Phospholamban knockout increases CaM kinase II activity and intracellular 
Ca\textsuperscript{2+} wave activity and alters contractile responses of murine gastric antrum

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Phospholamban knockout increases CaM kinase II activity and intracellular Ca\textsuperscript{2+} wave activity and alters contractile responses of murine gastric antrum. Am J Physiol Cell Physiol 294: C432–C441, 2008. First published November 28, 2007; doi:10.1152/ajpcell.00418.2007.—Phospholamban (PLB) is a sarcoplasmic reticulum (SR) Ca\textsuperscript{2+}-ATPase (SERCA), and this inhibition is relieved by Ca\textsuperscript{2+} calmodulin-dependent protein kinase II (CaM kinase II) phosphorylation. We previously reported significant differences in contractility, SR Ca\textsuperscript{2+} release, and CaM kinase II activity in gastric fundus smooth muscles as a result of PLB phosphorylation by CaM kinase II. In this study, we used PLB-knockout (PLB-KO) mice to directly examine the effect of PLB absence on contractility, CaM kinase II activity, and intracellular Ca\textsuperscript{2+} waves in gastric antrum smooth muscles. The frequencies and amplitudes of spontaneous phasic contractions were elevated in antrum smooth muscle strips from PLB-KO mice. Be
thanol increased the amplitudes of phasic contractions in antrum smooth muscles from both control and PLB-KO mice. Caffeine decreased and cyclopiazonic acid (CPA) increased the basal tone of antrum smooth muscle strips from PLB-KO mice, but the effects were less pronounced compared with control strips. The CaM kinase II inhibitor KN-93 was less effective at inhibiting caffeine-induced relaxation in antrum smooth muscle strips from PLB-KO mice. CaM kinase II autonomous activity was elevated, and not further increased by caffeine, in antrum smooth muscles from PLB-KO mice. Similarly, the intracellular Ca\textsuperscript{2+} wave frequency was elevated, and not further increased by caffeine, in antrum smooth muscles from PLB-KO mice. These findings suggest that PLB is an important modulator of gastric antrum smooth muscle contractility by modulation of SR Ca\textsuperscript{2+} release and CaM kinase II activity.

gastrointestinal smooth muscle; Ca\textsuperscript{2+} calmodulin-dependent protein kinase II

**PERISTALTIC CONTRACTIONS** of the gastric antrum accomplish the grinding, mixing, and gastric emptying functions of this part of stomach. Slow wave propagation from the greater curvature of the orad corpus to the pylorus initiates the phasic contractions of the antrum, which form the basis for gastric peristalsis (17). Conditions affecting the intrinsic pacemaker frequency of the antrum or producing arrhythmic activity in this region can result in a breakdown in functional coupling between the corpus and antrum and interfere with normal gastric emptying (4, 18). Insufficient gastric emptying produces pathophysiological problems such as functional dyspepsia, postsurgical gastroparesis, and diabetic gastroparesis (24, 41). Slow waves generated by myenteric interstitial cells of Cajal (ICC-MY) activate smooth muscle voltage-dependent Ca\textsuperscript{2+} channels to generate the phasic contractions of the antrum (5, 7, 33, 34). Thus, along with studies of the electrical coupling of ICC-MY to smooth muscle cells, studies of the Ca\textsuperscript{2+} handling mechanisms of antrum smooth muscles will provide insights into gastric smooth muscle physiology and pathophysiology.

Excitation-contraction (E-C) coupling is the process whereby electrical excitation of a myocyte leads to muscle contraction. Ca\textsuperscript{2+} is essential in E-C coupling as both a carrier of charge during electrical excitation and as an activator of myofilaments, causing contraction. Whereas extracellular Ca\textsuperscript{2+} ions can enter smooth muscle cells through mainly L-type Ca\textsuperscript{2+} channels during muscle action potentials, intracellular storage and release of Ca\textsuperscript{2+} continues even when smooth muscle cells are apparently quiescent (1, 36). The sarcoplasmic reticulum (SR) is the main organelle in smooth muscle cells that stores and releases Ca\textsuperscript{2+} and maintains a ∼1,000-fold Ca\textsuperscript{2+} gradient across the SR membrane by the SR Ca\textsuperscript{2+}-ATPase (SERCA) pump (29, 35). SERCA pump activation elevates the SR Ca\textsuperscript{2+} load or content (14) and can contribute to relaxation by lowering the intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) of the myoplasm and decreasing the amount of Ca\textsuperscript{2+} available for contraction (16). On the other hand, elevations in luminal SR Ca\textsuperscript{2+} load by SERCA pump activation increases the frequency of Ca\textsuperscript{2+} sparks and spontaneous transient outward currents (STOC’s), which also contributes to relaxation (3). In cardiac, skeletal, and smooth muscles, SERCA activity is under rheostat control by the SR membrane protein phospholamban (PLB) to continuously vary the rate of Ca\textsuperscript{2+} influx into the SR via its phosphorylation levels, from maximal SERCA inhibition of 50% by diphospho-PLB to maximal SERCA activity by phospho-PLB (23, 38). Phosphorylation of PLB by cAMP-dependent protein kinase or cGMP-dependent protein kinase at Ser16 or by CaM kinase II at Thr17 removes its inhibitory effects on SERCA, thereby increasing Ca\textsuperscript{2+} uptake into the SR and contributing to relaxation (8, 25, 32, 43). The role of PLB in the regulation of contractility by SR Ca\textsuperscript{2+} uptake and release has been investigated through the use of gene knock out and transgenic mouse models (32, 38, 43). In hearts from PLB knockout (PLB-KO) mice, cytosolic Ca\textsuperscript{2+} clearance is enhanced, and the rates of contraction and relaxation are increased (38). In bladder smooth muscle from PLB KO mice, Ca\textsuperscript{2+} clearance is also enhanced and the SR Ca\textsuperscript{2+} load is increased (32). In cerebral arteries of PLB-KO mice, Ca\textsuperscript{2+} spark frequency and transient large conductance K\textsuperscript{+} (BK) current frequency are increased (43). On the other hand, overexpression of PLB decreases SR Ca\textsuperscript{2+} uptake and cardiac contractility (31).
A number of studies show that low caffeine concentrations induce focal Ca^{2+} release from the SR through ryanodine-sensitive Ca^{2+} channels in various smooth muscles, including gastrointestinal smooth muscles (22, 39, 44). These localized Ca^{2+} transients activate outward K+ currents that promote membrane potential hyperpolarization and relaxation (42). Previously, we reported that caffeine hyperpolarized and relaxed gastric fundus smooth muscles via SR Ca^{2+} release. In addition, autonomous CaM kinase II activity and PLB-Thr17 phosphorylation was increased, suggesting that CaM kinase II activation is mediated by localized Ca^{2+} release events from the SR in murine gastric smooth muscles (19). Furthermore, the findings that PLB phosphorylation results in increased SERCA activity and increased SR Ca^{2+} release suggest that PLB phosphorylation would exert a positive feedback effect on CaM kinase II, by maintaining enhanced SR Ca^{2+} uptake and release (32, 43). At physiological temperatures, SR Ca^{2+} release occurs as a propagating wave (12) that is likely to be due to spatiotemporal recruitment of Ca^{2+} sparks from local release sites (2, 9). Ca^{2+} spark activity is clearly increased in smooth muscles from PLB-KO mice, but it is not known if intracellular Ca^{2+} wave activity is also increased by PLB-KO.

To assess the potential role of PLB in modulating the intracellular Ca^{2+} handling dynamics of gastric antrum smooth muscles, we measured contractile responses, CaM kinase II activities, and intracellular Ca^{2+} waves of gastric antrum smooth muscles from wild-type control and PLB-KO mice. We report here that the frequency of intracellular Ca^{2+} waves and CaM kinase II autonomous activity are both increased in PLB-KO antrum smooth muscles. In addition, PLB-KO increased the frequency of spontaneous phasic contractile activity and altered the mechanical responses to caffeine and cyclopiazonic acid (CPA). Our data support the hypothesis that PLB modulation of SERCA is an important site for regulation of cytosolic Ca^{2+} transients and gastric antrum smooth muscle contractility.

METHODS

Preparation of antrum smooth muscles for CaM kinase II assays. Wild-type control mice (SVJ129 X CF1, 6–8 wk old) were purchased from Charles River Laboratories (Wilmington, MA) (26). PLB-KO mice were purchased from the Mutant Mouse Regional Resource Center (Harlan, MO). Mice were maintained and the experiments were 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 anesthetized by isoflurane inhalation and killed by decapitation. The stomach was removed and cut open along the lesser curvature. The antrum was cut away from the rest of the stomach and pinned out in a Sylgard-lined dish containing oxygenated Krebs (in mM: 120 NaCl, 6 KCl, 15 NaHC03, 12 glucose, 3 MgCl2, 1.5 NaH2PO4, and 3.5 CaCl2; pH 7.2). The mucosa and submucosal layers were removed by using fine-tipped forceps. For determining the effects of caffeine on CaM kinase II activity, the antrum smooth muscles were equilibrated in Krebs buffer for 45 min at 37°C and then incubated at 37°C in the absence or presence of each compound for 15 min. The tissues were then collected, frozen in liquid nitrogen, and stored at −80°C. For the activity assays, each frozen tissue was homogenized at 4°C with a glass tissue grinder in 0.3 ml lysis buffer (50 mM MOPS, 0.2% Nonidet P-40, 100 mM Na2PO4, 100 mM NaF, 250 mM NaCl, 3 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor tablet) and centrifuged at 16,000 g for 15 min at 4°C, and the supernatant was aliquoted and stored at −80°C. Protein concentrations were determined using the Bradford assay with bovine γ-globulin as standard.

CaM kinase II assays. CaM kinase II activity was assayed using the specific CaM kinase II peptide substrate autocamtide-2 (KKALRRQETVDAL, 20 μM; BioMol, Plymouth Meeting, PA), as described previously (19). Total (Ca^{2+}/CaM-stimulated) and autonomous (Ca^{2+}/CaM-independent) CaM kinase II activities from the cytosolic fraction of control and treated gastric antrum smooth muscles from at least three animals were assayed in triplicate from each tissue. Kinase activity was calculated (expressed as nmol of P_i incorporated·min⁻¹·mg⁻¹ of protein).

SDS-PAGE and Western blot analysis of SERCA2 from gastric antrum smooth muscle SR fractions. Gastric antrum smooth muscles were obtained from adult CF-1 mice and PLB-KO mice (as described above), frozen in liquid nitrogen, and stored at −80°C. The SR fractions were obtained by high-speed centrifugation of smooth muscle lysates as described previously (19, 30). Protein concentrations were determined with the Bradford assay using bovine γ-globulin as standard. Smooth muscle SR proteins were separated by SDS-PAGE (12% gel) and transferred to nitrocellulose by Western blot analysis. The blots were incubated with primary and secondary antibodies, washed, and processed for enhanced chemiluminescence image detection using ECL Advantage (Amersham Biosciences, Piscataway, NJ). The SERCA2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and smooth muscle actin antibody (Sigma, St. Louis, MO) were used at 1:5,000 dilutions, and the horseradish peroxidase-conjugated secondary antibodies (Chemicon, Temecula, CA) were used at a 1:50,000 dilution. Protein bands were visualized with a CCD-camera-based detection system (Epi Chem II, UVP Laboratory Products). The collected TIFF images were analyzed using Adobe Photoshop. Denitrosometric analysis of the Western blot TIFF images was performed using Un-Scan-It software (Silk Scientific, Orem, UT). SERCA protein expression in PLB-KO antrum smooth muscle SR fractions is reported as the percentage of the SERCA protein pixel intensity values (minus background) from wild-type antrum smooth muscle SR fractions.

Mechanical responses of gastric antrum smooth muscles from CF-1 and PLB-KO mice. Standard organ bath techniques were employed to measure the force generated by antrum smooth muscle strips (~6 mm × 3 mm). One end of each smooth muscle strip was attached to a fixed mount and the opposite end to a Fort 10 isometric strain gauge (WPI, Sarasota, FL) in parallel with the circular smooth muscle layer. The muscle strips were immersed in organ baths containing Krebs buffer (3 ml) maintained at 37 ± 0.5°C. The pH was kept constant at a pH of 7.4 by bubbling the Krebs solution with 97% O2-3% CO2. A resting force of 6 mN (1 g = 9.8 mN) was applied to set the muscles at optimum length. This was followed by an equilibration period of at least 1 h with continuous perfusion of oxygenated Krebs solution. After equilibration, the muscle strips were incubated in Krebs solution containing the compounds as indicated in the figure legends. Mechanical responses were recorded, and the frequencies, amplitudes, and maximum rising slopes were analyzed with Acqknowledge 3.2.6 (BIOPAC Systems, Santa Barbara, CA). Decay constants were determined by taking the log10 of the decay in force after a contraction and fitting of a least squares regression line to calculate the slope of the exponential decay. The time constant (Tau) is defined as 1/slope.

Preparation of antrum smooth muscles for Ca^{2+} imaging. After the mucosa and submucosa were peeled from the antrum smooth muscles, each preparation was pinned down with the circular muscle layer topmost in an organ bath containing Krebs solution at 35 ± 0.5°C bubbled with 97% O2-3% CO2 (pH of 7.3–7.4). The temperature of
the bath was monitored during the experiment and was not changed when the microscope lens was immersed in the Krebs solution. The tissue was equilibrated for 1 h before dye loading.

Fluo-4 AM loading. After the 1-h equilibration, the smooth muscle preparation was incubated with the Ca\(^{2+}\) fluorescent dye fluo-4 AM (5 \(\mu\)M) (cell permeant, special packaging; Molecular Probes, Eugene, OR), 0.02% dimethyl sulfoxide, and 0.01% noncytotoxic detergent cremophor EL (Sigma), for 15 min at room temperature (12). As the tissue was pinned tight with longitudinal muscle (LM) side down and the dye was only applied to the circular muscle (CM), negligible amounts of dye penetrated the LM layer. After the dye incubation, the tissue was perfused with oxygenated Krebs solution (1 \(\mu\)M nicardipine was used to prevent tissue movement and block intercellular Ca\(^{2+}\) waves) for 15–20 min at 35 ± 0.5°C to allow for deesterification (11, 12).

Visualization of intracellular Ca\(^{2+}\) waves. The dye-loaded smooth muscles were imaged with a Nikon Eclipse E600FN microscope fitted with epifluorescence. A 175-Watt ozone-free xenon arc lamp (Lambda DG-5) was used to excite Fluoro-4, and only emitted light at wavelengths greater than 515 nm was captured. A ×60 water immersion lens was used (Nikon, CFI fluor 60XW) giving a field of view of 150 \(\mu\)m × 150 \(\mu\)m. Ca\(^{2+}\)-induced fluorescence was recorded using a Cascade 512 B CCD camera (Prontometrics, Tucson, AZ) using Universal Imaging’s Metamorph 6.1r6 onto a PC. To gauge any bleaching that occurred due to illumination of the preparation to high intensity light, we performed two control recording separated by 5 min. When drugs were used we used a similar protocol, except the drug of interest was added immediately after the first recording to control for any bleaching by comparing each second time-matched control recording to the recording obtained during the drug treatment.

Image analysis. Image sequences were imported into custom written software (Volumetry 6.6a, by G. W. Hemmig), and the pattern of Ca\(^{2+}\)-induced fluorescence during intracellular Ca\(^{2+}\) wave activity within single cells in the intact antrum smooth muscle was revealed using spatiotemporal maps (ST Maps) (12). After motion stabilization, individual cells were outlined and ST Maps of Ca\(^{2+}\)-induced fluorescence perpendicular to the long axis of antrum smooth muscle cells were constructed. The frequency, velocity, and waveform characteristics of intracellular waves were calculated. The amplitudes of Ca\(^{2+}\) waves were measured using the F/F\(_{avg}\) ratio as Ca\(^{2+}\)-induced fluorescence intensities were constantly oscillating, preventing an F\(_{0}\) from being determined (12).

Materials. Caffeine, nicardipine, and tetrodotoxin were obtained from Sigma. CPA and KN-93 were purchased from Biomol (Plymouth Meeting, PA). Mini-EDTA free protease inhibitor pills were obtained from Roche Applied Science, Indianapolis, IN. All other chemicals and materials were of reagent grade.

Statistical analysis. Data are expressed as means ± SD. Data sets were tested for significance using ANOVA to analyze multiple groups. Data were considered significantly different from control values when \(P < 0.05\).

RESULTS

Mechanical responses of gastric antrum smooth muscles from wild-type and PLB-KO mice. Representative force traces of antrum smooth muscle strips from wild-type CF-1 mice and PLB-KO mice are shown in Fig. 1. Muscle strips from the gastric antrum of wild-type CF-1 mice showed ongoing phasic contractions at a frequency of 3.3 ± 0.3 cycles per minute (cpm) and an average amplitude of 1.1 ± 0.3 mN. The average rising slope and decay constant were 0.3 ± 0.1 mN/s and 5.3 ± 0.8 s, respectively (\(n = 15\), Fig. 1A). Phasic contractions in antrum muscle strips from PLB-KO mice occurred at a higher frequency (4.5 ± 0.4 cpm, \(n = 15\), \(P < 0.001\)) and had larger amplitudes (2.1 ± 0.3 mN, \(n = 15\), \(P < 0.001\)) than those in wild-type antrum muscle strips (Fig. 1A). The higher frequency contractions had a higher rising slope (0.7 ± 0.2 mN/s, \(n = 15\)) and a faster decay constant (2.5 ± 0.5 s, \(n = 15\), \(P < 0.001\)). Receptor-mediated contractile responses were compared by incubation of the antrum smooth muscle strips with 10 \(\mu\)M bethanechol (BCh) of antrum smooth muscle strips from CF-1 or PLB-KO mice. The amplitudes of the phasic contractions from both wild-type and PLB-KO antrum smooth muscle strips were noticeably enhanced. Bethanechol increased the amplitudes of phasic contractions of wild-type antrum smooth muscle strips from 1.1 ± 0.3 to 7.6 ± 1.7 mN (\(n = 5\), \(P < 0.001\)) and increased the amplitudes of phasic contractions of PLB-KO antrum smooth muscle strips from 2.1 ± 0.3 to 13 ± 2.8 mN (\(n = 5\), \(P < 0.001\)). However, these results indicate that the increases in amplitudes were proportionally similar. Bethanechol treatment also increased an increase in tone of 0.8 ± 0.3 mN in wild-type and 0.9 ± 0.15 mN in PLB-KO antrum smooth muscle strips, respectively (\(n = 5\), Fig. 1B).

Effect of caffeine on the mechanical responses of gastric antrum smooth muscles from wild-type and PLB-KO antrum smooth muscle strips. We examined the effect of 1 mM caffeine on the mechanical responses of gastric antrum smooth muscles, because this concentration of caffeine has been reported to liberate Ca\(^{2+}\) from ryanodine-sensitive stores of stomach smooth muscles and cause relaxation (39, 44). The effects of caffeine were fully reversible upon washout and were
not sensitive to 1 μM tetrodotoxin (data not shown). Figure 1C shows that caffeine (1 mM) dramatically decreased the amplitudes of phasic contractions of wild-type and PLB-KO antrum smooth muscle strips and also decreased the average baseline tone, but the decrease was more pronounced in the wild-type antrum smooth muscles (0.9 ± 0.2 mN) than in PLB-KO antrum smooth muscles (0.4 ± 0.1 mN, n = 20, P < 0.05, Fig. 1C).

**Effect of CPA on caffeine-induced relaxation of gastric antrum smooth muscles from wild-type control and PLB-KO mice.** Since PLB acts as an inhibitor of SERCA in the resting state, once this inhibition is removed in PLB-KO mice, SR Ca$^{2+}$ load and localized Ca$^{2+}$ release events from the SR are expected to be elevated (32). To assess potential differences between SERCA activity in wild-type and PLB-KO antrum smooth muscles, we examined the effect of the SERCA inhibitor CPA on contractility. Figure 2A shows that CPA concentrations of 2 or 5 μM each evoked a rapid increase in basal tone of 3.9 ± 1.5 mN (n = 6) and 4.5 ± 1.3 mN, (n = 5), respectively, in wild-type antrum smooth muscle strips, which was associated with a decrease in the frequency and amplitudes of the phasic contractions. The addition of caffeine (1 mM) during treatment with 2 μM CPA suppressed phasic contractions and reduced the tone by 0.7 ± 0.15 mN (n = 6, P < 0.05). At the higher CPA concentration (5 μM), caffeine (1 mM) evoked a transient relaxation followed by an increase basal tone. In PLB-KO antrum smooth muscles, the increase in tone evoked by CPA was still lower than that of the CPA-evoked increase in tone of wild-type antrum smooth muscles. Furthermore, except for the 5 μM CPA treatment of wild-type antrum smooth muscle strips, the increase in tone evoked by CPA in the presence of caffeine was lower than the increase in tone evoked by CPA alone.

**Effect of KN-93 on caffeine-induced relaxation in gastric antrum smooth muscles from wild-type and PLB-KO mice.** We previously reported that PLB Thr17 phosphorylation by CaM kinase II contributes to caffeine-induced relaxation of murine gastric fundus smooth muscles (19). Here we used the CaM kinase II inhibitor KN-93 to investigate the role of PLB phosphorylation by CaM kinase II activation in caffeine-induced relaxation of wild-type and PLB-KO antrum smooth muscles. As shown in Fig. 3A, 10 μM KN-93 alone had no effect on the frequency of spontaneous phasic contractions of wild-type antrum smooth muscle strips but did slightly decrease the tone by 0.2 ± 0.1 mN (n = 5, P < 0.05) and the amplitudes of the phasic contractions from 1.0 ± 0.3 to 0.8 ± 0.1 mN (n = 5). The caffeine-induced inhibition of the amplitudes and frequencies of phasic contractions was unaffected KN-93; however, the caffeine-induced decrease in basal tone was attenuated by KN-93 (0.8 ± 0.2 mN by caffeine alone vs. 0.42 ± 0.2 mN by caffeine in the presence of KN-93, n = 5, P < 0.001). Similar to wild-type antrum smooth muscles,
KN-93 had no effect on the frequency of spontaneous phasic contractions of PLB-KO antrum smooth muscle strips but did slightly decrease the tone by 0.2 ± 0.1 mN (n = 5, P < 0.05) and the amplitudes of the phasic contractions from 2.0 ± 0.3 to 1.8 ± 0.2 mN (n = 5) (Fig. 3B). Similarly, the caffeine-induced inhibition of the amplitudes and frequencies of phasic contractions was unaffected KN-93. However, the caffeine-induced decrease in basal tone was not significantly attenuated by KN-93 (0.46 ± 0.2 mN by caffeine alone vs. 0.36 ± 0.2 mN by caffeine in the presence of KN-93, n = 5).

**Effect of caffeine on autonomous CaM kinase II activity in gastric antrum smooth muscles from wild-type control and PLB-KO mice.** We previously reported that caffeine activated CaM kinase II and increased PLB-Thr17 phosphorylation in gastric fundus smooth muscles (19). Since SERCA activity is enhanced by PLB phosphorylation or PLB knockout, autonomous (Ca<sup>2+</sup>/CaM-independent) CaM kinase II activities in the lysates of untreated and caffeine-treated gastric antrum smooth muscles from wild-type and PLB-KO mice were examined. Total CaM kinase II activity was not significantly affected by caffeine: 8.5 ± 1.4 and 8.8 ± 1.3 nmol·min<sup>-1</sup>·mg<sup>-1</sup> in untreated and caffeine-treated wild-type antrum smooth muscles, respectively (n = 5). Figure 4 shows that caffeine (1 mM) increased autonomous CaM kinase II activity in wild-type antrum smooth muscles from 1.9 ± 0.2 to 2.9 ± 0.2 nmol·min<sup>-1</sup>·mg<sup>-1</sup> (n = 5, P < 0.001). Thus caffeine increased CaM kinase II autonomous activity from 22 ± 2% to 33 ± 4% of CaM kinase II total activity (n = 5, P < 0.001). In contrast, even without caffeine treatment, autonomous CaM kinase II activity was significantly higher in PLB-KO antrum smooth muscles than in untreated wild-type antrum smooth muscles. In fact, the kinase activity of 2.6 ± 0.3 nmol·min<sup>-1</sup>·mg<sup>-1</sup> is similar to the kinase activity of 2.9 ± 0.2 nmol·min<sup>-1</sup>·mg<sup>-1</sup> evoked by caffeine treatment of wild-type antrum smooth muscles. Furthermore, caffeine treatment of PLB-KO antrum smooth muscles did not further increase autonomous CaM kinase II activity (2.6 ± 0.3 nmol·min<sup>-1</sup>·mg<sup>-1</sup> in untreated antrum vs. 2.5 ± 0.5 nmol·min<sup>-1</sup>·mg<sup>-1</sup> in caffeine-treated antrum, n = 5). Total CaM kinase II activity was not significantly affected by caffeine: 8.8 ± 1.7 and 8.9 ± 1.1 nmol·min<sup>-1</sup>·mg<sup>-1</sup> in untreated and caffeine-treated PLB-KO antrum smooth muscles, respectively (n = 5). Thus as a percentage of total CaM kinase II activity, autonomous activity levels from untreated PLB-KO antrum smooth muscles (30 ± 3%) were similar to autonomous activity levels in the presence of caffeine (28 ± 3%) (n = 5).

**SERCA2 expression in gastric antrum smooth muscle SR fractions from wild-type control and PLB-KO mice.** The previous results are consistent with increased SR Ca<sup>2+</sup> uptake and release due to uninhibited SERCA activity in PLB-KO antrum smooth muscles. To confirm that SERCA expression was not affected by PLB knockout, SERCA2 protein expression was monitored by Western blot analysis of antrum smooth muscle SR fractions from wild-type and PLB-KO mice. Figure 5 shows the levels of SERCA2 protein expression in wild-type and PLB-KO antrum smooth muscles are similar. Densitome-

### Table 1. CPA-evoked increases in basal tone of antrum smooth muscle strips from wild-type or PLB-KO mice

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<th>Increase in Basal Tone, mN</th>
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<td>2 μM CPA</td>
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<td>Wild-Type</td>
<td>3.9±1.5</td>
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<td>PLB-KO</td>
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Values are means ± SD, n = 4–6/group. PLB-KO, phospholamban knockout mice; CPA, cyclopiazonic acid. *P* < 0.05 for wild-type vs. PLB-KO and for CPA alone vs. CPA with caffeine, except for 5 μM CPA alone and 5 μM CPA with 1 mM caffeine for wild-type strips.
try of the SERCA protein bands indicates that the SERCA2 protein expression level in PLB-KO antrum smooth muscles is 98 ± 1% of SERCA2 wild-type protein in wild-type antrum smooth muscles.

Intracellular Ca²⁺ wave activities in intact antrum circular smooth muscle strips from wild-type and PLB-KO mice. At physiological temperatures, Ca²⁺ release from intracellular stores occurs as a propagating wave that is likely due to spatiotemporal recruitment of Ca²⁺ sparks from local release sites (2, 9, 12). Since the previous results suggest that increased SR Ca²⁺ release accounts for the differences between wild-type and PLB-KO antrum smooth muscles, we compared intracellular Ca²⁺ wave activity in wild-type and PLB-KO antrum smooth muscle cells. Intracellular Ca²⁺ waves were recorded in 150 μm × 150 μm regions of interest of intact antrum circular smooth muscles to generate spatiotemporal wave maps as described in Methods. During our preliminary experiments examining the effects of caffeine on intracellular Ca²⁺ waves, we found that subsequent exposure to the high-intensity fluo-4 AM excitation light decreased the amplitude and frequency, but not the propagation velocities, of intracellular Ca²⁺ waves. The propagation velocities of intracellular Ca²⁺ waves during the first and second exposures were not significantly different in wild-type (60 ± 10 vs. 56 ± 6 μm/s, n = 7, P > 0.05) and PLB-KO antrum smooth muscle cells (82 ± 7 vs. 77 ± 9 μm/s, n = 7, P > 0.05). However, in both wild-type and PLB-KO antrum smooth muscle cells, the second exposure decreased the average amplitudes of the intracellular Ca²⁺ waves by 19 ± 11% (n = 7, P < 0.05). The spatiotemporal maps in Fig. 6A show that the frequency of intracellular Ca²⁺ waves recorded from the first fluo-4 AM excitation exposure is markedly elevated in PLB-KO antrum smooth muscle cells compared with wild-type (49 ± 5 waves/min in wild-type vs. 78 ± 6 waves/min in PLB-KO, n = 7, P < 0.05). Figure 6, B and C, shows that the second exposure decreased the frequencies of intracellular Ca²⁺ waves to 36 ± 4 waves/min (n = 7, P < 0.05) and 56 ± 5 waves/min in wild-type and PLB-KO antrum smooth muscles, respectively (n = 7, P < 0.05).

Having established the intracellular Ca²⁺ wave frequencies in wild-type and PLB-KO antrum smooth muscle cells during the second exposure to fluo-4 AM excitation light, the effects of caffeine (1 mM) on intracellular Ca²⁺ wave activity were examined. All responses to caffeine treatment were compared with the second time-matched control recording (see Methods) to account for the depression of activity from the subsequent exposure to the fluo-4 AM excitation light. In both wild-type and PLB-KO antrum smooth muscles, caffeine did not affect the reduction in amplitude caused by the second fluo-4 AM excitation. In wild-type antrum smooth muscles, the average amplitudes of intracellular Ca²⁺ waves in the second time-matched controls and caffeine-treated samples were 81 ± 11% and 77 ± 17%, respectively, of the amplitudes recorded from the first exposure (n = 7). Similarly, in PLB-KO antrum smooth muscles, the average amplitudes of intracellular Ca²⁺ waves in the second time-matched controls and caffeine-treated samples were 81 ± 12% and 78 ± 15%, respectively, of the amplitudes recorded from the first exposure (n = 7). These findings indicate that the amplitudes of intracellular Ca²⁺ waves were not significantly changed by caffeine treatment. Caffeine did not change the propagation velocities of intracellular Ca²⁺ waves in wild-type and PLB-KO antrum smooth muscle cells. In wild-type antrum smooth muscle cells, the average propagation velocities in the second time-matched controls and caffeine-treated samples were 56 ± 6 and 45 ± 7 μm/s, respectively (n = 7). Similarly, in PLB-KO antrum smooth muscle cells, the average propagation velocities in the second time-matched controls and caffeine-treated samples were 77 ± 9 and 72 ± 6 μm/s, respectively (n = 7).

Fig. 4. Calmodulin-dependent protein kinase (CaM kinase II) activity is elevated in gastric antrum smooth muscles from PLB-KO mice and is not further activated by caffeine. Autonomous (Ca²⁺/CaM-independent) CaM kinase II activities were assayed as described in Methods. The average values ± SD of autonomous CaM kinase II activities were obtained from antrum smooth muscle strips incubated without (control) or with 1 mM caffeine for 15 min. **P < 0.001.

Fig. 5. Sarcoplasmic reticulum (SR) Ca²⁺-ATPase (SERCA2) expression in gastric antrum smooth muscle SR fractions from CF-1 mice or PLB-KO mice. SDS-PAGE and immunoblotting with SERCA2 antibodies was carried out as described in Methods. Representative immunoblot of smooth muscle actin as an additional control for equal protein loading (n = 4).
Fig. 6. The frequency of intracellular Ca\(^{2+}\) waves is elevated in gastric antrum smooth muscle strips from PLB-KO mice and is not further increased by caffeine. 

A: representative Z-compressed images (left) showing the region of interest and spatiotemporal maps of intracellular Ca\(^{2+}\) waves in antrum smooth muscle cells from constructed from CF-1 mice and PLB-KO mice. 

B: representative spatiotemporal maps of intracellular Ca\(^{2+}\) waves constructed from time-matched control or caffeine-treated antrum smooth muscle cells from CF-1 mice. 

C: representative spatiotemporal maps of intracellular Ca\(^{2+}\) waves constructed from time-matched control or caffeine-treated antrum smooth muscle cells from PLB-KO mice. 

D: summary graph showing the effect of caffeine (1 mM) on the frequencies of intracellular Ca\(^{2+}\) waves in antrum smooth muscle cells from CF-1 mice and PLB-KO mice. *P < 0.05.
However, Fig. 6B shows that caffeine prevented the decrease in frequency of intracellular Ca\(^{2+}\) waves caused by the second fluo-4 AM excitation in wild-type antrum smooth muscle cells (36 ± 4 waves/min in second time-matched control vs. 53 ± 5 waves/min with 1 mM caffeine, n = 7, P < 0.05). This value is similar to the value of 49 ± 5 waves/min obtained during the first exposure. These findings indicate that caffeine increased the intracellular Ca\(^{2+}\) wave frequency. Figure 6C shows that in PLB-KO antrum smooth muscle cells, the frequency of intracellular Ca\(^{2+}\) waves with caffeine treatment was similar to the second time-matched control (56 ± 5 waves/min in second time-matched control vs. 58 ± 6 waves/min with caffeine, n = 7). These frequencies of intracellular Ca\(^{2+}\) waves are higher than the frequency of Ca\(^{2+}\) waves induced by caffeine treatment of wild-type antrum smooth muscles (53 ± 5 waves/min). These findings suggest that caffeine did not further increase the frequency of intracellular Ca\(^{2+}\) waves because the frequency of intracellular Ca\(^{2+}\) waves in PLB-KO antrum smooth muscles is already elevated due to the absence of PLB in PLB-KO antrum smooth muscle cells. Figure 6D summarizes these findings.

DISCUSSION

The regulation of SR Ca\(^{2+}\) load by SERCA activity is an important factor regulating localized SR Ca\(^{2+}\) release events and smooth muscle contractility. In cardiac, skeletal, and smooth muscles, PLB phosphorylation/dephosphorylation states determine SERCA activity. PLB phosphorylation increases SERCA activity and elevates the SR Ca\(^{2+}\) load, which leads to elevated Ca\(^{2+}\) spark frequencies and amplitudes (43). Studies using PLB gene-targeted mice have directly shown that PLB is an important factor in regulating Ca\(^{2+}\) homeostasis and modulating smooth muscle contractility (32, 38, 43). We showed previously that the ryanodine receptor agonist caffeine hyperpolarized and relaxed gastric fundus smooth muscles and activated Ca\(^{2+}\) kinase II (19). This study also showed that PLB-Thr17 phosphorylation by CaM kinase II is involved in the mechanism of caffeine-induced relaxation of murine fundus smooth muscles (19). Thus the purpose of the present study was to use gene-targeted PLB-KO mice to directly determine the role of PLB in regulating mechanical responses, intracellular Ca\(^{2+}\) wave activity, and CaM kinase II activity in gastric antrum smooth muscles.

The PLB-KO mouse represents one limit on the modulation of SERCA activity, in which SERCA activity is not inhibited. In this study we used PLB-KO mice to address the significance of uninhibited SERCA activity on antrum smooth muscle contractility. Force development in PLB-KO antrum smooth muscles was increased, as was the frequency of spontaneous phasic contractions. The increased phasic contractile activity was associated with a higher average rising slope and faster decay constant of the phasic contractions. These findings are consistent with studies of hearts from PLB-KO mice showing that elevated SR Ca\(^{2+}\) levels lead to increased rates of contraction and relaxation due to increased rates of SR Ca\(^{2+}\) release and uptake (26, 38). However, the mechanism by which phasic contractile activity is increased by PLB-KO in smooth muscles may not be equivalent to cardiac muscle due to the predominant role of Ca\(^{2+}\)-induced Ca\(^{2+}\) release in cardiac contractility. For example, PLB-KO affects both steady-state and receptor-mediated SR Ca\(^{2+}\) release and uptake in the portal vein, a phasic vascular smooth muscle (40). However, in the present study the sensitivities of wild-type and PLB-KO antrum smooth muscles to bethanechol were similar. In contrast, the extent of caffeine-induced relaxation was diminished in PLB-KO antrum smooth muscles. These findings suggest that the presence of PLB is important for the modulation of antral smooth muscle basal tone and that in the absence of PLB, further adjustments to tone are blunted due to loss of modulation of SERCA activity. These findings support our previous study showing that caffeine relaxes gastric fundus smooth muscle through SERCA activation by PLB phosphorylation and CaM kinase II activation (19). However, PLB-KO mice lack the modulation of SERCA activity via the pathway of PLB phosphorylation by CaM kinase II, resulting in the loss of a key caffeine target and reducing the effect of caffeine on antrum smooth muscle tone. These findings are similar to the findings that the adenylyl cyclase activator forskolin increases Ca\(^{2+}\) sparks and transient BK currents from cerebral arteries of wild-type mice but had little effect on the already elevated frequency of Ca\(^{2+}\) sparks and transient BK currents from PLB-KO cerebral arteries (43). Furthermore, the findings that the CaM kinase II inhibitor KN-93 attenuated caffeine-induced relaxation by ~50% in wild-type antrum smooth muscles, but had only a slight effect in the PLB-KO smooth muscles, suggest that PLB phosphorylation and CaM kinase II activation are important for the mechanism of relaxation of murine antrum smooth muscles.

To further investigate whether the contractility differences between wild-type and PLB-KO antrum smooth muscles are due to altered SERCA activity, we examined the contractile responses of wild-type and PLB-KO antrum smooth muscles to the SERCA inhibitor CPA. CPA concentrations (2 μM, 5 μM) were used that do not result in complete inhibition of SERCA activity (27). CPA evoked a larger increase in tone of wild-type antrum smooth muscles. In addition, CPA was more effective at inhibiting the caffeine-induced relaxation of wild-type antrum smooth muscles. These findings suggest that the inhibitory effects of PLB and CPA on SERCA activity are additive, resulting in greater SERCA inhibition and slower rates of both Ca\(^{2+}\) clearance from the cytosol and localized Ca\(^{2+}\) release from the SR at a given CPA concentration.

The magnitude of autonomous CaM kinase II activity depends on the duration, amplitude, and frequency of Ca\(^{2+}\) transients (6). Thus different frequencies of Ca\(^{2+}\) transients can give rise to different levels of autonomous CaM kinase II activity. In the present study, PLB-KO antrum smooth muscles had a higher level of autonomous CaM kinase II activity. This finding is consistent with the findings that SR Ca\(^{2+}\) load and localized SR Ca\(^{2+}\) release are elevated in cardiac and smooth muscles from PLB-KO mice due to uninhibited SERCA activity (37, 43). In the present study, caffeine (1 mM) increased autonomous CaM kinase II activity in wild-type antrum smooth muscles but had no effect on the autonomous CaM kinase II activity in PLB-KO antrum smooth muscles. However, autonomous CaM kinase II activity was already high in PLB-KO antrum smooth muscles. These findings are consistent with previous reports in which the adenylyl cyclase activator forskolin had little effect on the already elevated frequency of Ca\(^{2+}\) sparks and transient BK currents from PLB-KO cerebral arteries but did increase Ca\(^{2+}\) sparks and
transient BK currents from wild-type control mice (43). These findings suggest that caffeine activates CaM kinase II by increasing the frequency of local Ca2+ release events from the SR in antrum smooth muscles. Similarly, the elevated CaM kinase II autonomous activity in PLB-KO antrum smooth muscles suggests that the frequency of local Ca2+ release events from the SR is increased; supporting the conclusion that PLB modulation of SERCA activity affects the pattern of localized SR Ca2+ release events.

Most studies of intracellular Ca2+ waves have been carried out using freshly dispersed single cells or intact vascular smooth muscle segments at room temperature (13, 15). However, Hennig et al. (12) demonstrated that intracellular Ca2+ waves also occur at the physiological temperature of 37°C in large intestine longitudinal smooth muscles. Several reports suggest that the role of intracellular Ca2+ waves in smooth muscles is similar to the role of Ca2+ sparks or Ca2+ puffs (2, 12, 15). The spatiotemporal recruitment of localized Ca2+ release events produces an intracellular Ca2+ wave that activates calcium-sensitive potassium channels (KCa) to cause membrane hyperpolarization (9, 10). On the other hand, intracellular Ca2+ waves have also been shown to activate ClCa
transients from wild-type control mice (43). These findings support the results of the CaM kinase II activity assays showing that caffeine treatment increased frequency of local SR Ca2+ release events from the SR and CaM kinase II activity in wild-type antrum smooth muscles.

In the basal state, the frequency of intracellular Ca2+ waves was higher in PLB-KO. These findings are consistent with previous reports in which basal Ca2+ sparks frequency was elevated in cardiac myocytes and cerebral arteries from PLB-KO mice compared with wild-type mice (37, 43) and provide a mechanism for increasing the basal autonomous CaM kinase II activity in PLB-KO. Caffeine increased the frequency of intracellular Ca2+ waves in wild-type antrum smooth muscles. However, the intracellular Ca2+ wave frequency in PLB-KO antrum smooth muscles was already elevated in the absence of caffeine and was not further increased by caffeine treatment, consistent with a previous report in which forskolin had little effect on Ca2+ sparks in PLB-KO cerebral arteries but did increase Ca2+ sparks from wild-type control mice (43). These findings suggest that PLB and CaM kinase II appear to be important components of the regulatory network of proteins that control SR Ca2+ load and local SR Ca2+ release events in murine gastric antrum smooth muscles. Although PLB may have additional physiological roles in gastrointestinal smooth muscles, the results from the present study indicate that modulation of SR Ca2+ uptake and release by PLB plays a key role in gastric antrum smooth muscle excitability and contractility.

**REFERENCES**