Mechanism of a Nitric Oxide Donor NOR 1-Induced Relaxation in Longitudinal Muscle of Rat Proximal Colon

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ABSTRACT—We previously suggested that nitric oxide (NO)-mediated relaxation of the rat proximal colon is not associated with change in cyclic GMP content. We further studied the intracellular mechanism of NO-induced relaxation by measuring changes in tension and intracellular Ca2+ concentration ([Ca2+]i), simultaneously. NOR 1, NO donor, relaxed the longitudinal muscle of the rat proximal colon, which was precontracted by carbachol, with a concomitant decrease in [Ca2+]i. ODQ, an inhibitor of soluble guanylate cyclase, partially inhibited the relaxant effect of only higher concentrations of NOR 1, but Rp-8-Br-cGMPS, an inhibitor of cyclic GMP-dependent protein kinase (PKG), did not have any effects on the relaxant effect of NOR 1. When the preparations were transferred to normal solution after the treatment with thapsigargin, an inhibitor of sarcoplasmic reticulum (SR) Ca2+-ATPase, in the absence of Ca2+, contraction with a concomitant increase in [Ca2+]i occurred. NOR 1 did not show significant effects on the tension and [Ca2+]i in thapsigargin-treated preparations. In high K+-precontracted preparations, NOR 1 relaxed the preparations with a slight change in [Ca2+]i. The relaxant effect was significantly inhibited by ODQ and Rp-8-Br-cGMPS. These results suggest that NO induces the relaxation preferentially by acting thapsigargin-sensitive function of SR and in turn decreasing [Ca2+]i, although a cyclic GMP-PKG pathway is suggested under the experimental conditions of a high K+ concentration.

Keywords: Nitric oxide-induced relaxation, NOR 1, Rat proximal colon, Decrease in intracellular Ca2+ concentration, Sarcoplasmic reticulum

Nitric oxide (NO) has been reported to mediate non-adrenergic, non-cholinergic (NANC) relaxation in various regions of the gastrointestinal tract (for reviews, see refs. 1–3). However, the intracellular mechanism of relaxation induced by NO is not clarified yet.

It was shown that an elevation in cyclic GMP content was associated with electrical field stimulation (EFS)-induced relaxation in the opossum (4) and human (5) lower esophageal sphincter and the canine internal anal sphincter (6). NO is known to increase the intracellular cyclic GMP content through an activation of soluble guanylate cyclase in vascular smooth muscles (7). It was also shown that NO or EFS which induces NO-mediated relaxation, increased the cyclic GMP content in the smooth muscle cells in the rat ileum (8) and proximal colon (9). These results suggest that NO induces the relaxation of smooth muscle by increasing the cyclic GMP content. On the contrary, an inhibitor of soluble guanylate cyclase inhibited the NO-mediated increase in cyclic GMP content, whereas it did not affect NO-mediated relaxation in the guinea pig and porcine trachea (10, 11), rat mesenteric artery (12) and canine aorta (13). Cyclic GMP-independent NO-mediated relaxation was also suggested in the gastrointestinal tract such as the rat duodenum (14) and proximal colon (15). There are also interesting reports that NO induces relaxation via cyclic GMP-dependent and -independent mechanisms in the rat aorta (16) and NO induces relaxation via a cyclic GMP-independent mechanism in the rabbit aorta when the cyclic GMP-dependent protein kinase (PKG) system is blocked (17). These results indicate that the involvement of cyclic GMP in NO-mediated relaxation differs among animal species and tissues.

The tension of the smooth muscle is mainly determined by the amount of phosphorylated myosin light chain (MLC), which is related to intracellular Ca2+ concentration.
PKG, which in turn increases uptake of Ca\textsuperscript{2+}. Cyclic GMP increased by NO donor activates activated K\textsuperscript{+} channels of the membrane Ca\textsuperscript{2+} by the sarcoplasmic reticulum (SR) and extrusion by membranes. Therefore, excised segment were flushed out with Tyrode solution.

### MATERIALS AND METHODS

Male Wistar rats (250 – 350 g) obtained from Clea Japan, Inc. (Osaka) were lightly anesthetized with ether and then stunned by a blow on the head and bled via the carotid artery. The proximal colon was removed and placed in Tyrode solution consisting of: 137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl\textsubscript{2}, 1.1 mM MgCl\textsubscript{2}, 0.42 mM NaH\textsubscript{2}PO\textsubscript{4}, 11.9 mM NaHCO\textsubscript{3}, and 5.6 mM glucose. The contents of the excised segment were flushed out with Tyrode solution.

#### Measurement of intracellular Ca\textsuperscript{2+} level of longitudinal muscle cells of proximal colon

Small strips (about 2.0 × 7.0 mm) of longitudinal muscle were incubated in Tyrode solution containing 5 \(\mu\)M fura-2 acetoxyethyl ester (fura-2 AM) and 0.02% cremophor EL for 5 – 6 h at 25°C under 5% CO\textsubscript{2} in O\textsubscript{2} and for about 18 h at 4°C. Then, the fura-2 loaded muscle was washed and mounted on a strain gauge transducer (U gauge, UL-2GR; Minebea, Tokyo) to record contractile responses isometrically. Changes in Ca\textsuperscript{2+} level were measured simultaneously in a CAF-100 spectrofluorometer (Nihonbunko, Tokyo), using 340/380 nm excitation with emission at 510 nm. A load of 0.25 g was applied as a resting tension. Contractions and increases in Ca\textsuperscript{2+} level of the muscle strips were induced by 1 \(\mu\)M carbachol (CCh) or 100 mM K\textsuperscript{+}. Relaxation and changes in Ca\textsuperscript{2+} induced by the NO donor OR1 were examined after the contraction reached a constant level. For the quantitative comparison of [Ca\textsuperscript{2+}], the tonic phase of [Ca\textsuperscript{2+}] level induced by 1 \(\mu\)M CCh or 100 mM K\textsuperscript{+} was taken as 100%. Changes in tension were expressed as percentages of the tonic contraction induced by 1 \(\mu\)M CCh or 100 mM K\textsuperscript{+}. K\textsuperscript{+} channel blockers or cyclic GMP-related drugs were applied for at least 15 min before the effect of OR1 was examined.

#### Drugs

Charybdotoxin, thapsigargin and 8-bromoguanosine-3',5'-cyclic monophosphate (8-Br-cyclic GMP) were purchased from Sigma Chemical Co., St. Louis, MO, USA. 8-Bromoguanosine-3',5'-cyclic monophosphorohioate, Rp-isormer (Rp-8-Br-cyclic GMPS), was from Biolog Life Sci, Inst., Bremen, Germany. Fura-2 acetoxyethyl ester (fura-2 AM), (±)-(E)-methyl-2-[((hydroxyiminio)]-5-nitro-6-methoxy-3-hexanamide (NOR 1), S-nitroso-N-acetyl-d,l-penicillamine (SNAP) and 1H-[1,2,4]oxadiazoild-[4,3-\(\alpha\)]quinoxalin-1-one (ODQ) were from Dojin, Kumamoto. Apamin was from the Peptide Institute, Osaka. Carbamyl-choline chloride (carbachol, CCh) was from Wako Pure Chemicals, Osaka. All other chemicals were of analytical grade. Thapsigargin, NOR 1, SNAP, ODQ and fura-2 AM were dissolved in dimethyl sulfoxide (DMSO) at stock solutions. The final DMSO concentration was 0.1%, which did not have any effect on preparations. Other drugs were added as redistilled-water solutions in volumes of less than 1.0% of the bathing solution. A similar volume of redistilled water alone had no effect on the muscle activity.

#### Statistical analyses

Data are expressed as means ± S.E.M. Results were analyzed by Student’s \(t\)-test and a \(P\) value of <0.05 was regarded as significant.
RESULTS

Effects of NOR 1 on tension and [Ca$^{2+}$]$_i$ of the precontracted preparation of the rat proximal colon by CCh

CCh induced a biphasic contraction of longitudinal muscle of the rat proximal colon, consisting of a rapid phasic contraction and subsequent tonic contraction, with concomitant increase in [Ca$^{2+}$]. The increase in [Ca$^{2+}$]$_i$ was also biphasic, a transient phasic component followed by a tonic sustained component. Both tonic sustained phases lasted for 20 – 30 min. Decreases in contraction and [Ca$^{2+}$]$_i$ 15 min after reaching the tonic phase were 7.0 ± 2.2% and 5.9 ± 2.7%, respectively. When the tonic phases of contraction and the increase in [Ca$^{2+}$]$_i$ reached constant levels, NOR 1, a NO donor, was added to the organ bath to examine its effects on the tension and [Ca$^{2+}$]$_i$. NOR 1 simultaneously decreased the tension and [Ca$^{2+}$]$_i$ in a concentration-dependent manner (Fig. 1A). Figure 1B shows a summary of the effects of NOR 1 on the tension and [Ca$^{2+}$]$_i$. The EDT$_{50}$ values of NOR 1 on the tension and [Ca$^{2+}$]$_i$ were 1.46 ± 0.15 and 2.54 ± 0.4 μM, respectively. SNAP, another NO donor, also induced relaxation and a decrease in [Ca$^{2+}$]$_i$ in a similar manner to that of NOR 1 (data not shown).

To know whether cyclic GMP is involved in the NOR 1-induced relaxation, effects of ODQ, an inhibitor of soluble guanylate cyclase, on the tension and [Ca$^{2+}$]$_i$ were examined. After the ODQ (1 μM) treatment, effects of NOR 1 at lower concentrations on the tension and [Ca$^{2+}$]$_i$ remained unchanged, but those of NOR 1 at higher concentrations than 1 μM were moderately inhibited (Fig. 2). Increasing the concentration of ODQ to 10 μM did not induce further effects on both parameters (n = 2, data not shown). Next, to know whether PKG is involved in the NOR 1-induced relaxation, effects of Rp-8-Br-cGMPS, an inhibitor of PKG, were examined. The drug at 30 μM, which maximally inhibited the relaxant effect of NOR 1 on high K$^+$-induced contraction as shown in the experiment described below, did not have any effect on the relaxation and the decrease in [Ca$^{2+}$]$_i$ induced by NOR 1 (Fig. 3). Rp-8-Br-cGMPS also did not have any effects on those induced by SNAP (data not shown). These results suggest that cyclic GMP itself somewhat decreases the tension and [Ca$^{2+}$]$_i$ in the precontracted preparations by CCh, but PKG has no role in them. Indeed, a membrane permeable analog of cyclic GMP, 8-Br-cGMP at 30 μM decreased the tension and [Ca$^{2+}$]$_i$ (Fig. 4).

Fig. 1. Relaxation and decrease in [Ca$^{2+}$]$_i$ of longitudinal muscle of rat proximal colon in response to NOR 1. Relaxant responses and the fura-2 fluorescence signals ([Ca$^{2+}$]$_i$) were simultaneously recorded. A) Muscle strips of the proximal colon preloaded with fura-2 AM were first contracted with 1 μM carbachol (CCh). When CCh-induced responses reached a constant level, the effects of various concentrations of NOR 1 indicated were examined. The top panel shows NOR 1-induced relaxation and the bottom panel shows the changes in [Ca$^{2+}$]$_i$, as indicated by the fluorescence ratio, F340/F380. Lines indicate the presence of 1 μM CCh. Arrows show the times of application of NOR 1. W, washout. B) Summary of effects of NOR 1. Changes in tension and [Ca$^{2+}$]$_i$ are expressed as percentages of the tonic phases induced by 1 μM CCh. Values are means ± S.E.M. for 7 experiments.
Apamin and charybdotoxin, inhibitors of a small conductance Ca\(^{2+}\)-activated K\(^+\) channel and a large conductance Ca\(^{2+}\)-activated K\(^+\) channel, respectively, did not affect NOR 1-induced relaxation (data not shown).

Examination of role of SR in NOR 1-induced relaxation

The preparations from the rat proximal colon were treated with 1 \(\mu\)M thapsigargin, an inhibitor of SR Ca\(^{2+}\)-ATPase, for 1 h in the absence of Ca\(^{2+}\). When the preparations were transferred to normal Tyrode solution, the lon-
A high concentration of K⁺ (100 mM) induced a biphasic contraction with concomitant increase in [Ca²⁺]. When the tonic phases of contraction and the increase in [Ca²⁺], reached constant levels, the addition of NOR 1 induced the relaxation with very slight decrease in [Ca²⁺]. (Fig. 5). ODQ at 1 μM completely inhibited this relaxation (Fig. 6). Rp-8-Br-cGMPS also inhibited the relaxation in a concentration-dependent manner, but its maximal inhibition at 30 μM was still partial, about 40% inhibition (Fig. 7). 8-Br-cGMP induced relaxation without any changes in [Ca²⁺]. (Fig. 4).

**DISCUSSION**

In the present study, we tried to clarify the mechanism by which NO, released from NOR 1, induced relaxation of longitudinal muscle of the rat proximal colon by measuring changes in muscle tension and [Ca²⁺], simultaneously. In CCh-precontracted preparations, NOR 1 induced the relaxation, with a concomitant decrease in [Ca²⁺], in a concentration-dependent manner. The relaxation and the decrease in [Ca²⁺] by NOR 1 seem quite parallel, suggesting that NO induces the relaxation through the decrease in [Ca²⁺].

It is well known that NO activates soluble guanylate cyclase and in turn increases cyclic GMP content (7, 29). NO was suggested to relax the smooth muscle via an increase in cyclic GMP content in various tissue preparations (4, 31). ODQ at 1 μM completely inhibited the relaxation induced by NO donors, but not that induced by cyclic GMP analogs (32). In the present study, ODQ had no effect on the inhibitory responses in the tension and [Ca²⁺], induced by NOR 1 at lower concentrations, but partially counteracted the effects of NOR 1 at concentrations higher than 1 μM. Therefore, it seems likely that NOR 1 at lower concentrations induces the relaxation via a cyclic GMP-independent mechanism and at higher concentrations (higher than 1 μM under the present experimental conditions) via a cyclic GMP-dependent as well as independent mechanism in the longitudinal muscle of the rat proximal colon. We previously suggested that NO-mediated relaxation of the rat proximal colon induced by EFS is not associated with change in cyclic GMP content of the tissue, since soluble guanylate cyclase inhibitors inhibited the NO-mediated increase in the cyclic GMP content, but did not affect the NO-mediated relaxation (15). Therefore, it is reasonable to postulate that EFS-induced relaxation of longitudinal muscle of the rat proximal colon is mediated by NO at a concentration that does not increase cyclic GMP content to such a level that the relaxation is induced. In other words, the cyclic GMP-dependent pathway is not involved in the relaxation induced by EFS at the supramaximal voltage. Furthermore, it should be reminded that ODQ was also suggested to inhibit the NANC relaxation in a cyclic GMP-PKG pathway-independent manner (33). The effects of exogenously added NOR 1 at the concentrations higher than 1 μM were partially cyclic GMP dependent. Cyclic GMP activates the PKG in the smooth muscle (34). Rp-8-Br-cGMPS inhibited the PKG purified from the porcine aorta with apparent Kᵢ values of 2 – 3 μM and completely inhibited the 8-Br-cGMP-induced responses at 10 μM in the rat mesenteric artery (35). However, activation of PKG does not seem to be involved in NOR 1-induced relaxation of longitudinal muscle of the rat proximal colon, since at 30 μM, it did not affect the responses induced by NOR 1 in the present study.

The relationship between a decrease in [Ca²⁺] and opening of Ca²⁺-activated K⁺ channels in the smooth muscle cell was reported in the rat duodenum (14) and rat pulmonary artery (36). However, apamin and charybdotoxin, inhibitors of Ca²⁺-activated K⁺ channels, did not affect the relaxation of NOR 1 in the present study. The results are consistent
with our previous results that exogenously added NO did not change the membrane potentials of the longitudinal smooth muscle cells of the rat proximal colon (22). It was suggested that NO decreased \([Ca^{2+}]_i\) by increasing the SR Ca\(^{2+}\) uptake in the guinea pig gastric fundus (28) and the rabbit (25) and rat (26) aorta. Thapsigargin depletes

\[\text{NO-Induced Relaxation in Colon}\]

**Fig. 5.** Effects of the treatment of the proximal colon muscle strip preparations with thapsigargin on NOR 1-induced relaxation and decrease in \([Ca^{2+}]_i\). A) Muscle strips of the proximal colon preloaded with fura-2 AM were first contracted with 1 \(\mu\)M carbachol (CCh). When CCh-induced responses reached a constant level, effects of various concentrations of NOR 1 were examined. Then, the preparations were treated with 1 \(\mu\)M thapsigargin for 1 h in the absence of Ca\(^{2+}\) and presence of 2 mM EGTA. When the preparations were transferred to normal Tyrode solution containing 1.8 mM Ca\(^{2+}\), contraction with concomitant increase in \([Ca^{2+}]_i\) occurred. The top panel shows NOR 1-induced relaxation and the bottom panel shows the changes in \([Ca^{2+}]_i\) as indicated by the fluorescence ratio, F340/F380. Lines indicate the presence of indicated drugs. Arrows show the times of application of NOR 1. W, washout. B) Summary of the relaxation (a) and decrease in \([Ca^{2+}]_i\) (b) in CCh-precontracted (control) or thapsigargin (TG)-treated preparations. Changes in tension and \([Ca^{2+}]_i\) are expressed as percentages of the tonic phases induced by 1 \(\mu\)M CCh or those by transferring the preparations to normal Tyrode solution after thapsigargin treatment, respectively. Values are means ± S.E.M. for 4 experiments. *Significantly different from the values in the corresponding controls at \(P<0.05\).
Ca\(^{2+}\) content of SR by inhibiting SR Ca\(^{2+}\)-ATPase (37). We examined the effect of thapsigargin on the NOR 1-induced responses in the rat proximal colon. NOR 1 lost its effects on the tension and [Ca\(^{2+}\)] \(i\) after the treatment of the preparations with thapsigargin. Although NO is recently suggested to decrease [Ca\(^{2+}\)] \(i\) by inhibiting Ca\(^{2+}\) release from SR in the canine cardiac muscle (38) and the porcine tracheal smooth muscle (39), our results suggest that NO may induce the relaxation by directly activating the uptake of Ca\(^{2+}\) to SR and indirectly via cyclic GMP, probably significantly increased by high concentrations of NO. NO is known to modulate the activity of SR Ca\(^{2+}\)-ATPase by a direct action on SR Ca\(^{2+}\)-ATPase protein in skeletal muscle (40, 41). However, details of the direct action of NO on the activity of SR Ca\(^{2+}\)-ATPase in smooth muscle cells of the rat proximal colon are not clear at present.

NOR 1 also induced the relaxation of the preparations that were precontracted by a high K\(^+\) concentration (100 mM), with very slight change in [Ca\(^{2+}\)] \(i\), suggesting Ca\(^{2+}\)-independent relaxation of longitudinal muscle of the rat proximal colon. ODQ completely and Rp-8-Br-cGMPS significantly inhibited the NOR 1-induced relaxation in contrast to their effects on CCh-precontracted preparations. 8-Br-cGMP also induced the relaxation without a change in [Ca\(^{2+}\)] \(i\) in high K\(^+\)-precontracted preparations. These results indicate that the relaxation by NO in the high K\(^+\)-precontracted preparations is cyclic GMP-PKG pathway dependent. In membrane-permeabilized preparations of the rat mesenteric artery (34) and proximal colon (42), cyclic GMP or cyclic GMP analogs relaxed the preparations via the PKG pathway, which in turn decreases Ca\(^{2+}\)-sensitivity of the contractile elements. Taking these results into account, NO seems likely to relax high K\(^+\)-precontracted prepara-

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**Fig. 6.** Effects of NOR 1 on tension and [Ca\(^{2+}\)] \(i\). Increased by a high K\(^+\) concentration and inhibitions of the effects by ODQ. When high K\(^+\)-induced responses reached a constant level, effects of 1 \(\mu\)M NOR 1 were examined. The top panel shows NOR 1-induced relaxation and the bottom panel shows the change in [Ca\(^{2+}\)] \(i\), as indicated by the fluorescence ratio, F340/F380. Lines indicate the presence of indicated drugs.

**Fig. 7.** Effects of Rp-8-Br-cGMPS on NOR 1-induced relaxation and decrease in [Ca\(^{2+}\)] \(i\). In high K\(^+\)-precontracted preparations. Relaxation and decrease in [Ca\(^{2+}\)] \(i\) were induced by 1 \(\mu\)M NOR 1 in the absence (control) or presence of 30 \(\mu\)M Rp-8-Br-cGMPS in high K\(^-\)-precontracted preparations. When 100 mM K\(^+\)-induced responses reached constant levels, the effects of various concentrations of NOR 1 indicated were examined (control). After the washing, the effects of NOR 1 in the presence of 30 \(\mu\)M Rp-8-Br-cGMPS were examined again. Changes in tension and [Ca\(^{2+}\)] \(i\), are expressed as percentages of the tonic phases induced by 100 mM K\(^+\). Values are means ± S.E.M. for 4 experiments. *Significantly different from the control at P<0.05.

Ca\(^{2+}\) content of SR by inhibiting SR Ca\(^{2+}\)-ATPase (37). We examined the effect of thapsigargin on the NOR 1-induced responses in the rat proximal colon. NOR 1 lost its effects on the tension and [Ca\(^{2+}\)] \(i\) after the treatment of the preparations with thapsigargin. Although NO is recently suggested to decrease [Ca\(^{2+}\)] \(i\) by inhibiting Ca\(^{2+}\) release from SR in the canine cardiac muscle (38) and the porcine tracheal smooth muscle (39), our results suggest that NO may induce the relaxation by directly activating the uptake of Ca\(^{2+}\) to SR and indirectly via cyclic GMP, probably significantly increased by high concentrations of NO. NO is known to modulate the activity of SR Ca\(^{2+}\)-ATPase by a direct action on SR Ca\(^{2+}\)-ATPase protein in skeletal muscle (40, 41). However, details of the direct action of NO on the activity of SR Ca\(^{2+}\)-ATPase in smooth muscle cells of the rat proximal colon are not clear at present.

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tions through a decrease in Ca$^{2+}$ sensitivity of contractile elements. However, this mechanism of relaxation was very slight in the CCh-precontracted preparations, if any. The discrepancy between the relaxant mechanisms of NOR 1 in CCh- and high K$^+$-precontracted preparations may be due to the difference in the extent of the increase in [Ca$^{2+}$]. Under the high K$^+$ condition, the plasma membrane of smooth muscle cells is depolarized and continuous influx of a large amount of Ca$^{2+}$ ions must occur. In present study, the change in [Ca$^{2+}$], level necessary for the contraction to a certain extent induced by a high K$^+$ concentration was three times than that induced by CCh in each preparation (data not shown).

These results suggest that NO induces the relaxation in CCh-precontracted longitudinal muscle of the rat proximal colon through the decrease in [Ca$^{2+}$], by acting on a thapsigargin-sensitive function of SR, probably by activating SERCA 2 (Fig. 8, pathway 1). NO only at higher concentrations may further activate the cyclic GMP-dependent mechanism (Fig. 8, pathway 2). On the other hand, under the experimental conditions of a high K$^+$ concentration, an additional mechanism of the cyclic GMP-PKG pathway may be developed, although its physiological significance is not clear (Fig. 8, pathway 3).

Fig. 8. Pharmacologically inferred mechanisms of relaxation mediated by NO in longitudinal muscle of the rat proximal colon. Excitation of the myenteric nerves induced by EFS or exogenously added NO at low concentrations induces the relaxation via activation of SERCA 2 which in turn decrease in [Ca$^{2+}$], (pathway 1). Cyclic GMP significantly increased by high concentrations of NO or membrane permeable cyclic GMP analog at high concentrations also activates SERCA 2, probably by a direct action (pathway 2). Cyclic GMP-PKG pathway which had been shown in permeabilized preparations was also suggested under the conditions of a high K$^+$ concentration (pathway 3).

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