CALCIUM SIGNALLING IN VASCULAR ENDOTHELIAL CELLS: Ca^{2+} ENTRY AND RELEASE

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INTRODUCTION

Endothelial cells serve both autocrine and paracrine functions within the cardiovascular system to modulate blood pressure and maintain tissue perfusion. Endothelial cells respond to a variety of humoral and physical stimuli to release endothelium-dependent vasodilators, such as prostacyclin (PGI_2) and endothelium-derived relaxing factor (EDRF), and vasoconstrictors such as endothelin (Furchgott and Vanhoutte, 1989). The synthesis and release of endothelium-derived vasodilators have been shown to be Ca^{2+}-dependent whereby the production of PGI_2 and EDRF is attenuated by the removal of extracellular Ca^{2+} (Singer and Peach, 1982; Long and Stone, 1985; Griffith et al., 1986; Lückhoff et al., 1988). It is now well established that an EDRF released from endothelial cells, both *in situ* and in culture, is nitric oxide (NO) or a nitroso compound that readily releases NO, and that NO is synthesized in endothelial cells by the oxidation of one of the two equivalent guanidino nitrogens of L-arginine via a cytoplasmic NADPH- and Ca^{2+}-dependent enzyme, termed NO synthase (see Moncada et al., 1991). Changes in the extracellular Ca^{2+} concentration around the physiological range have been shown to modulate the synthesis/release of NO by the vascular endothelium and consequently, vascular tone (Lopez-Jaramillo et al., 1990).

Both the entry of extracellular Ca^{2+} and the mobalization of Ca^{2+} from intracellular stores can contribute to the increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in endothelial cells, which is an essential step in the activation of NO synthase. Regulation of endothelial $[Ca^{2+}]_i$ is composed of activating mechanisms which supply Ca^{2+} to the cytoplasm and homeostatic mechanisms which maintain low $[Ca^{2+}]_i$ and remove cytoplasmic free Ca^{2+} after stimulation. Activation of Ca^{2+} entry from the extracellular space and Ca^{2+} release from intracellular stores (endoplasmic reticulum) occurs in response to humoral and physical stimuli. Although the importance of intracellular Ca^{2+} as a second messenger is unquestioned, its regulation in endothelial cells is not clearly delineated.

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The activation of endothelial cell-surface receptors by vasoactive substances evoke a biphasic increase in the cytoplasmic free Ca^{2+} concentration (Hallam and Pearson, 1986; Hallam et al., 1988a). An initial transient component reflects the release of Ca^{2+} from intracellular stores by the intracellular second messenger, inositol 1,4,5-trisphosphate ($InsP_3$) (Derian and Moskowitz, 1986; Lambert et al., 1986; Pirotten et al., 1987; Freay et al., 1989), whereas a subsequent sustained elevation in $[Ca^{2+}]_i$ results from the influx of Ca^{2+} from the extracellular space (Hallam and Pearson, 1986; Morgan-Boyd et al., 1987; Hallam et al., 1988; Schilling et al., 1988). Endothelial cells often respond to prolonged agonist stimulation with repetitive spikes or oscillations in $[Ca^{2+}]_i$ superimposed on the plateau phase (Jacob et al., 1988; Sage et al., 1989; Laskey et al., 1990).

PLASMALEMMAL Ca^{2+} ENTRY

Extracellular Ca^{2+} may enter the cell via at least five different mechanisms: [1] a passive Ca^{2+} leak pathway dependent on the electrochemical gradient for Ca^{2+}; [2] receptor-mediated Ca^{2+} influx via receptor-operated or second messenger activated non-selective cation channels; [3] a "capacitative Ca^{2+} influx" pathway dependent on the state of the intracellular Ca^{2+} stores; [4] mechanosensitive (stretch-activated) cation channels, and [5] Na-dependent Ca^{2+} entry via a Na^+-Ca^{2+} exchanger. Electrophysiological and unidirectional ^{45}Ca flux measurements in cultured endothelial cells derived from large blood vessels indicate an absence of the L-type Ca^{2+} channels which are sensitive to dihydropyridines and activated by membrane depolarization (Colden-Stanfield et al., 1987; Johns et al., 1987; Takeda et al., 1987). In recent studies on freshly dissociated endothelial cells from rabbit aorta, no evidence for depolarization-activated Ca^{2+} influx via voltage-dependent Ca^{2+} channels was found (Sakai, 1990; Rusko et al., 1992a), however, such channels may be present in capillary endothelium (Bossu et al., 1989; Bossu et al., 1992).

Passive Ca^{2+} Leak Pathway

A passive Ca^{2+} "leak" influx driven by the electrochemical gradient for Ca^{2+} (E_m - E_{Ca}) and ubiquitous to all eukaryotic cells is present in vascular endothelial cells (Johns et al., 1987; Schilling et al., 1989). Johns et al (1987) observed a resting ^{45}Ca influx into unstimulated, cultured bovine pulmonary artery endothelial cells which was postulated to mediate basal EDRF release. Lanthanum ions, but not other calcium channel blockers such as verapamil and diltiazem, inhibited the passive ^{45}Ca uptake into cultured endothelial cells. Depolarization reduced the driving force for Ca^{2+} entry through the leak pathway by reducing the electrochemical gradient. The rate of basal ^{45}Ca leak, corresponding to 16 pmol/10^6 cells/s, was reduced by approximately 15% in an isotonic KCl solution. Changes in extracellular pH also modulate the Ca^{2+} leak. The resting ^{45}Ca influx increased progressively as extracellular pH was raised from 5.2 to 9.2 (S. Purkerson, D.J. Adams and C. van Breemen, unpublished observations). "Leak" channels which open at negative membrane potentials and are permeable to divalent cations have been described in vascular smooth muscle cells (Benham and Tsien, 1986) and Duchenne human and *mdx* mouse myotubes (Fong et al., 1990). The nature of the Ca^{2+} leak pathway in endothelial cells is unknown but may play an important physiological role in the basal release of EDRF and thus regulation of vascular tone and peripheral resistance.

Receptor-mediated Ca^{2+} Entry via Non-selective Cation Channels

Direct support for a receptor-mediated Ca^{2+} influx pathway in endothelial cells

came from the finding that ^{45}Ca influx is enhanced by agonists such as bradykinin, histamine and thrombin (D'Amore and Shepro, 1977; Whorton et al., 1984; Johns et al., 1987). Thrombin and bradykinin evoked an inward nonspecific cation current in pulmonary artery endothelial cells which could account for the enhanced Ca^{2+} influx (Johns et al., 1987; Lodge et al., 1988). Recently, bradykinin-evoked inward currents carried by Na^+ and Ca^{2+} with amplitudes of 5-25 pA/cell were reported only in clusters of electrically coupled endothelial cells from bovine aorta (Mendelowitz et al., 1992). Histamine and A23187 (a calcium ionophore) also induced a $[Ca^{2+}]_i$ dependent inward current in human umbilical vein endothelial cells (Bregestovski et al., 1988). The existence of receptor-operated cation channels permeable to Ca^{2+} has been demonstrated in cultured endothelial cells from bovine pulmonary artery (Johns et al., 1987; Lodge et al., 1988), human umbilical vein (Bregestovski et al., 1988, Nilius, 1990; Nilius and Rienmann, 1990) and capillary endothelial cells (Popp and Gögelein, 1992). Bradykinin- and thrombin-activated single channels recorded in approximately 10% of cell-attached patches exhibited a slope conductance of ~15 pS with 100 mM Ba^{2+} (Lodge et al., 1988; Adams et al., 1989). A non-selective cation channel activated by histamine and thrombin was also described in cell-attached patches in cultured human umbilical vein endothelial cells and exhibited a single channel conductance of 26 pS with asymmetrical solutions (140 Na^+//140 K^+) and 8 pS with 110 mM Ca^{2+} pipette solution (Nilius, 1990; 1991). The permeation ratio for this receptor-operated non-selective cation channel is $P_K:P_{Na}:P_{Ca}$ = 1:0.9:0.2 (Nilius, 1990). Recently, a histamine-activated cation channel with a slope conductance of 22.5 pS in physiological solutions was reported in undispersed endothelial cells of the rat intrapulmonary artery (Yamamoto et al., 1992). The channel exhibited inward rectification, possibly due to block by intracellular Mg^{2+}, and the permeability ratios calculated from the reversal potentials were $P_K:P_{Na}:P_{Ca}$ = 1:1:15.7. The observation that apparently similar ion channels are activated by different agonists suggests a convergent intracellular messenger cascade between receptor activation and channel opening. Lückhoff and Clapham (1992) recently reported the activation of a Ca^{2+}-permeable channel by inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) and Ca^{2+} applied to the cytoplasmic surface of inside-out membrane patches from bovine aortic endothelial cells. These channels were not activated by GTPγS or $InsP_3$, although the phosphorylation of $InsP_3$ to generate $InsP_4$ would be an obvious final step in a convergent signalling pathway. These channels appear to have a lower conductance than those described above, that is, 2.5 pS with 110 mM Mn^{2+} in the pipette solution. The data presently available, therefore, suggest the presence of two types of receptor-operated Ca^{2+} permeable cation channels, a low conductance channel activated by $InsP_4$ and Ca^{2+}, and a channel approximately 10 times larger.

Capacitative Ca^{2+} Influx Pathway

An alternative mechanism proposed for the regulation of agonist-stimulated Ca^{2+} entry involves a pathway controlled by the content of the intracellular stores, as first proposed for rat parotid acinar cells (Putney, 1986; Merritt and Rink, 1987). According to this mechanism, termed "capacitative Ca^{2+} entry", the Ca^{2+} influx across the plasma membrane is also activated by emptying the internal Ca^{2+} stores. This may account for the phenomenon of receptor-mediated divalent cation entry reported in stimulated endothelial cells despite the removal of agonist (Hallam et al., 1988,1989; Jacob, 1990). This model has gained support from observations made in a variety of non-excitable cells using either thapsigargin (TG), or 2',5'-di(*tert*-butyl)-1,4-benzohydroquinone (BHQ). Thapsigargin and BHQ are known to selectively inhibit the Ca^{2+}-ATPase in the endoplasmic reticulum and prevent Ca^{2+} reuptake thereby mobilizing intracellular Ca^{2+} by a mechanism independent of receptor stimulation and $InsP_3$ formation (Takemura et al., 1989; Kass et al., 1989).

In contrast to the rapidly developing, transient rise in $[Ca^{2+}]_i$ initiated by bradykinin, TG and BHQ have been shown to induce a slow rise and prolonged elevation in $[Ca^{2+}]_i$ in cultured endothelial cells from bovine aorta and pulmonary artery (Dolor et al., 1992; Schilling et al., 1992). Mobilization of intracellular Ca^{2+} with either inhibitor depleted intracellular Ca^{2+} stores and greatly reduced subsequent mobilization of the $InsP_3$-sensitive Ca^{2+} store by bradykinin. Although TG and BHQ had no effect on phosphoinositide hydrolysis, both agents stimulated ^{45}Ca uptake and divalent cation permeability, as measured by enhanced Mn^{2+} quenching of intracellular fura-2, suggesting that depletion of the agonist-sensitive store is sufficient to activate Ca^{2+} influx (Dolor et al., 1992; Schilling et al., 1992). However, in a separate study on pig aortic endothelial cells (Lückhoff and Busse, 1990a), BHQ increased $[Ca^{2+}]_i$ in prestimulated and resting cells without stimulation of Mn^{2+} entry. The TG- and BHQ-induced elevation in $[Ca^{2+}]_i$ required extracellular Ca^{2+} and was inhibited by extracellular Ni^{2+} or La^{3+}, membrane depolarization and the putative receptor-operated channel blocker, SKF 96365 (Dolor et al., 1992; Schilling et al., 1992). These results are identical to those obtained for the agonist-stimulated increase in the plateau value of $[Ca^{2+}]_i$ suggesting that the influx pathway activated by depletion of the agonist-sensitive internal Ca^{2+} store is similar to the agonist-activated Ca^{2+} influx pathway. A Ca^{2+} current that is activated by depletion of intracellular Ca^{2+} stores has been recently identified in mast cells (Hoth and Penner, 1992). This "Ca^{2+} release-activated Ca^{2+} current" observed only when cytoplasmic free Ca^{2+} concentration was buffered to very low values, was voltage-insensitive and highly selective for Ca^{2+} over Mn^{2+}. This Ca^{2+} entry pathway may contribute to Ca^{2+} homeostasis and replenish empty Ca^{2+} stores in electrically non-excitable cells.

The original capacitative Ca^{2+} entry model which featured a direct pathway for Ca^{2+} entry into the ER lumen, was modified primarily due to the lack of additivity of the effects of carbachol and TG on steady-state $[Ca^{2+}]_i$ in parotid acinar cells (Putney, 1990). Depletion of ER Ca^{2+} was postulated to signal the opening of plasmalemmal Ca^{2+}-permeable channels connecting the extracellular space to the cytoplasm, however, no plausible candidate for this signalling pathway has been identified.

Mechanosensitive Ca^{2+}-permeable Channels

Mechanosensitive (stretch-activated) ion channels in endothelial cells may serve as transducers in detecting changes in blood pressure or flow. Depending on their sensitivity to these stimuli and on their ionic selectivity, mechanosensitive ion channels could, for example, change endothelial cell membrane potential, and thus the driving force for passive Ca^{2+} entry, if they are K^+ permeable, or directly allow the entry of Ca^{2+}, if they are Ca^{2+} permeable. Indeed, stretch-activated, single-channel currents have been described in cultured endothelial cells from neonatal pig aorta (Lansman et al., 1987). With the application of suction pulses (10-20 mm Hg, 300 ms) to cell-attached membrane patches and physiological ion concentrations in the patch pipette and external media, the frequency of opening of inward unitary currents increased for negative membrane potentials. These currents displayed an ohmic I-V relationship with a slope conductance of 40 pS and an extrapolated reversal potential of 9 mV. They were impermeable to Cl^-, but able to pass K^+ (elementary conductance of 56 pS with an isotonic KCl pipette solution) and Ca^{2+} (19 pS with isotonic $CaCl_2$). It was reported that the calculated P_{Ca}/P_{Na} ranged between 1.2 and 8.4. These suction-activated channels were not Ca^{2+}-sensitive. Stretch-activated unitary currents with similar characteristics have also been recently described in endothelial cells from porcine cerebral capillaries (Popp et al., 1992).

If membrane stretch is similar to wall shear stress generated by flowing blood, then activation of stretch-activated channels should elicit changes in membrane potential and

$[Ca^{2+}]_i$. In a more direct test of the effects of increased shear stress, a laminar flow-activated, whole-cell, inwardly rectifying K^+ current was described in cultured bovine aortic endothelial cells (Olesen et al., 1988). Half-maximal activation of this current in symmetrical K^+ solutions occurred at a flow rate producing wall shear stress of 0.7 dyn/cm^2, with saturation being reached at 15 dyn/cm^2. It was reported that flow caused cell hyperpolarization of 0 to 6 mV under physiological ionic conditions, where the average resting potential was -77 mV (E_K = -90 mV). This is in accord with another study where the flow-associated shift in the fluorescence of a potential-sensitive dye was consistent with membrane hyperpolarization (Nakache and Gaub, 1988). Shear stress-induced $[Ca^{2+}]_i$ transients have been described in cultured bovine aortic endothelial cells (Ando et al., 1988), however, this result has been attributed to a potentiation of the ATP-induced Ca^{2+} transient by shear stress (Mo et al., 1991). Simultaneous measurements of shear stress-induced membrane currents and $[Ca^{2+}]_i$ changes have been recently reported in cultured endothelial cells from human umbilical vein (Schwarz et al., 1992). In the presence of extracellular Ca^{2+} (10 mM), shear stress ($\sim$10 dyn/cm^2) evoked an inward current at a holding potential of 0 mV which was accompanied by a slow rise in $[Ca^{2+}]_i$. In the absence of extracellular Ca^{2+} no increase in $[Ca^{2+}]_i$ could be evoked and the reversal potential of the shear stress induced current was shifted by approximately -24 mV. A permeability ratio, P_{Ca}/P_{Cs} of 12.5 was calculated indicating that shear stress activated a Ca^{2+}-permeable channel in endothelial cells thereby inducing a Ca^{2+} influx and an increase in $[Ca^{2+}]_i$. Similarly, measurement of intracellular Ca^{2+} in cultured bovine aortic endothelial cells showed that upon the initiation of flow (shear stress 0.2 to 8 dyn/cm^2), $[Ca^{2+}]_i$ increased within 15-40 s to a peak and either declined to an elevated plateau that persisted for >5 min (Geiger et al., 1992) or back to baseline within 40-80 s (Shen et al., 1992). Removal of extracellular Ca^{2+}, blockade of Ca^{2+} entry with La^{3+} or depolarization with high K^+ did not eliminate this $[Ca^{2+}]_i$ response (Geiger et al., 1992; Shen et al., 1992). Taken together with the reported increase in $InsP_3$ levels in endothelial cells exposed to flow (Nollert et al., 1990) these findings lend support to the hypothesis that initial flow effects may be mediated by the release of Ca^{2+} from intracellular stores.

At low shear stresses, the flow-activated K^+ current would hyperpolarize endothelial cells, whereas at higher shear stresses, stretch-activated channels might produce depolarization. The dependence of current direction on stress magnitude, the location of a particular endothelial cell within the circulatory system and the channel type expressed could combine to modulate the release of various endothelium-derived vasoactive factors. This may be a possible mechanism by which vascular endothelium in intact vessels regulates smooth muscle tone in response to haemodynamic stimuli (Rubanyi et al., 1990).

Na^+-dependent Ca^{2+} Influx

The removal of extracellular Na^+ has been shown to have little effect on the basal and bradykinin-activated levels of intracellular Ca^{2+} in cultured endothelial cells (Schilling et al., 1988; Laskey et al., 1990). Similarly, Ca^{2+}-dependent K channel activity, an indirect estimate of $[Ca^{2+}]_i$, is unaffected by the removal of extracellular Na^+ (Sauvé et al., 1988). This suggests that Na^+/Ca^{2+} exchange does not contribute either to Ca^{2+} extrusion from the cultured endothelial cells or to the supply of Ca^{2+} during the plateau phase of agonist mediated activation. However, if the endothelial cells were Na^+-loaded using monensin and then exposed to a Na^+-free solution, a large transient increase in $[Ca^{2+}]_i$ ensued (Sage et al., 1991). Pretreatment of Na^+-loaded cells with ouabain doubled the transient $[Ca^{2+}]_i$ response. Removal of extracellular Na^+ stimulated a large transient increase in $[Ca^{2+}]_i$ in these cells due to Ca^{2+} influx because the Na^+-Ca^{2+} exchanger was operating in reverse. This Ca^{2+} influx mediated by Na^+/Ca^{2+}-exchange was diminished as

intracellular Na^+ was lost to the Na^+-free medium. Electrogenicity of the carrier was demonstrated by the observation that this Ca^{2+} influx was augmented by high external K^+ depolarization, as expected from the coupling by the exchanger of the transport of 3 Na^+ out for 1 Ca^{2+} in. Although the presence of the Na^+/Ca^{2+} exchanger could be demonstrated under these extreme experimental conditions, the lack of effects of Na^+ removal under physiological conditions leave its possible contribution to Ca^{2+} entry in doubt. A recent study in endothelial cells freshly dispersed from porcine arteries showed a >4-fold increase in $[Ca^{2+}]_i$ when the Na^+ gradient was reduced to 48% of normal, while subcultured cells showed a varied response with no $[Ca^{2+}]_i$ increase by the 7th passage (Sturek et al., 1991).

Role of K Channels in Ca^{2+} Influx

The rate of Ca^{2+} entry through plasmalemmal ion pathways is modulated by the resting membrane potential (E_m), which is regulated by membrane K^+ conductance. Membrane depolarization by elevation of extracellular K^+ or under voltage clamp reduces the agonist-stimulated Ca^{2+} influx in vascular endothelial cells (Adams et al., 1989; Laskey et al., 1990; Lückhoff and Busse, 1990b). There are at least four types of potassium channels present in endothelial cells: [1] an inwardly rectifying K channel activated upon hyperpolarization (Johns et al., 1987; Takeda et al., 1987; Silver and DeCoursey, 1990) or by shear stress (Olesen et al., 1988); [2] a transient (A-type) K channel (Takeda et al., 1987; Silver and DeCoursey, 1990); [3] an ATP sensitive K channel (Janigro et al., 1992) and [4] a Ca^{2+}-dependent K channel activated by membrane depolarization and a rise in $[Ca^{2+}]_i$ (Sauvé et al., 1988; Colden-Stanfield et al., 1990; Rusko et al., 1992a). The activation of Ca^{2+}-dependent K channels concomitant with the receptor-mediated increase in $[Ca^{2+}]_i$ is most likely to underlie agonist-induced changes in E_m of endothelial cells.

Agonist-induced changes in K^+ permeability, initially described by measuring the rate of ^{86}Rb efflux, were shown to be largely dependent on the presence of extracellular Ca^{2+} and inhibited by La^{3+} (Gordon and Martin, 1983; Schilling et al., 1988). Whole-cell outward currents evoked by agonists have been observed in cultured endothelial cells in the absence of significant intracellular Ca^{2+} buffering (Colden-Stanfield et al., 1987; Cannell and Sage, 1989; Lückhoff and Busse, 1990c; Takeda and Klepper, 1990). The agonist-induced outward K^+ current produces a transient hyperpolarization of the endothelial cell and has been observed in response to ACh in native endothelial cells from rabbit aorta (Busse et al., 1988; Sakai, 1990) and pig coronary artery (Chen and Cheung, 1992). Similarly, bradykinin, substance P, ATP and adenosine evoked a transient hyperpolarization in cultured endothelial cells from porcine coronary artery and bovine aorta (Brunet and Bény, 1989; Lückhoff and Busse, 1990b; Mehrke and Daut, 1990; Takeda and Klepper, 1990), as did histamine in cultured pig coronary artery and human umbilical vein endothelial cells (Mehrke and Daut, 1990). The peak amplitude of the agonist-induced hyperpolarizations in cultured endothelial cells from pig coronary artery is dependent on the extracellular concentrations of agonist and K^+ (Brunet and Bény, 1989; Mehrke and Daut, 1990). This transient hyperpolarization was attenuated by the removal of external Ca^{2+} and by blockers of Ca^{2+}-activated K^+ channels (Mehrke and Daut, 1990; Chen and Cheung, 1992).

The ACh-induced outward current observed in freshly dispersed rabbit aortic endothelial cells shows a similar dependence on extracellular $[K^+]$, and is attenuated in Ca^{2+}-free solution (Busse et al., 1988; Sakai, 1990; Rusko et al., 1992a). ACh, bradykinin and ATP each evoke a biphasic increase in the open probability (P_{open}) of Ca^{2+}-activated K^+ channels, while the removal of external Ca^{2+}, both in the presence and absence of agonist stimulation, failed to inhibit Ca^{2+}-activated K^+ channel activity (Sauvé et al., 1988;

Sakai, 1990; Rusko et al., 1992b). These data suggest that endothelium-dependent vasodilators produce a large initial Ca^{2+} transient due to Ca^{2+} release from intracellular stores together with Ca^{2+} influx via receptor-operated ion channels. This elevated $[Ca^{2+}]_i$ activates Ca^{2+}-dependent K channels, increasing K^+ permeability and thereby hyperpolarizing the endothelial cell. This membrane hyperpolarization provides an electrochemical gradient for maintained Ca^{2+} entry during agonist stimulation.

Ca^{2+} RELEASE FROM INTRACELLULAR STORES

The dynamic equilibrium between Ca^{2+} entry and extrusion at the plasma membrane and Ca^{2+} release and reuptake by internal stores, may be altered by agonist stimulation. Numerous studies have led to the view of two phases of Ca^{2+}-mobilization following receptor activation. Direct measurement of $[Ca^{2+}]_i$, using fluorescent Ca^{2+} indicator dyes, show that the initial, rapid increase in $[Ca^{2+}]_i$ is largely independent of the presence of extracellular Ca^{2+} and is relatively insensitive to blockers of the plasmalemmal Ca^{2+} entry pathways, while the sustained elevation in $[Ca^{2+}]_i$ has an absolute requirement for extracellular Ca^{2+} (Hallam et al., 1988; Lückhoff et al., 1988; Schilling et al., 1988). In the absence of extracellular Ca^{2+}, bradykinin evokes a transient increase in $[Ca^{2+}]_i$ and ^{45}Ca efflux from cultured bovine pulmonary artery endothelial cells (Peach et al., 1987; Johns et al., 1987; Freay et al., 1989). These results clearly indicate an agonist-induced release of Ca^{2+} from an intracellular store.

A biphasic increase in the P_{open} of the Ca^{2+}-dependent K channels was observed upon stimulation of cultured endothelial cells from bovine aorta with either bradykinin or ATP (Sauvé et al., 1988; 1990) and native endothelial cells from rabbit aorta with either bradykinin, ATP or ACh (Sakai, 1990; Rusko et al., 1992a). This finding is reminiscent of the biphasic $[Ca^{2+}]_i$ responses observed in endothelial cells following stimulation by bradykinin (Morgan-Boyd et al., 1987), ATP (Hallam and Pearson, 1986) or ACh (Danthuluri et al., 1988; see **Figure 1A**). Tetraethylammonium ions (TEA), which inhibited Ca^{2+}-activated K^+ currents, attenuated the bradykinin-induced biphasic increase in P_{open} of the channels in native endothelial cells. Thus, in addition to direct measurement of $[Ca^{2+}]_i$ using fluorescent indicator dyes, agonist-activated Ca^{2+} store release can be monitored using ionic current measurement of Ca^{2+}-dependent K channel activity.

Unitary and spontaneous transient outward currents (STOCs) observed in native endothelial cells are believed to represent the sporadic release of Ca^{2+} from intracellular stores adjacent to TEA-sensitive, Ca^{2+}-activated K channels (Rusko et al., 1992a). Involvement of receptor-mediated Ca^{2+} release from intracellular stores was also demonstrated by inhibition of the ACh-evoked outward currents by caffeine, ryanodine and heparin, agents which disrupt Ca^{2+} uptake and release from intracellular stores (Sakai, 1990). STOCs recorded in native endothelial cells were characterized by a large amplitude varying between 50 and 150 pA (at 0 mV) representing the simultaneous activation of several Ca^{2+}-dependent K channels. STOCs were initially described in vascular and visceral smooth muscle cells of rabbit (Benham et al., 1986; Benham and Bolton, 1986) and were either reduced in amplitude or abolished on exposure to Ca^{2+}-free, EGTA-containing external solutions (Benham and Bolton, 1986). In marked contrast, the absence of extracellular Ca^{2+} evoked an increase in both P_{open} and the amplitude of STOCs in endothelial cells freshly dissociated from rabbit aorta (Rusko et al., 1992b). The lack of effect of Ca^{2+}-free external solutions on unitary outward currents and STOCs suggests that Ca^{2+} influx is not necessary for maintaining the activity of unitary currents and STOCs in native endothelial cells. Therefore, the intracellular stores must be an important source of Ca^{2+} for activation of Ca^{2+}-dependent K channels.

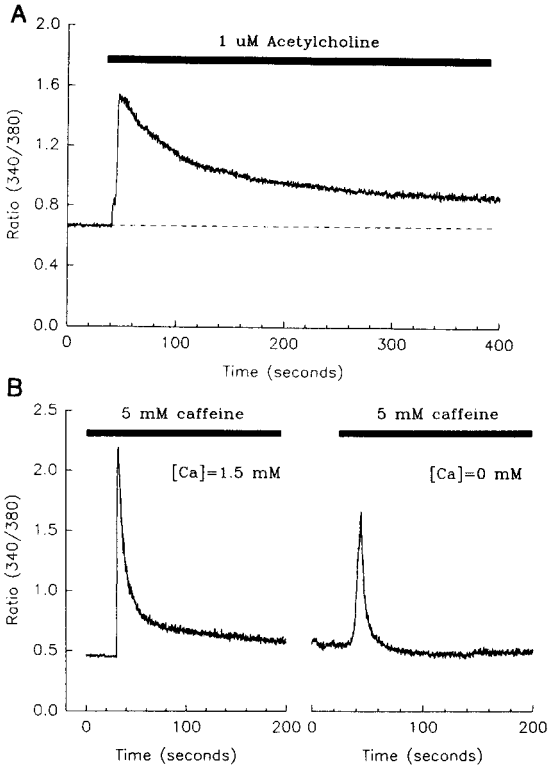

Figure 1. Acetylcholine- and caffeine-stimulated increases in $[Ca^{2+}]_i$ in endothelial cells freshly dissociated from rabbit aorta.
A. A biphasic $[Ca^{2+}]_i$ response to bath application of acetylcholine (1 μM) in physiological salt solution. Removal of extracellular Ca^{2+} abolished the $[Ca^{2+}]_o$-dependent plateau phase.
B. Caffeine-induced transient rise in $[Ca^{2+}]_i$ observed in the presence ($[Ca^{2+}]$ = 1.5 mM) and absence ($[Ca^{2+}]$ = 0 mM) of extracellular Ca^{2+}.

Ins(1,4,5)P_3-mediated Ca^{2+} release

In *myo*[^{3}H]inositol-labelled cultured human umbilical vein endothelial cells, both thrombin- and histamine-induced increases in $InsP_3$ occurred in less than 15 sec and were temporally correlated with $[Ca^{2+}]_i$ increases (Lambert et al., 1986; Pollock et al., 1988). Bradykinin also transiently stimulated $InsP_3$ production (up at 15 s, down at 90 s) (Derian and Moskowitz, 1986; Lambert et al., 1986). $InsP_3$ is released into the cytosol following the hydrolysis of phosphatidylinositol 4,5-bisphosphate in response to cell-surface receptor-mediated activation of phospholipase C. $InsP_3$, in micromolar concentrations, binds to specific membrane receptors to rapidly release Ca^{2+} from a non-mitochondrial store in a variety of cells (see Berridge and Irvine, 1989). $InsP_3$ has been shown to be the second messenger for agonist-induced Ca^{2+} release from an intracellular store by opening Ca^{2+}-permeable channels in the ER (Ferris et al., 1989; Bezprozvanny et al., 1991). This messenger was effective in releasing the ER Ca^{2+} with a high affinity (K_d = 1 μM) but had no effect on the mitochondrial Ca^{2+} stores in cultured endothelial cells (Freay et al., 1989). Flash photolysis of 'caged' $InsP_3$ in voltage-clamped aortic endothelial cells evoked a rise in $[Ca^{2+}]_i$ with a delay and time to peak which decreased with increasing concentrations of $InsP_3$ over the range 0.2-5 μM (Carter and Ogden, 1992), consistent with a direct binding and gating action of $InsP_3$ on the ER Ca channel.

The release of Ca^{2+} from intracellular stores of endothelial cells was investigated in both intact and saponin-permeabilized cultured cells (Freay et al., 1989). Mg-ATP dependent Ca^{2+} uptake by the intracellular organelles exhibited two compartments, a high Ca^{2+} affinity (half saturation at 0.8 μM) low capacity (2.5 nmoles/10^6 cells) compartment and a low affinity (half saturation at >8 μM) high capacity (>14 nmoles/10^6 cells) compartment. The low affinity Ca^{2+} uptake was inhibited by azide and could therefore be identified as mitochondrial Ca^{2+} uptake. Only 74% of the high affinity Ca^{2+} compartment was releasable by $InsP_3$ with an EC_{50} of 1 μM. The high-affinity Ca^{2+} compartment was identified as ER, allowing for the possibility that it is not homogeneous with respect to its Ca^{2+} release mechanisms. The $[Ca^{2+}]_i$ transient and efflux of ^{45}Ca were lost after a single exposure to a maximally effective bradykinin concentration in Ca^{2+}-free media and restored by addition of Ca^{2+} to the extracellular space (Morgan-Boyd et al., 1987; Freay et al., 1989). Once the ER store of Ca^{2+} has been discharged it must be refilled by extracellular Ca^{2+} although the precise Ca^{2+} entry pathway into the ER is not known. It appears that Ca^{2+} does not cycle between the ER and cytoplasm exclusively but follows a larger cycle involving the plasma membrane (see Capacitative Ca^{2+} Influx Pathway).

In addition to activating internal Ca^{2+} store release, $InsP_3$ has also been implicated in activating Ca^{2+} influx. Various mechanisms have been proposed to underlie $InsP_3$-mediated Ca^{2+} entry in non-excitable cells (see above). In most studies, direct application of $InsP_3$ to the plasma membrane failed to increase permeability to Ca^{2+} although $InsP_3$ has been reported to increase the plasmalemmal Ca^{2+} permeability in human T-lymphocytes (Kuno and Gardner, 1987). Exposure of mast cells to $InsP_3$ appeared to increase $[Ca^{2+}]_i$ by Ca^{2+} entry, but an $InsP_3$-activated Ca^{2+} channel could not be identified (Penner et al., 1988). Irvine and collaborators have suggested that the phosphorylated product of $InsP_3$ metabolism, $InsP_4$, plays a significant role in the regulation of Ca^{2+} entry (Irvine et al., 1988; Irvine, 1992). It was observed that alone neither $InsP_3$ nor $InsP_4$ in the patch pipette could evoke mobilization of Ca^{2+} in lacrimal cells. However, when added together a biphasic response was observed, which apparently resulted from Ca^{2+} release as well as entry (Morris et al., 1987; Changya et al., 1989).

The internal release of Ca^{2+} mediated by $InsP_3$ is from a discrete intracellular pool that probably does not include all of the non-mitochondrial sequestered Ca^{2+}. There is evidence that the size of this pool can be regulated in cells by a GTP-dependent mechanism (Mullaney et al., 1988; Ghosh et al., 1989). The organelle from which $InsP_3$ releases Ca^{2+} is probably a component of the ER and may also be an $InsP_3$-sensitive "calciosome" (Volpe et al., 1988). However, the heterogeneity of $InsP_3$ receptor distribution within cells (Ross et al., 1989) is inconsistent with a single specialized $InsP_3$-responsive organelle (the "calciosome") but rather suggests the occurrence of complex and dynamic interactions between $InsP_3$-sensitive and -insensitive organelles mediated by intracellular G proteins. Two non-mitochondrial Ca^{2+} compartments have been proposed to exist in rat pancreatic acinar cells (Streb et al., 1984). Similarly, evidence suggests that there are at least two functionally distinct non-mitochondrial Ca^{2+} compartments present in native endothelial cells: [1] an $InsP_3$-sensitive Ca^{2+} store, and [2] an $InsP_3$-insensitive Ca^{2+} store which may be coupled to the $InsP_3$-sensitive pool by a GTP-dependent pathway (see Freay et al., 1989).

Caffeine-induced Ca^{2+} Release

Caffeine translocates Ca^{2+} from intracellular storage sites, such as the sarcoplasmic reticulum in excitable cells, into the cytosol by an enhanced Ca^{2+}-induced Ca^{2+} release mechanism. There is considerable evidence to show that an intracellular Ca^{2+} store in muscle can be released by caffeine (Endo, 1985). In skinned smooth muscle cells of the

guinea-pig taenia caeci, it has been suggested that $InsP_3$-sensitive Ca^{2+} stores do not completely overlap with caffeine-sensitive stores (Iino et al., 1988). In contrast, pretreatment of cells with caffeine abolished the carbachol-induced Ca^{2+}-dependent K^+ currents in smooth muscle cells of rabbit small intestine, suggesting that caffeine had released the carbachol-sensitive Ca^{2+} stores (Bolton and Lim, 1989). Alternatively, caffeine inhibition of $InsP_3$-mediated responses in *Xenopus* oocytes has been attributed to caffeine antagonism of the binding of $InsP_3$ to its intracellular receptor (Parker and Ivorra, 1991).

In cultured bovine aortic endothelial cells, caffeine (5 mM) caused a small increase in $[Ca^{2+}]_i$ which was abolished following incubation in Ca^{2+}-free medium (Buchan and Martin, 1991). Furthermore, a recent study of the effects of caffeine on bradykinin-induced fluctuations of $[Ca^{2+}]_i$ by monitoring the activity of Ca^{2+}-dependent K channels in bovine aortic endothelial cells of confluent monolayers (Thuringer and Sauvé, 1992), provides evidence for the co-existence of $InsP_3$-sensitive and caffeine-sensitive Ca^{2+} stores. Caffeine-induced $[Ca^{2+}]_i$ transients which are abolished in the presence of ryanodine (10 μM) demonstrate the presence of functional ryanodine-sensitive Ca^{2+} release channels in the ER of freshly isolated endothelial cells (see **Figure 1B**). Caffeine (5-20 mM) also stimulated a dose-dependent increase in unitary current activity superimposed on a large, prolonged transient outward current. In the continued presence of caffeine, unitary and spontaneous outward currents were observed to occur at both a higher frequency and larger amplitude (see **Figure 2A**). External TEA inhibited unitary currents and STOCs but failed to completely inhibit the large, long-lasting transient outward current evoked by caffeine (Rusko et al., 1992b) suggesting the presence a TEA-insensitive component of the caffeine-induced outward current in native endothelial cells.

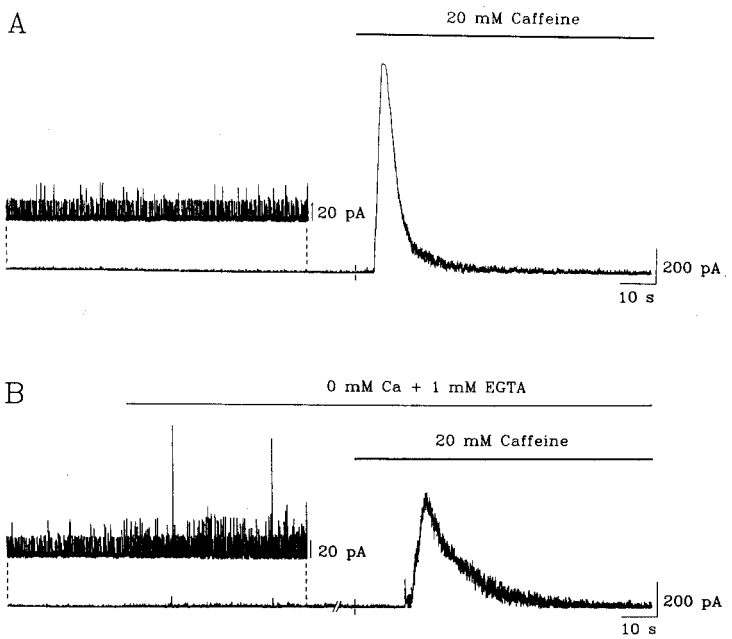

Figure 2. Caffeine-evoked outward currents in native endothelial cells obtained in the absence and presence of extracellular Ca^{2+}.
Outward currents recorded in normal (1.5 mM Ca^{2+}) PSS (A) and in Ca^{2+}-free (1 mM EGTA) external solution (B) before and during exposure to 20 mM caffeine. Holding potential, +20 mV. Horizontal bars indicate duration of exposure to caffeine and Ca^{2+}-free external solution.

In a Ca^{2+}-free, EGTA-containing solution, caffeine evoked a long-lasting transient outward current of smaller amplitude than that obtained in the presence of external Ca^{2+} (Rusko et al., 1992b; see **Figure 2B**). The ability of caffeine to evoke transient outward currents in the absence of external Ca^{2+} suggests that the intracellular Ca^{2+} store is an important source of Ca^{2+} for activation of K channels in native endothelial cells. The reduced amplitude of these currents, however, suggest that influx of extracellular Ca^{2+} is necessary for full development of transient currents evoked by agonists. Heparin, a relatively specific and potent competitive antagonist of the $InsP_3$ receptor, had no effect on caffeine-induced transient outward currents or STOCs in native endothelial cells suggesting the presence of another intracellular Ca^{2+} store (J. Rusko and D.J. Adams, unpublished observations).

CONCLUSIONS

The picture which has emerged from studies of Ca^{2+} entry pathways and Ca^{2+} release from intracellular stores in vascular endothelial cells is summarized in **Figure 3**. The intracellular ER Ca^{2+} stores play an important role in Ca^{2+} signalling and in creating intracellular Ca^{2+} gradients. Investigation of the proposed compartmentalization of the ER and the different types of Ca^{2+} release channels present in endothelial cells are required. The interpretation of studies to date on the relationship between the state of filling of the intracellular Ca^{2+} stores and Ca^{2+} influx, however, are limited by the specificity of agents (e.g., caffeine, thapsigargin, BHQ) used to perturb Ca^{2+} homeostasis in endothelial cells. The transfection of endothelial cells with the complimentary DNA for the Ca^{2+}-sensitive photoprotein, aequorin, fused in a frame encoding a non-mitochondrial organelle (ER) presequence as recently applied to monitor mitochondrial $[Ca^{2+}]$ (see Rizzuto et al., 1992), should provide insight into the the temporal response of changes in ER $[Ca^{2+}]$ upon receptor activation in endothelial cells. Similarly, improved temporal and spatial resolution of $[Ca^{2+}]_i$ in cells using fluorescent probes and digital imaging techniques (see Fay et al., 1989) may provide evidence for Ca^{2+} gradients in the endothelial cell as recently proposed for smooth muscle cells (van Breemen and Saida, 1989).

While considerable attention has focussed on Ca^{2+} entry pathways, little is known about the mechanisms of Ca^{2+} extrusion from endothelial cells. The removal of extracellular Na^+ has been reported to have little influence on $[Ca^{2+}]_i$ in cultured endothelial cells, indicating that Ca^{2+} extrusion is mediated primarily by the plasmalemmal Ca^{2+}-ATPase and not the Na^+-Ca^{2+} exchanger. However, further experiments are required to quantitatively describe the relative contributions of the Na^+-Ca^{2+} exchanger and Ca^{2+}-ATPase to Ca^{2+} extrusion during rest and excitation.

Much of our understanding of the ionic basis of cytoplasmic Ca^{2+} regulation in vascular endothelium has been obtained from studies on cultured endothelial cells. However, recent studies on freshly dissociated and intact endothelial cells have revealed striking differences between cultured and native cells with respect to the expression of the muscarinic ACh receptor, the inwardly rectifying K channel, and intracellular Ca^{2+} release mechanisms such as Ca^{2+}-induced Ca^{2+} release channels. These recently uncovered differences in the expression of cell surface receptors and ionic transport mechanisms imply that studies on cultured endothelial cells alone cannot provide a satisfactory understanding of Ca^{2+} signalling in the endothelium *in vivo*. Furthermore, many of the studies of endothelial cell physiology are based on cells derived from large conduit vessels, however, evidence suggesting specific properties of microvascular endothelial cells (e.g., Bossu et al., 1992) indicates a need for further studies to convincingly establish differences between endothelia derived from conductance and resistance arteries, veins and capillaries.

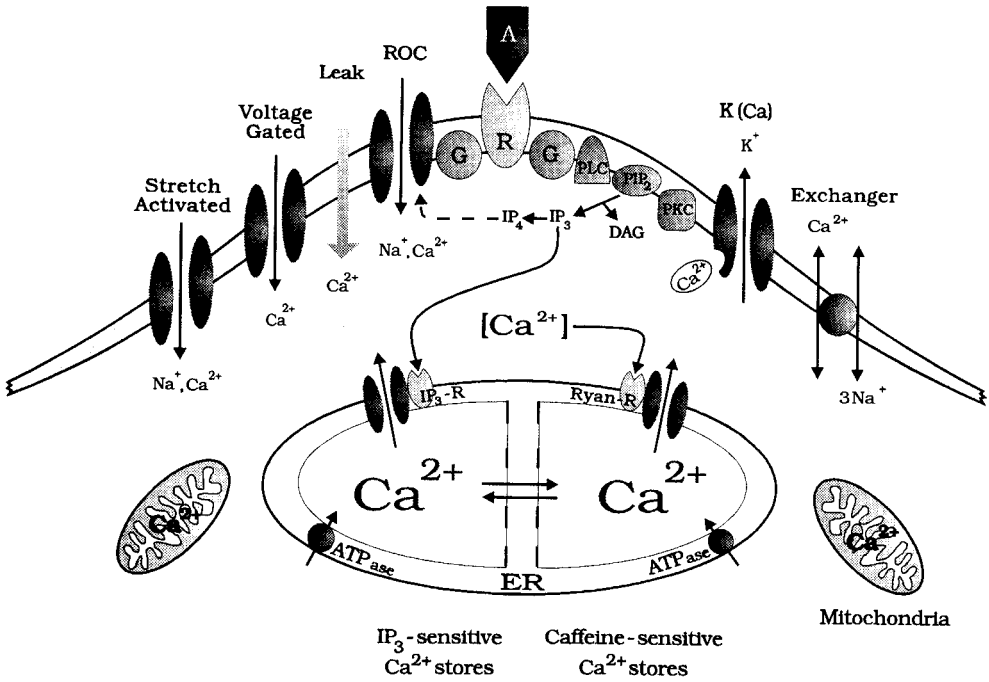

Figure 3. Schematic illustration of ionic transport pathways and intracellular Ca^{2+} stores mediating Ca^{2+} entry and release in vascular endothelial cells. Calcium entry may occur through a Ca^{2+} leak pathway, stretch-activated channels, and receptor-operated channels (ROC) according to the electrochemical gradient. The driving force for Ca^{2+} influx during activation is maintained primarily by the hyperpolarizing action of Ca^{2+}-activated K channels (K(Ca)). There is evidence to suggest Ca^{2+} influx may occur via the Na^+-Ca^{2+} exchanger operating in reverse mode and via voltage-gated Ca channels in capillary endothelium. Agonist (A) binding to a cell-surface receptor (R), which is coupled by a G-protein (G) to a specific phospholipase C (PLC), leads to the hydrolysis of phosphatidylinositol-4,5-bisphosphate(PIP_2) yielding diacylglycerol and inositol 1,4,5-trisphosphate ($InsP_3$). $InsP_3$ serves as a mediator of Ca^{2+} release from a compartment of the endoplasmic reticulum (ER). The caffeine-induced release of Ca^{2+} from the ER via activation of the ryanodine receptor (RyanR) suggests the presence of a Ca^{2+}-induced Ca^{2+} release mechanism in vascular endothelial cells. Elevated intracellular Ca^{2+} may be sequestered into the mitochondria (a low affinity, high capacity organelle) or into the ER compartments via an ATP-Ca^{2+} pump.

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