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RESEARCH PAPER

Amplified NO/cGMP-mediated relaxation and ryanodine receptor-to-BK_{Ca} channel signalling in corpus cavernosum smooth muscle from phospholamban knockout mice

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BACKGROUND AND PURPOSE

Relaxation of corpus cavernosum smooth muscle (CCSM) is induced by NO. NO promotes the formation of cGMP, which activates cGMP-dependent protein kinase I (PKGI). The large conductance calcium-activated potassium (BK_{Ca}) channel is regarded as a major target of NO/cGMP signalling; however, the mechanism of BK_{Ca} activation remains unclear. The aim of the present study was to determine whether sarcoplasmic reticulum (SR) Ca^{2+} load and Ca^{2+} release from the SR via ryanodine receptors (RyRs) is important for BK_{Ca} channel activation in response to NO/cGMP.

EXPERIMENTAL APPROACH

In vitro myography was performed on CCSM strips from wild-type and PLB knockout ($PLB^{-/-}$) mice to evaluate contraction and relaxation in response to pharmacological agents and electrical field stimulation (EFS).

KEY RESULTS

In CCSM strips from $PLB^{-/-}$ mice, a model of increased SR Ca^{2+} load, contractile force in response to EFS or phenylephrine (PE) was increased by nearly 100%. EFS of strips precontracted with PE induced transient relaxation in CCSM, an effect that was significantly larger in $PLB^{-/-}$ strips. Likewise, the relaxation of PE-induced contraction in response to SNP and cGMP was greater in $PLB^{-/-}$, as demonstrated by a shift in the concentration–response curve towards lower concentrations. Blocking RyRs and BK_{Ca} channels diminished the induced relaxations and eliminated the difference between wild-type and $PLB^{-/-}$.

CONCLUSIONS AND IMPLICATIONS

NO/cGMP activates BK_{Ca} channels through RyR-mediated Ca^{2+} release. This signalling pathway is responsible for approximately 40% of the NO/cGMP effects and is amplified by increased SR Ca^{2+} concentrations.

Abbreviations

BK_{Ca}, large conductance calcium-activated potassium channel; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CCSM, corpus cavernosum smooth muscle; ED, erectile dysfunction; EFS, electrical field stimulation; IBTX, iberiotoxin; IP_3Rs, 1,4,5-trisphosphate; ODQ, 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one; PE, phenylephrine; PKGI, protein kinase I; $PLB^{-/-}$, phospholamban knockout; RyRs, ryanodine receptors; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; sGC, soluble guanylate cyclase; *Slo*$^{-/-}$, BK_{Ca} channel knockout mice; SNP, sodium nitropruside; SR, sarcoplasmic reticulum; WT, wild-type

Introduction

Penile erection results from elevated pressure in the corpus cavernosum (CC), a spongy sinusoidal structure that runs the length of the penis, caused by smooth muscle relaxation and increased blood flow to the penis. NO released from parasympathetic NANC nerves as well as from the vascular endothelium is thought to be largely responsible for penile smooth muscle relaxation (Holmquist *et al.*, 1991; Andersson and Wagner, 1995; Hedlund *et al.*, 1999; Mizusawa *et al.*, 2001). The NO-producing enzyme, NOS, has been found in the dorsal penile nerve and its branches in the mouse penis (Burnett *et al.*, 1996) as well as in intrinsic nerves of the erectile tissue (Hedlund *et al.*, 2000). In corpus cavernosum smooth muscle (CCSM), most NO effects are mediated by the haem-containing soluble guanylate cyclase (sGC), which catalyses the formation of cGMP in response to NO binding (Holmquist *et al.*, 1991; Burnett *et al.*, 1992; Schmidt *et al.*, 1993; Andersson and Wagner, 1995). The second messenger cGMP can activate ion channels, PDEs and protein kinases (Lincoln and Cornwell, 1993). cGMP-dependent protein kinase type I (PKGI) phosphorylates and activates a variety of target proteins in smooth muscle to reduce the apparent Ca^{2+} sensitivity of the contractile apparatus and decrease intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ (Sauzeau *et al.*, 2000; Ammendola *et al.*, 2001). The significance of PKGI in erectile physiology is underscored by the observation that precontracted CCSM strips from PKGI knockout mice do not relax to nerve stimulation (Hedlund *et al.*, 2000).

The large conductance calcium-activated potassium (BK_{Ca}) channel is regarded as a major target of PKGI (Robertson *et al.*, 1993; Alioua *et al.*, 1998). Upon activation, the BK_{Ca} channel hyperpolarizes smooth muscle cell membranes and thereby promotes relaxation. BK_{Ca} channels play a key role in the regulation of CCSM contractility. Blocking the channel with tetraethylammonium ions or charybdotoxin increases the contractile force induced in CCSM strips by the α-adrenoceptor agonist phenylephrine (PE) *in vitro* (Spektor *et al.*, 2002). Consistent with the importance of BK_{Ca} channels in opposing smooth muscle contractility, precontracted CCSM strips from BK_{Ca} channel knockout mice (*Kcnma1*$^{-/-}$ or *Slo*$^{-/-}$) exhibit pronounced force fluctuations in the presence of PE and a reduced ability to relax in response to nerve stimulations compared with wild-type (WT) mice (*Slo*$^{+/+}$) (Werner *et al.*, 2005). Furthermore, in the absence of functional BK_{Ca} channels, the relaxant effect of sildenafil is markedly reduced, and the drug has no effect on the force fluctuations observed in *Slo*$^{-/-}$ mice (Werner *et al.*, 2008). Interestingly, injection of naked cDNA encoding the human BK_{Ca} channel into the corpora cavernosa reverses the apparent erectile dysfunction (ED) in aged and diabetic rats (Melman *et al.*, 2003; Christ *et al.*, 2004), and improves ED symptoms in humans (Melman *et al.*, 2005). Collectively, these observations establish the BK_{Ca} channel as an attractive target for therapeutic strategies to treat vascular and lower urinary tract disorders, including ED.

Direct injection of the vasodilator papaverine, a non-specific PDE inhibitor, into the CC has long been used to treat ED (Virag, 1982). The importance of cGMP in erectile function was further reinforced by the development of drugs such as sildenafil and vardenafil that specifically inhibit the cGMP-degrading PDE-5 (Boolell *et al.*, 1996; Turko *et al.*, 1999). Although the positive effect of PDE-5 inhibitors on erectile function is now well established, the downstream mechanisms through which cGMP and PKGI act to mediate relaxation of vascular and CC smooth muscle have remained elusive. PKGI could act directly on the BK_{Ca} channel to elevate its open probability, or indirectly through phosphorylation of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA)-associated protein, phospholamban (PLB) (Robertson *et al.*, 1993; Porter *et al.*, 1998), which acts as a negative regulator of SERCA activity. Phosphorylation of PLB inhibits the suppressive effect of PLB on SERCA-mediated Ca^{2+} uptake, leading to an increased Ca^{2+} concentration in the sarcoplasmic reticulum (SR). This elevated Ca^{2+} SR load can raise the frequency and amplitude of ryanodine receptor (RyR)-mediated Ca^{2+} release events (Ca^{2+} sparks) (Jaggar *et al.*, 1998; Porter *et al.*, 1998; Wellman *et al.*, 2001).

The primary focus of the current work is on the underlying mechanisms that lead to BK_{Ca} channel activation in CCSM in response to activation of the NO/cGMP signalling pathway. We hypothesized that intracellular Ca^{2+} signalling from RyRs to BK_{Ca} channels is an important contributor to CCSM relaxation. To test this hypothesis, we used WT mice as well as PLB knockout (PLB$^{-/-}$) mice, which are characterized by elevated SR Ca^{2+} load and increased Ca^{2+} spark frequency (Wellman *et al.*, 2001). Furthermore, using this PLB$^{-/-}$ model, we examined the effect of activating the NO/cGMP signalling pathway on the contractility of isolated CCSM strips in the absence and presence of the α-adrenoceptor agonist PE. Our results support the concept that RyR-to-BK_{Ca} channel signalling is a major component of NO/cGMP-induced relaxation of CCSM and it is more effective under conditions of elevated SR Ca^{2+} load.

Methods

Animal handling and tissue preparation

All animal procedures and experiments were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and conformed to institutional regulations at the University of Manchester. The generation and characterization of PLB$^{-/-}$ mice have been described previously (Luo *et al.*, 1994; Lalli *et al.*, 1997). All mice were bred at the University of Manchester, and C57BL/6 WT mice were used as controls. Adult male mice (10–20 weeks old; ~30 g bodyweight) were killed by cervical dislocation. For *in vitro* myography experiments, 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, 2 $MgCl_2$, pH 7.3).

In vitro *myography experiments*

Myography experiments were performed as described previously (Werner *et al.*, 2005; 2008). Briefly, the tunica albuginea was cut longitudinally starting at the most proximal point of the CC towards the penile shaft, and the erectile tissue was

partially dissected free from the tunica. One strip of tissue (0.3 × 0.3 × 5 mm) was obtained from each CC. The contractility of isolated CCSM strips was measured at 37°C using a tissue bath system (Radnoti Glass Technology Inc., Monrovia, CA, USA) containing 10 mL aerated (5% CO_2 in air) physiological saline solution (in mM: 119 NaCl, 4.7 KCl, 24 $NaHCO_3$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 0.023 EDTA, 11 glucose) in conjunction with a myograph muscle contractility data acquisition and analysis package (Catamount Research and Development Inc., St. Albans, VT, USA). Each strip was mounted in a thermostatically controlled tissue bath and stretched to a tension of 0.3 mN; after equilibrating for 1 h, a stable resting tension of ~0.1 mN was reached. Contractile and relaxation responses to nerve stimulation were analysed in strips exposed to electrical field stimulation (EFS) delivered continuously at 30 Hz every minute. Pulse amplitude was 300 mA, and polarity was reversed for alternating pulses. Pulse width was 0.5 ms, and stimulus duration was 2 s. Stimuli were delivered using a model PHM-152I stimulator (MED Associates, Georgia, VT, USA). In pharmacological experiments, contractile responses were measured in strips exposed to 10 μM PE, and relaxation responses were analysed in the presence of increasing concentrations of sodium nitroprusside or 8-pCPT-cGMP. All pharmacological agents were applied to the bath.

Drugs and data analysis

The compounds used were iberiotoxin (IBTX; 300 nM; Peptides International Inc., USA), 8-pCPT-cGMP (10 nM to 100 μM; BioLog Life Science Institute, Germany), 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 2 μM), PE (10 μM), phentolamine (10 μM), ryanodine (20 μM), sodium nitroprusside (SNP; 1 nM to 10 μM), all from Sigma, UK. Data were analysed and presented using MyoMed (MED Associates), MiniAnalysis (Synaptosoft), Origin (OriginLab) and PowerPoint (Microsoft) software. Statistical comparisons were made using paired or unpaired *t*-tests, as applicable, and data are expressed with standard errors. A *P*-value <0.05 was considered significant.

Results

EFS-induced contractions are augmented in CCSM strips from PLB$^{-/-}$ mice.

To investigate the role of PLB in erectile function, we examined the contractile properties of CCSM strips from WT and PLB$^{-/-}$ mice. Autonomic nerves innervate the CC, and transmural EFS has been widely used to induce neurotransmitter release from these nerves (Mizusawa *et al.*, 2001; Werner *et al.*, 2008). EFS induces contraction in CCSM strips through activation of sympathetic nerves and release of noradrenaline. Under experimental conditions in which the CCSM is precontracted with PE, EFS induces relaxation through stimulation of NANC nerves and release of NO. We first analysed the contractile response of isolated CCSM strips from WT and PLB$^{-/-}$ mice to the application of 30 Hz EFS pulses lasting 2 s and repeated every 60 s, an EFS protocol that has been shown to produce robust and reliable CCSM contraction (Werner *et al.*, 2008). Blocking α-adrenoceptors with phentolamine completely abolished these EFS-induced contractions (control: 0.2 ± 0.02 mN; phentolamine: 0.0 ± 0.0 mN, $n = 4$). EFS induced transient contractions in both WT and PLB$^{-/-}$ mice (Figure 1A), but the amplitude of the transient contrac-

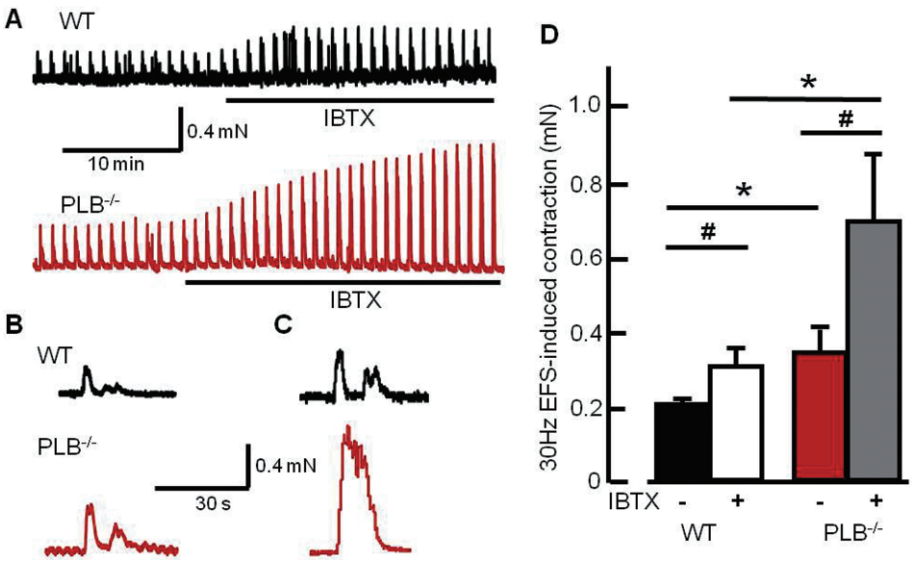

Figure 1

EFS-induced contraction in WT and PLB$^{-/-}$ CCSM strips. (A) Representative recordings of two consecutive force transients from a WT and a PLB$^{-/-}$ CCSM strip in response to EFS (2 s duration) before and after adding IBTX. (B) Force transients of a WT and a PLB$^{-/-}$ CCSM strip (as above, with a different time scale) under control conditions. (C) Force transients of a WT and a PLB$^{-/-}$ CCSM strip (as above, with a different time scale) in the presence of IBTX. (D) Average transient force induced by EFS in WT ($n = 6$) and PLB$^{-/-}$ strips ($n = 5$) in the presence and absence of IBTX. *$P <$ 0.05 Student's unpaired *t*-test; #$P < 0.05$ Student's paired *t*-test.

tion was about 1.7-fold greater in strips from $PLB^{-/-}$ mice than in those from WT mice (Figure 1D). The duration of the contraction, measured as the half-width of the force transients, was not significantly different between the two genotypes (WT: 1.98 ± 0.19 s, $n = 6$; $PLB^{-/-}$: 2.40 ± 0.21 s, $n = 5$; $P = 0.19$).

In CCSM strips, EFS-induced depolarization and elevation of smooth muscle $[Ca^{2+}]_i$ should activate BK_{Ca} channels to oppose nerve-induced contraction (Werner *et al.*, 2005; 2008). Indeed, blocking BK_{Ca} channels with IBTX increased EFS-induced contractions in strips from WT mice (Figure 1A–D), but it did not change the half-width of the force transient (control: 1.98 ± 0.19 s; IBTX: 1.97 ± 0.08 s; $P = 0.98$). In the absence of PLB, block of BK_{Ca} channels caused a significantly greater increase in EFS-induced force amplitude (WT: 149 ± 13%; $PLB^{-/-}$: 194 ± 13%; $P < 0.05$) (Figure 1A and D); here, IBTX also significantly prolonged the force transient, as evidenced by the increased half-width (control: 2.40 ± 0.21 s; IBTX: 3.31 ± 0.13 s; $P < 0.01$). The elevated EFS-induced force transients and effects of BK_{Ca} channel block in $PLB^{-/-}$ strips are consistent with the concept that the loss of PLB increases SR Ca^{2+} content, which upon EFS results in an increase in Ca^{2+} release through RyRs and greater activation of BK_{Ca} channels.

Sympathetic and NANC nerve activation shapes EFS-induced force transients

EFS-induced contractile force transients exhibited two peaks in both WT and $PLB^{-/-}$ strips (Figures 1B and C, 2C and D) (Werner *et al.*, 2008). The first peak occurred at the end of the 2 s stimulation, and decayed to nearly baseline levels within ~5 s. Following the first peak, a second force transient occurred after EFS and peaked within ~12 s of the stimulation start time. These two force transients could reflect simultaneous stimulation of sympathetic and NANC nerves, with the elevation in force reflecting the actions of noradrenaline, which is opposed by the release of NO. To test this hypothesis, we inhibited sGC, the target of NO in smooth muscle, with ODQ (2 μM), and examined the effects on EFS-induced force transients. Block of sGC effectively eliminated the relaxation phase of the force transient (Figure 2C and D). ODQ significantly elevated peak amplitude in CCSM strips from WT mice (Ctrl: 0.15 ± 0.01 mN; ODQ: 0.39 ± 0.04 mN; $P < 0.01$) and increased the duration of the force transient, measured as peak half-width (Ctrl: 1.5 ± 0.2 s; ODQ: 4.0 ± 0.1 s; $P < 0.01$). In the presence of ODQ, inhibiting the BK_{Ca} channels with IBTX did not significantly increase the amplitude of the force transients (ODQ + IBTX: 0.45 ± 0.06 mN; $P = 0.07$) and peak half-width (ODQ + IBTX: 5.0 ± 0.5 s; $P = 0.145$). In strips from $PLB^{-/-}$ mice, inhibition of sGC also increased the force amplitude from 0.4 ± 0.08 mN to 1.3 ± 0.1 mN ($P < 0.01$) and the duration (half-width) from 2.6 ± 0.07 s to 3.7 ± 0.01 s ($P < 0.01$). In contrast to the effects observed in WT strips, blocking BK_{Ca} channels in $PLB^{-/-}$ CCSM strips with IBTX in the presence of ODQ did further increase force amplitude (1.8 ± 0.1 mN; $P < 0.01$) and peak half-width (4.6 ± 0.04 s; $P < 0.01$). The effects of IBTX and ODQ suggest that enhancement of the force transient by BK_{Ca} channel block (as shown in Figure 1) reflects, in part, a reduction in the NO-mediated ability to oppose EFS-induced contraction; that is, BK_{Ca} channels have an important role in the regulation of CCSM contractility.

NO, RyRs and BK_{Ca} channels regulate CCSM contractility

The effects of NO, released in response to NANC nerve stimulation, on BK_{Ca} channels could be mediated through direct phosphorylation of the BK_{Ca} channel and increased open probability (Robertson *et al.*, 1993; Alioua *et al.*, 1998), or through increased RyR-mediated Ca^{2+} release with subsequent activation of juxtaposed BK_{Ca} channels (Robertson *et al.*, 1993; Porter *et al.*, 1998). On the other hand, increased RyR-mediated Ca^{2+} release might also contribute to the total $[Ca^{2+}]_i$, thereby promoting SM contraction. To investigate the role of RyRs in EFS-induced force transients, we applied 20 μM ryanodine to inhibit RyRs in the presence of ODQ and IBTX. Similar to Figures 1 and 2, ODQ and IBTX increased the EFS-induced force transient in WT and $PLB^{-/-}$ strips (Figure 3E and F). In the presence of ODQ (Figure 3A, C and E) as well as in the presence of IBTX (Figure 3D and F), inhibition of RyR in WT and $PLB^{-/-}$ strips raised the baseline to 80% of the maximum EFS-induced peak force (before RyR inhibition). While ryanodine caused a tonic contraction, the maximal force (peak force during EFS) was unchanged, except in WT strips in the presence of IBTX plus ryanodine (Figure 3F). Inhibition of NO signalling by adding ODQ in the presence of IBTX and ryanodine significantly increased the total force in WT and $PLB^{-/-}$ strips (Figure 3C, D and F).

EFS is necessary to change contractions from transient to tonic after inhibition of RyRs

As shown in Figure 3, inhibition of RyRs induced the development of a tonic force. To test whether this effect is mediated by ryanodine alone or by the combination of ryanodine and EFS, we stopped EFS prior to ryanodine application and restarted EFS after incubating for 20 min. Figure 4Aa and Ba shows that, in the absence of EFS, ryanodine had no significant effect on the force. Only after restarting EFS did tonic force develop; this response was prominent in $PLB^{-/-}$ strips (Figure 4Ba) but was near the detection limit in WT strips (Figure 4Aa). However, if WT strips were pre-incubated with either IBTX or ODQ, the tonic force in response to ryanodine was more pronounced (Figure 3A and C). Interestingly, stopping EFS again did not return tonic force back to baseline (data not shown) despite the lack of effects of ryanodine on the force in the absence of EFS (Figure 4Aa and Ba).

Ryanodine also changed the EFS-induced force transients by reducing the relaxation phase between the two peaks (Figure 4Ab). The tonic force produced by $PLB^{-/-}$ CCSM strips in response to ryanodine was greater than the force produced by WT strips (Figure 4Aa, Ba and C); moreover, there was a more pronounced loss of the relaxation phase between the two peaks (Figure 4Bb). The peak force production (i.e. the sum of tonic and transient force amplitudes) was unchanged in WT strips but was increased in $PLB^{-/-}$ strips by ryanodine (Figure 4D). The effects of ryanodine in $PLB^{-/-}$ strips suggest that RyR-mediated Ca^{2+} release is a major component in CCSM to oppose contraction and, if this is inhibited, sympa-

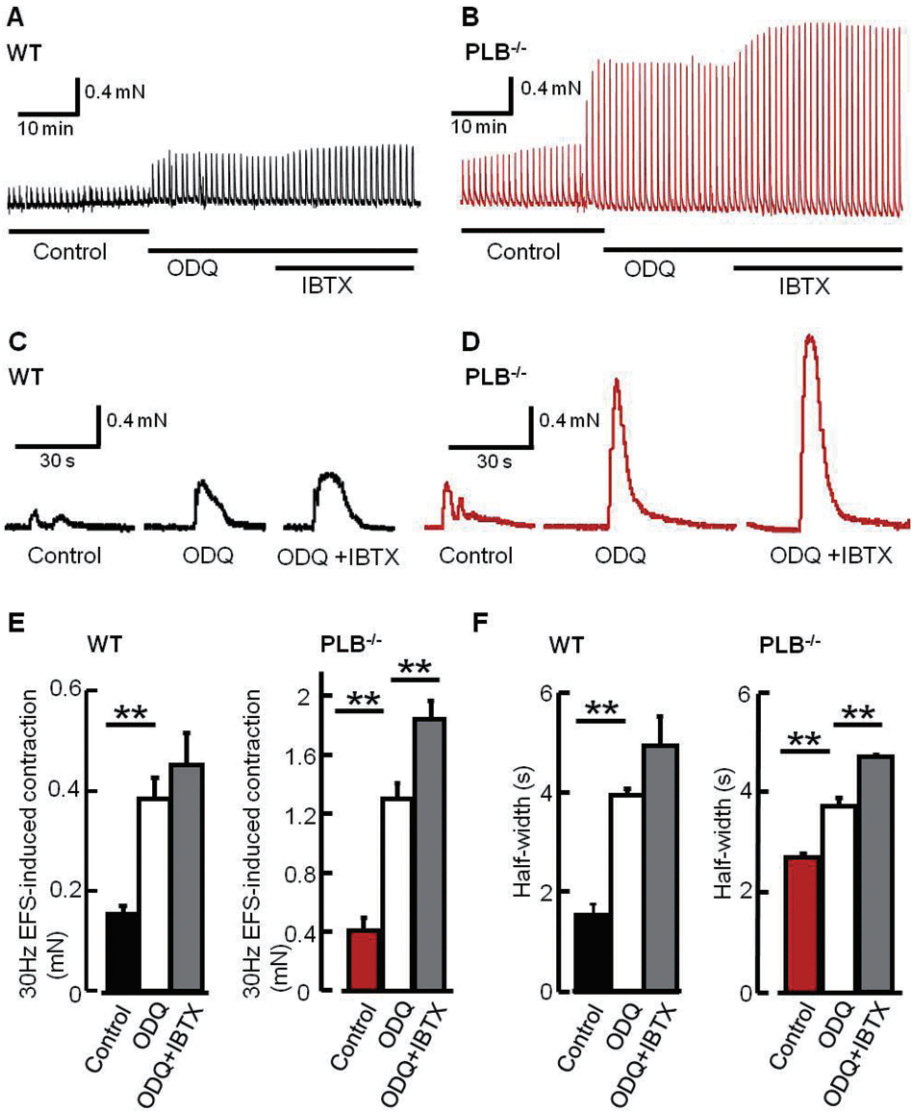

Figure 2

Effects of ODQ and IBTX on EFS-induced contraction in WT and PLB$^{-/-}$ CCSM strips. (A, B) Representative recordings of EFS-induced force transients from a WT and a PLB$^{-/-}$ CCSM strip in response to continuous EFS every 60 s under control conditions, and in the presence of ODQ (2 μM), or ODQ + IBTX (300 nM). (C, D) Force transients of CCSM strip (as above, with a different time scale). (E) Average amplitude of the EFS-induced force transient in WT and PLB$^{-/-}$ CCSM under control conditions, and in the presence of ODQ, or ODQ + IBTX. (F) Average half-width of the EFS-induced force transients in WT and PLB$^{-/-}$ CCSM under control conditions, and in the presence of ODQ, or ODQ + IBTX. $^{**}P < 0.01$, Student's paired *t*-test; $n = 4$.

thetic nerve stimulation dominates and produces a tonic contraction of CCSM. However, these findings reflect signal processing in the PLB$^{-/-}$ context, which does not necessarily resemble the WT physiological situation.

PE-induced contractile force is increased in CCSM strips from PLB$^{-/-}$ mice

The CC is under tonic sympathetic influence, which keeps penile smooth muscle contracted to maintain the flaccid state. To simulate this situation *in vitro*, we induced smooth muscle contraction by applying the α-adrenoceptor agonist PE (10 μM) to the organ bath. PE produced a biphasic contraction of CCSM strips, consisting of an initial peak component followed by a more slowly developing tonic component (Figure 5A). The contraction reached peak strength ~2–5 min after application of PE to the bath, and achieved a steady-state level after ~20 min. PE-induced contraction is thought to be partly dependent on Ca^{2+} release from the SR through inositol 1,4,5-trisphosphate receptors (IP_3Rs) (Christ and Hodges, 2006), and therefore might be influenced by the Ca^{2+} concentration in the SR. Previous work has shown that SR Ca^{2+} load is elevated in SM from PLB$^{-/-}$ mice (Wellman *et al.*, 2001); accordingly, we hypothesized that PE-induced contraction would be greater in CCSM from PLB knockout animals. Indeed, the contractile force measured at peak and steady state was significantly higher in CCSM strips from

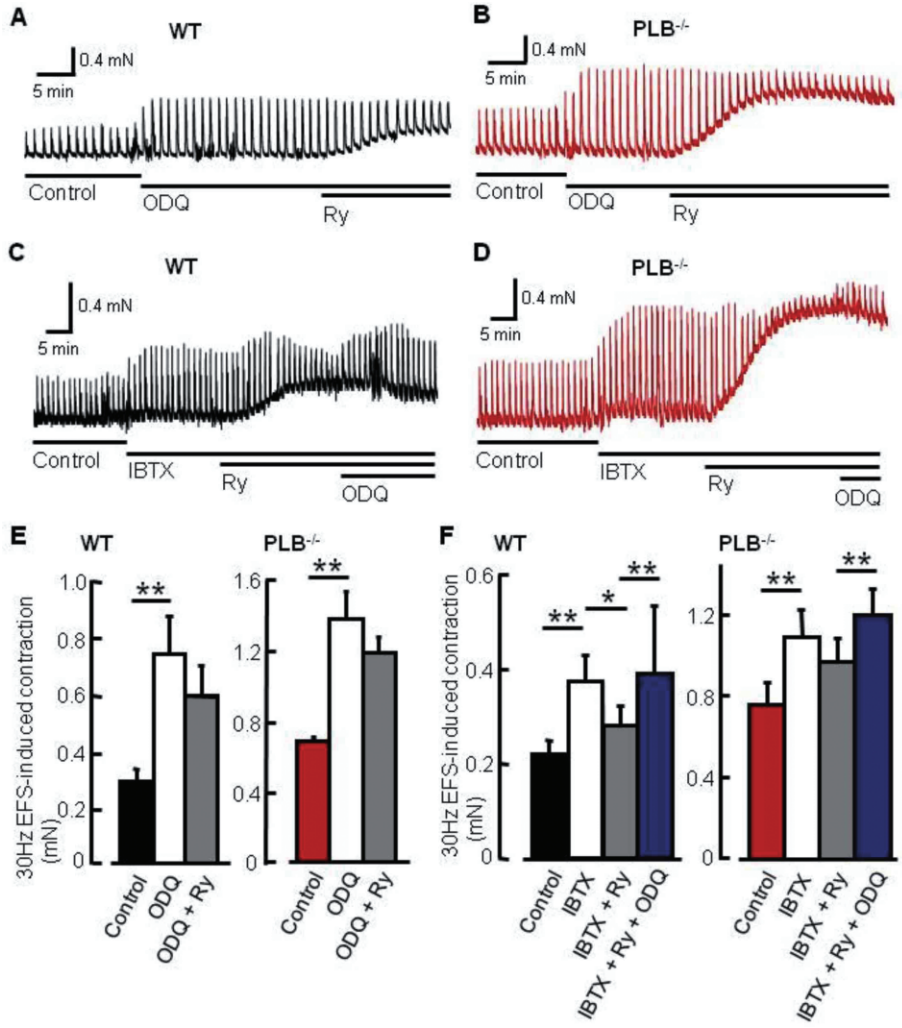

Figure 3

Effects of ODQ, IBTX and ryanodine (Ry) on EFS-induced contraction in WT and PLB$^{-/-}$ CCSM strips. (A, B) Representative recordings of EFS-induced force transients from a WT and a PLB$^{-/-}$ CCSM strip in response to continuous EFS every 60 s under control conditions, and in the presence of ODQ (2 μM), or ODQ + Ry (20 μM). (C, D) Representative recordings of EFS-induced force transients from a WT and a PLB$^{-/-}$ CCSM strip in response to continuous EFS every 60 s under control conditions, and in the presence of IBTX (300 nM), IBTX + Ry (20 μM) + IBTX, or Ry + ODQ (2 μM). (E) Average amplitude of the EFS-induced force transient in WT and PLB$^{-/-}$ CCSM under control conditions, and in the presence of ODQ, or ODQ + Ry. (F) Average amplitude of the EFS-induced force transient in WT and PLB$^{-/-}$ CCSM under control conditions, and in the presence of IBTX, IBTX + Ry, or IBTX + Ry + ODQ. $^{**}P < 0.01$, $^{*}P < 0.05$, Student's paired *t*-test; WT: $n = 6$, PLB$^{-/-}$: $n = 5$.

PLB$^{-/-}$ mice compared with the contractile force in similar tissue from WT mice (Figure 5B).

To test whether RyR-mediated Ca^{2+} release plays a pro- or anti-contractile role in the PE-induced contraction of CCSM, we tested the effects of 20 μM ryanodine on peak and steady-state force in CCSM strips from WT and PLB$^{-/-}$ mice. In WT strips, inhibition of RyR had no effect on peak (control: 0.55 ± 0.06 mN; ryanodine: 0.5 ± 0.06 mN; n = 10) or steady-state (control: 0.51 ± 0.06 mN; ryanodine: 0.45 ± 0.05 mN; n = 10) force. Similarly, in PLB$^{-/-}$ strips, ryanodine did not influence the PE-induced peak (control: 0.74 ± 0.05 mN; ryanodine: 0.66 ± 0.03 mN; n = 7) or the steady-state (control: 0.49 ± 0.03 mN; ryanodine: 0.54 ± 0.03 mN; n = 7) force. These results indicate that contractions induced by an exogenous α-adrenoceptor agonist are not dependent on RyRs.

EFS-induced relaxation of precontracted CCSM strips is enhanced in PLB$^{-/-}$ mice

To examine the effects of NANC nerve activation without the confounding effects of changes in α-adrenoceptor activation, we tested the effects of EFS in CCSM strips precontracted with PE (Andersson and Wagner, 1995; Werner *et al.*, 2005). We have shown previously that inhibition of sGC with ODQ completely abolishes EFS-induced relaxation. Likewise, we have shown that the absence of BK_{Ca} channel function significantly reduces, but does not abolish, EFS-induced relaxation (Werner *et al.*, 2005; 2008). In the present study, EFS (2 s

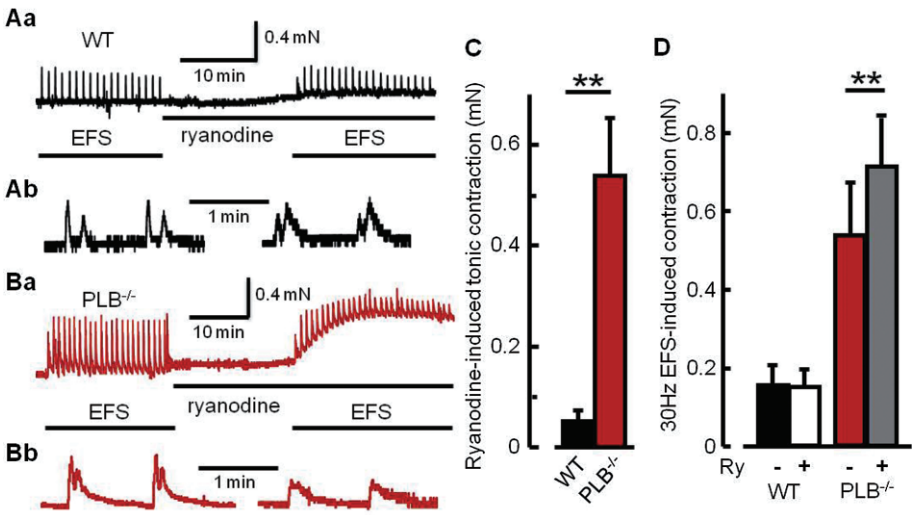

Figure 4

Effect of ryanodine (Ry) on EFS-induced contraction in WT and $PLB^{-/-}$ CCSM strips. (Aa, Ba) Representative recordings of EFS-induced force transients from a WT and a $PLB^{-/-}$ CCSM strip in response to continuous EFS every 60 s under control conditions and after incubation with 20 μM Ry. (Ab, Bb) Force transients of a WT and a $PLB^{-/-}$ CCSM strip (as above, with a different time scale). (C) Average Ry-induced tonic force amplitude. (D) Average Ry-induced total force amplitude (i.e. tonic + transient force amplitude). $^{**}P < 0.01$, Student's paired *t*-test; $n = 4$.

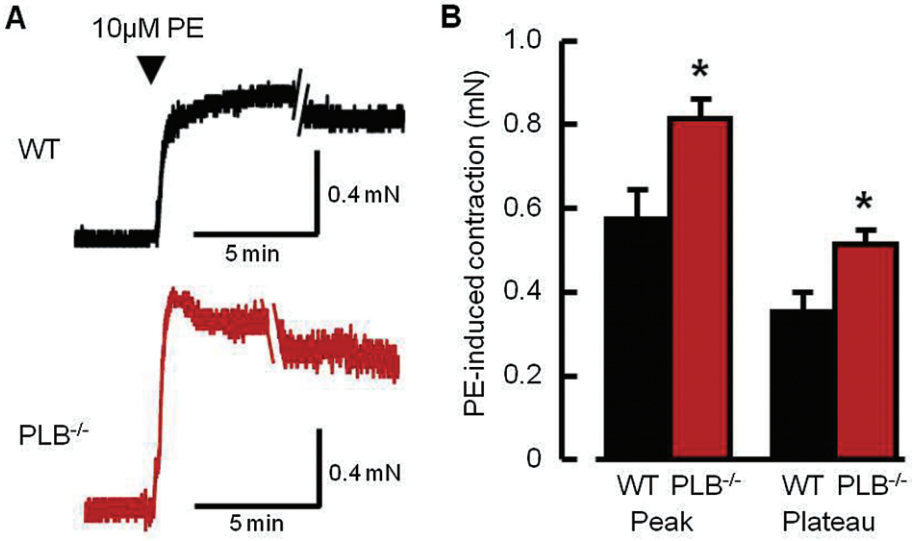

Figure 5

PE-induced contraction of CCSM strips. (A) Original traces showing peak and plateau phase of contraction induced by 10 μM PE. (B) Summary comparing differences in the peak and plateau phases of PE-induced contractile force between WT and $PLB^{-/-}$. $^{*}P < 0.05$ versus $PLB^{-/-}$, Student's *t*-test; $n = 6$.

duration) evoked relaxation of PE-precontracted strips from WT and $PLB^{-/-}$ mice (Figure 6A). Surprisingly, the relaxation was significantly greater in $PLB^{-/-}$ mice (WT: 0.2 ± 0.03 mN; $PLB^{-/-}$: 0.4 ± 0.05 mN; $P < 0.01$). To take into consideration that $PLB^{-/-}$ CCSM strips produce a greater contractile force in response to PE, we normalized the relaxation to the PE-induced force. Even when normalized, the EFS-induced relaxation was still significantly greater in $PLB^{-/-}$ mice; maximum normalized relaxations were 44 ± 3% in WT CCSM strips and 54 ± 3% in $PLB^{-/-}$ strips ($P < 0.05$) (Figure 6B).

This enhanced relaxing effect of nerve stimulation could be due to increased RyR-mediated Ca^{2+} release and concomitant activation of nearby BK_{Ca} channels (Wellman *et al.*, 2001). To test the hypothesis that RyRs are involved in EFS-induced relaxation and are responsible for the enhanced

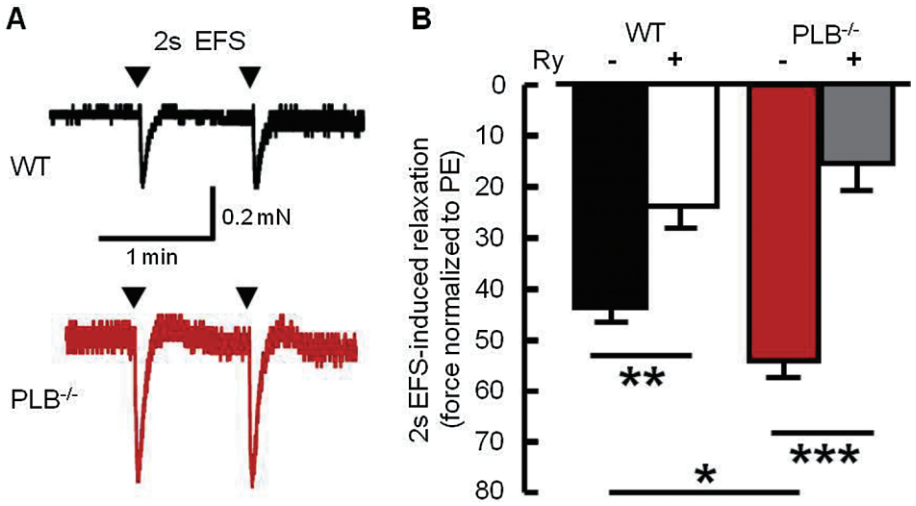

Figure 6

EFS-induced relaxation of PE-precontracted CCSM strips in WT and PLB$^{-/-}$. (A) Original traces showing relaxation induced by 30 Hz EFS for 2 s. (B) Summary comparing mean relaxation amplitudes (normalized to PE response) between CCSM strips from WT and PLB$^{-/-}$ in the absence (n = 10) and presence (n = 4) of ryanodine (Ry). *P < 0.05, **P < 0.01, ***P < 0.001; Student's unpaired t-test.

relaxation potential of PLB$^{-/-}$ CCSM strips, we applied EFS to PE-precontracted strips from WT and PLB$^{-/-}$ mice after blocking RyRs with 20 μM ryanodine. Indeed, in WT CCSM, ryanodine reduced the relaxation by ~45% (from 44 ± 3% to 24 ± 4%; Figure 6B). This effect was significantly greater in PLB$^{-/-}$ CCSM, where ryanodine reduced the relaxation by ~70% (from 54 ± 3% to 16 ± 5%; Figure 6B). Both results indicate that RyRs play an important role in nerve-mediated CCSM relaxation.

SNP-induced relaxation of precontracted CCSM strips is enhanced in PLB$^{-/-}$ mice

Release of NO from NANC nerves and the vascular endothelium causes relaxation of CCSM. The majority of NO effects are mediated through cGMP and PKGI, which can activate BK_{Ca} channels directly through phosphorylation of the channel's α subunit (Robertson *et al.*, 1993; Alioua *et al.*, 1998) or indirectly by increasing Ca^{2+} release (Robertson *et al.*, 1993; Porter *et al.*, 1998). To elucidate the indirect regulation of BK_{Ca} channel activity, we investigated the effect of the NO donor SNP on PE-precontracted CCSM strips from WT and PLB$^{-/-}$ mice. If NO signalling activates BK_{Ca} channels indirectly by increasing Ca^{2+} release from intracellular stores, then this effect should be amplified in the PLB$^{-/-}$ model, where SR Ca^{2+} is elevated.

Application of SNP reduced the contractile response to PE in a concentration-dependent manner in strips from WT and PLB$^{-/-}$ mice (Figure 7A and B). Responses reached a steady state within 5 min, and the highest concentration of SNP (10 μM) reversed the PE-induced contraction by ~90% in WT and ~100% in PLB$^{-/-}$. Figure 7C shows the concentration–response curve for SNP, constructed from the mean data obtained from four to six CCSM strips. Drug concentration was increased in steps after relaxation had appeared to reach a steady level; the measured force value corresponds to the level reached immediately before addition of the next higher drug concentration. The concentration–response curves obtained for each strip were fitted to a Hill equation to calculate the concentration that produced 50% of the maximum response (EC_{50}) (Table 1). The average EC_{50} values in strips obtained from WT mice (202 ± 17 nM) were significantly greater than those in PLB$^{-/-}$ (86 ± 9 nM; P < 0.05) (Table 1), indicating an increased responsiveness in PLB$^{-/-}$ CCSM strips. The relaxing effects of SNP were largely prevented by the sGC inhibitor, ODQ (Figure 7C), consistent with relaxation through the NO/cGMP/PKG pathway. SNP at 100 nM reduced PE-induced force in WT strips by 30.8 ± 2.3%, but produced an even more pronounced reduction in force in PLB$^{-/-}$ strips (51.4 ± 2.8%; P < 0.05).

To further investigate the cGMP-mediated relaxation of CCSM strips, we used the PDE-resistant cGMP analogue 8-pCPT-cGMP to bypass NO and the activation of sGC as well as to avoid possible off-target effects of SNP. Consistent with the effects of SNP on PE-precontracted CCSM strips, the application of 8-pCPT-cGMP (10 nM to 100 μM) reduced contractile force in a concentration-dependent manner (Figure 7D). The EC_{50} for 8-pCPT-cGMP, calculated as above, was 1.4 ± 0.4 μM (n = 4) in WT strips and 0.3 ± 0.06 μM (n = 6) in PLB$^{-/-}$ strips, indicating that 8-pCPT-cGMP was significantly more potent in PLB$^{-/-}$ strips (P < 0.05) (Table 1).

RyRs are important mediators of NO- and cGMP-induced relaxation in CCSM

If the relaxation-promoting effects of SNP were due to an indirect action of PKGI on the BK_{Ca} channel (i.e. through RyR-mediated Ca^{2+} sparks), then inhibiting RyRs should reduce the relaxing effect of SNP. To investigate this possibility, we tested the effect of 20 μM ryanodine on the SNP concentration–response curves. At this concentration, ryanodine has been shown to block RyRs and Ca^{2+} sparks (Wellman *et al.*, 2001). As shown in Figure 7C, ryanodine clearly shifted the concentration–response curves for SNP to the right in

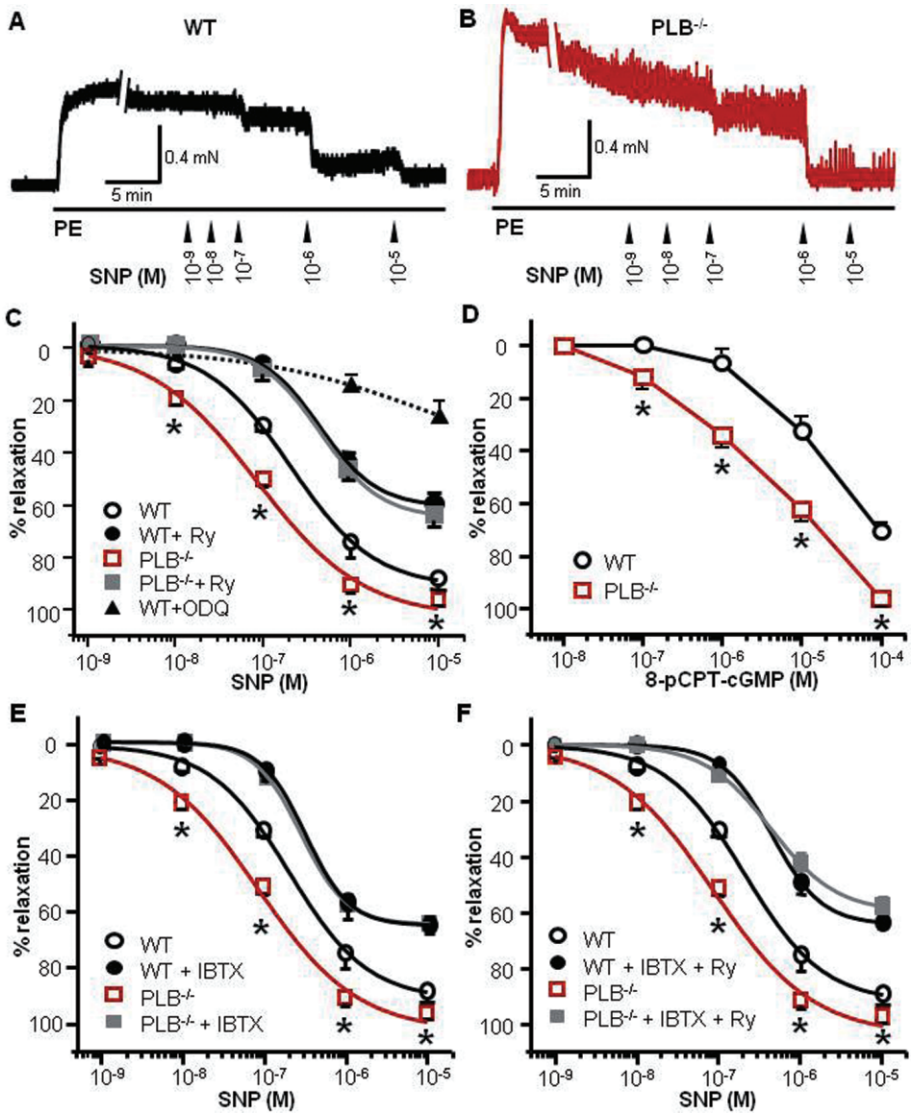

Figure 7

SNP-induced relaxation of PE-precontracted WT and $PLB^{-/-}$ CCSM strips. (A, B) Contraction induced by 10 μM PE followed by relaxation in WT (A) and $PLB^{-/-}$ (B) strips upon cumulative application of SNP at the indicated concentrations. (C) Concentration–response curves for SNP-induced relaxation in WT and $PLB^{-/-}$ strips in the presence and absence of ryanodine (Ry; 20 μM). Dotted line: the mean responses in WT in the presence of ODQ (2 μM). (D) Concentration–response curves for 8-pCPT-cGMP-induced relaxation in WT and $PLB^{-/-}$ strips. Each point represents the mean ± SEM of four to six experiments. (E) Concentration–response curves for SNP-induced relaxation in WT and $PLB^{-/-}$ in the presence and absence of IBTX (300 nM). (F) Concentration strips response curves for SNP-induced relaxation in WT and $PLB^{-/-}$ strips in the presence and absence of IBTX (300 nM) and Ry (20 μM). Each point represents the mean ± SEM of six to nine experiments. *$P < 0.05$, WT versus $PLB^{-/-}$; Student's unpaired *t*-test. The relaxation was expressed as a percentage of PE-induced contraction. For comparison, the same data from WT and $PLB^{-/-}$ strips without drugs are shown in panels (C), (E) and (F).

both WT and $PLB^{-/-}$ strips, and markedly decreased the maximum relaxation responses to SNP. The EC_{50} values for SNP calculated in the presence of ryanodine for WT (518 ± 45 nM) and $PLB^{-/-}$ (472 ± 36 nM) strips were also significantly different compared with control conditions ($P < 0.05$) (Table 1). At 100 nM, SNP reduced PE-induced force by 30.8 ± 2.3% in CCSM strips from WT mice and by 51.4 ± 2.8% in those from $PLB^{-/-}$ mice (see above). Application of ryanodine completely abolished this relaxing effect of 100 nM SNP in both genotypes (Figure 7C). Interestingly, ryanodine also eliminated the increased response to SNP in CCSM from $PLB^{-/-}$ mice, suggesting that this difference was indeed due to increased Ca^{2+} release via RyR.

BK_{Ca} channels are important mediators of SNP-induced relaxation in CCSM

It has been shown previously that the BK_{Ca} channel is an important target of PKGI, and genetic deletion of the pore-forming BK_{Ca} α-subunit or PKGI in mice leads to ED (Hedlund *et al.*, 2000; Werner *et al.*, 2005). If the relaxing effect of SNP

Table 1

EC_{50} values for SNP and 8-pCPT-cGMP in WT and $PLB^{-/-}$ CCSM strips, calculated from the concentration-response curves shown in Figure 7

Drug	EC_{50} WT	EC_{50} $PLB^{-/-}$
SNP	202 ± 17 nM (n = 6)	86 ± 9 nM (n = 6)#
Ryanodine – SNP	518 ± 45 nM (n = 9)†	472 ± 36 nM (n = 4)†, ns
IBTX – SNP	353 ± 46 nM (n = 6)†	279 ± 44 nM (n = 6)†, ns
Ryanodine + IBTX – SNP	462 ± 57 nM (n = 5)†	489 ± 108 nM (n = 5)†, ns
8-pCPT-cGMP	1.4 ± 0.4 μM (n = 4)	0.3 ± 0.06 μM (n = 6)#

#P < 0.05, WT versus $PLB^{-/-}$.
ns, not significant, WT versus $PLB^{-/-}$.
†P < 0.05, compared with SNP alone.

is due to activation of the BK_{Ca} channel, then blocking the channel with IBTX should reduce SNP-induced relaxation of PE-precontracted CCSM strips. Furthermore, if SNP effects on BK_{Ca} channels are mediated by RyRs, then inhibiting RyRs should have the same effect as blocking BK_{Ca} channels, and the effects should not be additive. To address this hypothesis, we performed SNP concentration–response experiments in the presence of IBTX in CCSM strips from WT and $PLB^{-/-}$ mice. Inhibition of BK_{Ca} channels with IBTX clearly inhibited SNP-induced relaxation in CCSM strips, shifting the concentration–response curves for SNP to the right in both WT and $PLB^{-/-}$ strips and markedly decreasing the maximum relaxation responses (Figure 7E), an effect similar to that of ryanodine (see Figure 7C). The calculated EC_{50} values for SNP in the presence of IBTX were 353 ± 46 nM in WT strips and 279 ± 44 nM in $PLB^{-/-}$ strips, both of which were significantly different compared with control conditions (P < 0.05) (Table 1). As shown above, 100 nM SNP reduced PE-induced force in WT strips and even more effectively in $PLB^{-/-}$ strips. IBTX almost completely abolished this relaxing effect of 100 nM SNP (Figure 7E) and also eliminated the difference between the genotypes, strongly suggesting that the increased relaxation-promoting effect of SNP in $PLB^{-/-}$ strips is dependent on BK_{Ca} channels. Pre-incubation of CCSM strips with IBTX and ryanodine together had an effect similar to that of either drug alone (compare Figure 7C and E) and their effects were not additive (Figure 7F and Table 1), indicating that they act on the same signalling pathway.

Discussion

The roles of SR Ca^{2+} and BK_{Ca} channels in CCSM contraction

The first major finding of this work is that eliminating PLB and thereby enhancing calcium re-uptake leads to increased force production, indicating that the level of SR Ca^{2+} is highly important in modulating the contraction response and regulating CCSM tone. Both direct agonist activation of α-adrenoceptors and the use of EFS to induce the release of endogenous noradrenaline from sympathetic nerves produced a larger force in $PLB^{-/-}$ CCSM strips than in WT strips (Figures 1–5). Under resting conditions, EFS induces a transient CCSM contraction through stimulation of sympathetic nerves (Mizusawa *et al.*, 2001; Werner *et al.*, 2008). We have shown previously that eliminating BK_{Ca} function with pharmacological blockers or by gene deletion elevates the amplitude of these EFS-induced force transients (Werner *et al.*, 2008). This effect of BK_{Ca} channel inhibition was significantly greater in $PLB^{-/-}$ CCSM strips (Figure 1), suggesting that in addition to the amplified force production, the increased Ca^{2+} release also leads to greater BK_{Ca} channel activation. This is in agreement with observations by Wellman and colleagues (Wellman *et al.*, 2001), who showed that augmented SR Ca^{2+} in vascular SM from $PLB^{-/-}$ mice was responsible for the increased frequency of Ca^{2+} sparks that led to increased BK_{Ca} channel-mediated spontaneous transient outward currents. α-Adrenoceptor stimulation in response to EFS also causes IP_3R-mediated Ca^{2+} release, which, like RyR-mediated Ca^{2+} release, can activate BK_{Ca} channels (Young *et al.*, 2001); this mechanism could contribute to the observed effects.

The NO/RyR/BK_{Ca} channel pathway mediates CCSM relaxation

The second major finding is that eliminating PLB and thereby enhancing calcium re-uptake leads to elevated NO/cGMP-mediated relaxation. Furthermore, both effects are most likely mediated by enhanced RyR-to-BK_{Ca} channel signalling. Although EFS is used to activate NANC nerves and assess NO-mediated relaxation in PE-precontracted CCSM strips, it is not known if simultaneous activation of NANC nerves opposes EFS-induced, noradrenaline-mediated force transients (Andersson and Wagner, 1995; Werner *et al.*, 2005). Inhibition of sGC significantly augmented EFS-induced force, indicating that NANC nerves are also activated under these conditions (Figure 2). In WT CCSM, blocking BK_{Ca} channels had no additive effect on sGC inhibition, suggesting that under these conditions most NO/cGMP effects that oppose noradrenaline-mediated contractions are indeed mediated by BK_{Ca} channels. However, in $PLB^{-/-}$ CCSM strips, blocking BK_{Ca} channels did further increase force after sGC inhibition. Similarly, without previous inhibition of sGC, the effect of IBTX on EFS-induced force amplitude and duration was more pronounced in $PLB^{-/-}$ CCSM strips (Figure 1). This effect of IBTX in the

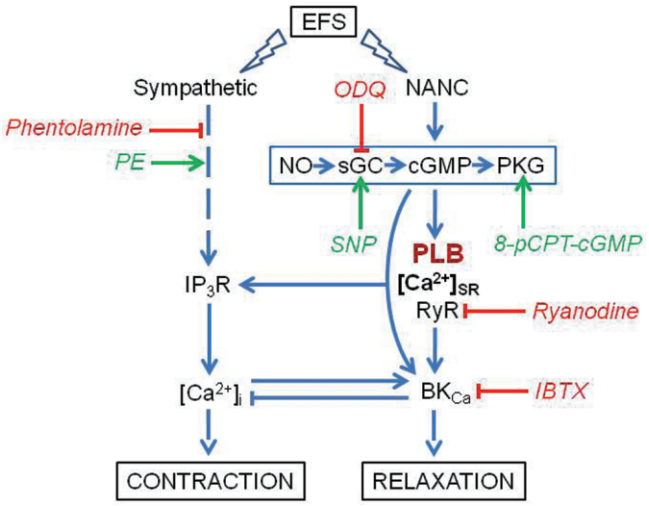

Figure 8

Effects of $PLB^{-/-}$ on CCSM contraction and relaxation. EFS activates both sympathetic and NANC nerve terminals in the CC. The first leads to CCSM contraction through inositol IP_3R activation, the latter leads to CCSM relaxation through NO/sGC/cGMP/PKG signalling. Loss of PLB elevates the SR Ca^{2+} concentration ($[Ca^{2+}]_{SR}$), leading to increased noradrenaline-induced contraction through elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) as well as to increased NO-induced relaxation mediated by (RyR) SR-Ca^{2+} release and BK_{Ca} channels. Antagonists used in this study are shown in red, agonists are shown in green.

absence of PLB is most likely the consequence of increased SR Ca^{2+} load and Ca^{2+} release, and is at least partly independent of the NO/cGMP signalling pathway. In $PLB^{-/-}$, RyR function is essential to return the contractile status of CCSM strips back to baseline after cessation of the electrical stimulus (Figures 3 and 4). If RyRs are inhibited in $PLB^{-/-}$, EFS-induced force is summed to produce a tonic contraction. In WT, however, RyR inhibition is not sufficient to produce a significant tonic force (Figure 4). Only if either NO/cGMP signalling or BK channel function is eliminated before RyR inhibition does EFS-induced force sum to produce a tonic contraction (Figure 3). These results support the idea that SR Ca^{2+} release through RyRs largely functions to oppose contraction by activating nearby BK_{Ca} channels (Figure 8).

Electrical field stimulation in the presence of PE (i.e. in precontracted CCSM strips) is used to investigate NO-dependent relaxation (Andersson and Wagner, 1995; Werner *et al.*, 2005). As demonstrated in Figure 6, relaxation under these conditions is more pronounced in $PLB^{-/-}$ strips. Others have shown that, in rabbit CCSM, ryanodine inhibits up to 50% of EFS-induced relaxation, indicating that this relaxation is at least partly mediated by RyRs (Levin *et al.*, 1997). Here, we showed a similar reduction in CCSM from WT mice. Inhibition of RyRs also reduced the enhanced EFS-induced relaxation in $PLB^{-/-}$ CCSM (up to 70%), thereby eliminating the difference between WT and $PLB^{-/-}$ strips. This result indicates that RyR-mediated Ca^{2+} release is responsible for the greater EFS-induced and NO-dependent relaxation of CCSM strips. The enhanced ability of precontracted $PLB^{-/-}$ strips to relax is further supported by the increased sensitivity to the NO donor SNP and the cGMP analogue 8-pCPT-cGMP (Figure 7). Relaxation of smooth muscle in response to NO/cGMP involves a number of mechanisms that reduce $[Ca^{2+}]_i$ and decrease Ca^{2+} sensitivity (Sausbier *et al.*, 2005). Our results support a mechanism by which EFS-evoked NO release enhances the functional coupling of RyR-mediated Ca^{2+} release to BK_{Ca} channel activation to cause a significant fraction of the total relaxation. The RyR-to-BK_{Ca} channel serial linkage is supported by the relaxation of CCSM to PDE-5 inhibitors, NO donors and cGMP analogues, and by the significant non-additive inhibition of EFS- and SNP-induced relaxation by BK_{Ca} channel block or RyR inhibition (Werner *et al.*, 2005; 2008; Figure 7). This functional coupling is enhanced by the loss of PLB, and thereby increased SR Ca^{2+} load. In WT, PLB is a potential target of PKGI; phosphorylated PLB dissociates from SERCA, leading to elevated SR Ca^{2+} load (Porter *et al.*, 1998). However, in $PLB^{-/-}$ CCSM strips, the enhanced relaxation and RyR-to-BK_{Ca} channel coupling in response to NO/cGMP signalling cannot be due to PLB phosphorylation by PKGI. The BK_{Ca} channel is also an important mediator of NO/cGMP-dependent relaxation, and it has been shown that PKGI increases the open probability of the BK_{Ca} channel (Robertson *et al.*, 1993; Alioua *et al.*, 1998), which would enhance its functional response to calcium release through RyRs. In addition, it is possible that PKGI has a direct effect on RyRs (Figure 8). The combination of increased contractility and improved relaxation ability in the absence of PLB is most likely the reason for the absence of an obvious breeding phenotype in $PLB^{-/-}$ mice.

In conclusion, the present study shows that increased Ca^{2+} concentration in the SR due to the lack of PLB-mediated inhibition of SERCA leads to increased CCSM contractility in response to α-adrenoceptor stimulation (Figure 8), a contractile response that is not dependent on RyRs. More importantly, it shows that RyR-to-BK_{Ca} channel signalling is indeed a central mechanism for opposing contraction in CCSM from both $PLB^{-/-}$ and WT mice. This pathway is further activated through NO/cGMP signalling, and both are amplified under conditions in which SR Ca^{2+} concentration is elevated.

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Conflict of interest

The authors state no conflicts of interest.

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