Ion Channels and Their Functional Role in Vascular Endothelium

BERND NILIUS AND GUY DROOGMANS

Department of Physiology, KU Leuven, Campus Gasthuisberg, Leuven, Belgium

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I. INTRODUCTION

Endothelial cells (EC) form a multifunctional signal-transducing surface that performs different tasks dependent on its localization in the vessel tree. Arterial EC provide a pathway for delivery of oxygen from blood to tissue. They modulate the tone of vascular smooth muscle cells (VSM), which in turn controls blood pressure and blood flow by adjusting the caliber of arteries and arterioles. In the microvascular bed, EC regulate the permeation of various metabolites, macromolecules and gases, as well as autocrine and paracrine factors and are involved in the regulation of cell nutrition. In all vessel types, EC are involved in blood coagulation, control of the transport between blood and tissue, movement of cells adhering to EC, wound healing, and angiogenesis. Several of these functions depend on constitutive properties of EC, e.g., presenting an anticoagulative surface for blood flow by secreting heparin sulfates and expressing ectonucleotidases and thrombomodulin (32, 33, 273, 295). Other functions require an active response of EC to various signals of mechanical, chemical, or neuronal nature. These signals originate from the blood or tissue side of EC or from a coupling with other cells, such as neighboring EC and adhering cells such as white blood cells and thrombocytes, but also with cells at the abluminal side, e.g., smooth muscle cells, mast cells, and fibroblasts. This signal transduction is impaired during vessel disease (atherosclerosis) and injury, inflammation, or hemodynamic disturbances (hypertension).

The role of ion channels in the transduction of these signals into cellular responses is still a matter of debate and has received substantial attention only in the last few years. Ion channels are mainly involved in “short-term responses” (in the second and minute range) and affect the synthesis or release of pro- and anticoagulants, growth factors, and the release of vasomotor regulators. Some of these compounds are produced “on demand” [nitric oxide (NO), PGI2, and platelet activating factor (PAF)], and others are released by exocytosis from storage granules [tissue plasminogen activator (tPA), tissue factor pathway inhibitor (TFPI), von Willebrand factor (vWF)] in response to various transmitters, such as acetylcholine, histamine, kinins (bradykinin), angiotensin, ATP, ADP, the coagulation factor thrombin, growth factors, but also in response to mechanical stimuli, such as shear stress and biaxial tensile stress. Fast responses related to the regulation of the permeability of the blood-tissue interface, controlled by changes in the contractile state of EC and their ability to modulate cell-cell contacts are also controlled by ion channels. The “slow responses” consist in the expression of several surface molecules, adhesion molecules, gene expression, cytoskeletal changes, remodeling, endothelial cell growth, and angiogenesis.

This review addresses the diversity of ion channels and their functional role in EC. Our current knowledge is limited to effects of ion channels on fast endothelial responses, these channels being mainly essential for the regulation of Ca2+ signaling. The major part of the review is therefore devoted to ion channels that function as pathways for Ca2+ entry. These channels are mainly responsible for a long-lasting increase in the free intracellular Ca2+ concentration ([Ca2+]i) during various types of stimuli. This Ca2+ entry is given by

\[ J_{Ca} = 2 \cdot F \cdot N \cdot p \cdot \gamma_{Ca} \cdot (V_M - E_{Ca}) \]  

where \( J_{Ca} \) is the Ca2+ influx, \( F \) is the Faraday constant, and \( N \) is the number of channels. The open probability \( p \) of the channel and its conductance \( \gamma_{Ca} \) for Ca2+ represent inherent features of Ca2+ entry channels, which are discussed in the first part of this review. In addition, Ca2+ entry is regulated by the driving force for Ca2+, i.e., the difference between membrane potential (\( V_M \)) and equilibrium potential for Ca2+ (\( E_{Ca} \)). Thus Ca2+ entry will be tuned by channels that modulate electrogenesis in EC, which will be discussed in the second part of the review. Figure 1 illustrates the dual function of ion channels on Ca2+ entry in EC. Agonist stimulation induces in the absence of extracellular Ca2+ only a fast release of intracellular Ca2+, which is accompanied by a transient hyperpolarization. In the presence of extracellular Ca2+ however, it also induces a plateau-like phase of [Ca2+]i due to Ca2+ entry which leads membrane hyperpolarization and an increased driving force for Ca2+ influx. Figure 1 gives also an overview of the most important channels in EC that are likely responsible for Ca2+ entry and modulation of membrane potential. The third part of this review discusses endothelial functions in which ion channels are likely involved.

II. VARIABILITY OF ION CHANNEL EXPRESSION IN ENDOTHELIAL CELLS

EC express a bewildering variety of ion channels. Unfortunately, it is not clear which channels may be of functional impact in the vascular tree in vivo because some of these channels have only been detected in cultured endothelial cell lines or in primary cultured cells, others in cells obtained by diverse cell dispersion methods, and only very few from in situ measurements in blood vessels. It is therefore not surprising that the extensive amount of data on channel physiology in EC is sometimes controversial and often questionable with regard to their physiological significance. In the future, it might therefore be necessary to identify the candidate channel in vivo. An elegant method to identify channels in
endothelium by cell-specific single cell RT-PCR has been published recently (337).

Another problem typical for EC is that ion channels in EC adapt their appearance to the external conditions. Apparently, ion channels or generally gene expression in EC are highly malleable and vary with cell isolation, culture, and growth conditions (136). As a striking example, human capillary EC grown in normal medium show TEA-sensitive outwardly rectifying K$_{1}$ currents and inwardly rectifying K$_{1}$ channels (IRK), but when grown in a "conditioned medium" (low cell density, medium changed only during passages at days 5–6 compared with controls in which the medium was changed every second day) expressed different channels, such as slo BK$_{ca}$ or 4-amino-pyridine-sensitive A channels (173). The quantitative features of volume-regulated anion currents in primary explants of EC are different, i.e., activation during cell swelling is delayed and inactivation at positive potentials is accelerated as compared with cultured EC (381). The expression of IRK channels in freshly isolated human umbilical vein EC (HUVEC) changes with the number of passages (290). The loss of different serpentine receptors in EC during culturing is well documented (389). Likely, EC might be especially predisposed for this variability.

**Ca$^{2+}$ entry channels**
- P2X4
- RACC
- CNG, HCN
- CCE, SOC
- mechano-sensitive Ca$^{2+}$ channels

**Ca$^{2+}$ entry via NCX ?**

**Channels controlling driving force**
- Ca$^{2+}$ impermeable NSC, CNG
- Kir - types
- BK$_{cap}$ IK$_{ca}$ SK$_{ca}$, K$_{ATP}$
- Cl$^{-}$ channels (ClCa, VRAC, CFTR, B$_{Cl}$)

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**FIG. 1. Dual function of ion channels for Ca$^{2+}$ entry in endothelial cells.** Simultaneous recordings of intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{i}$) and membrane potential ($V_{m}$) in a freshly isolated mouse aorta endothelial cell in the presence and absence of extracellular Ca$^{2+}$. Note the fast Ca$^{2+}$ peak followed by a long-lasting plateau, which disappears after washing out the agonist (10 $\mu$M ACh, 37°C). This plateau is absent when extracellular Ca$^{2+}$ is removed. Thus the functionally important plateau phase is mediated by Ca$^{2+}$ entry. The increase in [Ca$^{2+}$]$_{i}$ is accompanied by a hyperpolarization, which is maintained during the plateau phase, but transient in the absence of extracellular Ca$^{2+}$ ([Ca$^{2+}$]$_{ext}$). This hyperpolarization stabilizes the driving force for Ca$^{2+}$ entry, $V_{m} - E_{Ca}$ (see text). Candidates for the Ca$^{2+}$ entry pathway are listed at the right together with channels that mainly control the driving force for Ca$^{2+}$ entry (see text for further explanation). RACC, receptor-activated cation channel; CNG, cyclic nucleotide-gated channel; HCN, hyperpolarization and cyclic nucleotide-gated channel; CCE, capacitative Ca$^{2+}$ entry; SOC, store-operated channel; NCX, Na$^{+}$/Ca$^{2+}$ exchanger; NSC, nonselective cation channel; ClCa, Ca$^{2+}$-activated Cl$^{−}$ channel; VRAC, volume-regulated anion channel; CFTR, cystic fibrosis transmembrane conductance regulator.
EC also show regional heterogeneity, including differences in [Ca$^{2+}$], signaling, in immunological and metabolic properties, and in the pattern of released mediators for endothelium-dependent vasorelaxation (202, 245, 273, 386, 393).

The uncertainty as to which types of ion channels are present in a vessel type under physiological conditions is a complicating factor for a comprehension of ion channel function in EC.

III. CALCIUM ENTRY CHANNELS

Their role in Ca$^{2+}$ signaling is obviously a fundamental aspect of ion channels in EC. We therefore first describe the various influx routes of Ca$^{2+}$ in EC. Electrophysiological properties of the presently known endothelial Ca$^{2+}$ entry pathways are summarized in Table 1.

A. Nonselective Cation Channels

Nonselective cation channels (NSC) poorly discriminating between cations have been detected in several ion channel families. There was so far no compelling evidence for the existence of NSC members of the purinergic ligand-gated receptor-channel complexes (the P$_2$X family) in EC, but very recently P$_2$X4 receptors have been identified in several macrovascular primary cultured EC (448). This receptor, which is involved in ATP and shear stress induced Ca$^{2+}$ influx (447), seems to be part of a far more complex Ca$^{2+}$ entry system, because the ATP-induced Ca$^{2+}$ entry still persists in HUVECs treated with antisense P$_2$X4. NSCs belonging to the mammalian degenerins (MDEG) family are apparently not expressed in EC.

1. Receptor-activated cation channels

Various functionally different receptor-activated cation channels (RACCs), gated by binding of agonists to their membrane receptor, have been described in EC (123, 172, 177, 291, 295). Most of these channels are activated via signaling cascades involving activation of phospholipase C (PLC), but it is not clear which second messengers are involved. Interestingly, TRP3 and -6, which are members of the family of six transmembrane helix channels (STRPC, see below) that are present in endothelium (98), are nonselective RACCs (132). Probably, TRP4 and -5 are also nonselective RACCs (357), although it has been shown that TRP4 might be at least part of a more Ca$^{2+}$-selective, store-operated channel (99). Endothelial RACCs, activated by an increase in [Ca$^{2+}$], are also permeable for Ca$^{2+}$ and provide a Ca$^{2+}$-entry route that exerts a positive feedback on their own activation. Their molecular structure is not known, although members of the TRP family with Ca$^{2+}$-calmodulin binding sites in their primary structure (see sect. mC) are possible candidates.

Ca$^{2+}$-permeable NSC activated by vasoactive agonists, which have been already described in freshly isolated cells from umbilical vein (270, 271, 276, 289, 291), have recently been characterized in more detail in cultured cell lines. The channel has a conductance of ~25 pS and is permeable for Na$^+$, Cs$^+$, and Ca$^{2+}$ with a permeation ratio $P_{Ca}/P_{Na}$ that varies between 0.03 and 2 (172, 177, 267, 272, 296). The current slowly activates during agonist stimulation and has been observed only in the presence of physiological [Ca$^{2+}$]. Buffering of [Ca$^{2+}$] with 10 mM BAPTA completely prevents current activation. On the other hand, loading the cells with Ca$^{2+}$ via the patch pipette does not activate the current. Interestingly, application of a store-depleting inhibitor of sarco/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) pumps, such as tert-butyl-benzohydroquinone (tBHQ) or thapsigargin (TG), can also activate this NSC. The influx of Ca$^{2+}$ through this NSC is coupled to agonist stimulation and depends on inositol 1,4,5-trisphosphate [Ins(1,4,5)P$_3$] production. Block of PLC with the pyrrole-dione derivative U73122 rapidly inhibits Ca$^{2+}$ influx, whereas the pyrrolidin-dione derivative U73343, which does not inhibit PLC, is ineffective. Also 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), Ni$^{2+}$, ecanozole, and SKF-96365 inhibit the agonist-induced Ca$^{2+}$ entry (177). This channel shares some properties with the hTRP3 channels expressed in EC that do not express this NSC (178). Baron et al. (13) described another nonselective Ca$^{2+}$-permeable cation channel, activated by intracellular Ca$^{2+}$ and with a con-

TABLE 1. STRP channels in EC

<table>
<thead>
<tr>
<th>Type</th>
<th>Tissue</th>
<th>Reference No.</th>
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<tbody>
<tr>
<td>TRP1</td>
<td>BAEC</td>
<td>52, 107</td>
</tr>
<tr>
<td></td>
<td>RPAEC</td>
<td>257</td>
</tr>
<tr>
<td></td>
<td>HPAEC</td>
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<td></td>
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<td>123</td>
</tr>
<tr>
<td></td>
<td>BPAEC</td>
<td>177</td>
</tr>
<tr>
<td></td>
<td>EA.hy926</td>
<td>98, 177</td>
</tr>
<tr>
<td></td>
<td>MAEC</td>
<td>195</td>
</tr>
<tr>
<td></td>
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<tr>
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</tr>
<tr>
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<tr>
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BAEC and MAEC, bovine and mouse aorta endothelial cells (EC), respectively; RPAEC, HPAEC, and BPAEC, rat, human, and bovine pulmonary EC, respectively; HUVEC, human umbilical vein EC; EA.hy926, HUVEC-derived EC; HMAEC, human mesenteric artery EC.
ductance of 44 pS for monovalent cations. This channel, with a permeability ratio \( P_{Ca^2+}/P_{Na^+} \) of 0.7, is half-maximally activated at 0.7 \( \mu M \) \([Ca^{2+}+\]). A nonselective, agonist-induced current, which is also activated by \([Ca^{2+}+]\), and suppressed by inhibitors of the cyclooxygenase pathway, has been described in aortic EC (137).

2. Amiloride-sensitive NSC

An amiloride-sensitive, poorly selective cation channel (23 pS for \(Na^+\) and \(K^+\)) has been observed in brain microvessels (416). This channel seems to be important for regulation of cation fluxes across the blood-brain barrier. Constitutively open, nonselective \(Ca^{2+}\)-permeable cation channels have been detected in both luminal and abluminal membranes of endocardial EC. They consist mainly of two types of nonselective outwardly rectifying cation channels with a single-channel conductance of 36 and 11 pS, respectively. The permeability sequence for monovalent cations is \(K^+ > Rb^+ > Cs^+ > Na^+ > Li^+\) which corresponds to the Eisenmann sequence IV and indicates a weak-field strength binding site for these cations (83). \(Cd^{2+}\) and \(La^{3+}\) are efficient blockers, whereas \(Ca^{2+}\) and \(Ba^{2+}\) but neither \(Mg^{2+}\) nor \(Mn^{2+}\) are permeable. Vasoactive agonists induce a nonselective cation current in endocardial EC with different ion selectivity and rectification properties (234, 235).

3. Redox NSC

Oxidant stress, which induces superoxide anions, activates a 28-pS NSC in a calf pulmonary artery cell line. This channel is equally permeable for \(Na^+\) and \(K^+\) and also for \(Ca^{2+}\). Oxidized glutathione, a cytosolic product of oxidant metabolism, activates these channels, whereas reduced glutathione reverses activation (198). This channel opens in two gating modes that do not depend on intracellular \(Ca^{2+}\) stores or on \([Ca^{2+}]_i\). Activation of these channels and the concomitant membrane depolarization may limit \(Ca^{2+}\) influx (199). TRP3 has been proposed as a molecular candidate for this channel (10).

4. Cyclic nucleotide-gated channels

NSC gated by cyclic nucleotides (CNG) have been recently identified in cultured rat EC. NH\(_2\) and COOH termini of these channel proteins are cytoplasmic with the binding site for cyclic nucleotides located in the COOH terminus. These channels are directly gated by binding of cAMP or cGMP at the cyclic nucleotide-binding domain (CNBD) and also open at hyperpolarized potentials. The channels have a conductance of \(-20\) pS for monovalent cations and are also \(Ca^{2+}\) permeable.

A CNG1 member of the CNG family has been recently cloned in vascular endothelium. It is expressed in aorta, medium-sized mesenteric arteries, and small mesenteric arteries and forms a nonselective cation channel mediating entry of extracellular \(Ca^{2+}\) that might be involved in the regulation of arterial blood pressure (452).

Agonist stimulation of rat pulmonary artery EC in primary culture activates CNG channels, which resemble CNG2 subunits of rat olfactory neurons. This activation is subsequent to \(Ca^{2+}\) entry, probably via store-operated or RACC channels, and is prevented in the presence of specific inhibitors of soluble guanylate cyclase. It has been proposed that the increase of \([Ca^{2+}]_i\) upon agonist stimulation activates endothelial NO synthase (eNOS), which in turn enhances NO levels, stimulates soluble guanylate cyclase, and increases cGMP levels, which then leads to activation of CNG2 (see Fig. 2 and Ref. 441). This endothelial CNG is neither selective for \(K^+, Na^+, Cs^+\), nor \(Rb^+\). Its activation contributes to membrane depolarization and exerts a negative feedback on \(Ca^{2+}\) entry. Modulation of CNG by cAMP via G protein-coupled receptors (GPCR) has so far not been described in EC. It has to be mentioned that cGMP may also induce a negative feedback on SOC/RACC via a PKG pathway (see sect. mB).

In summary, EC lack typical ligand-gated channels, such as the \(P\_x\) receptor. They express several \(Ca^{2+}\)-permeable, receptor-operated NSC with diverse activation mechanisms, superoxide-gated NSC, and cyclic nucleotide-gated channels. Background NSCs are probably constitutively open. Some RACCs might be members of the STRPC family.

B. Store-Operated or Capacitative \(Ca^{2+}\) Entry (SOC or CCE)

It is well known that compounds, such as tBHQ, cyclopiazonic acid (CPA), and TG, which deplete intracellular \(Ca^{2+}\) stores without affecting Ins(1,4,5)P\(_3\) production, activate a \(Ca^{2+}\) influx pathway in EC (73, for a review see Refs. 295, 358, 359, 398, 399). This influx is apparently controlled by the filling degree of intracellular \(Ca^{2+}\) stores and is presumed to represent the major pathway for \(Ca^{2+}\) influx during agonist stimulation (318). Charged or filled stores prevent \(Ca^{2+}\) entry, whereas discharged or empty stores promote influx of extracellular \(Ca^{2+}\). We will use the term SOC for electrophysiological data and the term CCE for data derived from changes in intracellular \(Ca^{2+}\) upon readmission of extracellular \(Ca^{2+}\) to store-depleted cells. This distinction is not only semantic since the latter signals also depend on other mechanisms, such as \(Ca^{2+}\) sequestration and buffering which makes their interpretation often controversial. CCE signals are not much affected by mitochondrial \(Ca^{2+}\) buffering, but inhibition of the plasma membrane \(Ca^{2+}\) pump (PMCA) inhibits their secondary decay phase, indicating a modulating role of PMCA (194). Other reports also indicate a modulating role of a \(Na^+/Ca^{2+}\) exchanger on CCE.
signals: inhibition of the exchanger increases CCE and delays the decay of store depletion-induced Ca\(^{2+}\) signals after reapplication of extracellular Ca\(^{2+}\) (75, 118, 315). Changes in membrane potential in non-voltage-clamped cells will also affect the driving force for Ca\(^{2+}\) and hence the CCE signals.

Store-operated Ca\(^{2+}\) channels (SOC) are obviously distinct from classical voltage-operated Ca\(^{2+}\) channels and Ca\(^{2+}\)-permeable RACCs. In general, SOCs are much more selective for Ca\(^{2+}\) than the above-described Ca\(^{2+}\) entry channels. Only two highly Ca\(^{2+}\)-selective agonist-activated channels have been described in EC. The one is activated by ATP and bradykinin and has been characterized at both the whole cell and single-channel level in bovine aorta EC (BAEC) (227). It is very selective for Ca\(^{2+}\) and Mn\(^{2+}\) and has a conductance of 2.5 pS for Mn\(^{2+}\). Inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P\(_4\)], but not Ins(1,4,5)P\(_3\) increases its open probability, which is \(~50\%\) lower at 1 \(\mu\)M [Ca\(^{2+}\)]\(_i\) than at 1 mM [Ca\(^{2+}\)]\(_o\)].. The other highly Ca\(^{2+}\)-selective channel is gated by Ins(1,4,5)P\(_3\) and has been observed in cell-attached, inside-out, and outside-out patches in BAEC. Ins(1,4,5)P\(_3\) increases its open probability at a constant [Ca\(^{2+}\)]\(_i\), whereas Ins(3,4,5,6)P\(_4\) is ineffective. This channel is reversibly blocked by heparin added to the intracellular side of inside-out patches and might be directly gated by Ins(1,4,5)P\(_3\) binding (400).

The best-described SOC is the so-called “Ca\(^{2+}\)-release-activated Ca\(^{2+}\)-channel” (CRAC), which has been studied extensively in mast cells and T lymphocytes (145, 146, 318). CRAC is a highly Ca\(^{2+}\)-selective channel with a single-channel conductance of \(~30\) fS for Ca\(^{2+}\) and of \(~40\) pS for monovalent cations under divalent-free conditions (188). The first “CRAC-like” current in endothelium was published already in 1992. Bradykinin activates a whole cell current in BAEC (\(~10\) pA/cell at a potential of \(~60\) mV) with characteristics similar to CRAC (251), which is blocked by La\(^{3+}\) and inactivates rapidly if Ca\(^{2+}\) is the main charge carrier but not if it is carried by Na\(^{+}\). Inwardly rectifying CRAC-like currents have also been recorded in other endothelial cells after depleting intracellular Ca\(^{2+}\) stores by dialyzing them with either a high concentration of BAPTA to buffer [Ca\(^{2+}\)]\(_i\) at very low concentrations or with Ins(1,4,5)P\(_3\), by extracellular application of ionomycin, or administration of the SERCA blockers TG or tBHQ. (88, 109, 110, 306). Store depletion protocols also activate Ca\(^{2+}\) channels in BAEC with a conductance between 5 and 11 pS and which are not highly selective for Ca\(^{2+}\), i.e., \(P_{Ca/PNa} = 10\) (394, 398, 399). The density of this current is approximately \(~0.5\) pA/pF at 0 mV, which is \(~10–20\) times smaller than that reported for Jurkat and peritoneal mast cells (318). Its permeability sequence is Ca\(^{2+}\) > Na\(^{+}\) > Cs\(^{+}\) and Ca\(^{2+}\) > Ba\(^{2+}\) (88). Elevation of extracellular Ca\(^{2+}\) increases the current amplitude, whereas removal of extracellular divalent cations induces a large Na\(^{+}\) current that is blocked by micromolar concentrations of La\(^{3+}\).

In general, it seems that SOC currents in EC exhibit much weaker inward rectification, are less Ca\(^{2+}\) selective, and have a higher single-channel conductance than the typical CRAC currents. A typical example for activation of a SOC current in EA.hy926 cells, a cell line derived from human umbilical vein, is shown in Figure 3. Breaking into the cell with a patch pipette containing a high concentration of BAPTA, 30 \(\mu\)M Ins(1,4,5)P\(_3\), and 20 mM tBHQ activates within \(~30–60\) s an inward current, which reverses at a potential more positive than 50 mV and is
completely blocked by 1 μM La^{3+}. This protocol bypasses the agonist-mediated activation pathway via PLC activation described for RACC, but the exact activation pathway of this channel remains to be elucidated.

Pharmacological data on SOC currents in EC are rather scarce. They are almost completely blocked by 1 μM La^{3+} and inhibited by econazol, NPPB, Ni^{2+}, and carboxyamidotriazole (CAI), but a selective pharmacological blocker is not available. It has recently been shown that metabolites of the sphingomyelin pathway inhibit CRAC in RBL cells (244). However, sphingosylphosphorylcholine, which increases membrane capacitance in RBL cells, activates SOCs and NO production in EC (257). Likely, membrane lipids and metabolites of membrane breakdown are directly involved in regulation of CCE (see also sect. III).

FIG. 3. Store depletion activates a cation current in mouse aorta endothelial cells (MAEC). A: activation of Ca^{2+} entry can occur via depletion of intracellular Ca^{2+} stores. The mechanism of activation is unknown and may involve a diffusible messenger, direct coupling between SOC and the inositol 1,4,5-trisphosphate receptor (IP_{3}R), or store-dependent exocytosis and membrane incorporation. B: breaking into an isolated MAEC under the conditions shown in the inset of C activates a cation current. The current is blocked by 1 μM La^{3+} and shows a typical delayed unblock. C: the current reverses around +60 mV, indicating a Ca^{2+}-selective current. (From S. H. Suh and B. Nilius, unpublished data.)
Mechanisms of CCE modulation are also still not much elaborated. Stimulation of EC with vasoactive compounds, and application of TG results in tyrosine phosphorylation of 42- and 44-kDa isoforms of mitogen-activated protein kinase (MAP kinase). Inhibition of this phosphorylation attenuates CCE. Tyrosine kinases may therefore be associated with the regulation of CCE (94). Calmodulin seems to inhibit CCE (394), whereas activation of protein kinase C (PKC) inhibits CRAC (318). Activation of protein kinase G (PKG) has been shown (204) to inhibit both SOC (patch-clamp data) and CCE (Ca\textsuperscript{2+} entry).

The signal transduction mechanism linking changes in intraluminal Ca\textsuperscript{2+} to the opening of plasma membrane Ca\textsuperscript{2+} stores and membrane channels. Activation of cytochrome P-450 epoxygenase 2C, the endothelial isoform of the endoplasmic reticulum cytochrome P-450 monooxygenase (CytP-450 MO), by \beta-naphthoflavone induces the biosynthesis of 5,6-EET and potentiates CCE in BAEC, porcine aorta and human umbilical vein (119), whereas several P-450 inhibitors diminish CCE and induction of CytP-450 MO. In addition, depletion of endothelial Ins(1,4,5)P\textsubscript{3}-sensitive stores directly activates CytP-450 MO (139). It has therefore been proposed that CytP-450 MO-derived 5,6-EETs may constitute a signal for CCE activation.

It has also been suggested that Ins(1,3,4,5)P\textsubscript{4} or Ins(1,4,5)P\textsubscript{3} or a decrease of [Ca\textsuperscript{2+}], in a restricted subplasmalemmal space may directly gate the entry channels and that a direct coupling between CRAC and the Ins(1,4,5)P\textsubscript{3} receptor may control channel gating (8, 14, 58, 141, 144, 193, 318, 342). A direct physical contact between Ins(1,4,5)P\textsubscript{3} receptors in the endoplasmic reticulum and COOH-terminal contact sites of the putative plasma membrane TRP channels may be functionally involved (192, 193). Two short stretches of 54 (position 742–795) and 21 amino acids (position 777–797) that bind strongly to specific sequences in the Ins(1,4,5)P\textsubscript{3} receptor have been identified in hTRP3. The binding domain in the InsP\textsubscript{3} receptor comprises 289 amino acids downstream of the Ins(1,4,5)P\textsubscript{3} binding site (position 638–926) (37). This protein-protein complex may fulfill a similar function as the dTRP, PKC, receptor complex with the PDZ-domain protein InaD in Drosophila (258, 339). More recently, it has been shown that stimulation of vascular endothelial growth factor (VEGF) receptor-2 by VEGF-E, which induces store depletion probably via phosphorylation of PLC-γ, activates CRAC-like currents, which are blocked by micromolar concentrations of La\textsuperscript{3+} and are enhanced in amplitude if carried by Na\textsuperscript{+} in the absence of extracellular Ca\textsuperscript{2+} (single-channel conductance of ~45 pS; Lepple-Wienhues, personal communication), a value similar to that observed in T lymphocytes (36–40 pS, Ref. 188).

C. TRPCs: Diverse Ca\textsuperscript{2+} Entry Channels in EC?

The tremendous efforts in the last 3 years to identify mammalian genes encoding for Ca\textsuperscript{2+} entry channels have resulted in the discovery of the trp gene family (“transient receptor potential”) in photoreceptors of Drosophila. The genes of this still expanding family might be related to several types of Ca\textsuperscript{2+}-entry channels but in a much broader sense than encoding for store-operated Ca\textsuperscript{2+} entry channels only (22, 29, 144, 457–459). Tpr genes encode a superfamily of proteins with six transmembrane helices, which can be divided into three subfamilies, i.e., S(hort)-TRPCs, L(ong)TRPCs, and O(sm-9-like)TRPCs (Fig. 4, Ref. 132). The latter group, named after its homology to the osm-9 members in Caenorhabditis elegans which respond to changes in osmolarity, contains interesting members, such as the epithelial Ca\textsuperscript{2+} channel (ECaC) involved in Ca\textsuperscript{2+} reabsorption in the distal tubules of the kidney, NSc such as the vanilloid receptor VR1, VRL1, and the insulin-like growth factor regulated channel GRC (132, 140, 183, 408). The LTRPC group contains proteins of more than 1,600 amino acids, the function of which is presently not understood.

The STRPC group of proteins between 700 and 1,000 amino acids comprises the mammalian isoforms of TRP1–7, which are composed of six transmembrane helices with a pore-forming loop between the fifth and sixth helix (Fig. 4). The NH\textsubscript{2} terminus of all isoforms contains three highly conserved ankyrin domains. The NH\textsubscript{2}-terminal part of the COOH terminus bears a proline-rich motif (29, 132, 329, 333, 457). The TM4 region lacks the positive charges typical for voltage-gated ion channels (16, 29, 457). STRPCs contain several calmodulin-binding regions, which may mediate the Ca\textsuperscript{2+}-dependent modulation of store-related Ca\textsuperscript{2+} entry. However, the functional effect of calmodulin is difficult to evaluate because it influences many EC functions including the uptake of Ca\textsuperscript{2+} into intracellular stores (437). Some STRPCs (e.g., TRP1) have NH\textsubscript{2}-terminal coiled-coil structures and COOH-terminal dystrophin motifs (for details see Ref. 333). STRPCs are mainly activated by agonist stimulation of diverse receptors coupled to different PLC isoforms but some probably by store depletion.

It is not clear how these channel proteins are organized, but the extreme functional heterogeneity observed in expression experiments may point to a multiheteromeric structure. Proper heteromerization may depend on unidentified auxiliary proteins similar to the Drosophila InaD (from the mutant “inactivation no afterpotential”)...
protein. This protein contains five PDZ domains (comprising DTSL motifs) and is physically linked to the rhodopsin PLC (NorpA), a PKC (InaC), and the G\textsubscript{q,\alpha}-protein photoactivated by rhodopsin. The entire structure forms a signaling complex in which dTRP and dTRPL are integrated (258). PDZ domains probably mediate the clustering of membrane and membrane-associated proteins with TRP channels. A similar protein hINAl (human InaD like), which is twice as long as the \textit{Drosophila} InaD (1,525 vs. 675 amino acids), has been cloned in human (330) and has been detected in human EC (V. Flockerzi and B. Nilius, unpublished data). In this context, formation of structural complexes of Ca\textsuperscript{2+} entry channels could also be integrin dependent, since an integrin-associated protein (IAP-50) physically bound to integrin \beta\textsubscript{3} has been proposed to function as a Ca\textsuperscript{2+} entry channel (363). This signaling pathway seems to be linked to the generation of inositol phosphate and the production of Ins\textsubscript{(1,4,5)}P\textsubscript{3} (328). INAF is another 241-amino acid protein that is probably involved in Ca\textsuperscript{2+} signaling via STRPC proteins, since mutations in the gene encoding for this protein directly affect TRP channel function (215).

The activation steps following PLC stimulation are unclear for all STRPC isoforms. One group of STRPCs (still frequently described as TRPs) seems to be activated via production of DAG and by binding of polyunsaturated fatty acids (PUFAs; Refs. 58, 132, 141, 142). Stimulation of EC via activation of the pertussis toxin-insensitive G\textsubscript{q} protein-coupled PLC-\textbeta\textsubscript{ca} causes Ins\textsubscript{(1,4,5)}P\textsubscript{3} production that initiates store depletion and probably STRPC-mediated Ca\textsuperscript{2+} entry. Also PLC-\gamma, which is activated by agonist-stimulated receptor tyrosine kinases and growth factor receptor tyrosine kinases, may activate STRPCs (98). Figure 5 summarizes the activation pathways for STRPCs that may be relevant for EC. It has to be noted that only a few members of the putative STRPC family have been analyzed at the functional level.

Phylogenetic analysis of the mammalian STRPCs (TRPs) reveals four subfamilies (see Fig. 6A; Refs. 98, 132, 142, 333).
The first subfamily is a subclass that comprises TRP4 and TRP5. Heterogeneously expressed mTRP4 and mTRP5 are NSCs with a single-channel conductance of 42–66 pS that can be stimulated by guanosine 5′-O-(3-thiotriphosphate) (GTPγS). The activation pathway may integrate signaling pathways from G protein-coupled receptors and receptor tyrosine kinases independently of store depletion. Their biophysical properties are inconsistent with those of CRAC channels. In other experiments, however, TRP4 and -5 are clearly store dependent and have a much higher Ca^{2+} selectivity (331, 332, 434).

TRP1, which is closely related to TRP4 and -5 in terms of evolutionary distance, probably forms SOCs (223, 224, 431) but is probably less Ca^{2+} selective than TRP4 and -5 (329, 387, 434).

The short TRP3, TRP6, and TRP7 with ~800 amino acids form store-independent NSCs probably activated via the DAG pathway (141) and are also sensitive to [Ca^{2+}] (178, 307, 461). TRP3 and -6 have a conductance between 25 and 66 pS and are not very Ca^{2+} selective (141, 178). However, also a store-dependent activation mechanism has been considered for hTRP3 (124, 193).
The subfamily consisting of TRP2 is still uncertain and forms probably truncated channel proteins.

Several TRPs are expressed in endothelium, as illustrated by their expression pattern in mouse aorta in Figure 6B. Their expression varies, however, considerably between different types of EC, as shown in Table 2.

### Table 2. Ca^{2+} entry pathways in EC identified by current measurements

<table>
<thead>
<tr>
<th>Current</th>
<th>Conductance</th>
<th>Selectivity</th>
<th>Activation</th>
<th>Mol Cand</th>
<th>Remarks</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCE</td>
<td>-3 pA/pF at -50 mV (est.)</td>
<td>NSC, Ca^{2+} &gt; Na^{+}</td>
<td>Ins(1,4,5)P_{3}R, TG</td>
<td>TRP3</td>
<td>HUVEC</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>5 pA/pF at -80 mV</td>
<td>Ca^{2+} &gt; Ba^{2+} &gt;&gt; Na^{+}</td>
<td>BHQ?</td>
<td>TRP1</td>
<td>Pulmonary EC</td>
<td>257</td>
</tr>
<tr>
<td></td>
<td>-fs, 45 pS in Ca^{2+} free</td>
<td>Na^{+}</td>
<td>Ins(1,4,5)P_{3}R, TG, VEGF-E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOC</td>
<td>0.02 pA/pF at -40 mV, at 1 mM Ca^{2+}</td>
<td>Ca^{2+} &gt; Na^{+}</td>
<td>TG, Ins(1,4,5)P_{3}</td>
<td>?</td>
<td>HUVEC, BPAEC</td>
<td>110, 303</td>
</tr>
<tr>
<td></td>
<td>0.1–0.2 pA/pF at 0 mV</td>
<td>Ca^{2+} &gt; Ba^{2+} &gt;&gt; Na^{+}</td>
<td>TG, Ins(1,4,5)P_{3}P_{2}</td>
<td>BAPTA</td>
<td>Block by µM La^{3+}, BPAEC</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>5 pS in Ca^{2+}, 11 pS Ca^{2+} free, 0.3–2 pA/pF in Ca^{2+} at -60 mV (est.)</td>
<td>Na^{+}</td>
<td>Agonists, TG, BHQ</td>
<td></td>
<td>Inhibition by calmodulin, BAE</td>
<td>249, 390, 394, 395</td>
</tr>
<tr>
<td>NSC_{Ca}</td>
<td>28 pS, 6 pS in Ca^{2+}</td>
<td>Na^{+} &gt; Ca^{2+}</td>
<td>CPA, [Ca^{2+}]_{i}??</td>
<td>HUVEC</td>
<td>449</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28 pS, 13 pS in Ca^{2+}</td>
<td>Na^{+} = K^{+} &gt; Ca^{2+}</td>
<td>Agonist, [Ca^{2+}]_{i}</td>
<td>TRP3??</td>
<td>HUVEC</td>
<td>449</td>
</tr>
<tr>
<td></td>
<td>-25 pS</td>
<td>Na^{+} ~ K^{+} &gt; Ca^{2+}</td>
<td>Agonist, [Ca^{2+}]_{i}</td>
<td></td>
<td>BAE</td>
<td>596</td>
</tr>
</tbody>
</table>

### Other channels

| CNG Mech-NS | 24 pS, 16–19 pS in Ca^{2+} | K^{+} > Na^{+} > Ca^{2+} | NO donors, cGMP | CGN1, CGN2 | BBAEC | 154, 164, 446 |
|             | 21 pS, 6 pS in Ca^{2+} | Ca > Na = K | Pressure | RAEC | 239, 240 |
|             | 18 pS | Na^{+} = K^{+} > Ca^{2+} | Pressure | RAEC | 147, 148 |
| NCX         | 32 pS, 13 pS in Ca^{2+} | Na^{+} = K^{+} > Ca^{2+}, Ba^{2+} | Stretch | Endocardial EC | 146 |
|             | 40 pS, 19 pS in Ca^{2+} | Ca^{2+} < Na^{+} | Stretch | PAEC | 208 |
|             | | Ca^{2+} > Na^{+} = Cs^{+}, NSC | Shear | HUVEC, HCEC, HAEC | 170, 264, 363 |

SOC, store-operated channels; CCE, capacitative Ca^{2+} entry channel (STRPCs ?); NSC_{Ca}, nselective, Ca^{2+}-permeable channels; HCN, hyperpolarization activated cation channels, nucleotide sensitive; CNG, cyclic nucleotide-gated cation channels; NCX, Na^{+}/Ca^{2+} exchanger; HUVEC, human umbilical vein EC; PAEC, porcine aorta EC; BPAEC, cultured (calf) bovine pulmonary artery EC; BAEC, bovine aorta EC; EA, EA.hy926 HUVEC derived EC (81); MAEC, mouse aorta EC; CAEC, pig coronary EC; BBEC, blood-brain barrier EC; RAEC, excised rat aorta EC; RTAEC, rat aorta EC; HCEC, human coronary EC; HAEC, human aorta EC; SHR, spontaneously hypertensive; TG, thapsigargin; Ins(1,4,5)P_{3}P_{2}, inositol 1,4,5-trisphosphate; [Ca^{2+}]_{i}, free intracellular Ca^{2+} concentration; NO, nitric oxide; InsP_{2}R, inositol tetrakisphosphate receptor.

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may serve as a Ca\(^{2+}\)-influx pathway during agonist stimulation. These channels share several properties with an endogenous nonselective Ca\(^{2+}\) entry channel observed in TRP3-expressing EA.hy926 cells (177). Expression of an NH\(_2\)-terminal fragment of hTRP3, which exerts a dominant negative effect on TRP channel function presumably due to suppression of channel assembly (124), abolishes activation of a store-dependent, agonist-activated nonselective cation current in HUVEC cells (124). These results suggest a role of STRPC-related proteins as store-operated and/or agonist-activated cation channels identical to TRP3. Other studies show that \(\beta\)-estradiol significantly downregulates TRP4, and transretinoic acid dramatically upregulates TRP5, whereas these hormones have little effect on CCE (52). Expression of TRP1 and TRP4 in BPAEC induces a cation current that is activated by t BHQ and Ins(1,4,5)P\(_3\), suggesting that both STRPCs may form functional but not highly Ca\(^{2+}\)-selective channels (409). Interestingly, activation of TRP1 in rat pulmonary EC induces changes in EC shape and remodeling of the peripheral (cortical) filamentous actin (F-actin) cytoskeleton (259).

Another question refers to the nature of Ca\(^{2+}\) stores involved in CCE activation. In addition to Ins(1,4,5)P\(_3\)-mediated Ca\(^{2+}\) release, ryanodine-sensitive Ca\(^{2+}\) release (RsCR) seems also to be important for Ca\(^{2+}\) signaling in EC. Several studies have presented evidence for endothelial ryanodine receptors (1, 62, 213, 460). In human aortic EC, ryanodine-sensitive Ca\(^{2+}\) stores seem to be involved in the regulation of agonist-sensitive, Ins(1,4,5)P\(_3\)-depleted Ca\(^{2+}\) pools. Caffeine and ryanodine induce only a small Ca\(^{2+}\) entry if the Ins(1,4,5)P\(_3\)-sensitive stores are filled. However, after store depletion, caffeine but not ryanodine induces Ca\(^{2+}\) entry (62). One likely interpretation of these effects is that caffeine regulates CCE and/or that caffeine also controls the extent of store depletion by agonist stimulation. Also other experiments show that RsCR may modulate but not initiate CCE. By using deconvolution microscopy to monitor simultaneously cytosolic, perinuclear Ca\(^{2+}\) by fura 2 and membrane near, subplasmalemmal Ca\(^{2+}\) by the cell membrane impermeable dye FFP-18 in the same EC (315), it has been shown that Ca\(^{2+}\) release initiated by ryanodine increases subplasmalemmal Ca\(^{2+}\) but not perinuclear Ca\(^{2+}\). In addition, conditions that initiate RsCR also increase CCE. Although these data support the existence of local Ca\(^{2+}\) gradients, they are not indicative for CCE activation by the discharge of ryanodine-sensitive Ca\(^{2+}\) stores. Other findings do not support any effect of the discharge of ryanodine-sensitive stores on Ca\(^{2+}\) entry or Mn\(^{2+}\) quenching (354). However, subplasmalemmal Ca\(^{2+}\) elevation could dramatically change the driving force for Ca\(^{2+}\) entry by activating BK\(_{\text{Ca}}\) channels, which in turn would affect CCE (see sect. ivA2).

D. Ca\(^{2+}\) Entry Via a Na\(^+\)/Ca\(^{2+}\) Exchanger, NCX?

The modulation of endothelial Ca\(^{2+}\) signaling via a Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) is an incompletely resolved issue. Several groups have shown that a reduction of the Na\(^+\) gradient increases [Ca\(^{2+}\)]\(_i\), via an NCX-mediated Ca\(^{2+}\) entry (352, 369, 378, 385). Evidence that the exchanger may also shape Ca\(^{2+}\) transients activated by vasoactive agonists comes from experiments in which [Ca\(^{2+}\)]\(_i\), transients are modulated by changing Na\(^+\) gradients, loading EC with Na\(^+\) by using the ionophore monensin, application of NCX blockers, such as 3‘,4‘-dichlorobenzamil (in comparison with the NCX ineffective amiloride) or La\(^{3+}\) (369). Recently, a NCX protein was detected in caveolin-rich EC membrane fractions (385).
E. Conclusion

Ca\textsuperscript{2+} entry in EC occurs via different pathways, but the molecular nature of these different entry pathways and their mechanism of activation are still elusive. Likely, endothelial Ca\textsuperscript{2+} entry channels belong to the family of STRPC which comprise both receptor-activated, NSC and more Ca\textsuperscript{2+}-selective store-operated channels. Activation of these channels occurs via both GPCR and receptor tyrosine kinases. In addition, ligand-gated Ca\textsuperscript{2+}-permeable channels, such as CNG channels and P_{2X} receptors, and probably endothelial isoforms of the NCX provide alternative pathways for Ca\textsuperscript{2+} entry in EC. These mechanisms, which are controlled by various signaling cascades, are important for maintaining long-lasting responses of EC “on demand,” such as Ca\textsuperscript{2+}-dependent synthesis and release of NO, secretion of vasoactive compounds out of vesicular compartments, and probably also during stimulation of EC by growth factors, such as VEGF.

IV. ION CHANNELS CONTROLLING MEMBRANE POTENTIAL

A crucial role of ion channels in endothelial Ca\textsuperscript{2+} signaling is the fine-tuning of the electrochemical gradient for Ca\textsuperscript{2+} (176, 226, 271, 272). The influence of membrane potential, which as discussed in section viiiA is determined by the expression pattern of various ion channels, on Ca\textsuperscript{2+} entry in EC has been clearly demonstrated (170, 176, 226, 359). K\textsuperscript{+} channels, including Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels, inwardly rectifying K\textsuperscript{+} channels, and probably also voltage-dependent K\textsuperscript{+} channels, are the major class of ion channels in setting the membrane potential. In addition, it has been shown recently that Ca\textsuperscript{2+} entry in freshly isolated EC from rabbit aorta requires the presence of extracellular Cl\textsuperscript{−} to maintain a polarized membrane (433). Thus Cl\textsuperscript{−} channels, e.g., volume-regulated anion channels (VRAC), Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels (CaCC) and, interestingly, also cystic fibrosis transmembrane conductance regulator (CFTR) channels, recently detected in EC (388), might be important modulators of the inwardly driving force for Ca\textsuperscript{2+}. In addition, NSC may tune the membrane potential of resting and activated EC (178, 419).

A. NSC

Some NSC are obviously impermeable for Ca\textsuperscript{2+} (419). They may induce a negative feedback on Ca\textsuperscript{2+} entry by inducing depolarization of EC (see also Fig. 2). Little information is available at the molecular level. Probably, some TRP members might be candidates for background Ca\textsuperscript{2+}-impermeable NSC in resting endothelium.

Recently, a family of NSC has been cloned which includes channels activated by hyperpolarization and by cyclic nucleotides (HCN; Refs. 24, 25–27, 228). Four new members of this family with a length between 780 and >900 amino acids have been cloned recently. These channels consist of six transmembrane domains (TM) and are modulated by binding of cyclic nucleotides to a CNBD. TM4 is positively charged and contains at least 10 arginine and lysine residues. These channels show a high overall similarity with the cone CNG. They activate slowly at hyperpolarized potentials and binding of cyclic nucleotides. They are permeable for monovalent but not for divalent cations, Na\textsuperscript{+} being more permeable than K\textsuperscript{+} ($P_{Na}P_{K} \approx 0.15–0.3$). HCN channels play a role as mediators of NO- and cGMP-dependent cellular processes (25). Therefore, they might be of potential importance in EC. Indeed, in EC from the blood-brain barrier, a hyperpolarization-activated current carried by Na\textsuperscript{+} and K\textsuperscript{+} has been described (165) that is modulated by NO, cGMP, and cAMP. Its modulation by a cyclic guanosine nucleotide may also explain previous results about a role for NO in regulating blood-brain barrier function.

B. K\textsuperscript{+} Channels

1. Inwardly rectifying K\textsuperscript{+} channels (IRK or $K_{ir}$)

IRK is one of the most important channels for the control of the resting potential in nonstimulated cells. It conducts inward currents at potentials more negative than the K\textsuperscript{+} equilibrium potential but permits much smaller currents at potentials positive to that potential. However, this channel is quite heterogeneously expressed in different EC types. Some macrovascular EC express the inward rectifier channel (BPAEC, BAEC, coronary EC), others do not (microvascular EC, EA.hy926 cells), whereas in other cells only a fraction of the cells shows IRK activity (HUVEC, Ref. 290). In general, the channel appears to be more abundant in cultured than in primary EC. In intact endocardial endothelium, it is mainly confined to the luminal side of the cells (235), but it appears to be randomly distributed in monolayers of cultured BAEC (60). If present, this conductance together with the basal activity of the volume-regulated anion channel and a background NSC determine the resting membrane potential of EC (46, 96, 419). As discussed in section viiiA, the impact of these channels on the resting potential is often counteracted by that of Cl\textsuperscript{−} and background NSC channels.

In addition to its well-understood role in controlling electrogensis, IRK is a K\textsuperscript{+} sensor, as shown in Figure 8. A small increase in extracellular K\textsuperscript{+}, e.g., resulting from the activation of BK_{Ca} channels, shifts the reversal potential for K\textsuperscript{+} toward a more positive value and thereby increases the conductance of IRK. This enhanced IRK...
conductance is sufficient to overcome the depolarizing action of the nonselective cation and Cl\textsuperscript{−} conductance (see also Fig. 11) and shifts the membrane potential toward more negative potentials.

The conductance of single endothelial K\textsubscript{ir} channels ranges from 23 to 30 pS in symmetrical K\textsuperscript{+} solutions (84, 156, 180, 290, 295, 325, 372, 373). Typical features of this channel are the increase in single-channel conductance with the square root of the extracellular K\textsuperscript{+} concentration (289, 373, 462) and a permeation profile of P\textsubscript{Rb} > P\textsubscript{K} > P\textsubscript{Cs} (325, 373).

Extracellular Ba\textsuperscript{2+}, TEA, TBA, and Cs\textsuperscript{+} all block K\textsubscript{ir} (214, 322, 343, 419, 427). Extracellular Mg\textsuperscript{2+} causes a time-dependent block at negative potentials, which can be antagonized by extracellular K\textsuperscript{+}. At positive potentials, also intracellular Mg\textsuperscript{2+} induces a time- and voltage-dependent block (84).

The shear stress evoked hyperpolarization of EC has been attributed to an enhanced outward whole cell K\textsuperscript{+} current, which can be blocked by Cs\textsuperscript{+}. Shear stress also enhances the open probability of IRK channels in luminal cell-attached patches (312) and in inside-out patches (163).

IRK seems to be metabolically regulated. Intracellular ATP but not its nonhydrolyzable analogs adenosine 5′-O-(3-thiotriphosphate) (ATP\textsubscript{γS}) and adenylyl imidodiphosphate (AMP-PNP) prevent its rundown in whole cell mode. Reduction of intracellular ATP and induction of hypoxic conditions dramatically downregulate IRK (180). The phosphatase inhibitor okadaic acid also prevents rundown, but protamine, an activator of phosphatase 2A (PP2A), enhances the rate of rundown. Phosphorylation of the channel molecule therefore seems to be essential for maintaining its activity, its rundown probably being due to dephosphorylation by PP2A (180).

A similar inhibition has been observed if GTP\textsubscript{γS} is applied via the patch pipette (150, 180). This inhibition is probably due to activation of a pertussis toxin (PTX)-insensitive G protein, which may mediate these inhibitory actions by modulating PP2A.

IRK has been identified as Kir2.1, a member of the Kir family (95, 179). Two highly conserved transmembrane regions, consisting of 427 amino acids and a highly conserved TIGYG-H5 motif in the pore region, characterize this channel. pH insensitivity, which is also characteristic for the endothelial IRK, is conferred on the channel by a methionine at site 84 (M84). Mg\textsuperscript{2+} and spermine, spermidine, and putrescine block appears to be linked to D172. S425 in the COOH-terminal phosphorylation motif RRESEI, which is important for modulation of Kir2.1, might be a candidate for the observed modulation by phosphatases (180).

In addition to well-defined IRK channels, a number of poorly characterized K\textsuperscript{+} channels, apparently not belonging to any of the cloned subfamilies of K\textsuperscript{+} channels, have been observed in EC. An example is the 170-pS inward...
rectifier in aorta EC activated by isoprenaline, adenosine, forskolin, and membrane-permeable analogs of cAMP and inhibited by PKA inhibitors (117).

2. \(Ca^{2+}\)-activated \(K^+\) channels

The initial response to most EC stimuli is an increase in \([Ca^{2+}]_i\) that is often due to influx of \(Ca^{2+}\) and therefore depends on the electrochemical \(Ca^{2+}\) gradient, primarily set by the membrane potential. The depolarizing action of \(Ca^{2+}\) influx on the membrane potential is in various EC compensated by activation of \(Ca^{2+}\)-dependent \(K^+\)-channels (\(K_{Ca}\)), their increased open probability at elevated \([Ca^{2+}]_i\), causing membrane hyperpolarization. Different types of \(Ca^{2+}\)-activated \(K^+\) channels have been described, e.g., \(BK_{Ca}\) or maxi-K channels, \(IK_{Ca}\) or intermediate-conductance channels, and \(SK_{Ca}\) or small-conductance channels. However, the expression of these channels between different types of EC is also very heterogeneous (295).

High-conductance \(Ca^{2+}\)-activated \(K^+\) channels, \(BK_{Ca}\), are expressed in most freshly isolated EC and EC in primary culture. Their conductance ranges between 165 and 240 pS (129, 147, 176, 179, 220, 289, 349, 381). The fast elevation in \([Ca^{2+}]_i\) during stimulation with vasoactive agonists induces a fast increase in the probability of the channel being open. The open probability is enhanced at more positive potentials, suggesting that the apparent \(Ca^{2+}\) affinity of the channel is voltage dependent and that the open probability depends on both \([Ca^{2+}]_i\) and voltage. An increase in \([Ca^{2+}]_i\) shifts the voltage dependence of activation toward more negative potentials (13, 63, 129, 176, 317).

The endothelial \(BK_{Ca}\) is blocked by charybdotoxin (\(IC_{50} \approx 50\) nM), iberiotoxin, TEA (\(IC_{50} \approx 1\) mM, 100% block at 10 mM), d-tubocurarine, and quinine. Extracellular \(Mg^{2+}\) block is voltage dependent (13, 349, 350). The benzimidazolone compounds NS004 and NS1619 (in the \(\mu\)M range) increase the open probability of \(BK_{Ca}\) in EC and shift its activation curve toward more negative potentials (121, 316). Nanomolar amounts of intracellular dehydroasapoin (DHS-I) induce discrete episodes of high channel open probability interrupted by periods of apparently normal activity. DHS-I decreases the \(Ca^{2+}\) concentration for half-maximal activation of the channel (113, 247). \(BK_{Ca}\) activators may be functionally important, since it has been shown in many assays that inhibition of \(BK_{Ca}\) interferes with NO release (42). It has not yet been shown that cGMP-dependent kinases can activate \(BK_{Ca}\) in EC like in smooth muscle (166, 445).

\(Ca^{2+}\)-activated \(K^+\) channels in vascular smooth muscle cells are targets for various physiological factors released from the endothelium, including NO (31, 346, 384) and endothelium-derived hyperpolarizing factor (EDHF; probably a cytochrome \(P-450\)-derived arachidonic acid metabolite; Refs. 92, 334). This latter compound also exerts an autocrine effect on \(BK_{Ca}\) in inside-out patches of primary cultures of endothelial cells from pig coronary artery (12). A similar autocrine effect of NO release is however unlikely, since the NO donor S-nitrosoy cysteine does neither directly activate nor modulate \(BK_{Ca}\) channels activated by increased \([Ca^{2+}]_i\) in cultured endothelial cells (129). Interestingly, \(K^+\) efflux via endothelial \(BK_{Ca}\) might activate \(K^+\)-sensitive smooth muscle IRK channels and induce hyperpolarization. Thus \(K^+\) itself may act as EDHF (82).

\(BK_{Ca}\) has been identified by RT-PCR analysis as \(hslo\) in EA.hy926 cells (179). Mammalian slo channels, consisting of \(~1,200\) amino acids, share a high degree of homology with voltage-dependent \(K^+\) channels and consist of six transmembrane segments with a positively charged S4 segment. Five additional hydrophobic segments form a unique secondary structure in the COOH terminus of the mammalian slo channel. The origin of the voltage dependence is not completely clear, but \(hslo\) channels probably contain a \(Ca^{2+}\)-independent intrinsic voltage sensor (411). An extra segment (segment 0) in the NH\(_2\) terminal is probably a coupling site for the \(\beta\)-subunit. Four \(\beta\)-subunits have been cloned so far: \(\beta_1\) seems to be mainly expressed in smooth muscle cells; \(\beta_2\) in endocrine cells; \(\beta_3\) probably in epithelium, liver, pancreas, and intestinal tract; and \(\beta_4\) in the central nervous system (15). The unique COOH terminus possibly contains four additional helices, H7–H10, which represent almost 70% of the protein (80, 392). A 28-amino acid stretch between segment 9 and 10 contains highly conserved negatively charged amino acid residues that possibly form a highly selective \(Ca^{2+}\) binding site, the “\(Ca^{2+}\) bowl” (Fig. 9A, and Ref. 362). Expression of \(\alpha\)-\(hslo\) in EC that lack \(BK_{Ca}\) channels enhances the agonist-induced increase in \([Ca^{2+}]_i\) and shifts the membrane potential toward more negative values, a functional example in support of the role of the driving force for \(Ca^{2+}\) influx in EC. Figure 9B and C, shows an example of such an expression experiment in BPAEC, which does not express functional slo channels. The plateau phase of the \(Ca^{2+}\) transient in BPAEC cells transfected with \(hslo\) is much more pronounced than in non-transfected cells. This finding correlates with the hyperpolarizing action of vasoactive agonists in the transfected cells and indicates that activation of \(hslo-BK_{Ca}\) exerts a positive feedback on the \(Ca^{2+}\) signals by countering the negative (depolarizing) effect of \(Ca^{2+}\)-activated \(Cl^-\) channels (179).

The \(\beta\)-subunit of \(hslo\) could not be detected with RT-PCR methods in EC, which does not exclude the presence of other \(\beta\)-subunits. The \(BK_{Ca}\) opener DHS-I, which only works in the presence of the \(\beta\)-subunit, is ineffective in EC (248, 383), a functional indication for the absence of the \(\beta_1\)-subunit. Expression of the \(\beta\)-subunit in EA.hy926 cells induces a leftward shift of the activation curve and has a sensitizing effect on \(BK_{Ca}\) (316, 317).
Ryanodine-sensitive Ca\(^{2+}\) release during agonist stimulation is the main activator of BK\(_{\text{Ca}}\) in an umbilical vein-derived cell line, suggesting that these channels may form a subplasmalemmal complex with Ca\(^{2+}\) release units located in their close vicinity (100).

Intermediate-conductance, Ca\(^{2+}\)-activated K\(^{+}\)-channels, IK\(_{\text{Ca}}\), are inwardly rectifying and have a conductance between 30 and 80 pS in symmetrical K\(^{+}\) and 15 pS at physiological extracellular K\(^{+}\). IK\(_{\text{Ca}}\) are activated by Ins(1,4,5)P\(_3\)-sensitive Ca\(^{2+}\) release induced by agonists, such as bradykinin, acetylcholine, and ATP (355). ET-1 and ET-3 activate IK\(_{\text{Ca}}\) channels in brain microvascular endothelium via endothelin-A receptors (404). A Ca\(^{2+}\)-dependent 30-pS channel in cultured BAEC is regulated by G proteins (401). GTP together with Mg\(^{2+}\), as well as GTP\(\gamma\)S stimulates IK\(_{\text{Ca}}\) an effect that is reversed by guanosine 5’-O-(2-thiodiphosphate) (GDP\(\beta\)S). An agonist-activated IK\(_{\text{Ca}}\) with a single-channel conductance of 34 pS and a dissociation constant \((K_D)\) of 0.63 \(\mu\)M for Ca\(^{2+}\) has been described in intact endocardial cells (234).

The pharmacology of the endothelial IK\(_{\text{Ca}}\) is not very detailed. Charybdotoxin, quinine, and TBA are efficient blockers of IK\(_{\text{Ca}}\) channels (220, 355, 356, 404). Noxius-toxin, a toxin purified from Centruroides scorpion toxin (61, 396), and interestingly NS1619 (44), an activator of BK\(_{\text{Ca}}\) channels, are potent inhibitors of IK\(_{\text{Ca}}\) channels. The hydrophilic oxidative reagents 5,5\'-dithio-bis(2-nitrobenzoic acid) and thimerosal reduce IK\(_{\text{Ca}}\) channel activity in BAEC recorded in inside-out experiments but do not affect its unitary conductance. This activity is partly restored by the SH-group reducing agents dithiothreitol or reduced glutathione. The lipid-soluble oxidative agent 4,4\'-dithiodipyridine is less potent, suggesting that critical SH groups localized at the inner face of the cell membrane may be involved in channel gating (45).

The biophysical and pharmacological profiles of
these channels in EC are consistent with those of the recently cloned hIK (158, 168) and mIK (403) channel, which has an amino acid sequence related to, but distinct from, the six-TM helix, small-conductance SKCa channel subfamily (~50% conserved). Currents are inwardly rectifying, \( K_f \) for \( Ca^{2+} \) activation is 0.3 mM, and their single-channel conductance is 39 pS. The channels are reversibly blocked by charybdoxin (inhibitory constant \( = 2.5 \) nM) and clotrimazole, minimally affected by apamin, iberiotoxin, or ketoconazole (158). Importantly, this channel lacks putative \( Ca^{2+} \)-binding sites, but the proximal COOH terminus contains a \( Ca^{2+} \)-dependent calmodulin (CaM)-binding site that is probably associated with four CaM molecules (87, 190).

Small-conductance \( Ca^{2+} \)-activated \( K^+ \) channels, SKCa, with a conductance of ~10 pS in asymmetrical conditions have also been observed in EC (122, 265, 353). Unlike BKCa, these channels are not voltage sensitive. They are blocked by TBA, apamin, clotrimazole, and \( \alpha \)-tubocurarine (122). Recently, two types of SKCa channels were identified in excised patches of rat aortic EC with a conductance of 18 and 9 pS in symmetrical 150 mM K\(^+\), and 6.7 pS and 2.8 pS at physiological extracellular \( K^+ \) concentrations (241). The smaller one is completely blocked by 10 nM apamin and 100 \( \mu M \) \( \alpha \)-tubocurarine, and the larger one is insensitive to apamin but inhibited by charybdoxin at concentrations >50 nM. The topology of SKCa is similar to that of voltage-gated \( K^+ \) channels, exhibiting six TM helices and an intracellular COOH and NH\(_2\) terminus. Primary sequences, however, are very different. Likely, four subunits form the channel. No EF hand, C2A, or Ca\(^{2+}\) bowl motif indicative for \( Ca^{2+} \) binding has been found. Gating of SKCa involves a Ca\(^{2+}\)-independent interaction of calmodulin with the COOH-terminal domain of the \( \alpha \)-subunit and is mediated by binding of Ca\(^{2+}\) to the first and second EF hand motifs in the NH\(_2\)-terminal domain of CaM (187, 442).

3. ATP-sensitive \( K^+ \) channels, \( K_{ATP} \)

The reduced intracellular ATP content during ischemic/hypoxic conditions can be mimicked by intracellular dialysis with ATP-free solutions or application of extracellular glucose-free/NaCN solutions. Under these conditions, increased whole cell and single-channel currents have been observed in EC from rat aorta and brain microvessels. Currents are reversibly blocked by glibenclamide or ATP in inside-out patches and activated by the \( K_{ATP} \) channel opener pinacidil (164). Lowering intracellular ATP or applying the \( K^+ \) channel activator levcromakalim evokes unitary currents with a conductance of 25 pS in rabbit aortic EC. They are inhibited by glibenclamide or in inside-out patches by increasing the ATP concentration (184, 185). Low concentrations of the \( K^+ \) channel openers diazoxide and rilmakalim cause a pronounced glibenclamide-sensitive hyperpolarization in coronary capillary EC and induce a rapid \( Ca^{2+} \) transient followed by a sustained elevation of [\( Ca^{2+} \)]\(_i\). The high sensitivity to diazoxide has been interpreted as a novel type of \( K_{ATP} \) channel composed of a novel combination of \( K_r \) and sulfonylurea receptor (SUR) subunits (208). At present, Kir6.1 and Kir6.2 were detected in freshly isolated capillaries from guinea pig heart. It is not known whether Kir6.1 and/or Kir6.2 \( \alpha \)-subunits form the \( K_{ATP} \) channels together with the \( \beta \)-subunit SUR2B in EC (249).

Langheinrich et al. (207) have also reported a glibenclamide-sensitive hyperpolarization induced by \( K^+ \) channel openers and glucose deprivation in capillaries isolated from guinea pig hearts. Interestingly, evidence for a role of endothelial \( K_{ATP} \) channels in the flow- and shear stress-mediated vasodilation has been presented (152, 203). The endothelium-dependent vasodilatation of coronary microvessels in response to an abluminal increase in osmolarity is significantly attenuated by glibenclamide, but not by low concentrations of BaCl\(_2\) that inhibit the inward rectifier or by iberiotoxin, an inhibitor of \( K_n \) channels (159).

However, the contribution of \( K_{ATP} \) channels to EC function has been challenged in several reports (for a review see Ref. 295). Effects of \( K_{ATP} \) blockers or activators might instead be transmitted via myo-endothelial communication and originate from the vascular smooth muscle cells (264).

C. Amiloride-Sensitive \( Na^+ \) Channels

Amiloride-sensitive \( Na^+ \) channels are essential for controlling \( Na^+ \) and fluid transport in epithelia, but have also been described in endothelium (17). These channels form a superfamily consisting of two TM helices, a short intracellular \( NH_2 \) and COOH terminus, and a long extracellular loop comprising two cysteine-rich domains. They have been described in microvascular endothelium, where they might be connected with water transport through aquaporin-4, and in cornea endothelium (9, 254). However, the expression pattern and also the functional impact of these channels in endothelium are not clear at all.

D. \( Cl^- \) Channels

1. The volume-regulated anion channel, VRAC

Like most mammalian cells, EC also express anion channels, which are mainly permeable for \( Cl^- \) under physiological conditions and activated by classical “cell-swelling” protocols. They are referred to as volume-regulated anion channels (VRAC; Refs. 270, 278, 279, 308, 377). Cell shrinkage reduces the basal current in EC below its
value in control conditions, indicating that VRAC is partially activated in resting cells and therefore important for the electrogenesis of the resting potential in nonstimulated cells (419). VRAC is also activated by shear stress (11, 266).

The swelling-activated Cl\(^-\) current (\(I_{\text{Cl,swell}}\)) shows outward rectification and is time independent, except at potentials positive to \(+60\) mV where it slowly inactivates. An increase in volume but also a reduction of intracellular ionic strength or dialysis of EC with GTP\(\gamma\)S in isovolumic conditions are the main triggers for channel activation (281, 286, 423, 424). In hypotonic conditions, the current density of \(I_{\text{Cl,swell}}\) at \(+100\) mV ranges between 100 and 150 pA/pF in cultured endothelial cells (278). However, the density is much smaller (40 pA/pS) in freshly isolated EC pA/pF in cultured endothelial cells (278). However, the density is much smaller (40 pA/pS) in freshly isolated EC

\(\text{Mg}^{2+}\) is another blocker. Inactivation is accelerated by \(\text{Mg}^{2+}\) and a voltage-dependent recovery from inactivation. Its kinetic properties are modulated by extracellular divalent cations, extracellular pH, the permeating anion itself, and voltage-dependent open channel block by molecules, like calixarenes and suramin, which block VRAC channels but also amino acids and organic osmolytes permeate through the channel. The anion permeability sequence for VRAC is SCN\(^-\) > I\(^-\) > NO\(_3\) > Br\(^-\) > Cl\(^-\) > HCO\(_3\) > F\(^-\) > gluconate > glycine > taurine > aspartate, glutamate. The permeability sequence for amino acids is consistent with that for the rates at which these amino acids are lost from cells during hyposmotic swelling. The pore diameter estimated from the fit of the relative permeabilities of these anions to their Stoke’s diameter is \(\sim 11\) Å. A similar value has been obtained from the analysis of the voltage-dependent open channel block by molecules, like calixarenes and suramin, which block VRAC channels but also permeate at large driving forces (76, 297). VRAC is further characterized by inactivation at positive potentials and a voltage-dependent recovery from inactivation. Its kinetic properties are modulated by extracellular divalent cations, extracellular pH, the permeating anion itself, and various channel blockers. Inactivation is accelerated by acidic extracellular pH and by increasing the extracellular \(\text{Mg}^{2+}\) concentration (278, 308, 377, 421).

The mechanism of VRAC gating is a matter of extensive research. In most experimental approaches VRAC is activated by hyposmotic cell swelling, but it can also be activated in isovolumic conditions by reducing intracellular ionic strength at constant extracellular osmolality and intracellular Cl\(^-\) concentration, a possible interpretation being an influence of ionic strength on protein tyrosine kinases (424). Such a decrease in ionic strength is to be expected from the influx of water during cell swelling (286, 423). Because these conditions are rather nonphysiological, the isovolumic activation, e.g., by shear stress, may be more relevant for EC.

Intracellular dialysis with GTP\(\gamma\)S also activates VRAC without any increase in cell volume, an effect that is probably mediated via small G proteins (423, 424). A possible candidate is the GTP-hydrolyzing RhoA-GTPase, since VRAC is downregulated in cells pretreated with the p21RhoA inactivating \textit{Clostridium limosum} exoenzyme C3, and enhanced by cytotoxic necrosis factor (CNF1), a bacterial toxin that constitutively activates Rho GTPases. Inhibition of the RhoA-associated protein kinase, a possible downstream target of p21 RhoA, with the specific blocker Y-27632 also downregulates VRAC (277, 298). A tyrosine phosphorylation step downstream of the Rho-signaling cascade may be critical for the swelling-induced activation of the channel, since inhibitors of protein tyrosine kinases inhibit VRAC and the protein tyrosine phosphatase inhibitors Na\(_3\)VO\(_4\) and diphosphatidyl potentiate it. It has been shown recently that the tyrosine kinase p56lck mediates activation of the swelling-induced Cl\(^-\) currents in lymphocytes (212). Figure 10 shows a typical example for activation of VRAC and a tentative gating scheme of this channel.

Part of the activation cascade of VRAC seems to be coupled to specialized cytoskeletal structures. The annexin II-p11 complex, which is part of the cortical cytoskeleton and involved in the formation of caveolae, is important for activation of endothelial VRAC (280). The current density of VRAC correlates with the content of CAV-1 in various cell types. CAV-1 is the major protein in caveolae, and linked to many other signaling proteins (see sect. vuB3). In cells devoid of CAV-1, such as the cancer cells FRT, MCF-7, T47D, and CaCo2, VRAC has a very low current density (391), suggesting that targeting of VRAC to caveolae may provide a microdomain for activation.

The pharmacology of VRAC is quite extensively studied, and the search for more selective high-affinity blockers is still underway. There is a huge variety of compounds that inhibit VRAC with moderate affinity (EC\(_{50}\) between 1 and 1,000 \(\mu\)M) and specificity. Among these compounds are not only several classical Cl\(^-\) channel blockers, such as DIDS, SITS, N-phenylanthranilic acid (NPA), 9-aminoacamptothecic acid (9-AC), NPPB, and niflumic acid, but also several “surprising” substances, such as the P-glycoprotein inhibitors tamoxifen, 1,9-dideoxyforskolin, verapamil (281), quinine and quinidine (422), antiallergic drugs from the chromone family (134), Ins(3,4,5,6)P\(_4\) and Ins(1,4,5,6)P\(_4\) (285), the antihypertensive \(\text{Ca}^{2+}\) antagonist mibefradil (283), and the widely used antidepressant drug fluoxetine, supposed to be a selective serotonin (5-hydroxytryptamine) reuptake inhibitor (231). Most of these voltage-independent blockers are relatively hydrophobic aromatic compounds, which can easily permeate the cell membrane. It has been shown that quinine, quinidine, and fluoxetine, which can be both positively charged and neutral, exert their blocking effect at nanomolar concentrations of the neutral form, supporting the idea that these blockers interact with VRAC within the plasma membrane. Many of these blockers are propyramines with a -CF\(_3\) group, a group of compounds that
may comprise promising candidates for highly selective VRAC blockers (347). Additionally, phenol derivatives, such as gossypol, a polyphenolic pigment found in cotton plants, are generally rather potent blockers of VRAC (for a review, see Ref. 297).

In addition to these voltage-independent blockers, several compounds that contain sulfonyl groups have been shown to block VRAC almost exclusively at positive potentials. This group contains the classical Cl⁻ channel blockers DIDS and SITS, as well as the family of calix-arenes, para-substituted phenols conjugated by methylenes to form macrocyclic basket (calix)-like molecules. Calix[4]arenes induce a voltage-dependent inhibition of $I_{\text{Cl,swell}}$, probably by occluding the pore region at moderate positive potentials, but this block is relieved at more positive potentials where the negatively charged calix[4]arene permeates the channel. Lowering external pH potentiates the effect of calix[4]arenes, suggesting a role for positively charged residues, presumably histidine, within the channel pore. A similar voltage-dependent block can be induced by negatively charged compounds, including suramin, reactive blue (basilen blue), calix[6,8]-

FIG. 10. Activation of VRAC by cell swelling. A: time course of VRAC activation and deactivation at $-80$ mV in a BPAEC. The bar indicates the application of a solution with 25% lower osmolality than the control solution. This hypotonic challenge induces cell swelling (419). B: $I-V$ curves obtained at the time points 1 and 2 indicated in A. C: current traces under isotonic, hypotonic, or hypertonic conditions evoked by a step-voltage protocol. For all traces, a holding potential of 0 mV was used. Steps are 2 s long and range from $-80$ to $+100$ mV (increment of 20 mV). D: tentative gating scheme of VRAC, which is discussed in detail in the text and in References 275, 284, 286, 295, 296, 419.
arenes, and other compounds carrying sulfonic acidic sites. The voltage-dependent block of VRAC by ATP is probably also due to a similar mechanism. The blocking effect of these compounds is enhanced by lowering extracellular pH and at moderate positive membrane potentials. The relief of the blocking effect at positive potentials is only observed for compounds small enough to permeate the pore. Calix[6]arene and calix[8]arene are apparently not able to permeate the VRAC pore, from which a pore diameter between 1 and 1.6 nm has been estimated, a value in the same range as the values obtained above (76, 77).

The molecular nature of the VRAC channel is still unknown, and the putative candidates so far have been disputed. P-glycoprotein, the product of the multidrug resistance 1 gene which functions as a drug transporter and possibly as an anion channel, has been proposed as a putative candidate for VRAC, but now even its role as a regulator of VRAC is questioned.

A second candidate for VRAC is pICln, whereby n refers to a putative extracellular nucleotide-blocking site. Because the primary amino acid sequence of pICln lacks transmembrane helices, it has been proposed that the pICln “channel” is a homodimer consisting of four β-strands which form an eight-stranded, antiparallel β-barrel transmembrane pore similar to that of porins. The most compelling evidence for pICln being a plasma membrane channel is the presence of a GXGXG motif, being an essential component of the extracellular nucleotide binding site believed to be essential for the nucleotide sensitivity of the VRAC current. It has been reported that mutations of this site (G54A; G56A; G58A) prevent VRAC current sensitivity of the VRAC current. It has been reported that mutations of this site (G54A; G56A; G58A) prevent VRAC current. It has been reported that mutations of this site (G54A; G56A; G58A) prevent VRAC current.

CIC-3 is responsible for the swelling-activated Cl⁻ channel in EC.

CIC-2 is obviously volume sensitive, but its biophysical and pharmacological properties are completely different from those of VRAC. Also another candidate, the 72-amino acid intrinsic membrane protein phospholemman with a single membrane-spanning domain, is obviously not VRAC (detailed discussion in Ref. 278).

The enigmatic molecular nature of VRAC does however not prevent us from speculating about its possible functions in EC.

Because VRAC is a “housekeeping” Cl⁻ channel, its block induces changes in the membrane potential of EC. If these Cl⁻ channels are blocked, K⁺ channels may become dominant and strongly hyperpolarize the membrane with potentially beneficial effects on agonist or shear stress-induced Ca²⁺ influx. Block of Cl⁻ channels may therefore be a strongly underestimated tool to modulate the driving force for Ca²⁺ entry in EC and hence Ca²⁺ signaling (283, 297).

VRAC can also be activated by mechanical forces (shear stress, biaxial tensile stress) that induce changes in cell shape and possibly folding and unfolding of the plasma membrane (11, 266, 277, 278, 295, 297). This function might be especially important for EC that sense mechanical forces, a process in which a RhoA-mediated pathway may be involved (see sect. VI). Activation of RhoA inhibits myosin light-chain phosphatases (MLCP) via a Rho/Rho-associated kinase (ROK), a pathway that has been shown to modulate VRAC in cultured BPAEC. One of the downstream effects is an increased myosin light-chain phosphorylation, raising the possibility of a functional link between myosin light-chain phosphorylation and VRAC activation. Application of myosin light-chain kinase (MLCK) inhibitors, including AV25, a specific MLCK inhibitory peptide, inhibits preactivated VRAC. Cell dialysis with a specific inhibitory peptide of MLCP, NIPP1-(191-210), potentiates the activation of VRAC. These results indicate that myosin phosphorylation is a prerequisite for efficient activation of VRAC in BPAEC cells and might be involved in the mechano-sensing pathway of VRAC by connecting the gating machinery to the cytoskeleton (288).

VRAC is supposed to regulate EC proliferation. Chemically completely different compounds that inhibit VRAC also inhibit in the same concentration range serum-induced EC proliferation and [³H]thymidine incorporation (278, 279, 283, 425). As discussed in section VII E, VRAC blockers also inhibit angiogenesis. Interestingly, VRAC is downregulated if cells switch from proliferation to differentiation (237, 426), which may also explain the different current densities of VRAC in primary cultured and freshly isolated cells (381). Thus VRAC could assist in regulating EC growth and the transition from proliferation to differentiation.
In addition, VRAC may also contribute to the control of intracellular pH homeostasis (282, 351). Interestingly, activation of VRAC induces a decrease in intracellular pH (pHi) (282). A decrease in pHi stimulates prostanoid synthesis in EC from cerebral microvessel, which is possibly mediated directly via a protein phosphorylation-dependent mechanism involving protein tyrosine kinases and phosphatases as well as PKC. cPLA2 is the key enzyme affected by a decreased pHi (319). Therefore, this mechanism could also link channel activity (VRAC) to the production of vasoactive compounds.

2. Ca2+-dependent Cl− channels, CaCC or CLCA

The increase in [Ca2+]i during stimulation of EC depends on the cell type two distinct effects on membrane potential. In some EC, it is accompanied by a prominent hyperpolarization due to activation of Ca2+-dependent K+ channels, but in others, such as BPAEC, it evokes only small changes in membrane potential due to activation of Ca2+-dependent Cl− channels (CaCC; Refs. 123, 138, 179, 284, 287). These channels inactivate rapidly at negative potentials and activate slowly at positive potentials. Outward tail currents are slowly decaying, while inward tail currents decay much faster (284, 287). They show strong outward rectification. The permeability ratio for anions is I−:Cl−:gluconate = 1.7:10:4 (283). The single-channel conductance is ~7 pS at 300 mM extracellular Cl− but only ~3 pS at physiological intra- and extracellular Cl− concentrations (283, 287). Typical current densities of these Ca2+-activated Cl− currents range from 10–30 pA/pF at +100 mV and are thus much smaller than those of the volume-activated Cl− currents. The channel open probability is high at positive potentials, but very small at negative potentials. [Ca2+]i for half-maximal activation is voltage dependent and ranges from 400 to 600 nM at ~100 mV to 50–80 nM at +80 mV, pointing to high-affinity binding sites for Ca2+. Steady-state and kinetic behavior of this current can be described by a model which assumes activation of the channel by two identical, independent, sequential Ca2+ binding steps preceding a final Ca2+-independent transition from the closed to the open state of the channel. The putative binding site for Ca2+ is ~10–15% within the membrane electric field from the cytoplasmic side (7, 287).

Current activation requires intracellular ATP (438). NPA, DIDS, Zn2+, and calmodulin antagonists (123, 284, 287) block CaCC currents. Inhibition by DIDS and niflumic acid is voltage dependent and more potent at positive potentials. The block by NPA, NPPB, and tamoxifen is voltage independent. Niflumic acid and tamoxifen are the most potent blockers.

The molecular nature of this channel is not yet resolved. Recently, a number of related membrane proteins have been cloned including the endothelial adhesion protein Lu-ECAM, the bovine bCLCA1, murine mCLCA1, and human hCLCA1, -2, and -3 proteins that are believed to represent putative Cl− channels. Currents, showing some similarity with CaCC, have been observed in HEK cells expressing these proteins. These Ca2+-sensitive Cl− currents, activated by extremely high, nonphysiological concentrations of [Ca2+]i are outwardly rectifying and inhibited by DIDS, diisothiocetil, and niflumic acid. Cell-attached patch recordings of transfected cells reveal single channels with a slope conductance of 13.4 pS. These findings suggest that members of the CLCA family represent a Ca2+-activated Cl− conductance. Proteins of this family are characterized by a precursor of ~130 kDa consisting of between 900 and 940 amino acid residues. This precursor is cleaved to form heterodimers of ~90 and 35 kDa. The most likely topology is five TM with an extracellular glycosylated NH2 terminus, containing a number of conserved cysteine residues and an intracellular COOH terminus. The above-mentioned cleavage site is located in the intracellular loop between TM3 and TM4. The proteins contain several consensus sites for PK phosphorylation (105, 126–128). RT-PCR shows a high expression of mCLCA1 in mouse aorta EC. However, so far we failed to record CaCC-like currents in cells expressing mCLCA1 and bCLCA1 (287; B. Nilius and J. Papassotiriou, unpublished data).

A Ca2+-activated Cl− channel with properties clearly different from the above-described CaCC can be activated by a Ca2+/CaM-dependent protein kinase II. Interestingly, this channel is inhibited by Ins(3,4,5,6)P4, an endogenous inositol tetakisphosphate that also inhibits Ca2+-stimulated Cl− secretion. The effect seems to be specific for that particular isoform, since Ins(1,4,5,6)P4, Ins(1,3,4,5)P4, Ins(1,3,4,6)P4, and inositol 1,3,4,5,6-pentakisphosphate [Ins(1,3,4,5,6)P5] (the latter being the immediate precursor of Ins(3,4,5,6)P4) are all ineffective (444).

3. High-conductance Cl− channels

Occasionally, high-conductance Cl− channels (B3), which are obviously different from all other described Cl− channels, have been observed in EC. These channels are virtually silent in intact cells or in cell-attached patches, but become active in excised inside-out and outside-out patches. Their single-channel conductance ranges from 113 to 400 pS, and they exhibit several subconductance states, which probably refers to a multi-barrel structure (125, 310, 340, 395, 397). B3 is activated upon β-adrnergic stimulation in EC, which phosphorylates the channel and shifts its activation range toward more negative potentials (397). It is also activated by the antiestrogen tamoxifen and inhibited by 17β-estradiol. This effect has been discussed in relation to the protective effect of estrogens on several cardiovascular diseases (218). A prolonged increase in intracellular Ca2+, as well as inhibition
of PKC, seems to increase the incidence of the channel in cell-attached patches. Zn\(^{2+}\) from either side of the membrane blocks the channel (125). The physiological relevance of these channels is elusive.

4. CFTR in endothelium?

A multitude of \(\text{Cl}^{-}\) channel types has been characterized in EC, but until recently, no clear evidence for cAMP-stimulated CFTR channels had been presented. Tousson et al. (388) reported expression of CFTR in EC from umbilical vein and lung microvasculature at both the messenger and protein level, as well as functional expression from Cl\(^{-}\) efflux and whole cell patch-clamp data. cAMP-stimulated Cl\(^{-}\) currents in these cells were not blocked by DIDS, but sensitive to glibenclamide indicative of CFTR Cl\(^{-}\) channel activity.

CFTR channels have so far not been observed in cultured BPAEC cells (294), but a functional interaction with other Cl\(^{-}\) channels has been observed if they are heterologously expressed in these cells. Overexpression of CFTR downregulates VRAC in an activity-independent manner, but expression of the nonmaturating mutant ΔF508-CFTR does not. Because neither the single-channel conductance nor the kinetics of macroscopic currents are affected, CFTR probably downregulates the number of functional VRAC channels (410). Also CaCC is downregulated by CFTR, but this effect is potentiated by a cAMP-mediated activation of CFTR (439). A functional role for CFTR in EC biology is not clear, but like in epithelial cells, it could be involved in transendothelial transport and act as a regulator of other channels or transporters. Interestingly, CFTR has been associated with ATP transport (169, 367, 368). ATP and its metabolites, such as adenosine, are important autacoid and paracrine substances that affect many functions of the vessel wall (273).

E. Conclusion

The membrane potential of EC is modulated by a variety of ion channels. \(\text{K}^{+}\) channels, which are constitutively open (Kir type channels) or which are activated during EC stimulation (BK\(_{\text{Ca}}\), IK\(_{\text{ca}}\), SK\(_{\text{Ca}}\), K\(_{\text{ATP}}\)), induce hyperpolarization. Interestingly, EC express also a variety of Cl\(^{-}\) channels (VRAC, CLCA, CFTR) whose inhibition can also induce EC hyperpolarization. Activation of these channels during EC stimulation may clamp the EC membrane potential at negative potentials close to the equilibrium potential for Cl\(^{-}\) (\(E_{\text{Cl}}\)). Importantly, \(\text{K}^{+}\) and Cl\(^{-}\) channels interacting with cation channels induce sometimes dramatic stepwise or oscillatory changes in the EC membrane potential of up to 80 mV. Channels regulating EC electrogenesis or inducing changes in the membrane potential due to hormonal, neuronal, and mechanical stimuli importantly influence several mechanisms. 1) They regulate the driving force for Ca\(^{2+}\) entry via several pathways and add positive and negative feedback mechanisms to the numerous regulation mechanisms inherent to EC for controlling Ca\(^{2+}\) influx. 2) They might be the main players in transducing electrical signals such as flow-dependent hyperpolarization to neighboring EC and smooth muscle cells (SMC), thereby regulating the functional state in blood vessels upstream and downstream of sites of EC activation. 3) They control the driving force for several electrogenic transporters such as NCX. 4) An increasing number of events in EC are likely dependent on the polarization state of EC without interference of Ca\(^{2+}\), e.g., depolarization-induced superoxide formation (see sect. viiF). 5) Some channels, such as VRAC, might be important, in addition to regulation of electrogenesis, for the transport of amino acids, organic osmolytes, and HCO\(_{3}^{-}\), which is important for regulation of the intracellular pH in EC.

V. VOLTAGE-DEPENDENT ION CHANNELS IN ENDOTHELIUM?

EC are nonexcitable, and it is therefore difficult to reconcile the presence of voltage-gated ion channels with the slow and often small changes in membrane potential in these cells. Several reports have nevertheless clearly shown the incidence of voltage-gated ion channels in both cultured and freshly isolated EC. Voltage-activated Na\(^{+}\) channels with a low tetrodotoxin and high saxitoxin sensitivity have been observed in EC from human umbilical vein, in rat interlobar arterial EC, and in microvascular EC (114, 429). These currents are also present in primary cultures of microvascular EC, however at a rather small density (2.1 pA/pF at 0 mV). At the normal resting potential of EC, ~50% or more of these channels will be inactivated. Stimulation of PKC increases this current without any change in its voltage dependence (429).

Voltage-gated Ca\(^{2+}\) channels, which share some similarities with the classical L- and T-type Ca\(^{2+}\) channels, have been described in freshly dissociated capillary EC from bovine adrenal glands (34–36, 417). Their voltage dependence, kinetics, responses to BAY K 8644, nifedipine, amiloride, and Cd\(^{2+}\), and their different selectivity for Ba\(^{2+}\) and Ca\(^{2+}\) indicate that the observed currents are typical T and L channels resembling those of endocrine secretory cells (417). The “T-type” Ca\(^{2+}\) channels have a conductance of ~8 pS and are involved in depolarization-induced Ca\(^{2+}\) transients, which may suggest a role in endothelial Ca\(^{2+}\) signaling. L-type Ca\(^{2+}\) channels in these EC have a single-channel conductance of ~20 pS and are sensitive to dihydropyridines. In addition to these two voltage-dependent Ca\(^{2+}\) channels, a tiny conductance channel (SB Ca\(^{2+}\) channel, 2.8 pS in 110 mM Ba\(^{2+}\)) has been observed in capillary EC. It is sensitive to the Ca\(^{2+}\)
agonist BAY K 8644, but not to the Ca\(^{2+}\) antagonist nicardipine (35). This channel shows long openings at negative potentials and might provide a low-threshold Ca\(^{2+}\) entry pathway. A novel type of voltage-dependent Ca\(^{2+}\) channel, the R-type Ca\(^{2+}\) channel, has been proposed to be important during activation of EC by PAF (30). This channel, which is activated by a long-lasting depolarization and does not inactivate at depolarized potentials, has also been proposed as a possible candidate for a sustained Ca\(^{2+}\) influx.

A hyperpolarization-activated, nonselective cation channel that carries K\(^{+}\) and Na\(^{+}\) and is modulated by NO has been described in vascular brain EC (165), but its physiological role is completely unknown. A 4-aminopyridine-sensitive, voltage-dependent, transient outward current (A-type K\(^{+}\) channel) has been described in cultured EC (173, 382). In freshly isolated EC from resistance vessels, a delayed rectifier K\(^{+}\) current has been observed that is probably Kv1.5 and might be involved in release of vasoactive compounds (143). Dittrich et al. (72) describe a voltage-dependent current in freshly isolated EC from heart capillaries that activates at potentials positive to −20 mV and inactivates much slower than A currents at positive potentials. This current is blocked by TEA but is insensitive to charybdotoxin and apamin. This 12-pS channel is different from HERG channels or Shaker-type K\(^{+}\) channels. The functional role of this channel, supposed to be involved in the generation of oscillations in membrane potential and [Ca\(^{2+}\)]\(_i\), remains hypothetical.

VI. MECHANOSENSITIVE CHANNELS IN ENDOTHELIUM

EC are notorious for their ability to sense mechanical forces, such as biaxial tensile stress (stretch, pressure), a force component mainly vertical to the EC surface, and shear stress, a tangential force due to blood flow (for detailed reviews, see Ref. 211). Many endothelial responses are modulated by changes in blood flow and blood pressure: 1) the secretion of prostacyclin (PGI\(_2\)) and endothelium-derived relaxing factor (EDRF; and NO); 2) the expression of genes and proteins, such as tPA, plasminogen-activator inhibitor (PAI-1), tissue factor (TF), several adhesion molecules (VCAM-1, the intercellular adhesion protein-1), growth inhibitors (heparin), growth factors (platelet-derived growth factor, PDGF), endothelin, monocyte-chemoattractant protein (MCP-1), NO synthase, and activation of early response genes and small G proteins; 3) cell cycle entry; and 4) cytoskeletal rearrangement, long-term responses, such as adaptive changes in cytoskeleton, vessel remodeling, and others (64, 66, 232).

Shear stress also upregulates expression of the connexin (Cx) 43 (70, 69, 104, 182, 205).

Some mechanisms, such as the mechanically induced stimulation of DNA binding activities of nuclear factor κB (NFκB) and the nuclear factor activator protein-1 (AP-1, AP-2) (206), have been elucidated. Shear stress can directly activate cis-elements of different genes, e.g., the shear stress response element in the PDGF-B gene and the tPA-responsive element in the MCP-1 gene, and can stimulate the GC-rich transcription-initiation site of the specificity protein 1 (Sp-1) in the TF gene.

Protein kinases appear to be involved in an early step of mechanosensing. Shear stress induces a fast tyrosine phosphorylation at the luminal side of EC, primarily in caveolae and also supports translocation of signaling proteins to caveolae (345). Gene activation is modulated by a complex network of protein kinases (JNK, ERK, focal adhesion kinases FAK, c-src), which might be affected by changes in the ionic conditions and [Ca\(^{2+}\)]\(_i\). (23, 53–56).

Changes in [Ca\(^{2+}\)]\(_i\) are also important for the regulation of gene expression (189, 217), since the shear stress-mediated Ca\(^{2+}\) responses activate protein kinases, e.g., the functionally important upregulation of eNOS in response to shear stress depends on an initial Ca\(^{2+}\)-independent phosphorylation step (93). The initial fast release of NO in response to mechanical forces is therefore Ca\(^{2+}\)-dependent, whereas the sustained NO production is Ca\(^{2+}\)-independent (42, 71, 93, 263).

Receptor tyrosine kinases and integrins can serve as mechanosensors (e.g., the vitronectin receptor avb3) by acting through several kinases including FAK. Interestingly, shear stress induces the translocation of Cdc42 and Rho from the cytosol to the plasma membrane, a mechanism that might be also involved in VRAC gating (see sect. νCl and Refs. 53, 216). An intriguing example for the role of tyrosine phosphorylation and the interference of [Ca\(^{2+}\)]\(_i\) is the role of the p130 Crk-associated substrate (Cas), a c-Src substrate, which has been identified as a highly phosphorylated protein localized to focal adhesions and acting as an adapter protein. Cas is important for actin filament assembly and is tyrosine-phosphorylated by shear stress via activation of the Ca\(^{2+}\)-dependent c-Src. This involvement of Ca\(^{2+}\) in activation of c-Src and tyrosine phosphorylation of Cas defines a novel mechanosensing mechanism (309).

Ion channels may serve as effective mechanosensors to convert mechanical forces into electrical responses. It is not clear how ion channels may be directly involved in the responses to mechanical forces. Obviously, changes in [Ca\(^{2+}\)]\(_i\) might again be an important transmitter of the mechanical signal. We therefore first discuss the mechanically induced changes in [Ca\(^{2+}\)]\(_i\) and afterward the channels responding to mechanical forces. VRAC or VRAC-like channels may also belong to this class of channels because they can be obviously activated by shear stress and membrane stretch, induced by positive pressure in the patch pipette.
A. Mechanically Induced Ca$^{2+}$ Entry

Mechanostimulation of isolated EC by shear stress, stretch, or swelling in Ca$^{2+}$-free solutions induces an increase in [Ca$^{2+}$]; (206, 305). Acute changes in flow also induce Ca$^{2+}$ release from Ins(1,4,5)P$_3$-sensitive pools and a subsequent NO release (153). Pretreatment of EC with thapsigargin attenuates this Ca$^{2+}$ release, indicating that the mechanical stimulus induces a release of Ca$^{2+}$ from Ins(1,4,5)P$_3$-sensitive stores. However, neither the PLC pathway nor Ins(1,4,5)P$_3$ are involved, since the PLC inhibitor neomycin and the Ins(1,4,5)P$_3$ receptor antagonist heparin do not block this release. Also the PKA inhibitory peptide as well as cyclooxygenase and lipoxygenase inhibition is ineffective (305). Because PLA$_2$ blockers inhibit the release and arachidonic acid mimics the effect of mechanostimulation even in the presence of heparin and PLA$_2$ blockers, it has been proposed that the mechanically induced release of Ca$^{2+}$ is mediated via production of arachidonic acid.

The depletion of endothelial Ins(1,4,5)P$_3$-sensitive Ca$^{2+}$ stores by mechanical stimulation may also activate Ca$^{2+}$ entry via store-operated Ca$^{2+}$ entry channels (269, 305).

Recently, a mechanosensitive Ca$^{2+}$-permeable channel with a relative permeability of $P_{Na} / P_{Ca}$ = 5:1:1 has been described in freshly isolated EC from rat aorta and also in the ECV304 cell line (451). This channel is activated by stretch (negative pressure in inside-out patches), but its activity correlates with a shear stress-induced Ca$^{2+}$ signal. Single-channel conductance is 32 pS for monovalent cations and 9 pS for Ca$^{2+}$. This channel is inhibited by PKG, initiating a negative feedback by activation of eNOS.

1. Channels activated by tensile stress

Membrane stretch induced by negative pressure applied via the patch pipette to cell-attached patches on EC activates a channel that is permeable for monovalent cations ($\approx 50$ pS) and Ca$^{2+}$ (19 pS) (209). Stretch-activated NSC with a conductance of 20–30 pS for monovalent cations and 10–20 pS for Ca$^{2+}$ and Br$^{2-}$ have also been described in endocardial endothelium and microvascular EC (147, 335). Activation of these channels induces an increase in [Ca$^{2+}$], that is sufficient to activate BK$_{Ca}$ and to hyperpolarize the membrane. Interestingly, BK$_{Ca}$ itself is sensitized to [Ca$^{2+}$] when stretch (negative pressure) is applied to inside-out patches (148, 195, 233). Hoyer et al. (149) have described a K$^{+}$-selective stretch-activated channel in rat intact aortic endothelium and isolated aortic EC with a similar permeability for K$^+$ and Na$^+$. Its single-channel conductance is 22 pS for K$^+$ and Na$^+$ and 4 pS for Ca$^{2+}$. Channel activity is clearly pressure dependent in a physiological range and disappears during application of 20 $\mu$M gadolinium. Opening of the pressure-activated cation channel (PAC) is followed by the opening of a Ca$^{2+}$-dependent NSC, indicating that Ca$^{2+}$ influx through PAC may be sufficient to increase intracellular Ca$^{2+}$ and to activate thereby neighboring NSCs (see sect. mA). This channel is upregulated in renovascular hypertension and in spontaneous hypertensive rats (195). Marchenko and Sage (242, 243) described a similar pressure-activated NSC with a conductance of 34 pS for monovalent cations and 6 pS for Ca$^{2+}$, which provides an influx pathway for Ca$^{2+}$. Indirect evidence for a functional role of these stretch-activated channels has been obtained from experiments in which Ga$^{3+}$, an efficient blocker of mechanosensitive channels, has been used to inhibit NO synthesis (379).

Although not directly activated by biaxial tension, voltage-dependent K$^+$ channels are upregulated by application of static and cyclic stretch, indicating a role of these channels in mechanotransduction of cardiac microvascular EC (86).

2. Channels activated by shear stress

Shear stress-induced activation of endothelial ion channels represents one of the earliest responses in mechanosignal transduction, which is important for the regulation of vascular tone. Several examples of ion channels directly activated by shear stress have been reported, although most experimental conditions cannot exclude a stretch component. It is therefore sometimes difficult to distinguish between these two forces and their role in channel activation.

HAEC respond to flow with Ca$^{2+}$ entry, activation of NSC, Cl$^{-}$ channels, and Ca$^{2+}$-activated K$^+$ channels. Conversely, similar flow rates do not affect human capillary EC. The increase in [Ca$^{2+}$] in macrovascular EC depends on the presence of extracellular Ca$^{2+}$ (171), but it is not clear which entry channels are responsible for these changes in [Ca$^{2+}$]. A possible candidate is a shear stress-activated NSC, which is slightly more permeable for Ca$^{2+}$ than Na$^+$, and reversibly blocked by La$^{3+}$. It is insensitive to activators of protein kinase and inhibited by nonsteroidal anti-inflammatory drugs, such as mfenamic acid (266, 364, 365).

Other channels, modulated by shear stress, may regulate the driving force for Ca$^{2+}$. However, these channels can only be effective if at the same time a Ca$^{2+}$ pathway is activated, e.g., if mechanical stimulation opens CCE channels (305, 306). Changes in [Ca$^{2+}$] in human aorta EC are modulated by shear stress-activated Cl$^{-}$ channels, most likely VRAC. In BAEC, flow induces an initial hyperpolarization, which is followed by a sustained depolarization, caused by activation of Cl$^{-}$ channels. Thus shear stress-dependent Cl$^{-}$ channels may be important modulators of endothelial mechanosensitivity (11, 266). Changes in shear stress due to pulsatile flow or by varying
the viscosity activate SKCa and BKCa channels (64, 65, 152). Opening of these K+ channels induces hyperpolarization, which may increase Ca2+ influx and NO release, and also modulate the membrane potential in smooth muscle cells electrically coupled to the endothelium (63, 64, 295). An inwardly rectifying, 30-pS K+ channel has been described as a possible mechanosensor of shear stress (163, 312). Its single-channel conductance, its degree of rectification, and its Ca2+ sensitivity in excised patches suggest that this channel may belong to the class of SKCa channels. These channels share some properties with ROMK-like channels, and the previously described Ca2+- and GTP-modulated inwardly rectifying K+ channels (301, 302, 311, 401), but clearly differ from the classical IRK channels (see sect. ivA1). PTX-sensitive mechanisms, cGMP, and NO seem to be involved in the activation of shear stress-dependent K+ channels (133, 302). The observation that various blockers of K+ channels reduce shear stress-mediated release of NO provides indirect evidence for the involvement of these channels (5, 70, 376).

Another mechanosensitive ion channel participating in EC responses to shear stress is surprisingly a voltage-gated Na+ channel (see sect. v). The activation of the shear stress-mediated extracellular signal-regulated kinase (ERK1/2) is potentiated by substituting extracellular [Na+] with N-methyl-D-glucamine (NMDG+) and by the voltage-gated Na+ channel antagonist tetrodotoxin. Evidence for the expression of the Na+ channel genes SCN4a and SCN8a α-subunit genes in these EC has been obtained from RT-PCR analysis and is supported by Western blotting of a 250-kDa protein with Na+ channel antibodies (390). These results may indicate that Na+ channels are involved in regulation of shear stress-mediated ERK1/2 activation. Because of the low membrane potential and the slow changes in membrane polarization, it is difficult to reconcile this interpretation with activation of voltage-dependent Na+ channels.

B. Conclusion

A variety of mechanosensitive ion channels have been identified at the functional level in various types of EC. However, none of them has been identified at the molecular level. It is also difficult to identify such mechanosensitive channels with already described types of ion channels. Mechano- sensitivity is inherent to several types of ion channels among which Ca2+-permeable cation channels that provide Ca2+-entry pathways in EC, different types of K+ channels, and also Cl− channels. Therefore, mechanical forces modulate not only Ca2+-entry, but the membrane potential is also a target of the mechano-sensing machinery of EC. The signal transduction between mechanical stimulus and gating or modulation of the respective ion channels is not yet understood.

VII. GAP JUNCTIONAL CHANNELS

Gap junctions are agglomerates of cell-to-cell channels that allow direct electrical and metabolic communication between endothelial cells, between EC and SMC, and also between EC and lymphocytes/monocytes (67). An extensive description of EC-EC, EC-SMC, and EC-blood cell coupling is beyond the scope of this review. In short, coupling of EC consists of clublike protrusions, which contact neighboring smooth muscle cells through the basement membrane (344). The large variability of functional low-resistance cell-cell connections may depend on the presence of various isoforms of connexins. At least three isoforms (Cx37, -40, -43) are expressed in EC and may give rise to heterogeneous expression patterns. Cx43 is more abundant in macrovascular than in microvascular cells. Aortic and pulmonary arterial endothelia express all three types of connexin, whereas coronary artery endothelium expresses Cx40 and Cx37 but lacks Cx43 (68, 221, 453). Functional channels can be formed between the same or different isoforms. Interaction does not occur between the Cx40 and Cx43 isoforms, but possibly between Cx40 or Cx43 and Cx37 (40). Cx40 is prominent in cultured EC. The single gap junctional channel conductance in HUVEC is in the range of 19–75 pS, but in cultured human aorta EC, an additional conductance of 80–200 pS has been observed. The latter may possibly reflect the activity of Cx40 isoform channels, which are more abundant in cultured arterial endothelial cells (405). Expression of connexins depends on the functional state of EC and is modulated by growth factors, by factors that are present during inflammation (TNF-α), and by mechanical forces (68, 326, 327, 443). Connexins play an important role in the regulation of inflammatory responses with a main target on EC, including cell extravasation and changes in vessel permeability. The normal expression pattern of connexins is disturbed in the presence of TNF-α, a major regulator of inflammatory reactions, which downregulates Cx37 and Cx40 expression, but does not alter that of Cx43, and attenuates EC coupling (406). The electrical coupling between EC is the morphological substrate for the electrotonic spread of electrical signals along the vessel wall. Electrical coupling via high-conductance gap junctional channels between EC and smooth muscle cells may be functionally important in EC of small-terminal arterioles where a large total EC surface contacts a much smaller smooth muscle surface and allows an efficient modulation of the smooth muscle membrane potential by the EC (20, 63, 67). In contrast, other results show that the agonist-induced hyperpolarization
of EC does not spread electrotonically to smooth muscle cells, but rather the opposite happens, i.e., an electrotonic spread from smooth muscle to the lining EC (18–20).

VIII. FUNCTIONAL ROLE OF ION CHANNELS IN ENDOTHELIUM

A. Ion Channels and Electrogenesis in EC

The membrane potential of vascular endothelial cells is negative compared with the blood and tissue compartment. Its value, as well as that of cell capacitance and input resistance, varies considerably between different cell types and conditions of cell isolation and culturing. Resting membrane potentials between 0 and −80 mV, membrane capacitances between 30 and 80 pF, and input resistances between 1 and 10 GΩ have been reported for isolated, nonconfluent EC. Confluent cells have a higher membrane capacitance (up to 160 pF) and a lower input resistance 0.01–0.4 GΩ. Although these latter values are uncertain, they are consistent with the existence of intercellular electrical coupling via gap junctions. The membrane potential also depends on the EC type and is in general more negative in macrovascular than in microvascular EC (63, 295, 449, 462).

The main determinant of the resting potential in most cell types is a basal K+ conductance. However, the expression of K+ channels varies greatly between different EC types and even within the same strain of cultured EC. For example, inwardly rectifying K+ channels (IRK) are functional in some macrovascular EC (137, 138, 180, 290, 295) but seem to be preferentially expressed in cultured cells and absent in freshly isolated cells (349, 381). They have also been reported to be either present (427) or absent in freshly isolated coronary microvascular EC (72). This heterogeneous expression may contribute to the highly variable resting potential of EC.

The resting membrane potential in several endothelial cell types has a bimodal distribution, consisting of cells with a resting potential between −70 and −60 mV that is mainly controlled by IRK (K+–type EC) and cells in which the Cl− conductance is dominating (Cl−–type EC) with a resting potential between −40 and −10 mV. The latter values are close to $E_{CB}$, suggesting that a Cl− conductance is activated under resting conditions. This conductance, which is suppressed by cell shrinkage, apparently represents basal activity of swelling-induced Cl− channels, as described above. This bimodal distribution may be explained by the N-shaped current-voltage relationship observed in these cells (274, 419). In addition, the hyperpolarization induced by decreased extracellular Na+ is consistent with a contribution of either a Na+-selective or a nonselective cation conductance to the resting permeability. The relative contributions of the Na+, K+, and Cl− conductance to the resting potential range from 3 to 30%, 27 to 95%, and 9 to 35% (138, 240, 295, 407, 419). Figure 11 gives an example for the electrogenesis of the membrane potential in a “resting,” nonstimulated macrovascular BPAEC.

The hyperpolarization, induced by vasoactive stimuli, such as acetylcholine, bradykinin, and histamine, in cells that do not express inwardly rectifying K+ channels is mediated by the activation of Ca2+-dependent K+ currents and is often followed by a sustained depolarization due to activation of NSC (63, 177, 225, 239, 250, 274). Figure 12 shows an example of stimulation-dependent changes in membrane potential that are a mirror image of the concomitant changes in [Ca2+]. The electrogenic Na+-K+-ATPase contributes approximately −8 mV to the resting potential of EC (63, 303, 370). Although a NCX has...

![Image](http://physrev.physiology.org/Downloadedfromhttp://physrev.physiology.org/.../1440)
1. Ca$^{2+}$ pattern, waves, and release sites

Two phenotypes of Ca$^{2+}$ signals can be distinguished, i.e., [Ca$^{2+}$]$_{i}$ oscillations or a biphasic increase in [Ca$^{2+}$]$_{i}$, consisting of a fast peak followed by a long-lasting plateau (see Figs. 1 and 12 and Refs. 161, 162, 272, 295, 296). Low concentrations of various agonists, such as acetylcholine, ATP, histamine, and bradykinin, often induce oscillations of [Ca$^{2+}$]$_{i}$. These oscillations appear only in a small window of agonist concentrations (162, 304), whereas a biphasic rise in [Ca$^{2+}$]$_{i}$ prevails at higher concentrations (144, 271, 272, 291, 295, 296). The fast transient Ca$^{2+}$ peak represents release of Ca$^{2+}$ from InsP$_3$-sensitive intracellular Ca$^{2+}$ stores, whereas Ca$^{2+}$ influx from the extracellular space activated by store depletion accounts for the plateau-like phase. Plasmalemmal ion channels provide an influx route for Ca$^{2+}$ and tune the inwardly driving force for Ca$^{2+}$ influx (271, 272, 295, 318). The sustained plateau level can be further modulated at a constant driving force for Ca$^{2+}$ by mitochondrial Ca$^{2+}$ buffering, Na$^+$/Ca$^{2+}$ exchange, PMCA, and SERCA (predominantly SERCA-3; Refs. 222, 262).

Application of vasoactive agonists can also induce Ca$^{2+}$ waves that travel through the cell at a speed of 5–60 $\mu$m/s depending on the agonist concentration and cellular region (151, 255). Ca$^{2+}$ waves are regenerative, often arise at peripheral cytoplasmic processes, and propagate from the cell in which they originate to neighboring cells in the cell cluster without decrement of amplitude, kinetics, and speed of propagation (74, 151). These waves spread through gap junctions, mainly consisting of Cx40 and -43 (221). Regenerative propagation depends on PLC and likely also on the presence of calcium-induced calcium release (CICR). Extracellular Ca$^{2+}$ is required, which points to a role of Ca$^{2+}$ entry channels in wave propagation (74). Activation of a Ca$^{2+}$ transient and wave is preceded by small highly localized increases in [Ca$^{2+}$]$_{i}$ that originate from clusters of elementary Ca$^{2+}$ release events (blips). The amplitude of these blips of $\sim$23 nM corresponds to a Ca$^{2+}$ current of 0.3 pA and is consistent with a current through a single or a small number of InsP$_3$-sensitive release channels (151). The expression pattern of SERCA and inositol 1,4,5-trisphosphate receptor (InsP$_3$R) isoforms in the endothelial endoplasmic reticulum varies between different cell types. Most EC express only SERCA-3. During cell differentiation, the expression of InsP$_3$R-1 decreases, whereas that of InsP$_3$R-3 increases (262). Isshiki et al. (160) reported that the initial rise in [Ca$^{2+}$]$_{i}$ and Ca$^{2+}$ wave in EC stimulated with agonists originate at peripheral loci enriched in caveolin, suggesting that caveolae may be involved in the initiation of Ca$^{2+}$ waves. In addition, Ca$^{2+}$ stores in EC might also be compartmentalized, since Ca$^{2+}$ signals during submaximal stimulation were restricted to a subplasmalemmal region, which probably reflects functional units consist-
ing of superficial endoplasmic reticulum in close contact with the plasma membrane, such as caveolae and membrane near mitochondria. These restricted Ca\textsuperscript{2+} signals induce local subplasmalemmal Ca\textsuperscript{2+} gradients. According to Graier et al. (118), release from ryanodine-sensitive Ca\textsuperscript{2+} stores contributes to these local gradients.

The most important release sites in EC are Ins(1,4,5)P\textsubscript{3}-sensitive Ca\textsuperscript{2+} stores, and the release channel is most likely InsP\textsubscript{3}-R-3 (262). Other release sites, clearly distinct from mitochondria and from stores sensitive to ryanodine or Ins(1,4,5)P\textsubscript{3}, seem to respond to mechanical forces, such as stretch and shear stress (167). The intracellular messenger responsible for this mechanosensitive release may be an arachidonic derivative (305). The pharmacological properties of the ryanodine receptors mediating CICR in EC (213, 432) are clearly different from the CICR mechanism in other cell types. Ruthenium red inhibits and caffeine induces only a small release. The ryanodine-induced Ca\textsuperscript{2+} release is also different from the ruthenium red-blocked release, suggesting the existence of a ruthenium red-sensitive but ryanodine-insensitive CICR mechanism. CICR may operate to amplify the magnitude of the Ins(1,4,5)P\textsubscript{3} response (313, 315, 350, 432, 454). A mitochondrial CICR mechanism, insensitive to ryanodine and caffeine and blocked by cyclosporin A, an inhibitor of the mitochondrial permeability transition pore, has been described recently (440).

Equally important for Ca\textsuperscript{2+} signaling is the refilling of Ca\textsuperscript{2+} stores. It probably occurs via the SERCA-3 isoform of the endoplasmic Ca\textsuperscript{2+}-ATPase, which is abundantly expressed in EC. Because the acetylcholine-induced endothelium-dependent relaxation of aorta precontracted with phenylephrine was attenuated in SERCA-3-deficient mouse, it has been concluded that SERCA-3 plays a role in EC Ca\textsuperscript{2+} signaling involved in NO-mediated relaxation of vascular smooth muscle (222).

2. Ca\textsuperscript{2+} oscillations

EC exhibit oscillations in [Ca\textsuperscript{2+}], if stimulated with low agonist concentrations in a narrow concentration window (see Fig. 12 and Refs. 162, 272, 295, 296, 304). Synchronization of these Ca\textsuperscript{2+} oscillations in confluent EC depends on cell-cell coupling (268). These oscillations are frequently accompanied by oscillatory changes in membrane potential due to activation of BK\textsubscript{ca} channels (272, 295, 296, 314, 415). These oscillations are mainly due to periodic discharges of intracellular Ca\textsuperscript{2+} stores, since they are sustained for a long time in cells exposed to Ca\textsuperscript{2+}-free media (162, 272) and apparently not via activation of a Ca\textsuperscript{2+}-influx pathway since they are not affected by changes in the driving force for Ca\textsuperscript{2+} (272). It is therefore likely that low agonist concentrations do not activate Ca\textsuperscript{2+} entry channels and that these channels are not involved in Ca\textsuperscript{2+} oscillations. However, [Ca\textsuperscript{2+}] oscillations in EA.hy926 cells seem to depend on Na\textsuperscript{+} loading via NSC and Ca\textsuperscript{2+} entry via the endothelial NCX, a mechanism that also depends on membrane hyperpolarization via activation of BK\textsubscript{ca} channels (314). However, in the same cell type, similar Ca\textsuperscript{2+} oscillations were observed in voltage-clamped cells (272). Activation of Ca\textsuperscript{2+} entry channels at higher agonist concentrations is important for refilling of depleted intracellular stores. Activation of PKC, PLC blockers, and inhibitors of SERCA pumps strongly suppress Ca\textsuperscript{2+} oscillations (49, 272). Under pathological conditions, glucose overload and superoxide anions also impair Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} oscillations (191).

3. Structural organization of Ca\textsuperscript{2+} signaling in endothelium: a role for caveolae?

Caveolae, abundantly present in EC, are specialized plasmalemmal microdomains enriched in glycosphingolipids, cholesterol, sphingolipids, and lipid-anchored membrane proteins (321). They are omega-shaped invaginations with a diameter of 50–100 nm. Caveolae originate from “lipid raft domains” (LRD) in which sphingolipids and cholesterol are highly accumulated. Specific proteins attach to these LRD (101–103, 130). The major component of endothelial caveolar structures is caveolin-1 (CAV-1). It consists of 178 amino acid residues and has a molecular mass of 22 kDa. A Tyr residue at position 14 provides a target for protein tyrosine kinase (PTK). The stretch of residues 81–101 is the scaffolding region (CSD), which binds and “deactivates” several other proteins. Membrane insertion of CAV-1 occurs via interaction with the hydrophobic hairpin (residue 102–134). Caveolar structures contain a variety of signal transduction molecules, including GPCRs, G proteins, and adenyl cyclase, molecules involved in the regulation of intracellular Ca\textsuperscript{2+} homeostasis, such as the plasma membrane Ca\textsuperscript{2+}-ATPase and InsP\textsubscript{3}-R-3 (361), eNOS, multiple components of the tyrosine kinase mitogen-activated protein kinase pathway, Ca\textsuperscript{2+}-sensitive activated PKC-α, and numerous lipid signaling molecules (3, 371). Various membrane receptors, such as the BK\textsubscript{ca} receptor and the muscarinic m\textsubscript{2}-AchR, are targeted to caveolae upon agonist binding (21, 89). Vesicular transport systems in EC are in close contact with caveolae, which contain key proteins that mediate different aspects of exo- and endocytotic vesicle formation, docking, and/or fusion, including the vSNARE VAMP-2, monomeric and trimeric GTPases, annexins II and VI, and the NEM-sensitive fusion factor NSF with its attachment protein SNAP. VAMP is also sensitive to cleavage by botulinum B and tetanus neurotoxins. Caveolae in endothelium may therefore be considered as complex signaling and transport units (246, 360).

Mechanostimulation by shear stress induces rapid tyrosine phosphorylation of proteins located in caveolae, e.g., ERK activation (320, 345). Tyrosine phosphorylation influences Ca\textsuperscript{2+} entry (94) and is necessary for gating of VRAC
channels, which are probably associated with CAV-1 (391). Ca\(^{2+}\) waves are triggered from caveolin-rich edges of EC (160), and Ca\(^{2+}\) entry pathways are probably colocalized with caveolar sites in EC (210). Recently, TRP1 has been identified in caveolae and LRD in human submandibular glands and found to be associated with CAV-1, Ga\(_{q/11}\) proteins, and the InsP\(_3\) receptor type 3 (InsP\(_3\)R\(_3\)). The scaffolding domain of CAV-1 probably binds to NH\(_2\)- and COOH-terminal sites of TRP1 (224). An NH\(_2\)-terminal site downstream of the InsP\(_3\) binding site of the InsP3R associates with several COOH-terminal sites of TRP3 (37).

An NCX protein has been detected in caveolin-rich membrane fractions containing both eNOS and CAV-1. It is interesting to speculate that also transmembrane Na\(^+\) gradients may be involved in regulation of eNOS activity. This would provide a tight functional interaction between endothelial NCX and eNOS (385). A complex of Ca\(^{2+}\) entry channels (STRPCs), channels regulating the driving force for Ca\(^{2+}\) entry (e.g., VRAC) connected with Ca\(^{2+}\) release sites (InsP\(_3\)R), the responding elements for agonist stimulation (GPCR and G proteins) and NCX in a close vicinity to caveolin-bound eNOS would provide a powerful signaling unit in EC (Fig. 13).

**C. Secretion of Vasoactive Compounds: A Potential Role for Ion Channels**

Endothelial function and its role in hypertension, arteriosclerosis, and coronary diseases depend on a balance between the release of various factors (32). These factors promote or inhibit 1) vasodilatation and vasosclerosis, 2) blood coagulation and fibrinolysis, 3) thrombogenesis and thrombolysis.

![Diagram](http://physrev.physiology.org/)

**FIG. 13.** Scheme of the cholesterol- and sphingolipid-rich caveolar compartments in EC. This compartment contains various important proteins, such as signaling molecules and tools for the exocytotic machinery. VAMP-2, vesicle-associated membrane protein is a vesicular SNAP receptor similar to synaptobrevin; NSF, the N-ethylmaleimide-sensitive fusion factor with its soluble attachment protein SNAP. Caveolae are formed from “lipid raft domains.” It is depicted that possibly a functional unit comprising receptors for the agonist response (GPCR), Ca\(^{2+}\) entry channels (TRPs), channels for regulating the driving force for Ca\(^{2+}\) and being involved in electrogenesis (e.g., the volume-regulated anion channel, VRAC) and possible also the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) are components of such units (for a discussion see text). Probably TRPs could be coupled to the InsP\(_3\) receptor (InsP3R) in subplasmalemmal store. Coupling domain has been identified in the NH\(_2\)-terminus of the InsP3R downstream of the InsP3R binding site and the COOH terminus of TRPs. TRPs might couple to caveolin-1 (cav-1) as well as VRAC and GPRC (for discussion see text). The main structural component of the endothelial caveolae, caveolin-1, is shown at the bottom. CSD marks the caveolar scaffolding domain. The FEDVIA sequence is conserved in all known caveolins. The putative PTK phosphorylation site at position 14 is present in caveolin-1 but not in caveolin-2. The hydrophobic hairpin region links this protein to the caveolar membrane (for detailed description, see text).
Procoagulant, prothrombotic promoters and inhibitors.

It is generally agreed that synthesis and/or release of vasoactive compounds depends on or can be modulated by changes in \([Ca^{2+}]_i\) (41, 50, 154, 273, 295). Sources for an increase in \([Ca^{2+}]_i\), are primarily release of \(Ca^{2+}\) from intracellular stores via an Ins(1,4,5)P_3-dependent mechanism and influx of extracellular \(Ca^{2+}\). Ion channels play a role in the latter process by either providing an influx pathway for extracellular \(Ca^{2+}\) or by regulating the driving force for this influx. We first discuss some EC functions in which \(Ca^{2+}\) plays a regulatory role.

The release of NO is involved in the control of relaxation of vascular SMC, but also inhibits platelet aggregation, proliferation of SMC (an anti-atherosclerotic function), the migration of monocytes and leukocytes, and the expression of adhesion molecules. NO produced by eNOS is a fundamental determinant of cardiovascular homeostasis and regulates systemic blood pressure, vascular remodeling, and angiogenesis. The most important physiological stimulus for the continuous formation of NO is viscous drag (shear stress) exerted by streaming blood on the endothelial layer. This basal release seems to be \(Ca^{2+}\) independent. The serine/threonine protein kinase Akt/PKB mediates the activation of eNOS, leading to increased NO production and might be the target of shear stress probably involving a sensor function of platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31, 71). NO can also be released via activation of a \(Ca^{2+}\)/CaM-dependent pathway which requires sustained influx of extracellular \(Ca^{2+}\) (116, 157, 210). In EC, eNOS is localized in signal-transducing domains of the plasmalemma, the so-called caveolae (321). Acetylation of eNOS by myristic and palmitoyl acid enables targeting of the enzyme to the caveolae. Under resting conditions, eNOS is inactivated due to its binding to the caveolar scaffolding protein CAV-1. An increase in \([Ca^{2+}]_i\) promotes binding of calmodulin to eNOS and its dissociation from caveolin. A more prolonged exposure to increased levels of \([Ca^{2+}]_i\) redistributes eNOS to the cytosol and is accompanied by depalmitoylation and phosphorylation (for reviews, see Refs. 90, 252, 253, 336). Lin et al. (219) showed in a recent elegant study that NO production is mainly controlled by myristoylated, membrane-associated eNOS that senses CCE, and that it is less sensitive to intracellular \(Ca^{2+}\) release.

The rapid synthesis and release of the anticoagulant prostacyclin (PGI_2) requires a rise in \([Ca^{2+}]_i\) and phosphorylation of PLA_2 (323). PGI_2 is released “on demand.” An increase in \([Ca^{2+}]_i\) translocates cytosolic PLA_2 from the cytosol to phospholipid membranes, especially to the nuclear membrane and the plasmalemma. PLA_2 is supposed to be colocalized in these regions with cyclooxygenase and lipoxygenase, which are necessary to convert arachidonic acid to vasoactive compounds, such as PGI_2 and EET products. This translocation occurs via a \(Ca^{2+}\)-dependent phospholipid-binding domain at the NH_2-terminus of PLA_2 (CaLB; Refs. 59, 97). An additional phospholipase cPLA_2 is required for the synthesis of PGI_2. This phosphorylation is mediated by a p42/p44 MAP kinase, which is activated by PKC and probably also by an increase in \([Ca^{2+}]_i\). Stimulation of PGI_2 synthesis by vasoactive compounds (ACh, bradykinin, and ATP) is supported by \(Ca^{2+}\) influx via \(Ca^{2+}\) entry channels, including SOC (115, 181, 261). PGI_2 supports the vasodilating action of NO and is also produced in response to shear stress.

PAF is also produced on demand. Its synthesis is triggered by an increase in \([Ca^{2+}]_i\), which precedes activation of PLA_2. Importantly, released PAF also binds to EC and mediates \(Ca^{2+}\) influx into its generator cells (200).

Another factor that is synthesized and released in a \(Ca^{2+}\)-dependent way is the EDHF, which might be 5,6-EET. Another recent hypothesis suggests that EDHF, or at least one of its components, is K^+ released from EC by activation of BK_{ca} channels into the space between EC and smooth muscle cells (82). This effect would also require a sustained elevation of \([Ca^{2+}]_i\) in EC.

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**TABLE 3. Factors released from endothelial cells**

<table>
<thead>
<tr>
<th><strong>Vasoactive factors</strong></th>
<th><strong>Anticoagulant, thrombolytic factors</strong></th>
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</thead>
<tbody>
<tr>
<td>Vasoconstrictors</td>
<td>Anticoagulant, thrombolytic factors</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>Tissue factor plasminogen activator</td>
</tr>
<tr>
<td>Endothelium-derived</td>
<td>Tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>hyperpolarizing factor</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Prostacyclins, PGI_2</td>
<td>PGI_2</td>
</tr>
<tr>
<td>Natriuretic peptide</td>
<td>Protein S</td>
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<tr>
<th><strong>Eastern growth modulators</strong></th>
<th><strong>Inhibitors</strong></th>
</tr>
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<tbody>
<tr>
<td>Promoters</td>
<td></td>
</tr>
<tr>
<td>Tumor necrosis factor-α</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Superoxide radicals</td>
<td>PGI_2</td>
</tr>
<tr>
<td>Natriuretic peptide</td>
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<table>
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<tr>
<th><strong>Inflammatory agents</strong></th>
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<tbody>
<tr>
<td>Promoters</td>
</tr>
<tr>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>Superoxide radicals</td>
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<tr>
<td>Peroxynitrite, ONOO⁻</td>
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[Modified from Born et al. (32).]
In addition to synthesis and release on demand, several stored vasoactive compounds are secreted during EC stimulation. The best-studied example is the vWF, a large polymeric protein that binds coagulation factor VIII and protects it from cleavage, and which also contains binding sites for the platelet glycoproteins GPIb and GPIIb/IIIa to collagen, heparin, and vitronectin. It is stored in secretory granules, Weibel-Palade bodies, which are large vesicular organelles of ~200 nm in diameter and up to 4,000 nm in length. vWF is released by CaM-dependent exocytosis triggered by an increase in \([Ca^{2+}]_i\) (28). Weibel-Palade bodies also contain the leukocyte adhesion protein P-selectin (GMP-140), which appears at the cell surface as a result of exocytosis (428).

tPA, which activates fibrinolysis and thrombolysis in blood vessels, is another example of a stored anticoagulant molecule that is released in a \(Ca^{2+}\)-dependent way. The mechanisms involved in the secretion of vWF and tPA differ considerably. Thrombin stimulation of EC induces tPA secretion from small vesicles morphologically different from the larger Weibel-Palade bodies and from caveolae (85). Release of tPA from EC is regulated by histamine, which like thrombin elevates \([Ca^{2+}]_i\), but also by endothelins and cytokines. In addition, tPA is constitutively released.

TFPI is an important antithrombotic factor that inhibits complex "tissue factor (TF) VIIa," which in turn activates factors IXa and Xa and starts coagulation. TFPI-1, a 36-kDa glycoprotein, is a Kunitz-type serine protease inhibitor with an obvious role in the prevention of thrombosis. TFPI is released upon heparin administration. The heparin-binding site of TFPI (HBS-1) is located in its COOH-terminal basic portion. This Kunitz-type serine proteinase inhibitor contains three tandem KPI domains. TFPI-2 is another heparin-reaslease 33-kDa serine-protease inhibitor. TFPIs are located in well-defined granules evenly spread over the cell surface and in the apical part of the cytoplasm. These granules are distinct from Weibel-Palade bodies and have a smaller diameter of ~200 nm. TFPI colocalizes in EC with caveolin. It is bound by a specific glycosylphosphatidylinositol-anchorage mechanism that depends on the presence of cholesterol (229, 230). TFPI containing vesicles might be docked on caveolae and can therefore be rapidly translocated to the exocytic machinery (360). TFPI binds in a \(Ca^{2+}\)-independent way to a phosphatidylserine phospholipid surface depending on the presence of the TFPI COOH-terminal Kunitz domain. The interaction of TFPI with phosphatidylserine is decreased when \([Ca^{2+}]_i\) is increased, a mechanism important for TFPI release (402).

PAI-1, an inhibitor of fibrinolysis, is also released in a \(Ca^{2+}\)-dependent manner. However, the increase in \([Ca^{2+}]_i\) induces only a modest increase of PAI-1 but suppresses the action of much stronger release stimuli, such as TNF-\(\alpha\) (324). The release of PAI-1 is regulated by physiological factors that elevate \([Ca^{2+}]_i\), such as thrombin and histamine, but also by endothelins and cytokines. The regulation of tPA release and that of PAI-1 release are not necessarily coupled, and the exact role of \([Ca^{2+}]_i\) has to be elucidated (324, 446).

Another stored factor in the regulation of coagulation is protein S, a vitamin K-dependent nonenzymatic coagulation factor involved in the regulation of activated protein C that has a strong anticoagulant function. An increase in \([Ca^{2+}]_i\) releases protein S from cell organelles with a diameter of 0.1 \(\mu\)m (375).

Vasoconstrictor responses of EC are mainly mediated by the release of ET-1, which is produced during stimulating of EC with thrombin, transforming growth factor-\(\beta\) (TGF-\(\beta\)), interleukin-1, angiotensin II, and arginine vasopressin. It acts via ET\(_A\) receptors on subendothelial vascular SMC, but it can also act on ET\(_B\) receptors on EC and provide a negative feedback by stimulating NO release. ET-1 and its precursor big ET-1 are colocalized in granules with a diameter of ~0.1 \(\mu\)m, suggesting that they are not only release sites but also important subcellular compartments for ET processing (131). Small amounts of ET-3 increase NO release (436). \(Ca^{2+}\) is involved in this regulation probably via activation of PKC and an Ins(1,4,5)P\(_3\)-mediated increase in \([Ca^{2+}]_i\). Both ET-1 and ET-3 mobilize stored \(Ca^{2+}\) and activate \(Ca^{2+}\) entry pathways (4). The release of ETs is, however, not transient but persistent, and the relationship between \([Ca^{2+}]_i\) and ET release seems to be contradictory, since thrombin increases but bradykinin decreases ET release although both elevate \([Ca^{2+}]_i\) (120). The role of \(Ca^{2+}\) for ET release remains unclear.

All substances stored in granules are released by regulated exocytosis, e.g., vWF, tPA, TFPI, protein S, and ET-1 and ET-3. Exocytosis, probably except for the release of ET-1, depends on an increase in \([Ca^{2+}]_i\). Vesicular secretion of vasoactive mediators has been assessed from changes in membrane capacitance. Intracellular \(Ca^{2+}\) increases induced by flash photolysis of caged \(Ca^{2+}\) or \(Ins(1,4,5)P_3\) exert a biphasic effect on membrane capacitance, consisting of an initial transient increase followed by a much larger delayed component and which both depend on \([Ca^{2+}]_i\) (51). Probably, the late and large changes in membrane capacitance reflect the exocytic release of vWF, whereas the faster events might be related to tPA and TFPI release. NO release and vWF release clearly depend on the presence of extracellular \(Ca^{2+}\), indicating that \(Ca^{2+}\) influx is necessary to support this EC function (51), whereas prostacyclin, tPA, and TFPI depend to a larger extent on release of intracellular \(Ca^{2+}\) (50). In summary, several EC functions including the production of vasoactive compounds on demand and the release of stored compounds by exocytosis depend on an increase in \([Ca^{2+}]_i\). Two sources of \(Ca^{2+}\) are involved: an \(Ins(1,4,5)P_3\)-mediated \(Ca^{2+}\) release from intracellular...
stores, which in a long term must be replenished from extracellular sources, and Ca\(^{2+}\) entry from the extracellular side. Although not many details are known about the mechanisms that regulate exocytosis in endothelium, it is obvious that ion channels play an important role in these processes by modulating the changes in [Ca\(^{2+}\)]\(_i\).

### D. Ion Channels and Control of Vessel Permeation, Leukocyte Migration, and Cell-Cell Contacts

A major activity of EC is the regulation of exchange of solutes, proteins, and endocytosed particles between blood and tissue. Part of this transport is mediated by changes in the paracellular permeability of the endothelial layer. An increase in endothelial [Ca\(^{2+}\)]\(_i\) enhances the permeability of microvessels, which is abolished in Ca\(^{2+}\)-free solution, but also when the cells are depolarized. Agonists like thrombin or histamine, which all transiently increase [Ca\(^{2+}\)]\(_i\), will therefore induce a short increase in vascular permeability, whereas others, such as cytokines and growth factors, induce a more sustained change. This increase in permeability is due to changes in the cytoskeleton, cellular contraction, and cell-cell coupling (68), processes that are all controlled by myosin light-chain phosphorylation. EC contraction and regulation of hydraulic permeability depend on influx of Ca\(^{2+}\) (174) and involve a Ca\(^{2+}\)/CaM-dependent nonmuscle MLCK isoform that has recently been cloned in human endothelium (see below and Refs. 412, 413).

The regulation of permeability in macrovessels is more complex. An ionomycin-induced increase in [Ca\(^{2+}\)]\(_i\) enhances the permeability of porcine aorta endothelium, whereas a comparable increase in [Ca\(^{2+}\)]\(_i\) by ATP reduces its permeability. This increase is attenuated by the PLC inhibitor U-73122 (209).

Shrinkage of EC increases microvessel permeability if perfused with the Gly-Arg-Gly Asp-Thr-Pro (GRGDTP) peptide to disrupt integrin-dependent attachment of EC to the extracellular matrix (174, 175). In general, spreading of EC on their matrix and contact formation with their basement membrane has a selective requirement for Ca\(^{2+}\) influx (2).

[Ca\(^{2+}\)]\(_i\) and cAMP are two interactive signals that control vessel permeability. Increased [Ca\(^{2+}\)]\(_i\) promotes disruption of the macrovascular EC barrier but not the microvascular barrier. Increased cAMP enhances endothelial barrier function. During inflammation, elevated [Ca\(^{2+}\)]\(_i\) decreases cAMP to increase intercellular permeability (260). Changes in [Ca\(^{2+}\)]\(_i\) that regulate the permeability of the EC barrier possibly require activation of store-operated Ca\(^{2+}\) entry channels (259).

We have shown (see sect. vC1) that the VRAC regulates cell volume and provides a pathway for organic osmolytes and amino acids. Interestingly, its gating involves activation of RhoA (288, 298). Because inactivation of RhoA also enhances the endothelial barrier function due to enhanced myosin ribbon and stress fiber-focal adhesion formation (47), VRAC may also be involved in the control of endothelial barrier function. Also, the control of EC permeability via MLCK is tightly coupled to CCE-mediated Ca\(^{2+}\) entry (300).

EC permeability and neutrophilic leukocyte diapedesis through paracellular gaps are cardinal features of acute inflammation, which are mediated by myosin light-chain phosphorylation. This phosphorylation results from a Ca\(^{2+}\)/CaM-dependent activation of an EC-specific, 214-kDa, nonmuscle MLCK distinct from the smooth muscle MLCK isoforms (130–150 kDa) (106). This EC MLCK isoform binds biotinylated CaM in a Ca\(^{2+}\)-dependent manner and colocalizes with myosin, actin, and CaM. Thus MLCK in EC is a further target for changes in [Ca\(^{2+}\)]\(_i\). Inhibition of MLCK activity by activating cAMP-PKA reduced leukocyte transmigration. The myosin-associated phosphatase inhibitor calyculin induces accumulation of phosphorylated myosin light chains, EC contraction, and significantly enhances leukocyte migration. Ca\(^{2+}\)-dependent EC MLCK is therefore a key determinant of vascular permeability and transendothelial leukocyte migration (107, 412, 413).

### E. Ion Channels: A Role in EC Growth and Angiogenesis?

Extracellular angiogenesis inducers (VEGF, basic fibroblast growth factor, acidic fibroblast growth factor, PDGF, IL-8, PAF, etc.) that stimulate proliferation and migration of EC regulate the formation of new blood vessels (angiogenesis). Angiogenesis inhibitors, such as tumor suppressor genes, angiostatin, and endostatin, counteract these processes (for a review, see Refs. 43, 48). Surprisingly, blockers of VRAC, such as tamoxifen, clomiphene, \(\beta\)-estradiol, NPPB, IAA-94, DIDS, quinine, quinidine, mibebradil, and flufenamic acid, also suppress the growth of EC (238, 278, 283, 425). It is well known that ion channels modulate the progression through the cell cycle. Block of K\(^+\) channels and cell depolarization induce G\(_1\) arrest in several cells types (275). The cyclin-dependent kinase inhibitors p27(Kip1) and p21(CIP1) might be involved in this mechanism (112). In addition, angiogenesis is inhibited by various blockers of VRAC with unrelated chemical structure, such as NPPB, mibebradil, and the antiestrogens tamoxifen and clomiphene, as assessed in four assays of angiogenesis: the Matrigel and fibrin gel assays of tube formation by endothelial cells in vitro, the rat aorta-ring assay of spontaneous microvessel formation ex vivo, and the chick chorioallantoic membrane assay of neovascularization in vivo. These results suggest that anion channels may play a role in angiogen-
esis and have a potential therapeutic action in tumor growth and other angiogenesis-dependent pathophysiological processes (238). The mechanism of action is unknown, but it may be linked to the role of VRAC in volume and pH regulation or fine-tuning the driving force for Ca\(^{2+}\) entry, which is important at the Ca\(^{2+}\)-dependent cell cycle control point, i.e., between G\(_1\) and S. It has also been shown that the functional expression of VRAC changes when cells switch from proliferation to differentiation, further supporting a role of ion channels in the regulation of cell growth (237, 278, 295, 426).

Recently, clear evidence has been presented for a role of Ca\(^{2+}\) in the regulation of angiogenesis in vivo. VEGF-A, a member of the family of endothelial growth factors (VEGFs), plays a dominant role in the regulation of angiogenesis and vascular permeability (91). The endothelial vascular growth factor receptor, VEGFR-2, binds VEGF-A and VEGF-E and activates a signaling cascade involving autophosphorylation, activation of PLC-\(\gamma\) and elevation of [Ca\(^{2+}\)]\(_i\), and a VEGF-dependent Ca\(^{2+}\) influx (135, 341, 430). Activation of VEGFR-2 induces store depletion and triggers activation of CCE (CRAC) channels, resulting in Ca\(^{2+}\) influx that might be important for the control of angiogenesis. This example clearly demonstrates that ion channels are involved in a complex phenomenon like angiogenesis. Furthermore, CAI, a blocker of ligand-stimulated Ca\(^{2+}\) influx, inhibits EC proliferation, adhesion, and invasion into the basement membrane of EC. Likely, Ca\(^{2+}\)-regulated events are important in native and FGF\(_2\)-stimulated EC proliferation and invasion, perhaps through regulation of FGF\(_2\)-induced phosphorylation events (197).

**F. Role of Ion Channels in Dysfunction of Endothelium?**

Several disorders, among which hypertension, atherosclerosis and other cardiovascular diseases, diabetes, and hypercholesterolemia, are associated with a dysfunctional endothelium (33). Endothelial-dependent relaxation is impaired in essential hypertension because of a deficient release of NO. Release of ET-1 exacerbates hypertension. Obviously, defective Ca\(^{2+}\) signaling can be the cause of this deficient NO production. CCE is significantly reduced in aorta EC of trp4-deficient mice, and the NO-mediated vasorelaxation of aortic rings is impaired (99), indicating that a sustained influx of Ca\(^{2+}\) is important for endothelial NO production. It is well known that BK\(_{ca}\) channels modulate NO production (256), indicating that channels that control the driving force for Ca\(^{2+}\) entry are also essential for the control of NO synthesis.

Changes in membrane potential per se also induce significant changes in NO-mediated EC responses. An intriguing example is the depolarization-induced superoxide formation in HUVEC (374). Superoxide anions not only impair NO-mediated responses but are also involved in the development of hypertensive vascular hypertrophy. EC depolarization also enhanced tyrosine phosphorylation of as yet unidentified membrane proteins and induced membrane association of the small G protein Rac. Cation and anion channels are the main candidates for inducing depolarization and are therefore probably involved in this important pathogenic mechanism.

Hypoxic and ischemic conditions also mediate EC-dependent vasoconstriction by release of arachidonic acid, PGH\(_2\), thromboxane A\(_2\), and endoperoxides. The enhanced NO production under these conditions increases the amount of NO-derived peroxynitrite (ONOO\(^-\)), which becomes peroxinitrosic acid and forms hydroxyl ions. These vasoconstrictor superoxide anions are highly reactive, damage the cell membrane, and finally reduce NO production (32). A possible mechanism for the hypoxia-induced autacoid release, which seems to depend on extracellular Ca\(^{2+}\), could be an increase in [Ca\(^{2+}\)]\(_i\) (6, 10, 42, 93, 178, 348). It has been recently described that metabolic inhibition stimulates Ca\(^{2+}\) entry in EC probably via activation of TRP3, a NSC that is a member of the STRPC subfamily (see sect. \(\mu\)C and Ref. 380).

**G. Concluding Remarks**

EC form a multifunctional signal-transducing surface that regulates many fundamental processes in the vascular system, such as the control of blood flow and blood pressure, coagulation, platelet aggregation, vessel permeability, wound healing, and angiogenesis. It is rather surprising that these cells express such a huge variety of ion channels. We are still in the very beginning of identifying these channels at the molecular level, although some of the main players, such as trp gene-encoded channels, are distinctly emerging. One obvious functional target of EC ion channels is Ca\(^{2+}\) signaling. It is firmly established that many EC functions depend on Ca\(^{2+}\), but we do not understand at all how this signal, an increase in [Ca\(^{2+}\)]\(_i\), allows for specific EC responses on demand. The Ca\(^{2+}\) signal in EC is shaped by release of Ca\(^{2+}\) from intracellular pools, entry of Ca\(^{2+}\) via plasmalemmal channels, and the concerted action of several Ca\(^{2+}\) sequestration mechanisms, such as activation of PMCA and SERCA Ca\(^{2+}\) pumps and probably NCX. However, several EC functions, e.g., release of vasoactive compounds including NO, vWF, PAF, tPA, and TFPI, probably require activation of exocytosis and need a high and a long-lasting plateau-like increase in [Ca\(^{2+}\)]\(_i\), that mainly depends on Ca\(^{2+}\) entry. The molecular nature of Ca\(^{2+}\) entry channels is not yet known. However, there is increasing evidence that SOC and nonselective Ca\(^{2+}\)-permeable channels are the main...
players for this entry. Probably, members of the STRPC family are involved in functionally structuring this Ca\(^{2+}\) entry.

We have also shown that it is mandatory for EC not only to activate Ca\(^{2+}\) entry channels upon various stimuli, but also to provide a sufficiently large inwardly driving force for Ca\(^{2+}\). Among the channels that influence electrogensis in EC are various types of K\(^{+}\) channels. Especially Ca\(^{2+}\)-activated K\(^{+}\) channels, mainly belonging to the slo family, are important targets for regulating Ca\(^{2+}\) entry and Ca\(^{2+}\)-dependent EC functions. Surprisingly, Cl\(^{-}\) channels are also abundantly present in EC. Among these channels, especially the VRAC might be multifunctional. This channel can counteract the depolarizing action of other channels and contribute to a stabilization of the inwardly driving force for Ca\(^{2+}\). Interestingly, these channels might be localized in the caveolar compartment together with eNOS, signaling proteins, including receptors such as GPCRs, ATPases, small G proteins, kinases, the endo- and exocytotic machinery, and other ion channels, such as STRPCs, forming a complex that seems to play an important role in signal transduction in EC. Future work must focus on the functional role of these important cellular compartments.

Obviously, ion channels are also important players in other EC functions. VRAC channels are involved in transport of organic molecules including amino acids, and various channels influence cell proliferation and angiogenesis as well as control EC permeation and cell-cell coupling.

Another intriguing topic in EC biology is its role as a mechanosensor. Irrefutably, mechanosensing properties of EC are directly or indirectly coupled with ion channels, although clear mechanosensor channels are, after several preliminary candidates have been proposed, elusive. We discuss here the possibility that CCE channels are indirectly involved in mechanosensing and discuss channels with obvious mechanosensing properties, VRAC and inwardly rectifying K\(^{+}\) channels.

We are obviously at the verge of understanding the role of ion channels in EC and to appreciate that these channels might be important targets for the pharmacological modulation of fundamental EC functions. Indeed, we are at the very beginning of considering EC ion channels as targets of novel therapeutic compounds to control Ca\(^{2+}\) entry and its driving force. In addition, ion channels might be the cause for EC dysfunction linked to human disorders. To catalyze a further understanding of EC physiology and pathophysiology, it is necessary to include also the expanding field of ion channels expressed in EC in the general interest of vascular cell biologists.

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Address for reprint requests and other correspondence: B. Nilius, Laboratorium voor Fysiologie, KU Leuven, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium (E-mail: bernd.nilius@med.kuleuven.ac.be).

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