Chapter 6
Regional Variation in Arterial Myogenic Responsiveness: Links to Potassium Channel Diversity/Function

Michael A. Hill, Yan Yang, Zahra Nourian, Barry D. Kyle, Kwangseok Hong, and Andrew P. Braun

Abstract Regional variation in small artery myogenic responsiveness is associated with differences in relationships amongst intraluminal pressure, smooth muscle cell (SMC) membrane potential (Em) and vessel diameter. For example, under *in vitro* conditions, small arteries from cremaster muscle show a steeper relationship between Em and myogenic contraction compared with cerebral arteries. To explain this difference, we hypothesized that the function/regulation of the large conductance, Ca^{2+}-activated, K^+ channel (BK_{Ca}) differs between these vascular beds. This was based on previous observations by Nelson and colleagues that BK_{Ca}, activated by sarcoplasmic reticulum (SR)-generated Ca^{2+} sparks, exerts a hyperpolarizing influence that opposes myogenic constriction. To test this, studies were performed using Ca^{2+} imaging, vessel myography, isolated cell electrophysiology and molecular biology techniques on small resistance arteries from the cerebral and cremaster muscle vasculatures. While BK_{Ca} in SMCs of both small arteries showed a similar conductance and voltage sensitivity, Ca^{2+} sensitivity was 2–3-fold greater in cerebral SMCs. Single channel open times were greater in cerebral SMCs compared with those of cremaster SMCs. Conversely, closed times were significantly shorter in cerebral SMCs. In addition to variation in biophysical characteristics, β_1-BK_{Ca} subunit expression was decreased in cremaster SMCs. Further, siRNA-induced knockdown of the β1 subunit of the BK_{Ca} holo-channel shifted gating behavior of cerebral BK_{Ca} channels to resemble that observed in cremaster SMCs. Collectively, the data indicate that while BK_{Ca} is present in both vascular preparations expression levels and modes of regulation differ. In particular, BK_{Ca} in small cerebral arteries is configured to show a higher Ca^{2+} sensitivity resulting in greater opening at

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physiological levels of membrane potential. Heterogeneity in SMC ion channel function is not limited to BK_{Ca} as vascular bed differences are also apparent for other K^+ channels including the voltage-gated Kv and Kv7 families of channels. With respect to the latter, Kv7 channels appear to play a greater role in cerebral vasculature compared to the coronary circulation. From a physiological perspective it is suggested that differences in local ion channel function allow for regional differences in the regulation of myogenic tone and hence the control of tissue hemodynamics.

Keywords Arterioles • Vascular smooth muscle • Signal transduction • Ion channels • Mechanotransduction

The ability of arterioles to constrict to acute increases in intraluminal pressure has been recognized for well over 100 years and is commonly referred to as the myogenic response [1–4]. The physiological significance of this mechanically-induced contraction relates to its role in blood flow autoregulation, setting of basal vascular tone and its contribution to the regulation of capillary pressure and prevention of pressure-induced damage [2].

Although the basic pathways underlying myogenic contraction are known to lie within the vascular smooth muscle cells (VSMCs) of the arteriolar wall, there remains doubt as to the precise molecular mechanisms by which the pressure-mediated mechanical stimulus is sensed and ultimately converted to an activation of the contractile proteins. Substantial evidence exists for a pivotal role of membrane depolarization, opening of voltage-gated Ca^{2+} channels, increased intracellular Ca^{2+} ($Ca^{2+}{}_i$) and Ca^{2+}-dependent phosphorylation of the regulatory myosin light chain [5–11]. These events are supported by a number of other protein kinase-based mechanisms that involve pathways leading to alterations in Ca^{2+} sensitivity and the rearrangement of cytoskeletal elements [8, 12–14].

While the exact event(s) underlying the pressure-induced regulation of membrane potential are uncertain they appear to involve a number of different ion channels (including stretch/mechanogated channels, various TRP channels and possibly TMEM16A/ANO1 chloride channels) [15–20]. Activation of these channels occurs either by direct mechanical perturbation or in response to integrin and/or second messenger-mediated mechanisms [19, 21, 22]. In addition to contributing to the initial pressure-induced depolarization, various ion channels also underlie movement of Ca^{2+} from the extracellular space into VSMCs, release of Ca^{2+} from the intracellular stores [2, 4] and to repolarization both following restoration of the initial pressure level and as a feedback mechanism controlling the extent of myogenic constriction [23–25]. It has also become evident that considerable complexity surrounds ion channel structure and regulation. Rather than being simple pores, ion channels and their gating behavior are regulated through their expression levels, existence of isoforms, splice variants and regulatory subunits, post-translational modification, dynamic trafficking to and from the cell surface, and spatial location within signaling microdomains [26–32].

This chapter will present evidence suggesting that heterogeneity in K^+ channel expression and function may contribute to differences in how myogenic reactivity is controlled in different vascular beds. An underlying assumption is that this regional heterogeneity allows fine-tuning of the basic myogenic contractile mechanism to specifically serve local tissue requirements. A particular example of this is shown in small arteries from rat skeletal muscle (cremaster muscle) where there is a steeper relationship between Em and myogenic contraction compared with that in cerebral vessels (Fig. 6.1). It was suggested that such steeper relationship allows maintenance of a high peripheral resistance in resting skeletal muscle vasculature while at the same time maintaining adequate cerebral perfusion [11, 33]. An additional

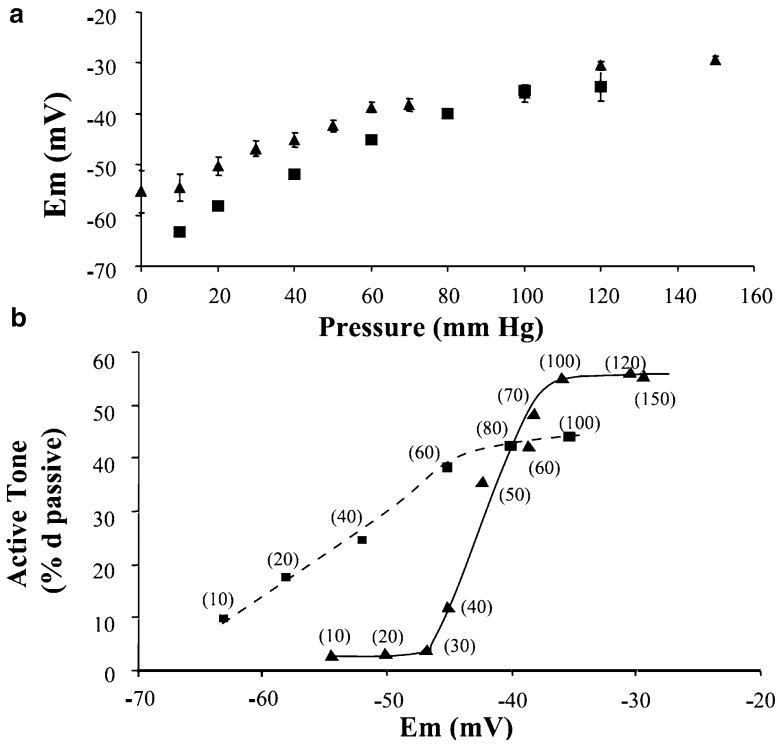

Fig. 6.1 Small arteries from skeletal muscle demonstrate a steeper Em-myogenic tone relationship compared with cerebral vessels. Comparison of data from cerebral vessels (*squares*) [data replotted from Knot et al. [24]] with skeletal muscle arterioles (*triangles*) from Kotecha and Hill[11] shows an upward shift in the pressure-Em relationship for skeletal muscle arterioles with largest differences between data sets evident at pressures below approx. 80 mmHg (**a**). Above this pressure, cerebral vessels (approx. 200 μm diameter) exhibit a lesser extent of myogenic constriction compared with skeletal muscle arterioles (approx. 150 μm diameter) (**b**). Note that for both vessel sets, active tone is plotted relative to the passive diameter at each pressure. Data are normalized as cerebral vessels are slightly larger than cremaster muscle vessels. *Numbers* in *parentheses* indicate intraluminal pressures (in mmHg). From Kotecha and Hill [11]. *Em* membrane potential

example of ion channel diversity affecting myogenic reactivity can be seen between branch orders within a vascular bed. Chen and colleagues thus showed that first order mesenteric arteries from rats express higher levels of the voltage-gated K^+ channel (Kv) than do the smaller, more myogenically reactive third order vessels [34]. Further, *in vitro* treatment of the first order vessels, for 48 h, with a targeted oligonucleotide decreased Kv channel expression at the protein level and increased myogenic reactivity. Similarly, Loutzenhiser and colleagues have shown variation in the expression and function of ion channels, including K_{ir} and voltage-operated Ca^{2+} channels (VOCC), in the renal vasculature of the rat [35, 36]. Thus, angiotensin-mediated constriction of the afferent arterioles was shown to be dependent on Ca^{2+} entry via VOCCs while that of the efferent arteriole showed a greater dependence on Ca^{2+} release from intracellular stores and subsequent store-operated Ca^{2+} entry [36]. In the interlobular artery the expression and function of K_{ir} increases along the length (proximal to distal) of the vessel. Interestingly, associated with this pattern of channel expression is a decrease in vessel diameter and increases in myogenic reactivity and vascular resistance [35].

Ion channel diversity may also occur in regard to intracellular channels. Westcott et al. have shown variation in the expression of SR ryanodine receptors (RyR; an intracellular Ca^{2+} release channel) between mouse (C57Bl/6 strain) feed arteries (arteries entering a tissue such as a specific skeletal muscle; diameter approximately 190 μm) and smaller intramuscular arterioles (diameter approximately 35 μm) [37, 38]. Thus, the feed arteries demonstrated significantly greater expression of the RyR2 isoform than did the smaller arterioles. From a functional perspective RyR contributed to Ca^{2+} signals and myogenic tone in the larger vessels but not in the smaller arterioles. Both vessels exhibited similar complements of inositol trisphosphate receptors (IP_3R1 dominant in both).

The following sections draw heavily on our comparisons of BK_{Ca} expression and function between cerebral and skeletal muscle circulations. Particular emphasis has been placed at the level of small arteries. It is, however, acknowledged that considerable heterogeneity may also exist within the microvascular bed of any tissue. Supporting this latter point, and as outlined above, Westcott et al. have demonstrated ion channel diversity within mouse skeletal muscle microvasculature.

Large Conductance, Ca^{2+}-Activated K^+ Channels (BK_{Ca})

The BK_{Ca} channel is widely expressed in the body and vascular smooth muscle BK_{Ca} channels exhibit a single channel conductance of approximately 240 pS under conditions of symmetric 150 mM KCl [33, 39–41]. Its large conductance, along with its relatively high level of cellular expression, makes BK_{Ca} a dominant K^+ channel. In excitable cells, including VSMCs, BK_{Ca} plays a significant role in the regulation of membrane potential. In doing so, BK_{Ca} contributes to the regulation of the open state of VOCCs, which are major contributors to Ca^{2+} influx, intracellular Ca^{2+} handling, contractile activity and thus artery/arteriolar tone [41]. In addition,

activation of BK_{Ca} by both depolarization and increased intracellular Ca^{2+} results in hyperpolarization that conceivably acts to limit agonist and mechanically-induced vasoconstriction in small arteries and arterioles [25].

An important question relates to whether the BK_{Ca} channel can be regulated locally or is simply ubiquitously expressed throughout the vasculature and regulated on a more global level. While channel opening is affected by membrane potential and cytosolic free Ca^{2+} the function of BK_{Ca} is regulated at a variety of other levels providing multiple mechanisms to fine tune channel activity in a differential manner to meet local needs. As outlined above these levels of regulation include differences in expression [33, 42, 43], the existence of splice variants [28], subunit composition [33, 44, 45], modulation by phosphorylation and small molecules [46], trafficking of subunits [27] and spatial distribution within the VSMC [47, 48].

In support of local differences in channel activity, comparison of VSMCs from cerebral and cremaster muscle arteries shows a higher expression for β1:α BK_{Ca} in the cerebral vasculature, both at the mRNA and protein levels [33, 43]. Further, using a cell surface biotinylation assay the α subunit of BK_{Ca} was expressed at significantly higher levels in cerebral vessel SMCs compared with that for cremaster VSMCs [43]. Coincident with this difference is a greater whole cell, iberiotoxin (a highly selective inhibitor of BK_{Ca})-sensitive, K^+ conductance in cerebral VSMCs. Importantly, this electrophysiological difference persists when K^+ current data are normalized for cellular capacitance indicating that the results cannot be explained by a difference in cell size between the two sites.

Molecular Heterogeneity BK_{Ca} Subunits

The functional BK_{Ca} channel is comprised of four α-subunits that assemble around a central axis forming a K^+-selective conduction pore (Fig. 6.2). Each α-subunit contains seven transmembrane-spanning segments, denoted S0–S6, with an extracellular amino terminus and a long intracellular carboxyl terminus. The S1 to S6 membrane-spanning segments are a common structural feature of voltage-gated K^+ channels, in general, while the S0 segment located at the amino terminus is found only in BK_{Ca} (Slo1) and the related H^+-gated large conductance K^+ channel encoded by Slo3 [49–51]. The latter are not, however, gated by physiological calcium levels. The S0 segment of BK_{Ca} appears to be important for modulation of the channel by the associated β1 subunit (described below; [52]). Contained within the membrane spanning segments are the voltage sensor domain (S1–S4) and conduction pore (S5–S6); these assignments are based largely on recent crystal structure data from closely related voltage-gated Kv channels [53, 54] and are supported by a low resolution (~20 Å) structure of the holo-channel in a cell membrane environment determined by electron cryo-microscopy [55]. The large intracellular domain accounts for approximately two-thirds of the α-subunit amino acid sequence and contains a number of known regulatory sites that are modulated by Ca^{2+}, phosphorylation and

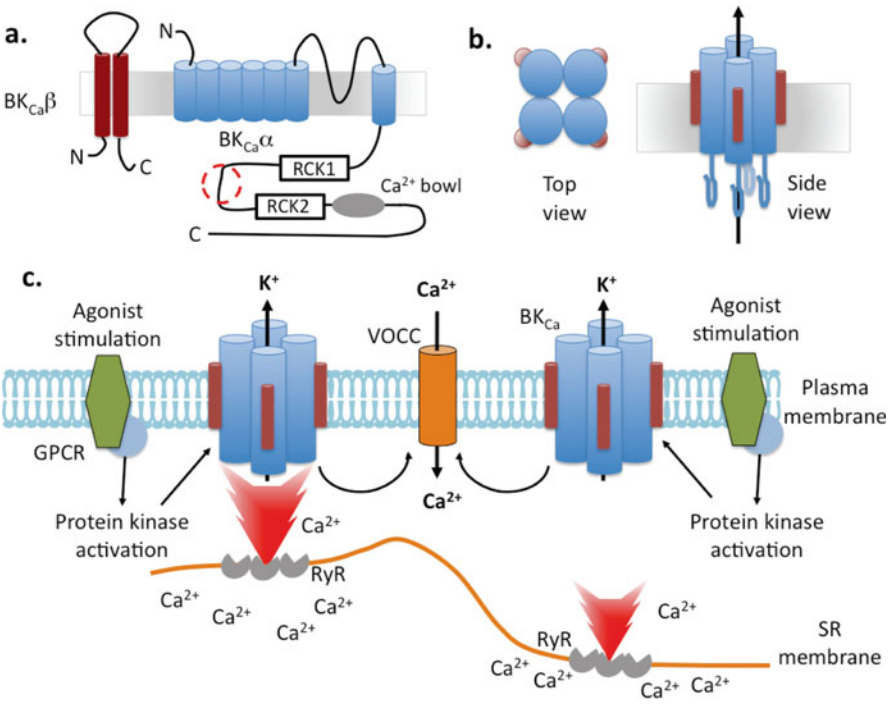

Fig. 6.2 Structure and regulation of BK_{Ca}. (**a**) Smooth muscle cell BK_{Ca} holo-channel consists of α pore-forming subunits and the regulatory β1 subunit. The α subunit is characterized by seven membrane spanning segments (S0–S6); a pore region between S5 and S6; a long cytoplasmic tail containing a number of regulatory sites; and an extracellular amino terminus. The *red circle* between RCK1 and two domains indicates the C2 region known to exhibit several splice variants including STREX (see text for more detail). The β1 subunit has two membrane spanning segments and intracellular amino and carboxy termini. (**b**) The functional smooth muscle cell channel typically consists of four α and four β subunits. (**c**) BK_{Ca} is regulated by Ca^{2+} released from the SR in the form of Ca^{2+} sparks and a number of intracellular signaling molecules including phosphorylation events mediated by various activated kinases. *BK_{Ca}* large conductance Ca^{2+}-activated K^+ channel, *RCK* regulator of K^+ conductance, *RyR* ryanodine receptor, *VOCC* voltage operated Ca^{2+} channel

a variety of small molecules (including lipids, reactive oxygen species, CO_2 and H^+) [46, 56–58]. Two RCK (regulators of K^+ conductance) domains have been described in the large, intracellular C-terminal domain of each BK_{Ca} alpha subunit and contain functionally relevant, high affinity Ca^{2+} binding sites (Kd 1–10 μM). The Ca^{2+} bowl region, denoted by its density of acidic amino acids, flanks the C-terminal end of RCK2 and appears to be responsible for much of the Ca^{2+} sensitivity exhibited by BK_{Ca} channels. Occupancy of these sites in the presence of micromolar levels of cytosolic Ca^{2+} promotes opening of the channel's C-terminal activation gate through an allosteric mechanism, leading to greater open probability. Electrophysiologically, this process is typically observed as a left-ward shift of the BK_{Ca} conductance-voltage (G-V) relationship to more negative voltages [59]. The proximal RCK1 domain

also contains a low affinity (mM range) binding site for intracellular Mg^{2+}; occupancy of this site by Mg^{2+} increases BK_{Ca} open probability and shifts the channel's G-V curve to more negative potentials. This effect of Mg^{2+} on channel gating appears to occur via an electrostatic interaction between the Mg^{2+} binding site in RCK1 and the intracellular face of the voltage sensor domain [59]; such coupling could allow for modulation of voltage-dependent gating by regulatory events within the C-terminal region. In the intact channel (containing four α-subunits) the 8 RCK domains form a 'Ca^{2+}-dependent gating ring' the mobility of which is involved in regulating channel opening [60]. Recent crystallographic structures of the intracellular gating ring apparatus in both the closed (Ca^{2+}-free) and open (Ca^{2+}-bound) configurations confirm the critical involvement of this structure in the regulation of the ion conduction pore [61, 62].

In VSMCs each α-subunit is often associated with a β1 subunit that, while not being absolutely necessary for channel activity, enhances Ca^{2+} sensitivity and increases channel opening probability [61]. The β1 subunit has also been suggested to influence trafficking of BK_{Ca} and hence its distribution between the membrane and intracellular compartments [63]. More recently, an additional γ subunit, LRRC26 (a leucine-rich repeating-containing peptide), has been demonstrated for BK_{Ca} channels [44, 64] and shown to markedly increase voltage sensitivity, thus allowing the channel to open at more hyperpolarized (and perhaps physiological) membrane potentials. A schematic diagram illustrating the overall structure of the BK_{Ca} channel and the association of its subunits is shown in Fig. 6.2.

The BK_{Ca} α-subunit is encoded by a single gene (also referred to as Slo 1 or KCNMA1) that contains 27 distinct exons [65]. Alternate splicing of BK_{Ca} α-subunit pre-mRNA allows considerable diversity of the ultimately translated protein and, to date, more than ten splice sites have been described for mammalian BK_{Ca} α subunit mRNA [66]. Particular focus has been placed on spliced variants involving the site designated C2 as at least five variants have been described at this position and this region lies close to C terminal regulatory domains including RCK 1 and 2 and the so-called "Ca^{2+} bowl" located at the distal end of RCK2 [67]. Diversity at the functional protein level results from the splice variants showing differences in voltage and Ca^{2+} sensitivity, regulation by phosphorylation and cellular localization/trafficking (including to the plasma and ER membranes) as described earlier.

Thus the existence of splice variants of αBK_{Ca} raises the possibility that these may impact channel function and that this could occur at a tissue-specific level. One splice variant that has received considerable attention is STREX (STRess axis regulated EXon) that encodes for a 59 amino acid insertion (relative to the ZERO variant that lacks the insertion) located between RCK1 and RCK2 in the C terminus of the α subunit. Importantly the STREX insert is of functional significance as STREX-containing channels open at more negative potentials compared with the ZERO form [68]. Further, Shipston and colleagues, in particular, have shown that the STREX insert changes the response of BK_{Ca} channels to cAMP signaling relative to the variant lacking this exon (ZERO) [69]. Thus, in BK_{Ca} channels heterologously expressed in HEK293 cells, the STREX variant channel

is inhibited by the cAMP/protein kinase A (PKA) signaling pathway, while in the ZERO form cAMP/PKA signaling is associated with increased channel activity and larger whole cell currents [68].

Studies in non-vascular tissues have examined the role of the STREX insert in the inherent mechanosensitivity demonstrated by BK_{Ca} [70–73]. In channels isolated from chick ventricular myocytes and heterologously expressed in CHO cells (referred to as stretch activated BK_{Ca} channel, $SAK_{Ca}C$), it has been suggested that the STREX sequence is critical for mechanosensitivity [70–73]. The mechanism by which STREX confers mechanosensitivity has been suggested to relate to the length of the linker region joining S6 and the RCK1 domain (see Fig. 6.2), but importantly occurs independently of intracellular Ca^{2+} [70–73]. In apparent contrast to these observations, in studies of freshly isolated murine colonic SMCs, Wang et al. demonstrated mechanosensitivity of native BK_{Ca} channels in freshly isolated murine colonic SMCs, in which only the ZERO variant was expressed and not STREX (as determined by end-point RT-PCR) [70–73]. Based on these observations, it was therefore concluded that the presence of the STREX insert was not an absolute requirement for BK_{Ca} mechanosensitivity.

In our own studies we have examined the relative mRNA expression levels of three splice variants within the C-terminus of the BK_{Ca} α-subunit; ZERO, STREX and SS4. ZERO is the predominant isoform of these variants in small arteries from both cremaster muscle and cerebral circulations of the rat although the STREX insert is significantly more highly expressed in the vessels from cremaster muscle (approximately 29 compared with 16 % of total αBK_{Ca} α-subunit) [43]. Sequencing of PCR products generated from isolated VSMCs confirmed that ZERO and STREX were identical in both vessel types [43]. While at this point in time, the functional significance of these differences in expression levels is unclear, particularly as they relate to the generation of differing levels of myogenic tone, observed differences in ZERO/STREX expression did not alter the distribution of BK_{Ca} alpha subunits between the membrane and the cytosol. [43]. Interestingly, the vascular preparations contain a mixed population of STREX and ZERO BK_{Ca} α-subunits raising the possibility of there being both homomeric and heteromeric holo-channels. Studies are required to recapitulate these differences in expression systems and in fully functional arterioles, where levels of these variants can be manipulated.

In regard to modulation of BK_{Ca} function by β accessory subunits, four genes have been identified and encode for the distinct proteins referred to as β1, β2, β3 and β4. As stated above, arterial VSMCs typically express the β1 subunit. At the mRNA level both cerebral and cremaster VSMCs express predominantly the β1 subunit although small amounts of β4 subunit can be detected. At the protein level the β1 subunit is seen in both vasculatures although significantly greater levels were seen in small cerebral arteries [33]. Despite the presence of small levels of β4 subunit mRNA measured using real time PCR, Western blotting analysis does not show measurable quantities of the protein in homogenates of cerebral arteries. Further, no evidence for β2 or β3 subunit mRNA expression was obtained in either vasculatures (unpublished observations).

A cautionary note is that quantitative mRNA and, in particular, protein expression data are often necessarily derived from homogenates of whole microvessels and not purely from VSMCs. As native arterial endothelial cells are generally not thought to contain significant levels of BK_{Ca} and they represent a relatively small component of the vessel wall, it is assumed that the bulk of the measured signal does, in fact, come from the VSMCs. This is, however, currently a matter of interest and debate, in part, because endothelial cell data have largely been derived from large vessels and expression of BK_{Ca} channels may be up-regulated when endothelial cells are cultured or in pathophysiological states [74, 75]. Importantly though, vessel homogenates contain other cell types (for example neurons, fibroblasts and immune cells), which are resident in the adventitial layer of the vessel wall. More stringent studies are required, therefore, using purified VSMCs and appropriate markers for non-smooth muscle cell contamination.

From a functional perspective the importance of the BK_{Ca} β1 subunit to arteriolar function was first demonstrated in the $\beta1^{-/-}$ mouse [76, 77]. Cerebral blood vessels from the homozygous global knockout animal showed increased arterial tone in the face of decreased coupling between BK_{Ca} and SR-derived Ca^{2+} sparks. The decrease in coupling between the Ca^{2+} sparks and BK_{Ca} was further shown to be a result of a decrease in the Ca^{2+} sensitivity of the channel resulting from the absence of the regulatory β1 subunit.

The BK_{Ca} $\beta1^{-/-}$ mouse has been reported to be either mildly hypertensive [76, 77] or not hypertensive at all [78]. This, in part, may relate to differences in methodologies for assessing blood pressure, namely direct cannulation, tail cuff, or telemetry. Interestingly, 24 h monitoring by telemetry (in the absence of anesthesia and stress due to handling) showed a mild increase in systemic blood pressure at night, but statistically unchanged when considered over the 24 h period [78]. It would be expected that, if BK_{Ca} activity were to oppose myogenic tone in all vascular beds, a higher blood pressure would be seen in the $\beta1^{-/-}$ mouse. Vascular heterogeneity in this feedback mechanism could conceivably, however, obscure such a relationship. Alternatively in the whole animal, compensatory mechanisms may develop in the knock-out model.

The complex molecular composition of BK_{Ca} also raises a number of questions as to whether channel subunit stoichiometry varies between cells or between channels within a given cell? For example in VSMCs is there always a 1:1 relationship between the α and β subunits in VSMCs and do heteromultimeric channels exist between the various α subunit splice variants (for example STREX and ZERO)? Are these relationships fixed or do they vary on a more dynamic basis, particularly given the recent work of Leo and colleagues showing stimulus-dependent trafficking of the β1 subunit? Substantial evidence exists for variation in β subunit expression in both physiological (for example pregnancy where both α and β subunits decrease prior to parturition [79]) and pathophysiological states (diabetes [80], hypertension [81] and increased vascular resistance associated with heart failure [82]). The existence of such variation raises additional questions as to their potential effects on electrophysiological properties and regulation by post-translational mechanisms (phosphorylation, palmitoylation, fatty acids, steroid hormones, redox state) and cellular location (trafficking).

BK_{Ca} Regulation by Voltage and Ca^{2+}

BK_{Ca} is a member of the KV superfamily as indicated by similarities in amino acid sequence and biophysical properties [83–85]. Together with Slo 2 and Slo3, the mammalian Slo1 gene and its orthologs encode for potassium channels of large conductance [50, 51]. These channels share structural similarities with Slo 1 and 3 having an S0 membrane spanning segment at its amino terminus while Slo 1, 2 and 3 all exhibit a long intracellular carboxy terminus containing two RCK domains [50, 51]. However BK_{Ca} is distinct from other members of the Kv family of channels due to its regulation by intracellular Ca^{2+} levels. A number of Ca^{2+} (and divalent cation) binding sites have been identified, particularly in the intracellular C-terminus of the α-subunit. Further, the presence of the β1 subunit facilitates Ca^{2+} sensitivity in VSMCs.

In comparative studies, BK_{Ca} of cerebral and cremaster muscle VSMCs showed similar responsiveness to applied voltage steps. Thus, Boltzmann-type analysis of single channel openings activated by negative and positive voltage pulses showed similar Boltzmann constants (Z) over a $[Ca^{2+}]$ range of 0.05–100 μM [45]. In contrast to voltage characteristics differences in BK_{Ca} Ca^{2+} sensitivity were observed such that skeletal muscle VSMCs required a 2–3-fold increase in cytosolic Ca^{2+} concentration compared with cerebral VSMCs to affect half maximal channel opening. Alternatively the differences in Ca^{2+} sensitivity can be compensated for by an additional depolarization of approximately 30 mV. As membrane potential is approximately −35 to −45 mV in both vessels (as measured under *in vitro* pressurized conditions [11, 24]) it is unlikely that voltage alone can compensate for the difference in Ca^{2+} sensitivity between skeletal muscle and cerebral BK_{Ca} channels. Alternatively, an argument can be made that in the presence of additional stimuli, particularly those resulting in the activation of PKA and PKG, may cause a leftward shift in the open probability-voltage relationship thereby allowing BK_{Ca} channels to open at more negative membrane potentials [39, 86].

Single channel studies have further showed that cremaster VSMC BK_{Ca} channels exhibit shorter opening times and longer closed times compared with BK_{Ca} of cerebral vessels. Based on studies of expression systems [87] these differences could relate to variation in the number of β1 subunits within the holo-channel. These biophysical observations are also consistent with the molecular data showing decreased BK_{Ca} β1 subunit expression in cremaster VSMCs.

Supporting variation in β1 subunit expression between cremaster and cerebral VSMCs, acute (48 h) *in vitro* knockdown of cerebral VSMC β1 BK_{Ca} using specific siRNA resulted in decreased expression of the β1 subunit, attenuated whole cell K^+ currents and decreased responsiveness of BK_{Ca} channels to estrogen [45]. Further, knockdown of the β1 subunit decreased single channel open time and increased closed time while also decreasing the amplitude and frequency of BK_{Ca}-dependent STOCs in whole cell recordings. From these data we concluded that following the decrease in BK_{Ca} β1 subunit expression the electrophysiological properties of cerebral VSMC BK_{Ca} activity more closely resembled those of cremaster muscle. This observation was taken to be further evidence that the cerebral VSMCs channel contains a higher relative proportion of functional β1 subunit.

While the modulatory roles of BK_{Ca} β-subunits in channel activity are well-recognized, recent data have revealed a novel set of γ-subunits that can also co-assemble with BK_{Ca} α-subunits and have a positive regulatory effect on channel activity [64, 88, 89]. These γ-subunits belong to a family of leucine-rich repeat containing (LRRC) proteins and Evanson et al. [44] have shown that the LRRC26 variant is endogenously expressed in cerebral VSMCs and enhances the Ca^{2+} and voltage-dependent gating of BK_{Ca} channels.

BK_{Ca} Regulation by Phosphorylation

As many vasoactive stimuli (including vasoactive hormones and locally acting factors including NO) result in the production of cyclic nucleotides and protein kinase activation (including protein kinases A, G and C), regional differences in phosphorylation could conceivably result in tissue-specific differences in BK_{Ca} activity. Using proteomics and radiolabeling approaches, numerous potential phosphorylation sites have been identified in BK_{Ca} [90, 91]. In VSMCs stimuli elevating cAMP and cGMP typically activate BK_{Ca} and lead to vasodilatation while PKC activation often causes channel closure and vasoconstriction [86]. It should be noted, however, that PKC effects may be isoform specific as evidence has also been presented for it activating BK_{Ca} [92]. Further variation may exist in these relationships, however, as the expression of different splice variants (for example the STREX insert referred to earlier) can markedly change modulation of BK_{Ca} by cyclic nucleotides and phosphorylation.

An important question is how, at a mechanistic level, do cyclic nucleotides and protein kinases alter conductivity of BK_{Ca}? In regard to the action of NO/cGMP/PKG signaling in VSMCs, Kyle, Braun and colleagues have identified, using biochemical, cellular and electrophysiological techniques, three specific serine phosphorylation sites (i.e. Ser691, Ser873 and Ser1111–1113) in the C-terminus of BK_{Ca} [91]. The data strongly suggested that maximal augmentation of BK_{Ca} current by NO/cGMP signaling in A7r5 smooth muscle cells requires phosphorylation of all three Ser locations and that disruption of any one of the three sites by Ser to Ala substitution could prevent NO/cGMP stimulation of BK_{Ca} current. In both A7r5 cells and rat cerebral VSMCs, BK_{Ca} channels co-localize with PKGIα, as determined by proximity ligation assay analysis, and this co-distribution can be further augmented in the presence of a NO donor (Fig. 6.3). In rat brain, BK_{Ca} channels appear to be constitutively phosphorylated at multiple Ser and Thr sites [90] and such modifications may help to stabilize the channel's tertiary structure or create interacting sites for auxiliary proteins. The extent to which BK_{Ca} channels in other tissues or following heterologous expression undergo constitutive phosphorylation is presently unknown, along with the specific protein kinases responsible for these modifications.

BK_{Ca} current is also augmented by cAMP/PKA signaling and Ser873 has been implicated as a putative phosphorylation site in the channel targeted by PKA [93].

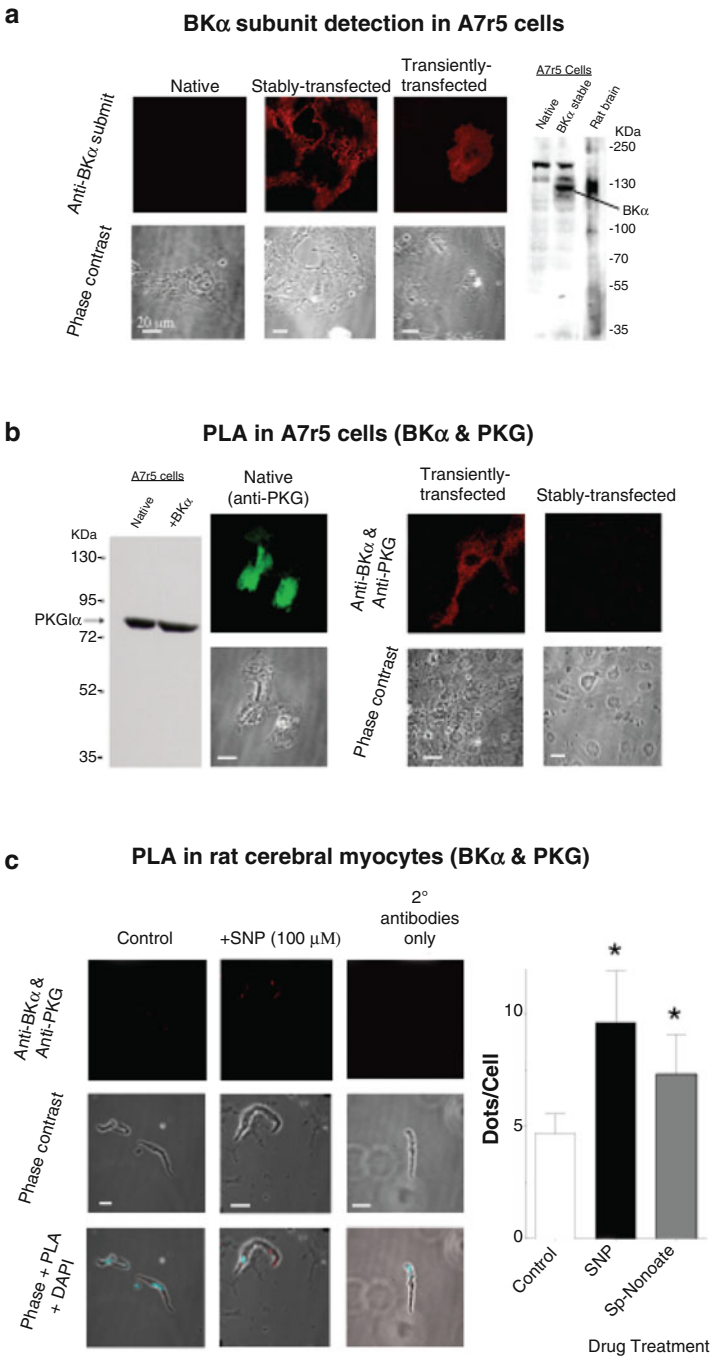

Fig. 6.3 Immuno-detection of BK_{Ca} channels and PKGIα in VSMCs. Native A7r5 aortic smooth muscle cells exhibit very low levels of BK_{Ca} channel expression, as revealed by immunostaining of the BK_{Ca} α subunit, however, channels are more readily detected in these cells following transient or stable expression of a cloned mammalian BK_{Ca} α subunit. The scale bar shown in each phase contrast micrograph represents 20 μm and also applies to the accompanying

In STREX-containing BK_{Ca} channels, PKA signaling has the opposite effect and decreases current amplitude by modifying a serine residue within the STREX insert [69]. Functionally, STREX phosphorylation by PKA is reported to have a dominant-negative effect on BK_{Ca} channel regulation and is able to override any potential stimulatory effect of PKA-mediated phosphorylation at additional sites (e.g. Ser873). Moreover, the presence of only a single STREX-containing α-subunit within a BK_{Ca} tetrameric channel is sufficient to change the effect of PKA-mediated phosphorylation on BK_{Ca} gating from positive to negative. This inhibitory effect of PKA signaling on STREX-containing BK_{Ca} channels also appears to require fatty acid modification (i.e. palmitoylation) of the cysteine-rich STREX insert [29, 30]. Additional aspects of BK_{Ca} channel phosphorylation are described by Schubert and Nelson [86] and Kyle and Braun [46].

Studies from our laboratory have shown that VSMCs from cremaster arterioles exhibit less activation of BK_{Ca} in response to forskolin (activator of adenylyl cyclase) as compared with cells isolated from small cerebral arterioles (Yang et al., unpublished data; Fig. 6.4). These observations at the whole cell level may thus reflect differences in BK_{Ca} channel density/expression as mentioned earlier or regulatory differences at the single channel level. Interestingly, the BK_{Ca} response to forskolin stimulation was blocked by a PKG inhibitor (KT5823), suggesting a 'cross over' between PKA and PKG-dependent pathways as reported earlier by Barman and colleagues [94]. The exact mechanism(s) by which evoked cAMP production promotes PKG activity (e.g. direct binding and stimulation of PKG, indirect elevation of cellular cGMP via reduced breakdown, etc.) has not been fully elucidated.

Additional post-translational enhancement of BK_{Ca} current occurs through direct tyrosine phosphorylation of the channel by c-Src and Pyk2 tyrosine

Fig. 6.3 (continued) fluorescent image immediately above. Western blotting demonstrates that the expressed BK_{Ca} α subunit (~130 kDa MW) is comparable to that seen in rat brain (panel **a**). As shown in panel **b**, endogenous PKGIα is readily detected in A7r5 cells by either western blotting or immunocytochemistry. Immunostaining of A7r5 cells using the PLA technique (*right-hand* images) demonstrates co-localization (<40 nm) of BK_{Ca} channels with endogenous PKGIα, as revealed by the appearance of distinct *red fluorescent dots* (panel **b**). Scale bar shown in each phase contrast micrograph represents 20 μm. BK_{Ca} channels and PKGIα co-localization can also be readily detected by PLA staining in acutely isolated rat cerebral myocytes (panel **c**). The *third row* of images shows merged detection of BKα + PKGIα by PLA (*red* fluorescence), along with DAPI staining for cell nuclei (*blue* fluorescence), superimposed on phase contrast micrographs of cerebral myocytes.The scale bar depicted in each phase contrast micrograph represents 20 μm and applies to the accompanying images within the column. As quantified in the histogram, the number of *red dots* was observed to increase following treatment with an NO donor, either SNP (100 μM) or sp nonoate (5 μM), suggesting that NO promotes BK_{Ca}-PKG co-localization. *Red dots* are not observed in the absence of primary antibody versus BK_{Ca} α subunit or PKGIα. Data are presented as mean ± SEM, n = 10–15 cells under each condition. *Asterisk* indicates a statistically significant difference compared with control, as determined by one-way ANOVA and a Dunnett's post-hoc test; $P < 0.05$. *BK_{Ca}* large conductance Ca^{2+}-activated K^+ channel, *PKGIα* protein kinase G type Iα, *NO* nitric oxide, *PLA* proximity ligation assay, *SNP* sodium nitroprusside, *sp nonoate* spermine nonoate

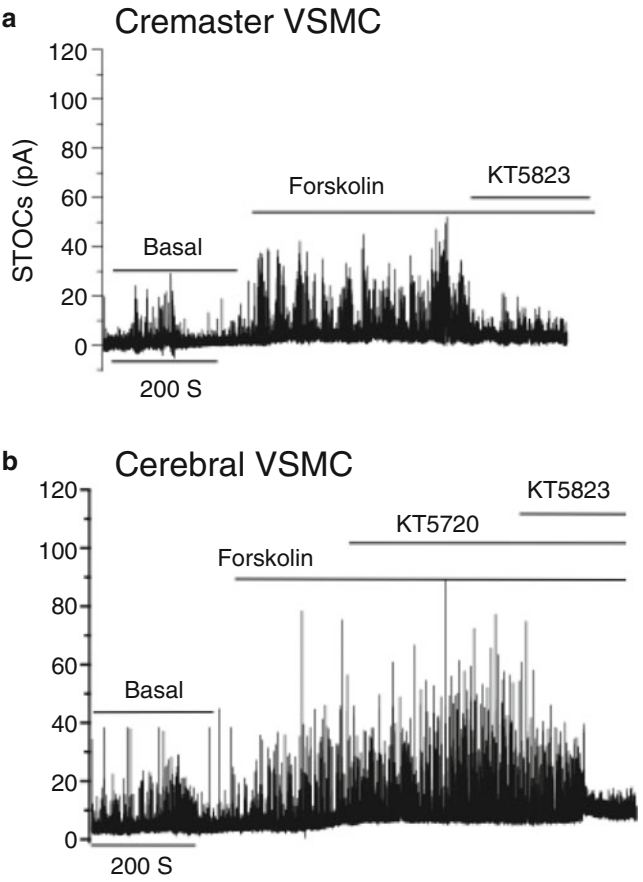

Fig. 6.4 Forskolin increases BK_{Ca}-mediated STOC activity in a PKG-dependent manner. Panels **a** and **b** show representative traces depicting STOC activity following forskolin (30 μM) stimulation in cremaster and cerebral VSMCs, respectively. STOCs were inhibited by the PKG inhibitor KT5823 (300 nM) but not the PKA inhibitor KT5720 (300 nM). Note the higher amplitude and frequency of STOCs consistent with earlier studies [33]. *PKA* protein kinase A, *PKG* protein kinase G, *STOC* spontaneous transient outward current, *VSMC* vascular smooth muscle cells

kinases [95, 96]. These kinases have been implicated in integrin-based signaling and the subsequent modulation of BK_{Ca} current [97, 98], thereby providing a mechanism by which ion channel conductance and membrane potential can be influenced by the extracellular environment and mechanical forces.

Whole cell BK_{Ca} currents (and STOCs) in VSMCs isolated from both cremaster and cerebral small arteries are increased by the PP1 and PP2 phosphatase inhibitor, okadaic acid, and decreased by the application of alkaline phosphatase

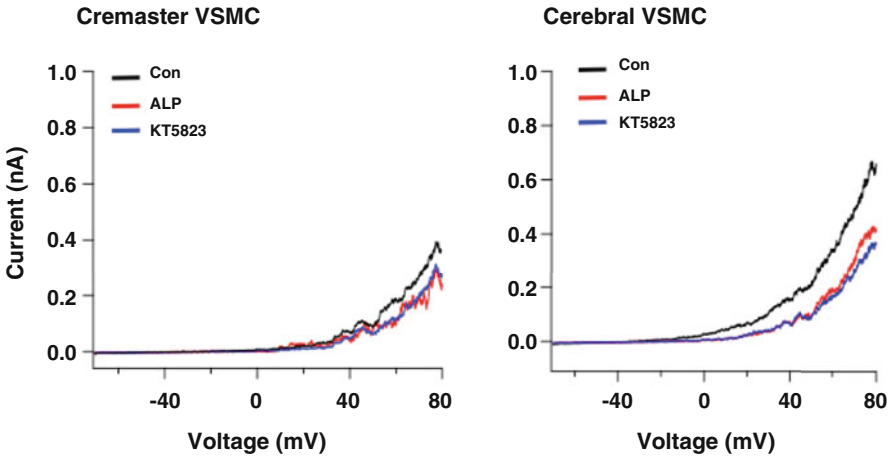

Fig. 6.5 Alkaline phosphatase decreases whole cell currents to a greater extent in cerebral VSMCs compared with cremaster VSMCs. Electrophysiological recordings were conducted in whole cell mode with voltage ramps applied. Alkaline phosphatase (ALP; 350 U/ml) was delivered intracellularly via the patch pipette while the PKG inhibitor, KT5823 (300 nM), was added to the bath. At 70 mV AP decreased current by 49.3 ± 0.9 % in cerebral VSMCs and 28.6 ± 2.1 % in cremaster VSMCs (n = 13 cells per group; $p < 0.05$). Following treatment with ALP VSMCs from both vessel types no longer showed significant inhibition by KT5823 consistent with a basal level of BK_{Ca} channel phosphorylation and PKG activity. *ALP* alkaline phosphatase, *BK_{Ca}* large conductance Ca^{2+}-activated K^+ channel, *PKG* protein kinase G, *VSMC* vascular smooth muscle cells

through a patch clamp pipette suggesting that channel activity can also be regulated by dephosphorylation (unpublished observations; Fig. 6.5). Interestingly the effect of alkaline phosphatase on BK_{Ca} current appeared greater in cerebral VSMCs suggesting a higher level of basal phosphorylation. This suggestion is line with recent proteomic data demonstrating that BK_{Ca} channels in rat brain exhibit a high level of constitutive phosphorylation [90]. Further consistent with a basal level of phosphorylation our studies have shown that the PKG inhibitor, KT5823, reduces baseline levels of BK_{Ca}-mediated STOCs (both in terms of amplitude and frequency). This effect occurred similarly in cremaster and cerebral VSMCs, although the absolute effect was greater in cerebral vessels due to their overall higher STOC activity.

BK_{Ca} Regulation by Its Local Environment

BK_{Ca} channel function is likely influenced by its local environment. For example, its regulation in VSMCs by elevated $[Ca^{2+}]_i$ is facilitated by close proximity to the SR and focal Ca^{2+} release. This arrangement is necessary for the channel to have access

to local [Ca^{2+}]s in the range of 5–10 μM that are required to activate BK_{Ca} (at physiological membrane potentials) while bulk cytosolic [Ca^{2+}] is typically <250 nM in myogenically active arteries [10] and only achieves levels of approximately 1 μM during maximal agonist stimulation. In addition to spatial considerations, BK_{Ca} is known to interact with ECM proteins [97, 98] and is also influenced by its local lipid environment [58]. As an example of the former fibronectin potentiates BK_{Ca} current at the single channel level through a mechanism involving integrin activation ($\alpha_5\beta_1$), the tyrosine kinase c-SRC and subsequent modulation of BK_{Ca} Ca^{2+} sensitivity [97, 99].

As stated above, regulation of BK_{Ca} by intracellular Ca^{2+} has been estimated to require local Ca^{2+} concentrations in the order of 5–10 μM [47, 100]. As such Ca^{2+} levels, on a global cellular basis, would cause maximal VSMC contraction and likely activate Ca^{2+}-dependent proteases and cell death pathways, a spatially restricted increase in Ca^{2+} would be required to activate BK_{Ca} channels, thereby preserving cell viability. In this regard, Nelson and colleagues first presented evidence in VSMCs for Ca^{2+} sparks as a regulatory process for BK_{Ca} activity. Sparks are spatially-restricted Ca^{2+} release events from the SR that utilize a ryanodine-sensitive release mechanism [25, 101].

Recent studies have shown, however, that while a number of vascular beds exhibit Ca^{2+} spark- dependent regulation of BK_{Ca} [25, 100, 102, 103], this situation may not always be the case [37, 42]. In addition other studies have suggested that in contrast to promoting hyperpolarization and relaxation, Ca^{2+} sparks may promote contraction, perhaps through summation to more global Ca^{2+} events such as Ca^{2+} waves [103].

In studies comparing VSMCs from cremaster muscle arterioles with those from cerebral vessels Ca^{2+} sparks were only observed in the latter. It would appear that this result was unlikely to simply reflect methodological differences as both cell preparations were studied using the same spinning disk confocal microscope system. That being said, both sets of VSMCs did exhibit an accepted electrophysiological correlate or readout of Ca^{2+} sparks (i.e. the presence of STOCs) and, further, the STOCs observed in cremaster VSMCs were attenuated by both ryanodine (at concentrations that would be expected to deplete SR Ca^{2+} stores) and by the BK_{Ca} channel inhibitor, iberiotoxin. These latter observations strongly suggest that communication does occur between ryanodine-sensitive Ca^{2+} stores in the SR and plasmalemmal BK_{Ca} channels in both cremaster and cerebral VSMCs.

As an additional example of BK_{Ca} regulation via the local cellular environment, BK_{Ca} channels are known to organize into macromolecular complexes consisting of various receptors, scaffolding proteins and specific membrane domains. For example, β_2-adrenoceptors, together with BK_{Ca}, associate with A-kinase anchoring proteins (AKAPs), PKA and voltage-gated Ca^{2+} channels [104]. Similarly, angiotensin II receptors (AT1R) have been reported to complex with BK_{Ca} through caveolin 1 [105]. Further, BK_{Ca} associates with specific lipids [58] providing a further possible mechanism for the targeting/trafficking of BK_{Ca} channels to spatially restricted domains/signaling complexes.

Conclusion

The activity of BK_{Ca} shows considerable complexity due to regulation of the channel at multiple levels. This complexity likely contributes to tissue diversity in channel function and its contribution to vasoregulation, particularly as this relates to the control of myogenic tone. Thus, in comparing BK_{Ca} function in cremaster and cerebral VSMCs, a number of differences are apparent. Overall, mRNA and protein expression levels are less in cremaster VSMCs, subtle differences exist with regard to splice variant expression, channels differ in their activation by Ca^{2+} (both in terms of channel Ca^{2+} sensitivity and reliance on Ca^{2+} sparks) and phosphorylation status of the channel appears to be relatively higher in cerebral VSMCs. Similarities and differences in their cellular spatial localization are yet to be examined in any detail. An obvious question is whether these regional differences in the BK_{Ca} channel properties are of physiological significance. Given the importance of this channel in the regulation of membrane potential and intracellular Ca^{2+} handling, along with vascular bed-specific differences in the relationships between membrane potential and myogenic tone, it is tempting to speculate that differences in BK_{Ca} contribute to the local control of vessel diameter, and hence blood flow, in resistance vessels. Thus, variation in the molecular make-up of the channel along with differences in post-translational regulation would allow the same basic ion channel to contribute to differing degrees to VSM function within a given vascular bed.

An additional question in understanding the relevance of K^+ channel distribution to vasoregulatory mechanisms relates to why BK_{Ca} is predominantly expressed in VSMCs while intermediate and small conductance, Ca^{2+}-activated, K^+ channels (IK_{Ca} and SK_{Ca}) are primarily located in endothelial cells. As endothelial cells do not typically exhibit sparks, it is conceivable that they express channels with higher Ca^{2+} sensitivity as a reflection of their more modest Ca^{2+} dynamics. Activation of IK_{Ca} and SK_{Ca} in the endothelium tends to support the vasodilatory effects of Ca^{2+}-mobilizing stimuli and thus these channels function closely with other pathways, including eNOS, towards a common goal (i.e. vasodilation). Further, the higher Ca^{2+} affinity of IK_{Ca} and SK_{Ca} will provide a prolonged signal to support vasodilation. Thus, endothelial cells do not appear to require two distinct Ca^{2+} operated mechanisms, while in VSMCs, Ca^{2+} evokes contraction at sub-micromolar levels and sparks evoke relaxation by locally increasing Ca^{2+} to higher levels (approximately 10 μM). As a consequence there is a dichotomy in Ca^{2+} signaling that requires two distinct pathways and sensors. The lower affinity Ca^{2+} sensor of BK_{Ca}, together with its spatial localization, will limit its activation/opening thereby preventing the channel from interfering with evoked contraction occurring at sub-micromolar Ca^{2+} levels. However, the much higher conductance will provide a rapid and robust Vm change to oppose voltage-gated Ca^{2+} channel activity when needed. As these mechanisms may also vary between tissues, understanding of the complexity of K^+ channel distribution, molecular structure and function requires considerable further work so that these properties can be exploited in the development of vascular bed-specific therapeutics.

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