INTRACELLULAR CALCIUM REGULATION IN THE INTACT ENDOTHELIAL CELLS FROM RABBIT AORTIC OR PULMONIC VALVES

by

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ABSTRACT

Intracellular Ca$^{2+}$ signal and nitric oxide (NO) release from the intact endothelial cells of rabbit aortic or pulmonic valves were measured by digital imaging microscopy and NO microsensor, respectively. Vasoactive agents such as agonists, Ca$^{2+}$ ionophore triggered an increase of cytoplasmic free calcium concentration ([Ca$^{2+}$]) and correspondent increase of NO release.

Inhibitors of endoplasmic reticulum (ER) Ca$^{2+}$-ATPase induced a [Ca$^{2+}$]$_i$ increase. SK&F 96365, a receptor-operated cation channel (ROC) blocker, and 2-nitro-4-carboxyphenyl-$N,N$-diphenyl-carbamate (NCDC), a postulated phospholipase C inhibitor and a ROC blocker, greatly reduce the agonist ATP induced sustained [Ca$^{2+}$]$_i$ increase, but not the ER Ca$^{2+}$-ATPase inhibitor CPA induced [Ca$^{2+}$]$_i$ increase. Ni$^{2+}$, a Ca$^{2+}$ entry blocker, blocked both the ATP- and CPA- induced [Ca$^{2+}$]$_i$ increases. Divalent cation entry measured as Mn$^{2+}$ quenching of fura-2 fluorescence was inhibited by Ni$^{2+}$, but enhanced by ATP. This enhancement was abolished by pretreatment with NCDC or SK&F 96365. In contrast, the rate of Mn$^{2+}$ quenching was unaffected by CPA. These results demonstrate that ATP stimulates a divalent cation influx through ROC, but interruption of ER Ca$^{2+}$ accumulation does not signal an increased Ca$^{2+}$ entry from the extracellular space. These results can be best explained by a "Superficial Buffer Barrier" (SBB) hypothesis where inhibition of Ca$^{2+}$ uptake into the ER disrupts the Ca$^{2+}$ buffer barrier function of ER, thus increasing the effectiveness of the Ca$^{2+}$ leak in raising [Ca$^{2+}$]$_i$.

A voltage gated Ca$^{2+}$ channel (VGC) blocker, diltiazem, did not affect the ATP-Induced sustained [Ca$^{2+}$]$_i$ increase. Depolarization of endothelial cells did not affect the
resting $[\text{Ca}^{2+}]_i$, but blocked the ATP-induced sustained $[\text{Ca}^{2+}]_i$ increase. These results indicate the absence of VGC in intact endothelial cells.

Decreasing the $\text{Na}^+$ gradient through either receptor stimulation (agonist), $\text{Na}^+,\text{K}^+$ pump inhibition (ouabain), $\text{Na}^+$ ionophore (monensin) or by reversing $\text{Na}^+$ gradient through $\text{Na}^+$ substitution all increased $[\text{Ca}^{2+}]_i$, implying the presence of a $\text{Na}^+-\text{Ca}^{2+}$ exchange as a mechanism for $\text{Ca}^{2+}$ entry on the plasmalemma of intact endothelium. This $\text{Ca}^{2+}$ entry component is enhanced when $[\text{Na}^+]_i$ is elevated.
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<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>CCh</td>
<td>carbachol</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BK</td>
<td>bradykinin</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet activating factor</td>
</tr>
<tr>
<td>[Ca$^{2+}$]$_i$</td>
<td>cytoplasmic free calcium concentration</td>
</tr>
<tr>
<td>[Na$^+$]$_i$</td>
<td>cytoplasmic free sodium concentration</td>
</tr>
<tr>
<td>[Ca$^{2+}$]$_i$</td>
<td>cytoplasmic free calcium concentration</td>
</tr>
<tr>
<td>[Ca$^{2+}$]$_o$</td>
<td>extracellular calcium concentration</td>
</tr>
<tr>
<td>[K$^+$]$_o$</td>
<td>extracellular potassium concentration</td>
</tr>
<tr>
<td>[Na$^+$]$_o$</td>
<td>extracellular sodium concentration</td>
</tr>
<tr>
<td>pHi</td>
<td>intracellular pH</td>
</tr>
<tr>
<td>Em</td>
<td>membrane potential</td>
</tr>
<tr>
<td>E$E_{Ca}$</td>
<td>reversal potential of Ca$^{2+}$</td>
</tr>
<tr>
<td>E$E_{Na}$</td>
<td>reversal potential of Na$^+$</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>EDRF</td>
<td>endothelium-derived relaxing factor</td>
</tr>
<tr>
<td>EDHF</td>
<td>endothelium-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>PGI$_2$</td>
<td>prostacyclin</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine 3', 5'-monophosphate</td>
</tr>
<tr>
<td>CTR</td>
<td>control</td>
</tr>
<tr>
<td>Phe</td>
<td>phenylephrine</td>
</tr>
<tr>
<td>SBB</td>
<td>superficial buffer barrier</td>
</tr>
<tr>
<td>CRAC</td>
<td>Ca$^{2+}$ release activated Ca$^{2+}$ increase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine 5'-triphosphatase</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic, endoplasmic reticulum Ca$^{2+}$ ATPase</td>
</tr>
<tr>
<td>ECS</td>
<td>extracellular space</td>
</tr>
<tr>
<td>PSS</td>
<td>physiological saline solution</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate-buffered saline solution</td>
</tr>
<tr>
<td>CPA</td>
<td>cyclopiazonic acid</td>
</tr>
<tr>
<td>Tg</td>
<td>thapsigargin</td>
</tr>
<tr>
<td>BHQ</td>
<td>2',5',-di(tert-butyl)-1,4,-benzohydroquinone</td>
</tr>
<tr>
<td>Kd</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>NCDC</td>
<td>2-nitro-4-carboxyphenyl-N,N-diphenyl-carbamate</td>
</tr>
<tr>
<td>DTZ</td>
<td>diltiazem</td>
</tr>
<tr>
<td>ROC</td>
<td>receptor-operated channel</td>
</tr>
<tr>
<td>VGC</td>
<td>voltage-gated channel</td>
</tr>
<tr>
<td>SAC</td>
<td>stretch-activated channel</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>CICR</td>
<td>Ca$^{2+}$ induced Ca$^{2+}$ release channel</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-Methyl D-Glucamine</td>
</tr>
<tr>
<td>DCB</td>
<td>dichlorobenzamyl</td>
</tr>
<tr>
<td>HMA</td>
<td>5-((N,N-hexamethylene)amiloride</td>
</tr>
<tr>
<td>EM</td>
<td>electron micrograph</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>AM</td>
<td>acetoxymethyl ester</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>HEPES</td>
<td>hydroxyethylpiperazine ethansulphonic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol bis-(β-aminoethyl)ether)N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>TEA</td>
<td>tetraethylammonium</td>
</tr>
<tr>
<td>IC50</td>
<td>half-inhibitory concentration</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
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PREFACE


To

My Parents
CHAPTER I

BACKGROUND

1.1. Introduction and history

The endothelium is not merely a simple barrier of varying permeability between the circulating blood and underlying smooth muscle or cardiac muscle, but also synthesize de novo many active substances, including large molecules such as fibronetin, interleukin-1, cytokines, tissue plasminogen activator and various growth-promoting factors, as well as smaller molecules such as nitric oxide, prostacyclin, platelet-activating factor and endothelin. These substances cause and/or modulate inflammation, platelet aggregation, thrombosis, fibrinolysis, extravasation, angiogenesis, mechanoreception, cerebral vascular autoregulation, vascular growth and repair, etc. Among these substances, nitric oxide (NO) and prostacyclin have received considerable attention, for they both have potent immediate actions on the underlying smooth muscle or myocardium and on platelets in the blood stream. In addition, they may have important roles in inflammation and vascular pathology such as atherosclerosis, thrombosis, hypertension, diabetes, septic (endotoxic) shock and dementia, etc. (reviews: Dusting, 1995; Adams et al., 1989, 1993; Shah, 1992; Moncada & Higgs, 1993; Jaffe, 1985; Moncada et al., 1991; Ganz & Alexander, 1985; Oyama et al., 1986; Lockett et al., 1986; Pearson, 1991).

1.2. Endothelium-Derived Relaxing Factor----Nitric Oxide

In 1980, Furchgott & Zawadzki discovered the essential role of endothelium lining on the intimal surface of the rabbit aorta for the vasodilatory action of acetylcholine (ACh) (Furchgott & Zawadzki, 1980). It was therefore concluded that ACh stimulated muscarinic receptors of the endothelium to release a low molecular weight non-prostanoid substance termed endothelium-derived-relaxing factor (EDRF) (Cherry et
Furchgott and Ignarro postulated that EDRF released by endothelium was nitric oxide (NO) or a closely related derivative of NO, acting in a similar way to the nitrovasodilator drugs (Furchgott et al., 1987; Ignarro et al., 1987). This speculation was subsequently confirmed (Palmer et al., 1987; Kelm & Schrader, 1988). Since then, many stimuli were found to require the presence of the endothelium to produce partial or complete relaxation of arteries, veins and microvessels. Among these are the calcium ionophore A23187, bradykinin, substance P, electrical stimulation, hypoxia, shear stress, and endogenous substances such as thrombin, ATP, serotonin and other inflammatory factors (Furchgott, 1983, 1984; Moncada et al., 1989; Lüscher & Vanhoutte, 1990).

1.3. The mechanisms of endothelial action

The primary target of NO in smooth muscle and platelets is the soluble guanylyl cyclase (GC). Activation of GC leads to elevation of the intracellular cyclic 3' 5' guanosine monophosphate (cGMP) which mediates muscle relaxation, and inhibits platelet aggregation and adhesion (Palmer, 1987; Furlong et al., 1987; Azuma, 1986, Radomski, 1987a, b, c, d).

A different vascular relaxation mechanism has also been proposed, involving the release of endothelium-derived hyperpolarizing factor (EDHF), a factor that is distinct from NO and prostacyclin (Feletou & Vanhoutte, 1988; Taylor & Weston, 1989). In addition, NO itself hyperpolarizes smooth muscle and accounts for most of the hyperpolarizing effect of ACh. The activation of K+ channels in the muscle membrane has been demonstrated to underlie the hyperpolarizing response (Tare et al., 1990; Dusting, 1995). The possible mechanism of endothelial functions is depicted in Figure 1.

1.4. Nitric Oxide Synthase

NO produced in mammalian cells is derived from L-arginine. A cytoplasmic NADPH-dependent enzyme system, NO synthase (NOS), converts L-arginine (ARG) to L-
citrulline (CITR). Several isoforms of NOS have been identified, with the "constitutive" isoforms being Ca\(^{2+}\)-dependent (Bredt & Synder, 1990; Luckhoff et al., 1988; Busse & Mulsch, 1990); whereas the "inducible" isoforms being functionally Ca\(^{2+}\)-independent (Stuehr & Marletta, 1987; Sessa, 1994). Although originally described as cytosolic (Moncada et al., 1991), most of the enzyme activity found in endothelium is in the particulate fraction (Fürstermann et al., 1991). Endothelial cells possess the Ca\(^{2+}\)-dependent "constitutive" NOS to synthesize and release NO within seconds in response to stimuli (Busse & Mulsch, 1990; Long & Stones, 1985). Relaxation of the rabbit aorta with an intact functional endothelium was demonstrated to be initiated by the Ca\(^{2+}\) ionophore A23187 and suppressed by the removal of Ca\(^{2+}\) from the incubation medium (Furchgott & Zawadzki, 1980; Furchgott, 1983). Cytoplasmic Ca\(^{2+}\) plays an important role in regulating the production and/or release of NO. The characteristics of \([\text{Ca}^{2+}]_i\) regulation are thus crucial for understanding the physiology of the endothelium as well as its role in diseases.

In the following sections, I will discuss the various mechanisms involved in the regulation of Ca\(^{2+}\) activation and homeostasis, and the role of Ca\(^{2+}\) in regulating the release of NO.

1.5. The structure of endothelial cells

The endothelium constitutes of a monolayer of cells lining the luminal surface of the blood vessels (vascular endothelial cell) and heart cavities (endocardial endothelial cell). The endothelial cell has a nucleus. It is thick in the nuclear region, and thinner toward the periphery. The cytoplasm contains a variable number of Weibel-Palade bodies (see chapter 1.7), endoplasmic reticulum, mitochondria, Golgi apparatus, vesicles and bundles of intermediate filaments. The plasmalemmal membrane consists of caveoli which are 50-100 nm membrane domains of various flat or invaginated morphology, increasing the surface area and functioning in the transcytosis of certain blood-borne macromolecules (Lisanti et al., 1994). The endoplasmic reticulum (ER), a
major intracellular Ca\textsuperscript{2+} store, is a highly convoluted meshwork of interconnected membranous tubules or flattened cisternae partly decorated with ribosomes. The ER constitutes more than half of the total membrane of the endothelial cell and extends throughout the cytoplasm (Albert et al., 1989). The ER membrane mediates rapid exchange of Ca\textsuperscript{2+} between the ER lumen and the cytoplasm. Although there is no direct histological support for the apposition of the plasmalemma and the ER in endothelial cells, a bridging structure has been observed between these two compartments in other types of cells such as vascular smooth muscle cells (Somlyo, 1985). It is therefore likely that these junctional regions may be present in endothelial cells that separate the cytoplasmic leaflets of the plasmalemma and the adjacent ER, with the "superficial ER" located in the cell periphery and the "deep or central ER" located in the deeper cytoplasm.

The continuous endothelial monolayer has an intact basal lamina and gap junctions between endothelial cells. However, gap junctions between endothelial cells and smooth muscle cells, the so-called myoendothelial junctions, are absent in endocardial endothelial cells. The junctional area in the endocardial endothelial cells is either a simple structure with straight intercellular clefts between two adjacent cells, or a complex organization with considerable overlapping between peripheral cell parts and membrane interdigitations. At some points there may be some close contacts such as tight junctions (Andries & Brutsaert, 1991; Laskey et al., 1994).

1.6. Endothelial cell sources:

Several endothelial cell types from different sources can be used for endothelial studies. Isolated, cultured and intact endothelial cells are mainly used. In certain situations, transformed endothelial cells are used (Jaffe, 1984; Ryan, 1984).

a. Isolated endothelial cells

Endothelial cells are freshly isolated from the vasculature and dispersed on a coverslip for study. There are several ways of harvesting cells from vasculature:
Mechanical harvesting: Endothelial cells on the surface of vessels can be mechanically isolated by scraping with a scalpel or peeling off the luminal surface of vessel. The endothelial sheets collected in this way preserve the original polarity. However, initial isolates are not suitable for studies requiring large numbers of uniform endothelial cells and have some inherent disadvantages such as contamination by smooth muscle cells. This method is not suitable for the study of endothelial cells from small vessels.

Proteolytic enzyme digestion: Due to the possibility of contamination by other cell types such as smooth muscle cells and fibroblast cells, researchers attempted to isolate endothelial cells from blood vessels by perfusing vessels with proteolytic enzymes (trypsin, collagenase, papain, etc.), making it possible to isolate relatively greater populations of endothelial cells. The enzymes, especially collagenase, selectively digest the subendothelial basement membrane, leaving the internal elastic lamina intact (Majno, 1970). This method markedly increases the yield of endothelial cells, decreases the contamination of smooth muscle cells or fibroblast cells and supports replication of endothelial cells. However these enzymes could affect membrane proteins which would be unfavorable for the studies of endothelial properties.

Microcarrier beads: Isolation of endothelial cells from small vessels can be achieved by perfusion with a cold solution and microcarrier beads without using proteolytic enzymes (Ryan, et al, 1982). Cold solutions can cause endothelial cells to round up and detach from the vessel wall. The loosened cells can be collected by perfusion. Microbeads in the perfusion solution provide a large surface area for endothelial adhesion and the yield of cells is enhanced. Cells can be removed from beads by vortexing.

b. Cultured endothelial cells:

The number of cells collected by isolation procedures is not sufficient for some studies. The endothelial cells can be subsequently seeded onto culture flasks containing tissue culture medium to proliferate. Monolayer growth and property of contact-
inhibition appear to be a fundamental characteristic of vascular endothelium. After transferring to a new flask, cells can then be replicated for more passages.

c. **Intact endothelial cells:**

To approximate the physiology of the intact vasculature, an intact endothelial preparation is used as a preparation that has not been exposed to any enzyme or culture medium. This preparation has the least mechanical damage.

1.7. Identification of endothelial cells

The vessel source for endothelial cells also contain smooth muscle cells, fibroblast, etc. It is therefore important to characterize the endothelial cells among these cells. The following is a list of the main markers that are frequently used for the positive identification of endothelial cells (Zetter, 1984):

a. **Microscopy**

Immediately after dispersion from an artery, the endothelial cells are often found to be flat and sheetlike, closely resembling their *in situ* appearance (Shimokawa & Vanhoutte, 1989; Sturek *et al.*, 1991). After being loaded with fura-2, the freshly isolated endothelial cells most often exhibit a morphology similar to a cluster of grapes. Placing the isolated endothelial cells in culture media for long periods yields a subcultured monolayer of cells that are grown to confluence with closely apposed (as they are joined by continuous tight junctions), homogeneous, typical "cobble-stone" appearance under low magnification phase contrast optics (Sturek *et al.*, 1991). Under differential interference contrast microscopy at higher magnification, the isolated endothelial cells reveal the characteristic tight cell-to-cell coupling with adhesion to a basement membrane that overlies two or three layers of collagen and elastin fibers with an oval, centrally located, nucleus (Jaffe *et al.*, 1973).

b. **Non-thrombogenic surface**

A critical function of the endothelium is to prevent platelets from adhering to the vessel wall. It is presumed that the presence or absence of specific molecules on the
luminal surface of the cell prevents platelet adhesion, but the nature of these molecules is not yet understood. Nonetheless, cultured endothelial cells growing in tissue culture maintain an upper surface to which platelets do not adhere. In contrast, platelets rapidly adhere to the upper surface of cultured fibroblasts or smooth muscle cells (Zetter, 1984).

c. **Factor VIII/von Willebrand (VIII/vWF) antigen**

Endothelial cells, megakaryocytes and platelets contain Factor VIII/von Willebrand (VIII/vWF) antigen when examined by immunofluorescence microscopy using specific antisera, whereas smooth muscle cells and fibroblasts both lack this antigen (Bloom et al., 1973; Jaffe, 1977).

d. **Weibel-Palade bodies**

Endothelial cells, either in situ or in culture, contain unique rod-shaped cytoplasmic inclusion bodies composed of longitudinally located tubules, Weibel and Palade (WP) bodies, which usually have an oval or circular outline when studied by electron microscopy (Weibel & Palade, 1964). The diameter of the WP bodies measures up to 0.4 μM. WP bodies are dispersed throughout the cytoplasm of the endothelial cell. These organelles are not present in either smooth muscle cells or fibroblast cells, thus they are an excellent marker of endothelial cells. Their function is not completely known. WP bodies contain the von Willebrand factors (vWF), which may play an important role in hemostatic regulation and are probably an adhesion factor for endothelial cells in injured vessels (Andries & Brutsaert, 1991; Sporn et al., 1989). Unfortunately, WP bodies are much less frequent or absent in endothelial cells derived from some species and are thus less useful in identifying endothelial cells in these species.

e. **Angiotensin Converting Enzyme (ACE)**

Angiotensin converting enzyme (ACE), an enzyme situated on the surface of endothelial cells, is commonly used as an endothelial marker although this enzyme can also be found in some types of macrophages or fibroblasts. The presence of ACE does not
guarantee that a cell is of endothelial origin but the absence of ACE activity in putative endothelial cells could raise some doubt.

f. **Low density lipoprotein (LDL)**

The presence of immobile receptors on the cell surface for acetylated low density lipoprotein (AcLDL) is often used as the positive identification of endothelial cells. Cells are incubated in growth media containing AcLDL labeled with the fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (dil). The uptake of fluorescent dil-AcLDL by cells can be visualized on an epifluorescence microscope equipped with rhodamine fluorescence filters. All endothelial cells incorporate dil-AcLDL in localized regions. A negative control is provided by contractile smooth muscle cells and fibroblasts.

g. **Production of prostacyclin (PGI2)**

Endothelial cells facilitate blood flow by producing prostacyclin (PGI2) to prevent platelet aggregation and cause vasodilation. The production of PGI2 by endothelial cells is an inducible phenomenon that is stimulated by a diverse groups of vasoactive agents (Moncada, et al., 1976).

1.8. **Regulation of [Ca2+]i**

Regulation of endothelial [Ca2+]i is composed of activating mechanisms which supply Ca2+ to the cytoplasm and homeostatic mechanisms which remove cytoplasmic Ca2+ after stimulation. The ionic transport mechanisms that underlie endothelial [Ca2+]i regulation are shown in Figure 2.

The activating mechanisms include Ca2+ entry from extracellular space (ECS) and Ca2+ release from the intracellular store(s).

a. **Ca2+ entry pathways:**

Ca2+ "leak": Under physiological conditions a passive non-regulated Ca2+ leak across the plasmalemma, driven by the electrochemical gradient for Ca2+ (E_m-E_Ca, E_m: membrane potential; E_Ca: the equilibrium potential for Ca2+), is present and increases
[Ca$^{2+}$]$_i$ in endothelial cells (Johns et al., 1987; Schilling, 1989; Demirel et al., 1993). Depolarization reduces the driving force for Ca$^{2+}$ entry through this leak pathway by reducing the electrochemical gradient. The nature of the leak pathway in endothelial cells is not known but it may play an important physiological role in the basal release of EDRF and other vasoactive mediators, thus regulating vascular tone and peripheral resistance.

Receptor-operated Ca$^{2+}$ channels (ROCs): The binding of an agonist to its receptor leads to an enhanced Ca$^{2+}$ influx (Whorton et al., 1984; Johns et al., 1987; Lodge et al., 1988). The existence of ROCs permeable to Ca$^{2+}$ has been demonstrated in endothelial cells (Bregestovski et al., 1988; Nilius, 1990; Yamamoto et al., 1992). The observation that apparently similar ion channels are activated by different agonists suggests a convergence of the intracellular messenger cascade between receptor activation and channel opening. The second messenger which activates ROCs remains unknown.

Stretch-activated Ca$^{2+}$ channels (SACs): Mechanosensitive (stretch-activated) ion channels in endothelial cells may serve as transducers in detecting changes in blood pressure or flow (Lansman, et al., 1987; Popp & Gögelein, 1992). Mechanosensitive ion channels could change endothelial cell membrane potential, and thus the driving force for passive Ca$^{2+}$ entry. This may be a possible mechanism by which the vascular endothelium in intact vessels regulates smooth muscle tone in response to haemodynamic stimuli (Rubanyi., et al., 1990).

Na$^+$.Ca$^{2+}$ exchange: The existence of Na$^+$-Ca$^{2+}$ exchange in endothelial cells has been a controversial issue. The Na$^+$-Ca$^{2+}$ exchanger functions reversibly so that Ca$^{2+}$ can be transported in either direction (inwardly or outwardly) across the plasmalemma in exchange for Na$^+$, depending on the electrochemical gradients of Na$^+$ and Ca$^{2+}$ on both sides of the membrane (Blaustein, 1977). Variations of the intracellular or extracellular Na$^+$ concentration thus affects the [Ca$^{2+}$]$_i$ level. The net Ca$^{2+}$ movement [JCa(Na/Ca)] mediated by the exchanger is determined by the membrane potential
(E_m), the reversal potential of the exchanger (E_{Na/Ca}) and the kinetic parameter (k) that controls the rate of exchange: J_{Ca(Na/Ca)} = k(E_m - E_{Na/Ca}). The stoichiometry for the exchanger has been shown to be 3Na\(^+\): 1Ca\(^{2+}\). Thus the reversal potential for the Na\(^+\)-Ca\(^{2+}\) exchange is E_{Na/Ca} = 3E_{Na} - 2E_{Ca}, where the equilibrium potential for Na\(^+\): E_{Na} = (RT/F)ln([Na\(^+\)]_o/[Na\(^+\)]_i), and the equilibrium potential for Ca\(^{2+}\): E_{Ca} = (RT/2F)ln([Ca\(^{2+}\)]_o/[Ca\(^{2+}\)]_i). R, T, and F are the gas constant (1.987 cal K\(^{-1}\) mol\(^{-1}\)), absolute temperature (273.16 K[°Celsius]) and Faraday's number (9.648 x 10\(^4\) C mol\(^{-1}\)), respectively. If E_{Na/Ca} is lower than the membrane potential (E_m), the exchanger in vivo will operate in the Ca\(^{2+}\) influx mode (inwardly); if the E_{Na/Ca} is higher than E_m, the exchanger will operate in the Ca\(^{2+}\) efflux mode (outwardly).

Voltage-gated Ca\(^{2+}\) channels (VGCs): Conflicting lines of indirect evidence were provided for (Rubanyi et al., 1985a; Rubanyi & Vanhoutte, 1988; Singer & Peach, 1982; Bossu et al., 1989; Bossu et al., 1992; Bkaily et al., 1993; Clain et al., 1994) and against (Colden-stanfield et al., 1987; Hallam & Pearson, 1986; Busse et al., 1988; Jacob et al., 1988; Morgan-Boyd et al., 1987; Olesen et al., 1988a, b; Retrosen & Gallin, 1986; Lansmen et al., 1987; Takeda et al., 1987; Jaffe et al., 1987; Brotherton & Hoak, 1982; Daut et al., 1988; Cannell & Sage, 1989; Lückhoff & Busse, 1990a; Sturek et al., 1991; Furchgott, 1983) the presence of VGCs in endothelial cells. For the studies that argue against the presence of VGC, the role of E_m in regulating Ca\(^{2+}\) entry is simply to determine the driving force for Ca\(^{2+}\) entry (E_m - E_{Ca}). Thus depolarization decreases and hyperpolarization increases [Ca\(^{2+}\)]_i in endothelial cells.

K\(^+\) channels are known to regulate E_m. Membrane depolarization of endothelial cells by elevating extracellular K\(^+\) or under voltage clamp conditions reduces agonist-stimulated Ca\(^{2+}\) influx in endothelial cells (Adams et al., 1989; Laskey et al., 1990b; Lückhoff & Busse, 1990b). There are at least four types of K\(^+\) channels present in endothelial cells: 1) an inwardly rectifying K\(^+\) channel (K_{IR}) activated upon hyperpolarization (Johns et al., 1987; Takeda et al., 1987) or by shear stress (Olesen et al., 1988a); 2) a transient (A-type) outward K channel (K_{A}) (Takeda et al., 1987);
3) a Ca$^{2+}$-dependent K$^+$ channel activated by membrane depolarization and a rise in [Ca$^{2+}$]$_i$ (K$_{Ca}$) (Sauvè et al., 1988; Colden-Stanfield et al., 1990; Rusko et al., 1992a); and 4) an ATP-sensitive K$^+$ channel (K$_{ATP}$) (Janigro et al., 1992). In addition, a muscarinic gated K$^+$ channel (K$_{ACh}$) was also proposed in endothelial cells (Olesen et al., 1988b; Busse et al., 1988).

b. Ca$^{2+}$ release from the ER

In the absence of extracellular Ca$^{2+}$, agonists evoke a transient release of EDRF associated with a transient [Ca$^{2+}$]$_i$ increase and $^{45}$Ca$^{2+}$ efflux (Frey et al., 1989). It clearly indicates an agonist-induced Ca$^{2+}$ release from intracellular store(s). The intracellular Ca$^{2+}$ stores might serve as an important source of Ca$^{2+}$ for activation of Ca$^{2+}$-dependent K$^+$ channels.

**IP$_3$-mediated Ca$^{2+}$ release from ER:** The intracellular inositol 1,4,5 trisphosphate (IP$_3$) level is enhanced upon agonist stimulation and is correlated with [Ca$^{2+}$]$_i$ increase (Lambert et al., 1986). IP$_3$ is released into the cytoplasm following the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP$_2$) in response to phospholipase C (PLC) activated by receptor-G protein. IP$_3$ is a second messenger for agonist-induced Ca$^{2+}$ release from the ER and binds to specific receptors to open Ca$^{2+}$-permeable channels on ER membranes to rapidly release Ca$^{2+}$ (Derian and Moskowitz, 1986). This messenger is effective in releasing the ER Ca$^{2+}$ with a high affinity (Kd = 1 μM) but has no effect on the mitochondrial Ca$^{2+}$ (Frey et al., 1989).

**Ca$^{2+}$-induced Ca$^{2+}$ release (CICR):** The finding that the fractional release of Ca$^{2+}$ from the nonmitochondrial intracellular Ca$^{2+}$ store was greater at lower than at higher [Ca$^{2+}$]$_i$ suggests that a portion of the ER may be specialized in this "Ca$^{2+}$-induced Ca$^{2+}$ release" function. The net effect is to release Ca$^{2+}$ from the ER into the cytosol. An enhanced Ca$^{2+}$-induced Ca$^{2+}$ release has been postulated for the effect of caffeine in cultured and freshly isolated endothelial cells (Buchan & Martin, 1991; Rusko et al., 1992b). A ryanodine receptor on the ER membrane of endothelium has been identified, which may be responsible for the action of caffeine (Lesh et al., 1993).
Ca\textsuperscript{2+} release from mitochondria: Under pathological condition, Ca\textsuperscript{2+} could be released from mitochondria to serve as a rescue step to maintain Ca\textsuperscript{2+} homeostasis. Ca\textsuperscript{2+} is released from mitochondria with a low affinity (Kd = 10 \mu M) and this release can be inhibited by azide.

c. Ca\textsuperscript{2+} extrusion pathways:

Ca\textsuperscript{2+} homeostasis mechanisms include Ca\textsuperscript{2+} extrusion towards the extracellular space (ECS) and Ca\textsuperscript{2+} uptake into intracellular stores.

ATP-driven Ca\textsuperscript{2+} pump (Ca\textsuperscript{2+}-ATPase) on the plasmalemmal membrane appears to be primarily responsible for Ca\textsuperscript{2+} extrusion towards the ECS (Hagiwara et al., 1983). Blockade of plasmalemmal Ca\textsuperscript{2+}-ATPase would raise \([Ca\textsuperscript{2+}]_i\).

Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange on the plasmalemmal membrane contributes to Ca\textsuperscript{2+} extrusion in smooth muscle (Chen & van Breemen, 1992), but its role in Ca\textsuperscript{2+} extrusion in endothelial cells has not yet been identified.

In addition, Na\textsuperscript{+}-H\textsuperscript{+} exchanger and Na\textsuperscript{+}, K\textsuperscript{+} pump might affect the \([Ca\textsuperscript{2+}]_i\) by affecting the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger (Adams et al., 1989; Kitazono et al., 1988).

d. Ca\textsuperscript{2+} sequestration into the ER.

ATP-driven Ca\textsuperscript{2+} pump (Ca\textsuperscript{2+}-ATPase) on the ER membrane pumps cytoplasmic Ca\textsuperscript{2+} into the ER, therefore decreasing \([Ca\textsuperscript{2+}]_i\).

These Ca\textsuperscript{2+} transport mechanisms on the plasmalemma and ER work in an integrated manner to maintain the Ca\textsuperscript{2+} homeostasis at rest and under stimulated conditions (see review Adams et al., 1993).

1.9. Methods used in investigating \([Ca\textsuperscript{2+}]_i\) regulation and endothelial cell function

a. \textsuperscript{45}Ca\textsuperscript{2+} flux measurement

Before fluorescent Ca\textsuperscript{2+} indicators were widely used to measure \([Ca\textsuperscript{2+}]_i\), \textsuperscript{45}Ca\textsuperscript{2+} flux measurement was a very frequently used technique to investigate Ca\textsuperscript{2+} regulation. It was useful in estimating the net cellular Ca\textsuperscript{2+} content and monitoring the \textsuperscript{45}Ca\textsuperscript{2+} influx.
and efflux across the cell membrane so as to provide information about Ca\textsuperscript{2+} mobilization in the cell (Hwang & van Breemen, 1987). By incubating digitonin-treated cells in \textsuperscript{45}Ca\textsuperscript{2+}-labelled solutions, it is also possible to measure \textsuperscript{45}Ca\textsuperscript{2+} release and uptake from the ER, and therefore to obtain information about Ca\textsuperscript{2+} regulation by the intracellular Ca\textsuperscript{2+} store (Missiaen et al., 1990).

b. Electron microscopy (EM)

Some potassium salts, e. g., potassium pyroantimonate and potassium oxalate, are used to precipitate Ca\textsuperscript{2+} in the cell. The precipitates are shown as electro-opaque deposits using electron microscope (Debbas et al., 1975). This technique is useful for Ca\textsuperscript{2+} localization and distribution in the cell, but it can only obtain Ca\textsuperscript{2+} data at a semi-quantitative level and is unable to detect [Ca\textsuperscript{2+}]\textsubscript{i} changes in a continuous fashion (e.g. during Ca\textsuperscript{2+} activation).

c. Electrophysiological studies (patch-clamping)

The patch-clamp method has contributed greatly to the understanding of the electrical properties of endothelial cells. Application of the voltage-clamp technique using a single electrode has made it possible to record membrane currents from a single cell or a membrane patch. This method is especially useful in examining the properties and regulation of different types of Ca\textsuperscript{2+} channels or other ion channels which are affected by [Ca\textsuperscript{2+}]\textsubscript{i} changes such as Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels on the cell membrane. Ionic current measurement of Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel activity is usually used to investigate Ca\textsuperscript{2+} release from the intracellular Ca\textsuperscript{2+} store (Rusko et al., 1992a).

The drawback of the patch-clamping method is the use of isolated single cells or a patch of membrane. Isolation of cells with digestive enzymes and alteration of the intracellular medium with the pipette solution or separation of a membrane patch might modify some membrane or ion transport properties. The method could be improved by the on-cell patch electrode which avoids breaking the membrane. Whole cell voltage-clamp can be performed by reducing the membrane resistance under the patch electrode.
by agent such as nystatin, which increases the membrane permeability only to ions, not to large molecules (Tomita, 1992).

d. \([Ca^{2+}]_i\) measurement with fluorescent dyes

The technique of measuring \([Ca^{2+}]_i\) with fluorescent indicators has been studied for many years (Tsien et al. 1982, 1985). When the molecules of certain fluorescent indicators, such as indo-1, quin-2, and fura-2, combine with \(Ca^{2+}\), either their fluorescence intensities change or their excitation wavelengths shift. Thus \([Ca^{2+}]_i\) can be measured by: 1) detecting the changes in fluorescence intensity of the fluorescent indicator after it combines with \(Ca^{2+}\); 2) upon shift of excitation wavelength, measuring the ratio of fluorescence intensity at the excitation wavelength of the indicator-\(Ca^{2+}\) complex to that at the excitation wavelength of the free form of the fluorescent indicator. The ratio of fluorescence intensities at two wavelengths provides a more accurate measurement of intracellular ion concentrations than the absolute intensity at a single wavelength (Bright et al., 1989). In the ratio method, a number of variables that may perturb the measurements are eliminated. In particular, ion-independent factors that affect the signal intensity, such as cell thickness, non uniform intracellular dye concentrations, probe leakage, photobleaching and dye leakage, are cancelled in the ratio measurements, since these parameters have a similar effect on intensities at both wavelengths.

Fluorescent \(Ca^{2+}\) indicators are available in both the free acid form and the acetoxy methyl ester (AM) form. The polar groups of acetoxy methyl ester are "protected" with ester groups, resulting in electrically neutral, lipophilic molecules that diffuse freely across the plasmalemma membrane into the cytoplasm, where they are cleaved by intracellular esterase into the active, more polar, membrane-impermeant polyanionic free acid form that is too hydrophilic to cross the plasmalemma and therefore accumulates intracellularly. It provides a less disruptive method to assess cellular processes and viability than microinjection techniques, osmotic shock,
electroporation, scrape loading, or other invasive methods. Examples of these permeant ion indicators include probes such as fura-2 AM and indo-1 AM.

Among these fluorescent Ca\(^{2+}\) indicators, fura-2, as a member of a new generation of fluorescent Ca\(^{2+}\) indicator, has many advantages compared to the previously used fluorescent dye such as quin-2: 1) fura-2 shows much stronger fluorescence (30 times stronger than quin-2); 2) it is more resistant to photobleaching; 3) its excitation wavelength shifts upon Ca\(^{2+}\) binding, so that it is the dye of choice for digital-imaging microscopy, where it is more practical to change excitation wavelengths. Alteration between two preferred wavelengths allows assessment of the ratio of Ca\(^{2+}\)-bound dye to free dye. At the excitation wavelength around 340 nm, the fluorescence intensity increases as [Ca\(^{2+}\)]\(_i\) increases; whereas at about 380 nm, the fluorescence intensity decreases as [Ca\(^{2+}\)]\(_i\) decreases. Therefore the ratio of fluorescence intensity at 340 nm excitation wavelength to that at 380 nm would be a relative measurement of [Ca\(^{2+}\)]\(_i\). This ratio measurement largely cancels out the effects of cell thickness, dye content, or instrumental efficiency as described above; 4) it has a weaker affinity, which makes it possible to sense [Ca\(^{2+}\)]\(_i\) changes at lower [Ca\(^{2+}\)]\(_i\); 5) it has better Ca\(^{2+}\) selectivity than Mg\(^{2+}\) and heavy metals (Tsien et al., 1985; Grynkiewicz et al., 1985); 6) the effect of pH changes on fura-2 is very small within the physiological range. All these properties of fura-2 make it a favorable dye for [Ca\(^{2+}\)]\(_i\) measurement.

At present, [Ca\(^{2+}\)]\(_i\) measured with fura-2 is usually calculated with Grynkiewicz's equation:

\[
[\text{Ca}^{2+}]_i = K_d \frac{b (R - R_{\text{min}})}{(R_{\text{max}} - R)}
\]

In the equation, "K\(_d\)" is the dissociation constant of the fura-2-Ca\(^{2+}\) complex; "R" is the ratio of fluorescence intensity due to excitation at 340 nm and 380 nm, and "R\(_{\text{min}}\)" and "R\(_{\text{max}}\)" are the ratios measured with free fura-2 and Ca\(^{2+}\)-saturated fura-2. "b" is the ratio of measured fluorescence signal for free dye and that for Ca\(^{2+}\)-bound dye at 380 nm.
This equation has to be used with three assumptions: 1) the dye forms a simple 1:1 complex with Ca$^{2+}$; 2) fura-2 behaves in cells as it does in the calibrating solution; and 3) the dye concentration is sufficiently diluted for fluorescence intensity to be linearly proportional to the fura-2 concentration (Gryniewicz, 1985).

e. **Measurement of NO release from endothelial cells**

*Indirect measurement of NO--- Bioassay*

A perfusion-superfusion bioassay method was described in detail by Rubanyi et al (1985b). Briefly, a segment of the vessel (donor ring) is fixed between two stainless steel cannulas and mounted vertically in an organ chamber. The segment is then perfused with warmed (37°C) solution (pH 7.4) oxygenated with 5% CO$_2$-95% O$_2$ gas mixture. A separate shorter aortic ring (bioassay ring) denuded of endothelium is suspended below the donor between a stationary stainless steel hook and a force-displacement transducer. After recording the resting tension, the bioassay ring is precontracted submaximally with phenylephrine perfused directly onto the bioassay ring. The bioassay ring was then superfused with donor effluent (*supply 1*) and an additional perfusion from below the donor (*supply 2*). Agents are infused either over the donor or under the donor segment for testing the bioassay system. Secretion of EDRF into the superfusate is monitored by the relaxation of a precontracted, endothelial cells-free arterial smooth muscle preparation (bioassay ring). Relaxation is expressed as percentage inhibition of the initial contractions. This technique has the advantage of measuring the activity of the product of manipulation (EDRF) by its physiological action (relaxation); however, it is not always possible to separate the actions of a drug or ionic environment on endothelial cells from the subsequent effect on the target bioassay tissue.

*Direct NO measurement----NO sensor*

Direct NO measurement can be carried out with a porphyrinic microsensor (Malinski & Taha, 1992). A three-electrode system is used for the measurement of NO release. It consists of a NO sensor working electrode, a platinum wire counter electrode,
and saturated calomel reference electrode. NO concentration can be determined from the measured current by means of a calibration curve and/or standard addition method.

1.10. Hypotheses for regulated Ca$^{2+}$ entry

There are three main hypotheses regarding the regulation of Ca$^{2+}$ entry.

a. The "Superficial Buffer Barrier (SBB)" hypothesis

In 1977, van Breemen (van Breemen, 1977) first proposed the "SBB" hypothesis from observations in smooth muscle. In essence the "SBB hypothesis" states:

1) Ca$^{2+}$ which enters the cell across the plasmalemma is in part pumped into the ER before it exerts its biological function. The peripherally located ER thus functions as a barrier for Ca$^{2+}$ diffusion into the deeper cytoplasm. The Ca$^{2+}$-ATPase on the superficial ER membrane (sarcoplasmic or endoplasmic reticulum Ca$^{2+}$-ATPase, SERCA) essentially contributes to the process of pumping Ca$^{2+}$ into the ER. In this fashion the superficial ER would play a specialized Ca$^{2+}$ entry buffer function. Inhibition of the ER buffering of Ca$^{2+}$ entry is thus postulated to raise [Ca$^{2+}]_i$.

2) The influx of Ca$^{2+}$ across the plasmalemma combined with its removal from the cytoplasm by the ER creates a Ca$^{2+}$ gradient in the peripheral cytoplasm. In other words, in the resting state, [Ca$^{2+}]_i$ in the peripheral cytoplasm is higher than in the deeper part of the cell.

3) Ca$^{2+}$ accumulation by the ER Ca$^{2+}$-ATPase contributes to Ca$^{2+}$ extrusion from the cell due to vectorial release of ER Ca$^{2+}$ towards a restricted space in the inner surface of the plasmalemma, from where it is extruded by the Na$^+$-Ca$^{2+}$ exchanger and the plasmalemmal Ca$^{2+}$-ATPase on the plasmalemmal membrane.

A pictorial model of this hypothesis as originally proposed (van Breemen, 1977, 1986, 1995) is illustrated in Figure 3A.

b. The "capacitative Ca$^{2+}$ entry" hypothesis

Casteels & Droogmans (1981), Cauvin & van Breemen (1985), the groups of Putney (putney, 1986) and Daniel (Bourreau et al., 1992) have proposed a direct Ca$^{2+}$
pathway between the ECS and the ER which bypasses the ER Ca$^{2+}$-ATPase (Fig. 3B) in their attempts to explain the replenishment of the ER. The essence of this hypothesis is that the depletion of the ER by any mechanism (IP$_3$ generation, SERCA blockade, treatment with caffeine and/or ryanodine to activate the ryanodine receptor, etc.) signals the opening of a pathway on the plasmalemma and thus increases Ca$^{2+}$ entry from the ECS into the cytoplasm. The notion of a direct coupling between the ER and plasmalemma was subsequently retracted and the generation of an unknown messenger from the depleted ER to open cell membrane Ca$^{2+}$ channels was proposed (Putney, 1990). This idea has been reinvestigated and the existence of a diffusible cytosolic factor has been presented (Randriamampita & Tsien, 1993; Parekh et al., 1993).

c. Receptor-mediated Ca$^{2+}$ entry

A direct coupling of receptors to plasmalemmal membrane channels mediated by one or more specific G proteins has been proposed for Ca$^{2+}$ entry (Fasolato et al., 1988, 1994; Graier et al., 1991). This type of G protein involved in Ca$^{2+}$ influx regulation is different from the G proteins responsible for the coupling of receptors to phospholipase C (Komori & Bolton, 1990). In addition, Ca$^{2+}$ could also enter through channels that are controlled by second messengers generated as a result of agonist-receptor binding (Perner et al., 1993; Meldolesi et al., 1991).
CHAPTER II

STATEMENT OF PROBLEMS AND THESIS OBJECTIVE

II.1. The originality of endothelial cells from different sources

Characterization of \([\text{Ca}^{2+}]_i\) regulation mechanisms in endothelial cell has been mostly carried out using cultured or isolated endothelial cells. However, treatment of isolated tissues with enzymes for the purpose of dislodging endothelial cells from the extracellular matrix and adjoining cells will modify the influence of surrounding tissue on the function of the endothelial monolayer (e.g. the interaction with smooth muscle cells is required). In addition, proliferation of endothelial cells in artificial media will alter the expression of cell-surface receptors, transport processes, and intercellular coupling. The ion channels present in endothelial cells in situ may be lost during enzymatic dissociation so that endothelial cells in culture may undergo changes in phenotype that render the cells insensitive to channel ligands (Adams et al., 1989; Whitmer et al., 1988). Therefore, the \([\text{Ca}^{2+}]_i\) dynamics in cultured cells do not always parallel the endothelium-dependent relaxation of pre-contracted arteries (Loeb et al., 1988; Peach, 1987). Three main findings concerning endothelial cell \([\text{Ca}^{2+}]_i\) regulation are inconsistent with physiological endothelial-dependent relaxation of intact arteries. First, overwhelming direct evidence from ligand binding, voltage-clamp, and fluorescence studies convincingly shows the absence of VGC in cultured vascular endothelial cells (Adams et al., 1989; Whitmer et al., 1988). In contrast, there is indirect evidence from studies on arterial contraction suggesting that VGCs exist in vascular endothelium in situ (Rubanyi et al., 1985a; Rubanyi & Vanhoutte, 1988). It is recognized that culture conditions or other physiological modulators could alter the expression of some voltage-gated Ca\(^{2+}\) regulatory mechanisms (Colden-Stanfield et al., 1987; Tekeda et al., 1987) (see chapter I.8); second, ACh-induced increases in \([\text{Ca}^{2+}]_i\) (Busse et al., 1988; Danthuluri et al., 1988), hyperpolarization of membrane potential
(Rusko et al., 1992a) and EDRF release (Cocks et al., 1985; Loeb et al., 1988; Peach et al., 1987) have not been noted consistently in subcultured endothelial cells, however, endothelium-dependent relaxation of arteries with an intact endothelium by receptor agonists such as ACh has been widely noted (Furchgott & Vanhoutte, 1989; Vanhoutte, 1989) and is diagnostic for a healthy intact endothelium; third, the physiological importance of Na+-Ca²⁺ exchange in endothelial cells is questionable because the Na⁺-dependent modulation of [Ca²⁺]ᵢ in cultured endothelial cells is apparent only under extreme experimental conditions where the cells have been highly Na⁺-loaded. In contrast, indirect evidence from contraction of arterial vessel segments shows that several Na⁺-Ca²⁺ exchange inhibitors alter vessel contraction in an endothelial-dependent manner (Winquist et al., 1985). Collectively, these data suggest that there are fundamental differences in [Ca²⁺]ᵢ regulation between cultured and intact endothelial cells. Although there are a lot of studies on [Ca²⁺] regulation in either cultured or isolated endothelial cells (review: Adams et al., 1989, 1993), only a few (Aoki et al., 1991; Laskey et al., 1994; Li & van Breemen, 1995) have been made in intact endothelial cells.

In this study, I have recorded the [Ca²⁺]ᵢ and [NO] signals in intact endothelial cells from the rabbit aortic or pulmonic valves; where applicable, I have recorded [Ca²⁺]ᵢ signals in isolated endothelial cells for the purpose of comparison to that in an intact endothelium. There are several reasons for choosing the rabbit cardiac valve as a source of endothelial cells: 1) the valve provides an intact endothelial preparation in which the [Ca²⁺]ᵢ and [NO] signal can be directly studied without using enzyme(s) or growth factor treatment; it circumvents the problems of studies in cultured endothelial cells as described above, i.e. cell surface receptor, ion channel expression, and intercellular coupling may be changed by enzymatic or mechanical treatments used to separate the endothelial cells from surrounding smooth muscle and subsequent culture procedures; 2) The valve is a relatively thin preparation and therefore avoids certain complexities brought by thick preparation (i.e. lower autofluorescence) and the
complete endothelial covering of this thin valve allows for selective loading with Ca$^{2+}$-sensitive dyes, as was first reported by Aoki et al (1991); 3) The digital imaging fluorescence microscopy used allows visualization and recording of fura-2 fluorescence signals in individual or groups of endothelial cells with their intercellular interactions on the surface of valve leaflet; and 4) It could also be used for comparative studies of endothelium-related pathophysiologies and readily adapted to studies of other facets of intracellular signaling pathways and intercellular communications via the use of immunohistochemical and other fluorescent indicators. The ability to record changes in [Ca$^{2+}$]i in the intact endothelial monolayer therefore provides a more realistic assessment of excitation-response coupling within individual cells and communication between cells.

II.2. The existence and role of Na$^+$-Ca$^{2+}$ exchange on [Ca$^{2+}$]i regulation

Among the Ca$^{2+}$ transport processes, the sodium-calcium (Na$^+$-Ca$^{2+}$) exchange in the plasmalemma, first identified in cardiac muscle (Reuter & Seitz, 1968) and nerve membrane (Baker & Blaustein, 1968), has controversial effects in endothelial cells. The voltage dependence of resting [Ca$^{2+}$]i which was consistent with a simple pump-leak model provided no evidence for the existence of Na$^+$-Ca$^{2+}$ exchange in cultured endothelial cells (Cannell & Sage, 1989). A similar conclusion was reached from the observation in cultured endothelium that isotonic substitution of extracellular Na$^+$ with K$^+$ (which would be expected to depolarize the cell) decreased [Ca$^{2+}$]i (Laskey et al., 1990b) and that application of the Na$^+$-K$^+$ pump inhibitor ouabain was without effect on [Ca$^{2+}$]i (Sage et al., 1991). Since the activity of the exchanger is expected to be highly dependent on [Na$^+$]i, it is possible that changes in [Ca$^{2+}$]i driven by the exchanger will not become apparent until [Na$^+$]i is sufficiently elevated. Agonist may increase Na$^+$ influx via non-selective surface membrane channels (Johns et al., 1987; Bregestovski et al., 1988), thereby increasing [Na$^+$]i. Such an increase in [Na$^+$]i, coupled to Na$^+$-Ca$^{2+}$ exchange, may contribute to the plateau phase of the [Ca$^{2+}$]i response to agonists.
(Cannell & Sage, 1989). However it was reported that isotonic lithium (Li+) substitution for extracellular Na+ in cultured endothelium had no effect on the [Ca\(^{2+}\)]\(_i\) regardless of whether the cells were at rest or activated by the agonist bradykinin (Laskey et al., 1989, 1990b). Similarly, bradykinin-stimulated changes in [Ca\(^{2+}\)]\(_i\) were unaffected by isotonic substitution of external Na+ with N-methyl D-glucamine (NMDG) (Schilling et al., 1988). These results suggested that Na+-Ca\(^{2+}\) exchange does not significantly contribute [Ca\(^{2+}\)]\(_i\) at either resting or activated condition in culture endothelial cells.

There are, however, other observations suggesting the existence of the Na+-Ca\(^{2+}\) exchanger in the endothelium. More recently it was reported that if cultured endothelial cells were first loaded with the Na+ ionophore monensin and then exposed to Li+, a large transient increase in [Ca\(^{2+}\)]\(_i\) ensued. Combined pretreatment with ouabain and monensin doubled this [Ca\(^{2+}\)]\(_i\) transient (Sage et al., 1991). This observation established the presence of Na+-Ca\(^{2+}\) exchange in cultured endothelial cells even though it was not shown to regulate [Ca\(^{2+}\)]\(_i\) either at rest or during activation. A similar conclusion was obtained from the observation that the rate of \(^{45}\)Ca\(^{2+}\) influx was increased by ouabain in bovine aortic cultured endothelium and was increased further when the ouabain-treated endothelium was transferred to a solution in which extracellular Na+ was substituted by choline (Hansen et al., 1991). Data obtained from an intact endothelium also supports a role for Na+-Ca\(^{2+}\) exchange. It has been reported that a putative blocker of the Na+-Ca\(^{2+}\) exchanger dichlorobenzamyl (DCB) blocked the relaxation responses to ACh and A23187 in rat aorta, which was interpreted in terms of the blocker decreasing [Ca\(^{2+}\)]\(_i\) by reducing Ca\(^{2+}\) influx via the exchanger (Winquist et al., 1985).

Although the removal of extracellular Na+ could be shown to affect [Ca\(^{2+}\)]\(_i\) under extreme experimental conditions (Sage et al., 1991), it has little effect on the basal and bradykinin-activated levels of [Ca\(^{2+}\)]\(_i\) and basal Ca\(^{2+}\)-dependent K+ channel activity (an indirect estimate of [Ca\(^{2+}\)]\(_i\)) in cultured endothelial cells under normal condition (Schilling, et al., 1988; Laskey et al., 1990a; Sauvé et al., 1988). These somewhat
conflicting data cast some doubt on the physiological role of Na+-Ca\textsuperscript{2+} exchange in intact endothelium. The existence of Na+-Ca\textsuperscript{2+} exchange in intact endothelial cells has not been studied. In this study, experiments were conducted to investigate the existence of Na+-Ca\textsuperscript{2+} exchange and its role in regulating [Ca\textsuperscript{2+}]	extsubscript{i} in intact endothelium of rabbit cardiac valve using digital imaging microscopy.

II.3. The role of ER on Ca\textsuperscript{2+} transport across the plasmalemma

There has been a recent surge of interest in the interaction between the plasmalemma and ER regarding Ca\textsuperscript{2+} transport across the plasmalemma, but the nature and control of Ca\textsuperscript{2+} transport through plasmalemma in relation to ER Ca\textsuperscript{2+} uptake and discharge remains elusive. The mechanisms whereby the ER modulates the [Ca\textsuperscript{2+}]	extsubscript{i} signal in the endothelium were explored in this study and discussed in terms of the above-mentioned hypotheses (see chapter I. 10).
CHAPTER III

METHODS AND MATERIALS

III.1. Cell preparation

a. **Intact endothelial cells**

Adult New Zealand white rabbits weighing 2.0-2.5 kg were killed by carbon
dioxide (CO₂) asphyxiation followed by exanguination from the carotid artery. The heart
was rapidly excised and placed in warmed (37°C) normal physiological saline solution
(N-PSS) buffered with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
(HEPES) (pH 7.4). The apex of the ventricle was then cut away to facilitate removal of
blood inside the heart. Both the aortic and pulmonic arteries are opened with a
longitudinal incision at their respective attachments to the left and right ventricles. The
semilunar aortic and pulmonic valves are situated at the summit of the outflow tract of
their corresponding ventricles, with the pulmonic valve being anterior, superior, and
slightly left to the aortic valve. Each valve is composed of three cusps. The U-shaped
convex lower edges of each cusp are attached to and suspended from the root of the aorta
or pulmonic artery, with the upper free valve edges projecting into the lumen. The
cusps circle the inside of the vessel root. Figure 4 is the anteriosuperior view of heart
with the atria removed.

The aortic and pulmonic valves were dissected, placed in Normal physiological
saline solution for fluorescent dye loading or in Hank's balanced salt solution (HBSS) for
NO measurement.

b. **Isolated endothelial cells**

For the preparation of freshly isolated endothelial cells, a 4 cm-long segment of
the rabbit aorta was isolated and cleaned of connective tissue and fat layers. It was then
opened longitudinally. The thin sheets of endothelium on the intimal surface of aorta
were gently peeled off with fine forceps under a dissecting microscope. Pieces of the
endothelial sheets were transferred to 2 ml Dulbecco's phosphate-buffered saline solution (DPBS, Gibco Laboratories, NY, USA) containing 0.9 mg/ml papain and 0.8 mg/ml dithiothreitol. After incubation at 37°C for 35 minutes in this dispersal DPBS solution, the tissue fragments were placed in fresh enzyme-free DPBS and were gently triturred with a fire-polished Pasteur pipette (tip diameter ~4 mm). Undispersed pieces of tissue were removed by nylon mesh filtration (mesh opening 62 μm) and the solution containing single cells was centrifuged at 2500 r.p.m for 2.5 min in 15 ml DPBS. The supernatant was discarded and the cell pellet was resuspended in DPBS containing 0.8 mM-Ca²⁺ and placed on glass cover slips. The cells were kept at 4°C until use on the same day.

The identification of endothelial cells was verified by their uptake of low-density lipoprotein (LDL). Freshly isolated endothelial cells were incubated with Medium 199 (Gibco Laboratories, NY, USA) containing 10 μg/ml of Dil-acetylated low-density lipoprotein (Dil-AcLDL; Biomedical Technologies, Inc. Stoughton, MA, USA). Cell-associated fluorescence was visualized with an epifluorescence microscope using standard rhodamine filters (excitation wavelength 340 nm, emission wavelength 380 nm).

III.2. Fluorescent dye loading and experimental setup

The valve preparation, never exposed to any digestive enzyme or cell culture medium, was loaded with a fluorescent dye fura-2 in a loading solution at room temperature in the dark for 60-75 minutes (min). The aortic or pulmonic valves were chosen for this study in part for the ease of its selective fura-2 loading. In the case of isolated endothelial cells, the endothelial cells attached to cover slips were loaded under similar conditions except that the loading time was 30 min. The loading solution was made of N-PSS containing 1 μM of membrane-permeant fura-2 acetoxymethyl ester (fura-2/AM). The fura-2/AM stock solution (1 mM) was made up by dissolving 50 μg fura-2/AM in 50 μl dimethyl sulfoxide (DMSO) so that the final concentration of DMSO
in the loading solution would be 0.1% by volume. At this concentration, DMSO neither influenced the fluorescence intensity of fura-2 nor caused a shift of excitation maxim. The fura-2/AM permeates the plasma membrane and is cleaved by intracellular esterase to the polarized free acid which is trapped inside the cells and available for Ca\(^{2+}\) binding (Gryniewicz et al. 1985). Although cells would be loaded with fura-2 more rapidly at 37\(^{\circ}\)C, fura-2 extrusion towards the ECS or sequestration into intracellular organelles is greatly reduced at room temperature. After fura-2 loading, the valve or coverslip was kept in warmed N-PSS (37\(^{\circ}\)C) for about 10-15 minutes to remove excess external fura-2, yielding cells with an even dye distribution. It was then mounted in a specially designed tissue chamber.

Figure 5 illustrates the design of this tissue chamber. It was made from a polystyrene tissue culture dish (35x10 mm style) (Corning Glass Works, Corning, NY, USA) with part of the bottom (diameter: 20 mm) cut out. A glass coverslip was glued to the bottom to seal the missing part. The bottom of the dish was then coated with Sylgard (Dow Corning Corporation, Midland, MI, USA) with a fine polytetrafluoroethylene (Teflon) tube (internal diameter, 0.28 mm) embedded in it. Through one end of the Teflon tube, warmed experimental solutions (37\(^{\circ}\)C) passed continuously to a channel (width, 1.5 mm; length, 4mm), which was made by cutting away some Sylgard at the center of the chamber bottom. The fura-2 loaded valve was mounted over the channel by pins and formed the ceiling of this narrow perfusion channel. On the other end of Teflon tube, an adjustable vacuum pump continuously removed the perfusion solution from the channel where the valve was exposed to so as to allow quick solution changes. The chamber was then placed on the stage of an inverted microscope (Nikon Diaphot). A x20 phase/fluor objective (numerical aperture, 0.75) was focused on the lower surface of endothelial monolayer of the valve leaflet. Warmed test solutions were perfused through a bubble trap directly into the channel bathing the lower surface of the valve with a dead space of \(\approx 0.6\) ml. To prevent tissue drying, the inflow rate (900 \(\mu l/min\)) was slightly higher than the outflow rate. In the mean time, a second inflow-and-outflow system was
established above the tissue to moisten the upper surface of the valve, keep the total volume of bath around 1.5 ml, and prevent overflow. The temperature in the chamber was maintained at 37°C by using a Bipolar Temperature Controller (Medical Systems Corp, Greenvale, NY, USA). The perfusion process was continuous and sometimes caused a small artifact in the fluorescence trace when switching to different test solutions. The test substances were dissolved in their respective solutions first and then applied into the chamber.

For the isolated endothelial cells, the coverslip with the fura-2 loaded endothelial cells was placed on the bottom of a tissue culture dish where part of the bottom (diameter: 20 mm) was made of a glass coverslip. The dish was then mounted on the stage of inverted microscope, similar to the procedures performed in intact endothelial cells. A Pasteur pipette tube was situated above the coverslip to infuse the fura-2 loaded coverslip (by gravity) with warmed experimental solutions. A total volume of 3 ml was used to change the solution. Total volume of the effective perfusion in the chamber was kept at ≈0.5 ml, which was constantly maintained by using a vacuum suction at the surface of the fluid.

III.3. Instrumentation for monitoring the fluorescence signal

Fluorescence signals were recorded as digital image data or simple plotting signals monitored under digital imaging fluorescence microscopy. The composition of this imaging microscopy system is shown in Figure 6. The light of a high-intensity Xenon lamp passes through the light-filter to provide the light wavelength of high spectral purity. The filter wheel contains 340 nm, 380 nm, and 360 nm wavelength filters (bandwidth 10 nm) of ultraviolet light. Qualitative observations of morphology simultaneously with fluorescence recordings was made possible by inserting onto the side port of the microscope a dichroic mirror that reflected the alternating excitation light onto the lower surface of valve pinned at the bottom of chamber. From the tissue, the light was then reflected to a mirror and passed through an 510 nm wavelength
(bandwidth 40 nm) cut-off emission filter prior to acquisition by ICCD camera (an intensified charge-coupled device, model 4093G, 4810 series, San Diego, CA, USA), permitting observation of the cell on a video monitor during fura-2 signal recording. Video signals were digitized by a Data Translation frame grabber (DT3861) housed in a PC 80286 computer connected with ICCD camera. An Inovision acquisition and analysis software in Sun Sparc1+ Workstation provides a menu-driven experimental control, filters alteration, imaging visualization, data collection and manipulation, data storage and post-experimental data management (Inovision, Research Triangle Park, NC, USA). Up to 64 regions of interest containing individual or groups of endothelial cells on the valve could be monitored simultaneously.

III.4. [Ca\textsuperscript{2+}]\textsubscript{i} measurement

Autofluorescence of unloaded valves was minimal and background images at 340 nm and 380 nm were obtained from a region of the chamber away from valve. Pairs of the fluorescence ratio signals were collected every 7 seconds at alternating 340 nm and 380 nm excitation wavelength (F340/F380) and plotted as background-subtracted ratio value versus time on line during the experimental procedure. Cytoplasmic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) can be calculated using the equation of Grynkiewicz et al (1985) (see chapter I.9).

Grynkiewicz's equation:

$$[\text{Ca}^{2+}]_i = K_d \cdot b \cdot \frac{R_{\text{min}} - R}{R_{\text{max}} - R}$$

In employing this equation, "K_d", the dissociation constant of Ca\textsuperscript{2+}-fura-2 complex, is taken as 224 nm; "R" is the above-mentioned fluorescence ratio at 340 nm and 380 nm (F340/F380), "R_{\text{min}}" and "R_{\text{max}}" are the ratios measured by the addition of 10 \(\mu\)M of the Ca\textsuperscript{2+} ionophore ionomycin to Ca\textsuperscript{2+}-free (10 mM EGTA) and Ca\textsuperscript{2+}-replete (2 mM CaCl\textsubscript{2}) solution, respectively. "b" is the ratio of the 380 nm signals in Ca\textsuperscript{2+}-free and Ca\textsuperscript{2+}-replete solution. The baseline ratio range of the rabbit intact valvular endothelial cells within the field of view varied between \(\approx 0.75\) and \(\approx 1.21\), with
a mean of 1.05 and SEM of 0.03 (N=50, 50 preparations). By using this equation, these baseline ratio values corresponded to \( \approx 34 \pm 8 \), \( 121 \pm 8 \) and \( 90 \pm 8 \) nM of \([\text{Ca}^{2+}]_i\), respectively. This range and variability appear to fall within the usual biological variability, and was compatible with the values reported by others (Laskey et al., 1992; Sage et al., 1989a).

There are several considerations (Sturek et al., 1991; Ziche et al., 1993; Moore et al., 1990) when using the equation to translate the ratio (F340/F380) into a \([\text{Ca}^{2+}]_i\) value: 1) It is difficult to determine the Kd value in the cytoplasm. Simply applying the Kd (224 nM) which is obtained in a salt solution to the above equation may cause systemic deviation when calculating \([\text{Ca}^{2+}]_i\) in biological tissue; 2) The intensity of fura-2 fluorescence in the cell-free solution might be different from that in the cytoplasm. Background-subtracted fluorescence intensity ratio signals (F340/F380) were therefore reported as relative indication of \([\text{Ca}^{2+}]_i\). The intensity threshold applied is between 15-25. The ratio range is between 0 and 2.55. The ratio range obtained for \([\text{Ca}^{2+}]_i\) was 0-500 nM.

The effectiveness of F340/F380 ratio as a reflection of \([\text{Ca}^{2+}]_i\) was tested using "Calcium Calibration Buffer Kit II" (Molecular Probes, Inc, Eugene, OR, USA). It shows that the ratio values (F340/F380) were positively proportional to \([\text{Ca}^{2+}]_i\) changes below the submicromolar range (Fig. 7).

III.5. \( \text{Mn}^{2+} \) quenching measurement

Points at which absorption, excitation or emission intensities are independent of the concentration of ionic species are called isosbestic points. At the 360 nm wavelength, a proposed isosbestic wavelength for fura-2, the fura-2 fluorescence signal is not influenced by \([\text{Ca}^{2+}]_i\) changes (Sage et al., 1989b). \( \text{Mn}^{2+} \) has been shown to quench fura-2 fluorescence after binding to the dye inside the cell (Gryniewicz's et al., 1985). Since \( \text{Mn}^{2+} \) and \( \text{Ca}^{2+} \) share common entry pathways in the plasmalemmal membrane and \( \text{Mn}^{2+} \) can not be taken into the ER by SERCA (Gomes de Costa & Madeira,
the quenching of fura-2 fluorescence by Mn\(^{2+}\) at 360 nm is used to measure Mn\(^{2+}\) influx. Mn\(^{2+}\) entry as recorded by its quenching of cytoplasmic fura-2 fluorescence can thus be used as a measure of Ca\(^{2+}\) influx pathways. Experiments were performed in the absence of external Ca\(^{2+}\) which excludes any competition for the divalent cation entry pathway and enhances the observed fluorescence quenching resulting from Mn\(^{2+}\) entry. The slope of Mn\(^{2+}\) quenching trace entry can be regarded as a measure of the rate of Mn\(^{2+}\) influx. Background fluorescence intensity at 360 nm was recorded from a region of the chamber away from valve.

III. 6. Bioassay Study

A perfusion-superfusion bioassay method described in detail elsewhere (Rubanyi et al., 1985b) was used (see chapter I.9). In brief, a 4- to 5-cm segment of the thoracic aorta (donor segment) was cleaned of adventitia and the branch vessels were tied off. It was then fixed between two stainless steel cannulas and mounted vertically in an organ chamber. The segment was perfused with warmed Krebs solution (37°C) which was continuously bubbled with a 5% CO\(_2\)-95% O\(_2\) gas mixture. The endothelium was removed from another 3 mm long aorta (bioassay segment) by rubbing the luminal surface with a wet filter paper. The bioassay ring was then suspended below the donor ring between a stationary stainless steel hook and a force-displacement transducer (Grass Instruments FT 03), and connected to a Grass Polygraph recorder (model 79). A resting tension of 2 g was applied to the bioassay ring at the beginning of an experiment, and allowed to stabilize for 1 hour. After this period, the bioassay ring was precontracted with phenylephrine (Phe, 0.1 \(\mu\)M) perfused directly onto the bioassay ring (under-the-donor) and washed with normal Krebs solution for 40 minutes. A second Phe (0.1 \(\mu\)M) dose was then applied to produce submaximal contraction of the bioassay ring. The bioassay ring was superfused with the donor effluent (over-the-donor) and additional Krebs solution from below the donor (under-the-donor) at 1.5 ml/min. The transit time between the distal end of the donor segment and the bioassay
ring was \( \approx 5 \)s. The total flow rate at the bioassay ring was maintained at 3 ml/min by means of a multichannel roller pump (Gilson minipulse 3) (Fig. 8A). ACh was infused at 0.09 ml/min by means of an infusion pump (Sage Instruments, syringe pump model 351) either over- or under- the donor for testing the bioassay system. All bioassay experiments were performed in the presence of indomethacin (10 \( \mu \)M) in over-the-donor perfusion to prevent the possible synthesis of prostanoids. Atropine (1 \( \mu \)M) was included in under-the-donor perfusion solution to inhibit the direct action of ACh on the smooth muscle.

The experimental design allowed determination of effects of \( K^+ \)-channel blockers Ba\(^{2+}\) and tetraethylammonium (TEA) on the release of relaxing factors such as EDRF from the donor segment. Both Ba\(^{2+}\) and TEA were perfused through over- or under- the donor for 10-15 min before ACh superfusion. Relaxation caused by ACh is expressed as percentage inhibition of the initial contraction. Data are shown as means±SEM, and statistical analysis of data was performed using Student's \( t \)-test with \( P < 0.05 \) considered significant.

III.7. [NO] measurement

EDRF (NO) released from the endothelial cells on the surface of the valve was measured by a NO microsensor. The NO microsensor was produced by threading perpendicularly an array of five carbon fibers (Amoco Performance Products, Inc., Greenville, SC, USA) through a pulled end of a L-shape glass capillary holder with 0.6 cm length of the carbon fiber left protruding. The tip of the glass capillary was sealed by bee's wax. Then a copper lead was inserted into the opposite side of the glass capillary and sealed by conductive silver epoxy (A. I. Technology. IWC, Princeton, NJ, USA). A conductive polymeric porphyrinic film was then deposited on the surface of the carbon fibers from a solution of nickel(II)tetrakis(3-methoxy-4-hydroxyphenyl) porphyrin (Malinski & Taha, 1992). The sensor's active tip was sequentially coated with a Nafion film (1% w/w in alcohol). The sensor placed on the surface of the valve. A three-
electrode system was used for the measurement of NO release. It consists of a NO sensor working electrode (Fig. 8A), a platinum wire counter electrode (diameter 0.5 mm) and a saturated calomel reference electrode.

Immediately before NO measurements, a dissected rabbit aortic or pulmonic valve was mounted onto the bottom of the tissue chamber in fresh Hank’s solution in which the electrodes were stable (2 ml, 37°C). Three electrodes were placed in the solution of the chamber. The active tip of the L-shaped NO microsensor touched the surface of the valve so that NO release from nearby endothelium could be immediately detected. Due to the limitation of perfusion and stability of electrode system, agents were applied by simply dropping their respective stock solutions onto the valve surface. Stock solutions (20 µl each) of BK (40 mM), CCh (100 mM), ATP (1 M) or ionomycin (100 mM) were dropped on the valve in the organ chamber (2 ml solution) to reach final concentrations of 4, 10, 100, 10 µM respectively. The concentration of NO released can be calculated by measuring the current change. Chronoamperometry was used to monitor an analytical current signal (Malinski et al., 1993). The amperometric method (with a response time less than 10 ms) provides rapid quantitative response to changes of NO concentration. Chronoamperometric experiments were performed using the EG&G PAR Model 273 potentiostat/galvanostat (EG&G PAR, Princeton, NJ, USA) interfaced to Gateway 2000 P4D-66 computer with custom data acquisition and control software.

III.8. Solutions and chemicals

The composition of different solutions is shown in Table 1. Normal PSS (N-PSS) contains (in mM): NaCl 140, KCl 5, CaCl₂ 1, MgCl₂ 1, Glucose 10, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) 5 (pH 7.4 at 37°C). N-PSS was adjusted to pH 7.4 at 37°C by using NaOH. 10Ca²⁺-PSS contained 10 mM external Ca²⁺. 0Ca²⁺-PSS was almost the same as N-PSS except that CaCl₂ was omitted from the solution. In Ca²⁺-free PSS, 0.2 mM ethylene glycol-bis(β-amino-ethyl ether) N,N,N',N'-tetraacetic acid (EGTA) replaced 1 mM CaCl₂. External Na⁺ substitution was
achieved by eqimolar substitution of external Na\(^+\) with Li\(^+\) (Li\(^+\)-PSS) or N-methyl d-glucamine (NMDG-PSS). NMDG-PSS was adjusted to pH 7.4 by using HCl. 2 Mg\(^{2+}\)-NMDG-PSS, 4 Mg\(^{2+}\)-NMDG-PSS contain 2 mM and 4 mM external MgCl\(_2\) in NMDG-PSS, respectively. For 80K\(^+\)-PSS, 75 mM NaCl was replaced with eqimolar KCl to make the final KCl concentration equal to 80 mM. 0K\(^+\)-PSS was almost the same as N-PSS except that KCl was omitted.

HBSS contains (in mM): NaCl 137, KCl 5, MgSO\(_4\) 0.8, NaH\(_2\)PO\(_4\) 0.33, K\(_2\)HPO\(_4\) 0.44, MgCl\(_2\) 1, CaCl\(_2\) 0.9, Tris-HCl 10, L-Arginine 0.1.

Krebs solution contains (in mM): NaCl 118, KCl 5, CaCl\(_2\) 2.5, MgCl\(_2\) 1.2, NaH\(_2\)PO\(_4\) 1.2, Na\(_2\)CO\(_3\) 25, EGTA 0.03, glucose 11.

The osmotic activity (280-300 mmol/kg) of the solutions was monitored with a vapor pressure osmometer (Wescor 5500) and was identical for all the experimental solutions used.

Analytical grade reagents for PSS, HBSS or Krebs solutions, acetylcholine (ACh), carbamylcholine chloride (carbachol, CCh), bradykinin (BK), adenosine 5'-triphosphate (ATP), PAF, cyclopiazonic acid (CPA), thapsigargin (Tg), 2',5',di(tert-butyl)-1,4,-benzhydroquinone (BHQ), ionomycin, monensin, ouabain, 17-8-estradiol, NMDG, 5-(N,N-hexamethylene)amiloride (HMA), 2-nitro-4-carboxyphenyl-N,N-diphenyl-carbamate (NCDC), MnCl\(_2\), NiCl\(_2\), and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemicals Co. (St Louis, MO, USA); TEA, from Eastman Kodak Co., (Rochester, NY, USA); Fura-2/AM, from Molecular Probes (Eugene, OR, USA); EGTA, from Fisher Scientific (Fair Lawn, NJ, USA). DCB was kindly provided by Merck Research Laboratory (NJ, USA). All agents were dissolved in their respective stock solutions. The stock solutions of CPA, Tg, BHQ, HMA, DCB, ionomycin and estradiol were made in DMSO; those of ACh, CCh, BK, ATP, PAF ouabain were in double distilled H\(_2\)O, where NCDC and monensin were made in 95% ethanol. Serial dilutions of all chemicals were made with buffered PSS. The experimental agents used did not affect the fura-2 fluorescence under the experimental conditions. The maximal
concentration of any vehicle to which preparations were exposed was 0.1%, which had no effect on the fura-2 signal.

III.9. Data analysis and statistics

The single trace line shown in each figure was the average of the simultaneously measured fluorescence ratio (F340/F380) or intensity (at 360 nm) of individual or groups of endothelial cells (1 - 70 cells) of interest chosen in the field of the same valve preparation, or coverslip with isolated endothelial cells. Where applicable, values were expressed as mean±SEM of these cells in the same preparation (n=number of cells). The trace was representative of similar responses obtained in at least 6 preparations. Where applicable, statistic values were summarized and expressed as mean±SEM of those preparations with the same experimental procedure (N=number of preparations). Comparison of the results was made by using the paired Student t test. P < 0.05 is considered as significantly different. Each trace was obtained from a different preparation. The experimental variables were introduced at various times after initiation of the recording cycle. Chemicals and/or drugs were applied as indicated by the horizontal bars or arrows in each figure and had no intrinsic autofluorescence. Each recording cycle started at t=0, but the relevant experimental procedure might start at a different time point in each experiment. The time period from 0 to the start of the experiment had no experimental significance. For comparison between figures, the magnitude of both X and Y axis scale was normalized for most of the traces.
CHAPTER IV

RESULTS

IV.1. Morphology of endothelial cells from aortic or pulmonic valves

Regions along the face of the valve leaflet where individual cells could be visualized under the inverted microscope using a x20 phase/fluor objective were chosen for recording. Figure 9A shows the outline of the monolayer of endothelial cells on the surface of the pulmonic valve leaflet after silver staining (magnification: 960x). Cells exhibit typical cobble-stone morphology. Cell borders were clearly shown, without overlapping of the cells. The cross sectional photograph of the haematoxylin stained pulmonic valve leaflet (Fig. 9B, magnification 384x) shows that both surfaces of the valve were covered only by a monolayer of endothelium which sandwiched a subendothelial layer of connective tissue. The nucleus of endothelial cells is seen on both sides of the valve leaflet, as shown in the photograph of electron micrograph (EM) of the valve leaflets. Figure 10 (9900 x) is an EM of part of the pulmonic valve leaflet where the continuous endothelial monolayer had an intact basal lamina with gap junctions between cells. The nuclear region of the cell had a height of ≈1.5 μm and width of ≈6 μm. The total length of the cell was as much as 30 μm, with apparent interdigitations between adjacent cells. Under the endothelial cell is a collagenous ground matrix of various components (elastic elements, collagen, fibronectin and laminin) that was sparsely populated by fibroblast-like cells. There were no smooth muscle cells between theses two endothelial monolayers, i.e., the absence of "trophic influence" of smooth muscle on endothelial cells signal which could result in alteration in [Ca^{2+}]i dynamics (Sturek et al., 1991).

Figure 11 shows the photograph of fluorescence emission from the lower surface of the fura-2-loaded pulmonic valve leaflet mounted in the above-mentioned chamber. Cells illuminated with 380 nm excitation wavelength were observed with a 20 times
phase/fluor objective (1 cm = 125 μm) of an inverted microscope. It was clear that only the endothelial monolayer on the surface of the valve was loaded with fura-2. No obvious disruption of the cell monolayer on the surface of the valve leaflet was observed. The photograph was taken directly from the camera port of inverted microscope, therefore corrections for variation in the field illumination were not possible. However, when the image is recorded as ratio of intensities at two wavelengths and illumination correction is subtracted, cells are shown to be uniformly loaded.

Precautions were made to ensure that all fluorescence recordings reflected responses from endothelial cells only. First, focusing through the thickness of the valve (∼100 μm) revealed the fura-2 fluorescence only on the surface endothelial layer; second, as shown in the EM picture in Figure 10, the surface endothelial layer with a continuous basement membrane overlays the subendothelial layer composed of connective tissue, without smooth muscle cells; the third assurance is that gently rubbing the surface of the valve leaflet or out of surface endothelial focus reduced the raw fura-2 fluorescence by more than 10 fold, and abolished the response to agonist acetylcholine (see chapter IV.2).

IV. 2. Characteristics of intact valvular endothelial cells
a. Responses to vasoactive agents

[Ca^{2+}]_i measurement:

The activation of endothelial cell-surface receptors by vasoactive substances evokes a biphasic [Ca^{2+}]_i increase (Hallam & Pearson, 1986; Hallam et al., 1988; Adams et al., 1989, 1993). The initial peak phase represents the release of Ca^{2+} from intracellular stores by the intracellular second messenger, IP3 (Derian & Moskowitz, 1986; Lambert et al., 1986; Freay et al., 1989); the accompanying sustained phase correlates with the influx of extracellular Ca^{2+} (Hallam & Pearson, 1986; Morgan-Boyd et al., 1987; Hallam et al., 1988; Johns et al., 1987; Schilling et al., 1988). The stimulated Ca^{2+} influx may serve two functions. First it prolongs the response beyond
the point when the ER Ca$^{2+}$ is depleted. Secondly, it may be linked to the mechanism for refilling of the ER after it is depleted. Similar [Ca$^{2+}$]$\text{i}$ response patterns in fura-2 loaded intact endothelial cells were observed upon stimulation with the agonists ATP, ACh, CCh, bradykinin or other vasoactive agents.

(1) ACh

Figure 12 (trace a) shows that continuous application of 10 μM ACh to intact endothelial cells caused an initially rapid increase of fluorescence ratio to a peak, which then declined slowly to a low plateau that was slightly higher than baseline. Similar results were obtained when using 2 μM or 20 μM ACh. The fura-2 fluorescence signal was not contaminated by a response form underlying cells, since gently rubbing the surface of the valve leaflet after loading effectively eliminated the fura-2 fluorescence and abolished any observable changes in fluorescence in response to ACh [Fig. 12 (trace b)]. The visual fluorescence was virtually abolished by the procedure, however, due to the low value assigned for background cut-off, the ratio of the image remained close to 1.

(2) CCh

A similar pattern of [Ca$^{2+}$]$\text{i}$ increase to that of ACh stimulation with a higher plateau occurred upon stimulation with 10 μM CCh, a non-hydrolyzable analogue of ACh, in the presence of extracellular Ca$^{2+}$ (Fig. 13A). Application of CCh in 0Ca$^{2+}$-PSS induced only a transient [Ca$^{2+}$]$\text{i}$ increase which returned to basal level and subsequent stimulation with CCh (Fig. 13B) or ATP (Fig. 13C) failed to induce a second [Ca$^{2+}$]$\text{i}$ peak. These results indicate that the application of physiological agonists in the absence of Ca$^{2+}$ is an effective way of depleting ER content and that both ATP and CCh affected the same intracellular store.

(3) ATP

Figure 14A shows that application of 100 μM ATP in the presence of 1 mM extracellular Ca$^{2+}$ induced a biphasic [Ca$^{2+}$]$\text{i}$ increase, consisting of an initially rapid but transient rise in [Ca$^{2+}$]$\text{i}$, which peaked within 20 seconds, followed by a prolonged elevation in [Ca$^{2+}$]$\text{i}$ that was higher than those induced by other agonists. The subsequent
removal of extracellular Ca\(^{2+}\) reversibly abolished the sustained [Ca\(^{2+}\)]\(_i\) elevation, which was most likely due to Ca\(^{2+}\) influx from the extracellular space; re-addition of Ca\(^{2+}\) to the perfusate returned [Ca\(^{2+}\)]\(_i\) to higher level (Fig. 14B). Placing the cells in 0Ca\(^{2+}\)-PSS before ATP stimulation only slightly reduced the initial response but abolished the sustained signal, and subsequent ATP stimulation failed to induce [Ca\(^{2+}\)]\(_i\) increase (Fig. 14C).

The colored image picture of individual endothelial cells is shown in Figure 15, reflecting the [Ca\(^{2+}\)]\(_i\) levels at resting conditions or upon stimulation with 100 \(\mu\)M ATP in the presence of extracellular Ca\(^{2+}\). According to the rainbow spectrum, at rest the color was nearly blue (F340/F380: \(\approx 0.8\)); after stimulated with ATP, it reached green (nearly yellow) (F340/F380: \(\approx 1.3\)).

(4) BK

Intact valvular endothelial cells responded to the continuous application of 2 \(\mu\)M BK in the presence of extracellular Ca\(^{2+}\) with a transient spike which gradually declined towards nearly basal level (Fig. 16A).

Serial applications of CCh and BK in the same preparation induced a [Ca\(^{2+}\)]\(_i\) signal with a magnitude similar to that obtained when either BK or CCh was applied alone (Fig. 16B), indicating that under these conditions BK and CCh have no lasting interactions.

In the absence of extracellular Ca\(^{2+}\), BK caused a transient [Ca\(^{2+}\)]\(_i\) spike (Fig. 16C).

(5) Ionomycin:

The Ca\(^{2+}\) ionophore ionomycin (4 \(\mu\)M), which transports Ca\(^{2+}\) along its gradient from the ER and the ECS to cytoplasm, was shown to induce a rapid and transient [Ca\(^{2+}\)]\(_i\) increase, followed by a sustained [Ca\(^{2+}\)]\(_i\) increase in the presence of extracellular Ca\(^{2+}\) as shown in Figure 17A. In the absence of extracellular Ca\(^{2+}\), ionomycin only transports from the ER to cytoplasm to induce a transient [Ca\(^{2+}\)]\(_i\) increase which returned to the baseline shortly after it reached a peak (Fig. 17B).
(6) PAF

As an inflammatory factor, platelet activating factor (PAF), has been reported to induce a sustained \([\text{Ca}^{2+}]_i\) increase in human and canine cultured endothelial cells (Bkaily et al., 1993). The application of 480 pM PAF induced a gradual increase in \([\text{Ca}^{2+}]_i\) to a plateau level (Fig. 18). A response could be detected when the PAF concentration was as low as 40 pM.

(7) Estrogen

The female sex hormone estrogen, 17 β-Estradiol, has been reported to cause relaxation of rabbit coronary arteries (Jiang et al., 1992). The role of the endothelium in mediating estradiol’s effect was investigated in both intact and isolated endothelial cells.

In intact valvular endothelial cells, 17 β-Estradiol (20 μM) elevated \([\text{Ca}^{2+}]_i\) to a sustained level. This effect was inhibited by 3 mM tetraethylammonium (TEA), a \(\text{Ca}^{2+}\)-activated \(\text{K}^+\) channel blocker (Fig. 19A). The steady state \([\text{Ca}^{2+}]_i\) increase can be observed in response to concentrations of estrogen as low as 1 μM. In the absence of extracellular \(\text{Ca}^{2+}\), 100 μM estradiol did not induce any \([\text{Ca}^{2+}]_i\) changes (results not shown).

In isolated endothelial cells from rabbit aorta, similar responses to that obtained in intact endothelium were observed in response to 20 μM 17 β-estradiol. This \([\text{Ca}^{2+}]_i\) increase was also inhibited by 3 mM TEA (Fig. 19B). The steady state \([\text{Ca}^{2+}]_i\) increase was observed in response to concentrations of estrogen as low as 5 μM.

Taken together, stimulation of muscarinic, purinergic, and kinin receptors elevated \([\text{Ca}^{2+}]_i\) in intact endothelial cells. The results are consistent with those reported in isolated or cultured endothelial cells (Busse et al., 1988; Hallam & Pearson, 1986; Morgan-Boyd et al., 1987). Exposure to an agonist in the absence of \(\text{Ca}^{2+}\) depleted the agonist-sensitive ER and prevented \([\text{Ca}^{2+}]_i\) increase in response to subsequent agonist stimulation. Figure 20 shows the quantitative evaluation of
F340/F380 responses to these agents. It indicates that compared to the resting level, ACh (10 \mu M) induces a maximal ratio increase of 31\pm 4\% (N=21), CCh (10 \mu M) 38\pm 4\% (N=22), ATP (100 \mu M) 41\pm 6\% (N=20), BK (2 \mu M) 38\pm 8\% (N=11), ionomycin (4 \mu M) 44\pm 11\% (N=10), PAF (480 pM) 19\pm 4\% (N=6) and estradiol (20 \mu M) 14\pm 3\% (N=7).

**Bioassay study:**

The biological consequence of activation of endothelial function was tested in a bioassay study. A rabbit aortic ring (bioassay ring) precontracted with 0.1 \mu M phenylepherine (Phe) was shown to elicit a variable extent of relaxation upon stimulation of donor aorta with ACh in the bioassay experiments.

The validity of the bioassay system was tested by applying ACh to the bioassay aortic ring, which did not induce relaxation since the endothelium was denuded. Application of 10 \mu M ACh to the donor aorta elicited a 53\pm 6\% (N=8 preparations from 8 rabbit) relaxation (expressed as the decrease of tension as \% of pre-relaxation tension) of the bioassay aorta that was precontracted with 0.1 \mu M Phe in the presence of 0.1 \mu M atropine. The role of K\textsuperscript{+} channels in endothelial activation was investigated using the blockers Ba\textsuperscript{2+} and TEA. The extent of relaxation in response to ACh was significantly reduced to 35\pm 6\%, and 25\pm 7\%, if 1 mM Ba\textsuperscript{2+}, at a concentration that can block Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels, was applied before ACh stimulation to the bioassay ring and donor aorta, respectively (N=8, P < 0.05, compared to the relaxation induced by ACh without Ba\textsuperscript{2+} treatment, paired Student t test) (Fig. 21A; Table 2). Similarly, relaxation was reduced to 33\pm 7\% and 21\pm 6\%, when TEA (5 mM) was applied to under- and over-the-donor, respectively (N=7). There were also significant differences between the degree of relaxation caused by ACh pretreatment with over-the-donor and under-the-donor application of Ba\textsuperscript{2+} (0.01 < P < 0.05) (Fig. 21B, Table 2).
[NO] measurement

Although intracellular Ca$^{2+}$ is required for activating NOS to cause NO release from endothelial cells, there are no reports on NO release measurements corresponding with the [Ca$^{2+}$]$_i$ measurement in intact endothelial cells. In a parallel study using imaging fluorescence microscopy to measure [Ca$^{2+}$]$_i$ and a NO microsensor to measure NO, similar patterns of [Ca$^{2+}$]$_i$ increases and NO release traces were obtained in intact valvular endothelial cells. Figure 22 shows a representative trace of NO concentration enhanced by ATP (100 μM) stimulation. Figure 23 shows the representative traces of NO concentration enhanced by CCh (10 μM) stimulation in the presence (N-PSS, A) or absence of extracellular Ca$^{2+}$ (0Ca$^{2+}$-PSS, B). Figure 24 shows the representative traces of NO concentration enhanced by BK (2 μM) stimulation in N-PSS (A) or 0Ca$^{2+}$-PSS (B). It is clear that the NO release in response to CCh in N-PSS is higher than that in 0Ca$^{2+}$-PSS. Figure 25 was a representative trace of NO concentration enhanced by ionomycin (4 μM) stimulation in N-PSS. The control experiments were done with the applications of agents in the absence of valve (shown as dash lines in figures). The results are summarized in Table 3.

b. Response to depolarization

The existence of VGC in endothelial cells is a controversial issue. In this study, depolarization by high extracellular K$^+$ (80K$^+$) did not induce an elevation in [Ca$^{2+}$]$_i$ in intact valvular endothelial cells (Fig. 26). However, 80K$^+$ depolarization inhibited the ATP (100 μM) -induced plateau phase of [Ca$^{2+}$]$_i$ increase, and upon switching back to normal PSS the signal returned to the previous ATP-induced plateau level (Fig. 27). In addition, diltiazem (1 μM), a VGC blocker, did not affect the ATP -induced sustained [Ca$^{2+}$]$_i$ increase (Fig. 28). These results demonstrate that in intact endothelium from rabbit valve, voltage-gated Ca$^2$ channels are not involved in [Ca$^{2+}$]$_i$ regulation.
IV.3. Identification of the effect of Na\(^+\)-Ca\(^{2+}\) exchange on [Ca\(^{2+}\)]\(_i\) signal in intact endothelial cells

a. Existence of Na\(^+\)-Ca\(^{2+}\) exchange

The experiment presented in Figure 29 was designed to investigate the existence of a Na\(^+\)-Ca\(^{2+}\) exchange in intact endothelial cells. The extracellular Na\(^+\) in N-PSS was isotonically replaced with lithium (Li\(^+\)). Since the activity of the exchanger is critically dependent on the electrochemical Na\(^+\) and Ca\(^{2+}\) gradients across the cell membrane, the Na\(^+\) substitution procedure would first reverse the Na\(^+\) gradient and after loss of internal Na\(^+\), abolish the Na\(^+\) gradient; correspondingly, it would cause Ca\(^{2+}\) entry in exchange for Na\(^+\) efflux and elevate [Ca\(^{2+}\)]\(_i\). This would slow down after loss of intracellular Na\(^+\) and cause [Ca\(^{2+}\)]\(_i\) to decline. There was a transient increase of the F340/F380 ratio following Li\(^+\) replacement of Na\(^+\) in N-PSS and a subsequent return to the basal level (Fig. 29A). However a ratio transient was often observed when Li\(^+\) replaced Na\(^+\) in the absence of extracellular Ca\(^{2+}\), an effect which can not be interpreted to be due to Na\(^+\)-Ca\(^{2+}\) exchange (Fig. 29B). Since Li\(^+\) has an inhibitory effect on the IP\(_3\) hydrolysis (Berridge et al., 1982; Sherman et al., 1985), this [Ca\(^{2+}\)]\(_i\) transient induced by Li\(^+\) in the absence of extracellular Ca\(^{2+}\) could result from ER Ca\(^{2+}\) release due to IP\(_3\) accumulation induced by Li\(^+\), an effect that has not been reported in cultured endothelial cells. Due to the effect of Li\(^+\) on metabolism, Li\(^+\) substitution for Na\(^+\) can not be used to investigate a Na\(^+\)-dependent transport process in intact endothelial cells.

Alternatively, NMDG, was used to replace Na\(^+\). Figure 30 shows that NMDG replacing Na\(^+\) in the presence of extracellular Ca\(^{2+}\) induced a [Ca\(^{2+}\)]\(_i\) peak which then declined to the basal level (trace a, solid line), while in the control experiment NMDG did not induce any [Ca\(^{2+}\)]\(_i\) peak in the absence of extracellular Ca\(^{2+}\) (trace b, dash line). The [Ca\(^{2+}\)]\(_i\) increase phase of the transient was consistent with the internal Na\(^+\)-dependent Ca\(^{2+}\) entry pathway, while the declining phase of [Ca\(^{2+}\)]\(_i\) during the period of exposure to Na\(^+\)-free solution might be due to the loss of intracellular Na\(^+\) during the exposure to Na\(^+\)-free solution, which abolishes the Na\(^+\) gradient across the membrane.
and diminishes Ca\textsuperscript{2+} entry via the exchange process. In addition, a Na\textsuperscript{+}-independent Ca\textsuperscript{2+} extrusion mechanism such as a Ca\textsuperscript{2+}-ATPase in the surface membrane is likely to extrude the Ca\textsuperscript{2+} brought into the cell by the exchanger (Hagiwara et al., 1983).

The reversal of the Na\textsuperscript{+} gradient, however, might possibly alter two Na\textsuperscript{+}-dependent processes: (i) the reversal of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange, thereby increasing \([\text{Ca}^{2+}]_i\); and (ii) the reversal of Na\textsuperscript{+}-H\textsuperscript{+} exchange, the presence of which has been demonstrated in brain capillary endothelial cells in vivo and in cultured bovine aortic endothelial cells (Kitazono et al., 1988), leading to a lowering of intracellular pH (pHi), which might affect \([\text{Ca}^{2+}]_i\) and Ca\textsuperscript{2+}-dependent intracellular events.

DCB, an amiloride analogue which selectively inhibits Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange (IC\textsubscript{50}, 17 \(\mu\)M) (Supplisson et al., 1991), was used to confirm the involvement of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange to elevate \([\text{Ca}^{2+}]_i\) signal in response to NMDG. Figure 31 show that the transient \([\text{Ca}^{2+}]_i\) increase in response to Na\textsuperscript{+} removal by NMDG (A) was abolished after the valve leaflet was pre-incubated with 25 \(\mu\)M DCB for 8 minutes in N-PSS (B). At this concentration of DCB, Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange can be effectively inhibited while the osmotic integrity of the plasma membrane, Na\textsuperscript{+},K\textsuperscript{+}-ATPase enzymatic activities, Na\textsuperscript{+}-H\textsuperscript{+} exchange, mitochondrial oxygen consumption, and Na\textsuperscript{+}, Ca\textsuperscript{2+}, or K\textsuperscript{+} conductance are not changed (Siegl et al., 1984).

Another inhibitor of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger is Mg\textsuperscript{2+} (Trosper & Philipson, 1983). Mg\textsuperscript{2+} is thought to compete with Ca\textsuperscript{2+} for the divalent binding site of the carrier protein on the plasmalemmal membrane without being transported (Smith et al., 1987). Figure 32 shows that the \([\text{Ca}^{2+}]_i\) transient caused by Na\textsuperscript{+} removal with NMDG in the presence of 2 mM Mg\textsuperscript{2+} was faster and larger than that in the presence of 4 mM Mg; furthermore it was faster and larger in the control experiment (1 mM Mg\textsuperscript{2+}) (NMDG-PSS) than that in the presence of 2 mM Mg\textsuperscript{2+}. The transient was abolished in the presence of 8 mM Mg\textsuperscript{2+} (data not shown). The inhibitory effect of Mg\textsuperscript{2+} on NMDG-induced transient can be reversed after removing high levels of extracellular Mg\textsuperscript{2+}. Since Mg\textsuperscript{2+} acts extracellularly, these results strongly support the idea that the Na\textsuperscript{+}-
Ca²⁺ exchanger is involved in the [Ca²⁺]ᵢ change induced by external Na⁺ removal. In control experiments made without addition of DCB or raised extracellular Mg²⁺, the intact endothelial cells demonstrated repetitive [Ca²⁺]ᵢ transients with similar amplitudes in response to consecutive applications of NMDG (data not shown).

Conversely, HMA, anotheramiloride analogue which selectively blocks Na⁺-H⁺ exchange (IC₅₀, 0.3 μM), did not appear to affect the NMDG-induced [Ca²⁺]ᵢ transient. In the presence of 10 μM HMA, the NMDG induced peak was slightly smaller than that in the absence of HMA (Fig. 33). At a concentration of 10 μM HMA, pHᵢ changes mediated by Na⁺-H⁺ exchange could be effectively blocked (Kitazono et al., 1988).

These results clearly indicate the presence of Na⁺-Ca²⁺ exchange in intact valvular endothelial cells. However it does not appear to influence the steady state [Ca²⁺]ᵢ, because [Ca²⁺]ᵢ finally returned to basal levels during Na⁺ substitution. Since the activity of the exchanger is expected to be steeply dependent on [Na⁺]ᵢ, it is possible that changes in [Ca²⁺]ᵢ due to the exchanger will not become apparent until [Na⁺]ᵢ is sufficiently elevated. Therefore, the contribution of Na⁺-Ca²⁺ exchange to the steady state [Ca²⁺]ᵢ increase was further investigated under elevated [Na⁺]ᵢ conditions in the following sections.

b. **Effect of Na⁺-Ca²⁺ exchange on Ca²⁺ entry**

Ouabain was used to increase [Na⁺]ᵢ by inhibiting the Na⁺, K⁺ pump thereby indirectly modulate the Na⁺-Ca²⁺ exchange. The increased [Na⁺]ᵢ due to Na⁺, K⁺ pump inhibition by ouabain will promote Na⁺ extrusion coupled to Ca²⁺ entry, an effect believed to be mediated by Na-Ca²⁺ exchange. Figure 34 shows that ouabain (100 μM) increased the steady state [Ca²⁺]ᵢ, consistent with the presence of an internal Na⁺-dependent Ca²⁺ entry.

Monensin, a Na⁺ ionophore, represents yet another way of increasing [Na⁺]ᵢ. Figure 35 shows that 50 μM monensin in N-PSS promoted a steady state increase of [Ca²⁺]ᵢ, consistent with [Na⁺]ᵢ increase coupled Ca²⁺ entry via Na⁺-Ca²⁺ exchange. The
elevated $[Ca^{2+}]_i$ increase was maintained because high $[Na^+]_i$ was sustained by the continued presence of monensin and external Ca$^{2+}$.

Removal of external Na$^+$ after $[Na^+]_i$ has first been elevated prolonged the Na$^+$ efflux mediated Ca$^{2+}$ entry. As shown in Figure 36, substituting NMDG for Na$^+$ in the presence of monensin induced a $[Ca^{2+}]_i$ transient which declined to a plateau level higher than the steady state $[Ca^{2+}]_i$ level induced by monensin alone.

Figure 37 shows what might be the physiological correlate of this type of experiment with the application of an agonist. Agonists may increase Na$^+$ influx via nonselective plasma membrane channels. Na$^+$ substitution after stimulation with the agonist CCh induced a further transient $[Ca^{2+}]_i$ increase, followed by an elevated level of $[Ca^{2+}]_i$.

The increased resting level of $[Ca^{2+}]_i$ induced by ouabain and monensin, and the higher steady state resulting from Na$^+$ removal after $[Na^+]_i$ was first elevated by monensin or agonist can thus be explained by stimulation of Ca$^{2+}$ entry via Na$^+$-Ca$^{2+}$ exchange due to elevated $[Na^+]_i$.

c. Effect of Na$^+$$-$$Ca^{2+}$ exchange on Ca$^{2+}$ extrusion

Prolonged elevation of $[Ca^{2+}]_i$ may stimulate Ca$^{2+}$ extrusion (Becker et al., 1989). The Ca$^{2+}$ extrusion process in intact endothelial cells was investigated after a large elevation of $[Ca^{2+}]_i$ was induced.

Figure 38 displays the representative "Ca$^{2+}$ decline trace" under control conditions and in Na$^+$-free medium. After $[Ca^{2+}]_i$ was elevated to a new steady state by exposing intact endothelial cells to Na$^+$ free (NMDG-PSS) solution in the presence of 10 mM Ca$^{2+}$, cells were then exposed to 0Ca$^{2+}$-PSS to wash out Ca$^{2+}$ in the presence or absence of external Na$^+$. The rate of decline in the presence of Na$^+$ appeared to be faster than that in the absence of Na$^+$.

In order to evaluate the effect of Na$^+$-Ca$^{2+}$ exchange inhibition on $[Ca^{2+}]_i$ decline quantitatively, the rates of $[Ca^{2+}]_i$ decline at F340/F380 value of 1.3 were compared under different conditions as shown in Figure 39. The ratio of 1.3 was chosen because:
(1) in most experiments the ratio raised by agonists in high external Ca\(^{2+}\) solution were higher than 1.3; (2) In most cases, ratio 1.3 was higher than the average resting ratio of about 1.05. Figure 39 shows that compared to the control, the rate of \([\text{Ca}^{2+}]_i\) decline was decreased by 63±7 % (N=5) in Na\(^+-\)free solution at F340/F380 ratio of 1.3.

These results demonstrate that inhibition of the Na\(^+-\)Ca\(^{2+}\) exchanger with Na\(^+\) gradient reversal slows the rate of Ca\(^{2+}\) removal in Ca\(^{2+}\) free external medium when [Ca\(^{2+}\)]\(_i\) is above the resting level.

IV. 4. Inhibition of ER Ca\(^{2+}\) accumulation on \([\text{Ca}^{2+}]_i\) signal

a. Inhibition of ER Ca\(^{2+}\) accumulation enhances \([\text{Ca}^{2+}]_i\)

Agonists which interfere with ER Ca\(^{2+}\) accumulation could raise the steady state \([\text{Ca}^{2+}]_i\) by stimulating Ca\(^{2+}\) release from ER. Another way of interfering with ER Ca\(^{2+}\) accumulation can be achieved by inhibiting ER Ca\(^{2+}\) ATPase (SERCA). Figure 40 shows that three SERCA inhibitors, CPA, Tg and BHQ, induced slow and gradual increases in \([\text{Ca}^{2+}]_i\) which reached sustained plateau within 3-6 min. This steady state level of \([\text{Ca}^{2+}]_i\) returned towards baseline if extracellular Ca\(^{2+}\) was omitted. The three agents were used in separate experiments to avoid possible misleading conclusions based on the side effects of any particular agent. The quantitative evaluations of the effects of CPA, Tg, and BHQ on steady state \([\text{Ca}^{2+}]_i\) are shown in Figure 41. It indicates that CPA increases the steady state \([\text{Ca}^{2+}]_i\) by 34±2% (N=19), BHQ by 62±3% (N=8), and Tg by 46±4% (N=5).

Caffeine represents an alternative way of interfering with ER Ca\(^{2+}\) accumulation through a Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) mechanism. About 50% of intact valve preparations have transient \([\text{Ca}^{2+}]_i\) responses to caffeine (20 mM) (Fig. 42A), while 50% of valve preparations do not have any \([\text{Ca}^{2+}]_i\) changes. When the caffeine response was absent, the intactness of the CCh response was confirmed in the same preparation (Fig. 42 B).
b. Inhibition of ER Ca\textsuperscript{2+} accumulation prevents ER Ca\textsuperscript{2+} refilling

By disrupting the balance between Ca\textsuperscript{2+} leak and Ca\textsuperscript{2+} uptake through the ER membrane at rest, SERCA inhibitors could empty the ER. As shown in Figure 43A, B, CPA or BHQ in the absence of extracellular Ca\textsuperscript{2+} induced a transient [Ca\textsuperscript{2+}]\textsubscript{i} increase, and subsequent agonist (CCh) activation did not induce any changes in [Ca\textsuperscript{2+}]\textsubscript{i} in intact endothelial cells, indicating that the IP\textsubscript{3}-sensitive intracellular Ca\textsuperscript{2+} store affected by CCh was overlapping with that affected by CPA or BHQ. Kass et al (1989) have shown that the BHQ induced [Ca\textsuperscript{2+}]\textsubscript{i} increase is due to mobilization of an IP\textsubscript{3}-sensitive store. Figure 43C shows that after a transient [Ca\textsuperscript{2+}]\textsubscript{i} increase induced by CCh, subsequent BHQ stimulation induced only a small [Ca\textsuperscript{2+}]\textsubscript{i} increase, confirming that most of the intracellular store affected by BHQ was a IP\textsubscript{3}-sensitive store.

The discharged ER has to be replenished by extracellular Ca\textsuperscript{2+}. In order to understand how the ER functions as a Ca\textsuperscript{2+} entry buffer barrier, the ER refilling mechanism has to be clarified. Experiments were conducted to find out whether the ER is refilled by Ca\textsuperscript{2+} entering directly from the ECS to ER or from the ECS to the restricted cytoplasmic space between the plasmalemma and superficial ER first and then into the ER.

The effects of ER Ca\textsuperscript{2+} ATPase inhibitors on ER Ca\textsuperscript{2+} refilling was investigated and representative results are shown in Figure 44. During the CPA-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase phase in the presence of extracellular Ca\textsuperscript{2+}, the first application of 10 μM CCh to endothelial cells induced a [Ca\textsuperscript{2+}]\textsubscript{i} transient and a subsequent application of CCh failed to induce further changes in [Ca\textsuperscript{2+}]\textsubscript{i}. The time interval between the applications of CCh was long enough for the ER to be refilled since in a control experiment repetitive applications of CCh in the absence of CPA caused repetitive [Ca\textsuperscript{2+}]\textsubscript{i} increases in the presence of extracellular Ca\textsuperscript{2+} (data not shown). These results indicate that the ER Ca\textsuperscript{2+}-ATPase is essential for ER Ca\textsuperscript{2+} refilling. The first transient induced by CCh stimulation in the presence of CPA may be due to the release of remaining ER Ca\textsuperscript{2+}. With
continued inhibition of ER Ca\textsuperscript{2+}-ATPase by CPA, the emptied ER could not be refilled, so that there was no Ca\textsuperscript{2+} available for release by the second stimulation with CCh.

Application of agonist in the absence of extracellular Ca\textsuperscript{2+} has been shown to be an effective means of depleting the ER and to estimate ER Ca\textsuperscript{2+} content (van Breemen, 1976). The experiment presented in Figure 45 provides further support for the role of the ER Ca\textsuperscript{2+}-ATPase in refilling of the ER. In the upper panel, after the ER was depleted by CCh in the absence of extracellular Ca\textsuperscript{2+}, the tissue was exposed to N-PSS containing 1 mM Ca\textsuperscript{2+} which induced a large Ca\textsuperscript{2+} influx. As the ER was fully refilled in this step, a second application of CCh in the absence of extracellular Ca\textsuperscript{2+} induced a [Ca\textsuperscript{2+}]\textsubscript{i} peak (Fig. 45A). In the lower panel, 5 μM BHQ was present in all solutions used. The ER therefore cannot be refilled with Ca\textsuperscript{2+} as shown by the lack of CCh-induced [Ca\textsuperscript{2+}]\textsubscript{i} signal following BHQ application even after the cells was exposed to N-PSS containing Ca\textsuperscript{2+} (Fig. 45B). Similar results were obtained with the application of CPA instead of BHQ (data not shown). In addition, as indicated in Figure 43, there were no additive effects between BHQ and CCh on releasing Ca\textsuperscript{2+} from the ER in intact endothelial cell. These results demonstrate that the ER Ca\textsuperscript{2+}-ATPase inhibitors and agonists affected the same intracellular Ca\textsuperscript{2+} store, and that ER Ca\textsuperscript{2+}-ATPase inhibitors could prevent ER refilling via Ca\textsuperscript{2+} influx from the ECS through the leak pathway. The results suggest that the ER has to be refilled via the ER Ca\textsuperscript{2+}-ATPase and do not provide support for the existence of a direct connection between the ER lumen and the ECS as preposed by the "capacitative model".

IV.5. The nature of steady state [Ca\textsuperscript{2+}]\textsubscript{i} increase

As described in chapter IV.2 and IV.4, both the ER inhibitors and agonists increase the steady state [Ca\textsuperscript{2+}]\textsubscript{i}. However, this steady state [Ca\textsuperscript{2+}]\textsubscript{i} increase could be due to either an increase in Ca\textsuperscript{2+} influx or a decrease in Ca\textsuperscript{2+} efflux. The nature of the [Ca\textsuperscript{2+}]\textsubscript{i} enhancement was therefore investigated by using Ca\textsuperscript{2+} entry blockers and Mn\textsuperscript{2+} quenching measurement.
a. Effects of Ca\(^{2+}\) entry blockers on steady state [Ca\(^{2+}\)]\(_i\) increase

NCDC, a proposed phospholipase C inhibitor which interferes with second messenger systems of ROC, was employed to investigate its effect on both ATP- and CPA-induced [Ca\(^{2+}\)]\(_i\) signals in intact endothelial cells. Figure 46A shows that pretreatment of endothelial cells with 100 \(\mu\)M NCDC, a concentration that blocks agonist-activated Ca\(^{2+}\) entry (Rüegg et al., 1989), greatly reduced the sustained component of the [Ca\(^{2+}\)]\(_i\) signal induced by ATP. This suggests that Ca\(^{2+}\) entry was linked to receptor activation. Interestingly, compared with the control response to ATP (Fig. 14A), the steady state [Ca\(^{2+}\)]\(_i\) increase was inhibited 66±9% by NCDC (N=8); while the transient [Ca\(^{2+}\)]\(_i\) increase was not affected by NCDC, indicating that NCDC was more effective in inhibiting ROC indices (sustained [Ca\(^{2+}\)]\(_i\) increase) than the indices of Ca\(^{2+}\) release from ER (transient [Ca\(^{2+}\)]\(_i\) increase) induced by ATP. The results were similar to those obtained in vascular smooth muscle cells (Rüegg et al., 1989) and myocytes (Blatter, 1994) stimulated with vasopressin and were consistent with an inhibitory effect of NCDC on agonist opening of ROC. In contrast to its effect on ATP-induced [Ca\(^{2+}\)]\(_i\) increase, NCDC did not affect the CPA-induced [Ca\(^{2+}\)] signal (Fig. 46B).

To confirm the involvement of ROC on agonist-induced [Ca\(^{2+}\)]\(_i\) increase, a putative ROC blocker, SK&F 96365, was used. Figure 47 shows that SK&F 96365 reduced the ATP- (A), but not the CPA- (B) induced [Ca\(^{2+}\)]\(_i\) increase.

Ni\(^{2+}\), a potent Ca\(^{2+}\) channel blocker which competes for common binding sites on the Ca\(^{2+}\) channels including "leak" and ROC was shown to block both the CPA- and ATP-induced sustained [Ca\(^{2+}\)]\(_i\) increases in intact valvular endothelial cells. Removal of Ni\(^{2+}\) allowed the [Ca\(^{2+}\)]\(_i\) signal to return to the previous plateau levels (Fig. 48).

b. Mn\(^{2+}\), as a probe for Ca\(^{2+}\) influx

Results obtained by using fura-2 to measure [Ca\(^{2+}\)]\(_i\) provide only indirect evidence for the existence of Ca\(^{2+}\) entry mechanisms. To further investigate the contribution of Ca\(^{2+}\) entry to [Ca\(^{2+}\)]\(_i\) enhancement, Ca\(^{2+}\) influx was estimated by measuring Mn\(^{2+}\) entry as an index of divalent cation influx. Since there is no endogenous
Mn\textsuperscript{2+} release (Missiaen et al., 1990), fluorescence quenching unambiguously signals the Mn\textsuperscript{2+} that enters the cytoplasm from the ECS.

Figure 49 shows that Mn\textsuperscript{2+} enters the cell and progressively quenches the fura-2 fluorescence at 360 nm in a nearly linear manner, at least until about 30% of the fluorescence is quenched. The slope of the linear part of the quenching trace can be regarded as a measurement of the rate of Mn\textsuperscript{2+} entry into endothelial cells (see Fig. 49 legend). This decline of fluorescence intensity at 360 nm presumably reflects Mn\textsuperscript{2+} entry through the leak, together with a small level of photobleaching of intracellular fura-2. No major effects due to addition of 250 \(\mu\)M Mn\textsuperscript{2+} on \([\text{Ca}^{2+}]_{i}\) were observed (Fig. 49, inset), indicating that the \([\text{Ca}^{2+}]_{i}\) signal was unaffected by the addition of Mn\textsuperscript{2+} at the concentration used.

As a further confirmation that Mn\textsuperscript{2+} quenching reflects divalent cation entry, the effects of Ca\textsuperscript{2+} entry blockers on the rate of quenching were examined. Ni\textsuperscript{2+}, an inorganic Ca\textsuperscript{2+} channel antagonist, was shown to diminish the rate of Mn\textsuperscript{2+} entry (Fig. 50). Since Ni\textsuperscript{2+} normally quenches fura-2 fluorescence, this experiment also suggests that Ni\textsuperscript{2+} cannot gain access to the cytoplasm of resting endothelial cells. Experimental confirmation was provided by the observation that addition of Ni\textsuperscript{2+} did not quench the fura-2 at 360 nm (Fig. 50, inset). A similar result was reached by Hallam et al. in their studies in cultured human umbilical-vein endothelial cells (Hallam et al., 1988). Both Figure 49 and 50 suggest that Mn\textsuperscript{2+} can enter the endothelial cells through a leak pathway.

Besides the leak pathway, Mn\textsuperscript{2+} also enters the plasmalemma through ROCs. As shown in Figure 51A, ATP caused an increase in the rate of Mn\textsuperscript{2+} influx. This quenching rate increase was not related to changes in \([\text{Ca}^{2+}]_{i}\) since ATP by itself did not affect the fluorescence intensity at 360 nm (Fig. 51A, inset). The degree of quenching following stimulation was expressed as a percentage of the unstimulated rate. In agreement with the reduction in the ATP-induced sustained plateau of \([\text{Ca}^{2+}]_{i}\) by NCDC and SK&F 96365
(Fig. 46A, 47A), the increased Mn$^{2+}$ quenching rate was also inhibited by pretreatment with NCDC (Fig. 51B) and SK&F 96365 (Fig. 51C).

CPA enhanced the steady state [Ca$^{2+}$]$_i$; however, it appears not to do so by increasing the divalent cation entry through the plasmalemmal membrane, as reflected by its failure to increase the rate of Mn$^{2+}$ entry (Fig. 52A). This suggests that ER depletion by itself is not sufficient to cause an increase in plasmalemmal divalent cation permeability in intact endothelial cells. To assure complete depletion of the ER and to confirm the lack of correlation between ER depletion and Ca$^{2+}$ entry, Mn$^{2+}$ quenching was measured at various time after the endothelium was pretreated with CPA. Figure 52B shows that the Mn$^{2+}$ quenching rate after the CPA application was not altered compared to the control quenching rate in the absence of CPA (Fig. 49). However at the end of a period which was sufficiently long to deplete the ER, addition of ATP still caused an increase in the quenching rate. Similar experiments were done with additions of Mn$^{2+}$ at 170, 200, 400, 500, 600, and 800 seconds after CPA application and the quenching was not changed within the time of recording, during which the ER was previously shown to be depleted (see Fig 43A). Therefore it can be concluded that ER depletion does not directly stimulate Ca$^{2+}$ entry in a manner analogous to that induced by ATP.

Taken together, these measurements of the decay in fura-2 fluorescence which is believed to be caused by Mn$^{2+}$ influx do not support the hypothesis that ER depletion leads to an increase in plasmalemmal permeability to Ca$^{2+}$ in endothelial cells.

I repeated each experiment at least 5 times and the average results of [Ca$^{2+}$]$_i$, and Mn$^{2+}$ quenching are depicted in Figure 53. It shows the quantitative evaluation of the effects of ATP, CPA and Ca$^{2+}$ entry blockers NCDC, Ni$^{2+}$ on steady state fura-2 ratio (F340/F380) and Mn$^{2+}$ entry rate. The results indicate that compared to the resting levels, ATP increases the steady state F340/F380 by 35±4% (N=7), CPA by 33±5% (N=8), ATP plus NCDC by 10±2% (N=5), CPA plus NCDC by 32±5% (N=5), ATP plus Ni$^{2+}$ by 5±1% (N=5) and CPA plus Ni$^{2+}$ by 4±1% (N=5). The average Mn$^{2+}$ quenching
rate was 10±3 (F. I Units/sec) (N=12 preparations), which was increased 27±3% by ATP (N=6), and decreased 22±4% by Ni²⁺ (N=5).

IV.6. Inhibition of ER Ca²⁺ accumulation on Ca²⁺ extrusion

The results described in chapter IV.4 and IV.5 show that inhibition of ER Ca²⁺ accumulation increased the steady state [Ca²⁺]ᵢ, which was not likely due to an increase in the plasmalemmal permeability to Ca²⁺. The results from this section will describe the role of inhibition of ER Ca²⁺ accumulation on the Ca²⁺ extrusion pathway. Figure 54 shows that CPA decreased the rate of [Ca²⁺]ᵢ decline in 0Ca²⁺-PSS after [Ca²⁺]ᵢ had first been raised. In the control experiment, [Ca²⁺]ᵢ was first raised to a high steady state by incubating the valve with CCh (10 µM) in the presence of 10 mM extracellular Ca²⁺. The valve was then exposed to 0Ca²⁺+PSS in the presence of muscarinic antagonist atropine (1 µM ) that washed out both Ca²⁺ and CCh. In the test solution, CPA (10 µM) was included in both the CCh, high Ca²⁺ PSS and atropine, 0Ca²⁺+PSS solutions. To facilitate comparison, the figure only shows the part of the [Ca²⁺]ᵢ trace after the valve was exposed to atropine, 0Ca²⁺-PSS solution. This trace was defined as "[Ca²⁺]ᵢ decline traces". By comparing the control and test decline traces, it appeared that the [Ca²⁺]ᵢ elevated by CCh in high Ca²⁺ PSS took longer time to return to the resting state after switching to 0Ca²⁺+PSS in the presence of CPA than that in the control solution.

To evaluate the result shown in Figure 54 in a more direct way, the "[Ca²⁺]ᵢ decline trace" (Fig. 54) was converted to a "rate-concentration" curve by measuring the slopes of the "[Ca²⁺]ᵢ decline trace" at 7 second intervals and plotting them against their respective F340/F380 ratio values and the results are shown in Figure 55. In the control curve it appeared to show that the rate of [Ca²⁺]ᵢ decline decreased as the F340/F380 ratio decreased and finally became zero when the F340/F380 ratio declined to resting levels. In the case of CPA, the rate of [Ca²⁺]ᵢ decline was slower than that under control conditions when the [Ca²⁺]ᵢ was above the resting levels and this effect was greater at higher F340/F380 ratios.
Taken together, the above results indicate that selective inhibition of ER Ca\(^{2+}\) accumulation by CPA slows the [Ca\(^{2+}\)]\(_i\) decline in Ca\(^{2+}\)-free PSS.

IV.7. Oscillations in [Ca\(^{2+}\)]\(_i\)

Fluctuations in [Ca\(^{2+}\)]\(_i\) are often observed in response to various situations in cultured endothelial cells (Laskey et al., 1992, 1994). This phenomenon was re-examined in the intact endothelial cells. Figure 56 shows that exposure to a K\(^+\)-free extracellular solution in the presence of agonist ACh (20 \(\mu M\)) and extracellular Ca\(^{2+}\) evoked [Ca\(^{2+}\)]\(_i\) fluctuations. The rhythmic oscillations in individual cells within the field of view of endothelial monolayer occur in synchrony.

Extracellular K\(^+\) concentration changes may affect the activity of the electrogenic Na\(^+\)-K\(^+\) pump. To evaluate the role of Na\(^+\)-K pump underlying the [Ca\(^{2+}\)]\(_i\) oscillations observed in K\(^+\)-free solution, the effect of Na\(^+\), K\(^+\) pump inhibitor, ouabain, on CCh-stimulated [Ca\(^{2+}\)]\(_i\) was examined. Figure 57 shows that application of CCh plus ouabain in the presence of extracellular Ca\(^{2+}\) also evoked synchronized oscillations in [Ca\(^{2+}\)]\(_i\).

The oscillations which became highly reproducible upon removal of extracellular K\(^+\) or ouabain application might implicate surface membrane mechanisms. Removal of K\(^+\) or inhibition of Na\(^+\)-K\(^+\) pump is known to depolarize cell (Daut et al., 1988; Sage & Cannell, 1989). The involvement of an intracellular Ca\(^{2+}\) store on [Ca\(^{2+}\)]\(_i\) oscillations was also examined. Figure 58 shows that 5 mM caffeine results in an initial transient [Ca\(^{2+}\)]\(_i\) response followed by asynchronous [Ca\(^{2+}\)]\(_i\) transients monitored in different cell clusters (Fig. 58).

All the oscillations did not occur over the entire field of view but were instead localized to clusters of adjacent cells, which represented ~45\% of the cells within the field of view. The cells in the field that did not show fluctuations in [Ca\(^{2+}\)]\(_i\) remained at pre-oscillation levels.
These results indicate that the oscillations in \([\text{Ca}^{2+}]_i\) could be generated by periodic changes in either \(\text{Ca}^{2+}\) release from the ER or surface membrane permeability to \(\text{Ca}^{2+}\).
Figure 1. The mechanisms of endothelial action.
Figure 2. Schematic representation of ion transport pathways underlying calcium homeostasis in endothelium. Cl\(^{-}\), chloride channel; 2K\(^{+}\)/3Na\(^{+}\), ATPase; KClNaCl, co-porter; Ca\(^{2+}\), ATPase; A, agonist; R, receptor; G, G-protein; PLC, phospholipase C; PKC, protein kinase C; ROC, receptor-operated channel; CRAC, Ca\(^{2+}\) release activated Ca\(^{2+}\) release; CIF, Ca\(^{2+}\) influx factor; L, leak; R, R-type of voltage-gated channel; K\(_{IR}\), inward rectifying K\(^{+}\) channel; K\(_{A}\), transient K\(^{+}\) channel; K\(_{Ca}\), Ca\(^{2+}\)-activated K\(^{+}\) channel; K\(_{ATP}\), ATP-activated K\(^{+}\) channel; K\(_{ACh}\), acetylcholine-activated K\(^{+}\) channel; 3Na\(^{+}\)/Ca\(^{2+}\), exchanger; Na\(^{+}\)/H\(^{+}\), antiporter; Cl\(^{-}\)/HCO\(_{3}^{-}\), exchanger; SAC, stretch-activated channel; ER, endoplasmic reticulum; CICR, Ca\(^{2+}\)-induced Ca\(^{2+}\) release channel; IP\(_{3}\), IP\(_{3}\) release channel; M, mitochondria; L-ARG, L-arginine; NOS, nitric oxide synthase; L-CITR, L-citrulline; NO, nitric oxide.
Figure 3. Schematic representation of the hypothetical superficial buffer barrier (SBB). Ca^{2+} entry through the basal Ca^{2+} leak and ligand- or stretch-gated channels is partially sequestered by the superficial endoplasmic reticulum (ER) from a restricted subplasmalemmal space by the ER Ca^{2+}-ATPase (SERCA). The superficial ER functions as a Ca^{2+} buffer barrier. Mobilization of ER Ca^{2+} will short circuit the SBB and enhance the flow of Ca^{2+} into the deeper cytoplasm (A). Schematic representation of the "capacitative Ca^{2+} entry" hypothesis: Ca^{2+}-release-activated-Ca^{2+} channels (CRAC). The depletion of ER sends unknown messengers to open the Ca^{2+} channels on the plasmalemmal membrane directly or indirectly (B).
Figure 4. Schematic anterosuperior view of the heart with the atria removed. The components of the fibrous skeleton and the orientation of the leaflets of each valve are demonstrated.
Figure 5. Schematic diagram of the experimental chamber specially designed for visualization of fura-2 loaded intact valvular endothelial cells. The valve leaflet was pinned flat over a narrow channel (1 mm width, 4 mm length). Only the lower surface of the valve was brought to focus and exposed to solution changes while the upper surface was continuously bathed in normal PSS. Teflon tubing (I.D: 0.28 mm) for perfusion was secured to the bottom of the chamber by the Sylgard (see method).
Figure 6. Experimental setup for fluorescence measurements
Figure 7. Fluorescence ratio at 340 nm and 380 nm excitation wavelengths (F340/F380) versus different $[\text{Ca}^{2+}]_i$. 
Figure 8  Experimental setup for bioassay (A); and the structure of NO microsensor (B).
Figure 9. Histological studies of cardiac valve. The outline of the endothelial cells on the surface of the valve leaflet was revealed following silver staining (magnification: x312) (A); A cross section photograph of the valve leaflet was shown by haematoxylin staining (magnification: x500) (B).
Figure 10. Cross-sectional electron micrograph of valve leaflet (magnification x9,900). Both intact basal lamina and gap junctions between endothelial cells (arrow) are visible. Central region of valve leaflet consists primarily of collagen fibers in a ground matrix with fibroblastoid cells dispersed throughout. Thickness of valve in this region is ≈ 60 μm (1 cm = 1 μm).
Figure 11. Fluorescence emission of the field of view of a fura-2 loaded valve leaflet illuminated with 380 nm excitation wavelength and observed with a x20 phase/fluor objective (1 cm = 125 μm). No correction was made for variation in illumination of field.
Figure 12. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to continuous perfusion of acetylcholine (ACh, 10 μM) in the presence of extracellular Ca2+ (trace a, solid line) and after the removal of endothelium by gently scraping the surface of the valve (trace b, dashed line). Each trace was obtained from a different preparation.
Figure 13. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to continuous perfusion of a nonhydrolyzable form of ACh, carbachol (CCh, 10 μM) in the presence of extracellular Ca²⁺ (A); in the absence of extracellular Ca²⁺ (B); and in the absence of extracellular Ca²⁺ followed by ATP application (C).
Figure 14. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to continuous perfusion of ATP (100 μM) in the presence of extracellular Ca²⁺ (A), subsequent removal of extracellular Ca²⁺ in the presence of ATP followed by re-addition of 1 mM Ca²⁺ to perfusate (B), in the absence of extracellular Ca²⁺ (C).
Figure 15. Color images of fluorescence ratio (F340/F380) of individual endothelial cells in the field of view of a fura-2 loaded intact rabbit cardiac valve at resting condition (A); and 30 seconds after stimulation with ATP (100 μM) in the presence of extracellular Ca\(^{2+}\) (B).
Figure 16. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to continuous perfusion of bradykinin (BK) (2 μM) in the presence of extracellular Ca^{2+} (A), following CCh (10 μM) stimulation (B) and in the absence of extracellular Ca^{2+} (C).
Figure 17. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to continuous perfusion of ionomycin (4 μM) in the presence of extracellular Ca$^{2+}$ (A), in the absence of extracellular Ca$^{2+}$ (B).
Figure 18. Representative trace of the average fluorescence ratio (F340/F380) signal of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to continuous perfusion of platelet-activating factor (PAF, 480 pM) in the presence of extracellular Ca$^{2+}$. 
Figure 19. Representative traces of the average fluorescence ratio (F340/F380) signals in response to continuous perfusion of 17-β-estradiol (20 μM) in the presence of extracellular Ca\(^{2+}\) followed by addition of 3 mM TEA to the intact endothelial cells from rabbit cardiac valve (A) and isolated endothelial cells from rabbit aorta (B).
Figure 20. A summary of the effects of ACh (10 μM), CCh (10 μM), ATP (100 μM), BK (2 μM), ionomycin (4 μM), PAF (480 pM) and 17-β-estradiol (20 μM) on the maximal increase of F340/F380 in intact endothelial cells from rabbit cardiac valve in the presence of extracellular Ca²⁺. Values on the ordinate scale indicate the percentage changes of maximal F340/F380 in response to all agents compared with the resting F340/F380 level (control). Each column represents mean±SEM of 5 valve preparations.
Figure 21. Relaxation of rabbit aorta in response to ACh (10 μM). The under-the-donor bioassay aorta, precontracted with 0.1 μM phenylephrine (Phe) in the presence of 0.1 μM atropine, was relaxed by ACh perfused over-the-donor in the control conditions; or following Ba$^{2+}$ (1 mM) perfusion to under-the-donor or over-the-donor (A); Similar protocol as in (A) except that TEA (5 mM) was used instead of Ba$^{2+}$ (B).
Figure 22. Nitric oxide release in the intact rabbit cardiac valve in response to ATP (100 μM) in the presence of extracellular Ca$^{2+}$. 
Figure 23. Nitric oxide release in the intact rabbit cardiac valve in response to CCh (10 μM) in the presence of extracellular Ca\(^{2+}\) (A); in the absence of extracellular Ca\(^{2+}\) (B). Dashed lines are recordings obtained without the valve tissues.
Figure 24. Nitric oxide release in the intact rabbit cardiac valve in response to BK (2 μM) in the presence of extracellular Ca²⁺ (A); in the absence of extracellular Ca²⁺ (B). Dashed lines are recordings obtained without the valve tissues.
Figure 25. Nitric oxide release in the intact rabbit cardiac valve in response to ionomycin (4 μM) in the presence of extracellular Ca$^{2+}$. Dashed line is recording obtained without the valve tissue.
Figure 26. Representative trace of the average fluorescence ratio (F340/F380) signal of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to depolarization induced by 80K⁺-PSS at resting condition.
Figure 27. Representative trace of the average fluorescence ratio (F340/F380) signal of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to depolarization induced by 80K⁺-PSS at the ATP (100 μM)-stimulated phase of F340/F380 increase.
Figure 28. Representative trace of the average fluorescence ratio (F340/F380) signal of individual endothelial cells in the field of view of intact rabbit cardiac valve in response voltage-gated channel (VGC) blocker diltiazem (DTZ, 1 μM) on the ATP (100 μM)-stimulated plateau phase of F340/F380 increase.
Figure 29. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to eqimolarly substituting external Na\(^+\) with lithium (Li\(^+\)) in the presence of extracellular Ca\(^{2+}\) (A) and in the absence of extracellular Ca\(^{2+}\) (B).
Figure 30. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to eqimolarly substituting external Na\(^+\) with N-methyl glucamine (NMDG) in the presence of extracellular Ca\(^{2+}\) (NMDG-PSS) (trace a, solid line) and in the absence of extracellular Ca\(^{2+}\) (trace b, dashed line). Each trace was obtained from a different preparation.
Figure 31. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to the Na\(^+\)-Ca\(^{2+}\) exchange inhibitor dichlorobenzamyl (DCB). After recording a control response to NMDG-PSS (A), the fura-2 loaded valve was exposed to N-PSS containing 25 \(\mu\)M DCB for 8 minutes. After a brief wash with N-PSS the valve was again exposed to NMDG-PSS (B). These two traces were obtained from the same valve preparation.
Figure 32. Representative trace of the average fluorescence ratio (F340/F380) signal of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to NMDG-PSS in the presence of the Na⁺-Ca²⁺ exchange inhibitor Mg²⁺ (2mM and 4 mM, respectively), the control response was subsequently recorded in NMDG-PSS.
Figure 33. Representative trace of the average fluorescence ratio (F340/F380) signal of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to the Na\(^+\)-H\(^+\) exchange inhibitor 5-(N,N-hexamethylene)amiloride (HMA). After recording a control response to NMDG-PSS, the fura-2 loaded valve was exposed to 10 mM HMA and subsequent NMDG-PSS in the presence of HMA.
Figure 34. Representative trace of the average fluorescence ratio (F340/F380) signal of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to ouabain (100 μM).
Figure 35. Representative trace of the average fluorescence ratio (F340/F380) signal of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to monensin (50 μM).
Figure 36. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to NMDG-PSS on the elevated F340/F380 signal induced by monensin (50 μM).
Figure 37. Representative trace of the average fluorescence ratio (F340/F380) signal of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to NMDG-PSS on the elevated F340/F380 signal induced by agonist CCh (10 mM).
Figure 38. Representative trace of the average fluorescence ratio (F340/F380) signal of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to external Na\(^+\) substitution with NMDG in the presence of 10 mM extracellular Ca\(^{2+}\), followed by exposure to 0Ca\(^{2+}\)-PSS in the presence of extracellular Na\(^+\) (control) (A) or in the absence of extracellular Na\(^+\) (B).
Figure 39. Inhibition of the rate of F340/F380 decline in the absence extracellular Ca$^{2+}$ by external Na$^+$ substitution at F340/F380 equal to 1.3. (1) CTR, control (2) 0Na$^+$, external Na$^+$ substituted by NMDG. The slope of the "F340/F380 decline trace (Fig 38)" at 1.3 were measured. The ordinate was the F340/F380 change. External Na$^+$ free solution significantly reduced the rate of F340/F380 decline at 1.3 (*P < 0.05, N=5 preparations).
Figure 40. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to three ER Ca^{2+} ATPase inhibitors: 10 μM cyclopiazonic acid (CPA) (A); 5 μM BHQ; (B) or 2 μM thapsigargin (Tg) (C) in the presence of extracellular Ca^{2+}, followed by removal of extracellular Ca^{2+}.
Figure 41. Percentage changes of average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in the presence of extracellular Ca\(^{2+}\) produced by (1) 10 \(\mu\)M cyclopiazonic acid (CPA); (2) 5 \(\mu\)M BHQ; (3) 2 \(\mu\)M thapsigargin (Tg).
Figure 42. Representative traces of the different average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to caffeine (20 mM). Each trace was obtained from a different preparation.
Figure 43. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to 10 μM cyclopiazonic acid (CPA) (A); 5 μM BHQ (B) followed by subsequent CCh stimulation, and 10 μM CCh followed by 5 μM BHQ in the absence of extracellular Ca^{2+} (C).
Figure 44. Representative trace of the average fluorescence ratio (F340/F380) signal of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to 10 μM cyclopiazonic acid (CPA) in the presence of extracellular Ca\(^{2+}\), followed by repetitive CCh stimulations.
Figure 45. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to respective exposure to OCa²⁺-PSS, CCh (10 μM), OCa²⁺-PSS, N-PSS, OCa²⁺-PSS, CCh (10 μM) (A); or all the same solutions as those in (A) plus that the BHQ (5 μM) was included in all the solutions used (B).
Figure 46. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to NCDC (100 μM) followed by ATP (100 μM) stimulation in the presence of extracellular Ca^{2+} (A); NCDC on the elevated F340/F380 level induced by CPA (10 μM) (B).
Figure 47. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to SK&F 96365 (40 μM) on the elevated F340/F380 level induced by ATP (100 μM) (A) or CPA (10 μM) (B) in the presence of extracellular Ca$^{2+}$,
Figure 48. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to Ni\(^{2+}\) (3 mM) on the elevated F340/F380 level induced by ATP (100 \(\mu\)M) (A) or CPA (10 \(\mu\)M) (B) in the presence of extracellular Ca\(^{2+}\).
Figure 49. Representative Mn$^{2+}$ quenching trace of the average fura-2 fluorescence intensity at excitation wavelength 360 nm of individual endothelial cells in the field of view of intact rabbit cardiac valve. The incubating solution (N-PSS) was first replaced with Ca$^{2+}$-free PSS. The fluorescence intensity decayed immediately upon addition of 250 $\mu$M Mn$^{2+}$. Determinations of the Mn$^{2+}$ quench rates were limited to a "psuedolinear" range (i.e. total quenching was no more than 30% of starting total fluorescence) and the slope of the nearly straight line portion of the fura-2 quenching trace was assumed to be proportional to the rate of Mn$^{2+}$ entry. All the subsequent Mn$^{2+}$ quenching recordings were terminated before the fluorescence intensities decayed to 70% of their original values. The ordinate scale indicates fura-2 fluorescence intensity excitation at 360 nm [Fluorescence Intensity Units, F. I. U (arbitrary units)]; inset. Effect of Mn$^{2+}$ (250 $\mu$M) on the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve.
Figure 50. Representative Mn²⁺ quenching trace of the average fura-2 fluorescence intensity at excitation wavelength 360 nm [Fluorescence Intensity Units, F. I. U (arbitrary units)] of individual endothelial cells in the field of view of intact rabbit cardiac valve following addition of Ni²⁺ (3 mM). The protocol was the same as in Fig 49 with addition of one more step when 3 mM Ni²⁺ was added; inset. Effect of Ni²⁺ (3 mM) on the average fura-2 fluorescence intensity at excitation wavelength 360 nm.
Figure 51. Representative Mn²⁺ quenching traces of the average fura-2 fluorescence intensity at excitation wavelength 360 nm (Fluorescence Intensity Units, F, L.U (arbitrary units)) of individual endothelial cells in the field of view of intact rabbit cardiac valve following addition of ATP (100 μM). The rabbit cardiac valve was exposed respectively to N-PSS, Ca²⁺-free PSS, 250 μM Mn²⁺ added to Ca²⁺-free PSS and addition of 100 μM ATP to the preceding solution (A). Inset. Effect of ATP (100 μM) on the average fura-2 fluorescence intensity at excitation wavelength 360 nm. The rabbit cardiac valve was exposed respectively to N-PSS, Ca²⁺-free PSS, 250 μM Mn²⁺ added to Ca²⁺-free PSS, 100 μM NCDC and subsequent addition of 100 μM ATP to the preceding solution (B); the same protocol as (B) except that SK&F 96365 was applied instead of NCDC (C).
Figure 52. Representative Mn$^{2+}$ quenching trace of the average fura-2 fluorescence intensity at excitation wavelength 360 nm [Fluorescence Intensity Units, F. I. U (arbitrary units)] of individual endothelial cells in the field of view of intact rabbit cardiac valve following addition of CPA (10 µM). The rabbit cardiac valve was exposed respectively to N-PSS, Ca$^{2+}$-free PSS, 250 µM Mn$^{2+}$ added to Ca$^{2+}$-free PSS and addition of 10 µM CPA to the preceding solution (A). The rabbit cardiac valve was exposed respectively to N-PSS, Ca$^{2+}$-free PSS, 10 µM CPA added to Ca$^{2+}$-free PSS, addition of 250 µM Mn$^{2+}$ to the preceding solution 300 seconds (s) after exposure to CPA followed by ATP (100 µM) stimulation (B). Similar experiments were done with the additions of Mn$^{2+}$ at 170, 200, 400, 500, 600, and 800 seconds after CPA application.
Figure 53. A summary of the effects of ATP (100 μM) and CPA (10 μM) on increasing the steady state F340/F380 in N-PSS (open columns) and the rate of Mn²⁺ quenching (closed column) in the absence or presence of NCDC (100 μM) or Ni²⁺ (3 mM). Values on the ordinate scale indicate the percentage change in steady state F340/F380 in response to all agents compared with the resting F340/F380 level and rate of Mn²⁺ entry compared with that obtained when only Mn²⁺ was applied. Each column represents mean±S.E.M of 5 experiments. *P < 0.05, compared with the control value. **P < 0.05, compared with the value of each other group.
Figure 54. Representative trace of the average fluorescence ratio (F340/F380) signal of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to CCh (10 μM) in the presence of 10 mM extracellular Ca²⁺ and CPA (10 μM), followed by exposure to atropine (1μM) in 0Ca²⁺-PSS (control, trace a) or all the same experimental solutions as in trace a except that CPA was not included (trace b).
Figure 55. Effect of CPA (10 μM) on the rate of decline of the average fluorescence ratio (F340/F380) signal of individual endothelial cells in the field of view of intact rabbit cardiac valve as function of F340/F380 ratio. The "rate-concentration" curve was converted from the "[Ca2+]i decline" trace displayed in Fig 54 by measuring the slope at every seven seconds and plotting them as a function of respective F340/F380. Dashed line: control condition; Solid line: in the presence of CPA.
Figure 56. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to continuous ACh (20 μM) perfusion and subsequent removal of extracellular K+.
Figure 57. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to combined application of ouabain (100 μM) and CCh (10 μM).
Figure 58. Representative traces of the average fluorescence ratio (F340/F380) signals of individual endothelial cells in the field of view of intact rabbit cardiac valve in response to caffeine (5 mM).
Table 1  COMPOSITIONS OF REPRESENTATIVE SOLUTIONS*

<table>
<thead>
<tr>
<th></th>
<th>NMDG</th>
<th>NaCl</th>
<th>CaCl₂</th>
<th>MgCl₂</th>
<th>LiCl</th>
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<tr>
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<td>0 Ca²⁺-PSS</td>
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<td>0</td>
<td>1</td>
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<tr>
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<td>10</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>NMDG-PSS</td>
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<td>1</td>
<td>1</td>
<td>0</td>
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<tr>
<td>NMDG,0 Ca²⁺PSS</td>
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<td>0</td>
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<tr>
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<td>1</td>
<td>1</td>
<td>140</td>
</tr>
<tr>
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<td>0</td>
<td>1</td>
<td>1</td>
<td>140</td>
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<td>2 Mg²⁺-NMDG-PSS</td>
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<td>1</td>
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</tr>
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<td>4 Mg²⁺-NMDG-PSS</td>
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<td>140</td>
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</tr>
<tr>
<td>Krebs solution</td>
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<td>118</td>
<td>2.5</td>
<td>1.2</td>
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</tr>
<tr>
<td>HBSS</td>
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<td>0.9</td>
<td>1</td>
<td>0</td>
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<tr>
<td>0Ca²-HBSS</td>
<td>0</td>
<td>137</td>
<td>0</td>
<td>1</td>
<td>0</td>
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</tbody>
</table>

* In mM. In addition, all solutions based on PSS contained 5 mM KCl, 10 mM glucose, 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) with pH 7.4 at 37°C except OK-PSS that did not contain KCl and 80K-PSS contained 80 mM KCl. Krebs solution contained 5 mM KCl, 11 mM glucose, 1.2 mM NaH₂PO₄, 2.5 mM Na₂CO₃, 0.03 mM EGTA; HBSS contained 5 mM KCl, 0.8 mM MgSO₄, 0.33 NaH₂PO₄, 0.44 K₂HPO₄, 10 mM Tris-HCl, and 0.1 mM L-arginine.
Table 2. Relaxation of bioassay aorta to ACh (10 μM) pretreated with K⁺ channel blockers under- or over-the donor aorta

<table>
<thead>
<tr>
<th>Relaxation (decrease of tension as % of pre-relaxation tension)</th>
<th>Ba²⁺ (1 mM)</th>
<th>TEA (5 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>under-the-donor</td>
<td>35±6*,**</td>
<td>33±7*,**</td>
</tr>
<tr>
<td>over-the-donor</td>
<td>25± 7*,**</td>
<td>21±6*,**</td>
</tr>
</tbody>
</table>

*: P< 0.05, compared to the relaxation induced by merely ACh;

**: 0.01 < P< 0.05, comparison between Ba²⁺ or TEA applied under- and over-the-donor.
Table 3.  NO release from intact endothelial cells following stimulation with different agents

<table>
<thead>
<tr>
<th>Agent</th>
<th>[NO] (nM) in N-PSS</th>
<th>[NO] (nM) in 0Ca(^{2+})PSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK (4 (\mu)M)</td>
<td>85.71±29.42 (N=4)</td>
<td>66.67±5.59 (N=7)</td>
</tr>
<tr>
<td>CCh (10 (\mu)M)</td>
<td>119.48±10.28 (N=14)*</td>
<td>33.33±13.27 (N=5)*</td>
</tr>
<tr>
<td>ATP (100 (\mu)M)</td>
<td>158.02±56.50 (N=5)</td>
<td></td>
</tr>
<tr>
<td>Ionomycin (10 (\mu)M)</td>
<td>238.09±40.23 (N=3)</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05, comparison between the data measured in N-PSS and in 0Ca\(^{2+}\)PSS.
CHAPTER V
DISCUSSION

V.1 The value of studying intact endothelial cells

This study using intact endothelial cells from rabbit cardiac valves provides new insight into $[\text{Ca}^{2+}]_i$ regulation, and is the first report on NO release from intact endothelial cells.

The special location of cardiac valves exposes them to mechanical stress. The force of systolic blood flow directed radially across the leaflets and the tension generated circumferentially by diastolic back-pressure creates localized intermittent turbulence, imposing complex stress patterns. The alterations of flow and stress would predispose valve leaflets to platelet adhesion and aggregation, and lesion formation. In addition, the valvular endothelium is easily damaged during catheterization and is a frequent target for pathological attacks such as acute rheumatic fever and endocarditis induced by Steptococcal infection. By releasing vasoactive substances such as EDRF and prostacyclin, the valvular endothelium, as continuation of endocardium, provides a nonthrombogenic surface that prevents platelet adhesion and aggregation, and thrombus formation under basal conditions as well as upon stimulation. The endothelium has also been shown to modulate inotropic responses of subjacent myocardium (Muelemans & Brutsaert, 1991). All these protective effects provided by valvular endothelium are regulated by cNOS, which is activated by intracellular $\text{Ca}^{2+}$ elevation. Therefore studying $\text{Ca}^{2+}$ signaling in the valve preparation is relevant to both physiology and clinical medicine.

As a continuation of the endocardium, the pulmonic or aortic valves are composed of thin flaps of dense fibrous connective tissue covered by monolayers of endothelium on both surfaces (endocardial endothelium). As shown in the histological (Fig. 9) and EM study (Fig. 10), smooth muscle cells were not found in the valve.
In addition to its considerable medical interest, the valve preparation also has a practical ease of visualization due to its relatively thin structure which reduces autofluorescence and its selective loading of fura-2 (Fig. 11). Except for the fact that the aortic valves are slightly thicker than the pulmonic valves, there are no major differences between these valves in terms of the structure and Ca\textsuperscript{2+} signaling. Therefore, it may not be necessary to indicate the specific valve (aortic or pulmonic) used in the text or legends.

V.2. The valvular endothelium preserves the characteristics of endothelial cells

a. [Ca\textsuperscript{2+}]\textsubscript{i} increase in response to vasoactive agents

The intact endothelium from cardiac valves was shown to preserve the characteristics of vascular endothelial cells. [Ca\textsuperscript{2+}]\textsubscript{i} was increased in response to vasoactive agents such as ACh, ATP, BK, the inflammatory factor PAF, the female sex hormone estrogen and the Ca\textsuperscript{2+} ionophore ionomycin (Figs. 12-19).

The [Ca\textsuperscript{2+}]\textsubscript{i} increase in response to agonists can be observed in the presence of extracellular Ca\textsuperscript{2+}, with a pattern of a transient peak followed by a sustained [Ca\textsuperscript{2+}]\textsubscript{i} increase. In the absence of extracellular Ca\textsuperscript{2+}, only the peak [Ca\textsuperscript{2+}]\textsubscript{i} response occurred. This indicates that both the intracellular store and Ca\textsuperscript{2+} entry from the ECS contribute to the [Ca\textsuperscript{2+}]\textsubscript{i} increase. The levels of the sustained phase of [Ca\textsuperscript{2+}]\textsubscript{i} increase induced by these agonists varied. Since supramaximally effective concentration of agonists were used to maximize the opportunity of discrimination between the mobilization of ER Ca\textsuperscript{2+} and the activation of Ca\textsuperscript{2+} influx pathways, and the same intracellular stores were shown to be affected by the different agonists, the difference in the plateau levels of ATP- and CCh-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase may indicate variations in the signal transduction pathways activated by muscarinic and purinergic receptor stimulation, variations in the receptor number or possible desensitization of receptors.
The $[\text{Ca}^{2+}]_i$ increase response produced by ACh indicates that the valvular endothelial preparation was functionally intact, since the ability to respond to ACh is a characteristic of healthy endothelial cells in vivo. Studies have shown that endothelial cells express m1, m2, and m3 subtypes of muscarinic receptor (Tracey & Peach, 1992; Marchenko & Sage, 1993). However in endothelial cells that have been cultured for several passages, a response to ACh is not observed (Adams et al., 1989), although ACh responses have been reported once for primary cultures of endothelial cells (Danathuluri et al., 1988) and for freshly dissociated endothelial cells (Rusko et al., 1992a). In the latter case intercellular communication is disrupted by the isolation process. Stimulation of purinergic or kinin receptor was also found to increases $[\text{Ca}^{2+}]_i$ in endothelial cells (Figs. 14-16), as reported in other studies (Morgan-Boyd et al., 1987; Gosink & Forsberg, 1993).

The $[\text{Ca}^{2+}]_i$ response to the inflammatory mediator PAF suggests an important role of the endothelium in inflammation and vascular pathology, since the endothelium may influence functions of other components present in circulating blood. It has been reported that PAF caused a concentration-dependent sustained $[\text{Ca}^{2+}]_i$ increase in human and canine cultured endothelial cells (Bkaily et al., 1993) and an endothelium-dependent relaxation in rat mesenteric artery (Chiba et al., 1990). Intravascular administration of PAF triggers a hypotensive response in the rat (Braquet et al., 1987).

Recently, great interest has focused on the contribution of circulating hormones to cardiovascular diseases. It has been reported that short- or long-term administration of the female sex hormone estrogen attenuates the constrictor response of coronary arteries to ACh in cynomolgus monkeys (William et al., 1992). A number of studies have shown that cardiovascular events are less prevalent in premenopausal women or women receiving estrogen replacement therapy than in men or postmenopausal women (Barrett-Connor & Bush, 1991; Stampfer & Colditz, 1991a; Stampfer et al., 1991b). However the exact role of estrogen in cardiovascular diseases remains unknown. The
sustained \([\text{Ca}^{2+}]_i\) increase in response to 17-\(\beta\)-estradiol in both the isolated and intact endothelial cells suggests a role of the endothelium in mediating estrogen's effect. The absence of an initial \([\text{Ca}^{2+}]_i\) peak associated with \(\text{Ca}^{2+}\) release from the ER and the dependence of the sustained \([\text{Ca}^{2+}]_i\) increase on the extracellular \(\text{Ca}^{2+}\) in response to 17 \(\beta\)-estradiol indicates that rapid release of \(\text{Ca}^{2+}\) from the ER does not play a dominant role in the action of estradiol. The sustained \([\text{Ca}^{2+}]_i\) increase induced by 17-\(\beta\)-estradiol was reduced upon addition of a \(\text{Ca}^{2+}\)-dependent \(\text{K}^+\) channel blocker TEA, indicating that \(\text{K}^+\) channels are involved in this sustained \([\text{Ca}^{2+}]_i\) increase. The \(\text{Ca}^{2+}\)-dependent \(\text{K}^+\) channel is characteristically sensitive to TEA (Rusko et al., 1992a). By blocking the \(\text{Ca}^{2+}\)-dependent \(\text{K}^+\) channel induced hyperpolarization, TEA decreases the electrical driving force for \(\text{Ca}^{2+}\) entry and therefore decreases \([\text{Ca}^{2+}]_i\).

b. Role of \(\text{Ca}^{2+}\)-dependent \(\text{K}^+\) channel

The existence of a \(\text{Ca}^{2+}\)-dependent \(\text{K}^+\) channel in intact endothelial cells was further shown in the bioassay study (Fig. 21, Table 2). TEA or \(\text{Ba}^{2+}\), at concentrations that can block the \(\text{Ca}^{2+}\)-activated \(\text{K}^+\) channel, applied either under- or over-the-donor, significantly inhibited the relaxation response of the bioassay aorta. This inhibitory effect was greater when \(\text{K}^+\) channel blockers were applied over-the-donor than under-the-donor. These results indicate that under control conditions, the endothelium of the donor aorta releases both EDRF and EDHF in response to ACh to cause the relaxation of bioassay aorta. EDRF causes vessel relaxation by activating guanylyl cyclase of smooth muscle and increasing cGMP level while EDHF causes relaxation by hyperpolarizing the smooth muscle to decrease \([\text{Ca}^{2+}]_i\). \(\text{K}^+\) channel blockers applied under-the-donor would block the effect of EDHF on bioassay aorta, so that the relaxation response to ACh was smaller than that in control conditions; but there was still some EDRF released from the endothelium of the donor aorta to act on the bioassay aorta to cause relaxation. When \(\text{K}^+\) channel blockers were applied over-the-donor, the effects of both EDRF and EDHF were inhibited, the relaxation in response to ACh was even smaller than that when \(\text{K}^+\) channel
blocker was applied under-the-donor. The extent of relaxation was significantly
different for the relaxation reduced by under- and over-the-donor applications of K+
channel blockers. These findings indicate that EDRF accounts for a significant relaxation
when the endothelium is stimulated by vasodilatory substances and that K+ activity is
important in mediating EDRF release. Similar results were obtained that TEA also
inhibited EDRF release induced by ACh (Demirel et al., 1994).
c. Oscillations of [Ca\textsuperscript{2+}]\textsubscript{i} occur in intact endothelial cells

[Ca\textsuperscript{2+}]\textsubscript{i} oscillations, where the [Ca\textsuperscript{2+}]\textsubscript{i} increases to a peak and then rapidly
declines to baseline, has been reported in cultured endothelial cells (Jacob et al., 1988;
Neylon & Irvine, 1990; Laskey et al., 1990b, 1992; Sage et al., 1989a) in response to
agonists. This study is the first report of [Ca\textsuperscript{2+}]\textsubscript{i} oscillations in an intact endothelial cell
layer. Similar to previously reported oscillations of [Ca\textsuperscript{2+}]\textsubscript{i} in cultured endothelial
monolayers (Laskey et al., 1990b), removal of extracellular K\textsuperscript{+} during the CCh-
induced [Ca\textsuperscript{2+}]\textsubscript{i} plateau caused oscillations of [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 56), suggesting a role of K+
conductance in [Ca\textsuperscript{2+}]\textsubscript{i} oscillations. Ouabain was also found to induce oscillations when
cells were stimulated with CCh (Fig. 57). A correlation between oscillations of [Ca\textsuperscript{2+}]\textsubscript{i}
and Em has been shown for cultured endothelial cell exposed to bradykinin stimulation
in K\textsuperscript{+}-free solution (Laskey et al., 1990b, 1992). In conjunction with the observation
that oscillations were inhibited by removal of extracellular Ca\textsuperscript{2+} (Sage et al., 1989a),
it is suggested that oscillatory changes in membrane conductance cause rhythmic
oscillations of Em (Laskey et al., 1990b).

Oscillations can also be induced by addition of caffeine to intact endothelial cells
(Fig. 58), indicating that a periodic Ca\textsuperscript{2+} release from the ER may also be a mechanism
for [Ca\textsuperscript{2+}]\textsubscript{i} oscillations. Whatever the cellular mechanism(s) of the oscillations, their
physiological role is of special interest, since an oscillating intracellular Ca\textsuperscript{2+} signal
may be an important determinant of signal transduction mechanisms that leads to
oscillatory release of endothelium-derived substances such as prostacyclin and nitric
oxide, which have potent effects on the rhythmic vasomotion and maintenance of optimal tissue perfusion.

d. NO release increases in response to vasoactive agents

The biological function of \([Ca^{2+}]_i\) changes was investigated by parallel EDRF measurements. The endothelial cells from valves release EDRF in a manner similar to those in the vasculature (Ku et al., 1990). NO release in intact endothelial cells was increased in response to CCh, ATP and BK (Fig. 22-25, Table 3), with a pattern similar to that of \([Ca^{2+}]_i\) increases discussed above. The fact that NO release can be increased in the absence of extracellular Ca\(^{2+}\) of indicates that the ER contains enough Ca\(^{2+}\) to stimulate NOS. These changes in endothelial signaling would result in the relaxation of smooth muscle by these agents (Palmer, 1987; Cocks et al., 1985; Arnold, 1977).

V.3. Receptor-operated divalent cation pathway is involved in agonist-induced \([Ca^{2+}]_i\) increase

The mechanism of agonist-activated Ca\(^{2+}\) entry was investigated. Studies have suggested that agonists induce a Ca\(^{2+}\) influx by stimulating surface membrane receptors coupled to ion channels either via G proteins or second messengers. Electrophysiological studies have demonstrated an inward nonspecific cation current induced by thrombin (Johns et al., 1987) and bradykinin (Colden-stanfield et al., 1987). The current correlates with an increase in Mn\(^{2+}\) entry (Hallam et al., 1988; Jacob, 1990) and \(^{45}\text{Ca}^{2+}\) uptake caused by ATP (Gosink & Forsberg, 1993) or bradykinin stimulation (Whorton et al., 1984). Two lines of evidence presented in this study suggest a role of a receptor-operated channel (ROC) in mediating Ca\(^{2+}\) influx following agonist activation. First, ACh, CCh, BK and ATP increased the steady state \([Ca^{2+}]_i\), which was completely dependent on the presence of extracellular Ca\(^{2+}\). In contrast to nonresponsiveness to the organic Ca\(^{2+}\) antagonist diltiazem (Fig. 28), the ATP-induced sustained phase of \([Ca^{2+}]_i\) was shown to be blocked not only by the removal of extracellular Ca\(^{2+}\) or addition of a
Ca$^{2+}$ entry blocker, Ni$^{2+}$ (Fig. 14B, 48A) but also by PLC blockers such as NCDC (Fig. 46A) and ROC inhibitors such as SK&F 96365 (Fig. 47A); second, corresponding with the $[\text{Ca}^{2+}]_i$ measurement data, the rate of Mn$^{2+}$ entry was increased by ATP and this effect could be blocked by the above mentioned blockers (Fig. 51). Since Mn$^{2+}$ was added extracellularly and is not taken up by SERCA (Gomes de Costa & Madeira, 1986), it can be concluded that ATP stimulates Mn$^{2+}$ entry and the increased Mn$^{2+}$ entry was due to opening of a receptor-operated divalent cation pathway.

V.4. Voltage-gated Ca$^{2+}$ channels are not present in endothelial cells

In exploring the mechanism of Ca$^{2+}$ entry due to agonist stimulation in endothelial cells, the role of VGC, which is a main Ca$^{2+}$ entry pathway in most cells, should be considered. Studies are somewhat conflicting about the existence of VGC in endothelial cells (see Chapter I. 8). In this study, the lack of effect of an organic Ca$^{2+}$ channel blocker diltiazem on the ATP-induced $[\text{Ca}^{2+}]_i$ increase (Fig. 28) indicates that agonist-stimulated Ca$^{2+}$ influx in intact endothelium is not mediated by the voltage-gated channels (VGCs). This conclusion was confirmed by the observations that depolarization with high extracellular K$^+$ did not increase $[\text{Ca}^{2+}]_i$ at rest (Fig. 26), but greatly reduced the ATP-induced sustained $[\text{Ca}^{2+}]_i$ increase (Fig. 27). The sustained $[\text{Ca}^{2+}]_i$ increase induced by agonist is related to hyperpolarization which increases the driving force for Ca$^{2+}$ (Marchenko & Sage, 1993), depolarization during ATP-induced sustained $[\text{Ca}^{2+}]_i$ increase would decrease the driving force, therefore reduces the sustained $[\text{Ca}^{2+}]_i$ increase. In the absence of evidence for the existence of VGC, the membrane potential $E_m$ is most likely to determine the driving force for Ca$^{2+}$ movement along its electrochemical gradient (Lückhoff & Busse, 1990b; Schilling, 1989). Hyperpolarization increases, while depolarization decreases, Ca$^{2+}$ entry. These results are in agreement with studies that did not show the existence of VGC. Ca$^{2+}$ channel blockers were found to be without effect, and depolarization with high K$^+$ decreased
[Ca^{2+}]_i and ^{45}\text{Ca}^{2+} uptake (Whorton et al., 1984; Colden-Standfield et al., 1987; Johns et al., 1987; Sturek et al., 1991). The ^{45}\text{Ca}^{2+} influx stimulated by bradykinin was insensitive to blockade by Ca^{2+} antagonists in cultured endothelial cells (Whorton et al., 1984). Electrophysiological studies have also failed to show the existence of VGCs in cultured endothelial cells (Colden-Standfield et al., 1987; Johns et al., 1987; Lansman et al., 1987). In agreement with the [Ca^{2+}]_i measurements, agonist-induced release of endothelium-derived relaxing factors (EDRF) was found to be reduced in high K\textsuperscript{+} solution (Furchgott, 1983) and was not inhibited by Ca^{2+} channel blockers (Adams et al., 1989).

However there are reports supporting the presence of VGCs. For example, endothelium-dependent relaxation of rabbit aorta was inhibited by verapamil (Singer & Peach, 1982), although it is difficult to separate its effects on the endothelium from direct effects on smooth muscle. Voltage-gated Ca^{2+} entry was reported in the endothelium of depolarized microvessels (Bossu et al., 1989). A R-type VGC in isolated endothelium was reported to respond to PAF and depolarization with 30 mM extracellular K\textsuperscript{+} ([K\textsuperscript{+}]_o) (Bkaily et al., 1993; Claing et al., 1994).

The difficulty in identifying the Ca^{2+} channel type responsible for the [Ca^{2+}]_i increase in endothelial cells has been attributed to damage of these cells during enzymatic dispersion and/or the use of long term culture preparations. Some of the variability encountered in the literature may be related to the difference in cell preparations, species and in vivo physiological state. It may be possible that putative VGCs which may be present in endothelial cells in situ are lost during the preparation of cells. Endothelial cells may undergo changes in phenotype in culture, so that channels are not sensitive to classic Ca^{2+} channel ligands or that an interaction with smooth muscle cells may be required. All these lead to a variable conclusion on the role of VGC in endothelial cells. The conclusions reached in this study are derived from an intact endothelium and provides convincing evidence for the absence of VGC in endothelial cells.
V. 5. The Ca$^{2+}$ leak is an important pathway in endothelial cells

The steady state [Ca$^{2+}$]$_i$ increase caused by CPA was inhibited by Ni$^+$ (Fig. 48B) or removal of extracellular Ca$^{2+}$ (Fig. 40) suggesting a role of the Ca$^{2+}$ leak pathway. Additional evidence for a Ca$^{2+}$ leak pathway in this study is the observation that Mn$^{2+}$ entered the cells under resting conditions (Fig. 49) and the rate of this process was decreased by Ni$^{2+}$ (Fig. 50). My data also indicates that Ca$^{2+}$ entry through leak pathway, not the ROCs, is the main mechanism for CPA-induced [Ca$^{2+}$]$_i$ increase. Demirel et al have demonstrated a nonstimulated Ca$^{2+}$ leak pathway in cultured porcine aortic endothelial cells that has a relatively high basal $^{45}$Ca$^{2+}$ uptake and can be differentiated from ATP- and stretch-activated Ca$^{2+}$ influx (Demirel et al., 1993).

V. 6. Ca$^{2+}$ release activated Ca$^{2+}$ entry pathway is not responsible for agonist-induced [Ca$^{2+}$]$_i$ increase

Receptor stimulation by agonists activates plasmalemmal PLC, which stimulates hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$), producing two physiologically active substances, diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP$_3$) (Berridge, 1993). DAG stimulates protein kinase C, but inhibitors of protein kinase C do not block ACh-evoked responses (Marchenko & Sage, 1993). Therefore agonists exert their intracellular effect mainly through the synthesis of IP$_3$, which elevates [Ca$^{2+}$]$_i$ by acting on specific receptors to release Ca$^{2+}$ from the ER. This mechanism was further supported by the effect of Li$^+$ on [Ca$^{2+}$]$_i$ in the absence of extracellular Ca$^{2+}$ in the intact endothelial preparation (Fig. 29B). Due to the agonist's effect on ER Ca$^{2+}$ release through IP$_3$ sensitive channels, an alternative explanation for agonist-induced Ca$^{2+}$ entry is considered, i.e. it is the depleted ER that induces Ca$^{2+}$ entry via unknown mechanism(s), the so called "CRAC" (Ca$^{2+}$ release activated Ca$^{2+}$ entry) pathway (Fasolato et al., 1994).
To test the assumption of "CRAC", SERCA blockers, which deplete the ER through other mechanism independent of IP3 formation, were used. It was found that even though both agonist stimulation (ATP) and SERCA inhibition (CPA) enhanced the steady state $[Ca^{2+}]_i$ in intact endothelial cells (Fig. 40, 41), the mechanisms involved were different as shown by their differential sensitivities to Ca$^{2+}$ entry blockers and their disparate effects on Mn$^{2+}$ entry. In contrast to the results with ATP stimulation described above, the CPA-induced $[Ca^{2+}]_i$ increase was only inhibited by Ni$^{2+}$ (Fig. 48B), but not by NCDC (Fig. 46B) or SK&F 85365 (Fig. 47B). Mn$^{2+}$ entry which was enhanced upon ATP stimulation, could not be affected by CPA (Fig. 52) even though the ER had been shown to be depleted (Fig. 43). These findings suggested that ATP raised the steady state $[Ca^{2+}]_i$ by stimulating Ca$^{2+}$ influx through ROCs, while CPA raised the steady state $[Ca^{2+}]_i$ through other mechanisms unrelated to stimulation of Ca$^{2+}$ influx. It indicates that the depletion of the ER per se did not appear to activate the same Ca$^{2+}$ channels as were activated by agonists.

These results are consistent with other studies indicating that depletion of the ER evoked an increase in $[Ca^{2+}]_i$ at rest, but did not accelerate Mn$^{2+}$ entry (Lückhoff & Busse, 1990a) and that $^{45}Ca^{2+}$ uptake was reduced rather than being enhanced by Tg (Gosink & Forsberg, 1993).

V.7. Evidence for the SBB hypothesis

Taken together, the data presented in this study can best be explained by a model of Ca$^{2+}$ entry through ROC and the "leak" plus a buffer barrier (SBB). The SBB hypothesis can be manifested in Ca$^{2+}$ entry and ER refilling.

a. Ca$^{2+}$ entry buffer

According to the SBB model, at rest or under steady state conditions, Ca$^{2+}$ that enters the cell across the plasmalemma through the leak is in part removed from the restricted cytoplasmic space between the plasmalemma and ER by the SERCA before
reaches the deeper cytoplasm (van Breemen et al., 1995). Cabelle & Schilling (1993) proposed the existence of a similar restricted sub-plasmalemmal space in the endothelium. Agents such as CPA and BHQ that selectively interfere with ER Ca\textsuperscript{2+} accumulation would interrupt this Ca\textsuperscript{2+} buffering function of the ER so as to raise steady state [Ca\textsuperscript{2+}]\textsubscript{i}. Removal of CPA restores this Ca\textsuperscript{2+} buffering function, so that [Ca\textsuperscript{2+}]\textsubscript{i} declines. The observation that this [Ca\textsuperscript{2+}]\textsubscript{i} increase was decreased by removal of extracellular Ca\textsuperscript{2+} or addition of Ni\textsuperscript{2+} (Fig. 48B, Fig. 40), but not by NCDC (Fig. 46B) or SK&F 96365 (Fig. 47B) at concentrations which could block ATP-induced ROC opening (Fig. 5, 6), further supports the notion that Ca\textsuperscript{2+} entry through the "leak" pathway, and not the ROCs, is the main Ca\textsuperscript{2+} entry pathway for the CPA-induced [Ca\textsuperscript{2+}]\textsubscript{i} response. Under resting conditions, this leak pathway is to a certain extent masked by SERCA pumping Ca\textsuperscript{2+} into the ER. When the Ca\textsuperscript{2+} buffering function of the ER is inhibited by CPA in the presence of extracellular Ca\textsuperscript{2+}, the Ca\textsuperscript{2+} leak becomes more effective in elevating [Ca\textsuperscript{2+}]\textsubscript{i} due to the redistribution of Ca\textsuperscript{2+} from ER to cytoplasm. In the absence of extracellular Ca\textsuperscript{2+}, CPA-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase is transient resulting from the movement of this redistributed portion of Ca\textsuperscript{2+} driven by the electrochemical gradient for Ca\textsuperscript{2+} towards the ECS. When receptors are activated by an agonist, the IP\textsubscript{3} mediated Ca\textsuperscript{2+} permeability of the ER increases and rapidly releases ER Ca\textsuperscript{2+}. This would short circuit the Ca\textsuperscript{2+} cycle between the ECS and ER so as to enhance [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 14). In addition, agonists stimulate Mn\textsuperscript{2+} entry through ROCs (Fig. 51). In view of the fact that VGCs were not identified in endothelial cells, and that stretch-activated channels (SACs) are not considered to be involved in [Ca\textsuperscript{2+}]\textsubscript{i} regulation in this study since all the experiments were performed at the same perfusion rate and multiple applications of same stimulating agent did not cause additional [Ca\textsuperscript{2+}]\textsubscript{i} changes, the recorded increases in [Ca\textsuperscript{2+}]\textsubscript{i} and Mn\textsuperscript{2+} influx following agonist stimulation were assumed to be mediated through the leak and ROC pathways. Interestingly, the ATP induced sustained [Ca\textsuperscript{2+}]\textsubscript{i} increase was not completely abolished by NCDC (Fig. 46A). The remaining portion of the
[Ca\(^{2+}\)]_i increase could be explained by the contribution of Ca\(^{2+}\) entry through the "leak", when buffering by the ER was inhibited by IP_3 induced Ca\(^{2+}\) release.

b. ER refilling

The depleted ER needs to be refilled by Ca\(^{2+}\) entering the cell from ECS. There are two possibilities for ER refilling:

1. The ER is refilled directly from the ECS according to the original "capacitative model"; (Casteels & Droogmans, 1981; Putney, 1986, 1990; Bourreau et al., 1992).

2. Ca\(^{2+}\) has to enter the restricted cytoplasmic space between the plasmalemma and the peripheral ER first before it is taken up into the ER by the ER Ca\(^{2+}\)-ATPase (SERCA) according to the SBB hypothesis (van Breemen et al., 1995).

My results demonstrate that not only at rest, but also during refilling of an empty ER, Ca\(^{2+}\) first enters the restricted cytoplasm and is then actively transported into ER lumen by SERCA. The SERCA is essential for the refilling process since repetitive applications of CCh, which could cause repetitive [Ca\(^{2+}\)]_i increases in the presence of extracellular Ca\(^{2+}\) under control conditions, did not induce repetitive [Ca\(^{2+}\)]_i increases in the presence of a SERCA blocker (Fig. 44) indicating that CCh and SERCA blocker affect the same intracellular store (Fig. 43). The role of SERCA is further shown in Figure 45. After being emptied by an agonist (CCh) in the absence of extracellular Ca\(^{2+}\), the ER was allowed to be refilled by repletion of Ca\(^{2+}\) from the ECS through the leak pathway. There was a relatively small increase in [Ca\(^{2+}\)]_i during the refilling phase since Ca\(^{2+}\) was taken up into ER; subsequent CCh application induced a [Ca\(^{2+}\)]_i transient due Ca\(^{2+}\) release from the refilled ER. However, when the same protocol was carried out in the presence of BHQ, even though a larger increase in [Ca\(^{2+}\)]_i occurred through 'leak' pathways during the refilling phase, subsequent application of CCh did still not induce a [Ca\(^{2+}\)]_i transient. It is speculated that if Ca\(^{2+}\) uptake occurred directly from the ECS, then no effect of BHQ on [Ca\(^{2+}\)]_i would be expected. The large [Ca\(^{2+}\)]_i increase during the refilling phase in the presence of BHQ could also be due to the disruption of the ER buffer
barrier function by BHQ, so that Ca^{2+} entering from the ECS effectively causes [Ca^{2+}]_i increase without being uptaken into the ER. Similar results were reported by Lückhoff et al. (1990a) in their studies defining the route of Ca^{2+} for refilling. The SERCA mediated ER refilling would be a requirement for the formation of an intracellular Ca^{2+} gradient during this period which means that more intracellular Ca^{2+} is in the peripheral area than that in the deeper cytoplasm, consistent with the "SBB".

However, there are some studies showing different results. For example, SERCA inhibitor-induced persistent [Ca^{2+}]_i elevation was concomitant with an increase in Mn^{2+} entry (Graier et al., 1995; Morgan & Jacob, 1994; Fasolato et al., 1993; Takemura et al., 1989) and transmembrane currents (Vaca & Kunze, 1993) which share the properties activated by substance P (Sharma & Davis, 1995), supportive of the "capacitativemodel".

It should be pointed that the notion addressed in this study is not totally at odds with the data supporting the capacitative model. First, the corollary of ER depletion to activate Ca^{2+} influx in the capacitative model is a spatially close proximity of the influx site to the Ca^{2+} store and a restricted space between the plasmalemma and Ca^{2+} store (Vaca & Kunze, 1993); second, an additional consequence of agonist induced ER depletion, which is thought to activate Ca^{2+} influx in the capacitative model, is the abolition of Ca^{2+} entry buffering by superficial ER, which according to SBB hypothesis would cause a [Ca^{2+}]_i increase. Therefore the function of the superficial ER as a physiologically regulated buffer barrier can be seen as an additional rather than an alternative means of regulating the movement of Ca^{2+} from ECS into the cytoplasm. The relative importance of the Ca^{2+} leak plus a SBB and I_{CRAC} may be related to species variability, different experimental paradigms, variability between different cell preparations, different cytosolic environments, or even between different agents on the same cell type (Sturek et al., 1991; Hallam et al., 1988; Sage et al., 1989b). The finding that caffeine induced a transient [Ca^{2+}]_i increase in only 50% of intact valve
preparations (Fig. 42) indicates a heterogeneity of the intracellular store(s) which is in agreement with a recent finding that caffeine only induced [Ca$^{2+}$]$_i$ transient in 37% of the cells; in cells that did not respond to caffeine, BK still increased [Ca$^{2+}$]$_i$ (Graier et al., 1994). It is also possible that store depletion may be linked to the activation of currents with characteristics rather different from those of prototypical ICRAC (Hoth & Penner, 1992). In addition, ICRAC-like currents may have limited permeability to Mn$^{2+}$ (Van Renterghem & Lazdunski, 1994); so the possibility that ICRAC might be impermeable to Mn$^{2+}$ is not ruled out. However, it can be concluded that the agonist ATP and the SERCA blocker CPA stimulate a [Ca$^{2+}$]$_i$ increase through different mechanisms: ATP activates Ca$^{2+}$ entry through ROCs and to some extent inhibits buffering of Ca$^{2+}$ entry by the ER; while the CPA mediated [Ca$^{2+}$]$_i$ increase depends on the Ca$^{2+}$ leak but not on a Mn$^{2+}$ permeable cation channels.

V.8. Na$^+$-Ca$^{2+}$ exchange plays a role in regulating [Ca$^{2+}$]$_i$ in endothelial cells

Besides the leak and ROC, another possible Ca$^{2+}$ transport process in the plasmalemmal membrane of endothelial cells is the Na$^+$-Ca$^{2+}$ exchanger. Although it has been demonstrated in cultured endothelial cells under extreme experimental conditions, its physiological importance is in doubt. In this study, changes in the Na$^+$ gradient in intact endothelial cells were found to induce a transient [Ca$^{2+}$]$_i$ increase. Since the removal of external Na$^+$ by substitution with NMDG did not cause a [Ca$^{2+}$]$_i$ increase due to mobilization from intracellular stores (Smith et al., 1989), this transient [Ca$^{2+}$]$_i$ increase is thought to be due to an internal sodium-dependent calcium entry pathway such as provided by Na$^+$-Ca$^{2+}$ exchange in the plasmalemma. It demonstrates the existence of Na$^+$-Ca$^{2+}$ exchange in intact endothelial cells under normal conditions (Fig. 30). This function was further confirmed by the applications of Na$^+$-Ca$^{2+}$ blockers such as DCB (Fig. 31) and Mg$^{2+}$ (Fig. 32). The possible role of intracellular pH$_i$
changes on \([\text{Ca}^{2+}]_i\) due to \(\text{Na}^+\)-\(\text{H}^+\) exchange when changing the \(\text{Na}^+\) gradient was not proven since application of \(\text{Na}^+\)-\(\text{H}^+\) exchange blocker (HMA) did not affect the response to \(\text{Na}^+\) gradient change (Fig. 33).

Interestingly, \(\text{IP}_3\) accumulation when substituting \(\text{Na}^+\) with \(\text{Li}^+\) was found to be sufficient to release \(\text{Ca}^{2+}\) from the ER in intact endothelium. The inactivation process of \(\text{IP}_3\) includes the hydrolysis of \(\text{IP}_3\) to inositol 1,4-bisphosphate \((\text{IP}_2)\), inositol 1-phosphate \((\text{IP})\), and then to inositol \((I)\) and inorganic phosphate \((P)\). Another means of \(\text{IP}_3\) inactivation is by phosphorylation to inositol 1,3,4,5-tetrakisphosphate \((\text{IP}_4)\), a putative second messenger activating ROC on the plasmalemmal membrane. \(\text{IP}_4\) is eventually broken down to inositol. \(\text{Li}^+\) directly inhibits inositol 1-phosphate \((\text{IP})\) monoesterase, an enzyme present in the cytoplasm of the cell which hydrolyzes \(\text{IP}\) to inositol, inorganic phosphate, and thus causing \(\text{IP}_3\) accumulation and \([\text{Ca}^{2+}]_i\) increases (Berridge et al., 1982). Acute administration of \(\text{Li}^+\) to rats has been found to result in increased endogenous \(\text{IP}\) levels especially in brain, and the small increase of \(\text{IP}\) that followed the administration of centrally acting agonists \textit{in vivo} was greatly enhanced in the presence of \(\text{Li}^+\) (Sherman et al., 1985). The effect of \(\text{Li}^+\) observed in intact endothelial cells (Fig. 29) was absent in cultured endothelial cells in both the presence and absence of extracellular \(\text{Ca}^{2+}\) (Laskey et al., 1990b).

As discussed above, the \(\text{Na}^+-\text{Ca}^{2+}\) exchanger was found to exist in intact endothelial cells. If it holds true that the resting membrane potential \((E_m)\) is -50 mV (Rusko et al., 1992a) and that \([\text{Na}^+]_i\) is 12 mmol/L (Hansen et al., 1991), while \([\text{Na}^+]_o\) is 140 mmol/L and \([\text{Ca}^{2+}]_o\) is mmol/L, according to the equation \(E_{\text{Ca}/\text{Na}}=3E_{\text{Na}}-E_{\text{Ca}}\), the reversal potential for the exchanger \((E_{\text{Ca}/\text{Na}})\) in intact endothelial cells is calculated to be about -110 mV. This is considerably more negative than the membrane potential \((E_m)\). Thus the \(\text{Na}^+-\text{Ca}^{2+}\) exchange \textit{in vivo} most likely operates in a net \(\text{Ca}^{2+}\)-influx mode at rest. However, the steady state \([\text{Ca}^{2+}]_i\) appeared not to be increased by external \(\text{Na}^+\) substitution and the transient ratio increase returned to baseline. This implies that
the Na⁺-Ca²⁺ exchanger might be relatively dormant under resting conditions (Fig. 30). Activation of Ca²⁺ entry through the exchanger requires internal Ca²⁺. This portion of internal Ca²⁺ is not transported by the exchanger and allosterically activates the exchanger operating in the Ca²⁺ influx mode. When [Ca²⁺]ᵢ is increased to a threshold level, the exchanger is activated, and the direction of net Ca²⁺ movement will be then be determined by whether Vₘ is more positive (Ca²⁺ influx) or more negative (Ca²⁺ efflux) than Eₙa/Ca (Blaustein, 1984).

For these reasons, the effect of Na⁺-Ca²⁺ exchange was tested under higher [Na⁺] and [Ca²⁺]ᵢ conditions. To increase [Na⁺]ᵢ, the Na⁺-K⁺ pump inhibitor ouabain and Na⁺ ionophore monensin were applied. Both electrophysiological measurements (Daut et al., 1988) and influx measurements (Nakagawa et al., 1987) have shown the existence of ouabain-sensitive Na⁺,K⁺-pump in endothelial cells. Inhibition of the Na⁺,K⁺-pump will cause depolarization (Daut et al., 1988) which could favor Ca²⁺ entry via Na⁺-Ca²⁺ exchange, especially when [Na⁺]ᵢ was increased (Eisner et al., 1983; Allen et al., 1983 Chapman, 1974). However, Na⁺,K⁺-pump inhibition would depolarize the cell so as to decrease the driving force for Ca²⁺ entry along its electrochemical gradient. In the cultured endothelium, ouabain was without effect on [Ca²⁺]ᵢ, indicating that the increase in Ca²⁺ entry (or inhibit Ca²⁺ extrusion) via the Na⁺-Ca²⁺ exchange possibly promoted by increase in [Na⁺]ᵢ induced by ouabain may be offset by the decrease in Ca²⁺ entry through a leak pathway resulting from the depolarization upon Na⁺,K⁺-ATPase inhibition. Without a sufficient increase in [Na⁺]ᵢ, the Na⁺-Ca²⁺ exchange may be unable to transport Ca²⁺ into the cell at a high enough rate to overcome other Ca²⁺ buffering systems. I found that in the intact endothelium both ouabain or monensin increased [Ca²⁺]ᵢ (Fig. 34, 35). The steady state [Ca²⁺]ᵢ increase was maintained since a high [Na⁺]ᵢ was sustained by the continuous presence of ouabain or monensin. Subsequent Na⁺ substitution in the presence of monensin or following the CCh induced [Ca²⁺]ᵢ increase caused both an additional transient as well as a steady state [Ca²⁺]ᵢ.
increase (Fig. 36, 37). These data indicate that Na\textsuperscript+-Ca\textsuperscript{2+} exchange has a more prominent role in elevating [Ca\textsuperscript{2+}]\textsubscript{i} in the intact endothelium than in the cultured endothelial cells when [Na\textsuperscript{+}]\textsubscript{i} was elevated.

V.9. Ca\textsuperscript{2+} extrusion pathways

Little is known about the mechanisms of Ca\textsuperscript{2+} extrusion in endothelial cells. There are several possibilities to extrude elevated intracellular Ca\textsuperscript{2+} towards the extracellular space.

a. ER unloading Ca\textsuperscript{2+} towards the ECS

It is reasonable to propose that if the superficial ER functions as a maintained buffer for Ca\textsuperscript{2+} entry, it is then required to continuously unload Ca\textsuperscript{2+} into the ECS. It is predicted in the SBB model that there exists a continuous vectorial Ca\textsuperscript{2+} extrusion pathway from the peripherally located ER lumen towards surface membrane, and then to the ECS. Inhibition of Ca\textsuperscript{2+} accumulation by the ER would interfere with this vectorial Ca\textsuperscript{2+} extrusion pathway, which enhances the steady state [Ca\textsuperscript{2+}]\textsubscript{i}.

This extrusion pathway was tested in the experiment shown in Figure 54 and 55. [Ca\textsuperscript{2+}]\textsubscript{i} was first raised by an agonist in a high Ca\textsuperscript{2+} solution. The monitored [Ca\textsuperscript{2+}]\textsubscript{i} decline, when the valve was exposed to 0Ca\textsuperscript{2+}-PSS external medium, reflects the process of Ca\textsuperscript{2+} removal from the cells. Both the original "[Ca\textsuperscript{2+}]\textsubscript{i} decline trace" displayed in Fig 54 and the converted "rate-concentration curve" shown in Fig. 55 show that the rate of [Ca\textsuperscript{2+}]\textsubscript{i} decline was slower in the presence of CPA than that in the absence of CPA, leading to the conclusion that inhibition of ER Ca\textsuperscript{2+} accumulation slows the rate of Ca\textsuperscript{2+} extrusion towards the ECS when the [Ca\textsuperscript{2+}]\textsubscript{i} was above the resting levels in the absence of extracellular Ca\textsuperscript{2+}. A possible direct contribution by ER Ca\textsuperscript{2+} accumulation to Ca\textsuperscript{2+} removal under these conditions seems unlikely since ER Ca\textsuperscript{2+} content declines in zero external Ca\textsuperscript{2+}.
b. Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange

It was reported that in smooth muscle cells, the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger is mainly concentrated on the plasmalemma-sarcoplasmic reticulum (SR) junctional areas and its activity appeared to be closely coupled to Ca\textsuperscript{2+} availability from intracellular store (Moore et al., 1992). Relating this finding to the "SBB" hypothesis in endothelial cells, it would be reasonable to predict that the exchanger may also play a role in extrusion of Ca\textsuperscript{2+} released from the ER Ca\textsuperscript{2+} towards the inner surface of the plasmalemma for extrusion to ECS. The experiment described in Figure 38 and 39 were designed to test this prediction.

Besides its contribution to Ca\textsuperscript{2+} entry, the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger was also found to operate in the Ca\textsuperscript{2+} extrusion mode in intact endothelial cells. Blockade of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange slowed the [Ca\textsuperscript{2+}]\textsubscript{i} decline after [Ca\textsuperscript{2+}]\textsubscript{i} was first elevated, indicating that rate of extrusion is partially inhibited by blockade of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange. This result supports the existence of the above-mentioned vectorial Ca\textsuperscript{2+} extrusion pathway in endothelial cells.

c. Plasmalemmal Ca\textsuperscript{2+}-ATPase

In cultured endothelial cells, however, several authors have noted that removal of extracellular Na\textsuperscript{+} had little influence on [Ca\textsuperscript{2+}]\textsubscript{i} signal and concluded that Ca\textsuperscript{2+} extrusion is mainly mediated by plasmalemmal Ca\textsuperscript{2+}-ATPase, which has been identified by Hagiwara et al (1983). The finding in this study that [Ca\textsuperscript{2+}]\textsubscript{i} returned to baseline after being transiently elevated following Na\textsuperscript{+} substitution confirms a Na\textsuperscript{+}-independent mechanism such as Ca\textsuperscript{2+}-ATPase.
CHAPTER VI

SUMMARY AND CONCLUSION

In summary, the methods employed in this study provide a new means of monitoring intracellular Ca^{2+} signaling and correspondent EDRF (NO) measurements in intact endothelial cells from rabbit aortic or pulmonic valves. [Ca^{2+}]_i signals in the intact endothelial cells, such as those which responded to agonist stimulation and Na\(^+\) gradient reversion, were found to be different from those obtained in cultured endothelial cells. The existence of a Na\(^+\)-Ca\(^{2+}\) exchange mechanism was identified in this intact endothelial preparation, and functioned to stimulate Ca\(^{2+}\) entry when [Na\(^+\)]_i is elevated and the Na\(^+\) gradient was reversed. It may also contribute to Ca\(^{2+}\) extrusion towards the ECS when [Ca\(^{2+}\)]_i is high.

At rest, Ca\(^{2+}\) entry through the leak is an important pathway. When cells were stimulated by agonist, by using ROC blockers and Mn\(^{2+}\) quenching of fura-2 fluorescence measurements, Ca\(^{2+}\) entry through the receptor-operated cation channels (ROCs) was demonstrated to be a main pathway contributing to the sustained [Ca\(^{2+}\)]_i increase. Voltage-gated cation channels (VGCs) were found not to be present. In addition, [Ca\(^{2+}\)]_i activated K\(^+\) channels were found to contribute to the estrogen-induced [Ca\(^{2+}\)]_i increase and ACh-induced vessel relaxation.

The results indicate that in endothelial cells, the ER functions as a Ca\(^{2+}\) entry buffer barrier. At rest or during ER refilling, the ER could accumulate part of Ca\(^{2+}\) from the restricted cytoplasmic space between the plasmalemma and the ER before it reaches the deep cytoplasm. Inhibition of ER Ca\(^{2+}\) accumulation would interrupt this barrier function so that the Ca\(^{2+}\) entry through the leak becomes more effective in
raising [Ca$^{2+}$]$i$. This was confirmed by the observation that CPA, Tg and BHQ increased steady state [Ca$^{2+}$]$i$. The results of this study do not indicate a direct pathway between the ECS and the ER.

The depletion of endoplasmic reticulum (ER) caused by agonist-stimulated Ca$^{2+}$ release from IP$_3$-sensitive channels, was found not to activate Ca$^{2+}$ entry from the extracellular space (ECS). Depletion of ER by ER Ca$^{2+}$-ATPase inhibitors, which prevented ER Ca$^{2+}$ reuptake and gradually emptied the IP$_3$-sensitive ER, did not signal an increase in Mn$^{2+}$ entry. The results do not support the idea that following depletion of ER, the permeability to Ca$^{2+}$ entry was increased. In addition, the rate of [Ca$^{2+}$]$i$ decline in the absence of extracellular Ca$^{2+}$ after [Ca$^{2+}$]$i$ was first elevated was found to be decreased by inhibition of ER Ca$^{2+}$ accumulation.
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