Dissociation of Cyclic GMP Level from Relaxation of the Distal, but Not the Proximal Colon of Rats

Naowarat Suthamnatpong, Tomofumi Maehara, Atsunori Kanada, Tadayoshi Takeuchi and Fumiaki Hata

Department of Veterinary Pharmacology, College of Agriculture, University of Osaka Prefecture, Sakai 593, Japan

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ABSTRACT—The role of cyclic GMP (cGMP) in nonadrenergic, noncholinergic (NANC) relaxation of the longitudinal muscle of rat proximal and distal colon was examined. Electrical field stimulation (EFS) of preparations of longitudinal muscle from the proximal region significantly increased the cGMP content. Nitro-L-arginine inhibited this increase, and L-arginine reversed the inhibitory effect of nitro-L-arginine. Exogenously added nitric oxide (NO) and atrial natriuretic peptide (ANP) also increased the cGMP content of preparations of the proximal colon and induced muscle relaxation. From these and our previous findings suggesting an essential role of NO in NANC inhibition in the proximal colon, we conclude that the mechanism of NANC inhibition in the proximal region of rat colon involves NO and a cGMP generating system. In contrast, although exogenously added NO and ANP increased the cGMP content in the distal colon to the same extent as in the proximal colon, they did not induce any muscle relaxation. Vasoactive intestinal peptide (VIP), the most likely candidate as a NANC neurotransmitter in rat distal colon, did not increase the cGMP content in this region. Furthermore, no participation of NO in the NANC inhibitory response was observed in the distal region, but EFS increased the cGMP content significantly. Thus we conclude that relaxation of longitudinal smooth muscle in the distal portion of rat colon is not associated with a change in the cGMP content.

Keywords: Cyclic GMP, Nitric oxide (NO), Vasoactive intestinal peptide (VIP), Nonadrenergic, noncholinergic (NANC) inhibition, Colonic motility

Several lines of evidence suggest that nitric oxide (NO) is a mediator of the nonadrenergic, noncholinergic (NANC) inhibitory response in various parts of the gastrointestinal tract, including the caecum (1) and stomach (2, 3) of guinea pigs; the gastric fundus (4, 5), duodenum (6) and proximal colon (7) of rats; and human ileum (8). The release of NO was suggested to occur during nerve stimulation of the canine ileocolonic junction (9) and rat gastric fundus (10). The high immunoreactivity of NO synthase in the myenteric plexus throughout the gut (11, 12) also supports the idea that NO is involved in the NANC inhibitory response.

At present, the exact mechanism of the action of NO is unknown. Some reports suggest that NO and other nitrovasodilators induce relaxation of vascular smooth muscle cells by increasing the cyclic GMP (cGMP) content (13–16). There is also recent evidence that cGMP formation is involved in the non-vascular smooth muscle relaxation elicited by NO or NO-producing compounds in preparations of taenia coli (17), corpus cavernosum (18, 19), tracheal (20) and ileal (21) smooth muscle. In these preparations, the relaxant effects of NO or NO-producing compounds were closely associated with an elevation of the intracellular cGMP level. NO was reported to activate soluble guanylate cyclase and increase the cGMP level in various tissue preparations (22). These observations strongly support the idea that cGMP is involved in smooth muscle relaxation, although some data, such as those on rat vas deferens (23), rat myometrium and guinea pig taenia coli (24) do not support this idea.

Our previous studies (25) indicated that the mediators of the NANC inhibitory response in longitudinal muscle are different in different regions of rat colon, and that other myenteric neurons, such as vasoactive intestinal peptide (VIP) neurons, are closely related to the NANC inhibitory response. We have obtained evidence that NO is a mediator of the inhibitory NANC response in the proximal colon and have suggested that VIP, but not NO, is involved in inhibitory neurotransmission in the distal colon.
Atrial natriuretic peptide (ANP) was found to activate the particulate form of guanylate cyclase specifically (26), and immunoreactive ANP was demonstrated in rat stomach and small intestine (27). ANP was also reported to induce relaxation of vascular (28), ileal (29), and rectal (30) smooth muscle. Thus, it is of interest to study the effect of ANP on colonic smooth muscle in relation to the cGMP content.

In the present work, we extended our previous studies by investigating whether the formation of cGMP is involved in smooth muscle relaxation. We examined the effects of NO, ANP and electrical nerve stimulation on the mechanical responses and cGMP levels of longitudinal muscle from the proximal and distal colon of rats. The results showed clear dissociation of relaxation of the muscle from the cGMP content in the distal colon, but their association in the proximal colon. The relationships between cGMP levels and the relaxation are discussed in connection with mediators of NANC inhibition. Some of the results presented in this paper have been reported in preliminary form (31).

MATERIALS AND METHODS

Materials

Nitro-L-arginine (N^5-nitroamidino-L-2,5-diaminopentanoic acid) and L-arginine hydrochloride were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Atrial natriuretic peptide and vasoactive intestinal peptide were from Peptide Institute, Inc., Osaka. All other chemicals were of analytical grade. Nitric oxide solution was prepared freshly before experiments from gaseous nitric oxide as described by Gillespie and Sheng (32).

Preparation of proximal and distal colonic segments

Male Wistar rats (250–380 g) were lightly anesthetized with ether and then stunned by a blow on the head and bled via the carotid artery. Segments of rat proximal and distal colon (2–3 cm in length) were prepared as described elsewhere (25).

Recording of responses of colonic longitudinal muscle to relaxants or electrical transmural stimulation (TMS)

Colonic segments were suspended in an organ bath filled with Tyrode solution consisting of 136.9 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl\(_2\), 1.05 mM MgCl\(_2\), 0.4 mM NaH\(_2\)PO\(_4\), 11.9 mM NaHCO\(_3\) and 5.6 mM glucose, aerated with 5% CO\(_2\) and 95% O\(_2\) and maintained at 37°C. During the 30-min equilibration period, the preparations were washed with fresh Tyrode solution every 10 min. The longitudinal muscle was subjected to a resting load of 1.0 g. Responses of the longitudinal muscle to TMS with trains of 100 pulses of 0.1-msec width at 30 V and 10-Hz frequency were recorded isotonically with a 10-min interval between tests in the presence of 1 µM atropine and 4 µM guanethidine (25). Drugs were added to the organ bath in volumes of less than 1.0% of the bathing solution. The vehicle of the drugs, redistilled water, at the volume used did not affect the spontaneous contractile activity or the muscle tone of the preparation.

Measurement of cGMP content of isolated longitudinal muscle from the proximal or distal colon

After an equilibration period of 30 min, the preparations were incubated for 30 sec at 37°C in the absence or presence of 10 µM nitric oxide, 100 nM ANP or 100 nM VIP. When the effects of nitro-l-arginine and l-arginine were examined, these compounds were also added during the equilibration period. After the incubation period, preparations were quickly frozen, and their cGMP contents were determined with a cGMP assay kit (Amersham Japan, Tokyo).

To determine the relationships between electrical field stimulation (EFS) and changes in cGMP levels, longitudinal muscle strips with adhering myenteric plexus were prepared from the proximal and distal colon as described by Paton and Zar (33). The strips were mounted between two plate electrodes in an organ bath: the parameters for EFS were the same as those used for TMS. After EFS for 10 sec, the preparations were quickly frozen within 5 sec after EFS for determination of their cGMP content. Every determination of cGMP was carried out in the absence of phosphodiesterase inhibitor.

RESULTS

Effects of nitro-l-arginine and l-arginine on the cGMP contents of longitudinal muscle preparations from the proximal and distal colon

Nitro-l-arginine (N^5-nitroamidino-l-2,5-diaminopentanoic acid) at a concentration of 10 µM significantly decreased the resting content of cGMP in longitudinal muscle preparations from the proximal and distal colon and the presence of 1 mM L-arginine restored the decreased cGMP content to the control level (Fig. 1). The resting contents of cGMP and effects of nitro-l-arginine and L-arginine in the two portions were similar. Neither drug had any significant effect on the spontaneous contractile activity nor muscle tone as shown previously (25).

Effects of NO and ANP on the longitudinal muscle of rat proximal and distal colon and their cGMP contents

With repeated application of ANP, the desensitization occurred, while having no effect on the relaxation induced by either electrical transmural stimulation or NO (data not shown). To prevent the desensitization to the drug, ap-
plication of a new dose of ANP was not performed until 10–15 min had elapsed after washing out the previous dose. By this method, ANP at 1–100 nM induced concentration-dependent relaxation in the proximal colon, but did not induce relaxation in the distal colon (Fig. 2). These results are consistent with previous findings that NO induced concentration-dependent relaxation of preparations of the proximal colon but had no effect on those of the distal colon (25). NO (10 μM) and ANP (100 nM) significantly increased the cGMP contents of the longitudinal muscle preparations from both the proximal and distal colon (Fig. 3). This increase in cGMP accumulation paralleled relaxation of the smooth muscle in the proximal colon but not the distal one. Namely, neither NO nor ANP induced smooth muscle relaxation of the distal colon, although they increased the cGMP content significantly (Fig. 2).

Fig. 1. Effects of nitro-L-arginine (N-L-A) and L-arginine (L-Arg.) on cGMP contents of longitudinal muscle preparations obtained from proximal and distal regions of rat colon. Longitudinal muscle preparations of the proximal and distal colon were incubated in the absence (Cont.) or presence of 10 μM nitro-L-arginine or nitro-L-arginine plus 1 mM L-arginine. Columns and bars represent means and standard errors for the numbers of experiments shown inside the columns. *Significantly different from the control value at P < 0.01 by Student's t-test. For further details, see Methods.

Fig. 2. Effects of ANP on contractile activity of longitudinal muscle of rat proximal and distal colon. A: Typical responses of the proximal and distal colonic segments to ANP. The downward direction represents relaxation. ANP was added at the times indicated by the arrows, and lines indicate the presence of ANP. In the distal colonic segments, transmural stimulation (TMS) (indicated by a small bar) induced transient relaxation. B: Dose-response curve of the proximal (○, n=5) and distal (●, n=4) colonic segments to ANP. The extent of relaxation was expressed as the area under the line of resting tone that was drawn on the center of resting spontaneous contractile activity. For further details, see Methods.
Effects of EFS on the cGMP content of longitudinal muscle preparations from the proximal and distal colon and effects of nitro-L-arginine and L-arginine during EFS on the cGMP content

EFS of the longitudinal muscle preparations from the proximal colon, increased the cGMP content about two-fold. Nitro-L-arginine at 10 μM significantly decreased the enhanced content to below the resting level, and L-arginine (1 mM) completely reversed this decrease (Fig. 4).

TMS induced NANC relaxation in the proximal colon, nitro-L-arginine (10 μM) inhibited the relaxation, and L-arginine (1 mM) reversed the inhibition, as shown previously (25). On the other hand, in the distal colon, TMS-induced relaxation was independent from the NO-generating system, as shown previously (25). Nevertheless, EFS also increased the cGMP content, although somewhat less than in the proximal region. In the distal colon, nitro-L-arginine (10 μM) also inhibited the increase in the cGMP content induced by EFS, and L-arginine (1 mM) reversed this inhibition (Fig. 4).

Effect of VIP on the cGMP content in longitudinal muscle of the distal colon

VIP at concentrations of 0.01–0.1 μM induced sustained relaxation of longitudinal muscles of the proximal and distal colon, as shown previously (25). However, VIP at 0.1 μM did not increase the cGMP level in the distal or proximal colon (Fig. 5).

DISCUSSION

Previously we found that TMS elicited relaxation of proximal colonic segments from rats, which was not affected by atropine or guanethidine, but was abolished by tetrodotoxin and nitro-L-arginine. Moreover, exogenously applied NO induced dose-dependent relaxation of the segments (25). From these results, we proposed that NO is a mediator of longitudinal muscle relaxation in the
proximal colon. In vascular (16, 34) and nonvascular (17–20, 34, 35) smooth muscle, NO is believed to stimulate soluble guanylate cyclase and induce relaxation through the formation of cGMP.

In the present study, we also examined the effect of ANP, which specifically activates the particulate guanylate cyclase (26). Both NO and ANP increased the cGMP level and induced relaxation of segments of the proximal colon, suggesting the existence of two separate and independently regulated pools of guanylate cyclase, soluble and particulate forms, in these preparations. Therefore, both pools seem to contribute to smooth muscle relaxation in the proximal colon. The relative roles of these forms of guanylate cyclase in cGMP elevation and relaxation of the smooth muscle are not known. However, because NO activated the soluble form of guanylate cyclase and nitro-L-arginine, a potent inhibitor of NO synthesis, inhibited the NANC inhibitory response induced by electrical nerve stimulation in the proximal colon, while desensitization of ANP did not inhibit it, we suggested that the NANC inhibitory response of the proximal colon is attributable to activation of soluble guanylate cyclase, resulting in an increase in the intracellular level of cGMP.

In this study we obtained the following strong evidence that cGMP is an intracellular mediator of relaxation of the longitudinal muscle of rat proximal colon. First, the relaxations induced by NO and electrical nerve stimulation were associated with a significant increase in the cGMP content. Second, the inhibitory effect of nitro-L-arginine on the cGMP level, and the reversal of this effect by L-arginine were parallel with the NANC responses to electrical nerve stimulation.

On the other hand, data on the role of cGMP in the relaxation of the longitudinal muscle of the rat distal colon were complex. In this portion, changes in cGMP levels caused by NO and ANP did not correlate with changes in tension. It is interesting that 10 μM NO and 100 nM ANP, which induced maximal relaxation of the proximal portion, elevated the cGMP level about 2.5- and 2-fold, respectively (Fig. 3), without appreciably affecting the muscle tone of the distal colon (ref. 25 and Fig. 2). If cGMP is a mediator of relaxation in this region, these large increases in the tissue levels of cGMP should have had relaxant effects. We found previously that in the distal colon, NANC relaxation induced by TMS was insensitive to nitro-L-arginine, suggesting an NO-independent mechanism of relaxation in this region. EFS of the distal colon increased the cGMP level, and the increase was inhibited by treatment with nitro-L-arginine (Fig. 4). If the increase of cGMP is responsible for NANC-relaxation, decrease of the cGMP content induced by nitro-L-arginine should have decreased or inhibited the NANC-relaxation induced by TMS. Thus, our findings all indicate that relaxation of the longitudinal muscle of the rat distal colon occurs by a cGMP-independent mechanism.

In the resting condition, nitro-L-arginine significantly decreased the cGMP content of the longitudinal muscle preparations of both proximal and distal regions of rat colon (Fig. 1). It did not affect the spontaneous contractile activity or muscle tone of the proximal or distal segments (25). Thus, it seems likely that cGMP in the resting condition or at a lowered level has no role in the spontaneous contractile activity or maintenance of muscle tone in either portion, but that its increase over the resting level has a relaxant effect in the proximal colon.

We have suggested that VIP mediated a part of the NANC relaxation of smooth muscle in the rat distal colon, because we found that alpha-chymotrypsin treatment and a VIP antagonist inhibited the NANC relaxation by about 40% (25). VIP had no significant effect on the cGMP level in the distal colon (Fig. 5). Therefore, VIP-induced relaxation is probably not mediated through the cGMP generating system.

The present findings on the mechanical responses and cGMP levels of the proximal and distal regions of rat colon are summarized in Fig. 6. The mechanism of the NANC inhibition in longitudinal muscle of the proximal

![Fig. 6. Summary of the present findings.](image-url)
portion of rat colon is concluded to involve an NO-cGMP generating system. However, in the distal portion, NANC relaxation is not associated with change in the cGMP level, indicating an NO-cGMP-independent mechanism involving VIP and some unknown mediator(s).

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