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Nitric oxide-mediated inhibitory response of rat proximal colon: independence from changes in membrane potential

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1 We studied the relation of nitric oxide-mediated relaxation of smooth muscle to changes in membrane potential of cells in the proximal colon of rats.

2 The resting membrane potential and electrical field stimulation (EFS)-induced junction potentials were recorded from the circular and longitudinal muscle cells.

3 Localized distension with a small balloon caused relaxation of the circular muscle on the anal side of the distended region (descending relaxation). Relaxation of the longitudinal muscle was also induced by EFS.

4 Inhibitory junction potentials (i.j.ps) were recorded from all circular muscle cells tested, but rarely from the longitudinal muscle cells.

5 The i.j.ps were recorded only in the presence of atropine but relaxations of both muscles were induced even in the absence of atropine.

6 Apamin (100 nM) completely abolished the i.j.ps recorded in both circular and longitudinal muscle cells, but had no significant effect on the relaxations of either.

7 In contrast to apamin, N^G nitro-L-arginine (10 μM) inhibited the relaxations of both muscles, but did not affect the i.j.ps.

8 Exogenously added nitric oxide (0.1–10 μM) induced relaxations of both muscles concentration-dependently, but did not affect the membrane potentials at these concentrations.

9 These data strongly suggest that nitric oxide-mediated relaxation of rat proximal colon is not associated with the i.j.ps of the cell membrane.

Keywords: Nitric oxide; rat proximal colon; apamin; nonadrenergic noncholinergic transmission; descending relaxation

Introduction

Nitric oxide has been reported to mediate an inhibitory response in various regions of the gastrointestinal tract, such as the ileocolonic junction (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1990; Ward *et al.*, 1992b), duodenum (Toda *et al.*, 1990) and the proximal colon (Dalziel *et al.*, 1991; Thornbury *et al.*, 1991; Ward *et al.*, 1992a) of dogs, proximal colon (Hata *et al.*, 1990a) and gastric fundus (Li & Rand, 1991; Boeckxstaens *et al.*, 1991) of rats, and the lower oesophagus (Murray *et al.*, 1991; Tottrup *et al.*, 1991) and internal anal sphincter (Rattan *et al.*, 1992) of opossums. The mechanism of the inhibitory action of nitric oxide is still unknown, but two possible mechanisms have been proposed. One is a pathway involving a guanosine 3′,5′-cyclic monophosphate (cyclic GMP) generating system. Increase in the cyclic GMP level is reported to be associated with the relaxation of the lower oesophageal sphincter of opossums (Torphy *et al.*, 1986; Barnette *et al.*, 1989) and man (Barnette *et al.*, 1991), and the internal anal sphincter of dogs (Grous *et al.*, 1991). Moreover, increase in the cyclic GMP content also seems to be associated with nitric oxide-induced relaxation in preparations of taenia coli of guinea-pigs (Shikano *et al.*, 1988), the proximal colon of dogs (Ward *et al.*, 1992) and rats (Suthamnatpong *et al.*, 1993b) and the ileum of rats (Kanada *et al.*, 1992). Nitric oxide has been reported to activate soluble guanylate cyclase and increase the cyclic GMP level in various preparations (Arnold *et al.*, 1977). These findings strongly suggest that a nitric oxide-cyclic GMP generating system is responsible for the inhibitory neuronal pathway in the gastrointestinal tract, though the mechanism beyond the production of cyclic GMP is still unclear.

The other possible mechanism of nitric oxide-mediated relaxation is based on studies on the membrane potentials of smooth muscle cells. A nitric oxide synthetase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) was found to inhibit the inhibitory junction potentials (i.j.ps) evoked by electrical field stimulation (EFS; Dalziel *et al.*, 1991) and hyperpolarization induced by exogenous nitric oxide in the proximal colon (Thornbury *et al.*, 1991) and small intestine (Stark *et al.*, 1991) of dogs. L-NAME also abolished the i.j.ps recorded by the double sucrose gap method from circular muscle of opossum esophagus and dog intestine (Christinck *et al.*, 1991). Thornbury *et al.* (1991) suggested that nitric oxide enhanced the open probability of Ca^{2+}-activated K^+ channels which mediate the hyperpolarization response to inhibitory neurotransmission in colonic muscle cells.

We previously suggested that nitric oxide mediates relaxation of longitudinal (Suthamnatpong *et al.*, 1993a) and circular (Hata *et al.*, 1990a) muscle of rat proximal colon. Thus, it was of interest to study whether nitric oxide-mediated relaxation of the rat proximal colon was related to the i.j.ps of the cell membrane. In the present work, we examined the effect of N^G-nitro-L-arginine, an inhibitor of nitric oxide synthase, and some K^+-channel antagonists on the i.j.ps induced by EFS and the inhibitory responses of both longitudinal and circular smooth muscles of the rat proximal colon. The data strongly suggest that nitric oxide-mediated relaxation of both muscles of the proximal colon of rats is independent of the i.j.ps. Some of the results present in this paper have been reported in preliminary form (Suthamnatpong *et al.*, 1993c).

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Methods

Male Wistar rats (250–350 g) were used. They were lightly anaesthetized with ether and then stunned by a blow on the head and bled via the carotid arteries. Proximal segments of the colon were removed and placed in Tyrode solution consisting of (in mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 5.6. The contents of the excised segments were gently flushed out with Tyrode solution.

Recording of i.j.ps in longitudinal and circular muscles of proximal colon induced by EFS

The segments of the proximal colon were mounted in a 1.5 ml organ bath maintained at 30°C and perfused continuously with Tyrode solution at a rate of 3 ml min^{-1}. Atropine (1 μM) and guanethidine (5 μM) were added to the bathing solution throughout the experiment to block cholinergic and noradrenergic responses, respectively. Membrane potentials were recorded with a conventional glass microelectrode filled with 3 M KCl with a resistance of 50–80 MΩ. The electrode impalement were made into the longitudinal muscle cells of the superficial layer or circular muscle cells of the deep layer from the serosal side (Takewaki & Ohashi, 1977). Intramural nerves within the segment were stimulated by a pair of Ag wire electrodes, one on the serosal surface 1–2 mm from the impaled glass microelectrode and the other in the solution. The distance between the two electrodes was about 20 mm.

Recording of responses of colonic longitudinal muscle to EFS

Segments of the proximal colon (2.5–3.0 cm in length) were suspended in an organ bath containing 20 ml of Tyrode solution maintained at 37°C and bubbled with 95% O_2:5% CO_2. After an equilibration period of 30 min, responses of the longitudinal muscle to EFS with trains of 100 pulses of 0.3 ms width and supramaximal voltage (20 V) at a frequency of 10 Hz were recorded isotonically, with 10 min intervals between tests.

Recording of responses of colonic circular muscle to the stimulus of distension

Colonic segments were held horizontally in a specially designed organ bath described elsewhere (Hata *et al.*, 1990b). The middle of the segment was connected by a hook at the joint of the mesentery to an anchor fixed to the bottom of the bath. A rubber balloon connected to a syringe by thin polyethylene tubing was introduced into the lumen and positioned in the middle of the segment. The balloon was inflated with 0.1 to 0.2 ml of warm water from the syringe to produce slightly greater local distension than that produced by a faecal bolus. The duration of distension was 20 or 30 s. The mechanical response of the circular muscle about 1.0 cm anal to the balloon was recorded by connecting a frog heart clip to a small area of the wall opposite the anchor and then connecting the clip via a thread to an isotonic transducer. Both ends of the segment were free. This arrangement allowed preferential recording of the response of the circular muscle. The circular muscle was subjected to a resting load of 0.5 g.

Drugs

Apamin, glibenclamide and N^G-nitro-L-arginine (L-NOARG) were purchased from Sigma Chemical Co., St. Louis, U.S.A. Charybdotoxin was from the Peptide Institute, Osaka, Japan. Gaseous nitric oxide was dissolved in Tyrode solution just before experiments, as described by Gillespie & Sheng (1988), and added to the organ bath in volumes of 0.3–300 μl. These volumes of Tyrode solution alone did not affect the spontaneous contractile activity or the muscle tone. Drugs were added to the organ bath as solutions in redistilled water in volumes of less than 1.0% of the bathing solution. A similar volume of redistilled water alone also had no affect on the muscle. Data are expressed as means ± s.e.

Results

Effects of EFS on the membrane potentials of the longitudinal and circular smooth muscle cells of rat proximal colon

The resting membrane potentials of longitudinal and circular muscle cells of the rat proximal colon were −62.7 ± 1.2 mV ($n = 87$) and −63.4 ± 1.1 mV ($n = 133$), respectively. In the absence of atropine and guanethidine, EFS (1–10 pulses) at 0.5 Hz induced excitatory junction potentials of the membrane of longitudinal and circular smooth muscle cells. In contrast, in the presence of atropine (1 μM) and guanethidine (5 μM), EFS induced transient hyperpolarization in all circular muscle cells tested. These hyperpolarizations were inhibitory junction potentials (i.j.ps), since they were abolished by tetrodotoxin (0.1 μM). The i.j.ps reached a peak in about 250 ms, and their total duration varied from 800 to 1300 ms. Repetitive nerve stimulation at frequencies of 0.5–1 Hz resulted in facilitation of the i.j.ps. At higher frequencies than 2 Hz, successive i.j.ps summed up resulting in large hyperpolarization (Figure 1). The maximal hyperpolarization was approximately 20 mV, and its latency was about 100 ms. Occasionally the hyperpolarization was followed by rebound depolarization. In the following experiments, atropine and guanethidine were added to the bathing fluid throughout the experiments for recording the i.j.ps. In contrast to the circular muscle, a single pulse of EFS did not affect the membrane potential in 82 of 87 longitudinal muscle cells in the presence of atropine and guanethidine and induced only a small transient hyperpolarization in the other 5 cells.

Effects of apamin and N^G-nitro-L-arginine on i.j.ps of circular and longitudinal muscle cells evoked by EFS

N^G-nitro-L-arginine (L-NOARG) even at the highest concentration tested (200 μM) did not have any significant effect on

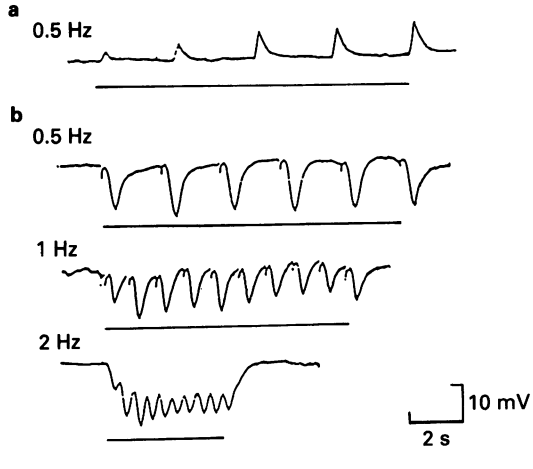

Figure 1 Facilitation or summation of junction potentials recorded from a circular muscle cell of the rat proximal colon in response to repetitive stimulation of intramural nerves. Junction potentials were induced by electrical field stimulation (EFS) at the indicated frequencies in the absence (a) or presence (b) of 1 μM atropine and 5 μM guanethidine. Lines indicate the duration of EFS.

the resting membrane potential or the i.j.ps recorded in either circular or longitudinal muscle (Figure 2). Apamin, a blocker of the small conductance Ca^{2+}-activated K^+-channel, did not have any significant effect on the resting membrane potential at 20–100 nM, but it concentration-dependently reduced the amplitude of the i.j.ps induced by a single pulse or train of pulses in both circular and longitudinal muscle cells and abolished them at 100 nM (Figures 2 and 3).

Effects of apamin and L-NOARG on descending inhibitory responses of circular muscle of rat proximal colon to local distension

On local distension, colonic segments showed relaxation of the circular muscle anal to the distended region in the absence or presence of atropine and guanethidine. Apamin at 0.1–1.0 μM gradually increased the amplitude of the spontaneous contractile activity, but did not affect the descending relaxation ($n = 6$; Figure 4). L-NOARG at 10 μM inhibited the descending relaxation, as shown previously (Hata *et al.*, 1990a).

Effects of apamin and L-NOARG on inhibitory responses of longitudinal muscle of rat proximal colon to EFS

EFS of the proximal segment induced rapid, transient relaxation and subsequent contraction of the longitudinal muscle, in the absence or presence of atropine and guanethidine. Apamin increased the tone of the longitudinal muscle and the frequency of spontaneous contractile activity, slightly at 0.1 μM and moderately at 1 μM, but did not have any significant effect on EFS-induced relaxation at these concentrations ($n = 6$; Figure 5). L-NOARG at 10 μM markedly inhibited EFS-induced relaxation, as shown previously (Suthamnatpong *et al.*, 1993a).

Effects of exogenous nitric oxide on contractile activity and membrane potential of circular muscle cells

Exogenous nitric oxide inhibited spontaneous contractile activity and decrease in tone of the circular muscle concentration-dependently. Significant inhibitions were observed in the micromolar range of nitric oxide (Figure 6). However, lower concentrations (<20 μM) of nitric oxide induced no detectable change in the membrane potential of circular muscle cells ($n = 6$). At 20 μM, it induced only weak hyperpolarization of 2.1 ± 0.7 mV ($n = 5$), whereas at 40–100

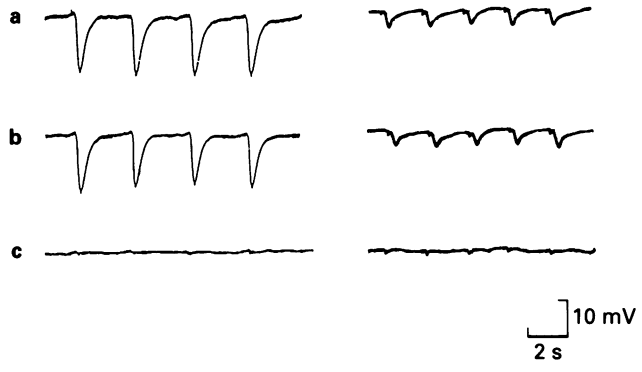

Figure 2 Effects of N^G-nitro-L-arginine (L-NOARG) and apamin on the inhibitory junction potentials (i.j.ps) induced by EFS in circular (left) and longitudinal (right) muscle cells of the rat proximal colon: i.j.ps induced by EFS at 0.5 Hz were recorded before (a) and 10 min after application of 200 μM L-NOARG (b) or L-NOARG plus 100 nM apamin (c). Atropine (1 μM) and guanethidine (5 μM) were added to the bathing fluid throughout the experiment.

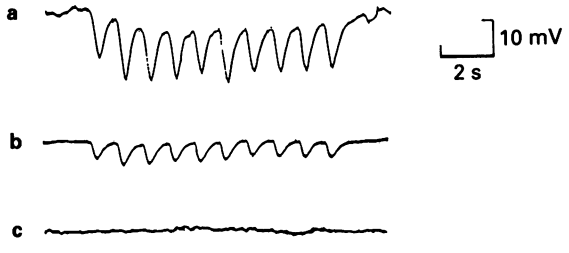

Figure 3 Concentration-dependent inhibition of i.j.ps by apamin in a circular muscle cell of the rat proximal colon: i.j.ps were induced by EFS at 1 Hz in the absence (a) or presence of 30 (b) or 100 (c) nM apamin. All records were from the same circular muscle cell in the presence of atropine and guanethidine.

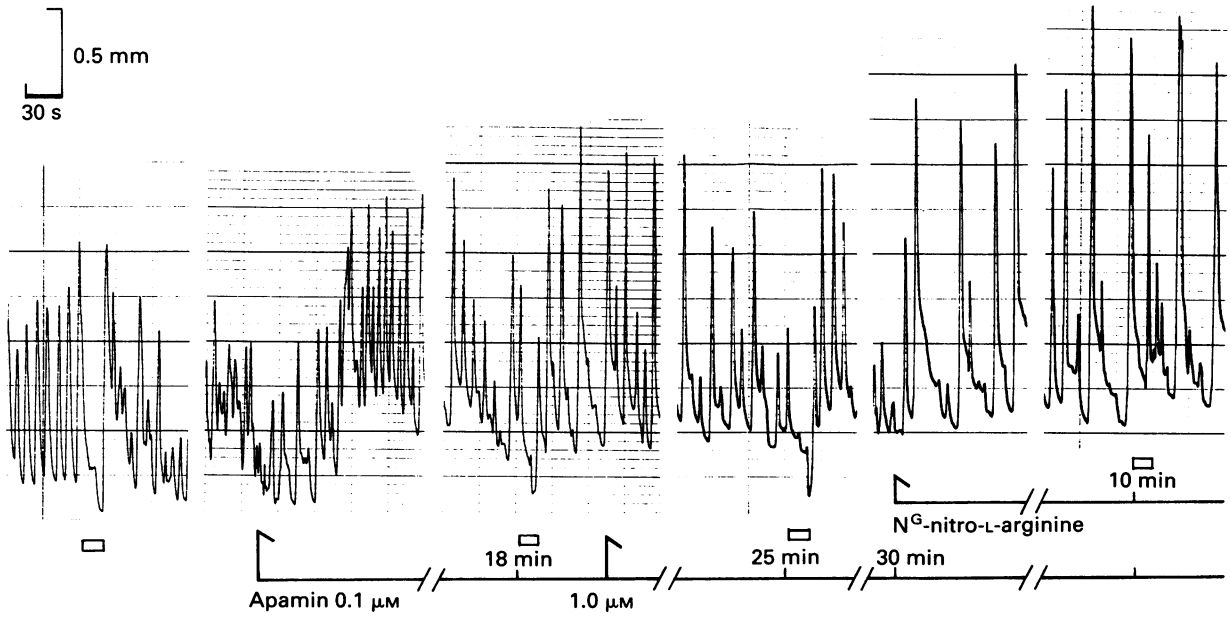

Figure 4 Effects of apamin and N^G-nitro-L-arginine (L-NOARG) on descending relaxation of rat proximal colon. Small rectangles indicate 20 s distension. Apamin at the indicated concentrations and L-NOARG at 10 μM were added at the times indicated by arrows. The continuous lines indicate the presence of apamin and L-NOARG in the bathing fluid. Times noted on the lines are those after addition of the drugs. Atropine (1 μM) and guanethidine (5 μM) were present throughout.

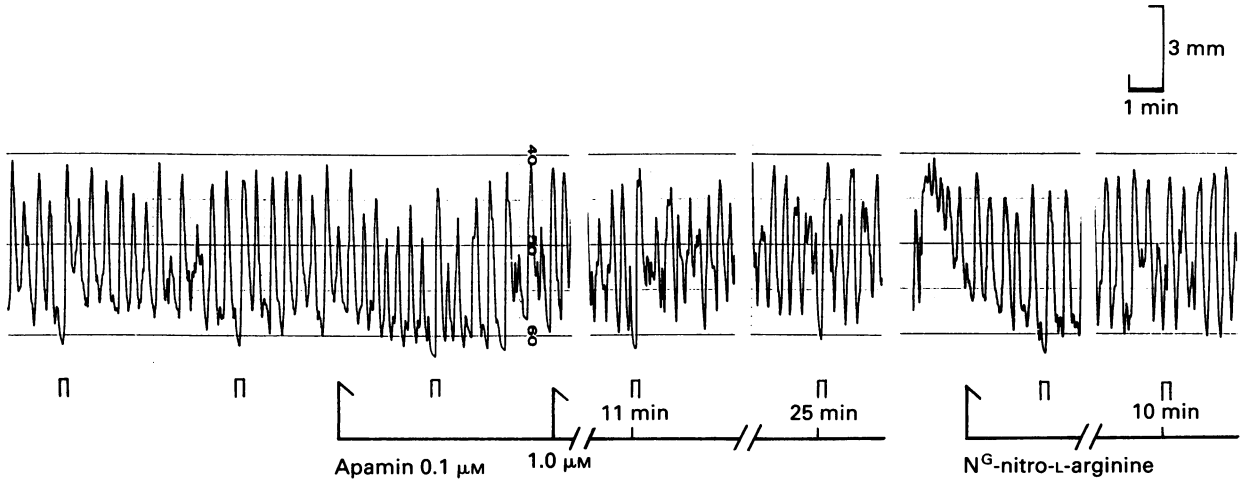

Figure 5 Effects of apamin and N^G-nitro-L-arginine on EFS-induced relaxation of longitudinal muscle of rat proximal colon. A segment of rat proximal colon was stimulated electrically at the times marked with small bars for 10 s at 10 Hz and relaxation of the longitudinal muscle was recorded. Further details were as for Figure 4.

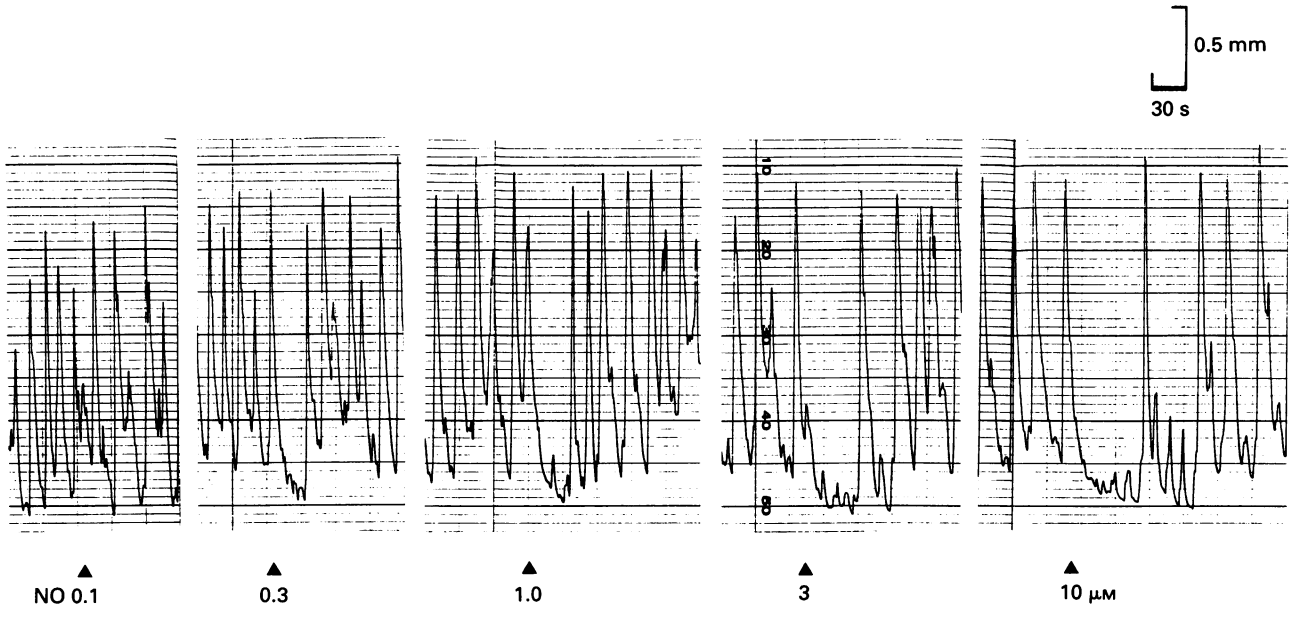

Figure 6 Relaxation of circular muscle of rat proximal colon in response to nitric oxide (NO). Spontaneous contractile activity of the circular muscle was recorded and various concentrations of NO were added at the times indicated by (▲). This record is typical of those from 6 preparations.

μM, it inhibited spontaneous discharges of action potentials, in addition to causing moderate hyperpolarization, and at 100 μM it induced further hyperpolarization of 16.1 ± 0.4 mV reproducibly ($n = 9$; Figure 7).

Effects of exogenous nitric oxide on the contractile activity and membrane potential of longitudinal muscle cells

The longitudinal muscle also responded dose-dependently to exogenous nitric oxide in the micromolar range, as observed previously (Suthamnatpong *et al.*, 1993a). However, 40 μM nitric oxide induced only slight hyperpolarization of the longitudinal muscle cell membrane, although a maximal amplitude of 15.3 ± 0.5 mV ($n = 7$) was recorded at 100 μM.

Effects of charybdotoxin and glibenclamide on inhibitory responses and the i.j.ps of circular muscle cells

Charybdotoxin, a blocker of the Ca^{2+}-activated large conductance K^+ channel, did not have any appreciable effects on EFS-induced relaxation of longitudinal muscle ($n = 4$) and descending inhibitory responses of circular muscle ($n = 3$) at 1 nM–1 μM. The EFS-induced i.j.ps recorded in circular muscle cells were also not affected by these concentrations of charybdotoxin ($n = 4$; not shown).

Glibenclamide, a blocker of the ATP-sensitive K^+ channel, at concentrations up to 1 μM had no effect on the relaxation of either circular or longitudinal muscle ($n = 4$) or on the i.j.ps of circular muscle ($n = 6$; not shown).

Discussion

In the presence, but not the absence, of atropine, i.j.ps in response to EFS were recorded from all circular smooth muscle cells of rat proximal colon tested, but rarely from longitudinal muscle cells. Furthermore, when recorded, the amplitudes of the i.j.ps from longitudinal muscle cells were small. Therefore, it seems likely that innervation of non-adrenergic, noncholinergic inhibitory neurones that induce

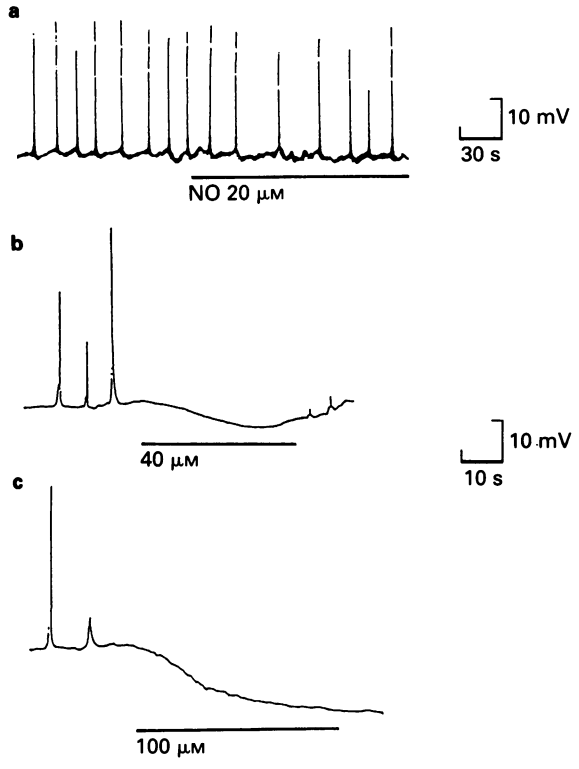

Figure 7 Effects of exogenous nitric oxide (NO) on discharges of spontaneous action potentials and the membrane potential in a circular muscle cell of the rat proximal colon. Membrane potentials of the cell which discharged spontaneously were recorded in the presence of atropine (1 μM) and guanethidine (5 μM). Lines (a), (b) and (c) indicate the presence of 20, 40 and 100 μM NO, respectively.

the i.j.ps is abundant in the circular muscle but scarce in the longitudinal muscle of the rat proximal colon.

In contrast to the i.j.ps, relaxations of the circular and longitudinal muscles were induced even in the absence of atropine. Apamin completely abolished the EFS-induced i.j.ps of the circular and longitudinal muscle cells, but did not affect relaxations of either induced by EFS or local distension. In contrast, L-NOARG inhibited the relaxations of both circular and longitudinal muscles as shown previously (Hata *et al.*, 1990a; Suthamnatpong *et al.*, 1993a), but did not affect the i.j.ps recorded in either. Furthermore, exogenously added nitric oxide induced relaxations of both muscles dose-dependently at 0.1–10 μM, but did not affect the membrane potential at these concentrations. These data strongly suggest that nitric oxide-mediated relaxation of rat proximal colon is not associated with the i.j.ps.

Several studies in vascular smooth muscle have suggested a dissociation of nitric oxide-mediated relaxation of smooth muscle from the i.j.ps: exogenous nitric oxide caused relaxation of canine isolated mesenteric (Komori *et al.*, 1988), rabbit femoral (Huang *et al.*, 1988), cerebral (Brayden, 1990) and basilar (Rand & Garland, 1992; Plane & Garland, 1993) artery without inducing hyperpolarization. Moreover L-NOARG partly inhibited relaxation, but not hyperpolarization of rat femoral veins induced by ACh (Nagao & Vanhoutte, 1991). Haemoglobin, which binds and inactivates nitric oxide, also inhibited relaxation, but not hyperpolarization induced by ACh in rat aorta and the main pulmonary artery (Chen *et al.*, 1988). Methylene blue inhibited ACh- and histamine-induced relaxation, but not hyperpolarization (Chen & Suzuki, 1989). The potassium channel blocker, glibenclamide, inhibited hyperpolarization but not relaxation induced by nitric oxide in the mesenteric artery of rats (Garland & McPherson, 1992) and it also completely inhibited hyperpolarization but only partly inhibited relaxation induced by ACh in the cerebral artery of rabbits (Brayden, 1990). All these data strongly suggest that the nitric oxide-mediated relaxation of smooth muscle of all kinds of blood vessels tested is independent of hyperpolarization of the smooth muscle cell membrane.

Many studies have indicated that ACh releases a hyperpolarizing factor (EDHF) from the endothelium which is different from the endothelium-derived relaxing factor (EDRF), and that hyperpolarization of the cell membrane results in relaxation of smooth muscle (Chen *et al.*, 1988; Komori *et al.*, 1988; Chen & Suzuki, 1989; Brayden, 1990; Nagao & Vanhoutte, 1991; Garland & McPherson, 1992; Rand & Garland, 1992). The results in most of these studies suggested that ACh-induced relaxation also involves a nitric oxide-mediated component that is independent of changes in the membrane potential, although the results in two studies (Garland & McPherson, 1992; Rand & Garland, 1992) suggested that nitric oxide does not participate in the relaxation. However, none of the results indicated that a nitric oxide-mediated component is involved in the coupling, activation of muscarinic cholinoceptors-hyperpolarization of the cell membrane-relaxation of the smooth muscle. Therefore, it is reasonable to postulate that in vascular smooth muscle cells, the agonist induces the production of EDRF and EDHF separately by activating the receptors on the membrane of endothelial cells, and these in turn induce relaxation of the smooth muscle.

One report is inconsistent with this postulation. Namely, L-NOARG was found to inhibit ACh-induced relaxation and hyperpolarization of the uterine artery of guinea-pigs, and L-arginine was found to reverse these inhibitions in a parallel fashion (Tare *et al.*, 1990). The reason for this discrepancy between uterine artery and other blood vessels is unknown. Possibly there may be differences in different regions of the blood vessels.

A few reports suggest an association of hyperpolarization with relaxation of the smooth muscles in the gastrointestinal tract: EFS induced nitric oxide-mediated i.j.ps and exogenous nitric oxide (0.1–30 μM) dose-dependently induced hyperpolarization of circular muscle cells of canine proximal colon (Thornbury *et al.*, 1991; Dalziel *et al.*, 1991). In a subsequent study in the same laboratory, a membrane permeable analogue of cyclic GMP, 8-bromo cyclic GMP, was shown to induce relaxation of the muscle as well as hyperpolarization of the cell membrane (Ward *et al.*, 1992a). These results suggested that cyclic GMP may be a second messenger that transduces the enteric inhibitory transmitter signal into the i.j.ps and subsequent relaxation of the proximal colon. However, as we pointed out above, definite evidence for the coupling of nitric oxide-(cyclic GMP)-hyperpolarization-relaxation has been obtained only from results on the uterine artery of guinea-pigs.

EDHF was suggested to increase the potassium conductance of smooth muscle cell membranes of rat arteries (Chen & Suzuki, 1989). It was also suggested that Ca^{2+}-activated K^+ channels were important in termination of electrical slow waves in canine colonic myocytes (Carl & Sanders, 1989). Various results have been obtained on the effects of several kinds of K^+-channel antagonist on agonist-induced hyperpolarization or relaxation of smooth muscles in different tissue preparations, but there are several reports of inhibitory effects of K^+ channel antagonists on nitric oxide-mediated responses: apamin was found to block partially nitric oxide-associated relaxation of canine intestine circular muscle (Christinck *et al.*, 1991), charybdotoxin to block nitrovasodilator-induced relaxation of bovine tracheal smooth muscle (Hamaguchi *et al.*, 1992) and glibenclamide to block ACh-induced hyperpolarization of rabbit cerebral artery (Brayden, 1990) and also nitric oxide-induced hyperpolarization of rat mesenteric artery (Garland & McPherson, 1992). Recently, it was also suggested that endothelium-dependent hyperpolarization in response to ACh was not important for

relaxation of the basilar artery, since glibenclamide abolished ACh-induced hyperpolarization, but not relaxation (Plane & Garland, 1993). Thus, it seemed interesting to examine the effects of K^+ channel antagonists on nitric oxide-mediated relaxation and on EFS-induced i.j.ps in the proximal colon of rats. However, apamin inhibited only EFS-induced i.j.ps that were insensitive to L-NOARG. The EFS-induced relaxation sensitive to L-NOARG and the exogenous nitric oxide-induced relaxation were not inhibited by the K^+ channel antagonists tested. These results strongly suggested that nitric oxide-mediated relaxation of the rat proximal colon does not involve activation of the K^+ channels that are sensitive to these antagonists.

The present findings in the rat proximal colon suggest that EFS activates two pathways separately: a nitric oxide-mediated pathway coupled to relaxation of the muscle, and a neurogenic pathway that induces hyperpolarization of the membrane of the muscle cells. In the rat proximal colon, unlike in blood vessels, where endothelial cells produce EDHF, some inhibitory neurotransmitter(s) released from the myenteric plexus activates apamin-sensitive K^+ channels. But the hyperpolarization is not associated *per se* with relaxation of the muscle. Thus, it is likely that the nitric oxide-mediated component is important in inducing relaxation of the rat proximal colon and that the hyperpolarization-related component has a minor role, if any.

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