CaM kinase II activation and phospholamban phosphorylation by SNP in murine gastric antrum smooth muscles

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Kim M, Perrino BA. CaM kinase II activation and phospholamban phosphorylation by SNP in murine gastric antrum smooth muscles. Am J Physiol Gastrointest Liver Physiol 292: G1045–G1054, 2007. First published December 21, 2006; doi:10.1152/ajpgi.00203.2006.—Elevations in the intracellular Ca2+ concentration activate the serine/threonine protein kinase Ca2+/calmodulin-dependent protein kinase II (CaM kinase II). We tested the hypothesis that increased sarcoplasmic reticulum Ca2+-ATPase activity by phospholamban (PLB) phosphorylation contributes to smooth muscle relaxation by elevating the sarcoplasmic reticulum (SR) Ca2+ load and increasing the frequency of Ca2+ release events from the SR. We have previously shown that caffeine or sodium nitroprusside (SNP) relaxes murine gastric fundus smooth muscles and increases PLB phosphorylation by CaM kinase II. These findings suggest that an increased SR Ca2+ load increases the frequency of Ca2+ transients from the SR and results in PLB phosphorylation by CaM kinase II, contributing to caffeine- or SNP-induced relaxation. The aim of the present study was to investigate the effects of SNP on CaM kinase II and PLB phosphorylation in gastric antrum smooth muscles. SNP or 8-bromo-cGMP decreased the basal tone and amplitudes of spontaneous phasic contractions and activated CaM kinase II. SNP-induced relaxation and CaM kinase II activation were blocked by [1,2,4]oxadizolo-[4,3-a]quinoline-1-one (ODQ) and inhibited by cyclopiazonic acid (CPA) or KN-93. SNP also increased PLBSer16 and PLBThr17 phosphorylation. Both PLBSer16 and Thr17 phosphorylation were ODQ sensitive. However, only PLBThr17 phosphorylation was inhibited by CPA or KN-93. These results suggest that CaM kinase II activation and PLB phosphorylation participate in the relaxant effect of SNP on murine gastric antrum smooth muscles through a nitric oxide/guanylyl cyclase/cGMP pathway. Ca2+/calmodulin-dependent protein kinase II; phospholamban; nitric oxide

THE GASTRIC ANTRUM undergoes spontaneous peristaltic contractions to accomplish the grinding, mixing, and gastric emptying functions of this part of the stomach. Slow wave propagation from the greater curvature of the oral corpus to the pylorus produces gastric peristalsis. Phasic contractions initiated by slow waves are the basis for gastric peristalsis (25). The normal pattern of slow wave propagation results from a gradient in intrinsic frequencies along the longitudinal axis of the stomach. The oral corpus is the dominant pacemaker in the human stomach and in animal models such as the dog and mouse (12, 25, 38) because it generates slow waves at the fastest rate. Conditions that affect the intrinsic pacemaker frequency in the antrum or produce arrhythmic activity in this region can result in a breakdown in functional coupling between the corpus and antrum and interfere with normal gastric emptying (6, 26).

Insufficient gastric emptying produces pathophysiological problems such as functional dyspepsia, postsurgical gastroparesis, and diabetic gastroparesis (29, 51). Slow wave generation by myenteric interstitial cells of Cajal results in the activation of voltage-dependent Ca2+ channels and the generation of phasic contractions of antrum smooth muscle cells (9, 11, 39, 40). Thus, in addition to studies of the electrical coupling of myenteric interstitial cells of Cajal to smooth muscle cells, studies of the Ca2+-handling mechanisms of antrum smooth muscles will provide insights into gastric smooth muscle physiology and pathophysiology.

The relaxant effects of nitric oxide (NO) and NO donors on smooth muscles are well established, but many details of the mechanism of NO-induced relaxation are still under investigation (54). One possible mechanism by which NO relaxes smooth muscle is by hyperpolarization of the smooth muscle cell plasma membrane. Porter et al. (43) reported that the NO donor sodium nitroprusside (SNP) increases Ca2+ spark frequency and activates spontaneous transient outward currents (STOCs) via a NO/soluble guanylyl cyclase (sGC)/cGMP pathway in cerebral and coronary artery myocytes. Furthermore, SNP-induced relaxation of the rat gastric fundus is ryanojodine sensitive and involves small-conductance Ca2+-activated K+ (KCa) channels (15). Similarly, in guinea pig gastric antrum myocytes, SNP increases intermediate-conductance KCa current and enhances STOCs, which are sensitive to inositol (1,4,5)-trisphosphate receptor (IP3,R) inhibitors (58). These findings suggest that sarcoplasmic reticulum (SR) Ca2+ release plays a role in the nitricergic relaxation of smooth muscles.

In several smooth muscles, it has been shown that nitricergic relaxation involves lowering the intracellular Ca2+ concentration ([Ca2+]i) and elevating the SR Ca2+ concentration ([Ca2+]SR) by the activation of sarcoplasmic reticulum Ca2+-ATPase (SERCA) (7, 24, 42, 45, 55). SERCA activity is regulated by the SR membrane protein phospholamban (PLB). Phosphorylation of PLB by PKA or PKG at Ser16 or Ca2+/calmodulin-dependent protein kinase II (CaM kinase II) at Thr17 removes its inhibitory effects on SERCA, thereby enhancing Ca2+ uptake into the SR. The increased SERCA activity elevates the SR Ca2+ load and lowers cytosolic Ca2+ levels, resulting in relaxation (19, 24). The SR Ca2+ load is important in the regulation of the frequency of Ca2+ release events from the SR (5, 59). Higher levels of SR Ca2+ are correlated with increased Ca2+ spark frequencies, which lead to increased STOC frequencies, membrane potential hyperpolarization.
larization, and relaxation (3, 36, 49, 59). PLB transgenic animal studies (47, 57) have supported the relationship between SR Ca\(^{2+}\) load and Ca\(^{2+}\) spark frequency and suggest that PLB plays a key role in the regulation of SR Ca\(^{2+}\) load and local Ca\(^{2+}\)-release events from the SR in smooth muscles. These findings suggest that CaM kinase II activation and PLB phosphorylation due to an increase in SR Ca\(^{2+}\)-release events could be involved in NO- or NO donor-induced relaxation of smooth muscles.

We (27, 28) have previously shown that 1 mM caffeine or 10 nM SNP relaxes gastric fundus smooth muscle strips in vitro. These studies (27, 28) also showed that CaM kinase II activation by caffeine or SNP in murine gastric fundus smooth muscles is blocked by SR Ca\(^{2+}\) channel inhibitors and suggested that PLB phosphorylation by CaM kinase II is involved in the relaxation induced by caffeine or SNP. The purpose of the present study was to extend these studies to a portion of the stomach that exhibits spontaneous phasic contractions and to investigate the roles of CaM kinase II and PLB phosphorylation in SNP-induced relaxation of murine antrum smooth muscles.

**METHODS**

*Tissue preparation for the CaM kinase II assay.* Gastric antrum smooth muscles were obtained from adult CD-1 mice (6–8 wk old, Charles River Laboratories, Wilmington, MA). Animals were anesthetized by isofluorane inhalation and euthanized by decapitation. Mice were maintained and 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 Institutional Animal Care and Use Committee. The gastric antrum tissues were pinned out in a Sylgard-lined dish containing oxygenated Krebs solution (120 mM NaCl, 6 mM KCl, 15 mM NaHCO\(_3\), 12 mM glucose, 3 mM MgCl\(_2\), 1.5 mM NaH\(_2\)PO\(_4\), and 3.5 mM CaCl\(_2\), pH 7.2). Mucosa and submucosa layers were removed using fine-tipped forceps. To determine the effects of SNP or 8-bromo-cGMP on CaM kinase II activity, tissues 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. To determine the effects of various blockers on SNP-induced CaM kinase II activity, tissues were equilibrated in Krebs buffer for 45 min at 37°C and then incubated at 37°C in the presence of each blocker for 20 min, followed by further incubations with SNP for 15 min in the presence of each blocker. After treatment, tissues were 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 Na\(_3\)P\(_2\)O\(_7\), 100 mM NaF, 250 mM NaCl, 3 mM EGTA, 1 mM DTT, 1 mM PMSF, and protease inhibitors) and centrifuged at 16,000 g for 15 min at 4°C, and the supernatant was aliquotted and stored at −80°C. Protein concentrations were determined using the Bradford assay with bovine \(\gamma\)-globulin as the standard.

*CaM kinase II activity assays.* CaM kinase II activity in the lysates was assayed in a total volume of 30 μl containing 50 mM HEPES (pH 7.4), 10 mM magnesium acetate, 0.2 mM \([\gamma-\text{P}]\)ATP (500–1,000 counts·min\(^{-1}\)·pmol\(^{-1}\)) (MP Biomedicals, Irvine, CA), and 20 μM autocamtide-2 (a specific CaM kinase II peptide substrate, KKLRRQETVDAL) (BioMol, Plymouth Meeting, PA) plus 600 nM CaM (EMD Biosciences, La Jolla, CA) and 0.8 mM CaCl\(_2\) (for total activity) or 1.0 mM EGTA (for autonomous activity) as previously described (27, 28). 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 and expressed as nanomoles of Pi, incorporated per minute per milligram of lysate protein.

*Mechanical responses of gastric antrum smooth muscles.* Standard organ bath techniques were employed to measure the changes in force provided by antrum smooth muscle strips (~6 × 3 mm). One end of each smooth muscle strip was attached to a fixed mount, and the opposite end of each muscle strip was attached to a Fort 10 isometric strain gauge (WPI, Sarasota, FL) in parallel with the circular smooth muscle layer. Muscle strips were immersed in organ baths containing Krebs buffer (3 ml) maintained at 37 ± 0.5°C. The pH was kept constant at 7.4 by bubbling the Krebs solution with 97% O\(_2\)-3% CO\(_2\). A resting force of 6 mN was applied to set the muscles at optimum length. This was followed by an equilibration period of at least 1 h, during which time the bath was continuously perfused with oxygenated Krebs solution. Following the equilibration period, muscle strips were incubated in Krebs solution containing the compounds as indicated in the figures. Mechanical responses were recorded on a personal computer running Acqknowledge 3.2.6 (BIOPAC Systems, Santa Barbara, CA).

*SDS-PAGE and Western blot analysis of PLB.* Phospho-Thr\(^{17}\) and phospho-Ser\(^{16}\) from gastric antrum smooth muscle SR fractions. Gastric antrum smooth muscles obtained from adult CD-1 mice (as described above) were placed into the following groups: oxygenated Krebs solution treated (control), SNP treated, or SNP treated in the presence of each blocker. All antrum smooth muscle strips were equilibrated in oxygenated Krebs buffer for 45 min at 37°C. For SNP treatment, smooth muscle strips were then perfused with oxygenated Krebs buffer containing SNP for 15 min at 37°C. To determine the effects of the various blockers, smooth muscle strips were perfused with oxygenated Krebs buffer containing the appropriate blocker for 20 min, followed by further incubations with SNP for 15 min in the presence of the appropriate blocker. Tissues were collected, frozen in liquid nitrogen, and stored at −80°C. Gastric antrum smooth muscle SR fractions were obtained from CD-1 mice as previously described (21). Briefly, frozen tissues were homogenized at 4°C with a glass tissue grinder in 6 volumes by weight of ice-cold lysis buffer [10 mM Tris·HCl (pH 6.8), 100 mM NaF, 20 mM sodium pyrophosphate, and protease inhibitor tablet], the homogenate was centrifuged at 1,000 g for 10 min at 4°C, and the supernatant was decanted and placed on ice. The pellet was resuspended in 4 volumes by weight of ice-cold lysis buffer and spun at 1,000 g for 10 min at 4°C. The two supernatants were combined and spun at 8,000 g for 20 min at 4°C. The supernatant was brought to a total volume of 2 ml with lysis buffer. Solid KCl was added to a final concentration of 0.6 M, and samples were placed on ice for 25 min. Samples were then centrifuged at 40,000 g for 60 min at 4°C to pellet the SR fraction. Pellets were resuspended in 200 μl of SR buffer [10 mM Tris·HCl (pH 6.8), 100 mM KCl, 100 mM NaF, 20 mM sodium pyrophosphate, and protease inhibitor tablet] and stored at −80°C. Protein concentrations were determined with the Bradford assay using bovine \(\gamma\)-globulin as the standard. Smooth muscle SR proteins were separated by SDS-PAGE (15% high-salt gel) and transferred to nitrocellulose membranes by Western blotting. Blots were incubated with primary and secondary antibodies, washed, and processed for enhanced chemiluminescence image detection using ECL Advantage (Amersham Biosciences, Piscataway, NJ). PLB-Po2-Thr\(^{17}\), PLB-Po2-Ser\(^{16}\), and PLB antibodies (Santa Cruz Biotechnolgy, Santa Cruz, CA) were used at 1:5,000 dilution, and horseradish-peroxidase-conjugated secondary antibody (Chemicon, Temecula, CA) was used at 1:50,000 dilution. Protein bands were visualized with a charge-coupled device camera-based detection system (Epi Chem II, UVP Laboratory Products). Collected TIFF images were analyzed using Adobe Photoshop. The Western blot data shown in each figure are representative of four separate experiments. Densitometry of the immunostained phospho-PLB and PLB protein bands was carried out with Un-Scan-It software (Silk Scientific, Orem, UT) using lane analysis for background subtraction. Signal intensities of anti-phospho-
PLBSer\textsuperscript{16} and anti-phospho-PLBThr\textsuperscript{17} immunoblots from 1 mM cGMP-treated antrum smooth muscle tissues were normalized to 1 \((n = 5)\). The ratio of SNP-evoked PLB phosphorylation to cGMP-evoked PLB phosphorylation was determined by densitometry of the signal intensities of the immunoblots.

**Materials.** The following drugs were used: TTX, iberiotoxin (IbTX), and apamin were obtained from Sigma (St. Louis, MO). Cyclopiazonic acid (CPA) was purchased from BioMol. KN-93, 8-bromo-cGMP, 1H-[1,2,4]oxadiazolo-[4,3-H]-quinoxalin-1-one (ODQ), and SNP were purchased from EMD Bioscience (La Jolla, CA). 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 \pm 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**

Effect of SNP on mechanical responses of murine antrum smooth muscles. As shown in Fig. 1A, 10 \(\mu\)M SNP reduced the average amplitude of contractions from 1.4 \pm 0.6 to 0.16 \pm 0.05 mN \((n = 8, \ P < 0.001)\), representing an 89 \% inhibition. SNP (10 \(\mu\)M) also decreased the basal tone by 0.6 \pm 0.1 mN \((n = 8, \ P < 0.001)\). The effects of SNP on the mechanical responses of the antrum smooth muscle strips were fully reversible upon washout. In addition, the effects of SNP on the mechanical activity of antrum smooth muscle strips were TTX (1 \(\mu\)M) insensitive (data not shown), indicating that the effects of SNP on the mechanical responses are myogenic in origin.

Effects of \(K_{Ca}\) channel blockers on SNP-induced mechanical responses of antrum smooth muscles. Since we (28) have previously reported that large-conductance \(K_{Ca}\) channels and small-conductance \(K_{Ca}\) channels are involved in the SNP-induced relaxation of gastric fundus smooth muscles, the effects of the large-conductance \(K_{Ca}\) channel inhibitor IbTX (100 nM) and the small-conductance \(K_{Ca}\) channel blocker apamin (300 nM) on SNP-induced mechanical responses were investigated in antrum smooth muscles. As shown in Fig. 1A, 10 \(\mu\)M SNP decreased the basal tone by 0.6 \pm 0.1 mN and almost completely abolished spontaneous phasic contractions. In the presence of IbTX, small spontaneous contractions were measured during treatment with SNP. The amplitudes of the spontaneous contractions decreased from 1.8 \pm 0.5 mN in the absence of SNP to 0.4 \pm 0.08 mN in the presence of both SNP and IbTX (i.e., contractile amplitudes were decreased to 20 \% of the original value, \(n = 4, \ P < 0.05\)). The frequency of the phasic contractions in the presence of IbTX and SNP (3.5 \pm 0.7 cycles/min) was slightly lower than the frequency of phasic contractions in the absence of IbTX and SNP (4.5 \pm 0.7 cycles/min, \(n = 4\)). IbTX alone increased the basal muscle tone by 0.11 \pm 0.06 mN without changing the amplitudes and frequency of the spontaneous contractions (Fig. 1A). In the presence of IbTX, the SNP-induced decrease in basal tone was not significantly changed compared with SNP alone (Fig. 1A).

As shown in Fig. 1B, apamin alone had no effect on the frequency of phasic contractions but did increase the antrum muscle tone by 0.40 \pm 0.1 mN \((n = 6, \ P < 0.001)\) and increased the frequencies of the spontaneous contractions from 1.3 \pm 0.4 to 2.1 \pm 0.2 mN \((n = 6, \ P < 0.05)\). In the absence of apamin, SNP decreased the contractile amplitudes from 1.3 \pm 0.4 mN to 0.21 \pm 0.07 mN (i.e., contractile amplitude was decreased to 16 \% of the original value, \(n = 6, \ P < 0.05\)). In the presence of apamin, SNP decreased the ampli-

![Fig. 1. Effects of large- and small-conductance \(Ca^{2+}\)-activated \(K^{+}\) channel blockers on sodium nitroprusside (SNP)-induced relaxation of gastric antrum smooth muscle strips. A and B: representative traces of the changes in mechanical responses of gastric antrum smooth muscle strips incubated with 10 \(\mu\)M SNP alone followed by incubation with 10 \(\mu\)M SNP and 100 nM iberiotoxin (IbTX; A) or with 10 \(\mu\)M SNP alone followed by incubation with 10 \(\mu\)M SNP and 300 nM apamin (B).](http://ajpgi.physiology.org/ "Downloaded from http://ajpgi.physiology.org/ by 10.220.33.3 on June 24, 2017")
since we (28) have previously reported antrum smooth muscles. Not affect the SNP-induced relaxation of basal tone (that SNP-induced SR Ca²⁺ release activates CaM kinase II in gastric antrum smooth muscle tissues were 8.7 ± 1.4 and 8.2 ± 0.7 nmol·min⁻¹·mg⁻¹, respectively (n = 6). However, as shown in Fig. 2A, 10 μM SNP increased autonomous CaM kinase II activity from 2.7 ± 0.2 to 3.6 ± 0.3 nmol·min⁻¹·mg⁻¹ (n = 6, P < 0.001). This represents an increase in autonomous CaM kinase II activity from 33% to 44% of the total CaM kinase II activity. KN-93 (10 μM), the inhibitor of Ca²⁺/CaM binding to CaM kinase II, prevented the SNP-induced increase in ³²P incorporation into the autocalmodulin-2 peptide substrate, supporting the conclusion that the kinase activity measured in response to SNP is due to CaM kinase II. As shown in Fig. 2B, by itself KN-93 decreased the muscle tone by 0.23 ± 0.06 mN but inhibited the SNP-induced reduction in basal tone by 84 ± 5% (n = 5, P < 0.001). In contrast, KN-93 did not affect the SNP-induced reduction in the amplitudes of phasic contractions but delayed the recovery of spontaneous phasic contractions (Fig. 2B).

Effects of CPA on SNP-induced mechanical responses and CaM kinase II activation. Since SR Ca²⁺ release is influenced by the SERCA inhibitor CPA (10 μM) was used to disrupt SR Ca²⁺ levels. As shown in Fig. 3A, CPA alone evoked a rapid increase in the basal tone of the antrum smooth muscle strips of 1.2 ± 0.2 mN and induced a gradual decrease in the frequency and amplitudes of phasic contractions. The frequency of phasic contractions decreased from 4.2 ± 0.6 to 1.7 ± 0.5 cycles/min, and the amplitudes of the phasic contractions decreased from 1.3 ± 0.6 to 0.4 ± 0.1 mN. CPA had no effect on the SNP-induced decrease in basal tone. In the presence of 10 μM CPA, SNP reduced the tone by 0.65 ± 0.2 mN, whereas in the absence of CPA, 10 μM SNP decreased the basal tone by 0.6 ± 0.1 mN. However, in contrast to the results obtained with 10 μM SNP alone, the inhibition of phasic contractions by SNP was attenuated in the presence of CPA. Instead of almost complete inhibition, the amplitudes of the phasic contractions were 0.8 ± 0.2 mN (n = 5). The frequency of these phasic contractions was 1.8 ± 0.5 cycles/min (Fig. 3A). Similar results were obtained when the order of addition of SNP and CPA was reversed. A recovery of spontaneous contractions occurred when CPA was added to smooth muscle strips incubated with SNP (Fig. 3B). The amplitudes of these phasic contractions were 1.0 ± 0.1 mN, and the frequency was 2.4 ± 0.6 cycles/min (n = 6). As shown in Fig. 3C, in contrast to the results obtained with 10 μM SNP alone, autonomous CaM kinase II activity was not increased by 10 μM SNP in the presence of CPA. CPA alone had no effect on autonomous CaM kinase II activity compared with control. Autonomous CaM kinase II activity measured as described in METHODS. Average values ± SD of autonomous CaM kinase II activities were obtained from gastric antrum smooth muscle strips incubated without (control) or with 10 μM SNP for 15 min, with 10 μM KN-93 for 20 min followed by 10 μM SNP with KN-93 for 15 min, or with 10 μM KN-93 alone for 20 min. **P < 0.001. B: representative traces of the changes in mechanical responses of gastric antrum smooth muscle strips incubated with 10 μM SNP alone or with 10 μM SNP in the presence of 10 μM KN-93.
activities in control and antrum smooth muscle tissues treated with SNP in the presence of CPA were 2.5 ± 0.2 and 2.2 ± 0.6 nmol·min⁻¹·mg⁻¹, respectively (n = 6). In contrast, autonomous CaM kinase II activities in antrum smooth muscle tissues treated with 10 μM SNP alone were 3.7 ± 0.3 nmol·min⁻¹·mg⁻¹ (n = 6).

Role of the NO/sGC/cGMP pathway in SNP-induced mechanical responses and CaM kinase II activation. Since we (28) have previously reported that the sGC inhibitor ODQ prevented SNP-induced relaxation and CaM kinase II activation in gastric fundus smooth muscles, the effect of ODQ on SNP-induced relaxation and CaM kinase activation in antrum smooth muscle strips was examined. As shown in Fig. 4A, ODQ (10 μM) alone had no effect on the amplitudes of phasic contractions (from 1.3 ± 0.5 to 1.1 ± 0.2 mN, n = 4) but decreased the frequency of phasic contractions from 4.3 ± 0.4 to 2.8 ± 0.9 cycles/min (n = 4, P < 0.001). In contrast to the results obtained with 10 μM SNP alone (Fig. 4A), 10 μM SNP in the presence of ODQ did not decrease the frequency or amplitudes of phasic contraction, suggesting that the effects of SNP on the frequency and amplitudes of phasic contraction are mediated by a NO/sGC pathway in antrum smooth muscles. In support of these findings, as shown in Fig. 4B, spontaneous phasic contractions were almost completely abolished by treatment of the antrum smooth muscle strips with the membrane-permeable cGMP analog 8-bromo-cGMP (1 mM, n = 4). In addition, in contrast to the results obtained with 10 μM SNP alone, autonomous CaM kinase II activity was not increased by 10 μM SNP in the presence of ODQ (Fig. 4C). ODQ alone had no effect on autonomous CaM kinase II activity compared with control. Autonomous CaM kinase II activities in control and antrum smooth muscle tissues treated with SNP in the presence of ODQ were 2.4 ± 0.2 and 2.6 ± 0.1 nmol·min⁻¹·mg⁻¹, respectively (n = 5). In contrast, autonomous CaM kinase II activities in antrum smooth muscle tissues treated with 10 μM SNP alone were 3.3 ± 0.3 nmol·min⁻¹·mg⁻¹ (n = 5). Furthermore, 1 mM 8-bromo-cGMP also increased autonomous CaM kinase II activity from 2.4 ± 0.3 to 3.4 ± 0.3 nmol·min⁻¹·mg⁻¹ (n = 5, P < 0.001).

Effect of SNP-induced CaM kinase II activation on PLB phosphorylation. We (28) have previously found that SNP increases PLB phosphorylation in gastric fundus smooth muscles, occurring at both Ser¹⁶ and Thr¹⁷. Thus, we examined PLB phosphorylation in SNP-treated antrum smooth muscles by Western blot analysis using antibodies specific for phospho-Thr¹⁷ or phospho-Ser¹⁸ of PLB. As shown in Fig. 5A, treatment of antrum smooth muscle strips with 10 μM SNP increased PLBSer¹⁸ phosphorylation. KN-93 (10 μM) or CPA (10 μM)
had no effect on the SNP-induced increase in PLBSer\textsuperscript{16} phosphorylation. In contrast, ODQ (10 \( \mu \)M) prevented the SNP-induced increase in PLBSer\textsuperscript{16} phosphorylation. As shown in Fig. 5B, treatment of antrum smooth muscle strips with 10 \( \mu \)M SNP also increased PLBThr\textsuperscript{17} phosphorylation. However, in contrast to the anti-phospho-PLBSer\textsuperscript{16} results, KN-93 (10 \( \mu \)M) or CPA (10 \( \mu \)M) prevented the SNP-induced increase in PLBThr\textsuperscript{17} phosphorylation. PLBSer\textsuperscript{16} and PLBThr\textsuperscript{17} phosphorylation were also increased by 1 mM 8-bromo-cGMP (Fig. 5, C and D). The ratio of SNP-evoked PLB phosphorylation to cGMP-evoked PLB phosphorylation was determined by densitometry of the signal intensities of the immunoblots. Signal intensities of anti-phospho-PLBSer\textsuperscript{16} immunoblots from antrum smooth muscle tissues treated with 10 \( \mu \)M SNP increased to 92 \( \pm \) 7% of the signal from cGMP-evoked anti-phospho-PLBSer\textsuperscript{16} immunoblots (\( n = 3 \)). Similarly, signal intensities of anti-phospho-PLBThr\textsuperscript{17} immunoblots from antrum smooth muscle tissues treated with 10 \( \mu \)M SNP increased to 85 \( \pm \) 4% of the signal from cGMP-evoked anti-phospho-PLBThr\textsuperscript{17} immunoblots (\( n = 4 \)).

DISCUSSION

NO relaxes smooth muscles by activating sGC and increasing cGMP levels, but the signaling cascades that ultimately trigger relaxation appear to vary depending on the smooth muscle type (2, 54, 58). Several mechanisms by which NO and cGMP relax smooth muscle have been investigated: 1) IP\textsubscript{3}R-associated cGMP kinase substrate (IRAG) phosphorylation by PKG (13); 2) a decrease in \([Ca^{2+}]_i\) sensitivity of the contractile apparatus (37, 49); 3) hyperpolarization of the smooth muscle cell membrane (48, 52); and 4) lowering of \([Ca^{2+}]_i\) and elevation of \([Ca^{2+}]_{SR}\) by SERCA pump activation (7, 8). In the present study, we focused on the possible mechanism of
increased SR Ca\(^{2+}\) release due to elevated \([\text{Ca}^{2+}]_\text{SR}\) and investigated the effects of SNP on mechanical responses, CaM kinase II activity, and PLB phosphorylation in murine gastric antrum smooth muscles.

The NO donor SNP (10 \(\mu\)M) almost completely abolished the spontaneous contractions of murine antrum smooth muscle strips. The amplitudes of spontaneous contractions were reduced by 90%. The involvement of SR Ca\(^{2+}\) levels in the relaxation of antrum smooth muscles by SNP is suggested by the results shown in Fig. 3 demonstrating that the SERCA inhibitor CPA partially inhibited SNP-induced relaxation. Lowering the level of SR Ca\(^{2+}\) by SERCA pump inhibition is correlated with decreased frequencies of Ca\(^{2+}\) sparks and a resistance to relaxation (3, 36, 49, 59). The amplitudes of the spontaneous contractions observed in the presence of CPA and SNP were fivefold higher than the amplitudes measured in the presence of SNP alone. The frequency of spontaneous contractions in the presence of CPA and SNP was 20% of the frequency observed in untreated antrum smooth muscle strips. Similar results were obtained when we incubated antrum smooth muscle strips with SNP and then added CPA. A recovery of spontaneous phasic contractions occurred when CPA was added to antrum smooth muscle strips in the presence of SNP. Studies (22, 23) in other muscle types have indicated that SERCA activity influences contractile behavior by modulating the rate of Ca\(^{2+}\) clearance from the cytosol and the rate of subsequent SR Ca\(^{2+}\) release. When cytosolic Ca\(^{2+}\) removal is inhibited by CPA, more Ca\(^{2+}\) is available for contraction (31). In addition, when SR Ca\(^{2+}\) refilling is inhibited by CPA, less Ca\(^{2+}\) is available for release (20). The findings shown in Fig. 3 suggest that SERCA pump activity is involved in the SNP-induced relaxation of antrum smooth muscle. However, the lack of complete inhibition of SNP-induced relaxation by CPA indicates that parallel cGMP-dependent pathways, such as increased myosin light chain phosphatase activity due to PKG inhibition of RhoA pathways, are likely contributing to the relaxation (35).

Localized Ca\(^{2+}\) transient release from intracellular stores can activate K\(_{\text{Ca}}\) channels in the plasma membrane where the SR and plasma membranes are in close apposition (12–20 nm) (56). Porter et al. (43) showed that SNP increased Ca\(^{2+}\) spark frequency twofold and STOC activities in isolated rat cerebral and coronary arterial smooth muscle cells. Thus, we treated antrum smooth muscle strips with IbTX or apamin to examine whether large- or small-conductance K\(_{\text{Ca}}\) channels were involved in mechanical responses to SNP. IbTX alone slightly increased the basal tone with no changes in the contractile amplitude and frequency of spontaneous phasic contractions, whereas apamin evoked larger increases in the basal tone and amplitudes of phasic contractions. These results suggest that small-conductance K\(_{\text{Ca}}\) channels may be more important than large-conductance K\(_{\text{Ca}}\) channels in setting the basal tone and amplitudes of phasic contractions of antral smooth muscle. These findings are different from our previous report (28), in which large-conductance K\(_{\text{Ca}}\) channels were more involved in setting the basal fundus tone. As shown in Fig. 1, neither K\(_{\text{Ca}}\) channel blocker was particularly effective at attenuating the inhibitory effects of SNP on the contractile activity of antrum smooth muscle strips, although small but measurable phasic contractions could be observed in strips treated with SNP in the presence of either K\(_{\text{Ca}}\) channel blocker. These results are consistent with findings from other smooth muscle tissues showing that removal of cytosolic Ca\(^{2+}\) by increased SERCA activity is more important than K\(_{\text{Ca}}\) channel activation in NO-induced relaxations (7).

CaM kinase II activation is dependent on the duration, amplitude, and frequency of Ca\(^{2+}\) oscillations (10, 14, 34). Although bulk increases in cytosolic Ca\(^{2+}\) can activate CaM kinase II, frequency-dependent activation by transient Ca\(^{2+}\) release resulting in localized high Ca\(^{2+}\) concentrations are likely to activate nearby CaM kinase II (1). Evidence from studies (1, 27, 28, 41) of tonic vascular and gastric fundus smooth muscles have suggested that Ca\(^{2+}\)-release events from the SR (Ca\(^{2+}\) sparks or puffs) activate CaM kinase II. These findings led us to investigate CaM kinase II activation by SR Ca\(^{2+}\) release in gastric smooth muscles that undergo spontaneous phasic contractions. The results shown in Fig. 2 demonstrate that incubation of gastric antrum smooth muscle strips

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**Fig. 5.** SNP increases phospholamban (PLB) phosphorylation at Ser\(^{16}\) and Thr\(^{17}\). SDS-PAGE and immunoblot analysis using phospho-PLBSer\(^{16}\) (P-Ser\(^{16}\)) and phospho-PLBThr\(^{17}\) (P-Thr\(^{17}\)) antibodies were carried out as described in METHODS. A and B: representative immunoblots of P-Ser\(^{16}\) (A) or P-Thr\(^{17}\) (B) from the sarcoplasmic reticulum (SR)-enriched fraction of gastric antrum smooth muscle strips incubated without (control) or with 10 \(\mu\)M SNP for 15 min, with 10 \(\mu\)M KN-93 for 20 min followed by 10 \(\mu\)M SNP with KN-93 for 15 min, or with 10 \(\mu\)M ODQ for 20 min followed by 10 \(\mu\)M SNP with ODQ for 15 min. C and D: representative immunoblots of P-Ser\(^{16}\) (C) or P-Thr\(^{17}\) (D) from the SR-enriched fraction of gastric antrum smooth muscle strips incubated without (control) or with 1 mM 8-Br-cGMP for 15 min. Each lane contained 50 \(\mu\)g protein. **Bottom** blots in A–D show samples immunostained with the anti-PLB antibody as an additional control for equal protein loading.
with 10 μM SNP activated CaM kinase II, as indicated by the increase in autonomous CaM kinase II activity. The CaM kinase II inhibitor KN-93 prevented CaM kinase II activation by SNP, indicating that the increase in kinase activity measured in lysates from SNP-treated antrum smooth muscle strips is due to CaM kinase II. The results shown in Fig. 3 demonstrate that CPA inhibited SNP-induced relaxation of antrum smooth muscle strips and suggest that SNP evokes SR Ca\(^{2+}\) release. Similarly, CPA prevented the activation of CaM kinase II by SNP. The lack of CaM kinase II activation by SNP in the presence of CPA is consistent with previous reports (5, 20, 47, 59) showing that decreasing the SR Ca\(^{2+}\) load by blockade of SERCA decreases the concentration of Ca\(^{2+}\) released from the SR.

Cardiac, skeletal, and smooth muscle SERCA activity is regulated by the integral SR membrane protein PLB (4, 44, 46, 50). Dephosphorylated PLB inhibits SERCA activity by 50% (53), PLB phosphorylation by PKA or PKG at Ser\(^{16}\), or CaM kinase II at Thr\(^{17}\), relieves its inhibitory effect on the SERCA pump, enhancing SERCA activity and contributing to relaxation by decreasing [Ca\(^{2+}\)], and increasing [Ca\(^{2+}\)]\(_{SR}\) (30). The Western blot analysis results shown in Fig. 5 demonstrate that the sGC inhibitor ODQ prevented the SNP-induced increase in PLBSer\(^{16}\) phosphorylation, suggesting that PLBSer\(^{16}\) phosphorylation is due to PKG activation by cGMP. KN-93 or CPA had no effect on PLBSer\(^{16}\) phosphorylation, indicating that the SNP-induced increase in PLBSer\(^{16}\) is not due to CaM kinase II and is not affected by the SR Ca\(^{2+}\) load. The results shown in Fig. 5 also demonstrate that PLBThr\(^{17}\) phosphorylation also increased in response to SNP and was inhibited by KN-93 or CPA. These findings are consistent with the results shown in Figs. 2 and 3, which demonstrated that KN-93 or CPA prevented CaM kinase II activation by SNP, and further strengthen the conclusion that CaM kinase II activation by SNP increased PLBThr\(^{17}\) phosphorylation. The SNP-induced increase in PLBThr\(^{17}\) phosphorylation was also prevented by ODQ, suggesting that PKG activation by the NO/sGC/cGMP pathway is a prerequisite for CaM kinase II activation. The levels of SNP-evoked PLB phosphorylation were comparable to the levels of PLB phosphorylation evoked by 1 mM cGMP. Signal intensities of anti-phospho-PLBSer\(^{16}\) and anti-phospho-PLBThr\(^{17}\) immunoblots from untreated antrum smooth muscle tissues increased from 31% to 92% and 85%, respectively, of the signal intensities of anti-phospho-PLBSer\(^{16}\) and anti-phospho-PLBThr\(^{17}\) immunoblots from cGMP-treated tissues. These findings suggest that the ODQ-sensitive PLB phosphorylation in antrum smooth muscles due to SNP is physiologically relevant. Furthermore, these findings are similar to those of a study (33) investigating β-adrenergic-induced PLB phosphorylation in the heart, which indicated about a fourfold increase in PLBSer\(^{16}\) phosphorylation.

The physiological role of CaM kinase II activation in antrum smooth muscles is not fully known. The CaM kinase II inhibitor KN-93 inhibited the relaxation of gastric fundus smooth muscle strips (27, 28). The results shown in Fig. 2 demonstrate that KN-93 also inhibited the SNP-induced decrease in basal tone of gastric antrum smooth muscle strips. KN-93 had no effect on the SNP-induced inhibition of phasic contractions but lengthened the time for recovery of spontaneous phasic contractions. The findings shown in Fig. 3 demonstrate that basal tone increased and phasic contractions were partially restored with CPA, suggesting that cytosolic Ca\(^{2+}\) is important for spontaneous contractions. Although CaM kinase II activity is inhibited by KN-93, PKG activity is not, and phosphorylation of PLB by PKG may be more important during SNP treatment. In cardiac myocytes, Ca\(^{2+}\) transients modulate the phosphorylation of PLB by CaM kinase II and, thus, SERCA pump activity and SR Ca\(^{2+}\) load (17). The ability of CaM kinase II to phosphorylate PLBThr\(^{17}\) in response to different stimulation frequencies warrants further investigations into the modulation of smooth muscle contractility by differential activation by CaM kinase II (17). In neuronal and cardiac tissues, autonomous CaM kinase II (Ca\(^{2+}\)-independent) activity persists after the initial Ca\(^{2+}\) stimulus has ceased (16, 32). Similarly, its Ca\(^{2+}\)-independent activity in antrum smooth muscles may provide a mechanism for prolonging the effects of NO/cGMP on SERCA activity after the cGMP and Ca\(^{2+}\) signals have ceased. Additional studies are underway to determine the respective contributions of SERCA activity, PLB phosphorylation, and CaM kinase II in the response of antrum smooth muscles to nitricergic relaxation.

In summary, the results of the present study suggest that the relaxant effect of SNP on antrum smooth muscles involves PLB phosphorylation by PKG through a NO/sGC/cGMP pathway. These results also suggest that the activation of CaM kinase II by SNP involves increased Ca\(^{2+}\)-release events from the SR through PLB phosphorylation by PKG and indicate that CaM kinase II may also play a role in the relaxant effect of SNP by phosphorylation of PLB at Thr\(^{17}\). Furthermore, these findings demonstrate a novel link between nitricergic and Ca\(^{2+}\)/calmodulin-dependent signaling pathways in murine antrum smooth muscle tissues.

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