REGULATION OF INTRACELLULAR CALCIUM CONCENTRATION IN VASCULAR ENDOTHELIAL CELLS AND VALVULAR MYOFIBROBLASTS

by

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ABSTRACT

Background

Some aspects of Ca^{2+} signaling in vascular endothelial cells (ECs) and cardiac valvular myofibroblasts (VMFs) were examined in this study. Over the last decade, the relationship between endothelial dysfunction, smooth muscle proliferation, and development of vascular diseases has been relatively well elucidated. Although the specific mechanisms remain unclear, it is generally agreed that alterations in endothelial Ca^{2+} regulation play an important role in vascular pathophysiology. However, information regarding the underlying causes of valvular heart diseases is very limited, despite the large number of clinical cases reported. In the cardiac valve, ECs serve as a physical barrier between the VMFs underneath and the circulating blood. In addition, ECs secrete vasoactive substances in response to the enormous hemodynamic forces generated by the cardiac cycle. The endothelial secretions may have a general effect on the whole body or they may act locally on the cardiac valve. The VMFs, being the most predominant cells in the cardiac valve, perform a variety of functions to maintain normal valvular physiology. These functions, such as contraction, proliferation, and wound repair, are all directly or indirectly mediated by intracellular Ca^{2+} concentrations ([Ca^{2+}]_{i}). Thus, knowing how [Ca^{2+}]_{i} is regulated by vasoactive agents in VMFs becomes a critical step towards understanding valvular biology in both health and diseases.

Methods

Both VMFs and ECs were loaded with Ca^{2+}-sensitive fluorescent indicators fura-2 and/or fluo-4. The cells were visualized with a fluorimeter-coupled microscope system. Measurements of [Ca^{2+}]_{i} upon different pharmacological stimulations were done at room temperature (23 °C). Raw [Ca^{2+}]_{i} data were analyzed with respect to i) their amplitudes and curve areas (as for the
VMF experiments) and ii) their decline rates (as for the EC experiments). Also in the VMF experiments, the Mn\textsuperscript{2+}-quenching method was used to study divalent cation entry. Quantitative data were subjected to the appropriate statistical tests including Student’s t-tests and one-way ANOVA.

**Results and Conclusions**

**Part I: Ca\textsuperscript{2+} Mobilization by Pharmacological Agents in Valvular Myofibroblasts**

The fundamentals of Ca\textsuperscript{2+} signaling properties in cultured human VMFs were studied. In VMFs, two agonist-induced and one store-operated Ca\textsuperscript{2+} signaling pathways were identified, and observations of spontaneous Ca\textsuperscript{2+} releases were made. The main findings are listed below.

Histamine, upon binding to H\textsubscript{1} receptors, elicited [Ca\textsuperscript{2+}]\textsubscript{i} increase solely from ER Ca\textsuperscript{2+} release in normal VMFs. In rheumatic VMFs, however, histamine also stimulated Ca\textsuperscript{2+} influx. The purinergic agonists ATP and UTP activated P\textsubscript{2Y2} receptors and induced both ER Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} influx. The ER Ca\textsuperscript{2+} emptying stimulated by histamine and ATP/UTP was limited to an IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} store. A second ER compartment, the IP\textsubscript{3}-insensitive Ca\textsuperscript{2+} store, released Ca\textsuperscript{2+} in response to SERCA blockade, which depletes the ER Ca\textsuperscript{2+} content and stimulates store-operated Ca\textsuperscript{2+} entry. There was also evidence for the presence of RyRs in VMFs, as shown by the effect of RyR modulation on spontaneous Ca\textsuperscript{2+} releases.

Data in support of the above findings are summarized in brief below. The selective H\textsubscript{1} antagonist pyrilamine, but not the selective H\textsubscript{2} antagonist cimetidine, abolished the histamine-induced [Ca\textsuperscript{2+}]\textsubscript{i} response, which showed no difference in the presence or absence of extracellular Ca\textsuperscript{2+} or following treatment of SKF-96365, a blocker of Ca\textsuperscript{2+} entry. In a Ca\textsuperscript{2+}-free environment (or in the presence of SKF-96365), the duration of ATP/UTP-induced [Ca\textsuperscript{2+}]\textsubscript{i} response was shorter than in normal Ca\textsuperscript{2+}-containing solution. This suggested a Ca\textsuperscript{2+} influx component
regulated by purinergic receptor stimulation, as confirmed by accelerated Mn$^{2+}$ entry as well. The purinergic receptor subtype was characterized based on the following results: i) [Ca$^{2+}]_i$ response elicited by the selective $P_{2Y2}$ agonist UTP; and ii) the lack of [Ca$^{2+}]_i$ response in VMFs challenged with the selective $P_{2Y1}$ agonists ADPβS and 2-Me-S-ATP. The presence of the IP$_3$-insensitive ER Ca$^{2+}$ store was revealed by additional Ca$^{2+}$ release stimulated by CPA, following repeated applications of histamine and ATP. Store-operated Ca$^{2+}$ entry was activated by application of CPA which increased the rate of Mn$^{2+}$ entry. Unlike in normal VMFs, the curve areas of histamine-elicited [Ca$^{2+}]_i$ responses in rheumatic VMFs showed a significant increase between Ca$^{2+}$-containing and Ca$^{2+}$-free environments, indicating that in the diseased cells histamine also induced Ca$^{2+}$ entry. Finally, in a small number of VMFs spontaneous Ca$^{2+}$ releases in localized areas were observed. Blockade of the RyR elongated the latency period between each Ca$^{2+}$ releasing event, demonstrating the presence of functional RyRs in VMFs.

Part II: Mechanisms of Ca$^{2+}$ Removal in Vascular Endothelial Cells

How endothelial Ca$^{2+}$ homeostasis is maintained is still an unresolved issue, an important one though since cardiovascular diseases often prevail in the absence of controlled [Ca$^{2+}]_i$ regulation. In contrast to most published studies that investigated Ca$^{2+}$ entry pathways in ECs, the Ca$^{2+}$ removal mechanisms, which have equally vital roles in Ca$^{2+}$ homeostasis, were examined in this study. In an effort to study cells that resemble physiological conditions as much as possible, a preparation of freshly isolated ECs was used. It was found that two pathways of extruding cytosolic Ca$^{2+}$ were present after agonist stimulation in ECs. The sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase (SERCA), ryanodine receptor (RyR), and Na$^+$/Ca$^{2+}$ exchanger (NCX) were components of the first pathway. The SERCA actively transported cytosolic Ca$^{2+}$ into the endoplasmic reticulum (ER). The RyR is a Ca$^{2+}$ release channel on the
ER membrane. Ca\(^{2+}\) exited the ER via the RyR and diffused across a subplasmalemmal restricted space to the plasmalemmal NCX, where Ca\(^{2+}\) was extruded to the extracellular space. In parallel to the SERCA-RyR-NCX pathway, Ca\(^{2+}\) extrusion also occurred via the plasma membrane Ca\(^{2+}\)-ATPase (PMCA), constituting the second Ca\(^{2+}\) removal pathway in ECs.

Data supporting the above findings are summarized as follows. Inhibitors of the SERCA, RyR and NCX, which respectively were cyclopiazonic acid (CPA), ryanodine (at the high concentration of 100 \(\mu\)M) and Na\(^+\)-free solution (ONaPSS), each slowed Ca\(^{2+}\) removal rate to the same extent. Combinations of these treatments, e.g. CPA+ONa and ryanodine+ONa, did not reduce Ca\(^{2+}\) removal rate any further, indicating that the SERCA, RyR, and NCX are arranged in series. When the RyR was activated with caffeine or the low concentration of ryanodine (1 \(\mu\)M), Ca\(^{2+}\) extrusion was facilitated. On the contrary, stimulation of RyR (with 1 \(\mu\)M ryanodine) and inhibition of SERCA (with CPA) together slowed down Ca\(^{2+}\) extrusion, showing the sequential delivery of Ca\(^{2+}\) from SERCA to RyR. When the SERCA-RyR-NCX pathway was blocked, Ca\(^{2+}\) extrusion could be further inhibited by blocking the PMCA with carboxyeosin, suggesting the contribution of the plasmalemmal Ca\(^{2+}\) pump in removing cytosolic Ca\(^{2+}\).
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<table>
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<tr>
<td>%F/s</td>
<td>unit of divalent cation (or Mn$^{2+}$) entry rate; F$^{360}$ per second</td>
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<tr>
<td>α-nPSS</td>
<td>antibiotic-containing normal physiological salt solution</td>
</tr>
<tr>
<td>[Ca$^{2+}$],</td>
<td>cytosolic Ca$^{2+}$ concentration</td>
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<tr>
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<td>zero-Ca$^{2+}$ physiological salt solution</td>
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<td>zero Na$^+$ zero Ca$^{2+}$ physiological salt solution</td>
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<td>0NaPSS; 0Na</td>
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<td>ADP$\beta$S</td>
<td>adenosine 5'-O-(2-thio-diphosphate)</td>
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<td>cimetidine</td>
</tr>
<tr>
<td>COX-1</td>
<td>cyclo-oxygenase-1</td>
</tr>
<tr>
<td>COX-2</td>
<td>cyclo-oxygenase-2</td>
</tr>
<tr>
<td>CPA</td>
<td>cyclopiazonic acid</td>
</tr>
<tr>
<td>CVD</td>
<td>combined valvular disease</td>
</tr>
<tr>
<td>CytD</td>
<td>cytochalasin D</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDCF</td>
<td>endothelium-derived contracting factor</td>
</tr>
<tr>
<td>EDHF</td>
<td>endothelium-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>EDRF</td>
<td>endothelium-derived relaxing factor</td>
</tr>
<tr>
<td>EET</td>
<td>epoxyeicosatrienoic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>ET$_A$R; ET$_A$</td>
<td>endothelin (subtype) A receptor</td>
</tr>
<tr>
<td>ET$_B$R; ET$_B$</td>
<td>endothelin (subtype) B receptor</td>
</tr>
</tbody>
</table>
F_{340}/F_{380} \quad \text{ratio of emitting fluorescence at 510 nm from \text{Ca}^{2+}\text{-bound over \text{Ca}^{2+}\text{-free}}}

F_{360} \quad \text{fluorescence intensity at 510-nm emission following excitation at 360 nm}

fGF-2 \quad \text{fibroblast growth factor 2}

G protein \quad \text{guanosine 5'-triphosphate binding protein}

H_1R; H_1 \quad \text{histaminergic (subtype) 1 receptor}

H_2R; H_2 \quad \text{histaminergic (subtype) 2 receptor}

HEPES \quad 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid

His \quad \text{histamine}

hVMF \quad \text{human valvular myofibroblast}

ICCD \quad \text{intensified charge-coupled device}

IL-1 \quad \text{interleukin-1}

iNOS \quad \text{inducible nitric oxide synthase}

Ion \quad \text{ionomycin}

IP_3 \quad \text{inositol 1,4,5-triphosphate}

IP_3R \quad \text{inositol 1,4,5-triphosphate receptor}

K_{Ca} \text{ channel} \quad \text{calcium-dependent potassium channel}

K_d \quad \text{dissociation constant}

LA \quad \text{local anesthetic}

LatA \quad \text{latrunculin A}

LSCM \quad \text{rapid laser-scanning confocal microscope}

LuEry \quad \text{lupus erythematosus}

MCDB-131 \quad \text{Molecular and Cellular Developmental Biology-131}

MDMA \quad \text{3,4-methylenedioxymethamphetamine}

Mn \quad \text{manganese chloride}

Mn^{2+} \quad \text{manganese ion}

MV \quad \text{mitral valve}

Na^{+} \quad \text{sodium ion}

NAADP \quad \text{nicotinic acid adenine dinucleotide phosphate}

NCX \quad \text{Na}^{+}/\text{Ca}^{2+} \text{ exchanger}

NHA \quad \text{sodium-hydrogen antiporter}

NMDG \quad \text{N-methyl-D-glucamine}

NO \quad \text{nitric oxide}

defined physiological salt solution

NSCC \quad \text{non-selective cation channel}

P_{2X} \quad \text{purinergic (subtype) 2X receptor}

P_{2Y_1}R; P_{2Y_1} \quad \text{purinergic (subtype) 2Y1 receptor}

P_{2Y_2}R; P_{2Y_2} \quad \text{purinergic (subtype) 2Y2 receptor}

PGI_2 \quad \text{prostacyclin}

PLB \quad \text{phospholamban}

PLC \quad \text{phospholipase C}

PM \quad \text{plasma membrane}

PMCA \quad \text{plasma membrane Ca}^{2+}\text{-ATPase}

PMT \quad \text{photomultiplier tube}

PPADS \quad \text{pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid}

Pyr \quad \text{pyrilamine}
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Term</th>
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<tbody>
<tr>
<td>Rs</td>
<td>unit of area under curve; F340/F380 ratio × seconds</td>
</tr>
<tr>
<td>RhMVD</td>
<td>rheumatic mitral valve disease</td>
</tr>
<tr>
<td>ROC</td>
<td>receptor-operated Ca^{2+} channel</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SBB</td>
<td>superficial buffer barrier</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco(endo)plasmic reticulum Ca^{2+}-ATPase</td>
</tr>
<tr>
<td>SKF-96365; SKF</td>
<td>1-{α-[3(4-methoxyphenyl)propoxy]-4-methoxy-phenethyl}-1H-imidazole hydrochloride</td>
</tr>
<tr>
<td>SOC</td>
<td>store-operated Ca^{2+} channel</td>
</tr>
<tr>
<td>spCa^{2+} release</td>
<td>spontaneous subcellular Ca^{2+} release</td>
</tr>
<tr>
<td>t_{50}</td>
<td>time required for peak F340/F380 (or [Ca^{2+}]_i level) to decay by half</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>TRP channel</td>
<td>transient receptor potential channel</td>
</tr>
<tr>
<td>TV</td>
<td>tricuspid valve</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine 5′-triphosphate</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VMF</td>
<td>valvular myofibroblast</td>
</tr>
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</table>
LIST OF PUBLICATIONS AND ABSTRACTS

Material from this dissertation has been published or accepted for publication:


4. W Liang, M Buluc, C van Breemen, X Wang. 2004. Vectorial Ca\textsuperscript{2+} release via ryanodine receptors contributes to Ca\textsuperscript{2+} extrusion from freshly isolated rabbit aortic endothelial cells. *Cell Calcium*, in press.

Material from this dissertation is currently under preparation for submission:


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


2. W Liang, C van Breemen, X Wang. 2003. Role of ryanodine receptors in Ca\textsuperscript{2+} extrusion in fresh endothelial cells. *Frontiers in Cardiovascular Science*, Vancouver, Canada. (also in poster form)


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

1. W Liang, P McDonald, B McManus, C van Breemen, X Wang. 2002. Ca\textsuperscript{2+} compartments
in the endoplasmic reticulum of human mitral valvular myofibroblasts. *XIVth World Congress of Pharmacology*, San Francisco, USA.


Material from this dissertation has also been presented orally at the Graduate Student Seminar Series in the Department of Pharmacology and Therapeutics, University of British Columbia, and at the *Research in Progress* Seminars at the James Hogg iCAPTURE Centre for Cardiovascular and Pulmonary Research.
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CHAPTER I: INTRODUCTION

Myofibroblasts and endothelial cells are the two most prevalent cell types in the cardiac valve. Endothelial cells act as a barrier between the myofibroblasts and the circulating blood. With an average 50 mL stroke volume and 70 to 80 heartbeats per minute, the cardiac valves, and especially ECs, are consistently subjected to enormous hemodynamic forces. In response to different physiological requirements, ECs secrete various vasoactive substances to the surrounding tissues and circulation. Valvular myofibroblasts (VMFs), located beneath the ECs, are direct recipients of different endothelial secretions. These secretions may regulate numerous functions of VMFs including contraction and wound repair, etc. These processes likely relate to interactions between VMFs and ECs. In cases of valvular heart diseases, damages to ECs may expose VMFs to the circulating substances, altering the valvular biology at the same time. On the other hand, VMFs may proliferate, migrate, and attempt to remodel the functions of a healthy valve. Thus, ECs and VMFs are perhaps independently and cooperatively responsible for the maintenance of valvular physiology in both health and disease. However, many of the cellular signaling events involved are currently unknown, in spite of the growing incidence of valvular heart diseases in the world. The research field of signaling mechanisms in the cardiac valve is therefore a novel one, with it requires fundamental understanding of how ECs mediate the synthesis of vasoactive substances and how VMFs may respond to the endothelial secretions. This study will first look in detail at the agonist-induced Ca\(^{2+}\) signaling properties of VMFs. Then the Ca\(^{2+}\) removal mechanisms in ECs, which play significant roles in intracellular Ca\(^{2+}\) homeostasis and consequently regulating releases of secretory agents, are investigated. This introductory chapter will provide information relevant to the background, design, and outcomes of the current study.
In the following sections, the anatomical features of the cardiac valve are introduced first. Valvular myofibroblasts (VMFs) are multi-functional cells making up the bulk of the cardiac valve, and their various roles in valvular physiology, in both health and disease, will be summarized. Also discussed are the pharmacological characteristics of major vasoactive substances potentially modulating valvular function. Another type of valvular cells, the endothelial cells (ECs), lining the surface of cardiac valves, serves many functions: protective barrier for underlying tissues, control of valvular tone, and maintenance of blood fluidity. These functions of endothelial cells (ECs) are made possible by a number of secretory substances. Precise regulation of the amount and type of endothelial secretions is in large part mediated by controlled Ca\(^{2+}\) fluxes via various Ca\(^{2+}\) transporters. An overview will thus be given on the substances released by ECs and the entities responsible for Ca\(^{2+}\) homeostasis in ECs. Effects of damaged ECs on tissue physiology will also be discussed. Finally, the introductory chapter will close with a discussion of some examples of interactions between endothelial cells and myofibroblasts in other tissues, which provide supportive evidence for similar cell-cell communications in the cardiac valve.

1.1. Organization of the Cardiac Valve

During embryonic development, mesenchyma, descended from endocardial cells, grow out of the cardiac cushion to become the cardiac valve (Taylor et al. 2003). The cardiac valve is a multi-layered structural entity composed of four main cell types embedded in connective tissue matrix. The outermost valvular layers are the atrialis facing the atrial side and the ventricularis facing the ventricular side (Mulholland and Gotlieb 1996). Semi-lunar valves, also known as aortic and pulmonary valves, lack the atrialis layer that is only present in atrio-ventricular, or mitral and tricuspid, valves (Ferrans and Rodriguez 1991). Elastic fibres and smooth muscle
cells make up the atrialis layer, which only covers the proximal end of the valve. The free or distal end of the valve consists mainly of the spongiosa layer, and it is where most of the extracellular matrices are (Mulholland and Gotlieb 1996). Collagen is also present in the spongiosa, but most is located in the fibrosa, which is the thickest layer in the valvular leaflet (Mulholland and Gotlieb 1996). Chordae tendineae extend from the fibrosal collagen and into the endocardium.

Four types of cells are found in the cardiac valve. Figure 1.1.1 shows an illustration of a cross-sectional view of a cardiac valve. In the figure, bright-field images of enzymatically dissociated ECs and VMFs are also shown. The surface is lined with a continuous layer of ECs that extends along the atrial and ventricular endocardial surfaces (Mulholland and Gotlieb 1996). Cardiac myocytes are often present at the bases of atrio-ventricular valves (Mulholland and Gotlieb 1996). The base of the valvular leaflet contains blood vessels, and thus smooth muscle cells are always found in that region (Mulholland and Gotlieb 1996). Myofibroblasts are the most prominent cells in the valve and are present in all tissue layers (Taylor et al. 2000). VMFs are mostly arranged loosely \textit{in vivo}, with much intercellular space filled with extracellular matrices (ECMs) (Mulholland and Gotlieb 1996). Integrins form links between VMFs and ECMs and mediate cell proliferation and cell motility (Taylor et al. 2003). Communications among VMFs also occur where there are close contacts between cells, via adherens junctions (Lester et al. 1988) and gap junctions (Filip et al. 1986).
Figure 1.1.1. **Organization of the cardiac valve.** The diagram shows the main components of a cardiac valve. The cell types of interest in this study are the endothelial cells (ECs) and valvular myofibroblasts (VMFs), which are the most predominant cells in the valve. Cardiac and smooth muscle cells may be present near the valvular root. Brightfield microscopic images of enzymatically-dissociated ECs and VMFs are shown as reference. VMFs are arranged loosely *in vivo* and where there are close contacts, gap or adherens junctions form links between the cells. The majority of the space between VMFs are filled with extracellular matrices (ECMs), the synthesis of which are one of the important functions of VMFs.
1.2. Functional Roles of Valvular Myofibroblasts

Staining and functional studies on VMFs have revealed the various functions of these cells. Characteristics of smooth muscle cells are found in VMFs. Fluorescent labeling with anti-cGMP-dependent protein kinase, usually used as a smooth muscle marker, stains VMFs, but not fibroblasts and endothelial cells (Filip et al. 1986). Moreover, VMFs contains α-smooth muscle actin, although not as much as in smooth muscle cells (Zacks et al. 1991). Contractility of VMFs was first proposed when actin microfilaments, anchored in electron-dense bodies (Filip et al. 1986), colocalized with myosin in microfilament bundles (Lester et al. 1988). When VMFs are grown on a silicone rubber substratum, application of vasoactive substances results in wrinkling of the substratum, indicating contraction of VMFs (Filip et al. 1986; Messier et al. 1994). Studies of whole valvular leaflets have also demonstrated isometric force generation upon agonist stimulation (Filip et al. 1986; Taylor et al. 2000). Thus, VMFs probably contract in vivo to counter the enormous hemodynamic forces (Mulholland and Gotlieb 1997).

The structure and function of the cardiac valve are maintained by ECMs, and their secretion is another function of VMFs (Mulholland and Gotlieb 1997). The main constituents of valvular ECMs are collagen, elastin, and proteoglycans. The proteoglycans provide an attachment base for collagen and elastin (Mulholland and Gotlieb 1996). There is also suggestion that the proteoglycans alleviate friction between the fibrosa and ventricularis (Tamura et al. 1995). Types I and III collagens are present in the greatest amounts, but traces of Type V collagen are also found (Bashey and Jimenez 1988; Lis et al. 1987). Collagen helps the valve to maintain its shape in response to pressure. Elastin, on the other hand, provides flexibility to the valve so that it can distend accordingly to the pressure. Thus, the collagen-rich fibrosa, being the thickest layer in the valve, is able to provide strength while the elastin-rich atrialis and
ventricularis gives elasticity on the free ends of the valve. The ECMs, via regulating growth factors, mediate valvular growth and differentiation (Flaumenhaft and Rifkin 1991; Carey 1991). The distribution and organization of ECMs are altered in disease conditions, and these are reflected in valvular dysfunction. For example, ECM remodeling as a result of increased matrix metalloproteinase activities, is observed in valves of patients with Marfan syndrome (Segura et al. 1998). Elsewhere, Lis et al. reported that both rheumatic and floppy valves have higher collagen contents than normal tissues (Lis et al. 1987). The increase in collagen has different consequences between the two pathological conditions, however. In floppy valves, interactions between proteoglycans and collagen cease as well as cross-linking within core collagens, resulting in fragility of the tissue (Lis et al. 1987). Fibrosis due to overproduction of surface collagen is often observed in floppy but not rheumatic valves (Lis et al. 1987). In contrast to floppy valves, rheumatic valves contain a wealth network of core collagen that results in tissue thickening (Lis et al. 1987).

The modifications to valvular anatomy mentioned above are potential outcomes of valvular diseases, and the developments of which are triggered by endocardial injury. The injury may arise from surgical interventions in the treatment of cardiac and valvular diseases (Mulholland and Gotlieb 1996). As the immediate cells beneath the endocardial or endothelial layer, VMFs participate actively in valvular wound repair. The repair process does not end up with the normal endothelial monolayer but overlapping of cells is observed (Lester et al. 1988). It is the property of the newly formed valvular surface that suggests that VMFs, and not ECs, are involved in injury repair. A study used an organ culture model with the dissection site resembling the edge of the wound (Lester et al. 1992). Proliferating cells were stained with anti-Proliferating Cell Nuclear Antigen (anti-PCNA), since PCNA accumulates during active mitosis.
(Kurki et al. 1988). Lester et al. found that only VMFs were positively stained with anti-PCNA and that the immunolabeling only occurred near the dissection site, i.e. where the wound was located (Lester et al. 1992). Therefore, it is concluded that VMFs proliferate and migrate in the event of valvular injury.

1.3. Overview of Valvular Heart Diseases

Since many functions of VMFs revolve around disease conditions, it will be of interest here to discuss the indications of the most clinically relevant valvular heart diseases, with references to structural and cellular modifications. For detailed explanation on all types of valvular pathologies, the readers are advised to refer to Alpert et al. (2000). The occurrence of valvular heart diseases is much less often than coronary artery disease, but the former still contributes significantly to the cause of heart failure and sudden deaths (Wilson 1997; Mangion and Tighe 1995; Grigioni et al. 1999). Both valve-associated hospitalizations and surgical interventions in the New York State from 1983-2000 have seen a three-fold increase (Supino et al. 2002). The incidences of degenerative, drug-induced, and microorganism-induced valvular heart diseases are all on the rise (Soler-Soler and Galve 2000). People in the developed world are most seriously affected by age- and lifestyle-related valvular conditions whereas in developing countries, people are hit hardest by valvular problems arisen from hygienic issues.

In brief, valvular heart diseases may be classified according to their sites of occurrence. In the mitral valve (MV), prolapse and rheumatic disease are two common conditions. MV prolapse is a result of degeneration of the fibrosal tissue, and in severe cases can cause valvular thickening and chordal elongation, leading to poor structural support of the valvular leaflet (Farb et al. 2000). Calcification is also a symptom of MV prolapse, as well as in chronic rheumatic MV disease (RhMVD) (Farb et al. 2000). Figure 1.3.1 shows an image of a calcified mitral
valve from a 58-year-old male (Manabe and Yutani 1998). In chronic RhMVD, valvular scarring due to fusion of valvular structures results in mitral stenosis (Farb et al. 2000). Inflammatory cells and VMFs are present concurrently with capillary formation (Farb et al. 2000). Secondary effects of RhMVD and stenosis include congestive heart failure, dyspnea, and atrial fibrillation (Farb et al. 2000). Chronic RhMVD mostly happens in individuals with acute RhMVD previously. Acute RhMVD is usually a consequence of pancarditis, which is inflammation of all three layers of the heart (Farb et al. 2000). Pancarditis is often a result of an altered immune response to Group A hemolytic Streptococcus infections, and thus acute RhMVD remains prevalent in developing countries (Farb et al. 2000; Soler-Soler and Galve 2000). Other valvular pathologies may arise indirectly from RhMVD. In both aortic valve (AV) and tricuspid valve (TV), post-rheumatic valvular disease is a frequent problem, leading to symptoms similar to those in mitral stenosis (Farb et al. 2000). Chordal thickening is usually less severe in TV than in MV and AV (Farb et al. 2000). In AV, calcification, inflammation, and neovascularization may occur. Calcification is also seen in degenerative AV stenosis, which is associated with coronary atherosclerosis, increasing age, smoking, hypertension, and high low-density lipoprotein levels (Farb et al. 2000). AV regurgitation may derive from both post-rheumatic and degenerative AV diseases (Farb et al. 2000). Figure 1.3.2 shows an image of an aortic valve with fibrotic thickening causing it to close improperly (Manabe and Yutani 1998). Regurgitating valves of the left heart are particularly significant in causing dyspnea and fatigue.
Figure 1.3.1. Image of a calcified mitral valve. The valve was isolated from a 58-year-old male. There is prominent fibrotic thickening of the valvular leaflets (white portions near where the arrows are), in contrast to the transparent portions near the top of the figure. There are also obvious calcified deposits (yellow-appearing structures pointed by arrows), which are often observed in valvular heart diseases. [Figure reproduced from Manabe and Yutani 1998]
Figure 1.3.2. Image of an improperly closed aortic valve. The valve was isolated from a 54-year-old female. Fibrotic thickening of the valvular leaflets is evident and fusion of structures are observed in the distal (free) ends of the valve. The opening in the middle of the figure indicates improper closing of the valve, resulting in aortic regurgitation. [Figure reproduced from Manabe and Yutani 1998]
Besides valvulopathies specific to a particular valve, combined valvular disease (CVD) occurs in a small number of patients (Paraskos 2000). Rheumatic heart disease is the most common cause of CVD, but it may be associated with systemic and drug-induced diseases as well (Paraskos 2000). Conditions involving all four valves are extremely rare. Simultaneous MV stenosis and AV regurgitation/stenosis has much higher incidence than combined TV and MV stenosis (Paraskos 2000). There have been reports noting cardio-protective effects in CVD. In one example, MV stenosis protects the left ventricle from back pressure of AV regurgitation (Paraskos 2000). In another example, TV stenosis protects the right ventricle from the extensive effects of MV stenosis (Paraskos 2000).

As mentioned above, systemic conditions such as metastatic carcinoid tumor and lupus erythematosus (LuEry) are likely to result in CVD. Carcinoid tumor is vaguely defined as a tumor with characteristics not distinguishable from gastro-entero-pancreatic neuroendocrine tumor (Quaedvlieg et al. 2002). In both carcinoid tumor and LuEry-associated valvular dysfunctions, EC injury plays a significant role in disease development (Farb et al. 2000; Quaedvlieg et al. 2002). In carcinoid valvular disease, plagues of smooth muscle cells, proteoglycans, and probably VMFs adhere to valvular surfaces where ECs normally are (Farb et al. 2000). The presence of plagues results in thickening of valvular leaflets and stenosis.

Connolly et al. reported structural changes in valves, similar to those observed in carcinoid valvular diseases, in patients taking appetite suppressants (Connolly et al. 1997). This report shares a common aspect with studies that showed valvular dysfunction caused by ergot alkaloids (Redfield et al. 1992) and by 3,4-methylenedioxymethamphetamine (MDMA) in that the drugs tested in all three studies are serotonin (5-HT) agonists (Setola et al. 2003). These 5-HT mimetic agents are believed to activate 5-HT2 receptors, eliciting their effects on migraine
headache and on appetite suppression (Fitzgerald et al. 2000; Xu et al. 2002). Complicated signaling pathways downstream of 5-HT receptor stimulation have been elucidated that involves phospholipase C (PLC), protein kinase C, mitogen-activated protein kinase and extracellular signal-related kinase pathways. These pathways regulate hundreds of cellular functions that require detailed discussion of each. Nevertheless, the fact that carcinoid tumors also releases 5-HT may explain why Connolly et al. observed similar morphological changes in anorexigen-induced valvular disease.

Despite the diverging signaling cascades induced by 5-HT, the PLC pathway raises particular interests. This is partly due to the PLC-regulated intracellular Ca\textsuperscript{2+} mobilization common to many pharmacological agonists. More importantly, many valvulopathies discussed in this section have no known 5-HT-related effects, but display structural modifications including calcification. No report has explored the relationship between calcified valves and Ca\textsuperscript{2+} signaling properties in VMFs. However, there were studies showing evidence for the role of Ca\textsuperscript{2+} mobilization in mediating calcification in canine fibroblasts (Kim 1994; Kim et al. 1999). Therefore, associations between VMFs’ intracellular Ca\textsuperscript{2+} responses to agonists may provide clues to the development of valvular heart disease.

1.4. Circulating Vasoactive Substances and Their Pharmacological Aspects

Considering the many activities of VMFs in maintaining a healthy valve and in remodeling a diseased valve in the previous sections, it is unclear why research has not been emphasized in investigating the underlying signaling mechanisms. The finding that VMFs are contractile was rather significant because of its implications on structural support, cell motility and wound closing. However, there were only limited research efforts to examine agonist-induced contractions of valvular leaflets and VMFs. Filip et al. reported for the first time ever
the contractility of single VMFs under treatments of epinephrine and angiotensin II (Filip et al. 1986). Almost eight years later Messier et al. reported that bradykinin, carbachol, and KCl also induced contractions in VMFs (Messier et al. 1994). Neither study attempted to pharmacologically characterize the receptors involved in contracting the cells though. It was until the year of 2000 that the first comprehensive study was conducted on agonist-induced valvular contractions (Chester et al. 2000). A variety of vasoactive substances and their respective receptor antagonists were applied to intact valvular leaflets. Their force generations were measured and concentration-response relationships were established. In the study, epinephrine, norepinephrine, endothelin-1, thromboxane A$_2$, 5-HT, and histamine, all of which are important vaso-modulators, were used to elicit the contractions. However, by looking at the entire leaflet, the drug effects on VMFs alone were not examined, since it has been documented that smooth muscle cells might also be present near the valvular root (Mulholland and Gotlieb 1996). In view of this shortfall, and the regulatory role of histamine and adenosine 5'-triphosphate (ATP), respectively, in inflammation and ischemia, the following paragraphs will discuss about the pharmacological characteristics of these two agents.

Three subtypes of histamine receptors have been identified, but only two, H$_1$ and H$_2$, are present in the heart. H$_1$ receptor (H$_1$R) activation reduces atrio-ventricular node conduction (Hattori 1999). Stimulation of H$_2$ receptors (H$_2$R) increases heart rate (Hattori 1999). Increased cardiac contractility is a result of combined effects of H$_1$ and H$_2$ receptors, depending on the species (Hughes and Coret 1972; Verma and McNeill 1977). Both H$_1$R and H$_2$R are coupled to GTP-binding proteins (G proteins): H$_1$R with the G$_q$ subunit, and H$_2$R with the G$_s$ subunit. Stimulation of H$_1$R leads to the activation of phospholipase C, production of IP$_3$ and increase in $[Ca^{2+}]_i$ (Hill 1990). H$_2$R-induced $[Ca^{2+}]_i$ increase is also present in certain cells (Chew 1985,
Mitsuhashi et al. 1989), but the most common effect of H$_2$R activation is the stimulation of adenylate cyclase and production of cAMP (Hill 1990). It should be noted that a hypothesis for the presence of an intracellular histamine receptor is supported by some studies (Saxena 1989; Brandes 1990, 1992). The purpose of the proposed intracellular histamine receptor is in the mediation of tumor cell proliferation (Bartholeyns and Bouclier 1984; Bartholeyns and Fozard 1985; Watanabe et al. 1981), although the existence of such a receptor has not been widely accepted. Noting the proliferating role of VMFs in the cardiac valve, there may be a function for the intracellular histamine receptor in these cells.

ATP increases cardiac contractility via actions on both P$_{2X}$ and P$_{2Y}$ receptors (Vassort 2001). The lack of specific ligands has slowed the progress on the characterization of purinergic receptors and generated conflicting reports on receptor subtypes. In the heart, P$_{2X1-7}$ receptors have been found and are all ionotropic, meaning the receptors function as nonselective cation channels, with permeability to Na$^+$, K$^+$, and Ca$^{2+}$ (Bean 1992, Dubyak and el-Moatassim 1993). All P$_{2Y}$ receptors are coupled to G-proteins. Five of a total 11 P$_{2Y}$ receptor subtypes are present in the heart: P$_{2Y1}$, P$_{2Y2}$, P$_{2Y4}$, P$_{2Y6}$, and P$_{2Y11}$ (Vassort 2001). ATP is fully agonistic on all P$_{2Y}$ subtypes except P$_{2Y4}$, and UTP is only active on P$_{2Y2}$ and P$_{2Y4}$ (Vassort 2001). P$_{2Y2}$ and P$_{2Y4}$ were formerly known as P$_{2U}$ receptors because of their activation by UTP (Ralevic and Burnstock 1998). It is possible to distinguish P$_{2Y2}$ from P$_{2Y4}$ receptors by the latter’s nonresponsiveness to ATP. Activation of P$_{2Y2}$ receptors results in PLC-mediated IP$_3$ production and increased [Ca$^{2+}$], although cAMP and cGMP production have also been reported (Post et al. 1996; Buvinic et al. 2002).
1.5. Pathophysiology of Endothelial Cells – Implications on Valvular Heart Diseases

The discussion so far has been limited to the roles of VMFs in valvular (patho)physiology. Indeed VMFs are the key players in the progression of valvular heart diseases. However, it is often the ECs which become dysfunctional or injured that initiate signaling events leading to the activation and proliferation of VMFs in valvular heart diseases. Such interactions between ECs and VMFs are implied because of suggestive roles of excessive nitric oxide (NO), fibroblast growth factor-2 (fGF-2), and endothelin-1, all secreted by injured ECs, in repairing valvular and vessel damages (Durbin and Gotlieb 2002; Siney and Lewis 1993; Villaschi and Nicosia 1993). Specifically, the modified secretions from ECs are likely the results of dysfunctional Ca\(^{2+}\) homeostasis. In a number of vascular abnormalities such as reperfusion injury, atherosclerosis, and post-operative grafts, there are associations with altered Ca\(^{2+}\) signaling mechanisms in both ECs and smooth muscle cells (Lounsbury et al. 2000; Irani 2000; Mehta et al. 1997). Direct evidence for modified endothelial Ca\(^{2+}\) signaling properties has not yet been shown in valvular heart diseases, but pathological changes to the structure and functions of ECs similar to those in vascular diseases are observed. A few examples are cited here. In endocarditis, thrombosis attracts adhesion molecules and valvular thickening and stenosis develop (Lopez et al. 1987). Inflammatory responses arisen from rheumatic valvular disease render the ECs antigenic and subject to attack by antibodies (Galvin et al. 2000). Floppy valves may lose ECs due to improper valvular closing and results in activation of cell proliferation signals and direct exposure of the sub-endothelial cells (i.e. valvular myofibroblasts) to vasoactive agents (Stein et al. 1989). In view of the damages to ECs in valvular heart diseases, modifications to endothelial Ca\(^{2+}\) signaling and resultant secretory products may be detected in future studies. Meanwhile, it is
important to first understand the relationship between \( \text{Ca}^{2+} \) homeostasis in ECs and their releases of vasoactive substances, which will be discussed next.

### 1.6. Pharmacology and Endocrinology of Endothelial Cells

The vaso-modulatory function of ECs has been extensively studied and reviewed. Although there is very little information about endothelial influences on the VMFs, it is postulated that interactions similar to those between ECs and vascular smooth muscle also exist in the cardiac valve. In the vasculature, ECs secrete a variety of substances that bind to membrane receptors of smooth muscle cells or diffuse across the vessel wall with an ultimate effect on contractility and in *in vivo* conditions, result in a change in blood pressure. Secretions by ECs for this purpose may be classified into endothelium-derived contracting factors (EDCFs) and endothelium-derived relaxing factors (EDRFs). Each EDCF and EDRF not only elicits its own independent effects but also interacts with each other to mediate vascular smooth muscle function. Figure 1.6.1 summarizes the most common endothelial secretions and their actions on the underlying vascular smooth muscle or valvular myofibroblasts.
Figure 1.6.1. **Endothelial secretions and their actions on tissue contractility.** This schematic diagram shows the most common secretions by the endothelial cell (EC) that contribute to the contraction or relaxation of the underlying vascular smooth muscle (VSM) or valvular myofibroblast (VMF). The EC synthesizes relaxing factors: i) nitric oxide (NO) by endothelial nitric oxide synthase (eNOS); ii) prostacyclin (PGI₂) by cyclo-oxygenases 1 & 2 (COX-1,2); and iii) epoxyeicosatrienoic acid (EET) by cytochrome P450 (CYP). EET activates $\text{Ca}^{2+}$-sensitive potassium ($K_{\text{Ca}}$) channels on EC or VSM, allowing $K^+$ efflux and membrane hyperpolarization. An alternative theory suggests that $K^+$ efflux from EC diffuse to VSM and activates the inward-rectifying $K^+$ ($K_{\text{IR}}$) channels, causing relaxation. There is also evidence for the function of myo-endothelial gap junctions between EC and VSM where hyperpolarizing signals (or electrical charges, $q$) are transmitted across the cellular layers. Contracting factors synthesized by EC are: i) excessive NO from eNOS and iNOS; ii) ROS including $\text{H}_2\text{O}_2$; iii) endothelin-1 (ET-1) from epinephrine, thrombin, interleukin-1, and angiotensin II (AngII) stimulations of EC – ET-1 then acts on $\text{ET}_A$ receptor ($\text{ET}_A$R); and iii) autocrine release of AngII acting on $\text{AT}_1$ receptor ($\text{AT}_1$R).
Nitric oxide (NO) is a well-established example of EDRF. Endothelial nitric oxide synthase (eNOS) oxidizes L-arginine to form NO and citrulline. In normal physiological conditions, a small amount of eNOS is constitutively expressed, contributing to basal NO release (Nishida et al. 1992; Weiner et al. 1994). Low levels of NO facilitates cyclic 3'-5'-guanosine monophosphate (cGMP) production via guanylate cyclase. cGMP-dependent protein kinases in vascular smooth muscle induce vasodilatation (Huang et al. 2000). It was mentioned in Section 1.2 that cGMP-dependent protein kinases are present in valvular myofibroblasts (VMFs). Thus, NO from the endothelium may influence the VMFs underneath in the cardiac valve. Both shear stress and pharmacological agents such as acetylcholine and histamine can elicit \([\text{Ca}^{2+}]_i\) increase in ECs. The associations between \([\text{Ca}^{2+}]_i\) and NO are strengthened by the elucidation of a negative feedback system which shows enhanced cytosolic \([\text{Ca}^{2+}]_i\) removal following application of cGMP (Lau et al. 2003). Increase in endothelial \([\text{Ca}^{2+}]_i\) is followed by transient NO production in the same time frame, i.e. several seconds. However, continuous NO release is maintained by another mechanism. Studies have shown that activation of phosphatidylinositol-3-kinase, serine kinases Akt, and protein kinase A leads to the phosphorylation of the reductase domain of eNOS, increasing its activity (Dimmeler et al. 1999; Go et al. 1998). Another NOS, the inducible NOS (iNOS), is expressed when ECs are stimulated with cytokines. Large amounts of NO are produced by iNOS. However, the excessive NO is also a source of reactive oxygen species, which are regarded as EDCFs.

Besides inducing its own direct effects on vascular relaxation, NO also modulates the production of another EDRF – prostacyclin (PGI₂) (Marcelin-Jimenez and Escalante 2001). Aside from NO regulation, PGI₂ production is mediated by cyclooxygenases 1 and 2 (COX-1, COX-2). In ECs, COX-1 is constitutively present while COX-2 is only expressed in
inflammatory reactions (Davidge 2001). Similar to NO, PGI₂ synthesis is dependent on endothelial \([Ca^{2+}]_i\), except with two differences. Firstly, higher \([Ca^{2+}]_i\) than in NOS activation is necessary for the activation of phospholipase A₂ and generation of arachidonic acid, which is the substrate of COX. Secondly, unlike NO release upon shear stress, cyclic stress of the vessel stimulates PGI₂ production (Rosales et al. 1997).

The third group of EDRFs is endothelium-derived hyperpolarizing factor (EDHF). The identity of EDHF(s) is still debatable, but three major theories have been developed with all showing relationships with endothelial \(Ca^{2+}\)-dependent \(K^+\) (\(K_{Ca}\)) channels. Campbell et al. proposed that endothelial cytochrome P450 epoxygenase generates epoxyeicosatrienoic acids (EETs), activating \(K_{Ca}\) channels on either ECs or smooth muscle cells (Campbell et al. 1996). Another theory claims \(K^+\) exiting endothelial \(K_{Ca}\) channels diffuses to smooth muscle cells and hyperpolarizes the cells by opening inward-rectifying \(K^+\) channels (Edwards et al. 1998). Finally, Segal et al. proposed tightly coupled myo-endothelial gap junctions transmit the hyperpolarizing signal across the vessel wall (Emerson and Segal 2000).

It was mentioned earlier that excessive NO could be a source of reactive oxygen species (ROS), which is an example of EDCF. Though, the primary ROS released by ECs are superoxide anions and \(H_2O_2\) (Hishikawa et al. 1997). Although ROS synthesis is often related to oxidative stress-induced vascular diseases such as atherosclerosis, EC production of ROS occurs in normal conditions as well. To further complicate the issue, ROS do not always cause vasoconstriction. Matoba et al. suggested that \(H_2O_2\) has vasodilatory effects and is a potential EDHF (Matoba et al. 2002). In addition, \(H_2O_2\), by increasing endothelial \([Ca^{2+}]_i\), can activate eNOS, producing NO that stimulates guanylate cyclase in smooth muscle cells, and the increased cGMP levels cause vasorelaxation (Yang et al. 1999; Iesaki et al. 1999).
Endothelin-1 (ET-1) is another main EDCF having vasodilatory effects. ECs usually release ET-1 when stimulated with angiotensin II, interleukin-1, thrombin, and epinephrine (Drexler 1997). When bound to ET<sub>A</sub> receptors on smooth muscle cells, ET-1 causes potent vasoconstriction (Busse and Fleming 2003). However, ET-1 also binds to endothelial ET<sub>B</sub> receptors which, upon activation, have stimulatory effects on NO and PGI<sub>2</sub> syntheses (Busse and Fleming 2003; Lavallee et al. 2001). Vasodilatation is undoubtedly caused by the increased NO and PGI<sub>2</sub> production, as a consequence of the ET-1-mediated negative feedback (Ullrich and Bachschmid 2000; Wagner et al. 2001). Moreover, NO and PGI<sub>2</sub> enhance, respectively, the production of cGMP and cAMP in ECs, and these cyclic nucleotides inhibit additional ET-1 synthesis and release (Boulanger and Luscher 1990).

1.7. Endothelial Ca<sup>2+</sup> Transporters: Channels, Pumps, Exchangers

It is clear from the above discussion of EDCFs and EDRFs that intracellular Ca<sup>2+</sup> plays a central role in either the synthesis or modulation of the secretory substances. In the following paragraphs, the main endothelial Ca<sup>2+</sup> transporters are introduced, with an emphasis on those involved in unloading cytosolic Ca<sup>2+</sup>. Details on all Ca<sup>2+</sup> transporters are extensively reviewed in Nilius and Droogmans (2001), Brini and Carafoli (2000), and Tran et al. (2000).

Membrane-bound Ca<sup>2+</sup> entry channels and endoplasmic reticulum (ER) inositol 1,4,5-triphosphate receptors (IP<sub>3</sub>Rs) are the two main endothelial Ca<sup>2+</sup> channels responsible for generating [Ca<sup>2+</sup>]<sub>i</sub> increases in response to physiological and pharmacological stimuli. Most stimuli bind to G-protein-coupled receptors and activate phospholipase C (PLC) with a production of IP<sub>3</sub>. The IP<sub>3</sub> opens IP<sub>3</sub>R and stimulates ER Ca<sup>2+</sup> release. In ECs, the IP<sub>3</sub>R3 isoform is most commonly expressed (Mountian et al. 1999). Slightly after or concurrently with IP<sub>3</sub>R activation, Ca<sup>2+</sup> entry is often facilitated via a variety of channels. These include the
nonselective cation channels (NSCCs) and store-operated Ca\textsuperscript{2+} channels (SOCs). Agonist-induced Ca\textsuperscript{2+} influx may occur via both NSCCs and SOCs, although the latter also transport Ca\textsuperscript{2+} when the ER store is depleted and is more Ca\textsuperscript{2+}-permeable than NSCCs (Nilius and Droogmans 2001). In some cells, however, there is not a clear distinction between the function of NSCCs and SOCs, as both are activated upon agonist stimulation (Wang and van Breemen 1997). The molecular identities of NSCCs and SOCs vary greatly among species and types of ECs. Transient receptor potential (TRP) channels are proposed as the major pathway via which Ca\textsuperscript{2+} enters ECs, and TRP-1 to TRP-6 isoforms have been found in different species (Nilius and Droogmans 2001). A minor endothelial Ca\textsuperscript{2+} entry pathway may be via the reverse-mode Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) (Teubl et al. 1999).

The NCX and plasma membrane Ca\textsuperscript{2+}-ATPase (PMCA) are the two main membrane-bound transporters that extrude Ca\textsuperscript{2+} to the extracellular space. The NCX has an affinity to Ca\textsuperscript{2+} at $K_D > 1 \mu M$, compared to that of PMCA at $K_D < 0.5 \mu M$. The $K_D$ difference between NCX and PMCA provides some insights to their localization and function. The PMCA, although very sensitive to Ca\textsuperscript{2+}, is not the optimal machinery for Ca\textsuperscript{2+} removal after agonist stimulation, which generally results in $[Ca^{2+}]_i$ of several hundred nanomolars. Most of the elevated Ca\textsuperscript{2+} is thus drawn towards the NCX for extrusion purposes. Because of the relative insensitivity to Ca\textsuperscript{2+}, NCX is thought to cluster in parts of the plasma membrane (PM) where there is high subplasmalemmal local $[Ca^{2+}]_i$ (Philipson 1999). With reference to the superficial buffer barrier (SBB) theory proposed by van Breemen et al. (van Breemen et al. 1995), effective Ca\textsuperscript{2+} sequestration by the ER is only possible when there is continuous Ca\textsuperscript{2+} extrusion via NCX. Thus, large amounts of Ca\textsuperscript{2+} pass from the ER to the NCX in any given period. The restricted space between the PM and ER, with a width of about 20 nm, is therefore a likely area in the cell with a
localized Ca$^{2+}$ concentration of a few micromolars. Indeed, modulation of NCX activity influences Ca$^{2+}$ release from the ER (Paltauf-Doburzynska et al. 1998), suggesting the close proximity between NCX and the ER.

If NCX and ER are located near each other, Ca$^{2+}$ uptake and release by the ER becomes important in ensuring that Ca$^{2+}$ is properly delivered to the exchanger. The sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase (SERCA) on the ER takes up Ca$^{2+}$ at a faster rate than Ca$^{2+}$ leaks out spontaneously (Tran et al. 2000). Two isoforms, SERCA1 and SERCA3, are found in ECs and both are sensitive to blockade by cyclopiazonic acid (CPA) (Mountian et al. 1999). In the presence of CPA, SERCA stops pumping in Ca$^{2+}$, and there is a net Ca$^{2+}$ loss from the ER, causing [Ca$^{2+}$]$_i$ increase and activation of SOCs. Store-operated Ca$^{2+}$ entry has also been demonstrated in ECs with the opening of ryanodine receptors (RyRs) (Paltauf-Doburzynska et al. 1998). The presence of RyRs in ECs has been shown implicitly in several reports (Wang et al. 1995; Frieden and Graier 2000; Ziegelstein et al. 1994). These receptors, unlike the IP$_3$Rs, are unable to elicit recordable [Ca$^{2+}$]$_i$ changes in ECs, despite their Ca$^{2+}$ releasing properties. Recently the RyR3 isoform is found in human ECs (Kohler et al. 2001), but other studies have also shown endothelial RyRs having similar functions to the cardiac RyR2 in modulating Ca$^{2+}$ oscillations (Jacob et al. 1988; Neylon and Irvine 1990; Laskey et al. 1992). RyRs may have a variety of functions in different ECs. For example, besides having an effect on SOCs, RyR opening also activates K$_{Ca}$ channels in human ECs (Frieden and Graier 2000). On the other hand, the role of RyRs in removing cytosolic Ca$^{2+}$ from native rabbit ECs will be demonstrated in the Results Section.
1.8. Examples of Interactions Between Endothelial Cells and Myofibroblasts

The ultimate goal of finding the link between endothelial Ca$^{2+}$ homeostasis and its influences on the functions of VMFs is largely based on a wealth of information on endothelial cell-smooth muscle interactions. One of the most investigated topics in cardiovascular physiology is the regulation of vascular smooth muscle tone by endothelial cells (ECs). A recent example is the use of a bioassay system where ECs act as the donor of secretory agents, and the detection of nitric oxide-, prostacyclin-, and EDHF-mediated contractions in a de-endothelialized vessel (Kuang, et al. 2003). Myofibroblasts, which themselves possess smooth muscle-like contractile properties, are known to interact with various cell types. In the cornea, injury to the epithelium results in keratocyte apoptosis mediated by interleukin-1 (IL-1) and tumor necrosis factor-α (TNFα) (Wilson et al. 2003). The apoptotic events also promote myofibroblast migration to the site of injury (Mohan et al. 2003), thus indicating potential communicative pathways between epithelial cells and myofibroblasts. Interestingly corneal endothelial cells also secrete IL-1 and TNFα, both of which are key players in the motility of myofibroblasts, suggesting possible interactions with ECs as well (Wilson et al. 1994). In another example, myofibroblasts and ECs lie in proximity in the portal circulation. In the healthy (or quiescent) state, myofibroblasts in the liver are more commonly referred as hepatic stellate cells (Dooley et al. 2000). In liver cirrhosis, the fenestrae between ECs are gradually lost, making diffusion across the endothelial layer more difficult (Ramadori and Saile 2002). At the same time, accumulation of cytokines stimulates proliferation and movement of myofibroblasts, in attempts to repair the injured tissue (Knittel et al. 2000). This EC barrier later becomes a basal membrane for the adhesion of more myofibroblasts, thus forming a septum between the hepatocytes and the circulation (Ramadori and Saile 2002). Although not investigated in detail, EC-myofibroblast
interactions are thought to partially trigger the series of events in the damaging liver (Ramadori and Saile 2002). Rather than isolating tissues from blood vessels, myofibroblasts facilitate neovascularization in tumor cells. In an ovarian carcinoma cluster, co-culture of ECs has no effects whereas in the presence of myofibroblasts, both cells invade the tumor (Walter-Yohrling et al. 2003). The EC-myofibroblast coupled invasion is prevented by angiogenesis inhibitors (Walter-Yohrling et al. 2003). It is speculated that myofibroblasts secrete growth factors and extracellular matrices that promote contacts with ECs and the migration of both cell types into the tumor (Walter-Yohrling et al. 2003). Yet interactions between ECs and myofibroblasts are not always only implicated in pathological conditions. In valvular transplant, immune responses elicited by the recipient’s T-cells against the donor valvular ECs have long been known for their roles in graft rejection (Hengstenberg et al. 1990). A study by Batten et al. shows that although the surface antigens are similar on both valvular myofibroblasts and ECs, only the latter are immunogenic and a proliferation of T-cells is detected (Batten et al. 2001). A co-culture of valvular ECs and myofibroblasts also abolishes the T-cell response, suggesting that VMFs interact with and protect ECs from inducing an immune response (Batten et al. 2001). This finding will likely contribute positively to the design of improved preparative procedures for donor valves. From a developmental point of view, the argument for valvular EC-myofibroblast communication is strengthened by the way the heart tube is formed. The heart tube is the embryonic stage of the vascular system, and two endocardial cushions are believed to have significant roles in the looping of vessels and segmentation of the heart (Mulholland and Gotlieb 1997). The endocardial cushions are where protrusions occur later in development, eventually forming the valves (Moore 1988). In summary, despite limited evidence, interactions between ECs and myofibroblasts play significant roles in multiple areas of medicine.
CHAPTER II: Rationale of Study, Specific Aims, and Hypothesis

As explained earlier, the cardiac valve is made up of mostly endothelial cells and myofibroblasts. Both normal functioning and diseased alterations of the valve likely depend heavily on endothelial cell-myofibroblast interactions. Myofibroblasts possess contractile properties, and their interactions with ECs may in part resemble those between smooth muscle and endothelium in the blood vessel. Decades of work has been devoted to the elucidation of signaling mechanisms within the vasculature, but with a significant clinical prevalence of valvular heart diseases, there is pressing need for the study of cardiac valvular cell physiology. Thus, the ultimate goal of research in valvular biology, in connection with the published articles, is to foster further investigation in the underlying causes of valvular heart diseases. In order to achieve this goal, the pharmacology and physiology of the myofibroblast, a major functional constituent of the cardiac valve, must be well understood first. Unfortunately, information regarding ionic (e.g. Ca$^{2+}$) signaling mechanisms in valvular myofibroblasts (VMFs) is seriously lagging behind the research on endothelial cells. Therefore, before designing experiments aiming at resolving myofibroblast-endothelial cell cross-talk in the cardiac valve, attempts are put in to investigate the fundamental signaling properties of VMFs. With that in mind, the first specific aim of this study is developed as follows:

**Specific Aim #1**

*Characterization of Plasma Membrane and Endoplasmic Reticular Receptor-Mediated Ca$^{2+}$ Signaling Pathways in Valvular Myofibroblasts – Identifying*

i) Histaminergic & Purinergic Receptor Subtypes and

ii) Functional Store-Operated Ca$^{2+}$ Channels and Ryanodine Receptors
The findings from Specific Aim #1 will yield basic but vital knowledge about VMF physiology. In the long run, follow-up studies will enrich the information database of VMFs. Only until then will studies aiming at examining valvular cell-cell interactions achieve their maximal potential, especially since much is already known about endothelial cells (ECs). Interesting enough, with over 100,000 publications ranging from single-cell to whole-tissue studies, there are still knowledge gaps in some areas of Ca\(^{2+}\) signaling and secretory pathways in ECs. With growing interests in intracellular Ca\(^{2+}\) regulation and its correlation with endothelium-related diseases, it becomes essential to contribute to the full understanding of endothelial Ca\(^{2+}\) signaling. Ca\(^{2+}\) homeostasis depends equally on ion influx and extrusion, and the attention for the second part of this study is drawn to the removal mechanisms of cytosolic Ca\(^{2+}\) after agonist stimulation of ECs. Freshly isolated ECs are used here since they resemble *in vivo* physiology much closer than cultured ECs do. It is also noted that aortic ECs from rabbits are used because of the immense challenge in obtaining healthy human valvular endothelium. In spite of this, the findings here are readily applicable to ECs in general since an earlier publication from our laboratory has found that the physiological characteristics of rabbit valvular ECs (Li and van Breemen 1995) resembled those from the aorta. Having justified the use of freshly isolated aortic ECs, the second specific aim is developed as follows:

**Specific Aim #2**

*Elucidation of Ca\(^{2+}\) Extrusion Pathways in Vascular Endothelial Cells –*

*Contribution of Sarco(Endo)plasmic Reticulum Ca\(^{2+}\)-ATPase, Ryanodine Receptor, Na\(^{+}\)-Ca\(^{2+}\) Exchanger and Plasma Membrane Ca\(^{2+}\)-ATPase*

It is evident from the specific aims that two seemingly distantly related research questions are being pursued at the same time. It should be emphasized again that the objective of
this study is to innovate further research on both ECs and VMFs, leading to the eventual understanding of the development of valvular diseases. Keeping the objective in mind, the working hypothesis also reflects different levels of investigation in both types of cells. With very little being known about VMFs other than their smooth muscle-like contractile functions, it is reasonable to propose that VMFs possess Ca\textsuperscript{2+} signaling properties similar to those in smooth muscle. It has long been established that smooth muscle cells respond to vasoactive substances by eliciting [Ca\textsuperscript{2+}]\textsubscript{i} increases and the sources of Ca\textsuperscript{2+} are from intracellular releases and/or extracellular entry. Thus, this phenomenon of smooth muscle forms the basis of the first hypothesis:

**Working Hypothesis #1**

*Ca\textsuperscript{2+} mobilization in valvular myofibroblasts is regulated by ER Ca\textsuperscript{2+} release and extracellular Ca\textsuperscript{2+} influx, with varying sensitivities to different agents.*

With regards to the endothelial cells, SERCA, NCX and PMCA are all known Ca\textsuperscript{2+} transporters involved in removing cytosolic Ca\textsuperscript{2+} in other cell types, and their roles in ECs will be confirmed here. NCX, having a low Ca\textsuperscript{2+} affinity, is activated by high [Ca\textsuperscript{2+}]\textsubscript{i} and is thus probably situated near the ER where the majority of Ca\textsuperscript{2+} is. As a result, it is postulated that SERCA and NCX function in one unit in removing Ca\textsuperscript{2+}. Opening of RyR will provide continual feeding of Ca\textsuperscript{2+} from the ER to NCX for extrusion. In contrast, PMCA has a high Ca\textsuperscript{2+} affinity and will respond to small fluctuations in cytosolic [Ca\textsuperscript{2+}]\textsubscript{i}, apart from influences from the ER. In summary, the second working hypothesis is formulated as follows:

**Working Hypothesis #2**

*Two parallel Ca\textsuperscript{2+} removal pathways are present in vascular endothelial cells: one consisting of SERCA, RyR and NCX in series, and the other involving PMCA.*
CHAPTER III: MATERIALS AND METHODS

3.1. Preparation of Valvular Myofibroblasts

Human cardiac valves were collected by the Cardiovascular Registry at St. Paul's Hospital, Vancouver, Canada, from explanted hearts obtained at the time of transplantation according to guidelines set forth by the UBC-Providence Health Care Care Research Ethics Board. A total of five valve specimens (four mitral valves, one tricuspid valve) from five patients ranging from age 45 to 64 years were used as sources of normal human valvular myofibroblasts (hVMFs). The clinical cause of heart failure was ischemic cardiomyopathy in three of five patients and dilated cardiomyopathy in the remaining two patients. None of the patients were suffering from valvular diseases. Nor were the patients receiving anti-migraine medications which, according to some studies can potentially cause valvular dysfunction. The valvular leaflets used to prepare the cellular preparations were examined by a pathologist and were determined to be grossly normal in that there was neither thickening of tissue nor evidence of calcium deposits.

Valves were procured from the explanted hearts as soon as possible after transplantation and stored briefly in cold, sterile medium. A section of the valvular leaflet immediately adjacent to the piece taken for culture was fixed in 10 % neutral-buffered formalin, paraffin-embedded and histochemically stained for microscopic analysis. Only the distal two-thirds of the valvular leaflet was used to extract the hVMFs. This was done to avoid contamination with cardiac myocytes positioned toward the base or proximal end of the valve. In addition, valve chordae were removed prior to culture to prevent fibroblast contamination of the cultures. Endothelial cells on the surface of the valve were removed by gentle rubbing on filter paper. The valve tissue was then minced and placed in a sterile container with 1 % Type I collagenase at 37 °C for
4 hours. The hVMFs were dissociated from the valve tissue by gentle vortexing, and the cell-tissue mixture was separated using a 70-μm filter. The hVMFs were small enough to pass through the filter and were collected in Molecular and Cellular Developmental Biology-131 (MCDB-131) medium supplemented with 1.18 g/L sodium bicarbonate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 20 % newborn calf serum. The hVMFs were grown to confluence in an incubator humidified with 95 % O₂ / 5 % CO₂ at 37 °C. Cell medium was changed every 48 hours. The hVMFs were subcultured for one to two passages to expand cell numbers, aliquoted into cryovials, and frozen in liquid nitrogen for long-term storage. At one week prior to the experiments, hVMFs were thawed and maintained in culture using standard tissue culture techniques. The hVMFs used in this study have undergone four to eight passages in secondary culture. At the time of the experiments, hVMFs were at 70 % confluence, mimicking the loose arrangement of these cells in vivo.

3.2. Preparation of Vascular Endothelial Cells

Use of animals was governed by guidelines set forth by the University of British Columbia (UBC) Committee on Animal Care, UBC, Vancouver, Canada. Adult female New Zealand White rabbits weighing between 2.0 to 2.5 kg were sacrificed by CO₂ asphyxiation. Pinching and blinking reflexes were checked before any surgical invasion. A surgical blade was used to make a cut at the rabbit’s neck, allowing exsanguination via the carotid arteries. The thoracic cavity was cut open after all the blood has been removed from the rabbit. The heart and lungs were removed, exposing the thoracic aorta. A 2-inch segment of the aorta near the distal end was dissected out and immediately placed in warm (37 °C) normal physiological salt solution (nPSS). Using a dissecting microscope (Olympus SZ 30), all surrounding connective and fat tissues of the vessel segment were carefully removed.
When the vessel was thoroughly cleaned, it was placed in nominal Ca\(^{2+}\)-free PSS (Ca\(^{2+}\)-free PSS) containing the enzyme solution at 37 °C for 30 minutes. The enzyme solution used for dissociation of endothelial cells consisted of 0.01 % Type IV collagenase, 0.5 % Type I elastase, 1 % bovine albumin, and 1 % trypsin inhibitor. After the enzyme treatment, the vessel was first washed in nPSS a few times and then was placed in antibiotic-containing nPSS (α-nPSS). The α-nPSS consisted of 90 % nPSS, 9.99 % fetal bovine serum, and 0.01 % antibiotic mixture of penicillin, streptomycin and amphotericin B. A Pasteur pipette was used to disperse the endothelial cells by the method of trituration. During trituration, small force was applied to flush solution into and out of the vessel. As a result, clusters of endothelial cells (ECs) were separated from the vessel. The ECs were seeded onto glass-based experimental chambers and were left for attachment at room temperature (23 °C) for 2 hours. All ECs were used within 6 hours after preparation to ensure viability of cells and quality of data.

3.3. Measurements of Intracellular Ca\(^{2+}\) Concentration

3.3.1. Conventional Fluorescence Microscopy

Valvular myofibroblasts (VMFs) and endothelial cells (ECs) in glass-based chambers were loaded with 1 μM of the fluorescent Ca\(^{2+}\) indicator fura-2-acetoxymethylester (fura-2/AM) and 1 μM pluronic acid (F-127) for 30 minutes at room temperature (23 °C). Figures 3.3.1.1 and 3.3.1.2 show sample images of fura-2-loaded VMFs and ECs. Upon entering the cells, the ester bond of Fura-2/AM is cleaved, trapping the fluorophore in the cytosol. Uncleaved fura-2 or fura-2/AM, i.e. in the ester form, emits maximal fluorescence when excited at 380 nm (Scanlon et al. 1987). As discussed later, Ca\(^{2+}\)-free fura-2 also fluoresce at 380-nm excitation. Thus, fura-2/AM in the cytosol does not affect the measurement of net changes in [Ca\(^{2+}\)]. Proper cleavage of ester bonds also prevents penetration of fura-2 into subcellular organelles such as ER and
mitochondria. Compartmentalization of fluorescent dyes into organelles present a source of experimental error in $[\text{Ca}^{2+}]_{i}$ measurements, since fluorescent signals are detected in locations other than the cytosol. In this study, potential influences from organellar Ca$^{2+}$ signals are greatly diminished also because of the relatively low $K_D$ (224 nM) of fura-2, compared to other dyes such as mag-fura-2 which, with a high $K_D$ of 25 μM, specifically binds to Ca$^{2+}$ in the ER. The high Ca$^{2+}$ affinity (or low $K_D$) of fura-2 enables detection of nanomolar changes in Ca$^{2+}$ concentration in the cytosol. If small amounts of fura-2 are loaded into the ER and mitochondria, the dye would be over-saturated by the concentrated Ca$^{2+}$ in these compartments and would not detect changes of Ca$^{2+}$ levels in these locations.

After the loading period, the chamber was mounted on an inverted microscope (Nikon, Diaphot) with a 20x phase/fluor and an 100x oil-immersion objective and washed with nPSS via a solution perfusion system for 15 minutes to remove any fura-2/AM remaining in the extracellular space. Solution was infused into the system via polyethylene tubing. The volume of the chamber was kept constant at 0.5 mL by suction. A total of 3 mL was used during each solution change to ensure that the chamber contained the designated solution only. All experiments were performed at room temperature (23°C).

The cells were excited with alternating ultraviolet (UV) light of 340 nm and 380 nm at 1-second intervals and fluorescence was recorded at the emission wavelength at 510 nm. The 340 nm / 380 nm wavelengths were chosen due to fura-2's fluorescent characteristics. When fura-2 is bound with Ca$^{2+}$ in the cytosol, maximal fluorescence is emitted when the cell is excited with 340 nm light. On the contrary, maximal light is emitted under 380-nm excitation when fura-2 is free from Ca$^{2+}$, i.e. in low $[\text{Ca}^{2+}]_{i}$ environment. Thus, cytosolic Ca$^{2+}$ levels ($[\text{Ca}^{2+}]_{i}$) were expressed as the fluorescence ratio of the excitation wavelengths: 340 nm and 380 nm ($F_{340}/F_{380}$).
Fluorescence intensities of EC clusters containing 5 to 10 cells were recorded by a PMT fluorimeter (Photon Technology International (PTI), London, ON) coupled to the microscope. The PMT fluorimeter was connected via an interface to a computer, and data were recorded on Felix® Fluorescence Analysis Software v.1.21 (PTI, London, ON). For the hVMF experiments, the cells were exposed to 340- and 380-nm UV light, and images at 510 nm emission were taken with a digital camera (DVC-1300, Digital Video Camera Company, Austin, TX) that was connected to the computer via an interface. The fluorescence ratio ($F_{340}/F_{380}$) of individual hVMF (by means of regions of interests (ROIs)) were recorded using the Northern Eclipse® software (Empix Imaging, Toronto, ON). Each ROI included the entire cellular area, with no distinction between cytosolic and nuclear areas. Using the method shown by Grynkiewicz et al. (1985) and the equation: $[Ca^{2+}]_i = K_D (R - R_{\text{min}})/(R_{\text{max}} - R)(S_{f380}/S_{b380})$, absolute $[Ca^{2+}]_i$ values were calculated from the maximal and minimal fluorescence values under treatment of the $Ca^{2+}$ ionophore ionomycin (10 μM) in nPSS and zero $Ca^{2+}$ PSS (0CaPSS), respectively. Baseline $[Ca^{2+}]_i$ in hVMFs was around 25 nM while a typical agonist (e.g. 100 μM histamine) could elicit a peak $[Ca^{2+}]_i$ up to 980 nM. Although it is uncommon to record milli-molar levels of $[Ca^{2+}]_i$ in most cells, this has been reported in hVMFs before (Taylor et al. 2000). Baseline $[Ca^{2+}]_i$ in ECs was approximately 35 nM and maximal $[Ca^{2+}]_i$ upon a typical agonist stimulation, i.e. 10 μM acetylcholine (ACh), could reach close to 760 nM. $[Ca^{2+}]_i$ values calculated by this method, however, were subjected to significant experimental uncertainties because the dissociation constant ($K_D$) of fura-2 used by Grynkiewicz et al. (1985) corresponds to the dye in solution rather than in endothelial cells. Thus, for the purpose of this study, the fluorescence ratio was not converted to absolute $[Ca^{2+}]_i$ because of the uncertainty for in situ calibrations (Wang et al. 1995).
Figure 3.3.1.1. Image of fura-2-loaded valvular myofibroblasts (VMFs). VMFs were loaded with the Ca$^{2+}$-sensitive fluorescent indicator fura-2 acetoxymethylester (1 μM) for 30 minutes at 23 °C. This image was taken when the VMFs at rest were exposed to 380-nm ultraviolet light.
Figure 3.3.1.2. Image of fura-2-loaded endothelial cells (ECs). ECs were loaded with the Ca^{2+}-sensitive fluorescent indicator fura-2 acetoxymethylester (1 μM) for 30 minutes at 23 °C. This image was taken when the ECs at rest were exposed to 380-nm ultraviolet light.
3.3.2. Rapid Laser-Scanning Confocal Microscopy

Some hVMF experiments were performed using a rapid laser-scanning confocal microscope (LSCM) in order to capture subcellular Ca\textsuperscript{2+} signals which occurred in millisecond intervals. The LSCM consisted of the Olympus BX50WI upright microscope and a Nipkow confocal disc. The pinhole on the confocal disc filters the out-of-focus light and allows for maximal sensitivity of the fluorescence acquisition at a given focal plane. This is especially important in the detection of basal, subcellular Ca\textsuperscript{2+} signals that are usually of weaker amplitudes than global [Ca\textsuperscript{2+}], increase elicited by stimuli. The high speed of the LSCM mechanics here also is an asset to capture [Ca\textsuperscript{2+}], signals occurring rapidly. hVMFs were loaded with the Ca\textsuperscript{2+}-sensitive fluorescent indicator fluo-4 (1 \mu M) and Pluronic F-127 (1 \mu M) for 30 minutes at room temperature (23 °C). Design of the experimental chambers and perfusion system was similar to that described above. A water-dipping 60\times objective was used to visualize hVMFs which were excited by a Argon-Krypton laser at 488 nm. Images of hVMFs were captured at 525-nm emission, at a rate of 25-30 frames per second by an ICCD (PerkinElmer Olympix) controlled by the Ultraview® 4.0 software (PerkinElmer, NJ). Fluorescent intensities of selected ROIs were recorded on an Excel spreadsheet.

3.4. Mn\textsuperscript{2+}-Quenching Experiments

The Mn\textsuperscript{2+}-quenching method was used occasionally to measure the rate of divalent cation entry (Wang and van Breemen 1997). The cells were exposed to UV light at 360 nm, which is the isobestic point of fura-2, MnCl\textsubscript{2} (50 \mu M) in nominal Ca\textsuperscript{2+}-free PSS (Ca-free PSS) was perfused into the chamber. Upon entering the cells, Mn\textsuperscript{2+} binds to the intracellular fura-2 and quenches its fluorescence. The basal Mn\textsuperscript{2+} entry rate was determined for 200 seconds prior to the addition of drugs. A more negative slope of fluorescent intensity at 360 nm (F\textsubscript{360}) during
stimulation indicates an increased rate of Mn\textsuperscript{2+} entry, which is a sign of Ca\textsuperscript{2+} entry under normal circumstances. At the end of each experiment, ionomycin (10 \SI{}{\mu M}) was added to the cells to achieve maximal quenching, at which point the intensity was arbitrarily set as 0 \% fluorescence. To express Mn\textsuperscript{2+} entry rate, the unit \% F\textsubscript{360}/second (%F/s) was used.

### 3.5. Fluorescent Labeling of Ryanodine Receptors

The fluorescent indicator BODIPY TR-X ryanodine was used to specifically label ryanodine receptors. The cells were loaded with 1 \SI{}{\mu M} BODIPY TR-X ryanodine and 1 \SI{}{\mu M} pluronic acid (F-127) for 30 minutes at 23 °C. After the loading period, the cells were washed with nPSS for 15 minutes to remove any excessive dye remaining in the extracellular space. The chambers were then mounted on an inverted microscope (Nikon Eclipse TE300) with a 60× oil-immersion objective, coupled to a pinhole confocal scanning head (BioRad Radiance 2000). A krypton laser was used to excite the cells at 589 nm and images were captured with an ICCD at 616 nm. To obtain an image stack for three-dimensional composition later, images were taken at the bottommost focal plane first and moved upward in 0.15-\textmu m steps.

### 3.6. Data Analysis and Statistics

All acquired data were exported to Microsoft® Excel where they were analyzed. Statistical tests were performed using GraphPad® Prism. In figures where representative tracings are shown, responses from an individual VMF or an EC cluster were illustrated. Data on line and bar graphs were shown as mean ± standard error of the mean (SEM). Where applicable, one-way ANOVA and Student’s t-test were used to determine statistical significance. The Neumann-Keuls post-hoc test was used in cases of overall differences in one-way ANOVA. P values of less than 0.05 (P < 0.05) were considered to be statistically different.
3.6.1. Ca$^{2+}$ Contents in Valvular Myofibroblasts

When VMFs were stimulated in a Ca$^{2+}$-free environment, the recorded Ca$^{2+}$ signals (i.e. expressed as fura-2 fluorescence – $F_{340}/F_{380}$ ratio) indicated Ca$^{2+}$ mobilized only from intracellular compartments. The amount of Ca$^{2+}$ stored in these compartments could be estimated by calculating the area under curves (AUCs) of the drug-induced [Ca$^{2+}$]i responses. It must be noted that the AUC may underestimate the true amounts of Ca$^{2+}$ released from the ER because the amplitudes of [Ca$^{2+}$]i responses may be suppressed by elevated Ca$^{2+}$-activated Ca$^{2+}$ removal processes. An example of such is the activation of PMCA by the Ca$^{2+}$-calmodulin complex (Berridge et al. 2003). In this study, enhanced Ca$^{2+}$ removal affecting the sizes of [Ca$^{2+}$]i responses was implicated by [Ca$^{2+}$]i decaying below baseline after stimulation in several occasions (some examples are illustrated in Figures 4.2.1.1 and 4.2.1.3). In contrast, when Ca$^{2+}$ removal was inhibited, as in Figures 5.2.1.1 and 5.2.2.1, [Ca$^{2+}$]i elevations were much larger. Despite the shortcoming of using AUC as a measurement of Ca$^{2+}$ content, this method allows for a present a way of comparing the apparent sizes of different ER Ca$^{2+}$ compartments.

Each AUC was the sum of individual sub-AUC in one time unit. A time unit was defined as the time between any two data points, and was assigned to be the height of the sub-AUC. The sub-AUC was in trapezoidal shape, and the lengths of the top and bottom surfaces of the trapezoid were represented by $F_{340}/F_{380}$ ratios of each of the two data points. Thus, AUC was expressed in terms of $F_{340}/F_{380}$ ratio·seconds (R·s).

3.6.2. Spontaneous Ca$^{2+}$ Release Rates and Duration in Valvular Myofibroblasts

Two methods were used in the analysis of spontaneous Ca$^{2+}$ releases in VMFs. First, the percentage of time each Ca$^{2+}$ release site’s fluorescence intensity spent below baseline (< 0 %) and at 0-25 %, 25-50 %, 50-75 %, and 75-100 % above baseline within a 30-second period were
calculated. The maximal \([\text{Ca}^{2+}]_i\) level achieved in a given cell was considered to be 100 % above baseline. The data were plotted as a histogram with % above & below baseline on the abscissa and % of 30-second period on the ordinate. The histogram would provide an indicator of the distribution of data under basal, unstimulated conditions and during pharmacological modulation.

Taken into account the signal-to-noise relationship and consistency of all data, fluorescence intensities of > 25 % above baseline were further analyzed. Spontaneous \(\text{Ca}^{2+}\) release that resulted in \([\text{Ca}^{2+}]_i\) increases of > 25 % above baseline were termed arbitrarily as substantial spontaneous \(\text{Ca}^{2+}\) releases in this study. The peak-to-peak duration of each substantial spontaneous \(\text{Ca}^{2+}\) release site provided a measurement of the frequencies of \(\text{Ca}^{2+}\) release occurrence.

3.6.3. \(\text{Ca}^{2+}\) Removal Rates in Vascular Endothelial Cells

As mentioned above, the \(F_{340}/F_{380}\) ratio was used to represent \([\text{Ca}^{2+}]_i\) levels. In order to group data from different experiments, all \(F_{340}/F_{380}\) ratios were normalized to the maximal fluorescence achieved by ACh stimulation in the control experiments, or in the 0Na experiments when comparing effects of simultaneous NCX + RyR and NCX + PMCA blockades. The relative rates of \([\text{Ca}^{2+}]_i\) decline were determined by comparing the downstroke of the \([\text{Ca}^{2+}]_i\) responses measured under different experimental conditions. Two approaches were used to quantify the effects of \(\text{Ca}^{2+}\) channel/pump modulators on \(\text{Ca}^{2+}\) extrusion. Firstly, by dividing the discrete normalized \([\text{Ca}^{2+}]_i\) values by time, we obtained instantaneous \([\text{Ca}^{2+}]_i\) decaying rates by calculating between each data pair. The values were then binned into different groups: 0-5, 5.01-10, 10.01-15, 15.01-20, 20.01-40, 40.01-60, 60.01-80, and 80.01-100 % of normalized \([\text{Ca}^{2+}]_i\) levels (Wang et al. 2002). \([\text{Ca}^{2+}]_i\) declining rates were then plotted against the binned % of normalized \([\text{Ca}^{2+}]_i\) levels. Secondly, decaying \([\text{Ca}^{2+}]_i\) responses were fitted with an one-
component exponential decay function of the form \( y = ae^{-bt} \) (Wang et al. 2002). The Ca\(^{2+}\) extrusion rate constants, \( b \), were compared among different experimental conditions.

### 3.6.4. Three-Dimensional Images of Ryanodine Receptors in Endothelial Cells

Individual x-y slices (in 0.15 µm thickness) of BODIPY TR-X-ryanodine-labeled images of ECs were stacked into a two-dimensional (2-D) projection image using ImageJ® (National Institutes of Health, MD). The original gray-colored 2-D projection image was assigned with a red color. A duplicate of the 2-D projection image was created, rotated by 6° to the right and assigned with a green color. The two 2-D projection images were superimposed on each other using Adobe Photoshop® and a three-dimensional composite image was generated.

### 3.7. Solutions and Chemicals

The Ca\(^{2+}\)-sensitive fluorescent indicator fura-2/AM, the fluorescent label for ryanodine receptor BODIPY TR-X ryanodine, and the PMCA inhibitor carboxyeosin were purchased from Molecular Probes Inc. (Eugene, OR). All other drugs and chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO). The solutions used in this study were constituted as follows [in mM] at pH 7.4:

1) normal physiological saline solution (nPSS): NaCl [126], KCl [5], MgCl\(_2\) [5], HEPES [10], D-glucose [10], and CaCl\(_2\) [1];
2) zero Ca\(^{2+}\) PSS (0CaPSS): NaCl [126], KCl [5], MgCl\(_2\) [5], HEPES [10], D-glucose [10], and EGTA [0.1];
3) nominal Ca\(^{2+}\)-free PSS (Ca-free PSS): NaCl [126], KCl [5], MgCl\(_2\) [5], HEPES [10], and D-glucose [10];
4) zero Na\(^{+}\) PSS (0NaPSS): N-methyl-D-glucamine [126], KCl [5], MgCl\(_2\) [5], HEPES [10], D-glucose [10], and CaCl\(_2\) [1];
5) zero Na\(^{+}\)-zero Ca\(^{2+}\) PSS (0Na0CaPSS): N-methyl-D-glucamine [126], KCl [5], MgCl\(_2\) [5], HEPES [10], D-glucose [10], and EGTA [0.1].
CHAPTER IV: $\text{Ca}^{2+}$ MOBILIZATION BY PHARMACOLOGICAL AGENTS IN VALVULAR MYOFIBROBLASTS (VMFs) – ER $\text{Ca}^{2+}$ STORES AND MEMBRANE RECEPTORS

4.1. Overview

Despite evidence suggesting the importance of VMFs in regulating many of the cellular and molecular processes involved in valvular injury and remodeling, very few pharmacological studies of VMFs have been conducted to date. In one study, epinephrine- and angiotensin II-induced contractions were examined in isolated rabbit VMFs (Filip et al. 1986). In addition, transient increases in $[\text{Ca}^{2+}]_i$ levels induced by serotonin (Hafizi et al. 2000), endothelin-1, angiotensin II, and U46619 were shown in human mitral VMFs (Taylor et al. 2000). However, little has been reported on the possible mechanisms involved in $\text{Ca}^{2+}$ signaling in VMFs.

In the following sections, cultured human VMFs were examined with respect to the characteristics of their $[\text{Ca}^{2+}]_i$ responses to histamine, ATP & UTP, and cyclopiazonic acid (CPA). First, experiments were conducted on normal VMFs. Sample pseudocolored $F_{340}/F_{380}$ images of fura-2-loaded VMFs before and after histamine stimulation are shown in Figure 4.1.1. Both histamine and ATP/UTP are important vaso-modulators in healthy and diseased tissues. Identifying the $\text{Ca}^{2+}$-signaling pathways induced by these two agonists would provide valuable insights in the understanding of VMF physiology. On the other hand, CPA is a widely used, reversible inhibitor of SERCA, and its use in this study would reveal information regarding the distribution and size of intracellular $\text{Ca}^{2+}$ stores in VMFs. Finally, a comparative study was done where VMFs isolated from rheumatic valves were subjected to the above pharmacological agents and their responses were evaluated against those of normal VMFs.
Figure 4.1.1. $F_{340}/F_{380}$ images of fura-2-loaded VMFs before and after histamine stimulation. As explained in Section 3.3.1, intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) was expressed as the ratiometric fluorescent intensity at 340-nm and 380-nm excitation. The original grayscale images were converted to pseudocolored ones with references to the color scale shown in the lower right of each panel. White color represents high [Ca$^{2+}$]$_i$ while violet represents low [Ca$^{2+}$]$_i$. The unstimulated VMFs (left panel) had [Ca$^{2+}$]$_i$ values towards the lower end of the scale. Upon stimulation with 100 µM histamine (right panel), [Ca$^{2+}$]$_i$ elevations were indicated by the color changes of the image.
4.2. Results

4.2.1. Comparable $[\text{Ca}^{2+}]_i$ Responses in VMFs Originated from Different Patients

As mentioned in Section 3.1, grossly normal atrioventricular valves from 5 patients diagnosed with ischaemic or dilated cardiomyopathy were used as a source of normal VMFs. Because of the potential biovariability among human tissues, preliminary experiments with selected pharmacological agents were done to determine whether VMFs from different individuals produced similar $[\text{Ca}^{2+}]_i$ elevations upon stimulation. In particular, $[\text{Ca}^{2+}]_i$ responses elicited by pharmacological agents 100 µM histamine & 100 µM ATP and by the reversible SERCA blocker cyclopiazonic acid (30 µM, CPA) were compared.

Representative agonist- and CPA-induced $[\text{Ca}^{2+}]_i$ responses are shown in Figures 4.2.1.1 to 4.2.1.3. As shown in Figure 4.2.1.1, a typical histamine response in nPSS consisted of a rapid $[\text{Ca}^{2+}]_i$ increase followed by decay to baseline within 300 seconds. In most cases, there were several $\text{Ca}^{2+}$ oscillations after the initial upstroke until the response eventually died out. The inset table of Figure 4.2.1.1 shows that peak $F_{340}/F_{380}$ ratios elicited by histamine among the 5 valves tested were not statistically different.

In Figure 4.2.1.2, typical CPA-induced gradual $[\text{Ca}^{2+}]_i$ increases in nPSS are shown. In about half of the VMFs tested, sustained $[\text{Ca}^{2+}]_i$ elevation was observed after several minutes of CPA stimulation. In the remaining half, $[\text{Ca}^{2+}]_i$ returned to baseline eventually. This heterogeneity, seen in VMFs from all 5 valves, was probably not due to variability among individual patients. The inset table of Figure 4.2.1.2 shows that peak $F_{340}/F_{380}$ ratios of CPA-induced responses were not statistically different among valves from the 5 patients.

Some variations in the ATP-induced $[\text{Ca}^{2+}]_i$ responses were also seen in VMFs from different origins. ATP increased $[\text{Ca}^{2+}]_i$ to similar maximal values in all VMFs but in some the
[Ca\textsuperscript{2+}]_i decay occurred slowly in the range of minutes. In other VMFs, [Ca\textsuperscript{2+}]_i elevation was maintained at a plateau phase with little sign of decay during the ATP treatment. Typical ATP-induced [Ca\textsuperscript{2+}]_i responses mentioned above are shown in Figure 4.2.1.3. The inset table of Figure 4.2.1.3 shows that despite the ATP-induced [Ca\textsuperscript{2+}]_i plateau was not present in all VMFs, the maximal F\textsubscript{340}/F\textsubscript{380} ratios achieved were not significantly different among the 5 valves.

The [Ca\textsuperscript{2+}]_i responses shown in this section suggested that VMFs demonstrate heterogeneous Ca\textsuperscript{2+} signaling upon ATP and CPA stimulation. The heterogeneity in VMF responses was present in all of the 5 valves tested, and was not a property of any particular individual. Mulholland and Gotlieb (1996) discussed the two morphological states of VMFs in culture. Cultured VMFs may appear in spindle and cobblestone shapes, both of which were seen in this study. In the initial stage of the study, preliminary analysis comparing the two VMF morphologies revealed that shape is not a factor in the heterogeneity of ATP and CPA responses. Despite the noticeable differences in the geometry of [Ca\textsuperscript{2+}]_i responses, all VMFs showed similar [Ca\textsuperscript{2+}]_i elevations in their initial responses to the stimulus (i.e. peak F\textsubscript{340}/F\textsubscript{380} ratios were close within errors). In the subsequent experiments where the Ca\textsuperscript{2+} signaling mechanisms of the various pharmacological agents were elucidated, only VMFs from 2 of the 5 patients were used.
Figure 4.2.1.1. Sample histamine-induced $[\text{Ca}^{2+}]_i$ responses in VMFs. 100 μM histamine was applied to a VMF in either normal Ca$^{2+}$-containing physiological salt solution (nPSS) (solid trace) or zero Ca$^{2+}$ PSS (0CaPSS) (dotted trace) for the duration of the response. The inset table shows the peak $[\text{Ca}^{2+}]_i$ levels (expressed in F$_{340}$/F$_{380}$ ratio) of the histamine responses in nPSS and 0CaPSS of VMFs from 5 patients. The average peak values indicated that VMFs from different individuals responded similarly to histamine stimulation, in both nPSS and 0CaPSS.

<table>
<thead>
<tr>
<th>Valve No.</th>
<th>Mean Peak Level</th>
<th>nPSS</th>
<th>0CaPSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ($n = 17$)</td>
<td>1.36</td>
<td>1.323</td>
<td></td>
</tr>
<tr>
<td>2 ($n = 13$)</td>
<td>1.245</td>
<td>1.048</td>
<td></td>
</tr>
<tr>
<td>3 ($n = 13$)</td>
<td>0.846</td>
<td>0.852</td>
<td></td>
</tr>
<tr>
<td>4 ($n = 29$)</td>
<td>1.291</td>
<td>1.278</td>
<td></td>
</tr>
<tr>
<td>5 ($n = 17$)</td>
<td>1.215</td>
<td>1.251</td>
<td></td>
</tr>
<tr>
<td>Average ± SEM</td>
<td>1.191 ± 0.090</td>
<td>1.150 ± 0.088</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.2.1.2. Sample CPA-induced $[\text{Ca}^{2+}]_i$ responses in VMFs. The sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase (SERCA) blocker cyclopiazonic acid (CPA, 30 μM) was applied to VMFs in nPSS. The inset table shows the peak $[\text{Ca}^{2+}]_i$ levels of VMFs from 5 patients. Heterogeneity of CPA responses was displayed among VMFs and patients. The solid trace shows the plateau phase of the response in some VMFs that is absent from other VMFs (dotted trace). In spite of this, the peak sizes among valves from different patients were generally the same.
Figure 4.2.1.3. Sample ATP-induced $[Ca^{2+}]_i$ responses in VMFs. Adenosine 5'-triphosphate (ATP, 100 µM) was applied to VMFs in nPSS. The inset table shows peak $[Ca^{2+}]_i$ levels of VMFs from 5 patients. In some VMFs, ATP elicited sustained $[Ca^{2+}]_i$ elevation (solid trace) whereas in other VMFs, $[Ca^{2+}]_i$ gradually decayed towards baseline levels (dotted trace). In any case, the average peak values among different valves were equal to each other, suggesting individual biovariability was rather insignificant in generating $[Ca^{2+}]_i$ responses.
4.2.2. **Histamine-Induced [Ca$^{2+}$]$_i$ Increase is Independent of Extracellular Ca$^{2+}$**

Shown in Figure 4.2.2.1 is a sample His-induced [Ca$^{2+}$]$_i$ response in 0CaPSS (representative of n = 159). When compared with the response shown in Figure 4.2.1.1, both histamine responses with and without extracellular Ca$^{2+}$ shared similar upstroke and decay characteristics. Blockade of receptor-operated Ca$^{2+}$ channels (ROCs) and store-operated Ca$^{2+}$ channels (SOCs) with 50 µM SKF-96365 did not alter the shape and time course of the histamine response in nPSS (Figure 4.2.2.2, representative of n = 68 cells). Areas under the [Ca$^{2+}$]$_i$ responses elicited by histamine with and without SKF-96365 were compared and were not significantly different. In support of the data that histamine did not induce Ca$^{2+}$ entry in VMFs, it was also found that fura-2 quenching by Mn$^{2+}$ was not increased by histamine stimulation (data not shown).
Figure 4.2.2.1. Histamine-induced $[\text{Ca}^{2+}]_i$ transient in VMFs in 0CaPSS. In the absence of extracellular $\text{Ca}^{2+}$ (i.e. 0CaPSS), the histamine (His, 100 μM)-induced $[\text{Ca}^{2+}]_i$ response in VMFs (representative of $n = 159$ cells) was not distinguishable to that in nPSS (Figure 4.2.1.1).
Figure 4.2.2.2. Histamine response of VMF in the presence of SKF-96365. Blockade of receptor- and store-operated Ca\textsuperscript{2+} channels by SKF-96365 (SKF) (50 μM), applied for 200 seconds in nPSS prior to histamine stimulation, had no effect on the shape and time course of the histamine response (representative of n = 68 cells). Together with Figure 4.2.2.1, the data suggested that histamine did not induce Ca\textsuperscript{2+} entry, and only elicited [Ca\textsuperscript{2+}]\textsubscript{i} increase via activating intracellular Ca\textsuperscript{2+} release.
4.2.3. Evidence for H₁ Receptor-Mediated Ca²⁺ Signaling in VMFs

Selective receptor antagonists were used to identify the subtypes of histamine receptors in VMFs. Pyrilamine (selective H₁ antagonist, 0.1 μM) completely abolished the histamine-induced [Ca²⁺]ᵢ response (Figure 4.2.3.1, representative of n = 77 cells). After 20 minutes of continuous washout in nPSS, the antagonizing effect of pyrilamine was reversed, as shown by the second histamine response in Figure 4.2.3.1. Knockout of H₂ receptor function, however, did not alter the histamine-induced [Ca²⁺]ᵢ response in any way. Figure 4.2.3.2 shows a representative trace of n = 74 VMFs under histamine stimulation, before and after the incubation of cimetidine (selective H₂ antagonist, 1 μM).
Figure 4.2.3.1. Effects of pyrilamine on histamine-induced \([\text{Ca}^{2+}]_i\) response in VMFs. Pyrilamine (Pyr) (0.1 μM), a selective \(H_1\) antagonist, completely abolished the histamine (His, 100 μM)-induced \([\text{Ca}^{2+}]_i\) response in nPSS (representative of \(n = 77\) cells). The \(H_1\) blockade was reversible after 20 minutes of continuous perfusion with nPSS.
Figure 4.2.3.2. Effects of cimetidine on histamine-induced $[Ca^{2+}]_i$ response in VMFs. Cimetidine (Cim) (1 μM), a selective H\textsubscript{2} antagonist, did not alter the histamine (His, 100 μM)-induced $[Ca^{2+}]_i$ response (representative of $n = 74$ cells). This suggested that no functional H\textsubscript{2} receptor-mediated Ca\textsuperscript{2+} signaling is present in VMFs.
4.2.4. ER Ca\(^{2+}\) Refilling is Essential in Regenerative Histamine Responses

Two histamine stimulations in nPSS at 15 minutes apart produced consistent \([\text{Ca}^{2+}]_i\) responses in VMFs, as illustrated in the representative trace of \(n = 78\) cells in Figure 4.2.4.1. The slight run-down effect, i.e. gradually diminishing responses after repetitive histamine stimulations in VMFs, was observed at times. Incomplete refilling of \(\text{Ca}^{2+}\) into the ER and, to a lesser extent, receptor desensitization are possible causes for the run-down effect. In the absence of extracellular \(\text{Ca}^{2+}\), i.e. in 0CaPSS, however, VMFs did not respond to the second histamine stimulation (Figure 4.2.4.2, representative trace of \(n = 66\) cells). Based on the different results from nPSS and 0CaPSS, it appears that refilling of the ER with \(\text{Ca}^{2+}\) derived from the extracellular space is essential for repetitive histamine responses.

When VMFs were treated with the reversible SERCA blocker cyclopiazonic acid (CPA, 30 \(\mu\text{M}\)), \([\text{Ca}^{2+}]_i\) oscillations that usually followed the initial peak of a histamine response was abolished (Figure 4.2.4.3, representative of \(n = 58\) cells). In this figure, the CPA and histamine responses can be distinguished by their kinetics. CPA caused an initial slow \([\text{Ca}^{2+}]_i\) increase. Subsequent application of histamine induced a more rapid \([\text{Ca}^{2+}]_i\) upstroke. In addition, the rapid \([\text{Ca}^{2+}]_i\) decay of the histamine response was different from the shoulder phase of the response attributed to CPA stimulation. In the continuous presence of CPA, a second histamine application failed to elicit \([\text{Ca}^{2+}]_i\) elevation. In total, findings in this section suggested that a single pulse of histamine depletes ER \(\text{Ca}^{2+}\) that depends on SERCA activity for replenishment and for subsequent responses.
Figure 4.2.4.1. Regenerative histamine responses in VMFs. Histamine (His) (100 µM) was applied twice, 15 minutes apart, in nPSS (representative of n = 78 cells).
Figure 4.2.4.2. Second histamine application did not elicit a response in 0CaPSS. Histamine (His) (100 μM) was applied twice, 15 minutes apart, in 0CaPSS (representative of n = 66 cells). Only the first stimulation resulted in $[Ca^{2+}]_i$ increase.
Figure 4.2.4.3. SERCA blockade abolished regenerative histamine responses in VMFs. 30 μM cyclopiazonic acid (CPA), which elicited a slow [Ca^{2+}]_{i} increase (shown between the dotted vertical lines) by inducing Ca^{2+} release from the ER via SERCA blockade, was added 100 seconds prior to the first histamine (His) (100 μM) application in nPSS. With CPA present throughout the experiment, the second His application did not induce a Ca^{2+} response (representative of n = 58 cells). Prolonged incubation with CPA revealed a small maintained [Ca^{2+}]_{i} elevation above the dashed line, which denotes resting [Ca^{2+}]_{i}. Upon removal of CPA, [Ca^{2+}]_{i} returned to baseline.
4.2.5. Histamine-Insensitive Ca\(^{2+}\) Release from the ER

Despite the failure to elicit repetitive histamine-sensitive [Ca\(^{2+}\)_i] responses in 0CaPSS, CPA was still able to induce a [Ca\(^{2+}\)_i] transient, as shown in Figure 4.2.5.1 (representative of n = 112 cells). Here 5 minutes of washout (instead of 15 minutes as in previous experiments) were allowed in between histamine applications to minimize undesirable effects due to prolonged incubation in a Ca\(^{2+}\)-free environment. As a control for possible receptor desensitization, VMFs were repeatedly stimulated with histamine in nPSS. Figure 4.2.5.2 shows that histamine induced [Ca\(^{2+}\)_i] responses in repeated stimulations in nPSS (representative of n = 80 cells), indicating that the lack of subsequent histamine responses in 0CaPSS (Figure 4.2.5.1) was the result of a depleted ER Ca\(^{2+}\) store. After emptying the histamine-sensitive ER Ca\(^{2+}\) content, additional ER Ca\(^{2+}\) release activated by CPA was likely related to a portion of the ER insensitive to histamine-induced signaling cascades, i.e. generation of IP\(_3\) and opening of IP\(_3\) receptors. This histamine-insensitive ER compartment is probably also free of or possess only sparse amount of ryanodine receptors, since no [Ca\(^{2+}\)_i] response was seen when caffeine (activator of ryanodine-mediated ER Ca\(^{2+}\) release channels, 20 mM) was added to VMFs (data not shown).
Figure 4.2.5.1. Histamine-insensitive ER Ca$^{2+}$ compartment in VMFs. After three histamine (His) (100 μM) applications to completely deplete the histamine-sensitive Ca$^{2+}$ store in 0CaPSS, 30 μM CPA induced an additional transient [Ca$^{2+}$], response (representative of n = 112 cells).
Figure 4.2.5.2. A control experiment showing 3 histamine responses in VMFs. These traces were obtained in nPSS (representative of n = 80 cells), as opposed to 0CaPSS in the previous figure. The fact that a $[\text{Ca}^{2+}]_i$ response was seen here with each histamine (His) (100 μM) stimulation indicated that receptor desensitization did not explain the lack of responses to second and third application of histamine in Figure 4.2.5.1.
4.2.6. Two Compartments of ER Ca\(^{2+}\) Stores in VMFs

It is clear from the data in Section 4.2.5 that there are histamine-sensitive and histamine-insensitive ER Ca\(^{2+}\) stores in VMFs. As mentioned in Section 3.6.1, area under curves (AUCs) of histamine- and CPA-induced [Ca\(^{2+}\)]\(_i\) responses were calculated to determine the sizes of the two ER Ca\(^{2+}\) compartments.

In Figure 4.2.5.1, CPA revealed the histamine-insensitive ER Ca\(^{2+}\) store after the VMFs were subjected to 0CaPSS for 1400 seconds. In order to compare the size of the histamine-insensitive store with the histamine-sensitive store, VMFs must be exposed to 0CaPSS for the same duration prior to the addition of histamine (Figure 4.2.6.1, representative trace of \(n = 69\) cells). The initial 0CaPSS treatment in Figure 4.2.6.1 allowed direct comparison of histamine-sensitive and histamine-insensitive ER Ca\(^{2+}\) contents in the existing protocol because basal Ca\(^{2+}\) loss was taken into consideration. Similarly, after subtracting basal Ca\(^{2+}\) loss, the sum of the two Ca\(^{2+}\) compartments was represented by the AUC of the CPA-induced [Ca\(^{2+}\)]\(_i\) response in Figure 4.2.6.2 (representative trace of \(n = 62\) cells).

Values of AUCs from Figures 4.2.5.1, 4.2.6.1 and 4.2.6.2 are as follows. The unit of AUC is expressed as \(F_{340}/F_{380}\) ratio-seconds (R-s) (as described in Section 3.6.1). The size of the histamine-sensitive ER Ca\(^{2+}\) compartment was 66.30 ± 2.48 R-s. The size of the histamine-insensitive compartment was 57.45 ± 4.63 R-s. The experimentally derived size of both Ca\(^{2+}\) compartments was 93.45 ± 11.34 R-s – this value was comparable to the arithmetic sum of 66.30 ± 2.48 R-s and 57.45 ± 4.63 R-s, which equals 123.75 ± 7.11 R-s, within statistical error.

The sizes of ER Ca\(^{2+}\) compartments calculated thus far were valid after VMFs basally lost ER Ca\(^{2+}\) in 0CaPSS for 1400 seconds. It must be noted that the protocol design for the purpose of the findings here makes the extended 0CaPSS treatment inevitable. To precisely
determine the sizes of ER Ca\(^{2+}\) stores in normal conditions, the amount of basal Ca\(^{2+}\) loss in the experiments shown in Figures 4.2.5.1, 4.2.6.1 and 4.2.6.2 was also calculated. Figure 4.2.6.3 shows a sample trace of CPA-induced [Ca\(^{2+}\)], response in 0CaPSS (with only 100 seconds of 0CaPSS pre-incubation) in \(n = 55\) cells. The 100-second 0CaPSS treatment was standard in all Ca\(^{2+}\)-free experiments and its purpose was to ensure complete chelation of any free Ca\(^{2+}\) in the extracellular space by EGTA, thereby eliminating possible Ca\(^{2+}\) influx. The AUC of Figure 4.2.6.3 was 126.12 ± 4.63 Rs, which represented the actual total ER Ca\(^{2+}\) content, i.e. the total ER Ca\(^{2+}\) content without the effect of basal Ca\(^{2+}\) loss. Expressing the AUC of Figure 4.2.6.2 as a percentage of 126.12 ± 4.63 R·s (AUC of Figure 4.2.6.3) would notice that 25.9 ± 19.6 % of Ca\(^{2+}\) was lost from the ER basally during the long 0CaPSS treatment in the protocol. Knowing the amount of non-stimulated Ca\(^{2+}\) loss, it was possible to extrapolate the actual Ca\(^{2+}\) content in each compartment by adding a basal loss factor of 25.9 ± 19.6 % to the AUCs of Figure 4.2.5.1 and 4.2.6.1. Thus, the sizes of the revised Ca\(^{2+}\) contents were: 89.47 ± 17.54 R·s for the histamine-sensitive compartment and 77.53 ± 15.20 R·s for the histamine-insensitive compartment. The relative Ca\(^{2+}\) distribution in the two ER compartments is shown in Figure 4.2.6.4. In summary, assuming basal Ca\(^{2+}\) loss occurs at equal rates from both ER Ca\(^{2+}\) stores, the histamine-sensitive compartment makes up 53.6 % of total ER Ca\(^{2+}\), and the histamine-insensitive compartment makes up the remaining 46.4 %.
Figure 4.2.6.1. Histamine-sensitive ER Ca\(^{2+}\) content in VMF after basal Ca\(^{2+}\) loss. In consideration of the basal ER Ca\(^{2+}\) loss due to extended period of incubation in 0CaPSS (as shown in Figures 4.2.5.1 and 4.2.5.2), an experiment was done where histamine (His) (100 µM) was applied to VMFs that had been sitting in the absence of extracellular Ca\(^{2+}\) for the same amount of time (representative of \(n = 69\) cells). The area under curve (AUC), as explained in Section 3.6.1, was calculated from the histamine response, and constituted to the size of the histamine-sensitive ER Ca\(^{2+}\) compartment after basal Ca\(^{2+}\) loss.
Figure 4.2.6.2. Total ER Ca$^{2+}$ content in VMFs after basal ER Ca$^{2+}$ loss. Similar to Figure 4.2.6.1, VMFs were first incubated in OCaPSS for 1400 seconds (representative of n = 62 cells). Based on Figure 4.2.5.1, cyclopiazonic acid (CPA) (30 μM) was able to deplete the entire ER Ca$^{2+}$ store. Thus, CPA was applied to VMFs here, and the AUC represented the size of the histamine-insensitive ER Ca$^{2+}$ compartment after basal Ca$^{2+}$ loss.
Figure 4.2.6.3. Actual ER Ca\textsuperscript{2+} content in VMFs (without basal ER Ca\textsuperscript{2+} loss). The cyclopiazonic acid (CPA, 30 \( \mu \)M)-induced [Ca\textsuperscript{2+}]\( _i \) response obtained without the prolonged 0CaPSS incubation represented the total Ca\textsuperscript{2+} released from the ER (representative of \( n = 55 \) cells). The AUC of this response was the size of the actual ER Ca\textsuperscript{2+} content.
Figure 4.2.6.4. Relative sizes of ER Ca\(^{2+}\) compartments in VMFs. The sizes of the histamine-sensitive and histamine-insensitive ER Ca\(^{2+}\) compartments were expressed as a percentage of the total Ca\(^{2+}\) content. The two compartments were about the same size within statistical error in a total of \(n = 112\) cells subjected to the protocol illustrated in Figure 4.2.5.1.
4.2.7. Activation of Ca\(^{2+}\) (and Mn\(^{2+}\)) Entry by SERCA Blockade

It was shown in both Figures 4.2.1.2 and 4.2.4.3 that CPA stimulation could induce maintained \([\text{Ca}^{2+}]_i\) elevation in VMFs. The \([\text{Ca}^{2+}]_i\) plateau suggested continuous Ca\(^{2+}\) influx due to opening of SOCs. This speculation was further supported by the data in Figure 4.2.7.1 (representative of \(n = 48\) cells), where addition of extracellular Ca\(^{2+}\) in place of OCaPSS in the presence of CPA caused a maintained \([\text{Ca}^{2+}]_i\) increase above the resting level (shown by the dotted line). It is also noted that when VMFs were treated with CPA for over 10 minutes, \([\text{Ca}^{2+}]_i\) eventually went below the baseline in most cases. This was due to removal of basal Ca\(^{2+}\) influx into the cell, and VMFs incubated in OCaPSS also showed a slightly depressed baseline \([\text{Ca}^{2+}]_i\) (\(n = 37\) cells, data not shown).

Effects of SERCA blockade were also determined using the Mn\(^{2+}\)-quenching method. In 56 of 75 cells tested, CPA increased the rate of Mn\(^{2+}\) entry significantly (Figure 4.2.7.2). As described in Section 3.4, the Mn\(^{2+}\)-influx rate was expressed in terms of \% F\(_{360}\)/second (\%F/s). Basal Mn\(^{2+}\)-influx rate was determined to be 0.0227 ± 0.0037 \%F/s. Mn\(^{2+}\) influx was increased to a maximum of 0.0587 ± 0.0034 \%F/s, i.e. a 260 \% increase, after 300 seconds of CPA treatment. Mn\(^{2+}\) influx remained faster than baseline for at least another 300 seconds. The bar graph in Figure 4.2.7.3 compares the Mn\(^{2+}\) influx rates at different time points.
Figure 4.2.7.1. Store-operated Ca\(^{2+}\) entry in VMFs. Store-operated Ca\(^{2+}\) entry demonstrated by restoring extracellular Ca\(^{2+}\) after the initial Ca\(^{2+}\) release by 30 µM cyclopiazonic acid (CPA) in 0CaPSS (representative of n = 48 cells). The elevated \([\text{Ca}^{2+}]_i\) was maintained until the removal of CPA. The dashed line shows resting \([\text{Ca}^{2+}]_i\) level.
Figure 4.2.7.2. Store-operated divalent cation entry in VMFs. The manganese-quenching method was used here to monitor Mn\textsuperscript{2+} entry from the extracellular space. Fluorescent intensities at 360 nm, which is the isobestic point of fura-2, were recorded. Manganese chloride (Mn) (50 μM) was present throughout the experiment. 30 μM cyclopiazonic acid (CPA) accelerated Mn\textsuperscript{2+} influx, as shown by the increased negative slope of the trace, compared to the basal Mn\textsuperscript{2+} influx rate (dashed line) (representative of n = 56 cells). Maximal quenching was obtained with the addition of ionomycin (Ion) (10 μM).
Figure 4.2.7.3. Mn^{2+} entry rates before and after CPA addition to VMFs. The rate of Mn^{2+} entry was calculated from the experiment illustrated in Figure 4.2.7.2. In a total of n = 56 cells, Mn^{2+} entry rate increased by a maximum of 257 ± 22.5 % (mean ± SEM) after addition of CPA for 300 seconds. Mn^{2+} influx was maintained for a total of 600 seconds in the presence of CPA. (*P < 0.05 compared to basal Mn^{2+} entry rate. #P < 0.05 compared to Mn^{2+} influx rates at 0 and 600 seconds.)
4.2.8. \([\text{Ca}^{2+}]_i\) Responses Elicited by ATP and UTP

After resolving the two ER \(\text{Ca}^{2+}\) compartments in VMFs, it was further tested if the histamine-sensitive \(\text{Ca}^{2+}\) store is also responsive to other agonists that mobilize intracellular \(\text{Ca}^{2+}\) via the IP\(_3\) signal transduction pathway. Purinergic receptor agonists, ATP and UTP, were applied to VMFs to monitor \([\text{Ca}^{2+}]_i\) changes. ATP (\(P_2\) agonist, 100 \(\mu\)M) induced a longer-lasting \([\text{Ca}^{2+}]_i\) increase in nPSS than in oCaPSS (Figure 4.2.8.1, representative of \(n = 203\) cells). Similarly, application of UTP (selective \(P_{2Y2}\) agonist, 100 \(\mu\)M) also resulted in \([\text{Ca}^{2+}]_i\) increase with longer duration in nPSS than in oCaPSS (Figure 4.2.8.2, representative of \(n = 78\) cells). Qualitatively the wider \([\text{Ca}^{2+}]_i\) responses in the presence of extracellular \(\text{Ca}^{2+}\) indicated the role of \(\text{Ca}^{2+}\) influx during ATP/UTP stimulation.
Figure 4.2.8.1. ATP-induced $[\text{Ca}^{2+}]_i$ responses in VMFs with and without extracellular $\text{Ca}^{2+}$. 100 μM adenosine 5'-triphosphate (ATP) induced a longer-lasting $[\text{Ca}^{2+}]_i$ response in nPSS than in 0CaPSS (representative of $n = 203$ cells). The data suggested ATP-induced $\text{Ca}^{2+}$ influx in VMFs.
Figure 4.2.8.2. UTP-induced \([Ca^{2+}]_i\) responses in VMFs with and without extracellular \(Ca^{2+}\). 100 μM uridine 5'-triphosphate (UTP) induced a longer-lasting \([Ca^{2+}]_i\) response in nPSS than in 0CaPSS (representative of \(n = 78\) cells). The data suggested UTP-induced \(Ca^{2+}\) influx in VMFs.
4.2.9. **Ca\(^{2+}\) Influx Component of ATP- and UTP-Induced \([\text{Ca}^{2+}]_i\) Responses**

Two methods were used to reveal the Ca\(^{2+}\)-influx component of the ATP/UTP-induced \([\text{Ca}^{2+}]_i\) responses in VMFs. Treated with the ROC/SOC blocker SKF-96365 (50 μM), ATP-induced \([\text{Ca}^{2+}]_i\) increase decayed faster than control (Figure 4.2.9.1, representative of n = 47 cells). The time required for the peak \([\text{Ca}^{2+}]_i\) level to decay to 50 % of the peak (t\(_{50}\)) was calculated. According to the bar graph in Figure 4.2.9.2, t\(_{50}\) was 73.3 ± 5.7 seconds for the control (i.e. without SKF-96365) ATP-induced \([\text{Ca}^{2+}]_i\) response. The t\(_{50}\) was significantly shorter when Ca\(^{2+}\) influx was blocked with SKF-96365, which was 48.0 ± 6.8 seconds.

Secondly, Mn\(^{2+}\)-quenching experiments were conducted as described in Section 3.4. Figure 4.2.9.3 shows representative traces of Mn\(^{2+}\)-quenched F\(_{360}\) values during stimulation of VMFs with ATP (n = 90 cells), UTP (n = 146 cells), and UTP + SKF-96365 (n = 79 cells). In Figure 4.2.9.3, both lines [b] ATP and [c] UTP traces showed accelerated Mn\(^{2+}\) entry rates, as illustrated by the faster F\(_{360}\) decay when compared to the basal rate [a]. The dotted UTP + SKF-96365 trace showed slower F\(_{360}\) decay than the basal rate, suggesting that both UTP-stimulated and basal Mn\(^{2+}\) influx was inhibited. Mn\(^{2+}\) influx rates were also calculated (as described in Section 3.4) for the various experimental conditions (Figure 4.2.9.4). The Mn\(^{2+}\) entry rate of 0.0374 ± 0.0011 %F/s at baseline was significantly increased to 0.0471 ± 0.0015 %F/s and 0.0530 ± 0.0019 %F/s during ATP and UTP stimulation, respectively. When Mn\(^{2+}\) influx was abolished with SKF-96365, the rate of Mn\(^{2+}\) entry was 0.0170 ± 0.0014 %F/s, significantly slower than the basal rate of 0.0374 ± 0.0011 %F/s.
Figure 4.2.9.1. Effect of SKF-96365 on ATP response in VMFs. Abolition of Ca$^{2+}$ influx by SKF-96365 (50 μM) treatment shortened the duration of the ATP-elicited [Ca$^{2+}$], response in nPSS (representative of n = 47 cells).
Figure 4.2.9.2. Time taken for ATP (+/- SKF-96365)-induced \([\text{Ca}^{2+}]_i\) response to decay by half. The data represented in Figure 4.2.9.1 were compared with respect to \(t_{50}\), which is the time needed for \([\text{Ca}^{2+}]_i\) to decrease 50% from the peak. In a total of \(n = 47\) cells, \(t_{50}\) was shorter when VMFs were treated with SKF-96365 during ATP stimulation. (* \(P < 0.05\) vs. ATP)
Figure 4.2.9.3. Mn\textsuperscript{2+} quenching data of ATP/UTP application to VMFs. Basal Mn\textsuperscript{2+} entry rate was indicated by the dotted line [a]. Stimulation of VMFs with either ATP (100 µM) (representative of n = 90 cells) and UTP (100 µM) (representative of n = 146 cells) accelerated the rate of Mn\textsuperscript{2+} entry [b, c]. In the presence of SKF-96365 (SKF) (50 µM), Mn\textsuperscript{2+} entry was reduced (representative of n = 79 cells).
Figure 4.2.9.4. Mn$^{2+}$ entry rates under basal and ATP/UTP-stimulated conditions. Mn$^{2+}$ entry rates from the data represented in Figure 4.2.9.3 were calculated. Both ATP (in a total of n = 90 cells) and UTP (in a total of 146 cells) treatments significantly increased Mn$^{2+}$ entry rates. On the contrary, SKF-96365 together with UTP (in a total of n = 79 cells) resulted in very little Mn$^{2+}$ entry, the amount of which was even below the basal level. (* P < 0.05 vs. all bars)
4.2.10. More Evidence for P$_{2Y_2}$ Receptor-Mediated Ca$^{2+}$ Signaling in VMFs

In Section 4.2.8, [Ca$^{2+}$]$_i$ responses elicited by the selective P$_{2Y_2}$ agonist UTP and the non-selective P$_2$ agonist ATP were shown to have similar shapes and time courses. However, it is still possible for the presence and subsequent activation of P$_{2Y_1}$ receptors when they are stimulated preferentially with ADPβS or 2-Me-S-ATP, both of which are selective P$_{2Y_1}$ agonists. As illustrated in the representative traces of Figures 4.2.10.1 and 4.2.10.2, VMFs did not respond to either 100 μM ADPβS (n = 68 cells) or 100 μM 2-Me-S-ATP (n = 77 cells). To ensure viability of cells, VMFs were subsequently challenged with 100 μM ATP and [Ca$^{2+}$]$_i$ increases were observed.
Figure 4.2.10.1. Lack of \([\text{Ca}^{2+}]_i\) response under ADPβS stimulation. Adenosine 5'-O-(2-thio-diphosphate) (ADPβS) (100 µM), a selective P_{2Y1} agonist, was applied to VMFs. No \([\text{Ca}^{2+}]_i\) increase was observed (representative of \(n = 68\) cells). ATP (100 µM) was applied afterward to ensure viability of the cells.
Figure 4.2.10.2. Lack of $[\text{Ca}^{2+}]_{i}$ response under 2-Me-S-ATP stimulation. 2-methyl-thio-adenosine 5'-triphosphate (100 μM), another selective P2Y1 agonist, was applied to VMFs. Same as in Figure 4.2.10.1, no $[\text{Ca}^{2+}]_{i}$ increase was observed with P2Y1 stimulation by 2-Me-S-ATP (representative of n = 77 cells). ATP (100 μM) was applied afterward to ensure viability of the cells.
4.2.11. Sizes of Histamine- and ATP/UTP-Sensitive ER Ca\(^{2+}\) Stores

Having identified the histaminergic and purinergic receptor subtypes and the presence of separate ER Ca\(^{2+}\) compartments in VMFs, the next step was to determine if the histamine- and ATP/UTP-sensitive Ca\(^{2+}\) stores are inclusive of each other. As shown earlier in Figure 4.2.5.1, after exhausting the histamine-sensitive Ca\(^{2+}\) store, CPA was able to activate the release of additional Ca\(^{2+}\) from the ER. Experiments were done where ATP was added after histamine could no longer induce an [Ca\(^{2+}\)]\(_i\) increase (Figure 4.2.11.1, representative of n = 102 cells). The purpose of these experiments was to investigate whether ATP and histamine share the same ER Ca\(^{2+}\) compartment. Based on the data in Figure 4.2.11.1, ATP did not stimulate additional ER Ca\(^{2+}\) release after the abolition of the histamine response in 0CaPSS, but application of CPA afterward still induced a [Ca\(^{2+}\)]\(_i\) response. Since both histamine and ATP induced intracellular Ca\(^{2+}\) mobilization via activation of IP\(_3\)Rs, the CPA-induced [Ca\(^{2+}\)]\(_i\) response in Figure 4.2.11.1 can be further characterized as Ca\(^{2+}\) release from an IP\(_3\)-insensitive ER Ca\(^{2+}\) compartment. Using the same logic, the histamine-sensitive Ca\(^{2+}\) compartment described in Section 4.2.5 should more precisely be called the IP\(_3\)-sensitive ER Ca\(^{2+}\) compartment.

It is now clear that the ER Ca\(^{2+}\) releasable by histamine is inclusive of the ATP-sensitive Ca\(^{2+}\) store. The question remains whether repetitive ATP stimulation is able to deplete the entire IP\(_3\)-sensitive Ca\(^{2+}\) store, making subsequent histamine-induced [Ca\(^{2+}\)]\(_i\) responses impossible. As shown in Figure 4.2.11.2 (representative of n = 39 cells), after repeated ATP stimulations, application of histamine induced further ER Ca\(^{2+}\) release. ATP only stimulated partial Ca\(^{2+}\) release from the IP\(_3\)-sensitive store. Therefore, despite both ATP and histamine are IP\(_3\)-mobilizing agents, only the latter was able to deplete the entire IP\(_3\)-sensitive ER Ca\(^{2+}\) store.
Figure 4.2.11.1. ATP and histamine shared the same ER Ca\(_{2+}\) compartment. Histamine (His) (100 μM) was applied for three times in order to deplete the histamine-sensitive ER Ca\(_{2+}\) store. Subsequent application of adenosine 5'-triphosphate (ATP) (100 μM) failed to elicit a \([\text{Ca}^{2+}]_i\) response, suggesting that ATP and histamine, both IP\(_3\)-mobilizing agents, shared the same IP\(_3\)-sensitive Ca\(_{2+}\) store (representative of n = 102 cells). Treatment of cyclopiazonic acid (CPA) (30 μM) stimulated Ca\(_{2+}\) release from the IP\(_3\)-insensitive Ca\(_{2+}\) store.
Figure 4.2.11.2. Partial ER Ca$^{2+}$ emptying by ATP. VMFs were first repeatedly stimulated with adenosine 5'-triphosphate (ATP) (100 μM) until [Ca$^{2+}$]$_i$ increases were no longer present (representative of n = 39 cells). Application of histamine (His) (100 μM) afterward induced additional ER Ca$^{2+}$ release. The data suggested that although histamine and ATP shared a Ca$^{2+}$ compartment, the latter only partially depleted the Ca$^{2+}$ in that store.
4.2.12. Selected Parameters of Ca\(^{2+}\) Signaling in Normal and Rheumatic VMFs

The data presented so far only dealt with VMFs isolated from grossly normal cardiac valves. It would be of interest to make a cursory comparison between some Ca\(^{2+}\)-signaling properties in VMFs from normal and diseased valves. As indicated in Section 1.3, one of the most common valvular abnormalities is rheumatic valvular disease. In this part of the study, VMFs from 6 rheumatic and 5 normal valves were evaluated based on their responsiveness to pharmacological agents. In particular, peak \([\text{Ca}^{2+}]_i\) values (or peak \(F_{340}/F_{380}\) ratios) and AUCs of the histamine, ATP, and CPA responses were measured. For the CPA experiments, the \([\text{Ca}^{2+}]_i\) plateau was also compared between normal and rheumatic VMFs because SOC activity is implied when elevated \([\text{Ca}^{2+}]_i\) persists during SERCA blockade.

Tables 4.2.12.1 lists all the data and findings. In the table, experiments done in nPSS were indicated by "\(+\text{Ca}^{2+}\)" and those done in 0CaPSS were indicated by "\(-\text{Ca}^{2+}\)". In normal VMFs, histamine did not induce Ca\(^{2+}\) influx (see Section 4.2.2). This finding was confirmed here by the equal AUCs in Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free environments. In 0CaPSS, the AUC represents the total amount of Ca\(^{2+}\) release from the ER whereas in nPSS, the AUC is equal to the sum of Ca\(^{2+}\) release and Ca\(^{2+}\) influx. Thus, if the AUCs in nPSS and 0CaPSS are the same, no Ca\(^{2+}\) influx is present, as in the case with histamine stimulation in normal VMFs. In rheumatic VMFs, however, the AUC in nPSS was significantly larger than that in 0CaPSS, suggesting Ca\(^{2+}\) influx elicited by histamine in diseased cells.

Data in Section 4.2.9 showed that ATP induced Ca\(^{2+}\) influx in normal VMFs. Also shown in Table 4.2.12.1, the ATP AUC in nPSS was significantly larger than that in 0CaPSS. The AUC difference was also detected in rheumatic VMFs. Moreover, in nPSS, the AUC of rheumatic VMFs tended to be larger than the normal VMFs (\(P = 0.187\)). Thus, rheumatic VMFs
may have stronger Ca\textsuperscript{2+} influx machinery than normal VMFs, although the small sample size could not reveal the difference here.

According to Table 4.2.12.1, for either histamine or ATP responses, the peak sizes were not different between normal and rheumatic VMFs with or without extracellular Ca\textsuperscript{2+}. However, CPA in 0CaPSS elicited a significantly smaller peak than in nPSS. The CPA AUC in 0CaPSS was also smaller than in nPSS. As a result, CPA differed from histamine or ATP in that the SERCA blocker induced Ca\textsuperscript{2+} influx before the [Ca\textsuperscript{2+}]\textsubscript{i} response reached the peak. On the contrary, both histamine (in rheumatic VMFs) and ATP (in both normal and rheumatic VMFs) induced Ca\textsuperscript{2+} influx only after the [Ca\textsuperscript{2+}]\textsubscript{i} reached the maximum, as demonstrated by AUC differences and peak size equalities. Similar to ATP, CPA induced slightly more Ca\textsuperscript{2+} influx in rheumatic VMFs (P = 0.0734) than in normal VMFs. The stronger Ca\textsuperscript{2+} influx may in part be associated with the marginally higher plateau level in rheumatic VMFs (P = 0.241).
Table 4.2.12.1. Comparison of histamine-, ATP-, and CPA-elicited \([\text{Ca}^{2+}]_i\) responses in normal and rheumatic valvular myofibroblasts. The table lists mean ± SEM values from experiments on VMFs from 5 normal and 6 rheumatic valves.

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<th>Histamine</th>
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<td>Normal</td>
<td>Rheumatic</td>
<td>Normal</td>
</tr>
<tr>
<td><strong>Peak Level</strong></td>
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<tr>
<td>+\text{Ca}^{2+}</td>
<td>1.19 ± 0.10</td>
<td>1.16 ± 0.09</td>
<td>0.84 ± 0.05</td>
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<tr>
<td>–\text{Ca}^{2+}</td>
<td>1.13 ± 0.10</td>
<td>1.19 ± 0.07</td>
<td>0.86 ± 0.11</td>
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<tr>
<td><strong>Plateau Level</strong></td>
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<td>(% Peak)</td>
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<tr>
<td><strong>Area Under Curve</strong></td>
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<tr>
<td>+\text{Ca}^{2+}</td>
<td>137.49 ± 28.54</td>
<td>129.45 ± 22.23</td>
<td>79.38 ± 14.71</td>
</tr>
<tr>
<td>–\text{Ca}^{2+}</td>
<td>112.6 ± 28.52</td>
<td>96.66 ± 20.77*</td>
<td>65.83 ± 14.14*</td>
</tr>
</tbody>
</table>

* P < 0.05 vs. +\text{Ca}^{2+} values;
† P = 0.187 vs. Normal;
* P = 0.0734 vs. Normal;
& P = 0.241 vs. Normal.
4.2.13. Spontaneous Ca\textsuperscript{2+} Release in VMFs

In a small number of VMFs, spontaneous Ca\textsuperscript{2+} (spCa\textsuperscript{2+}) releases were observed without any application of drugs. Subcellular Ca\textsuperscript{2+} signals from individual Ca\textsuperscript{2+} release sites were visible using rapid scanning laser confocal microscopy. A total of 101 subcellular Ca\textsuperscript{2+} release sites from 10 unstimulated VMFs were analyzed. These VMFs were treated with either 15 mM caffeine or 100 μM ryanodine because of their potential actions on ryanodine receptors (RyRs), which mediate Ca\textsuperscript{2+} sparks in both cardiac and smooth muscles. Sample time-lapsed images of fluo-4-loaded VMFs displaying subcellular Ca\textsuperscript{2+} signals are shown in Figure 4.2.13.1.

In Figure 4.2.13.2, sample traces of spontaneous [Ca\textsuperscript{2+}]\textsubscript{i} signals from one subcellular Ca\textsuperscript{2+} release site in a VMF are shown. Spikes of Ca\textsuperscript{2+} release were present in the basal condition, whereas spCa\textsuperscript{2+} release was abolished during caffeine treatment. Figure 4.2.13.3 shows sample traces from another spCa\textsuperscript{2+} release site of a different VMF. The basal spCa\textsuperscript{2+} release patterns were similar in both Figures 4.2.13.2 and 4.2.13.3. However the baseline [Ca\textsuperscript{2+}]\textsubscript{i} level was depressed when VMFs were treated with ryanodine.

The histogram in Figure 4.2.13.4 shows the time distribution of [Ca\textsuperscript{2+}]\textsubscript{i} levels as a function of the baseline as described in Section 3.6.2. During the 30-second period when data were analyzed, [Ca\textsuperscript{2+}]\textsubscript{i} levels were near or below baseline (i.e. < 25 % above baseline) for over 90 % of the time. Compare with the unstimulated condition, when VMFs were treated with RyR modulators (caffeine or ryanodine), significantly more data fell below the baseline - 63 % (unstimulated) vs. 98 to 99 % (modulated RyR). For approximately one-third of the 30-second period, [Ca\textsuperscript{2+}]\textsubscript{i} levels were < 25 % above baseline in the basal condition, compared to only 0.5 – 1.6 % of data during caffeine or ryanodine treatment. Thus, a generally depressed [Ca\textsuperscript{2+}]\textsubscript{i}
baseline was observed when RyR activity was modulated, although caffeine may also suppress IP₃R activity.

The bar graph in Figure 4.2.13.5 compares the peak-to-peak time between spikes of Ca²⁺ releases. Under basal condition, the average latency period between Ca²⁺ release peaks was 6.37 ± 0.30 seconds. When treated with ryanodine, the time between Ca²⁺ release peaks was significantly increased to 9.01 ± 2.14 seconds. The ryanodine treatment data clearly showed that RyRs regulate spontaneous Ca²⁺ releases in VMFs. The suppression of spontaneous Ca²⁺ releases by caffeine, which has dual actions on RyRs and IP₃Rs, suggested possible roles of IP₃Rs in generating subcellular [Ca²⁺]ᵢ signals that can only be confirmed with more extensive studies.
Figure 4.2.13.1. Pseudocolored fluo-4-loaded VMF images showing various spontaneous Ca\(^{2+}\) (spCa\(^{2+}\)) release sites recorded over time. The top and bottom panels show spCa\(^{2+}\) releases of two different VMFs. The VMFs here were not stimulated and were viewed under a rapid laser-scanning confocal microscope acquiring images at 25-30 frames per second. Only representative images showing spCa\(^{2+}\) releases over the course of the experiments are illustrated here. The original grayscale images were converted with reference to the color scale according to the arbitrary \([\text{Ca}^{2+}]_i\) values.
Figure 4.2.13.2. Effects of caffeine on spontaneous Ca\textsuperscript{2+} releases in VMFs. The solid trace shows the basal spCa\textsuperscript{2+} release events of one site in a VMF. The ryanodine receptor opener caffeine (15 mM) abolished the spCa\textsuperscript{2+} releases and slightly depressed the baseline [Ca\textsuperscript{2+}]\textsubscript{i} (dotted trace).
Figure 4.2.13.3. Effects of ryanodine on spontaneous Ca\(^{2+}\) releases in VMFs. The solid trace shows the basal spCa\(^{2+}\) release events of one site in another VMF. The specific ryanodine receptor blocker ryanodine (100 μM) preserved the spCa\(^{2+}\) releases but prolonging the time between each spCa\(^{2+}\) release while also slightly depressed the baseline [Ca\(^{2+}\)], (dotted trace).
Figure 4.2.13.4. Distribution of spCa\(^{2+}\) release data as a function of [Ca\(^{2+}\)]. In experiments where spontaneous Ca\(^{2+}\) releases were examined (i.e. a total of 101 spCa\(^{2+}\) release sites in \(n = 10\) cells), data points from a 30-second period were grouped according to their [Ca\(^{2+}\)] values (as % of the peak).
Figure 4.2.13.5. Latency period between spCa$^{2+}$ release events. The time between the peaks of each spCa$^{2+}$ release was calculated. For the purpose of analysis, a spCa$^{2+}$ release event was defined as [Ca$^{2+}$]$_i$ > 25 % above baseline (please refer to Section 3.6.2 for details). Only data from basal and ryanodine (100 μM) treatments were included for calculation of the peak-to-peak latency, since caffeine treatment completely abolished the spontaneous Ca$^{2+}$ releases. Blockade of the RyR with ryanodine significantly increased the latency period between spCa$^{2+}$ releases. (* P < 0.05 vs. basal)
4.2.14. Schematic Model of Membrane Receptors and Ca\(^{2+}\) Transporters in VMFs

The current understanding of both spontaneous and drug-induced Ca\(^{2+}\) signaling pathways in human VMFs is summarized in Figure 4.2.14.1. Briefly, agonists binding to H\(_1\) and P\(_{2Y2}\) receptors stimulate ER Ca\(^{2+}\) release from one of two Ca\(^{2+}\) compartments while P\(_{2Y2}\) receptor activation also induces Ca\(^{2+}\) influx. Ca\(^{2+}\) influx also occurs via SOC opening when both IP\(_3\)-sensitive and IP\(_3\)-insensitive ER Ca\(^{2+}\) compartments are deprived of Ca\(^{2+}\). Although no caffeine-induced \([\text{Ca}^{2+}]_i\) response is present in VMFs, the presence of functional RyRs is implicated by their modulatory actions on spontaneous Ca\(^{2+}\) releases.
Figure 4.2.14.1. Schematic model of Ca\(^{2+}\) signaling in VMFs. Histamine, upon binding H\(_1\) receptor, activates intracellular Ca\(^{2+}\) release from the ER. Stimulation of P\(_{2Y2}\) receptors also induces Ca\(^{2+}\) release from the same store, albeit at a lesser extent. A secondary effect of P\(_{2Y2}\) receptor activation is the stimulation of Ca\(^{2+}\) influx, a phenomenon not observed with histamine application. Both histamine and ATP fail to elicit Ca\(^{2+}\) release from the entire ER, since inhibition of the SERCA reveals an additional ER Ca\(^{2+}\) compartment that is free of IP\(_3\)Rs. By completely depleting the ER of Ca\(^{2+}\), store-operated Ca\(^{2+}\) channel is activated, facilitating Ca\(^{2+}\) influx to refill the ER. Opening of RyRs, and possibly of IP\(_3\)Rs as well, are responsible for the fast, localized spontaneous Ca\(^{2+}\) releases observed in some cells.
4.3. Discussion

Numerous reports have suggested that VMFs, which are the predominant cells in cardiac valves, are the principal targets in the development of valvular diseases (Roberts 1973; Rose 1996; Becker 1998; Virmani 1998; McDonald et al. 2002). Evidence points to the roles of VMFs in valvular contraction (Filip et al. 1986), injury repair (Lester and Gotlieb 1988), and ECM secretion (Lester and Gotlieb 1988), all of which may be extensively controlled by Ca\(^{2+}\) signaling. There are a few reports on agonist-induced transient [Ca\(^{2+}\)]\(_i\) increases in VMFs (Taylor et al. 2000; Hafizi et al. 2000), but no information is available regarding the mechanisms involved. Thus, the present study investigated drug-induced Ca\(^{2+}\) signaling pathways in VMFs.

4.3.1. Histamine as an Intracellular Ca\(^{2+}\) Mobilizing Agent in VMFs

Histamine is one of the most important mediators of inflammatory and allergic reactions. The roles of histamine in the cardiovascular system have been well studied. Constrictions in conduit arteries are governed by H\(_1\) receptors, while peripheral vessels are relaxed by activation of both H\(_1\) and H\(_2\) receptors (Novak and Falus 1997). In the heart, histamine is inotropic, chronotropic, and arrhythmogenic, via activation of H\(_1\) and H\(_2\) receptors (Novak and Falus 1997). Little is known about the effects of histamine on cardiac valves, but with about 30% of hospitalized cardiac patients suffering from valvular heart disease (Soler-Soler and Galve 2000) this topic is certainly relevant to cardiac medicine.

In this study, hVMFs were stimulated consistently with 100 μM histamine due to the lack of reproducible responses at lower concentrations. At first glance, this histamine concentration seems high but its use was justified for two reasons. Firstly, the concentration of histamine in mammalian tissue ranges between 0.01 and 1 mM histamine (Babe Jr and Serafin 1996). Secondly, numerous studies of [Ca\(^{2+}\)]\(_i\) regulation in vascular smooth muscle cells (Krsti et al. 1996).
1996) and fibroblasts (Johnson et al. 1990; Niisato et al. 1996) have employed histamine concentrations in the 100 micromolar range to investigate Ca\(^{2+}\) signaling mechanisms at maximal levels of response.

It was shown in Figures 4.2.3.1 and 4.2.3.2 that the transient histamine-induced \([\text{Ca}^{2+}]_i\) increase in hVMFs is due to activation of \(H_1\) receptors, i.e. the \([\text{Ca}^{2+}]_i\) increase was selectively blocked by pyrilamine (\(H_1\) antagonist) but not by cimetidine (\(H_1\) antagonist). This finding agrees with an earlier study which showed that activation of \(H_1\) receptors is responsible for generating valvular contractions in pigs (Chester et al. 2000). The histamine-induced \([\text{Ca}^{2+}]_i\) responses were indistinguishable when elicited in either \(\text{Ca}^{2+}\)-containing or \(\text{Ca}^{2+}\)-free solution, as shown in Figures 4.2.1.1 and 4.2.2.1. In addition, blockade of \(\text{Ca}^{2+}\) influx by SKF-96365 did not alter \(\text{Ca}^{2+}\) signals elicited by histamine (Figure 4.2.2.2) nor did histamine accelerate the rate of \(\text{Mn}_{2+}\) entry in hVMFs. It is possible that the cell-culturing process may cause hVMFs to lose the expression of membrane-bound \(\text{Ca}^{2+}\) channels but this has not been the case in studies where cultured human smooth muscle cells were used (L'Heureux et al. 2001; Stepien et al. 2002). Therefore, the evidence suggests that histamine activation of \(H_1\) receptors induces an increase in \([\text{Ca}^{2+}]_i\) solely by \(\text{Ca}^{2+}\) release from the ER.

4.3.2. Intracellular \(\text{Ca}^{2+}\) Release and \(\text{Ca}^{2+}\) Influx Stimulated by Purinergic Agents in VMFs

Besides histamine, the other key vasoactive substances belong to the family of 5'-nucleotide triphosphates. Representative examples of this class of agents include endogenous ATP and UTP. In response to physiological stimuli, endothelial cells release ATP (Bodin et al. 1992) and UTP (Saiag et al. 1995). The actions of ATP and UTP may be autocrine or on surrounding cells. For example, activation of endothelial \(P_{2Y}\) receptors results in \([\text{Ca}^{2+}]_i\) increases and production of NO and EDHF (Marrelli 2001). Stimulation of \(P_{2X}\) and \(P_{2Y}\)
receptors increases the contractility of cardiac myocytes as a result of ER Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} influx (Vassort 2001). The ionotropic properties of P\textsubscript{2X} receptors (Vassort 2001) mean that they open to allow influx of Ca\textsuperscript{2+} (and other ions) but have no effects on intracellular Ca\textsuperscript{2+} release. On the contrary, P\textsubscript{2Y} receptors are G-protein-coupled and are upstream of the IP\textsubscript{3} signal transduction cascade. As shown in Figures 4.2.8.1 and 4.2.8.2, the purinergic agonists ATP and UTP induced ER Ca\textsuperscript{2+} release in VMFs in the absence of extracellular Ca\textsuperscript{2+}, suggesting minimal, if any, contribution of P\textsubscript{2X} receptors in intracellular Ca\textsuperscript{2+} mobilization. In some cells such as microglia, opening of P\textsubscript{2X} receptors prevents Ca\textsuperscript{2+} influx via P\textsubscript{2Y} receptors (Wang et al. 2000). This phenomenon is not present in VMFs, since preliminary data showed unaltered ATP-induced Mn\textsuperscript{2+} entry in the presence of pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), a selective P\textsubscript{2X} receptor antagonist. Thus, the emphasis of purinergic receptor-mediated Ca\textsuperscript{2+} signaling in VMFs is on the study of P\textsubscript{2Y} receptors.

It has been reported that ATP is rapidly degraded by endothelial ecto-ATPases to adenosine in whole tissue preparations (Bernardi et al. 1997; Fleetwood et al. 1989; Kuzmin et al. 1998; Meghji et al. 1992). Adenosine has cardioprotective effects during myocardial ischemia and reperfusion (Ely and Berne 1992). Vasodilatory actions by adenosine are also observed in smooth muscle (Shryock and Belardinelli 1997). The generation of adenosine complicates the study of ATP actions on whole tissues but not in the experiments with VMFs here, since ecto-ATPases and endothelial cells are not present in the specimens. The potential effects of adenosine on VMFs represent an entirely new research area that is beyond the scope of this study, and thus are not discussed here. Readers are referred to reviews by Ralevic and Burnstock (1998) and Shryock (1997) for details about adenosine and its pharmacological actions on the cardiovascular system.
Several reports have documented the use of high concentration of ATP and UTP (i.e. 100 μM) that elicits maximal responses of \([\text{Ca}^{2+}]_i\) and membrane potential (Mateo et al. 1996; Viana et al. 1998; Wang et al. 2000; Moccia et al. 2001). Therefore, 100 μM ATP and UTP, were applied to VMFs throughout this study. Similar efficacies of \([\text{Ca}^{2+}]_i\) responses elicited by ATP and UTP were seen in VMFs, and clear \(\text{Ca}^{2+}\) influx components were demonstrated in the presence of both agonists. As shown in Figures 4.2.8.1 and 4.2.8.2, the longer durations of \([\text{Ca}^{2+}]_i\) responses in nPSS than in 0CaPSS first suggested \(P_2\gamma\) receptor-stimulated \(\text{Ca}^{2+}\) influx. Secondly, Figure 4.2.9.3 showed acceleration of \(\text{Mn}^{2+}\) entry rates during the ATP and UTP treatments, indicating agonist-stimulated divalent cation influx. Lastly, incubating VMFs with the ROC/SOC blocker SKF-96365 significantly suppressed the \(P_2\gamma\) receptor-stimulated \(\text{Ca}^{2+}\) influx (and \(\text{Mn}^{2+}\) influx).

The choice of pharmacological agents in the characterization of \(P_2\gamma\) receptor subtypes in VMFs depends largely on existing literature documenting studies with other cell types in various species. According to a number of reviews (Ralevic and Burnstock 1998; Vassort 2001; Williams and Jarvis 2000; Alexander and Peters 1999), there is great heterogeneity in the potencies and efficacies of purinergic drugs on receptor subtypes between animals and cell types. The general consensus after consulting the literature is that ADPβS and 2-Me-S-ATP are widely accepted as selective \(P_2\gamma_1\) receptor agonists whereas UTP is frequently used to stimulate \(P_2\gamma_2\) receptors specifically. As mentioned before, activation of \(P_2\gamma_2\) receptors with UTP elicited \([\text{Ca}^{2+}]_i\) responses similar to those with ATP. In addition, Figures 4.2.10.1 and 4.2.10.2 showed that neither ADPβS nor 2-Me-S-ATP induced a \([\text{Ca}^{2+}]_i\) response. Thus, the present data indicate that \(P_2\gamma_2\) receptors are responsible for both ER \(\text{Ca}^{2+}\) release and \(\text{Ca}^{2+}\) influx in VMFs.
With regards to purinoreceptor antagonists, the conclusion from literature sources is that no selective P$_{2Y}$ antagonists are commercially available. Suramin is one of the more commonly used broad-spectrum P$_2$ receptor blockers (Dunn and Blakeley 1988). However, its P$_2$-antagonistic property is overcome by non-specific activities (Ralevic and Burnstock 1998). A report has shown that suramin non-competitively blocked serotonin and thromboxane receptors in platelets (Hourani et al. 1992). Most importantly, suramin increases skeletal and cardiac RyR opening probabilities and conductances (Sitsapesan and Williams 1996). Knowing from Section 4.2.13 that RyRs may contribute to spontaneous Ca$^{2+}$ releases in VMFs, suramin was not used in this study to block P$_2$ receptors. In fact, the use of suramin, minus its side effects, would not provide additional information on P$_{2Y}$ receptors in VMFs, amid the lack of selective purinoreceptor antagonists.

4.3.3. Distinction between IP$_3$-Sensitive and IP$_3$-Insensitive ER Ca$^{2+}$ Compartments

Both histaminergic (Bakker et al. 2002) and purinergic P$_{2Y2}$ receptors (Ralevic and Burnstock 1998) are coupled to G-proteins which, upon activation, stimulates IP$_3$ production and Ca$^{2+}$ release via IP$_3$Rs. There are other modes of Ca$^{2+}$ release from the ER – mediated by RyR (or cyclic ADP-ribose) and by nicotinic acid adenine dinucleotide phosphate (NAADP). Evidence in various cell types indicates that different modes of ER Ca$^{2+}$ release are not necessarily inclusive of each other. ACh-insensitive ER Ca$^{2+}$ store has been shown in fresh endothelial cells (Wang et al. 1995). In the pancreatic acinar cells, ACh-insensitive ER Ca$^{2+}$ store is prone to release by SERCA blockade (Camello-Almaraz et al. 2000), a phenomenon similar to the histamine- and ATP-insensitive ER Ca$^{2+}$ store in VMFs shown in Sections 4.2.5 and 4.2.11. Studies on smooth muscle and secretory gonadotropes have reported the existence of two independent, SERCA-containing ER Ca$^{2+}$ stores – one with IP$_3$Rs and RyRs and the other
with RyRs only (Johnson et al. 2000; Flynn et al. 2001). It is not unusual that ER Ca$^{2+}$ release via RyRs is not observed as easily as that mediated by IP$_3$Rs, as is the case in fresh endothelial cells (Wang et al. 1995). In the present study, a direct, measurable [Ca$^{2+}$]$_i$ response from opening RyRs was not visualized in VMFs. However, the presence of functional RyRs could be demonstrated by their modulatory actions on spontaneous Ca$^{2+}$ releases (please refer to Section 4.2.13 and 4.3.6 for details).

A series of experiments led to the finding of IP$_3$-sensitive and IP$_3$-insensitive (or SERCA-sensitive) ER Ca$^{2+}$ stores in VMFs. In Figure 4.2.4.2, it was shown that removal of Ca$^{2+}$ from the extracellular space had no effect on the histamine-induced [Ca$^{2+}$]$_i$ signal, but subsequent responses to histamine were abolished. In Ca$^{2+}$-containing solution, repeated histamine-induced [Ca$^{2+}$]$_i$ responses were seen, only interrupted by the application of CPA (Figure 4.2.4.3). It is worth mentioning here that CPA was preferred to thapsigargin in the experiment due to the latter’s irreversible blockade on the SERCA and subsequent cytotoxic events (Bai et al. 1999). Based on Figures 4.2.4.2 and 4.2.4.3, it is clear that refilling of the ER via Ca$^{2+}$ influx and SERCA-mediated sequestration is part of histamine-induced Ca$^{2+}$ signaling in VMFs. However, the fact that neither 0CaPSS (Figure 4.2.1.1) nor SKF-96365 (Figure 4.2.2.2) had any effect on the first [Ca$^{2+}$]$_i$ transient suggested that histamine did not activate receptor-operated or voltage-gated Ca$^{2+}$ channels. Therefore, refilling from the extracellular space was dependent on the presence of a basal Ca$^{2+}$ leak of the plasma membrane (Alderton and Steinhardt 2000). The observation that CPA increased the rate of decay of the histamine-induced [Ca$^{2+}$]$_i$ response and abolished the oscillatory component indicated in normal conditions, there is partial recycling of Ca$^{2+}$ from the cytosol to the ER.
When Ca\textsuperscript{2+} recycling is prohibited, not by SERCA inhibition, but by incubating VMFs in 0CaPSS as in Figure 4.2.4.2, the histamine-insensitive ER Ca\textsuperscript{2+} store is revealed. After VMFs no longer responded to histamine stimulation in 0CaPSS, additional Ca\textsuperscript{2+} release was induced by CPA (Figure 4.2.5.1). Based on the computational results explained in Section 4.2.4.6, the conclusion of two ER Ca\textsuperscript{2+} compartments in VMFs is reached. The histamine-sensitive Ca\textsuperscript{2+} compartment contains 53.6 % of total ER Ca\textsuperscript{2+} content, and the remaining 46.4 % Ca\textsuperscript{2+} makes up the histamine-insensitive Ca\textsuperscript{2+} compartment.

The previous paragraphs showed how histamine-insensitive (or CPA-sensitive) ER Ca\textsuperscript{2+} release was unresponsive to repetitive histamine stimulations. It is not clear until after the following experiments that the Ca\textsuperscript{2+} compartments (in)sensitive to histamine could be generalized in terms of their sensitivity to IP\textsubscript{3}. Experiments in Figure 4.2.11.1 illustrated that CPA, but not ATP (an IP\textsubscript{3} and Ca\textsuperscript{2+} mobilizing agent), was able to stimulate additional ER Ca\textsuperscript{2+} release after exhausting the histamine-sensitive Ca\textsuperscript{2+} store. A different result was seen when ATP was applied first, however. Figure 4.2.11.2 shows that following exposure to ATP, histamine was able to induce more Ca\textsuperscript{2+} release from the ER, with CPA stimulating the remaining ER Ca\textsuperscript{2+} release. It is paradoxical that both IP\textsubscript{3}-mobilizing agonists (i.e. histamine and ATP) would generate a response depending on the sequence they were applied in. With no known cross-desensitizing effects between H\textsubscript{1} and P\textsubscript{2Y2} receptors, one explanation for the observation in Figure 4.2.11.2 may rest in the distribution and number of P\textsubscript{2Y2} receptors in VMFs. For instance, if P\textsubscript{2Y2} receptors are localized to certain portions of the PM, the IP\textsubscript{3} produced probably diffuses only to the ER nearby and fails to activate all the IP\textsubscript{3}R. On the other hand, if P\textsubscript{2Y2} receptors are sparsely but evenly populated, globally only small amounts of IP\textsubscript{3} is produced upon ATP stimulation. The concentration of IP\textsubscript{3} maybe too diluted to stimulate full release of
the IP3-sensitive Ca\(^{2+}\) store. Nevertheless, the speculations above can only be verified by 1) immunofluorescent studies where the H\(_1\)R and P\(_{2Y2}\)R are labelled and 2) comparing both global and localized IP\(_3\) concentrations upon histamine and ATP application. To summarize, the data presented here are consistent with the notions of an IP3-sensitive Ca\(^{2+}\) store fully releasable upon stimulation by histamine, and an IP3-insensitive Ca\(^{2+}\) store that is susceptible to CPA.

4.3.4. Store-Operated Ca\(^{2+}\) Channel Activity in VMFs

Depletion of Ca\(^{2+}\) from the IP3-sensitive ER Ca\(^{2+}\) compartment alone is not sufficient to activate store-operated Ca\(^{2+}\) entry, as demonstrated by the lack of Ca\(^{2+}\) influx during histamine stimulation. Application of histamine only partially empties the ER of Ca\(^{2+}\). In VMFs, SOCs are activated when both IP3-sensitive and IP3-insensitive ER Ca\(^{2+}\) compartments are free of Ca\(^{2+}\) after SERCA blockade. SOCs, identified as Ca\(^{2+}\) channels which open in response to ER Ca\(^{2+}\) depletion (Clapham 1995), have been observed in many cell types, including vascular smooth muscle (Gibson et al. 1998; Zacks et al. 1991) and endothelium (Wang and van Breemen 1997). The slow rising phase of \([\text{Ca}^{2+}]_i\) (Figure 4.2.1.2) elicited by CPA is the net result of basal ER Ca\(^{2+}\) release and loss through SERCA locked in the open state. Prolonged CPA incubation eventually depletes the ER Ca\(^{2+}\) stores, and SOCs are activated subsequently. Activation of SOCs in VMFs is supported by three observations. Firstly, CPA caused a maintained elevation of \([\text{Ca}^{2+}]_i\), that is indicative of store-operated Ca\(^{2+}\) entry (Figures 4.2.1.2 and 4.2.4.3). Secondly, with CPA throughout the experiment, when 0CaPSS was substituted with nPSS, an elevated \([\text{Ca}^{2+}]_i\), above baseline was seen, again representing continuous Ca\(^{2+}\) influx (Figure 4.2.7.1). The last evidence of SOC activity is revealed by Mn\(^{2+}\) quenching experiments, which are commonly used in mimicking Ca\(^{2+}\) influx (Wang and van Breemen 1997; Trepakova and Bolotina 2001; Wang et al. 1995b). The rate of Mn\(^{2+}\) influx was significantly increased shortly after CPA was
added (Figure 4.2.7.2). The slight delay between CPA application and increase in Mn\(^{2+}\) influx rate maybe best explained by the time necessary for depletion of ER Ca\(^{2+}\), which is a prerequisite for the activation of SOCs.

4.3.5. \([\text{Ca}^{2+}]_i\) Responses of Normal and Rheumatic VMFs

The characterization of intracellular Ca\(^{2+}\) signaling mechanisms in the above sections allows the understanding of the physiology of normal VMFs. In response to the growing incidence of valvular heart diseases and their correlation with VMFs, the logical next step was to investigate the Ca\(^{2+}\) signaling properties of diseased VMFs. In this work of thesis, VMFs of the rheumatic origin were examined as a starting point towards more extensive and thorough studies on other valvular diseases in the future. As outlined in Table 4.2.12.1, comparisons between normal and rheumatic VMFs were made with respect to the peak sizes and AUCs of the histamine-, ATP- and CPA-induced \([\text{Ca}^{2+}]_i\) responses in nPSS and 0CaPSS. The most notable characteristic of rheumatic VMFs was the evidence for Ca\(^{2+}\) influx during histamine stimulation. In addition, there was marginally more Ca\(^{2+}\) influx induced by ATP stimulation in rheumatic VMFs \((P = 0.187)\). Slightly stronger store-operated Ca\(^{2+}\) influx \((P = 0.241)\) and lower ER Ca\(^{2+}\) content \((P = 0.0734)\) were detected in rheumatic VMFs. The data from rheumatic VMFs could be broadly interpreted as alterations in the diseased cells' handling of extracellular Ca\(^{2+}\). Ca\(^{2+}\) influx changes in rheumatic VMFs may have connections with reports 1) suggesting the alleviation of atrial fibrillations, associated with rheumatic heart disease, by Ca\(^{2+}\) channel blockers (Levy 2001), and 2) showing Ca\(^{2+}\) deposits in the myocardium of rheumatic hearts (Li 1993). Nevertheless, limited sample size and individual bio-variability in the present study may have hampered the statistics of some of the findings but they provide valuable leads in future experiments.
4.3.6. Spontaneous Ca\textsuperscript{2+} Releases and Functional Evidence for RyRs in VMFs

Spontaneous, subcellular Ca\textsuperscript{2+} releases were visualized in a number of VMFs in this study (Figure 4.2.13.1). The functional role of these Ca\textsuperscript{2+} releases in VMFs has yet to be determined, and it was unclear why not all the VMFs fire spontaneous [Ca\textsuperscript{2+}] \textsubscript{i} signals. However, the presence of Ca\textsuperscript{2+} sparks in cardiac (Eisner et al. 1998) and smooth muscles (Jaggar et al. 2000) may shed some light on the importance of spontaneous Ca\textsuperscript{2+} releases in VMFs. Ca\textsuperscript{2+} spark is a localized [Ca\textsuperscript{2+}] \textsubscript{i} increase caused by spontaneous opening of RyRs. It is believed that Ca\textsuperscript{2+} sparks mediate excitation-contraction coupling in cardiac myocytes and modulate vasorelaxation via the activation of Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channels (Imaizumi et al. 1999). In VMFs, there is evidence for Ca\textsuperscript{2+} sparks based on the data shown in Figures 4.2.13.2 and 4.2.13.3. These Ca\textsuperscript{2+} sparks probably contribute to the pulsatile movements of valvular leaflets in each cardiac cycle.

Both caffeine and 100 μM ryanodine treatments suppressed the [Ca\textsuperscript{2+}] \textsubscript{i} baseline and spontaneous [Ca\textsuperscript{2+}] \textsubscript{i} signals. Although known for its positive action on RyRs, caffeine has also been shown to inhibit IP\textsubscript{3}Rs in some cells (Bezprozvanny et al. 1994). The lack of a caffeine-induced [Ca\textsuperscript{2+}] \textsubscript{i} response in VMFs may suggest that the number or density of RyRs is relatively low. Thus, in VMFs the RyR-activating effect of caffeine may not overcome its potential IP\textsubscript{3}R-inhibitory effect. The abolition of spontaneous Ca\textsuperscript{2+} releases during caffeine treatment in Figure 4.2.13.2 implicitly suggests a role of IP\textsubscript{3}Rs in generating subcellular [Ca\textsuperscript{2+}] \textsubscript{i} signals. Experiments with selective IP\textsubscript{3}R modulators are required to confirm this speculation. In contrast, the ryanodine effect was straightforward since RyR blockade by 100 μM ryanodine inhibited RyR-mediated Ca\textsuperscript{2+} release, increasing the latency period of Ca\textsuperscript{2+} sparks (Figure 4.2.13.5). Therefore, the ryanodine data not only illustrate Ca\textsuperscript{2+} sparks via RyR opening, but also reveal the presence of functional RyRs in VMFs, previously unknown from global [Ca\textsuperscript{2+}] \textsubscript{i} measurements.
CHAPTER V: MECHANISMS OF CA\textsuperscript{2+} REMOVAL IN VASCULAR ENDOTHELIAL CELLS (ECs) – ROLE OF CYTOSKELETON AND CA\textsuperscript{2+} TRANSPORTERS

5.1. Overview

Having discussed several aspects of Ca\textsuperscript{2+} signaling in VMFs in the previous chapter, the following will concentrate on the maintenance of intracellular Ca\textsuperscript{2+} homeostasis in ECs, which are most likely to interact with VMFs in the cardiac valve. Numerous reports and reviews have discussed Ca\textsuperscript{2+} entry mechanisms in endothelial cells (ECs). One example is a review by Nilius and Droogmans (Nilius and Droogmans 2001). In contrast, only a handful of studies focused on how Ca\textsuperscript{2+} is removed from ECs. The lack of documentation on endothelial Ca\textsuperscript{2+} extrusion does not undermine the significance of this study. This is because a few studies have correlated abnormal Ca\textsuperscript{2+} removal in the endothelium with transplant vascular disease (Skarsgard et al. 2000; Demers et al. 2001) and oxidative stress-related conditions such as atherosclerosis (Grover and Sampson 1997). Even in the studies where endothelial Ca\textsuperscript{2+} removal was investigated, the conclusions were based on findings in cultured ECs (Paltauf-Doburzynska et al. 1999; Goto et al. 1996; Sedova and Blatter 1999). It has long been shown that cultured cells generally do not reflect \textit{in vivo} cell physiology as well as freshly isolated cells or intact tissues do. Using intact valvular endothelium may be ideal in this study; however, physiological influences by the underlying myofibroblasts, which to date are not well understood at all, may interfere with attempts to fully understand how endothelium functions on its own. Thus, a native preparation of enzymatically-dissociated ECs was used to elucidate mechanisms of cytosolic Ca\textsuperscript{2+} removal in this study. This native EC preparation enabled the study of single cell physiology without changes in expression of receptors and ion transport molecules so often seen during tissue culture.
In this study, although aortic ECs were used owing to the already established cell isolation protocols (Wang et al. 1995), the findings here would be applicable to ECs in general (i.e. valvular ECs) since an earlier report from our laboratory showed similar Ca$^{2+}$ signaling properties between aortic and valvular ECs (Li and van Breemen 1995).

In all experiments, acetylcholine (ACh) was used as the physiological stimulus on the freshly isolated ECs. Figure 5.1.1 shows sample $F_{340}/F_{380}$ images of fura-2-loaded ECs before and after ACh stimulation. The acquired data supported the idea of an in-series SERCA-NCX-linked Ca$^{2+}$ extrusion pathway. The importance of an intact cytoskeleton in facilitating this pathway was implicated. RyRs were shown to act as an intermediate component of the SERCA-NCX pathway. Functional couplings between SERCA & RyR, and NCX & RyR were demonstrated by simultaneous inhibition of both Ca$^{2+}$ transporters. Fluorescently labelled RyRs were visualized as additional evidence for the presence of functional RyRs in ECs. Lastly, the role of carboxyeosin as a PMCA blocker was observed. The use of carboxyeosin on top of NCX blockade effectively inhibited two parallel Ca$^{2+}$ removal pathways in ECs, one involving SERCA-RyR-NCX and the other involving PMCA.
Figure 5.1.1. F_{340}/F_{380} images of fura-2-loaded ECs before and after acetylcholine stimulation. As explained in Section 3.3.1, intracellular Ca^{2+} concentration ([Ca^{2+}]_{i}) was expressed as the ratiometric fluorescent intensity at 340-nm and 380-nm excitation. The original grayscale images were converted to pseudocolored ones with references to the color scale shown in the lower right of each panel. The [Ca^{2+}]_{i} values of the unstimulated ECs (left panel) increase upon stimulation with 10 μM acetylcholine (right panel).
5.2. Results

Note: Sections 5.2.1 and 5.2.2 reflected parts of a collective work (Wang et al. 2002) and represented integral components of this thesis.

5.2.1. Sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) and Sodium-Calcium Exchanger (NCX) Contribute in Series to Ca\(^{2+}\) Removal in ECs

In the control condition, freshly isolated ECs were stimulated with the physiological agonist acetylcholine (ACh, 10 μM) in nPSS. When [Ca\(^{2+}\)]\(_i\) level reached a plateau, both ACh and extracellular Ca\(^{2+}\) were removed and replaced with 0CaPSS. [Ca\(^{2+}\)]\(_i\) decline rates were used as a parameter of measuring Ca\(^{2+}\) extrusion in ECs. Besides the control, ECs were subjected in random order to three other treatments: SERCA inhibition, NCX inhibition, and simultaneous SERCA and NCX inhibition. Cyclopiazonic acid (CPA, 15 μM) was used to inhibit SERCA. Inhibition of NCX was achieved by substituting nPSS with 0NaPSS, which consisted of equimolar of N-methyl-D-glucamine (NMDG) in exchange of NaCl. Both CPA and 0NaPSS had no known adverse effects and their actions on SERCA and NCX were completely reversible. [Ca\(^{2+}\)]\(_i\) decline rates were compared among the control and CPA- &/or NCX-treated experiments. Figure 5.2.1.1 shows representative traces of ACh-induced [Ca\(^{2+}\)]\(_i\) increases in control and treated conditions (as reproduced from Wang et al. 2002). In this figure, the [Ca\(^{2+}\)]\(_i\) decaying phase of the control response was superimposed on the subsequent responses as dashed traces, demonstrating that ECs extrude Ca\(^{2+}\) slower when SERCA or NCX was inhibited. The rate of [Ca\(^{2+}\)]\(_i\) decay with combined CPA-0Na treatment was the same as that with CPA alone (superimposed as long dashed trace). Binned [Ca\(^{2+}\)]\(_i\) decline rates from all experiments were plotted against [Ca\(^{2+}\)]\(_i\) levels in Figure 5.2.1.2. Samples of exponentially fitted [Ca\(^{2+}\)]\(_i\) decaying phases are shown in Figure 5.2.1.3 and the average Ca\(^{2+}\) removal rate constants, as a percentage
of the maximum, i.e. the control rate constant, are shown in Figure 5.2.1.4. Both the raw \([\text{Ca}^{2+}]_i\) decline rates and calculated \(\text{Ca}^{2+}\) removal rate constants supported the finding in Figure 5.2.1.1 that SERCA and NCX handle \(\text{Ca}^{2+}\) in series for extrusion since blockade of either or both \(\text{Ca}^{2+}\) transporters reduces \(\text{Ca}^{2+}\) extrusion rate to the same extent.
Figure 5.2.1.1. Acetylcholine (ACh)-induced $[Ca^{2+}]_i$ elevations in ECs. ACh (10 μM) was applied to ECs in nPSS. Both ACh and nPSS was removed and substituted with Ca$^{2+}$-free solution (0CaPSS) once $[Ca^{2+}]_i$ has reached a plateau. Traces from control and 3 treatment conditions are shown below (representative of n = 6 experiments). The 3 treatments were: Na$^+$-free solution (0Na), cyclopiazonic acid (CPA), and a combination of both. 0Na was used to inhibit the activity of Na$^+/Ca^{2+}$ exchanger. CPA was used to block the SERCA. The $[Ca^{2+}]_i$ decline rates from different experiments were compared. The control $[Ca^{2+}]_i$ decline phase was reproduced in the subsequent traces (in short dashed form). The long dashed trace on top of the CPA+0Na-treated $[Ca^{2+}]_i$ decline phase was reproduced from the CPA experiment.
Figure 5.2.1.2. Summarized data of $[\text{Ca}^{2+}]_i$ decline rates at given ranges of $[\text{Ca}^{2+}]_i$. Instantaneous slopes of the raw traces (as exemplified in Figure 5.2.1.1) were calculated and binned into groups according to the % normalized $[\text{Ca}^{2+}]_i$. The $[\text{Ca}^{2+}]_i$ groups were 0-5 %, 5-10 %, 10-15 %, 15-20 %, 20-40 %, 40-60 %, 60-80 %, and 80-100 % normalized $[\text{Ca}^{2+}]_i$. From a total of $n = 6$ experiments in each treatment, data within each group were averaged and plotted as follows. Detailed explanation of the analytical method was given in Section 3.6.3. (* $P < 0.05$ vs. all treatments)
Figure 5.2.1.3. Sample one-component exponential fits of $[Ca^{2+}]_i$ decline phases in ECs. The kinetics of $[Ca^{2+}]_i$ declines in ECs was arbitrarily set to follow a 1st-order exponential decay: Normalized $% \text{[Ca}^{2+}\text{]}_i = e^{k \text{(time)}}$ where $k$ is the rate constant. Based on the raw data, computational iterations were made to determine the fitted curve that best represented the data. As a result of the fitting, $[Ca^{2+}]_i$ decay rate constants were calculated, and examples of which are shown in the inset table below.
Figure 5.2.1.4. Mean fitted Ca\textsuperscript{2+} decay rate constants as a percentage of the control value. Fitted 0Na-, CPA-, and 0Na+CPA-treated Ca\textsuperscript{2+} decay rate constants from different experiments were normalized to the control values (at 100 %). In a total of n = 6 experiments for each treatment, the control rate constant was significantly greater than the ones under NCX and/or SERCA inhibition. (* P < 0.0003 vs. control)
5.2.2. Serial Arrangement of SERCA and NCX is Maintained by Intact Cytoskeleton

The functional, serial arrangement of SERCA and NCX described above contributed significantly – about one-half – to Ca\(^{2+}\) extrusion in ECs, as shown by the 50 % reduction of Ca\(^{2+}\) removal rate constants in Figure 5.2.1.4. It is unlikely that such organization of Ca\(^{2+}\) transporters occurs completely by chance. Betz (1983) has shown that in brain capillary ECs the cytoskeleton may play a role in maintaining the functional linkage between IP\(_3\) receptors and the plasma membrane (PM). Cytochalasin D (CytD) is an actin-depolymerizing agent and was used here to disrupt the cytoskeleton in order to elucidate the mechanism of SERCA-NCX apposition.

The same control and (CPA &/or NCX) treated conditions were used as in Section 5.2.1, except the ECs were incubated with 50 μM CytD for 90 minutes prior to the experiments. It is noted that only 3 ACh responses could be obtained consistently after the CytD treatment, as opposed to the 4 responses in ECs without CytD incubation. The disruption of the cytoskeleton may render the ECs less responsive to repetitive agonist stimulation associated with altered spatial PM-ER arrangement. Due to this limitation, data from different experiments were pooled together for analysis. Figure 5.2.2.1 shows representative traces from one experiment where the control, CPA-treated, and 0Na-CPA-treated protocol was done. Binned Ca\(^{2+}\) decline rates are shown in Figure 5.2.2.2. Similar to untreated ECs, inhibition of either SERCA or NCX in CytD-treated ECs showed reduction in Ca\(^{2+}\) removal rates when compared to control. However, simultaneous SERCA and NCX inhibition resulted in further slowing of Ca\(^{2+}\) extrusion. This finding was supported by the Ca\(^{2+}\) removal rate constants (Figure 5.2.2.4) calculated from the exponentially fitted [Ca\(^{2+}\)] decaying phases (Figure 5.2.2.3). Another agent that disrupts the cytoskeleton via a different mechanism was also used. Latrunculin A (LatA, 30 μM) (De Oliveria and Mantovani 1988) works by inhibiting microfilament processes. Representative
traces from a separate experiment where the ECs was treated with LatA also showed that combined SERCA-NCX blockade slowed Ca\(^{2+}\) removal more than individual blockade of NCX or SERCA (Figure 5.2.2.5). Thus, it is evident that by dissociating the cytoskeleton, the serial arrangement of SERCA and NCX in fresh ECs is lost.
Figure 5.2.2.1. ACh-induced \([\text{Ca}^{2+}]_i\) elevations and declines in cytochalasin D (CytD)-treated ECs. CytD is an actin-polymerizing agent that disrupts the cytoskeleton of ECs. ECs were pretreated with 50 µM CytD for 90 minutes prior to ACh stimulation. In the sample shown below, CPA- and 0Na+CPA-treated \([\text{Ca}^{2+}]_i\) responses were obtained after the control (representative of \(n = 4\) experiments). This is in contrast to the total of 4 responses (1 control + 3 treatments) in ECs without prior CytD treatment (as in Figure 3.2.1.1). The CytD treatment might structurally alter the ECs in a way such that only 3 consecutive ACh responses could be recorded in the same experiment. Of the 3 responses in each experiment, the 0Na+CPA treatment was always present; the remaining responses were obtained from control, 0Na, and CPA, in random order. The peak of the 0Na+CPA-treated \([\text{Ca}^{2+}]_i\) response in each experiment represented 100 % of the normalized % \([\text{Ca}^{2+}]_i\). This enabled data from all the experiments to be pooled together for analysis. In this figure, the dotted trace was reproduced from the control response, and the long dashed trace from the CPA-treated response.
Figure 5.2.2.2. Summarized data of [Ca$^{2+}$]$_i$ decline rates in CytD-treated ECs. Data were analyzed in a similar manner as in Figure 5.2.1.2 for a total of $n = 4$ experiments.
Figure 5.2.2.3. Sample one-component exponentential fits of [Ca$^{2+}$]$_i$ decay phases in CytD-treated ECs. The inset table shows the fitted Ca$^{2+}$ removal rate constants, determined in the same manner as in Figure 5.2.1.3.
Figure 5.2.2.4. Comparison of the mean Ca\(^{2+}\) removal rate constants in CytD-treated ECs.
Fitted Ca\(^{2+}\) removal rate constants from different treatments were normalized to the control values in a total of n = 4 experiments. As in Figure 5.2.1.4, the control rate constant was significantly larger than those under 0Na or CPA treatment. However, a disrupted cytoskeleton further reduced the Ca\(^{2+}\) removal rate constant under combined NCX and SERCA blockades. (* P < 0.05 vs. control; # P < 0.05 vs. all bars)
**Figure 5.2.2.5.** Sample $[\text{Ca}^{2+}]_i$ responses in Latrunculin A (LatA)-treated ECs. LatA is another cytoskeleton dissociating agent. LatA (30 μM) was applied to ECs 90 minutes prior to the experiments. This figure shows representative control, 0Na-treated, and 0Na+CPA-treated ACh-induced $[\text{Ca}^{2+}]_i$ responses after the LatA treatment (in a total of n = 3 experiments). The dotted trace was reproduced from the control response, and the thin solid trace (superimosed on the CPA+0Na response) was reproduced from the 0Na-treated ACh response.
5.2.3. Ca^{2+} Removal is Facilitated by RyR Opening and Inhibited by RyR Blockade

After identifying the SERCA-NCX pathway of Ca^{2+} removal in ECs, the next step was to determine the ER Ca^{2+}-release channel in between the two Ca^{2+} transporters. The two major ER Ca^{2+} release channels in ECs are IP_{3}Rs and RyRs. IP_{3}Rs are involved in intracellular Ca^{2+} mobilization during agonist stimulation and thus, the role of these receptors in removing cytosolic Ca^{2+} is probably minimized. On the contrary, no known effects of RyRs have been found before although there have been reports of the presence of RyRs in ECs (Ziegelstein et al. 1994; Wang et al. 1995).

[Ca^{2+}]_i decline rates of control and ryanodine- or caffeine-treated ECs were compared. The control 10 \mu M ACh response was obtained first, and the treated response was done after 15 minutes of nPSS perfusion. The 15-minute washout allowed the ECs to fully recover from the previous stimulation, as was confirmed by two identical ACh responses obtained in 15 minutes apart in separate time control experiment (data not shown). Both 15 mM caffeine and 1 \mu M ryanodine were used as RyR activators. In Figures 5.2.3.1 to 5.2.3.4, the black solid trace denotes the control ACh response while the gray dotted trace denotes the caffeine- or ryanodine-treated ACh response. Caffeine (15 mM) facilitated the [Ca^{2+}]_i decline rate, as shown by the more rapid [Ca^{2+}]_i downstroke (Figure 5.2.3.1, representative of n = 8 experiments). The specific RyR ligand ryanodine exerts its activating effect at low concentrations, i.e. between 0.1 to 10 \mu M, depending on the tissue (Fill and Copello 2002). In the present study, 1 \mu M ryanodine increased [Ca^{2+}]_i decay rates in ECs (Figure 5.2.3.2, representative of n = 12 experiments). The data in Figures 5.2.3.1 and 5.2.3.2 showed that by increasing the RyR opening probability, cytosolic Ca^{2+} is removed more effectively.
Besides acting as a RyR agonist at low concentrations, ryanodine also blocks RyR at high concentrations (Fill and Copello 2002). RyRs in ECs were blocked by 100 μM ryanodine here. By comparing the ryanodine-treated [Ca\textsuperscript{2+}]\textsubscript{i} decay rates to those of the control cells, Ca\textsuperscript{2+} removal was slower when the RyR was blocked (Figure 5.2.3.3, representative of n = 8 experiments).

As mentioned in Section 3.6.3, two approaches were used to quantitate the [Ca\textsuperscript{2+}]\textsubscript{i} decline rate data. Average [Ca\textsuperscript{2+}]\textsubscript{i} decline rates from different experimental conditions: control, caffeine (15 mM), and ryanodine (1 and 100 μM) were plotted as a function of binned % normalized [Ca\textsuperscript{2+}]\textsubscript{i} levels, as described in Section 3.6.3 (Figure 5.2.3.4). At 15, 20 and 40 % normalized [Ca\textsuperscript{2+}]\textsubscript{i}, [Ca\textsuperscript{2+}]\textsubscript{i} declines were significantly faster in the presence of 15 mM caffeine or 1 μM ryanodine than in the control experiments (P < 0.05). On the other hand, Ca\textsuperscript{2+} extrusion in the 100 μM ryanodine treatment was significantly slower than control values (P < 0.05).

The experimental data were also analyzed by fitting the decaying phase of the [Ca\textsuperscript{2+}]\textsubscript{i} responses with the one-component exponential fit equation: \( y = a e^{bt} \) where b is the Ca\textsuperscript{2+} removal rate constant. Sample exponential fits are shown in Figure 5.2.3.5. In Figure 5.2.3.6, the bar graph shows average fitted Ca\textsuperscript{2+} removal rate constants. Under control conditions, Ca\textsuperscript{2+} removal rate constant after ACh stimulation was 0.063 ± 0.003 s\textsuperscript{-1}. The rate constants of the 1 μM ryanodine and 15 mM caffeine treatments were 0.090 ± 0.014 s\textsuperscript{-1} and 0.096 ± 0.008 s\textsuperscript{-1}, respectively, and were both significantly larger than control values (P < 0.05). When RyRs were blocked by 100 μM ryanodine, the rate constant was 0.028 ± 0.004 s\textsuperscript{-1}, significantly smaller than control values (P < 0.05). Based on both raw (Figure 5.2.3.4) and fitted (Figure 5.2.3.6) data, it can be concluded that RyR is an intermediate component of a cytosolic Ca\textsuperscript{2+} extrusion pathway in ECs.
Figure 5.2.3.1. Effects of caffeine on ACh-induced $[\text{Ca}^{2+}]_i$ responses in ECs. A control ACh-induced $[\text{Ca}^{2+}]_i$ response was first obtained, and $[\text{Ca}^{2+}]_i$ decay was monitored in $0\text{CaPSS}$ after removal of the agonist (representative of $n = 8$ experiments). The protocol was repeated except that the ryanodine receptor (RyR) activator caffeine (15 mM) was added together with $0\text{CaPSS}$. The solid line superimposed on the caffeine-treated response was reproduced from the control response.
Figure 5.2.3.2. Effects of 1 μM ryanodine-treated ACh-induced [Ca$^{2+}$]$_i$ responses in ECs. As in Figure 5.2.3.1, a control ACh-induced [Ca$^{2+}$]$_i$ response was first obtained, and [Ca$^{2+}$]$_i$ decay was monitored in 0CaPSS after removal of the agonist (representative of n = 12 experiments). ECs were pre-incubated with the specific RyR activator 1 μM ryanodine before repeating the protocol. The solid line superimposed on the 1 μM ryanodine-treated response was reproduced from the control response.
Figure 5.2.3.3. Effects of 100 µM ryanodine-treated ACh-induced [Ca\textsuperscript{2+}]\textsubscript{i} responses in ECs. The experiment was identical to that shown in Figure 5.2.3.2. The only difference was that ECs were pre-incubated with the RyR-inhibiting concentration of 100 µM ryanodine here (representative of n = 8 experiments). The solid line superimposed on the 100 µM ryanodine-treated response was reproduced from the control response.
Figure 5.2.3.4. Ryanodine receptor (RyR) modulating effects on Ca\(^{2+}\) removal rates in ECs. The summarized data were calculated as explained in Section 3.6.3. Data plotted below were obtained from treatments with caffeine (n = 8 experiments), 1 μM ryanodine (n = 12 experiments), and 100 μM ryanodine (n = 8 experiments). At any given [Ca\(^{2+}\)]\(_i\) from 15 % to 40 %, Ca\(^{2+}\) removal was facilitated when RyR was activated, and diminished when RyR was blocked.
Figure 5.2.3.5. Sample one-component exponential fits of RyR modulator-treated [Ca^{2+}]_i decaying phases in ECs.
Figure 5.2.3.6. Fitted Ca$^{2+}$ removal rate constants in RyR-modulated ECs. The data here again supported the claim that RyR mediated Ca$^{2+}$ removal. Opening of RyR with 15 mM caffeine (n = 8 experiments) or 1 μM ryanodine (n = 12 experiments) yielded Ca$^{2+}$ removal rate constants that were significantly greater than that of the control condition. On the contrary, control rate constant was larger than when RyR was inhibited with 100 μM ryanodine (n = 8 experiments).
5.2.4. RyR Opening Accelerates ER Ca\(^{2+}\) Loss in an Extracellular Ca\(^{2+}\)-Free Environment

Pharmacological actions of caffeine and ryanodine on RyRs were shown above. Using the same tools, RyR was again modulated and passive ER Ca\(^{2+}\) loss was monitored. To ensure that only Ca\(^{2+}\) release from the ER was measured, experiments were conducted in 0CaPSS. Data were acquired after 1, 5 and 15 minutes in 0CaPSS and ECs were stimulated with 10 µM ACh, which has been shown previously to completely deplete the ER of Ca\(^{2+}\) (Wang et al. 1995). The peak size of the ACh-induced Ca\(^{2+}\) response in 0CaPSS was used as a crude measurement of the amount of Ca\(^{2+}\) in the ER, from which the rate of ER Ca\(^{2+}\) loss could be inferred. The graph in Figure 5.2.4.1 shows peak sizes at 5 and 15 minutes of each treatment. Values were expressed as percentages of the control ACh response after 1 minute in each experiment. The relatively horizontal control and 100 µM ryanodine treatment (n = 6 experiments) curves indicated that ER Ca\(^{2+}\) content remained constant over time. The curves for 1 µM ryanodine (n = 8 experiments) and 15 mM caffeine (n = 3 experiments) treatments, however, showed much steeper slopes overall. Opening RyRs by either 1 µM ryanodine or 15 mM caffeine stimulated cumulatively a 30 % Ca\(^{2+}\) loss from the ER in 15 minutes in 0CaPSS. This finding suggested that in addition to increasing the Ca\(^{2+}\) removal rate at an elevated [Ca\(^{2+}\)]\(_{\text{i}}\) level (i.e. after ACh stimulation, as shown in Section 5.2.3), RyR opening also promotes basal ER Ca\(^{2+}\) depletion in 0CaPSS. The data here also confirmed that ryanodine exerts its agonistic effects at 1 µM and antagonistic effects at 100 µM.
Figure 5.2.4.1. Effects of RyR modulation on ER Ca\(^{2+}\) content of ECs. ECs were stimulated with acetylcholine (ACh) in 0CaPSS with and without pre-treatment of caffeine or 1 μM / 100 μM ryanodine in 0CaPSS for 1, 5, and 15 minutes. The [Ca\(^{2+}\)]\(_i\) peak was used as an indication of the relative ER Ca\(^{2+}\) content. For each drug treatment, [Ca\(^{2+}\)]\(_i\) peak levels were expressed as percentages of the ACh response after 1 minute of incubation. 100 μM ryanodine treatment (n = 6 experiments) had no significant effect on ER Ca\(^{2+}\) content while 1 μM ryanodine (n = 8 experiments) or 15 mM caffeine (n = 3 experiments) treatments induced a total of about 30 % Ca\(^{2+}\) loss from the ER over 15 minutes.
5.2.5. Ryanodine Receptors and NCX Are Functionally Coupled in Extruding Ca\(^{2+}\)

The data thus far showed that RyR (Section 5.2.4) and NCX (Section 5.2.1) are both involved in removing cytosolic Ca\(^{2+}\) after agonist stimulation. The next question was whether or not RyR and NCX handle Ca\(^{2+}\) in series for eventual extrusion. [Ca\(^{2+}\)]\(_i\) decline rates were thus compared under control, RyR-inhibited, NCX-inhibited, and RyR+NCX-inhibited conditions. Sample traces of ACh-induced [Ca\(^{2+}\)]\(_i\) responses in nPSS (black solid trace), 0NaPSS (light gray dashed trace), and 100 \(\mu\)M ryanodine-0NaPSS (dark gray dotted trace) from a total of 7 experiments are shown in Figure 5.2.5.1. For the control trace shown in Figure 5.2.5.1, sample [Ca\(^{2+}\)]\(_i\) decay under 100 \(\mu\)M ryanodine treatment from an earlier experiment was superimposed for comparison (shown as gray solid trace). Results of these experiments were summarized in Figure 5.2.5.2. The [Ca\(^{2+}\)]\(_i\) levels indicated in Figure 5.2.5.2 were normalized to the peak [Ca\(^{2+}\)]\(_i\) obtained in 0NaPSS. This was done to ensure that ECs were subjected to two ACh stimulations only, i.e. control vs. 0Na, 0Na vs. ryanodine, or 0Na vs. ryanodine + 0Na. When NCX was inhibited, [Ca\(^{2+}\)]\(_i\) decline was slower than control. This finding agreed with the data shown in Section 5.2.1. Also in Figure 5.2.5.2, 100 \(\mu\)M ryanodine treatment alone showed the same degree of [Ca\(^{2+}\)]\(_i\) decline rate reduction as the 0Na treatment. This indicated that by inhibiting RyRs, the same effect on Ca\(^{2+}\) removal rate was achieved as if NCX were blocked instead. Moreover, [Ca\(^{2+}\)]\(_i\) decline rates under 0Na treatment completely overlapped with those under 100 \(\mu\)M ryanodine + 0Na treatment, indicating that combined NCX and RyR blockades did not inhibit Ca\(^{2+}\) extrusion in an additive fashion. As a result, it may be concluded that RyR and NCX are coupled in series, sequentially transporting Ca\(^{2+}\) to be extruded to the extracellular space.
Figure 5.2.5.1. Evidence of functional NCX and RyR coupling in ECs. Representative traces (from left to right) of control (black solid trace), ONa-treated (light gray dashed trace), and 100 μM ryanodine + ONa-treated (dark gray dotted trace) ACh-induced $[Ca^{2+}]_i$ responses (representative of $n = 7$ experiments). At the control trace, the slower $[Ca^{2+}]_i$ decline under 100 μM ryanodine treatment (gray solid trace) from another experiment is shown here as a reference. The downstroke of the control response was superimposed on the ONa- and 100 μM ryanodine + ONa-treated responses for comparison.
Figure 5.2.5.2. **Summarized data of Ca$^{2+}$ removal rates of RyR- &/or NCX-inhibited ECs.** Data from experiments illustrated in Figure 5.2.5.1 were pooled together and were normalized to the maximal [Ca$^{2+}$]$_i$ achieved in 0Na treatment. Compared to control, [Ca$^{2+}$]$_i$ removal rates were reduced to the same extent under RyR &/or NCX blockade (total of n = 7 experiments for each treatment).
5.2.6. Ryanodine Receptors Act As a Link Between SERCA and NCX

Having identified the relationship between RyR and NCX in extruding Ca\textsuperscript{2+} from ECs, the next step was to confirm whether RyR is a component of the SERCA-NCX Ca\textsuperscript{2+} extrusion pathway reported in Section 5.2.1. More specifically, experiments were conducted to determine if SERCA serves as the Ca\textsuperscript{2+} transporter upstream of RyR, which then delivers Ca\textsuperscript{2+} to NCX for extrusion. The reversible SERCA blocker cyclopiazonic acid (CPA, 15 μM) was added to ECs and [Ca\textsuperscript{2+}]\textsubscript{i} decline rates with and without additional treatment of the RyR activator ryanodine (1 μM) were compared. In Figure 5.2.6.1 (representative of n = 10), the CPA-treated response (light gray dashed trace) showed slower [Ca\textsuperscript{2+}]\textsubscript{i} decay when compared to control (black solid trace). Despite opening RyRs (dark gray dotted trace) by 1 μM ryanodine, Ca\textsuperscript{2+} removal was slower when SERCA was inhibited altogether. Summarized data were shown in Figure 5.2.6.2. The finding in this section suggested that the proposed RyR-mediated Ca\textsuperscript{2+} extrusion pathway is effectively blocked once SERCA is inhibited. In addition to the data in Section 5.2.5 which states that RyR and NCX are part of the same Ca\textsuperscript{2+} extrusion pathway, it can be concluded that this pathway also consists of SERCA.
Figure 5.2.6.1. Effects of SERCA blockade and RyR opening on Ca$^{2+}$ extrusion from ECs. Representative traces (from left to right) of control (black solid trace), CPA-treated (light gray dashed trace), and 1 µM ryanodine + CPA-treated (dark gray dotted trace) ACh-induced [Ca$^{2+}$]$_{i}$ responses (representative of n = 10 cell clusters). For the CPA-treated responses, the CPA-induced gradual [Ca$^{2+}$]$_{i}$ increases (shown between the thin dotted vertical lines) were achieved prior to addition of ACh. The downstroke of the control ACh response was superimposed on the CPA- and CPA + 1 µM ryanodine-treated responses for comparison.
Figure 5.2.6.2. Summarized data of Ca\(^{2+}\) removal rates of CPA (+/- 1 μM ryanodine)-treated ECs. Blockade of SERCA with CPA significantly reduced Ca\(^{2+}\) removal rate, as also shown earlier in Figure 5.2.1.2. In the presence of 1 μM ryanodine, although RyR was activated, the SERCA-inhibited Ca\(^{2+}\) removal was still slower than control.
5.2.7. Fluorescent Labeling of Ryanodine Receptors

Ziegelstein et al. (1994) and Wang et al. (1995) have reported evidence for the presence of RyRs in ECs. In the above sections, data for the functional roles of RyRs have been presented. Up until now, RyR localization in freshly isolated ECs have not been visualized fluorescently. In this study, ECs were loaded with the fluorescent RyR indicator BODIPY TR-X-ryanodine (1 μM for 15 minutes, n = 4 experiments). Images of BODIPY TR-X-ryanodine-loaded ECs were taken with an ICCD-coupled confocal microscope at 0.15-μm horizontal sections, and for each stack of images the middle slice is shown (please refer to Section 3.5 for more details on the microscope system). Sample images of BODIPY TR-X-ryanodine-loaded ECs were shown in Figure 5.2.7.1 (left panels). As a negative control (n = 4 experiments), ECs were pretreated with an inhibitory concentration of ryanodine (100 μM) for 15 minutes prior to the application of BODIPY TR-X-ryanodine. At identical confocal microscope settings (e.g. laser power, black level, gain, iris size), prior treatment of 100 μM ryanodine prevented BODIPY TR-X-ryanodine from binding to RyRs and emitting fluorescence (Figure 5.2.7.1, right panels), demonstrating the binding specificity of the RyR fluorescent indicator. Three-dimensional composites of the same BODIPY TR-X-ryanodine-loaded ECs were shown in Figure 5.2.7.2.
Figure 5.2.7.1. BODIPY TR-X-ryanodine labeling of RyRs in ECs. ECs were treated with the specific fluorescent RyR ligand (1 μM BODIPY TR-X ryanodine) for 15 minutes with or without prior incubation of 100 μM ryanodine (representative of n = 4 experiments). These images were captured with an ICCD-coupled confocal microscope at a slice thickness of 0.15 μm. The images on the left illustrate BODIPY TR-X ryanodine fluorescence on RyRs. On the right are negative controls; ryanodine pre-treatment prevented BODIPY TR-X ryanodine binding to RyRs, thus no fluorescence was visible. All images were captured with the same microscope settings.
Figure 5.2.7.2. Three-dimensional composites of the BODIPY TR-X-ryanodine-labeled ECs. The images shown here were compiled from the stack of images taken from the same cells in Figure 5.2.7.1. Details on the preparation of the three-dimensional images were explained in Section 3.6.4.
5.2.8. Ca\(^{2+}\) Removal via Plasma Membrane Ca\(^{2+}\)-ATPase (PMCA) after NCX (and RyR) Blockade

It has been shown earlier that significant amount of Ca\(^{2+}\) was extruded, albeit at a slower rate, from ECs even after blockade of the SERCA-RyR-NCX pathway. The NCX-independent Ca\(^{2+}\) extrusion mechanism is probably via PMCA. As described in Section 5.2.5, the reduction of \([\text{Ca}^{2+}]_i\) decline rates in 0Na, 100 µM ryanodine, and 0Na + 100 µM ryanodine treatments were not distinguishable. The 0Na\(^{+}\) environment is sufficient in abolishing the entire SERCA-RyR-NCX pathway. Thus, for this part of the experiments, 0NaPSS was used as the tool to investigate non-NCX-associated Ca\(^{2+}\) extrusion. ACh-induced \([\text{Ca}^{2+}]_i\) responses were first recorded in 0NaPSS. Subsequently ECs were treated with the PMCA inhibitor carboxyeosin (CE, 25 µM) in the continual absence of extracellular Na\(^{+}\). Figure 5.2.8.1 shows representative traces (from \(n = 5\) experiments) of the inhibition of Ca\(^{2+}\) removal by CE on top of NCX blockade. It is clear that ACh-induced \([\text{Ca}^{2+}]_i\) increase was higher in the presence of CE, indicating a compromised Ca\(^{2+}\) removal system when PMCA is blocked. Average Ca\(^{2+}\) removal rates, binned as in the previous sections and summarized in Figure 5.2.8.2, showed marked inhibition of Ca\(^{2+}\) extrusion by simultaneous NCX and PMCA blockades.
Figure 5.2.8.1. Contribution of PMCA to Ca\(^{2+}\) removal in ECs. Representative traces showing ACh-induced [Ca\(^{2+}\)]\(_i\) responses in 0NaPSS and the slower [Ca\(^{2+}\)]\(_i\) decay phase under 25 µM carboxyeosin treatment in 0NaPSS (representative of n = 5 experiments). Carboxyeosin is an inhibitor of the PMCA. Only 0Na treatment was used here to compare to the actions of carboxyeosin since it has been established in the earlier experiments that 0NaPSS effectively blocked the RyR-NCX Ca\(^{2+}\) extrusion pathway.
Figure 5.2.8.2. Summarized data of Ca\textsuperscript{2+} removal rates of 0Na (+/-carboxyeosin)-treated ECs. By inhibiting the NCX, Ca\textsuperscript{2+} removal rate was significantly reduced. Treatment of carboxyeosin on top of 0Na resulted in further slowing of Ca\textsuperscript{2+} removal in a total of n = 5 experiments. Note: Ca\textsuperscript{2+} removal rates from control experiments were reproduced from Figure 5.2.5.2 and shown here for comparison.
5.2.9. Schematic Model of Ca\textsuperscript{2+} Removal Pathways in ECs

The current understanding of how elevated Ca\textsuperscript{2+} is removed from the cytosol of freshly isolated endothelial cells is shown in Figure 5.2.9.1. In brief, the ER and the PM are tightly associated by an intact cytoskeleton. The cytoskeletal network is essential in maintaining an in-series Ca\textsuperscript{2+} removal pathway after agonist stimulation. This pathway involves the following Ca\textsuperscript{2+} handling sequences: 1) Ca\textsuperscript{2+} uptake into the ER by SERCA; 2) ER Ca\textsuperscript{2+} release via RyR; and 3) Ca\textsuperscript{2+} extrusion via NCX to the extracellular space. PMCA functions in a parallel mechanism to extrude Ca\textsuperscript{2+} when the SERCA-RyR-NCX pathway is blocked.
Figure 5.2.9.1. Schematic model of Ca^{2+} removal pathways in ECs. Cytosolic Ca^{2+} is removed by sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) into the endoplasmic reticulum (ER), released from the ER via ryanodine receptors (RyRs), and extruded into the extracellular space via the Na^{+}/Ca^{2+} exchanger (NCX). This mode of Ca^{2+} removal is dependent on an intact cytoskeleton. When the SERCA-RyR-NCX pathway is inhibited, the plasma membrane Ca^{2+}-ATPase (PMCA), which acts in parallel to extrude Ca^{2+}, becomes the sole Ca^{2+} transporter to deliver Ca^{2+} to the extracellular space.
5.3. Discussion

The importance of Ca$^{2+}$ extrusion in endothelial Ca$^{2+}$ homeostasis has not been emphasized enough until recently. Much effort has been devoted in examining Ca$^{2+}$ entry mechanisms, but relatively poor understanding of the mechanisms of removing cytosolic Ca$^{2+}$ in the endothelium remains. Known Ca$^{2+}$ transporters in ECs, including SERCA, NCX and PMCA, were evaluated with respect to their contribution to Ca$^{2+}$ removal in this study. ER Ca$^{2+}$ release channels such as IP$_3$R and RyR also function to regulate [Ca$^{2+}$]$_i$, although the former mainly mediate agonist-induced [Ca$^{2+}$]$_i$ increase. Elevations in cytosolic [Ca$^{2+}$]$_i$ are generally not detected by RyR opening in ECs. In bovine ECs, RyRs have been shown to localize in the ER membrane facing the subplasmalemmal space, mediating store-operated Ca$^{2+}$ entry and endothelial nitric oxide synthase activity (Paltayf-Doburzynska et al. 1998). The localization of RyRs near the PM probably coincides with the dependence of the SERCA and NCX on the intact cytoskeleton in facilitating Ca$^{2+}$ extrusion. Thus, the roles of various endothelial Ca$^{2+}$ transporters in removing cytosolic Ca$^{2+}$ were thoroughly investigated in this study.

5.3.1. Structural Arrangement of SERCA and NCX in Removal of Ca$^{2+}$ from ECs

One mechanism via which elevated Ca$^{2+}$ is removed from the cytosol in ECs is through the NCX, as shown by the slower [Ca$^{2+}$]$_i$ decline in the 0NaPSS treatment in Figure 5.2.1.1. Blockade of NCX activity by removing extracellular Na$^+$ (and substituting with equimolar of NMDG to maintain a constant osmolarity), despite having non-specific effects, is preferred over other more selective agents. Bepridil and benzamil are two examples of selective NCX inhibitors; however, reports have documented the use of both drugs to block K$^+$ channels (Yumoto et al. 2004; Su et al. 2000). In contrast, the only side effect of 0NaPSS is its potential inhibition of the Na$^+/H^+$ antiporter (NHA). It has been shown in another study on rabbit
endothelium that NHA blockade has no effect on intracellular pH and \([Ca^{2+}]_i\) (Li and van Breemen 1995). Thus, resulting \([Ca^{2+}]_i\) decline rate reduction from ONaPSS treatment in this study was solely attributed to the inhibitory effect on the NCX.

The NCX is the final structure where \(Ca^{2+}\) passes through before being extruded to the extracellular space. The route of \(Ca^{2+}\) transport before reaching the NCX from the cytosol was resolved by experiments manipulating SERCA activity. CPA constitutes a better choice for blocking SERCA than thapsigargin because of the latter’s irreversible binding to SERCA and the induction of apoptotic events (Bai et al. 1999). As shown in Figures 5.2.1.1 and 5.2.1.2, the sizes of \([Ca^{2+}]_i\) decline rate decrease were the same in ONaPSS, CPA, and ONaPSS + CPA treatments. This finding implies that the amount of \(Ca^{2+}\) passing through either or both of SERCA and NCX is equal. The only explanation for this phenomenon is that SERCA and NCX are arranged in series, sequentially handling \(Ca^{2+}\) to be eventually extruded. When one component of this in-series arrangement is taken away, the entire pathway fails to function properly, thus slowing \(Ca^{2+}\) extrusion.

In order for the SERCA-NCX \(Ca^{2+}\) removal pathway to be effective, the two \(Ca^{2+}\) transporters must lie relatively close to each other. It is clear that SERCA is located on the ER membrane while NCX is situated on the PM. The mechanism underlying the correct alignment of the two independent membranes is worthy of investigation. During \(Ca^{2+}\) influx in smooth muscle, the superficial sarcoplasmic reticulum sequesters considerable amounts of \(Ca^{2+}\) (van Breemen et al. 1995). The SBB relies on the proximity between \(Ca^{2+}\) influx channels and the SERCA, very much analogous to the proposed apposition of SERCA and NCX in ECs. Thus, a similar SBB model may be applicable to endothelial \(Ca^{2+}\) regulation, with partial sequestration of cytosolic \(Ca^{2+}\) into the superficial ER prior to extrusion via NCX. The distribution of superficial
and central ER in the cell is unlikely a result of occurrence by chance. Subcellular organelles are known to be held in their respective locations by means of a cytoskeletal network, consisting of microtubules and actin filaments (Alberts et al. 1998). Experiments in Section 5.2.2 showed that an intact cytoskeleton maintains the SERCA-NCX alignment in ECs. Both cytochalasin D and latrunculin A, disrupting the cytoskeletal components via different mechanisms, broke apart the sequential linkage of SERCA and NCX. As a result, \([Ca^{2+}]_i\) decline rate was reduced further when SERCA and NCX were blocked simultaneously. With the modified cytoskeleton, SERCA and NCX no longer act in series but remove \(Ca^{2+}\) independently of each other.

5.3.2. Modulation of RyR Activity by Selective Pharmacological Agents

The SERCA-NCX \(Ca^{2+}\) extrusion pathway is not complete without a \(Ca^{2+}\)-release channel that exports the \(Ca^{2+}\) from the ER. In this study, RyR was proposed as the link between SERCA and NCX. Before presenting data supporting this claim, the pharmacology of RyRs is first discussed. Caffeine and ryanodine were used here to modulate RyR activity. Caffeine has been extensively used to stimulate \(Ca^{2+}\)-induced \(Ca^{2+}\) release via RyRs in both skeletal, cardiac, and smooth muscle (Copello et al. 2002; Kettlun et al. 2003). However, the effect of caffeine is not limited to RyRs, since IP$_3$R activity is also inhibited (Bezprozvanny et al. 1994). Despite caffeine’s IP$_3$-inhibitory effects, its use did not pose any problem in this study. In the experiments, the IP$_3$-mobilizing agonist ACh was removed after \([Ca^{2+}]_i\) reached a maximum. Extracellular \(Ca^{2+}\) was also removed at the same time. Theoretically \(Ca^{2+}\) release and influx were both abolished soon, if not immediately afterward. As illustrated in Figures 5.2.3.1 and 5.2.3.4, the caffeine-induced increase in \([Ca^{2+}]_i\) decline rate occurred towards the lower intracellular \(Ca^{2+}\) concentrations, i.e. when \([Ca^{2+}]_i\) has been decaying for some time, and \([Ca^{2+}]_i\) has almost returned to baseline level. By that time, it was speculated that IP$_3$Rs and IP$_3$-
mediated Ca$^{2+}$ release contributed minimally to the measured [Ca$^{2+}$]. Besides, 1 μM ryanodine and caffeine treatments shared a similar pattern in increasing [Ca$^{2+}$] decline rates (Figure 5.2.3.4). Since ryanodine is a highly selective RyR modulator (see the following paragraph) with no known IP$_3$R effects, it can be assumed that the common effects of ryanodine and caffeine on Ca$^{2+}$ clearance is due to the same RyR activating effects in ECs.

Ryanodine was the other drug used to modulate RyR activity in this study. Despite its specificity at RyRs, ryanodine has opposite effects on the RyR, owing to the complicated pharmacology of the receptor. Several reports and review articles have documented the concentration dependency of ryanodine’s actions in different tissues (Paltauf-Doburzynska et al. 1998; Fill and Copello 2002; Meissner 1986; Buck et al. 1992). It is generally agreed that ryanodine in the micromolar range tends to increase RyR opening probability. In this configuration, the RyR remains active and consistently releases ER Ca$^{2+}$. At higher concentrations, e.g. 100 μM, ryanodine initially opens RyR, subsequently locks it in a low-conductance or closed state, and Ca$^{2+}$ release is inhibited eventually (Fill and Copello 2002). This characteristic of ryanodine was also revealed in the data shown in Section 5.2.3 and is discussed in Section 5.3.5.

5.3.3. Other Potential RyR Modulators

There are many other commercially available pharmacological agents that elicit specific effects on RyRs. However their use was not practical in this study because of various reasons. Cyclic-ADP-ribose (cADPR) is a physiological RyR activator, but its inability to stimulate skeletal and cardiac RyRs has also been demonstrated, thus raising the possibility of multiple physiological stimuli for RyRs (Copello et al. 2001). Besides, cADPR is membrane-impermeable and its administration requires permeabilised cells, which will not allow the
measurement of \([\text{Ca}^{2+}]_i\) decline. Local anesthetics (LAs) such as tetracaine and procaine are not membrane impermeant, but are probably very poorly diffusible across the PM. The poor permeability may be the result of the LA’s cationic properties in solution. In order to reach the cytosol, the LA must first become nonionic. Thus, a charged-uncharged equilibrium governs the permeability of LAs. However, after getting across the PM the LA still needs to bind to the RyRs in the ER. According to several reports using LAs to inhibit ER \(\text{Ca}^{2+}\) release in various cell types (Lukyanenko et al. 2001; Lilly and Gollan 1995; Inoue et al. 2003; Turner et al. 2001), the effective concentrations of tetracaine and procaine are, respectively, 0.1 – 0.5 mM and 5 – 10 mM. The poor permeability of LAs is not only suggested by the use of millimolar concentrations in these studies, but is also illustrated by the fact that permeabilized cells were used in the above cited reports. It is highly questionable that similar concentrations of tetracaine or procaine would be effective in blocking RyRs in the intact ECs in this study. Most importantly, the LAs may induce non-specific side effects at such high concentrations. Another drug, ruthenium red was shown by Hoogduijn et al. to block RyRs in intact embryonic kidney cells (Hoogduijn et al. 2002). Unfortunately ruthenium red also blocks mitochondrial \(\text{Ca}^{2+}\) transport and requires membrane permeabilization in most other cells.

5.3.4. Role of RyR in Removing Cytosolic \(\text{Ca}^{2+}\) and Depleting ER \(\text{Ca}^{2+}\)

In Figures 5.2.3.2 and 5.2.3.3, 1 \(\mu\)M ryanodine treatment, by opening RyRs, was shown to result in a faster \(\text{Ca}^{2+}\) removal rate while 100 \(\mu\)M ryanodine inhibited \(\text{Ca}^{2+}\) extrusion by blocking RyRs. Low and high concentrations of ryanodine also produced different extents of ER \(\text{Ca}^{2+}\) depletion when ECs were left in \(0\text{Ca}^{2+}\)PSS for a 15-minute period (Figure 5.2.4.1). Both 15 mM caffeine and 1 \(\mu\)M ryanodine activated RyRs, but the higher \(\text{Ca}^{2+}\) removal rates (Figures 5.2.3.1 and 5.2.3.2) occurred before significant ER \(\text{Ca}^{2+}\) loss was detected (Figure 5.2.4.1). The
different onset times of caffeine or ryanodine effects may be attributed to two main factors: 1) different parameters were being measured and 2) measurements were done under different conditions. The effect of caffeine or ryanodine was seen quickly when the Ca\textsuperscript{2+} removal rate was measured, and for these measurements [Ca\textsuperscript{2+}]i was elevated as a result of ACh stimulation with RyRs actively handling ER Ca\textsuperscript{2+}. On the contrary, when caffeine or ryanodine was incubated in 0CaPSS in the measurement of ER Ca\textsuperscript{2+} loss, RyRs were activated under basal [Ca\textsuperscript{2+}]i conditions, i.e. ECs were not stimulated with ACh in the process. Thus, the difference in observed effect times of 1 µM ryanodine and 15 mM caffeine in Figures 5.2.3.1, 5.2.3.2 and 5.2.4.1 did not contradict each other, but rather converged to the same conclusion that open RyRs promote both ER Ca\textsuperscript{2+} depletion and Ca\textsuperscript{2+} extrusion. It is interesting to note that the concentration range of the biphasic ryanodine effects in the fresh rabbit ECs differed somewhat from those previously reported. For example, Paltauf-Doburzynska et al. (1998) showed that 25 µM ryanodine blocks RyRs in cultured bovine ECs whereas Turner et al. (2001) reported that in cultured guinea pig neurons, 1 µM ryanodine is already an inhibiting dose. It is possible that heterogeneity of RyR isoforms underlies this tissue- and species-dependent variability in ryanodine sensitivity.

5.3.5. Position of RyR in the SERCA-NCX Ca\textsuperscript{2+} Removal Pathway

The relationships between RyR & SERCA and RyR & NCX in Ca\textsuperscript{2+} removal were shown in Figures 5.2.5.2 and 5.2.6.2. Before these experiments were done, all is known is that both SERCA-NCX and RyR contribute to Ca\textsuperscript{2+} extrusion in ECs. Although the data so far agree with the proposed SERCA-RyR-NCX pathway, no concrete evidence has been shown yet. ECs were treated with 100 µM ryanodine and 0NaPSS so that both RyR and NCX were blocked. If RyR and NCX were not functionally coupled in an in-series manner, similar to that between NCX and SERCA, the result in Figure 5.2.5.2 should indicate a further slowing of [Ca\textsuperscript{2+}]i decline. The fact
that \([\text{Ca}^{2+}]_i\) decline rate was reduced to the same degree in treatments of 0NaPSS, 100  \(\mu\text{M}\) ryanodine, and 0NaPSS + 100  \(\mu\text{M}\) ryanodine suggests that RyR and NCX are components of the same \(\text{Ca}^{2+}\) removal pathway.

The next issue is between SERCA and RyR – whether these two \(\text{Ca}^{2+}\) transporters function in series. Instead of blocking RyRs here, RyRs were activated by 1  \(\mu\text{M}\) ryanodine. The purpose of combining RyR activation and SERCA blockade was to determine if SERCA is the first component of the proposed pathway. From Section 5.2.1, it is clear that SERCA inhibition decreased \(\text{Ca}^{2+}\) removal rate while in separate experiments RyR activation facilitated \(\text{Ca}^{2+}\) removal. If, during SERCA blockade, active RyR opening still increases \(\text{Ca}^{2+}\) removal rate, then either SERCA is not the only ER \(\text{Ca}^{2+}\) uptake mechanism or RyR does not belong to the same SERCA-NCX pathway. Figure 5.2.6.2 shows that SERCA inhibition with or without actively opening RyRs resulted in the same reduction of \([\text{Ca}^{2+}]_i\) decline rate. Thus, the conclusion is that SERCA, RyR and NCX 1) remove cytosolic \(\text{Ca}^{2+}\) in this order; 2) are arranged in series; and 3) are components of the same \(\text{Ca}^{2+}\) extrusion pathway in ECs.

5.3.6. Visualization of Ryanodine Receptors in Fresh Endothelial Cells

Prior to this study, RyRs in fresh ECs have not been visualized with fluorescence microscopy. In order to gain more information related to the localization of these receptors, RyRs were labelled with BODIPY TR-X-ryanodine. In other cells, BODIPY TR-X-ryanodine has been documented as a selective RyR ligand (Varadi and Rutter 2002). The specificity of BODIPY TR-X-ryanodine in fresh ECs is demonstrated in Figure 5.2.7.1, where pre-incubation of 100  \(\mu\text{M}\) ryanodine completely prevented receptor binding of the RyR label. The main conclusion from these micrographs is that the ECs do possess RyRs and that they may well be located peripherally. However to prove this point in these relatively small cells would require a
much better spatial resolution as could be obtained with immunogold electron microscopy. Despite the evidence for RyRs in ECs, caffeine failed to induce a \([\text{Ca}^{2+}]_i\) transient as shown by Wang et al (1995). This apparent paradox could be explained by the fact that most RyRs are located on the peripheral ER. Most of the \(\text{Ca}^{2+}\) released via RyRs is probably directed towards the NCX and extruded before reaching the cytosol. In another study, Lesh et al. (1993) showed extensive staining of anti-RyR antibody in the cytosol of guinea pig aortic endothelium. The variations in endothelial RyR distribution among different species may be the reason for tissue-specific caffeine-induced \([\text{Ca}^{2+}]_i\) responses, e.g. in bovine (Buchan and Martin 1991) and cultured human ECs (Neylon and Irvine 1990). Based on existing data, it may be concluded that RyR distribution varies among different tissues and species.

5.3.7. Effect of Combined PMCA- and NCX-Inhibition on \(\text{Ca}^{2+}\) Extrusion

In addition to demonstrating the in-series arrangement of SERCA, RyR and NCX in a common \(\text{Ca}^{2+}\) removal pathway, another ER-independent \(\text{Ca}^{2+}\) extrusion mechanism was also uncovered. This mode of \(\text{Ca}^{2+}\) extrusion acts in parallel to NCX and is undoubtedly the PMCA. No specific PMCA blocker is available at present, but some studies have reported PMCA-inhibitory effects of carboxyeosin (Moccia et al. 2002; Bassani et al. 1995) and caloxin (Chaudhary et al. 2001). Carboxyeosin (CE) was the choice of PMCA blocker in this study. As shown in Figure 5.2.8.1, CE further inhibited \(\text{Ca}^{2+}\) removal on top of NCX blockade. This marked inhibition of \(\text{Ca}^{2+}\) extrusion after blockade of NCX is consistent with \(\text{Ca}^{2+}\) extrusion via two parallel pathways: the PMCA and the composite of SERCA-RyR-NCX. The gradual \([\text{Ca}^{2+}]_i\) decay seen in the \(0\text{NaPSS} + \text{CE}\) treatment could be due to incomplete blockade by CE or may be complicated by cell mortality after this extreme intervention (unpublished observations). It is also possible that mitochondria become more active in \(\text{Ca}^{2+}\) removal, compensating for the
prolonged [Ca\textsuperscript{2+}], elevation. Future experiments where only the PMCA blocker (CE or other potential PMCA inhibitory agents) is used will be necessary in order to evaluate the percent contribution of PMCA alone in extruding Ca\textsuperscript{2+} from ECs.
CHAPTER VI: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The current work represented the first attempt to jointly study the Ca\(^{2+}\) signaling properties in endothelial cells and valvular myofibroblasts. These two types of cells are important regulators of a normal cardiac valve, and are modified significantly in a diseased valve. Much is known about the physiological and signaling aspects of ECs, but very few reports have examined the biology of VMFs. The different levels of knowledge about ECs and VMFs necessitated an experimental design with two foci. Without any previous work done on the signaling properties of the VMFs, the first focus of the study was to consider the fundamentals, i.e. the characteristics of receptor/channel-mediated Ca\(^{2+}\) signals in these cells. In cultured human VMFs, functional H\(_1\)R, P\(_{2Y2}\)R, and SOCs were identified pharmacologically and were found to elicit different Ca\(^{2+}\) mobilizing patterns. For instance, P\(_{2Y2}\)R activation, but not that of H\(_1\)R, induced Ca\(^{2+}\) entry. In addition, IP\(_3\)-sensitive and IP\(_3\)-insensitive intracellular Ca\(^{2+}\) compartments were identified. The second focus of the study was to elucidate the details of cytosolic Ca\(^{2+}\) removal from ECs, building upon a wealth of existing literature on Ca\(^{2+}\) entry pathways. By using an established model of freshly isolated aortic ECs, which are physiologically representative of ECs in general, two parallel Ca\(^{2+}\) removal mechanisms were elucidated. After agonist stimulation, cytosolic Ca\(^{2+}\) was extruded from the ECs via the PMCA and the composite of SERCA-RyR-NCX. This study has thus contributed to our understanding of endothelial Ca\(^{2+}\) homeostasis, as well as Ca\(^{2+}\) signaling mechanisms in VMFs. A more comprehensive investigation of Ca\(^{2+}\) regulation in VMFs is certainly required in future valvular research. This can be achieved, for example, by exploring the Ca\(^{2+}\) signaling characteristics of other vasoactive substances. Regarding the histaminergic and purinergic signaling pathways, efforts can be devoted in outlining the activation/deactivation of enzymes secondary to Ca\(^{2+}\).
mobilization. It is interesting to note that what is now known about the VMFs is somewhat similar to the findings in smooth muscle cells a couple decades ago. Thus, future studies with VMFs may also follow some of the approaches used with smooth muscle cells before. A good method is to use a bioassay system to measure VMF functions (e.g. contractility) in response to endothelial secretions. In the bioassay system, different endothelial secretions may be synthesized by challenging the ECs with different agonists or by manipulating the activity of Ca$^{2+}$ transporters responsible for extrusion. A better insight into the effects of endothelial releases on VMF responses may thus be gained. In addition to measuring contractile responses, the production of ECMs by VMFs (with or without endothelial influences) may also be determined by histochemical techniques. However, in vivo interactions between ECs and VMFs are resembled only when the intact valve is studied. With the advancement of imaging and electrophysiological techniques, simultaneous measurements of ionic concentrations and currents in ECs and VMFs in an intact valve should be a realistic goal in resolving the mystery around the development of valvular heart diseases.


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