NO donor sodium nitroprusside inhibits excitation-contraction coupling in guinea pig taenia coli

SEONG-CHUN KWON, HIROSHI OZAKI, AND HIDEAKI KARAKI

Department of Physiology, Yonsei University College of Medicine, Seoul 120-752, Korea; and Department of Veterinary Pharmacology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan

Received 19 November 1999; accepted in final form 28 June 2000


NO donor sodium nitroprusside inhibits excitation-contraction coupling in guinea pig taenia coli. Am J Physiol Gastrointest Liver Physiol 279: G1235–G1241, 2000.—In guinea pig taenia coli, the nitric oxide (NO) donor sodium nitroprusside (SNP, 1 μM) reduced the carbachol-stimulated increases in muscle force in parallel with a decrease in intracellular Ca2+ concentration ([Ca2+]i). A decrease in the myosin light chain phosphorylation was also observed that was closely correlated with the decrease in [Ca2+]i. With the patch-clamp technique, 10 μM SNP decreased the peak Ba2+ current, and this effect was blocked by an inhibitor of soluble guanylate cyclase. Carbachol (10 μM) induced an inward current, and this effect was markedly inhibited by SNP. SNP markedly increased the depolarization-activated outward K+ currents, and this current was completely blocked by 0.3 μMiberiotoxin. SNP (1 μM) significantly increased cGMP content without changing cAMP content. Decreased Ca2+ sensitivity by SNP of contractile elements was not prominent in the permeabilized taenia, which was consistent with the [Ca2+]i-force relationship in the intact tissue. These results suggest that SNP inhibits myosin light chain phosphorylation and smooth muscle contraction stimulated by carbachol, mainly by decreasing [Ca2+]i, which resulted from the combination of the inhibition of voltage-dependent Ca2+ channels, the inhibition of nonselective cation currents, and the activation of Ca2+-activated K+ currents.

intracellular calcium concentration; calcium channel; potassium channel; nonselective cation channel; myosin light chain phosphorylation

EVIDENCE FROM FUNCTIONAL and morphological studies supports the involvement of nitric oxide (NO) in non-adrenergic, noncholinergic inhibitory neurotransmission in gastrointestinal (GI) smooth muscles, including guinea pig ileum (29) and taenia coli (9). Although the mechanism of NO action is not fully understood, its action is considered to be mediated by the activation of guanylate cyclase and the resultant increase in cGMP content in smooth muscle cells (14). Several studies have suggested that NO may decrease the intracellular Ca2+ level ([Ca2+]i) and also decrease the Ca2+ sensitivity of the contractile elements, resulting in smooth muscle relaxation (18, 19, 26). In other studies, cGMP is proposed to act directly on ion channels (13) to activate protein kinase, which directly phosphorylates proteins to regulate their activity (31), or to work via a cGMP-stimulated phosphodiesterase (11).

As for the mechanisms of decreasing [Ca2+]i, there is evidence that NO causes an inhibition of Ca2+ entry through voltage-dependent Ca2+ channels (33). On the other hand, the plasma membrane of intestinal smooth muscle cells contains large-conductance Ca2+-activated K+ (KCa) channels, and charybdotoxin, a specific inhibitor of KCa channels, inhibits the relaxation of smooth muscle due to NO (4). Furthermore, the inhibition of nonselective cation channels, which is activated by muscarinic stimulation in GI smooth muscle (5, 15) by NO, may also be responsible for the decreasing [Ca2+]i.

Although NO has multiple actions that may be involved in the inhibition of smooth muscle contraction, there has been no report investigating how these actions contribute to relaxation in smooth muscle. The aim of this study was to investigate systematically the mechanism of NO-induced relaxation in muscarinic-stimulated guinea pig taenia coli by measuring [Ca2+]i, myosin phosphorylation, ionic currents, and cyclic nucleotide content.

MATERIALS AND METHODS

Fura 2 loading and simultaneous measurements of muscle force and [Ca2+]i. Male guinea pigs (300–400 g) were killed by a sharp blow to the neck and exsanguination. The taenia coli was isolated and cut into 8- to 10-mm-long strips and placed in physiological salt solution (PSS). EDTA (0.01 mM) was also added to chelate the contaminated trace amount of heavy metal ions. High-K+ solution was made by replacing NaCl with equimolar KCl. These solutions were saturated with 100% O2 at 37°C to maintain pH at 7.2. The force of contraction was recorded isometrically. Muscle preparations were attached to a holder under a resting force of 5 mN and equilibrated for 60–90 min. During this period, 40 mM K+ was repeatedly applied until the sustained force became reproducible. [Ca2+]i was measured according to the method described by Kwon et al. (21) using the fluorescent Ca2+...
**EFFECT OF SNP ON INTESTINAL SMOOTH MUSCLE**

Muscle strips were exposed to 10 μM fura 2-AM in the presence of 0.02% cremophor EL for 5–6 h at room temperature. The muscle strips were then transferred to the muscle bath integrated in the fluorometer (model CAF-100, Jasco, Tokyo, Japan). The muscle strips were illuminated alternately (48 Hz) with 340- and 380-nm light. The light emitted from the muscle strips was collected by a photomultiplier through a 500-nm filter.

**cAMP and cGMP content.** cAMP or cGMP content in taenia was measured by RIA. After an incubation, muscle strips were frozen in liquid nitrogen and homogenized in 6% TCA, which was removed after centrifugation by washing with water-saturated ether. cAMP or cGMP was succinylated and assayed by a competitive RIA with [125I]-succinyl-cAMP or cGMP-tyrosine methyl ester. Radioactivity was counted with an Auto-Gamma counter (Packard).

**Myosin light chain phosphorylation.** Tissues were frozen by immersion in a dry ice-acetone slurry (20 g, 20 ml) at −78°C. The frozen tissue samples were slowly warmed, first in a −20°C freezer for 1 h and then for 1 h at room temperature, and air dried for 15 min. Phosphorylation of the smooth muscle-specific 20-kDa myosin light chain (MLC) was determined by two-dimensional electrophoretic separation (8). Phosphorylation is reported as moles of P, per mole of total smooth muscle-specific light chain.

**Preparation of cells for patch-clamp analysis.** The muscle layer of taenia coli was cut into small pieces and placed in Ca2+-free PSS. The Ca2+-free PSS was then replaced with PSS containing 30 μM Ca2+ (low-Ca2+ PSS), and 30-min incubations at 37°C were carried out in fresh low-Ca2+ PSS containing collagenase (1 mg/ml), papain (1 mg/ml), and BSA (2 mg/ml). After this enzyme digestion, tissue fragments were suspended in fresh 120 mM Ca2+-containing PSS and gently agitated. The resulting suspension was centrifuged at 1000 g for 2 min, and the cells were resuspended in 0.5 mM Ca2+-containing PSS. Cell suspensions were placed on glass coverslips and stored in a moist atmosphere at 4°C. Experiments were carried out at room temperature (22–24°C).

**Whole cell voltage clamp.** Whole cell membrane current and potential were recorded at room temperature using standard patch-clamp techniques. The patch pipette had a resistance of 3–6 MΩ when filled with a pipette solution. Membrane currents were measured with an Axopatch 1C voltage-clamp amplifier (Axon Instrument). Command pulses were applied using an IBM-compatible computer and pCLAMP (version 5.5) software. The data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and a pen recorder.

**Permeabilization preparation.** Permeabilized smooth muscle was prepared as described by Nishimura et al. (26) and Kitazawa et al. (20). A thin bundle of taenia coli (~0.5 mm wide, 3 mm long) was dissected parallel to the longitudinal muscle fiber under a binocular microscope. The muscle strip was soaked for 5–10 min at room temperature (20–22°C) in a relaxing solution containing Staphylococcus aureus α-toxin (10 μg protein/ml). One end of the muscle strip was fixed to the chamber, and the other end was attached to a force-displacement transducer. The contractile force of the permeabilized muscle was recorded isometrically. Ca2+ concentrations were changed by adding an appropriate amount of CaCl2. The apparent binding constant of EGTA for Ca2+ was considered to be 1 μM at pH 6.8 and 20°C.

**Solutions.** The PSS used for the bath solution had the following composition (mM): 126 NaCl, 6 KCl, 2 CaCl2, 1.2 MgCl2, 14 glucose, and 10.5 HEPES (pH adjusted to 7.2 with NaOH). The patch pipette solution for the outward K+ currents had the following composition (mM): 134 KCl, 1.2 MgCl2, 1 ATP, 0.1 GTP, 0.05 EGTA, 14 glucose, and 10.5 HEPES (pH adjusted to 7.2 with KOH). Inward currents were isolated by suppressing K+ currents using a patch pipette filled with 140 mM CsCl, 14 mM glucose, 0.05 mM EGTA, 10.5 mM HEPES, and 4 mM Na2ATP (pH adjusted to 7.2 with CsOH). In experiments dealing with Ca2+ currents, CaCl2 in the bath solution was replaced by BaCl2. The relaxing solution used for permeabilized tissue contained (mM) 130 potassium propionate, 4 MgCl2, 5 Na2-ATP, 2 creatine phosphate, 10 creatine phosphokinase, 20 Tris-maleate (pH 6.8), and 2 EGTA. Added to the relaxing solution were the mitochondrial inhibitor carbonyl cyanide p-trifluoromethoxyphenylhydrazone (1 μM), the cholinesterase inhibitor diisopropyl fluorophosphates (10 μM), the protease inhibitor E-64 (1 μM), and 1 μM leupeptin.

**Statistics.** Values are means ± SE. Student’s t-test was used for the comparison of means (P < 0.05 was considered significant).

**RESULTS**

**Effect of SNP on muscle force and [Ca2+]i.** Figure 1A shows the inhibitory effect of SNP on spontaneous rhythmic changes in [Ca2+]i, and contraction in taenia coli. SNP (1 μM) completely inhibited these changes without affecting basal [Ca2+]i. Addition of 4 mM EGTA decreased [Ca2+]i below the basal level. Figure 1B shows the inhibitory effects of SNP on 0.3 mM carbachol-induced changes in [Ca2+]i, and contraction. Addition of 1 μM SNP inhibited the carbachol-
induced contraction by 72.2 ± 3.4% (n = 7), with a sustained decrease in [Ca\(^{2+}\)]\(_i\) of 54.6 ± 3.0% (n = 7).

**Effect of SNP on myosin phosphorylation.** Application of 0.3 μM carbachol increased MLC phosphorylation from a resting level of 0.08 ± 0.02 mol PO\(_4\)/mol MLC (n = 5) to 0.29 ± 0.04 mol PO\(_4\)/mol MLC (n = 5) at 10 min. Examination of the relationship between [Ca\(^{2+}\)]\(_i\) and MLC phosphorylation indicated that 1 μM SNP significantly decreased MLC phosphorylation, with decreasing [Ca\(^{2+}\)]\(_i\) in the carbachol-stimulated muscle (Fig. 2A). Verapamil (0.1 and 1 μM) also decreased [Ca\(^{2+}\)]\(_i\) and MLC phosphorylation in parallel (Fig. 2A). Figure 2B shows the relationship between [Ca\(^{2+}\)]\(_i\) and force, indicating a close correlation between [Ca\(^{2+}\)]\(_i\) and force, as observed in the relationship between [Ca\(^{2+}\)]\(_i\) and MLC phosphorylation.

**Effects of SNP on voltage-dependent Ca\(^{2+}\) currents and agonist-induced nonselective cation currents.** The effects of 10 μM SNP on Ca\(^{2+}\) currents through voltage-dependent Ca\(^{2+}\) channels were examined in single taenia coli smooth muscle cells in a solution containing 2 mM Ba\(^{2+}\). When Ba\(^{2+}\) was used instead of Ca\(^{2+}\) as the charge carrier, \(I_{Ba}\) was elicited by depolarization from a holding potential of −60 mV to a test potential of +10 mV for 80 ms at 0.1 Hz. Application of 10 μM SNP reduced the peak \(I_{Ba}\) amplitude (by 27.8 ± 5.4%, n = 6). \(I_{Ba}\) was significantly decreased within 1 min from the start of the application (Fig. 3A). After washout of SNP, \(I_{Ba}\) recovered almost completely (data not shown). 1H-(1,2,4)oxadiazole[4,3-a]quinoxaline-1-one (ODQ, 1 μM), a soluble guanylate cyclase inhibitor, alone showed no effect on basal \(I_{Ba}\), but it almost completely abolished the SNP-induced inhibition of \(I_{Ba}\) (Fig. 3). A membrane-permeable cGMP analog, 1 mM 8-bromo-cGMP, showed effects similar to that of SNP (data not shown).

In taenia coli smooth muscle cells clamped at a holding potential of −40 mV, application of 10 μM carbachol induced a slow inward current with an increase in baseline fluctuation (Fig. 4A). In most cells, the response continued for >10 min. To know whether this response represents a cationic current, we mea-

---

**Fig. 2.** A: relationship between [Ca\(^{2+}\)]\(_i\) and myosin light chain (MLC) phosphorylation induced by carbachol. Strips were contracted by carbachol for 10 min with or without verapamil (Ver, 0.1 and 1 μM) or SNP (1 μM) and then frozen for phosphorylation analysis. [Ca\(^{2+}\)]\(_i\) is expressed as a fraction of the high K\(^+\) at 10 min. B: relationship between [Ca\(^{2+}\)]\(_i\) and force. Values are means ± SE (n = 4–7).

**Fig. 3.** Effects of SNP on Ba\(^{2+}\) current (\(I_{Ba}\)) through Ca\(^{2+}\) channels in single smooth muscle cells of the guinea pig taenia coli. \(I_{Ba}\) was elicited by 80 ms of depolarization from −60 to +10 mV at 0.1 Hz. A: time course of change in peak amplitude of \(I_{Ba}\). SNP (10 μM) was applied during the period indicated by a horizontal bar. Time 0, start of \(I_{Ba}\) recording under conditions where K\(^+\) current was blocked by internal diffusion of Cs\(^+\) from the recording pipette for ~3 min after rupture of the patch membrane. ODQ, 1H-(1,2,4)oxadiazole[4,3-a]quinoxaline-1-one. *Inset:* current traces recorded at times corresponding to 1, 2, and 3 in A. B: current-voltage relationships for the peak inward \(I_{Ba}\) current activated from a holding potential of −60 mV. Peak amplitude of \(I_{Ba}\) evoked by stepping to different potentials from the holding potential is plotted against the potential.
sured the steady-state current-voltage relationship for carbachol-activated cationic current by the ramp method (39). The reversal potential of this current was \(-5\) mV (Fig. 4, inset), and the current was completely inhibited by Cd\(^{2+}\) (100 \(\mu\)M, \(n = 4\)). These results are consistent with those with nonselective cation current obtained in guinea pig ileum (15, 39). As also shown in Fig. 4A, the carbachol-induced inward current was inhibited by 10 \(\mu\)M SNP. At a holding potential of \(-40\) mV, 10 \(\mu\)M SNP inhibited the current by 54.5 \(\pm\) 7.1\% \((n = 4)\). The effect of SNP was markedly inhibited by 1 \(\mu\)M ODQ (Fig. 4B).

**Effects of SNP on the outward \(K^+\) currents.** Cells dialyzed with high-\(K^+\) solution showed large outward current during 900-ms depolarizing steps from a holding potential of \(-60\) mV to potentials positive to \(-30\) mV. To determine whether the \(K^+\) current is activated by SNP, the amplitude of the whole cell \(K^+\) current was measured before and after exposure to SNP. SNP (10 \(\mu\)M) increased the net outward current (Fig. 5A). Maximum effects were seen at 10 min after administration. The current amplitude at the last voltage step (from \(-60\) to +50 mV) increased from 1,565 \(\pm\) 264 to 2,094 \(\pm\) 295 pA \((n = 5)\) with the addition of SNP to the bath. These effects were reversible with washing. Pretreatment with 0.3 \(\mu\)M iberiotoxin blocked the SNP-activated \(K^+\) current (Fig. 5B) but had no significant effect on the delayed rectifier current. The effect on the SNP-activated current was reversible.

**Effects on the contents of cyclic nucleotides.** SNP (1 \(\mu\)M) increased cGMP content by 602\% but did not change cAMP content (Table 1). Forskolin (1 \(\mu\)M), on the other hand, increased cAMP content by 759\% but had no effect on cGMP content.

**Effect on \(Ca^{2+}\)-induced contraction in permeabilized muscle.** In the permeabilized taenia treated with \(\alpha\)-toxin, the cumulative addition of 0.03–10 \(\mu\)M \(Ca^{2+}\)-induced graded contractions in a concentration-dependent manner. Contractile responses were scarcely affected by pretreatment of muscle fiber with 10 \(\mu\)M SNP, except the contraction at 0.3 \(\mu\)M \(Ca^{2+}\) (Fig. 6).

**DISCUSSION**

In the guinea pig taenia, SNP inhibited the increase in [\(Ca^{2+}\)], and muscle force induced spontaneously or by stimulation with carbachol. In smooth muscles, there are at least three possibilities for the inhibitory effect of NO on [\(Ca^{2+}\)]; 1) inhibition of a voltage-dependent (L-type) \(Ca^{2+}\) channel, 2) inhibition of a nonselective cation channel that mediates membrane depolarization or directly passes \(Ca^{2+}\) into cytoplasm, and 3) activation of a voltage- or \(Ca^{2+}\)-dependent \(K^+\) channel.

Guinea pig taenia coli smooth muscle cells contain at least two types of voltage-dependent \(Ca^{2+}\) channels (T and L types) (38). Since dihydropyridine \(Ca^{2+}\) antagonists inhibited the \(Ca^{2+}\) channel current in guinea pig taenia coli smooth muscle cells, it is suggested that the L-type current predominates (25, 28). SNP partially but significantly decreased the inward current in the taenia and was recovered after removal of SNP. Previous studies showed that NO or cGMP decreases [\(Ca^{2+}\)] through inhibiting \(Ca^{2+}\) entry through voltage-gated \(Ca^{2+}\) channels in other smooth muscles (3, 6, 16). These findings suggest that the inhibition of L-type \(Ca^{2+}\) currents may be at least partly responsible for the decrease in [\(Ca^{2+}\)] in the taenia. Akbarali and Goyal (1) reported that SNP has a direct inhibitory effect on L-type \(Ca^{2+}\) currents in opossum esophageal circular muscle cells. However, this mechanism may not be responsible for the inhibition in taenia coli, since ODQ, a soluble guanylate cyclase inhibitor, almost completely restored the SNP-induced inhibition of \(I_{\text{Ba}}\) (Fig. 3). Any SNP-induced decrease in \(Ca^{2+}\) channel activity may be mediated by the phosphorylation of \(Ca^{2+}\) channels, since it has been demonstrated that calycin A, a phosphatase inhibitor, inhibits \(Ca^{2+}\) channel activity in canine GI smooth muscle (36). The phosphorylation may be catalyzed by cGMP-dependent, but not cAMP-dependent, protein kinase (17).

Whole cell recordings using the patch-clamp technique showed that carbachol activates nonselective cation channels in guinea pig taenia coli smooth mus-
The present results showed that SNP reversibly inhibited this cation current activated by carbachol (Fig. 4). Because the effect of SNP can be antagonized by ODQ, it is likely that this mechanism is also mediated by the guanylyl cyclase system coupled to NO. It has also been reported that, in vascular smooth muscles, NO donors and cGMP inhibit the nonselective cation currents (24). These results indicate that SNP reduces the carbachol-induced inward current by inhibiting the activity of a nonselective cation channel in the taenia.

Table 1. Effect of SNP on cyclic nucleotide contents of taenia coli

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cyclic Nucleotide Content, pmol/g wet wt</th>
<th>n</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cGMP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.5 ± 3.1</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>Forskolin (1 μM)</td>
<td>8.2 ± 3.0</td>
<td>6</td>
<td>86</td>
</tr>
<tr>
<td>SNP (1 μM)</td>
<td>57.2 ± 14.7</td>
<td>6</td>
<td>602</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cAMP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>455.2 ± 24.7</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>Forskolin (1 μM)</td>
<td>3,457.2 ± 70.5</td>
<td>6</td>
<td>759</td>
</tr>
<tr>
<td>SNP (1 μM)</td>
<td>538.1 ± 69.7</td>
<td>6</td>
<td>118</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of cells. SNP, sodium nitroprusside. In experiments 1 and 2, muscle strips were treated with each concentration of agents for 10 min.

An increase in the K⁺ current that balances the inward current during the stimulation would tend to...
decrease the level of depolarization, which in turn inhibits L-type Ca$^{2+}$ channel activity. In many types of smooth muscle cells, the major outward current in response to membrane depolarization is carried by Ca$^{2+}$-sensitive K$^+$ channels (4, 32). In the present experiment, SNP increased the outward K$^+$ current in all ranges, and these effects were completely inhibited by pretreatment withiberiotoxin, a highly selective and potent blocker of the K$_{ca}$ channel (10) (Fig. 5). Previously, He and Goyal (12), using single cells of the guinea pig ileum, showed an increase in outward current when SNP or 8-bromo-cGMP was added to the bath solution. It has also been reported that nitrovasodilator and cGMP induce outward currents by activating charybotoxin-sensitive K$^+$ channels in vascular smooth muscle (2). Other electrophysiological studies have also indicated that K$_{ca}$ channels are activated by nitrovasodilators and NO in many types of smooth muscle cells (22, 30, 35, 37). Although many studies have demonstrated that NO donors activate K$_{ca}$ channels, Comport et al. (7) reported that, in human bronchi, the opening of K$_{ca}$ channels does not functionally participate in the relaxation in response to SNP. Further study is needed to clarify the relative contribution of this mechanism.

In rat mesenteric artery, Nishimura and van Breemen (27) reported that cGMP inhibits the Ca$^{2+}$-induced contraction by the decrease in Ca$^{2+}$ sensitivity of contractile elements stimulated by norepinephrine. The present study demonstrated that, in permeabilized taenia coli, SNP also inhibited the Ca$^{2+}$-induced contraction, but the degree of inhibition was much smaller than in the vascular smooth muscle strips (Fig. 6). In intact taenia coli, SNP inhibited the MLC phosphorylation and contraction in parallel with the decrease in [Ca$^{2+}$]i, and the change in the Ca$^{2+}$ sensitivity of contractile elements due to SNP was not evident (Fig. 2). Thus we concluded that the decrease in force is attributable mainly to the decrease in [Ca$^{2+}$]i. In swine carotid arteries, McDaniel et al. (23) reported that nitrovasodilators can inhibit vascular contraction by reduction of [Ca$^{2+}$]i or uncoupling of stress from MLC phosphorylation. In the same preparation, Van Riper et al. (34) reported that nitrovasodilator induces site A MLC kinase phosphorylation by cGMP-dependent activation of cAMP-dependent protein kinase to decrease Ca$^{2+}$ sensitivity of the contractile element. The difference in the effects of SNP may be due to the difference in preparations.

In summary, we demonstrated that SNP, through cGMP-dependent signaling pathways, inhibits MLC phosphorylation and smooth muscle contraction by decreasing [Ca$^{2+}$]i, which resulted from the combination of activation of K$_{ca}$ currents, the inhibition of nonselective cation currents, and the inhibition of voltage-dependent Ca$^{2+}$ currents in guinea pig taenia coli. The decrease in the Ca$^{2+}$ sensitivity of contractile elements caused by SNP may not play a role in the muscarinic-stimulated taenia coli.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

REFERENCES


20. Kitazawa T, Kobayashi S, Horiiuti K, Somlyo AV, and Somlyo AP. Receptor-coupled, permeabilized smooth muscle: role of the phosphatidylinositol cascade, G-proteins, and modulation of...


