EXCITATION-CONTRACTION COUPLING IN
THE HUMAN SAPHENOUS VEIN

by

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B.Sc., The University of British Columbia, 1996

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We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 7, 2001

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Department of PHARMACOLOGY & THERAPEUTICS

The University of British Columbia
Vancouver, Canada

Date April 15, 2002

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ABSTRACT

**Background:** Human vascular preparations are most suitable to study vascular physiology and pathophysiology. Fresh tissues are difficult to obtain and may be damaged from the surgical procedure, limiting their use or introducing variability in the experimental findings. The purpose of this study was two-fold: (1) to demonstrate that a functional human saphenous vein (HSV) preparation may be obtained from coronary artery revascularisation procedures; (2) to elucidate excitation-contraction coupling mechanisms in functionally intact segments of HSV.

**Methods:** Isometric contraction experiments were performed using contractile stimuli, such as high K$^+$ depolarising solution (high K$^+$) and the $\alpha_1$-adrenergic agonist phenylephrine (PE), and an endothelium-dependent vasodilator, acetylcholine (ACh). Contraction was also combined with *in situ* smooth muscle intracellular Ca$^{2+}$ ([Ca$^{2+}]_i$) imaging by confocal microscopy in HSV stimulated by high K$^+$ and PE.

**Results & Conclusions:** HSV segments that were undistended showed far greater responsiveness to contractile and relaxing stimuli than portions of HSV segments that were distended and surgically prepared. Hence, all subsequent experiments were performed on undistended HSV segments. High K$^+$ elicited a sustained rise in [Ca$^{2+}]_i$ and contraction. Contractions in nominally Ca$^{2+}$-free physiological salt solution (PSS) were not attenuated and decayed many orders of magnitude slower in the HSV than in animal vascular preparations. High K$^+$ contractions in both normal and nominally Ca$^{2+}$-free PSS were nearly completely blocked by the Ca$^{2+}$ antagonist nifedipine. These results suggest the presence of an extracellular source of tightly bound Ca$^{2+}$ in HSV segments.

Stimulation of HSV segments with PE resulted in a transient rise in [Ca$^{2+}]_i$ that returned to baseline during sustained contractions. Nominally Ca$^{2+}$-free PSS, pre-incubation with nifedipine or the non-specific cation channel blocker SK&F 96365, significantly depressed the
maximum amplitude of these contractions. The sustained phase of the contraction was not sensitive to Ca\textsuperscript{2+} antagonists, but was attenuated by Ca\textsuperscript{2+} removal, suggesting Ca\textsuperscript{2+} influx through the leak pathway during this phase. The rho kinase inhibitor HA-1077 completely abolished while the tyrosine kinase inhibitor genistein only attenuated the plateau phase. Protein kinase C and mitogen-activated protein kinase were not involved. Hence, calcium, rho kinase and tyrosine phosphorylation mediate PE contractions in the HSV.
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<tr>
<td>2-APB</td>
<td>2-aminoethoxydiphenyl borate</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>[Ca(^{2+})](_i)</td>
<td>intracellular free Ca(^{2+}) concentration</td>
</tr>
<tr>
<td>[Ca(^{2+})](_o)</td>
<td>extracellular free Ca(^{2+}) concentration</td>
</tr>
<tr>
<td>[X](_i)</td>
<td>concentration of X inside the cell</td>
</tr>
<tr>
<td>[X](_o)</td>
<td>concentration of X outside the cell</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>CABG</td>
<td>coronary artery bypass graft</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
</tr>
<tr>
<td>cADP</td>
<td>adenosine 3',5'-cyclic diphosphate</td>
</tr>
<tr>
<td>CamKII</td>
<td>Ca(^{2+})-calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>cGMP</td>
<td>guanosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CICR</td>
<td>Ca(^{2+})-induced Ca(^{2+})-release</td>
</tr>
<tr>
<td>CPA</td>
<td>cyclopiazonic acid</td>
</tr>
<tr>
<td>dH(_2)(_O)</td>
<td>distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>EDHF</td>
<td>endothelium-derived hyperpolarising factor</td>
</tr>
<tr>
<td>EDRF</td>
<td>endothelium-derived relaxing factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
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<tr>
<td>eNOS</td>
<td>endothelium-derived nitric oxide synthase</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellularly-responsive kinase subfamily of MAPKs</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>GAGs</td>
<td>glycosaminoglycans</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
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<td>GDP(_\beta)S</td>
<td>guanosine 5'-O-(3-thiodiphosphate)</td>
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<td>G protein</td>
<td>GTP binding protein</td>
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<td>GTP(_\gamma)S</td>
<td>guanosine 5'-O-(3-thiotriphosphate)</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
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<td>High K(^+)</td>
<td>high K(^+) depolarisation solution</td>
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<td>HSV</td>
<td>human saphenous vein</td>
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<tr>
<td>HVSMC</td>
<td>human vascular smooth muscle cells</td>
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<tr>
<td>IICR</td>
<td>IP3-induced Ca(^{2+}) release</td>
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<tr>
<td>IMA</td>
<td>internal mammary artery</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol 1,4,5-trisphosphate</td>
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<tr>
<td>L-NNAME</td>
<td>N(^o)-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>N(^G)-monomethyl L-arginine</td>
</tr>
<tr>
<td>L-NNA</td>
<td>N(^o)-nitro-L-arginine</td>
</tr>
<tr>
<td>LPA</td>
<td>lysophosphatidic acid</td>
</tr>
<tr>
<td>M20</td>
<td>small non-catalytic subunit of MLCP</td>
</tr>
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<td>Abbreviation</td>
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<td>--------------</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBS</td>
<td>myosin binding subunit, same as MYPT</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK kinase isoform</td>
</tr>
<tr>
<td>MLC_{20}</td>
<td>20 kDa regulatory light chains of myosin</td>
</tr>
<tr>
<td>MLCK</td>
<td>myosin light chain kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix-degrading metalloproteinase</td>
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<tr>
<td>MYLP</td>
<td>myosin light chain phosphatase</td>
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<tr>
<td>MYPT</td>
<td>myosine phosphatase target subunit, same as MBS</td>
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<tr>
<td>MYPT1</td>
<td>MYPT isoform present in smooth muscle</td>
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<td>NA</td>
<td>noradrenaline</td>
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<td>NPY</td>
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</tr>
<tr>
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<td>phenylephrine</td>
</tr>
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<td>PKC</td>
<td>protein kinase C</td>
</tr>
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<td>PKG</td>
<td>protein kinase G</td>
</tr>
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<td>PKN</td>
<td>protein kinase N</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>phospholipase D</td>
</tr>
<tr>
<td>PP1c</td>
<td>protein phosphatase type 1</td>
</tr>
<tr>
<td>PP1c_{δ}</td>
<td>PP1c delta isoform, also referred to as β isoform</td>
</tr>
<tr>
<td>pp60_{src}</td>
<td>cellular proto-oncoprotein non-receptor tyrosine kinase</td>
</tr>
<tr>
<td>PSS</td>
<td>physiological salt solution</td>
</tr>
<tr>
<td>RCH</td>
<td>Royal Columbian Hospital</td>
</tr>
<tr>
<td>ROC</td>
<td>receptor-operated non-specific cation channel</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase</td>
</tr>
<tr>
<td>SK&amp;F 96365</td>
<td>1-{β-[3-(4-methoxyphenyl) propoxy]-4-methoxy-phenethyl}-1H-imidazole hydrochloride</td>
</tr>
<tr>
<td>SNP</td>
<td>sodium nitroprusside</td>
</tr>
<tr>
<td>SPH</td>
<td>St. Paul's Hospital</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>SV</td>
<td>saphenous vein</td>
</tr>
<tr>
<td>VOC</td>
<td>voltage-operated Ca^{2+} channel</td>
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PREFACE

Material from this dissertation is being prepared for submission to two different journals and are tentatively entitled as follows: *Phenylephrine-induced signalling and contraction in the undistended human saphenous vein*; and *Novel source of tightly bound Ca\(^{2+}\) contributes to high K\(^{+}\) depolarisation-induced contractions in undistended human saphenous vein*.

Material from this dissertation has been presented in oral form at the following meetings:

Lee CH, **Crowley CM**, van Breemen C. Calcium signalling in animal and human venous smooth muscle. Joint program for clinical scientist in training and MD-PhD students. Canadian Institutes of Health Research & Canadian Society for Clinical Investigation. Ottawa (Canada), September 20, 2001


Material from this dissertation has also been presented in abstract form at the following international and local meetings:


Material from this dissertation has been presented orally for the Graduate Student Seminars Series in the Department of Pharmacology and Therapeutics at UBC, for Research in Progress seminars in the McDonald Research Laboratories, and in research meetings for the Vancouver Vascular Biology Research Centre.
The dissertation author was responsible for the majority of the research work, data analysis and interpretation, figure and text preparation. The other authors contributed as follows: Casey van Breemen was the research supervisor. Cheng-Han Lee carried out the research presented in CHAPTER V and added much helpful discussion. Stephanie Gin and Albert M. Keep participated in data collection and analysis as summer research students. Samuel Lichtenstein and Richard C. Cook contributed to the clinical dimensions inherent to using human tissues in biomedical research.

Casey van Breemen

[Signature]

April 15, 2022
Date
ACKNOWLEDGEMENTS

My undergraduate training in the Dept. of Pharmacology & Therapeutics introduced me to the use of classical pharmacological techniques to understand the mechanisms of drug action and the use of drugs as pharmacological tools in the elucidation of physiological phenomena in a wide range of test systems. When the opportunity arose to work on human tissues, I was immediately excited by the possibility of applying the techniques I had learned as an undergraduate student to the elucidation of regulatory mechanisms of the human vasculature. I could not think of a better way to apply my efforts as doctoral research student. However, little was I aware of both the challenges and opportunities for growth that lay ahead. This graduate experience has allowed me to expand the breadth of my scientific and technical knowledge, as well as develop and improve many skills, such as written and oral communication, experimental design, leadership, organisation and mentoring. It provided the opportunity to interface with basic scientists, clinician, and surgeons, thereby expanding the scope of my learning experiences. The opportunities afforded to me were made possible through the combined efforts of many people in the Vancouver Vascular Biology Research Centre (VVBRC), the Cardiovascular Registry (CVR), the UBC/MRL/iCapture Centre (MRL) and the operating rooms at St. Paul’s Hospital (SPH) and Royal Columbian Hospital (RCH).

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of the VVBRC also gave me the opportunity to work on a research project for Eli Lilly & Co., a project unrelated to my dissertation, but which provided an interesting connection with industry.

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There are many people in the Cardiovascular Registry and Cardiovascular Research Laboratory who helped in many aspects of tissue supply. I am grateful for the assistance of Shelley Wood and Salima Harji, who also created the database, in establishing and making improvements to tissue collection protocols over the years. Timely collection of tissue samples and database upkeep was made possible by the efforts of many who have shared this responsibility (Shelley Wood, Kevin Naphtali, Stephanie Lee, Cheryl Jackson, Jennifer Kelly, Iva Kulic, Ying Li, Kunjumon Vadakkan, Vivienne Guy, Jenny Soo, and Patricia Aldea).

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[Ca$^{2+}$], imaging and use of the confocal microscope, and his helpful discussions regarding my research and this dissertation. I also thank Dr. Dietrich Ruehlmann, a former post-doctoral fellow in our laboratory, for his contributions to making the confocal microscope a working entity and creating macros for data analysis. I am extremely grateful for all the hard work of the summer students and co-op students who participated in my research project: Stephanie Gin, Michael Keep, and Damon Poburko. I also thank Elena Okon and Jennifer Kelly for proofreading this dissertation.

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Funding for this work was sponsored by many sources. I thank the SPH Hospital Foundation and Eli Lilly Company for funding many aspects of my research activities. I thank my sources of personal funding granted from the National Science and Engineering Research Council, the Heart and Stroke Foundation, the Killam Foundation, and the Faculty of Medicine for various scholarships, awards and top-ups.
In memory of my aunt

Maureen Pesant (1953 – 1971)

Valedictorian and Governor-General Medal (1970)

Convent of the Sacred Heart, Montreal, QC

“Life’s most treasured gifts take time to possess.”
CHAPTER I: INTRODUCTION

The study of vascular function in human tissues

Human vascular preparations are undoubtedly the best model for the study of vascular physiology and pathophysiology. Unfortunately, studies using human blood vessels lag behind those in animal preparations. Surgical tissues are difficult to obtain and are limited to procedures involving the removal of a vessel for grafting or a diseased organ (containing resistance or conduit vessels) for disposal. The bulk of these tissues may not be considered normal or may be damaged due to the surgical procedure. Because of these difficulties, vascular research has depended almost entirely on laboratory animals as a reliable and reproducible source of tissues whereby many factors can be controlled, such as diet, genetic strain, and sex. Consequently laboratory animals (swine, rodents and other mammals) have made enormous contributions to our understanding of normal physiology and to disease expression. If rats and mice never predicted what we would find in humans, then there would be little rationale for studying mechanisms in experimental animals (165).

Animals have also been used as models of cardiovascular disease. Over the past decades, numerous rat genetic models of hypertension have been developed, each strain having a different aetiology of hypertension and different degrees of susceptibility to the associated end-organ damage (88, 190). The use of these strains enabled the identification of markers (92) closely linked to CAD (311), myocardial infarction (311), and blood pressure (415). However, these models are not always reliable. Many physiological and pharmacological study designs have depended on control strains assuming that they differed only at 'hypertensive genes.' This assumption has been proven false because these strains, although normotensive, also exhibit other phenotypic differences besides blood pressure that were 'fixed' during the process of inbreeding.
In recent years, the mouse has come to represent the mammalian organism of choice for the study of gene function in physiological and diseased states. Clever applications of molecular biology techniques have enabled creation of genetically modified animal models of cardiovascular disease (ob/ob, apoE-deficient mice, etc., ...). The quest for linking the genotype to phenotype, including function, relies heavily on breeding these gain-of-function or loss-of-function animals (147, 320). However, these animal models are also limited. At a time when the human genome has been 'completely' sequenced in draft form, we only know the function of a small fraction of these genes. One major challenge is expected to be that of assessing the biological relevance of novel gene sequences, as well as those that are highly conserved. The distinctiveness of genomic sequences from humans, in comparison with those of mouse, *Drosophila melanogaster* and other organisms, highlights the challenges of studying human gene function in lower model organisms. In addition, current research has shown that whether a transport molecule (such as a RyR channel) performs a certain function or not (such as contraction or relaxation) depends on its micro-structural environment (334). It will be virtually impossible to recreate a human blood vessel using animal models because many genes and their processed proteins determine these ultrastructural domains. Hence, there is no perfectly satisfactory model other than the human vascular preparation itself to answer the question of how a human blood vessel is controlled. A detailed investigation of the activating mechanisms of human blood vessels is therefore pertinent to linking the human phenotype to functional mechanisms. This fact is particularly well borne out by the present study, which shows a specific relationship between \([Ca^{2+}]_i\) and force that diverges from most vascular smooth muscle preparations studied to date.
Human saphenous vein

The human saphenous vein is used extensively for revascularisation of occluded coronary arteries. It is one of the major trunks of the superficial venous circulation in the leg, is classified as a medium sized vein (diameters ranging from 1 to 10 mm) (379). The HSV is not really a normal vein. It is considered specialised (379) or differentiated (77) insofar as it is functionally adapted. It contains longitudinally oriented smooth muscle in all three layers (intima, media, and adventitia) (379) and the media is usually larger than that of other veins of similar calibre that are associated with maintaining hydrostatic pressure (77). A poorly defined internal elastic lamina is found in the HSV, thought partly because it conducts blood against the force of gravity (379).

The HSV has an intimal layer consisting of an endothelium that lines a thin subendothelial connective tissue of collagen, elastin and proteoglycans (336). The media is made up of layers of circular smooth muscle separated by connective tissue (77). The adventitia is composed of connective tissue and contains nerves and vasa vasora (297).

CABG surgery is the most effective means of revascularisation for patients with multi-vessel coronary artery disease (163, 464) to relieve the symptoms of angina, to prevent myocardial infarction, and to improve chances of survival. The number of surgeries has been increasing each year (1) and as many as a million CABG procedures are performed annually in the United States (451). There is no suitable alternative to the use of SV grafts when multiple coronary arteries must be bypassed (41). Because of the clinical importance of the HSV, it is important to know how the patency of this vessel is regulated. In addition, the large number of CABG surgeries makes it readily available and a desirable conduit for the study of vascular function.
Saphenous vein injury during surgical isolation

Because the experimenter does not have control over the isolation of the HSV segment, it is important to understand intra-operative handling of this vessel. Observation of the surgical procedure reveals that the vein undergoes considerable trauma during the complicated procedure of surgical preparation for grafting (Table 1).

Table 1. Trauma inflicted on the saphenous vein during preparation for grafting.

<table>
<thead>
<tr>
<th>Trauma Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanical trauma due to careless handling with instruments</td>
<td>such as pulling in all directions and stretching by direct vein grasping</td>
</tr>
<tr>
<td>Ligation with clips and avulsions of side-branches</td>
<td></td>
</tr>
<tr>
<td>Adventitial stripping</td>
<td></td>
</tr>
<tr>
<td>Wall ischemia due to disruption of vasa vasora &amp; prolonged hypoxic storage</td>
<td></td>
</tr>
<tr>
<td>Chemical and thermal injury due to exposure to non-physiological salt</td>
<td>solutions with variable pH, osmolarity, temperatures, and oncotic pressures</td>
</tr>
<tr>
<td>Manual pressure-distension at high, uncontrolled pressures to overcome</td>
<td>areas of focal spasm</td>
</tr>
<tr>
<td>Exposure to cocktails of vasodilators (nifedipine, verapamil, papaverine,</td>
<td>nitroglycerine, etc., ...)</td>
</tr>
<tr>
<td>Pressure-distension of the harvested vein segment is performed to overcome</td>
<td>spasm</td>
</tr>
<tr>
<td>incurred during harvest, to check for leaks from side-branches, and to</td>
<td>determine graft orientation and length. This procedure elicits a range of</td>
</tr>
<tr>
<td>determine graft orientation and length. This procedure elicits a range of</td>
<td>morphological and functional damage.</td>
</tr>
<tr>
<td>Microscopic evaluation of the endothelium after barotrauma (~300 mmHg)</td>
<td>demonstrates separation of endothelial cells, endothelial cell elevation,</td>
</tr>
<tr>
<td>(31, 273, 344) and types of irrigation solutions (3, 388).</td>
<td>with endothelial slough proportional to</td>
</tr>
</tbody>
</table>
distending pressure (143, 273, 344). Mural oedema, characterised by separation of the muscle bundles, has also been observed (44, 45, 107). It is thought that distension with cold solutions can be especially harmful to endothelial cell morphology (259). Very high pressures cause marked stiffening of the venous graft thought to be due to changes in the ratio of collagen to elastin (3), as well as altering endothelial cell fibrinolytic activity (273) and enhancing lipid accumulation by the vein wall (40). Biochemical changes are also associated with pressure-distension, such as a reduction in [ATP]j and the [ATP]j/[ADP]j in HSV (17) and porcine IMA (71), as well as in the synthesis of the cyclic nucleotides cAMP and cGMP in smooth muscle (196) and endothelial cells (399). Vascular reactivity (both endothelium and smooth muscle function) is also markedly depressed due to barotrauma (18, 20, 29, 65, 81, 245, 324, 367, 456). Pressure-distension need not always be injurious if performed gradually in the presence of papaverine and with a colloidal solution at body temperature (151).

In addition to the acute effects of surgical trauma, organ culture studies have revealed evidence for changes in gene expression, as well as vessel wall remodelling in response to human SV graft preparation. Surgical distension significantly increased the expression of genes such as c-fos, apparently regulated by mechanical factors. Increased c-fos expression was temporally associated with increased apoptosis in the media within 24 hours (115). After 14 days in organ culture, surgical preparation of human SV grafts stimulated medial (21, 167) or medial and intimal proliferation (21, 389), intimal thickening (167, 390), and a decrease in DNA concentrations, implying cell necrosis (389). It is thought that the increased smooth muscle cell proliferation observed with surgical preparation of vein segments may be associated with increased production and activation of MMP-2 and MMP-9 (120). It also has been clearly demonstrated that the damage caused by surgical preparation of vein segments reduces patency rates in vein grafts (19, 340).
The solution used to rinse, distend, and store veins prior to implantation may also have a profound influence on morphology and reactivity. A variety of crystalloid (normal saline, lactated Ringer’s, Plasma-Lyte, etc., ...) and colloidal solutions (whole blood, albumin, and balanced salt solutions to which serum has been added) have been tested (323). Many groups have shown that normal saline and lactated Ringer’s solution, when compared with either tissue culture medium or heparanised blood, evoked more severe endothelial cell disruption and oedema, mural oedema, and cytologic derangement of smooth muscle (143, 258, 272, 344, 370, 388). In addition, Hanks balanced salt solution provided more preservation of normal stress-strain relationship than normal saline or Ringer’s solution (3). And prostacyclin activity in vein endothelium is better preserved in tissue culture medium or heparanised normal saline (49). However, in some studies, crystalloid solutions are superior to colloidal ones. Microscopy revealed better endothelial cell coverage after room temperature storage in saline solution as compared to blood (168). The addition of papaverine to a heparinised Plasma-Lyte solution was superior to autologous blood insofar as there was less endothelial cell loss and more vessel dilation (31).

Temperatures may also influence preservation quality regardless of the storage solution used. Endothelium and smooth muscle functions were better preserved at low temperatures. In one study, contractility was best preserved after 36 hours at low (0.5°C) as compared to warmer temperatures in University of Wisconsin solution. Endothelial cell function was depressed after storage regardless of temperature, but was least affected at 4°C and 8.5°C (280). In another study, a comparison of a wide range of crystalloid solutions varying in the concentrations of electrolytes, buffers, glucose, and at different pH values demonstrated that contractility was preserved independent of buffer solution at low (4°C) and room temperature for 2 hours. In addition, endothelium-dependent relaxations were better preserved at low temperatures regardless of preservation solution (360).
In contrast, the application of endothelium-independent vasodilators is more effective after preservation at a warmer temperature (37°C). For example, papaverine was more potent and effective at relaxing NA-pre-contracted rings at 37°C than at cooler temperatures (357). In addition, Plasma-Lyte, which was more effective than normal saline at relaxing NA-pre-contracted canine SV at 37°C and room temperature, had a more pronounced effect at body temperature. This effect was attributed to the low Ca$^{2+}$ content of Plasma-Lyte (360).

With respect to biophysical experiments, the surgical and isolation procedures may introduce variability in the results, which are not related to the in vivo physiology and/or pathology. Some investigators have alluded to the effects of surgical trauma on the responses to HSV as early as 1975 (123). However, these effects have not been accounted for in pharmacological and physiological studies using human blood vessels. In many reports studying excitation-contraction coupling in the HSV, its integrity is unclear (52) or the extent of vein preparation is not indicated (432, 433). Other studies have specified the use of “remnants” (295), “leftovers” (174), “discarded” (2, 391), or “unused” HSV segments (72). It is possible that signal transduction in these tissues may be affected, (e.g., mechanisms underlying endothelial cell and smooth muscle reactivity); hence, the results and conclusions derived from these early studies must be evaluated with a new level of scrutiny and experimental depth. In light of the foregoing findings, part of this thesis work was committed to obtaining fresh samples of HSV as intact as possible to ensure the success of in vitro tissue bath experiments. Control over these variables may be imperative in order to yield experiments with reproducible physiological data.

**Excitation-contraction coupling in the human saphenous vein**

Saphenous vein graft spasm in the perioperative and/or postoperative period (12, 25, 91, 153, 274, 400, 434) and thrombosis (84, 137) are the main threats to maintenance of optimal cardiac perfusion by the bypass graft. Although the exact mechanism is unknown, spasm results
from the abnormal contraction of the medial layer of the vessel wall. The central messenger of smooth muscle contraction is [Ca\(^{2+}\)]\(_i\) (207); however, the nature and relative importance of this signal in smooth muscle contraction differs between vascular preparations. Graft spasm needs urgent treatment because it increases morbidity and mortality. An understanding of the mechanistic basis for HSV activation will be beneficial in the management of graft spasm.

In this study, Ca\(^{2+}\) signalling patterns were compared with force development in individual smooth muscle fibres of the intact HSV observed with the confocal microscope during stimulation with high K\(^+\) or the \(\alpha\)-adrenergic receptor agonist PE. There are different pathways that couple receptor activation to vasoconstriction. The initial stimulus may operate through changes in surface membrane potential (electromechanical coupling) or mediate changes in force independent of changes in membrane voltage (pharmacomechanical coupling) (384). The effectiveness of this coupling may be regulated by the degree of elevation in [Ca\(^{2+}\)]\(_i\), by the sensitivity of the myofilaments to Ca\(^{2+}\), or by certain kinases that may be independent of [Ca\(^{2+}\)]\(_i\) (387).

**Calcium regulation**

Decades of research in smooth muscle excitation have revealed a number of mechanisms governing the regulation of [Ca\(^{2+}\)]\(_i\) and their respective roles in smooth muscle activation, reviewed extensively elsewhere (38, 160, 169, 207, 244, 325). Mechanisms which increase [Ca\(^{2+}\)]\(_i\) stimulated by contractile stimuli are briefly discussed below.

Plasma membrane channels and exchangers govern the influx of Ca\(^{2+}\) (see Figure 1). Of the six subtypes of voltage-gated Ca\(^{2+}\) channels, only the L-type Ca\(^{2+}\) channel (referred to as VOC) is considered to be a major Ca\(^{2+}\) influx pathway (230). Receptor-activation may also result in the opening of non-selective cation channels (referred to as ROC). ROCs are defined as
Ca\(^{2+}\) channels located in the plasma membrane other than VOCs that are opened as a result of the binding of an agonist to its receptor, which is separate from the channel proper. Mechanisms of activation of these channels cannot be depolarisation and therefore must imply an intracellular messenger, a G protein, or the involvement of the ER (30). Agonists open VOCs by depolarising the cell membrane through activation of ROCs, inhibition of K\(^{+}\) channel and/or activation of a Cl\(^{-}\) channel (207, 330). For example, Ca\(^{2+}\) released from the sarcoplasmic reticulum can activate Ca\(^{2+}\)-activated K\(^{+}\) and Cl\(^{-}\) channels. Activation of Ca\(^{2+}\)-activated channels may lead to hyperpolarisation (K\(^{+}\) channels) and depolarisation (Cl\(^{-}\) channels) (384). Passive Ca\(^{2+}\) entry or 'leak' is also a possible Ca\(^{2+}\) influx pathway (263). The Na\(^{+}\)/Ca\(^{2+}\)-exchanger may mediate Ca\(^{2+}\) influx in some smooth muscle preparations under physiological conditions (207, 244). Na\(^{+}\) influx through ROCs may stimulate the influx of Ca\(^{2+}\) via the reverse mode of the Na\(^{+}\)/Ca\(^{2+}\) exchanger in the rabbit inferior vena cava (247).

The SR is an intracellular Ca\(^{2+}\) storage site and releases Ca\(^{2+}\) upon activation (see Figure 1) (424) via two different mechanisms: CICR (38, 131, 177, 179) and IICR (34, 339). CICR is activated by an increase in [Ca\(^{2+}\)]\(i\) and IICR is regulated by IP3 and Ca\(^{2+}\) (207).

Each of these mechanisms may contribute to increasing the [Ca\(^{2+}\)]\(i\), leading to activation of MLCK and smooth muscle contraction (384). However, the combination of specific Ca\(^{2+}\) transport molecules involved differs not only between stimuli, but also between species and vascular preparations within a given species. In this light, it is important to understand the mechanisms regulating [Ca\(^{2+}\)]\(i\) and the role of Ca\(^{2+}\) in contraction in the HSV.

**Calcium sensitisation**

Simultaneous recording of [Ca\(^{2+}\)]\(i\) and contraction revealed that Ca\(^{2+}\) sensitisation of smooth muscle contraction often occurs following agonist stimulation. At any given [Ca\(^{2+}\)]\(i\),
agonists elicit greater contractions than high K+ (206, 363). This finding supports the view that agonists can increase the Ca\(^{2+}\) sensitivity of the contractile apparatus. Because of possible artefacts relating measurements of [Ca\(^{2+}\)]\(_i\) to contraction (207), it is necessary to measure the [Ca\(^{2+}\)]\(_i\)–force relationship using a different method.

Ca\(^{2+}\) sensitisation of force was first examined when preparations were permeabilised with *Staphylococcus aureus* α-toxin (219, 316). Skinned fibres lose intracellular signalling mechanisms linking agonist-receptor activation to contraction (351). With α-toxin, it is possible to precisely regulate the cytosolic concentrations of Ca\(^{2+}\) as well as other substances of small molecular weight without disrupting the intracellular signalling machinery that mediates contraction. The increased force at a given [Ca\(^{2+}\)]\(_i\) with receptor stimulation (as compared to K\(^{+}\) depolarisation) required GTP and was reversed by GDP\(_j\)S, demonstrating G protein dependence (218, 219, 314, 316).

Smooth muscle contraction is regulated by Ca\(^{2+}\)-dependent MLCK activation and MLCP (203). Mechanisms affecting the activity levels of MLCK and MLCP will affect the Ca\(^{2+}\) sensitivity of the contractile machinery. Ca\(^{2+}\)-dependent activation of CamKII leads to phosphorylation of MLCK thereby decreasing its activity (396). Agonists may somehow inhibit this negative feedback pathway to elicit Ca\(^{2+}\) sensitisation, although this mechanism is not likely an important pathway under physiological conditions (207). There are two other main mechanisms thought to mediate Ca\(^{2+}\) sensitisation in smooth muscle: one converges on MLCP, and the other on actin-linked regulatory mechanisms.

Studies by Somlyo and co-workers (218, 220, 382, 383, 386) support the notion that Ca\(^{2+}\) sensitisation is regulated by inhibition of MLCP activity. Cellular signalling pathways mediating agonist- or GTP\(_y\)S-induced increased force at constant [Ca\(^{2+}\)]\(_i\); result from inhibition of MLCP (220, 227) via G protein coupled mechanisms (384), resulting in increased levels of MLC\(_{20}\) phosphorylation (110, 218). MLCP is a trimeric enzyme associated with myosin filaments (148),
and therefore its inhibition by a surface membrane bound receptor G protein complex has to be signalled to the contractile apparatus by second messengers. MLCP is composed of three subunits: a catalytic subunit, PP1cδ, and two putative regulatory subunits, termed MYPT1 and M20 (149). MYPT is the key subunit involved in binding to and activation of PP1c and in targeting myosin. Phosphorylation of this subunit inhibits phosphatase activity (176). Both rho kinase and PKC have been associated with inhibition of MLCP (385-387). Rho kinase phosphorylates MYPT and inhibits MLCP activity (215). PKC phosphorylates CPI-17, a potent inhibitor of MLCP when phosphorylated (96, 221). Recent evidence suggests that rho kinase and PKN can also phosphorylate CPI-17 (146, 224) (also see CHAPTER VIII: DISCUSSION and Figure 38).

Thin-filament (actin)-linked Ca\(^{2+}\) sensitisation mechanisms are independent of MLC\(_{20}\) phosphorylation (207) and involve thin filament associated protein, caldesmon and calponin (53, 436). Phorbol esters and a variety of agonists activate PKC. Phosphorylation of calponin by PKC reduces affinity of calponin for actin thereby alleviating its inhibition of cross-bridge cycling rate (438). Similarly, activated PKC may stimulate MAPK, which phosphorylates caldesmon, thereby removing its inhibition of actin-activated MgATPase activity as well (436, 437). Hence, phosphorylation of either calponin or caldesmon or both may regulate smooth muscle contraction via Ca\(^{2+}\) sensitisation of the contractile filaments (121).

Tyrosine kinases are involved in smooth muscle contraction (47, 164, 200, 291, 408). The exact nature and physiological significance of tyrosine kinase activity in vascular smooth muscle cells have yet to be identified. Historically, tyrosine kinase activity has been associated with cell growth, proliferation and transformation regulated by receptors with intrinsic tyrosine kinase activity. More recently, it has been observed that GPCR also stimulate growth thereby implicating cytosolic tyrosine kinases, such as pp60\(^{c-src}\), as the common link between the two types of receptors (see references in (199)). Tyrosine kinases have been implicated in Ca\(^{2+}\)
sensitisation largely because of the effects of tyrosine kinase inhibitors; however, tyrosine phosphorylation has yet to be linked to the activities of MLCP, MLCK, or to actin-associated proteins (387). Hence, its role in Ca\(^{2+}\) sensitisation mechanisms is currently unknown.

**Calcium regulation and calcium sensitisation in the HSV**

Little is known about such mechanisms in human blood vessels (278). Calcium signalling in the HSV has been studied in response to a variety of growth factors and to ATP (67, 68, 145, 260, 312); however, these studies were performed in enzymatically digested and/or cultured cells. Non-selective proteolysis may destroy membrane-associated proteins integral to the cellular response and prevent intercellular communication through gap junctions. These alterations may modify the behaviour of single cells as compared to cells in the intact tissue. In addition, when smooth muscle cells are cultured, they may undergo phenotypic changes, such as a loss of VOCs (124). A range of studies have characterised contractions in HSV pharmacologically (73, 87, 118, 307, 335, 350, 414); however, there have been few reports of excitation-contraction coupling mechanisms in this tissue, studies being limited to Ca\(^{2+}\)-activated K\(^{+}\) channels (294, 463) and effects of Ca\(^{2+}\) antagonists on NA contractions (328, 433). There are no studies to date demonstrating the relationship between [Ca\(^{2+}\)]\(_i\) and force in the intact HSV tissue. The degree to which this vascular preparation depends on Ca\(^{2+}\) sources for contraction and other signalling pathways has not yet been elucidated.

*Figure 1 to Figure 2 to follow*
Figure 1. Possible Ca\(^{2+}\) movements in vascular smooth muscle.

Plasma membrane channels and exchangers govern the influx of Ca\(^{2+}\). The L-type Ca\(^{2+}\) channel (referred to as VOC) is considered to be a major Ca\(^{2+}\) influx pathway. Receptor-activation may also result in the opening of non-selective cation channels (referred to as ROC). Mechanisms of activation of ROCs channels cannot be depolarisation and therefore must imply an intracellular messenger, a G protein, or the involvement of the SR. Agonists open VOCs by depolarising the cell membrane through activation of ROCs, inhibition of the K\(^{+}\) channel and/or activation of the Cl\(^{-}\) channel (e.g., Ca\(^{2+}\) release from the SR activates Ca\(^{2+}\)-activated Cl\(^{-}\) channels resulting in depolarisation. Note that Ca\(^{2+}\) release from the SR activates Ca\(^{2+}\)-activated K\(^{+}\) channels leading to hyperpolarisation.). Passive Ca\(^{2+}\) entry or 'leak' is also a possible Ca\(^{2+}\) influx pathway. The Na\(^{+}/Ca^{2+}\)-exchanger may mediate Ca\(^{2+}\) influx in some smooth muscle preparations under physiological conditions. Na\(^{+}\) influx through ROCs may stimulate the influx of Ca\(^{2+}\) via the reverse mode of the Na\(^{+}/Ca^{2+}\) exchanger. The SR is an intracellular Ca\(^{2+}\) storage site and releases Ca\(^{2+}\) upon activation via two different mechanisms: CICR and UICR. CICR is activated by an increase in [Ca\(^{2+}\)]\(i\); and UICR is regulated by IP3 and Ca\(^{2+}\).
Figure 2. Ca\(^{2+}\) sensitisation mechanisms in vascular smooth muscle.

There are two other main mechanisms thought to mediate Ca\(^{2+}\) sensitisation in smooth muscle: one converges on MLCP, and the other on actin-linked regulatory mechanisms. Cellular signalling pathways mediating agonist- or GTP\(_{y}\)S-induced increased force at constant \([Ca^{2+}]_i\) result from inhibition of MLCP via G protein coupled mechanisms, resulting in increased levels of MLC\(_{20}\) (myosin) phosphorylation. Rho kinase phosphorylates MLCP directly. And rho kinase, PKC, and PKN phosphorylate CPI-17, a potent inhibitor of MLCP when phosphorylated.

Thin-filament (actin)-linked Ca\(^{2+}\) sensitisation mechanisms are independent of MLC\(_{20}\) phosphorylation and involve thin filament associated protein, caldesmon and calponin. Phosphorylation of calponin by PKC reduces affinity of calponin for actin thereby alleviating its inhibition of cross-bridge cycling rate. Similarly, activated PKC may stimulate MAPK, which phosphorylates caldesmon, thereby removing its inhibition of actin-activated MgATPase activity as well. Hence, phosphorylation of either calponin or caldesmon or both may regulate smooth muscle contraction via Ca\(^{2+}\) sensitisation of the contractile filaments.
MLCP\textsubscript{inactive} ➔ P

Rho kinase
PKN
PKC
CPI-17

MLCP\textsubscript{active}

Myosin ➔ Myosin ➔ MLCK ➔ Myosin + Actin ➔ Cross-bridge cycle & Force development

Calponin ➔ P + Actin

PKC
MAPK

Calponin ➔ Actin
CHAPTER II: RATIONALE, HYPOTHESIS AND SPECIFIC AIMS

Knowledge of species differences in vascular reactivity and the desire to understand the mechanism of vascular function in humans were the reasons for focussing this thesis on excitation-contraction coupling in human blood vessels.

The HSV was chosen as the human vascular preparation of choice because it is readily available from CABG operations. As many as 2-6 cases may be performed on any given day at SPH. By virtue of the fact that the HSV must be obtained from patients, harvest is necessarily limited to the medical staff. One must take the necessary precautions to account for as many variables as possible because of the lack of control by the experimenter. And in light of the documented deleterious effects of intra-operative manipulations on the integrity of HSV segments (see CHAPTER I: INTRODUCTION), it becomes essential to demonstrate that a fresh and functionally useful preparation may be obtained for physiological and pharmacological experiments. Hence, our first working hypothesis was that with both careful dissections during CABG operations and optimal preservation, a functionally intact segment of HSV, which is suitable for pharmacological and physiological experiments, could be obtained. To test this hypothesis, our first set of specific aims were:

1. To ensure daily and timely collection of optimally harvested samples of HSV suitable for a long-term research project, without hindering the activities of the surgical staff; and
2. Using isometric tension experiments, to determine the most optimally preserved HSV segment for use in pharmacological and physiological experiments.

The HSV is also a clinically relevant vascular preparation insofar as it is used to bypass occluded coronary arteries and the mechanisms of vein graft failure have not been completely elucidated (304). During the perioperative period, spasm limits the viability and usefulness of
the vein graft (420). And as early as one month to several years post-CABG surgery, the vein
graft may fail due to thrombosis, intimal thickening or atherosclerotic occlusion (304). A better
understanding of the mechanisms underlying HSV reactivity becomes necessary in order to
understand the pathological basis of various mechanisms governing vein graft failure. However,
as indicated in CHAPTER I: INTRODUCTION, very little is known about the excitation-
contraction coupling mechanisms in this preparation. Many combinations and permutations of
the various Ca\(^{2+}\) transporters and Ca\(^{2+}\) sensitisation mechanisms may be involved in force
generation in the HSV and may differ between agonists. But the role of these various
mechanisms can only be demonstrated experimentally. Our second working hypothesis was
that both Ca\(^{2+}\) regulation and Ca\(^{2+}\) sensitisation mechanisms might contribute to excitation-
contraction coupling mechanisms in the HSV. In particular, it was hypothesised that Ca\(^{2+}\) influx
and SR Ca\(^{2+}\) release, resulting in changes in [Ca\(^{2+}\)]\(_i\) and MLCK activity, may be involved. And
in light of the finding that changes in force do not always parallel changes in Ca\(^{2+}\) with receptor
stimulation in animal blood vessels, it was also hypothesised that Ca\(^{2+}\) sensitisation mechanisms
may also be participate. These hypotheses led to our second set of specific aims:

1. Using confocal microscopy and isometric contraction experiments on fluo-4 loaded HSV
segments, to determine the force to [Ca\(^{2+}\)]\(_i\) ratio by measuring changes in [Ca\(^{2+}\)]\(_i\) in response
to high K\(^+\) and PE and comparing these findings to observed parallel changes in force.

2. During stimulation of optimally preserved segments of HSV with PE (as compared to high
K\(^+\)) under isometric tension recording conditions, to use well-characterised pharmacological
inhibitors to determine the relative roles of:

   2.1. Ca\(^{2+}\) influx through membrane channels (VOC, ROC, leak);

   2.2. Ca\(^{2+}\) release from the SR; and

   2.3. Ca\(^{2+}\) sensitisation mechanisms (rho kinase, PKC, MAPK and tyrosine phosphorylation).
CHAPTER III: MATERIALS AND METHODS

Isolation of human saphenous vein segments.

Segments of HSV were harvested from patients undergoing CABG operations at SPH (Vancouver) and RCH (New Westminster), who were sampled from a population of elderly patients afflicted with coronary artery disease in British Columbia, Canada. Institutional approval for the use of these tissues was obtained.

Isolation at SPH

The general procedure for isolating and preparing the vein for grafting at SPH may be described as the 'open' technique, i.e., the vein is harvested from a single incision. In this procedure, the SV was isolated from surrounding connective tissues. The side-branches were ligated and severed. Once the vein was completely freed from areolar tissues, it was divided proximally and distally and placed in an irrigation solution. This solution may be saline (0.9% NaCl) or a solution called Plasma-Lyte® A Injection. The vein was then prepared for grafting. A balanced electrolyte solution (pH 7.4), such as Plasma-Lyte, or the patient’s heparanised blood, was used to flush any clot or platelet deposits. The vein was then distended with pressures of 300-600 mm Hg achieved by injection of Plasma-Lyte or the patient’s heparanised blood with one hand, while manually compressing the lumen of the vessel with the other. This procedure enabled the surgeon to verify leaks and overcome areas of focal spasm. Portions of vein were sectioned according to the desired lengths for bypassing the occluded coronary arteries. The segment was first grafted distal to the occluded region of the coronary artery. After the anastomosis was complete, either warm or cold blood-cardioplegia (depending on the surgeons’ preferences) was infused into the vein graft to verify patency. Leftover portions of HSV segments not used for grafting or in excess after blood-cardioplegia infusion were
packaged in their respective collection vials and termed 'distended' and 'cardioplegic' HSV segments, respectively (see below).

**Isolation at RCH**

At RCH, vein removal was performed by minimally invasive harvest. Multiple incisions were made in lieu of one long incision, as in the open technique. The vein was isolated at multiple incision sites and then pulled through the skin tunnels. Excess traction on the vein during harvesting is thought to damage the integrity and function of these SV segments.

**Blood vessel collection protocol**

Specimen collection by SPH staff was performed according to an established protocol (see **APPENDIX I. BLOOD VESSEL COLLECTION PROTOCOL**).

**Types of HSV segments collected**

Initially, any piece of HSV segment in excess of lengths required for grafting was collected for experiments. After performing a basic characterisation of these tissues (see **CHAPTER IV: BASIC PHYSIOLOGICAL AND PHARMACOLOGICAL CHARACTERISATION OF HSV REACTIVITY**), three types of HSV segments were collected from SPH: 'undistended', 'distended', and 'cardioplegic' segments. An undistended SV segment was sectioned from the distal end as soon as the vein had been harvested and before preparation for grafting. A distended SV segment was a leftover portion of vein sectioned after the vein had been flushed, pressurised, and divided for the number of coronary arteries to be bypassed. A cardioplegic segment was also in excess of that required for grafting sectioned after...
infusion of the blood-cardioplegia solution. Only undistended SV segments were collected from RCH.

Packaging & paging

Each specimen was placed in its own 50 mL-centrifuge tube containing RPMI cell culture medium stored in a designated refrigerator located in the operating room core. A member of the laboratory who was notified by pager then retrieved the freshly packaged specimen placed in the refrigerator.

Case, patient and specimen information

Each vial was carefully labelled with the identity of the specimen and the time it was placed in the vial. An addressograph label containing patient information such as age, sex, and a hospital ID# was also added to the vial for tracking purposes. A patient information sheet (see APPENDIX I. BLOOD VESSEL COLLECTION PROTOCOL) was also filled out with case information, such as the operating room # and surgery time; specimen information, such as time of leg incision and the physician performing the harvest; and patient information, such the diabetic status of the patient.

Blood vessel collection from RCH

Only undistended segments were collected from RCH. Each vial was carefully labelled with the identity of the specimen and the time it was placed in the vial. An addressograph label containing patient information such as age, sex, and a hospital ID# was also added to the vial for
tracking purposes. There was no patient information sheet accompanying specimens from these cases.

Database

All the foregoing information (case, patient, and specimen) was entered into a database in Microsoft Access©.

Other vascular preparations

On three occasions, blood vessels were also harvested from infants, rats, rabbits, and swine to verify certain findings, to test hypothesis regarding phenomena in the HSV, or to verify certain drug effects. (1) In CHAPTER VI: HIGH K⁺-DEPOLARISATION-INDUCED CONTRACTIONS IN THE HSV, high K⁺ contractions in nominally Ca²⁺-free PSS were observed in HSV. This finding was unusual insofar as it is not thought to occur in animal blood vessels. Hence, a series of experiments was designed to examine further this phenomenon in the HSV and in vascular preparations harvested from swine, rabbit and infants. (2) Also in CHAPTER VI: HIGH K⁺-DEPOLARISATION-INDUCED CONTRACTIONS IN THE HSV, it was hypothesised that high K⁺ contractions in nominally Ca²⁺-free PSS in the HSV may be due to the cardiovascular impairments as a result of the CAD status of the patient. This hypothesis was tested using a porcine model of diabetes. (3) The effects of nifedipine and SK&F 96365 on PE contractions in the HSV were compared to those in the rat thoracic aorta as elaborated in CHAPTER VII: α₁-ADRENERGIC RECEPTOR-STIMULATED CONTRACTIONS IN THE HSV.
Porcine renal artery and femoral vein

Segments of swine femoral vein and renal artery were obtained from Dr. Michael Sturek's Laboratory at the University of Missouri. Procedures involving swine were approved by the Animal Care and Use Committee of the University of Missouri (Protocol number 2676). The animals were treated similarly to previously published methods (85). Sexually mature male Yucatan miniature swine between 9-12 months of age were obtained from the Sinclair Research Centre (Columbia, MO, USA). The animals were anaesthetised with the following drugs (in mg/kg): atropine 0.05, telazol 6.6, and xylazine 2.2; the level of anaesthesia was subsequently maintained with isoflurane gas (up to 4%) during in vivo experiments; pigs were euthanised by isoflurane overdose and removal of the heart. Renal arteries and femoral vein segments were removed and placed in cold RPMI cell culture medium. Vascular preparations were air shipped overnight to Vancouver and used the next day. Freshly harvested smooth muscle cells were not different from cells harvested from 'cold stored' pig arteries (157, 158).

There were three experimental groups. In one group, insulin action was impaired by simply decreasing plasma insulin levels through the destruction of pancreatic β cells with alloxan (85). These alloxan-treated diabetic pigs were fed a high fat, high cholesterol, atherogenic diet (Minipig chow supplemented with cholesterol, coconut oil, corn oil, and sodium cholate)(85). The term 'diabetic' is often used for readability. Because a previous study from the same laboratory indicated that there were minimal effects of diabetes alone on plasma lipids, an alloxan-treated group on normal pig chow was not included in the present study. A second group of pigs ('high fat') was fed the atherogenic diet, and a third group ('control') was fed only Minipig chow. Pigs were fed twice daily and had free access to water.
Rabbit femoral artery

Seven New Zealand white rabbits (~2 kg) were anaesthetised with 5% isofluorane. Approximately 4-5 cm lengths of femoral artery were isolated from the upper thigh and immediately placed in a cold physiological salt solution (PSS).

Rabbit inferior vena cava

Four female New Zealand white rabbits (1.5 to 2.5 kg) were obtained from the University of British Columbia Animal Care Centre. They were killed by CO₂ asphyxiation and then exsanguinated in accordance with local regulations. The inferior vena cava was removed and immediately placed in PSS solution.

Rat thoracic aorta

Four male Sprague-Dawley rats (200 – 300 g) were anaesthetised with intra-peritoneal injections of sodium pentobarbital (Somnotol, 30 mg/kg) and heparin (Hepalean; 500 U/kg), and then killed by decapitation. The thoracic aorta was removed and placed in cold PSS.

Human thymus veins

The thymus was isolated from infants undergoing open-heart surgery at British Columbia Children’s Hospital (Vancouver). Institutional approval for the use of these tissues was obtained.
Experiments

Dissection

Samples of HSV retrieved from the morning cases were used the same day. Those from the afternoon cases were stored in the refrigerator overnight. Excess adventitia and fat were removed from blood vessels in cold PSS, and cut into rings 4-mm in length. Rings were earmarked for isometric tension experiments or [Ca^{2+}]; imaging using confocal microscopy. Only HSV segments devoid of any obvious lesions were used in this study.

Isometric tension experiments

Data acquisition

Rings were mounted on pairs of stainless steel metal hooks and placed in glass jacketed tissue baths containing 10-mL of PSS solution (pH 7.4), warmed to 37°C and oxygenated with 100% O2. One end was attached permanently to a tissue bath hook, while the other was connected with suture to a force-displacement transducer (FT03E, Grass Instrument Division, Astro-Med, Inc, Longueil, PQ). The output from force transducers was fed to analog signal ETH-400 amplifiers (CB Sciences, Milford, MA). The voltage signals were converted to digital signals and recorded by both MacLab 8/s and PowerLab 8/s computer based recorders on a Power Macintosh (7200/90) and a PC (Pentium 133 MHz, Trison), respectively. Chart© recording software (ADInstruments, Mountain View, CA) was used for data acquisition. Tissues were equilibrated under zero tension for 90 – 120 min, while the bathing medium was changed every 15 min. Passive tension was applied by stretching the ring three times over a 45 min period to overcome stress relaxation (374) such that a final resting tension of 2 - 2.5 g was achieved for the HSV. Each stretch was preceded by a 15 min wash. A resting tension of 2 - 2.5 g was the minimum stretch shown to produce maximum active tension in response to contractile
stimuli (see Figure 3). The resting tension used for the other vascular preparations were 0.5 g for
the rabbit inferior vena cava and human thymus vein, 0.75 g for the rabbit thoracic aorta, and 2 g
for the swine renal artery and rabbit femoral vein. Rings were then challenged with 1-3
exposures to high K⁺ depolarising solution (high K⁺ or 80 mM K⁺) prior to effecting a given
protocol, unless otherwise indicated.

*Experimental design*

Wherever possible, individual rings from a CABG patient or laboratory animal were
assigned to different treatment groups such that each patient or animal acted as his or her own
control. Sometimes, this design was not possible because of insufficient material, especially
from CABG patients; therefore, there were cases where control and test rings were from different
patients. Sometimes, because of tissue excess from a given patient, more than one ring from a
patient was assigned to a treatment group and were counted as individual rings from the same
patient. There were also some designs were the different experimental groups contained
mutually exclusive sets of patients.

*Data analysis*

Off-line analysis was performed using the Data Pad window in Chart©. Data were
imported into a Microsoft Excel© spreadsheet. Rings were excluded if control high K⁺
contractions were less than 0.5 g. This rule did not apply to distended and cardioplegic HSV
segments. Data were normalised by control high K⁺ or PE contractions were appropriate and as
indicated in the text. Statistical analysis was performed using JMP© (SAS Institute, Inc., Cary,
NC) software. The theoretical assumptions for the use of parametric statistical tests, such as that
of normality and homoscedasticity, were verified. Outliers were excluded. The two sample t-
test and one-way ANOVA test (sometimes a Welch ANOVA test because of unequal variances
between groups), followed by multiple comparisons with the Tukey–Kramer HSD test, were used for most comparisons. If paired samples from the same patients were available for a given data set, paired sample t-tests were performed. The probability of an α error was set at 5%. Responses were expressed as mean ± SEM with ‘n’ representing the number of rings and/or patients as indicated. A 4-parameter logistic curve fit was applied to dose-response data using SigmaPlot© (SPSS Inc., Chicago, IL).

**Confocal microscopy**

Confocal microscopy experiments were performed in collaboration with Cheng-Han Lee, a MD-PhD student in Dr. Casey van Breemen’s laboratory.

**Data acquisition**

Detailed methods have been previously described regarding confocal [Ca\(^{2+}\)]\(_i\) imaging of in situ vascular smooth muscle cells within intact blood vessels(354). Briefly, inverted rings of HSV were loaded with the Ca\(^{2+}\)-binding dye fluo-4 AM (10 µM, with 10 µM Pluronic F-127, dissolved in PSS for 90 min at 25°C), followed by a 30 minute equilibration period in normal PSS. The rings were isometrically mounted on a custom-made microscope stage. [Ca\(^{2+}\)]\(_i\) imaging was accomplished with the use of a Noran Oz™ laser scanning confocal microscope (Noran Instruments, Middleton, WI) with a 100 µm slit through an air 20X lens (numerical aperture 0.45) on an inverted Nikon microscope. The 488 nm line of an argon-krypton laser illuminated the lumen side of the vessel, while a high-gain photo-multiplier tube collected the emission after it had passed through a 525/25 bandpass filter. The scanned region corresponds to a 232 µm × 217 µm area on the tissue and yields an image 512 pixels × 479 pixels in size. Image
acquisition was set at a rate of 1.07 frames/s. A higher image acquisition rate was unnecessary, as it did not yield any additional information.

**Data analysis**

Data analysis was performed in Image-Pro Plus© (Media Cybernetics Silver Spring, MD) using customised macros. The representative experimental fluorescence traces reflected the average fluorescence signal from a region 3 pixels ×3 pixels (or 1.36μm²) in size in a single cell. The changes in fluorescence (F525) in this region directly reflected changes in [Ca²⁺]. The 1.36μm² region was positioned towards the midline of the spindle-shaped smooth muscle cell that was delineated by the basal fluorescence level prior to stimulation. Numerical data were analysed in Microsoft Excel©. The theoretical assumptions for the use parametric statistical tests were verified and paired sample t-tests were performed using JMP© (SAS Institute, Inc., Cary, NC). The probability of an α error was set at 5%. Responses were expressed as mean ± standard error of the mean (SEM) with n representing the number of cells and/or tissues from different patients.

**Histology**

The two ends of each segment were sectioned and archived immediately. All rings used for isometric tension experiments or confocal microscopy were fixed by immersion in 10% neutral (pH 7.4) buffered formaldehyde phosphate solution for 12-24 hours and embedded in paraffin. Sections were cut at 4 μm onto microscope slides and stained with modified Movat’s pentachrome or for endothelial cells using an antibody (dilution factor of 1:2000) to Factor VIII-related antigen. Stained sections were examined by light microscopy. Staff of the Clinical Laboratory at St. Paul’s Hospital performed tissue embedding and tissue staining. Image acquisition and processing was performed with the expert assistance of Mr. Stuart Greene from
the McDonald Research Laboratories. Pictures were taken using a Nikon Ecclipse 800 microscope (Nikon Corporation, Tokyo, Japan) and a JVC KY-F70 camera (Victor Company of Japan Ltd, Tokyo, Japan). Image processing was performed using Adobe Photoshop 6 (Adobe, San Jose, California).

**Composition of solutions**

Plasma-Lyte® A Injection contained (in mg per 1000 mL): sodium chloride, 526; sodium gluconate, 502; sodium acetate trihydrate, 368; potassium chloride, 37; magnesium chloride hexahydrate, 30; and pH adjusted with sodium hydroxide to 7.4. Blood-cardioplegia was prepared by mixing four parts of oxygenated blood with one part Plasma-Lyte. Depending on the final concentration on K\textsuperscript{+} required for maintenance of cardiac arrest, the K\textsuperscript{+} concentration could vary from 4 to 27 mM. RPMI 1640 cell culture medium was prepared as per the instructions provided by the supplier and contained penicillin (5,000 units/L) and streptomycin (5,000 μg/L). The ionic composition of the PSS was (in mM): NaCl, 140; KCl, 5.9; MgCl\textsubscript{2}•6H\textsubscript{2}O, 1.2; CaCl\textsubscript{2}•2H\textsubscript{2}O, 2.5; glucose, 11; HEPES, 10. For the 80 mM K\textsuperscript{+} PSS (high K\textsuperscript{+}), 75 mM NaCl was replaced by an equimolar amount of KCl. For the nominally Ca\textsuperscript{2+}-free PSS, Ca\textsuperscript{2+} was omitted from the normal PSS and 1 mM EGTA was added. For the nominally Ca\textsuperscript{2+} free 80 mM K\textsuperscript{+} PSS, 75 mM NaCl was replaced by an equimolar amount of KCl, Ca\textsuperscript{2+} was omitted from the normal PSS, and 1 mM EGTA was added. In some cases, 0.1 and 10 mM EGTA, or 1 mM BAPTA were used as indicated in the text. For the Ca\textsuperscript{2+} dose-response curve in high K\textsuperscript{+} depolarisation solution, an 8 mM CaCl\textsubscript{2} containing high K\textsuperscript{+} PSS was serially diluted until the concentration of CaCl\textsubscript{2} was 0.0156 mM.
Drug dissolution information

PE, ACh, and HA-1077 were dissolved in dH2O and frozen in 1 mL aliquots of 0.1 M, 0.1 M, and 0.01 M, respectively. SK&F 96365 was dissolved in dH2O and frozen in 1 mL aliquots of 10 mM. CPA was dissolved in DMSO in 0.74 mL aliquots of 20 mM. Genistein, calphostin C, PD098,059 were dissolved in DMSO and frozen in aliquots of 0.1 M, 0.001 M, and 0.1 M, respectively. Dilutions were made fresh daily using PSS and drug additions were always less than or equal to 50 µL. Nifedipine was dissolved in ethanol at a stock concentration of 10 mM. All stock solutions were prepared in advance and stored at -20°C until use. Caffeine solutions were made fresh before prior to each experiment. The final concentrations of DMSO and ethanol to which the tissues were exposed were 0.5% and 0.0475%, respectively. The vehicles did not affect responses to PE, ACh and 80 mM K⁺.

Materials

Plasma-Lyte® A Injection was prepared by Baxter Corporation (Toronto, ON). RPMI 1640 cell culture medium and penicillin-streptomycin were purchased from Canadian Life Technologies (Burlington, ON). Minipig chow was obtained at Purina Mills, Inc (85). Isoflurane was obtained from the Hospital pharmacy. Somnotol and hepalean were obtained from MCT Pharmaceutical (Cambridge, ON). 10% buffered formaldehyde phosphate solution was purchased from Fisher Scientific (Napean, ON). Antibody to factor VIII-related antigen was purchased from Dako (Carpinteria, CA). All electrolytes, glucose, HEPES, EGTA, dimethylsulfoxide (DMSO), caffeine, ACh, nifedipine, L-NAME, HA-1077, genistein, calphostin C, and PD098,059, were purchased from Sigma-Aldrich (Oakville, ON). SK&F 96365 and CPA were ordered from Rose Scientific (Edmonton, AL). PE was obtained from Research Biochemicals International (Natick, MA). Fluo-4 AM, pluronic acid, and Hoechst 33342 were purchased from Molecular Probes (Eugene, OR). Fluo-4 AM and pluronic acid were
dissolved in DMSO and prepared fresh daily. Anhydrous ethanol was obtained from the Clinical Laboratory at SPH. LY379196 was a generous gift from Eli Lilly & Co (Indianapolis, IN).
CHAPTER IV: BASIC PHYSIOLOGICAL AND PHARMACOLOGICAL CHARACTERISATION OF HSV REACTIVITY

No spontaneous contractions

When HSV rings were first mounted under in vitro conditions in organs baths, no spontaneous activity was observed. This finding is in contrast to spontaneous contractions observed in human umbilical vessels (166) and human coronary arteries (352).

Determination of optimal pre-load

Smooth muscle contractility varies with the initial length of the tissue and may be governed by the amount of overlap between myosin thick filaments and actin thin filaments or by other mechanisms (86). The equipment for generating active and passive length-tension relationships was not available, but it was possible to generate a high K⁺-stimulated passive tension vs. active tension curve (452). The independent variable was passive tension instead of length: the greater the stretch, the greater the length, and the greater the pre-load. The following terms were used interchangeably: pre-load, resting tension, and baseline tension. The pre-load that generated the maximum response to stimulation with high K⁺ would be used for all future experiments.

Two segments from the same patient differed in the degree of intra-operative handling during surgical preparation for grafting. Each segment was divided into four rings. After an initial equilibration period in the absence of tension, the pre-load was set to 1 g and the tissues were stimulated successively with high K⁺ three times with wash periods at 30 min intervals. The tissues were then stretched in increments of 0.25 to 0.5 g and challenged with high K⁺ at each new pre-load (or length) separated by 30 min wash periods. As shown in Figure 3, both preparations reached maximum activation around 2 – 2.5 g and increasing the baseline tension
beyond this pre-load did not yield further increases in tension with high K\(^+\) stimulation over a broad range of baseline tensions. The preparations differed in the amount of developed force, most likely due to the difference in handling, but the response pattern did not differ with the contractility of each preparation and the optimal resting tensions were the same. Similar data were obtained in other tissues. Hence, all further experiments were performed at resting tensions in that range.

**No recovery of tissue responsiveness**

Occasionally, some HSV preparations did not respond at all to high K\(^+\) at the optimal pre-load. It was hypothesised that the surgical preparation represented a disruption in filament organisation, resulting in a possible shift in the length-tension curve. It has been recently demonstrated that rabbit tracheal smooth muscle adapted to a longer length could not generate tone unless stretched twice its optimal length (440). Hence, it was thought that increasing the pre-load may elicit contractions in these tissues. Tissues with no reaction to contractile stimuli at 2 – 2.5 g did not recover responsiveness to high K\(^+\) when the resting tension was increased to accommodate the possible shift (Figure 3B – grey line).

In the absence of a shift in the length-tension curve, it was thought that perhaps the time required for any structural alteration to occur due to the surgical handling could be influenced by the rate of high K\(^+\) stimulation. Wang and colleagues (439) have previously demonstrated that recovery from a length oscillation (perturbation in filament organisation) can recover within 30 min and is influenced by stimulation frequency. Experiments were performed to determine whether tissue responsiveness was altered by the frequency of high K\(^+\) challenges. Tissues were first equilibrated with three exposures to high K\(^+\) separated by 30 min wash periods. Then, tissues were stimulated repeatedly with high K\(^+\) at intervals of 20, 30, 40, and 60 min (Figure 4); a minimum wash period of 20 min was required to allow the tissues to return to baseline after a
high K\(^+\) contraction. As can be seen from the data, there was no difference between the third high K\(^+\) (the control response after the tissues had equilibrated) and successive high K\(^+\) responses at all the time intervals tested. It was concluded varying the high K\(^+\) stimulation rate did not recover tissue responsiveness. A 30 min wash was arbitrarily chosen as the wash period to be used between challenges with high K\(^+\) for all future experiments.

**Standardisation of HSV tissue supply**

Many preparations were obtained that did not respond to contractile stimuli, and based on the foregoing experiments, *in vitro* conditions could not be determined to recover reactivity in these vessels. Part of the observed variability could be explained by inter-patient variability. However, based on the numerous studies confirming the deleterious effects of intra-operative manipulations on the integrity and reactivity of HSV segments (see CHAPTER I: INTRODUCTION), the variability may best be explained by the surgical handling of these vessels during preparation for grafting.

In order to establish that surgical handling impaired the integrity and function of HSV samples collected from SPH, three classes of HSV segments were defined according to the stage of preparation of the vein for grafting (see CHAPTER III: MATERIALS AND METHODS). Segments termed ‘undistended’ were sectioned immediately after removal from the leg, prior to further manipulation. ‘Distended’ segments were obtained after pressurisation with the patient’s heparanised blood or a non-physiological salt solution. ‘Cardioplegic’ segments were sectioned, if in excess of the length required for grafting, after infusion with warm or cold blood-cardioplegia. Distended and cardioplegic vessels were considered ‘leftover’ segments.
Tests with high K⁺, PE and ACh

Samples were mounted and tested with two types of commonly used contractile stimuli, high K⁺ and PE, and with a vasodilator, ACh, a classical agonist that tests the integrity of the endothelium by eliciting endothelium-dependent relaxation after a pre-contraction (112).

Cell membrane depolarisation with high K⁺ and receptor stimulation with PE (50 μM) elicited responses that were far greater in undistended than in ‘leftover’ HSV samples (Figure 5A). The latter responses were marginal or non-existent in distended and cardioplegic segments, respectively (Figure 5C). Hence, undistended HSV segments were the best preparations in which to study mechanisms of smooth muscle reactivity.

Endothelial cell function was compared in the three sample types by adding ACh to the peak of PE (50 μM)-pre-contracted rings in two high doses (1 & 10 μM; Figure 5B & Figure 5D). ACh relaxed rings from undistended segments at both concentrations used. The frequent lack of adequate responses to PE in distended and cardioplegic samples of HSV precluded reproducible evaluations of endothelial cell function. When PE pre-contraction was observed, ACh stimulation did not relax most distended HSV samples, although in a few rings a decrease in tension was observed. The responses to ACh were not reproducible between segments. Based on these observations, endothelial cell function was best preserved in undistended HSV samples.

Dose-response curves to PE and ACh

Cumulative dose-response curves (427) to PE and ACh were also effected in undistended, distended, and cardioplegic tissues to gain insight into the mechanism(s) responsible for the depressed contractions in leftover segments.

PE elicited dose-dependent increases in tension in undistended and distended HSV samples, but responses were very depressed in distended rings, without any obvious shift in
EC50 (Figure 6A). A dose-response curve could not be elicited in cardioplegic HSV segments (Figure 6A). Hence, responses to PE in distended and cardioplegic segments resulted from a loss of contractile ability rather than a change in the sensitivity to PE.

Normalising PE dose-response curves in undistended HSV rings by high K\(^+\) (Figure 6A) partially removed some of the variability in the raw data (Figure 6B). Dose-response curve parameters were determined based on individually normalised dose-response curves. PE elicited 95% of the dose-response curve between 1 and 55 \(\mu\)M and the mean EC50 was 11.8 ± 0.2 \(\mu\)M (n=16 rings from 11 patients; Figure 6B). The 50 \(\mu\)M dose used in the preceding experiments corresponded to an EC93 and was used for all mechanistic experiments with PE.

Dose-response curves to ACh were also elicited under two conditions: at the peak of a pre-contraction with PE (50 \(\mu\)M) and with U46619 (1 \(\mu\)M) in undistended, distended and cardioplegic tissues. As can be seen from Figure 7, ACh elicited dose-dependent relaxations only in tissues from undistended HSV rings pre-contracted with PE, but not in distended or cardioplegic tissues. ACh-induced relaxation was not observed in any tissues pre-contracted with U46619. At doses for ACh beyond 10 \(\mu\)M, a constrictor response was observed. A significant relaxation as compared to control was observed at the 30 \(\mu\)M dose for ACh when the data was normalised by PE (p < 0.025; n = 4 rings from 4 patients). This finding contrasts with the relaxations observed with the limited addition of 1 and 10 \(\mu\)M ACh (Figure 5) that were both highly significantly different from control. Similarly, distended vein segments were significantly relaxed with ACh at 1 and 10 \(\mu\)M but not during the Ach dose-response curve.

**Histology of HSV samples**

Undistended, distended, and cardioplegic HSV samples were stained with a Movat pentachrome stain (Figure 8A,C,E) and an antibody to Factor VIII-related antigen (Figure 36...
Each segment type was composed of intimal, medial, and adventitial layers. As can be seen from the illustrations (Figure 8B, D, and E), each segment type had dark antigen staining indicating that the endothelial cell layer was morphologically intact and could not account for differences in ACh responses between undistended, distended, and cardioplegic tissues. Each ring differed in wall thickness, cross-sectional area, and organisation. The undistended ring had the smallest cross-sectional area as compared to the distended and cardioplegic segments because it was sampled from the distal region of the leg, but had several smooth muscle layers and a diffusely thickened intima. The distended segment was almost devoid of adventitia and did not have any diffuse intimal thickening. However, several smooth muscle layers were also present. The cardioplegic segment was similar to the undistended ring insofar as it had several smooth muscle layers and a thickened intima, but it also had a greater wall thickness. Despite these similarities and differences between each segment type, none could explain the difference in contractility observed between these segment types. Adventitial stripping has been shown to decrease smooth muscle cell function (128), but the cardioplegic segment had a thick adventitial layer and did not elicit a response to high K⁺ or PE.

In light of the foregoing functional and morphological findings, undistended HSV samples were the most intact physiologically and pharmacologically, and elicited the most reproducible responses, as compared to distended and cardioplegic vein segments. The superior reactivity of these vessels were not attributed to any morphological characteristics not present in distended and cardioplegic segments, but were reflected in the fact that they were sectioned as soon as the vein was removed from the leg and prior to further surgical handling.

**Undistended HSV segments from RCH**

HSV samples from RCH were removed by the minimally invasive harvest technique. This method is thought to be deleterious to the integrity of the vein graft because the tissue is
isolated from surrounding areolar tissues at only a few incisions and then removed from the leg by pulling it through an open site. High K⁺, PE and ACh responses were elicited in undistended HSV samples from RCH (Table 2) and compared with those from SPH (Figure 5). There was no difference in the magnitude of high K⁺ and PE responses between tissues from RCH and SPH (p > 0.05). In RCH tissues, ACh responses at both 1 and 10 µM were not significantly different from control responses (p > 0.05), and at 10 µM, a net contraction was observed. Control responses represented the level of PE-induced force had ACh not been added to the tissue. Hence, ACh relaxations in RCH tissues were depressed as compared to those in tissues from SPH.

Table 2. Responses in RCH HSV tissues.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Response (g)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>High K⁺</td>
<td>3.79 ± 0.63</td>
</tr>
<tr>
<td>PE 50 µM</td>
<td>3.19 ± 0.58</td>
</tr>
<tr>
<td>ACh 1 µM</td>
<td>0.69 ± 0.15</td>
</tr>
<tr>
<td>Control for ACh 1 µM</td>
<td>0.64 ± 0.20</td>
</tr>
<tr>
<td>ACh 10 µM</td>
<td>0.66 ± 0.22</td>
</tr>
<tr>
<td>Control for ACh 10 µM</td>
<td>0.91 ± 0.22</td>
</tr>
</tbody>
</table>

†n = 26 rings from 26 patients

Use of overnight tissues

Occasionally tissues from afternoon cases were stored overnight in a refrigerator and used the next morning. Reactivity in overnight stored undistended HSV samples was compared to that of freshly isolated tissues (Table 3). Responses to high K⁺ and PE after overnight cold
storage were significantly depressed as compared to freshly isolated tissues, although PE responses normalised by high K$^+$ were not significantly different between groups. Hence, cold storage may result in a fractional loss of viable smooth muscle cells, but not a selective depression in receptor-mediated responses. This finding is unlike that in the gastroepiploic artery where contractions to noradrenaline increased in force and sensitivity of contraction after storage at 4°C in Krebs solution for up to 14 days (166).

Table 3. Comparison of fresh and overnight HSV segments.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Fresh†</th>
<th>Overnight‡</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>High K$^+$</td>
<td>4.50 ± 0.38</td>
<td>3.13 ± 0.43</td>
<td>0.026</td>
</tr>
<tr>
<td>PE 50 μM</td>
<td>3.09 ± 0.32</td>
<td>1.97 ± 0.29</td>
<td>0.028</td>
</tr>
<tr>
<td>PE as % high K$^+$</td>
<td>67.52 ± 4.18</td>
<td>64.56 ± 7.71</td>
<td>0.71</td>
</tr>
</tbody>
</table>

†n=23 rings from 5 patients
‡n=12 rings from 4 patients

Figure 3 to Figure 8 to follow
Figure 3: Isometric passive vs. active tension relationships in HSV.

Tissues were equilibrated with three high K$^+$ challenges at a resting tension of 1 g. The baseline tension was then increased in increments of approximately 0.25-0.5 g. The segment in (A) reacted more forcefully to high K$^+$ at each new resting tension than the segment in (B). However, the response pattern did not differ between these two tissues. A tissue that did not contract at the optimal length or passive tension did not recover responsiveness with increasing stretch (grey). Values represent the change in tension from baseline (g).
Figure 4. Effect of varying the stimulation rate with high K\(^+\) depolarisation.

Tissues with different abilities to respond to high K\(^+\) were first equilibrated at the optimal resting tension with three high K\(^+\) challenges separated by 30 min wash periods. The third high K\(^+\) response was the control response to be compared with subsequent challenges. Successive high K\(^+\) contractions were separated by (A) 20 min, (B) 30 min, (C) 40 min, and (D) 60 min wash periods. Changing the duration of the washing interval had no impact of the reactivity of the tissue to high K\(^+\) contractions.
Figure 5. Smooth muscle and endothelial cell functions in undistended, distended, and cardioplegic segments.

A. Sample traces of high K\(^+\) and PE (50 \(\mu\)M) responses in all three sample types. Contractions in undistended segments were greater in magnitude than in distended and cardioplegic HSV segments. Exposures to high K\(^+\) or PE are indicated by the bar under each trace. B. Sample traces of 1 & 10 \(\mu\)M ACh-mediated endothelium-dependent relaxations after pre-contraction with PE (50 \(\mu\)M). ACh dose-dependently relaxed undistended HSV but had no effect in distended and cardioplegic HSV segments. The addition of ACh is indicated by arrows. Stimuli remained in contact with the tissues for the duration of the responses. C. Mean responses for high K\(^+\) & PE. On average, responses were significantly depressed or absent in distended (n = 10 rings from 4 patients), and cardioplegic (n = 8 rings from 4 patients) segments as compared to undistended (n = 42 rings from 12 patients) HSV segments (p < 0.0001 for high K\(^+\) & PE). D. Mean ACh relaxation responses in undistended, distended, and cardioplegic HSV segments. On average, responses were significantly depressed or absent in distended (n = 10 rings from 4 patients), and cardioplegic (n = 8 rings from 4 patients) samples as compared to undistended (n = 37 to 39 rings from 12 patients) HSV segments (p < 0.005 for 1 \(\mu\)M ACh, and p < 0.0146 for 10 \(\mu\)M ACh). It should be noted that responses to ACh were corrected for the declining plateau phase with PE stimulation. For undistended HSV samples, ACh-mediated endothelium-dependent relaxation was significantly greater than the control PE responses in undistended (p < 0.0001 for 1 and 10 \(\mu\)M ACh) and distended (p < 0.03 for 1 \(\mu\)M ACh and p < 0.008 for 10 \(\mu\)M ACh) HSV samples.
A

Undistended  Distended  Cardioplegic

High K⁺  PE 50 μM

2 g  2.5 min

B

ACh 1 μM  ACh 10 μM

ACh 1 μM  ACh 10 μM

PE 50 μM  PE 50 μM  PE 50 μM

C

D

Change in tension (g)

High K⁺  PE 50 μM

ACh 1 μM  ACh 10 μM

UNDIST  DIST  CARDIOPL

UNDIST  DIST  CARDIOPL
Figure 6. Dose-response curves to PE in undistended, distended, and cardioplegic HSV samples.

A. Average dose-response curves to PE in undistended (n=18 rings from 12 patients), distended (n=29 rings from 9 patients), and cardioplegic (n=29 rings from 11 patients) HSV segments. Dose-response curves to PE were depressed in distended as compared to undistended HSV samples with no apparent shift in EC50. Dose-response curves were not measurable in cardioplegic HSV segments. B. Average dose-response curve to PE in undistended HSV normalised by high K⁺ responses.
Figure 7. Dose-response curves to ACh in undistended, distended and cardioplegic segments.

ACh dose-response curves were effected in half-log increments on HSV samples that were pre-contracted with PE (50 μM) or U46619 (1 μM). A. Dose-response curves in undistended HSV segments pre-contracted with PE (n = 4 rings from 4 patients). B. Same as in (A) but normalised by the PE pre-contraction. C. Dose-response curves in distended HSV segments pre-contracted with PE (n = 4 rings from 2 patients). D. Dose-response curves in cardioplegic HSV segments pre-contracted with PE (n = 13 rings from 6 patients). E. Dose-response curves in undistended HSV segments pre-contracted with U46619 (n = 6 rings from 6 patients for ACh and n = 5 rings from 5 patients for control). F. Same as in (E) but normalised by the U46619 pre-contraction. G. Dose-response curves in distended HSV segments pre-contracted with U46619 (n = 15 rings from 7 patients for ACh and n = 10 rings from 6 patients for control). H. Dose-response curves in cardioplegic HSV segments pre-contracted with U46619 (n = 10 rings from 4 patients for ACh and n = 6 rings from 4 patients for control).
Figure 8. Movat- and factor VIII- stained rings of undistended, distended, and cardioplegic segments.

Movat stained undistended (A), distended (C), and cardioplegic (E) HSV segments. Same segments as in A, C, and E labelled with Factor-VIII related antigen to identify endothelial cell staining in undistended (B), distended (D), and cardioplegic (F) HSV segments. Each segment type is from a different patient.
CHAPTER V: CHANGES IN INTRACELLULAR $[\text{Ca}^{2+}]$ AND FORCE MEDIATED BY HIGH K$^+$ AND PE IN THE HSV

Confocal microscopy experiments were performed to gain insight into the intracellular Ca$^{2+}$ signalling patterns underlying contractile responses to high K$^+$ and PE in the HSV. This chapter illustrates and compares the Ca$^{2+}$ responses elicited by each stimulus. Studies to elucidate the mechanistic basis for the Ca$^{2+}$ response and other aspects of excitation-contraction coupling mechanisms are presented in CHAPTER VI for high K$^+$ and CHAPTER VII for PE.

Hoechst 33342-stained endothelial and smooth muscle cells.

The endothelium was mechanically removed to facilitate the examination of agonist-induced Ca$^{2+}$ signalling in the smooth muscle layer of the HSV and the effectiveness of endothelium removal was examined. In endothelium-intact preparations, both endothelial and smooth muscle cell nuclei could be identified using the DNA stain Hoechst 33342, a dye commonly used to stain the nuclei of living cells (354) (Figure 9A). Endothelial cell nuclei were oblong shaped and smooth muscle cell nuclei appeared elongated. After endothelium-denudation, only smooth muscle nuclei were present (Figure 9B), thus confirming endothelium removal.

Force versus Ca$^{2+}$ in response to high K$^+$ and PE.

Using laser scanning confocal microscopy on four fluo-4 loaded HSV segments, changes in $[\text{Ca}^{2+}]$, were measured at a rate of approximately 1 frame per sec for 5-6 min. Figure 10 illustrates a series of confocal images depicting intact HSV smooth muscle cells loaded with fluo-4. The first image in each series represents the tissue at baseline (time = 0 sec). Subsequent images represent the response of the tissue to high K$^+$ and PE at successive time points recorded.
from the same tissue region. High K\(^+\) elicited a sustained increase in [Ca\(^{2+}\)]\(_i\), which lasted as long as the stimulus was present (Figure 10A). In contrast, PE (50 \mu M) resulted in a transient rise in [Ca\(^{2+}\)]\(_i\) (Ca\(^{2+}\) transient), which returned to near baseline for the duration of the recording (Figure 10B). Fluo-4 loading did not affect contractile responses to high K\(^+\) or PE stimulation (data not shown). Nearly all cells responded uniformly with only a marginal number (~3%) of cells in a few tissues exhibiting [Ca\(^{2+}\)]\(_i\) oscillations in response to PE.

Changes in [Ca\(^{2+}\)]\(_i\) were compared to changes in force as shown in Figure 11. Stimulation with high K\(^+\) resulted in parallel elevations in force (Figure 11A) and fluorescence (Figure 11B). Thus, contractions elicited by high K\(^+\) were dependent on increases in [Ca\(^{2+}\)]\(_i\). In contrast, sustained elevations in tension (Figure 11C) in response to PE were observed in the absence of maintained elevations in [Ca\(^{2+}\)]\(_i\) (Figure 11D). The fluorescence response rose rapidly to a peak, while force was still rising, and then fell rapidly to a lower level, where it declined more slowly to baseline levels (Figure 11D). Meanwhile, force maintained a steady plateau (Figure 11C). Thus, a transient increase in [Ca\(^{2+}\)]\(_i\) may play a role in the initial part of the PE response.

The duration of the PE transient from each region was quantified by calculating the time between 5 and 100% of the peak for both the upstroke and the decline of the fluorescence signal. On average, as little as 7 ± 2 sec were required to span 95% of the upstroke and 18 ± 5 sec for the decline to reach near basal levels (5% of the peak). In total, as little as 26 ± 7 sec (n = 109 cells from 4 tissues) were required to span 95% of the Ca\(^{2+}\) transient.

Figure 12 summarises and compares average fluorescence and force measurements at 4 min post-stimulation with high K\(^+\) and PE. Although the average amplitude of contractions at 4 min elicited by high K\(^+\) was greater than that elicited by PE (p < 0.0001; n = 25 tissues), these values represented 85% of the peak force developed for each stimulus, respectively (Figure 12). The average [Ca\(^{2+}\)]\(_i\) for high K\(^+\) at 4 min was significantly increased as compared to baseline (p <
0.0001), while that for PE was not significantly different from basal levels (p > 0.05; Figure 12), indicating that mechanisms other than Ca$^{2+}$ were responsible for force maintenance during exposure to PE.

Figure 9 to Figure 12 to follow
Figure 9. Hoechst 33342-stained endothelial and smooth muscle cells.

A. In intact tissues, the Hoechst dye stained both endothelium and smooth muscle DNA. The elongated smooth muscle cells have their long axis oriented diagonally (i.e., perpendicularly to the direction of flow). Endothelial cells are the oblong-shaped cells scattered on top of the smooth muscle layer. B. After endothelium-denudation, Hoechst 33342 staining identified only smooth muscle cell DNA, thereby confirming the absence of endothelium.
Figure 10. Series of confocal images depicting changes in \([Ca^{2+}]_i\) in individual smooth muscle cells in the HSV elicited by high K\(^+\) and PE.

Fluo-4 loaded tissues were challenged with high K\(^+\) and PE (50 µM). Fluorescence responses were measured at a rate of about 1 frame per sec for about 5 min using a fast laser scanning confocal microscope. Select images depict the tissue at baseline and at successive time points recorded from the same tissue region.  

**A.** High K\(^+\) elicited a sustained increase in \([Ca^{2+}]_i\), which lasted the duration of the recording.  

**B.** In contrast, the change in \([Ca^{2+}]_i\) in response to PE (50 µM) was only transient and the \([Ca^{2+}]_i\) returned to baseline within 30 sec after stimulation, remaining there for the duration of the recording.
Figure 11. Parallel changes in force and [Ca$^{2+}$]i in response to high K$^+$ and PE in the HSV. High K$^+$- and PE-elicited responses were obtained in the same preparation to facilitate a comparison between these two stimuli. A. High K$^+$ elicited sustained increases in force. B. Sustained increases in fluorescence elicited by high K$^+$ in three areas of the tissue as depicted in panel E. C. Maintained contractions stimulated by PE (50 μM). D. Transient increases in fluorescence stimulated by exposure to PE (50 μM) in three areas of the tissue as depicted in panel E.
Figure 12. Average fluorescence and force measurements at 4 min post-stimulation with high $K^+$ and PE.

High $K^+$ and PE responses were elicited in the same preparations to facilitate comparison. The average amplitude of high $K^+$ and PE contractions at 4 min were significantly different from each other ($p < 0.0001$) but represented $84.6 \pm 1.3\%$ and $84.6 \pm$ $^+$ was significantly increased as compared to baseline ($n = 101$ cells from 4 tissues, $p < 0.0001$) while that for PE was not significantly different from basal levels ($n = 111$ cells from 4 tissues, $p > 0.05$).
Force Calcium

[Graph showing comparison of Force and Calcium with different conditions: High K and PE]
CHAPTER VI: HIGH K⁺-DEPOLARISATION-INDUCED CONTRACTIONS IN THE HSV

Isometric tension experiments were performed to investigate further excitation-contraction coupling mechanisms in the HSV in response to high K⁺-depolarising solution. Surprising differences were observed between human and animal vascular preparations, which may be related to morphological differences between species due to ageing and/or vascular pathophysiology.

High K⁺-depolarisation-induced Ca²⁺-entry through VOCs

High K⁺ elicited sustained contractions that were inhibited by pre-incubation with the selective VOC antagonist nifedipine (122) (Figure 13). Contractions were also inhibited by the addition of nifedipine (5 - 10 μM) at the peak of the response (Figure 14). Similar to most vascular preparations, high K⁺ contractions stimulated sustained elevations in Ca²⁺ (see Figure 10 and Figure 11 in CHAPTER V) by the influx of Ca²⁺ through VOCs. However, high K⁺ contractions persisted in a nominally Ca²⁺-free solution (0 Ca²⁺ and containing 1 mM EGTA, a Ca²⁺ chelator; Figure 13A & Figure 13B). This finding is a novel property of high K⁺ contractions. Previous reports have demonstrated the resistance of receptor-mediated contractions to Ca²⁺ removal (425), but not to high K⁺-induced contractions, which are typically abolished in Ca²⁺-free medium (161, 455). The high K⁺ contractions in 0 Ca²⁺ 1 mM EGTA PSS were also inhibited by pre-incubation with nifedipine (5 μM), confirming that Ca²⁺ entered through VOCs despite the removal of extracellular free Ca²⁺ and the presence of a high concentration EGTA (1 mM). The residual, nifedipine-insensitive high K⁺ responses were not due to the release of intramural noradrenaline from sympathetic nerve terminals because phenolamine pre-incubation (50 μM) did not significantly attenuate high K⁺ contractions (high
K⁺ controls 130 ± 10% n= 7 rings from 6 patients, high K⁺ after phentolamine 100 ± 10% n = 9 rings from 6 patients; p ~ 0.11).

**Tightly bound extracellular Ca²⁺ in the HSV**

**Prolonged exposure to nominally Ca²⁺-free PSS**

Based on the observation that high K⁺ contractions in 0 Ca²⁺ 1 mM EGTA solution were almost entirely due to influx of Ca²⁺ through VOCs, it was hypothesised that the source of Ca²⁺ was from an extracellular store of tightly bound Ca²⁺ protected from, or resistant to, exposure to high concentrations of EGTA. A tightly bound source of extracellular Ca²⁺ is thought to be absent from animal blood vessels, (161) or present but protected by caveolae (300). The hypothesis was tested by measuring the magnitude of high K⁺ contractions after increasing the incubation time in 0 Ca²⁺ 1 mM EGTA in the HSV and in a series of arteries and veins harvested from commonly used laboratory animals. The swine was chosen as an example of a large species and the rabbit as an example of a smaller animal. As can be seen from Figure 15A, a 20 min incubation period in nominally Ca²⁺-free solution was necessary to produce marked inhibition of the high K⁺ response as compared to control. In contrast, in the rabbit femoral artery, the response to high K⁺ disappeared completely after only 2 min in the same nominally Ca²⁺-free medium (Figure 15B). The difference in the time courses between the HSV and vascular preparations from swine and rabbit is summarised in Figure 15C. The slowest time course was observed in the HSV. On average, high K⁺ contractions in 0 Ca²⁺ 1 mM EGTA solution were abolished twice as fast in the pig renal artery and 25 times faster in the rabbit femoral artery. The rate of decay in the pig femoral vein was nearly indistinguishable from that in the rabbit femoral artery.
The foregoing curves were derived in the absence of phentolamine. Although intramural release of noradrenaline was not a significant component of high K⁺ contractions in the HSV, it was in porcine renal arteries (Figure 16B & C) and femoral veins (Figure 17B & C); this component was larger in femoral veins than renal arteries. Phentolamine (10 µM) pre-incubation significantly depressed high K⁺ contractions in nominally Ca²⁺ free PSS in pig renal artery (p < 0.0001; Figure 16C) and femoral vein (p < 0.0001; Figure 17). The phentolamine-resistant component was abolished by pre-incubation with nifedipine (5 µM) in both preparations (Figure 16 & Figure 17); hence, these phentolamine-resistant contractions were due to Ca²⁺ influx through VOCs. Although these measurements were only made at the 30 sec time point, it is conceivable that the time course curves for the decay of the high K⁺ contractions in nominally Ca²⁺-free PSS in Figure 15 have been overestimated for the swine renal artery and femoral vein. The effects of phentolamine were not tested in the rabbit femoral artery.

Attempts were also made to obtain time course curves for high K⁺ contractions in nominally Ca²⁺-free solution in the rabbit inferior vena cava and veins isolated from the thymus of infants and adolescents. However, contractions in these preparations were inhibited by more than half after merely 30 sec in nominally Ca²⁺-free PSS in the presence of phentolamine (10 µM). Contractions were 36.2 ± 10.4% (n = 3 rings) in the human thymus vein and 17.8 ± 4.3 % (n = 4 rings) in the rabbit inferior vena cava. It is conceivable that high K⁺ would be abolished within 1 min in nominally Ca²⁺-free PSS. These phentolamine-resistant contractions were completely abolished by nifedipine (5 µM; data not shown).

Properties of the vessel wall

Based on the foregoing observations, it was hypothesised that the differences between the decay curves in Figure 15 could be related to differences in the properties of the vessel walls.
Figure 18 contains images of each vascular preparation challenged with high K\(^+\) in 0 Ca\(^{2+}\) 1 mM EGTA solution. Upon visual inspection of these images, the vessels differed greatly in their wall properties. Although all vessels consisted of intimal, medial, and adventitial layers, there were differences in these layers between preparations. The thickest vessel was the HSV (Figure 18A) consisting of several smooth muscle layers and a thickened intima. None of the other vessels exhibited a thickened intimal layer. The cross-sectional area of the HSV was much greater than that of other vessels. The medial layers of both swine renal artery (Figure 18C) and rabbit femoral artery (Figure 18E) consisted of several smooth muscle layers but the thickness was less than that of the HSV. The swine femoral vein (Figure 18D) had fewer smooth muscle layers and a smaller wall thickness than the renal artery. Also shown are the rabbit inferior vena cava (Figure 18F) and human thymus vein (Figure 18B). These were the thinnest preparations amongst this group and high K\(^+\) contractions were most sensitive in nominally Ca\(^{2+}\) free PSS when tested in these preparations.

The discrepancies between the dimensions of the different preparations tested suggest these vessel properties may explain the differences observed in Figure 15. An attempt was made to relate the dimensions of the vessel wall to the time course curves for the decay of the high K\(^+\) contractions in 0 Ca\(^{2+}\) 1 mM EGTA. The wall thickness of each representative image in Figure 18 was compared to the average time required for nominally Ca\(^{2+}\)-free PSS to inhibit contractions by 50\% (IT50) in each tissue. Wall thickness, limited to intimal and medial layers, and IT50 values (Table 4) were tightly correlated (correlation coefficient of 0.998). The thickness of medial and intimal layers of the HSV was in agreement with values in the literature (336). The tight relationship between wall thickness and IT50 values suggests that the inhibitory effect of 0 Ca\(^{2+}\) 1 mM EGTA solutions on the high K\(^+\) contractions may be affected by diffusion distance. The thicker the preparation, the more time required for EGTA to permeate the medial layer and for Ca\(^{2+}\) to diffuse out of the tissue. However, the HSV was only 5-6 times thicker, but
the IT50 value was 12-13 times greater, than the rabbit femoral artery (Table 4). This discrepancy suggests that factors other than diffusion distance may account for the observed differences between the HSV and the animal vascular preparations.

Table 4. Comparison between wall thickness and IT50 for the high K+ decay curves.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Thickness (µm)</th>
<th>IT50 (min)</th>
<th>Relative thickness*</th>
<th>Relative IT50^</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV</td>
<td>779</td>
<td>12.05</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Renal artery</td>
<td>302</td>
<td>3.037</td>
<td>2.6</td>
<td>4.0</td>
</tr>
<tr>
<td>Femoral vein</td>
<td>154</td>
<td>1.088</td>
<td>5.1</td>
<td>11.1</td>
</tr>
<tr>
<td>Femoral artery</td>
<td>138</td>
<td>0.9611</td>
<td>5.6</td>
<td>12.5</td>
</tr>
</tbody>
</table>

*HSV wall thickness/Tissue wall thickness

^HSV IT50/Tissue IT50

Figure 19 depicts high power images of the HSV (A) and the rabbit femoral artery (B). The adventitia of the HSV was composed of a fibro-elastic coat consisting of loose connective tissue such as collagen and elastin. It contained vasa vasora and longitudinal smooth muscle bundles surrounded by GAGs. The external elastic lamina was clearly visible. The fibromuscular media contained circular smooth muscle layers separated by matrix components, such as depositions of elastin, collagen, and GAGs. A thick layer of longitudinal bundles surrounded by collagen separated the media from the intimal layer. The intima was also fibrotic. A thickened subendothelium was composed of longitudinal smooth muscle or myofibroblasts, collagen and GAGs, but did not contain elastin. The internal elastic lamina was not clearly visible. A well-defined elastic lamina is rarely seen (275), it is often fragmented in tissues with intimal thickening (337), or may be duplicated (62). These descriptions are consistent with
histological surveys of HSV segments obtained from patients undergoing CABG surgery or at autopsy (62, 77, 402, 435).

The rabbit femoral artery differed from the HSV in many ways. The adventitia contained loose connective tissue composed of strands of elastin interspersed with collagen, but lacked vasa vasora. The media contained mainly circular smooth muscle layers with some evidence of GAGs, but virtually no collagen and elastin. Most of the connective tissue was located in the adventitia as compared to the media, a property of smaller arteries. The intimal layer consisted of the internal elastic lamina and endothelial cells.

In summary, the media and intima in the HSV (Figure 19A) were not only much thicker but also contained more connective tissue (elastin, collagen, myofibroblasts) and GAGs in the HSV than in the rabbit femoral artery (Figure 19B). Collagen and elastin bind to and exchange Ca\(^{2+}\) with the extracellular space (430). Also, GAGs, covalently bound to a core protein forming proteoglycans, or free in the extracellular space (hyaluronic acid), contain negatively charged residues (150, 448). Hence, the increase in collagen, elastin, and GAGs in the HSV represent a repository of anionic sites, not found in the rabbit femoral artery, which may bind Ca\(^{2+}\) and/or repel EGTA. In addition, the increased connective tissue and GAGs may act as barriers to the diffusion of EGTA through the media (36).

\textbf{Ca}^{2+} \textit{release from the SR by high K}^+ \textit{in nominally Ca}^{2+} \textit{free PSS}

Previous findings have shown that in vascular smooth muscle Ca\(^{2+}\) release from the SR can occur in response to Ca\(^{2+}\) binding to the RyR channel located on the membrane of the SR (177). It is possible that Ca\(^{2+}\) entering through VOCs can induce Ca\(^{2+}\) release from the SR, as in cardiac muscle (99) and that this additional supply of Ca\(^{2+}\) contributes to the high K\(^+\) contraction in nominally Ca\(^{2+}\) free medium in the HSV. The SR Ca\(^{2+}\) store was unloaded with a brief
exposure (2 min) to caffeine (10 mM), which induces Ca^{2+}-release through RyR channels (173, 364), and with the maintained addition of CPA (10 μM), which inhibits SERCA (80, 419) in smooth muscle and the re-uptake of Ca^{2+} into the SR. Unloading took place in nominally Ca^{2+}-free medium. After unloading the SR, the high K^+ contraction in nominally Ca^{2+}-free solution was reduced as compared to a contraction in nominally Ca^{2+}-free PSS in the absence of CPA and caffeine (Figure 20A). The high K^+ contraction after 15 min in nominally Ca^{2+}-free PSS was also inhibited by nifedipine (5 μM) pre-incubation, confirming that the SR Ca^{2+} release component was due to the influx of Ca^{2+} and not due to the release of intramural NA, or the co-transmitters, ATP and NPY (341, 356). On average, contractions were significantly lower after SR Ca^{2+} unloading than in nominally Ca^{2+}-free PSS alone, regardless of the order in which these high K^+ contractions were effected (p < 0.023 and p < 0.0025; Figure 20C). It was concluded from these experiments that a component of the high K^+ contraction in nominally Ca^{2+}-free medium was due to the release of Ca^{2+} from the SR.

Experiments were performed to ensure that caffeine exposure did not inhibit subsequent high K^+ contractions as caffeine decreases the sensitivity of the myofilaments to Ca^{2+} (364). A set of paired rings from the same patient was challenged with high K^+. At the peak of the contraction, one ring was exposed to caffeine (10 mM) for two minutes and then rinsed for 13 min, prior to a second challenge with high K^+. The control ring was simply rinsed for 15 min. This experiment was repeated in another set of paired rings from a different patient. In both cases, there was no difference in the magnitude of successive contractions between the two rings (Figure 21); hence, brief exposure to caffeine had no direct inhibitory effect on the magnitude of high K^+ contractions.

Attempts were made to determine whether CICR was also a component of high K^+ contractions in 0 Ca^{2+} 1 mM EGTA solution in swine femoral vein and renal artery. The protocol used for the HSV required an incubation period in 0 Ca^{2+} 1 mM EGTA solution with or
without SR Ca\(^{2+}\) depletion in the presence of caffeine and CPA. As can be seen from Figure 15, high K\(^+\) contractions after 15 min in nominally Ca\(^{2+}\)-free PSS were abolished in the femoral vein and renal artery. Hence, the same protocol was attempted, but in the presence of extracellular free Ca\(^{2+}\), without EGTA, and using higher concentrations of caffeine and CPA (20 mM and 50 \(\mu\)M, respectively). In addition, the effects of nifedipine and phentolamine were also tested on high K\(^+\) contractions after attempts to deplete the SR with caffeine and CPA. However, SR Ca\(^{2+}\) depletion under these conditions could not be achieved, and the response pattern (data not shown) was identical to that observed in Figure 16 & Figure 17.

**Effect of age and disease on extracellular bound Ca\(^{2+}\)**

It was hypothesised that the putative extracellular tightly bound Ca\(^{2+}\) in the HSV resulted from ageing and/or disease. Patients supplying SV tissues were relatively old and suffered from coronary artery disease, whereas the other vascular preparations were obtained from animals that were young and healthy. Attempts were made to test the effects of age in vascular preparations from humans, but a suitable supply of comparable veins from infants could not be obtained. Instead, it was possible to test the effects of disease on vascular preparations obtained from swine allocated to one of three different experimental groups: control, high fat fed, and alloxan-fed diabetic pigs on a high fat diet. Alloxan kills pancreatic \(\beta\) cells and decreases plasma insulin levels thereby decreasing insulin action (85).

The effects of prolonged incubation in 0 Ca\(^{2+}\) 0.1, 1, and 10 mM EGTA on high K\(^+\) contractions were tested in both renal artery and femoral vein. High K\(^+\) contractions were abolished in 10 mM EGTA in all tissues tested (data not shown). There was no difference between experimental groups in either tissue at EGTA concentrations of 0.1 and 1 mM, except for 0.1 mM EGTA-containing solution in the femoral vein (Figure 22). The time course curve
for the hyperlipidemic pigs, but not the diabetic pigs on a high fat diet, was significantly faster during the first 5 min in 0.1 mM EGTA as compared to control animals (Figure 22). In addition, the time course curve for the hyperlipidemic pigs in 0.1 mM EGTA declined at a rate similar to the time course curve in 1 mM EGTA (depicted for all groups combined in Figure 15). In contrast, the time course curves at 0.1 mM EGTA were significantly slower than those at 1 mM EGTA for both the diabetic pigs on a high fat diet (Figure 23) and control pigs (data— similar to Figure 23 – not shown). Differences between treatment groups were not attributed to alterations in Ca\(^{2+}\) handling by the myofilaments as no difference was observed in the Ca\(^{2+}\) dose-response curves in K\(^+\)-depolarised rings of swine femoral vein (Figure 24). In addition, no difference was observed in influx of Ca\(^{2+}\) through VOCs or the intramural release of NA between experimental groups (data not shown).

Based on these findings, it is thought that the hyperlipidemic environment interfered with the Ca\(^{2+}\) binding properties of the extracellular matrix by decreasing the number of anionic Ca\(^{2+}\) binding sites and/or decreasing their affinity for Ca\(^{2+}\). A treatment group of alloxan-fed diabetic pigs on a normal diet was missing and the effects of diabetes alone could not be tested. However, it is possible to speculate that the hyperlipidemic environment counteracted the effects of decreased insulin action by alloxan. In other words, the effects of diabetes may have increased the affinity and/or number of Ca\(^{2+}\) biding sites through vascular wall remodelling, but these effects were inhibited by the concomitant high fat diet.

**Access to extracellular Ca\(^{2+}\) binding sites**

EGTA, considered a ‘slow’ Ca\(^{2+}\) chelator, may be unable to bind Ca\(^{2+}\) before Ca\(^{2+}\) derived from the extracellular Ca\(^{2+}\) binding sites reached VOCs. BAPTA, a much faster Ca\(^{2+}\) chelator, could possibly intercept Ca\(^{2+}\) diffusing to the VOCs resulting in greater inhibition of
high K\(^+\) contractions in nominally Ca\(^{2+}\)-free solution. However, no difference was observed between 1 mM EGTA and 1 mM BAPTA in HSV and swine renal artery (data not shown). It is possible that chelation speed is not, but diffusional access to Ca\(^{2+}\) binding sites or the affinity of the anionic sites for Ca\(^{2+}\), is the limiting factor.

**Figure 13 to Figure 24 to follow**
Figure 13. Effects of pre-incubation with nifedipine and nominally Ca\(^{2+}\)-free PSS on high K\(^+\) contractions.

A. Sample traces of high K\(^+\) contractions in normal PSS, in 0 Ca\(^{2+}\) 1 mM EGTA solution, and after a 15 min pre-incubation period in 5 μM nifedipine. B. Sample traces of high K\(^+\) contractions in normal PSS and in 0 Ca\(^{2+}\) 1 mM EGTA solution with or without a 15 min pre-incubation period in 5 μM nifedipine. C. Mean responses to high K\(^+\) contractions under the foregoing conditions. High K\(^+\) control contractions (n = 8 rings from 4 patients) were inhibited by nifedipine in the presence of 2.5 mM Ca\(^{2+}\) (n = 6 rings from 6 patients) or in 0 Ca\(^{2+}\) 1 mM EGTA solution (n = 7 rings from 7 patients; p < 0.0001). However, high K\(^+\) contractions were resistant to 0 Ca\(^{2+}\) 1 mM EGTA PSS (n = 24 rings from 13 patients). Responses were normalised by control high K\(^+\) contractions in each ring.
A

Control

0 Ca^{2+} 1 mM EGTA

Nifedipine 5 μM

High K^+

B

Control

0 Ca^{2+} 1 mM EGTA

0 Ca^{2+} 1 mM EGTA

Nifedipine 5 μM

High K^+

C

High K^+ force (% control)

2.5 mM Ca^{2+}

0 Ca^{2+} 1 mM EGTA

Control

Nifedipine
Figure 14. Sensitivity of high $K^+$ contractions to nifedipine added at the peak of the response.

Nifedipine (5 or 10 µM) abolished high $K^+$ contractions when added at the peak of the response (n = 13 rings from 8 patients). Contractions were normalised by the maximum amplitude for each high $K^+$ challenge.
Control

Nifedipine

High K⁺

5 min

15%

Nifedipine 10 µM

Control

Nifedipine
Figure 15. High K\(^+\) contractions after increasing incubation time in 0 Ca\(^{2+}\) 1 mM EGTA in arteries and veins from humans and animals.

A. Sample traces of high K\(^+\) contractions after 30 sec, 10, and 20 min in 0 Ca\(^{2+}\) 1 mM EGTA in the HSV. The black bars indicate addition of high K\(^+\) solution. B. Sample traces of high K\(^+\) contractions after 30 sec, 1, and 2 min in 0 Ca\(^{2+}\) 1 mM EGTA in the rabbit femoral artery. The black bars indicate addition of high K\(^+\) solution. C. Time courses curves for the decline of high K\(^+\) contractions with increasing incubation time in 0 Ca\(^{2+}\) 1 mM EGTA in the HSV (n = 6 to 24 rings from 14 patients in total – per data point), the swine renal artery (n = 10 to 26 rings from 22 pigs in total) and femoral vein (n = 9 to 19 rings from 19 pigs in total), and the rabbit femoral artery (n = 3 - 15 rings from 7 rabbits in total). The decline of high K\(^+\) responses was fastest in the femoral vein from swine and the femoral artery from the rabbit. The slowest time course was in the HSV. All high K\(^+\) contractions in 0 Ca\(^{2+}\) 1 mM EGTA were normalised by control high K\(^+\) responses.
A. Human saphenous vein

B. Rabbit femoral artery

C. Graph showing High K⁺ contraction (% control) over time in 0 Ca²⁺ 1 mM EGTA (min) for different tissues:
- Human SV
- Swine renal artery
- Rabbit femoral artery
- Swine femoral vein
Figure 16. Effects of phentolamine and nifedipine on high K\textsuperscript{+} contractions in 0 Ca\textsuperscript{2+} 1 mM EGTA in porcine renal arteries.

A. High K\textsuperscript{+} contractions in nominally Ca\textsuperscript{2+}-free PSS were attenuated by nifedipine (5 \mu M) pre-incubation.  B. Phentolamine (10 \mu M) pre-incubation also attenuated high K\textsuperscript{+} contractions in nominally Ca\textsuperscript{2+}-free PSS. Both phentolamine and nifedipine pre-incubation together completely abolished high K\textsuperscript{+} contractions in nominally Ca\textsuperscript{2+}-free PSS.  C. Mean responses to high K\textsuperscript{+} contractions in nominally Ca\textsuperscript{2+}-free PSS (n = 19 rings from 19 pigs) were significantly inhibited (p < 0.0001) after pre-incubation with phentolamine (n = 12 rings from 12 pigs), with nifedipine (n = 25 rings from 25 pigs), and with both inhibitors in combination (n = 19 rings from 19 pigs). All responses were normalised by control contractions in nominally Ca\textsuperscript{2+} free PSS. All groups were significantly different from each other (p < 0.0001).
Figure 17. Effects of phentolamine and nifedipine on high K\(^+\) contractions in 0 Ca\(^{2+}\) 1 mM EGTA in porcine femoral veins.

A. High K\(^+\) contractions in nominally Ca\(^{2+}\)-free PSS were attenuated by nifedipine (5 \(\mu\)M) pre-incubation. B. Phentolamine (10 \(\mu\)M) pre-incubation also attenuated high K\(^+\) contractions in nominally Ca\(^{2+}\)-free PSS. Both phentolamine and nifedipine pre-incubation together completely abolished high K\(^+\) contractions in nominally Ca\(^{2+}\)-free PSS. C. Mean responses to high K\(^+\) contractions in nominally Ca\(^{2+}\)-free PSS (\(n = 18\) rings from 18 pigs) were significantly inhibited (\(p < 0.001\)) after pre-incubation with phentolamine (\(p < 0.0001\); \(n = 9\) rings from 9 pigs), with nifedipine (\(n = 17\) rings from 17 pigs), and with both inhibitors in combination (\(n = 16\) rings from 16 pigs). All responses were normalised by control contractions in nominally Ca\(^{2+}\)-free PSS. High K\(^+\) contractions in nominally Ca\(^{2+}\)-free PSS in the presence of phentolamine were abolished in the presence of nifedipine (\(p < 0.01\)).
Figure 18. Low power views of Movat-stained cross-sections of arteries and veins from humans, swine and rabbits.

A. Human saphenous vein showing diffuse intimal thickening as well as thickening of the longitudinal and circular smooth muscle layers. B. Human thymus vein. C. Swine renal artery.
D. Swine femoral vein. E. Rabbit femoral artery. F. Rabbit inferior vena cava. Scale bar is 0.5 mm in each image. It should be noted that the rabbit inferior vena cava was turned inside-out to facilitate proper paraffin-embedding. Attempts to embed a non-inverted ring resulted in folded arrangements not suitable for cross-sectional area sectioning.
Figure 19. High power images of Movat-stained HSV and rabbit femoral artery.

A. Top right hand section of the HSV segment depicted in Figure 18A. Scale bar is 0.5 mm.  
B. Top part of rabbit femoral artery ring illustrated in Figure 18B. Scale bar is 0.25 mm.
Figure 20. Ca\(^{2+}\)-release from the SR during high K\(^{+}\) contractions in nominally Ca\(^{2+}\)-free medium in HSV.

A. Sample traces of high K\(^{+}\) contractions in normal PSS, after 15 min in nominally Ca\(^{2+}\)-free PSS, and after unloading of SR Ca\(^{2+}\). The SR Ca\(^{2+}\) store was unloaded with exposure to 10 mM caffeine, during the first 2 min of a 15 min incubation period in nominally Ca\(^{2+}\)-free medium and with the maintained addition of 10 \(\mu\)M CPA. B. Mean responses to high K\(^{+}\) contractions under the foregoing experimental conditions. C. High K\(^{+}\) contractions in nominally Ca\(^{2+}\)-free PSS after unloading the SR were significantly depressed as compared to those elicited with an intact SR (p < 0.023; n = 8 rings from 7 patients). The same finding was observed when the order of the two high K\(^{+}\) contractions in nominally Ca\(^{2+}\)-free medium with and without SR Ca\(^{2+}\) unloading was reversed (p < 0.0025; n = 6 rings from 6 patients).
A

Control
0 Ca²⁺ 1 mM EGTA (15 min)
0 Ca²⁺ 1 mM EGTA (15 min)
10 mM caffeine and 10 μM CPA
High K⁺

B

Control
0 Ca²⁺ 1 mM EGTA (15 min)
0 Ca²⁺ 1 mM EGTA (15 min)
5 μM nifedipine
High K⁺

C

0 Ca²⁺ 1 mM EGTA
0 Ca²⁺ 1 mM EGTA
10 mM caffeine
10 μM CPA

High K⁺ force (% control)

Order 1 Order 2
Figure 21. Effect of caffeine on the magnitude of high K⁺ contractions in the HSV.

Sample traces of high K⁺ contractions in paired rings from the same patient.  

A. Caffeine (10 mM) was added at the peak of the control high K⁺ contraction (control) for two minutes and then rinsed for 13 min, prior to a second challenge with high K⁺ (caffeine).  

B. The control ring was simply rinsed for 15 min prior to a second high K⁺ challenge. There was no difference in the magnitude of successive contractions between the two rings; hence, brief exposure to caffeine had no direct inhibitory effect on the magnitude of high K⁺ contractions.
Figure 22. Effect of diabetes and high fat diet on high K\(^+\) contractions in 0 Ca\(^{2+}\) 0.1 mM EGTA contractions in swine femoral vein.

Time courses curves for the decline of high K\(^+\) contractions with increasing incubation time in 0 Ca\(^{2+}\) 0.1 mM EGTA in swine femoral vein from 3 different experimental groups: control (n = 6 to 39 rings from 7 pigs in total), high fat diet (n = 1 to 8 rings from 7 pigs in total), diabetic on a high fat diet (n = 3 to 5 rings from 5 pigs in total). The decline of high K\(^+\) responses was fastest in the rings from the high fat fed pigs (p < 0.0001 at 30 sec; p < 0.0007 at 5 min) as compared to diabetic and control pigs. Rings from diabetic pigs on a high fat diet elicited a decline in the high K\(^+\) contraction at a rate similar to controls. These findings suggest that femoral vein from hyperlipidemic swine have less extracellular bound Ca\(^{2+}\) than control and diabetic hyperlipidemic swine.
Figure 23. Difference between 0.1 and 1 mM EGTA on high K⁺ contractions in 0 Ca²⁺ solution in swine femoral vein from diabetic high fat fed pigs.

Time courses curves for the decline of high K⁺ contractions with increasing incubation time in 0 Ca²⁺ solution containing 0.1 mM (n = 3 to 5 rings from 5 pigs in total) or 1 mM EGTA (n = 3 to 5 rings from 5 pigs in total) in diabetic swine femoral vein. The decline of high K⁺ responses was slower in 0.1 mM as compared to 1 mM EGTA at 30 sec (p < 0.0006) and 5 min (p < 0.0001). A similar difference was found in control swine (data not shown).
Figure 24. Ca\textsuperscript{2+} dose-response curves in high K\textsuperscript{+}-depolarised swine femoral vein.

Rings were pre-contracted with high K\textsuperscript{+} PSS and then relaxed with a 0 Ca\textsuperscript{2+} (EGTA-free) high K\textsuperscript{+} depolarising solution. Ca\textsuperscript{2+} was then added back in a cumulative fashion. Responses were normalised by the control high K\textsuperscript{+} contraction prior to Ca\textsuperscript{2+} removal. No difference was observed in the Ca\textsuperscript{2+} dose-response curve indicating that the Ca\textsuperscript{2+} handling by the myofilaments was the same in the three different experimental groups.
CHAPTER VII: \(\alpha_1\)-ADRENERGIC RECEPTOR-STIMULATED CONTRACTIONS IN THE HSV

Isometric tension experiments were performed to elucidate the sources of activator Ca\(^{2+}\) responsible for generating the transient rise in \([\text{Ca}^{2+}]_i\) observed with PE stimulation (see CHAPTER V: CHANGES IN INTRACELLULAR \([\text{Ca}^{2+}]\) AND FORCE MEDIATED BY HIGH K\(+\) AND PE IN THE HSV) as well as the intracellular signalling pathways mediating sustained force development at basal \([\text{Ca}^{2+}]_i\). The data suggests several mechanisms operating with \(\alpha_1\)-adrenergic receptor activation: an initial Ca\(^{2+}\)/MLCK-dependent increase in tension followed by mechanisms that suppress MLCP activity thereby allowing low levels of MLCK activation to maintain MLC\(_{20}\) phosphorylation and force when \([\text{Ca}^{2+}]_i\) is at baseline.

Role of Ca\(^{2+}\) influx and Ca\(^{2+}\) release from the SR in PE-induced contractions

Experiments were performed to determine the source(s) of activator Ca\(^{2+}\) mediating \(\alpha_1\)-adrenergic receptor-mediated contractions. The amplitude of PE (50 \(\mu\)M) responses were depressed by pre-incubation with the VOC blocker nifedipine (5 \(\mu\)M; Figure 25A) and the ROC blocker SK&F 96365 (50 \(\mu\)M; Figure 25B), as well as by a solution of nominally Ca\(^{2+}\)-free PSS (Figure 25C). For the latter responses, the solution was switched to 0 Ca\(^{2+}\) 1 mM EGTA solution for 30 sec prior to the addition of PE. All three methods of inhibition of Ca\(^{2+}\) influx resulted in a significant depression of PE contractions (p < 0.0001; Figure 25D); however, there was no significant difference in the magnitude of the inhibitory effects between nifedipine, SK&F 96365, and nominally Ca\(^{2+}\)-free PSS.

Nifedipine at a concentration that completely abolished contractions to high K\(^+\) did not completely inhibit contractions to PE. Hence, there is only a partial involvement of VOCs in PE-
stimulated contractions in the HSV. SK&F96365, a relatively non-selective inhibitor of ROCs with affinity for VOCs (189, 290), did not result in further inhibition of the PE response. This finding was unexpected. To verify the specificity of SK&F 96365, the effect of this compound was tested on high K$^+$ contractions in the HSV. Pre-incubation with SK&F 96365 (50 µM) significantly attenuated high K$^+$ contractions (Figure 26B; $p < 0.0001$). Hence, SK&F96365 may also inhibit VOCs in the HSV as exemplified in other cell types (290) and vascular preparations (189). In light of these findings, it was concluded that inhibition by SK&F 96365 of PE-induced contractions was limited to VOCs and did not involve ROCs.

These inhibitors of Ca$^{2+}$ entry did not completely inhibit PE contractions in the HSV unlike PE contractions in the inferior vena cava (247). Therefore, positive control experiments using identical solutions & drugs were effected in the rat thoracic aorta to verify whether our SK&F96365 sample had any inhibitory effects on ROCs. Pre-incubation with SK&F96365 (50 µM) attenuated PE contractions to a greater extent than nifedipine (5 µM) in the rat thoracic aorta, as shown in Figure 26A ($p < 0.0001$). Both inhibitors depressed PE contractions in the rat aorta more than in the HSV. Hence, PE contractions in the HSV were less dependent on Ca$^{2+}$ influx through VOCs and ROCs than animal preparations, such as the rabbit thoracic aorta.

In the presence of nifedipine or SK&F 96365, PE contractions were sustained during the plateau phase of the response, suggesting that Ca$^{2+}$ influx through VOCs did not occur during this phase of the contraction. Consistent with this finding, the addition of nifedipine and SK&F 96365 at the peak of the response did not inhibit the plateau phase of PE contractions (Figure 27A&B). Surprisingly, the plateau phase of the PE contraction rose above control levels soon after the addition of SK&F 96365 (Figure 27B), which may be due to other non-selective effects of this compound (191). Hence, influx of Ca$^{2+}$ through VOCs was only important during the initial phase of PE contractions in the HSV.
PE contractions were less resistant to nominally Ca\(^{2+}\)-free PSS (Figure 25C) as compared to high K\(^+\) (see Figure 13). This finding was unlike other preparations where high K\(^+\) contractions are generally more sensitive to Ca\(^{2+}\)-free solutions than NA (161). It is generally accepted that α\(_{1}\)-adrenoceptor activation of vascular smooth muscle in the absence of extracellular Ca\(^{2+}\) is mediated by Ca\(^{2+}\) release from the SR, which can be completely refilled by the entry of extracellular Ca\(^{2+}\) (55). In nominally Ca\(^{2+}\)-free PSS, the maximum amplitude of contractions was significantly depressed and the plateau phase was not sustained. Nevertheless, more than 70% of the PE-contraction was elicited in 0 Ca\(^{2+}\) 1 mM EGTA solution; therefore, Ca\(^{2+}\) release from the SR was an important component of the PE response. An additional Ca\(^{2+}\) influx pathway was most likely involved in sustaining PE contractions during the slow phase of the response, such as the putative ‘Ca\(^{2+}\) leak’ pathway. This pathway may be important for directly supplying Ca\(^{2+}\) to the myofilaments and/or for replenishing the SR Ca\(^{2+}\) store.

Experiments were performed to determine the sensitivity of PE contractions to prolonged incubation time in 0 Ca\(^{2+}\) 1 mM EGTA PSS, a non-pharmacological means of depleting Ca\(^{2+}\) storage sites (138). It is thought that EGTA removes the superficially bound Ca\(^{2+}\) and subsequently reduces the intracellular Ca\(^{2+}\) pool via extraction of intracellular Ca\(^{2+}\) at the cell membrane surface. Segments of HSV were pre-incubated in nominally Ca\(^{2+}\)-free PSS for various periods of time prior to challenges with PE. Similar experiments were performed in the rabbit femoral artery. As can be seen from Figure 28A, increasing the pre-incubation period in nominally Ca\(^{2+}\)-free PSS resulted in a progressive decrease in the magnitude of PE-induced contractions, presumably due to depletion of SR Ca\(^{2+}\). Prolonged incubation times in nominally Ca\(^{2+}\) free PSS also depressed PE contractions in the rabbit femoral artery (Figure 28B). Responses in nominally Ca\(^{2+}\)-free PSS were normalised by control PE contractions and reported in Figure 28C. The decay of PE contractions with prolonged incubation in nominally Ca\(^{2+}\)-free
PSS was mono-exponential with a half-time of approximately 11 min in the HSV and 12 min in
the rabbit femoral artery.

In the HSV, mean responses at the initial time point in nominally Ca\(^{2+}\)-free PSS were
different from those reported in Figure 25D. The reason for this discrepancy relates to the
difference in the protocols used in each set of experiments. In the experiments for Figure 25D,
tissues were equilibrated with high K\(^+\) prior to effecting control PE contractions. These latter
contractions were overestimated as compared to the subsequent PE contraction in nominally Ca\(^{2+}\)
free PSS because they were performed immediately after loading the SR with Ca\(^{2+}\). Prior
exposure to high K\(^+\) can increase the size of the agonist-releasable Ca\(^{2+}\) pool (264). In contrast,
the HSV tissues used for Figure 28 were equilibrated with PE; hence, control PE contractions
were performed with a normal SR Ca\(^{2+}\) content and PE control responses were not
overestimated. In fact, they may have been underestimated; the amplitude of subsequent PE
contractions in nominally Ca\(^{2+}\)-free PSS was sometimes greater than control PE contractions.
Had the control PE contractions been performed after equilibrating with high K\(^+\), then the 30 sec
time point would have been the same as in Figure 25, i.e., approximately 71%. This change
would result in a downward shift in the initial time points of the curve plotted in Figure 28C, and
superimposition with the femoral artery plot. In light of these protocol differences, both HSV
and rabbit femoral artery preparations exhibited similar rates of decay of the PE contraction with
increasing pre-incubation times in nominally Ca\(^{2+}\)-free solution and the sensitivity of
intracellular Ca\(^{2+}\) store to nominally Ca\(^{2+}\)-free PSS. Other preparations also exhibit decay of PE
responses with prolonged incubation in 0 Ca\(^{2+}\) and low EGTA concentrations (within 400 μM),
such as the rat aorta and dog mesenteric artery (138).

The contribution of SR Ca\(^{2+}\) release to PE contractions in the HSV was also studied by
examining the effect of pharmacological unloading of the SR on the magnitude of PE
contractions without Ca\(^{2+}\) removal or the addition of EGTA. The SR Ca\(^{2+}\) store was unloaded

100
with PE in the presence of CPA (50 μM), a SERCA inhibitor (80, 419). The use of CPA was to prevent the sequestration of released Ca\(^{2+}\) back into the SR, thereby attenuating Ca\(^{2+}\) release during the subsequent PE challenge. The tissues were rinsed for 30 min in the presence of CPA, and the PE (50 μM) challenge was repeated in nominally Ca\(^{2+}\)-free PSS to test SR Ca\(^{2+}\) release without the confounding effects of Ca\(^{2+}\) influx. According to this protocol (Figure 29B), PE responses in 0 Ca\(^{2+}\) 1 mM EGTA solution plus CPA were significantly depressed (Figure 30A) as compared to control rings subjected to a similar protocol without exposure to CPA (Figure 29A & Figure 30A). An additional set of rings was tested in the same manner as in Figure 29B, except that L-NAME (400 μM), an inhibitor of eNOS (345), was included to control for possible endothelium-dependent relaxation by CPA (465). In the presence of CPA and L-NAME, PE challenges in nominally Ca\(^{2+}\)-free PSS were also significantly depressed as compared to control PE contractions (see Figure 30C).

It is possible that the presence of CPA during the PE washout in protocols from Figure 29B and Figure 29C prevented [Ca\(^{2+}\)]\(_i\) and tension from returning to baseline; however, no difference was observed in ring tensions after PE washout between the three different protocols (p > 0.05, n = 6 rings sets of rings from 6 patients). It is also conceivable that prior exposure to CPA and/or L-NAME potentiated control PE contractions. The effects of prior exposure to CPA and L-NAME, either alone or combined, were tested on PE contractions in the HSV (Figure 30B). The presence of CPA, L-NAME, or both CPA and L-NAME together did not significantly (p > 0.05) increase PE responses as compared to control.

The role of the SR in PE contractions was also examined by unloading the SR with a brief pulse of caffeine (10 mM) with the maintained addition of CPA (10 μM). Unloading and subsequent PE contractions took place in nominally Ca\(^{2+}\)-free solution. Experiments were performed according to the same protocol summarised in Figure 20. After unloading the SR, PE
contractions in nominally Ca\(^{2+}\)-free solution (13.6 ± 4.2 %) were not significantly reduced as compared to contractions in nominally Ca\(^{2+}\)-free PSS without exposure to CPA and caffeine (26.9 ± 8.4%) (p > 0.05; n = 7 rings from 7 patients). Either the SR was not an important component of PE contractions after 15 min in 0 Ca\(^{2+}\) 1 mM EGTA solution or the amount of SR Ca\(^{2+}\) depletion did not differ with or without prior unloading with caffeine and CPA exposure.

The foregoing data demonstrated roles for Ca\(^{2+}\) influx through VOCs, but not ROCs, and Ca\(^{2+}\) release from the SR were important components of PE contractions. And although elevated Ca\(^{2+}\) levels were not detected during sustained PE contractions, PE responses were inhibited by prolonged exposures to 0 Ca\(^{2+}\) and 1 mM EGTA-containing PSS; hence, coupling of \(\alpha_1\)-adrenergic receptor activation to force development was not completely independent of cellular Ca\(^{2+}\). However, because PE contractions were sustained in the absence of elevated [Ca\(^{2+}\)], levels, other mechanisms may be involved in mediating the slow phase of the PE response. Excitation contraction-coupling in the HSV may require an initial Ca\(^{2+}\) dependent effect followed by other non-Ca\(^{2+}\)-dependent processes, such as Ca\(^{2+}\)-sensitisation mechanisms.

**Ca\(^{2+}\)-sensitisation of the contractile proteins by PE**

The potential involvement of Ca\(^{2+}\) sensitisation pathways was examined by using a variety of inhibitors of protein kinases previously shown to be implicated in Ca\(^{2+}\) sensitisation mechanisms: rho kinase (111), protein kinase C (436), MAPK (387), and tyrosine phosphorylation (171).

**Effect of addition of inhibitors at peak of contractions**

HA-1077 (50 µM), a rho kinase (310) and protein kinase N (14) inhibitor, completely abolished the sustained phase of PE-induced contractions (p < 0.000 1; Figure 31), previously
ascribed to maintained Ca\textsuperscript{2+} influx, while high K\textsuperscript{+} contractions were only marginally affected (p < 0.000 1; Figure 31). A component of the plateau phase of the PE contractions was also mediated by tyrosine phosphorylation because genistein (50 \mu M) addition at the peak force resulted in significant attenuation of the response (p < 0.002 at 18 min and p < 0.000 4 at 35 min; Figure 32). In contrast, genistein had no effect on high K\textsuperscript{+} contractions (p > 0.05 at both 18 and 35 min; Figure 33). Surprisingly, dose-response curves to calphostin C, a protein kinase C inhibitor, or PD098,059, an inhibitor of MAPK, had no effect on the sustained phase of PE contractions (Figure 34).

From these findings, it appears that a rho kinase-associated pathway may account for the sustained phase of PE-induced force, and that tyrosine phosphorylation may be associated with a component of this pathway.

**Pre-incubation of inhibitors**

HA-1077 (50 \mu M) and genistein (50 \mu M) were pre-incubated for 30 min prior to exposure to PE in order to determine whether either of these inhibitors affected the peak amplitude of \alpha\textsubscript{1}-adrenergic receptor-induced contractions. Pre-incubation with HA-1077 (50 \mu M) or genistein (50 \mu M) resulted in approximately 40\% (p < 0.001) and 33\% (p < 0.01) inhibition, respectively (Figure 35), of the peak amplitude of force generation by PE. When both inhibitors were pre-incubated together, the PE contraction was completely abolished (p < 0.000 1; Figure 35). The same experiments were also performed on high K\textsuperscript{+}. Again, genistein (50 \mu M) had no effect on high K\textsuperscript{+} contractions (Figure 36); however, pre-incubation with HA-1077 (50 \mu M), or HA-1077 & genistein together, significantly attenuated responses to high K\textsuperscript{+} (p < 0.0001; Figure 36). Pre-incubation with calphostin C (1 \mu M) or MAPK (50 \mu M) had no effect.
(Figure 37). Pre-incubation with the specific PKCβ isoform inhibitor, LY379196, also had no effect (data not shown).

Because pre-incubation with HA-1077 inhibited both high K⁺ and PE to the same extent, MLCK rather than rho kinase may be important during the initial phase of contraction. Ca²⁺ and tyrosine phosphorylation were also important.

Figure 25 to Figure 37 to follow
Figure 25. Inhibitory effects of pre-incubation with nifedipine, SK&F 96365, and nominally Ca$^{2+}$-free PSS on PE contractions in the HSV.

A. Sample traces of PE (50 μM) contractions in normal (control) PSS and after a 30 min pre-incubation period with nifedipine (5 μM). B. Sample traces of PE (50 μM) contractions in normal (control) PSS and after a 30 min pre-incubation period with SK&F 96365 (50 μM). C. Sample traces of PE (50 μM) contractions in normal PSS (control) and in nominally Ca$^{2+}$-free PSS. Unlike nifedipine and SK&F 96365, the sustained phase of the PE contraction was not maintained in nominally Ca$^{2+}$-free PSS. D. Mean responses to PE contractions under the foregoing conditions. On average, pre-incubation with nifedipine (n = 19 rings from 15 patients), SK&F 96365 (n = 20 rings from 16 patients), and nominally Ca$^{2+}$-free PSS (n = 27 rings from 15 patients) significantly inhibited the maximal amplitude of PE (n = 35 rings from 18 patients) responses (p < 0.000 1), indicating that Ca$^{2+}$ influx contributed to the magnitude of the maximum amplitude of PE contractions.
Figure 26. Inhibitory effects of nifedipine and SK&F 96365 on PE contractions in the rat thoracic aorta, and of SK&F 96365 on high K⁺ contractions in the HSV.

A. On average, pre-incubation with nifedipine (5 μM; n = 6 rings from 4 rats) and SK&F 96365 (50 μM; n = 5 rings from 4 rats) significantly inhibited the maximal amplitude of PE (50 μM) control responses (n = 6 rings from 4 rats; p < 0.0001). SK&F 96365 elicited a significantly greater inhibitory effect than nifedipine (p < 0.05), indicating that SK&F96365 inhibited both VOCs and ROCs in this preparation. B. Pre-incubation with SK&F 96365 (50 μM) significantly attenuated high K⁺ contractions in the HSV (n = 6 paired rings from 5 patients; p < 0.0001). Hence, SK&F 96365 inhibits VOCs in this preparation. Responses were expressed as a percentage of the control PE and high K⁺ responses in each ring.
Figure 27. Addition of nifedipine or SK&F 96365 at the peak of the PE response in HSV.

The addition of (A) nifedipine (10 μM; n = 8 rings from 5 patients) or (B) SK&F 96365 (50 μM; n = 7 rings from 5 patients) at the peak PE (50 μM) contractions had no inhibitory effect on the plateau phase of the response. Contractions increased with the addition of SK&F 96365. Hence, Ca$^{2+}$ influx through VOCs and ROCs was not important in excitation-contraction coupling during this phase of the response. Responses were expressed as a percentage of the maximal response to PE.
A

Nifedipine 10 μM

15%

PE 50 μM

5 min

Control

Nifedipine 10 μM

B

SK&F 96365 50 μM

15%

PE 50 μM

5 min

Control

SK&F 96365 50 μM
Figure 28. Effect of different incubation periods in nominally Ca$^{2+}$-free PSS on PE contractions in undistended HSV and rabbit femoral artery.

A. Representative traces of PE (50 µM) contractions in normal PSS, and after 30 sec, 5 and 15 min in nominally Ca$^{2+}$- free PSS in the HSV. 

B. Representative traces of PE (50 µM) contractions in normal PSS, and after 30 sec, 5 and 15 min in nominally Ca$^{2+}$- free PSS in the rabbit femoral artery. 

C. Mean responses to PE contractions in the HSV (n = 5-14 rings from 10 patients in total) and rabbit femoral artery (n = 8-13 rings from 6 rabbits in total) after various time intervals in nominally Ca$^{2+}$-free PSS normalised by control contractions in normal PSS. Although PE contractions in the HSV were more resistant to nominally Ca$^{2+}$-free PSS as compared to the rabbit FV for short incubation periods, this difference merely reflects a difference in the protocols used for each tissue and a difference in the magnitude of control contractions to PE (see text).
Figure 29. Ca\textsuperscript{2+} release from the SR during PE contractions in nominally Ca\textsuperscript{2+}-free PSS in the HSV.

Parallel, time-controlled protocols were conducted in sets of 3 rings from each patient. A. A 10 min control contraction was elicited in response to PE (50 µM), followed by three 10 min washes. The solution was then switched to nominally Ca\textsuperscript{2+}-free PSS for 1 min prior to a second PE challenge. B. The same protocol as in (A) was effected, but in addition, CPA (50 µM) was added 5 min prior to the control PE contraction. C. The same protocol as in (B), but to control for the possibility of CPA-mediated endothelium-dependent relaxation (see text), L-NAME (400 µM) was pre-incubated for 30 min and included throughout. The PE contractions in nominally Ca\textsuperscript{2+}-free PSS were more depressed when CPA (B) or CPA & L-NAME (C) were added during unloading of the SR Ca\textsuperscript{2+} store as compared to control (A). Traces were normalised by the maximum amplitude of the initial PE contraction in each ring.
Figure 30. Effect of SR Ca$^{2+}$ depletion with PE and CPA on the magnitude of PE contractions in nominally Ca$^{2+}$-free PSS.

A. Pharmacological unloading of SR Ca$^{2+}$ with PE in the presence of CPA (50 µM), or both CPA & L-NAME (400 µM), significantly (p < 0.0001) depressed subsequent PE contractions in nominally Ca$^{2+}$ free PSS. B. There was no effect of CPA (50 µM; n = 6 rings from 6 patients) or L-NAME (400 µM; n = 6 rings from 6 patients), alone or in combination (n = 6 rings from 6 patients), on the magnitude of PE contractions in the HSV (p > 0.05).
Figure 31. Sensitivity of PE and high K$^+$ contractions to HA-1077, a rho kinase inhibitor.

A. HA-1077 (50 μM) completely abolished the sustained phase of PE (50 μM)-induced contractions. B. High K$^+$ contractions were marginally inhibited by HA-1077. C. Average responses were calculated approximately 18 min after the point of HA-1077 addition, which occurred during the plateau phase approximately 10 min after stimulation with high K$^+$ or PE. HA-1077 abolished the plateau phase of PE contractions (p < 0.000 1, n = 12 rings from 8 patients), and marginally inhibited the contractions to high K$^+$ (p < 0.000 1; n= 15 rings from 11 patients). Responses were plotted and expressed as a percentage of the maximum amplitude of the PE or high K$^+$ contraction.
Figure 32. Sensitivity of PE contractions to genistein, a tyrosine kinase inhibitor.

Responses were plotted and expressed as a percentage of the maximum amplitude of the PE or high K⁺ contraction. A. Genistein (50 μM) addition at the peak of PE (50 μM)-induced contractions resulted in attenuation of sustained force. B. Average responses were calculated approximately 18 and 35 min after the point of genistein addition, which occurred during the plateau phase approximately 10 min after stimulation with PE. Force was significantly attenuated at both 18 min (p < 0.002; n = 9 rings from 6 patients) and 35 min (p < 0.0004; n = 9 rings from 6 patients) after genistein addition.
Figure 33. Sensitivity of high K⁺ contractions to genistein, a tyrosine kinase inhibitor.

Responses were plotted and expressed as a percentage of the maximum amplitude of the PE or high K⁺ contraction. A. Genistein addition at the peak of high K⁺-induced contractions had no effect. B. Average responses calculated approximately 18 and 35 min after the point of genistein addition, which occurred during the plateau phase approximately 10 min after stimulation with high K⁺. Force was not significantly attenuated at both 18 min (p > 0.05; n = 8 paired rings from 8 patients) and 35 min (p > 0.05; n = 8 paired rings from 8 patients) after genistein addition.
Genistein
20°/c
Control
Genistein
High K
18 min
35 min

A

B

High K$^+$ force as % max

Control
Genistein

18 min
35 min
Figure 34. No involvement of protein kinase C or MAPK during the slow phase of PE-induced contractions in HSV rings.

A. Dose-response curves to calphostin C, a protein kinase C inhibitor, and vehicle were effected at the peak of a PE (50 µM) pre-contraction (n = 4 rings from 2 patients). B. Dose-response curves to PD098,059, a MAPK inhibitor, and vehicle were also effected at the peak of a PE (50 µM) pre-contraction. A significant difference was observed for the 100 µM dose (p < 0.02; n = 5 rings from 2 patients). Data were expressed as a percentage of the amplitude of the PE pre-contraction level.
Figure 35. Effect of HA-1077 and genistein pre-incubation on PE contractions in HSV rings.

A. Pre-incubation with HA-1077 (50 μM) attenuated the magnitude of PE (50 μM)-induced contractions. B. Pre-incubation with genistein (50 μM) also inhibited the magnitude of PE (50 μM)-induced contractions. C. Pre-incubation with HA-1077 (50 μM) and genistein (50 μM) together completely abolished the response to PE. D. Average responses were calculated and expressed as a percentage of control PE contractions. On average, pre-incubation with HA-1077 or genistein resulted in approximately 40% (p < 0.001; n = 10 rings from 10 patients) and 33% (p < 0.01; n = 10 rings from 10 patients) inhibition, respectively, of the peak amplitude of force generation by PE as compared to control (n = 23 rings from 20 patients). When both inhibitors were pre-incubated together, the PE contraction was completely abolished (p < 0.0001; n = 8 rings from 7 patients).
Figure 36. Effect of HA-1077 and genistein pre-incubation on high K⁺ contractions in HSV rings.

A. Pre-incubation with genistein (50 μM) had no effect on the magnitude of high K⁺-induced contractions. B. Pre-incubation with HA-1077 (50 μM) attenuated the magnitude of high K⁺-induced contractions. C. Pre-incubation with HA-1077 (50 μM) and genistein (50 μM) together also inhibited the magnitude of high K⁺ contractions. D. Average responses were calculated and expressed as a percentage of control high K⁺ contractions. Pre-incubation with genistein had no effect on the magnitude of high K⁺ contractions (n = 11 rings from 7 patients); however, pre-incubation with HA-1077 resulted in approximately 36% (p < 0.0001; n = 8 rings from 7 patients). When both inhibitors were pre-incubated together, the high K⁺ contraction was inhibited by about 50% (p < 0.0001; n = 10 rings from 9 patients). There was no significant difference between the effects of HA-1077 alone or in combination with genistein.
Figure 37. Effect of calphostin C and PD098,059 pre-incubation on PE contractions in HSV rings.

A. Pre-incubation with calphostin C (1 µM) had no effect on the magnitude of PE (50 µM)-induced contractions. B. PD098,059 (50 µM) pre-incubation also had no effect. C. PE-induced contractions were not significantly affected by calphostin C (n = 5 rings from 5 patients) or PD098,059 (n = 7 rings from 6 patients). PE control data were reproduced from Figure 35 as all data were part of same data set and were analysed together.
CHAPTER VIII: DISCUSSION

Basic physiological and pharmacological characterisation of HSV reactivity

Determination of optimal experimental conditions

The HSV was unlike other vessels such as human umbilical vessels (166) and human coronary arteries (352) insofar as spontaneous contractile activity was not observed. In one study, phasic contractile activity was described in HSV segments; these contractions were dependent on the presence of Ca$^{2+}$ in the external medium (51). However, the segments used in that study were ‘leftover’ segments from the surgical preparation, indicating that the force oscillations may have resulted from trauma to the vein segment.

Beyond a pre-load of 2.5 g, active force generation did not exhibit the “Starling curve” observed by heart (309) or the length-tension curve demonstrated by skeletal muscle (132). Given that veins are very compliant tissues, it is possible that much greater lengths would be required to reach the stiffest portion of the passive length-tension curve (not generated in this study). Also, smooth muscle can function over a long range of lengths conferring a broad plateau on the length-tension curve (338). Alternatively, successive high K$^+$ contractions increase with time because of Ca$^{2+}$ buffering by the SR (422). In other words, some of the Ca$^{2+}$ entering the cells during initial high K$^+$ depolarisations may be removed from the cytoplasm by the SR before reaching the myofilaments, but with each successive challenge, this effect is reduced thereby delivering more Ca$^{2+}$ to the myofilaments and generating greater force. Hence, decreases in tension with increasing length could be counteracted by the increase in tension from successive high K$^+$ challenges.

It should be noted that a passive tension of 2 g in rings measuring about 4 mm corresponds to an intravascular pressure of approximately 10 mmHg given the approximate
dimensions of HSV segments according to the Law of Laplace (295). This pressure corresponds to in vivo conditions experienced by the HSV. The unit pre-load used in our experiments, 0.5 to 0.6 g/mm, was in agreement with other baseline tensions reported in the literature: 0.4 - 0.5 g/mm (98), 0.5 g/mm (28), but higher than some studies, such as 0.1 to 0.2 g/mm (52), or 0.2 g/mm (208). The variability could be explained by differences in experimental conditions or the compliance of components in the test system, such as the surgical silk often used to connect the vein segment to the force transducer, as per our system. In other studies, baseline tensions were reported but the unit length was not indicated (1 – 1.5 g (341), 2 g (271), 6 g (13)), or optimal length tension relationships were performed but the final parameters were not specified (381, 457, 459, 460).

The same optimal pre-load was found for HSV segments independent of their contractile ability. Although smooth muscle can adapt after a perturbation (439, 440), changing the pre-load or stimulation rate with high K$^+$ did not allow contractility to recover in segments which did not respond to an initial high K$^+$ challenge at the optimal resting tension. It thus appears that the main effect of intra-operative manipulations was to decrease the number of contractile elements, or disrupt excitation-contraction coupling elements, thereby weakening the contractile ability of the muscle, rather than to change the properties of the active length-tension relationship or myofilament structural organisation.

The elastic components of the vessel wall, the radius, and the thickness of the tissue are known to influence both active and passive length-tension properties of vascular smooth muscle preparations (86). As illustrated in the various photomicrographs, HSV segments varied greatly in these attributes. Because it is not possible to measure length-tension relationships for each ring for all experiments, an important caveat is that these experiments may not have always been performed at the optimal resting tension in each ring. In addition, the optimal resting tension in any given ring may change throughout the experiment due to changes in the elastic components.
or plasticity of the smooth muscle. In order to control for these discrepancies, all comparisons were made using normalised data including time controls for each drug effect.

**Standardisation of HSV tissue supply**

A great number of HSV preparations that did not respond to high $K^+$ in spite of attempts to recover contractility in these tissues. As indicated above, it was thought that intra-operative trauma impaired contractile function in these tissues. Some studies have avoided the damage from the surgical process by using SV branches (33, 74, 381), or by specifying the use of ‘undilated’ segments (59). The deleterious effects of intra-operative handling were confirmed by comparing smooth muscle function and endothelium-dependent relaxation in three classes of HSV segments. There has been no attempt until now to systematically demonstrate the superiority of undistended HSV for functional experiments.

In this study, freshly isolated, ‘undistended’ segments of HSV contracted much more forcefully to high $K^+$ and PE as compared to surgically prepared segments, such as those obtained after uncontrolled distension (‘distended’), or segments in excess after distal anastomosis and infusion of blood-cardioplegia solution (‘cardioplegic’). Dose-response curves to PE in undistended segments were also more forceful than that in distended and cardioplegic segments. Distension resulted in a depression without any apparent shift in EC50 suggesting that surgical preparation impaired contractility, rather than a change in the affinity of PE for the $\alpha_1$-adrenergic receptor or in the receptor-effector coupling mechanisms stimulated by PE. Other studies have also shown significantly attenuated high $K^+$ responses in distended (65, 324) or surgically prepared HSV segments (29), as well as significant reductions of PE contractions after distension (65, 245). Pressurisation may also lead to other types of medial damage. Some investigators have reported biochemical changes, such as a reduction in the [ATP], and the
In a study using canine jugular veins, distension to pressures of 300 – 1000 mmHg resulted in a significant increase in stiffness of the vein segments, indicating a loss in the elastic components of the tissue (3).

There is much variability in the literature with respect to PE dose-response curves in the HSV. The maximal response in our study was 3.1 ± 0.5 g, as compared to 1-1.5 g (28), 3 ± 0.3 g (405), 4.4 ± 0.7 g (32), 5.4 ± 0.3 g (95) and approximately 8 g (152). Maximal responses normalised by high K+ also varied. In one study, PE contractions were nearly 100% of 80 mM K+ responses (405), while maximal responses in this study were only 64%. The EC50 is this study (11.8 ± 0.2 μM) was in agreement with the EC50 value (10.9 ± 2.0 μM) in one study by Beattie (32). However, it was shifted to the right relative to other studies where the EC50 varied from 1.2 ± 0.7 μM (380), 1.6 ± 0.9 μM (28), 1.8 ± 0.2 μM (444), 2.8 ± 0.6 to 3.6 ± 0.3 μM (406), 2.9 μM (95), 5.7 ±0.3 μM (393), 6.3 ± 0.8 μM (405). Much of the variability in the foregoing studies may be explained by differences in the experimental conditions, such as components in the bathing solution which may have contained indomethacin (28), imipramine (95), propranolol (95, 380, 393), or cocaine (380, 393), different exclusion criteria (393, 444), endothelium removal (406, 444), or use of circular strips (393, 444) instead of rings. For example, it is known that cocaine and pindolol may affect potency of PE dose-response curves and the effects of α-adrenoceptor antagonists on these responses in the rabbit SV (369). In addition, responses to α-adrenergic receptor activation in HSV may be altered with disease. Specifically, contractile responses to NA may be depressed with diabetes (51, 409) or potentiated with hypertension (295). Hence, relative proportion of diabetics and hypertensives in any given study may affect the amplitude of reported PE responses.

ACh dose-dependently relaxed PE pre-constricted undistended HSV segments, whether ACh was added in two high doses or in a full dose response curve, and these responses, in
agreement with other studies, were attenuated by distension (65, 245) or surgical preparation (81, 367). ACh has been shown to lead to the release of nitric oxide (9, 269, 460), and of EDHF (456) in the HSV. The release of both substances was impaired with surgical preparation of the HSV (20, 456). Consistent with impaired release of EDRFs by ACh, surgical preparation has been shown to decrease eNOS staining (65) and ACh-stimulated cyclic GMP production (399). Pressurisation with 300 mmHg with the patient's heparinised blood decreased prostacyclin production as compared to freshly isolated vein (18).

ACh responses in the HSV were depressed as compared to those in human IMA, obtained from patients with CAD, where ACh relaxed NA-precontracted rings by nearly 95% (460). In veins, ACh released less NO than in arteries, and the effects of NO were inhibited by the concomitant release of autacoids, thought to be thromboxane A2, derived from the cyclooxygenase pathway (460). Unlike dog femoral veins (296), NO was the only relaxing factor released by ACh in the HSV (460), although EDHF responses have been reported elsewhere (456). The weaker endothelium-dependent relaxation in the HSV cannot be attributed to an impaired ability of the smooth muscle to relax to NO because the effects of exogenous NO, such as nitrovasodilators, were slightly enhanced in the HSV relative to the human IMA (269, 458).

It is not known why ACh relaxation was greater when given in two large bolus doses, rather than in cumulative doses. It was also surprising that ACh-mediated relaxations were not observed when undistended HSV rings were pre-incubated with U46619. In the HSV, responses to nitrovasodilators were significantly reduced when added at the peak of ET-1 as compared to NA pre-contractions (420). It is possible that U46619-induced contractions in the HSV were elicited by mechanisms which are not sensitive to ACh-induced release of NO. For instance, U46619 elicited contractions in porcine coronary arteries (43) and in canine pulmonary artery (192) without increases in [Ca^{2+}]. NO stimulates the cGMP –PKG pathway which
phosphorylates target proteins mediating a decrease in $[\text{Ca}^{2+}]_i$ and smooth muscle relaxation (54). In the absence of an increase in $[\text{Ca}^{2+}]_i$, NO may not elicit relaxation. On the other hand, the cGMP-PKG pathway may reduce the sensitivity of the contractile apparatus to $\text{Ca}^{2+}$ (54), for example, via the phosphorylation of MLCK (37).

In light of the foregoing findings, undistended samples of HSV segments were best preserved in terms of contractile function and endothelium-dependent relaxation, and exhibited the most consistent and reproducible responses as compared to distended and cardioplegic segments. Hence, physiological characterisation of the HSV was performed in undistended HSV segments.

Histology of HSV samples

Obvious morphological differences between undistended, distended, and cardioplegic segments as detected by light microscopy were not observed in this study. However, there are reports on the effects of surgical preparation on the integrity of HSV segments in the literature. Morphological intimal and medial changes have been demonstrated: thinning of the vessel wall, smooth muscle and elastic tissue, loss of vessel wall invaginations (210), stretching of medial elements (65), flattening of the intima (65), endothelial cell loss (65, 90, 142, 210, 324, 367), oedema (142), and increased platelet and red blood cell adherence in denuded areas (90).

In this study, the evaluation of undistended, distended, and cardioplegic segments was best-performed using functional methods over morphological studies. In addition, morphological differences between segments do not necessarily implicate normal function or dysfunction in HSV segments (324, 326).
Undistended HSV segments from RCH and use of overnight tissues

Undistended HSV samples were obtained from RCH to supplement the supply of tissues. Most of these segments were harvested via a minimally invasive harvesting technique thought to impair vein graft function due to excess traction and increased manipulation of the vein, which may compromise vein quality. Although thought to be more traumatic, these techniques reduce complications from the open technique and reduce postoperative morbidity (175). High K$^+$ and PE (this study) or NA (other studies) responses were not affected by the minimally invasive procedure (101, 326, 349). However, ACh responses were significantly attenuated relative to the open technique, unlike reports in other studies (101, 326, 349); some methodological differences are noteworthy. In one study, ACh responses were assessed at the peak of high K$^+$ depolarisation responses (101), while in another, responses were assessed after preservation for 2 days at 4°C in University of Wisconsin solution (349). It is unclear whether minimally invasive harvest results in endothelial damage. Different methods resulted in either intact endothelial cell lining (100) or partial denudation (410).

Contractile function was depressed in tissues obtained in the afternoon after overnight storage at 4°C; however, a specific loss of receptor-mediated contractions was not observed. Normalised data from overnight and same day tissues were therefore pooled because the loss of function was a non-specific decrease in contractility. Similar findings were observed with cold storage in cell culture medium with 80 mM KCl stimulation in a study by Milesi and colleagues (295). Some reports have published data using solely tissues incubated overnight (95) and claim that responses to vasoconstrictor and vasodilator agents were not affected based on studies using canine SV segments (94).
Changes in intracellular $[\text{Ca}^{2+}]$ and force mediated by high $K^+$ and PE in the HSV

Much attention has focussed on the elucidation of intracellular $\text{Ca}^{2+}$ signalling (384) in a wide variety of vascular and non-vascular smooth muscle preparations. Very little is known about such mechanisms in human blood vessels (111, 278). Imaging of $[\text{Ca}^{2+}]_i$ in HSV has been reported for growth factors and ATP in enzymatically digested and/or cultured cells (67, 68, 145, 260, 312). This study is the first to examine patterns of changes in $[\text{Ca}^{2+}]_i$ elicited by contractile stimuli in intact HSV samples.

Pairs of high $K^+$ and PE fluorescence responses were generated in the same ring preparations to facilitate a qualitative comparison of the $\text{Ca}^{2+}$ signals. Both high $K^+$ and PE stimulated comparable tonic contractions; however, the fluorescence signal was sustained in response to high $K^+$, and transient in response to PE. The difference in intracellular fluorescence patterns was not due to the inability of the dye to detect changes in $[\text{Ca}^{2+}]_i$ or to the experimental conditions. Fluo-4 was used because it is a new fluorescent dye that is ideally suited to measure agonist-induced physiological changes in $[\text{Ca}^{2+}]_i$ (100 nM to 1 $\mu$M range). It has certain advantages over its widely used congener fluo-3 insofar as it has higher fluorescence emission intensity, making it useful at lower intracellular concentrations (119), and it is more photo-stable (403). With respect to the experimental conditions, the intensity of the laser and the magnification of the objective lens (20X) were kept low to minimise bleaching. In addition, empirical evidence demonstrated that fluo-4 was capable of measuring tonic increases in $[\text{Ca}^{2+}]_i$, as observed with high $K^+$, and that it did not interfere with excitation-contraction coupling mechanisms, as PE contractions were identical in the presence or absence of fluo-4 (data not shown). Hence, the transient PE response was not due to bleaching or loss of fluorescence intensity, and the high $K^+$ and PE fluorescence signals reflected true differences in the $\text{Ca}^{2+}$ signalling patterns generated by these two stimuli.
High K⁺ depolarisation elicited parallel increases in both force and \([\text{Ca}^{2+}]_i\), which remained elevated as long the depolarising stimulus was present; therefore, high K⁺ contractions were \(\text{Ca}^{2+}\)-dependent. These findings were consistent with force-\([\text{Ca}^{2+}]_i\) relationships measured with aequorin in the rat portal vein (302) and epicardial coronary arteries (43), as well as with fura-2 in the rat aorta (363).

The rise in force elicited by PE initially followed the rise in \([\text{Ca}^{2+}]_i\); however, the \([\text{Ca}^{2+}]_i\) signal was transient declining to basal levels. This pattern is different from those observed with \(\alpha\)-adrenergic agonists in animal vascular preparations. One pattern is a sustained response with a slowly declining plateau phase, such as NA in the fura-2 loaded rat aorta (363) and PE in the pulmonary artery (159). The other is a biphasic signal where the second phase is a sustained elevation in \([\text{Ca}^{2+}]_i\), just above basal levels, such as responses after NA-stimulation in portal vein smooth muscle cells with either indo-1 (136, 331) or fura-2 (249). Similar \([\text{Ca}^{2+}]_i\) signals were recorded in response to PE with fura-2 (250, 299) and aequorin (302).

In the HSV, nearly all cells exhibited \([\text{Ca}^{2+}]_i\) transients simultaneously, in a synchronised fashion, unlike the asynchronous single-cell \([\text{Ca}^{2+}]_i\) oscillations observed in response to PE in the rabbit inferior vena cava (354) and in pressurised rat mesenteric resistance arteries (298), as well as in the response to NA in the rat tail artery (178). The global \([\text{Ca}^{2+}]_i\) response was sustained in each of the foregoing examples. It should be noted, however, that the first oscillation elicited by PE in the rabbit vena cava is synchronised because the individual cells are in contact with PE at the same time (354). It appears that for a given agonist or receptor subtype, there is biological variability in the nature of the \([\text{Ca}^{2+}]_i\) response observed in tissues from healthy, young animals as compared to tissues from the elderly population of CABG patients.
High K+-depolarisation-induced contractions in the HSV

High K+-depolarisation-induced Ca2+-entry through VOCs

High K+ contractions were nearly completely abolished by the dihydropyridine VOC channel inhibitor nifedipine added prior to depolarisation; also, the addition of nifedipine at the peak of the high K+ contraction elicited complete relaxation of the HSV. Hence Ca2+ entered the cell through voltage-gated Ca2+ channels. This finding confirms other studies demonstrating the sensitivity of high K+ contractions to nifedipine (293, 433) as well as to other Ca2+ entry blockers, such as verapamil (292, 432, 433), diltiazem (433), and nisoldipine (328) in the HSV and are consistent with the idea that high K+ activates smooth muscle through electromechanical coupling (384). Phentolamine attenuated high K+ contractions by 30% but the effect was not significant, indicating that intramural release of NA was not an important component of high K+ contractions in the HSV. Mikklesen and colleagues showed that phentolamine inhibited high K+ contractions by 22% (292); it is unclear whether this reduction was significant.

Tightly bound extracellular Ca2+ in the HSV

Contractions to high K+ were not inhibited with acute removal of extracellular Ca2+. This unforeseen and surprising observation was unlike that of a variety of smooth muscle preparations, such as the rabbit SV (75), aorta (455), and uterus (42), as well as bovine tracheal smooth muscle (216), and frog stomach (42), except for the turtle aorta (42). Also, this finding was not specific to human vascular preparations as high K+ contractions in nominally Ca2+-free PSS were only marginal in the human thymus vein (this study) and not observed in the human renal artery (262). This response was also nearly completely inhibited by nifedipine indicating that Ca2+ influx through VOCs was required to elicit the contraction. Intramural release of NA was ruled out because of the non-significant inhibition of phentolamine on high K+ in normal
PSS. Hence, an extracellular source of tightly bound Ca\(^{2+}\) not sensitive to acute changes in the extracellular Ca\(^{2+}\) concentration or to the presence of EGTA may be present in the HSV.

The possible role of Ca\(^{2+}\) bound to the external surface of the membrane as a source of activating Ca\(^{2+}\) or as a factor regulating the permeability of the membrane to Ca\(^{2+}\) has been the subject of much speculation (263). An extracellular bound pool of Ca\(^{2+}\) contributes to high K\(^{+}\) induced contractions and the phasic component of muscarinic activation in the guinea pig ileum. It is thought that this pool may lie on the outer surface of the plasma membrane (60, 61, 172, 412). NA also stimulates the release of membrane bound Ca\(^{2+}\) in the rat aorta (455). Direct evidence that agonist stimulation led to Ca\(^{2+}\) influx stimulated by prostaglandin endoperoxide was demonstrated in the rabbit aorta (265). And an extracellular bound source of Ca\(^{2+}\) supplied the influx of Ca\(^{2+}\) required for an ‘initial burst’ in [Ca\(^{2+}\)] elicited by both NA and high K\(^{+}\) in the rabbit aorta (257). The function of agonist-induced Ca\(^{2+}\) release from binding sites on the outer membrane surface may be to increase the inward Ca\(^{2+}\) gradient (232) or as a mechanism involved in receptor binding and activation of the contractile response (423).

Fixed anionic sites on the smooth muscle membrane have been proposed as Ca\(^{2+}\) binding sites (129, 426). Electron microscopy and \(^{45}\)Ca autoradiography provided evidence for anionic sites at the plasma membrane in the guinea pig taenia coli (130) and guinea pig vas deferens (286), respectively, although the exact nature and location of these Ca\(^{2+}\) binding sites have yet to be determined. The glycocalyx has been suggested as a likely repository of extracellular Ca\(^{2+}\) binding sites in canine coronary artery smooth muscle (445), quail oviductal smooth muscle (22), and in cardiac muscle (231). A role for the negatively charged carboxyl groups in sialic acid molecules in the glycocalyx were involved in Ca\(^{2+}\) exchange in cardiac muscle cells (239). And a role for sialic acid residues in smooth muscle excitation-contraction coupling cannot be excluded in smooth muscle (346). However, it has been proposed that membrane phospholipids are more likely candidates for plasma membrane Ca\(^{2+}\) binding sites in smooth muscle (257),
where NA increased membrane fluidity by releasing a small pool of Ca\(^{2+}\) bound to phospholipids relieving the Ca\(^{2+}\) membrane stabilising effect (46). Phospholipids are also thought to be the main site for the fast exchangeable Ca\(^{2+}\) fraction in cardiac muscle (233, 234, 237, 238, 242).

More recently, an extracellular source of bound Ca\(^{2+}\) was proposed in canine bronchial smooth muscle because of the resistance of agonist-induced contractions in Ca\(^{2+}\)-free medium containing 50 \(\mu\)M EGTA. High EGTA concentrations and nifedipine abolished these contractions (300). It was originally thought that the source of Ca\(^{2+}\) was the cartilage (144, 342, 343), but sustained contractions were only maintained with a functional SR (300). It was hypothesised that caveolae was the source of extracellular Ca\(^{2+}\) (76, 300); the SR lies in close relationship to caveolae (113), which contain Ca\(^{2+}\) handling mechanisms (76).

**Prolonged exposure to nominally Ca\(^{2+}\)-free PSS**

Extended incubation periods in nominally Ca\(^{2+}\)-free solution depleted the putative extracellular store of bound Ca\(^{2+}\) and inhibited high K\(^+\) contractions in undistended HSV segments: after 30 min in nominally Ca\(^{2+}\)-free, 1 mM EGTA-containing PSS, the high K\(^+\) contraction was 13% of control. Other studies have also shown the resistance of high K\(^+\) responses in Ca\(^{2+}\)-free PSS solutions in the HSV. After 30 min in Ca\(^{2+}\)-free 10 \(\mu\)M EGTA solution, 127 mM K\(^+\) contractions were 21% of control (292, 293). Verapamil (292) and nifedipine (293) further reduced these responses, but phentolamine did not (292). Although responses to 64 mM K\(^+\) were 54% of control after 30 min in Ca\(^{2+}\)-free medium containing 1 mM EDTA (2), a Ca\(^{2+}\) and Mg\(^{2+}\) chelator, these contractions were most likely due to increased permeability of the membrane to Ca\(^{2+}\) caused by Mg\(^{2+}\) chelation by EDTA.

In contrast, brief incubation periods were required to abolish high K\(^+\) contractions in the vascular preparations from swine and rabbit. The rates of decay for the animal preparations tested in Figure 15 were also underestimated because of the role of intramural release of NA in
these preparations (Figure 16 & Figure 17). Hence, only the HSV appeared to contain this putative extracellular bound pool of Ca\(^{2+}\). It should be noted that this extracellular source of Ca\(^{2+}\) exchanged slowly with the extracellular solution, unlike the membrane bound Ca\(^{2+}\) thought to be important in the high K\(^+\)- and agonist-stimulated smooth and cardiac muscle contraction, discussed above. It is thought that the extracellularly bound Ca\(^{2+}\) in the HSV may be related to histological vessel wall properties characteristic of the HSV and that these characteristics were not observed in the animal preparations examined in the present study.

**Properties of the vessel wall**

The histological appearance of HSV segments described in Figure 19 was consistent with reports in the literature (63, 77, 251, 394). Intimal thickening (62, 63, 337, 402, 435) may be focal (also known as cushions (337)) or diffuse thereby affecting the entire circumference of the vein wall (62, 297, 337). Some veins may exhibit a ten-fold increase in intimal thickness (336). As many as 80\% (297) to 90\% (435) of clinically normal HSV have intimal fibrosis (297), which may be the result of increased wall tension (63, 211, 276, 340) or senescence (77, 275, 402), although the degree of lumenal narrowing does not decrease with increasing age (435).

The thickened intima consists of endothelium, abundant extracellular matrix, connective tissue, and longitudinal smooth muscle (62, 63, 77, 275, 297, 336, 337, 402, 435). Desmin-positive (297) smooth muscle cells are the main cellular component of thickened intima, and may be isolated or in fascicles with different orientations, longitudinal and circular (275, 337, 402). Fascicles of longitudinal smooth muscle were surrounded by abundant connective tissue (336). Most of the increased thickness in the HSV can be attributed to excess connective tissue matrix (275, 402, 435) and may be in the form of collagen bundles (337) surrounding smooth muscle cells (297, 402). Elastic fibres tend to lay parallel to the internal elastic lamina (297).

The amounts of collagen and elastin thickness may vary (62). Variable amounts of GAGs may
also be found in the extracellular matrix of the thickened intima (62). The basal lamina of endothelial and smooth muscle cells from tissues with focal to diffuse intimal thickening contain type IV collagen and laminin. A thickened basal lamina of the endothelium thought to be the hallmark of smokers exhibit specific accumulations of fibronectin (156). The basal lamina of the endothelium and smooth muscle also contained heparan sulfate proteoglycans (156).

The medial smooth muscle layer in almost all HSV segments is well developed (63). Smooth muscle cells, constituting more than 40% of the media (123), may be hypertrophied but regularly arranged and interspersed with collagen and elastin (62, 63, 275, 297, 394) and GAGs (62). The adventitial layer also exhibits bundles of smooth muscle cells lying longitudinally associated with elastic (62, 275, 336) and collagen fibres (63). Vasa vasora may also be present (275).

It is thought that vessel wall characteristics of the HSV may contribute in various ways to the resistance of high K\textsuperscript{+} contractions to 0 Ca\textsuperscript{2+} 1 mM EGTA solutions. The increased thickness of the intimal and medial layers may lengthen the diffusion distance for Ca\textsuperscript{2+} to exit and EGTA to penetrate the tissue (as suggested in CHAPTER VI: HIGH K+-DEPOLARISATION-INDUCED CONTRACTIONS IN THE HSV). On the other hand, the increased amounts of collagen and elastin in the media and intima may represent a physical barrier for EGTA penetration. Indeed, the slow rate of diffusion of NA through the rabbit aortic wall is thought to be due to elastic components of the media (36). Also, the increase in type IV collagen in the thickened basement membrane around endothelial cells and smooth muscle cells affect the permeability of the vessel wall. Excessive amounts of these substances may trap and retain large molecules and/or Ca\textsuperscript{2+} (447).

In addition to steric interactions, ionic interactions may also impede movement through the extracellular matrix or create a reservoir of anionic sites for Ca\textsuperscript{2+} binding. Collagen, elastin and and GAGs have significant Ca\textsuperscript{2+} binding capacities (430). GAGs are complex
carbohydrates, usually chondroitin sulfate or keratan sulfate, consisting of linear repeats of disaccharides; these sugars contain carboxylate or sulfate ester moieties resulting in a linear array of anionic groups giving GAGs an overall negative charge (376). GAGs usually do not occur as free components of tissues but are usually bound to core proteins forming proteoglycans, except for hyaluronic acid (150, 448). Proteoglycan content in the HSV is increased as compared to the IMA (372). Changes in the proteoglycan and hyaluronan content of the extracellular matrix of the vascular wall form a network of highly charged macromolecules that regulate the movement of small and large macromolecules by retention through ionic or steric interactions (447). Also, laminin and fibronectin, which are increased in the thickened basement membranes of endothelial and smooth muscle cells of the HSV (as indicated above), are two glycoproteins with sialic acid residues (453). Hence, the multitude of anionic sites resulting from extracellular matrix and basement membrane components in the HSV, in addition to the negative surface charges of the plasma membrane (135), may impede diffusion of EGTA, a negatively charged molecule, and increase the number of anionic binding sites for Ca$^{2+}$.

It is thought that the foregoing morphological properties contributed to the prolonged incubation times in nominally Ca$^{2+}$-free solutions required to inhibit high K$^+$ contractions in the HSV. As illustrated with the rabbit femoral arteries, vessel wall properties characteristic of the HSV were not apparent in animal blood vessels. Accordingly, high K$^+$ contractions in the animal preparations were rapidly abolished by brief exposure to the nominally Ca$^{2+}$-free PSS. This finding may represent an important species difference with yet unknown implications for vascular physiology, remodelling, ageing, and disease.
Ca2+ release from the SR by high K+ in nominally Ca2+ free PSS

In smooth muscle, the SR has two well recognised functions for the regulation of [Ca2+]i. One is to amplify the Ca2+ signal by CICR through RyR channels, while the other is to take up Ca2+ from the cytoplasm via SERCA. Hence, the peripheral SR may act by amplifying or buffering Ca2+ influx. The role of CICR has been demonstrated in a variety of skinned preparations, such as the rabbit mesenteric artery (248), and human uterine smooth muscle (270, 301). Few studies have demonstrated a role for CICR in intact smooth muscle. The role of the SR was investigated during high K+ contractions in nominally Ca2+-free PSS, conditions where Ca2+ influx is reduced thereby facilitating SR Ca2+ buffering (461). A component of the high K+ contraction after 15 min in nominally Ca2+-free PSS was dependent on SR Ca2+ release indicating that release was more important than buffering under these conditions. Ca2+ entering through VOCs was thought to activate RyR channels located on the SR membrane and stimulate the release of Ca2+ from the SR via CICR, a crucial mechanism in cardiac muscle (99, 240) and of some importance in smooth muscle (177). The residual contraction was most likely mediated by direct activation of the myofilaments by Ca2+. In other intact preparations, CICR was demonstrated in Ca2+ contractions in K+-depolarised intact guinea pig thoracic aorta (187); in this latter study, the CICR component of high K+ contractions was inversely related to the amount of Ca2+ influx. In other words, during high K+ contractions in normal PSS, the [Ca2+]i was high enough to directly activate the myofilaments or inactivate CICR. Hence, CICR may not play a role during high K+ contractions in normal PSS in the HSV.

Montaño and colleagues suggested that the sustained agonist-induced contraction in Ca2+-free solution depended on continuous cycling of Ca2+ between the SR and the extracellular membrane pools (300); such mechanism may also operate during sustained high K+ contractions in nominally Ca2+-free PSS in the HSV. Intramural release of NA could not account for the SR Ca2+ release component because phenotolamine non-significantly attenuated high K+ contractions
in the HSV. But, it has recently been demonstrated that modulation of the membrane potential in guinea pig coronary myocytes resulted in a voltage-dependent increase in the liberation of IP3 and increase in the [Ca\(^{2+}\)]\(_i\), presumably due to release of Ca\(^{2+}\) from the SR (116). This effect also modulated ACh-induced increases in IP3 in the same preparation (117). Also, KCl caused a progressive stimulation of contractile activity in guinea-pig jejunal longitudinal smooth muscle strips, accompanied by increased production of IP3 in smooth muscle fragments (35). Hence, high K\(^{+}\)-induced depolarisation and increase in IP3 could mediate the SR Ca\(^{2+}\) release component of the nominally Ca\(^{2+}\)-free high K\(^{+}\) contraction.

**Effect of age and disease on extracellular bound Ca\(^{2+}\)**

It was hypothesised that the putative increase in extracellular tightly bound Ca\(^{2+}\) in the HSV resulted from ageing and/or disease. Patients supplying SV tissues were relatively old and suffered from coronary artery disease, whereas the other vascular preparations were obtained from animals that were young and healthy. Young people and adolescents do not undergo CABG surgery; therefore, an alternative source of HSV segments was necessary. The only available source of fresh HSV segments from young people would be obtained from those who donate their organs for transplantation. Although ethical approval was obtained, it was very difficult to obtain tissues from these sources because the time required to harvest a portion of the HSV could delay the harvesting of other organs, thereby putting the recipient(s) of the transplanted organs at risk.

Instead, it was possible to test the effects of disease on vascular preparations obtained from swine allocated to one of three different experimental groups: control, high fat fed, and alloxan-fed diabetic pigs on a high fat diet. Alloxan kills pancreatic \(\beta\) cells and decreases plasma insulin levels thereby decreasing insulin action (85). The time course for the decay of the high
K+ contractions in the hyperlipidemic pigs was significantly faster during the first 5 min in the 0.1 mM EGTA solution as compared to control animals, while that for the diabetic pigs on a high fat diet was no different from control. These results were unexpected because hyperlipidaemia and diabetes are both risk factors that would be expected to stimulate vessel wall remodelling such that the number or affinity of Ca2+ binding sites would increase or access to bound Ca2+ by EGTA would be restricted. These findings could not be attributed to differences in the release of intramural NA, or to differences in Ca2+ influx through VOCs because the effects of phentolamine or nifedipine pre-incubation did not differ between groups. In addition, there were no alterations in Ca2+ handling by the myofilaments because Ca2+ dose-response curves were identical for all groups. Although the effect of hyperlipemia was unexpected, the data suggests that femoral vein segments from high fat fed pigs had a smaller or more EGTA-accessible pool of extracellular bound Ca2+ than control pigs. Hence, for a given amount of time in 0 Ca2+ 0.1 mM EGTA solution, less Ca2+ entered upon K+-depolarisation thereby attenuating the contraction as compared to control. These findings are in contrast to studies showing an increase in Ca2+ current density and a decrease in inactivation kinetics of VOCs in rats fed a high fat diet (450).

A treatment group of alloxan-fed diabetic pigs on a normal diet was missing and the effects of diabetes alone could not be tested. However, it is possible to speculate that the hyperlipidemic environment counteracted the effects of decreased insulin action by alloxan. In a study by Kravtsov and colleagues (226), the effects of high K+ contractions in the presence or absence of Ca2+ (and variable concentrations of Na+ and EDTA) were tested in aorta taken from rats of three different genetic strains, including the spontaneously hypertensive rat. Differences in contractility in the various solutions reflected strain differences and were not related to the hypertensive state of the animal. Hence, it is possible that the anionic binding sites for Ca2+ (as
well as K\(^+\), Na\(^+\), and Mg\(^{2+}\)) are not dependent on the disease status of the animal but may be determined by other factors.

**Access to extracellular Ca\(^{2+}\) binding sites**

Both EGTA and BAPTA have similar Ca\(^{2+}\) binding affinities but BAPTA has a faster on-rate for Ca\(^{2+}\) (78). Hence, it was hypothesised that EGTA may be too slow to bind Ca\(^{2+}\) before Ca\(^{2+}\) derived from the extracellular Ca\(^{2+}\) binding sites reached the VOCs. BAPTA, a much faster Ca\(^{2+}\) chelator, could possibly intercept Ca\(^{2+}\) diffusing to the VOCs resulting in greater inhibition of high K\(^+\) contractions in nominally Ca\(^{2+}\) free solution. However, no difference was observed between EGTA and BAPTA in HSV and swine renal artery (data not shown). Hence, chelation speed was not a limiting factor. This finding supports the view that diffusional access to Ca\(^{2+}\) binding sites by EGTA or the high affinity of the anionic sites for Ca\(^{2+}\) (close to the VOCs) is responsible for the observed high K\(^+\) contractions in nominally Ca\(^{2+}\)-free PSS.

**α\(_1\)-adrenergic receptor-stimulated contractions in the HSV**

PE, a relatively selective α\(_1\)-adrenergic receptor agonist, contracted the undistended HSV as observed in other studies (95, 380, 393, 444), indicating the presence of functional α\(_1\)-adrenergic receptors in our preparation. The receptor subtype in question was not explored; however, a recent study has demonstrated mRNA expression of the α\(_{1a}\)- and α\(_{1b}\)-receptor subtypes in the HSV (353). PE-induced responses were sustained, but high K\(^+\) contractions always developed faster and had greater amplitudes. This observation was consistent with other studies using intact HSV rings (292, 293) and denuded rings of discarded HSV (2), where contractions to high K\(^+\) and noradrenaline were compared.
Other HSV studies (95, 155, 174, 350, 380, 392, 393) have pharmacologically characterised α-adrenergic receptor-mediated contractions, but this report is the first to examine the sources of Ca\(^{2+}\) and Ca\(^{2+}\) sensitisation mechanisms elicited with α\(_1\)-adrenergic receptor stimulation.

**Role of Ca\(^{2+}\) influx and Ca\(^{2+}\) release from the SR in PE-induced contractions**

The role of Ca\(^{2+}\) influx through two separate Ca\(^{2+}\) influx pathways (289, 423), ROCs and VOCs, on the amplitude of PE contractions was examined by the use of the selective VOC blocker nifedipine (122) and the relatively selective ROC blocker SK&F 96365 (290). In this study, pre-incubation with nifedipine or SK&F 96365 significantly attenuated the amplitude of PE contractions. However, SK&F 96365 did not further inhibit PE responses as compared to nifedipine, unlike the rat thoracic aorta (this study). In the latter preparation, the inhibitory effects of nifedipine or SK&F 96365 pre-incubation were greater than in the HSV and SK&F 96365 significantly inhibited PE contractions more than nifedipine. And our laboratory has shown that in the rabbit inferior vena cava, the nifedipine-resistant PE contraction was completely abolished by SK&F 96365 (247). These findings suggest that the inhibitory effects of SK&F 96365 in the HSV were restricted to VOCs. In this study, SK&F 96365 inhibited high K\(^+\)-induced contractions in the HSV, which were dependent on Ca\(^{2+}\) influx through VOCs. SK&F 96365 also significantly attenuated or nearly completely abolished high K\(^+\) contractions in human airways (133), guinea pig bronchial smooth muscle (26), and canine pulmonary artery (189). Hence, in the HSV, Ca\(^{2+}\) influx occurred through VOCs, not ROCs, was important during force development of PE-induced contractions. NA contractions in human omental arteries elicited the same sensitivity pattern to nifedipine and SK&F 96365 (278). In contrast, in the
human renal artery, NA contractions were not dependent on Ca\(^{2+}\) influx through VOCs, but through ROCs (262).

The degree of involvement of VOCs in PE contractions amount to over 30% in HSV was similar to some studies using the canine SV preparations, where nitrendipine and nifedipine attenuated maximum PE responses by 20-36% (140, 154, 197). However, there were also some studies where nifedipine had no inhibitory effects on PE contractions in the canine (306, 308) and rabbit (369) SV. In other studies using dog SV, nimodipine attenuated the \(\alpha_1\)-adrenoceptor component of NA contractions in one study (70) but nifedipine had no effect on NA contractions in another (306).

Differences between nifedipine sensitivity of PE and NA contractions in rabbit and dogs could be due to the degree of opening of VOCs (140) resulting from differences in plasma membrane depolarisation (283, 284, 355) or in the opening of ROCs (57), or species variation (57). On the other hand, it has been demonstrated that the variability could not be due to tissue differences in receptor reserve (140) or \(\alpha_1\)-adrenoceptor subtype (308). A note of caution should be made when comparing the VOC-dependent component of \(\alpha_1\)-adrenoceptor responses in tissues harvested from ‘young’ laboratory animals and ‘elderly’ patients. In the rat thoracic aorta, age-dependent changes in potency of NA and the sensitivity of maximum responses to the dihydropyridine isradipine were related to age-dependent changes in receptor density or population of Ca channels (365). On the other hand, nifedipine itself does not exhibit differential effects due to ageing (228).

In the HSV, nifedipine and verapamil addition at the plateau phase of NA contractions almost completely abolished the response (292, 293). In addition, pre-incubation with verapamil, nifedipine, and diltiazem reduced the peak amplitude of NA contractions by 55-70% (292, 293, 432, 433). The greater sensitivity of NA contractions to Ca\(^{2+}\) antagonism as compared to PE may be related to the fact that NA activates both \(\alpha_1\)- and \(\alpha_2\)-adrenergic
receptors. While stimulation of both receptor types may be linked to both Ca\(^{2+}\) influx and release of Ca\(^{2+}\) from intracellular stores (5, 70, 93, 139, 193, 194, 197, 355, 428, 429), the relative involvement of Ca\(^{2+}\) influx and Ca\(^{2+}\) release for each receptor type (and respective subtypes) may depend on species variation, in addition to dose of the agonist (57). Hence, NA activation of \(\alpha_{1}\)- and \(\alpha_{2}\)-adrenoceptors in the HSV may involve a larger Ca\(^{2+}\) influx component through VOCs than PE activation of \(\alpha_{1}\)-adrenoceptors in the same preparation.

There were no studies examining the effects of ROCs in human, canine and rabbit SV preparations using SK&F 96365. In the canine SV, saponins extracted from Panax notoginseng, putative ROC blockers, significantly attenuated PE (10 \(\mu\)M) responses by 19% (141). In contrast, ROCs play an important role in the rabbit inferior vena cava as PE contractions were completely abolished with SK&F 96365 (247).

The addition of nifedipine and SK&F 96365 did not inhibit the sustained phase of PE contractions in the HSV suggesting that Ca\(^{2+}\) entry through VOCs was not important during this phase. This finding was unlike the inhibitory effect of nifedipine on the slow phase of PE contractions in the rat aorta (317, 368) and NA in the HSV (292, 293) but consistent with the observation that \([\text{Ca}^{2+}]_{i}\) levels were not sustained during the plateau phase of the PE-induced contraction. In the case of SK&F 96365, there was a tendency for PE contractions to get stronger. SK&F 96365 elicits multiple and complex effects on \([\text{Ca}^{2+}]_{i}\) signalling in addition to its non-selective inhibition of Ca\(^{2+}\) influx. For instance, SK&F 96365 causes Ca\(^{2+}\) release from the SR (184, 191) followed by capacitative Ca\(^{2+}\) entry (191). In addition, it blocks inwardly rectifying K\(^{+}\) currents causing depolarisation (371). Hence, it is possible that SK&F 96365 elicited a combination or all of the foregoing effects in the HSV resulting in an increase in levels of \([\text{Ca}^{2+}]_{i}\) and an amplification of the PE contraction.

It is generally believed that \(\alpha_{1}\)-adrenergic stimulation is more dependent on mobilisation of intracellular Ca\(^{2+}\) stores and is only partly inhibited by nifedipine (58, 122). The role of the
SR was examined by eliciting PE contractions in nominally Ca\(^{2+}\)-free PSS. Contractions were significantly attenuated but only to the same extent as nifedipine and SK&F 96365, indicating the lack of involvement of Ca\(^{2+}\) entry through non-stimulated Ca\(^{2+}\) influx pathways during PE force development. The residual PE contraction in nominally Ca\(^{2+}\)-free PSS was thought to be due to release of Ca\(^{2+}\) from SR Ca\(^{2+}\) stores, consistent with findings in the canine SV (139, 154, 193, 194, 283).

Unlike nifedipine or SK&F 96365 pre-incubation, the plateau phase during PE contractions was completely abolished in nominally Ca\(^{2+}\)-free PSS, indicating a Ca\(^{2+}\) influx pathway important in mediating the sustained phase of the response. This finding also confirms successful removal of free Ca\(^{2+}\) in the external medium and extracellular space. Because nifedipine and SK&F 96365 did not abolish the plateau phase, Ca\(^{2+}\) influx did not occur through known stimulated Ca\(^{2+}\) influx pathways, such as VOCs or ROCs. Excluding the possibility of an unknown stimulated Ca\(^{2+}\)-influx channel, the only other mechanism that could account for Ca\(^{2+}\) influx during this phase is the passive Ca\(^{2+}\) leak pathway (excluding the Na\(^{+}/Ca^{2+}\) exchanger).

A Ca\(^{2+}\) leak has been demonstrated in a number of cell types, including smooth muscle cells (56, 285), and is thought to play a role in the regulation of contractile activity (268). The Ca\(^{2+}\) leak pathway is not thought to be due to low activity of VOCs (201) or ROCs (79), and is not blocked by calcium entry blockers (see references in Loutzenhiser and colleagues, 1985 (263)). Under resting conditions, the amount of Ca\(^{2+}\) entering through the Ca\(^{2+}\) leak pathway is of sufficient magnitude to support a contraction (263), but is sequestered into the SR (422) and is extruded from the cell (64) before activating the myofilaments. During PE stimulation, the permeability of the SR is increased thereby reducing the ability of the SR to sequester Ca\(^{2+}\) (264, 424) and making the Ca\(^{2+}\) that enters through the leak pathway available to activate the myofilaments. Hence, the Ca\(^{2+}\) required during the tonic phase of PE contractions was likely mediated by Ca\(^{2+}\) entry through the leak pathway.
The rate at which the amplitudes of PE contractions decayed in nominally Ca\(^{2+}\) free PSS was determined to further characterise the extent to which SR Ca\(^{2+}\) contributed to these responses. The amplitude of the contraction decreased mono-exponentially with time, as in other studies (222, 248). It has been previously shown that the tonic component does not change with pre-incubation in 0 Ca\(^{2+}\) solution (222). At 30 min in nominally Ca\(^{2+}\)-free PSS, the contractions to PE were similar to those of NA in 0 Ca 10 \(\mu\)M EGTA in the HSV (292, 293), indicating similar decay rates for PE and NA. However, decay rates were faster in human as compared to canine SV. PE contractions after 1 hour in 0 Ca\(^{2+}\) 2 mM EGTA containing solution where nearly 50% (154) to 80% of control (359). These findings are in contrast to other studies (193, 194) where PE contractions were merely 10% of control after 1 hour in the same Ca-EGTA solution. No explanation can be offered for these differences.

The effect of SR Ca\(^{2+}\) unloading on the magnitude of PE contractions was examined to confirm that the intracellular source of Ca\(^{2+}\) was the SR. The SR was unloaded with a PE challenge in the presence of CPA, a putative SERCA inhibitor (80, 418). In the presence of CPA, Ca\(^{2+}\) released from the SR with PE-stimulation cannot be taken back up by the SR, thereby ensuring SR Ca\(^{2+}\) unloading. The subsequent PE-stimulated contraction in nominally Ca\(^{2+}\)-free PSS (to isolate the SR component) was nearly completely abolished by unloading SR Ca\(^{2+}\) indicating that Ca\(^{2+}\) release from the SR was the major contributor of Ca\(^{2+}\) for activation of the contractile apparatus. A significant contribution by SR Ca\(^{2+}\) to PE (10 \(\mu\)M) contractions has also been observed in both large and small arteries of the rat, guinea pig, dog and rabbit, although this contribution is greater in conduit than in resistance arteries (266).

CPA has been shown to relax PE-contracted rat aorta via the release of NO from the endothelium (303, 465), and L-NAME, an eNOS inhibitor (345), prevented the CPA-induced relaxation (465). In our experiments, the inhibitory effect of CPA and PE unloading was not due
to the release of NO from the endothelium, as the same result as the foregoing was observed in the presence of L-NAME.

Acute periods of CPA pre-incubation prevents buffering of Ca\(^{2+}\) entering the cell via the inhibition of SERCA (64) thereby allowing Ca\(^{2+}\) to diffuse to the contractile filaments, increase resting tension (377), and elicit contraction (4, 80). Therefore, CPA pre-incubation may amplify control PE contractions. In contrast, the potential CPA-induced release of NO from endothelial cells (303) would tend to decrease PE contractions, which can be prevented by L-NAME, as indicated above. The effects of CPA and L-NAME alone or in combination were tested on the magnitude of the control PE-induced contractions but these treatments tended to increase the magnitude of these responses, albeit non-significantly. CPA non-significantly increased NA contractions in the ferret portal vein (4). And in the HSV, the effects of eNOS inhibition on \(\alpha\)-adrenoceptor stimulated responses were potentiated by L-NNA pre-incubation (97), or had no effect when L-NMMA was used (460).

Attempts were also made to deplete the PE-sensitive intracellular Ca\(^{2+}\) store with caffeine, which releases Ca\(^{2+}\) by binding to RyR channels, and CPA, but in a nominally Ca\(^{2+}\)-free medium. However, PE contractions were not significantly reduced as compared to contractions in nominally Ca\(^{2+}\)-free PSS without exposure to CPA and caffeine. It is possible that the amount of SR Ca\(^{2+}\) depletion after 15 min in nominally Ca\(^{2+}\)-free PSS did not differ with or without prior unloading with caffeine and CPA exposure. Alternatively, the SR may play little role in mediating PE contractions after 15 minutes in the absence of extracellular Ca\(^{2+}\). It is still not known whether the PE sensitive SR store is also sensitive to caffeine, and vice-versa, in the HSV.
Ca²⁺-sensitisation of the contractile proteins by PE

Because the [Ca²⁺]ᵢ signal was near or at baseline during the slow phase of PE-induced contractions, [Ca²⁺]ᵢ was not likely to solely regulate force during this phase. In the absence of elevated [Ca²⁺]ᵢ levels, MLCK activity is low and myosin would be dephosphorylated if MLCP activity remained unaltered. A reduction in phosphorylated myosin would result in a decline in the number of cross-bridges and relaxation. But, in spite of the return of [Ca²⁺]ᵢ to baseline values, force remained elevated. Therefore other mechanism(s) must be activated to allow both cross-bridge cycling and the observed tonic contraction.

In some preparations, such as smooth muscle of the ferret aorta, a significant part of the contraction to PE persists at resting [Ca²⁺]ᵢ (69) and recently, Ca²⁺-independent MLC2₀ phosphorylation activity was discovered in rat caudal artery and chicken gizzard at pCa 9 (443). Although Ca²⁺ independent contractions were possible and cannot be excluded, it is likely that other mechanisms mediated the sustained phase of PE-induced contractions. An increasing number of studies point towards a role for MLCP inhibition as an important mediator of excitation-contraction coupling mechanisms in many vascular smooth muscle preparations (386). The most important mechanism today is thought to be the rhoA activated rho kinase pathway.

Rho targets: rho kinase and PKN

The small monomeric GTPase protein rho, a member of the rho subfamily of the Ras superfamily of monomeric GTPases (421), has been implicated in GTPγS- and agonist-induced Ca²⁺ sensitisation (127, 162), and is thought to act by increasing MLC₂₀ phosphorylation (319) by inhibiting MLCP activity (319, 413). There are many effectors of rho: protein kinase N (16, 305, 442), rho kinase (185, 215, 252, 282), and MBS (215), a subunit of MLCP. Although rho binds MBS, it does not directly modulate its activity (215). Instead, rho may inhibit MLCP via phosphorylation of MBS, also known as MYPT (149), by rho kinase (102, 149, 202, 215, 386).
Rho kinase also directly phosphorylates MLC20 in vitro (15) and in situ (229), although direct phosphorylation of MLC20 is not thought to be the mechanism of rho kinase-mediated Ca\textsuperscript{2+} sensitisation in vivo (386).

Rho and rho kinase have been implicated in GTP\textsubscript{S}- and agonist-induced Ca\textsuperscript{2+} sensitisation in a variety of smooth muscle preparations: vascular (11, 106, 108, 125, 127, 162, 223, 416), tracheal (416), intestinal (125, 127, 186, 267, 327, 398) and vas deferens (109) smooth muscle.

The role of rho has been elucidated by using inhibitory toxins specific for rho (11, 108, 109, 127, 162, 186, 223, 267, 319, 327). These methods involve ADP-ribosylation or glucosylation of rho but may have non-specific effects, such as inhibition of high K\textsuperscript{+} contractions (108), shifts in the pCa-force relationships (186) and non-specific ribosylation of other rho-like proteins (347, 348). Another strategy has been to introduce rho to the permeabilised preparation as the GTP\textsubscript{S}-bound form or the constitutively active mutant (127, 162, 327). However, the effects of wild-type rho may be due to the release of GTP\textsubscript{S} (366), while the effects of mutated proteins may differ from their normal counterparts (431). Also, some of these foregoing procedures depend on permeabilisation, which may introduce artifacts (431). The availability of specific rho kinase inhibitors, Y27632 (416) and HA-1077 (310), has enabled the evaluation of the physiological roles of rho kinase in intact smooth muscle, thereby avoiding some of the foregoing side effects of use of inhibitors of rho. In this study, the commercially available compound HA-1077, a serine/threonine protein kinase inhibitor, was selected. HA-1077 has good selectivity for rho kinase over protein kinase C and MLCK (310, 398, 416) and does not affect intracellular Ca\textsuperscript{2+} handling (398).

The addition of HA-1077 to the peak of PE-induced contractions resulted in complete inhibition of the sustained phase of the response, and only a slight decrease in the high K\textsuperscript{+}-induced tension. Although the affinity of HA-1077 for rho kinase is two orders of magnitude
greater than for MLCK (310), the small inhibitory effect of HA-1077 on the high K\(^+\) response was most likely due to inhibition of MLCK. The effects of HA-1077 on the high K\(^+\) contraction cannot be due to inhibition of Ca\(^{2+}\) sensitisation by rho kinase because the addition of nifedipine at the peak of the high K\(^+\) contraction completely abolished the response. These effects were in contrast to the absence of any effect of nifedipine added to the peak of PE contractions.

Inhibition of the PE contraction by HA-1077 in the HSV was similar to other studies where Y27632 dose-dependently inhibited the contraction to PE, histamine, ACh, serotonin, endothelin, and U46619 in intact rabbit aortic strips, pig coronary artery and guinea pig trachea (416), and to NA in intact human omental arteries (278). Studies show that rho kinase inhibition of the tonic phase of agonist-induced contractions was associated with inhibition of myofilament Ca\(^{2+}\) sensitisation (108, 327). Therefore, inhibition of the tonic phase of PE-induced force in the undistended HSV was most likely due to inhibition of rho kinase-mediated Ca\(^{2+}\) sensitisation mechanisms. Rho- or rho kinase-mediated Ca\(^{2+}\) sensitisation was also observed in response to PE-stimulation in the rabbit portal vein (108) and in response to NA in both the rabbit thoracic aorta (223) and the guinea pig vas deferens (109), but not in the rabbit mesenteric artery (127, 361, 362). Rho kinase-mediated Ca\(^{2+}\) sensitisation depends on the species and tissue type.

HA-1077 also inhibits PKN (14), one of the targets of rho (16, 442), mentioned previously. PKN phosphorylates CPI-17 (146), a novel phosphoprotein selectively expressed in smooth muscle tissues that specifically inhibits MLCP (96). PKN phosphorylation of CPI-17 has not yet been implicated in Ca\(^{2+}\) sensitisation mechanisms; however, a PKN-mediated effect cannot be excluded. CPI-17 is also phosphorylated by rho kinase (224) and mediates Ca\(^{2+}\)-sensitisation of the myofilaments in rabbit femoral artery (217). Hence, there are two mechanisms by which activation of the monomeric G protein rho may stimulate Ca\(^{2+}\) sensitisation in the HSV (see Figure 38). Rho may activate rho kinase, which phosphorylates
MLCP directly or via phosphorylation of CPI-17. Phosphorylated CPI-17 inhibits MLCP. Alternatively, rho may activate PKN, which also phosphorylates CPI-17.

**PKC**

Protein kinase C may also mediate Ca$^{2+}$ sensitisation (315) by phosphorylation of thin-filament associated proteins (436) or inhibition of MLCP (281, 386), independent of the rho kinase pathway (126). In the rabbit femoral artery, PKC increased the Ca$^{2+}$ sensitivity of MLC$_{20}$ phosphorylation and force production through inhibition of MLCP via phosphorylation of an inhibitory protein (281), later discovered to be CPI-17 (253). Phosphorylation of CPI-17 by PKC stimulated the binding of CPI-17 to and subsequent inhibition of MLCP (221, 253) (see Figure 38). However, some investigations have failed to illustrate that PKC activation is involved in G protein-mediated Ca$^{2+}$ sensitisation, such as in the guinea pig vas deferens (109) and the rabbit mesenteric artery (188). Indeed, our experiments showed no evidence for the involvement of PKC in PE-induced contractions. Addition of calphostin C at the peak of PE response had no effect. Hence, CPI-17 phosphorylation may be involved in PE-induced contractions in the HSV; however, it was not phosphorylated by PKC. In some preparations, $\alpha_{1}$-adrenergic receptor-induced Ca$^{2+}$ sensitisation may involve PKC. PE stimulated Ca$^{2+}$ sensitisation via phosphorylation of CPI-17 in the rabbit femoral artery (217). NA-induced PKC-mediated Ca$^{2+}$ sensitisation was elicited in rat intrarenal (103) and mesenteric arteries (50, 103), and in human omental arteries (278). Also, a minor role for PKC in PE-induced contractions has also been demonstrated in intact rabbit aorta (214). Evidence also suggests a role for PKC in Ca$^{2+}$-independent contractions in response to PE in ferret aortic cells (69, 213) although the physiological relevance of such contractions is questionable. It should be noted that many studies that have demonstrated a role for PKC in Ca$^{2+}$ sensitisation mechanisms used inhibitors such as staurosporine (6, 48) and H-7 (358, 378), which do not exhibit more selectivity.
for PKC over MLCK. Hence, interpretation of studies using these inhibitors should be done with caution and involvement of PKC in response from those studies may be questionable.

Tyrosine protein phosphorylation and MAPK activation

Tyrosine kinases have been implicated in GPCR-induced Ca\textsuperscript{2+} sensitisation in a variety of smooth muscle preparations, such as rat (322), rabbit (361), and guinea pig (366) mesenteric arteries, as well as of the guinea pig ileum (395). However, the underlying mechanisms are still unknown because tyrosine kinases have yet to be linked to the activities of MLCK or MLCP (387). The involvement of tyrosine kinases in vascular contractile responses to α-adrenoceptor stimulation was reported by Di Salvo and colleagues (83) who found that genistein and other kinase inhibitors attenuated receptor-mediated contractions in guinea pig mesenteric microvessels. The tyrosine kinase pp60\textsuperscript{c-src} was implicated because tyrosine kinase inhibitors attenuated the activity of this enzyme, but not that of MLCK or cAMP-dependent protein kinase (83). In ferret aortic differentiated smooth muscle cells, PE elicited a Ca\textsuperscript{2+}-independent contraction involving tyrosine phosphorylation-dependent MAPK translocation to the myofilaments consistent with a role for pp60\textsuperscript{c-src} involvement in PE-mediated contractions (212). Although pp60\textsuperscript{c-src} is a likely candidate for the tyrosine kinase-mediated α-adrenoceptor-stimulated contractions, its involvement has not yet been conclusively demonstrated. In addition, studies indicate that while pp60\textsuperscript{c-src} activity is high in bovine coronary artery (82) and more abundant than any other tyrosine kinase in porcine aortic smooth muscle, it was not the tyrosine kinase responsible for angiotensin II contractions in porcine coronary artery (321).

In nearly all studies, involvement of tyrosine kinases in excitation-contraction coupling mechanisms has relied largely upon the use of tyrosine kinase inhibitors. Genistein is a highly specific inhibitor of tyrosine kinases, such as the EGF receptor kinase and pp60\textsuperscript{v-src} tyrosine kinases. Genistein is competitive with respect to ATP and non-competitive with respect to the
substrate (10). Genistein was used in this study to gain insight into whether tyrosine kinase activity is involved in PE-induced contractions in the HSV. The addition of genistein to the peak of the PE contraction inhibited the plateau phase by 50%, suggesting a role in Ca$^{2+}$ sensitisation mechanisms in the HSV. Genistein had no effect on the high K$^+$ contraction, indicating the absence of non-specific inhibition of VOCs and MLCK. In addition, genistein at concentrations smaller or equal to 100 μM had no inhibitory effect on rho kinase activity (398), suggesting that the effects of genistein were not attributed to inhibition of rho kinase.

Although tyrosine kinase activity mediated a component of the plateau phase in PE-induced contractions, the mechanism of Ca$^{2+}$ sensitisation is not known. However, evidence suggests the involvement of tyrosine kinases with the MAPK and rho kinase pathways, both mediating different aspects of excitation-contraction coupling in smooth muscle.

Tyrosine phosphorylation has been linked to vascular smooth muscle contraction stimulated by GPCRs via activation of the MAPK pathway (66, 397). Signalling pathways involving sequential activation of ras, raf-1, and ultimately the protein kinase cascade termed MAPK (105, 333, 454) have been shown to be very important in mediating receptor tyrosine kinase regulation of cell growth and differentiation (see references in Hu, and colleagues, 1999 (170)). Activation of the MAPK pathway by GPCRs has also been associated with sensitisation of the myofilaments to Ca$^{2+}$ by phosphorylating caldesmon, thereby removing its inhibition of actomyosin ATPase activity (7, 209). Crosstalk between the ras-raf-MEK-ERK pathway and the MLCK pathway in response to PE stimulation (198) suggests that tyrosine kinase-dependent activation of MAPK component of PE contractions may occur via this pathway. However, recent work suggests that tyrosine kinase-ras-MAPK signalling pathways may also be signalled directly by GPCRs (see reviews in Hu, and colleagues, 1999 (170)). In the rat aorta, 5-HT$_{2A}$-receptor stimulation results in activation of the MAPK kinase pathway via src (27). It is unknown whether α$_4$-adrenergic receptor stimulated contraction also occurs via this pathway.
However, it is known that $\alpha_1$-adrenoceptor stimulated mitogenic responses involve tyrosine protein kinases and activation of the MAPK pathway because inhibitors of tyrosine kinase and MAPK block these responses (404). A recent study confirmed this finding in HVSMC (170) and PC12 cells (466). It is not clear how tyrosine kinases contribute to the overall signal transduction of specific GPRCs; however, it is thought that $\alpha$ and/or $\beta\gamma$ subunits of heterotrimeric G proteins may activate the ras/raf signalling pathway through cytosolic tyrosine kinases (261), such as src, pyk2, csk, and lyn (467). In PC12 cells, the cytosolic tyrosine kinase src played an obligatory role in the mitogenic response to $\alpha_1$-adrenoceptor stimulation (466). In human vascular smooth muscle cells, $\alpha_1$-adrenoceptor stimulation of mitogenesis was $Ca^{2+}$-dependent and may be mediated by src- or pyk2-dependent tyrosine phosphorylation of PLC$\gamma$1 (170).

The selective MEK inhibitor, PD098,059, was used to determine whether the MAPK pathway mediated the effect of tyrosine phosphorylation-induced contraction in our preparation. However, a dose-response curve elicited with PD098,059 had no effect on PE contractions, except for the 100 $\mu$M dose. Drug effects at concentrations above 50-75 $\mu$M were not considered a selective pharmacological effect. Hence PE-induced contractions did not involve the MAPK pathway in the HSV. This finding is in contrast to the possible role of tyrosine kinase/MAPK pathway in PE-induced contractions in rat aorta and pulmonary artery (198) and in NA-stimulated guinea pig mesenteric arteries (366).

The other possibility involves rho kinase. A component of the rho kinase-mediated $Ca^{2+}$ sensitisation may involve tyrosine kinase phosphorylation. Crosstalk between rho and tyrosine kinase pathways have been suggested in response to several agonists in a variety of smooth muscle preparations (278, 361, 362, 395, 398), although the mechanism of the interaction remains unknown. Evidence suggests that tyrosine kinase phosphorylation events occur up- or
downstream of rho kinase stimulation in cytoskeletal rearrangements. Specifically, in fibroblasts, LPA stimulation leads to tyrosine kinase-mediated activation of rho, resulting in focal adhesions and stress fibre formation (318, 348). In neuronal cells, exposure to LPA activates rho to induce growth cone collapse and neurite retraction through a G_{12,13}-initiated pathway that involves protein tyrosine kinase activity (225). Alternatively, GTP\_S (373) or LPA (182) stimulated rho-dependent tyrosine phosphorylation of focal adhesion kinase in fibroblasts and in tumour cells, respectively.

**Tyrosine kinases and cytoskeletal reorganisation**

Tyrosine kinases may also be involved in the reorganisation of cytoskeletal-membrane interactions during contractile stimulation (332). Tyrosine phosphorylation of paxillin, an integrin-associated protein located at dense plaques, has been associated with agonist- and KCl-stimulated contractions in tracheal smooth muscle (441), and does not depend on Ca^{2+} mobilisation or activation of contractile filaments (288). It was recently demonstrated that paxillin, as well as FAK, an integrin-associated tyrosine kinase, mediated (length-sensitive) mechanotransduction of tracheal smooth muscle. Tyrosine phosphorylation of FAK and paxillin may elicit changes in smooth muscle cell structure and contractility via remodelling of the actin filaments and/or via the mechanosensitive regulation of signalling molecules involved in contractile protein activation (401). Tyrosine phosphorylation of paxillin was independent of rho kinase activity (287). It is possible that inhibition of tyrosine kinase phosphorylation with genistein attenuated the roles of paxillin and FAK in cytoskeletal reorganisation thereby attenuating force elicited by PE stimulation in the HSV.
Effects of pre-incubation with inhibitors prior to PE contractions

The effects of the various protein kinase inhibitors were also tested by incubation prior to the PE contraction to verify their respective roles, if any, during the phase of force development, although findings from these studies may be confounding.

Pre-incubation with HA-1077 resulted in approximately 40% inhibition of the peak tension response to both PE and high K⁺. High K⁺ contractions were nearly completely abolished by pre-incubation with nifedipine. Therefore, Ca²⁺ sensitisation mechanisms were most likely not involved during the initial phase of K⁺ depolarisation-induced contractions. Given the similar degree of inhibition of HA-1077 on high K⁺ and PE contractions, one would conclude that the effects of HA-1077 on high K⁺ contractions were most likely attributed to incomplete inhibition of MLCK and not to inhibition of rho kinase or PKN. However, other studies showed that pre-incubation with an inhibitor of rho or rho kinase resulted in incomplete inhibition of agonist-induced contractions or Ca²⁺ sensitisation (108), even though drug addition during the plateau phase resulted in complete inhibition of force (106, 181). A satisfactory explanation for this effect was not offered, but it is possible that other Ca²⁺ sensitisation pathways mediated the residual contraction, such as PKC (180). In this study, PKC or MAPK were not involved in generating the maximum amplitude of PE contractions. Nevertheless, it cannot be excluded that PE-stimulated rho kinase or PKN activation during force development, which was inhibited by HA-1077, in addition to a partial effect on MLCK activity. But given the importance of Ca²⁺ influx through VOCs and Ca²⁺ release from the SR during the initial phase of PE-induced contractions, it is believed that the predominant mechanism mediating PE-induced force development was Ca²⁺-dependent MLCK activity.

Pre-incubation with genistein alone resulted in approximately 31% inhibition of peak tension and abolished the HA-1077-insensitive component of the PE contraction. In contrast, genistein had no effect on high K⁺ contractions either alone or in combination with HA-1077.
Hence, the inhibitory effects of genistein were not attributed to non-specific inhibition of VOCs or MLCK. Many studies have reported a role for tyrosine kinase phosphorylation in agonist-induced increase in $[\text{Ca}^{2+}]_i$ (134, 408). The tyrosine kinase-mediated rise in $[\text{Ca}^{2+}]_i$ may occur as a result of $\text{Ca}^{2+}$ influx and/or $\text{Ca}^{2+}$ release from the SR (104, 134, 205, 254, 313, 375). Mechanistic studies suggest that tyrosine phosphorylation may regulate the availability of VOCs (255, 256, 449) or receptor-operated channel conductance (183). Alternatively, tyrosine phosphorylation may regulate $\text{Ca}^{2+}$ release from the SR by phosphorylation of the IP3 receptor channel (195) or by increasing the level of IP3 synthesis (254, 407, 462) through phosphorylation of rasGAP (375), $\text{G} \alpha_{q/11}$ (417), PLC$_\gamma$ (277), or PLC$_\beta$ (407). These facts point towards a possible role for tyrosine phosphorylation in mediating the initial $\text{Ca}^{2+}$ transient. The effects of HA-1077 pre-incubation on MLCK and/or rho kinase/PKN, in synergy with the putative inhibitory effects of genistein pre-incubation on the initial $\text{Ca}^{2+}$ transient, may explain the complete inhibition of PE contractions in the HSV when these two inhibitors were present together.

Consistent with the previous data with calphostin C and PD098,059, the addition of these inhibitors prior to a PE contractions had no effect.

In summary, PE-induced tonic contractions were mediated by rho kinase, which most likely suppressed MLCP activity. But suppression of MLCP activity alone cannot lead to contraction. Although resting $[\text{Ca}^{2+}]_i$ levels were observed during the period of force maintenance, this level of cytosolic $\text{Ca}^{2+}$ was crucial for basal activation of MLCK activity and MLC$_{20}$ phosphorylation. A role for tyrosine kinase phosphorylation was also observed during the sustained phase of PE contractions, responsible for crosstalk with the rho kinase pathway or involved in cytoskeletal rearrangements. The initial phase of PE contractions were most likely dependent on $\text{Ca}^{2+}$-dependent MLCK, and partial rho kinase activation, with a putative role of tyrosine phosphorylation in mediating the initial $\text{Ca}^{2+}$ transient.
Summary of high K⁺- and PE-induced signalling and contraction

A summary of high K⁺- and PE-induced signalling and contraction may be found in Figure 39 and Figure 40, respectively.

Figure 38 to Figure 40 to follow
Figure 38. Relationships between rho kinase, PKN, PKC, CPI-17 and MLCP.

There are two mechanisms by which activation of the monomeric G protein rho may stimulate $\text{Ca}^{2+}$ sensitisation in the HSV. Rho may activate rho kinase, which phosphorylates MLCP directly or via phosphorylation of CPI-17. PKN also phosphorylates CPI-17. Phosphorylated CPI-17 inhibits MLCP. HA-1077 inhibits both rho kinase and PKN. PKN phosphorylation of CPI-17 has not yet been implicated in $\text{Ca}^{2+}$ sensitisation mechanisms. PKC may also mediate $\text{Ca}^{2+}$ sensitisation by inhibition of MLCP, independent of the rho kinase pathway.

Phosphorylation of CPI-17 by PKC stimulates the binding of CPI-17 to and subsequent inhibition of MLCP. Calphostin C (Cal C) inhibits PKC. The effect of rho kinase, PKN, and PKC is to inhibit MLCP activity, resulting in an increase in the levels of phosphorylated MLC$_{20}$ and increased contraction.
Rho kinase → PKN → PKC

HA-1077 → Cal C

HA-1077 → CPI-17 → P

P → MLCP_{active} → MLCP_{inactive} → P

P → Myosin → P

↓ Myosin → Relaxation

↑ Myosin → Contraction
Figure 39. Summary of high K\(^+\)-induced signalling and contraction in the HSV

High K\(^+\) elicited sustained increases in \([\text{Ca}^{2+}]_i\) resulting from depolarisation of the plasma membrane and opening of VOCs. An extracellular source of tightly bound Ca\(^{2+}\) not sensitive to acute changes in the extracellular Ca\(^{2+}\) concentration or to the presence of EGTA may be present in the HSV. This pool may lie on the outer surface of the plasma membrane at anionic sites, such as sialic acid in the glycocalyx, or in phospholipids. The caveolae may also be a source of extracellular Ca\(^{2+}\). Vessel wall characteristics of the HSV may contribute in various ways to the resistance of high K\(^+\) contractions to 0 Ca\(^{2+}\) 1 mM EGTA solutions. The increased thickness of the intimal and medial layers may lengthen the diffusion distance for Ca\(^{2+}\) to exit and EGTA to penetrate the tissue or the increased amounts of collagen and elastin in the media and intima may represent a physical barrier for EGTA penetration. Ionic interactions may also impede movement through the extracellular matrix or create a reservoir of anionic sites for Ca\(^{2+}\) binding. Collagen, elastin and and GAGs have significant Ca\(^{2+}\) binding capacities. The extracellular matrix of the vascular wall composed of proteoglycans and hyaluronic acid may form a network of highly charged macromolecules that regulate the movement of small and large macromolecules by retention through ionic or steric interactions. A component of the high K\(^+\) contraction after 15 min in nominally Ca\(^{2+}\)-free PSS was dependent on SR Ca\(^{2+}\) release indicating that release was more important than buffering under these conditions. Ca\(^{2+}\) entering through VOCs was thought to activate RyR channels located on the SR membrane and stimulate the release of Ca\(^{2+}\) from the SR via CICR. Other explanations may include continuous cycling of Ca\(^{2+}\) between the SR and the extracellular membrane pools. It is also possible that modulation of the membrane potential may result in a voltage-dependent increase in the liberation of IP3 and increase in the \([\text{Ca}^{2+}]_i\), presumably due to release of Ca\(^{2+}\) from the SR. See text for details.
High K$^+$-induced membrane depolarisation

VOC

$\text{Ca}^{2+}$

High K$^+$-induced membrane depolarisation

$\text{PL-C}$

$\text{IP}_3$

SR

$\text{Ca}^{2+}$

$\text{Ca}^{2+}$

$\uparrow [\text{Ca}^{2+}]$

MLCK

Myosin

Myosin

MLCP

Contraction

$\text{Ca}^{2+}$ = Bound Ca$^{2+}$
Figure 40. Summary of PE-mediated signalling and contraction in the HSV

PE contracted the undistended HSV acting at α1-adrenergic receptors. The rise in force elicited by PE initially followed the rise in [Ca\(^{2+}\)]\(_i\); however, the [Ca\(^{2+}\)]\(_i\) signal was transient declining to basal levels. Ca\(^{2+}\) influx through VOCs, not ROCs, was important during initial force development but not during the sustained phase of PE contractions. Ca\(^{2+}\) influx during this latter phase also occurred through the passive Ca\(^{2+}\) leak pathway. Inhibition of the tonic phase of PE-induced force in the undistended HSV was most likely due to inhibition of rho kinase- and or PKN-mediated Ca\(^{2+}\) sensitisation mechanisms. Rho may activate rho kinase, which phosphorylates MLCP directly or via phosphorylation of CPI-17. Phosphorylated CPI-17 inhibits MLCP. Alternatively, rho may activate PKN, which also phosphorylates CPI-17. Although tyrosine kinase activity mediated a component of the plateau phase in PE-induced contractions, the mechanism of Ca\(^{2+}\) sensitisation is not known (see dotted arrows). A component of the rho kinase-mediated Ca\(^{2+}\) sensitisation may involve tyrosine kinase phosphorylation. Crosstalk between rho and tyrosine kinase pathways has been suggested, although the mechanism of the interaction remains unknown. Evidence suggests that tyrosine kinase phosphorylation events occur up- or downstream of rho kinase stimulation in cytoskeletal rearrangements. Alternatively, tyrosine phosphorylation of FAK and paxillin may elicit changes in smooth muscle cell structure and contractility via remodelling of the actin filaments and/or via the mechanosensitive regulation of signalling molecules involved in contractile protein activation, independent of the rho kinase pathway. Tyrosine phosphorylation may regulate agonist-induced increase in [Ca\(^{2+}\)], by affecting the availability of VOCs or ROC conductance, Ca\(^{2+}\) release from the SR by phosphorylation of the IP3 receptor channel or by increasing the level of IP3 synthesis through phosphorylation of rasGAP, Go\(_{q/11}(417)\), PLC\(_\gamma\), or PLC\(_\beta\).
CHAPTER IX: CONCLUSIONS AND FUTURE DIRECTIONS

Prior to my thesis work, the effects of intra-operative manipulation on the integrity of HSV segments were widely reported in clinical and surgical journals. On the other hand, most functional studies using this vascular preparation were performed on segments that were surgically prepared or the extent of surgical preparation was not indicated. Only a handful of studies specified that undilated segments or branches of SV segments were used, without demonstrating that these segments were the most optimal preparations that could be obtained from the surgical procedure. This study is the first to emphasise the importance of standardising the supply of human saphenous vein segments obtained from CABG operations and explicitly demonstrate that the most optimally preserved segments were used in functional studies.

The mechanistic basis of the poor reactivity of ‘distended’ and ‘cardioplegic’ segments is not clearly understood. It is thought that distension stiffens vein segments due to disruption of elastic components (3), and the present study demonstrated that reactivity to contractile stimuli is reduced or abolished, cannot recover over the short-term, and is not likely due to a rearrangement of the contractile filaments. Further studies are required to determine the loci that are impaired with surgical preparation.

In light of the unknown effects of surgical preparation on the underlying physiology of the tissue, it is recommended that functional experiments using HSV segments be performed using the most optimally preserved segment in order to reduce variability in experimental findings and/or avoid the derivation of invalid conclusions. Surgical impairment of vein graft function may also deleteriously affect the lifetime of the bypass graft (as indicated in CHAPTER I: INTRODUCTION). Work in support of this hypothesis is currently in progress as the topic of a recently funded grant. It is hoped that information gained from this study will provide insights
into the mechanistic basis of impaired SV function of the transplanted and non-transplanted vein segments resulting from surgical preparation.

A surprising observation in this study was the refractoriness of high K\(^+\) contractions in nominally Ca\(^{2+}\)-free PSS in HSV segments and the data suggests the presence of an extracellular pool of tightly bound Ca\(^{2+}\). This finding appears to be a property of the HSV in particular, and not of other human blood vessels, such as the thymus vein and renal artery, nor of animal vascular preparations, such as those examined in this study and others. The vessel wall properties of the HSV suggest that extracellular matrix components (collagen, elastin, and GAGs), plasma membrane components (glycoproteins or phospholipids), or structural characteristics (caveolae), may represent sources of anionic Ca\(^{2+}\) binding sites or provide steric interactions preventing the free exchange of Ca\(^{2+}\) and EGTA molecules. Alternatively, these wall properties may simply increase the diffusion distance relative to other animal vascular preparations. Further characterisation of the HSV may be necessary to determine the basis of high K\(^+\) contractions in nominally Ca\(^{2+}\)-free PSS. The amount of collagen, elastin and GAGs in HSV can be quantified using Image Pro Plus© of Movat stained slides and compared with similar measurements performed in animal preparations, such as those in this study. There are also a variety of cytochemical detection methods that can be used to identify ionised groups (376) for Ca\(^{2+}\) binding or Ca\(^{2+}\) binding sites can be identified by electron microscopy using electron dense ions (22) or \(^{45}\)Ca\(^{2+}\) autoradiography (446). Extracellular matrix components (collagen, elastin, GAGs) and purified plasma membrane fractions could be isolated and characterised in terms of their Ca\(^{2+}\)-binding characteristics and compared with the characteristics of the high K\(^+\) decay curve in nominally Ca\(^{2+}\)-free PSS. Methods can be used to disrupt, or enhance anionic binding sites, or to displace bound Ca\(^{2+}\) and the effects of these treatments on the high K\(^+\) decay curve can be assessed. For example, enzymes, such as neuraminidase (346), elastase, collagenase, and PLD (241), remove sialic acid residues, breakdown elastin and
collagen, and increase the number of phosphatidic acid residues in the plasma membrane, respectively. And treatments such as varying the pH and trivalent/divalent cations (La$^{3+}$, Cd$^{2+}$, Mn$^{2+}$, Mg$^{2+}$) uncouple excitation from contraction and displace Ca$^{2+}$ from binding sites (235, 236, 243). Finally, the rates of diffusion of EGTA and BAPTA through the HSV and other blood vessels could also be compared (36), or the effects of these chelators can be tested on a thin HSV preparation.

One may speculate that the ability of the HSV to retain Ca$^{2+}$ may be the result of ageing and/or disease. Although intimal thickening and fibrosis may be observed in people of all ages, even in children, it occurs more frequently with ageing (251). A comparison of HSV samples from infants or adolescents with HSV samples from elderly patients would be useful to test this hypothesis.

Another major finding from this study is the relationship between force and cytosolic Ca$^{2+}$ in the undistended human saphenous vein. Stimulation with the $\alpha_1$-adrenergic receptor agonist PE produced a sustained contraction but only a transient rise in [Ca$^{2+}$]$_i$, due to Ca$^{2+}$ influx through VOCs and SR Ca$^{2+}$ release, indicating that force generation was only transiently signalled by the initial rise in [Ca$^{2+}$]$_i$ and that other mechanisms must be activated in order to maintain the tonic response when [Ca$^{2+}$]$_i$ returned to resting levels.

The transient nature of the Ca$^{2+}$ signal is unusual and not representative of any other PE- or NA- stimulated Ca$^{2+}$ signal in other vascular preparations, which are usually sustained. The transient nature of changes in [Ca$^{2+}$]$_i$ appears to be a property of the HSV as similar responses were observed after stimulation with endothelin-1 and angiotension II (246).

The nature of the Ca$^{2+}$ transient and its relationship to force, as well as findings with Ca$^{2+}$ antagonists, suggests that the roles of Ca$^{2+}$ influx through VOCs and Ca$^{2+}$ release from the SR were only components of the initial rise in force stimulated by PE. In order to verify the role of Ca$^{2+}$ influx through passive leak channels, the effects of La$^{3+}$ (79) on PE contractions could be
assessed. The role of VOCs could be confirmed with the use of other Ca\(^{2+}\) antagonists (411) and the mechanism(s) by which PE depolarises the cell membrane leading to the opening of VOCs must be elucidated. The SR Ca\(^{2+}\) release mechanism should also be further explored using agents such as dantrolene, to inhibit the release of Ca\(^{2+}\) from the SR, 2-APB (24, 279) and xestospongins (114), to examine IICR, as well as inhibitors of the endogenous RyR agonist, cADP ribose (329). Further experiments must elucidate the relative roles of Ca\(^{2+}\) influx and SR Ca\(^{2+}\) release in the initial Ca\(^{2+}\) transient and determine the role Ca\(^{2+}\) relative to Ca\(^{2+}\) sensitisation mechanisms during initial force development.

The slow phase of the PE response was not mediated by Ca\(^{2+}\) influx through VOCs unlike other vascular preparations, although Ca\(^{2+}\) influx through Ca\(^{2+}\) leak channels was important for maintaining basal [Ca\(^{2+}\)] during this phase. While the sustained phase did not depend on stimulated Ca\(^{2+}\) influx, it was mediated by rho kinase. This work underscores the importance of rho kinase in excitation-contraction coupling mechanisms in the HSV and suggests that this enzyme may be a useful therapeutic target for the alleviation of vein graft spasm, as it has been suggested for the treatment of hypertension (416), as well as cerebral (23) and coronary vasospasm (204). The importance of rho kinase in vein grafts can also be further emphasised by the recent finding that oxidised LDL reduces the responses to vasodilators such as ACh in NA-precontracted resistance arteries by increasing the sensitivity of the contractile apparatus to Ca\(^{2+}\) via the rho kinase pathway (39).

Finally, it should be noted that these findings do not necessarily reflect the normal physiology of the HSV but in fact may be limited to a subset of the population who is mostly elderly and suffers from coronary artery disease. In the latter scenario, this data may be characteristic of blood vessels with an altered physiology resulting from ageing and multifactorial disease processes. On the other hand, the data might reflect the vascular physiology of a specialised blood vessel that is associated with hydrostatic pressure and that conducts blood
against the force of gravity (379). The unique character of the HSV can also be exemplified by the fact that this preparation elicits a myogenic response with increases in intralumenal pressures which are greater than that observed in canine femoral and saphenous veins (33).
### Specimen & Patient Information

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**Diabetes status**

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<th>Type II (NIDDM)</th>
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**Saphenous vein**

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<tr>
<td>Resident performing harvest:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assistant performing harvest:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Internal mammary artery**

<table>
<thead>
<tr>
<th>Time IMA is cut:</th>
<th>Left: ____</th>
<th>Right: ____</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgeon performing the harvest:</td>
<td>As per addressograph label</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Resident in training</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Surgical assistant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other: ____</td>
<td></td>
</tr>
</tbody>
</table>

**Other blood vessel (identify vessel):** ____

<table>
<thead>
<tr>
<th>Time of incision:</th>
<th>Left: ____</th>
<th>Right: ____</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgeon performing harvest:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Addressograph label:** Place here

### Packaging and Paging

1. Place specimen in **vial with blue cap** (in core mini-fridge) as soon as possible after it becomes available.
2. Please be sure to complete the label on the vial with:
   a) time specimen is placed in vial;
   b) name of specimen (if a saphenous vein, indicate whether undistended, distended or cardioplegia - see below);
3. Place addressograph label on vial.
4. Page 54462 once the specimen is ready for pick-up (enter 31 and the OR# after the tone). If no one responds, page 54362.
5. Store the specimen in the mini-fridge located in the OR core while it awaits pick-up.
6. Specimen Information (this sheet) will be collected with the last specimen from each case.

### Types of Specimens Collected

Any vessels retrieved from patients will be collected (internal mammary artery, saphenous vein, radial artery). Three types of saphenous vein segments are collected:

1. **Undistended vein (U)** - the most critical segment for collection: segment removed from the distal end of the vein segment as soon as vein has been harvested from the leg and before distention.
2. **Distended vein (D):** segment has been flushed and distended/pressurized.
3. **Cardioplegia (C):** segment has been grafted and exposed to cardioplegia, typically has a tip at one extremity.
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