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Modulation of murine gastric antrum smooth muscle STOC activity and excitability by phospholamban

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We investigated intracellular Ca^{2+} waves, spontaneous transient outward currents (STOCs), and membrane potentials of gastric antrum smooth muscle cells from wild-type and phospholamban-knockout mice. The NO donor sodium nitroprusside (SNP) increased intracellular Ca^{2+} wave activity in wild-type antrum smooth muscle cells, but had no effect on the constitutively elevated intracellular Ca^{2+} wave activity of phospholamban-knockout cells. STOC activity was also constitutively elevated in phospholamban-knockout antrum smooth muscle cells relative to wild-type cells. SNP or 8-bromo-cGMP increased the STOC activity of wild-type antrum smooth muscle cells, but had no effect on STOC activity of phospholamban-knockout cells. Iberiotoxin, but not apamin, inhibited STOC activity in wild-type and phospholamban-knockout antrum smooth muscle cells. In the presence of SNP, STOC activity in wild-type and phospholamban-knockout antrum smooth muscle cells was inhibited by ryanodine, but not 2-APB. The cGMP-dependent protein kinase inhibitor KT5823 reversed the increase in STOC activity evoked by SNP in wild-type antrum smooth muscle cells, but had no effect on STOC activity in phospholamban-knockout cells. The resting membrane potential of phospholamban-knockout antrum smooth muscle cells was hyperpolarized by approximately −6 mV compared to wild-type cells. SNP hyperpolarized the resting membrane potential of wild-type antrum smooth muscle cells to a greater extent than phospholamban-knockout antrum smooth muscles. Despite the hyperpolarized membrane potential, slow wave activity was significantly increased in phospholamban-knockout antrum smooth muscles compared to wild-type smooth muscles. These results suggest that phospholamban is an important component of the mechanisms regulating the electrical properties of gastric antrum smooth muscles.

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The stomach generates myogenic activity in which electrical slow waves generate rings of contraction that spread from the corpus towards the pyloric sphincter (Hirst & Edwards, 2006). Slow waves generated by interstitial cells of Cajal (ICC) conduct into smooth muscle cells and depolarize the membrane, causing a transient contraction due to mechanically productive Ca^{2+} entry via voltage-gated (L-type) Ca^{2+} channels (Costa *et al.* 2005). Removal of Ca^{2+} from the cytoplasm by the plasma membrane Ca^{2+}-ATPase and the sarcoplasmic reticulum (SR) membrane Ca^{2+}-ATPase (SERCA) repolarizes the membrane and relaxes the smooth muscle cells (Sanders, 2008). Refilling of the SR by SERCA also ensures that sufficient Ca^{2+} will be available for regulatory SR Ca^{2+} release in the form of sparks or puffs (Sanders, 2008). These spontaneous release events activate a number of effectors including CaM kinase II and Ca^{2+}-sensitive ion channels, which regulate a variety of smooth muscle contractile responses (Amberg *et al.* 2002; Kim & Perrino, 2007). At physiological temperatures the spatio-temporal recruitment of Ca^{2+} puffs or sparks results in an intracellular Ca^{2+} wave that activates STOCs to hyperpolarize the membrane and reduce muscle excitability (Gordienko *et al.* 1998; Jaggar *et al.* 2000; Hennig *et al.* 2002).

Modulation of SR Ca^{2+} uptake by SERCA is an important regulator of smooth muscle phasic and tonic behaviour (Ishida & Paul, 2005). SERCA activation elevates the SR Ca^{2+} load (Inesi *et al.* 1990), which contributes to relaxation by lowering the Ca^{2+} concentration of the myoplasm $[Ca^{2+}]_c$ (Karaki *et al.* 1997), and increasing Ca^{2+} spark frequency and STOC

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activity (Wellman & Nelson, 2003). Inhibiting SERCA activity with thapsigargin results in diminished Ca^{2+} clearance from the cytosol, and decreases in the amplitudes of slow waves and the associated Ca^{2+} transients and phasic contractions of gastric antrum smooth muscles (Ozaki *et al.* 1992). SERCA activity is endogenously regulated by the SR membrane protein phospholamban (PLB) to vary the rate of Ca^{2+} influx into the SR via its phosphorylation levels, from maximal SERCA inhibition of 50% by dephospho-PLB to no SERCA inhibition by phospho-PLB (Paul *et al.* 2002). Cardiac, skeletal, vascular and bladder smooth muscles from phospholamban-knockout (PLB-KO) mice demonstrate clear differences in contractile properties from the corresponding wild-type muscles (Kadambi & Kranias, 1997).

In smooth muscle cells, NO or NO donors increase cGMP levels, resulting in the activation of several downstream signalling cascades which ultimately result in Ca^{2+} removal from the myoplasm, membrane hyperpolarization and relaxation (Petkov & Boev, 1996; Cohen *et al.* 1999; Yu *et al.* 2003). In gastric motility disorders where enteric neuropathies are implicated, such as impaired gastric accommodation, directly targeting smooth muscle cGMP levels with NO donors or phosphodiesterase inhibitors improves symptoms and gastric smooth muscle function (Tack *et al.* 2002; Sarnelli *et al.* 2004; van den Elzen & Boeckxstaens, 2006). We previously reported that SNP increased cGMP-dependent PLB phosphorylation, and relaxed murine gastric fundus and antrum smooth muscles (Kim *et al.* 2006; Kim & Perrino, 2007). Ryanodine, 2-APB, or cyclopiazonic acid inhibited the relaxation of gastric fundus and antrum smooth muscles (Kim *et al.* 2006). These findings suggest that increased SERCA activity due to PLB phosphorylation leads to increased SR Ca^{2+} release and higher STOC activity resulting in hyperpolarization and relaxation. Similarly, SNP hyperpolarizes and relaxes cerebral and coronary artery smooth muscles by increasing the frequency of Ca^{2+} sparks and STOCs via a NO–cGMP pathway (Porter *et al.* 1998). In support of these findings, we found that the frequency of intracellular Ca^{2+} waves in gastric antrum smooth muscles from PLB-KO mice are constitutively elevated and are unaffected by caffeine (Kim *et al.* 2008). In this study, we extended these findings and investigated the function of PLB in mediating the effects of SNP on intracellular Ca^{2+} waves, STOC activity, and membrane potential of murine gastric antrum smooth muscles using gene-targeted PLB-KO mice. Our results show that PLB is an important determinant of the electrical properties of gastric antrum smooth muscles, and plays a critical role in the hyperpolarization of antrum smooth muscles in response to the NO donor SNP.

Methods

Preparation of gastric antrum smooth muscles

Wild type control mice (SVJ129 × CF1, 6–8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA, USA). PLB-KO mice were purchased from the Mutant Mouse Regional Resource Center (Harlan, MO, USA). Mice were maintained and the experiments carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All protocols were approved by University of Nevada, Reno Institutional Animal Care and Use Committee.

The animals were anaesthetized by isofluorane inhalation and killed by decapitation. The stomach was removed and cut open along the lesser curvature. The stomach contents were flushed with Krebs solution, and the antrum was cut away from the rest of the stomach and pinned out in a Sylgard-lined dish containing oxygenated Krebs solution containing (in mM): 120 NaCl, 6 KCl, 15 $NaHCO_3$, 12 glucose, 3 $MgCl_2$, 1.5 NaH_2PO_4 and 3.5 $CaCl_2$, pH 7.2. The mucosa and submucosa layers were removed using fine-tipped forceps.

Imaging of intracellular Ca^{2+} waves

After the mucosa and submucosa were peeled from antrum of wild-type control and PLB-KO mice, the smooth muscle tissue was pinned with the circular muscle layer facing up in an organ bath containing Krebs solution at $35 \pm 0.5^{\circ}$C bubbling with 97% O_2–3% CO_2 (pH of 7.3–7.4). After a 1 h equilibration, the smooth muscle preparation was incubated with the Ca^{2+} fluorescent dye, fluo-4 AM (5 μM) (cell permeant, special packaging; Molecular Probes, Eugene, OR, USA), 0.02% dimethyl sulfoxide (DMSO), and 0.01% non-cytotoxic detergent cremophor EL (Sigma, St Louis, MO, USA), for 15 min at room temperature (Hennig *et al.* 2002). After the dye incubation, the tissue was perfused with oxygenated Krebs solution (1 μM nicardipine was used to prevent tissue movement and block intercellular Ca^{2+} waves) for 15–20 min at $35 \pm 0.5^{\circ}$C to allow for de-esterification (Hennig *et al.* 2002; Kim *et al.* 2008). Ca^{2+} imaging and image analyses were performed as previously described (Hennig *et al.* 2002; Kim *et al.* 2008).

Cell preparation for patch clamp experiments

Gastric antrum smooth muscle tissues obtained from wild-type and PLB-KO mice (as described above) were washed with Ca^{2+}-free Hanks' solution (pH 7.4) containing (in mM): 125 NaCl, 5.36 KCl, 15.5 NaOH, 0.336 Na_2HPO_4, 0.44 KH_2PO_4, 10 glucose, 2.9 sucrose, and 11

Hepes. The tissues were incubated in 1 ml of Ca^{2+}-free Hanks' solution supplemented with 0.8 mg collagenase (Worthington, Lakewood, NJ, USA), 2 mg bovine serum albumin (BSA), 2.5 mg trypsin inhibitor (Sigma-Aldrich), 0.154 mg L-DTT, and 2 mg papain for 20–25 min at 37°C. Following incubation in the enzyme solution, tissues were washed with Ca^{2+}-free solution and gently agitated to create a cell suspension. Dispersed cells were stored at 4°C in Ca^{2+}-free Hanks' solution.

Patch clamp recordings

Membrane currents were recorded from dissociated gastric antral cells using the amphotericin B-perforated whole-cell patch-clamp technique. Amphotericin B (Sigma) was dissolved in dimethyl sulphoxide (Sigma) as a stock solution (0.08 mg μl^{-1}) and added to the pipette solution (0.5 mg ml^{-1}). Currents were amplified with an Axopatch 200B (Axon Instruments, Union City, CA, USA), recorded using pCLAMP software (version 9.0, Axon Instruments), and digitized on-line at 1 kHz. All experiments were performed at room temperature (22–25°C). STOC amplitudes were measured with Mini Analysis program (Synaptosoft, Leonia, NJ, USA) with a threshold for detection set at 15 pA.

Patch clamp solutions

The bathing solution used in whole cell patch clamp studies contained (mM): 5 KCl, 135 NaCl, 2 $CaCl_2$, 10 glucose, 1.2 $MgCl_2$, and 10 Hepes adjusted to pH 7.4 with Tris. The pipette solution used in whole cell patch clamp experiments contained (mM): 135 KCl, 0.1 EGTA, 10 glucose, 3 MgATP, 0.1 NaGTP, 2.5 creatine phosphate disodium, and 10 Hepes.

SDS-PAGE and Western blot analysis of large conductance Ca^{2+}-activated K^+ channel (BK) expression from gastric antrum smooth muscles from wild-type and PLB-KO mice

Gastric antrum smooth muscles obtained from wild-type CF-1 and PLB-KO mice were frozen in liquid nitrogen and stored at −80°C. Each frozen tissue was homogenized at 4°C with a glass tissue grinder in 0.8 ml lysis buffer (50 mM Mops, 0.2% Nonidet P-40, 100 mM $Na_4P_2O_7$, 100 mM NaF, 250 mM NaCl, 3 mM EGTA, 1 mM dithiothreitol, 1 mM phenyl-methylsulphonyl fluoride, and protease inhibitor tablet), and centrifuged at 1000 *g* for 15 min at 4°C, and the supernatant aliquotted and stored at −80°C. Protein concentrations were determined with the Bradford assay using bovine γ-globulin as standard. Smooth muscle lysate proteins were separated by SDS-PAGE (12% gel) and transferred to nitrocellulose by Western blotting. The blots were incubated with the primary and secondary antibodies, washed, and processed for enhanced chemiluminescence image detection using ECL Advantage (Amersham Biosciences, Piscataway, NJ, USA). The BK α subunit and β subunit antibodies (Santa Cruz Biotechnology, Santa Cruz, CA USA, and Affinity Bioreagents, Golden, CO, USA, respectively) and the smooth muscle α-actin antibody (Sigma) were used at 1 : 5000 dilutions. The horseradish-peroxidase-conjugated secondary antibodies (Chemicon, Temecula, CA, USA) were used at 1 : 50 000 dilutions. Protein bands were visualized with a CCD camera based detection system (Epi Chem II, UVP Laboratory Products), and the collected TIFF images analysed using Adobe Photoshop. Densitometry analysis of the Western blot TIFF images was performed using Un-Scan-It software (Silk Scientific, Orem, UT, USA). The average pixel intensity values (minus background) of the BK α subunit and β subunit protein bands from wild-type antrum smooth muscle lysates were each normalized to 1. BK α subunit and β subunit protein expression in PLB-KO antrum smooth muscle lysates are each reported relative to the normalized values from the wild-type antrum smooth muscles.

Intracellular microelectrode recording

Gastric antrum smooth muscles obtained from wild-type and PLB-KO mice were pinned to a Sylgard (Dow Corning Corp., Midland, MI, USA) coated recording chamber with the circular muscle layer facing upward and maintained at 37.5 ± 0.5°C by a flowing Krebs solution bubbled with 97% O_2–3% CO_2. Tissues were equilibrated for 1 h before recordings were begun. Circular smooth muscle cells were impaled with glass microelectrodes filled with 3 M KCl having resistances between 50 and 80 MΩ. Transmembrane potentials were measured with a high input impedance amplifier (WPI S-7071, Sarasota, FL, USA). Electrical signals were recorded with a PC-style computer running AxoScope 9.2 data acquisition software (Axon Instruments) and hard copies printed using Clampfit analysis software (Axon Instruments). All experiments were performed in the presence of nifedipine (1 μM) to reduce movement and facilitate impalements of cells for extended periods of time.

Materials

The following drugs were used: nicardipine, tetrodotoxin, ryanodine, 2-APB, L-DTT, papain, BSA, iberiotoxin (IbTx), apamin, and trypsin inhibitor were obtained from Sigma. SNP, 8-bromo-cGMP was obtained from EMD Bioscience (La Jolla, CA, USA). Cyclopiazonic acid (CPA), KT5823, and KN-93 were purchased from Biomol

(Plymouth Meeting, PUSA). mini-EDTA free protease inhibitor pills were obtained from Roche Applied Science (Indianapolis, IN, USA). All other chemicals and materials were of reagent grade.

Statistical analysis

Data are expressed as means ± S.D. Data sets were tested for significance using ANOVA with Tukey's *post hoc* tests to analyse multiple groups. Data are considered significantly different from control values when $P < 0.05$.

Results

Effect of SNP on intracellular Ca^{2+} wave activity in gastric antrum smooth muscle cells from wild type and PLB-KO mice

We previously reported that SNP increases PLB phosphorylation, suggesting a mechanism by which SR Ca^{2+} release and intracellular Ca^{2+} waves can be increased due to increased SERCA activity (Kim & Perrino, 2007). Since intracellular Ca^{2+} wave frequency is higher in PLB-KO antrum smooth muscle cells, we examined whether SNP would increase intracellular Ca^{2+} wave frequency in wild-type antrum smooth muscle cells. Intracellular Ca^{2+} waves were recorded in 150 μm × 150 μm regions of interest (ROIs) of intact antrum circular smooth muscles before and after SNP treatment to generate spatiotemporal wave maps as described (Hennig *et al.* 2002; Kim *et al.* 2008). As we previously reported, the amplitudes, but not the propagation velocities, of intracellular Ca^{2+} waves were decreased by the second exposure to the fluo-4 AM excitation light, which serves as the time-matched control for experiments performed in the presence of SNP (Kim *et al.* 2008). SNP did not affect the reduction in amplitudes caused by the second fluo-4 AM excitation in both wild-type and PLB-KO antrum smooth muscles. The average amplitudes of intracellular Ca^{2+} waves recorded from time-matched untreated and SNP-treated wild-type antrum smooth muscles were 80 ± 11% and 81 ± 8%, respectively, of the amplitudes recorded from the first exposures ($n = 6$). Similarly, the average amplitudes of intracellular Ca^{2+} waves in time-matched untreated and SNP-treated PLB-KO antrum smooth muscle cells were 86 ± 9% and 84 ± 11%, respectively, of the amplitudes recorded from the first exposures ($n = 6$). SNP also did not affect the propagation velocities of intracellular Ca^{2+} waves in wild-type and PLB-KO antrum smooth muscle cells. In addition, as we previously found, the average propagation velocity of intracellular Ca^{2+} waves was higher in PLB-KO antrum smooth muscle cells than in wild-type cells. The average propagation velocities of intracellular Ca^{2+} waves recorded from time-matched untreated and SNP-treated wild-type antrum smooth muscles were $51 \pm 5\ \mu m\ s^{-1}$ and $59 \pm 7\ \mu m\ s^{-1}$, respectively ($n = 6$). The average propagation velocities of intracellular Ca^{2+} waves recorded from time-matched untreated and SNP-treated PLB-KO antrum smooth muscles were $75 \pm 7\ \mu m\ s^{-1}$ and $73 \pm 8\ \mu m\ s^{-1}$, respectively ($n = 6$).

Figure 1*A* and *B* shows that, as we previously reported, the frequencies of intracellular Ca^{2+} waves recorded from both wild-type and PLB-KO antrum smooth muscle cells during the second exposure to high-intensity fluo-4 AM excitation light, which serves as the time-matched control for experiments performed in the presence of SNP, were 70 ± 9% and 73 ± 10%, respectively, of the first response ($n = 6$) (Kim *et al.* 2008). However, the frequencies of intracellular Ca^{2+} waves in PLB-KO antrum smooth muscles recorded during both the first and second exposures were higher than the wild-type controls. The frequencies of intracellular Ca^{2+} waves recorded from wild-type antrum smooth muscle cells during the first and second exposures were 49 ± 5 waves min^{-1}, and 35 ± 3 waves min^{-1}, respectively. The frequencies of intracellular Ca^{2+} waves recorded from PLB-KO antrum smooth muscle cells during the first and second exposures were 78 ± 6 waves min^{-1}, and 57 ± 6 waves min^{-1}, respectively.

Incubation of wild-type antrum smooth muscle strips with SNP increased the frequency of intracellular Ca^{2+} waves (Fig. 1*A*). The average frequency of 35 ± 3 waves min^{-1} in the time-matched untreated controls was increased to 52 ± 4 waves min^{-1} in SNP-treated antrum smooth muscle cells ($n = 6$, $P < 0.05$). In contrast, SNP did not increase the frequency of intracellular Ca^{2+} waves in PLB-KO antrum smooth muscle cells (57 ± 6 waves min^{-1} in time-matched control *versus* 59 ± 3 waves min^{-1} with SNP ($n = 6$) (Fig. 1*B*). The frequency of 52 ± 4 waves min^{-1} in SNP-treated wild-type antrum smooth muscle cells is similar to the frequencies of 57 ± 6 waves min^{-1} and 59 ± 3 waves min^{-1} in untreated, and SNP-treated PLB-KO antrum smooth muscle cells, respectively (Fig. 1*C*).

STOC activities in antrum circular smooth muscle cells from wild type and PLB-KO mice

Since increased SR Ca^{2+} release and intracellular Ca^{2+} wave frequencies are associated with increased STOC activity (Gordienko *et al.* 1998), we measured STOCs in freshly isolated antrum smooth muscle cells from wild-type and PLB-KO mice using the perforated-patch whole cell configuration of the patch-clamp technique. As shown in Fig. 2*A*, the frequencies and amplitudes of STOCs were higher in PLB-KO antrum smooth muscle cells than in wild-type cells. The average amplitude of

STOCs from PLB-KO antrum smooth muscle cells was 195 ± 51 pA compared to 101 ± 26 pA from wild-type smooth muscle cells (-10 mV, $n = 20$, $P < 0.05$). The average frequency of STOCs in PLB-KO antrum smooth muscle cells was 199 ± 40 events min^{-1} compared to 64 ± 18 events min^{-1} in wild-type cells (-10 mV, $n = 20$, $P < 0.05$). To determine whether STOCs are from large conductance (BK), or small conductance Ca^{2+}-activated K^+ channels (SK), cells were incubated with the BK channel blocker IbTx or the SK channel blocker apamin. As shown in Fig. 2*B*, 100 nM IbTx decreased the amplitudes and frequency of STOCs by $84 \pm 10\%$ and $81 \pm 9\%$, respectively ($P < 0.01$). However, apamin (300 nM) did not affect STOC activity, suggesting that STOCs are mostly from BK channels in antrum smooth muscle cells. Similar results were obtained with antrum smooth muscle cells from PLB-KO mice (data not shown). The Western blot in Fig. 2*C* shows that the protein expression levels of the BK channel α and β subunits in wild-type and PLB-KO antrum smooth muscles are similar. PLB-KO : wild-type BKα and BKβ subunit ratios of 1.2 ± 0.4 and 0.88 ± 0.14 ($P > 0.05$), respectively, were calculated from the densitometric analyses of the immunostained protein bands. These findings suggests

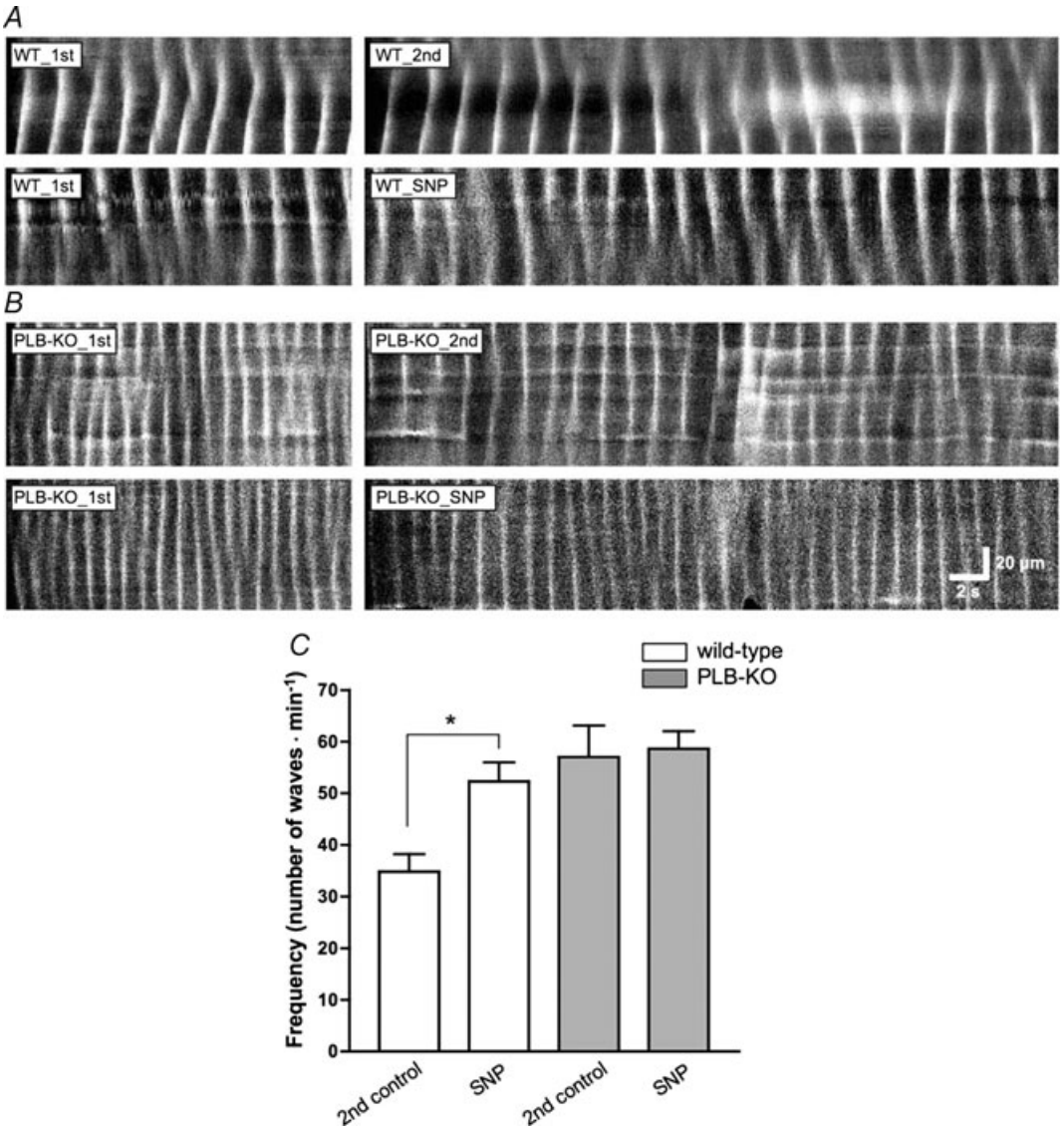

Figure 1. SNP increases intracellular Ca^{2+} wave activity in wild-type but not in PLB-KO gastric antrum smooth muscles

A and *B*, representative spatio-temporal maps of intracellular Ca^{2+} waves constructed from wild-type CF-1 (*A*) and PLB-KO (*B*) time-matched controls and SNP-treated (10 μM) antrum smooth muscles. *C*, summary graph showing the effect of SNP (10 μM) on the frequencies of intracellular Ca^{2+} waves in antrum smooth muscle cells from wild-type CF-1 mice and PLB-KO mice. $^*P < 0.05$.

that the increased STOC activity measured in PLB-KO antrum smooth muscle cells is not due to altered BK subunit composition or expression levels.

Effect of SNP on STOC activity in gastric antrum smooth muscle cells from wild-type control and PLB-KO mice

PLB phosphorylation by SNP, or PLB-KO elevates SR Ca^{2+} uptake and enhances regulatory SR Ca^{2+} release (Kim *et al.* 2006, 2008; Kim & Perrino, 2007). Since relaxation due to increased localized SR Ca^{2+} release is coupled to increased STOC activity, we measured the effect of SNP on STOC activity in wild-type and PLB-KO antrum smooth muscle cells. As shown in Fig. 3*A*, SNP increased the average STOC frequency and amplitude twofold in wild-type antrum smooth muscle cells. The average STOC amplitude of 101 ± 26 pA increased to 217 ± 54 pA in the presence of SNP (-10 mV, $n = 20$, $P < 0.05$). The average STOC frequency of 64 ± 18 events min^{-1} increased to 136 ± 41 events min^{-1} in the presence of SNP (-10 mV, $n = 20$, $P < 0.05$). In contrast, SNP did not significantly affect STOC activity in PLB-KO antrum smooth muscle cells (Fig. 3*B*). Average STOC amplitudes

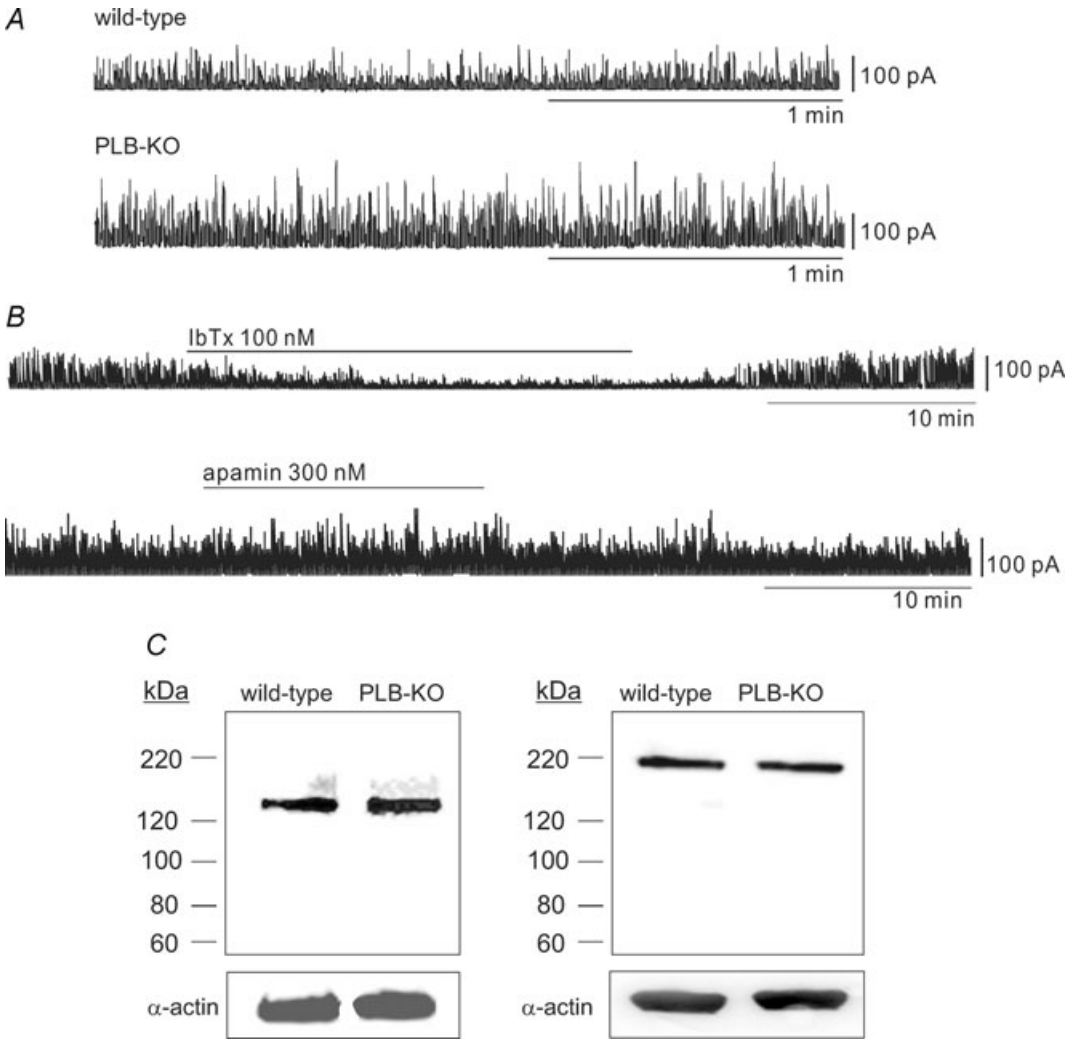

Figure 2. STOC activity and BK channel expression in wild-type CF-1 and PLB-KO gastric antrum smooth muscle cells
Similar cell capacitance values of 41.3 ± 4 pF and 41.1 ± 5 pF were measured from wild-type and PLB-KO antrum smooth muscle cells, respectively ($n = 20$). *A*, representative traces of STOC activity from wild-type and PLB-KO antrum smooth muscle cells ($n = 20$). *B*, representative traces of STOC activity in wild-type antrum smooth muscle cells in the presence of 100 nM IbTx or 300 nM apamin. *C*, representative immunoblots (of 4 separate smooth muscle homo-genates) of BKα (left panel) and BKβ (right panel) subunits from wild-type CF-1 and PLB-KO antrum smooth muscle homogenates. Each lane contains 25 μg of protein. The lower panels are smooth muscle α-actin immunoblots as an additional control for equal protein loading ($n = 4$).

of 195 ± 51 pA and 224 ± 67 pA were measured from untreated and SNP-treated PLB-KO smooth muscle cells, respectively ($n = 20$). Average STOC frequencies of 199 ± 40 events min^{-1} and 210 ± 29 events min^{-1} were measured from untreated and SNP-treated smooth muscle cells, respectively ($n = 20$). These findings are summarized in Fig. 3*C*.

Effect of SR Ca^{2+} channel blockers on STOC activity in gastric antrum smooth muscle cells from wild type control and PLB-KO mice

The previous findings suggest that STOC activity is due to BK channel activation by the spatio-temporal recruitment of SR Ca^{2+} release events in the form of sparks or puffs to form intracellular Ca^{2+} waves. To investigate the source of activating Ca^{2+}, we examined the effects of the IP_3 receptor inhibitor 2-APB and the ryanodine receptor inhibitor ryanodine on STOC activity. As shown in Fig. 4*A*, 2-APB had no effect on the stimulation of STOC activity by SNP in antrum smooth muscle cells from wild-type mice.

However, ryanodine significantly inhibited the stimulatory effect of SNP on STOC activity (Fig. 4*B*). The average amplitude and frequency of STOCs were decreased $70 \pm 11\%$, and $75 \pm 15\%$ by 10 μM ryanodine, respectively ($n = 6$, $P < 0.05$). Ryanodine, but not 2-APB, also inhibited the STOC activity of antrum smooth muscle cells from PLB-KO mice (data not shown). In murine proximal colon smooth muscle cells, STOC activity is coupled to IP_3 receptor-mediated Ca^{2+} release (Kong *et al.* 2000). Figure 4*C* shows that, unlike the results obtained with murine gastric antrum (Fig. 4*A*), 30 μM 2-APB inhibited STOC activity in proximal colon smooth muscle cells. The average STOC amplitude was decreased by 43%, from 76 ± 11 pA to 32 ± 5 pA in the presence of 2-APB (-10 mV, $n = 3$, $P < 0.05$). In addition, the average STOC frequency was decreased by 47%, from 40 ± 7 events min^{-1} to 19 ± 4 events min^{-1} (-10 mV, $n = 3$, $P < 0.05$). These findings suggest that Ca^{2+} release from ryanodine receptors (sparks) is the major source of STOC activity evoked by SNP in murine gastric antrum smooth muscle cells.

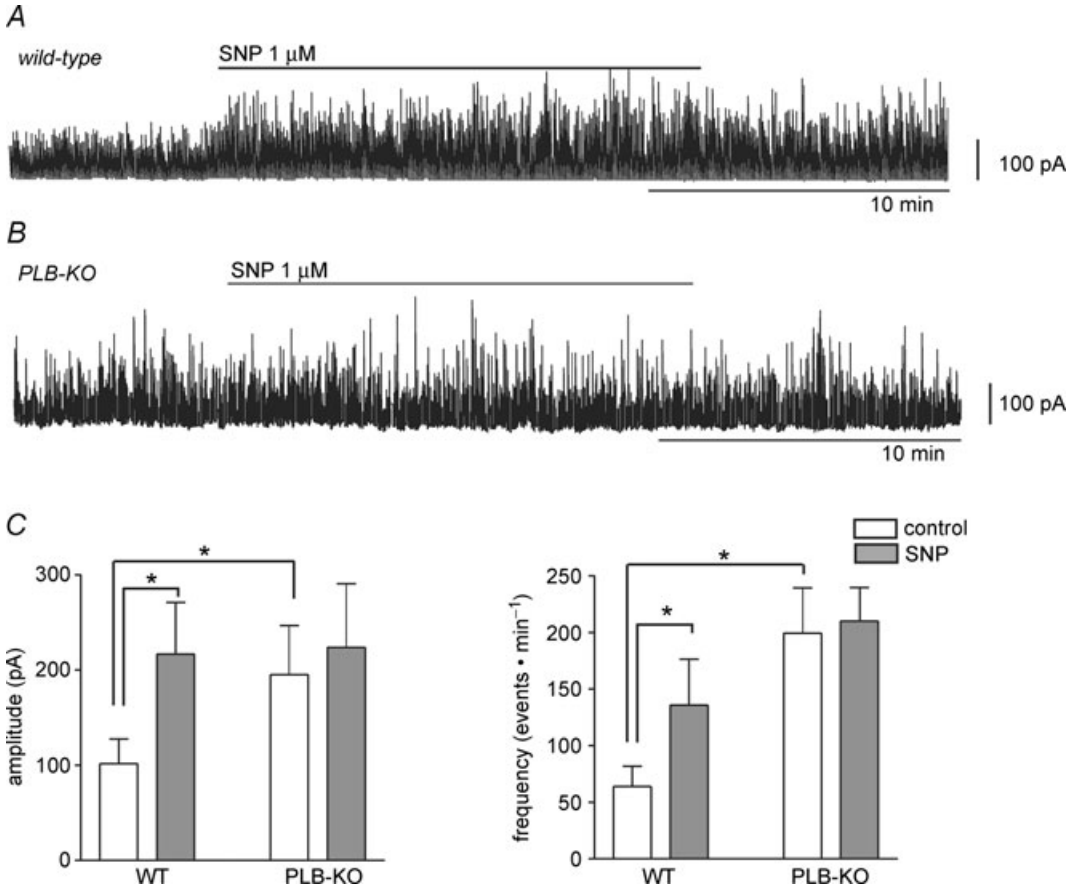

Figure 3. SNP increases STOC activity in wild-type but not in PLB-KO gastric antrum smooth muscle cells
A and *B*, representative traces of STOC activity from CF-1 wild-type (*A*) and PLB-KO (*B*) antrum smooth muscle cells in the absence or presence of 1 μM SNP ($n = 20$). *C*, average values of STOC amplitudes and frequencies from wild-type and PLB-KO antrum smooth muscle cells in the absence or presence of 1 μM SNP. WT, wild-type. $^*P < 0.05$

Effect of 8-bromo-cGMP (8-Br-cGMP) on STOC activity in gastric antrum smooth muscle cells from wild type and PLB-KO mice

Since most effects of SNP in smooth muscle cells are mediated by an increase in cGMP levels, the effect of the membrane permeant cGMP analogue 8-Br-cGMP on STOC activity was examined (Fig. 5). Similar to SNP, 8-Br-cGMP increased the average STOC frequency and amplitude by twofold in wild-type antrum smooth muscle cells (Fig. 5*A*). In these experiments, the average STOC amplitude of 125 ± 26 pA increased to 224 ± 50 pA in the presence of 8-Br-cGMP (-10 mV, $n = 5$, $P < 0.05$). The average

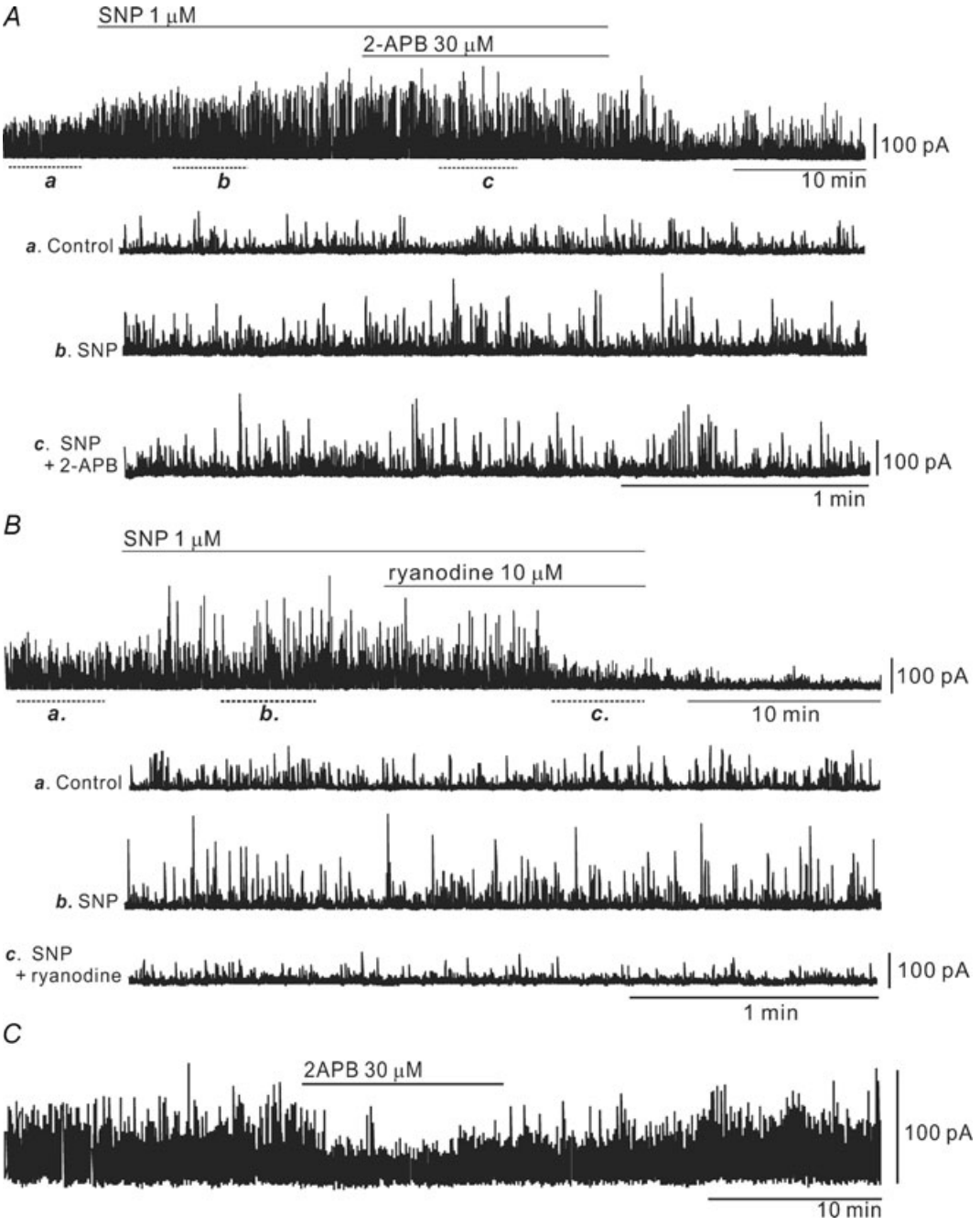

Figure 4. Ryanodine but not 2-APB blocks the SNP-evoked increase in STOC activities of wild-type gastric antrum smooth muscle cells
A and *B*, representative traces of STOC activity from wild-type antrum smooth muscle cells treated with 1 μM SNP in the absence or presence of 30 μM 2-APB (*A*) or 10 μM ryanodine (*B*) ($n = 6$). *C*, representative trace of STOC activity from wild-type proximal colon smooth muscle cells in the absence or presence of 30 μM 2-APB ($n = 3$).

STOC frequency increased from 56 ± 14 events min^{-1} to 122 ± 31 events min^{-1} in the presence of 8-Br-cGMP (-10 mV, $n = 5$, $P < 0.05$). In contrast, 8-Br-cGMP did not significantly affect STOC activity in PLB-KO antrum smooth muscle cells (Fig. 5*B*). Average STOC amplitudes of 210 ± 46 pA and 226 ± 40 pA were measured from untreated and 8-Br-cGMP-treated smooth muscle cells, respectively ($n = 5$). Average STOC frequencies of 195 ± 47 events min^{-1} and 206 ± 51 events min^{-1} were measured from untreated and 8-Br-cGMP-treated smooth muscle cells, respectively (Fig. 5*C*) ($n = 5$).

Effect of KT5823 on STOC activity in gastric antrum smooth muscle cells from wild type and PLB-KO mice

We previously reported that the guanylyl cyclase inhibitor [1,2,4]oxadizolo-[4,3α]quinoxaline-1-one (ODQ) inhibits SNP-induced PLB Ser16 phosphorylation and gastric smooth muscle relaxation, suggesting that SNP elevates cGMP levels and activates cGMP-dependent protein kinase (Kim *et al.* 2006; Kim & Perrino, 2007). Since the findings in Figs 1 and 5 show that SNP and 8-Br-cGMP increase STOC activity, we examined the effect of the cGMP-dependent protein kinase inhibitor KT5823 on STOC activity in the presence of SNP in wild-type and PLB-KO antrum smooth muscle cells. As shown in Fig. 6*A*, KT5823 reversed the increase in STOC activity evoked by SNP in wild-type antrum smooth muscles. Average STOC frequencies of 48 ± 12, 115 ± 30 and 77 ± 14 events min^{-1} were measured from untreated, SNP-treated and SNP plus KT5823-treated wild-type smooth muscle cells, respectively. Average STOC amplitudes in untreated, SNP-treated and SNP plus KT5823-treated wild-type smooth muscle cells were 84 ± 13, 183 ± 49 and 79 ± 18 pA, respectively. In contrast, STOC activity in PLB-KO antrum smooth muscle cells was unaffected by SNP or KT5823 (Fig. 6*B*). Average STOC frequencies of 178 ± 54, 198 ± 64 and 175 ± 45 events min^{-1} were measured from untreated, SNP-treated and SNP plus KT5823-treated PLB-KO smooth muscle cells, respectively ($n = 6$). Average STOC amplitudes in untreated, SNP-treated and SNP plus KT5823-treated PLB-KO smooth muscle cells were 180 ± 44, 201 ± 48 and 168 ± 54 pA, respectively (Fig. 6*C*) ($n = 6$).

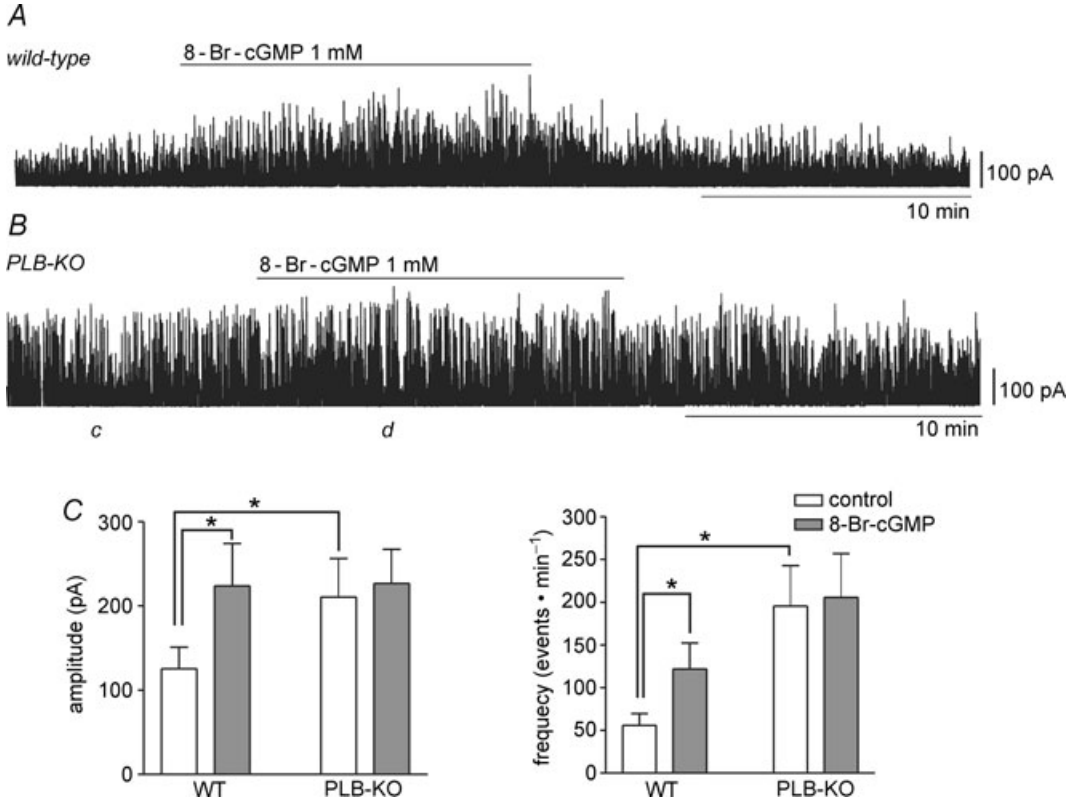

Figure 5. 8-Bromo-cGMP increases STOC activity in wild-type but not in PLB-KO gastric antrum smooth muscle cells
A and *B*, representative traces of STOC activity from CF-1 wild-type (*A*) and PLB-KO (*B*) antrum smooth muscle cells in the absence or presence of 1 mM 8-Br-cGMP. C. Average values of STOC amplitudes and frequencies from wild-type and PLB-KO antrum smooth muscle cells in the absence or presence of 1 mM 8-Br-cGMP. WT, wild-type. $^*P < 0.05$.

Effect of SNP on resting membrane potential of gastric antrum smooth muscles from wild-type and PLB-KO mice

The above findings strongly implicate PLB in the modulation of STOC activity and suggest that PLB affects the resting membrane potential (RMP) and excitability of antrum smooth muscles. Thus, we compared the RMP of wild-type and PLB-KO antrum smooth muscle cells in the absence or presence of SNP. As shown in Fig. 7*A*, SNP abolished slow waves in both wild-type and PLB-KO antrum smooth muscles. However, in the absence of SNP, the RMP of PLB-KO antrum smooth muscle cells is hyper-polarized compared to wild-type cells. An average RMP of -54 ± 2 mV was measured in wild-type cells compared to -60 ± 3 mV in PLB-KO antrum smooth muscle cells ($n = 6$, $P < 0.05$). The hyperpolarized membrane potential of the PLB-KO smooth muscle cells was associated with an altered electrical response to SNP. The magnitude of the SNP-induced hyperpolarization of PLB-KO smooth muscle cells was 50% of the hyperpolarization evoked in wild-type cells ($n = 6$, $P < 0.05$). As shown in Fig. 7*B*, 10 μM SNP hyperpolarized the membrane potential by -10 ± 2 mV in wild-type antrum smooth muscle cells and by -5 ± 2 mV in PLB-KO cells. The BK channel inhibitor IbTx (100 nM) inhibited SNP-induced hyperpolarization by $56 \pm 3\%$ ($n = 4$, $P < 0.05$) in wild-type antrum smooth muscles. Similar results were obtained with antrum smooth muscle cells from PLB-KO mice.

Slow wave frequency and amplitude are increased in PLB-KO gastric antrum smooth muscle cells

Figure 8*A* shows that the amplitudes and frequencies of slow waves were both increased in PLB-KO antrum smooth muscle cells compared to wild-type cells, despite the hyperpolarized RMP of cells from PLB-KO mice (Fig. 8*B*). The average amplitude of slow waves was 29 ± 6 mV in cells from PLB-KO mice and 20 ± 3 mV in wild-type antrum smooth muscle cells ($n = 6$, $P < 0.05$). The average frequency of slow waves was 7 ± 0.7 cycles min^{-1} in cells from PLB-KO mice and 4 ± 0.8 cycles min^{-1} in wild-type antrum smooth muscle cells ($n = 6$, $P < 0.05$) (Fig. 8*B*).

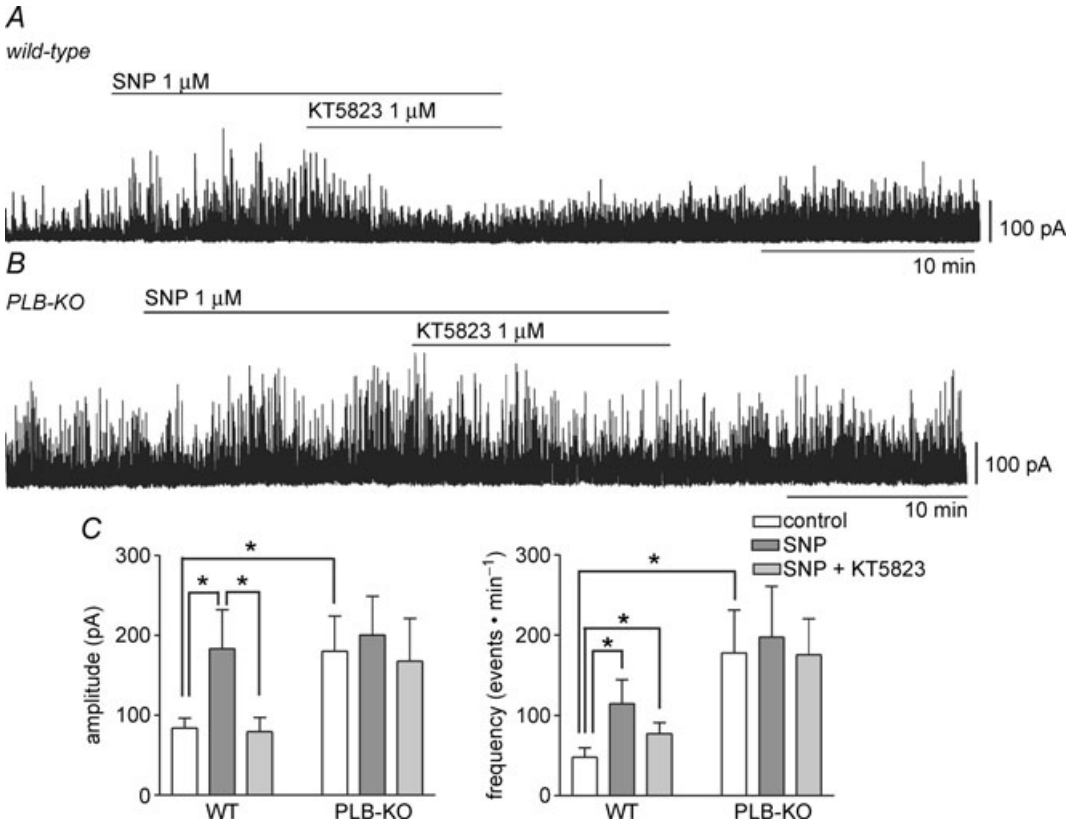

Figure 6. KT5823 reverses the SNP-evoked increase in STOC activities in wild-type but not in PLB-KO gastric antrum smooth muscle cells
A and *B*, representative traces of STOC activity from wild-type (*A*) or PLB-KO (*B*) antrum smooth muscle cells treated with 1 μM SNP in the absence or presence of 1 μM KT5823. C. Average values of STOC amplitudes and frequencies from wild-type and PLB-KO antrum smooth muscle cells treated with 1 μM SNP in the absence or presence of 1 μM KT5823. WT, wild-type ($n = 6$). *$P < 0.05$.

Discussion

It is well established that cardiac, skeletal, vascular, gastric antrum and bladder smooth muscles from PLB-gene targeted mice demonstrate clear differences in Ca^{2+} homeostasis and contractile properties compared to the corresponding wild-type muscles (Kadambi & Kranias, 1997; Kim *et al.* 2008). In this study we investigated the role of PLB in regulating STOC activity and the membrane potential of PLB-KO gastric antrum smooth muscles.

STOC activity is dependent on the SR Ca^{2+} level (ZhuGe *et al.* 1999). Our data consistently shows that the average frequency and amplitude of STOCs were elevated in antrum smooth muscle cells from PLB-KO mice compared to wild-type cells, supporting the conclusion that enhanced SERCA activity due to lack of inhibition by PLB lead to the increased STOC activity. The data in Fig. 2 showing that IbTx, but not apamin, inhibited STOCs suggests that STOC activity in murine antrum smooth muscle cells is mainly due to BK channels. The data in Fig. 4 showing that ryanodine, but not 2-APB, inhibited STOCs evoked by SNP suggests that BK channel activation in murine gastric antrum smooth muscle cells is due to localized Ca^{2+} release from ryanodine receptor-operated stores (Ca^{2+} sparks). In contrast, Yu *et al.* (2003) reported that the BK channels activated by SNP in guinea pig gastric antral circular myocytes are sensitive to IP_3 receptor inhibitors. STOCs in murine colonic smooth muscle cells are associated with localized Ca^{2+} release from inositol IP_3 receptor-operated stores (Ca^{2+} puffs), and are due to activation of both BK channels and small conductance SK channels (Bayguinov *et al.* 2001). In vascular smooth muscles, localized Ca^{2+} release from ryanodine receptors (Ca^{2+} sparks) activates BK channels (Wellman & Nelson, 2003). These differences in the currents responsible for STOCs could be due to species differences, differences between smooth muscle types, or differences in the primary source of activating Ca^{2+}. The difference in STOC activity between wild-type and PLB-KO antrum smooth muscles that we documented in this study is not likely to be due to a difference in BK channel expression or subunit composition, since Western blot and densitometric analysis showed that protein expression of the BK channel α and β subunits is similar in wild-type and PLB-KO antrum smooth muscles (Fig. 2).

As shown in Figs 2, 3 and 5, the basal amplitudes and frequencies of STOCs in PLB-KO antrum smooth muscle cells were higher than wild-type cells, and were not further increased by SNP or 8-Br-cGMP. However, SNP or

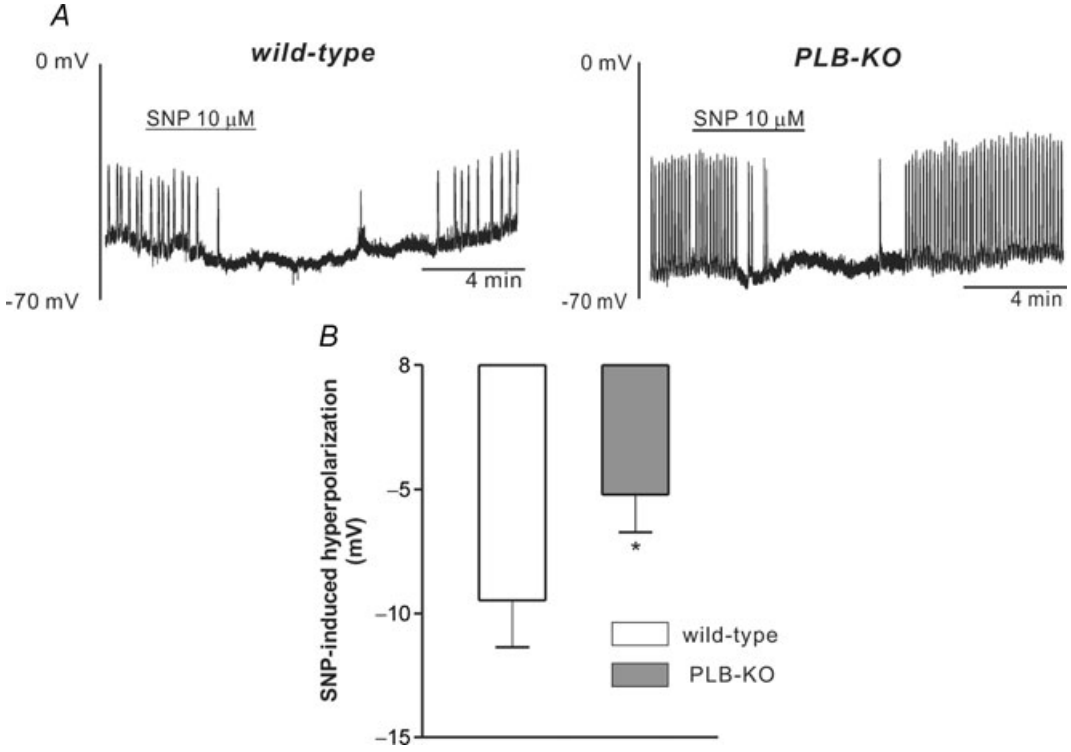

Figure 7. SNP-induced hyperpolarization is reduced in antrum smooth muscles from PLB-KO mice
A, representative traces of the change in resting membrane potential of wild-type and PLB-KO gastric antrum smooth muscle strips incubated with 10 μM SNP. *B*, summary data of SNP-induced hyperpolarization in gastric antrum smooth muscles from wild-type and PLB-KO mice ($n = 6$). $^*P < 0.05$.

8-Br-cGMP did increase the amplitude and frequency of STOCs approximately 2-fold in wild-type antrum smooth muscle cells. These findings suggest that STOC activity is constitutively near maximum levels in PLB-KO mice. Consistent with this conclusion, Wellman *et al.* (2001) showed that both Ca^{2+} spark frequency and BK current in the basal state were elevated in cerebral arteries from PLB-KO mice. They also showed that while the adenylyl cyclase activator forskolin had little effect on Ca^{2+} sparks and transient BK currents from PLB-KO cerebral arteries, forskolin increased Ca^{2+} sparks and transient BK currents in cerebral arteries from wild-type mice.

At physiological temperatures, the spatiotemporal recruitment of transient Ca^{2+} release events from the SR produces intracellular Ca^{2+} waves that activate Ca^{2+}-sensitive K^+ channels to hyperpolarize the plasma membrane (Gordienko *et al.* 1998; Hennig *et al.* 2002; Kim *et al.* 2005) In the present study we found that SNP increased the frequency of STOCs in wild-type antrum smooth muscle cells, but had no effect on the constitutively elevated STOC activity in PLB-KO smooth muscle cells (Fig. 3). The findings presented in Figs 2–4 demonstrating that ryanodine-sensitive STOC activity is elevated in PLB-KO antrum smooth muscle cells and that

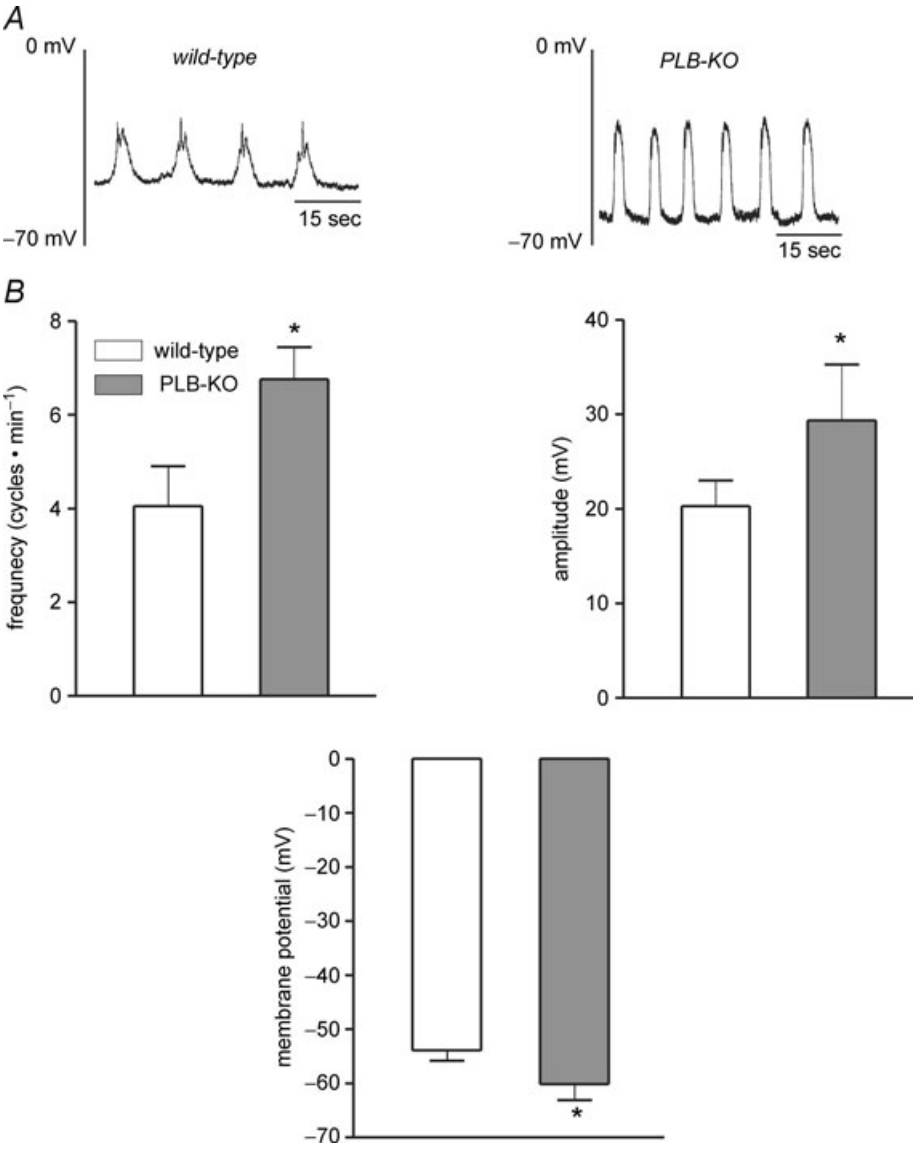

Figure 8. The amplitude and frequency of slow waves are increased in PLB-KO gastric antrum smooth muscles

A, representative traces of slow waves in wild-type and PLB-KO gastric antrum smooth muscle strips. *B*, summary data of the frequencies, amplitudes, and resting membrane potentials of slow waves in gastric antrum smooth muscles from wild-type and PLB-KO mice ($n = 6$). $^*P < 0.05$.

SNP increases STOC activity in wild-type smooth muscle cells indicate that the frequencies of transient SR Ca^{2+} release events (Ca^{2+} sparks) and intracellular Ca^{2+} waves are increased by PLB-KO or SNP treatment. The findings that SNP increased the frequency of intracellular Ca^{2+} waves in wild-type murine antrum smooth muscle cells, but had no effect on the constitutively elevated activity of intracellular Ca^{2+} waves in PLB-KO smooth muscle cells (Fig. 1), support the conclusion that the increased STOC activity of PLB-KO antrum smooth muscle cells and SNP-treated wild-type smooth muscle cells is due to increased intracellular Ca^{2+} wave frequencies.

We previously reported that SNP increases PLB phosphorylation in wild-type antrum smooth muscles (Kim & Perrino, 2007). Together with the findings from the present study, these findings suggest that STOC activity is increased in response to the increase in intracellular Ca^{2+} wave frequencies resulting from increased SR Ca^{2+} uptake due to SERCA activation by PLB phosphorylation. This conclusion is further supported by the findings that the frequencies of both intracellular Ca^{2+} waves and STOCs are higher in PLB-KO antrum smooth muscle cells, and that SNP or 8-bromo-cGMP had no effect on intracellular Ca^{2+} waves or STOCs in PLB-KO antrum smooth muscle cells. In our previous study we found that SNP or 8-bromo-cGMP increased PLB phosphorylation, and that the guanylyl cyclase inhibitor ODQ blocked SNP-induced PLB phosphorylation in antrum smooth muscles, suggesting that PLB phosphorylation by SNP is mediated by cGMP-dependent protein kinase (Kim & Perrino, 2007). In the present study, Fig. 6 shows that the cGMP-dependent protein kinase inhibitor KT5823 reversed the SNP-induced increase in STOC activity in wild-type antrum smooth muscle cells, but had no effect on STOC activity in PLB-KO antrum smooth muscle cells. These findings support the conclusion that the NO donor SNP increases STOC activity by cGMP-dependent protein kinase phosphorylation of PLB.

Smooth muscle cells have negative resting potentials that are important in regulating their excitability and contractility (Sanders, 2008). Smooth muscle cells in the gastro-intestinal (GI) tract display a range of resting potentials from approximately −40 to −80 mV (Sanders, 2008). The mechanisms for setting and regulating membrane potential, including the relative contributions of the variety of K^+ channel subtypes expressed in GI myocytes, are still not fully understood. Most of the previously characterized K^+ conductances expressed by these cells contribute only a few millivolts to resting membrane potentials in intact smooth muscles (Horowitz *et al.* 1999). In this study, we found that SNP hyperpolarized the RMP of wild-type antrum smooth muscle cells by −10 mV, from −55 mV to −65 mV. We also found that STOC activity was increased by SNP, and was sensitive to IbTx, suggesting that SNP hyperpolarized the membrane potential by increasing BK channel activity. At negative potentials of −55 mV to −65 mV, the open probability of BK channels would be expected to be low (Horowitz *et al.* 1999). However, our results show that SNP increased the frequency and amplitudes of intracellular Ca^{2+} waves. BK channel open probability is increased at negative potentials when $[Ca^{2+}]_c$ is increased (Carl *et al.* 1990). Together, these findings suggest that the voltage block on BK channel activity at these negative potentials was removed by the increased intracellular Ca^{2+} wave activity. These findings also provide a possible explanation for the more negative RMP of PLB-KO antrum smooth muscle cells relative to wild-type cells, since intracellular Ca^{2+} wave activity was increased in PLB-KO antrum smooth muscles.

In our studies of the RMP of wild-type and PLB-KO antrum smooth muscles, we unexpectedly noticed that the frequency and amplitudes of slow waves in PLB-KO smooth muscles were larger, despite the more negative RMP of PLB-KO smooth muscles (Figs 7 and 8). The amplitude of slow waves recorded from smooth muscle cells is highly dependent upon the input resistance and the resting membrane potentials of the smooth muscle cells (Kito *et al.* 2005). Typically, K^+ channel opening hyperpolarizes smooth muscle cells and diminishes the degree of depolarization achieved during slow waves (Sanders, 2008). However, slow waves are generated by the pacemaker activity in ICC, which form low resistance connections with smooth muscle cells via gap junctions to conduct slow wave depolarizations into smooth muscle cells (Sanders, 2008). Hyper-polarization of ICC would be likely to increase the amplitude of unitary potentials, because these are due to inward currents with equilibrium potentials positive to the resting potential (Kito *et al.* 2005). In addition, the dihydropyridine-resistant (mibefradil sensitive) Ca^{2+} current expressed in ICC activates and inactivates at potentials more negative than those of dihydropyridine-sensitive current (Kito *et al.* 2005). It has been shown that only 50% of the dihydropyridine-resistant Ca^{2+} channels in ICC are available at the resting membrane potential (Kito *et al.* 2005). However, hyperpolarization by −10 mV increased the availability of these channels to 80% (Kito *et al.* 2005). The increase in dihydropyridine-resistant Ca^{2+} channel availability could help to preserve the frequency and would tend to increase the amplitude and dV/dt_{max} of the slow wave (Kito *et al.* 2005). These findings suggest that the larger amplitudes and higher frequency of slow waves in PLB-KO antrum smooth muscle cells may be due to an effect of PLB-KO on pacemaker activity and slow wave generation in ICC. Recently, gene chip analysis of sorted ICC devoid of smooth muscle cells revealed that both SERCA and PLB are expressed in ICC (Chen *et al.* 2007), suggesting that loss of SERCA inhibition by PLB-KO could alter smooth

endoplasmic reticulum Ca^{2+} uptake and release dynamics in ICC and amplify slow wave activity. Ca^{2+} release from IP_3 receptor-operated stores is the initiating event that triggers the ICC pacemaker current (Sanders *et al.* 2006). Ca^{2+} release from the IP_3 receptor opens Ca^{2+} uptake channels in the mitochondria, causing a drop in $[Ca^{2+}]_c$ that triggers the opening of non-selective cation channels in the plasma membrane which are responsible for the unitary potentials and pacemaker activity. The inward current depolarizes the membrane potential and activates a dihydropyridine-resistant voltage-dependent Ca^{2+} conductance in ICC that appears to be responsible for the upstroke depolarization of slow waves and for active slow wave propagation (Sanders *et al.* 2006).

Thus, in the absence of PLB, increased Ca^{2+} uptake would be expected to elevate smooth endoplasmic reticulum Ca^{2+} levels, and increase the frequency of Ca^{2+} release events through IP_3 receptors. Increased Ca^{2+} puffs could open more mitochondrial Ca^{2+} uptake channels, causing a more severe drop in $[Ca^{2+}]_i$ and trigger the opening of more non-selective cation channels in the plasma membrane. A larger inward current would result in a larger depolarization and the activation of more voltage-dependent Ca^{2+} channels, resulting in the conduction of larger amplitude and higher frequency slow waves into smooth muscle cells, which could overcome the hyperpolarized membrane potential of PLB-KO smooth muscle cells. In support of this conclusion, agonists or conditions that increase IP_3 production or Ca^{2+} entry through voltage-dependent Ca^{2+} channels in ICC have significant chronotropic effects on slow waves in antrum smooth muscles (Sanders *et al.* 2006). Furthermore, our previous report demonstrates that the amplitudes and frequency of spontaneous phasic contractions are increased in PLB-KO antrum smooth muscles (Kim *et al.* 2008). These findings suggest that further investigations of the intracellular Ca^{2+} transport mechanisms of both gastric antrum ICC and smooth muscle may provide additional targets for novel therapeutic strategies aimed at relieving the symptoms and pathophysiology associated with gastroparesis.

In summary, the results of the present study show that the SERCA regulatory protein PLB plays an important role in modulating intracellular Ca^{2+} waves and the associated STOC activities of murine gastric antrum smooth muscle. Intracellular Ca^{2+} wave frequency and STOC activities were increased, and the membrane potential hyperpolarized by the NO donor SNP in wild-type smooth muscles, but not in PLB-KO smooth muscles, suggesting that PLB phosphorylation is a key event in NO-induced modulation of antral smooth muscle excitability. In PLB-KO antrum smooth muscles, intracellular Ca^{2+} wave frequency and the associated STOC activities were constitutively elevated. In addition, a hyperpolarized RMP was associated with the increased STOC activity in PLB-KO antrum smooth muscle cells. Despite the hyperpolarized membrane potential, the amplitudes and frequency of slow waves in PLB-KO smooth muscles were increased. These findings suggest that regulation of intracellular Ca^{2+} transport by PLB contributes to the gastric antrum functions of grinding, mixing, and gastric emptying. Future studies of the activities of Ca^{2+} sensitive proteins in PLB-KO antrum smooth muscle cells, and regulation of the pacemaker unit by PLB in ICC will provide additional understanding of the myogenic mechanisms that coordinate the electrical and mechanical activities of the gastric antrum.

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