Roles of CaM kinase II and phospholamban in SNP-induced relaxation of murine gastric fundus smooth muscles

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Submitted 5 August 2005; accepted in final form 22 February 2006

Kim, Minkyung, In Soo Han, Sang Don Koh, and Brian A. Perrino. Roles of CaM kinase II and phospholamban in SNP-induced relaxation of murine gastric fundus smooth muscles. Am J Physiol Cell Physiol 291: C337–C347, 2006. First published March 1, 2006; doi:10.1152/ajpcell.00397.2005.—The mechanisms by which nitric oxide (NO) relaxes smooth muscles are unclear. The NO donor sodium nitroprusside (SNP) has been reported to increase the Ca\(^{2+}\) release frequency (Ca\(^{2+}\) sparks) through ryanodine receptors (RyRs) and activate spontaneous transient outward currents (STOCs), resulting in smooth muscle relaxation. Our findings that caffeine relaxes and hyperpolarizes murine gastric fundus smooth muscles and increases phospholamban (PLB) phosphorylation by Ca\(^{2+}\)/calmodulin (CaM)-dependent protein kinase II (CaM kinase II) suggest that PLB phosphorylation by CaM kinase II participates in smooth muscle relaxation by increasing sarcoplasmic reticulum (SR) Ca\(^{2+}\) uptake and the frequencies of SR Ca\(^{2+}\) release events and STOCs. Thus, in the present study, we investigated the roles of CaM kinase II and PLB in SNP-induced relaxation of murine gastric fundus smooth muscles. SNP hyperpolarized and relaxed gastric fundus circular smooth muscles and activated CaM kinase II. SNP-induced CaM kinase II activation was prevented by KN-93. Ryanodine, tetracaine, 2-aminoethoxydiphenylborate, and cyclopiazonic acid inhibited SNP-induced fundus smooth muscle relaxation and CaM kinase II activation. The Ca\(^{2+}\)-activated K\(^{+}\) channel blockers iberiotoxin and apamin inhibited SNP-induced hyperpolarization and relaxation. The soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo-[4,3-a]quinolin-1-one inhibited SNP-induced relaxation and CaM kinase II activation. The membrane-permeable cGMP analog 8-bromo-cGMP relaxed gastric fundus smooth muscles and activated CaM kinase II. SNP-induced CaM kinase II activation was prevented by KN-93. Ryanodine, tetracaine, 2-aminoethoxydiphenylborate, and cyclopiazonic acid inhibited SNP-induced fundus smooth muscle relaxation and CaM kinase II activation. The Ca\(^{2+}\)-activated K\(^{+}\) channel blockers iberiotoxin and apamin inhibited SNP-induced hyperpolarization and relaxation. The soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo-[4,3-a]quinolin-1-one inhibited SNP-induced relaxation and CaM kinase II activation. The membrane-permeable cGMP analog 8-bromo-cGMP relaxed gastric fundus smooth muscles and activated CaM kinase II. SNP increased phosphorylation of PLB at Ser\(^{16}\) and Thr\(^{17}\). Thr\(^{17}\) phosphorylation of PLB was inhibited by cyclopiazonic acid and KN-93. Ser\(^{16}\) and Thr\(^{17}\) phosphorylation of PLB was sensitive to 1H-[1,2,4]oxadiazolo-[4,3-a]quinolin-1-one. These results demonstrate a novel pathway linking the NO-soluble guanylyl cyclase-cGMP pathway, SR Ca\(^{2+}\) release, PLB, and CaM kinase II to relaxation in gastric fundus smooth muscles.
PLB phosphorylation in NO- or NO donor-induced relaxation of smooth muscles.

It is well established that CaM kinase II mediates many Ca^{2+}-dependent physiological responses (12, 15, 18, 47, 59). We previously showed that 1 mM caffeine, which increases the frequency of Ca^{2+} release from the RyRs in the SR, activates CaM kinase II in a ryosamide-sensitive manner and suggested that PLB phosphorylation by CaM kinase II is involved in caffeine-induced relaxation in murine gastric fundus smooth muscle (28). The purpose of the present study was to investigate the effect of the NO donor SNP on smooth muscle tone, membrane potential, CaM kinase II activity, and PLB phosphorylation in murine gastric fundus smooth muscles.

METHODS

Tissue preparation for CaM kinase II assay. Gastric fundus smooth muscles were obtained from adult (6- to 8-wk-old) CD-1 mice (Charles River Laboratories, Wilmington, MA). The animals were anesthetized by isoflurane inhalation and euthanized by decapitation. The 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 the University of Nevada, Reno, Institutional Animal Care and Use Committee. The gastric fundus tissues were pinned out in a Sylgard-lined dish containing oxygenated Krebs solution (in mM: 120 NaCl, 6 KCl, 15 NaHCO3, 12 glucose, 3 MgCl2, 1.5 NaH2PO4, and 3.5 CaCl2, pH 7.2). The mucosal and submucosal layers were removed using fine-tipped forcesps. For determination of the effects of SNP or 8-bromo-cGMP on CaM kinase II activity, the tissues were equilibrated in oxygenated Krebs buffer for 45 min at 37°C and then incubated in the absence or presence of each compound for 15 min. For determination of the effects of various blockers on SNP-induced CaM kinase II activity, the tissues were equilibrated in oxygenated Krebs buffer for 45 min at 37°C, and then incubated in the presence of each blocker for 20 min, and then incubated further with SNP for 15 min in the presence of each blocker. After treatment, the tissues were collected, frozen in liquid nitrogen, and stored at −80°C. When needed for assays, the frozen tissues were homogenized at 4°C with a glass tissue grinder in 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 inhibitors). The homogenates were centrifuged at 16,000 g for 15 min at 4°C. The supernatant was divided into aliquots and stored at −80°C. Protein concentrations were determined using the Bradford assay, with bovine γ-globulin as standard.

CaM kinase II activity assays. CaM kinase II activity in the lysates was assayed using the specific CaM kinase II peptide substrate autocamtide-2 (KKALRRQETVDAL, 20 mM; BioMol, Plymouth Meeting, PA), as described elsewhere (28). Kinase activity was calculated and expressed as nanomoles of Pi incorporated per minute per milligram of lysate protein. Total (Ca^{2+}/CaM-stimulated) and autonomous (Ca^{2+}/CaM-independent) CaM kinase II activities from the cytosolic fraction of control and treated gastric fundus smooth muscles from at least three animals were assayed in triplicate from each tissue. Autonomous activity is expressed as percentage of the total Ca^{2+}/CaM-dependent activity.

Mechanical responses of gastric fundus smooth muscles. Standard organ bath techniques were employed to measure the changes in force provided by fundus smooth muscle strips (6 × 3 mm). One end of the smooth muscle strip was attached to a fixed mount and the opposite end to an isometric strain gauge (Fort 10, World Precision Instruments, Sarasota, FL) in parallel with the circular smooth muscle layer. The muscle strips were immersed in organ baths (3 ml) maintained at 37 ± 0.5°C with oxygenated Krebs solution. To maintain the pH of the organ bath at 7.4, the solution was bubbled with 97% O2-3% CO2. A resting force of 600 mg was applied to set the muscles at optimum length, and the muscles were allowed to equilibrate for 30 min, during which the bath was continuously perfused with oxygenated Krebs solution. After the equilibration period, the muscle strips were incubated in oxygenated Krebs solution containing the compounds to be tested. Mechanical responses were recorded on a personal computer running Aćnçknowlege 3.2.6 (Biopac Systems, Santa Barbara, CA).

SDS-PAGE and Western blot analysis of Thr^{77}- and Ser^{166}-phosphorylated PLB from gastric fundus smooth muscle SR fractions. Gastric fundus smooth muscles from adult CD-1 mice were assigned to groups as follows: oxygenated Krebs control, SNP treated, and SNP treated in the presence of each blocker. All fundus smooth muscle strips were equilibrated in oxygenated Krebs buffer for 45 min at 37°C. For SNP treatment, the smooth muscle strips were perfused with oxygenated Krebs buffer containing SNP for 15 min. For determination of the effects of the various blockers, the smooth muscle strips were perfused with oxygenated Krebs buffer containing the appropriate blocker for 20 min and further incubated with SNP for 15 min in the presence of the appropriate blocker. The tissues were then collected, frozen in liquid nitrogen, and stored at −80°C. Gastric fundus smooth muscle SR fractions were obtained from CD-1 mice as described previously (25). Protein concentrations were determined with the Bradford assay, with bovine γ-globulin as standard. The smooth muscle SR proteins were separated by SDS-PAGE (15%) and transferred to nitrocellulose membranes for Western blotting. The blots were incubated with primary and secondary antibodies, washed, and processed for enhanced chemiluminescence image detection (ECL Advantage, Amersham Biosciences, Piscataway, NJ). PLB-PO4-Thr^{77} and PLB-PO4-Ser^{166} antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were each used at a 1:5,000 dilution and the horseradish peroxidase-conjugated secondary antibody (Chemicon, Temecula, CA) at a 1:50,000 dilution. Protein bands were visualized with a charge-coupled device camera-based detection system (Epi Chem II, UVP Laboratory Products). The TIFF images were analyzed using Adobe Photoshop. The Western blotting data are representative of six separate experiments. Un-Scan-It software (Silk Scientific, Orem, UT) was used for densitometry of immunostained pentameric phosphorylated PLB protein bands; lane analysis was used for background subtraction. The control signal values were normalized to a value of 1.

Intracellular microelectrode recordings. Gastric fundus smooth muscles from adult CD-1 mice were maintained at 37 ± 0.5°C by a flowing Krebs solution with 97% O2-3% CO2. Smooth muscle cells were impaled with glass 50- to 80-MΩ-resistance microelectrodes filled with 3 M KCl. Transmembrane potential was measured with a high-input-impedance amplifier (model S-7071, World Precision Instruments). Tissues were equilibrated for 1 h before recordings were begun.

Materials. Ryanodine, tetracaine, iberiotoxin, apamin, and tetrodotoxin were obtained from Sigma (St. Louis, MO); cyclopiazonic acid (CPA) from Biomol; and KN-93, 2-aminoethoxydiphenylborate (2-APB), 8-bromo-cGMP, 1H-[1,2,4]oxadiazolo-[4,3-a]quinazolin-1-one (ODQ), BAY K 8644, and SNP 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. Values are means ± SD. Data sets were tested for significance using ANOVA to analyze multiple groups. Data are considered significantly different from control values when P < 0.05.

RESULTS

Effects of SNP on gastric fundus smooth muscle tone and membrane potential. We characterized the effects of SNP on the basal tone and membrane potential of murine gastric fundus smooth muscles. The effects of SNP on tone and membrane...
been reported to induce Ca\textsuperscript{2+} relaxation and membrane hyperpolarization, because SNP has sensitive to 1 M 2-APB, 30 μM 2-APB induced a sustained decrease in fundus smooth muscle tone of 0.17 ± 0.04 mN. In the presence of 30 μM 2-APB, 10 μM SNP decreased muscle tone by 0.10 ± 0.01 mN (n = 4, P < 0.05), which represents an 80 ± 3% inhibition of SNP-induced relaxation (Fig. 1D).

As shown in Fig. 2A, 10 μM SNP hyperpolarized RMP in fundus smooth muscles from −45 ± 4 to −66 ± 4 mV (n = 8, P < 0.001). Ryanodine (10 μM) alone depolarized RMP from −45 ± 3 to −40 ± 5 mV, and after the addition of 10 μM SNP, a membrane potential of −44 ± 6 mV (n = 5) was recorded (Fig. 2B). Similarly, tetracaine (100 μM) alone depolarized RMP from −45 ± 4 to −41 ± 5 mV, and after addition of 10 μM SNP, the membrane potential remained unchanged (−42 ± 6 mV, n = 4; Fig. 2C). In contrast, the RMP of −48 ± 5 mV was unaffected by 30 μM 2-APB (−49 ± 5 mV), and after addition of SNP, the membrane potential remained unchanged (−50 ± 6 mV, n = 5; Fig. 2D).

Effects of KC\textsubscript{a} channel blockers on SNP-induced hyperpolarization and relaxation. The results shown in Figs. 1 and 2 are consistent with previous studies showing that SNP-induced SR Ca\textsuperscript{2+} release activates KC\textsubscript{a} channels and induces hyperpolarization and relaxation of rat gastric fundus (16) and guinea pig gastric antral (60) smooth muscles. Thus we examined the effects of the large-conductance K\textsuperscript{+} (BK) channel inhibitor iberiotoxin (IbTx, 100 nM) and the small-conductance K\textsuperscript{+} (SK) channel blocker apamin (300 nM) on SNP-induced hyperpolarization and relaxation. As shown in Fig. 3A, 100 nM IbTx alone depolarized RMP from −42 ± 2 to −38 ± 2 mV. In the presence of 100 nM IbTx, 10 μM SNP hyperpolarized the membrane potential to −44 ± 1 mV (n = 4, P < 0.05; Fig. 3A), representing a 71 ± 7% inhibition of SNP-induced hypopolarization and relaxation of gastric fundus smooth muscles. Typical changes in membrane potential and resting membrane potential were fully reversible on washout and were not sensitive to 1 μM tetrodotoxin (data not shown), indicating that the effects of SNP on muscle tone and resting membrane potential (RMP) are myogenic in origin. Typical mechanical and electrical responses are shown in Figs. 1 and 2, respectively. SNP (10 μM) rapidly decreased basal gastric fundus tone by 0.51 ± 0.08 mN (n = 15, P < 0.001; Fig. 1A). We also examined the effects of the RyR inhibitors ryanodine and tetracaine and the IP\textsubscript{3}R inhibitor 2-APB on SNP-induced relaxation and membrane hyperpolarization, because SNP has been reported to induce Ca\textsuperscript{2+} release from the SR through RyRs or IP\textsubscript{3}Rs (16, 60). In the presence of 1 μM ryanodine, 10 μM SNP relaxed fundus tone by 0.49 ± 0.04 mN (n = 5). Ryanodine at 5 μM induced a sustained elevation in tone of 0.31 ± 0.03 mN (n = 4). In the presence of 5 μM ryanodine, SNP relaxed the muscle by 0.48 ± 0.12 mN (n = 4). In contrast to 1 and 5 μM ryanodine, 10 μM ryanodine caused an initial transient increase, followed by a sustained elevation of muscle tone by 0.34 ± 0.07 mN (n = 6). In the presence of 10 μM ryanodine, SNP decreased muscle tone by 0.26 ± 0.09 mN, which represents a 49 ± 8% inhibition of SNP-induced relaxation (n = 6, P < 0.05; Fig. 1B). In the presence of 10 μM tetracaine, SNP decreased fundus smooth muscle tone by 0.41 ± 0.08 mN (n = 4). In contrast to 10 μM tetracaine, 100 μM tetracaine induced a sustained elevation in tone of 0.14 ± 0.04 mN (n = 4). In the presence of 100 μM tetracaine, SNP relaxed the muscles by 0.19 ± 0.01 mN (n = 4, P < 0.05), which represents a 62 ± 2% inhibition of SNP-induced relaxation (Fig. 1C). In the presence of 1 μM 2-APB, SNP decreased muscle tone by 0.47 ± 0.03 mN (n = 3). In contrast to
perpolarization (Fig. 2A). Apamin (300 nM) did not affect RMP (−49 ± 4 vs. −48 ± 3 mV) but inhibited the SNP-induced hyperpolarization by 71 ± 7%, as indicated by hyperpolarization to −54 ± 3 mV (n = 6, P < 0.05; Fig. 3B). IbTx and apamin slightly increased fundus smooth muscle tone by 0.08 ± 0.01 and 0.03 ± 0.01 mN, respectively. In the presence of IbTx, SNP-induced relaxation was inhibited by 54 ± 9% (n = 4, P < 0.05), as indicated by the 0.24 ± 0.05 mN decrease in tone compared with the 0.51 ± 0.1 mN decrease in tone caused by SNP alone (Fig. 3C). Similarly, in the presence of apamin, SNP-induced relaxation was inhibited by 75 ± 12% (n = 5, P < 0.05), as indicated by the 0.13 ± 0.06 mN decrease in tone (Fig. 3D). Together, these results suggest that BK and SK channels activated by Ca2+ release from the SR are involved in SNP-induced hyperpolarization and relaxation in gastric fundus smooth muscles.

**Effect of SNP on autonomous CaM kinase II activity in fundus smooth muscle.** Because the results in Figs. 1 and 2 suggest that SNP induces Ca2+ release from the SR, we examined the effects of SNP on CaM kinase II by measuring autonomous CaM kinase II activities in lysates of untreated and SNP-treated fundus smooth muscles. SNP increased autonomous CaM kinase II activity from 2.2 ± 0.3 to 3.4 ± 0.4 nmol·min⁻¹·mg⁻¹ (n = 15, P < 0.001; Fig. 4). Total CaM kinase II activities were not significantly affected by SNP: 7.3 ± 1.1 and 8.2 ± 0.7 nmol·min⁻¹·mg⁻¹ in untreated and SNP-treated fundus smooth muscles, respectively (n = 15). Thus SNP increased Ca2+/CaM-independent (autonomous) activity of CaM kinase II from 29 ± 4 to 42 ± 5% of total CaM kinase II activity (n = 15, P < 0.001). The RyR inhibitors ryanodine and tetracaine prevented SNP-induced activation of CaM kinase II in fundus smooth muscles. Autonomous CaM kinase II activities of 2.1 ± 0.2 and 1.8 ± 0.4 nmol·min⁻¹·mg⁻¹ (n = 5) measured in fundus smooth muscle lysates from ryanodine- and tetracaine-treated smooth muscles, respectively, are essentially unchanged from autonomous activity levels measured in untreated smooth muscles. Ryanodine or tetracaine alone had no effect on autonomous CaM kinase II activities. The IP3R inhibitor 2-APB also prevented SNP-induced activation of fundus smooth muscle CaM kinase II and decreased basal autonomous activity levels, as indicated by autonomous activity levels of 1.2 ± 0.3 nmol·min⁻¹·mg⁻¹ (n = 6). 2-APB alone did not affect autonomous CaM kinase II activity. Total CaM kinase II activities were unaffected by the SR Ca2+ channel inhibitors. We then examined the effects of SR Ca2+ channel inhibitors on CaM kinase II activation induced by extracellular Ca2+ influx with the L-type channel agonist BAY K 8644 to validate their specificity. BAY K 8644 (1 μM) increased fundus smooth muscle tone by 0.53 ± 0.08 mN (n = 6) and increased autonomous CaM kinase II activity from 2.1 ± 0.3 to 3.1 ± 0.3 nmol·min⁻¹·mg⁻¹ (n = 5, P < 0.001). Ryanodine or tetracaine did not affect BAY K 8644-induced CaM kinase II activation: 3.1 ± 0.2 and 3.2 ± 0.2 nmol·min⁻¹·mg⁻¹ in the presence of 10 μM ryanodine and 100 μM tetracaine, respectively (n = 4 each). Similarly, 30 μM 2-APB did not affect BAY K 8644-induced CaM kinase II activation: 2.9 ± 0.2 nmol·min⁻¹·mg⁻¹ (n = 4).

**Effect of CPA on CaM kinase II activation.** Because SR Ca2+ release is influenced by the SR Ca2+ load (34, 61), we used CPA (10 μM), a SERCA pump inhibitor typically used to decrease SR Ca2+ levels, to examine the role of SERCA in SNP-induced relaxation of fundus smooth muscles (2, 38).
After an initial transient increase, fundus tone was stably elevated by CPA by 0.6 ± 0.2 mN, and addition of SNP caused a relaxation of 0.3 ± 0.04 mN (n = 5, P < 0.05; Fig. 5A), i.e., 38 ±% inhibition of SNP-induced relaxation. In the presence of 10 μM CPA, SNP did not increase autonomous CaM kinase II activity: 1.7 ± 0.3 nmol·min⁻¹·mg⁻¹ (n = 6; Fig. 5B). CPA alone had no effect on autonomous CaM kinase II activity. The effect of CPA on BAY K 8644-induced CaM kinase II activation was examined. In contrast to ryanodine, tetracaine, and 2-APB, CPA prevented BAY K 8644-induced CaM kinase II activation: 2.1 ± 0.2 nmol·min⁻¹·mg⁻¹ (n = 4).

Role of the NO-sGC-cGMP pathway in SNP-induced relaxation and CaM kinase II activation. To examine the role of cGMP production by sGC in SNP-induced relaxation of gastric fundus smooth muscles, fundus smooth muscle strips were treated with 10 μM SNP in the absence or presence of the sGC inhibitor ODQ (10 μM) (16, 46). ODQ alone increased fundus smooth muscle tone by 0.12 ± 0.05 mN and completely inhibited SNP-induced relaxation (n = 4, P < 0.001; Fig. 6A). Furthermore, SNP alone increased CaM kinase II autonomous activity from 2.1 ± 0.3 to 3.3 ± 0.2 nmol·min⁻¹·mg⁻¹, increasing autonomous activity from 26 ± 5 to 40 ±% of total CaM kinase II activity (n = 6, P < 0.001; Fig. 6B). However, in the presence of ODQ, SNP did not increase CaM kinase II autonomous activity: 2.1 ± 0.3 nmol·min⁻¹·mg⁻¹ (n = 6). ODQ alone did not affect autonomous CaM kinase II activity. To further examine the role of cGMP in gastric fundus smooth muscle CaM kinase II activation, we examined the effect of the membrane-permeable cGMP analog 8-bromo-cGMP (1 mM) on fundus smooth muscle tone and autonomous CaM kinase II activity. 8-Bromo-cGMP decreased fundus muscle tone by 0.55 ± 0.14 mN (n = 4) and increased autonomous CaM kinase II activity from 2.1 ± 0.3 to 3.1 ± 0.2 nmol·min⁻¹·mg⁻¹ (n = 5, P < 0.001; Fig. 6C), suggesting that SNP-induced activation of CaM kinase II is mediated by an NO-sGC-cGMP pathway in gastric fundus smooth muscles.

Effect of SNP-induced CaM kinase II activation on PLB phosphorylation. Because previous studies suggest that NO-induced relaxation in smooth muscles involves lowering [Ca²⁺], by activating the SERCA through PLB phosphorylation (9, 38, 40, 56), we obtained an enriched SR fraction from fundus smooth muscle after treatment of intact gastric fundus smooth muscles with SNP and monitored PLB phosphorylation by Western blot analysis using specific antibodies for phosphorylation of PLB at Thr17 or Ser16. Similar to PLB phosphorylation at Ser16 by PKA or PKG, PLB phosphorylation at Thr17 by CaM kinase II relieves SERCA inhibition by PLB (18, 26, 31). As shown in Fig. 7A, SNP increased PLB phosphorylation at Thr17 in fundus smooth muscles. The SNP-induced increase in immunostaining of phosphorylated Thr17 was inhibited by the CaM kinase II inhibitor KN-93 (10 μM).
or CPA (10 μM), suggesting that CaM kinase II activation by SNP increases PLB phosphorylation at Thr17. Similarly, 10 μM ODQ inhibited SNP-induced PLB phosphorylation at Thr17, suggesting that cGMP production and PKG activation are required for SNP-induced CaM kinase II activation.

These results and those in Fig. 6 showing that 8-bromo-cGMP relaxes fundus smooth muscles, whereas ODQ prevents SNP-induced fundus smooth muscle relaxation, suggest that cGMP levels are elevated by SNP and raise the possibility that Ser16 phosphorylation of PLB is increased by PKG. As shown in Fig. 7B, SNP treatment of fundus smooth muscles increased PLB phosphorylation at Ser16. However, the SNP-induced increase in Ser16 phosphorylation was not inhibited by KN-93 or CPA. In contrast, SNP-induced PLB phosphorylation at Ser16 was inhibited by ODQ, suggesting that Ser16 phosphorylation of PLB involves PKG activation by cGMP. The immunostaining intensities of phosphorylated Thr17 and Ser16 were quantified by densitometry and normalized to the immunostaining intensities measured from untreated fundus smooth muscles (Fig. 7C, n = 6).

Effect of KN-93 on CaM kinase II activity and SNP-induced hyperpolarization and relaxation. Because KN-93 inhibited the SNP-induced increase in PLB Thr17 phosphorylation, the effects of KN-93 on SNP-induced hyperpolarization and relaxation were examined. KN-93 (10 μM) treatment of fundus smooth muscles prevented CaM kinase II activation by SNP (1) (Fig. 8A). KN-93 (10 μM) alone slightly depolarized membrane potential from -44 ± 2 to -43 ± 2 mV and SNP hyperpolarized the membrane potential by -10 mV to -53 ± 4.1 mV in the presence of KN-93 (Fig. 8B). SNP alone hyperpolarized the membrane potential by -22 mV from -45 ± 4 to -66 ± 4 mV. Thus, in the presence of KN-93, SNP-induced hyperpolarization was inhibited by 53 ± 6% (n = 5, P < 0.05). KN-93 (10 μM) decreased fundus muscle tone by 0.12 ± 0.01 mN and SNP relaxed tone by 0.21 ± 0.03 mN in the presence of KN-93 (Fig. 8C). SNP alone reduced fundus smooth muscle tone by 0.51 ± 0.08 mN. Thus, in the presence of KN-93, SNP-induced relaxation was inhibited by 58 ± 8% (n = 5, P < 0.05), suggesting that CaM kinase II activation and PLB Thr17 phosphorylation contribute to SNP-induced relaxation of gastric fundus smooth muscles.

DISCUSSION

The functions of the mammalian stomach are storage, acidification, grinding, mixing, and delivery of ingested food to the duodenum. The stomach can be divided into two regions on the basis of motility patterns. The upper stomach, which does not show spontaneous activity, consists of the fundus and upper body and is characterized primarily by tonic contractions (4, 30, 52). The gastric accommodation reflex depends on receptive and adaptive reflexes of the fundus, which allow it to

Fig. 7. SNP increases phospholamban (PLB) Thr17 and Ser16 phosphorylation. A and B: representative immunoblots of phosphorylated PLB Thr17 or Ser16 in the sarcoplasmic reticulum (SR)-enriched fraction from gastric fundus smooth muscle strips incubated without (control) or with 10 μM SNP alone for 15 min, 10 μM KN-93 for 20 min and 10 μM SNP for 15 min, 10 μM CPA for 20 min and 10 μM SNP for 15 min, or 10 μM ODQ for 20 min and 10 μM SNP for 15 min. p, PLB pentamer; m, PLB monomer. C: densitometry of anti-phosphorylated PLB Thr17 (open bars) and Ser16 (solid bars) immunostaining signal intensities. *P < 0.05.
function as an expandable reservoir without a significant increase in pressure (30). The lower stomach consists of the lower body and antrum; the strong peristaltic contractions accomplish the grinding, mixing, and gastric emptying functions of this part of the stomach. Insufficient gastric accommodation and gastric emptying underlie common pathophysiological problems, such as functional dyspepsia, rumination, and postsurgical and diabetic gastroparesis (30, 52). Thus, studies of the problems, such as functional dyspepsia, rumination, and post-surgical gastroparesis, require a better understanding of stomach smooth muscle physiology and pathophysiology.

NO has been intensively studied because of its ability to relax smooth muscles, but the exact mechanism of NO-induced relaxation has not been resolved. Several possible mechanisms of NO- and cGMP-induced relaxation of smooth muscles that are not mutually exclusive, including hyperpolarization of the smooth muscle cell membrane (53), IP3R-associated cGMP kinase substrate phosphorylation by PKG (14), lowering of [Ca2+], by SERCA activation (10), and reduction in the Ca2+ sensitivity of the contractile apparatus (35, 50), have been investigated. In the present study, we found that ryanodine and 2-APB inhibited the hyperpolarization and relaxation induced by the NO donor SNP, suggesting that Ca2+ release events from the SR are involved in SNP-induced hyperpolarization and relaxation. Ryanodine and tetracaine inhibited SNP-induced relaxation by ~50 and 60%, respectively (Fig. 1). The IP3R inhibitor 2-APB inhibited SNP-induced relaxation by ~80%, suggesting that Ca2+ release through IP3Rs may predominate in SNP-induced relaxation of gastric fundus smooth muscles. Ryanodine or tetracaine each slightly increased fundus smooth muscle tone, whereas 2-APB did not, suggesting that an increase in tone, by itself, does not oppose relaxation. The interaction between IP3Rs and RyRs is complicated, because both channels appear to induce Ca2+ release from a common pool (43). Ca2+ release from one type of channel might increase the open probability (Po) of the other channel, because IP3Rs and RyRs are sensitive to cytoplasmic Ca2+ (33, 43). On the other hand, Ca2+ release from IP3Rs or RyRs can adversely affect Ca2+ sparks or Ca2+ puffs, respectively, because of a reduction of the SR Ca2+ load (61).

In the present study, we used ryanodine and tetracaine to affect Ca2+ release through RyRs and disrupt SR function. Low-micromolar (<10 μM) ryanodine tends to act as an RyR agonist (23, 42). Ryanodine at 1 μM did not inhibit SNP relaxation and slightly decreased fundus smooth muscle tone. Similarly, although 5 μM ryanodine slightly increased tone, SNP relaxation was not inhibited. However, 10 μM ryanodine induced a large transient increase in tone, suggesting that as the concentration of ryanodine increased, efflux of Ca2+ from the SR increased (23, 62). Higher concentrations of ryanodine increase the Po of the RyR and tend to place the RyR into a subconducting state (42, 62). Inhibition of SNP-induced relaxation by 10 μM RyR could thus be due to insufficient Ca2+ release from depleted SR stores (42, 62). Tetracaine was used, because it blocks RyRs by a different mechanism by inducing a long closed state of the channel without greatly increasing fundus smooth muscle tone (17, 32, 62). Tetracaine also inhibited SNP relaxation, further suggesting that Ca2+ release through RyRs also plays a role in SNP-induced relaxation of fundus smooth muscles.

Because localized Ca2+ release from the SR can activate plasma membrane Kca channels, IbTx or apamin was used to determine whether BK or SK channels are involved in SNP-induced hyperpolarization and relaxation. These Kca channel inhibitors depolarized RMP, increased fundus tone, and inhibited SNP-induced hyperpolarization and relaxation, suggesting that activation of BK and SK channels by Ca2+ sparks and Ca2+ puffs is involved in SNP-induced hyperpolarization and relaxation. In the present study, IbTx alone depolarized RMP by ~4 mV and slightly increased resting tone, whereas apamin had no significant effects on RMP or resting tone. However, IbTx and apamin inhibited SNP-induced relaxation by 54 ± 9 and 75 ± 12%, respectively, suggesting that BK channels may be more involved in setting RMP and fundus tone, whereas SK channels may be more involved in SNP-induced relaxation of gastric fundus smooth muscles. Geeson et al. (16) showed that SNP relaxed rat fundus smooth muscle in a ryanodine- and SK channel-sensitive, but BK channel-insensitive, manner. It has also been reported that cGMP/PKG directly increases BK channel Po (41, 45). However, the results from the present study suggest that SNP also leads to membrane potential hyperpolarization and relaxation of murine gastric fundus...
smooth muscles through activation of BK and SK channels by Ca\(^{2+}\) release events from the SR stores.

Because SR Ca\(^{2+}\) release can activate CaM kinase II (28, 37), we investigated whether CaM kinase II is activated by SNP and contributes to SNP-induced relaxation in gastric fundus smooth muscles. After SNP treatment, autonomous CaM kinase II activity increased in fundus smooth muscles from 29 to 42% of the total Ca\(^{2+}\)/CaM-stimulated activity. CaM kinase II activation was blocked by ryanodine, tetracaine, or 2-APB, suggesting that an increased frequency of localized Ca\(^{2+}\) release events from RyRs or IP3Rs activated CaM kinase II. It is unclear why ryanodine, tetracaine, or 2-APB alone had no effect on autonomous CaM kinase II activity. After the initial autophosphorylation, autophosphorylation of subunits is maintained by other autophosphorylated subunits, and Ca\(^{2+}\) is not required (11, 20, 48). In addition, the level of autophosphorylated CaM kinase II is an equilibrium between kinase and phosphatase activities (5), and in untreated fundus smooth muscles, the levels of phosphatase activity toward CaM kinase II may not be able to completely dephosphorylate CaM kinase II. This may also explain why the level of autonomous CaM kinase II activity was not affected by KN-93. KN-93 competitively inhibits CaM kinase II by binding to the CaM-binding domain and preventing activation by Ca\(^{2+}\)/CaM (51). Because autonomous CaM kinase II does not require Ca\(^{2+}\)/CaM, it is not inhibited by KN-93 (1, 51). Some of the Ca\(^{2+}\)-independent activity may be due to other kinases but is likely to be minimal, because autacamtide-2 is a highly selective substrate for CaM kinase II (19).

In addition to KCa channel activation, SNP can relax smooth muscles by activating SERCA and lowering [Ca\(^{2+}\)]\(_i\) (9, 38, 40, 56). In the present study, CPA was used to inhibit SERCA activity and deplete Ca\(^{2+}\) stores. As shown in our previous study, CPA increased [Ca\(^{2+}\)]\(_i\) in fundus smooth muscles but inhibited caffeine-induced CaM kinase II activation (28). Similarly, in the present study, CPA inhibited SNP-induced CaM kinase II activation. These findings suggest that blocking SR Ca\(^{2+}\) uptake with CPA lowers the luminal SR Ca\(^{2+}\) stores to a level that is insufficient to support SR Ca\(^{2+}\) release events, which activate CaM kinase II. Similarly, Abraham et al. (1) showed that thapsigargin prevented the threefold increase in autonomous CaM kinase II activity induced by angiotensin II.

Fig. 9. Model of CaM kinase II activation by SNP in gastric fundus smooth muscles. Nitric oxide (NO) produced by SNP activates soluble guanylyl cyclase (sGC), elevating cGMP levels and activating PKG. PLB Ser\(^{16}\) phosphorylation by PKG activates SERCA, leading to increased Ca\(^{2+}\) uptake into the SR and elevated SR Ca\(^{2+}\) levels (22, 27, 31). Higher levels of SR Ca\(^{2+}\) lead to an increase in SR Ca\(^{2+}\) release events through ryanodine receptors (RyRs) and inositol trisphosphate receptors (IP,R). CaM kinase II is activated by increased frequency of Ca\(^{2+}\) release events from the SR, leading to PLB Thr\(^{17}\) phosphorylation. VDCC, voltage-dependent Ca\(^{2+}\) channel. P, a phosphorylated Ser or Thr; PM, plasma membrane.
in vascular smooth muscle cells, suggesting that the enzyme could be localized in the vicinity of the SR and activated by localized Ca$^{2+}$ release events. However, it is unclear why CPA, in contrast to ryanodine, tetracaine, or 2-APB, prevented CaM kinase II activation by BAY K 8644.

SERCA activation by PLB phosphorylation lowers [Ca$^{2+}$]$_i$ (26, 31). Unphosphorylated PLB inhibits SERCA activity by lowering its affinity for Ca$^{2+}$ (6, 21). Phosphorylated PLB dissociates from SERCA and increases the turnover rate ($V_{\text{max}}$) of SERCA by increasing its affinity for Ca$^{2+}$ (6, 21). CaM kinase II phosphorylates PLB at Thr$^{17}$; thus, examining PLB phosphorylation at Thr$^{17}$ is another way of monitoring CaM kinase II activity and SERCA activation. Western blot analysis with a Thr$^{17}$-phosphorylated PLB antibody showed that Thr$^{17}$ phosphorylation of PLB increased in response to SNP in fundus smooth muscles. Similar to the results showing that KN-93 or CPA inhibited CaM kinase II activation, KN-93 or CPA inhibited Thr$^{17}$ phosphorylation, suggesting that CaM kinase II activated by SNP phosphorylates PLB at Thr$^{17}$. Furthermore, the finding that ODQ prevented SNP-induced relaxation (Fig. 6) strongly suggests that SNP elevates cGMP levels, raising the possibility that PLB Ser$^{16}$ phosphorylation could be increased by PKG (22, 27). Western blot analysis showed that SNP treatment of fundus smooth muscles increased PLB Ser$^{16}$ phosphorylation. The SNP-induced increase in PLB Ser$^{16}$ phosphorylation was KN-93 or CPA insensitive, indicating that phosphorylation of PLB Ser$^{16}$ is independent of CaM kinase II activation and SR Ca$^{2+}$ load. However, Thr$^{17}$ and Ser$^{16}$ phosphorylation was ODQ sensitive, suggesting that PKG and CaM kinase II activation by SNP is mediated by an NO-sGC-cGMP pathway. A model for the activation of CaM kinase II by SNP via elevation of cGMP and activation of PKG is presented in Fig. 9. NO produced by SNP activates sGC, elevating cGMP levels and activating PKG (22). PLB Ser$^{16}$ phosphorylation by PKG activates SERCA, leading to increased Ca$^{2+}$ uptake into the SR and elevated SR Ca$^{2+}$ levels (22, 27, 31). The increased rate of Ca$^{2+}$ uptake into the SR reduces the amount of Ca$^{2+}$ available for contraction and contributes to smooth muscle relaxation. The higher levels of SR Ca$^{2+}$ lead also to an increase in SR Ca$^{2+}$ release events through RyRs and IP$_{3}$Rs (34, 61). CaM kinase II is activated by the increased frequency of Ca$^{2+}$ release events from the SR, leading to PLB Thr$^{17}$ phosphorylation (28). In addition, the increased frequency of Ca$^{2+}$ release events through RyRs and IP$_{3}$Rs contributes to smooth muscle relaxation by activating K$_{Ca}$ channels, resulting in hyperpolarization of the membrane potential. The SNP-induced release of SR Ca$^{2+}$ may also potentiate the direct regulation of K$_{Ca}$ channels by PKG and CaM kinase II (29, 41, 45). The physiological significance of CaM kinase II activation remains unclear; however, its Ca$^{2+}$-independent activity may provide a mechanism to prolong the effects of NO/cGMP on SERCA K$_{Ca}$ channel activities after the cGMP and Ca$^{2+}$ signals have ceased.

In summary, the present results suggest that the relaxant effect of SNP on gastric fundus smooth muscles involves PLB phosphorylation by PKG and CaM kinase II via an NO-sGC-cGMP pathway. These results also suggest that elevation of cGMP levels by SNP activates CaM kinase II by increasing Ca$^{2+}$ release events from the SR through PLB phosphorylation by PKG and indicate that PLB phosphorylation by CaM kinase II plays an important role in SNP-induced relaxation. To our knowledge, these findings appear to be the first indication that CaM kinase II has a role in SNP-induced relaxation and that cGMP appears to activate CaM kinase II by increasing the frequency of Ca$^{2+}$ release events from the SR due to increased PLB phosphorylation by PKG. Thus, these findings demonstrate a novel link between nitroglycerin and Ca$^{2+}$/CaM-dependent signaling pathways in murine gastric fundus smooth muscle tissues.

ACKNOWLEDGMENTS

A preliminary version of this work was presented at the 20th International Symposium on Neurogastroenterology and Motility, 3–7 July 2005, Toulouse, France.

GRANTS

This work was supported by National Institutes of Health Grant RR-018751.

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