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Review

Endothelium as target for large-conductance calcium-activated potassium channel openers

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The endothelium is a highly active organ responsible for vasculatory tone and structure, angiogenesis, as well as hemodynamic, humoral, and inflammatory responses. The endothelium is constantly exposed to blood flow, sheer stress and tension. Endothelial cells are present as a vasculature in every tissue of the body and react to and control its microenvironment. A variety of ion channels are present in the plasma membranes of endothelial cells. These include potassium channels such as inwardly rectifying potassium (K_{ir}) channels, voltage-dependent (K_v) channels, ATP-regulated potassium (K_{ATP}) channels and three types of calcium-activated potassium channels (K_{Ca}), the large (BK_{Ca}), intermediate (IK_{Ca}), and small (SK_{Ca}) -conductance potassium channels. Potassium current plays a critical role in action potentials in excitable cells, in setting the resting membrane potential, and in regulating neurotransmitter release. Mitochondrial isoforms of potassium channel contribute to the cytoprotection of endothelial cells. Prominent among potassium channels are families of calcium-activated potassium channels, and especially large-conductance calcium-activated potassium channels. The modulation of BK_{Ca} channels, which are voltage- and calcium-dependent, has been intensively studied. The BK_{Ca} channels show large expression dynamics in endothelial cells and tissue-specific expression of large numbers of alternatively spliced isoforms. In this review, a few examples of the modulatory mechanisms and physiological consequences of the expression of BK_{Ca} channels are discussed in relation to potential targets for pharmacological intervention.

Keywords: endothelium, potassium channels, endothelium-derived hyperpolarising factor

INTRODUCTION

The endothelium is a monolayer of the cells that line the entire internal surface of the blood vessels and lymphatic system. The term endothelium was introduced by the anatomist Wilhelm His in 1865, and for a long time it was considered to be an inert "layer of nucleated cellophane" serving only as a non-reactive barrier (Galley & Webster, 2004). The important internal part of the blood vessels is the glycocalyx, discovered after the introduction of light and electron microscopy techniques. The glycocalyx is a layer of endothelial membrane-bound macromolecules composed of a variety of extracellular polysaccharide coating on cells. The membrane-bound glycocalyx with adsorbed plasma components plays a role in microvessel permeability. Most proteins at the endothelial surface are glycoproteins

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Abbreviations: BK_{Ca} channel, large conductance Ca^{2+}-activated potassium channel**;** ChTx, charybdotoxin**;** ECs, endothelial cells; EDHR, endothelium-derived hyperpolarising factor; IbTx, iberiotoxin; ICAM-1, intercellular adhesion molecule-1; 5-HD, 5-hydroxydecanoic acid; K_{Ca} channel, ATP-regulated potassium channel**;** MEGJ, myoendothelial gap junction; mitoBK_{Ca} channel, mitochondrial large conductance calcium-activated potassium channel**;** mitoK_{ATP} channel, mitochondrial ATP-regulated potassium channel**;** mitoK_v1.3 channel, mitochondrial voltage gated potassium channel**;** NS1619, 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2*H*-benzimidazole-2-one**;** oxo-LDL, oxidised low-density lipoproteins; PGI_2, prostacyclin; RCK1, RCK2, regulators of the conductance of K^+; RNS, reactive nitrogen species; ROS, reactive oxygen species; TNF-α, tumour necrosis factor α; SOD, superoxide dismutase; VCAM-1, vascular cell adhesion molecule-1.

(e.g., selectins and integrins) (Pries *et al.*, 2000). Interactions between highly specialised adhesion molecules are modified by sulfated glycans (e.g. heparin sulfate), the most abundant components of the glycocalyx (Skinner *et al.*, 1991). The layer of endothelial glycocalyx seems to play a significant role in the modulation of angiogenesis (Brown *et al.*, 1996; Pries *et al.*, 2000). It has also been shown that ischemia-reperfusion can damage the glycocalyx layer of endothelial cells and impair endothelial vasodilatation. These changes, related to reactive oxygen species (ROS), are reversed by superoxide dismutase (SOD) treatment. Oxidised low-density lipoproteins (oxo-LDL) can also severely damage the glycocalyx layer (Abrahamsson *et al.*, 1992; Czarnowska & Karwatowska-Prokopczuk, 1995; Beresewicz *et al.*, 1998). It is now well established that the endothelium is a very important active component of the cardiovascular system and has autocrine and paracrine activities (Galley & Webster, 2004). Endothelial cells (ECs) regulate vascular tone and blood flow, thrombosis and thrombolysis, and platelet adherence processes. The main role of the endothelium is to regulate vascular tone by releasing vasodilator and vasoconstrictor substances (Table 1). The endothelial cells in the vascular tree are not uniform in shape, thickness or expression of cell adhesion molecules (e.g., ICAM, VCAM, PECAM). The endothelium differs among the leukocyte trafficking between the skin, muscle mesentery (with classical multistep for leukocyte recruitment), and the lung, liver, and mescendrinc lymph nodes (Aird, 2007a; Dietmar, 2007; Aghajanian *et al.*, 2008; Wittchen, 2009). Each EC is a dynamic structure that responds to the extracellular environment, which may include mechanical (e.g., shear stress and tension) or biochemical factors (e.g., cytokines, hormones, growth factors, ROS, NO). These environmental factors cause endothelial phenotypic changes which can alter as cell shape, calcium influx, protein expression, mRNA levels, migration, proliferation, apoptosis and survival, vasomotor tone, inflammatory response, leukocyte adhesion and migration. Because the endothelium is distributed through the body and has contacts with every tissue, its dysfunction can influence the state of each tissue in the body (Aird, 2007b). A rapid progress in the documentation of the phenotypic heterogeneity of the endothelium with the use of different approaches has been achieved recently (e.g., immunohistochemistry, *in situ* hybridisation, real-time microscopy, and proteomic techniques) (Pasqualini & Arap, 2002; Aird, 2003; Shibata *et al.*, 2005; Shin & Anderson, 2005; Sandow & Grayson, 2009). The endothelial phenotypic changes related to the environment can clearly be seen in the formation of endothelium in the blood-brain barrier, where endothelium is under the regulation of astroglial-derived paracrine factors. Another example are the ECs lining microvessels in the heart, which are exposed to the mechanical forces generated by contracting cardiomyocytes and to their paracrine and electrical factors (Hsieh *et al.*, 2006). In embryogenesis, the mesoderm is the exclusive source of ECs precursors, which are in close colocalisation with haematopoietic precursor cells, and this has suggested that both arise from hemangioblasts. In the adult body, ECs in quiescent vasculature are proliferatively inactive with a relatively long life-span (Hobson & Denekamp, 1984; Ferran, 2006; Langenkamp & Molema, 2009). It is important to note that tumours depend on new vasculature supply for their growth, and it is crutial to characterise the phenotype of the ECs to understand the action of anti-angiogenic drugs that can affect tumours through their vasculature (Aird, 2009; Langenkamp & Molema, 2009). The endothelium is in constant balance between vasodilatation and vasoconstriction, proliferation and its inhibition, activation of smooth muscle cell migration, and activation and inhibition of adhesion, thrombogenesis and fibrinolysis. Shifts in the metabolism of the endothelium toward reduced vasodilatation, a proinflammatory state, and prothrombic characteristics lead to endothelial dysfunction (Feletou & Vanhoutte, 2006a; Vanhoutte *et al.*, 2009). The actions of endothelial vasoactive components very often involve the activation of potassium channels, especially calcium-activated potassium channels (K_{Ca}), a key component in the regulation of membrane potential in endothelial and smooth mus-

Table 1. Major components of endothelial metabolic activities

Endothelial function	Mediators
Vasodilators	Nitric oxide (NO), prostacyclin $(PGI)_2$, endothelium-derived hyperpolarizing factor (EDHF), adrenomedullin (AM), natriuretic peptide (CNP)
Vasoconstrictors	Angiotensin II (AgII), endothelin (ET), thromboxane A_2 (TxA_2), leukotrienes, free radicals
Growth factors	Transforming growth factor, colony stimulating factor, insulin like growth factor
Antithrombotic factors	Thrombomodulin (TM), antithrombin, plasminogen activator, heparin
Inflammatory mediators	Interleukins 1, 6, 8 (IL-1, IL-6, IL-8), leukotrienes, MHC class II
Procoagulant factors	Von Wilebrand factor (vWF), thromboxane A2, thromboplastin, factor V, paltelet activating factor, plasminogen activator inhibitor (PAI-1)
Lipid metabolism	LDL-receptor, lipoprotein lipase
Matrix components	Fibronectin, laminin, collagen, proteoglycans, proteases

cle cells that are among the regulatory components of vascular tone (Nelson & Quayle, 1995; Nilius & Droogmans, 2001). The endothelium-dependent response to aggregating platelets is not present to the same extent in all arteries, but is most prominent in the coronary and cerebral circulation. When analysing the architecture of the vasculature, the internal elastic lamina (IEL), with the fenestration required for myoendothelial gap junctions (MEGJ), is worthy of note, as it is probably related to the presence of sites of low resistance passage for the diffusion-mediated release of vasoactive endothelial and smooth muscle substances. The MEGJ are specialised structures with microdomains representing a selective target for the control of endothelial and vascular functions (Sandow *et al.*, 2009a; 2009b). The most important component of the control of vascular tone is regulation by potassium channels, which are themselves regulated by the best known endothelial releasing factors nitric oxide (NO) and prostacyclin (PGI_2). There are also other regulatory factors released from endothelium, known as endothelium-derived hyperpolarising factors (EDHF), which are not fully characterised and are associated with hyperpolarisation of the underlying endothelium smooth muscle cells (Feletou & Vanhoutte, 2006b). A variety of ion channels are present in the plasma membranes of endothelial cells. These include potassium channels such as Ca^{2+}-activated K^+ channels (BK_{Ca} channels), inwardly rectifying K^+ channels (K_{IR} channels), and voltage-dependent K^+ channels (K_V channels). Endothelial potassium channels have been implicated in endothelium-dependent vasodilation. Setting the membrane potential (V_m) leads to modulation of endothelial Ca^{2+} signalling and the synthesis of vasodilating factors. Different levels of potassium ion channel expression and a variety of alternative splicing particularly in variants of BK_{Ca} channels have multiple interactions with tissue-specific proteins and a large diversity of interactions with the microenvironments of ECs in the vasculature. Expression of specific channels responsible for stabilisation of the resting membrane potential and its changes are the paramount task for specific parts of endothelium (Nilius *et al.*, 1997; Nilius & Droogmans, 2001; Schmidt *et al.*, 2008).

ENDOTHELIAL POTASSIUM CHANNELS

Although ECs are not electrically excitable, a large number of the signalling functions performed by the vascular endothelium depend on the modulation of activity of endothelial cell ion channels. ECs secrete a variety of endothelium-derived vasoactive molecules and endothelium-derived hyperpolarising factors (EDHF) required for rapid calcium entry (Carter *et al.*, 1988; Lantoine *et al.*, 1998). Additionally, gap junction proteins (connexins) functionally couple ECs in an electrical fashion in some specific regions to smooth muscle cells. These connections permit the spreading of changes in membrane potential (V_m) in ECs to the underlying excitable tissue (De Wit *et al.*, 2006; De Wit & Wolfle, 2007). Changes in ECs membrane potential occur in response to a variety of stimuli (e.g., shear stress, hypertension, cytokines) (Mehrke & Daut, 1990; Barakat *et al.*, 1999; Chauhan *et al.*, 2003). The nature of the intracellular calcium dynamics and signalling in the ECs of the native endothelium are still unclear in comparison to the vascular myocytes (Tran & Watanabe, 2006). Potassium channels are the most diverse class of ion channels underlying electrical signalling in the cell, especially in excitable cells where they play a fundamental role in the regulation of action potential (AP). Potassium channels are ion-selective cation channels with an equilibrium potential near the typical potential of resting cells. A multiplicity of ion channels are present in the plasma membranes of ECs, including inwardly rectifying potassium (K_{ir}) channels, voltage-dependent (K_v) channels and ATP-regulated potassium (K_{ATP}) channels and a group of channels also responsible for modulation of the membrane potential in endothelial cells are Ca^{2+}-activated K^+ channels (K_{Ca} channels) (Nilius & Droogmans, 2001; Taylor *et al.*, 2003).

Endothelial calcium-activated potassium channels (K_{Ca})

Elevation of intracellular calcium concentration $[Ca^{2+}]_i$ in ECs is the first response to most stimuli experienced by the cell. The Ca^{2+} influx into the ECs depends on the electrochemical gradient set primarily by membrane potential. Influx of Ca^{2+} into the ECs leads to depolarisation of the membrane, which is compensated by the activation of K_{Ca} channels. An increased opening probability of K_{Ca} at elevated $[Ca^{2+}]_i$ causes ECs membrane hyperpolarisation and a driving force for Ca^{2+} entry through the opened calcium channels. Three types of calcium-activated potassium channels (K_{Ca}), the large (BK_{Ca}), intermediate (IK_{Ca}), and small (SK_{Ca}) conductance potassium channels, have been identified in the vascular wall (Table 2).

LARGE-CONDUCTANCE CALCIUM-ACTIVATED POTASSIUM CHANNELS (BK_{CA})

The discovery in many tissues of a large outward K^+ current with a dependence on calcium influx and membrane depolarisation has led to the identification of large conductance calcium-activated

Table 2. Calcium-activated potassium channels and its modulators

Type	Subtype	Conductance	Other names	Auxiliary subunits	Modulators	
					Activators	Inhibitors
BK_{Ca}; subfamily M (*KCNMA1*)	large number of alternative splicing products	100–300 pS	$K_{Ca}1.1$; maxi K^+ channel; BK channel; Slo1; Slo; hSlo; MaxiK; mSlo; mSlo1	β1(*KCNMB1*); β2(*KCNMB2*); β3(*KCNMB3*); β4(*KCNMB4*)	membrane electrical potential; intracellular Ca^{2+}; NS1619; NS004; DHS-1; NS1608; Maxi-K diol; CGS7184; Pimaric acid; S(+)-Niguldipine; TEA; Limbatoxin; PKA, PKG; estrogen; H_2O_2;	Iberiotoxin; Charybdotoxin; Slotoxin; Paxilline; Verruculogen; Penitrem A; BmBKTx1; Neuropeptide Y; TEA; PKC; H_2O_2;
IK_{Ca} (*KCNU1*)		50–100 pS		Calmodulin (CaM)	Riluzole; 1-EBIO/DC-EBIO; NS309; Chlorzaxazone	Charybdotoxin; TRAM-34; Clotrimazole; TEA
SK_{Ca}	SK1 (*KCNN1*) SK2 (*KCNN2*) SK2 (*KCNN3*)	8–20 pS		Calmodulin (CaM)	CKII; Riluzole; 1-EBIO/DC-EBIO; NS309; Chlorzaxazone	Apanmin; UCL1684; Bicuculine; Dequalinium; TEA

potassium (BK_{Ca}) channels (Heyer & Lux, 1976; Gorman & Thomas, 1980; Pallotta *et al.*, 1981). A mammalian ortholog of *Drosophila slo, Slo1,* was cloned by hybridisation of a mammalian cDNA library using the *Drosophila* 'slowpoke' (*slo*) cDNA (Pallanck & Ganetzky, 1994). The BK_{Ca} channel encoded by the *Slo1* gene (*KCNMA1*) is expressed in many excitable and nonexcitable cells. BK_{Ca} channels play a role in the control of vascular tone, coupling local increases in intracellular Ca^{2+} to membrane hyperpolarisation and vascular relaxation. BK_{Ca} channels can be activated by membrane depolarisation or intracellular calcium $[Ca^{2+}]_i$ separately or by both factors synergistically (Magleby, 2003). The BK_{Ca} channel belongs to the group of six/seven-transmembrane potassium-selective channels and consists of four α- and four auxiliary β-subunits (Knaus *et al.*, 1994a; Tanaka *et al.*, 1997). The pore-forming α subunit is encoded by the *KCNMA1* gene, which produces multiple isoforms through alternative splicing. The *KCNMA1* gene is located in the chromosome region 10q22.3 (Pallanck & Ganetzky, 1994; Du *et al.*, 2005). The α subunit and four β (1–4) subunits are encoded by different genes that show tissue-specific expression (Higgins *et al.*, 2008; Latorre & Brauchi, 2006; Sausbier *et al.*, 2004; Yu *et al.*, 2006). Different combinations of the β-subunits with α-subunit splice variants generate a physiologically diverse complement of BK_{Ca} channels that differ dramatically in their tissue distribution, trafficking, and regulation (e.g. individual splice variants are differentially sensitive to phosphorylation by cAMP-dependent protein kinase), whose parameters provide the kinetic range needed for electrical fine-tuning (Chen *et al.*, 2005; Langer *et al.*, 2003; Ma *et al.*, 2007; Ramanathan *et al.*, 1999). For a long time the expression of BK_{Ca} channels in ECs was questioned (Nilius & Droogmans, 2001). Currently, however, it is accepted that ECs express BK_{Ca} channels at the mRNA and protein levels (Haburcak *et al.*, 1997; Chiang & Wu, 2001; Wang *et al.*, 2005; Dong *et al.*, 2007). It has also been shown that the BK_{Ca} channel opener CGS7184 can cause endothelium-dependent vasodilatation in isolated aorta rings in a dose-dependent manner, increase NO production, and influence on mitochondrial membrane potential in the endothelial cell line EA.hy 926 (Wrzosek *et al.*, 2009). The discrepancies regarding the existence of BK_{Ca} channels in ECs were likely caused by the tremendous diversity of ECs along the blood arteries and differences in the preparations obtained for studies of BK_{Ca} channel expression and function from freshly isolated and cultured vascular ECs, which are known to exhibit phenotypic drift. There is evidence for the presence of endothelial BK_{Ca} channels that have a potential for rapid upregulation in some intact vessels, which may occur in disease (Sandow & Grayson, 2009). Because the BK_{Ca}

channels have a high conductance, it is conceivable that they could play an important role in the generation of membrane potential in ECs when BK_{Ca} channels are activated and intracellular Ca^{2+} is elevated. Indeed, it has been shown that the expression of BK_{Ca} channels in cultured ECs causes a transient hyperpolarisation induced by ATP (Kamouchi *et al.*, 1997). Studies have shown that polymorphism of the β_1 regulatory subunit of the BK_{Ca} channel modulates the risk of diastolic hypertension in humans.

Modulation of BK_{Ca} channel activity by ROS and RNS

It is well documented that reactive oxygen (ROS) and reactive nitrogen (RNS) species are produced in ECs, and they are very important factors in controlling the cardiovascular function (Droge, 2002; Gutterman *et al.*, 2005; Pacher *et al.*, 2007; Wolin, 2009). The endothelium can generate ROS and RNS through the enzymatic activity of nitric oxide synthases (NOS) (i.e., endothelial NOS (eNOS or NOS3 or cNOS) and inducible NOS (iNOS or NOS2)), xanthine oxidases, NAD(P)H oxidases, cyclooxygenases, cytochrome P450-dependent oxygenases, and leakage of electrons from mitochondria to generate superoxide ($O_2^{\bullet-}$) (Basuroy *et al.*, 2009; Gutterman *et al.*, 2005; Turrens, 2003). NO (also known earlier as endothelium-derived relaxing factor (EDRF)) was discovered as a compound that causes vascular smooth muscle relaxation in the presence of endothelium after stimulation by acetylcholine (ACh) (Furchgott & Zawadzki, 1980). Endothelium-derived RNS and ROS have been proposed to regulate vascular tone *via* complex mechanisms, one of them being the modulation of BK_{Ca} channel function (Matalon *et al.*, 2003). Most information regarding the actions of NO on BK_{Ca} channels comes from studies of vascular smooth muscle preparations. The relaxation caused by NO and NO donors (e.g., NTG, NONOate, SIN-1) and prostacyclin (PGI_2) and its synthetic analogues (e.g., beraprost, iloprost, cicaprost) is associated with concomitant hyperpolarisation of smooth muscle cells (Tanaka *et al.*, 2004). The important features of NO cellular actions are its high membrane permeability and short half-life, which is in the range of seconds. Other free radicals, metal-containing proteins, thiols, and oxygen are the major targets for NO. The NO released from ECs and many nitrovasodilators (e.g., nitroglycerine (NTG)) has been proposed to mediate smooth muscle relaxation *via* the stimulation of soluble guanylate cyclase (sGC) (Gruetter *et al.*, 1981; Cayabyab & Daniel, 1995). It is also well documented that muscle relaxation and membrane hyperpolarisation in smooth muscle can be induced by released NO in a manner independent of cyclic guanosine monophosphate (cGMP) (Bolotina *et al.*, 1994; Watson *et al.*, 1996). At least three possible mechanisms by which NO activates BK_{Ca} channels and leads to vascular smooth muscle relaxation have been proposed, including direct activation by NO *via* modulation of –SH groups, phosphorylation of the BK_{Ca} channel by cGMP-dependent protein kinase (PKG), and inhibition of NO formation by 20-hydroxyeicosatetraenoic acid (20-HETE), an inhibitor of BK_{Ca} channel activity. It is also possible that NO can modulate proteins that interact *in vivo* with BK_{Ca} channels. NO was shown to activate BK_{Ca} channels in a cGMP-independent manner *via* a direct modification of BK_{Ca} channels from vascular smooth muscle (Bolotina *et al.*, 1994; Abderrahmane *et al.*, 1998; Mistry & Garland, 1998; Ahern *et al.*, 1999; Lang *et al.*, 2000). Direct modulation of BK_{Ca} channel activity by NO and ROS has been demonstrated in renal artery endothelium (Brakemeier *et al.*, 2003). Those authors identified BK_{Ca} channels in the endothelium of porcine renal arteries using the patch-clamp technique *in situ*. The activity of the channel was controlled by calcium concentration and membrane potential and was inhibited by Ba^{2+} and iberiotoxin (IbTx), a potent and specific blocker of BK_{Ca} channels. NO donors also activated the channel. It is interesting that hydrogen peroxide led to a dose-dependent inactivation of BK_{Ca} and caused inhibition of vasodilatation of isolated porcine artery after bradykinin treatment. It has been shown that in contrast to NO, intracellular and extracellular challenge of endothelial BK_{Ca} channels with H_2O_2 and ROS results in a dose-dependent and irreversible channel inactivation (Brakemeier *et al.*, 2003). Such an inhibition by H_2O_2 has also been reported for another type of K_{Ca} channel, the intermediate-conductance K_{Ca} (IK_{Ca}), in bovine aortic ECs (Cai & Sauve, 1997). Thus, it is likely that other intracellular second messengers in addition to $[Ca^{2+}]_i$ co-stimulate endothelial BK_{Ca} channel activity. Therefore, such a stimulatory effect on whole-cell currents through BK_{Ca} channels might be a result of such an H_2O_2-induced influx of Ca^{2+}, which presumably overrides the direct inhibitory effects of H_2O_2 on the channel activity (Gupta *et al.*, 2001). There are also studies that support the hypothesis that NO cannot directly modulate BK_{Ca} channel activity. It was shown using whole cell and patch-clamp techniques, that BK_{Ca} channels are present in the endothelial cell line EA.hy 926 and are not stimulated directly by NO (Haburcak *et al.*, 1997). Hydrogen peroxide is produced in endothelial and smooth muscle cells from $O_2^{\bullet-}$, primarily enzymatically by superoxide dismutase. As previously mentioned, H_2O_2 can act as a vasoconstrictor or, depending on the tissue and the experimental conditions, can have dilatory properties that lead to hyperpolarisation of the vascular smooth muscle membrane (Ellis & Triggle, 2003). Oxidative stress is

also recognised as a significant determinant of BK_{Ca} channel function (DiChiara & Reinhart, 1997; Wang *et al.*, 1997; Liu & Gutterman, 2002). It has been proposed that the mechanisms of these changes involve the oxidation of cysteine residues located in the intracellular and C-terminal regions of the channel that alter its voltage- and calcium-dependence (DiChiara & Reinhart, 1995).

Moreover, it has been documented that both redox modulation and nitrothiosylation of cysteine residues on the cytosolic surface of the BK_{Ca} channel protein can alter channel gating (Lang *et al.*, 2000).

Modulation of BK_{Cs} channel activity by carbon monoxide

Carbon monoxide is an endogenous gaseous messenger that regulates physiological function in a variety of tissues in a paracrine and autocrine manner. CO is a deadly poisonous gas physiologically produced during heme catabolism by heme oxygenases (HOs) and is recognised as a biological signalling molecule (Jaggar *et al.*, 2005; Abraham & Kappas, 2008). CO is important in the regulation of vascular tone, synaptic plasticity, and tumour proliferation (Kim *et al.*, 2006). HO-1 and CO play roles in various aspects of vascular disorders, cancer, vascular restenosis, hypertension-impaired wound healing, ischemia/reperfusion, peripheral vascular disease, and atherosclerosis (True *et al.*, 2007; Abraham & Kappas, 2008; Dulak *et al.*, 2008). One target of CO modulation is ECs, where it can modulate the BK_{Ca} channel directly as well as *via* a mechanism involving NO or the cGMP-dependent pathway (Dong *et al.*, 2007). BK_{Ca} channels are involved in the hypoxia-signalling cascade of a number of cellular systems. It has been shown that knockdown of HO-2 expression leads to a reduction in BK_{Ca} channel activity, and the CO production by HO-2 reduces this loss of function (Williams *et al.*, 2004). Specificity to hypoxia is conferred by a highly conserved motif in the stress-regulated exon (STREX) of the BK_{Ca} channel α-subunit splice variant. Expression of the STREX splice variant is tissue-specific and can provide the control mechanism for cellular responses to hypoxia. Mutation of the serine (S24) residue abolished the hypoxia sensitivity of the STREX splice variant (McCartney *et al.*, 2005). Recently, a structural motif that acts as a sensor of CO was localized to the C-terminal tail of the human BK_{Ca} channel within the RCK1 domain and a high-affinity Ca^{2+} sensor (Hou *et al.*, 2008; Williams *et al.*, 2008). In BK_{Ca} channels, motifs that bind reduced heme have been recognised. The data support the hypothesis that reduced heme is a functional CO receptor for BK_{Ca} channels and could provide a mechanism by which gaseous messengers regulate the channel activity (Jaggar *et al.*, 2005). In fact, CO-mediated activation of BK_{Ca} channels can participate in the mesenteric arterial vasodilatation of ascetic cirrhotic rats (Bolognesi *et al.*, 2007). It has also been documented that CO and biliverdin can prevent endothelial cell sloughing in diabetic rats, probably by decreasing oxidative stress (Rodella *et al.*, 2006). The role of the CO and HO-2 pathway in astrocyte signalling is to activate BK_{Ca} channels in smooth muscle arterioles and dilate them (Li *et al.*, 2008a). Recently, it was shown that ECs respond to sheer stress by producing a sustained increase in NO, and a transient increase in ROS production can activate the HO-1 gene. This process is regulated by mitochondria-derived H_2O_2 that diffuses into the cytosol, leading to HO-1 up-regulation and maintenance of ECs protection (Li *et al.*, 2008b). The protective role of CO was demonstrated using the tricarbonylchloro(glycinato)ruthenium (II) (CORM-3) CO carrier in mice with lethal sepsis. Delivery of a controlled amount of CO dramatically reduced mortality in septic mice by supporting mitochondrial energetic metabolism (Lancel *et al.*, 2009). Variuos CO-releasing molecules have been tested for their potency in cell-protective mechanisms (Masini *et al.*, 2008; De Backer *et al.*, 2009). The protective role of CO against hypoxia could, at least in part, be related to activation of BK_{Ca} channels located in the plasma or inner mitochondrial membranes.

Role of the auxiliary β subunits in BK_{Ca} activation

BK_{Ca} channels are accompanied by four types of regulatory auxiliary β-subunits, β1-β4, which are 191 to 235 amino-acid residues long (Knaus *et al.*, 1994b; Wang *et al.*, 2002). The β-subunits have two putative transmembrane segments and an extracellular loop that contains glycosylation sites and cysteine residues capable of forming disulfide bonds. The N- and C-termini of the β-subunits are oriented intracellularly. In mammals, the β-subunits are encoded by the genes *KCNMB1-4* (Brenner *et al.*, 2000; Orio *et al.*, 2002; Liu *et al.*, 2008). Alternative splicing of transcripts encoding the β-subunits, especially the β3-subunit, leads to expression of a large number of proteins that modify cellular function. It seems that β-subunits are not uniformly expressed in every tissue in the body, but their expression is very precisely regulated (Torres *et al.*, 2007). It is especially remarkable that ECs do not express the regulatory β-subunit at the mRNA and protein levels (McManus *et al.*, 1995; Tanaka *et al.*, 1997; Papassotiriou *et al.*, 2000), while the α-subunit of the channel is fully expressed in ECs (Kamouchi *et al.*, 1997; Brakemeier *et al.*, 2003). The BK_{Ca} channel β4-subunit is preferentially localized to brain neurons, not only in the plasma membrane, but also in the inner mitochondrial membrane (Torres *et al.*, 2007; Piwonska *et al.*,

2008). It has been shown that the β4-subunit of BK_{Ca} channels has a role in charibtotoxin (ChTx) and iberiotoxin (IbTx) resistance (Meera *et al.*, 2000; Gan *et al.*, 2008). It seems that the main role of β-subunits is the regulation of sensitivity to $[Ca^{2+}]_i$ and membrane potential. It was shown that β-subunits also have a protective role against digestion of BK_{Ca} channels by trypsin, and the N-termini of the auxiliary β2-subunit causes inactivation of the channel through its pore-blocking position (Zhang *et al.*, 2009). Mice with deleted genes for β1-subunits show impairment in endothelium-dependent smooth muscle relaxation and are characterised by increased vascular superoxide production, which is probably caused by expression of vascular NADPH oxidase and leads to a reduction in cGMP-dependent kinase activity (Oelze *et al.*, 2006). This also enhances the oxidative regulation of BK_{Ca} channels and considerably alters the physiological voltage range at lower $[Ca^{2+}]_i$. Those authors have shown that the M177 β1-subunit is crucial for channel activation and oxidative sensitivity (Santarelli *et al.*, 2004). The β1-subunit enhances the internalisation of the *α*-subunit of the channel (Toro *et al.*, 2006) as well as the β-subunit *via* endocytic trafficking signals that can regulate surface expression of the BK_{Ca} channel (Zarei *et al.*, 2007). The Glu65Lys polymorphism of β1-subunit is associated with reduced systolic blood pressure in middle-aged men (Nielsen *et al.*, 2008). The BK_{Ca} channel is responsible for ethanol tolerance at the molecular and behavioural levels (Martin *et al.*, 2008). It was shown that β-subunit-specific modulation of BK_{Ca} channels and their different distribution in the brain can contribute to the pathophysiologies of epilepsy and dyskinesia (Lee & Cui, 2009). These differences in distribution and expression in different tissues can be critical for the development of β-subunit-selective drugs, as has been shown (Morimoto *et al.*, 2007). Those authors presented data demonstrating drug specificity for the β1- and β4-subunits of BK_{Ca} channels, but not for the β2-subunit.

Proteins interacting with the BK_{Ca} channels

There have been many observations suggesting that a large number of proteins can interact with and modulate BK_{Ca} channel activity. Recently, studies by Kathiresan *et al*. (2009) using coimmunoprecipitation and 2-dimensional PAGE combined with mass spectrometry have revealed 174 putative BK_{Ca} channel-associated proteins (BKAP) from the cytoplasmic and membrane/cytoskeletal fractions of mouse cochlea. The data revealed that 50% of these proteins have affiliations with potassium and calcium channels. It is very interesting that about 20% of the proteins are related to mitochondria. Compartmentalisation of BK_{Ca} channels to the mitochondria has been found to be splice variant-specific for the BK_{Ca}-DEC channel isoform cloned from cochlea. Those authors have identified novel BK_{Ca} channel complexes with important roles in development, calcium binding, chaperone activity and hearing loss. The presented observations also support earlier studies that revealed a wide range of interacting proteins with cellular localisations that regulate BK_{Ca} channel activity. Caveolae are membrane microstructures to which BK_{Ca} channels were found to localise in bovine aortic endothelial cells (Wang *et al.*, 2005). Caveolin-1 interacts directly with BK_{Ca} channels and exerts a negative regulatory effect on their function. Under control conditions, it was shown that BK_{Ca} channels could be activated by cholesterol depletion (Wang *et al.*, 2005). In HEK293T cells, BK_{Ca} channels have a caveolin binding motif that facilitates tethering of the channels to the membrane (Alioua *et al.*, 2008). A possible link between BK_{Ca} channels and the inositol 1,4,5-trisphosphate receptor (IP_3R) *via* lipids rafts in the membrane has also been shown (Weaver *et al.*, 2007). Data presented by those authors suggests a preferential association of BK_{Ca} channels with the lipid raft domain and provides evidence for a novel structure coupling to the source of calcium. Another well-documented interaction was observed between BK_{Ca} and IK_{Ca} channels co-localised in membranes rich in cholesterol (Romanenko *et al.*, 2009). These two channels work in tandem, where the IK_{Ca} channel plays a role as a modulator for the BK_{Ca} channel because of its higher Ca^{2+} sensitivity. Membrane depletion of cholesterol disturbed the interactions between the BK_{Ca} and IK_{Ca} channels, which was restored by disruption of the actin cytoskeleton. In fact, an actin binding domain (ABD) were identified in BK_{Ca} channels, and an interaction between BK_{Ca} and actin is necessary for trafficking of BK_{Ca} channels to the plasma membrane. This interaction is different from that with F-actin that is responsible for stretch-sensitive gating (Zou *et al.*, 2008; Romanenko *et al.*, 2009). LDL, and especially the oxidised form oxo-LDL, can change not only the glycocalyx, but can also modulate BK_{Ca} channel activity. It is possible that oxo-LDL can remove cholesterol from the plasma membrane and thus modulate BK_{Ca} channels. In the rat brain, BK_{Ca} channels were co-purified with voltage-gated calcium channels of the L-type, P/Q-type, and N-type as macromolecular complexes. Complex formation in neurons with different types of voltage-gated calcium channels allows for rapid responses by mediating membrane hyperpolarisation that controls the neuronal release of hormones and neurotransmitters and firing patterns in the central nervous system (Berkefeld *et al.*, 2006). In EA.hy 926 endothelial cells, the existence of BK_{Ca} channel complexes with subplasmalemmal

endoplasmic reticulum (the concept of subplasmalemmal control units (SCCU)) has been detected. These structures are responsible for local activation of BK_{Ca} channels through the release of Ca^{2+} into a limited space, leading to an increase in the local concentration of calcium ions (Frieden & Graier, 2000; Frieden *et al.*, 2002). In many cell types, BK_{Ca} channels are co-expressed with canonical transient receptor potential channels (TRPCs). In podocytes and human embryonic kidney (HEK293T) cells, TRPC6 and TRPC3 channels bind to BK_{Ca} channels, and this microorganisation can serve as an increased source of Ca^{2+} for the activation of BK_{Ca} channels. Additionally, TRPC6 channels can regulate the surface expression of a subset of podocyte BK_{Ca} channels (Larsen *et al.*, 2007; Kim *et al.*, 2009). Experiments employing a yeast two-hybrid screen to identify proteins that interact with BK_{Ca} channels have detected an essential adhesion and scaffolding molecule called nephrin. From the presented data, it was suggested that nephrin plays a role in organising the surface expression of ion channel proteins in podocytes, and may be involved in outside-in signalling to adapt stimuli from neighbouring cells (Kim *et al.*, 2008). In addition to β-subunits, Mink and the Mink-Related peptides 3, which play a role in the human heart, can directly modulate channels (Levy *et al.*, 2008). Nordilysin convertase, a Zn^{2+}-dependent metalloprotease, interacts in human myometrium with a specific BK_{Ca} channel splice variant with a 44 amino-acid insertion (mK44), and is part of the molecular mechanism that regulates the excitability of smooth muscle cells (Korovkina *et al.*, 2009). Modulation of BK_{Ca} channel activity and direct binding have been shown for receptor of activated C kinase 1 (RACK1). RACK1 was discovered as a PKC target, and recent studies suggest that this protein acts as a scaffolding protein (Isacson *et al.*, 2007). In addition to proteins, metabolites of arachidonic acid and other lipids can act as endothelium-derived hyperpolarising factors, which makes the regulatory picture for BK_{Ca} channels very complex (Denson *et al.*, 2006; Campbell & Falck, 2007; Medhora *et al.*, 2008; Vaithianathan *et al.*, 2008; Dhanasekaran *et al.*, 2009).

FINAL REMARKS

The endothelium along the vasculature displays different patterns of adhesion molecule expression and different patterns of leukocyte (macrophage) penetration. The three-dimensional organisations of the vessel and the lining ECs are also varied along the vasculature (Aird, 2007a; 2007b). There is a large variety of ECs along the vascular bed that leads to different expression patterns of different isoforms of BK_{Ca} channels. A number of BK_{Ca} channel isoforms in ECs are expressed only during diseased endothelial states. Considerable data exists supporting the contributions of the BK_{Ca} channel to the development and growth of cancer, and researchers still lack highly specific modulators of this channel. The molecular heterogeneity of normal endothelium and tumour endothelium might represent an opportunity to identify specific and high-potency modulators of specific isoforms of the channel (Kunzelmann, 2005; Aird, 2009). Thus, the endothelium may constitute an attractive target for potassium channel openers that act on BK_{Ca} channels in the plasma membrane, or even as a specific compound that can act on BK_{Ca} channels in the mitochondrial inner membrane. Currently, a large number of potent BK_{Ca} channel modulators are available (see: Wu *et al.*, 2006; Bentzen *et al.*, 2007; Nardi & Olesen, 2008; and Table 2) and future experiments showing their influence on ECs function are needed.

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