Chapter 5
Ca^{2+}/Calmodulin-Gated Small- and Intermediate-Conductance K_{Ca} Channels in Cardiovascular Regulation: Targets for Novel Pharmacological Treatments

Ralf Köhler and Aida Olivan-Viguera

Abstract In vascular biology, the Ca^{2+}/calmodulin-gated K^+ channels, $K_{Ca}3.1$ and $K_{Ca}2.3$, produce membrane hyperpolarization in response to Ca^{2+} mobilization events and thereby initiate endothelium-derived hyperpolarization (EDH)-type of arterial dilation. The physiological relevance of this system in-vivo is evidenced by the observation that genetically encoded loss of $K_{Ca}3.1$ and $K_{Ca}2.3$ caused channel-subtype specific cardiovascular phenotypes characterized by endothelial dysfunction to receptor stimulation or mechanical stress and blood pressure alterations. From the translational perspective, $K_{Ca}3.1$ and $K_{Ca}2.3$ dysfunctions are a feature of idiopathic cardiovascular disease, chronic inflammation, atherosclerosis and organ fibrosis and $K_{Ca}2.3$ has been implicated in atrial fibrillation. Accordingly, $K_{Ca}3.1$ and $K_{Ca}2.3$ emerge as possible drug targets. In this chapter, we would like to highlight our recent advances in $K_{Ca}3.1$ and $K_{Ca}2$ biology, pharmacology, as well as consequences of pharmacological manipulating $K_{Ca}3.1$ and $K_{Ca}2.3$ for systemic cardiovascular regulation and cardiovascular health. Moreover, we explore impacts of innovative channel modulators on cardiac function, physical activity and behavior in keeping with the expression of $K_{Ca}2$-subtypes in the heart and neurons.

Keywords Endothelium • EDH • $K_{Ca}2$ • $K_{Ca}3.1$ • KCNN3 • KCNN4 • Vasodilation • Hypertension • Hypotension • Heart • Behavior

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Introduction

Ca^{2+}-activated K^+ channels (K_{Ca}) are familiar to most electrophysiologists because K_{Ca} channels produce afterhyperpolarizations following an action potential (AP) in neurons and thereby influence firing pattern and neurotransmission [1]. Cardiovascular researchers recognize K_{Ca} as important effector proteins in endothelial control of arterial tone [2–5]. This is based on observations that subtype-selective inhibition of the major vascular K_{Ca}, **K_{Ca}3.1**, **K_{Ca}2.3**, and **K_{Ca}1.1** impair the ***endothelium-derived hyperpolarization factor*** (**EDHF**)-type vasodilation (involving smooth muscle K_{Ca}1.1 as target of a diffusible factor) and the ***endothelium-derived hyperpolarization*** (**EDH**)-type (involving endothelial K_{Ca}3.1/K_{Ca}2.3 as initiators and gap-junctional coupling of endothelium and smooth muscle) (Fig. 5.1). Both mechanisms have in common that they converge on hyperpolarization of smooth muscle leading to closure of voltage-gated Ca^{2+} channels and the resulting drop in intracellular Ca^{2+} causes finally myocyte relaxation and vasodilation. Hence, EDH or EDHF pathways are mechanistically different from pathways involving other

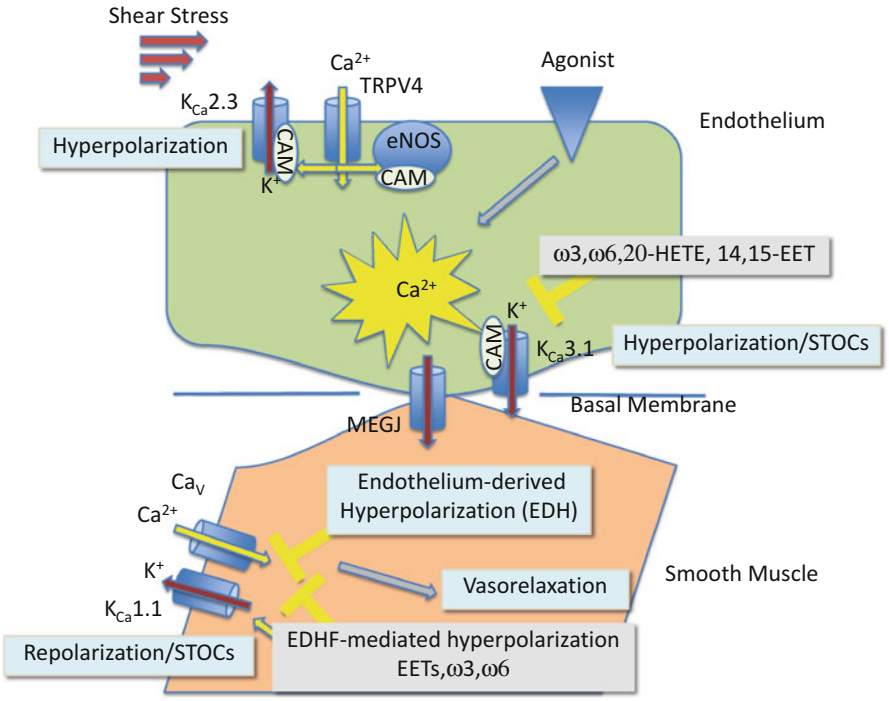

Fig. 5.1 Schematic overview of putative mechanisms of endothelium-derived-hyperpolarization mediated dilation(EDH) and endothelium-derived hyperpolarizing Factor (EDHF)-mediated dilation. *CAM* calmodulin, *eNOS* endothelial nitric oxide synthase, *MEGJ* myo-endothelial gap junction, *STOCs* spontaneous transient outward currents

diffusible molecules like NO or prostaglandins, although the latter paracrine factors are also capable in some cases to induce smooth muscle hyperpolarization and thus to act as *EDHFs* and endothelial hyperpolarization stimulates Ca^{2+}-dependent NO-synthesis by enhancing Ca^{2+} dynamics after receptor stimulations.

At present, the relative physiological significance of either EDHF or EDH systems is still debated, when discussing the input of the respective systems to human vasculature and in cardiovascular disease [4]. Yet, it is clear that the EDHF- or EDH-triggered hyperpolarization of smooth muscle is a potent mechanism that can stand on its own. However, it is not fully understood when and under what conditions EDHF and EDH mechanisms contribute alone, in parallel, or co-operate (or not) with NO-signaling or other diffusible dilating or contracting factors to vasoregulation in the different vascular beds. But it is likely that they integrate in a very complex fashion with NO being without any doubt a major player (for recent extensive review see [6]).

Here, we review mainly the contributions of the authors to their specific field according to the Editors' wish and start with a short introduction into K_{Ca} genes and their expression in the vascular wall, defects of EDH signaling and blood pressure alterations caused by $K_{Ca}3.1/K_{Ca}2.3$ deficiency, followed by K_{Ca}-pharmacology. In the following, the main focus will be on the cardiovascular actions of novel small molecule modulators (activators and inhibitors) targeting endothelial $K_{Ca}3.1$ and $K_{Ca}2.3$ channels in arteries and systemically. Finally, we explore potential therapeutic possibilities and briefly elaborate on possible impacts on cardiac and neurological functions. For additional in-depth and complete reviews and to not diminish previous work by others and us, we wish to re-direct the reader to [4, 7, 8].

K_{Ca} Genes, General Biophysical Properties of K_{Ca}, and Genetic Variations Relevant for Cardiovascular Disease

The gene family of K_{Ca} channels is divided into two subgroups according to phylogenetic relationships, small or large unitary conductance, and the mechanisms of Ca^{2+} activation [9] (Fig. 5.2a). The first group comprises four subtypes, of which three have a small unitary conductance of 5–10 pS (gene names: KCNN1, KCNN2, and KCNN3, protein names SK1-SK3, or KCa2.1-KCa2.3) and one member with an intermediate conductance of 20–40 pS (gene name: KCNN4; protein name: IK, IK_{Ca}, or KCa3.1). The functional channels are tetramers formed by four subunits and the four pore loops located between S5 and S6 of each subunit form the ion conduction pathway. Ca^{2+} sensitivity is conferred by four calmodulin molecules constitutively bound to the four CAM-bindings domains located in the cytosolic c-terminus.

An important feature to understand the roles of these channels in cardiovascular biology is that the channels lack a voltage sensor and are thus voltage-independent, which means that the channels do not inactivate at negative membrane potential like e.g. voltage-gated K_V channels. This feature enables $K_{Ca}2$ and $K_{Ca}3.1$ channels to produce long-standing K^+-outward currents at potentials more negative than the

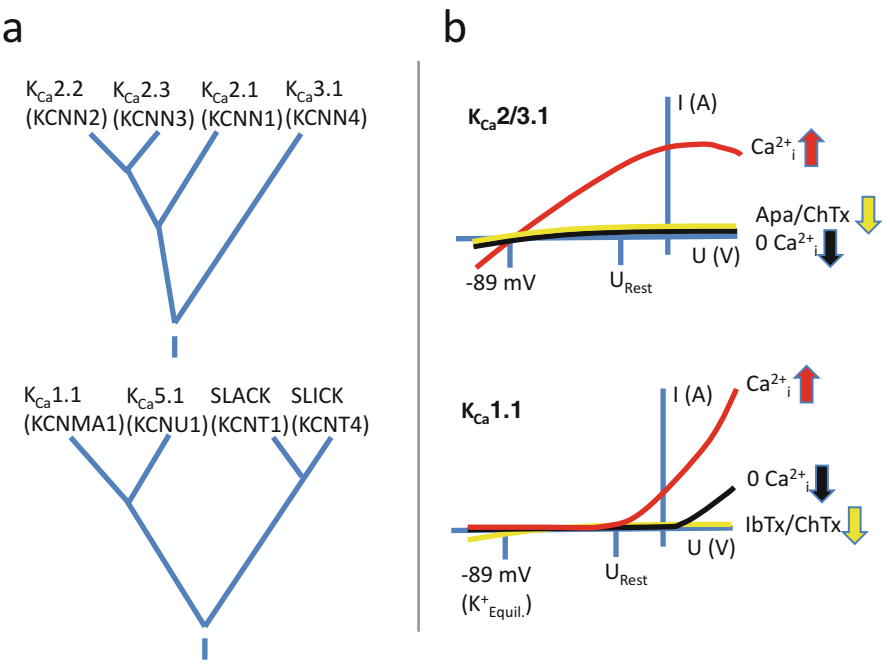

Fig. 5.2 (**a**) Phylogenetic relationships of Ca^{2+}-activated K^+ channel genes. (**b**) Schematic representation of K_{Ca} currents and their sensitivity to peptide toxins. *Upper* panel: Illustration of voltage-independent Ca^{2+}-activated $K_{Ca}2.3/K_{Ca}3.1$ currents in endothelial cells and inhibition by the peptide toxins, apamin (Apa) and charybdotoxin (ChTx). U_{Rest} resting membrane potential; −89 mV is the K equilibrium potential. Note that the $K_{Ca}2.3/K_{Ca}3.1$ channel activation produces voltage-independent currents shifting the reversal potential towards the K equilibrium potential. *Lower* panel: Ca^{2+} activation of voltage-dependent $K_{Ca}1.1$ currents and inhibition by iberiotoxin (IbTx) or ChTx. Note that dual Ca^{2+} and voltage activation occurs at near the U_{Rest}, stabilizing the membrane potential

resting potential[1] and by this they produce persisting membrane hyperpolarization (e.g. to −50 up to max. −89 mV (K^+ equilibrium potential) (Fig. 5.2b). In vascular biology, such a durable hyperpolarization is required for complete closure of smooth muscle voltage-gated Ca^{2+} channels and thereby for the endothelium-dependent hyperpolarization (EDH)-type of arterial dilation [8].

In other tissues, $K_{Ca}2$ (and possibly $K_{Ca}3.1$) produce afterhyperpolarization in neurons and thereby regulate firing frequency [1] and—in the heart—co-determine the length of the atrial action potential and thus atrial repolarization and the contraction cycle [10]. In particular, the link between $K_{Ca}2.3$ and the heart is of interest

[1] Values for endothelial resting potentials vary considerably (from 0 to −89 mV), which very much depends on the preparation (intact vessel vs. cultured cells; observation by the authors' group). In current-clamp experiments on morphologically intact endothelium of murine and human arteries we found values ranging from −25 to −45 mV that mirror the potential in smooth muscle of the same preparation (measured by sharp electrode techniques).

since a genome-wide association study (GWAS) identified an association of the $K_{Ca}2.3$-encoding KCNN3 gene with lone atrial fibrillation in humans [11]. In addition, pharmacological blocking of atrial $K_{Ca}2$ is currently studied as a way to prolong selectively atrial action potential length and thereby to stop atrial fibrillation [12].

In respect to $K_{Ca}3.1$, single-nucleotide polymorphisms (SNP) in the KCNN4 gene have been associated with cardiac infarction in a Japanese cohort [13] and in Crohn's disease in the Australian and New Zealand population [14, 15]. Moreover, there are several reports, which have been reviewed in depth elsewhere, on reduced, increased, or conserved functions of $K_{Ca}2.3$ and $K_{Ca}3.1$ in cardiovascular disease and other diseases such as chronic inflammation, allergy, organ fibrosis, and neurodegeneration [2–4, 16–18]. This assigns $K_{Ca}2.3$ and $K_{Ca}3.1$ as disease-relevant channels in idiopathic disease and targets in translational medicine.

The second subgroup of K_{Ca} channels, which is phylogenetically distantly related to the first subgroup [9, 19], consists of the well known K_{Ca} with large unitary conductance (200–300 pS; gene name KCNMA1, protein name: BK_{Ca}, BigK, Maxi K, or $K_{Ca}1.1$), and three other members (KCNU1, KCNT1, and KCNT2), of which KCNT1 and KCNT2 (protein names: SLACK and SLICK) are in fact more distantly related Na-activated K channels (K_{Na}) with large conductance (150–300 pS) [20].

In contrast to $K_{Ca}2$ and $K_{Ca}3.1$ channels, $K_{Ca}1.1$ contains a voltage-sensor in S4 producing gating by depolarization, particularly in a membrane potential range, in which voltage-dependent L-type Ca^{2+} channels operate (Fig. 5.2b). The $K_{Ca}1.1$ channel complex recruits four beta-1 subunits that sensitize channel gating to Ca^{2+} and voltage by favoring channel opening at physiological internal Ca^{2+} levels reached in the vicinity of the $K_{Ca}1.1$ channel Ca^{2+} sensors.

In respect to gene expression of channels of this subfamily in the vasculature, $K_{Ca}1.1$ is predominant in smooth muscle and the CNS while other members (SLICK and SLACK) are predominantly expressed in neuronal tissues. $K_{Ca}1.1$ forms complex functional units ("signalosomes") with physical interactions with voltage-regulated L-type Ca^{2+} channels [21, 22]. The electrical membrane events of such co-operations are spontaneous-transient-outward-currents (STOCs, mediated by $K_{Ca}1.1$) that are triggered by spatially co-localized Ca^{2+} influx through L-type Ca^{2+} channels and small Ca^{2+} release events from the sarcoplasmic reticulum, termed "sparks" [22, 23]. Physiologically, these $K_{Ca}1.1$-mediated STOCs are considered to provide a negative feedback on Ca^{2+} influx through L-type Ca^{2+} channels and thereby to limit depolarization and contractions or vasospasm [21, 24]. Presumably, diffusible EDHFs, like epoxyeicosanoids augment $K_{Ca}1.1$-mediated STOCs and by this produce their vasorelaxing actions.

In contrast to $K_{Ca}3.1$ and $K_{Ca}2.3$ channels, $K_{Ca}1.1$ does not seem to be expressed under physiological conditions in human, rat, and murine arterial endothelium, although porcine endothelium could be an exception [25, 26].

From the epidemiological perspective, SNPs in the $K_{Ca}1.1$-encoding KCNMA1 gene causing "loss-of-function" or in the associated beta-subunit gene (KCNMB1) causing "gain-of-function" are associated with systolic hypertension (KCNMA1) or resistance to hypertension, respectively [14, 27, 28].

K_{Ca} and Their Functions in Arterial Endothelium

In early nineties, the genes encoding for channels were still awaiting cloning ($K_{Ca}3.1$ in 1997 [29] and $K_{Ca}2$ in 1996, [30]) and K_{Ca} channels were pharmacologically characterized in endothelium and differentiated according to their sensitivity to the peptide toxins, apamin ($K_{Ca}2$), charybdotoxin ($K_{Ca}3.1$ and $K_{Ca}1.1$), and iberiotoxin ($K_{Ca}1.1$), and the small molecules, clotrimazole ($K_{Ca}3.1$) [31], and paxilline ($K_{Ca}1.1$) [32]. $K_{Ca}2$-blocking compounds like UCL-1684 [33] have not been invented yet. Notably, in 1996, Marchenko and Sage were first on providing comprehensive data on functional $K_{Ca}3.1$ and $K_{Ca}2$ by elegant in-situ patch-clamp electrophysiology on intact endothelium of isolated rat aorta [34]. Almost contemporaneously, $K_{Ca}3.1$ currents were identified in endothelia from different species and different vascular beds [2, 3, 35], for instance, in native bovine and rat aortic endothelial cells [36, 37], and rat brain capillaries [35]. In general, expression of $K_{Ca}3.1$ and $K_{Ca}2.3$ is a common finding for endothelia of several species and is documented throughout the entire vascular tree, with no considerable differences (at least qualitatively) between arterioles, arterioles of the microcirculation, and veins.

Moreover, alterations of $K_{Ca}3.1$ currents have been documented in a model of experimental hypertension, the spontaneously hypertensive rat [37]. This provided early electrophysiological evidence that endothelial $K_{Ca}3.1$ channels were altered in a hypertension-associated fashion. Moreover, these findings point to a capacity of endothelial channels to respond in a probably compensatory way to hypertension in this model. A few years later, the genes encoding for $K_{Ca}3.1$ and $K_{Ca}2.3$ currents were identified by others [26, 38] and our group as KCNN4 and KCNN3 using a single-cell RT-PCR approach combined with patch-clamp in endothelium from rats [39] and—importantly from the translational perspective—in human mesenteric endothelium [40, 41].

K_{Ca} and Endothelium-Dependent Vasodilation

In the eighties, combinations of peptide toxins in cardiovascular pharmacology were widely used to study endothelium-dependent vasodilator mechanisms by classical in-vitro myography on large rodent arteries. A contribution of $K_{Ca}3.1$ and $K_{Ca}2$ to the "EDHF" has been deduced from the blocking actions of the combination of charydotoxin ($K_{Ca}3.1$ and $K_{Ca}1.1$) and apamin ($K_{Ca}2$). A contribution of $K_{Ca}1.1$ has been discarded as iberiotoxin ($K_{Ca}1.1$) cannot replace charybdotoxin [4]. Yet, these peptide blockers are not fully selective and e.g. charybdotoxin—besides $K_{Ca}3.1$ and $K_{Ca}1.1$—blocks some Kv channels that are present in arterial tissue. Interestingly, in coronary arteries of pigs, "EDHF" required reportedly smooth muscle $K_{Ca}1.1$ as the response was abrogated by iberiotoxin, which suggested $K_{Ca}1.1$ as target of a diffusible EDHF (Fig. 5.1) that has been later identified as a P450-derived eicosanoids [42]. Still, vasorelaxation that involves endothelial $K_{Ca}3.1$ and $K_{Ca}2.3$ is also present in porcine coronary artery as concluded from more recent studies by others and us [43, 44].

It is noteworthy that P450-products and ω3 and ω6 fatty acids that activate $K_{Ca}1.1$ and have been used as antagonist of EDHF-signaling—like 20-HETE [45]—inhibit

$K_{Ca}3.1$ [46], suggesting interactions between putative EDHF and EDH mechanisms (Fig. 5.1). Despite this evidence from in-vitro experimentation, the true contribution of EDH or possibly EDHF to circulatory regulation in humans is not established at present and a matter of ongoing debate. A main reason for the lack of knowledge is that we miss sufficiently selective, potent, safe, and ethically approved pharmacological tools for in-vivo investigation. Yet, in the following paragraphs we explore potential utilities of novel small molecule activators and inhibitors.

Endothelial Dysfunction Caused by Genetic $K_{Ca}3.1/K_{Ca}2.3$-Deficiency

Since 2003, the availability of gene-targeted mice as tools provided more definite experimental evidence for the significance of $K_{Ca}3.1$ and $K_{Ca}2.3$ channels for EDH-type dilation in-vitro and in-vivo [47–49]. Indeed, myography on conduit arteries and intra-vital microscopy on resistance-size skeletal muscle arterioles of $K_{Ca}3.1{-}/{-}/K_{Ca}2.3^{T/T}$ + Dox mice with genetically encoded deficiency of both endothelial channels revealed that loss of $K_{Ca}3.1$ and $K_{Ca}2.3$ impairs strongly endothelial and smooth muscle membrane hyperpolarization and EDH-type dilations in both vessel types [50–52] (Fig. 5.3a). Interestingly, also NO-type dilations were disturbed in the mice, suggesting that $K_{Ca}3.1$ and $K_{Ca}2.3$-mediated hyperpolarization promotes Ca^{2+} influx and thereby stimulates also Ca^{2+}-dependent NO production [50, 51, 53]. A candidate channel mediating the Ca^{2+} influx during hyperpolarization is the TRPV4 channel [54–56], which conducts considerably Ca^{2+} at clearly negative potentials while it shows negative-feedback regulation by Ca^{2+} at potentials near zero. In support of the above-mentioned, loss of $K_{Ca}3.1$ in $K_{Ca}3.1{-}/{-}$ and pharmacological inhibition of TRPV4 in mesenteric artery impaired Ca^{2+} dynamics to acetylcholine [55].

Moreover, our studies on single and double $K_{Ca}3.1/K_{Ca}2.3$-deficient mice [50] revealed subtype specific roles of the channels for EDH-type dilation since $K_{Ca}3.1$ is of greater importance than $K_{Ca}2$ for acetylcholine-induced dilations while $K_{Ca}2.3$ is more important in dilations caused by mechanical stress acting on the endothelium (shear stress), for hyperemia during skeletal muscle twitching [52] and for a basal tonic dilator input from the endothelium [49]. Thus, a $K_{Ca}2.3$-EDH system is likely needed to regulate basal blood flow and the higher blood flow demand during exercise (metabolic demand) while $K_{Ca}3.1$-EDH is linked to muscarinic receptor stimulation and ER Ca^{2+} release and to possibly sympathetic drive on resistance arteries.

Some of these subtype specific roles can be explained by a differential intracellular localization of $K_{Ca}3.1$ and $K_{Ca}2.3$ in the endothelial cell (Fig. 5.1). However, there are some caveats because of the uncertain specificity of antibodies used to localize $K_{Ca}3.1$ and $K_{Ca}2.3$ and of lack of approach validation in KO-controls. Regardless of these uncertainties, $K_{Ca}3.1$ has been localized in proximity to protrusions of the endoplasmic reticulum (ER) [57] where it senses Ca^{2+} release events after e.g. muscarinic receptor (M3) stimulation by acetylcholine. Moreover, $K_{Ca}3.1$ has been reported to co-localize with myo-endothelial gap-junction proteins thus forming a functional unit for electro-tonic spread of EDH to smooth muscle (for review [2, 58–60]).

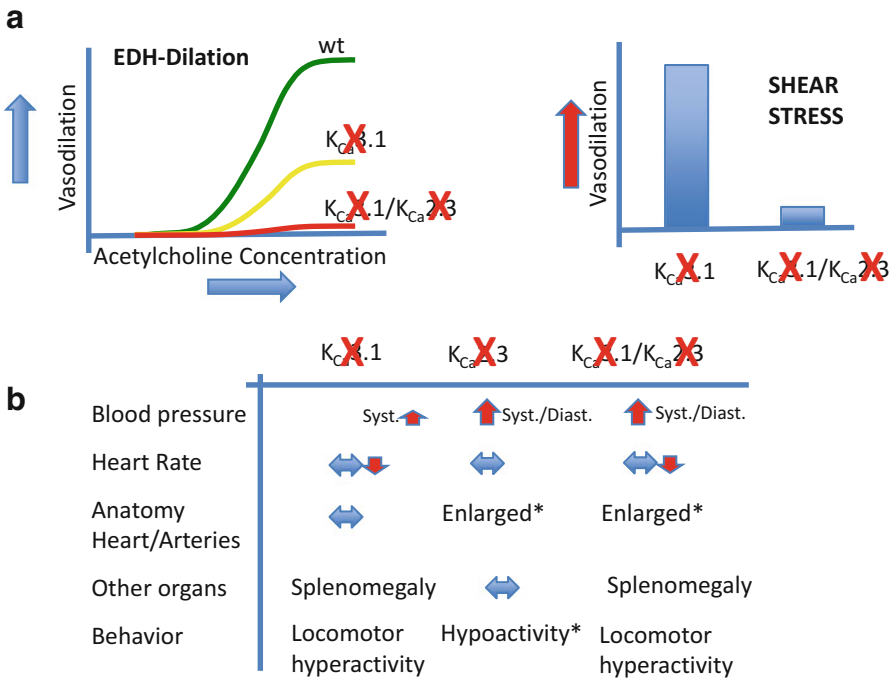

Fig. 5.3 (**a**) On *left*: Schematic illustration of the impaired EDH-type arterial dilation to acetylcholine in $K_{Ca}3.1$ and/or $K_{Ca}2.3$-deficient mice. On *right*: Impaired shear stress-induced EDH-type dilation in $K_{Ca}2.3$-deficient mice. (**b**) Major cardiovascular, anatomical, and behavioral phenotypes. The *asterisks* (*) indicate phenotypes that are unaltered by subchronic treatment with doxycycline to suppress $K_{Ca}2.3$-gene expression. Note that $K_{Ca}3.1$−/− exhibit mild systolic hypertension only during locomotor activity

$K_{Ca}2.3$ has been found at intercellular junctions [61] where it is suggested to form a functional unit with TRPV4 [62] or possibly with another Ca^{2+}-permeable channels. Interestingly, such a functional unit has recently been suggested to occur in the tubular system of the kidney [63] as well as in pulmonary and mesenteric arteries [62, 64], where TRPV4 uses $K_{Ca}2.3$ to produce vasodilation. Nevertheless, we need more definite visualization of $K_{Ca}3.1$ and $K_{Ca}2.3$ using gold-labeled AB, KO-controls, and transmission electron microscopy to ascertain the present view.

Interplay of $K_{Ca}3.1/K_{Ca}2.3$ Channels with Ca^{2+}-Permeable Channels

Evidence from patch-clamp studies point at close functional interactions of endothelial K_{Ca} with Ca^{2+}-permeable channels (Fig. 5.1): While patch-clamping the endothelium of intact vessel preparations or bovine endothelial cells in a flow chamber, we frequently observed that $K_{Ca}3.1$ gating and membrane hyperpolarization occurs

following stimulation of shear stress-activated or hyposmotic stress-activated Ca^{2+} permeable channels [65]. Moreover, these studies showed temporally fluctuating Ca^{2+} release events that amplified $K_{Ca}3.1$ activation. These fluctuating Ca^{2+} release events and concomitant $K_{Ca}3.1$-mediated currents and hyperpolarization were modulated in frequency and amplitude by the degree of shear force acting on the cells. Interestingly, these fluctuating currents shared some similarities with spontaneous transient outward currents (STOC) seen in smooth muscle, although the endothelial STOCS were of longer duration and largely voltage-independent and did not involve $K_{Ca}1.1$ and $K_{Ca}1.1$-interacting L-type Ca^{2+} channels as in smooth muscle. Today, one of the candidate MSCs triggering Ca^{2+} signaling events and co-activation of $K_{Ca}3.1$ (and $K_{Ca}2.3$) to flow or shear stress stimulation is (again) TRPV4 as concluded from elegant in-situ imaging in cerebral and mesenteric arteries [66] and by the loss of shear stress-induced dilation in carotid arteries from TRPV4−/− mice [67].

Thus, from the perspective of integrative cardiovascular physiology, the interplay of TRPV4 and $K_{Ca}2.3$ ($K_{Ca}3.1$) can be mechanistically important to fine-tune EDH and arterial tone in response to alterations of blood flow in e.g. the working skeletal muscle or the brain with their metabolic demands [52]. In terms of cross-reactivity with other vasodilator systems, TRPV4 has been proposed to physically interact with the scaffold protein caveolin-1 and thus the endothelial nitric oxide synthase (eNOS) [68] and the Ca^{2+} influx through TRPV4 further stimulates Ca^{2+}-dependent NO synthesis [54] in addition to EDH, producing some redundancy in endothelial vasoregulator capacity and during exercise.

It should be noted that $K_{Ca}3.1$ and $K_{Ca}2$ channels are not considered "smooth muscle" channels. In fact, according to our electrophysiological studies, $K_{Ca}3.1$ channels are absent in contractile i.e., differentiated, vascular smooth muscle of rodents and this makes some physiological sense because the channels are highly Ca^{2+}-sensitive because of the Ca^{2+} sensor, calmodulin, and, moreover, are voltage-independent. Functioning of $K_{Ca}3.1$ in smooth muscle would almost immediately abrogate any contraction because the relatively high Ca^{2+} signaling in contracting smooth muscle would cause strong $K_{Ca}3.1$ activation and lasting hyperpolarization, which in turn causes cessation of Ca^{2+} influx. In other words, $K_{Ca}3.1$ would provide a too strong negative feedback on Ca^{2+}-dependent contraction. In contrast, the voltage-dependent and less Ca^{2+}-sensitive $K_{Ca}1.1$—known as smooth muscle channel—is the better channel to mediate negative feedback during depolarization. With respect to $K_{Ca}2$ it is—because of the same theoretical considerations—rather unlikely that these closely related and similarly voltage-independent $K_{Ca}2$ channels are functionally expressed in contractile smooth muscle. We have not been successful in detecting $K_{Ca}2$ channel functions in vascular smooth muscle of the rat and mouse.

Systemic Cardiovascular Alterations in $K_{Ca}3.1/K_{Ca}2.3$-Deficient Mice

That $K_{Ca}3.1$ and $K_{Ca}2.3$ channels add significantly to systemic cardiovascular regulation has been suggested by the higher blood pressure in $K_{Ca}3.1$−/− mice [50] and $K_{Ca}2.3$-deficient mice ($K_{Ca}2.3^{T/T}$ + Dox) [49, 50] as found by blood pressure

telemetry in the freely moving animals (Fig. 5.3b). The hypertension has been reported to be, however, mild and overt only during physical activity (for review see [7]). Still, the latter observation was particularly interesting as it could mean that $K_{Ca}3.1$ and $K_{Ca}2.3$ and the EDH-system are needed to adjust tone during exercise and sympathetic input while they are of less or no importance during rest. Recently, $K_{Ca}3.1-/-$ and connexin(Cx)37−/− mice—Cx37 that has been considered a major constituent of myo-endothelial gap junctions—have been shown to exhibit lower ADP/ATP-receptor ($P2Y_{2/4}$)-mediated fast depressor responses and secondary increases in pressure [69] in anesthetized mice, suggesting decreased responsiveness to evoked endothelial function. Responses to acetylcholine-infusion has been reported to also be reduced at low but not high concentrations, suggesting endothelial dysfunction in vivo as it has been observed so far only in isolated $K_{Ca}3.1-/-$ arteries.

Regarding the contribution of nitric oxide in these mice, inhibition by L-NAME has been shown to be still capable to increase blood pressure in $K_{Ca}3.1-/-/K_{Ca}2.3^{T/T}$ + Dox mice [7], indicating that NO signaling is largely intact in these mice and it is therefore likely that NO- and EDH mechanisms act independently from each other to a large extent but are likely additive.

Other Phenotypes

It is worth to mention that $K_{Ca}3.1-/-$ mice have been shown to exhibit spontaneous physical hyperactivity (but without signs of general distress) and exhibited complex alteration in monoamine levels, with increased noradrenalin turnover in frontal cortex and lower serotonin-turnover in frontal cortex, striatum and brain stem, and no change in dopamine turnover [70]. Plasma corticosterone has been found to be normal but increased to higher levels in $K_{Ca}3.1-/-$ under stress as reported by others [71]. This suggests substantial alteration of neurotransmission and behavior in $K_{Ca}3.1$, which can add to the cardiovascular phenotypes in these mice. Another solid phenotype in $K_{Ca}3.1-/-$ is progressive splenomegaly (Fig. 5.3b) caused presumably by defects in erythrocyte volume regulation and a resulting increased erythrocyte degradation and turnover [72], producing a higher workload for the spleen and secondary adaptation of size to meet the demand.

Also $K_{Ca}2.3^{T/T}$ (+Dox/−Dox) have complex behavioral and morphological phenotypes (Fig. 5.3b) [1, 70], which can be linked in $K_{Ca}2.3^{T/T}$−Dox to larger afterhyperpolarization, refractory periods, and thus firing frequencies. Locomotor hypoactivity is evident in $K_{Ca}2.3^{T/T}$+Dox and −Dox (Fig. 5.3b). Moreover, there are significant anatomical (developmental) changes in the vasculature and the heart, i.e. visible enlargement of diameters of mesenteric and uterine arteries and an enlarged heart with increased right ventricular wall thickness and altered atrioventricular morphology [49, 73, 74]. Postnatal development of retinal vessels has been found to be normal, although deficiency of $K_{Ca}2.3$ ($K_{Ca}2.3^{T/T}$+Dox) increased branching (D. Rappert, 2011, German MD thesis; archiv.ub.uni-marburg.de/diss/z2011/0306/

pdf/ddr.pdf). In sum, these anatomical changes suggest that alterations of $K_{Ca}2.3$ expression levels can have a substantial impact on the development of the vasculature. As possible pathophysiological consequences, the mice experience parturition defects [48] and sudden cardiac death [75] when over-expressing the channels ($K_{Ca}2.3^{T/T}$–Dox).

$K_{Ca}3.1$ and $K_{Ca}2.3$ in Cardiovascular and Other Disease States

There is growing evidence that $K_{Ca}3.1$ and $K_{Ca}2.3$ channels are differentially regulated in disease. In fact, up-regulated $K_{Ca}3.1$ functions have been found to promote many disease states characterized by abnormal proliferative organ remodeling and inflammation such as neointima formation [76, 77], atherosclerosis[78], fibrosis of lungs [79], fibrosis in damaged and diabetic kidneys [80, 81], possibly liver cirrhosis and portal hypertension [82], allograft vasculopathy [83], kidney allograft rejection [84], as well as in obliterated trachea transplants [85]. Such up-regulation of $K_{Ca}3.1$ in diseased tissues reflected infiltration of $K_{Ca}3.1$-expressing T cells and macrophages and on de-novo mRNA-expression of $K_{Ca}3.1$ in the tissue itself. The de-novo mRNA-expression was induced by actions of classical growth factors on smooth muscle, fibroblast, and endothelial cells *in vitro* [80, 86, 87]. Moreover, shear stress has been shown to induce $K_{Ca}3.1$ in human endothelial cells [88]. In general, the induction of $K_{Ca}3.1$-mRNA synthesis requires activation of the MEK/ERK MAP kinase cascade and transcriptional mechanisms involving activator protein-1 (c-jun) and repressor protein-1 as negative regulator of gene expression [87]. Also the Ca^{2+}/calmodulin-dependent protein kinase kinase/Akt/p300 cascade has recently been reported to mediate the up-regulation of $K_{Ca}3.1$ in endothelial cells during shear stress stimulation [89].

With respect to endothelial functions and cardiovascular disease, endothelial $K_{Ca}2.3$ function and EDH were reduced in regenerated endothelium [90], renal insufficiency [91], ageing [92], and ovariectomy [93], which could explain some aspects of the endothelial dysfunction reported in these conditions. However, up-regulation of gene expression of the channel has been documented in obese rats [94, 95], in pulmonary hypertensive mice [74], and by shear stress [89] in vitro, suggesting here compensatory roles of the channel to maintain/improve endothelial vasodilator function under these conditions. The precise mechanisms of the altered gene expression in endothelium are not understood. However, estrogen is considered a major regulator of $K_{Ca}2.3$ expression and this positive regulation can be an additional explanation for pre-menopausal cardiovascular protection in women.

Interestingly, low $K_{Ca}2.3$ mRNA expression in mammary arteries correlated negatively ($r > -0.5$) with blood pressure in a small Danish cohort of patients with or without renal failure (uremia) or of diabetic and non-diabetic patients (all, $n = 55$) (unpublished data provided by Dr. Lars Melholt Rasmussen, Director of the Odense Artery Biobank SDU, Denmark).

In contrast, $K_{Ca}3.1$ expression did not correlate with blood pressure.

Regarding other tissue and disease states, it is worth to mention that, both, $K_{Ca}2.3$ and $K_{Ca}3.1$ are highly expressed in some cancers [96–99] and have been implicated in cancer progression and metastasis (for review of the field see also [100, 101]). In addition, $K_{Ca}3.1$ has been found to be up-regulated during immune responses and in chronic inflammation such as in T cells within the chronically inflamed mucosa in ulcerative colitis [102] and in microglia after ischemic stroke [103], which is in line with the notion that $K_{Ca}3.1$ is an important regulator of immune cell function [18].

Endothelial $K_{Ca}3.1$ and $K_{Ca}2.3$, Their Pharmacological Manipulation, and Systemic Cardiovascular Consequences

From the pharmacological viewpoint, the introduction of TRAM-34 as a potent and selective $K_{Ca}3.1$ blocker in early 2000 by the laboratory of Heike Wulff provided a "modernized" pharmacological tool to define mechanisms of EDH-type or EDHF-type dilation, in addition to TRAM-34's value for testing roles of $K_{Ca}3.1$ in inflammatory and autoimmune disease [104]. TRAM-34 derived from the fungicide and P450-blocker, BayerAG compound clotrimazole that was the first well studied small molecule blocker of $K_{Ca}3.1$ (Fig. 5.4a). TRAM-34 is more selective for $K_{Ca}3.1$ since the imidazole ring in clotrimazole—being required for P450-blockade—was replaced by a pyrazole ring. TRAM-34 was initially developed as possible immunosuppressant as it blocks $K_{Ca}3.1$ in T cells and macrophages and thereby proliferation, migration, and cytokine production of lymphocytes [104].

The introduction of TRAM-34 into the cardiovascular field greatly helped to define with more precision the requirement of $K_{Ca}3.1$ for EDH-type dilation in several vascular beds and from different species, including human arteries. While it is clear that TRAM-34 reduces acetylcholine-induced vasodilations, this does not have any systemic consequences in vivo since TRAM-34 injections into mice or rats did not cause a change in blood pressure. Interestingly, blocking $K_{Ca}3.1$ in the blood-brain-barrier by TRAM-34 has been reported to reduce water movements into the brain and early edema formation after ischemic stroke [105]. Another structurally different $K_{Ca}3.1$-blocker developed by the BayerAG was effective in reducing brain edema caused by trauma [106].

Senicapoc (ICA-17043; Fig. 5.4a), another $K_{Ca}3.1$-blocker similar to TRAM-34 advanced into clinical trials as a treatment for sickle cell disease [107] and has been found to be cardiovascular-safe since it did not increase blood pressure in humans. Senicapoc has been proposed to be efficient in sickle cell disease because blocking of the over-active $K_{Ca}3.1$ may hinder irreversible erythrocyte sickling (shrinkage) by blocking volume reduction by $K_{Ca}3.1$-mediated K efflux. In Phase IIb and III clinical trials, Senicapoc was found to improve erythrocyte parameters and hemoglobin content, but, unfortunately, did not prevent but rather increased painful vaso-occlusive crisis as primary end point for unknown reasons. The disappointing outcome terminated the development of Senicapoc at least for this indication. Whether endothelial

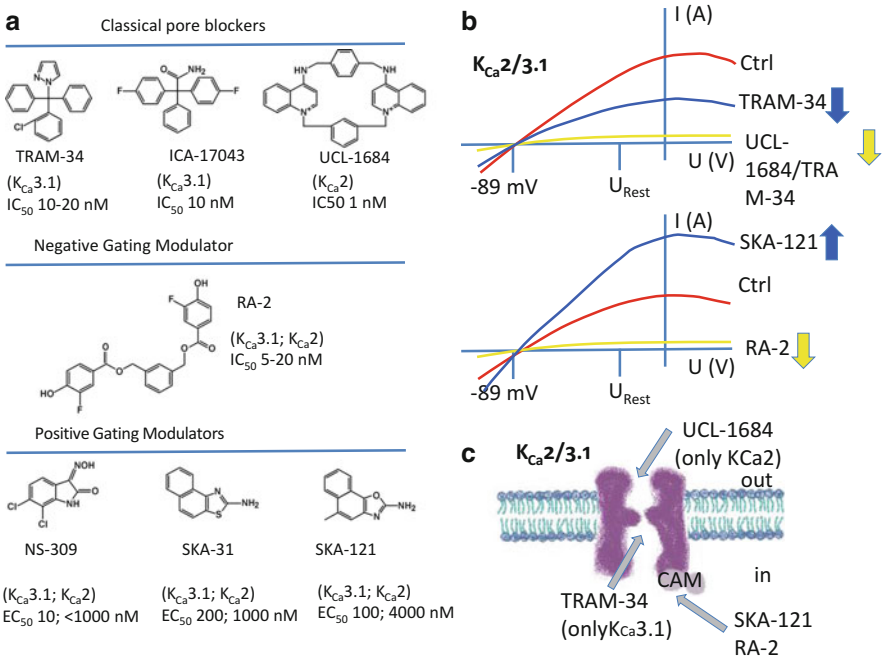

Fig. 5.4 (**a**) Chemical structures of selected $K_{Ca}2/3.1$ modulators with nanomolar potencies. (**b**) *Upper* panel: Schematic illustration of inhibition of mixed voltage-independent Ca^{2+}-activated $K_{Ca}2.3/K_{Ca}3.1$ currents by TRAM-34 ($K_{Ca}3.1$) and UCL-1684 ($K_{Ca}2.3$). *Lower* panel: SKA-121-evoked potentiation of mixed $K_{Ca}2.3/K_{Ca}3.1$ currents and antagonism by the pan-negative gating modulator, RA-2, at equimolar concentration. (**c**) Hypothetical interaction sides of blockers and gating modulators

dysfunction was one reason why Senicapoc failed is not known and has not been studied further.

From a more general perspective, we may consider that—during $K_{Ca}3.1$-blockade—the other potent vasodilator system, the NO system and $K_{Ca}2.3$ channels are still functional and have the capability to compensate the inhibition of $K_{Ca}3.1$. Still, in the case of reduced NO-synthesis and/or NO-availability, inhibition of endothelial $K_{Ca}3.1$ (and $K_{Ca}2.3$) may aggravate the impaired endothelial vasodilator influence on tone and could represent a cardiovascular safety issue.

Senicapoc has been also tested as treatment for exercise and allergic asthma and failed. Still, this $K_{Ca}3.1$ blocker—albeit safe—may have other utilities as mild in the case of immunosuppressant in chronic inflammatory disease, e.g. chronic inflammatory bowel disease, in reducing astrocyte-mediated secondary damage after spinal cord injury [108] and microglia-mediated neurotoxicity [109] and neurodegenerative disease such as Alzheimer's disease [110].

Recent Developments: Positive-Gating Modulators

From the perspective of cardiovascular translational pharmacology, activators of $K_{Ca}2.3$ and $K_{Ca}3.1$ may have more therapeutic utilities in cardiovascular disease characterized by endothelial dysfunction than obviously blockers. However, the existing activators with reasonable potency have not been tested in humans. At present all experimental evidence for their potential therapeutic utilities in cardiovascular disease relies on in-vitro experimentation and few in-vivo cardiovascular monitoring in rats, mice, and dogs [111–114].

The Neurosearch compound NS309 (Fig. 5.4a) has widespread use in in-vitro pharmacological experimentation. It has a high potency but a disadvantageous selectivity profile as the compound, besides activating $K_{Ca}3.1$ and $K_{Ca}2$ channels, has additional blocking effects on voltage-gated Ca^{2+} channels. Moreover, NS309 blocks cardiac hERG channels that may impair ventricular repolarization, which can be regarded a severe safety issue.

The development of SKA-31 (Fig. 5.4a), using the ALS-drug Riluzole (a mixed glutamate antagonist, Na^+ channel blocker and unselective K^+ channel activator with low potency) as template, provides first evidence that systemic activation of $K_{Ca}3.1$/$K_{Ca}2.3$ improves endothelial vasodilator function, particularly in vivo. In fact, SKA-31 displays fivefold selectivity for $K_{Ca}3.1$ over $K_{Ca}2$, channels and has been shown to potentiate endothelial $K_{Ca}3.1$/$K_{Ca}2.3$ currents as well as EDH-vasodilation to acetylcholine in carotid arteries of mice [53, 111]. In the microcirculation of skeletal muscle (cremaster muscle) SKA-31 has be shown to induce vasodilation in its own right [115], suggesting that there is some basal Ca^{2+} signaling or "Ca^{2+} pulsar" activity that gives rise to some $K_{Ca}3.1$ activation, which can be potentiated by SKA-31. Moreover, SKA-31 has been found to improve coronary blood flow in rats [112], to potentiate bradykinin-induced relaxation and to reduce serotonin-induced contraction in large porcine coronary artery [44]. In a model of severe fatal hypertension, SKA-31 has been found to increase renal blood flow and to increase survival in mouse model of fatal hypertension [16, 116]. In other tissues, SKA-31 modulated bladder contractility and SKA-31 decreased human detrusor muscle excitability and contractility, suggesting utility of the activator in overactive bladder [117].

Mechanistically, it should be noted, that SKA-31 and recently developed other SKAs [114] are not simple channel openers like other channel openers that activate the channel in the closed configuration (no gating). Rather, SKAs act as positive-gating modulators that keep the Ca^{2+}-gated channel in the open-configuration and thereby shift Ca^{2+} dependence of the channel to the left [114] (Fig. 5.5a). In other words, the channel has a higher activity (open probability) at Ca^{2+} concentrations that are normally not sufficient to produce large channel activation on its own.

The very elegant work by Michael Zhang's group on crystals of the $K_{Ca}2.2$ c-terminus has provided first insight into molecular details and potential bindings domains. They showed that 1-EBIO, an early less potent precursor of DC-EBIO, fits into a pocket between calmodulin and the c-terminus [118] (Fig. 5.4c). Moreover, they showed that NS309, the other activator of this compound class, stabilizes such interactions by stabilizing an intrinsically unordered linker between the CAM-binding

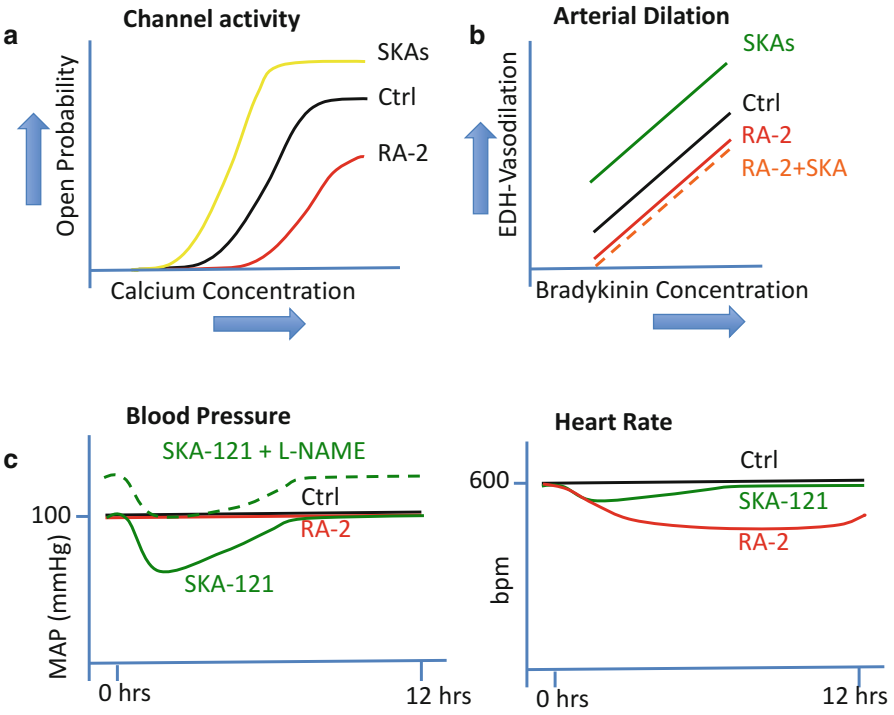

Fig. 5.5 (**a**) Schematic illustration of increase of Ca^{2+} sensitivity of $K_{Ca}3.1$-activity (open probability) by SKAs (*left* shift of the concentration-response curve). RA-2 produces a *right* shift of the curve, indicating reduced Ca^{2+} sensitivity. (**b**) Schematic illustration of SKA-induced potentiation of bradykinin-induced dilation of porcine coronary artery and antagonism by RA-2. (**c**) On *left*: Illustration of blood pressure lowering effects of SKA-121. RA-2 has no appreciable effect. On *right*: Mild bradycardia induced by SKA-121 and appreciable bradycardia evoked by RA-2

domain to S6, which can explain the molecular mechanics of channel activation ([119] for details; for review see [120]).

What would be the advantage of a positive-gating modulator compared to a more classical activator such as e.g. activators of K_{ATP} channels in cardiovascular disease? A mechanistic advantage of the $K_{Ca}2/3$ activators is presumably that they act only when there is Ca^{2+} mobilization, e.g. in form of "Ca^{2+} pulsars" in the endothelium [57] and thus initiation of endothelial function. Here, we would stimulate the active endothelium or potentiate endothelial function "when it is needed". Indeed, at present we understand well how the endothelium regulates arterial tone *in-vitro* and inhibition of NO synthesis *in-vivo* clearly elevates blood pressure while NO-donors are in the clinics since long to alleviate angina pectoris and hypertensive crisis. This fosters the pivotal role of NO in the systemic circulatory regulation. However, we do not know when endothelial EDH-vasodilator function occurs in the organisms and under physiological conditions and what are the consequences systemically or locally.

In situation of endothelial dysfunction/degeneration with loss of both channels an activator would not make sense at all while some conserved $K_{Ca}3.1/K_{Ca}2.3$ functions could be potentiated and improve thereby endothelial vasodilator function.

Systemic Effects of Positive-Gating Modulation

What do we learn from cardiovascular telemetry? In freely moving unstressed mice that have a blood pressure similar to ours (around 120/85 mmHg Systole/Diastole), acute administrations of SKA-31 (30–100 mg/kg; giving plasma levels above the EC50 for $K_{Ca}3.1$ and $K_{Ca}2$ activation) caused a rapid drop (by approx. −30 mmHg) in systolic and diastolic pressures that persisted over 1–4 h, pending on dose [111, 115]. In hypertensive mice, SKA-31 was similarly effective and lowered pressure to normotensive levels (mice treated with L-NAME or connexin(Cx)40−/− with angiotensin-II hypertension). Moreover, intra-vital microscopy on the microcirculation of cremaster skeletal muscle in anesthetized mice reveals that SKA-31 was capable to produce substantial arteriolar dilation from basal tone that did not require NO, but was abolished by genetic $K_{Ca}3.1$ deficiency [115]. This effect in resistance-size arteries explains to some extent the efficiency of SKA-31 to produce hypotension or normotension from hypertensive levels. Thus, this positive-gating modulator of $K_{Ca}3.1$ and $K_{Ca}2.3$ is active and causes the expected decrease in blood pressure.

A major concern is, however, that the pressure drop in the mice is accompanied by strong bradycardia (300 bpm at 100 mg/kg vs. 600 bpm (normal)) [115]. This is also seen in $K_{Ca}3.1$−/−, suggesting that this bradycardia is not related to activation of $K_{Ca}3.1$ but was rather caused by $K_{Ca}2$ activation in atria of the heart ensuing loss of sinus rhythm, and/or block of transmission within the conduction system. Interestingly, genetically encoded $K_{Ca}2.3$ over-expression has been shown to increase atrioventricular refractory period in young $K_{Ca}2.3$-overexpressor ($K_{Ca}2.3^{T/T}$) but decreased it in old $K_{Ca}2.3^{T/T}$ mice because of anatomical alterations related to $K_{Ca}2.3$-over-expression [75]. Moreover, genetic deficiency of the $K_{Ca}2.2$ subtype causes prolonged the PR and RR intervals [121] and caused prolongation of atrioventricular transmission, while over-expression of $K_{Ca}2.2$ had opposite effects.

Together, the data makes it likely that SKA-31 at $K_{Ca}2$-activating plasma concentrations affects the conduction system of the murine heart, which, unfortunately, may hinder a further development of this compound. Another, drawback is that SKA-31 causes severe sedation (immobility) in a $K_{Ca}3.1$-independent fashion [70], which relies likely on the bradycardia and/or the activation of central $K_{Ca}2$ channels, slowing neurotransmission, or—possibly—skeletal muscle $K_{Ca}2$, producing paresis.[2]

Interestingly, dogs that have a heart rate similar to us respond differently to SKA-31 (injected i.v.) because they show at $K_{Ca}3.1$/$K_{Ca}2$-activating plasma levels profound but short-lived hypotension and reflex tachycardia [113], which may point to

[2] *So far, there is no evidence that $K_{Ca}3.1$ is expressed in cardiomyocytes. In contrast, $K_{Ca}2$ channels are expressed cardiomyocytes. Moreover, $K_{Ca}3.1$ has been considered a non-neuronal channel as concluded form the absence of $K_{Ca}3.1$-mRNA in central neurons* [9, 29]. *Interestingly, $K_{Ca}3.1$ protein has recently been documented in rat brain by immunohistochemical approaches* [122]. *However, we could not clearly detect $K_{Ca}3.1$ in neuronal structures (but in the blood brain barrier) in murine brain and in human post mortem material using the current IHC approaches* [70]. *Thus, there are apparently species differences and it remains still possible that $K_{Ca}3.1$ in neurons add to cardiovascular effects and sedation described here.*

substantial species differences and request the need for thorough cardiovascular safety monitoring in large mammals.

Yet, SKA-31 and an analogue of SKA-31, SKA-19 has been found effective in the NINDS-funded anti-convulsant screening program [123]. Nonetheless, the likely disadvantageous cardiovascular profile of SKA-31 generates the need of a more selective $K_{Ca}3.1$-activator. Heike Wulff's group at UC-Davis synthesized a series of SKA-analogues [123], of which SKA-121 (5-methylnaphtho[2,1-d]oxazol-2-amine; Fig. 5.4a) has an improved selectivity profile for $K_{Ca}3.1$ (approx. 40-fold higher for $K_{Ca}3.1$ over $K_{Ca}2$). At the level of endothelium-dependent vasorelaxation, we find that SKA-121 does not act as vasorelaxant on its own in large coronary artery of the pig but potentiates the response elicited by bradykinin (Fig. 5.5a). This can be reversed by TRAM-34 [123]. Moreover, the compound does not affect endothelium-independent contraction or relaxation. In telemetry (Fig. 5.5b), SKA-121 reduced blood pressure in normotensive mice and L-NAME-treated hypertensive mice over approx. 2 h and 6 h, respectively, but did not cause blood pressure alterations in $K_{Ca}3.1-/-$ mice. Importantly, heart rate was insignificantly affected in this study, suggesting substantial improvement of selectivity and cardiovascular safety of SKA-121. Neurological effects still need to be investigated in details, but sedation can still be an issue because of the higher brain/plasma concentration ratio [123]. In sum, positive gating modulators like SKA-121 are likely to have some therapeutic utilities in cardiovascular disease (hypertensive crisis, vasospasm, and central and peripheral ischemia) (Fig. 5.6).

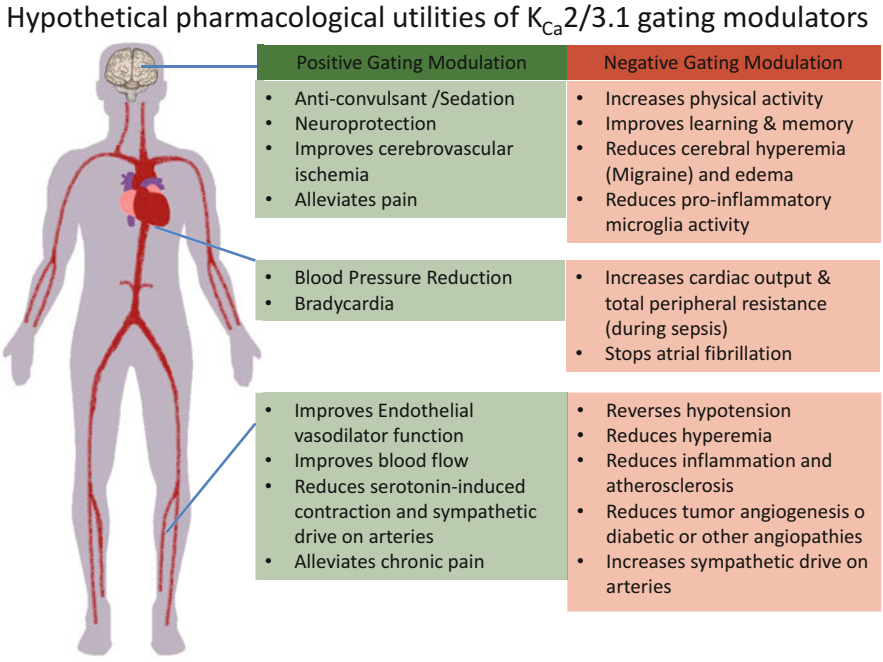

Fig. 5.6 Schematic illustration of systemic actions of the positive- and negative gating modulation of $K_{Ca}2/K_{Ca}3.1$ channels

Recent Developments: Negative-Gating Modulators

Besides the potential utility of positive-gating modulators of $K_{Ca}3.1/K_{Ca}2$ in cardiovascular pathophysiology—albeit counterintuitive at first—also negative-gating modulators of $K_{Ca}3.1/K_{Ca}2$ channel activators are of potential pharmacological value, for instance in atrial fibrillation, hypotension and sepsis, hyperemia, atherosclerosis, and restenosis after balloon catheter intervention, but perhaps also in neurological disorders associated with microglia-activation, chronic inflammation, and possible some cancer over-expressing $K_{Ca}3.1/K_{Ca}2$ channels.

The present selective $K_{Ca}3.1/K_{Ca}2$ inhibitors are mainly blockers that obstruct ion flow at the intracellular cavity below the selectivity filter (such as TRAM-34 on $K_{Ca}3.1$, Fig. 5.4c) or from the outside as classical pore blockers such as UCL-1684 (Fig. 5.4a, c), structurally mimicking the peptide blocker, apamin. The utility of UCL-1684 for in-vivo experimentation is not clear, but this very large molecule is still not drug-like. TRAM-34 has been used frequently in experimental in-vivo intervention trials and has been proven to be effective to inhibit BCI-induced neointima formation in rats and pigs [76, 77], neo-angiogenesis in Matri-gels [124], and experimental fibrosis in normal and diabetic kidneys [80, 81] and lungs [79, 125], suggesting utilities of $K_{Ca}3.1$-inhibitors for the treatment of disease characterized by pathological cell proliferation and pathological organ remodeling (for review see [8, 126]). However, TRAM-34 has been reported to be an inducer of P450 enzymes and looses selectivity for $K_{Ca}3.1$ over some other K channels at μmolar concentrations. Therefore, we have recently performed a small screening campaign focusing on another compound class (phenols and polyphenols with beneficial properties as anti-oxidants) and find 13b, tri-fluoro-benzoate ester [44, 127] as reasonably potent. Moreover, this compound turned out to act as negative-gating modulator because it competes (in this regard unlike TRAM-34) with the positive-gating modulators, SKA-31 and SKA-121 (Fig. 5.4a, c). Unfortunately, 13b is a large and lipophilic molecule and bioavailability of 13b is presumably poor. To generate a new more drug-like analogue, we synthesized smaller and less lipophilic variants, of which RA-2 (1,3-phenylenebis(methylene)bis(3-fluoro-4-hydroxybenzoate) (Fig. 5.4a) has acceptable drug likelihood and conserved potency (IC50 approx. 20 nM), a good selectivity over other K channel families, but also blocks with similar potencies all three $K_{Ca}2$-subtypes [128]. This can be explained by the negative-gating modulation at the level of calmodulin-activation (Fig. 5.4c), which is alike in the complete $K_{Ca}3.1/K_{Ca}2$ family. As to be expected for a negative gating modulator, RA-2 shifts the concentration-response curve for Ca^{2+} activation to the right (Fig. 5.5a).

Thus, RA-2 is the first pan-negative gating modulator of $K_{Ca}3.1$ and $K_{Ca}2$. In in-vitro experimentation (Fig. 5.5b), RA-2 has been found to inhibit bradykinin-induced EDH-type relaxation in porcine coronary artery and antagonizes potentiation of the bradykinin response by the positive-gating modulator, SKA-121 [128].

Systemic Effects of Negative-Gating Modulation

In respect to systemic cardiovascular regulation, RA-2 appears to be relatively safe since acute i.p. injections of up to 100 mg/kg did not produce hypertension or any disability in the mice (Fig. 5.5b). Still, RA-2 caused lasting bradycardia (drop by 150 bpm from the high levels of approx. 600 bpm, Fig. 5.5c). The simplest explanation for this bradycardia is that it mirrors baroreceptor-reflex to a higher peripheral resistance caused by $K_{Ca}3.1$ and $K_{Ca}2.3$ inhibition in small resistance-size arteries (Fig. 5.6). Other possibilities are a direct effect on the heart conduction system, caused by action potential prolongation as a result of $K_{Ca}2$ inhibition. However, we do not wish to exclude other changes in sympathetic or parasympathetic drive on the heart or central and peripheral mechanisms. Interestingly, bradycardia was absent in $K_{Ca}3.1-/-$ suggesting an effect that is selectively mediated by $K_{Ca}3.1$. Besides these cardiovascular actions of RA-2, our unpublished data demonstrate a higher locomotor activity in RA-2 treated mice, which is, interestingly, also a feature of $K_{Ca}3.1-/-$ mice and fosters the view of a participation of $K_{Ca}3.1$ in control of behavior and/or physical activity (Fig. 5.6).

Concluding Remarks

The current evidence derived from experimentation on gene-targeted mice and advance $K_{Ca}3.1/K_{Ca}2.3$ pharmacology assigns substantial impact of endothelial $K_{Ca}3.1$ and $K_{Ca}2.3$ in the endothelium on local arterial regulation as well as systemic cardiovascular regulation. Here, EDH and to some extent also NO are likely the downstream effectors. However, with respect to humans, we still need to be careful since the contribution of the channels to human systemic cardiovascular regulation remains unexplored although there is no doubt that human endothelium expresses these channels.

$K_{Ca}3.1$ and $K_{Ca}2.3$ are differentially regulated by cardiovascular disease at the functional and gene expression level and by other disease states. However, "loss-of-function" or "gain-of-function" (in the sense of monogenetic channelopathies) are unknown so far. Still, the $K_{Ca}2.3$-gene is linked to atrial fibrillation and $K_{Ca}2$ blockers are currently developed by the Danish Spin-off *Acesion* as novel types of atria-selective antiarrhythmic drugs.

Idiopathic forms of cardiovascular dysfunction in, particularly, human subjects go along with altered EDH and variable changes of $K_{Ca}3.1/K_{Ca}2.3$ gene expression and functions that could point at compensatory or pathogenic roles. This offers venues for endothelial selective treatment of endothelial dysfunction by activators like the positive-gating modulator, SKA-121 (Fig. 5.6). While the development of $K_{Ca}3.1/K_{Ca}2$ channel activators as antihypertensive is unlikely considering the availability of several established medications including Ca^{2+} antagonists and the—for activators—

potentially problematic tachyphylaxia, they may still be of use as alternative for the treatment of angina pectoris, local peripheral ischemia and pain, intra-surgical hypertension, and vascular protection of transplants. Not to forget that $K_{Ca}3.1/K_{Ca}2$-activators may be of advantage in early ischemic stroke and cause neuroprotection by impeding Ca^{2+}/Na^{+} overload in hypoxic neurons. Besides, such utilities in cardiovascular and cerebrovascular disease, they may also serve as targets in neurons to treat epilepsy and pain (Fig. 5.6).

In contrast, $K_{Ca}3.1$ and $K_{Ca}2$ inhibitors such as the pan-negative gating modulator of this channel class, RA-2, could have therapeutic value in situation of systemic hypotension as it occurs during sepsis, anesthesia accident, or after resurrection (Fig. 5.6). Moreover, it may have utilities for chronic inflammatory processes and proliferative angiopathies and, if considering $K_{Ca}2/3.1$ expression in brain and skeletal muscle, for the treatment of motivation loss and muscle weakness (Fig. 5.6).

Yet, these gating-modulators of $K_{Ca}3.1$ and $K_{Ca}2$ channels may be of help to shed new light on physiological and pathophysiological roles of $K_{Ca}3.1$ and $K_{Ca}2$ channels in the organisms. Considering the complex tissue expression pattern of the channels, the efficacy or undesired side effects of pharmacological manipulation remains to be tested in more detail. Still, the broad therapeutic utilities of $K_{Ca}3.1/K_{Ca}2$ gating modulation offers several attractive venues for further pharmaceutical development.

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