, with Ca\(^{2+}\)/calmodulin-activated myosin light-chain kinase responsible for phosphorylation of myosin, and initiation or maintenance of contraction (Somlyo & Somlyo, 1994; Horowitz, Menice, Laporte & Morgan, 1996). Briefly, Ca\(^{2+}\) binds to calmodulin and this complex binds to myosin light-chain kinase, activating the enzyme. Myosin light-chain kinase phosphorylates serine on the regulatory light-chain of myosin, permitting myosin ATPase
to become activated by actin and leading to contraction. Calmodulin-dependent protein kinase II can also phosphorylate the myosin light-chain, but the rate of this reaction is only approximately 5% of that of myosin light chain kinase, suggesting that this pathway does not participate in the initiation of contraction (Somlyo & Somlyo, 1994).

In most smooth muscles, [Ca\(^{2+}\)]\(_i\) is maintained at ~100 nM (e.g., Williams, Becker & Fay, 1987; Becker, Singer, Walsh, Fay, 1989; Ganitkevich & Isenberg, 1991; Pacaud & Bolton, 1991; Vogalis, Publicover, Hume & Sanders, 1991) by mechanisms which extrude Ca\(^{2+}\) from the cell and sequester it into the sarcoplasmic reticulum (SR) or mitochondria (Drummond & Fay, 1996). The [Ca\(^{2+}\)] in internal stores is estimated to be in the millimolar range (Iino, 1989; Golovina & Blaustein, 1997) and so, at rest there is a large gradient tending to move Ca\(^{2+}\) into the cytosol. Several neurotransmitters act through membrane delimited and second messenger signalling cascades to regulate [Ca\(^{2+}\)]\(_i\) in smooth muscle. The role of the receptor-mediated G protein signalling pathways in the regulation of [Ca\(^{2+}\)]\(_i\) has been reviewed (Berridge, 1993; Somlyo & Somlyo, 1994; Wickman & Clapham, 1995; Eglen, Hedge & Watson, 1996) and is discussed below (see Muscarinic Signalling in Smooth Muscle). Excitatory neurotransmitters, such as ACh, elevate [Ca\(^{2+}\)]\(_i\), with both transient and sustained components (Sims, Jiao & Zheng, 1996) with elevation of [Ca\(^{2+}\)]\(_i\) occurring due to both Ca\(^{2+}\) influx and release from intracellular stores. The relative contributions of each pathway varies among cell types and excitatory stimuli.

Opening of Ca\(^{2+}\) channels results in influx of Ca\(^{2+}\) that is estimated to elevate [Ca\(^{2+}\)]\(_i\) to 8-100 μM, assuming uniform distribution of the Ca\(^{2+}\) throughout the cytosol (e.g. Williams, Becker & Fay, 1987; Vogalis et al., 1991). However, measurements of [Ca\(^{2+}\)]\(_i\) using fluorescent indicator dyes indicate that the elevation of [Ca\(^{2+}\)]\(_i\) is markedly smaller than
predicted to arise from influx, with peak $[Ca^{2+}]_i$ of only ~1 μM (Becker et al., 1989; Vogalis et al., 1991; Ganitkevich & Isenberg, 1991). The discrepancy between the estimated and actual increases likely indicates that much of the Ca$^{2+}$ entering the cell is rapidly buffered, sequestered into intracellular compartments and/or extruded across the membrane. Fluorescence measurements from whole cells emphasize changes of $[Ca^{2+}]_i$ in the bulk cytosol. However, more recent studies using fluorescence imaging methods reveal regional specialization of endoplasmic reticulum Ca$^{2+}$ stores (Golovina & Blaustein, 1997) and localized changes of $[Ca^{2+}]_i$ that could control ion channel activity. Localized spontaneous release of Ca$^{2+}$ from SR causes Ca$^{2+}$ "sparks" that give rise to spontaneous transient outward currents (STOCs) due to the activation of Ca$^{2+}$-dependent K$^+$ channels (Nelson, Cheng, Rubart, Santana, Bonev, Knot & Lederer, 1995; Mironneau, Arnaudeau, Macrez-Lepretre & Boittin, 1996; Bolton & Imaizumi, 1996). Ca$^{2+}$ sparks are believed to originate from release of Ca$^{2+}$ from ryanodine receptors in the SR. It has also been suggested that the released Ca$^{2+}$ can lead to Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) from adjacent ryanodine receptors, giving rise to larger sparks (Bolton & Imaizumi, 1996). The activation of K$^+$ current by Ca$^{2+}$ from this localized release is apparent in many smooth muscles as spontaneous transient outward currents (STOCs), and has been suggested to contribute to relaxation of vascular muscle (discussed below; Nelson et al., 1995). Many smooth muscles also possess Ca$^{2+}$-activated inward currents, and therefore, hyperpolarization due to Ca$^{2+}$ sparks would not be expected to be a universal feature of smooth muscles if these currents are activated by Ca$^{2+}$ sparks. Indeed, it has recently been discovered that Ca$^{2+}$ sparks also underlie the signalling responsible for spontaneous transient inward currents, which arise from activation of Ca$^{2+}$-dependent Cl$^-$ currents.
(ZhuGe, Sims, Tuft, Fogarty, & Walsh, 1998). $\text{Ca}^{2+}$-ATPases in the SR actively sequester $\text{Ca}^{2+}$, contributing to restoration of basal $[\text{Ca}^{2+}]_i$ (Sims, Jiao & Zheng 1996). Therefore, the stores can contribute to regulation of $[\text{Ca}^{2+}]$ both globally within the cell and locally within a specialized region immediately below the plasma membrane, as described by the superficial buffer barrier model of van Breemen and colleagues (1995). Clearly the dynamic and regional specialization of $\text{Ca}^{2+}$ handling are emerging as topics of great relevance to control of ion channels and contraction in smooth muscle.

1.4 Muscarinic Signalling in Visceral Smooth Muscles

Acetylcholine is the principal neurotransmitter of the parasympathetic nervous system and acts on muscarinic receptors to cause depolarization and contraction of many visceral smooth muscles (reviewed in Janssen & Sims, 1997). ACh-mediated relaxation of vascular muscles occurs due to the actions on endothelial cells leading to liberation of nitric oxide. Early studies of the effects of ACh on visceral smooth muscle suggested that it caused depolarization by activating channels permeable to $\text{Na}^+$, $\text{K}^+$, and $\text{Ca}^{2+}$. The development of the voltage-clamp technique, molecular biological approaches and pharmacological tools has initiated a process to elucidate the role of muscarinic receptor subtypes in the regulation of ion channels, kinases and cytosolic $\text{Ca}^{2+}$ stores in smooth muscle (reviewed in Eglen et al., 1996 and discussed below).

Muscarinic receptors belong to the class of proteins with seven membrane-spanning regions and that act through heterotrimeric G proteins to regulate a variety of enzymes, other receptors, and ion channels. Muscarinic receptors are encoded by 5 intronless genes. The receptors are highly homologous with the exception of the large cytoplasmic loop between the fifth and sixth transmembrane spanning region, suggesting that this region
confers selectivity between the receptor subtype and its associated G proteins. ACh acts on M1, M3 and M5 receptors coupled to second messenger signalling cascades which result in the release of Ca\(^{2+}\) from intracellular stores, whereas ACh binding to M2 and M4 receptors inhibits adenylyl cyclase, lowering cAMP levels.

1.4.1 M3-Mediated Signalling

In smooth muscle, M3 receptors are believed to be coupled to phospholipase C-β (PLC-β) via the pertussis toxin-insensitive G protein G\(_{q/1}\). Activation of PLC-β initiates hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP\(_3\)) (Berridge, 1993). IP\(_3\) activates receptors on intracellular stores to release Ca\(^{2+}\) while DAG activates protein kinase C (PKC) to initiate phosphorylation of a variety of substrates. PLC-β activity is sensitive to intracellular Ca\(^{2+}\), providing the opportunity for interactions between this signalling pathway with others that regulate [Ca\(^{2+}\)]. IP\(_3\)-mediated signalling is responsible for rapid contraction of smooth muscle, with contraction occurring within seconds of the interaction of ACh with its receptors. DAG-mediated activation of protein kinase C (PKC) is proposed to lead to the phosphorylation of contractile proteins possibly regulating Ca\(^{2+}\)-independent contraction, sensitization of the contractile machinery to Ca\(^{2+}\) and/or the development of prolonged contraction (Berridge, 1993, Somlyo & Somlyo, 1994).

1.4.2 M2-Mediated Signalling

M2 and M4 receptors are coupled through pertussis toxin-sensitive G proteins to adenylyl cyclase. M2 receptors are believed to predominate in smooth muscle with binding of ACh to M2 leading to activation of the muscarinic nonselective cation current (see below) and inhibition of adenylyl cyclase leading to reduced cytosolic cAMP levels. Adenylyl cyclase
catalyses formation of cAMP which in turn binds to the regulatory subunit of PKA, causing its dissociation from the catalytic subunit leading to activation of the kinase. PKA phosphorylates PKA consensus sequences containing serine or threonine residues.

Although agents that elevate cAMP levels and activate PKA directly have been shown to both inhibit the effects of excitatory transmitters and cause relaxation of many smooth muscles, the molecular mechanisms underlying such effects are not fully appreciated at this time. Tolloczko and co-workers (Tolloczko, Jia, & Martin, 1997) report forskolin causes a significant elevation of cAMP in cultured rat tracheal smooth muscle and mediated 60% relaxation of muscle rings contracted with serotonin. This effect was accompanied by a decrease in the sustained phase of elevation of \([\text{Ca}^{2+}]_i\) during the serotonin response. The effects of forskolin were antagonized by both thapsigargin and an inhibitor of PKG, leading the authors to conclude the presence of a cAMP-PKG-dependent role for intracellular stores in mediating sequestration of \([\text{Ca}^{2+}]_i\). A role for cAMP mediating its effect through sequestration of \(\text{Ca}^{2+}\) by intracellular stores is further supported by the observation that CPA prevents forskolin-mediated decreases in \([\text{Ca}^{2+}]_i\) in rat anococcygeus muscle strips (Raymond & Wendt, 1996). Inhibition of excitatory signalling by cAMP as determined by the accumulation of inositol phosphates was investigated in cultured canine tracheal smooth muscle cells (Yang, Hsu, Tsao, Chiu, Ong, Hsieh & Fan, 1996). Forskolin inhibited carbachol-mediated increases in both \([\text{Ca}^{2+}]_i\) and inositol phosphates. PKA-antagonists reduced this effect but direct activation of G proteins by AlF\(^-\) did not overcome the effects of forskolin, suggesting that PKA dependent phosphorylation uncouples G proteins from the effector enzymes responsible for liberation of inositol
phosphates. There are also several reports of the regulation of ion channels by cAMP-dependent mechanisms in smooth muscle (see below).

1.5 Inward Currents In Smooth Muscle
1.5.1 Voltage-dependent Ca\(^{2+}\) Current
Voltage-dependent Ca\(^{2+}\) channels have been identified in virtually all smooth muscles and play a key role in initiating contraction (Bolton, 1989; Sanders, 1989; Somlyo & Somlyo, 1994). In early studies, Ca\(^{2+}\) entry was thought to be essential for excitation-contraction coupling in many tissues. This view was based on the findings that neurotransmitter-evoked excitation was accompanied by depolarization, and depolarizing the muscle (for example, with elevation of extracellular K\(^+\)) also produced contraction. Concurrent recording of membrane potential and cell length in a guinea-pig gastric smooth muscle demonstrates that depolarization initiates action potentials, accompanied by contraction (Fig. 1.). When a longer train of action potentials is elicited, greater contraction occurs, illustrating that changes of membrane potential can provide graded control over Ca\(^{2+}\) entry. Therefore, agents that regulate membrane potential or Ca\(^{2+}\) channels will have a profound effect on the contraction of smooth muscles.
Figure 1. Action potentials elicit contraction of a single gastric smooth muscle cell. Top. Current clamp recording of membrane potential with contraction simultaneously recorded on video (assessed as cell length, Below). Positive charge injected into the cell (5 pA, 1 s) caused action potentials (shown expanded in inset) and was accompanied by contraction. Right. A second more prolonged depolarization (16.5 s) resulted in a train of action potentials and greater cell shortening. Below. Contraction was quantified by measuring cell length.
The biophysical properties of L-type Ca\(^{2+}\) currents have been extensively studied in isolated cells and reviewed elsewhere (McDonald, Pelzer, Trautwein & Pelzer, 1994). Ca\(^{2+}\) channels are multisubunit complexes comprised of the pore-forming \(\alpha_1\) subunit associated with \(\beta\) subunits and a disulfide-linked \(\alpha_2\delta\) component (Birnbaumer, Campbell, Harpold, Hofmann, Horne, Mori, Schwarz, Snutch, Tanabe & Tsien, 1994; Hofmann, Biel & Flockerzi, 1994). The \(\alpha_{1C}\) subunit is common to both smooth and cardiac muscles. Several characteristics of the channel provide regulatory opportunities including putative phosphorylation (Dolphin, 1996) and ion binding sites, and sites for G protein interactions (Wickman & Clapham, 1995). The voltage-sensitivity of the Ca\(^{2+}\) channel provides a mechanism permitting regulation of \([\text{Ca}^{2+}]_i\) by graded Ca\(^{2+}\) entry. For example, voltage-dependent activation and inactivation characteristics permit a persistent Ca\(^{2+}\) current (window current, e.g., Fleischmann, Murray & Kotlikoff, 1994) possibly contributing to the maintenance of muscle tone. As such, activation of K\(^+\) channels causes hyperpolarization, reducing Ca\(^{2+}\) entry and leading to relaxation (e.g., Nelson et al., 1995; Quayle, Nelson & Standen, 1997).

1.5.1.1 Inhibition Of Ca\(^{2+}\) Current
Several neurotransmitters activate receptors coupled to G proteins, which may lead to regulation of Ca\(^{2+}\) channels directly, via membrane-delimited pathways, or indirectly, by the actions of cytosolic second messengers (Hille, 1994; Wickman & Clapham, 1995; Dolphin, 1996). While several early reports found that excitatory agents increase Ca\(^{2+}\) currents (see below), elevation of \([\text{Ca}^{2+}]_i\) is also known to inhibit Ca\(^{2+}\) channels. Could excitation of smooth muscle and the accompanying increases of \([\text{Ca}^{2+}]_i\) result in direct modulation Ca\(^{2+}\) channels? Muscarinic stimulation does cause acute suppression of Ca\(^{2+}\)
current in many smooth muscles, including ileum, stomach and trachea (Unno, Komori, Ohashi, 1995). A role for Ca²⁺ stores in regulation of Ca²⁺ currents is supported by the finding that flash photolysis-induced release of IP₃ inhibits Ca²⁺ currents in ileal muscle (Komori & Bolton, 1991b). Similarly, Yoshikawa and co-workers (1996) found that sequestration of Ca²⁺ by stores participates in the regulation of Ca²⁺ currents, since blockade of the SR Ca²⁺-ATPase with cyclopiazonic acid (CPA) causes a decrease in Ca²⁺ current in guinea-pig urinary bladder cells. Inward currents that elevate [Ca²⁺], such as ATP-gated nonselective cation current and stretch-activated current, also inhibit Ca²⁺ current.

Schumann and co-workers (1997) find that in addition to cytosolic Ca²⁺ mediating inhibition of Ca²⁺ currents directly, elevation of [Ca²⁺] at the cytosolic face of the membrane activates protein phosphatase 2B leading to reduced channel open probability, likely due to phosphorylation of the channel. Such regulation demonstrates that intracellular Ca²⁺ participates in two distinct negative feedback effects on L-type Ca²⁺ channels in smooth muscle.

The site of Ca²⁺ sensitivity on the L-type Ca²⁺ channel is located at an EF-hand Ca²⁺ binding motif on the α₁C subunit (de Leon, Wang Jones, Perez-Reyes, Wei, Soon, Snutch & Yu, 1995; Zong & Hofmann, 1996). This was determined by replacing the carboxy terminus of the α₁C subunit with that from the non-Ca²⁺-sensitive product of the α₁E gene, which gives rise to an α₁CE-1 chimera that lacks Ca²⁺-dependent inactivation.

In addition to Ca²⁺-dependent inhibition mediated by elevation of [Ca²⁺], neurotransmitters may also inhibit Ca²⁺ currents directly by G protein-dependent mechanisms. In ileal
smooth muscle, histamine and bradykinin cause both acute and persistent suppression of Ca$^{2+}$ current (Beech, 1993). Persistent bathing of cells in Ca$^{2+}$-free solution abolishes the acute suppression (indicating involvement of Ca$^{2+}$), while GTP$\gamma$S facilitates the persistent component, consistent with G protein-mediated regulation of Ca$^{2+}$ current. Pemberton and Jones (1997) report that NIH 3T3 cells transfected with each of the M1-M5 receptors demonstrate a reduction in L-type Ca$^{2+}$ current in the presence of ACh. ACh-mediated inhibition in cells transfected with M2 and M4 receptors was sensitive to pertussis toxin.

1.5.1.2 Enhancement of Ca$^{2+}$ Current
Enhancement of Ca$^{2+}$ currents has been characterized in a variety of excitable tissues (reviewed in Dolphin, 1996) including smooth muscle, suggesting that such regulation is of widespread and general significance. L-type Ca$^{2+}$ current enhancement was first demonstrated in gastric smooth muscle cells of *Bufo marinus* (Clapp, Vivaudou, Walsh & Singer, 1987), and has since been found in visceral and vascular smooth muscles (reviewed in McDonald *et al.*, 1994; Janssen & Sims, 1997). When studied in airway muscle at the level of single channels, cholinergic agonists cause increased L-type Ca$^{2+}$ channel open probability at depolarized potentials, due to a shift of the Ca$^{2+}$ channel activation range to more negative potentials. This effect is not accompanied by a change in the unitary conductance or the number of available channels (Kamishima, Nelson & Patlak, 1992; Tomasic, Boyle, Worley & Kotlikoff, 1992).

Enhancement of Ca$^{2+}$ current with excitatory neurotransmitters likely involves various protein kinases, including protein kinase C and protein kinase A and may also be due to G proteins acting directly on the channel. GTP-$\gamma$S and GTP increase Ca$^{2+}$ current in canine jejunal cells (Farrugia, 1997), an effect blocked by the non-specific kinase inhibitor
staurosporine, suggesting that it is due to channel phosphorylation. Noradrenaline causes
elevation of Ca\(^{2+}\) current in colonic myocytes through a pathway involving several kinases
(Koh & Sander, 1996). Low levels of cyclic AMP (cAMP) activate protein kinase A
(PKA), leading to facilitation of Ca\(^{2+}\) current, while higher levels appear to activate protein
kinase G, leading to inhibition of Ca\(^{2+}\) current (Koh & Sanders, 1996). In addition, other
pathways can contribute to enhancement of Ca\(^{2+}\) current in smooth muscle, including
calcium-dependent enhancement of Ca\(^{2+}\) current mediated by calmodulin-dependent
kinase II (McCarron, McGeown, Reardon, Ikebe, Fay & Walsh, 1992). Metabolites of the
arachidonic acid pathway, 20-hydroxyeicosatetraenoic acid (20-HETE) cause contraction
of cat cerebral arteries. This effect is antagonized by nifedipine suggesting 20-HETE
activates L-type Ca\(^{2+}\) channels (Gebremedhin, Lange, Narayanan, Aebly, Jacobs & Harder,
1998).

L-type Ca\(^{2+}\) channels may also be sensitive to mechanical changes in smooth muscle. Xu
and co-workers demonstrate that hyposmotic-induced stretching increases L-type Ca\(^{2+}\)
currents in guinea-pig gastric myocytes (Xu, Kim, So & Kim, 1997). Such regulation was
antagonized by the cytoskeletal disruptor cytochalasin-D and facilitated by phalloidin, an
actin filament stabilizer. These findings suggest that the actin cytoskeleton may, in some
way, couple to L-type Ca\(^{2+}\) channels, potentially leading to positive feedback during
excitation of smooth muscle. As other excitatory neurotransmitters (see above) would be
expected to activate the contractile machinery of cells, investigators should be careful in
their interpretation of the mechanisms underlying increased Ca\(^{2+}\) current by these agents.
1.5.1.3  

**Ca$^{2+}$-induced Ca$^{2+}$ Release (CICR)**

The presence of ryanodine receptors in many smooth muscles raises the possibility that, like in heart, Ca$^{2+}$ entry can elevate [Ca$^{2+}$]$\text{_{i}}$ and elicit release of Ca$^{2+}$ from intracellular stores (Ca$^{2+}$-induced Ca$^{2+}$ release, CICR; Iino, 1989). Functional evidence for CICR has been obtained from the relationship between Ca$^{2+}$ entry and the resulting elevation of [Ca$^{2+}$]$\text{_{i}}$ in cells (Ganitkevich & Isenberg, 1995). Depletion of Ca$^{2+}$ stores with ryanodine or caffeine reduced the elevation of [Ca$^{2+}$]$\text{_{i}}$, consistent with depletion of stores abolishing CICR. However, it was noted that physiologic levels of Ca$^{2+}$ entry are insufficient to activate CICR in dissociated smooth muscle cells. To elicit CICR, it is necessary to elevate extracellular Ca$^{2+}$ or augment Ca$^{2+}$ current. Part of the difficulties in demonstrating CICR in smooth muscle may be due to the challenges of distinguishing subsarcolemmal changes in [Ca$^{2+}$] from global changes within the cytosol (Ganitkevich & Isenberg, 1996). Evidence is emerging that changes in [Ca$^{2+}$] in a restricted region between the SR and plasmalemma contribute to regulation of ion channels (Bolton & Imaizumi, 1996; Mironneau *et al.*, 1996; van Breemen, Chen & Laher, 1995; Imaizumi, Torii, Ohi, Nagano, Atsuki, Yamamura, Muraki, Watanabe & Bolton, 1998). Channels could both regulate, and be regulated by, release of Ca$^{2+}$ from intracellular stores. These complex interactions in cellular signalling pathways represent an emerging area for research into the cellular physiology of smooth muscle.

1.5.1.4  

**Physiological Roles of Ca$^{2+}$ Current**

Signalling via excitatory efferents in smooth muscles causes depolarization by a number of different mechanisms, but is commonly accompanied by activation of voltage-dependent Ca$^{2+}$ channels and contraction. Like the suppression of K$^{+}$ conductance, enhancement of voltage-activated Ca$^{2+}$ currents would contribute to prolongation and increased amplitude
of the plateau of the action potential (e.g. in gastrointestinal smooth muscle, Sanders, 1989). Persistent Ca\textsuperscript{2+} current (window current) can contribute to physiologically relevant Ca\textsuperscript{2+} entry, as described in vascular and tracheal muscles (Ganitkevich & Isenberg, 1990; Fleischmann et al., 1994), which could maintain contraction or participate in the refilling of Ca\textsuperscript{2+} stores. Indeed, activation of L-type Ca\textsuperscript{2+} channels leads to partial refilling of stores following depletion by cyclopiazonic acid (CPA) in tracheal smooth muscle (Janssen & Sims, 1993b). This refilling occurs in the presence of CPA, suggesting that L-type Ca\textsuperscript{2+} channels may be in direct contact with the stores. This may represent an important process for recovery following stimulation with agents such as ACh, since the depolarization (caused in part by Ca\textsuperscript{2+} release from the SR) would promote Ca\textsuperscript{2+} entry and refilling of the stores.

1.5.2 Cl\textsuperscript{-} Current
Ca\textsuperscript{2+}-activated chloride channels contribute to excitation in vascular and visceral smooth muscles in response to various excitatory agonists (reviewed by Large & Wang, 1996). For example, ACh induces inward current in tracheal smooth muscles, and noradrenaline induces the current in many vascular muscles. Evidence that the current was Cl\textsuperscript{-} selective include findings that the current persists with removal of extracellular Na\textsuperscript{+} (ruling out nonselective cation currents), and has a reversal potential that is dependent on the Cl\textsuperscript{-} gradient. As well, the ACh- and noradrenaline-activated currents are reversibly blocked by Cl\textsuperscript{-} channel blockers such as SITS and niflumic acid (Janssen & Sims, 1992; Large & Wang, 1996).
1.5.2.1 $Ca^{2+}$-activation of Cl$^-$ current

Several observations support the conclusion that the Cl$^-$ current is $Ca^{2+}$-activated. Cl$^-$ current is accompanied by contraction, and depletion of stores (in $Ca^{2+}$-free solution, or using agents that inhibit the SR $Ca^{2+}$-ATPase, e.g., CPA) abolishes the current (Janssen & Sims, 1993b). Furthermore, caffeine and ryanodine activate a current resembling the ACh-elicited current, with regards to reversal potential, latency and duration (Clapp, Turner & Kozlowski, 1996; Janssen, 1996; Wang & Kotlikoff, 1997; Large and Wang, 1996; Janssen & Sims, 1997). Receptor occupation is not required for channel opening based on several observations. Spontaneous transient inward currents (STICs) are observed which reverse direction around the equilibrium potential for Cl$^-$ and which are coincident with STOCs (Large & Wang, 1996; Janssen & Sims, 1992; 1994a). STIC activity has recently been found to arise from localized elevation of $Ca^{2+}$ at the cytosolic face of the membrane due to release of $Ca^{2+}$ from the SR, in a similar manner to the activation of STOCs (ZhuGhe et al., 1998). Cl$^-$ currents are also activated by $Ca^{2+}$ influx (Janssen & Sims, 1995; Greenwood & Large, 1996). Finally, muscarinic agonists can cause oscillations of $Ca^{2+}$ and Cl$^-$ current in several smooth muscles, consistent with oscillatory release of $Ca^{2+}$ from intracellular stores by an IP$_3$-dependent pathway (Liu & Farley, 1996; Nuttle & Farley, 1996).

Release of $Ca^{2+}$ from intracellular stores, leading to activation of Cl$^-$ channels, is a point of convergence for excitatory signalling in many smooth muscles (Large & Wang, 1996). Examples include histamine, acting on H1 receptors, which act in tracheal muscle to elicit Cl$^-$ currents with similar characteristics to those elicited by ACh (Janssen & Sims, 1993a). More recently, neurokinins and substance P have also been found to cause release of $Ca^{2+}$ from stores and activation of Cl$^-$ conductances in the same muscles (Janssen & Sims,
Thus, receptor-signalling through elevation of \([\text{Ca}^{2+}]_i\) can elicit stereotyped activation of Cl\(^-\) currents.

Wang and co-workers (1997) have utilized antibodies selective to G proteins to elucidate muscarinic signalling pathways responsible for the activation of Cl\(^-\) currents in equine tracheal muscle. Antibodies to G\(_q/G_{11}\) attenuated the Ca\(^{2+}\)-activated Cl\(^-\) currents whereas antibodies directed at G\(_{i-1/i-2}\) and G\(_{i-3/Go}\) were without effect. They conclude that stimulation of phospholipase C via M\(_3/G_q\) proteins is the predominant signalling pathway for the activation of the Ca\(^{2+}\)-dependent Cl\(^-\) current.

While the correlation of Cl\(^-\) current and elevation of \([\text{Ca}^{2+}]_i\), recorded with fluorescent dyes reveals the key role for Ca\(^{2+}\) in activation of channels, the time course of currents is consistently briefer than accompanying changes of \([\text{Ca}^{2+}]_i\). A recent report provides preliminary evidence that CaM kinase may contribute to the inactivation of Cl\(^-\) channels, accounting for decline of the current while \([\text{Ca}^{2+}]_i\) is still elevated (Wang & Kotlikoff, 1997). Understanding the details of channel regulation would be greatly aided by molecular identification of the Cl\(^-\) channel types. Earlier reports described low conductance channels that are activated by excitatory agonists and elevated \([\text{Ca}^{2+}]_i\) (Klockner, 1993; Van Renterghem & Lazdunski, 1993). While several subtypes of Cl\(^-\) channels have been cloned and characterized (Jentsch, 1996), the identity of the smooth muscle channel(s) has not yet been determined.

### 1.5.2.2 Physiological Roles of Cl\(^-\) Current.

Cl\(^-\) currents play important roles in the excitation-contraction coupling of smooth muscle. The Cl\(^-\) equilibrium potential in smooth muscles ranges from -33 to -8 mV (Aickin, 1990).
and therefore, activation of Cl⁻ current at the resting potential would lead to an efflux of Cl⁻ from the cytosol resulting in inward, depolarizing current. Therefore, Cl⁻ current may be part of a positive feedback loop, where Ca²⁺ released from internal stores causes inward current and depolarization, which in turn leads to influx of Ca²⁺ through voltage-dependent Ca²⁺ channels. However, agonists cause contraction of muscles even when Cl⁻ current is largely eliminated using Cl⁻ channel blockers (Janssen & Sims, 1992), and so it does not appear to be essential for contraction. Several studies support a role for Cl⁻ current in regulating the membrane potential of smooth muscles. For example, Cl⁻ currents contribute to setting the resting membrane potential of dog trachea (Daniel, Jury, Bourreau & Jager, 1993). Recent studies reveal that spontaneous depolarizations and slow waves recorded in circular muscle of rabbit urethra arise due to activation of Cl⁻ current (Hashitani, Van Helden & Suzuki, 1996). Such regulation of Cl⁻ currents requires release of Ca²⁺ from SR, as it is inhibited by CPA, caffeine and loading of cells with the Ca²⁺ chelator BAPTA-AM. This provides functional evidence that rhythmic changes of membrane potential can be initiated by Ca²⁺ release from the SR, as also described for single muscle cells (Janssen & Sims, 1994a).

1.5.3 Nonselective Cation Currents

Nonselective cation channels are activated in response to a variety of stimuli, including binding of neurotransmitters, or by mechanical deformation of the membrane. Inward current through nonselective cation channels causes depolarization of the cell membrane, resulting in Ca²⁺ influx due to opening of voltage-dependent Ca²⁺ channels. Some nonselective cation channels can also allow Ca²⁺ entry directly. The properties of the ACh-
activated nonselective cation current, followed by ATP- and stretch-activated channels are described below.

1.5.3.1 Muscarinic nonselective cation current.
Perhaps the most thoroughly studied nonselective cation currents in smooth muscle are those activated by muscarinic agonists or neuropeptides (Lee, Shuttleworth & Sanders, 1995). Agonist stimulation of many smooth muscles initiates inward current that reverses close to 0 mV, indicating channels that select poorly among monovalent cations (reviewed in Isenberg, 1993). Under physiological conditions, the inward current is largely carried by Na⁺, although there are indications that divalent cations may also permeate the channel (see below). Reduction of extracellular Na⁺ (replacement with large impermeant ions) shifts the reversal potential to more negative potentials, although replacement of extracellular Na⁺ with Li⁺, K⁺, or Cs⁺ does not cause the reversal potential to change, evidence the channels do not discriminate between these cations (Inoue & Isenberg, 1990a). These characteristics confirm the nonselective permeability of this current. However, when extracellular Na⁺ is replaced with Ca²⁺ or Ba²⁺, the reversal potential is displaced approximately 20 mV in the positive direction (i.e., towards the equilibrium potential for Ca²⁺), suggesting that these channels are permeable to divalent cations as well as monovalent cations. Nonselective cation current in guinea-pig ileal smooth muscle is blocked potently by Cd²⁺ and less potently by Ni²⁺, Mn²⁺, Co²⁺, and Mg²⁺ (Chen, Inoue & Ito, 1993). Nonselective cation current in canine pyloric circular smooth muscle, however, was reported to be blocked by internal Cs⁺ or by extracellular TEA (Vogalis & Sanders, 1990).

Unlike the Ca²⁺ and Cl⁻ channels described above, the muscarinic nonselective cation channel requires receptor occupation for opening. Muscarinic receptors in smooth muscle
are coupled to G proteins (for review, Berridge, 1993; Eglen et al., 1996). Of four receptors identified, M2 and M3 subtypes predominate in smooth muscle. Although M2 receptors are numerous in most smooth muscles, evidence points to muscarinic contraction being mediated by the M3 receptor (see above; for review, Eglen et al., 1996). M2 receptors are coupled to Gi / Go (sensitive to pertussis toxin), and M3 receptors are coupled to phospholipase C by Gs (which is insensitive to pertussis toxin). While the muscarinic nonselective cation channel is clearly not ligand-gated (like the nicotinic ACh-gated nonselective cation channel), selective pharmacological tools (Komori, Unno, Nakayama & Ohashi, 1998) and antibodies (see below) have been used to determine that the channel is activated by M2 receptors coupled to specific G proteins. Pretreatment of ileal smooth muscle cells with pertussis toxin prevents receptor-mediated activation of nonselective cation current (Inoue & Isenberg, 1990c), but does not prevent release of Ca\(^{2+}\) from SR and activation of K\(^+\) current (Komori, Kawai, Takewaki & Ohashi, 1992). Furthermore, dialysis of smooth muscle cells with the nonhydrolysable GTP analogue GTP\(_y\)S (introduced into cells using the whole-cell recording configuration) results in irreversible activation of inward cation current, even in the absence of agonist (Inoue & Isenberg, 1990a; Komori et al., 1992).

Recent pharmacological evidence supports the hypothesis that muscarinic nonselective cation channels are activated by M2 receptors (Zholos & Bolton, 1997b). This finding suggests an important role for M2 receptors in the regulation of membrane potential and excitation of smooth muscle. Also, in a recent study, non-selective cation current was inhibited in guinea-pig myocytes dialyzed with antibodies to G\(_{ao}\) but not G\(_{ai}\). The expression of G\(_{ao}\) in these cells was confirmed by Western blot analysis and therefore
these finding strongly support a role for \( G_0 \) in muscarinic regulation of nonselective cation current. Wang and others (1997) report in studies of horse airways, intracellular dialysis with antibodies directed at \( G_{11-1}/G_{11-2} \) and \( G_{11-3}/G_{11} \) abolished methacholine-induced nonselective cation current but not the \( \text{Ca}^{2+} \)-activated \( \text{Cl}^- \) current. Antibodies to \( G_q/G_{11} \) attenuated the \( \text{Ca}^{2+} \)-activated \( \text{Cl}^- \) current but not the non-selective cation current. They conclude that activation of \( M_2 \) receptors leads to opening of non-selective cation channels through \( G_i/G_o \) proteins in smooth muscle and that an increase in \([\text{Ca}^{2+}]_i\) is necessary but not sufficient to activate non-selective cation currents in these muscles.

Non-selective cation currents recorded in smooth muscle tend to desensitize with repeated muscarinic stimulation (Wade & Sims, unpublished observations; Kim, Sim, Jun, Kang, Suh, So, Kim, 1998). Kim and co-workers report that such desensitization is removed with high intracellular ethylene glycol-bis(\( \beta \)-aminoethyl ether)-N, M, N', N'-tetraacetic acid (EGTA) or inclusion of the PKC antagonist chelerythrine in the electrode. These findings were interpreted to mean that muscarinic desensitization of non-selective cation current arises, in part, from \( \text{Ca}^{2+} \)-dependent activation of PKC.

Cytosolic \( \text{Ca}^{2+} \) is also known to facilitate the muscarinic nonselective cation current. Inoue and Isenberg (1990c) were the first to demonstrate that \( \text{Ca}^{2+} \) influx through voltage-dependent \( \text{Ca}^{2+} \) channels facilitates the cation current, and such regulation has been confirmed by several other workers (e.g., Pacaud & Bolton, 1991). \( \text{Ca}^{2+} \) release from intracellular stores facilitates nonselective cation current (Komori & Bolton, 1990; Sims, 1992; Lee, Baguinov & Sanders, 1993), although receptor occupancy appears to be essential for channel opening. Facilitation of the cation current in ileal cells is estimated to be approximately half-maximal between 100 and 300 nM \([\text{Ca}^{2+}]_i\), with maximal facilitation
at greater than 1 μM [Ca\textsuperscript{2+}]; (Inoue & Isenberg, 1990b). The mechanism of Ca\textsuperscript{2+}-mediated facilitation is uncertain. Facilitation is specific for Ca\textsuperscript{2+}, since it is not mimicked by Ba\textsuperscript{2+} or Sr\textsuperscript{2+} (Inoue & Isenberg, 1990b). These authors also showed that facilitation is blocked by Ca\textsuperscript{2+} channel blockers D-600, nitrendipine or Cd\textsuperscript{2+}. However, a number of divalent cations also rapidly block nonselective cation current through direct interaction with the channel protein (Chen et al., 1993).

It is important to elaborate on studies of the permeability of nonselective cation channels to Ca\textsuperscript{2+}, as this issue is of great physiological relevance. Inoue and Isenberg (1990a) provided evidence that Ca\textsuperscript{2+} can permeate the ACh-activated non-selective cation channels comparably or more readily than Na\textsuperscript{+}. Loirand et al. (1991) also found that, in the absence of all extracellular Na\textsuperscript{+}, Ca\textsuperscript{2+} contributes to the ACh-activated nonselective cation current in rat portal vein. These observations provide evidence that, in the absence of extracellular Na\textsuperscript{+}, nonselective cation channels can provide entry pathways for Ca\textsuperscript{2+}. In regular Na\textsuperscript{+}-containing solution, Ca\textsuperscript{2+} has been estimated to contribute approximately 10% of the ACh-activated nonselective cation current (Inoue & Isenberg (1990a). Combined electrophysiological and fluorescence studies of [Ca\textsuperscript{2+}]; led Pacaud and Bolton (1991) to conclude that Ca\textsuperscript{2+} entry through ACh-activated cation channels was very small under normal conditions in smooth muscle. However, species and tissue differences may exist, as clear evidence for Ca\textsuperscript{2+} entry through muscarinic nonselective cation current was reported for tracheal muscle from airway myocytes (Fleischmann, Wang & Kotlikoff, 1997).

A common feature of muscarinic nonselective cation currents is that the current-voltage (I-V) relationships of the macroscopic currents are nonlinear (Benham, Bolton & Lang, 1985; Inoue, Kitamura & Kuriyama, 1987; Inoue & Isenberg, 1990a; Nakazawa, Inoue,
Fujimori & Takanaka, 1990; Vogalis & Sanders, 1990; Sims, 1992). ACh-induced current is largest between approximately -50 and -20 mV and decreases with depolarization and with hyperpolarization. The I-V relationship of the peak ACh-induced current reverses direction close to 0 mV and illustrates the non-linear behavior of the macroscopic currents. The voltage-dependence of gating of the ACh-activated cation current was investigated in guinea-pig ileum using voltage-jump experiments (Inoue & Isenberg, 1990a). The steady-state conductance-voltage relationship is described by a Boltzmann distribution with half-maximal activation at -50 mV (Inoue & Isenberg, 1990a). Wang and Sims (1998) report that pretreatment of cells with the neuropeptide cholecystokinin (CCK), which also activates non-selective cation current, causes an increase in ACh-mediated non-selective cation current in guinea-pig gastric smooth muscle. This effect was most apparent at more negative membrane potentials and appeared to be due to linearization of the current voltage relationship.

The unitary I-V relationships for nonselective cation channels is linear, with slope conductance of 20 to 25 pS in guinea-pig ileum (Inoue et al., 1987) and rabbit portal vein (Inoue & Kuriyama, 1993), 30 pS in canine pylorus (Vogalis & Sanders, 1990) and around 200 pS in rat portal vein (Loirand et al., 1991). Since the single channel I-V relationship is linear, the decrease in macroscopic current at negative potentials must be due to a decrease in channel open probability rather than the unitary channel conductance. Early studies of the kinetics of single channel currents at various potentials support this voltage-dependence of opening (Inoue et al., 1987), although further studies of other cell types are required to more generally test the validity of this model.
Recent studies have revealed several additional ways that nonselective cation channels are regulated. Zholos and Bolton (1994) demonstrated that the voltage-dependence of the nonselective cation current is influenced by the level of activation of G proteins. Extracellular protons have also been found to regulate gating of the channel, with acidification causing suppression of the current (Inoue, Waniishi & Ito, 1995; Zholos & Bolton, 1997a). Further studies are needed to understand the general nature and physiological significance of these types of regulation.

1.5.3.2 Protein kinase regulation of nonselective cation current

Many growth factors and some hormones exert their actions by binding to cell surface receptors with intrinsic protein tyrosine kinase activity. Effector molecules include PLC-γ, phosphatidylinositol 3-kinase, and GTPase activating protein. The cascade of events initiated by receptor tyrosine kinases may involve various non-receptor tyrosine kinases and influence diverse processes such as Ca\(^{2+}\) homeostasis and gene transcription. Growth factors have long been known to act on the gastrointestinal tract, not only regulating growth and differentiation of many cell types, but also acutely regulating smooth muscle contractility (Hollenberg, 1994). Recent studies reveal that tyrosine kinases influence ion channels.

Evidence for involvement of tyrosine kinases in muscarinic signalling in smooth muscle comes from a study of nonselective cation current in ileal muscle, where the tyrosine kinase blocker genistein, but not the inactive analog daidzein, reduces ACh-induced current (Inoue, Waniishi, Yamada & Ito, 1994). This finding led the authors to conclude that tyrosine phosphorylation may be involved in Ca\(^{2+}\) mobilization, thereby influencing
channels. Further studies to determine the role of tyrosine kinases in mediating actions of growth factors and neurotransmitters in smooth muscle are a high priority.

There is also evidence that calmodulin participates in the regulation of muscarinic nonselective cation current. In guinea pig gastric muscle cells, addition of calmodulin to the electrode solution prevents the run-down of this current that is typically observed during whole-cell recording (Kim, Ahn, So & Kim, 1995). The calmodulin antagonist W-7 reduced the nonselective cation current activated by receptor ligation, but did not reduce nonselective cation current activated by GTPγS. Kim and coworkers (1995) suggest that these findings explain in part the Ca\(^{2+}\)-sensitivity of the muscarinic nonselective cation channel.

1.5.4 Other nonselective cation channels

A number of other types of nonselective cation channels have been described in smooth muscle, with distinct forms of channel activation. For example, ATP-activated channels were first described in vascular smooth muscle by Benham and Tsien (1987). Benham later characterized the role of these ligand-gated channels in directly causing Ca\(^{2+}\) entry (Benham, 1989). Ca\(^{2+}\) entry through ATP-gated channels leads to contraction, as well as the regulation of a number of channels, including suppression of voltage-dependent Ca\(^{2+}\) channels (Schneider, Hopp & Isenberg, 1991; Nakayama, 1993). Extracellular nucleotides activate a class of ion channels known as P2X purinoceptors (for review, see North, 1996). P2X receptors were first cloned from smooth muscle (Valera, Hussy, Evans, Adam, Suprenant, North & Buel, 1994) and have since been demonstrated in several other cell types (North, 1996; Ugur, Drummond, Hui, Sheng, Singer & Walsh, 1997). Caffeine has also been reported to directly activate a ligand-gated nonselective cation, that permits Ca\(^{2+}\)
entry (Guerrero, Fay & Singer, 1994), but this action was distinct from the action of caffeine on ryanodine receptors.

Mechanosensitive (stretch-activated) channels have also been described in smooth muscle (Kirber, Walsh & Singer, 1988), and are reported to cause elevation of [Ca^{2+}], by both directly mediating Ca^{2+} influx and depolarizing cells, which causes opening of voltage-dependent Ca^{2+} channels (Wellner & Isenberg, 1994). In contrast to the channels described above, stretch-activated channels are not sensitive to [Ca^{2+}], since the Ca^{2+} chelator BAPTA did not affect the stretch current (Wellner & Isenberg, 1994). Hypotonic cell swelling potentiates the nonselective cation current in guinea-pig ileum (Waniishi, Inoue & Ito, 1997). It will be important to determine the mechanism of this effect and whether it occurs in other smooth muscles.

1.5.4.1 Physiological Roles of Nonselective Cation Channels
ACh-activated nonselective cation channels play a role in excitation-contraction coupling in smooth muscle in at least two ways. Since the reversal potential for nonselective cation currents is approximately 0 mV under physiological conditions, opening of these channels gives rise to inward (depolarizing) current, leading to activation of voltage-dependent Ca^{2+} channels, influx of Ca^{2+} and contraction (Cousins, Edwards, Hirst & Wendt, 1993; Lee et al., 1993). The voltage-dependence for activation, as well as the facilitation of muscarinic nonselective cation current by intracellular Ca^{2+}, provides positive feedback mechanisms to initiate or prolong depolarization, and thereby promote Ca^{2+} influx and contraction. Furthermore, Ca^{2+} may enter through nonselective cation channels, also contributing to contraction.
1.5.5 Future Directions of Inward Current Research

A number of the channels carrying inward current have been identified at the molecular level, including P2X purinoceptors and voltage-dependent Ca\textsuperscript{2+} channels. The properties of K\textsuperscript{+} channels are also emerging (see below, also Carl et al., 1996). Molecular characterization of Cl\textsuperscript{-} and nonselective cation channels remains to be achieved. Ion channels represent potential targets for therapeutic intervention in pathological states, such as hypertension or gastrointestinal dysmotility, so identification of smooth muscle-specific channels is an important area for further work.

Newer imaging techniques are revealing features of Ca\textsuperscript{2+} handling with relevance to regulation of ion channels. These include the demonstration of localized changes of [Ca\textsuperscript{2+}], Ca\textsuperscript{2+} sparks, which activates both K\textsuperscript{+} and Cl\textsuperscript{-} currents (Nelson et al., 1995, ZhuGe et al., 1998). Tantalizing details of regional localization of membrane transporters and the SR have been described (e.g. Moore, Etter, Philipson, Carrington, Fogarty, Lifshitz & Fay, 1993) as well as localized release of Ca\textsuperscript{2+} from the SR (Golovina & Blaustein, 1997). Further understanding of the subcellular organization of channels and Ca\textsuperscript{2+} release mechanisms are needed to understand the ways in which Ca\textsuperscript{2+} carries out such diverse roles in smooth muscle. For example, elevation of [Ca\textsuperscript{2+}], is excitatory, initiating contraction, yet causes activation of K\textsuperscript{+} channels, which is inhibitory. Details of the temporal and spatial organization of Ca\textsuperscript{2+} signalling may help to account for these diverse actions.

1.6 Outward Currents In Smooth Muscle

A variety of inwardly rectifying, voltage-, Ca\textsuperscript{2+}- and ATP-dependent K\textsuperscript{+} channels play key roles in controlling the excitability of smooth muscles (see reviews Quayle et al., 1997; Kuriyama, Kitamura, Ito & Inoue, 1998). K\textsuperscript{+} conductances are the predominant
conductance in smooth muscle and therefore strongly determine the membrane potential that in turn determines the level of \( \text{Ca}^{2+} \)-entry into cells via \( \text{Ca}^{2+} \) channels. Many \( K^+ \) channels in smooth muscle are regulated by neurotransmitters directly, via membrane-delimited signalling pathways (reviewed in Wickman & Clapham, 1995), or indirectly, by changes in intracellular \([\text{Ca}^{2+}]\) due to release of \( \text{Ca}^{2+} \) from stores (Janssen & Sims, 1997; Carl et al., 1996).

In visceral smooth muscles, including gastrointestinal (Vogalis & Sanders, 1991) and airway (Muraki, Imaizumi, Kojima, Kawai & Watanabe, 1990; Fleischmann, Washabau & Kotlikoff, 1993), channels from the delayed rectifier family (below) are thought to play an important role in the regulation of resting membrane potential. Studies of spontaneous transient outward currents suggest the large-conductance \( \text{Ca}^{2+} \)-dependent \( K^+ \) (\( K_{\text{Ca}} \)) channel can also participate in the regulation of membrane potential and excitability of smooth muscle (Nelson et al., 1995). There are few studies of inwardly rectifying (\( K_{\text{ir}} \)) and ATP-dependent \( K^+ \) channels (\( K_{\text{ATP}} \)) in visceral smooth muscles and therefore these conductances are only briefly considered below.

### 1.6.1 Molecular Basis of \( K^+ \) Channels

\( K^+ \) channels are encoded by a superfamily of potassium channel genes (reviewed in Jan & Jan, 1997), which may be sub-divided into large families containing voltage-dependent \( K^+ \) channels and inwardly rectifying \( K^+ \) channels. Voltage-dependent \( K^+ \) channels are formed by tetramers of \( \alpha \) subunits, each with 6 transmembrane spanning domains (S1-S6), with the region between the S5 and S6 domains contributing to pore formation. The known \( \beta \) subunits associated with the voltage-gated \( K^+ \) channels do not have sequences consistent with membrane spanning proteins, while the \( \beta \) subunits associated with the maxi \( K^+ \)
channels have 2 putative transmembrane spanning regions. Inwardly rectifying channels contain two transmembrane spanning regions (M1, M2) and a pore forming region. There are at least 6 distinct families of voltage-dependent K\(^+\) channels including *Shaker* (Kv1.1-1.7), *Shab* (Kv2.1,2.2), *Shaw* (Kv3.1-3.4) *Shal* (Kv4.1-4.3), *ether-a-go-go* (HERG) and *slopoke* (Ca\(^{2+}\)-dependent K\(^+\) channels).

Recent studies have determined the molecular identity of some voltage-dependent K\(^+\) channels from smooth muscle. mRNA for Kv1.1, 1.2 and 1.5 has been isolated from visceral and vascular smooth muscles (Hart, Overturf, Russell, Carl, Hume, Sanders & Horowitz, 1993; Overturf, Russell, Carl, Vogalis, Hart, Hume, Sanders & Horowitz, 1994; Adda, Fleischmann, Freedman, Yu, Hay & Kotlikoff, 1996) and Kv2.2 (*Shab*) has been cloned colonic smooth muscle (Schmalz, Kinsella, Koh, Vogalis, Schneider, Flynn, Kenyon & Horowitz, 1998). K\(_{Ca}\) channels have been cloned from vascular and visceral smooth muscles (McCobb, Fowler, Featherstone, Lingle, Saito, Krause & Salkoff, 1995; Vogalis, Vincent, Qureshi, Schmalz, Ward, Sanders & Horowitz, 1996).

### 1.6.2 Ca\(^{2+}\)-Dependent K\(^+\) Channels

The large conductance Ca\(^{2+}\)-dependent K\(^+\) (K\(_{Ca}\)) channel has been demonstrated in a variety of smooth muscles (see reviews, Tomita, 1988; Walsh & Singer, 1987, Quayle et al., 1997). While some charybdotoxin-insensitive (ChTX) intermediate conductance K\(_{Ca}\) channels have been observed in smooth muscle, little is known about these channels and therefore the discussion here is limited to the maxi- or large conductance Ca\(^{2+}\)-dependent K\(^+\) channel.
The central pore forming protein of the $K_{Ca}$ channel is assembled as a heteromultimer from the $Slo$ gene. Although there is only one gene encoding $K_{Ca}$, there are multiple splice variants (Beech, 1997) possibly explaining some of the heterogeneity observed in native $K_{Ca}$ channels from regions of tissues and organs. The S4 transmembrane spanning region is homologous with the voltage-sensor region of channels from the $Shaker$ gene family, and gives the channel its voltage-dependent characteristics.

The channel’s unitary conductance (>100 pS in physiological $[K^+]$ gradients), voltage-dependence and sensitivity to cytosolic free $[Ca^{2+}]$ ([Ca$^{2+}$]i) is well documented (Benham, Bolton, Lang & Takewaki, 1986; Carl, McHale, Publicover & Sanders, 1990; reviewed in Carl et al., 1996; Singer & Walsh, 1987). The channel is believed to be voltage-gated, with $Ca^{2+}$ serving to shift the voltage-activation range to less positive membrane potentials. Such sensitivity permits the channel to open when $Ca^{2+}$ is elevated at the cytosolic face but without a change in the membrane potential. $K_{Ca}$ channels from different tissues share similar voltage-dependence, conductance, and pharmacological sensitivity. Notably however, the sensitivity of $K_{Ca}$ channels to [Ca$^{2+}$]i varies among tissues, regions, organs and species (Carl et al., 1996). The channel is blocked by 1 to 100 nM charybdotoxin (ChTX) or iberiotoxin (IbTX) and 0.1 to 10 mM TEA at the extracellular side.

1.6.2.1 Regulation of $K_{Ca}$

$K_{Ca}$ channel activity in smooth muscles at rest where the macroscopic $[Ca^{2+}]$, is ~100 nM and membrane potential is -40 to -70 mV is expected to be virtually non existent. However, recent findings suggest the regulation of $K_{Ca}$ by stores permits the channels to contribute outward current under these conditions (Nelson et al., 1995). Heretofore, it was difficult to explain how the regulation of $K_{Ca}$ channels in smooth muscle could cause their
physiologic effects. Now, such physiologic regulation including for example, small changes in the current voltage relationship for the channel, can be considered in terms of the effect on channels activated by \(\text{Ca}^{2+}\) sparks at physiologic membrane potentials (e.g. -40 to -70 mV).

\(\text{K}_{\text{Ca}}\) channels are activated by cAMP-dependent protein kinase (reviewed in Beech 1997) indicating that adenylyl cyclase activity participates in regulation of the channel. Satake and colleagues found that NKH477, a forskolin derivative, caused relaxation of guinea-pig trachealis pre-constricted with histamine (Satake, Takagi, Kodama, Honjo, Toyama & Shibata, 1998). This effect was antagonized by IbTX and TEA, suggesting the involvement of \(\text{K}_{\text{Ca}}\) channels. Nifedipine, a blocker of L-type \(\text{Ca}^{2+}\) channels, prevented the IbTX mediated inhibition of NKH477 actions on relaxation of the muscle. These results indicate that NKH477 causes relaxation of muscle by both hyperpolarization and other mechanism(s). Isoprenaline, forskolin and dibutyryl cAMP increase \(\text{K}_{\text{Ca}}\) open probability in single aortic smooth muscle cells, an effect observed in many smooth muscles and likely due to direct phosphorylation of the channel (Beech, 1997). To that point, okadaic acid, a phosphatase inhibitor, increases single \(\text{K}_{\text{Ca}}\) channel open probability in excised inside out patches from rabbit gastric antrum (Lee, Bang, Lim, Uhm, Rhee, 1994) and rabbit trachea (Kume, Takai, Tokuno & Tomita, 1989). Evidence also exists for direct regulation of \(\text{K}_{\text{Ca}}\) by the \(\alpha\) subunit of \(\text{G}_{\text{s}}\) (Beech, 1997).

Evidence that the free radical nitric oxide- (NO') mediated relaxation arises from the activation of \(\text{K}_{\text{Ca}}\) channels is supported by studies in which ChTX and IbTX antagonize the effects NO' on smooth muscle (Beech, 1997). While some studies support roles for cGMP-mediated activation of cGMP dependent protein kinase in the activation of \(\text{K}_{\text{Ca}}\),
other studies suggest that GMP and NO can activate $K_{Ca}$ channels directly, possibly due to redox state effects on the channel (Beech, 1997).

Evidence that protein kinase C inhibits $K_{Ca}$ channel activity is found in studies in which DAG and phorbol esters reduced $K_{Ca}$ channel activity and staurosporin, a non-specific PKC inhibitor, activated $K_{Ca}$ channel activity in the presence of PKC activators (Beech, 1997).

1.6.2.2 Muscarinic Regulation of $K_{Ca}$

There have been several reports of the regulation of $K_{Ca}$ currents in smooth muscle by neurotransmitters (Toro & Stefani, 1991). ACh reduces $K_{Ca}$ current in several smooth muscle cell types (reviewed in Sims & Janssen, 1997), an effect caused by shifting the voltage-activation range of the current to more positive membrane potentials. Studies of the receptor coupling mechanisms responsible for regulating $K_{Ca}$ in which non-hydrolyzable GTP analogues (e.g. GTPγS or GppNHp) cause an irreversible shift in the voltage-activation range, and pertussis toxin abolishes the effects of ACh suggest that a pertussis sensitive G protein is responsible for membrane delimited signalling between the muscarinic receptor and $K_{Ca}$ channel. Non-membrane delimited regulation is considered below in the discussion of the role of intracellular stores in the regulation of $K_{Ca}$ channels.

1.6.2.3 Regulation of $K_{Ca}$ by Intracellular Ca$^{2+}$ Stores

The sensitivity of $K_{Ca}$ channels to [Ca$^{2+}$]$_i$ combined with the presence of significant intracellular Ca$^{2+}$ stores in smooth muscle offers the opportunity for stores to influence $K_{Ca}$ channel activity. Such regulation may come about due to excitatory signalling, resulting in release of Ca$^{2+}$ from stores, or spontaneous release of Ca$^{2+}$ from intracellular stores (Sims & Janssen, 1997). A clear example of the potential for excitatory signalling of $K_{Ca}$ channels through stores is provided in studies by Muraki and co-workers (1990) on bladder
and tracheal smooth muscle using cells permeabilized with β-escin. They demonstrated that ACh- and substance P- mediated increases in \(K_{Ca}\) channel activity was mimicked by introduction of \(Ca^{2+}\) or IP\(_3\) into the cytosol. These findings are consistent with excitatory ligands causing elevation of \([Ca^{2+}]_i\) due to release of \(Ca^{2+}\) from intracellular stores by the cytosolic second messenger IP\(_3\).

### 1.6.2.4 Physiological Roles of \(K_{Ca}\)

This channel type has been suggested to play a role in repolarization of the membrane of smooth muscle following action potentials (Carl & Sanders, 1990; Walsh & Singer 1987) and more recently in causing relaxation of arteriolar smooth muscle (Nelson et al., 1995). Localized elevation of \(Ca^{2+}\) activates \(K_{Ca}\) leading to hyperpolarization of cells reducing \(Ca^{2+}\) entry by L-type \(Ca^{2+}\) channels. The role of \(K_{Ca}\) channels in smooth muscle has been investigated using the pharmacological antagonist iberiotoxin (IbTX). IbTX causes phasic contractions of increased force and frequency in human myometrium (Carl et al., 1996). The role of intracellular \(Ca^{2+}\) stores in the physiological regulation of \(K_{Ca}\) channels is suggested by studies in which depolarization of cells from the longitudinal layer of the colon caused transient outward currents that were inhibited both by TEA and pretreatment of cells with ryanodine (Carl et al., 1996). Ryanodine pre-treatment would be anticipated to prevent release of \(Ca^{2+}\) from intracellular stores. In summary, \(K_{Ca}\) channels can participate in both the regulation of resting membrane potential and negative feedback during excitation of smooth muscle.

### 1.6.3 Delayed Rectifier Currents (\(K_V\))

Delayed rectifier currents have been described in a variety of vascular and visceral smooth muscles. The currents carry the majority of outward current in smooth muscles and
therefore are believed to strongly participate in regulation of resting membrane potential, maintenance of slow wave activity and repolarization following action potentials (see Chapter 4 below, Beech, 1997). There are fewer studies of the regulation of Kv currents by neurotransmitters as compared to the regulation K_Ca channels. Molecular and electrophysiological techniques were used by Huang and coworkers (1993) to demonstrate that muscarinic suppression of delayed rectifier K^+ channels is mediated by both Ca^{2+} and diacylglycerol. Surprisingly however, tyrosine phosphorylation of the channels also occurs, leading the authors to conclude that muscarinic signalling involved non-receptor tyrosine kinase(s), which may be activated by increased [Ca^{2+}]. M-current, a non-inactivation delayed rectifier from amphibian smooth muscle is inhibited by ACh, substance P and isoprenaline (Sims, Singer, & Walsh, 1988; Sims, Clapp, Walsh, & Singer, 1990a). Histamine, isoprenaline, vasoactive intestinal peptide and angiotensin II also regulate Kv currents (Beech, 1997). Kv currents are activated by hypoxia, cytosolic ATP and cAMP-dependent protein kinase, and inhibited by PKC (see chapter 4 below, Beech, 1997).

1.6.4 Inward Rectifier K^+ Currents

The molecular basis and roles of inwardly rectifying K^+ channels in smooth muscle are reviewed by Quayle and co-authors (1997). To date, these channels have only been described in vascular smooth muscle. The IV relationship shows strong inward rectification, a characteristic that is abolished with extracellular Ba^{2+}. These currents are suggested to potentially play a significant role in the maintenance of resting membrane potential, a hypothesis that is supported by the observation that Ba^{2+} causes depolarization of segments of vascular smooth muscle (reviewed in Quayle et al., 1997). The presence and role of inward rectifiers in other smooth muscles remains to be determined.
1.6.5 ATP-Sensitive K+ Currents

ATP-sensitive K+ channels have been identified with small to medium conductances (7-50 pS) and large conductances (100-258 pS) (reviewed in Quayle et al., 1997). $K_{\text{ATP}}$ channels are identified by their inhibition by ATP, activation by metabolic inhibition, nucleoside diphosphates and sensitivity to K+ channel openers and glibenclamide. These channels are insensitive to membrane potential. The sensitivity of the channels to blockade by cytosolic ATP makes them sensitive to the metabolic state of the cell, thus underlying their probable physiological role. Inhibition of cellular metabolism causes an increase in $K_{\text{ATP}}$ activity in a variety of tissues (Quayle et al., 1997). $K_{\text{ATP}}$ channels are also sensitive to agents that cause PKA, cGMP and PKC dependent effects on smooth muscle. PKA dependent vasodilators including CGRP, prostacyclins, β-adrenergic agonists, VIP and adenosine have been found to activate $K_{\text{ATP}}$ (Quayle et al., 1997). Such regulation represents the convergence of second messenger signalling initiated by membrane receptors coupled to Gs and adenylyl cyclase.

1.7 Rational And Objectives of the Research

Depolarization, elevation of $[\text{Ca}^{2+}]_i$ and contraction are the predominant features of muscarinic excitation of smooth muscle. However, ACh-mediated release of Ca$^{2+}$ from intracellular stores can regulate a variety of membrane conductances, some of which could lead to effects that limit depolarization and contribute to recovery of muscle (Fig. 2).
Figure 2. Schematic diagram showing some of the molecular participants involved in regulating excitation and recovery of smooth muscle. Binding of ACh to M2 receptors (MACHR2) permits opening of nonselective cation channels, causing inward current that depolarizes the cell. Binding of ACh to M3 receptors (MACHR3) causes generation of IP$_3$ that elicits release of Ca$^{2+}$ from intracellular stores by opening of IP$_3$ receptors (IP$_3$R). Opening of nonselective cation channels is facilitated by depolarization and elevation of [Ca$^{2+}$]. Depolarization of cells leads to opening of L-type Ca$^{2+}$ channels, causing Ca$^{2+}$ entry, which further facilitates nonselective cation channels and supports contraction. Ca$^{2+}$ released from intracellular stores may regulate membrane currents sensitive to [Ca$^{2+}$]$_i$ (K$_{Ca}$, L-type Ca$^{2+}$ channels). K$_{Ca}$ channels and delayed rectifier K$^+$ channels may contribute to recovery of cells from ACh-mediated excitation. Channels are shown as tetramers for the purpose of simplicity only.
We examined the functional contribution of $K^+$ and $Ca^{2+}$ currents by studying the effects of $K^+$ and $Ca^{2+}$ channel antagonists on contraction of esophageal muscle strips. We investigated the acute regulation of $Ca^{2+}$ current by acetylcholine in guinea-pig gastric smooth muscle cells. ACh has been reported to cause both an increase (Clapp et al., 1987; Kamishima et al., 1992; Tomasic et al., 1992) and decrease (Ozaki, Zhang, Buxton, Sanders, & Publicover, 1992) in L-type $Ca^{2+}$ currents in smooth muscles. We tested the hypothesis that ACh acutely inhibited L-type $Ca^{2+}$ channels in freshly isolated guinea-pig gastric smooth muscle cells. ACh caused contraction and transient inhibition of both macroscopic $Ca^{2+}$ currents and single $Ca^{2+}$ channels. This inhibition was abolished by persistent bathing of cells in $Ca^{2+}$-free solution or exposure to cyclopiazonic acid, an inhibitor of SR $Ca^{2+}$-ATPase. We concluded that ACh inhibits $Ca^{2+}$ currents and that this effect is dependent upon release of $Ca^{2+}$ from intracellular stores. Such regulation of $I_{Ca}$ by $Ca^{2+}$ stores is an example of negative feedback signalling that may limit $Ca^{2+}$ entry during muscarinic excitation of smooth muscle.

The aim of the second study, Chapter 3, was to investigate regulation of the large-conductance $Ca^{2+}$-dependent $K^+$ channel by ACh in canine tracheal smooth muscle cells. ACh depolarizes airway smooth muscle (Janssen & Sims, 1992, Janssen & Sims, 1993b) and causes elevation of $[Ca^{2+}]_i$ due to release of $Ca^{2+}$ from intracellular stores. Depolarization and elevation of $[Ca^{2+}]_i$ could lead to activation of $K_{Ca}$ channels. We tested the hypothesis that ACh activates $K_{Ca}$ channels in airway smooth muscle cells. ACh caused a transient increase in $K_{Ca}$ activity, an effect that was mimicked by caffeine and occurred in the absence of extracellular $Ca^{2+}$. We concluded that ACh increases $K_{Ca}$ activity by elevation of $[Ca^{2+}]_i$ due to release of $Ca^{2+}$ from intracellular stores. Such
regulation of $K_{Ca}$ channels by Ca$^{2+}$ stores is an example of negative feedback that may contribute to limiting depolarization during muscarinic excitation of smooth muscle.

Little is known of the expression and contribution of outward currents in excitation contraction coupling in human esophageal muscles. In our third study, Chapter 4, we took the opportunity investigate a novel smooth muscle system and characterized macroscopic currents from human esophageal muscle. We characterized $K_{Ca}$ and $K_v$ currents in human esophageal smooth muscle cells and tested the hypothesis that these currents participated in regulation of contraction of smooth muscle strips by blocking the currents with pharmacological antagonists. We found that the delayed rectifier current appears to play a dominant role in regulating resting tension of esophageal muscle, whereas $K_{Ca}$ current serves to limit contraction during excitation. These studies provide the first description of these currents and their roles in the contraction of human esophageal smooth muscle.

1.8 References


PEMBERTON, K.E. & JONES, S.V.P. (1997). Inhibition of the L-type calcium channel by the five muscarinic receptors (m1-m5) expressed in NIH 3T3 cells. *Pflügers Archives* 433, 505-514.


ZONG, X., & HOFMANN, F. (1996). Ca\textsuperscript{2+}-dependent inactivation of the class C L-type Ca\textsuperscript{2+} channel is a property of the \(\alpha_{1a}\) subunit. *FEBS Letters*. 378, 121-125.
CHAPTER 2

2 CHOLINERGIC INHIBITION OF CA$^{2+}$ CURRENT IN GUINEA-PIG GASTRIC AND TRACHEAL SMOOTH MUSCLE CELLS

This chapter has been adapted from;


Reproduced here with permission from The Journal of Physiology.

G. Wade designed experiments, recorded and analyzed whole-cell currents and cell length and prepared the figures. Based upon G. Wade’s findings, a summer student, J. Barbera recorded and analyzed single Ca$^{2+}$ channels. All experiments were carried out within S. Sims’ laboratory who co-wrote the publication with G. Wade.
2.1 Introduction

Acetylcholine (ACh) acts on muscarinic receptors in many smooth muscles to cause depolarization and contraction (Bolton, 1981). Several conductance changes contribute to this depolarization, including activation of nonselective cation or chloride channels and suppression of K+ channels (Sanders, 1989; Sims and Janssen, 1993). Depolarization of smooth muscle causes opening of voltage-dependent Ca2+ channels, leading to increased cytosolic free Ca2+ concentration ([Ca2+]i) (e.g., Becker, Singer, Walsh & Fay, 1989; Schneider, Hopp & Isenberg, 1991; Vogalis, Publicover & Sanders, 1992).

Muscarinic receptors belong to the class of seven membrane-spanning receptors that act through heterotrimeric G proteins. The M1, M3 and M5 subtypes activate phospholipase C-β and initiate hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (Berridge, 1993). IP3 activates receptors on intracellular stores (sarcoplasmic reticulum in muscle, SR) to release Ca2+ while DAG activates protein kinase C (PKC) to initiate phosphorylation of a variety of substrates. Considerable evidence implicates these signalling pathways in excitation of smooth muscle (Somlyo & Somlyo, 1994).

[Ca2+]i regulates the activity of many types of ion channels (Hille, 1992). For example, elevation of [Ca2+]i due to Ca2+ entry facilitates the muscarinic nonselective cation current in smooth muscles (Inoue & Isenberg, 1991) while Ca2+ released from stores is sufficient to activate Cl- and K+ channels in tracheal smooth muscle (Janssen & Sims, 1992; 1993a; Wade & Sims, 1993). L-type Ca2+ channels are well known to be inhibited by increased [Ca2+]i (reviewed in Eckert & Chad, 1984; McDonald, Pelzer, Trautwein & Pelzer, 1994).
In smooth muscle, both IP$_3$-mediated release of Ca$^{2+}$ from stores and agonist-mediated entry of Ca$^{2+}$ suppressed Ca$^{2+}$ current (Komori & Bolton, 1991, Schneider et al., 1991).

ACh has been reported to increase Ca$^{2+}$ current in some smooth muscles, including gastric (Clapp, Vivaudou, Walsh & Singer, 1987) and airway types (Kamishima, Nelson & Patlak, 1992; Tomasic, Boyle, Worley & Kotlikoff, 1992). However, studies of cholinergic actions on intact muscle have led other workers to suggest that ACh reduces Ca$^{2+}$ entry (Ozaki, Zhang, Buxton, Sanders, & Publicover, 1992). Although not the primary focus of their studies, Inoue & Isenberg (1990) and Lee, Bayguinov and Sanders (1993) reported that ACh reduced Ca$^{2+}$ current in ileal and colonic muscle cells, a finding recently confirmed in ileal smooth muscle cells (Unno, Komori & Ohashi, 1995). Thus, the regulation of Ca$^{2+}$ channels by ACh remains controversial. Our previous studies have shown that the Ca$^{2+}$ released from stores in smooth muscle is sufficient to regulate both contraction and ion channel activity (Sims, 1992a; Janssen & Sims, 1992; 1993a; Wade & Sims, 1993). The objectives here were to investigate the acute regulation of Ca$^{2+}$ channels by ACh in gastric and tracheal smooth muscle cells. We used the perforated patch and cell-attached patch configurations to maintain cytosolic second messenger systems and Ca$^{2+}$ stores intact.

2.2 Methods

2.2.1 Isolation of single smooth muscle cells

Muscle cells were isolated from guinea-pig corpus and trachea using methods essentially as described previously (Janssen & Sims, 1993a,b; Sims, 1992a), but with several modifications. Guinea-pigs (200-300 g, either sex) were killed by stunning and then bleeding. Trachealis and stomach were removed, dissected and strips of muscle were
placed into 2.5 ml of dissociation solution (see below for composition) containing (for tracheal and gastric muscle, respectively) collagenase (0.4 mg/ml; blend type F, Sigma), papain (2.5 and 1 mg/ml; type IV, Sigma), bovine serum albumin (2 and 1.2 mg/ml; ICN Biomedicals Inc., Cleveland, OH, USA), and 1,4-dithio-L-threitol (0.4 and 0.1 mg/ml; Sigma). Tracheal tissues were first stored at room temperature 22 to 25°C for 60 minutes and then placed in a gently shaking water bath at 31°C for 60 to 90 minutes or stored at 4°C and dispersed 24 hrs later. Tissues stored at 4°C required only 20 to 40 min for dispersion. Gastric cells were maintained in the enzyme solution for 45 minutes at room temperature and then placed into the water bath at 31°C for 45 to 60 minutes, after which cells were dispersed by trituration with fire-polished Pasteur pipettes. Cells were studied within 8 hrs of dispersion.

2.2.2 Patch-clamp recording
All recordings were performed at room temperature (22 to 25°C). Whole-cell recordings of membrane potential or current employed the nystatin-perforated patch technique (Horn & Marty, 1988), and single channels were recorded in the cell-attached patch configuration, using an Axopatch 1D amplifier (Axon Instruments). Recording of whole-cell Ca\(^{2+}\) currents was initiated when the access resistance had stabilized at <40 MΩ, while Ca\(^{2+}\) channel currents (Na\(^{+}\) as the charge carrier) were recorded with access resistance <20 MΩ and using 70% series resistance compensation. Currents were filtered at 500 Hz and recorded at 2 kHz and. Capacitive currents were compensated for online using the amplifier circuitry. Peak inward Ca\(^{2+}\) current occurred approximately 50 ms after initiation of the voltage jumps which was well after the settling of capacitive current. In some cases the data has been corrected for leakage, as assessed at negative potentials. Cells were
observed in a bath perfusion chamber (1 ml volume, perfused at approximately 1 to 3 ml/min) with a Nikon diaphot phase contrast microscope. Contraction of cells was recorded using a charge coupled device camera and video recorder. Contraction of cells was quantified off-line from the video recording as described previously (Sims, 1992a). Data are presented as mean ± standard error of the mean. When responding cells indicated (n of n cells), non-responders showed neither an increase or decrease in the activity indicated.

2.2.3 Solutions
Dissociation solution contained the following (mM): NaCl, 125; KCl, 5; CaCl₂, 0.25; MgCl₂, 1.0; HEPES, 10; EDTA, 0.25; D-glucose, 10; taurine, 10, pH set to 7.0 with NaOH. For whole-cell recordings cells were bathed in Ringer solution containing (mM): NaCl, 130; KCl, 5; CaCl₂, 1; MgCl₂, 1; HEPES, 20; D-glucose, 10; pH set to 7.4 with NaOH. To prepare Ca²⁺-free bathing solution, CaCl₂ and MgCl₂ were omitted and 0.25 mM EGTA was added. Macroscopic Ca²⁺ currents were recorded using electrode solution containing (mM): CsCl, 130; EGTA, 1; HEPES, 20; MgCl₂, 1; TEA, 10; pH set to 7.2 with CsOH, with 250 ng/ml nystatin. Action potentials were recorded with the electrode containing (mM): KCl, 140; EGTA, 1; HEPES, 20; MgCl₂, 1 (pH set to 7.2 with KOH); and with 250 ng/ml nystatin. For single Ca²⁺ channel studies, cells were bathed in a solution in which NaCl was replaced with KCl and the electrode contained (mM): BaCl₂, 90; EGTA, 10; HEPES, 20; pH adjusted to 7.4 with N-methyl-D-glucamine free base. Test agents were prepared from stock solutions, diluted into the appropriate bathing solution and applied focally to cells (Picospritzer II, General Valve Corp., Fairfield, NJ, USA). All chemicals were from Sigma or BDH unless otherwise stated. Acetylcholine was prepared as a 20 mM
aqueous stock solution. Cyclopiazonic acid (CPA), nifedipine (Research Biochemicals) and phorbol 12, 13-dibutyrate (PdBu) was each prepared as a 1 mM stock solution in dimethylsulfoxide (DMSO). Bay K 8644 (Research Biochemicals) was prepared as a 1 mM stock solution in 95% ethanol. Chelerythrine was prepared as a 400 μM aqueous stock solution and bisindolyl-maleimide was prepared from a 2.4 mM stock solution in DMSO (both from Calbiochem).

2.3 Results
2.3.1 Action potentials elicit contraction of gastric smooth muscle cells.
Current-clamp recording revealed resting membrane potentials of gastric cells between -40 mV and -50 mV. Cells did not display spontaneous action potentials but action potentials could be elicited with depolarizing current. Action potentials were accompanied by reversible contraction (12 cells) that was graded, with more prolonged trains of action potentials initiating greater cell shortening. Action potentials in gastric smooth muscle are largely due to voltage-dependent Ca^{2+} channels (Sanders, 1989; Sims, 1992b), and therefore these results provide evidence that Ca^{2+} entry causes elevation of [Ca^{2+}]; which is sufficient to initiate contraction of these cells.

2.3.2 L-type calcium channel current in gastric smooth muscle.
We will briefly describe the characteristics of L-type Ca^{2+} channel currents (I_{Ca}) of gastric smooth muscle cells recorded in the perforated-patch configuration with Ca^{2+} or Na^{+} as charge carriers (Fig. 1). With K^+ currents blocked using Cs^+ electrode solution, depolarization from -60 mV to potentials between -40 mV and 20 mV elicited transient inward currents (Fig. 1) which were blocked by nifedipine and enhanced by Bay K 8644 (8 cells, not shown). To investigate the regulation of Ca^{2+} channels in the absence of
extracellular Ca\textsuperscript{2+}, we removed the divalent cations Ca\textsuperscript{2+} and Mg\textsuperscript{2+} from the bathing solution. Under these conditions, Na\textsuperscript{+} permeates the channel (McDonald \textit{et al.}, 1994).

These currents, which we will refer to as Ca\textsuperscript{2+} channel currents, inactivated less rapidly (Fig. 1A, inset) and were typically 10 to 20 times larger than Ca\textsuperscript{2+} currents recorded in the same cells (Fig. 1B). Removal of the extracellular divalent cations Ca\textsuperscript{2+} and Mg\textsuperscript{2+} was also accompanied by a negative shift in the current-voltage relationship of the Ca\textsuperscript{2+} channel current, consistent with cations screening surface charge (Hille, 1992; McDonald \textit{et al.}, 1994).

Ca\textsuperscript{2+} currents and Ca\textsuperscript{2+} channel currents showed similar voltage dependence of activation and inactivation, with the exception that Ca\textsuperscript{2+} current demonstrated some recovery from inactivation with more positive prepulses (Fig. 1C). This finding, together with the differences in the rate of inactivation (above), is consistent with Ca\textsuperscript{2+}-dependent inhibition of Ca\textsuperscript{2+} current (Ganitkevich, Shuba, & Smirnov, 1987; McDonald \textit{et al.}, 1994). In summary, Ca\textsuperscript{2+} currents recorded from gastric smooth muscle cells using the perforated patch technique were qualitatively similar to those described previously in gastric cells studied using conventional whole-cell recording (Katzka & Morad, 1989; Noack, Deitmer & Lammel, 1991; Sims, 1992b). We note that Ca\textsuperscript{2+} currents described here are somewhat smaller in magnitude than others have reported. This difference in the size of the currents appears to be due to the recording configurations, since we also record larger Ca\textsuperscript{2+} currents in guinea-pig gastric smooth muscle cells when using conventional whole-cell recording.
Figure 1. L-type calcium channel current in gastric smooth muscle with 1 and 0 mM Ca\(^{2+}\) solutions. 

**A, Left:** depolarization from -60 mV to membrane potentials between -40 and 10 mV (above) elicited inward Ca\(^{2+}\) current with peak inward current occurring at 10 mV; **Right:** Ca\(^{2+}\) channel currents (0 Ca\(^{2+}\), 0 Mg\(^{2+}\), 0.25 mM EGTA, Na\(^+\) as the charge carrier) were 10 to 20 times larger than Ca\(^{2+}\) currents in the same cells, with peak inward current occurring at -10 mV. The shift in the current-voltage relationship to more negative potentials is consistent with that expected for a divalent cation-free bathing solution. **Inset:** peak Ca\(^{2+}\) (▲) and Ca\(^{2+}\) channel (■) currents were scaled to equal levels and overlapped showing a more rapid time course of inactivation when Ca\(^{2+}\) was the charge carrier. 

**B, Current-voltage relationship of Ca\(^{2+}\) current (left) and Ca\(^{2+}\) channel current (right).** 

**C, Voltage dependence of inactivation (I/I\(_{\text{max}}\) , ○) and conductance (■) are plotted for Ca\(^{2+}\) current (left) and Ca\(^{2+}\) channel current (right).** Voltage dependence of inactivation was determined with prepulses from -80 to 20 mV (duration 1024 ms) followed by test pulses to 0 mV. Data plotted are peak inward currents elicited by the test pulse divided by maximum peak inward current occurring during the test pulses (3 cells). Half-maximal activation of Ca\(^{2+}\) and Ca\(^{2+}\) channel current occurred at -7 and -22 mV respectively.
2.3.3 Acetylcholine causes a reduction in Ca\textsuperscript{2+} current.

ACh (50 μM, 30 s) caused a reduction in peak Ca\textsuperscript{2+} current in gastric cells by 37 ± 3% (36 of 43 cells) when Ca\textsuperscript{2+} currents were elicited both by step depolarization (Fig. 2) or by applying a linearly varying potential, (voltage ramp protocol, see below). ACh also caused inward current which was more apparent upon repolarization of cells to -60 mV (Fig. 2B, middle). This inward tail current is consistent with the muscarinic non-selective cation current previously characterized in several smooth muscles (Inoue & Isenberg, 1990; Sims, 1992a; Lee et al., 1993; G. Wade & S.M. Sims, unpublished observations). The onset of ACh-mediated inhibition was rapid, with approximately half of the total effect occurring within 5 s of stimulation (Fig. 2C, and see below). Peak inhibition occurred within 40 s of the onset of ACh, with Ca\textsuperscript{2+} currents recovering to approximately 50% of control levels after 2 to 3 minutes (Fig. 2C). ACh did not cause a consistent change in the time course of inactivation of Ca\textsuperscript{2+} currents (Fig. 2D). We have observed similar acute reduction of Ca\textsuperscript{2+} current with a lower concentration of ACh (10 μM, more than 20 cells) and in several other smooth muscle types, including canine gastric and canine and guinea-pig tracheal muscles (see below), indicating the widespread nature of this phenomenon.
Figure 2. Acetylcholine inhibits Ca\(^{2+}\) current in gastric smooth muscle A, step depolarization from -60 to 0 mV elicited Ca\(^{2+}\) currents (shown expanded in B). ACh (30 s, 50 μM) reduced peak Ca\(^{2+}\) current (→) and activated an inward current apparent upon repolarization to -60 mV (tail current, *). B, selected Ca\(^{2+}\) currents shown leak corrected on an expanded scale for control (a), ACh (b), and recovery (c), showing the reduction in peak inward current and the inward tail current elicited by ACh. C, the time course of reduction in peak inward Ca\(^{2+}\) current is displayed with open symbols indicating current levels for traces expanded above. D. ACh caused little change in the time course of inactivation of Ca\(^{2+}\) current as apparent from currents that were scaled and overlapped.
We investigated the possibility that ACh was shifting the current-voltage relationship of Ca\(^{2+}\) currents by using voltage ramps from -100 to 50 mV. ACh inhibited Ca\(^{2+}\) current in the presence and absence of Ca\(^{2+}\) over the entire voltage activation range, with no shift in the current-voltage relationship (Fig. 3, representative of results in 15 cells).

### 2.3.4 Mechanisms Underlying Cholinergic Regulation of Ca\(^{2+}\) Current.

Muscarinic suppression of Ca\(^{2+}\) current could involve DAG activation of PKC, as suggested earlier from tissue studies of gastric smooth muscle (Ozaki et al., 1992). When the PKC activator phorbol 12, 13-dibutyrate (PdBU) was applied to single muscle cells (2 nM to 1 µM, 30 s, 14 cells, bath solution 1 mM Ca\(^{2+}\)), I\(_{Ca}\) was only reduced by 10 to 20%. This effect was rapid in onset (peak effect within 20 s), showed recovery within ~1 minute, and failed to affect subsequent inhibition of I\(_{Ca}\) by ACh (not shown). To further investigate a role for PKC, we pretreated cells for 90 s with the selective PKC inhibitors chelerythrine (2 µM) or bisindolylmaleimide (50 nM). Neither of these inhibitors prevented ACh-induced inhibition of I\(_{Ca}\), with mean reduction of 35 ± 6 % (n=5) in response to ACh. Furthermore the time course of onset and recovery was not different from control responses. Although these negative results do not allow us to totally discount the participation of PKC in this muscarinic signalling, these findings are inconsistent with a mechanism in which PKC is the predominant mediator of muscarinic inhibition of I\(_{Ca}\).

We next investigated the participation of Ca\(^{2+}\) stores in muscarinic inhibition of Ca\(^{2+}\) current by removing extracellular Ca\(^{2+}\). Cells were first bathed in solutions containing 1 mM Ca\(^{2+}\) and then the bathing solution was exchanged for Ca\(^{2+}\)-free solution. Initially, Ca\(^{2+}\) current was accompanied by contraction of cells (Fig. 4A, below, 12 cells).
Figure 3. Acetylcholine inhibits $I_{\text{ca}}$ over all potentials. Membrane potential was varied linearly between -100 and +50 mV (voltage ramp protocol, 1 s duration). A, In 1 mM Ca$^{2+}$, ACh (30s, 50 μM) reduced $I_{\text{ca}}$ over the entire range of membrane potentials during which Ca$^{2+}$ channels were open. B, ACh also inhibited Ca$^{2+}$ channel current in 0 Ca$^{2+}$ over the range of potentials during which Ca$^{2+}$ channels were open. Traces were leak corrected (see methods).
Figure 4. \( \text{Ca}^{2+} \) currents and contraction of smooth muscle cells in 1 and 0 mM \( \text{Ca}^{2+} \) solutions. Membrane currents and cell length are shown over two panels (A & B) for the same cell. Depolarization from -60 to 0 mV caused inward \( \text{Ca}^{2+} \) current (shown expanded to the right) and was accompanied by contraction. The bathing solution was changed to 0 mM \( \text{Ca}^{2+} \) (0.25 mM EGTA), which resulted in a decrease and then an increase in the \( \text{Ca}^{2+} \) channel current and was accompanied by relaxation. ACh (30 s, 50 \( \mu \)M) caused contraction, a brief inward current and reduction in the \( \text{Ca}^{2+} \) channel current followed by recovery. \( \text{Ca}^{2+} \) channel current is shown expanded to the right. B., a second application of ACh in 0 \( \text{Ca}^{2+} \) failed to cause contraction and little change in the \( \text{Ca}^{2+} \) channel current (expanded to the right). Restoration of 1 mM \( \text{Ca}^{2+} \) to the bath resulted in a decrease and then an increase in the \( \text{Ca}^{2+} \) current (expanded at right) and was accompanied by contraction of the cell.
Upon removal of extracellular Ca\textsuperscript{2+}, cells relaxed and I\textsubscript{Ca} decreased and then stabilized with Na\textsuperscript{+} as the charge carrier. The first application of ACh in Ca\textsuperscript{2+} free solution inhibited I\textsubscript{Ca} and was accompanied by contraction of cells (Fig. 4A), consistent with release of Ca\textsuperscript{2+} from intracellular stores contributing to elevation of [Ca\textsuperscript{2+}]\textsubscript{i}. Following recovery, the second application of ACh in Ca\textsuperscript{2+}-free solution failed to elicit contraction (Fig. 4B, below) and there was little change in the Ca\textsuperscript{2+} channel current (Fig. 4B). These findings could be explained by ACh causing release of Ca\textsuperscript{2+} from a depletable store which initiates both contraction and inhibition of Ca\textsuperscript{2+} current.

2.3.5 \textit{ACh repeatedly inhibits Ca\textsuperscript{2+} current in the presence of 1 mM Ca\textsuperscript{2+}.}  
To quantify the dependence of the ACh-mediated inhibition of Ca\textsuperscript{2+} current on Ca\textsuperscript{2+} we confirmed that at least 3 successive responses could be obtained from cells in 1 mM Ca\textsuperscript{2+} (Fig. 5A, left). In Ca\textsuperscript{2+}-free solution the first application of ACh reduced Ca\textsuperscript{2+} channel current by 45\% (9 cells) but the response to a second application of ACh was significantly attenuated (Student's t-test, P<0.05). Finally, when Ca\textsuperscript{2+} was returned to the bath, ACh-mediated inhibition was restored to levels not significantly different from control (Fig. 5A, middle). This finding is consistent with the involvement of a depletable cytosolic Ca\textsuperscript{2+} store. The inhibitory responses reported in Fig. 5 (middle panel) are from a subset of cells that were each stimulated 3 times. When all cells are considered, the first application of ACh in Ca\textsuperscript{2+}-free solution caused a 53 \pm 4\% reduction in Ca\textsuperscript{2+} channel current (mean reduction the 18 of 25 responding cells). A possible reason for the somewhat greater suppression in Ca\textsuperscript{2+}-free solution could be that removal of Ca\textsuperscript{2+} from the bath relieves some Ca\textsuperscript{2+}-dependent inhibition permitting greater inhibition in response to ACh.
Figure 5. Ca\textsuperscript{2+} stores participate in ACh-mediated inhibition of Ca\textsuperscript{2+} channel current. A, ACh regulation of $I_{Ca}$ was quantified as the maximal percentage reduction of control peak Ca\textsuperscript{2+} or Ca\textsuperscript{2+} channel current. In 1 mM Ca\textsuperscript{2+}, ACh repeatedly caused contraction and reduced Ca\textsuperscript{2+} current by ~30% (n=5) with each of 3 applications (30 s, 50 μM, 3 minutes apart for recovery). In 0 Ca\textsuperscript{2+} solution (middle panel) the first application of ACh caused contraction and reduced Ca\textsuperscript{2+} channel current by ~45% (n=9). ACh applied a second time (3 min later) failed to cause contraction and reduction in Ca\textsuperscript{2+} channel current was significantly attenuated (ANOVA). Introduction of 1 mM Ca\textsuperscript{2+} to the bath restored the ACh-mediated inhibition of Ca\textsuperscript{2+} current. Depletion of Ca\textsuperscript{2+} stores with cyclopiazonic acid (CPA; right panel) abolished the effect of ACh. CPA was applied to cells (1 μM, 90 s) after which ACh (50 μM, 30 s) inhibited peak Ca\textsuperscript{2+} current by only 15%. After a further 3 minutes CPA, ACh caused a small increase in Ca\textsuperscript{2+} current. After 5 to 8 minutes recovery and washout of CPA, ACh again inhibited Ca\textsuperscript{2+} current. *P<0.05. B, the time course of ACh-mediated reduction in $I_{Ca}$ currents was determined for 7 cells both in 0 and 1 mM Ca\textsuperscript{2+}. Maximal reduction in each of 1 and 0 mM Ca\textsuperscript{2+} solution was considered 100%. The onset of ACh-mediated inhibition of $I_{Ca}$ was rapid in both bathing conditions with recovery towards basal currents occurring earlier and more rapidly in 0 Ca\textsuperscript{2+} solution. C, cells were depolarized every 10 s to reduce the possible effect of Ca\textsuperscript{2+} entry during depolarization on the time course of inhibition. Responses were normalized as in B. Onset and time course of inhibition were similar to observations in B, but recovery was more complete.
2.3.6 Cyclopiazonic acid reversibly suppresses ACh inhibition of Ca\textsuperscript{2+} currents.

We further investigated the participation of Ca\textsuperscript{2+} stores using CPA to inhibit the SR Ca\textsuperscript{2+}-ATPase (Seidler, Jona, Vegh, & Martonosi, 1989), which depletes stores in these cells (Janssen & Sims, 1993b). Brief exposure to CPA (1 μM, 90 s) caused no change in the peak Ca\textsuperscript{2+} current or the muscarinic tail currents (see Fig. 2), indicating the G protein-linked muscarinic signalling system was functional. Moreover, the first challenge with ACh in the presence of CPA caused a reduction in Ca\textsuperscript{2+} current (Fig 5A, right). However, in the continued presence of CPA, a second application of ACh failed to inhibit Ca\textsuperscript{2+} current, with a slight increase in $I_{Ca}$ noted in 4 of 5 cells. Tail current was not inhibited. Following 5 to 8 minutes for washout of CPA and recovery, Ca\textsuperscript{2+} current was again inhibited by ACh (Fig. 5A, right). Thus, CPA did not directly interfere with the Ca\textsuperscript{2+} current or muscarinic receptor activation, and its effects were fully reversible. These findings complement the studies described above where stores were depleted using Ca\textsuperscript{2+}-free solution, providing evidence that Ca\textsuperscript{2+} stores are required for muscarinic inhibition of $I_{Ca}$.

2.3.7 Time course of ACh-mediated inhibition of $I_{Ca}$.

ACh-mediated reduction of $I_{Ca}$ was rapid and partially reversible under all experimental conditions. To quantify the time course of ACh-mediated inhibition of $I_{Ca}$, responses were normalized, with peak inhibition being 100% and basal $I_{Ca}$ currents being 0% inhibition (Fig. 5B). The onset of ACh-mediated inhibition was rapid under our experimental conditions. Half of the maximal suppression occurred within 5 s for cells in Ca\textsuperscript{2+}-containing solution while inhibition was maximal within 5 s in the Ca\textsuperscript{2+}-free solution. Recovery commenced within 10 s in Ca\textsuperscript{2+}-free solution, whereas a more persistent
component of inhibition was evident in Ca\(^{2+}\) containing solution. We investigated the possibility that Ca\(^{2+}\) entry due to depolarization was contributing to the differences in the time course of the response between the Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free bathing solutions. When the time between depolarizing pulses was increased from 5 to 10 s, (Fig. 5C) the time course of inhibition was similar to that described above, although recovery under these conditions was more complete (Fig. 5C). Ca\(^{2+}\) entry therefore appears to contribute to the prolonged inhibition of I\(_{\text{Ca}}\). The time course of inhibition was not affected by PKC antagonists (see above), so the mechanism underlying this component remains uncertain.

2.3.8 ACh decreases Ca\(^{2+}\) channel open probability.

Cholinergic inhibition of I\(_{\text{Ca}}\) was further studied using cell-attached patch recording of single Ca\(^{2+}\) channels (Ba\(^{2+}\) as the charge carrier). K\(^+\) (135 mM) was included in the bathing solution to set the membrane potential near 0 mV and prevent cholinergic depolarization of cells from contributing to changes in the channel open probability (P\(_{\text{o}}\)) or unitary current amplitude. Depolarization of patches to potentials more positive than \(-40\) mV activated inward currents (Fig. 6A). The unitary current-voltage relationship for 5 cells revealed a mean slope conductance of 22 pS (Fig. 6B). The voltage-dependence of channel activity was quantified by calculating P\(_{\text{o}}\) using the relationship \(I = i \cdot N \cdot P_{\text{o}}\) where \(I\) was the average current recorded in 16 to 32 steps to varying potentials, \(i\) was the unitary current amplitude at each voltage, and \(N\) was the number of channels in each patch (taken as the maximal number of channels open simultaneously at positive potentials). In 4 cells analyzed, P\(_{\text{o}}\) increased at more positive potentials, with half-maximal activation at \(-14\) mV. Channel opening was blocked when nifedipine was applied to the outside of the cell (8 cells).
Figure 6. Single Ca\textsuperscript{2+} channels in gastric smooth muscle. A, representative single Ca\textsuperscript{2+} channel currents (Ba\textsuperscript{2+} as the charge carrier, 1\textmu M Bay K 8644 in the electrode) recorded in cell-attached patch configuration from a gastric smooth muscle cell. Cells were bathed in solution with 135 mM K\textsuperscript{+} to depolarize the membrane to 0 mV. Patches were held at -100 mV and depolarized to various test potentials to elicit inward currents through single Ca\textsuperscript{2+} channels. B, unitary current-voltage relationship is plotted below for 5 cells, illustrating a mean slope conductance of 22 pS. C, the probability of observing channels in the open state (P\textsubscript{o}) was voltage dependent, increasing with depolarization to a maximal level (P\textsubscript{o} determined for the first 200 ms of depolarization). Data fitted with a Boltzmann distribution, giving half-maximal P\textsubscript{o} at -14 mV, with a slope factor of 3.4 mV (4 cells).
We investigated regulation of Ca\textsuperscript{2+} channels by recording in the cell-attached patch configuration and applying ACh to the rest of the cell. Control channel activity was recorded for at least 1 minute, after which ACh caused a reversible decrease in P\textsubscript{0} (Fig. 7A, representative of responses obtained in 22 of 36 cells). The time course was characterized in 5 cells, in which significant suppression of channel opening occurred within 10 s of ACh, with recovery to basal levels observed after ~ 1 min (Fig. 7B, one way ANOVA). Since the channels were isolated from ACh, such inhibition is consistent with involvement of a diffusible cytosolic messenger.

### 2.3.9 Inhibition of Ca\textsuperscript{2+} current in tracheal smooth muscle cells.

We investigated cholinergic regulation of Ca\textsuperscript{2+} current in tracheal smooth muscle, in which Ca\textsuperscript{2+} release from stores regulates several ion channel types, including Cl\textsuperscript{-} and K\textsuperscript{+} (Janssen & Sims, 1992; 1993a,b; Wade & Sims, 1993). L-type Ca\textsuperscript{2+} currents have been characterized previously in tracheal smooth muscle (Hisada, Kurachi, & Sugimoto, 1990). ACh also caused profound inhibition of Ca\textsuperscript{2+} channel current in guinea-pig tracheal muscle cells (Fig. 8A). Inhibition of Ca\textsuperscript{2+} current was rapid (peak suppression within 10 s), reversible and occurred over all potentials (Fig 8A, inset, 77 ± 3% inhibition in Ca\textsuperscript{2+}-free solution, 7 cells, 38 ± 6% inhibition in 1 mM Ca\textsuperscript{2+}, 4 cells). The Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} current often oscillates in response to cholinergic excitation, both in the presence or absence of extracellular Ca\textsuperscript{2+} (Janssen & Sims, 1993b). Fluorescence recordings confirm that ACh causes oscillation of [Ca\textsuperscript{2+}], in tracheal cells, with Ca\textsuperscript{2+} stores playing a primary role (Sims, Jiao & Zheng, 1996). In cells where ACh elicited oscillation of Cl\textsuperscript{-} current, recurrent inhibition of Ca\textsuperscript{2+} current was also observed (Fig. 8B, 3 cells).
Figure 7. Cholinergic regulation of single Ca\(^{2+}\) channels in gastric cells. Ca\(^{2+}\) channels were recorded in the cell-attached patch configuration by depolarizing cells from -100 to -10 mV every 3 s (Ba\(^{2+}\) as the charge carrier, 0.1 \(\mu\)M Bay K 8644 in electrode). A: Control, 5 representative traces show basal Ca\(^{2+}\) channel activity, with up to 3 channels open at one time; ACh, 5 consecutive traces beginning ~5 s after onset of ACh (100 \(\mu\)M) showing suppression of Ca\(^{2+}\) channels (unitary current amplitude remained constant during ACh, providing evidence that the membrane potential of the cell was effectively clamped at 0 mV by the bathing solution); Recovery, Ca\(^{2+}\) channel activity recovered within ~1 minute of ACh onset. B, \(P_o\) determined from 5 cells showing the time course of the reduction in Ca\(^{2+}\) channel activity. Mean values of \(P_o\) above are given on the figure for the periods indicated by the dashed lines. Suppression of channel opening occurred within 10 s of the application of ACh, with recovery to basal levels observed after ~ 1 min. Similar suppression of Ca\(^{2+}\) channel activity was observed in 22 of 36 cells investigated.
Figure 8. Acetylcholine inhibits \( I_{Ca} \) in guinea-pig tracheal muscle cells. A, membrane potential was varied between -100 and +50 mV (0 Ca\(^{2+}\) bathing solution, peak Ca\(^{2+}\) channel current indicated by arrows, \( \rightarrow \)). ACh (30s, 50 \( \mu \)M) activated a Ca\(^{2+}\)-activated Cl\(^{-}\) current that was inward at negative potentials and outward at positive potentials (peak currents off scale). Ca\(^{2+}\) channel current was profoundly inhibited. The current-voltage relationship is shown in inset to the right, prior to (a), during ACh (b) and ~3 minutes from the onset of ACh (c) (traces leak corrected as described in methods). Ca\(^{2+}\) channel current was reversibly diminished over the entire range of potentials. B, in a cell in which ACh caused oscillations of Ca\(^{2+}\)-activated Cl\(^{-}\) current (control trace shown above), recurrent inhibition of Ca\(^{2+}\) channel current was also observed.
2.4 Discussion
We investigated the regulation of L-type Ca\(^{2+}\) channels in smooth muscle cells using perforated-patch and cell-attached recording configurations. Ca\(^{2+}\) entry during action potentials or under voltage clamp was sufficient to initiate contraction of single cells, exemplifying the importance of Ca\(^{2+}\) entry in excitation of smooth muscle (Somlyo & Somlyo, 1994). ACh also caused excitation and contraction of cells; however, cholinergic stimulation acutely inhibited I\(_{\text{Ca}}\). The inhibition was rapid in onset, repeatable and reversible. However, depletion of Ca\(^{2+}\) stores either by persistent bathing of cells in Ca\(^{2+}\)-free solution, or by blockade of the SR Ca\(^{2+}\)-ATPase, attenuated the ACh-mediated inhibition. Release of Ca\(^{2+}\) from stores may be an essential step in the cholinergic inhibition of I\(_{\text{Ca}}\) reported here. ACh reduced the open probability of Ca\(^{2+}\) channels when recorded in cell-attached patches, providing additional evidence for the involvement of a cytosolic signalling pathway. Inhibition of I\(_{\text{Ca}}\) was characterized in guinea-pig gastric corpus and observed as well in canine and guinea-pig tracheal muscles, indicating that such regulation may be a fundamental feature of cholinergic signalling in mammalian smooth muscle.

The regulation of smooth muscle Ca\(^{2+}\) channels by ACh is controversial. ACh is reported to increase Ca\(^{2+}\) current in toad gastric smooth muscle (Clapp et al., 1987). When studied at the single channel level in airway smooth muscles, carbachol or methacholine increased Ca\(^{2+}\) channel current by shifting the voltage activation range to more negative potentials (Kamishima et al., 1992; Tomasic et al., 1992). What factors could account for the difference in the effect of ACh we report here? Species or experimental differences (such as the use of conventional whole-cell configuration) may account for the divergent findings.
between guinea-pig and toad stomach. The experiments reported by Tomasik and coworkers (1992), performed on acutely dissociated dog, pig and ferret tracheal muscle, were carried out in the persistent absence of extracellular Ca\(^{2+}\). Under such conditions, Ca\(^{2+}\) release from stores would be minimal. Indeed, we found that inhibition of \(I_{Ca}\) was attenuated in Ca\(^{2+}\)-free solution. The enhancement of Ca\(^{2+}\) channel activity described by Kamishima et al. (1992) was recorded after approximately 3 min exposure to cholinergic agonist, so an initial acute effect may not have been observed.

Several findings seem inconsistent with a membrane delimited mechanism (Hille, 1994) contributing to the inhibition of Ca\(^{2+}\) current. 1) Ca\(^{2+}\) channel open probability was reduced in patches that were isolated from ACh. 2) Brief stimulation of cells with ACh caused persistent inhibition of \(I_{Ca}\). 3) Oscillatory inhibition of Ca\(^{2+}\) current was observed in the continued presence of ACh. Our results contrast with those of Beech (1993), who proposed that a large, prolonged component of the inhibition of Ca\(^{2+}\) channels by histamine and bradykinin, was due to a Ca\(^{2+}\)-independent, membrane delimited G protein-mediated mechanism. However, elevation of [Ca\(^{2+}\)]\(_i\) due to release from stores participated in an initial phase of Ca\(^{2+}\) channel inhibition (Beech, 1993), an effect that is likely due to IP\(_3\)-mediated release of Ca\(^{2+}\) from the SR (Komori & Bolton, 1991).

Muscarinic signalling involves several second messenger cascades (Berridge, 1993; Somlyo & Somlyo, 1994). Ozaki and coworkers (1992) provide evidence that ACh reduces Ca\(^{2+}\) influx in gastrointestinal muscle, an effect that they speculate may be due to PKC-mediated suppression of Ca\(^{2+}\) current. We found no evidence for PKC participating in the acute inhibition of \(I_{Ca}\) since: 1) PdBU did not clearly mimic the effect of ACh, and 2) pretreatment of cells with PKC antagonists failed to prevent inhibition of \(I_{Ca}\). A role for
Ca\(^{2+}\) stores was supported by the observations that depletion of Ca\(^{2+}\) stores with CPA or by persistent bathing of cells in 0 mM Ca\(^{2+}\), reversibly abolished ACh-mediated inhibition.

Ca\(^{2+}\) stores could participate in the regulation of I\(_{\text{Ca}}\) by direct Ca\(^{2+}\)-dependent inhibition of L-type channels and/or by initiating other signalling pathways. We first consider the evidence that Ca\(^{2+}\) may act directly on Ca\(^{2+}\) channels. Ca\(^{2+}\)-dependent inhibition of L-type Ca\(^{2+}\) channels occurs in many cell types, including neuronal, cardiac and smooth muscle cells (reviewed in Eckert & Chad, 1984; McDonald \textit{et al}., 1994). In smooth muscle, Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels contributes to their inactivation (e.g. Ganitkevich \textit{et al}., 1987; Vogalis \textit{et al}., 1992) probably as a result of the local elevation of intracellular Ca\(^{2+}\) at the cytosolic face of the channel. Ca\(^{2+}\) influx through ATP-activated non-selective cation channels also causes inhibition of Ca\(^{2+}\) current in urinary bladder smooth muscle (Schneider \textit{et al}., 1991). IP\(_3\)-mediated release of Ca\(^{2+}\) from stores leads to acute inhibition of I\(_{\text{Ca}}\) in intestinal smooth muscle cells (Komori & Bolton, 1991). A recent report by Unno \textit{et al} (1995) has elegantly confirmed muscarinic inhibition of Ca\(^{2+}\) channel current in gastrointestinal smooth muscle. Using conventional whole cell recording they found that carbachol suppresses I\(_{\text{Ca}}\) in a biphasic manner. Several of their findings support the interpretation that the initial rapid suppression was due to release of Ca\(^{2+}\) from stores. Moreover, no role for PKC was found in mediating the prolonged phase of inhibition. We have reached essentially the same conclusions while using different experimental strategies (e.g. perforated patch recording, use of CPA to deplete stores).

Thus, inhibition of I\(_{\text{Ca}}\) due to elevation of [Ca\(^{2+}\)]\(_i\) is a widespread phenomenon in smooth muscle. The rapid onset, correlation with contraction and oscillatory of I\(_{\text{Ca}}\) that we observed here are consistent with Ca\(^{2+}\)-dependent inhibition due to elevation of [Ca\(^{2+}\)]\(_i\) by
release from stores. We have previously provided evidence that elevation of $[Ca^{2+}]_i$ regulates Cl$, K^+$ and non-selective cation channels as well as contraction of gastric and tracheal muscle cells (Janssen & Sims, 1992, 1993a,b, 1994; Sims, 1992a; Wade & Sims, 1993). Thus, there is precedent for the regulation of ion channels by elevation of $[Ca^{2+}]_i$ (Sims & Janssen, 1993). Combined patch-clamp and fluorescence (e.g. Becker et al., 1989; Schneider et al., 1991; Vogalis et al., 1992) have previously provided a way of correlating changes in channel activity with $[Ca^{2+}]_i$.

The more prolonged phase of suppression was dependent upon the presence of extracellular Ca$^{2+}$, but we do not presently understand the signalling pathways contributing to this phase of suppression. Elevation of $[Ca^{2+}]_i$ may initiate a number of signalling cascades, including, for example, nitric oxide synthase (Han, Shimoni & Giles, 1994) or tyrosine kinases (Huang, Morielli & Peralta, 1993; Inoue, Waniishi, Yamada & Ito, 1994), although the involvement of such pathways in the inhibition reported here is at present uncertain.

ACh and other agonists cause contraction of smooth muscle, but some of the events initiated can provide negative feedback. For example, ACh and ATP cause contraction of gastric muscle, but are thought to reduce Ca$^{2+}$ entry (Ozaki et al., 1992). Elevation of $[Ca^{2+}]_i$ due to release from stores also participates in negative feedback regulation of smooth muscle. Cholinergic activation of Ca$^{2+}$-dependent K$^+$ channels (Wade & Sims, 1993) and inhibition of Ca$^{2+}$ channels would serve to limit depolarization and reduce Ca$^{2+}$ entry. Signalling pathways for a number of excitatory agents converge to release Ca$^{2+}$ from stores (Berridge, 1993) and activate different channel types (Janssen & Sims, 1993a; 1994).
Spatial and temporal features of the elevation of \([\text{Ca}^{2+}]\), may be critical for integrating and coordinating the different actions of \(\text{Ca}^{2+}\).

### 2.5 References


CHAPTER 3

3 MUSCARINIC STIMULATION OF TRACHEAL SMOOTH MUSCLE CELLS ACTIVATES THE LARGE CONDUCTANCE Ca\textsuperscript{2+}-DEPENDENT K\textsuperscript{+} CHANNEL.

This chapter adapted from;


Reprinted here with permission from The American Physiological Society.

Experimental design, current recordings and data analysis and preparation of figures was carried out by G. Wade in the lab of S. Sims. G. Wade and S. Sims co-wrote the publication.
3.1 Introduction

Ca\(^{2+}\)-dependent K\(^+\) channels have been described in many smooth muscles (see reviews, Tomita, 1988; Toro & Stefani, 1991). The unitary conductance (>200 pS), voltage-dependence and sensitivity to cytosolic free \([\text{Ca}^{2+}]\) ([(\text{Ca}^{2+})]) of the large conductance Ca\(^{2+}\)-dependent K\(^+\) (K\(_{\text{Ca}}\)) channel has been well documented (Benham, Bolton, Lang & Takewaki., 1986; Carl, Carl & Sanders, 1989; Singer & Walsh, 1987). This channel type has been suggested to play a role in repolarization of the membrane of smooth muscle following action potentials (Carl, McHale, Publicover & Sanders, 1990; Walsh & Singer, 1987).

There have been several reports of the regulation of K\(_{\text{Ca}}\) currents in smooth muscle by neurotransmitters (review, Toro & Stefani, 1991). For example, ACh suppresses K\(_{\text{Ca}}\) channels in colonic smooth muscle, possibly contributing to excitation of this muscle (Cole, et al., 1989). Suppression of K\(_{\text{Ca}}\) channels was investigated in excised outside-out patches from tracheal smooth muscle (Kume & Kotlikoff, 1991), where it was demonstrated that cholinergic agonist shifted the voltage-activation curve to more positive membrane potentials, without changing single channel conductance. Cholinergic inhibition of K\(_{\text{Ca}}\) channel activity was shown to be due to a G protein-dependent mechanism in tracheal smooth muscle cells. The effects of cholinergic stimulation in colonic and tracheal smooth muscle were not thought to be mediated by changes in [\text{Ca}^{2+}]\(_i\). However, regulation of K\(_{\text{Ca}}\) channels indirectly through changes in [\text{Ca}^{2+}]\(_i\) has also been demonstrated. For example, substance P causes activation of K\(_{\text{Ca}}\) channels in colonic smooth muscle, due largely to Ca\(^{2+}\) entry (Mayer, Loo, Snape & Sachs, 1990). The release of Ca\(^{2+}\) from intracellular stores by ACh elicits Ca\(^{2+}\)-dependent K\(^+\) current in several
smooth muscles (e.g. Benham & Bolton, 1986; Ganitkevich & Isenberg, 1990; Sims, Vivaudou, Hillemeier, Biancani, Walsh, & Singer, 1990), but in these studies the single channels underlying the currents were not demonstrated. A more recent study by Muraki and co-workers (Muraki, Imaizumi & Watanabe, 1992) investigated the regulation of large conductance $K_{Ca}$ channels in bladder and tracheal smooth muscle. Using cells permeabilized with $\beta$-escin, they demonstrated that ACh and substance P mediated increases in $K_{Ca}$ channel activity, which was mimicked by introduction of Ca$^{2+}$ or inositol 1,4,5-trisphosphate (IP$_3$) into the cytosol. These findings are consistent with excitatory ligands causing elevation of [Ca$^{2+}$]$_i$ due to release of Ca$^{2+}$ from intracellular stores by the cytosolic second messenger IP$_3$.

The effects of ACh on membrane currents in canine smooth muscle have been investigated at the whole cell level (Janssen & Sims, 1992). ACh depolarizes the cells by activating Cl$^-$ and nonselective cation conductances. The cells also contract in response to ACh, even when the membrane is voltage clamped at negative potentials, preventing activation of voltage-dependent Ca$^{2+}$ channels, or in the absence of extracellular Ca$^{2+}$ (Janssen & Sims, 1993b). These observations are consistent with ACh causing elevation of [Ca$^{2+}$]$_i$ due to release from intracellular stores. Depolarization and elevation of [Ca$^{2+}$]$_i$ would favor activation of $K_{Ca}$ channels. We have investigated the regulation of $K_{Ca}$ channels by ACh in canine tracheal smooth muscle cells and have tested the hypothesis that Ca$^{2+}$ released from intracellular stores activates $K_{Ca}$ channels. Here we report that ACh causes transient activation of $K_{Ca}$ channels due to a shift of the voltage-activation range to less positive membrane potentials. This effect can largely be accounted for by release of Ca$^{2+}$ from intracellular stores.
3.2 Methods

3.2.1 Preparation of isolated tracheal smooth muscle cells

Mongrel dogs (15-35 kg) of either sex were tranquilized with xylazine (2.2 mg/kg I.M.) and killed by intravenous injection of sodium pentobarbitone (30 mg/kg). The trachea was removed and placed in physiologic saline solution (see solutions below). Connective tissue, vasculature and innervation were removed and smooth muscle strips approximately 1 mm wide and 1 cm long were dissected from the trachea, leaving the epithelium. The smooth muscle was then transferred into 25 ml Erlenmeyer flasks (pretreated with prosil-organosilane concentrate to reduce cell adhesion) containing 5 ml of dissociation solution (see solutions below). The tissues were then either placed in a shaking water bath at 37°C for 40 minutes or stored at 4°C and digested 24 or 48 hrs later. The tissues stored for 24 and 48 hrs required less time (20-30 min) in the shaking water bath for dissociation. No observable difference was evident for function or appearance between cells dissociated immediately or after refrigeration. After enzymatic digestion, the tissue was placed into a small volume of standard bathing solution (see solutions below) and cells were dispersed by trituration with a fire-polished Pasteur pipette. Cells were studied within 12 hours of dispersion.

3.2.2 Solutions

Physiologic saline solution contained (in mM) NaCl, 130; KCl, 5; CaCl₂, 1; MgCl₂, 1; HEPES, 20; D-glucose, 20; pH 7.4. Dissociation solution contained (in mM): NaCl, 130; KCl, 5; HEPES 10; glucose, 10; taurine, 10; CaCl₂, 1; MgCl₂, 1; EDTA, 0.25; collagenase, 400 units/ml (Type I, Sigma); bovine serum albumin, 1 mg/ml (ICN Biomedicals, Cleveland, OH); papain, 30 units/ml (Type IV, Sigma); 1,4 dithio-L-threitol, 1 mM
Standard bathing solution for electrophysiological studies contained (in mM): KCl, 135; HEPES, 20; D-glucose, 10; CaCl₂, 1; MgCl₂, 1; pH set to 7.2 using N-methyl-D-glucamine. Low-Ca²⁺ solution was standard bathing solution with 1 mM ethylene glycol-bis(β-aminoethyl ether)-N, M, N’, N’-tetraacetic acid (EGTA) and 0.2 mM CaCl₂ (free Ca²⁺ calculated to be <100 nM). For these studies the electrode contained standard bathing solution filtered (0.2 μm) before use. We substituted NaCl for KCl in experiments in which [K⁺] in the electrode (referred to as [K⁺]₀) was changed. Each patch was studied at only one [K⁺] gradient. Unless otherwise indicated, chemicals were from Sigma or BDH. Acetylcholine and caffeine were prepared from aqueous stock solutions. A23187 (Molecular Probes) was prepared in dimethylsulfoxide and diluted in standard bathing solution to 20 μM. Nifedipine (Research Biochemicals, Natick, MA) was prepared in ethyl alcohol and diluted to 20 μM in standard bathing solution.

3.2.3 Patch-clamp methods
Cells were allowed to settle and adhere to the bottom of a perfusion chamber (1 ml bath volume, perfused at ~3 ml/min) mounted on the stage of an inverted phase-contrast microscope. Cells selected for study appeared phase dense, relaxed, and contracted in response to acetylcholine, muscarine or caffeine. Current recordings were made in the cell-attached patch configuration using standard patch-clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Single channel currents were filtered at 1 kHz and recorded at room temperature (22 to 25°C) with Axopatch 1B or 1D amplifiers (Axon Instruments, Foster City, CA), low-pass, sampled at 2 kHz using pClamp 5.51 software (Axon Instruments), and stored on disk. Current and voltage information was displayed on a chart recorder (Gould 3200S) and stored digitally using a pulse code modulator (PCM-2,
Medical Systems, Greenvale, NY). Test agents were applied to cells by pressure ejection (Picospritzer II, General Valve, Fairfield, NJ) from a micropipette. Contractile responses were recorded on videotape using a charge-coupled device camera and videocassette recorder. Patch electrodes had ~1 μm tip diameter and 7-10 MΩ tip resistance.

The bathing and electrode solutions usually contained 135 mM K⁺, which depolarized the membrane potential of the cells to approximately 0 mV, assuming an intracellular [K⁺] ([K⁺]ᵢ) of 135 mM. Under these conditions, stimulation of the cell with ACh was not expected to cause any change in cell membrane potential thus the patch potential (referred to as Vₑ) was the negative of the electrode potential. Upward deflections indicate outward current across the membrane.

Because the number of channels in patches was generally not known we have used NPO, where N is the number of channels in the patch available to open and PO is the probability of a channel being in the open state, as an index of channel-open probability. NPO for steady-state membrane potentials was determined from the average current above baseline divided by the unitary current amplitude (program kindly provided by Dr. Michel Vivaudou, Grenoble, France). NPO for voltage ramps was calculated from the average current above baseline (adjusted for leak) divided by the unitary current value at each voltage, as previously described (Carl & Sanders, 1990). Voltage-ramp commands changed voltage at 100 mV/s, a value reported to be sufficiently low as to not affect voltage-dependent channel kinetics (Carl & Sanders, 1990). Sample sizes indicate the number of cells studied, with only one patch obtained per cell. All data are presented as means ± standard error of the mean (SE).
3.3 Results

3.3.1 Acetylcholine causes reversible cell contraction.

Enzymatic digestion of tracheal smooth muscle yielded spindle-shaped cells that were phase dense when studied using phase-contrast microscopy. Cells chosen for study were initially relaxed (from 50 to 150 μm in length) and contracted reversibly in response to ACh (20 or 100 μM ACh in application pipette), muscarine (20 μM in application pipette), or caffeine (5 mM in application pipette; greater than 95 cells were studied). In most of the experiments described here the cells were not expected to depolarize in response to the excitatory agonists, as they were bathed in 135 mM K⁺, so that the cell membrane potential was chemically clamped near 0 mV. The contraction demonstrated under these conditions (Janssen & Sims, 1992) is similar to that obtained in cells bathed in physiologic K⁺ solutions (Janssen & Sims, 1993b) and is consistent with ACh elevating [Ca²⁺], due to release from intracellular stores.

3.3.2 Identification of large-conductance Kᵦ channels.

The predominant single-channel activity recorded in tracheal smooth muscle cells was due to large-conductance Kᵦ channels, as previously described (Green, Foster & Small, 1991; McCann & Welsh, 1986). When studied in the cell-attached patch configuration, channels were not open near the reversal potential. To facilitate determination of slope conductance and reversal potentials we studied the channels in excised inside-out patches or after treatment of cells with Ca²⁺ ionophore. In excised inside-out patches (1 mM Ca²⁺ at the cytosolic face), large unitary currents that were outward at positive potentials, inward at negative potentials, and reversed at 0 mV were recorded (Fig. 1A). The current-voltage relationship, determined using voltage-ramp commands, revealed a slope conductance of
240 pS (Fig. 1B) with symmetric 135 mM K⁺. When recorded in the cell-attached patch configuration single-channel currents were only apparent at very positive potentials. Application of the Ca²⁺ ionophore A23187 caused contraction of cells (not shown) and increased channel activity at less positive potentials (Fig. 1C). The Ca²⁺-dependent channel had a linear current-voltage relationship with 135 mM [K⁺]₀ and a slope conductance of 242 ± 5 pS (n=19), and unitary currents reversed at -1 ± 1 mV. NPo was calculated from average currents above baseline from 3 cell-attached patches described (METHODS). A23187 increased NPo at less positive potentials (Fig. 1D) consistent with the known effects of [Ca²⁺]ᵢ on the PO versus voltage relationship of Kᵥ channels (Carl & Sanders., 1990; Green et al., 1991; McCann and Welsh., 1986; Singer and Walsh., 1987). Reversal potentials of the unitary currents were studied in inside-out excised patches or cell-attached patches after treatment with A23187. When [K⁺] in the pipette ([K⁺]₀) was changed in different cells, the reversal potential of the unitary currents was found to be dependent upon the [K⁺] gradient, shifting 56 ± 3 mV per ten-fold change in [K⁺]₀ (determined by least-squares regression) (Fig. 1E). The large unitary conductance, sensitivity to [Ca²⁺]ᵢ, voltage-dependent activation, and K⁺ selectivity of the observed channel are all consistent with the Kᵥ channel.

3.3.3 ACh causes a transient increase in Kᵥ channel activity.
We next investigated the effect of ACh on Kᵥ channel activity. Before application of ACh, channel activity was infrequent (at Vₘ = 50 mV). Within 0.5 to 1 s of application of ACh to the cell, single channel currents were apparent, with up to 6 channels open simultaneously in the example shown (Fig. 2). This response was observed in 57 of 70 cells studied with ACh. Channel activity usually returned to baseline within 10 s of the
application of ACh. The unitary amplitude of the ACh-activated currents is close to that expected for $K_{Ca}$ channels (see below for further description of the ACh-activated channel).

From this experimental configuration, the channels being studied were isolated from ACh by the high resistance seal between the patch-pipette and the cell membrane. Therefore, the increased $K_{Ca}$ channel activity elicited by ACh probably indicates the involvement of a cytosolic second messenger.
Figure 1. Identification of the large-conductance Ca\textsuperscript{2+}-dependent K\textsuperscript{+} (K\textsubscript{Ca}) channels in canine tracheal smooth muscle cells. A: single-channel currents recorded from an excised inside-out patch in which only one channel was present. Membrane potential is indicated at left of each trace. Currents were outward at positive potentials, inward at negative potentials, and reversed at 0 mV. [K\textsuperscript{+}] was 135 mM in bath and electrode. Bath solution contained 1 mM Ca\textsuperscript{2+}. B: current-voltage relationship for the channel in A indicates a slope conductance of \~220 pS. Patch potential was varied linearly from -100 to +100 mV over 2 s. C: when recorded in the cell-attached patch configuration single-channel currents were only apparent at very positive potentials. Application of the Ca\textsuperscript{2+} ionophore A23187 (20 \mu M in the application pipette) caused contraction of cell (not shown) and increased channel activity at less positive potentials. Reversal potential is approximately -25 mV ([K\textsuperscript{+}]\textsubscript{o} = 40 mM). Patch potential was varied linearly as in B. Three overlapping current traces are shown for each condition. D: index of channel-open probability (N\textsubscript{Po}, where N is number of channels and P\textsubscript{o} is probability of channel being open) was calculated from average currents of 30 sweeps from 3 cells. A23187 increased N\textsubscript{Po} at less positive potentials. E: reversal potential of unitary currents was dependent upon [K\textsuperscript{+}]\textsubscript{0}. Reversal potentials were obtained from excised inside-out patches or cell-attached patches following treatment of cells with A23187. Reversal potential shifted 56 \pm 3 mV per tenfold change in [K\textsuperscript{+}]\textsubscript{0} (determined by least-squares regression), consistent with channel being selective for K\textsuperscript{+}. The number of cells studied is indicated in parentheses for each value of [K\textsuperscript{+}]\textsubscript{0}. Large unitary conductance, Ca\textsuperscript{2+} sensitivity, voltage-dependent activation, and K\textsuperscript{+} selectivity of the observed channel are consistent with this being the large-conductance K\textsubscript{Ca} channel.
Similar responses to ACh were also observed when cells were bathed in physiologic solution containing 5 mM K⁺ (5 cells). However, studies under these conditions were complicated by two factors. At the normal resting potential, the input resistance of these cells was greater than 1 GΩ, and as a consequence, K⁺ currents in the patch caused a change in the cell potential, resulting in time-dependent changes in unitary current amplitude. In addition, cells depolarized in response to ACh (Janssen and Sims, 1992), resulting in a change in patch potential and reduction of unitary current amplitudes. All subsequent studies on Kᵥ channels were performed in 135 mM K⁺ solutions to avoid these complications, an approach used by some others when investigating Kᵥ channels (e.g. Cole et al., 1989).

To quantify the effect of ACh, amplitude histograms were constructed from current recordings made before and during the increased channel activity elicited by ACh. Analysis of the response in Fig. 2 reveals that, prior to ACh, channel openings were infrequent, corresponding to the small peak in the amplitude distribution close to 15 pA. After ACh, the amplitude distribution showed multiple peaks, indicating up to six channels open simultaneously (Fig. 3B). The peaks of the ACh amplitude histogram were equally spaced, indicating only multiple channels with similar unitary current amplitude were recorded. In most cases activation of other channels was not evident in response to ACh. Fitting the first open peak (O₁) with a Gaussian curve revealed a unitary current amplitude of 14.8 pA. Amplitude histograms were constructed in 0.5 s increments throughout the response in Fig. 2.
Figure 2. ACh causes a transient increase in channel activity. A: before ACh, K\text{Ca} channel activity was infrequent and brief (see three upward deflections at left). ACh (2 s, 100 \mu M in application pipette) caused contraction of cell (not shown) and increased channel activity, resulting in up to 6 channels being open simultaneously. Channel activity had returned to baseline by 10 s after ACh. B: effect of ACh shown on an expanded time scale. Increased channel opening was apparent within 1 s of the onset of ACh, with peak outward current within 2 s of onset. Cell had relaxed to near resting length by 5 min after stimulation. Patch (cell membrane) potential (V_m) = 50 mV. Currents were filtered at 1 kHz and digitally sampled at 2 kHz. Upward deflections represent outward current across membrane. As cell was depolarized to \sim 0 mV (bathed in 135 mM K⁰) and the patch was isolated from ACh, contraction and increased K\text{Ca} channel activity are consistent with a cytosolic second messenger-mediating the effect of ACh.
The amplitude of open channel currents did not vary during the increased activity elicited by ACh (Fig. 3C), providing evidence that the driving force on K⁺ was constant throughout the response. This indicates that chemically clamping the cell potential close to 0 mV with 135 mM K⁺ bath solution prevented changes in cell potential in response to ACh. We therefore rule out changes in membrane potential contributing to the changes in channel open probability (N₉ₒ) described in this report.

Channel open probability was calculated as N₉ₒ from average current above the baseline divided by the unitary current amplitude. N₉ₒ for the cholinergic response in Fig. 2 shows the increase in channel open probability, reaching maximal level approximately 2.5 s after the onset of ACh and returning to baseline levels within 10 s (Fig. 4A). Average N₉ₒ determined from 5 cells (Vₘ = 50 mV) showed significant increase within 1 s of ACh onset (Fig. 4B). While there was some variability between cells in the time course of recovery, N₉ₒ was not significantly different from baseline within 10 s of the application of ACh.
Figure. 3. Amplitude distribution of the ACh-elicited current shown in fig. 2. A: before ACh channel openings were infrequent, corresponding to the small peak in the distribution close to 15 pA (O₁). Closed-channel current is indicated by letter C above peak at zero current. B: after ACh, the current distribution shows multiple peaks indicating, up to six channels were open simultaneously. Number of channels open is indicated above each peak (O₁ to O₆). Unitary current amplitude (14.8 pA) was determined by fitting first open peak (O₁) with a Gaussian curve; 5.12 s of control current and after ACh were sampled at 2 kHz. C: time course of ACh activation of Kᵣ. Amplitude histograms were constructed in 0.5 s increments throughout the response. The amplitude of open-channel currents did not vary throughout the response, indicating the driving force on K⁺ was constant.
Figure 4. Quantification of the ACh-induced K_\text{Ca} current. NP_0 was calculated from average current above the baseline in 0.5 s segments. A: NP_0, determined for response in Fig. 2, was increased to a maximal level approximately 2.5 s after onset of ACh and returned to baseline levels within 9 s. Bar beneath the curve indicates the application of ACh. B: average NP_0 for 5 cells ± SE, calculated as in A, with all patches held at 50 mV (20 or 100 μM ACh in the application pipette). NP_0 was significantly increased within 1 s of ACh onset. Although variability in time course of recovery existed between responses, NP_0 was not significantly different from baseline by 10 s after application of ACh.
Figure 5. ACh shifts potential at which $K_{Ca}$ channels are open to less positive potentials. A: before application of ACh, unitary $K_{Ca}$ currents became apparent at $\sim 50$ mV, with 2 channels open simultaneously at $90$ mV. ACh (2 s, 100 $\mu$M in application pipette) resulted in unitary $K_{Ca}$ currents at approximately $-25$ mV, with up to 7 channels open simultaneously at $50$ mV. Slope conductance of ACh-activated channel was $240$ pS ($[K^+]_o = 135$ mM). $V_m$ was varied linearly between $-100$ and $100$ mV over 2 s. A single sweep is shown before and after ACh. B: average current response from 5 cells shows ACh shifts voltage at which $K_{Ca}$ channels are open to less positive potentials. Control trace is average of 50 sweeps and ACh trace is average of 5 sweeps. Voltage protocol was same as in A.
We investigated the voltage-activation range of the ACh-activated channels using voltage-ramp commands. Before application of ACh, unitary $K_{Ca}$ currents were apparent only at potentials positive to $-50$ mV, with two channels open simultaneously at $90$ mV in the patch illustrated in Fig. 5A. ACh shifted the voltage at which unitary $K_{Ca}$ currents were observed to less positive membrane potentials (Fig. 5A), resulting in $K_{Ca}$ channel opening at $-25$ mV and up to 7 channels open simultaneously at $50$ mV in the example shown in Fig. 5A. The slope conductance of the ACh-activated channel in Fig. 5A was 240 pS ($[K^+]_o = 135$ mM). The average slope conductance in 5 cells for ACh-activated channels was $247 \pm 10$ pS, similar to that reported above for the large conductance Ca$^{2+}$-dependent $K^+$ channels. Furthermore, the ACh-activated channel currents reversed direction close to the predicted $K^+$ equilibrium ($-1 \pm 1$ mV with $135$ mM $[K^+]_o$, n=5). Average current responses determined using ramp commands in 5 cells reveal that the voltage at which $K_{Ca}$ channels were open was shifted to less positive potentials (Fig. 5B), consistent with ACh causing elevation of $[Ca^{2+}]_i$. Thus the effect of ACh on $K_{Ca}$ channels was similar to elevation of $[Ca^{2+}]_i$ by Ca$^{2+}$ ionophore in Fig. 1 above.

We investigated the hypothesis that the effects of ACh were mediated by muscarinic receptors using two approaches. Pretreatment of cells with atropine reversibly blocked the effects of ACh in all four cells tested (Fig. 6) and the selective muscarinic agonist muscarine activated $K_{Ca}$ channels (6 of 6 cells tested) just as shown for ACh (Fig. 7).

**3.3.4 ACh causes increased $K_{Ca}$ channel activity independent of Ca$^{2+}$ entry.**

The results provided to this point are consistent with ACh causing elevation of $[Ca^{2+}]_i$, with resultant activation of $K_{Ca}$ channels. To investigate the possible involvement of voltage-dependent Ca$^{2+}$ channels (Kotlikoff, 1988) in the response to ACh, we used the
dihydropyridine Ca\textsuperscript{2+} channel blocker nifedipine. Nifedipine did not alter basal $K_{Ca}$ channel activity (Fig. 7A) and subsequent application of ACh or muscarine caused contraction and typical increases in the activity of $K_{Ca}$ channels in all 4 cells tested (Fig. 7B).

The role of ACh-induced Ca\textsuperscript{2+} release from intracellular stores was studied by bathing cells in low Ca\textsuperscript{2+} solution (containing 1 mM EGTA, 0.2 mM Ca\textsuperscript{2+}, estimated <100 nM free Ca\textsuperscript{2+}) to eliminate the entry of Ca\textsuperscript{2+} from the extracellular solution.
Figure 6. ACh acts on muscarinic receptors to elicit $K_{Ca}$ channel current. A: ACh (2 s, 20 μM in application pipette) caused contraction and a transient increase in channel activity. B: 7 minutes after the response in A, atropine was applied to cell (30 s, 20 μM in the application pipette), after which application of ACh did not elicit contraction or changes in channel activity. C: after 10 min washout of atropine, ACh again caused contraction and a transient increase in channel activity. D: patch was later determined to have only two $K_{Ca}$ channels by excision into bath solution containing 1 mM Ca$^{2+}$, permitting calculation of $P_0$ (2 s segments) for the representative response shown.
Figure 7. ACh causes increased $K_{Ca}$ activity independent of $Ca^{2+}$ entry into cell. A: nifedipine (20 μM) was applied to cell for 30 s, with subsequent application of muscarine (2 s, 20 μM in application pipette) causing contraction and a typical increase in unitary $K_{Ca}$ currents. $NP_o$ was determined for 2 s segments, and is shown in inset (Nif, nifedipine; Mus, muscarine). $V_m = 50$ mV. B: in another cell, bathing solution was exchanged with low $Ca^{2+}$ solution (1 mM EGTA, 0.2 mM $Ca^{2+}$; therefore <100 nM free $Ca^{2+}$). Application of ACh (2 s, 20 μM in low $Ca^{2+}$ solution) caused contraction (not shown) and a typical transient increase in unitary $K_{Ca}$ currents.
Under these conditions, muscarinic agonists continued to cause typical increases in $K_{Ca}$ channel activity in 12 of 14 cells studied (Fig. 7C). These findings are consistent with entry of Ca$^{2+}$ not being necessary to cause increased $K_{Ca}$ channel activity.

### 3.3.5 Caffeine causes activation of $K_{Ca}$ channels.

We further investigated the signalling pathways contributing to activation of $K_{Ca}$ channels using caffeine, which releases Ca$^{2+}$ from intracellular stores in a variety of smooth muscles (Benham & Bolton, 1986; Janssen & Sims., 1992; Pacaud & Bolton, 1991; Somlyo & Himpens, 1989). Caffeine is thought to act at ryanodine receptors on intracellular Ca$^{2+}$ stores (Berridge & Irvine, 1989). Like ACh, caffeine elicited increased $K_{Ca}$ channel activity in tracheal muscle in 14 of 19 cells studied (Fig. 8). When examined over a range of voltages, caffeine caused $K_{Ca}$ channels to be open at less positive potentials (Fig. 8B and C). The caffeine-activated channels had a slope conductance of $243 \pm 13$ pS (measured in 3 of 14 cells) when $[K^+]_o$ was 135 mM. The average of current responses from these 3 cells shows that caffeine reproducibly shifted channel opening to less positive potentials (Fig. 8C). These results indicate that release of Ca$^{2+}$ from intracellular stores is sufficient to cause increased $K_{Ca}$ channel activity. The interaction between caffeine and ACh was also studied. In 5 cells where caffeine caused increased $K_{Ca}$ channel activity, subsequent application of ACh (5 to 20 seconds after caffeine) caused little or no increase in $K_{Ca}$ channel activity. In each of these 5 cells, ACh on its own was found to cause increased $K_{Ca}$ channel activity.
Figure 8. Release of Ca\textsuperscript{2+} from intracellular stores is sufficient to increase unitary $K_{Ca}$ currents. 

A: caffeine (2 s, 5 mM in application pipette) caused increased $K_{Ca}$ channel activity resulting in up to 4 channels being open simultaneously. $V_m = 50$ mV. 

B: before caffeine, single channel currents were apparent at $\sim$60 mV. After caffeine, unitary $K_{Ca}$ currents were apparent at $\sim$0 mV. $V_m$ was varied linearly between -100 and 100 mV over 2 s. Single sweep is shown before and after caffeine. 

C: average current response from 5 cells show that caffeine shifted voltage at which $K_{Ca}$ channels were open to less positive potentials. Control trace is the average of 50 sweeps, and caffeine trace is average of 5 sweeps. Voltage protocol was same as in B. These results indicate that release of Ca\textsuperscript{2+} from intracellular stores is sufficient to cause increased $K_{Ca}$ currents.
It has been reported that ACh inhibits $K_{Ca}$ channel activity in colonic and tracheal smooth muscle cells (Cole et al., 1989; Kume & Kotlikoff, 1991). We investigated the possible suppression of $K_{Ca}$ channels by ACh in several ways. Cells were held at a potential suitable to elicit $K_{Ca}$ channel opening in the absence of muscarinic stimulation ($V_m \geq 50$ mV). After the ACh-induced transient increase in $K_{Ca}$ channel activity (as described above), there was no evidence for suppression of $K_{Ca}$ channels after either brief or prolonged applications of ACh. Because ACh has been reported to act directly upon $K_{Ca}$ channels in excised patches (Kume & Kotlikoff, 1991), we also included 20 or 100 μM ACh in the patch pipette while recording in the cell-attached patch configuration. In 5 cells in which ACh was present in the patch pipette, the voltage-dependence of $K_{Ca}$ channel opening was not noticeably different from control cells (e.g. Fig. 1C and D, 5B, and 8C). Furthermore, application of ACh to the rest of the cell caused typical activation of the $K_{Ca}$ channels in the patch (not shown). On the basis of these results, we found no evidence that ACh inhibited $K_{Ca}$ channels directly or indirectly in intact canine tracheal smooth muscle cells.

3.4 Discussion

We have demonstrated that muscarinic agonists cause transient activation of large-conductance Ca$^{2+}$ dependent $K^+$ channels ($K_{Ca}$) in freshly dissociated tracheal smooth muscle cells. This effect on $K_{Ca}$ channels is indirect, since channel activity was recorded from cell-attached patches isolated from the ACh applied to the rest of the cell. The effect of ACh was to cause channel opening at less positive potentials, consistent with a transient elevation of [Ca$^{2+}$]. ACh could elicit increased $K_{Ca}$ channel activity in the presence of organic Ca$^{2+}$ channel blockers, in the absence of extracellular Ca$^{2+}$, and with the cell
membrane potential clamped near 0 mV, suggesting that ACh effects involved the elevation of [Ca$^{2+}$]$_i$ due to release from intracellular stores.

The predominant channel activity recorded in cell-attached patches from canine tracheal smooth muscle cells was the large-conductance $K_{Ca}$ channel. We identified $K_{Ca}$ channel current based on four characteristics (Fig. 1). First, the channel had a large unitary conductance of 242 ± 5 pS when recorded with 135 mM K$^+$ in the extracellular solution (Fig. 1B). Second, the channel was voltage-dependent, with increased channel activity at more positive potentials (Fig. 1C and D). Third, ion substitution experiments revealed the channel was selective for K$^+$, with the reversal potential of the unitary currents shifting 56 ± 3 mV per ten-fold change of [K$^+$]$_o$ (Fig. 1E). Fourth, elevation of [Ca$^{2+}$]$_i$ caused increased channel activity (Fig. 1A, C, and D). The Ca$^{2+}$ sensitivity of the $K_{Ca}$ channels was demonstrated both by excision of patches into solution containing 1 mM Ca$^{2+}$ and by recording from cell-attached patches on cells treated with A23187 to elevate [Ca$^{2+}$]$_i$. These $K_{Ca}$ channel characteristics resemble those described in many types of smooth muscle cells (e.g. Benham et al., 1986; Kume, et al., 1989; McCann & Welsh, 1986; Singer & Walsh, 1987; reviewed in Tomita, 1988; Toro & Stefani, 1991). The channel that we show to be activated by muscarinic agonists was the $K_{Ca}$ channel based upon several characteristics, including large unitary conductance (247 ± 10 pS), voltage sensitivity, and current reversal (-1 ± 1 mV with 135 mM [K$^+$]$_o$) (Fig. 3 and 5). ACh also caused increased $K_{Ca}$ channel activity in cells bathed in physiologic solutions containing 5 mM K$^+$. However, under such conditions, ACh depolarizes the cell (Janssen & Sims, 1992). Depolarization of the cell results in changes of the patch potential and may also lead to activation of voltage-dependent Ca$^{2+}$ channels.
The regulation of \( K_{Ca} \) channels by neurotransmitters and neuropeptides has been demonstrated by others. Excitation of smooth muscle by cholinergic agonists causes transient activation of outward \( K^+ \) current when recorded in the whole-cell configuration (Benham & Bolton, 1986; Sims et al., 1990), consistent with transient elevation of \([Ca^{2+}]_i\) and subsequent activation of \( Ca^{2+}\)-dependent \( K^+ \) currents. ACh was shown to cause transient hyperpolarization of guinea pig coronary smooth muscle due to activation of \( Ca^{2+}\)-dependent \( K^+ \) channels (Ganitkevich & Isenberg, 1990). There have been several reports describing the modulation of large conductance \( K_{Ca} \) channels in smooth muscle by excitatory agents. Substance P causes activation of \( K_{Ca} \) channels in rabbit colonic smooth muscle, an effect attributed to entry of \( Ca^{2+} \) through dihydropyridine-sensitive \( Ca^{2+} \) channels (Mayer et al., 1990). Recent studies of \( \beta\)-escin-skinned bovine tracheal and guinea pig bladder smooth muscle cells revealed that ACh, caffeine, and substance P cause activation of \( K_{Ca} \) channels (Muraki et al., 1992). This study further demonstrated that the introduction of IP\(_3\) into the cytosol caused activation of \( K_{Ca} \) channels which mimicked that seen in response to ACh, substance P, and caffeine (Muraki et al., 1992).

Activation of \( K_{Ca} \) channels has been reported to underlie the relaxant effects of \( \beta\)-adrenergic agonists on smooth muscle cells (Kume et al., 1989; Sadoshima, Akaike, Kanaide & Nakamura, 1988). In these reports, the effects of \( \beta\)-agonists were mimicked by adenosine 3', 5'–cyclic monophosphate (cAMP) analogues and by cAMP-dependent protein kinase itself, providing evidence that phosphorylation of \( K_{Ca} \) channels can increase their open probability (see also Carl, Kenyon, Uemura, Fusetani & Sanders, 1991). The mechanism of ACh-activation of \( K_{Ca} \) channels reported here (Fig. 2, and 5-7) is different.
from that reported for adrenergic activation (Kume et al., 1989; Sadoshima et al., 1988) in both the latency and time course.

Cholinergic agonists have been reported to suppress $K_{Ca}$ currents in smooth muscle (Cole et al., 1989; Kume & Kotlikoff, 1991), which would contribute to depolarization and excitation of these cells (Sims & Janssen, 1992). Cholinergic stimulation is reported to suppress $K_{Ca}$ channel activity by shifting the voltage-activation range of $K_{Ca}$ channels to more positive potentials, so that at any voltage the channels spend less time open (Cole et al., 1989; Kume & Kotlikoff, 1991). Many smooth muscles also exhibit spontaneous transient outward currents, thought to be due to spontaneous release of Ca$^{2+}$ from intracellular stores causing activation of a Ca$^{2+}$-dependent K$^+$ current (Benham & Bolton, 1986). ACh suppresses spontaneous transient outward currents in a variety of cells (e.g. Benham & Bolton, 1986, Sims et al., 1990), including these tracheal smooth muscle cells (Janssen & Sims, 1992). We saw no evidence for cholinergic suppression of $K_{Ca}$ channel activity when ACh was present at the extracellular face of the channel. However, in contrast to the studies by Kume and Kotlikoff (1991), who used the excised outside-out patch configuration and clamped Ca$^{2+}$ at the cytosolic face, we investigated the effects of ACh in cell-attached patches, where changes of $[Ca^{2+}]_i$ could obscure suppression of $K_{Ca}$ channels by ACh.

Stimulation of muscarinic receptors in many cell types involves G protein-mediated activation of phospholipase C, which in turn leads to generation of at least two second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (IP$_3$)(Berridge & Irvine, 1989). A large body of evidence supports the hypothesis that IP$_3$ participates in excitation-contraction coupling in smooth muscle by releasing Ca$^{2+}$ from the sarcoplasmic reticulum.
(SR) (Muraki et al., 1992; reviewed in Somlyo & Himpens, 1989). Several results presented here are consistent with ACh releasing Ca\(^{2+}\) from intracellular stores, with subsequent activation of K\(_{Ca}\) channels. ACh and muscarine activated K\(_{Ca}\) channels when Ca\(^{2+}\) entry was blocked with nifedipine (Fig. 7A) or in the absence of extracellular Ca\(^{2+}\). Furthermore, caffeine activated K\(_{Ca}\) channels (Fig. 8), mimicking the effects of ACh and muscarine, as also reported in other smooth muscles (Akbarali, Nakajima, Wyse & Giles, 1989; Muraki et al., 1992). We also found that caffeine diminished or inhibited subsequent effects of ACh. Similar interaction between ACh and caffeine have been previously demonstrated for ACh activated K\(^+\) currents in coronary smooth muscle cells (Ganitkevich & Isenberg, 1990). These findings are consistent with the hypothesis that depletion of intracellular Ca\(^{2+}\) stores prevents activation of K\(_{Ca}\) channels by ACh.

Changes in Ca\(^{2+}\)-dependent channel activity reflect alterations in [Ca\(^{2+}\)]\(_i\) in many cell types (Marty, 1989). The effect of ACh on K\(_{Ca}\) channels reported here was to shift the voltage-activation range to less positive potentials (Fig. 5). This is precisely what would be expected for K\(_{Ca}\) channels upon elevation of [Ca\(^{2+}\)]\(_i\) (Benham et al., 1986; Carl & Sanders, 1989; McCann & Welsh, 1986; Singer & Walsh, 1987). In the presence of ACh, K\(_{Ca}\) currents became apparent near 0 mV (Fig. 5B) and in some cases, as negative as -30 mV (Fig 5A). On the basis of the Ca\(^{2+}\) sensitivity reported by Carl and Sanders (1989), we can speculate about the [Ca\(^{2+}\)]\(_i\) in the smooth muscle cells used for this study. At rest, K\(_{Ca}\) channel activation threshold was near 50 mV, consistent with resting [Ca\(^{2+}\)]\(_i\) of approximately 100 nM. During peak activation with ACh, [Ca\(^{2+}\)] immediately beneath the membrane may be elevated to between 500 and 750 nM.
Previous studies have shown that ACh depolarizes tracheal muscle cells to approximately -20 mV (Janssen & Sims, 1992). This effect is due to activation of nonselective cation and chloride currents, mediated in part by release of Ca\(^{2+}\) from intracellular stores. More recent studies have demonstrated that the muscarinic signalling pathway converges with that of another excitatory agent. Both histamine and ACh cause activation of Ca\(^{2+}\)-dependent Cl\(^-\) currents, due to release of Ca\(^{2+}\) from intracellular stores (Janssen & Sims, 1993a). In addition, whole cell studies reveal that an outwardly rectifying K\(^+\) current is elicited at potentials positive to -20 mV. The activation of \(K_{Ca}\) channels described in this report occurs over a similar range of membrane potentials, and could therefore account for the macroscopic K\(^+\) currents elicited by ACh and histamine. The physiologic role of \(K_{Ca}\) channels may be to limit depolarization of cells in response to excitatory agonists. \(K_{Ca}\) channels have also been proposed to participate in repolarizing the cell membrane of smooth muscle (Carl et al., 1990; Walsh & Singer, 1987). It appears then that elevation of \([Ca^{2+}]_i\) in smooth muscle cells initiates a number of different events, including contraction (Sims, 1992) and activation of several types of Ca\(^{2+}\)-dependent ion channels, just as described for other cell types (Marty, 1989).

In conclusion we have provided evidence that muscarinic stimulation of tracheal smooth muscle cells causes activation of large-conductance \(K_{Ca}\) channels. This is mediated in part by release of Ca\(^{2+}\) from intracellular stores and may provide negative feedback to limit depolarization in response to muscarinic excitation.

3.5 References


CHAPTER 4

4 ROLES OF DELAYED RECTIFIER AND CA\textsuperscript{2+}-DEPENDENT K\textsuperscript{+} CURRENTS IN REGULATING CONTRACTION OF HUMAN ESOPHAGEAL SMOOTH MUSCLE

This chapter has been submitted for publication;

Wade, G.R., Laurier, L.G., Preiksaitis, H.G. & Sims, S.M. (Submitted). Roles Of Delayed Rectifier And Ca\textsuperscript{2+}-Dependent K\textsuperscript{+} Currents In Regulating Contraction Of Human Esophageal Smooth Muscle.

G. Wade recorded and analyzed all electrophysiological data, prepared all figures and co-wrote the manuscript with S. Sims with comments from H. Preiksaitis and L. Laurier. L. Laurier undertook molecular biological studies of the expression of delayed rectifier channels in human esophageal muscle cells. Muscle strip studies were carried out in the lab of H. Preiksaitis by T. Chrones and were planned and analyzed by G. Wade.
4.1 Introduction
A variety of inwardly rectifying, voltage-, Ca\textsuperscript{2+}- and ATP-dependent K\textsuperscript{+} channels play key roles in controlling the excitability of smooth muscles (see reviews Quayle, Nelson & Standen, 1997; Kuriyama, Kitamura, Ito & Inoue, 1998). In visceral smooth muscles, including gastrointestinal (Vogalis & Sanders, 1991) and airway (Muraki, Imaizumi, Kojima, Kawai & Watanabe, 1990; Fleischmann, Washabau & Kotlikoff, 1993), studies support an important role for channels from the delayed rectifier family (see below) in the regulation of resting membrane potential. Studies of spontaneous transient outward currents suggest the large-conductance Ca\textsuperscript{2+}-dependent K\textsuperscript{+} (K\textsubscript{Ca}) channel can also participate in the regulation of membrane potential and excitability of smooth muscle (Nelson, Cheng, Rubart, Santana, Bonev, Knot & Lederer, 1995).

K\textsuperscript{+} channels are encoded by a superfamily of potassium channel genes (reviewed in Jan & Jan, 1997). Recent studies have determined the molecular identity of some voltage-dependent K\textsuperscript{+} channels from smooth muscle, including a number of delayed rectifier (K\textsubscript{v}) channel types from the Shaker family. For example, mRNA for Kv1.1, 1.2 and 1.5 has been identified in visceral and vascular smooth muscles (Hart, Overturf, Russell, Carl, Hume, Sanders & Horowitz, 1993; Overturf, Russell, Carl, Vogalis, Hart, Hume, Sanders & Horowitz, 1994; Adda, Fleischmann, Freedman, Yu, Hay & Kotlikoff, 1996). Additionally, a voltage-dependent channel from the Shab family (Kv2.2) has recently been cloned and expressed from colonic smooth muscle (Schmalz, Kinsella, Koh, Vogalis, Schneider, Flynn, Kenyon & Horowitz, 1998). K\textsubscript{Ca} channels have been cloned from vascular and visceral smooth muscles (McCobb, Fowler, Featherstone, Lingle, Saito,
Studies of esophageal muscle from a number of animal models have identified several types of $K^+$ channels. $K_V$ and $K_{Ca}$ currents have been observed in circular muscle from opossum esophagus and rabbit esophageal muscularis mucosae (Akbarali, 1993; Akbarali, Hatakeyama, Wang & Goyal, 1995; Jury, Boev & Daniel, 1996). Blockade of a transient outward $K^+$ current in opossum esophageal muscle cells with 4-aminopyridine (4-AP) caused depolarization, leading Akbarali and co-workers to conclude that this current contributes to setting the resting membrane potential. In early studies of opossum esophagus, tetraethylammonium (TEA), a blocker of $K_{Ca}$ currents was shown to cause development of myogenic contractions (Sarna, Daniel & Waterfall, 1977). At the time of those studies, the range of $K^+$ channel types expressed in esophageal muscle had not yet been described, and still, little is known of their role in human esophagus.

The objectives of this study were to characterize $K^+$ currents in cells from the body of human esophagus, and to examine their functional role in regulation of contraction. We have identified two types of $K^+$ currents, a $K_V$ current and a $K_{Ca}$ current. mRNAs encoding the Kv1.2 and Kv1.5 genes were identified. Functional studies of contraction of human smooth muscle strips revealed that $K^+$ channels serve distinct roles in the regulation of esophageal contraction. Portions of this work have been presented in abstract form (Wade, Preiksaitis & Sims, 1997).
4.2 Methods

4.2.1 Tissue retrieval and isolation of cells

Tissue collection was carried out in accordance with guidelines of the University Review Board for Research Involving Human Subjects and conformed to the Helsinki Declaration. Patient consent was obtained for removal of tissues. Tissues were obtained from 29 patients undergoing esophageal resection because of cancer. Upon resection, specimens were immediately cooled on ice. A sample of the entire thickness of the muscularis propria (~1 cm²) was removed from a disease-free region of the distal third of the esophagus and placed in ice-cold, oxygenated Krebs solution (see below) for transport to the laboratory. Portions of muscle were dissected for preparation of dispersed cells and others for tissue strip studies (see below).

Muscle cells were dispersed as previously described (Sims, Jiao & Preiksaitis, 1997). Briefly, segments of esophagus (~1 mm wide by 1 cm length) were placed in 2.5 ml of dissociation solution consisting of (in mM): NaCl, 5; KCl, 135; CaCl₂, 0.5; MgCl₂, 1; Hepes, 20; D-glucose, 10; plus the following: collagenase, 0.2 mg/ml (Sigma blend type F); bovine albumin, 2 mg/ml (ICN Biomedicals Inc.); papain, 2.5 mg/ml; 1,4 dithio-L-threitol, 0.4 mg/ml; taurine, 10 mM and EDTA, 0.5 mM, adjusted to pH 7.0 with NaOH. Tissues were stored in dissociation solution at 4°C overnight. The following day tissues were warmed to room temperature for 30 to 60 min then placed in a gently shaking water bath at 31°C for 60 min. Tissues were rinsed with warm Ringer's solution (31°C) and dispersed by gentle trituration with fire-polished pipettes. Cells used for RNA isolation (below) were filtered through a 210 μm mesh and then washed with ~4 ml of sterile ice-cold phosphate-buffered saline by repeated centrifugation (1000 RPM for 5 min at 4°C). The
cell pellet was re-suspended in 250 µl of Medium-199, gently layered on Percol and sedimented at 30,000 x G for 15 min. The cells formed a clearly visible band and were collected and stored at −70°C until RNA isolation was carried out.

4.2.2 Electrophysiology
Electrophysiological studies were performed at room temperature (22 to 25°C) within 8 h of dispersion. Whole-cell recordings of current employed the nystatin perforated-patch technique using an Axopatch 1D amplifier (Axon Instruments, Foster City, California). Recording of whole-cell currents was initiated when the access resistance had stabilized at <20 MΩ and series resistance compensation up to 80% was often used. Currents were filtered at 500 Hz and recorded at 2 kHz. Capacitive currents were compensated online using amplifier circuitry. Currents were corrected for linear leakage as assessed at negative potentials. Cells were observed in a perfusion chamber (1 ml volume, perfused at 1 to 3 ml/min) with a Nikon phase contrast microscope.

4.2.3 Solutions
Electrophysiological studies were performed with cells bathed in Ringer's solution containing (in mM): NaCl, 130; KCl, 5; CaCl₂, 1; MgCl₂, 1; HEPES, 20; D-glucose, 10; pH adjusted to 7.4 with NaOH. The recording electrode solution contained: KCl, 30; K aspartate, 100; NaCl, 10; HEPES, 20; MgCl₂, 1; EGTA, 1; CaCl₂, 0.4; adjusted to pH 7.2 with NaOH. Electrodes were filled at the tip with filtered solution then back-filled with solution containing 250 µg/ml nystatin. Krebs bicarbonate solution contained (in mM): NaCl, 116; KCl, 5; NaH₂PO₄, 2.2; NaHCO₃, 25; MgSO₄, 1.2; CaCl₂, 2.5; D-glucose, 10; EDTA, 0.25; equilibrated with 5% CO₂ and 95% O₂.
4.2.4 Reverse Transcription-PCR (RT-PCR)

Total RNA was isolated from human esophageal longitudinal and circular muscle layers or from dispersed cells, using the method of Chomczynski and Sacchi (1987) using polyinosinic acid (20 μg) as a carrier. RNA samples were run out on 1% TAE-agarose to verify integrity. 2 μg of total RNA from each sample was reverse transcribed for 60 min at 42°C using random hexamers and Superscript RNase H- (GibcoBRL, Gaithersburg, MD). The cDNA was diluted 2.5 times, and 5-8 μl was used in each 50 μl PCR reaction.

The cDNA coding sequences for human Kv1.2 (Ramashwami, Gautam, Kamb, Rudy, Tanouye & Mathew, 1990; GenBank accession no. L02752), Kv1.5 (Curran, Landes, & Keating, 1992; accession no. M83254) and β-actin genes (Ponte, Ng, Engel, Gunning & Kedes, 1984; accession no. M10278) were used to design specific PCR primers (Primer Designer). Actin primers were designed to span an intron so that genomic DNA contamination of the samples could be assessed. Because of the close sequence homology between Kv1.2 and Kv1.5 and other members of the Kv channel family, non-homologous regions were targeted for primer selection. For Kv1.2 the upstream primer was 5'-AGACCACGAGTGCTGTGAGA-3'; the downstream primer was 5'-GGAATAGGTGTGGAAGGTCA-3' (corresponding to nucleotides 81-618; predicted PCR product size: 538 bp). For Kv1.5 the upstream primer was 5'-GTGTAACGTCAAGGCCAAGACCA-3'; the downstream primer was 5'-AGACAGAGGCTTGGAGACAGGAA-3' (nucleotides 1909-2593; predicted product size: 685 bp). For β-actin the upstream primer was 5'-CACTCTTCCAGCCTTCTCTC-3'; the downstream primer was
5'-CTCGTCATACCTGCTGGTGC-3’ (nucleotides 820-1133; predicted product size: 314).

PCR reactions were carried out for 40 cycles in a GeneAmp 2400 PCR thermal cycler (Perkin-Elmer Corp., Norwalk, CT) using 2.5 mM MgCl₂, 0.2 mM dNTPs, 100 μM of primers, and 0.2 μl of Taq DNA polymerase (Gold Taq, Perkin Elmer). Each cycle was 0.5 min at 94°C, 0.5 min at 58°C, and 1 min at 72°C, followed by a final 7 min extension at 72°C. PCR products (13 μl) were analyzed by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. Product identity was confirmed by restriction digest.

4.2.5 Muscle contraction
Muscle strips were prepared as previously described (Sims et al., 1997). Briefly, muscle strips (~2 x 10 mm) were dissected from circular and longitudinal layers of the tissue, ensuring that cells were oriented along the long axis. Strips were mounted in water-jacketed tissue baths containing 10 ml of Krebs bicarbonate solution (see below) and bubbled with 5% CO₂ and 95% O₂ at 37°C. One end of the strip was attached to a Grass FT03 isometric force transducer coupled to a Grass 79E chart-recorder (Grass Instruments, Quincy, MA). Following 1 hour equilibration, the tension of each strip was adjusted so that maximum isometric tension increase was achieved with 10⁻⁶ M carbachol. Electrical stimulation was applied via two platinum wire electrodes that encircled the muscle strip, 1 cm apart. To activate intrinsic nerves, 0.5 ms square wave pulses in 5 s trains applied at 10 Hz and 40-60 volts were used. Smooth muscle cells are not significantly activated by 0.5 ms pulses because of their longer time constant. Hence, in order to study myogenic
responses directly, single square wave pulses of 500 ms duration were applied after cholinergic nerve-mediated responses were blocked by 1 μM tetrodotoxin (TTX) and 1 μM atropine.

4.2.6 Chemicals
Unless otherwise stated chemicals were from Sigma or BDH. Drugs were prepared from stock solutions in distilled water and diluted into the appropriate bathing solution. In the electrophysiology studies, drugs were applied focally to cells (Picospritzer II, General Valve Corp, Fairfield, NJ, USA) with the concentration reported being that in the application pipette. For the tissue strip studies, stock solutions of drugs were added directly to the bath to achieve the concentration reported.

4.2.7 Statistics
Values provided are the mean ± standard error of the mean. Comparisons were made using the Student's t-test, with P<0.05 considered to indicate significant difference. All traces are representative of at least three experiments on muscle from two or more preparations.

4.3 Results
Freshly isolated human esophageal smooth muscle cells were phase-dense, spindle-shaped and varied from 50 to 150 μm in length. Cells isolated from circular or longitudinal layers were the same size, with capacitance of 69 ± 5 pF in circular muscle cells (n=11) and 61 ± 4 pF (n=49) in longitudinal muscle cells. Cells reversibly contracted in response to acetylcholine (ACh) (see Sims et al., 1997). Electrophysiological recording was carried out on 110 cells from 29 specimens.
4.3.1 Outward currents in freshly isolated human esophageal smooth muscle cells

When studied under voltage-clamp, depolarization of cells elicited a transient outward current that peaked and then declined with time (Fig. 1A). At more positive potentials a sustained, noisier current became apparent, suggesting the presence of two distinct conductances. Outward current was abolished when CsCl was substituted for KCl in the electrode solution (data not shown) and was sensitive to several K⁺ channel blockers (below), leading us to conclude that the outward currents were due to K⁺ channels.

The transient current was apparent with depolarization to potentials more positive than -40 mV, greater in amplitude at more positive membrane potentials and delayed in both its onset and time to peak (Fig. 1A, inset). Based on these characteristics we refer to this current as a delayed rectifier (Kᵥ) current. The sustained current was apparent at more positive potentials and accompanied by additional noise on the current traces.
Figure 1. Outward currents in human esophageal smooth muscle cells. A) Series of overlapping traces illustrate currents elicited by prolonged depolarization, where outward current peaked then decayed to a steady-state level. Sustained current exhibited increased noise at more positive voltages. On an expanded time scale, delayed activation of the initial current was apparent, with delay to peak reduced at more positive potentials (inset, panel A). We will refer to the transient current as delayed rectifier K⁺ (Kᵥ) current and the sustained current as Ca²⁺-dependent K⁺ (KCa) current. B) Current-voltage relationship is plotted for peak outward current and sustained current, the latter measured as the average over 200 ms, 4 s after the onset of depolarization. Values are mean ± SEM for currents recorded in five cells. C) Voltage-dependent inactivation of Kᵥ was assessed using conditioning pulses of 5 s and test pulses to 10 mV. Transient current inactivated with depolarizing pre-pulses. D) Summary of activation and inactivation of K⁺ currents. The voltage-dependence of inactivation of Kᵥ current is plotted as a fraction of peak current (I/Ipeak, ▲), indicating mean ± SEM, n=5 longitudinal muscle cells. The solid line represents the best fit Boltzmann relation, with half-inactivation at -42 mV. Conductance of KCa (●) was determined as current at positive potentials when stepping from a holding potential of 0 mV. Conductance of Kᵥ (■) was determined as peak current recorded from -90 mV holding potential after subtraction of KCa. The conductance of Kᵥ was fit with a Boltzmann relation with half-maximal activation at 4 mV and slope factor of 13 mV. Traces are from longitudinal muscle cells.
As will be described below, the characteristics of the sustained current are consistent with that of the large conductance Ca\(^2+\)-dependent K\(^+\) (K\textsubscript{Ca}) channel. The current-voltage relationship for the two outward currents indicated different voltage-sensitivities (Fig. 1B). As well, the transient current exhibited voltage-dependent inactivation, as assessed by recording outward current at 10 mV following depolarization to potentials from -90 mV to 0 mV (Fig. 1C), with half-maximal inactivation at -43 mV (Fig. 1D). The voltage-dependence of activation of Kv and K\textsubscript{Ca} is plotted as conductance (Fig. 1D), assuming a K\(^+\) equilibrium potential of -84 mV. K\textsubscript{Ca} conductance was determined from the mean outward current over 200 ms elicited by step depolarization of cells from a holding potential of 0 mV to potentials from 10 mV to 50 mV. Persistent depolarization of cells to 0 mV for >2 min inactivated the transient current, permitting isolation of the K\textsubscript{Ca} current (see e.g. below, Fig. 2B). The conductance of Kv was determined in the same cells from peak outward current elicited by step depolarization from a holding potential of -90 mV to potentials from -60 mV to 40 mV after subtraction of K\textsubscript{Ca} current (see above). The conductance of Kv plotted in Figure 1D was fit by a Boltzmann equation with half maximal activation at 4 mV. The overlap of the inactivation and activation curves for the Kv conductance is consistent with the presence of a "window current".
Figure 2. Delayed rectifier and Ca\(^{2+}\)-dependent K\(^+\) currents in longitudinal and circular esophageal smooth muscle layers. A) \(K_v\) current was elicited with depolarization from a holding potential of -60 mV to test potentials more positive than -20 mV in cells from longitudinal and circular layers, as indicated. Inactivation is less apparent on this time scale. B) Persistent depolarization (>2 min) of same cells as in A to 0 mV inactivated \(K_v\), with remaining noisy current elicited by depolarization due to \(K_{Ca}\).
4.3.2 Delayed rectifier and Ca\(^{2+}\)-dependent K\(^{+}\) currents from longitudinal and circular muscle cells.

We compared \(K_V\) and \(K_{Ca}\) currents from cells of the longitudinal (Fig. 2, left) and circular (right) layers. Although variability in the proportion of \(K_V\) and \(K_{Ca}\) currents among cells was evident, mean current density calculated from the peak \(K_V\) current elicited by step depolarization from -60 mV to 0 mV was 2.7 ± 0.6 pA/pF for longitudinal cells and 2.9 ± 0.4 pA/pF for circular cells (n=7). \(K_{Ca}\) current was isolated in longitudinal (Fig 2B, left) and circular (right) cells by holding cells at 0 mV to inactivate \(K_V\) currents. Mean current density at 50 mV was 3 ± 0.5 pA/pF for longitudinal cells (n=8) and 5.7 ± 0.9 pA/pF (n=10) for circular cells (P< 0.05), which may contribute to some of the functional differences between layers noted below.

4.3.3 Pharmacological isolation of \(K_V\) and \(K_{Ca}\) currents.

\(K^{+}\) channel blockers were used to distinguish components of the outward current. Step depolarization of cells elicited both transient and sustained components (Fig. 3A). Outward currents in the presence of TEA (10 mM, n=5) were reduced in amplitude, transient in nature and had diminished current noise (Fig. 3B, TEA) consistent with blockade of \(K_{Ca}\) current revealing the \(K_V\) current. Outward currents in the presence of 4-AP (5 mM) were reduced in amplitude, non-inactivating and noisier (Fig. 3B, 4-AP), consistent with 4-AP blockade of \(K_V\) current. The concentration-dependent blockade of \(K_{Ca}\) currents by TEA was determined from the amplitude of \(K_{Ca}\) currents elicited by depolarization at 50 mV from a holding potential of 0 mV (Fig. 3C). \(K_{Ca}\) was half-maximally blocked at 0.1 mM TEA as determined from the best fit of a logistic equation. The concentration-dependent blockade of \(K_V\) current by 4-AP was determined for currents elicited at 0 mV (to minimize contributions from \(K_{Ca}\)), revealing half-maximal inhibition at 3.3 mM (Fig. 3D).
4.3.4 Pharmacological sensitivity of $K_{Ca}$ and insensitivity of $K_V$ currents to ChTX and IbTX.

The sensitivity of esophageal $K^+$ channels to the peptide channel blockers charybdotoxin (ChTX) and iberiotoxin (IbTX) was investigated. When the sustained current was studied in isolation (depolarization from holding potential of 0 mV), both ChTX and IbTX reduced the amplitude and the noise on the current trace (Fig. 4A) providing direct evidence for the large-conductance $Ca^{2+}$ dependent $K^+$ current in human esophagus. In contrast to that seen with TEA, high concentrations of these peptide-blockers incompletely blocked outward current (Fig. 4B). This finding raises the possibility of additional $K^+$ currents in these cells that are TEA sensitive and ChTX and IbTX insensitive. As expected, the transient current was insensitive to IbTX. Notably, ChTX did not block the transient component of the outward current (Fig. 4C) indicating that the $K_V$ current in human esophagus cannot be due solely to the gene products of Kv1.2, which is blocked at low concentrations of ChTX (Russell, Overturf & Horowitz, 1994).
Figure 3. Pharmacological separation of $K_V$ and $K_{Ca}$ currents in human esophagus. A) Depolarization from $-60$ mV to potentials more positive elicited $K_V$ and $K_{Ca}$ currents (control). $K_V$ current was apparent at less positive potentials and declined with time. Further depolarization of cells revealed $K_{Ca}$ current as noisy traces. B) Addition of $K^+$ channel blockers reveals two components of the outward current. Tetraethylammonium (TEA, 10 mM) reduced $K_{Ca}$, leaving the transient current. 4-aminopyridine (4-AP, 5 mM) blocked $K_V$, leaving only the non-inactivating, noisy $K_{Ca}$ current. C,D) Concentration-dependence of $K^+$ channel blockers. $K_{Ca}$ current was studied in isolation using a holding potential of 0 mV. TEA reduced $K_{Ca}$ evaluated at 50 mV, with half-maximal inhibition of 0.1 mM TEA. D) Depolarization from $-60$ mV to 0 mV elicited transient $K_V$ current, which was reduced by 4-AP, with half-maximal inhibition of the peak current at 3.3 mM 4-AP. Solid lines indicate best fit curves with number of cells studied indicated near each point. Traces are from a longitudinal muscle cell.
Figure 4. Charybdotoxin (ChTX) and iberiotoxin (IbTX) block $K_{Ca}$ current. $K_{Ca}$ was isolated by holding cells at 0 mV and stepping positively. A) Both ChTX (300 nM, left panel) and IbTX (200 nM, right panel) reduced outward current compared to control, with concentration-dependence shown in B. C) Depolarization from -60 mV to 80 mV elicited both $K_V$ and $K_{Ca}$ currents. ChTX (300 nM, left) and IbTX (100 nM, right) reduced a sustained component of the outward current, with no effect on the transient current. Traces are from longitudinal muscle cells.
4.3.5 **STOCs in human esophageal smooth muscle cells.**

Studies of $K_{Ca}$ current presented to this point examined the current only at positive potentials. However, we also found evidence for activation of $K_{Ca}$ currents at less positive potentials. Spontaneous transient outward currents (STOCs) were apparent in 20 cells. Spontaneous currents were abolished by TEA and ChTX (Fig. 5A,B). When studied in current clamp, TEA-sensitive spontaneous transient hyperpolarizations were observed at negative resting membrane potentials (Fig. 5C, 3 cells), likely due to STOCs. The spontaneous hyperpolarizations illustrated in Fig. 5C were similar in amplitude to voltage deflections elicited by 10 pA of current. These results indicate that $K_{Ca}$ current can participate in regulation of the resting membrane potential in human esophagus.

4.3.6 **RT-PCR identification of $K_{V}$ channels.**

Several families of delayed rectifier $K^+$ channels have been identified in gastrointestinal smooth muscle, including $Kv1.1$, $1.2$ and $1.5$ (Hart et al., 1993; Overturf et al., 1994; Adda et al., 1996). The $K_{V}$ current in human esophagus was insensitive to ChTX (above) suggesting that $Kv1.2$ alone does not account for the delayed rectifier. Heteromultimers of $Kv1.2$ and $Kv1.5$ channels show reduced sensitivity to ChTX (Russell, Overturf & Horowitz, 1994), perhaps accounting for the insensitivity of native delayed rectifier in esophageal cells. We considered the possibility that co-expression of an insensitive delayed rectifier channel contributed to ChTX insensitivity. RT-PCR of RNA derived from circular and longitudinal layers of human esophageal smooth muscle was amplified using unique primers specific for $Kv1.2$ and $Kv1.5$ yielded the predicted 538 bp and 685 bp products (Fig. 6). Similar results were obtained using 3 different specimens of intact muscle tissue. Rat brain, human heart and colon, tissues previously found to express $Kv1.2$
and Kv1.5 were used as positive controls (Hart et al., 1993; Overturf et al., 1994). The cDNA samples were also tested with primers for human β-actin to monitor the fidelity of the PCR reaction and to screen for genomic DNA contamination of the sample.
Figure 5. Spontaneous transient outward currents (STOCs) in esophageal muscle. A) The potassium channel blocker TEA (10 mM) reversibly abolished STOCs recorded at 0 mV, with recovery at right after 1 min wash. B) STOCs were also blocked by ChTX (100 nM), consistent with involvement of $K_{Ca}$ channels. C) Current-clamp recording from human esophageal cell revealing spontaneous transient hyperpolarizations. Periodic injection of current (10 pA) also hyperpolarized the cell. TEA (4 mM) abolished spontaneous transient hyperpolarizations and depolarized the cell.
Figure 6. Kv1.2 and Kv1.5 potassium channel mRNA expressed in circular and longitudinal layers of human esophageal smooth muscle. A. RNA was extracted from tissues as described in the Methods. Following reverse transcription, cDNA was probed using primers specific for the 5'-coding region of Kv1.2 and Kv1.5. PCR amplification revealed products of the expected size for circular (CM) and longitudinal (LM) muscle, as visualised on an ethidium bromide-stained agarose gel. For control lanes, no cDNA was added to the reaction mixture. For positive controls, mRNA was isolated from human heart and colon, tissues previously demonstrated to express Kv1.2 and Kv1.5. B. Similar results were obtained using RNA from 3 different preparations of dispersed muscle cells (CM1-3 & LM1-3, see Methods) confirming the localization of Kv1.2 and Kv1.5 message to the esophageal smooth muscle.
To exclude the possibility that RNA transcripts detected were due to cells other than esophageal smooth muscle (see Schmalz et al., 1998) we carried out RT-PCR using RNA isolated from purified esophageal smooth muscle cells. Transcripts from Kv1.2 and Kv1.5 were also identified in 3 different cell preparations (Fig. 6B).

### 4.3.7 Functional effects of K⁺ channel blockers.

We investigated the effects of K⁺ channel blockers on contraction of muscle strips. Electrical stimulation of circular muscle (see methods) elicited contraction of muscle strips (Fig. 7A, control). TTX (1 μM, n=6, not shown) blocked this response, confirming the nerve-mediated (neurogenic) basis of the excitation of the muscle. 4-AP caused a reversible increase in the peak contractile force elicited by these neurogenic and spontaneous oscillatory contractions (Fig. 7A). Similarly, TEA caused a reversible, concentration-dependent increase in the amplitude of neurogenic contractions (Fig. 7B). Moreover, TEA also prolonged the duration of these contractions. These findings indicate that Kv and KCa channels can regulate nerve-evoked contraction of esophageal muscle.

However, we could not exclude the possibility that these K⁺ channel blockers acted presynaptically to alter neurotransmitter release from endogenous nerve varicosities. In order to assess the direct effects of K⁺ channel blockers on muscle, atropine (1 μM) and TTX (1 μM) were included in the bathing solution to block neuronal action potentials and muscarinic signalling. Myogenic excitation was evoked with a single broad-pulse electrical stimulation (see methods).
Figure 7. Effects of K⁺ channel blockers on nerve-mediated contraction of human esophagus. Muscle contraction was monitored during periodic field stimulation of the muscle strip (0.5 ms pulses, 10 Hz, 53 s, at times indicated by ↑). A) 4-AP increased the amplitude of nerve-mediated contractions and elicited oscillatory contractions. Reversibility of the effects was apparent during periods of washout (wash). B) TEA caused a concentration-dependent increase in both amplitude and duration of nerve-evoked contractions. Effects of TEA were also reversible (wash). Responses illustrated are from the strips isolated from the circular muscle layer.
4-AP caused a concentration-dependent increase in the resting tension, but did not cause an increase in these electrically evoked contractions (Fig. 8A). As summarized in TABLE 1, the effect of 4-AP on resting tension occurred in all strips tested from both circular and longitudinal muscles. Although the resting tension induced by 4-AP was greater in longitudinal muscle than in circular, the control electrically evoked responses were also greater, precluding direct comparison between the muscle layers. TEA consistently increased the amplitude and duration of electrically evoked contractions, and also caused a small increase in the resting tension in both layers (half of that seen with 4-AP, Fig. 8B, TABLE 1). To account for the effect of TEA on the prolongation of contraction, we measured the area under the tension trace (standardized for control response), revealing half-maximal augmentation of contraction with 1.9 mM TEA for circular muscle and 2.9 mM TEA for longitudinal muscle (Fig. 8C). This augmentation by TEA occurred in both longitudinal and circular muscle (TABLE 1), and was inhibited by nifedipine (10 μM, Fig. 8D, n=4), consistent with a key role for K$_{Ca}$ channels in limiting depolarization during excitation of esophageal muscle.

We also noted that 4AP and TEA caused spontaneous contractions in a fraction of the esophageal strips similar to that described previously in opossum esophagus (Helm, Bro, Dodds, Sarna, Hoffman & Arndorfer, 1991). 4-AP caused oscillatory contraction in 5 of 6 circular muscle strips and 0 of 7 longitudinal muscle strips, with "high frequency" oscillatory contractile activity shown in Figure 9A. TEA caused a lower frequency oscillatory contractile activity in 5 of 8 circular muscle strips and 1 of 8 longitudinal muscle strips (Fig. 9B).
Figure 8. Myogenic contraction of human esophageal smooth muscle. Inclusion of atropine (1 μM) and TTX (1 μM) abolished nerve-mediated contractions and a single broad-pulse stimulus (500 ms, ↑) caused direct activation of smooth muscle. A) 4-AP increased resting tension with no consistent change in the twitch contraction in response to electrical stimulation. B) In contrast, TEA caused a concentration-dependent increase in the amplitude and duration of the contraction, with little change in resting tension. C) Augmentation of the myogenic contraction by TEA was quantified as the change in the area of the contractile responses, standardised relative to the control response. Solid lines indicate the best fit by a logistic equation revealing half-maximal augmentation at 1.9 mM for circular muscle (●), and 2.9 mM for longitudinal muscle (□). Data represent mean responses from 8 circular and 6 longitudinal muscle strips. D) Blockade of L-type Ca²⁺ channels with nifedipine (10 μM) abolished TEA-mediated augmentation of contraction.
TABLE 1. Summary of the effects of K⁺ channel blockers on contraction of human esophageal muscle. A, peak electrically evoked contraction in the presence and absence of K⁺ channel blockers. TEA augmented electrically evoked contraction compared to control in longitudinal and circular muscle layers (*p<0.05, Student's paired T-test) whereas 4-AP had no significant effect. B, change (Δ) in the resting tension, and number of strips responding (n of n) in the presence of K⁺ channel blockers. TEA increased resting tension consistently in longitudinal layer, but only in a portion of circular muscles. 4-AP increased resting tension in all of both longitudinal and circular muscle strips. All studies carried out in the presence of atropine and TTX (see text). The control electrically evoked tension in the absence of K⁺ channel blockers is given in order to illustrate the relative capabilities for tension development between the muscle layers.
Figure 9. Examples of K⁺ channel blockers causing oscillatory contraction of circular esophageal muscle. A) 4-AP caused elevation of resting tension and high-frequency oscillatory contractions. B) TEA increased resting tension and caused low frequency oscillatory contractions.
4.4 Discussion
Little is known of the expression and function of $K^+$ channels in human esophageal muscle. Using freshly isolated smooth muscle cells, we identified delayed rectifier and $Ca^{2+}$-dependent $K^+$ currents. Functional studies of the effects of $K_v$ and $K_{Ca}$ current blockers on human smooth muscle strips reveal distinct roles for these currents in the regulation of contraction. The delayed rectifier current appears to play a dominant role in regulating resting tension of esophageal muscle, whereas $K_{Ca}$ current serves to limit contraction during excitation.

The transient current was identified as a delayed rectifier based upon a number of properties. This current was apparent with depolarization to potentials more positive than -40 mV, greater in amplitude at more positive membrane potentials and delayed in both its onset and time to peak. Plots of the voltage-dependence of conductance and inactivation overlapped, giving rise to a window current over a physiological range of potentials. The transient current was blocked in a concentration-dependent manner by 4-AP, with half-maximal inhibition at 3.3 mM. This delayed rectifier was less sensitive to 4-AP than the cloned $K_v 1.2$ channel which was half-maximally inhibited at 74 $\mu$M 4-AP (Hart, Overturf, Russell, Carl, Hume, Sanders & Horowitz, 1993). This difference may reflect differences between native and expressed channels or as considered below, the delayed rectifier current we observe may be due to heteromultimeric channels. Delayed rectifier currents with similar voltage-dependent characteristics and pharmacological sensitivity have been characterized in a variety of other smooth muscles, including airway (e.g., Boyle, Tomasic, Kotlikoff, 1992; Fleischmann, et al., 1993; Snetkov, Hirst, Twort & Ward, 1995) and
gastrointestinal muscles (e.g. Vogalis et al., 1991; Carl, 1995). A similar $K^+$ current was reported for opossum esophageal smooth muscle (Akbarali et al., 1995).

$K_v1.2$ transcripts have been found in several types of smooth muscle (Hart et al., 1993; Adda et al., 1996). RT-PCR analysis of RNA isolated from human esophageal muscle revealed $K_v1.2$ in these studies. Currents from cloned $K_v1.2$ channels are sensitive to ChTX (Ramashwami et al., 1990), in contrast to our findings for the $K_v$ currents in human esophagus. Co-expression of $K_v1.2$ with $K_v1.5$ channels gives rise to ChTX-insensitive heteromultimeric $K^+$ currents (Russell et al., 1994). Indeed, as found in other smooth muscles (Overturf et al. 1994), mRNA for $K_v1.5$ was also identified in human esophageal muscle, providing a possible explanation for the ChTX-insensitivity of the $K_v$ currents here. More recent studies found evidence for $K_v2.2$ in several types of smooth muscles (Schmalz et al., 1998), raising the possibility that this too may be expressed in esophagus. Studies of the quinine sensitivity of delayed rectifier currents will help to resolve the presence of $K_v2.2$ and other delayed rectifiers in human esophageal tissues.

In addition to $K_v$ currents, we also identified a sustained outward current with characteristics of $K_{Ca}$. The current activated at quite positive voltages and was blocked in a concentration dependent manner by TEA and the selective $K_{Ca}$ peptide antagonist IbTX. These characteristics are consistent with the presence of large conductance Ca$^{2+}$-dependent $K^+$ channels, which have been characterized at the single channel level in these cells (Hurley, Preiksaitis & Sims, 1999). Both IbTX and ChTX failed to completely inhibit the sustained outward current, suggesting the presence of an additional conductance, not yet identified. $K_{Ca}$ currents are identified in most smooth muscles, and have been cloned and expressed from vascular and colonic muscles (McCobb et al., 1995; Vogalis et al., 1996).
Although in voltage-clamp recordings macroscopic $K_{Ca}$ currents were not active at physiologic potentials, STOCs and their corresponding transient hyperpolarizations were observed in some esophageal cells. These observations, combined with functional studies of the effects of $K_{Ca}$ channel blockers on contraction of strips (discussed below), suggest that $K_{Ca}$ can participate in the regulation resting membrane potential, as described in other cell types (e.g., Carl, McHale, Publicover, Sanders, 1990). Elevation of $[Ca^{2+}]$ in restricted regions ($Ca^{2+}$ sparks) due to release of $Ca^{2+}$ from intracellular stores underlies STOCs in vascular and esophageal smooth muscle (Nelson et al., 1995; Kirber, Etter, Singer, Fay & Walsh, 1997). We have previously described the contribution of $Ca^{2+}$-stores in cholinergic excitation of these muscles (Sims et al., 1997), as well as regulation of $K_{Ca}$ channels (Hurley et al., 1999). TEA-sensitive STOCs have previously been suggested to participate in the control of resting membrane potential in cat esophageal smooth muscle cells (Sims, Vivaudou, Hillemeier, Biancani, Walsh & Singer, 1990).

Functional roles for $K_{V}$ currents were investigated in muscle strips using channel blockers. 4-AP consistently caused an increase in resting tension, in some tissues accompanied by spontaneous oscillations. While 4-AP augmented nerve-mediated contraction, little effect was observed on myogenic response when nerves were blocked with TTX and in the presence of atropine. Thus, the effects of 4-AP on nerve-mediated contraction were likely due to pre-synaptic actions. These findings suggest the role for $K_{V}$ currents in human esophagus lies in setting the resting membrane potential, thereby regulating activation of voltage-dependent $Ca^{2+}$ channels and $Ca^{2+}$ influx, that has previously been shown to contribute to excitation of these muscles (Sims et al., 1997). Delayed rectifier $K^{+}$ channels control resting membrane and muscle tone in a variety of muscles, including opossum...
esophagus (Akbarali et al., 1995) and airway muscles (Muraki et al., 1990; Fleischmann et al., 1993; Adda et al., 1996).

K_{Ca} currents had distinct functional roles in regulating the contraction of human esophagus. Most notably, TEA caused marked augmentation of electrically evoked contractions, increasing both their peak tension and duration. The effects of TEA were concentration dependent, blocked by nifedipine, and not affected by blockade of nerves in the tissues. Together, these findings strongly suggest a role for K_{Ca} channels in limiting excitation and contraction. Elevation of Ca^{2+} will initiate contraction, as well as activation of K_{Ca} channels causing outward K^{+} current and hastening repolarization of the membrane, thereby limiting Ca^{2+} entry and contraction. Consistent with this model, K_{Ca} has been shown to be activated during muscarinic excitation of human esophageal cells (Hurley et al., 1999).

Blockade of K_{Ca} with TEA in some strips resulted in small increases in resting tension and spontaneous oscillatory contractions. This occurred most frequently in longitudinal muscle, indicating that K_{Ca} channels may also participate in regulating the resting membrane potential and resting tension. While macroscopic K_{Ca} currents were not apparent at resting potentials in unstimulated muscles, STOCs can contribute to setting the resting membrane potential (Sims et al., 1990; Nelson et al., 1995). K_{Ca} channels have previously been implicated in the control of esophageal smooth muscle. Although peristalsis is primarily a nerve-mediated phenomenon, TEA induces waves of contraction that propagate in both directions in an intact esophagus, even with intrinsic nerves blocked (Sarna et al. 1977; Helm, Bro, Dodds, Sarna & Hoffman, 1992). While differences between circular and longitudinal layers have been noted in some smooth muscles, there
were no qualitative differences between layers in esophageal muscle. We base this on the characteristics of cell capacitance, mRNA expression of delayed rectifiers and functional effects on contraction of muscle strips. However, circular muscle did have a larger $K_{Ca}$ current density and muscle strips from this layer exhibited oscillatory contractions in response to $K^+$ channel blockers, although the relationship between these two variables is not presently understood.

In summary, we have identified $K_V$ and $K_{Ca}$ currents in human esophageal muscle and found that they serve distinct physiological roles. $K^+$ channels in many tissues are targets for modulation by inhibitory as well as excitatory factors. Agents that cause relaxation of esophageal muscle activate $K^+$ currents, contributing to hyperpolarization. For example, NO' activates $K_{Ca}$ current in opossum esophagus, contributing to inhibitory junction potentials (Cayabyab & Daniel, 1995; Murray, Shibata, Buresh, Picken, O'Meara & Conklin, 1995) and delayed rectifier $K^+$ channels are activated by cAMP-dependent pathways (Koh, Sanders & Carl, 1996). In addition, $K^+$ currents are targets for suppression by excitatory pathways. Cholinergic excitation suppresses STOCs in esophageal muscle (Sims et al., 1990) and inhibits some cloned smooth muscle $K_V$ currents (Vogalis, Ward & Horowitz, 1995). Further studies are required to characterize the physiological regulation of $K^+$ currents in human esophagus.

4.5 References


5.1 Summary And Conclusions

These studies were undertaken to investigate the regulation and roles of membrane channels in excitation contraction coupling of smooth muscle. We investigated the regulation of L-type Ca\(^{2+}\) and large conductance Ca\(^{2+}\)-dependent K\(^{+}\) currents by excitatory neurotransmitters and characterized two distinct K\(^{+}\) currents and their roles in esophageal excitation contraction coupling in human esophageal muscle strips. A number of the features investigated were common to all the muscles studied revealing their fundamental role in the regulation of excitation contraction coupling in smooth muscle.

ACh caused contraction of guinea pig gastric cells and acutely inhibited I\(_{\text{Ca}}\). Several findings led us to the conclusion that the effects of ACh were mediated by release of Ca\(^{2+}\) from intracellular stores. The inhibition was rapid in onset, repeatable and reversible. Depletion of Ca\(^{2+}\) stores either by persistent bathing of cells in Ca\(^{2+}\)-free solution, or by blockade of the SR Ca\(^{2+}\)-ATPase, attenuated the ACh-mediated inhibition. ACh reduced the open probability of Ca\(^{2+}\) channels when recorded in cell-attached patches, providing additional evidence for the involvement of a cytosolic signalling pathway. Inhibition of I\(_{\text{Ca}}\) was also observed in canine and guinea-pig tracheal muscles, indicating that such regulation may be a fundamental feature of cholinergic signalling in mammalian smooth muscle. We proposed that elevation of Ca\(^{2+}\) concentration at the cytosolic face of the membrane elicits direct Ca\(^{2+}\) dependent inhibition of the channel, although, other Ca\(^{2+}\)-dependent inhibitory mechanisms may also participate in the observed inhibition that was observed.

ACh caused transient activation of K\(_{\text{Ca}}\) in freshly dissociated tracheal smooth muscle cells. This effect was due to the actions of a cytosolic second messenger, since channel activity
was recorded from cell-attached patches isolated from the ACh applied to the rest of the cell. The effect was to cause channel opening at less positive potentials, consistent with a transient elevation of [Ca$^{2+}$]$_i$. ACh could elicit increased $K_{Ca}$ channel activity in the presence of organic Ca$^{2+}$ channel blockers, in the absence of extracellular Ca$^{2+}$, and while the cell membrane potential was clamped near 0 mV, suggesting that ACh effects involved the elevation of [Ca$^{2+}$]$_i$ due to release from intracellular stores.

In studies of single cells isolated from human esophagus, we identified delayed rectifier and Ca$^{2+}$-dependent $K^+$ currents based upon their activation and inactivation characteristics and sensitivity to pharmacological antagonists. In functional studies of contraction, blockade of $K_{Ca}$ augmented and prolonged contraction, while blockade of $K_v$ caused an increase in resting tension. These studies reveal distinct roles for these currents in the regulation of contraction. The delayed rectifier current appears to play a dominant role in regulating resting tension of esophageal muscle, whereas $K_{Ca}$ current serves to limit contraction and participate in recovery.

5.2 Physiological Roles For Ca$^{2+}$ and K$^+$ Channels In Smooth Muscle
When considered as a whole, these studies reveal the importance of ionic currents in the regulation of excitation, contraction and recovery in smooth muscle. How does the inhibition of Ca$^{2+}$ currents and activation of $K_{Ca}$ contribute to limiting excitation and contribute to recovery of smooth muscle, and, what is the evidence that these pathways have functional effects on the contraction of smooth muscles?

The schematic diagram in figure 1 provides an overall view of the cellular processes studied in this thesis. ACh causes release of Ca$^{2+}$ from intracellular stores and
depolarization of the membrane by activating nonselective cation and Cl\(^-\) currents. Depolarization leads to further elevation of \([Ca^{2+}]_i\), due to the opening of L-type Ca\(^{2+}\) channels and resultant Ca\(^{2+}\) influx. The net result of the signalling pathways initiated by ACh is elevation of \([Ca^{2+}]_i\) and contraction, with contraction providing an index of both the duration and rise of \([Ca^{2+}]_i\).

In studies of single cells, we observed that ACh caused contraction, but this effect was accompanied by inhibition of Ca\(^{2+}\) current and activation of K\(^+\) channels as summarized in figure 1. We proposed that the effects of ACh on Ca\(^{2+}\) and K\(^+\) currents contribute to limiting excitation and promoting recovery of smooth muscle. Thus the effects reported in this thesis play an integral role in the excitation/contraction recovery cycle of smooth muscles. To extend the electrophysiological data, we also studied the functional role of K\(^+\) channels in contraction. We observed that inhibition of \(K_{Ca}\) currents augmented and prolonged electrically induced contraction of smooth muscle strips while blockade of \(K_v\) currents caused an increase in the basal level of muscle tension. The effects of K\(^+\) channel blockers on contraction of smooth muscle strips were abolished by Ca\(^{2+}\) channel blockers, confirming that regulation of Ca\(^{2+}\) entry is also important to the level of excitation. Therefore, the functional studies of K\(^+\) and Ca\(^{2+}\) channel blockers on esophageal smooth muscle strips provide complementary evidence for the physiological participation of these currents in excitation of smooth muscle.
Figure 1. Schematic diagram showing some of the molecular participants involved in regulating excitation and recovery of smooth muscle. Binding of ACh to M2 receptors (MACHR2) permits opening of nonselective cation channels leading to depolarization ($\Delta V$) and opening of Ca$^{2+}$ channels. Binding of ACh to M3 receptors (MACHR3) causes generation of IP$_3$ that elicits release of Ca$^{2+}$ from intracellular stores. Together Ca$^{2+}$ influx and release from intracellular stores causes contraction, inhibition of ICa, activation of KCa and IC$_{Cl}$. KCa and KV currents participated in limiting excitation and recovery of the membrane potential to the resting level.
5.3 New literature supporting the findings in the published studies.
Several researchers have investigated the regulation of Ca$^{2+}$ currents by excitatory release of Ca$^{2+}$ from intracellular stores in smooth muscle. Using conventional whole-cell recording, Yamashita and Kokubun (1997) found that ACh caused both an acute and prolonged suppression of $I_{\text{Ca}}$ in guinea pig tracheal smooth muscle cells. Dialyzation of the cells with 50 nM BAPTA to prevent elevation of [Ca$^{2+}$]$_i$ abolished the transient inhibition. They concluded the acute inhibition was due to elevation of [Ca$^{2+}$]$_i$ while the prolonged suppression was due to activation of PKC. Similarly, caffeine was found to cause transient and prolonged inhibition of $I_{\text{Ca}}$ in guinea pig urinary bladder smooth muscle cells, with the transient component, abolished by inclusion of EGTA in the electrode (Yoshino, Matsufuji & Yabu, 1996). Yoshikawa and co-workers (1996) found that sequestration of Ca$^{2+}$ by stores participates in the regulation of Ca$^{2+}$ currents, since blockade of the SR Ca$^{2+}$-ATPase with CPA causes a decrease in Ca$^{2+}$ current in guinea-pig urinary bladder cells.

The regulation of $K_{\text{Ca}}$ channels by excitatory transmitters has also been the subject of investigation since the publication of our findings. Hurley et al. (1999) found that cholinergic release of Ca$^{2+}$ from intracellular stores increased $K_{\text{Ca}}$ activity in human esophageal smooth muscle cells, further supporting a role for muscarinic release of Ca$^{2+}$ from stores regulating ion channels in visceral smooth muscles. Kim and co-workers noted in rabbit cerebral basilar artery cells, that histamine and caffeine induced oscillatory changes in [Ca$^{2+}$]$_i$ and $K_{\text{Ca}}$ activity (Kang, So & Kim, 1995). Although this group did not record channel activity and [Ca$^{2+}$]$_i$ simultaneously, they concluded that oscillatory changes in Ca$^{2+}$ likely was responsible for the regulation of $K_{\text{Ca}}$ in these cells. Lee and Earm (1994) recorded $K_{\text{Ca}}$ and [Ca$^{2+}$]$_i$ simultaneously in rabbit pulmonary artery smooth muscle cells.
and observed that caffeine and depolarization caused oscillatory increases in K_Ca activity that were correlated with elevated [Ca^{2+}]_i. Thus release of Ca^{2+} from stores by excitatory transmitters appears to be of widespread significance in smooth muscle.

The use of high resolution scanning confocal microscopy combined with Ca^{2+}-fluorescence imaging permits visualization of regions of intracellular Ca^{2+} release or "hot spots" (Imaizumi, Torii, Ohi, Nagano, Atsuki, Yamamura, Muraki, Watanabe & Bolton, 1998). Hot spots and K_Ca activity were blocked by caffeine and cyclopiazonic acid, and increased in size in response to depolarization of cells. The authors concluded that Ca^{2+} entry leads to release of Ca^{2+} from intracellular stores that activates K_Ca in guinea pig vas deferens and urinary bladder smooth muscle. These findings further support the role of stores in mediating release of Ca^{2+} and regulation of currents in smooth muscle.

### 5.4 Limitations of the Studies and Suggestions For Future Experiments

In our studies of the regulation of L-type Ca^{2+} channels by ACh, we could not definitively conclude that Ca^{2+-}dependent inhibition was the mechanism underlying the reduction in Ca^{2+} currents. Schumann and co-workers (1997) find that in addition to intracellular Ca^{2+} mediating inhibition of Ca^{2+} currents directly, elevation of [Ca^{2+}] at the cytosolic face of the membrane activates protein phosphatase 2B leading to reduced channel open probability, likely due to dephosphorylation of the channel. Such regulation demonstrates that intracellular Ca^{2+} participates in two distinct negative feedback effects on L-type Ca^{2+} channels in smooth muscle. The potential role of Ca^{2+-}dependent phosphatases was not investigated in the studies of muscarinic regulation of I_Ca presented here. Experiments to investigate this potential signalling pathway may further distinguish the mechanism underlying the muscarinic inhibition we observed.
ACh caused contraction of the guinea pig gastric muscle cells under investigation and reports suggest that L-type Ca\(^{2+}\) channels may also be sensitive to mechanical changes in smooth muscle. Xu and co-workers demonstrate that hyposmotic induced stretching increases L-type Ca\(^{2+}\) currents in guinea-pig gastric myocytes (Xu, Kim, So & Kim, 1997). Such regulation was antagonized by the cytoskeletal disruptor cytochalasin-D and facilitated by phalloidin, an actin filament stabilizer. These findings suggest that the actin cytoskeleton may, in some way, couple to L-type Ca\(^{2+}\) channels, potentially leading to positive feedback during excitation of smooth muscle. As other excitatory neurotransmitters (see above) would be expected to activate the contractile machinery of cells, investigators should be careful in their interpretation of the mechanisms underlying increased Ca\(^{2+}\) current by these agents. This presence of this regulatory pathway, should it have been active under our experimental conditions, would have lead to an underestimate of the inhibition of I\(_{\text{ca}}\) due to release of stores by ACh. In light of these findings, it would be interesting to investigate the concomitant effects of actin stabilizers and disrupters on muscarinic inhibition of I\(_{\text{ca}}\). Similarly, it would be of interest to simultaneously record [Ca\(^{2+}\)]\(_{\text{i}}\), using fluorescence imaging, cell-length and I\(_{\text{ca}}\) in order to characterize the muscarinic changes in [Ca\(^{2+}\)]\(_{\text{i}}\) associated with contraction and decreased I\(_{\text{ca}}\) in smooth muscle.

Phosphorylation of K\(_{\text{ca}}\) channels may participate in regulation of their activity (Kume, Takai, Tokuno & Tomita, 1989; Lee, Bang, Lim, Uhm, Rhee, 1994) with elevation of [Ca\(^{2+}\)] at the cytosolic face of the membrane activating phosphatases and resulting in a decrease in K\(_{\text{ca}}\) channel activity. Such regulation would lead to an underestimate to the effects of ACh on increases in K\(_{\text{ca}}\) activity due to release of Ca\(^{2+}\) from intracellular stores.
Therefore, it would be of interest to investigate the effects of okadaic acid, a phosphatase inhibitor on ACh-mediated increases in $K_{Ca}$.

In functional studies of human esophageal muscle strips, dissection of the roles of each of the two current types was accomplished by using the $K_v$ channel blocker 4AP and $K_{Ca}$ channel blocker TEA. Of interest would be experiments using other pharmacological antagonists, e.g. IbTX and perhaps blockers of small conductance $K_{Ca}$ channels (quinine). Additionally, molecular biological studies presented here are limited to descriptions of mRNA for $Kv1.2$ and $Kv1.5$. Other delayed rectifier channel types may be expressed in this muscle and therefore investigating these cells with a broader array of delayed rectifier probes may elucidate other channel types.

5.5 References


