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Ca^{2+} Signalling in Endothelial Cells: Role of Ion Channels

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Ca^{2+}-signals in endothelial cells are determined by release from intracellular stores and entry through the plasma membrane. In this review, the nature of Ca^{2+} entry and mechanisms of its control are reviewed. The following ion channels play a pivotal role in regulation of the driving force for Ca^{2+} entry: an inwardly rectifying K^+ channel, identified as Kir2.1, a big-conductance, Ca^{2+}-activated K^+ channel (hslo) and at least two Cl^- channels (a volume regulated Cl^- channel, VRAC, and a Ca^{2+} activated Cl^- channel, CaCC). At least two different types of Ca^{2+}-entry channels exist: 1. A typical CRAC-like, highly selective Ca^{2+} channel is described. Current density for this Ca^{2+} entry is approximately 0.1pA/pF at 0 mV and thus 10 times smaller than in Jurkat or mast cells. 2. Another entry pathway for Ca^{2+} entry is a more non-selective channel, which might be regulated by intracellular Ca^{2+}. Although detected in endothelial cells, the functional role of trp1,3,4 as possible channel proteins is unclear. Expression of trp3 in macrovascular endothelial cells from bovine pulmonary artery induced non-selective cation channels which are probably not store operated or failed to induce any current. Several features as well as a characterisation of Ca^{2+}-oscillations in endothelial cells is also presented.

Key Words: Endothelium-Ca^{2+}-entry channels-potassium channels-Ca^{2+}-oscillations

INTRODUCTION

Endothelial cells (ECs) form an ideal surface for blood flow. They prevent blood clotting but can also trigger ir in response to various signals, and can exert thrombolytic as well as thrombogenic activity. As antigen-presenting cells, they are also involved in immune responses. By changing the contractile state and cell-cell contacts, they control the permeability of the blood-tissue interface. ECs initiate angiogenesis and vessel repair. They help adjust the vessel diameter to the hemodynamic needs. These multiple functions are mediated by the production and release of a variety of vasoactive agents, which affect the cells in the vessel wall or its immediate vicinity, including the endothelial cells themselves.

These substances include nitric oxide (NO or endothelium-derived relaxing factor, EDRF), endothelium-derived hyperpolarizing factor (EDHF), various prostaglandins, endothelins (ET), natriuretic peptide, small signaling molecules such as substance P, ATP, growth factors, steroids, and even larger proteins, such as receptors and proteins involved in the blood clotting cascade (for a review, see Inagami et al, 1995; Nilius & Casteels, 1996; Nilius et al, 1997h). ECs respond not only to humoral substances, which bind to receptors, but also to mechanical forces, due to changes in flow rate (shear stress) or blood pressure (biaxial tensile stress) (Davies, 1995; Davies & Tripathi, 1993; Malek & Izumo, 1994). Ca^{2+} plays an important role in initiation of production and release of most of these agents. For these processes, ion channels are involved as follows; 1. They provide influx routes for Ca^{2+} (Nilius, 1991); 2. They control the membrane potential which in turn regulates the inwardly driving force for this Ca^{2+}-influx (Nilius et al, 1997h). This paper will refer to channels responsible for Ca^{2+} entry, for the control of the driving force for Ca^{2+} and will present some data on Ca^{2+}

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oscillations in endothelial cells.

METHODS

All work has been done on cultured bovine pulmonalis artery CPAE and an immortalised HUVEC (human umbilical vein) derived cells line, EA 926. We are using a combined method for patch clamping the cells and monitoring of the free intracellular Ca^{2+}-concentration, $[Ca^{2+}]_i$, with a Fura-II method. In addition, we track capacitance changes of the endothelial cells during cell stimulation. All the methods as well as solution, manoeuvres to clamp $[Ca^{2+}]_i$ when necessary and the apply shear stress or stretch has been described in detail elsewhere (Heinke et al, 1997; Nilius et al, 1994; Nilius et al, 1993b; Oike et al, 1994b; Schwarz et al, 1992).

RESULTS

Mechanically activated Ca^{2+}-entry

One of the fascinating properties of endothelial cells (ECs) is their function as mechano-sensors. They are constantly exposed to changes of mechanical forces within a blood vessel and can respond to such forces. Responses to mechanical stimuli develop at different time scales. The best studied fast response to mechanical stimulation is a release of nitric oxide, NO, which is preceded by a Ca^{2+} transient. Other fast responses comprise channel activation, synthesis of second messengers, activation of G-proteins. Intermediate responses are gene expression which are initiated by sensing of shear forces at shear stress response elements-SSRE in the promoter region of the PDGF gene, the tPA gene and others. Slow responses concern vessel remodelling and cytoskletal changes (for a reviews see Davies, 1995; Davies & Barbee, 1994; Davies & Tripathi, 1993; Malek & Izumo, 1994).

Fig. 1 shows a typical group of results. If an endothelial cell is mechanically stimulated in a Ca^{2+} free solution, it responds with activation of a Ca^{2+} transient (Fig. 1A). Because of the absence of extracellular Ca^{2+}, it must be related to Ca^{2+} release. This release can be activated by all kinds of mechano-stimulation: shear stress, cell swelling, and cell stretch (Oike et al, 1994b). If after mechano-stimulation an agonist is applied, no further Ca^{2+} signal can be activated, pre-treatment of the cells with thapsigargin also attenuates any further release of Ca^{2+}. This indicates that a) the mechanical stimulus releases Ca^{2+} from IP_3 sensitive stores and b) this stimulation supposedly depletes these stores. This store depletion probably activates Ca^{2+}-entry cannels (store operated Ca^{2+}-current, SOC, or Ca^{2+}-release activated-calcium current, CRAC). We have described that this release of Ca^{2+} might be mediated via production of arachidonic acid (AA) because a) PLC not involved, b) heparin does not block this release, c) PLA2 blockers inhibit this release, d) AA application mimics the effects of mechano-stimulation even in the presence of heparin and PLA_2 blockers. The PKA inhibitory peptide is inefficient, as well as cyclooxygenase and lipoxygenase inhibition (for de-

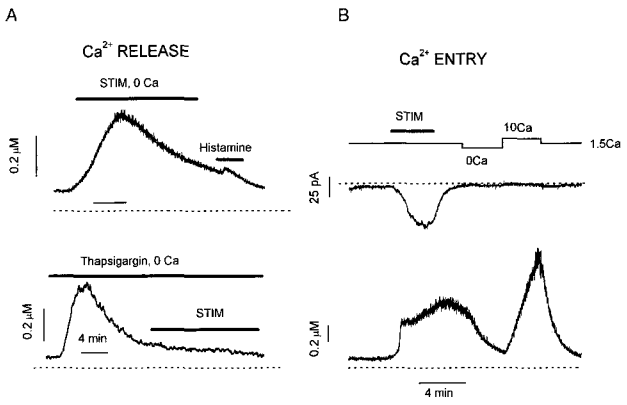

Fig. 1. Events in endothelial cells following mechano-stimulation. (A): Exposure of endothelial cells to hypotonic Ca^{2+}-free bath solutions activates an inward current (upper trace) and induces a transient increase in $[Ca^{2+}]_i$ (lower trace). The holding potential was −40 mV. Additional application of 100 µM histamine did not induce a further release of Ca^{2+} indicating that the stores are emptied (top). HTS, applied after depletion of intracellular Ca^{2+}-stores with 2 µM thapsigargin, does not evoke a Ca^{2+}-transient (below). The same Ca^{2+}-signals can be evoked by direct stretch or shear stress. Cell swelling by HTS is induced by changing from a 290 mOsm/1 to a 185 mOsm/1 Kre's solution (Oike et al, 1994b). (B): Swelling of endothelial cells by HTS increases $[Ca^{2+}]_i$ and activates a Cl^- current (VRAC). The recovery of $[Ca^{2+}]_i$ in isotonic solution is accelerated in Ca^{2+}-free solution, but $[Ca^{2+}]_i$ increases again after resubmission of extracellular Ca^{2+}. Apparently, a Ca^{2+}-entry pathway is activated by the exposure to HTS, but it is not accompanied by a significant change in transmembrane current (Oike et al, 1994b).

tails see Oike et al, 1994b).

Mechanical stimulation of EC probably depletes the Ins(3,4,5)P_3 sensitive Ca^{2+} stores. As a consequence of this depletion, Ca^{2+}-entry is activated. Currents which are activated by this mechanism will be described in detail later. Experimental evidence for activation of Ca^{2+}-entry due to mechano-stimulation is depicted in Fig. 1B. Mechano-stimulation induces a Ca^{2+}-transient as already shown in Fig. 1A but also activates a current which is independent on the changes in $[Ca^{2+}]_i$. This current is identical with the volume-regulated, Ca^{2+}-independent anion (Cl^-) current (VRAC) and has been described in detail elsewhere (Nilius et al, 1997a; Nilius et al, 1996; Nilius et al, 1997g). After switching to a Ca^{2+} free solution and reapplication of Ca^{2+} (10 mM) a large Ca^{2+}-signal is appeared which reflect Ca^{2+}-entry.

Without the mechanical stimulation, stepwise elevation of extracellular Ca^{2+} does not induce a Ca^{2+} transient (Oike et al, 1994b). No current can be detected during reapplication of Ca^{2+}.

Under the chosen buffering conditions for $[Ca^{2+}]_i$ (0.1 mM EGTA in the pipette solution), currents which are activated by store depletion, are in the range of 0.05 pA/pF at −40 mV and thus not detectable (Gericke et al, 1994; Oike et al, 1994c).

Ca^{2+} pattern after agonist stimulation

Fig. 2 shows an example of a typical Ca^{2+} pattern after stimulation of an EA endothelial cell with two different concentrations of UTP which binds to the P_{2Y2} (P_{2U}) receptors present in these cells (Viana et al, 1997) and another agonist, histamine. At low concentrations typical oscillations can be observed which are accompanied by in-phase oscillations of the membrane potential. At higher concentrations, a typical plateau-like response in $[Ca^{2+}]_i$ can be observed together with a hyperpolarization. Obviously, Ca^{2+}-oscillations appear in a small window of low agonist concentrations, whereas at higher agonist concentrations typically plateau responses are evoked (Jacob et al, 1988; Oike et al, 1994a). Application of vasoactive agonists also induced Ca^{2+}-waves which travel with a concentration dependent speed of between 5 μm/sec and ATP 40 μm/sec through the cells (Missiaen et al, 1996). Typically, Ca^{2+}-response in endothelial cells to different agonists consists in a fast initial peak which is mainly due to intracellular release of Ca^{2+} and a somewhat delayed plateau like

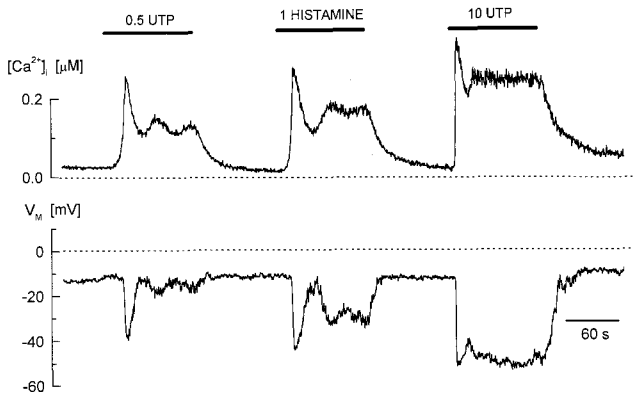

Fig. 2. Calcium response to agonist stimulation, Example of the complex pattern of $[Ca^{2+}]_i$ and membrane potential response in a, endothelial cell (EAhy 926, an umbilical vein derived cell line) to different agonists concentrations (0.5 μM and B: 20 μM UTP) and to 100 μM histamine. These cells react to low concentrations with an oscillatory Ca^{2+} and membrane potential response. At high concentrations the response is plateau-like, and a mirrored hyperpolarisation of the membrane is visible during the elevated Ca^{2+} phase (whole-cell current-clamp recording, the cell was pre-incubated in 2 μM fura-2 AM, the K-Aspartate internal solution contained 50 μM fura-2) (Nilius et al, 1997g).

phase which depends on Ca^{2+} entry (Jacob 1990; Nilius et al, 1997h). The release phase is not voltage-dependent. However, the plateau phase strongly depends on the driving force for Ca^{2+}.

The Role of Ion Channels in the Control of the Driving Force for Ca^{2+}-entry

Membrane potential in resting endothelial cells

Ion channels play the crucial role in ECs for the regulation of Ca^{2+}-signalling: they provide entry pathways for Ca^{2+} and they thune electro-chemical driving forces for Ca^{2+}. In CPAE cells, the resting potential is controlled under non-stimulated conditions by at least three ion channels: 1. an inwardly rectifying K^+channel, 2. An outwardly rectifying Cl^- channel, which is also activated by cell swelling (volume-regulated anion channel, VRAC) and 3. a non-selective cation channel (Voets et al, 1996). If the Cl^- channels are blocked, K^+ channels are now the dominating channels in the resting cells and the membrane potentials is strongly hyperpolarized. Such an example is shown in Fig. 3A. Mibefradil, which has been described as an efficient novel Ca^{2+} an-

tagonist, also inhibits endothelial Cl^- channels thereby inducing strong hyperpolarisation (Nilius et al, 1997c). This in turn might induce beneficial effects on agonist or shear stress induced Ca^{2+} influx. Presence of the highly non-linear inwardly rectifying K^+ channel togehter with a Cl^- channel is a sufficient condition for appearance of a bistable membrane potential, e.g. a fast switch between potentials close to the Cl^--(E_{Cl}) or-vice versa-close to the K^+ equilibrium potential (E_K) (Fig. 3B). Interestingly, sometimes dramatic hyperpolarisation can not only be induced by activation of a Ca^{2+}-dependent K^+ channel, as discussed later (see also Fig. 1), but also very efficiently by only modest inhibition of Cl^- channels. Likely, block of Cl^- channels seems to be a still underestimated tool to modulate the driving force for Ca^{2+}- entry in endothelial cells and thus Ca^{2+}-signalling.

The endothelial inwardly rectifying K^+channel, IRK

One of the most important channels for the control of the resting potential in non-stimulated cells is the

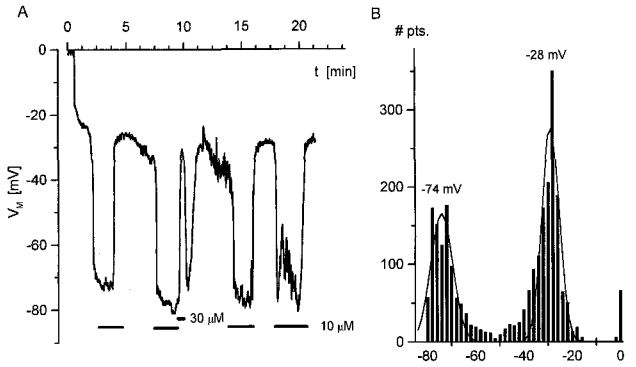

Fig. 3. Effects of a chloride channel blocker (mibefradil) on the membrane potential of CPAE cells. (A): Block of a Cl^- channel (VRAC) induced fast hyperpolarisation. Membrane potential was measured in current clamp mode after breaking into the cell. Mibefradil (10 µM) induced a fast and reversible hyperpolarisation of the cells. Shown is a typical cell with a large Cl^- conductance and a resting potential fluctuating between −22 and −27 mV. (B): Distribution of the membrane potentials sampled at 2 Hz from the cell shown in panel A. The two peaks represent the membrane potential in the absence (control) and the presence of mibefradil. The mean values were taken from the Gaussian fits. Data obtained from the fits are: −28 mV for control, −74 mV for 10 µM mibefradil (bin width 2 mV). Details, see (Nilius et al, 1997b).

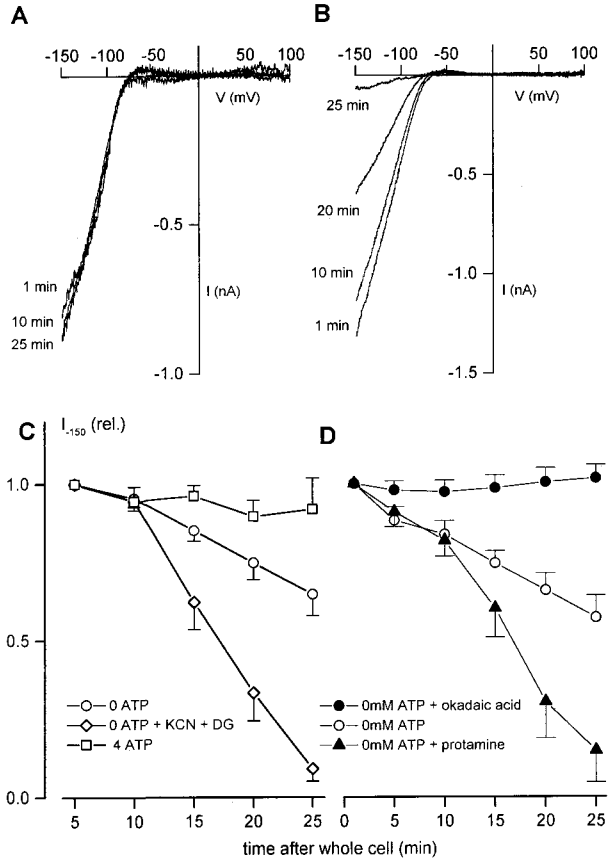

Fig. 4. Modulation of the inwardly rectifying K^+ channel, Kir2.1. (A): Representative current-voltage relationships for IRK measured from voltage ramps in the presence. Currents were obtained from differences in the absence and presence of 1 mM Ba^{2+}, which completely block Kir2.1. ATP is present in the patch pipette (4 mM). IV curves where obtained from voltage-ramps (−150 to +100 mV). (B): The same protocol as used in A but now in the absence of ATP in the patch pipette. The times are indicated which elapsed from getting whole cell access to the depicted current-voltage trace. Additionally, 2 mM KCN and 5 mM 2-DG were applied to the external solution immediately after the establishment of whole-cell configuration. Note the complete run-down of the current in approximately 25 minutes. (C): Run down of the activity of Kir2.1 is shown in the absence of ATP and in the absence of ATP but during application of 1 mM KCN and 5 mM 2-D-glucose (2DG). In the presence of ATP no run-down is observed. (D): Run-down of Kir2.1 can be accelerated by protamine (10 µg ml^{-1}), an activator of protein phosphatase PP2A. Okadaic acid (1 µM) completely prevented the run-down. Likely, inhibition of Kir2.1 is due to activation of PP2A. For details see also (Kamouchi et al, 1997b).

K^+-inward rectifier, IRK. We have identified the molecular identity of the channel. It is a member of the Kir-family, Kir2.1(Kamouchi et al, 1997b). The full length clone is also available (Forsyth et al, 1997). This channel is a strongly inwardly rectifying channel with a sigle channel conductance 30 pS. The two transmembrane regions are highly conserved. The channel has 427 AA, two membrane spanning regions, a highly conserved TIGYG-H5 motif in the pore region. The M84 site confers pH insensitivity which is also a characteristics of the endothelial channel (Kamouchi et al, 1997b). Mg^{2+}- and spermine, spermidine, putrescine block appears to be connected to D172. In the C-terminus a phosphorylation motive, PRESEI, is present in which S425 seems to be important for channels regulation. We have tested the possible modulation of Kir2.1 by phosphorylation. In the presence of intracellular ATP, channels activity is very stable (Fig. 4A). The channel can be dramatically inhibited by the absence of intracellular ATP and a run-down of channel activity will be accelerated under hypoxic condition (Fig. 4B, C). This is probably due to a PP2A dependent regulation because channel inhibition can be accelerated by PP2A activators (protamine) and can be inhibited by a PP2A inhibitor (okadaic acid, Fig. 4D).

Ca^{2+}-dependent channels, CaCC and BK_{Ca}

Under condition of cell stimulation which induce an increase in $[Ca^{2+}]_i$, mainly two types of responses can be observed. First, CPAE cells respond with only small changes in the resting potential during an increase in $[Ca^{2+}]_i$. These cells only activate a Ca^{2+}-dependent Cl^- channel, CaCC(Nilius et al, 1997d; Nilius et al, 1997e; Nilius et al, 1997f). Activation of CaCC induces a shift towards a mix-potential of E_{Cl} and the reversal potential of co-ctivated (probable small conductance) non-selective- or Ca^{2+}-selective cation channels (see below).

Another channel that is important for the regulation of the driving force for Ca^{2+}-influx is a big conductance, Ca^{2+}-activated K^+ channel (BK_{Ca}). Activation of these channels during an increase in $[Ca^{2+}]_i$ shifts the membrane potential towards the equilibrium potential for potassium, E_K. (see also Fig. 2, 3). We have characterised this channel in human EA cells.

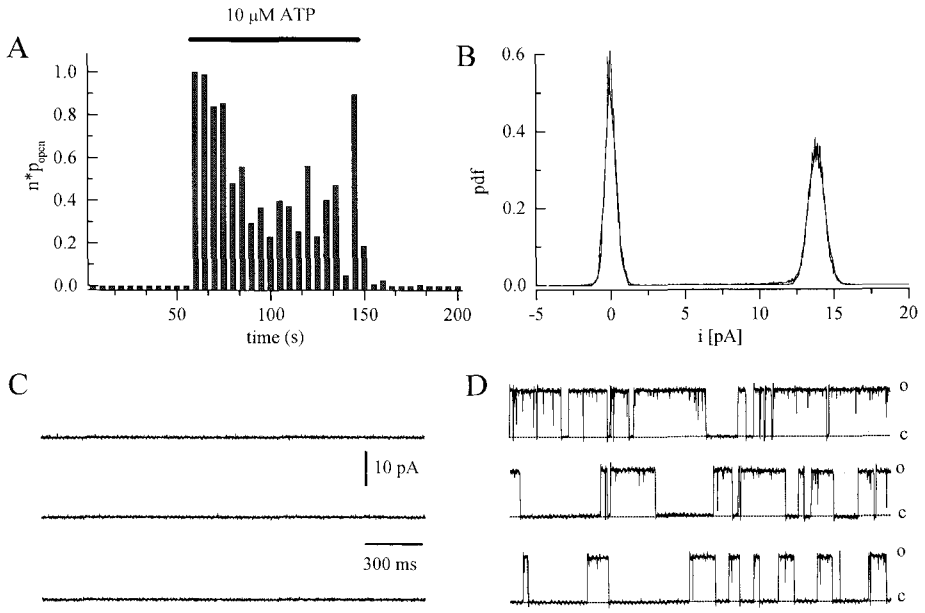

Fig. 5. Single channel currents through a Ca^{2+}-activated K^+ channel evoked by ATP in EA cells. (A): Change in open probability of the BKCa-channel after application of 10 μM ATP (cell-attached configuration at a holding potential of +40 mV, 140 mM KC1 in the patch pipette). (B): Amplitude histogram. Single channel amplitude is 13.9 pA at +60 mV indicating a channel conductance of 231 pS. (C): No channel activation is seen before ATP application. (D): Single channels traces obtained during superfusion of the cell with ATP (10 μM).

It is an approximately 230 pS channel when measured under conditions of symmetrical K^+ concentrations. Fig. 5 shows a measurement from a cell-attached patch. application of the agonist induces a fast increase in the probability of the channel being open (Fig. 5A).

Amplitude of the single channel current is 13.9 pA at a holding potential of +60 mV(Fig. 5 B, D). No channel activity is observed before application of ATP (Fig. 5C). The open probability increases with more positive potentials. The apparent Ca^{2+}-affinity of the channel is increased at positive potentials, and decreases at negative potentials. These properties already define an oscillator. Pharmacologically, the endothelial BK_{Ca} is blocked by charybdotoxin (IC_{50} approximately 50 nM), by iberiotoxin, TEA (IC_{50} approximately 1 mM, 100% block at 10 mM), and quinine. Mg^{2+} blocks vltage-dependently from outside.

Surprisingly, the endothelial BK_{Ca} is in contrast to the smooth muscle BK_{Ca} (Bolotina et al, 1994) insensitive against NO (Haburcak et al, 1997). From RT-PCR analysis, we have identified the channels as hslo. The structure of this channel is not completely known. At the N-terminus, six or even seven transmembrane helices have been suggested. A segment 0 in the N-terminal is probably a couplings site for β -subunit. The unique C-terminus contains possibly four additional helices, H7-H10. The channel has approximately 1200 amino acids (Dworetzky et al, 1994; Tseng Crank et al, 1994).

Interestinly, *hslo* is not expressed in CPAE cells. These cells respond completely different to cell activation than EA cells shown in Fig. 1. In these cells no hyperpolarization is observed during agonist. Cells with a rather negative membrane potential are depolarised (Fig. 6A). This depolarization approaches the equilibrium potential for Cl^-, E_{Cl}, and/or-due to activation of a non-selective ion channel-an even even more positive value (Fig. 6B). Ca^{2+} release is followed by a very low plateau (Fig. 6A and B). These cells mainly control their resting potential during agonist stimulation by activation of a Cl^-- and a non-selective cation channel. To directly evaluate the effects of the functional presence of BKCa channels, we have transfected the CPAE cells with hslo. In non-transfected control CPAE cells, ATP activates CaCC. The most prominent current component during ATP stimulation in *hslo* espressing cells is BKCa. An increase in $[Ca^{2+}]_i$ resultes in a pronounced transient hyperpolarization, which is

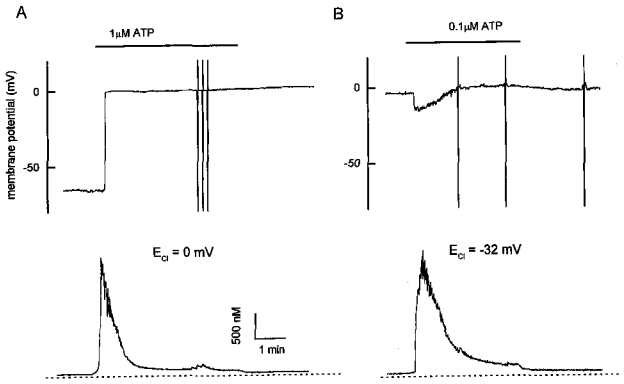

Fig. 6. Calcium and membrane potential responses of bovine pulmonary artery cells (CPAE) after stimulation with an agonist. (A): Simultaneous measurement of the membrane potential and $[Ca^{2+}]_I$ in a CPAE cells during agonist stimulation (unclamped cell, loaded with Fura-2/AM, 0.1 mM EGTA in the patch pipette). The Cl^- equilibrium potential is adjusted at 0 mV. During stimulation the cell is depolarised. Note the very low plateau and the low plateau of the Ca^{2+}-signal. (B): Examle of a CPAE cell, which is already depolarised. Since E_{Cl} is adjustment at more negative values, stimulation with 0.1 μM ATP first induces a small hyperpolarisation towards E_{Cl} followed by a depolarisation. Note the completely different pattern in comparison to Fig. 2.

absent in non-transfected cells. This hyperpolarization is enhanced if CaCC is blocked by niflumic acid. The sustained component of the Ca^{2+} response during ATP stimulation is significantly larger in *hslo* transfected cells than in non-transfected cells. This plateau level correlates well with the corresponding effects of ATP on the membrne potential, indicating that the expression of cloned BK_{Ca} exerts a positive feedback on Ca^{2+} signals in endothelial cells by counteracting the negative (depolarizing) effect of a stimulation of Ca^{2+}-activated Cl^- channels (Fig. 7A, B).

Expression of *hslo* in the plasma membrane of CPAE cells was performed by using a novel transient expression method with a novel bicistronic vector pCINeo/IRES-GFP containing *hslo* cDNA. This vector utilises a red-shifted variant of Green Fluorescent Protein as an *in vivo* cell marker. Incorporation of an ion channel cDNA in this bicistronic unit allows coupled expression of the ion channel and GFP. Bicistronic means, transcription results in two open reading frames. The second open reading frame for hslo is preceded by an Internal Ribosomal Entry Site

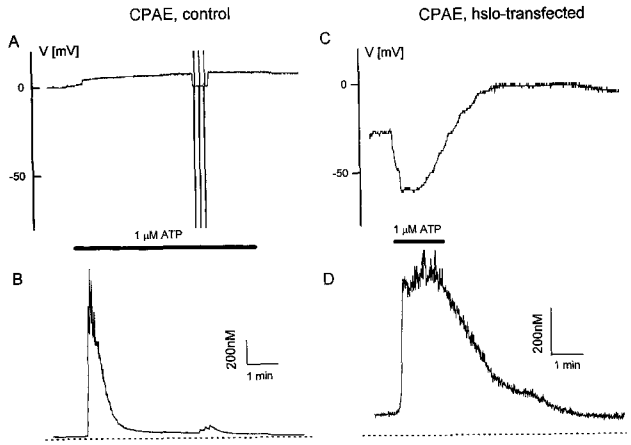

Fig. 7. Calcium and membrane potential responses of bovine pulmonary artery cells after expression of the BK_{Ca}, Ca^{2+}-activated K^+ channel, hslo. (A): Measurement of the membrane potential and $[Ca^{2+}]_i$ in a CPAE cells during agonist stimulation (unclamped cell, loaded with Fura AM, 0.1 mM EGTA in the patch pipette, 1 μM ATP, Cl^- equilibrium potential 0 mV). Note the very low plateau and the low plateau of the Ca^{2+}-signal and the depolarisation toward positive potentials. (B): Example of a CPAE cell which is transfected with hslo (identified by co-expression with GFP, see Fig. 8). Note the increased Ca^{2+} plateau and the large transient hyperpolarisation beyond the Cl^- equilibrium potential (E_{Cl}=0 mV). More details in (Kamouchi et al, 1997a).

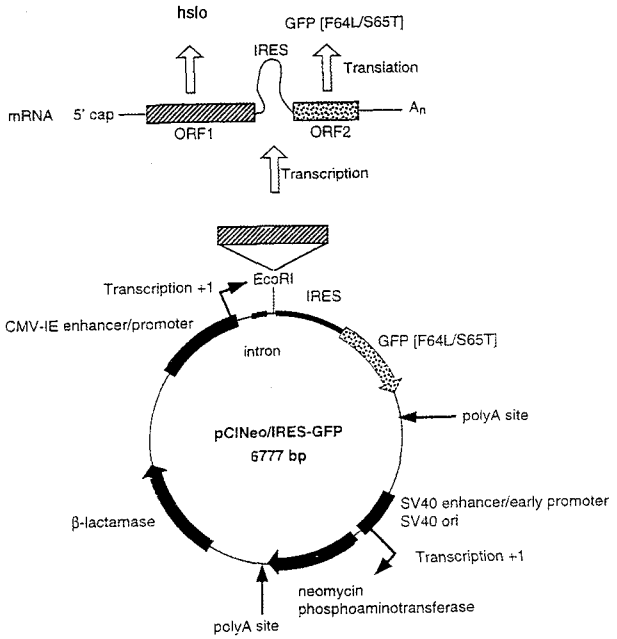

Fig. 8. Vector used for identification of endothelial cells which coexpress hslo and green flourescence protein, GFP. A pCINeo/IRES-GFP vector contains the Cytomegalovirus Immediate-Early Promoter/enhancer followed by an artifical intron, an EcoRI site, an Internal Ribosomal Entry Site (IRES) from the Encephalomyocarditis virus (EMCV), a GFP cDNA containing the Phe64Leu/Ser65 Thr double mutation and a poly-adenylation site. Cloning of a cDNA in the unique EcoRI site upstream of IRES creates a bicistronic expression unit. A cDNA encoding hslo (kindly provided by Dr. L. Pallanck, Mereck Research, New Jersey), a human BKCa channel, was inserted thereby generating the pCINeo/ IRES/GFP /hslo vector. For details see (Trouet et al, 1997).

(IRES, a RNA sequence of picornaviruses, encephalomyocarditis, which provides ribosomal attachment so that translation can initiate at an internal site in the mRNA). A scheme of the vector is given in Fig. 8 (for detalils see Trouet et al, 1997). After transient transfection of CPAE cells with pCINeo/IRES-GFP containing hslo cDNA clone, only 2~3% of the transfected cells acquired green fluorescence. All green cells, however, contained hslo-currents. This result is identical with co-expression of GFP and a another K^+ channel, Kv1.1. Details of this efficient method is described elsewhere (Trouet et al, 1997).

Ca^{2+} influx pathways

Until now, ion channels have been described which play an important role for the regulation of the driving force for Ca^{2+}-influx. Obviously, different pathways exist which are reviewed in detail elsewhere (Nilius & Droogmans, 1995). Recently, two pathways have been described which might be of special interest for both agonist-induced - and mechanically-induced Ca^{2+} entry.

Non-selecive cation channels

Mechanical stimulation of endothelium activates a non-selective cation channel with a conductance of 34 pS for monovalents and 6 pS for Ca^{2+} which thus provides an influx pathway for Ca^{2+} (Marchenko & Sage, 1996). Another non-selective cation channel, with a conductance of 44 pS for monovalents, is also permeable for Ca^{2+} and is itself activated by intracellular Ca^{2+} (Baron et al, 1996). Probably, typical receptor-operated Ca^{2+} channels (ROC) such as the P_{2X} receptor are not present in endothelial cells. We have described Ca^{2+}-influx pathways which are related to a non-selective cation channel which is Ca^{2+} permeable and probably also activated by Ca^{2+} itself (Nilius, 1990; Nilius & Droogmans, 1995; Nilius et

al, 1993a; Nilius & Riemann, 1990; Nilius et al, 1993b). Fig. 9 gives an example for whole cell currents. In EA cells after block of the BK_{Ca} channel, but also in CPAE ells after block of Ca^{2+}-activated Cl^-- and volume-regulated anion channels, a current can be activated by agonists which is reverses at potentials between +3 and +10 mV (Fig. 8A, B). This current has been only observed under conditions of increased $[Ca^{2+}]i$ (Fig. 8B). Fast substitution of extracellular NaCl by NMDG-Cl induces a current showing an reversal close to the theoretical K^+-equilibrium potential, E_K, indicating that both Na^+ and K^+ permeate the channel (Fig. 8C). An increase of extracellular Ca^{2+} induces a reduction of the current but also a small shift towards more positive potential indicating the Ca^{2+} may also permeate through the channel, however, with a reduced permeability (Nilius, 1990; Nilius et al, 1993a; Nilius et al, 1993b). Interestingly, a non-selective pathway possibly has been related to the Ins(3, 4, 5)P_3-type 3 receptor (Putney, 1997). In EA and in HUVEC cells but less in CPAE cells, the non-selective influx of Ca^{2+} is strikingly coupled to agonist stimulation and depends on Ins(1, 4, 5)P_3 production. Block of PLC with U73122-a pyrrole-dione derivative-rapidly inhibits the Ca^{2+}-influx whereas the pyrrolidine-dione derivative, which is PLC insensitive, is ineffictive. Also NPPB, Ni^{2+}, ecanozole, and SKF 96365 inhibit the agonist induced Ca^{2+}-entry (Viana, Nilius, unpublished). In CPAE cell, however, U73122 does not inhibit Ca^{2+}-influx during agonist stimulation (Kamouchi, Nilius, unpublished). Interestingly, we have no yet been able to detected the Ins(3, 4, 5)P_3-type 3 receptor in CPAE cells. It is, however, present in EA and HUVEC cells (Parijs, Nilius, unpublished). In addition, voltage-clamped CPAE cells show a much smaller slope of the correlation between driving force and Ca^{2+} -entry than EA cells. This result indicates that in CPAE cells the Ca^{2+} -entry per unit driving force is less in CPAE than in EA or HUVEC cells. One possible explanation is the non-selective entry pathway in CPAE.

SOC or CRAC entry channels

Another pathway influx pathway first discovered in mast cells (Hoth & Penner, 1993) and later on also described in endothelium (Gericke et al, 1993; Gericke et al, 1994; Oike et al, 1994c) is probably related to depletion of intracellular Ca^{2+}-stores. It is therefore termed, Ca^{2+}-release-activated Ca^{2+} current,

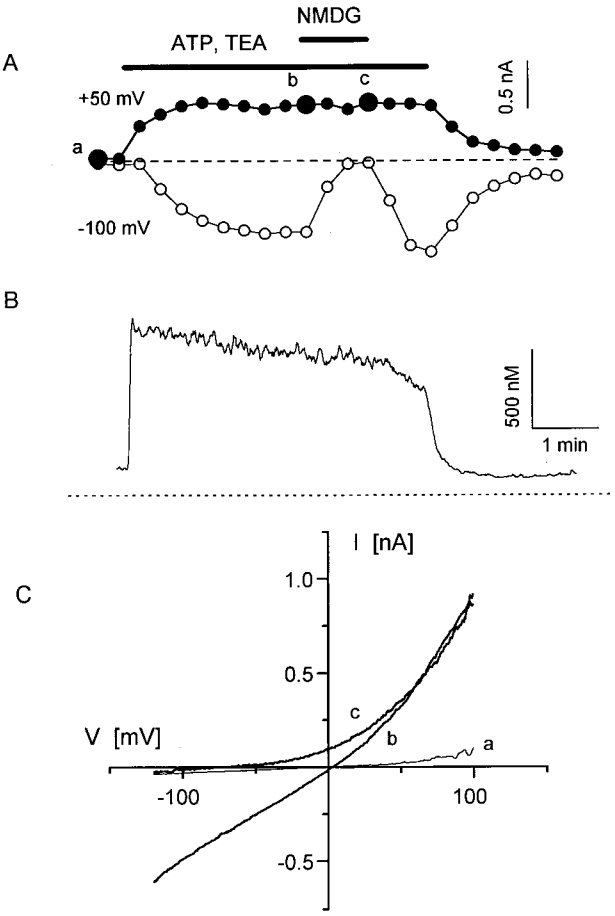

Fig. 9. Activation of a non-selective cation current after agonist stimulation. (A): Time course of the current activation after application of 1 μM ATP in the presence of 10 mM TEA. At the time indicated, NaCl was exchanged with NMDG-Cl. Currents were obtained from voltage ramps and depicted as IV curves from which the values at −100 mV and +50 mV were measured. (B): Ca^{2+} transients during ATP application. Holding potential from which ramps are applied is 0 mV. (C): Instantaneous IV curves from voltage ramps. a) depicts the current before application of ATP, b) is the activated non-selective cation current from which a K^+ component (c) is left as the main current after changing to Na^+-free bath solution (NMDG).

CRAC(Hoth & Penner, 1993). It was a long debate whether EN cells have the typical CRAC. Fig. 10 shows a typical example for such a CRAC current in CPAE cells. Typically for such experiments, intracellular Ca^{2+} is strongly buffered with 10 mM BAPTA. Inclusion of Ins(1,4,5)P_3 in the patch pipette, extracellular application of ionomycin, or administration of the SERCa blockers thapsigargin and BHQ slowly activate a tiny inward current (Fig.

10A). At 0 mV, the current is only in the range of some pA. An elevation of extracellular Ca^{2+} increases the current. Removal of extracellular divalents induced a large, inactivating current that is blocked by micromolar concentrations of lanthanum (Fig. 10A). From voltage ramps, the current at high extracellular Ca^{2+} reverses at very positive potentials indicating the main charge carrier is Ca^{2+} (Fig. 10B). In the absence of Ca^{2+}, the current is carried by monovalents. This current is much larger than the Ca^{2+} current, reverses at potentials between 0 and 15 mV and typically shows a slow deactivation (Fig. 10C).

This deactivation might be due to the reversed slow Ca^{2+}-dependent activation of CRAC at low extracellular Ca^{2+} concentration, which is seen as a slow inactivation of the Na^+ current through CRAC channels (Lepple Wienhues & Cahalan, 1996). Current density at 0 mV is between 10 and 20 times smaller as reported for Jurkat cell and peritoneal mast cells (Fasolato & Nilius, 1997). This endothelial current shares all the now well-described properties of the typical CRAC current present in a variety of non-excitable cells (Hoth et al, 1993; Penner et al, 1993).

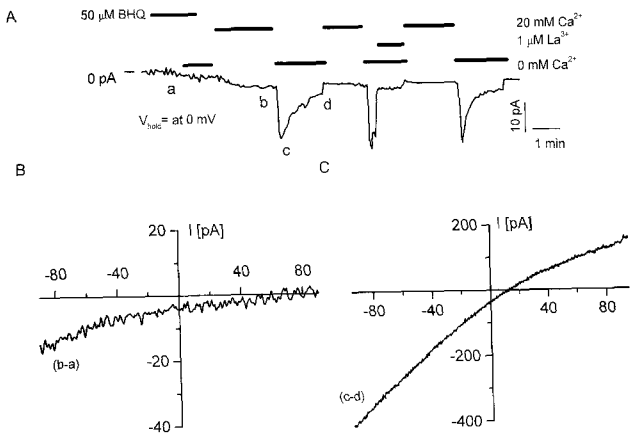

Fig. 10. Activation of a store-depletion dependent current (CRAC). (A): time course of CRAC activation at 0 mV holding potential. The current is activated by application of the SERCa inhibitor BHQ. for the three different cell types. Cells were perfused with different external standard solution containing 20 mM $CaCl_2$ or 0 mM as indicated in the Fig. Cells were dialysed with the standard internal solution containing 12 mM BAPTA. Currents were blocked by 1 μM La^{3+} added to either the standard, or the Ca^{2+}-free bath solution. (B): IV relationship in high Ca^{2+} medium, obtained by subtracting ramps recorded in (a) and (b). (C): IV relationship in divalent-free medium, obtained by subtracting ramps recorded in the same medium containing 1 μM La^{3+} (d) from ramps at the peak Na^+ current (c). Note the block of the current by 1 μM $LaCl_3$. For details, see (Fasolato & Nilius, 1997.)

Trp-a Ca^{2+} entry channel?

Recently a gene family has been described which might be related to Ca^{2+}-entry (Birnbaumer et al, 1996; Zhu et al, 1996). Members of this so-called trp-family (from transient receptor potential) are supposed to form functional Ca^{2+} channels, which can be activated by store depletion. This family is expanding. So far, at least 4 human isoforms are known (Birnbaumer et al, 1996; Philipp et al, 1996; Zhu et al, 1996). Human trp1 is a 793 amino acid (aa) protein with 62% similarity and 37% identity to drosophila trp and trp1. Human trp3 has 848 aa. In all isoforms an ankyrin motif is conserved. This motif is three times repeated. Another molecular feature is the proline rich region at the C-terminus. All trp's are characterised by six membrane spanning helixes and a putative pore region between TM5 and TM6. TM4 is not charged as for voltage-gated ion channels (Bennett et al, 1995; Birnbaumer et al, 1996; Philipp et al, 1996; Zhu et al, 1996). How the channels gate is unknown. One hypothesis discusses possible physical contacts between the trp's aaand the IP3 receptor in intracellular Ca^{2+} stores possibly via the ankyrin-like motifs(Bennett et al, 1995; Berridge, 1995). We have now identified for the first time these members in humans EC by the RT-PCR technique. Primers where designed which differentiate between the isoforms. For htrp1 a short fragment of 134 nucleotides which relates to a C terminal stretch right after TM6, was amplified. For trp3, we used a 465 nucleotide primer from the N-terminus. Trp4 was identified with a 276 nucleotides related to a region between TM5 and TM6.

All these three trp's are present in HUVEC cells in which we can also identify CRAC currents (Oike et al, 1994c). We have now inserted trp3 into the above described bicystronic mammalian expression vector, which utilises the red-shifted GFP bright mutant (F64L, S65T) in CPAE cells and HEK cells. So far, we have not observed CRAC like currents. If at all, appearance of currents in the positively transfected cells refers to rather non-selective cationic currents. These current have similarities with the non-selective cationic currents which we have described in non-transfected, non-stimulated CPAE cells

(Voets et al, 1996). Yet, we have no evidence that in ECs, trp's might be CRAC-channels (for a review on endothelial cells and Ca^{2+}-entry channels, see Nilius et al, 1997h)

Ca^{2+} oscillations in endothelial cells

We have so far discussed the role of ion channels in regulation of the driving force for Ca^{2+}-entry and have identified possible entry pathways. However, the intracellular Ca^{2+} signals are much more complex. At low agonist concentrations, responses scatter statistically. Some cells repond with oscillations, some with plateau-like Ca^{2+}-signals. EC always exhibit a small concentration window in which the main responses to agonists are Ca^{2+} oscillations. At higher concentrations, EC respond more homogeneously with plateau-like Ca^{2+} signals. Fig. 11 shows an example for single, unclamped EA cells. This Fig. depicts from the same cell response to 0.5, 2 and μM UTP, which bind to an identified P_{2y2} receptor present in these cells(Viana et al, 1997). The oscillations in membrane potential are due to the activation of BK_{Ca}. Although the membrane potential exerts this fluctuation in driving force, the oscillations per se are still present in clamped cells where the membrane potential is kept constant. The BK_{Ca} channel itself exhibit properties of an oscillator by its Ca^{2+}- and voltage-dependence. This feature seems to induce only modulation of Ca^{2+} oscillation but is not causing it. For agonists, which binds to the P_{2y2} receptor, oscillations are strongly inhibited at negative potentials. However, this is not the case for other agonists such as histamine or bradykinin. Our interpretation for this paradoxical effect is that the nucleotide-binding site might be locared within the electric field of the membrane. Unbinding of the negatively charged active agonist, UTP^{4-}, ATP^{4-}, occurs at very negarive potentials and may interupt PLC activation and Ca^{2+}transients.

The Ca^{2+} oscillations per se are also independent on extracellular Ca^{2+}. Fig. 12 shows an example. Oscillations are maintained even in the absence of extracellular Ca^{2+}. However, the maintenance of the oscillations seems to depend on Ca^{2+} influx. Supposedly, Ca^{2+} entry is not necessary per se to allow Ca^{2+} -oscillations but probably modulates and sustains them via its role in re-filling of the stores. In the EA cells used in this study, Ca^{2+} -oscillations are strongly inhibited by activation of protein kinase C. The biologically active phorbolester PMA but not its inactive form, αPDD, attenuated Ca^{2+} oscillations. Micromolar concentrations of lanthanum also inhibited Ca^{2+} oscillations (Viana, Nilius, unpublished).

The puzzle still remains in EC: what causes the oscillations. Likely, $Ins(1,4,5)P_3$ must oscillate. If EA cells are loaded with the non-metabolizable agonist, $Ins(2,4,5)P_3$, no oscillations can be evoked. In addition, UTP administration is unable to induce further

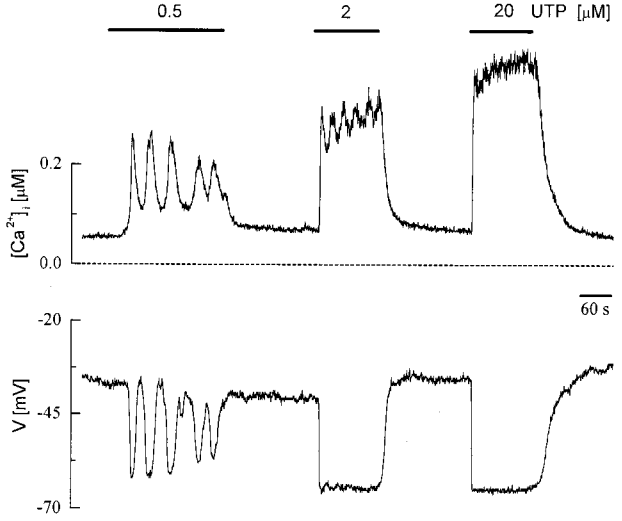

Fig. 11. Oscillations of $[Ca^{2+}]_I$ after application of the agonist UTP. Simultaneous measurements of membrane potential and $[Ca^{2+}]_i$ in a non-clamped EA cell, Note the concentration dependence of the Ca^{2+}-oscillations (application of 0.5, 2, and 20 μM UTP at the times indicated).

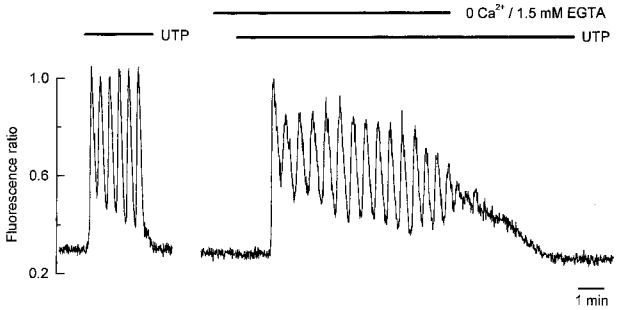

Fig. 12. Calcium oscillations persist in extracellular Ca^{2+} free solutions. A fluorescence imaging system was used (laser scanning Bio-Rad MRC-1000 confocal system, Bio-Rad, Hemel Hempsted, UK). Ca^{2+}-oscillations were monitored in non-voltage clamped EA cells loaded with indo-1 AM (5 μM in cell culture medium). UTP (0.5 μM) induced $[Ca^{2+}]_i$-oscillation which persist in the nominal absence of $[Ca^{2+}]_e$ (1.5 mM EGTA added).

Table 1. Ca^{2+}-oscillations in EA cells (viana, nilius unpublished)

Features of Ca^{2+} oscillations in human EA endothelium
Independent on membrane potential, probably voltage-modulated
Dependent on IP3 production and binding
Dependent on calcium release
calcium entry not necessary for oscillation itself but required for sustained oscillations
inhibited by lanthanum, magnesium
blocked by PKC
blocked by PKC inhibition
Substantial cell-cell variation
Only present at low agonist concentration
several feedback mechanisms are involved:
· $[Ca^{2+}]_I$
· Biphasic Ca^{2+} dependence of the IP3-receptor
· PKC
· PLC
· SERCa
· CRAC likely involved for re-filling of stores
likely due to $Ins(3,4,5)P_3$ oscillations

oscillations in the presence of $Ins(2,4,5)P_3$. Other properties of the oscillation process in endothelial cells refer to a significant involvement of SERCa pumps. Blockers of these pumps inhibit oscillations. Obviously, pump action is necessary to maintain Ca^{2+} oscillations. The explanation of this complex process still difficult and so far we have no endothelial specific nature of these channels is not yet known. Supposedly, some of these entry channels are non-selective cation channels and even probably regulated by intracellular Ca^{2+} itself. Other channels might be regulated by still unknown signals, which are connected to the filling state of intracellular Ca^{2+} stores. Mechanism of Ca^{2+} release and sequestration, which might be the crucial elements for Ca^{2+} oscillations in EC, have not been described in this review. Ca^{2+} oscillations are frequently observed but comprise not an obligatory response pattern in EC.

These signals are only little understood. Their physiological role and impact as an intrinsic signal is not yet elucidated.

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