Hypercontractility and impaired sildenafil relaxations in the BK<sub>Ca</sub> channel deletion model of erectile dysfunction

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Werner ME, Meredith AL, Aldrich RW, Nelson MT. Hypercontractility and impaired sildenafil relaxations in the BK<sub>Ca</sub> channel deletion model of erectile dysfunction. Am J Physiol Regul Integr Comp Physiol 295: R181–R188, 2008. First published May 14, 2008; doi:10.1152/ajpregu.00173.2008.—Erectile dysfunction (ED) can be elicited by a variety of pathogenic factors, particularly impaired formation of and responsiveness to nitric oxide (NO) and the downstream effectors soluble guanylate cyclase (sGC) and cGMP-dependent protein kinase I (PKGI). One important target of PKGI in smooth muscle is the large-conductance, Ca<sup>2+</sup>+-activated potassium (BK<sub>Ca</sub>) channel. In our previous report (42), we demonstrated that deletion of the BK<sub>Ca</sub> channel in mice induced force oscillations and led to reduced nerve-evoked relaxations and ED. In the current study, we used this ED model to explore the role of the BK<sub>Ca</sub> channel in the NO/sGC/PKGI pathway. Electrical field stimulation (EFS)-induced contractions of corpus cavernosum smooth muscle strips were significantly enhanced in the absence of BK<sub>Ca</sub> channel function. In strips precontracted with phenylephrine, EFS-induced relaxations were converted to contractions by inhibition of sGC, and this was further enhanced by loss of BK<sub>Ca</sub> channel function. Sildenafil-induced relaxations were decreased to a similar extent by inhibition of sGC or BK<sub>Ca</sub> channels. At concentrations >1 μM, sildenafil caused relaxations independent of inhibition of sGC or BK<sub>Ca</sub> channels. Sildenafil did not affect the enhanced force oscillations that were induced by the loss of BK<sub>Ca</sub> channel function. Yet, these oscillations could be completely eliminated by blocking L-type voltage-dependent Ca<sup>2+</sup> channels (VDCCs). These results suggest that therapeutically relevant concentrations of sildenafil act through cGMP and BK<sub>Ca</sub> channels, and loss of BK<sub>Ca</sub> channel function leads to hypercontractility, which depends on VDCCs and cannot be modified by the cGMP pathway.

Erectile dysfunction (ED) is described as the persistent inability to achieve or maintain an erection sufficient for satisfactory sexual performance (44). It affects 30 million men in the United States (5), and its prevalence increases with age and diseases like diabetes and hypertension (4, 24, 31). Erectile function and a proper penile erection occurs in response to nerve stimulation and the release of nitric oxide (NO) from parasympathetic nonadrenergic-noncholinergic (NANC) nerves as well as from the vascular endothelium (2, 13, 16, 27). In mice, the NO-producing enzyme NO synthase has been found in the dorsal penile nerve and its branches in the mouse penis (9) as well as in intrinsic nerves of the erectile tissue (14). The majority of NO effects are mediated through the soluble guanylate cyclase (sGC) and its product, cGMP (2, 8, 16, 33). cGMP acts as a modifying agent on ion channels, phosphodiesterases, and protein kinases (19). One of the protein kinases, the cGMP-dependent protein kinase I (PKGI), phosphorylates various proteins, including ion channels and pumps, which are known to reduce intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) and smooth muscle contraction. The importance of this kinase in erectile tissue is supported by the observation that precontracted corpus cavernosum smooth muscle (CCSM) strips from mice lacking PKGI did not relax to nerve stimulation (14). It is known that PKGI activates large-conductance Ca<sup>2+</sup>+-activated potassium (BK<sub>Ca</sub>) channels (1, 32), which hyperpolarize smooth muscle cell membranes, causing muscle relaxation. Relaxation of arterial and CCSM is necessary to increase blood flow into the corpora cavernosa that leads to penile tumescence.

BK<sub>Ca</sub> channels in CCSM clearly have an important role in the regulation of function. Blocking the BK<sub>Ca</sub> channel with tetraethylammonium ions or charybdotoxin led to an increase of phenylephrine (PE)-induced contractions of CCSM strips in vitro (34). In aged or diabetic rats, intracavernous injection of cDNA encoding the human BK<sub>Ca</sub> channel led to a reversal of ED (11, 25). Consistent with the significant role of BK<sub>Ca</sub> channels to oppose smooth muscle contractility, precontracted CCSM strips from mice lacking the BK<sub>Ca</sub> channel (Kcnma1<sup>−/−</sup>, also called Slo<sup>−/−</sup>) exhibited pronounced force fluctuations in the presence of the α-adrenergic receptor agonist PE and reduced relaxations in response to nerve stimulations compared with wild-type mice (Slo<sup>+/+</sup>) (42). The BK<sub>Ca</sub> channel is an attractive target in the treatment of vascular and lower urinary tract disorders, including urinary incontinence and ED. Kcnma1 (Slo) gene therapy has been shown to ameliorate ED in rodents (11), and recently in humans (22).

Early pharmacological studies on erectile function demonstrated that direct injection of vasodilator drugs into the corpus cavernosum, such as the phosphodiesterase inhibitor papaverine, resulted in erection (41). Later, the importance of cGMP was reinforced by the development of drugs such as sildenafil, which inhibit the cGMP-specific type 5 phosphodiesterase (PDE5) (6, 39). Even though the positive effect of sildenafil on erectile function is well established, the downstream mechanisms through which cGMP and PKGI mediate relaxation have not been elucidated.

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The primary goal of the current study was to explore the BK_{Ca} channel deletion model of ED and investigate the nature of its hypercontractile phenotype. Furthermore, we used this model to examine the effect of activating the cGMP signaling pathway with the PDE5 inhibitor sildenafil on the contractility of isolated CCSM strips in the absence and presence of the α-adrenergic receptor agonist PE.

**METHODS**

Animal handling and tissue preparation. Slo^{−/−} mice were generated as previously published (26). All procedures performed in the course of this study were approved by the Office of Animal Care Management at the University of Vermont. Adult male mice (10–20 wk old; ∼30 g body wt) were killed with intraperitoneal injection of pentobarbital sodium (150 mg/kg) followed by thoracotomy. For the in vitro contractility studies, the penis was removed and immediately placed in ice-cold dissection solution (in mM: 80 monosodium glutamate, 55 NaCl, 6 KCl, 10 glucose, 10 HEPES, and 2 MgCl₂, pH 7.3 adjusted with NaOH).

In vitro contractility studies. Contractility studies were performed as described earlier (42). Briefly, the contractility of isolated CCSM strips was measured using a MyoMED myograph system (MED Associates, Georgia, VT). Each strip was mounted in a thermostatically controlled tissue bath and stretched to a resting tension of 0.1 mN. After a 1-h equilibration period, the force generation of the strips was analyzed by applying electrical field stimulation (EFS) in the absence or presence of 10 μM PE in the bath. EFS was delivered either continuously at 30 Hz every minute or at increasing frequencies (1, 2, 3.5, 5, 7.5, 10, 12.5, 15, 20, 30, 40, and 50 Hz) every 3 min. Pulse amplitude was 20 V, and polarity was reversed for alternating pulses. Pulse width was 0.5 ms, and stimulus duration was 2 and/or 60 s (see Fig. 2). To apply stimuli, a model PHM-152V stimulator (MED Associates) was used.

Drugs and data analysis. The compounds used were: iberiotoxin (IBTX; 300 nM; Peptides International), sildenafil (10 nM-10 μM; Pfizer), PE (10 μM), 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM), and nisoldipine (100 nM; all from Sigma). Data were analyzed and presented using MyoMed (MED Associates), MiniAnalysis (Synaptosoft), Origin (OriginLab), Prism (GraphPad), and CorelDraw (Corel) software. Statistical comparisons were made using paired or unpaired t-tests, as applicable, and data are expressed with SEs. *P < 0.05 was considered significant.

**RESULTS**

**EFS-induced contractions are increased in CCSM strips from Slo^{−/−} mice and from Slo^{+/+} mice in the presence of IBTX.** Transmural EFS has been used to stimulate all autonomic nerves that innervate the smooth muscle of the corpus cavernosum (2, 14, 27). Depending on the contractile state of the CCSM, EFS can induce smooth muscle contraction through the stimulation of sympathetic nerves present in the CCSM strips, or it can induce relaxation through the stimulation of NANC nerves and the release of NO. We analyzed the contractile response of isolated CCSM strips from Slo^{+/+} and Slo^{−/−} mice to the application of EFS with increasing stimulation frequencies from 1 Hz to 50 Hz. (Fig. 1, A and C). In both genotypes, contractions were observed at the lowest frequencies. Above 12.5 Hz, EFS-induced force was up to 1.6 times greater in strips from Slo^{−/−} than Slo^{+/+}. The results with Slo^{−/−} mice suggest that blocking BK_{Ca} channels with IBTX should also elevate nerve-evoked force above 12.5 Hz.
Indeed, in Slo\(^{++}\) strips, IBTX lead to a 170% increase of the 30 Hz-induced contractions, an effect that was absent in Slo\(^{-/-}\) strips, supporting the role of BKCa channel to oppose sympathetic nerve-mediated contractions (Fig. 1, B and D). These results are consistent with BKCa channels opposing CCSM excitability.

**Inhibition of sGC converts EFS-induced relaxations into contractions that are increased in Slo\(^{-/-}\).** In the flaccid state, the corpus cavernosum is under tonic sympathetic influence to keep the smooth muscle contracted. As in our previous study, this condition was simulated by adding the \(\alpha\)-adrenergic receptor agonist PE (10 \(\mu\)M) to induce contraction of isolated CCSM strips (42). Electrical stimulation would stimulate both sympathetic and NANC nerves. However, under these conditions, the \(\alpha\)-adrenergic receptors are already activated by the presence of the externally added PE, and the NO release from NANC nerves then leads to relaxation of the CCSM strips.

In precontracted CCSM strips, EFS has been widely used to analyze the activation of NANC nerves and the subsequent relaxation of the cavernous tissue (2, 14). In our previous study (42), the following two stimulation protocols were used: one lasting for 2 s to induce a brief and transient relaxation and the other for 60 s to induce a prolonged and more physiological activation of nerves. Here we used these stimulation protocols to analyze the importance of cGMP for EFS-induced relaxations in animals and in humans that the PDE5-specific inhibitor sildenafil relaxes CCSM through elevating cGMP levels (6, 35, 40). One of the possible effectors of the elevated cGMP levels is the BKCa channel (3), but functional data supporting a role of BKCa channels in the actions of sildenafil have been lacking. We used Slo\(^{++}\) and Slo\(^{-/-}\) mice to investigate the role of BKCa channel in sildenafil-mediated relaxations of CCSM strips.

**Involvement of BKCa channels in sildenafil-mediated relaxations of CCSM.** It has been well established in animals and in humans that the PDE5-specific inhibitor sildenafil relaxes CCSM through elevating cGMP levels (6, 35, 40). One of the possible effectors of the elevated cGMP levels is the BKCa channel (3), but functional data supporting a role of BKCa channels in the actions of sildenafil have been lacking. We used Slo\(^{++}\) and Slo\(^{-/-}\) mice to investigate the role of BKCa channel in sildenafil-mediated relaxations of CCSM strips.
Two consecutive contractions with PE (10 μM) were induced, and sildenafil (10 μM) was added after the first contraction (Fig. 3). In the absence of sildenafil, there was no difference between first and second contractions (data not shown). In Slo\textsuperscript{+/+} strips, sildenafil (10 μM) reduced the PE-induced contractile force to 20.7 ± 3.5% of the control (compared with a second contraction in the absence of sildenafil). In strips from Slo\textsuperscript{−/−} mice, sildenafil was less effective (Fig. 3, B and C). Furthermore, force oscillations were observed in Slo\textsuperscript{−/−} strips, even in the presence of sildenafil (Fig. 3B, right).

Consistent with the effects of sildenafil on PE-induced force (Fig. 3), the application of sildenafil (10 μM) to precontracted Slo\textsuperscript{+/+} strips led to a 79.0 ± 1.7% relaxation (Fig. 4, A and E). Blocking the BK\textsubscript{Ca} channel with IBTX before the sildenafil treatment had a small, but significant, effect, reducing the relaxation by ~7% (72.1 ± 2.4% relaxation of the PE-induced force) (Fig. 4, B and E). Similar relaxation was observed in Slo\textsuperscript{−/−} strips (70.7 ± 2.9% relaxation), whereas IBTX had no effect. The amplitudes of the force oscillations in the presence of IBTX or in Slo\textsuperscript{−/−} strips were not altered by 10 μM sildenafil (Fig. 4C). However, in the absence of BK channel function, sildenafil was able to reduce the frequency of the force oscillations (Fig. 4D).

Although sildenafil is commonly used at concentrations 10 μM and greater in studies on rabbit and human tissue (35, 40), this concentration is high relative to inhibition of PDE5 (35, 40). At very high concentrations, sildenafil is thought to act on other pathways independent of the NO pathway (17, 21). Moreover, since we observed only a small difference between the two genotypes at a relatively high concentration of sildenafil (10 μM), the relaxing effects of sildenafil from 1 nM to 10 μM were examined, using precontracted Slo\textsuperscript{+/+} and Slo\textsuperscript{−/−} CCSM strips. At all concentrations, the relaxing effect of sildenafil was significantly reduced in Slo\textsuperscript{−/−}, with the largest difference at 100 nM (Slo\textsuperscript{+/+} 54.5 ± 2.8% and Slo\textsuperscript{−/−} 29.1 ± 4.1%; Fig. 4F) along with a right shift of the dose-response curve to higher concentrations in the absence of the BK\textsubscript{Ca} channel (Slo\textsuperscript{+/+}: logEC\textsubscript{50} = −7.60 ± 0.79; Slo\textsuperscript{−/−}: logEC\textsubscript{50} = −5.75 ± 0.22; P < 0.05).

cGMP is synthesized by guanylate cyclases, and broken down by PDE5, which is inhibited by sildenafil. Inhibition of the sGC with ODQ increased smooth muscle tone and attenuated but did not prevent sildenafil-induced relaxation entirely (21) (Fig. 4F). ODQ reduced the relaxation to sildenafil (100 nM) from 54.5 ± 2.8 to 22.5 ± 1.5% in Slo\textsuperscript{+/+} strips and from 29.1 ± 4.1 to 16.4 ± 1.6% in Slo\textsuperscript{−/−} strips. In the presence of ODQ, the sildenafil response was altered by the loss of the BK\textsubscript{Ca} channel at 10 and 100 nM (Fig. 4F), but without a significant shift of the dose-response curve (Slo\textsuperscript{+/+} + ODQ: logEC\textsubscript{50} = −3.99 ± 0.67; Slo\textsuperscript{−/−} + ODQ: logEC\textsubscript{50} = −3.51 ± 1.09; P = 0.31). These results indicate that blocking sGC or loss of the BK\textsubscript{Ca} channel caused a similar reduction in sildenafil’s action. This is consistent with the idea that BK\textsubscript{Ca} channel is one of the major downstream targets of the elevation of cGMP by sildenafil.

Blocking of voltage-dependent Ca\textsuperscript{2+} channels inhibits force oscillations in CCSM strips from Slo\textsuperscript{−/−} and from Slo\textsuperscript{+/+} plus IBTX. Loss of BK\textsubscript{Ca} channel function by IBTX or in the Slo\textsuperscript{−/−} mice leads to prominent force oscillations in the presence of PE, and sildenafil does not prevent these oscillations. It is likely that these force oscillations reflect an unstable membrane potential, and thus modulation of Ca\textsuperscript{2+} influx through voltage-dependent Ca\textsuperscript{2+} channels (VDCC). To examine this possibility, the effects of the VDCC blocker nisoldipine (100 nM) on PE-induced oscillations were tested. In Slo\textsuperscript{+/+} strips, the small-amplitude oscillations were unaffected by nisoldipine (Fig. 5, A and C). However, the larger oscillations observed in strips from Slo\textsuperscript{−/−} mice or from Slo\textsuperscript{+/+} mice in the presence of IBTX were completely abolished by blocking VDCCs (Fig. 5, A–C). In addition to the block of force oscillations, nisoldipine relaxed CCSM strips up to 25%, irrespective of genotypes or the presence of IBTX (Fig. 5C).

**DISCUSSION**

The role of BK\textsubscript{Ca} channels in controlling CCSM tone. The two principal findings of this study are that, in the absence or blockade of the BK\textsubscript{Ca} channel, CCSM exhibit enhanced contractility to stimulations of sympathetic nerves present in CCSM strips (Fig. 1) and, when precontracted with PE, display a reduced ability to relax in response to sildenafil (Fig. 4). Indeed, we found that relaxation of CCSM strips by sildenafil was blocked to a similar extent by loss of BK channel function or inhibition of sGC (Fig. 4), suggesting a major role of BK channels as a downstream target of sildenafil’s actions. In particular, the reduced relaxation is accompanied by continuous oscillatory contractions, which could not be prevented by sildenafil. In a very significant way, this study extends our...
previous findings (42), which demonstrated that a lack of BKCa channels leads to hypercontractility and reduced relaxation abilities to nerve stimulation in precontracted CCSM.

The enhancement of nerve-evoked contractions by IBTX and in Slo\textsuperscript{-/-} mice in this study could be explained by inhibition and absence of the BKCa channel in the smooth muscle, respectively. Nerve-evoked contractions are mediated by the release of norepinephrine from sympathetic nerves, are blocked by the \( \alpha \)-adrenergic receptor blocker prazosin (13), and, without the BKCa channel to counteract these contractions, they are very likely to be enhanced (Fig. 1). Furthermore, BKCa channels were only found in the smooth muscle of the penis (42).

In our previous study, loss of BK channel function reduced the relaxation to 60 s stimulation but had no effect on 2 s stimulation (42). Therefore, it is possible that the longer-duration stimulation engages additional relaxation pathways. However, inhibition of sGC in WT strips dramatically affected both the 2-s and the 60-s relaxations, converting relaxation to contraction. These results indicate that the 2- and 60-s nerve stimulations cause relaxation through the NO/sGC/cGMP pathway; however, the 2-s stimulation seems to recruit different downstream effector mechanisms of this pathway of which BKCa is only one (for review, see Ref. 15). Moreover, although inhibiting this pathway, EFS still activates sympathetic pathways, which leads to further contractions in precontracted strips.
NO activates sGC to synthesize cGMP, and sildenafil inhibits the breakdown of it. Thus sildenafil acts on the NO/sGC/cGMP pathway and amplifies its relaxing effect in smooth muscle. cGMP-dependent protein kinase directly activates the BKCa channel (32, 36), and indirectly through phosphorylation of phospholamban and elevation of local Ca2+ release events through ryanodine receptors (30). We found that the relaxing effect of sildenafil (10 μM) was reduced by ~10% in Slo−/− CCMS. However, loss of BKCa channel function has a very substantial effect on nerve-evoked relaxations (Fig. 2) and increases in intracavernous pressure (37). Furthermore, sildenafil’s relaxing effects were almost equally affected by the loss of BKCa channel function or inhibition of sGC (Fig. 4), suggesting that sildenafil largely acts through cGMP and activation of BKCa channel. ODQ and loss of BKCa channel function had an identical, but small, effect (10%) on sildenafil-induced relaxation at 10 μM sildenafil (Fig. 4F). This result suggests that sildenafil has additional effects (non-cGMP, non-BKCa channel) at 1 μM and greater. Indeed, sildenafil has been shown to inhibit VDCCs in cardiac muscle >1 μM (10). In CCMS, inhibition of VDCCs reduced smooth muscle contraction; particularly, it entirely eliminated the increased phasic contractions induced by the absence of BKCa function (Fig. 5). Sildenafil, however, did not affect the phasic contraction amplitude (Fig. 4), suggesting that, in CCMS, high sildenafil concentrations act through mechanisms other than inhibition of VDCCs. These results indicate that therapeutically relevant concentrations (<1 μM) of sildenafil act through cGMP and BKCa channels and that higher concentrations act through other, yet unknown mechanisms.

The lack of effects of sildenafil on the force oscillations when the BKCa channel is blocked or absent is noteworthy. These results indicate that, in a situation of compromised or diminished BKCa channel function, the CCMS cannot be kept in a relaxed state, and consequently the penis in an erect state, even in the presence of the potent ED drug sildenafil. BKCa channel expression has been shown to decrease with age in coronary arteries (38), and, if this occurs in the CCMS, it may diminish the efficacy of a PDE5 inhibitor therapy.

**BKCa channel and intracellular Ca2+.** Previous investigations established that the BKCa channel has a central role in the modulation of nonvascular (26, 37) and vascular contractility (18, 28), as well as in the corpus cavernosum (11, 12, 25). The suggested mechanism of BKCa channel function is linked to the hyperpolarization of smooth muscle cells and a concomitant decrease in transmembrane Ca2+ flux through L-type VDCC (7, 18). These observations indicate the importance of membrane potential in the control of smooth muscle tone and [Ca2+]i. Therefore, ablation of the BKCa channel should lead to an unstable membrane potential, increased Ca2+ influx, and finally to a reduced ability of the smooth muscle to oppose contraction. Indeed, CCMS strips from Slo−/− mice showed an increased contractile response to sympathetic nerve stimulations (Fig. 1). Furthermore, in the presence of the α-adrenergic receptor agonist PE, deficiency or blockade of the BKCa channel induced continuous force oscillations that were not present in Slo+/+ mice. We provide evidence that these oscillations are enhanced in Slo−/− comparable to the previous experiment in nonprecontracted strips (compare Figs. 1 and 2). Under these conditions, the EFS-induced contractions again are enhanced in Slo−/− comparable to the previous experiment in nonprecontracted strips.

Fig. 5. Blocking of voltage-dependent Ca2+ channels (VDCC) inhibits force oscillations in Slo−/− CCMS strips and in Slo+/+ with IBTX. A and B: representative recordings from a PE-precontracted Slo+/+ (left) and a Slo−/− (right) CCMS strip in the absence (A) and presence (B) of IBTX that demonstrate the effect of blocking VDCC with nisoldipine. C: average oscillation amplitudes of Slo+/+ and a Slo−/− CCMS strip in the absence and presence of IBTX, before and after adding nisoldipine. D: summary of total relaxation of PE-precontracted CCMS strips induced by nisoldipine as shown in A and B. NIS, nisoldipine; N, no. of mice; *P < 0.05 vs. Slo+/+ untreated.
lations are caused by increased Ca\(^{2+}\) influx through VDCCs because a specific inhibition of the Ca\(^{2+}\) channels completely suppressed force oscillations (Fig. 5). No difference was observed in the overall relaxation through the VDCC inhibition. The small total relaxation of \(-25\%\) is consistent with other reports where transient PE-induced Ca\(^{2+}\) influx was not reduced by removing extracellular Ca\(^{2+}\) but dependent on release from intracellular Ca\(^{2+}\) stores (43). Thus the observed increase of force oscillations is very likely due to membrane potential instability and increased opening of VDCCs but not due to a change in Ca\(^{2+}\) channel expression in CCSM strips from Slo\(^{−/−}\) mice or from Slo\(^{+/+}\) mice in the presence of IBTX.

**Perspectives and Significance**

In vascular smooth muscle of aged rats (20, 29) as well as of humans (38), the BK\(_{Ca}\) channel \(\alpha\)- and \(\beta_1\)-subunit expression is decreased. Correlated with this downregulation are increased risks of cardiovascular diseases such as coronary artery vasospasm, myocardial ischemia, and infarct (20, 38). Recently, reports where transient PE-induced Ca\(^{2+}\) sparks is involved in regulation of arterial diameter by cyclic nucleotides.

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