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Modulation of Outward Potassium Currents by Nitric Oxide in Longitudinal Smooth Muscle Cells of Guinea-pig Ileum

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To investigate the possible involvement of outward potassium (K^+) currents in nitric oxide-induced relaxation in intestinal smooth muscle, we used whole-cell patch clamp technique in freshly dispersed guinea-pig ileum longitudinal smooth muscle cells. When cells were held at −60 mV and depolarized from −40 mV to +50 mV in 10 mV increments, sustained outward K^+ currents were evoked. The outward K^+ currents were markedly increased by the addition of 10 μM sodium nitroprusside (SNP). 10 μM S-nitroso-N-acetylpenicillamine (SNAP) and 1 mM 8-Bromo-cyclic GMP (8-Br-cGMP) also showed a similar effect to that of SNP. 1 mM tetraethylammonium (TEA) significantly reduced depolarization-activated outward K^+ currents. SNP-enhanced outward K^+ currents were blocked by the application of TEA. High EGTA containing pipette solution (10 mM) reduced the control currents and also inhibited the SNP-enhanced outward K^+ currents. 5 mM 4-aminopyridine (4-AP) significantly reduced the control currents but showed no effect on SNP-enhanced outward K^+ currents. 0.3 μM apamin and 10 μM glibenclamide showed no effect on SNP-enhanced outward K^+ currents. 1 μM 1H-[1,2,4]oxadiazolo [4,3-a]quinoxaline-1-one (ODQ), a specific inhibitor of soluble guanylate cyclase, significantly blocked SNP-enhanced K^+ currents. We conclude that NO donors activate the Ca^{2+}-activated K^+ channels in guinea-pig ileal smooth muscle via activation of guanylate cyclase.

Key Words: Guinea-pig ileum, Outward K^+ currents, Sodium nitroprusside, cGMP, TEA, 4-AP, ODQ

INTRODUCTION

Nonadrenergic, noncholinergic (NANC) inhibitory nerve innervation is important in gastrointestinal (GI) tract because it mediates physiological GI reflexes such as relaxation of the lower esophageal sphincter after swallowing, receptive relaxation of the proximal stomach during eating, and descending inhibition in response to distension (Sanders & Ward, 1992; Sanders et al, 1992). Relaxation of the GI tract is at least in part due to a membrane hyperpolarization response to nerve stimulation, termed an inhibitory junction potentials (IJPs). IJPs hyperpolarize membrane potential and reduce or disrupt rhythmic electrical and mechanical activity that occurs spontaneously in many intestinal smooth muscles. Although relatively little is known about the ionic mechanisms of IJPs, the amplitude of these events is related to the resting potentials of cells and IJPs are due to a transient increase in potassium conductance (Smith et al, 1989; Tomita, 1972).

There has been overwhelming evidence that nitric oxide (NO) may play an important role in inhibitory transmission in GI smooth muscles including guinea-pig ileum (Osthamus & Galligan, 1992; Shuttleworth et al, 1991; Sanders & Ward, 1992). Previous studies have shown that an inhibitor of NO synthases such as N-nitro-L-arginine methyl ester (L-NAME) abolished the IJPs and the relaxation to electrical stimulation in the canine colon and that this inhibition could be restored by L-arginine but not by D-arginine (Dalziel et al, 1991). Thornbury et al (1991) found that exogenous NO and NO releasing agents mimicked IJPs caused by electrical field stimulation and that

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compounds inhibiting NO synthesis also inhibit IJPs. NO and NO-releasing compounds have been known to activate soluble guanylate cyclase, thereby elevating cGMP (Katsuki et al, 1977). Cyclic GMP is generally considered to be an intracellular messenger that mediates the vasodilatory effects of NO in vascular smooth muscle although the mechanisms of cGMP-induced relaxations remain elusive (Murad, 1990). In GI muscles, NO or NO releasing agents have been shown to increase cGMP while membrane permeable analogues of cGMP have been shown to mimic the hyperpolarization response to NO (Dalziel et al, 1991). These reports suggest that at least a portion of responses to NANC nerve stimulation may be mediated by a cGMP-dependent mechanism in intestinal as well as vascular smooth muscles.

There is evidence that drugs such as SNP and nitric oxide which stimulate production of cGMP, which cause hyperpolarization of intestinal smooth muscles by inhibiting Ca^{2+} entry through voltage-dependent Ca^{2+} channels, and produce relaxation (Thornbury et al, 1991). Membrane hyperpolarization and muscle relaxation typically result from opening of K^+-selective channels (Tomita, 1989). Because the plasma membrane of intestinal smooth muscle cells contains many large conductance Ca^{2+}-activated K^+ (K_{Ca}) channels and charybdotoxin, a specific inhibitor of K_{Ca} channels, inhibits the relaxation of smooth muscle (Carl & Sanders, 1989), K_{Ca} channels activation may be involved in NO-induced responses. In rabbit aortic cells, 8-Br-cGMP increased delayed outward rectifier K^+ currents (Bkaily, 1990). Furthermore, Shinbo & Iijima (1997) reported that NO activate ATP-sensitive K^+ currents. Therefore, it is possible that other K^+ channels also contribute to these responses.

We hypothesized that the NO donors induces intestinal smooth muscle relaxation by activating K^+ channels. The aim of this study is to see the effects of NO donors on K^+ currents, especially on K_{Ca} currents, and whether these effects are mediated by the activation of cGMP, using whole-cell voltage clamp technique in freshly dispersed guinea-pig ileum longitudinal smooth muscle cells.

METHODS

Preparation of cells

The ileum was isolated from male guinea-pigs and placed in 37°C physiological salt solution (PSS). The longitudinal muscle layer was peeled from the underlying circular muscle. The muscle layers were cut into small pieces, placed in Ca^{2+}-free PSS. The Ca^{2+}-free PSS was replaced PSS containing 30 μM Ca^{2+} (low Ca^{2+} PSS). 30 min incubations at 37°C was carried out in fresh low-Ca^{2+} PSS containing collagenase (0.3 mg/ml), papain (0.3 mg/ml) and bovine serum albumin (5 mg/ml). After this enzyme digestion, tissue fragments were suspended in a fresh 120M Ca^{2+}-containing PSS and gently agitated. The resulting suspension was centrifuged at $100 \times g$ for 2 min and the cells were resuspended in a 0.5 mM Ca^{2+}- containing PSS. Cell suspension was placed on glass cover-slips and stored in a moist atmosphere at 4°C.

Whole-cell voltage clamp

Whole-cell membrane current and potential were recorded at room temperature by using standard patch-clamp techniques. Patch pipette had a resistance of 3~6MΩ when filled with a pipette solution. Membrane currents were measured with an Axopatch 1C voltage-clamp amplifier C (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 a oscilloscope (Tektronix), a computer monitor, and a pen recorder (Universal Oscillograph, Harvard).

Solutions

PSS used for the bath solution had following composition (mM): NaCl 126; KCl 6; $CaCl_2$ 2; $MgCl_2$ 1.2; glucose 14; HEPES 10.5 (pH 7.2 with NaOH). The patch pipette solution had the following composition (mM): KCl 134; $MgCl_2$ 1.2; ATP 1; GTP 0.1; EGTA 0.05; glucose 14; HEPES 10.5 (pH 7.2 with KOH). In order to decrease the intracellular free Ca^{2+} concentration less than 10 nM, in some experiment EGTA in the pipette solution was increased from 0.05 to 10 mM.

Chemicals

The following chemicals were used: apamin, 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br- cGMP), glibenclamide, tetraethylammonium (TEA), 4-aminopyridine (4-AP), S-nitroso-N-acetylpenicillamine

(SNAP), sodium nitroprusside (SNP) were products of Sigma Chemical Co (St Louis, MO, USA). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was purchased from Tocris Cookson (Lanford, Bristol, UK).

Statistics

All values are expressed as means ± standard errors of means (SEM). Differences between two groups were determined by Student's t test and were considered to be significant when P values are less than 0.05.

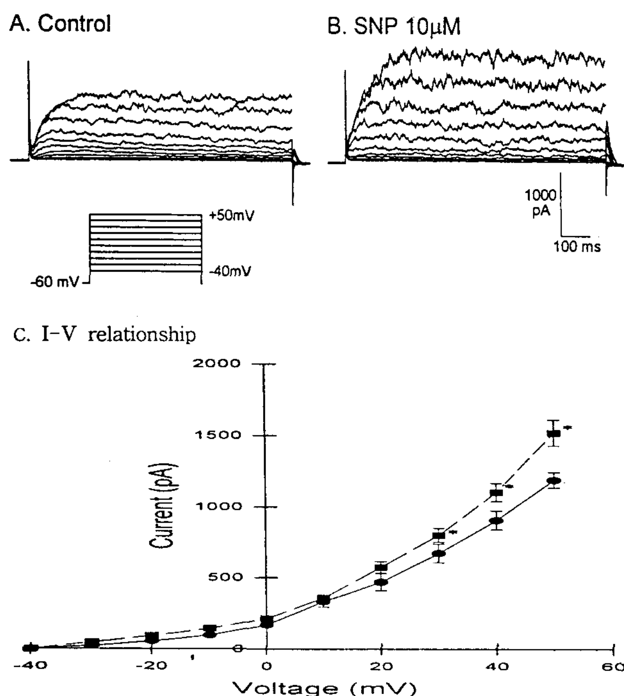


Fig. 1. Effects of SNP on outward K^+ currents in single ileal smooth muscle cells. In each panel the cell was held at −60mV, and test depolarization with durations of 900 ms were applied from −40 mV to +50 mV in 10 mV increments. Currents are recorded using a pipette containing 0.05 mM EGTA. A: whole cell currents under control conditions. B: effect of 10 μM SNP on the whole cell currents. C: current-voltage relationships show outward current is increased by SNP, recorded when maximum effects were seen at 10 min after addition to bath (means ± SEM, n=5, * Significantly different at $P<0.05$). ●; Control, ■; SNP presence.

RESULTS

Effects of SNP and SNAP on the outward K^+ currents

Because NO is highly unstable molecule, more stable NO-releasing molecule would be useful in the voltage clamp studies. Therefore, after recording control currents, SNP or SNAP was used as a NO donor. Fig. 1A shows 10 superimposed current traces elicited by 900 ms test pulses that ranged from −40 to +50 mV in 10 mV increments from a holding potential of −60 mV. Cells were dialysed intracellularly with KCl-rich solution containing 0.05 mM EGTA. The threshold for activation of the net outward current was −30 mV. Larger depolarizing voltage steps elicited faster and larger outward currents that was sustained during maintained depolarizations. In 5 of 5 cells, 10 μM SNP increased the net outward current (Fig. 1B). The SNP-induced increase in the outward currents was readily reversed to control level by

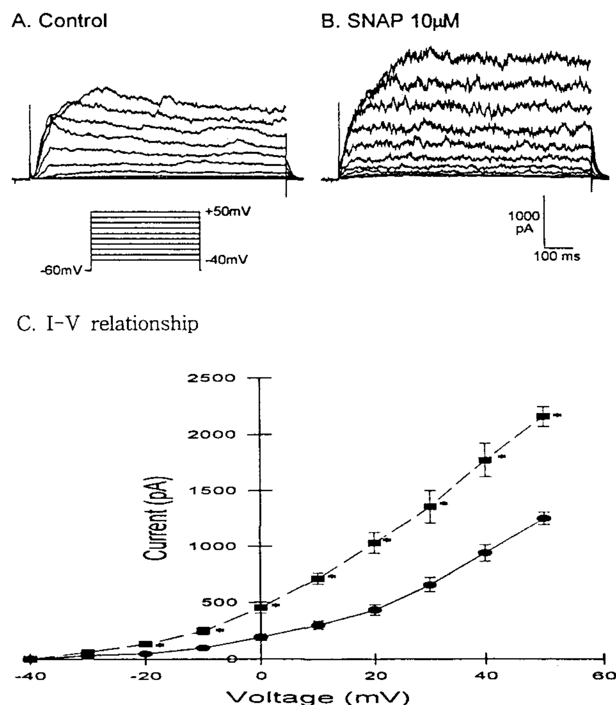


Fig. 2. Effects of nitric oxide-releasing agent SNAP on outward K^+ currents. A: control currents recorded from protocol shown in inset. B: increase in outward K^+ currents recorded from same cell after addition of 10 μM SNAP to the bath. C: current-voltage relationships showing effects of SNAP on sustained outward K^+ current recorded from 5 cells (means ± SEM, *$P<0.05$). ●; Control, ■; SNAP presence.

removing SNP. Fig. 1C displays the current-voltage relationship of the effect of SNP.

The NO-liberating agent SNAP (10 μM) increased the amplitude of the sustained outward current (illustrated in Fig. 2). Within 5 min of the addition of SNAP to the bath, the activation time of the outward currents was decreased and the outward currents was increased. Maximum effects were seen by 5 min. The current amplitude at the last step voltage (from −60 mV to +50 mV) increased from 1200±150 to 1788 ±250 pA (SEM, n=5) with the addition of SNAP to the bath. These effects were reversible with washing.

Effect of K^+ channel blockers on the SNP-enhanced K^+ currents

To determine whether SNP acts through the activation of K^+ channels, we explored the effects of K^+ channel blockers on the SNP-enhanced outward currents. It is well known that low concentrations of TEA (1 mM) block large conductance Ca^{2+}-activated K^+ channels (Carl et al, 1995), we applied 1 mM TEA to the bathing medium. TEA significantly decreased the net outward currents (Fig. 3B) compared to the control (Fig. 3A). When SNP was added to the bath in the presence of TEA, SNP-enhanced effect on the outward current was significantly inhibited by TEA pretreatment (Fig. 3C). Apamin (0.1 μM) did not affect the increase in outward currents due to SNP (data not shown, n=4).

Fig. 4 shows the effect of 4-AP on the outward currents. 5 mM 4-AP, a specific delayed rectifier K^+ channel inhibitor, decreased the basal outward currents, indicating that some of the sustained outward current was voltage-dependent (Fig. 4B). Exposure of the cells to SNP in the presence of 4-AP, showed no effect on the SNP-enhanced outward currents (Fig. 4C). Fig. 4D displays aggregate data of the effect of SNP on the current-voltage relationship (n=5, P< 0.05). Glibenclamide (10 μM), a specific ATP-sensitive K^+ channel inhibitor, did not affect the basal and SNP- activated outward currents (data not shown, n=4).

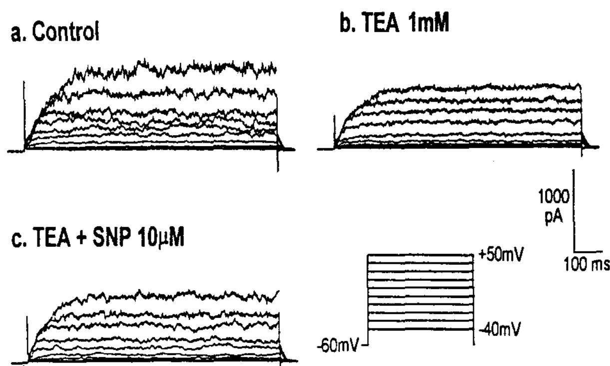
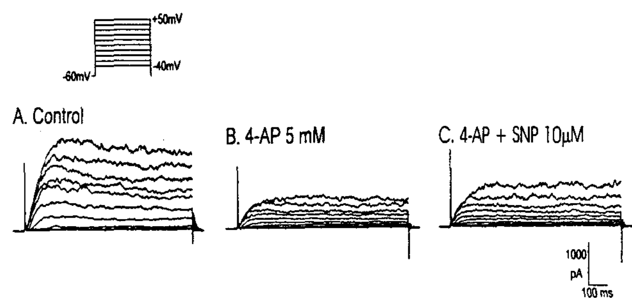


Fig. 3. Effects of SNP on outward K^+ currents in the presence of TEA. Current traces before (A) and after application of 1 mM TEA (B) and after application of 10 μM SNP in the presence of 1 mM TEA (C).

Effect of high EGTA on the SNP-enhanced K^+ currents

Currents recorded with pipette containing 10 mM EGTA were significantly smaller (measured at a depolarization of 50 mV; 1289 137 pA, SEM, n=5) than those recorded with pipette solution containing 0.05 mM EGTA (2050±355 pA, SEM, n=5) in different



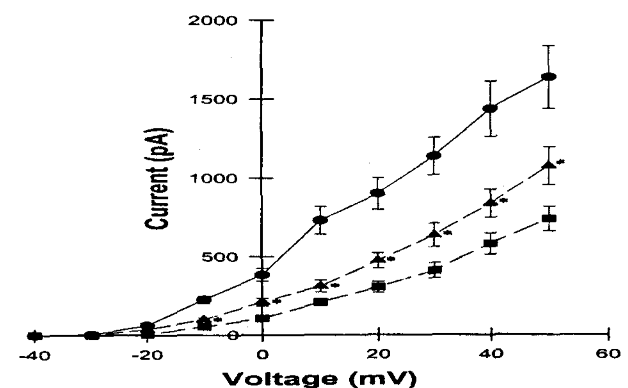


Fig. 4. Effects of SNP on outward K^+ currents in the presence of 4-AP. Current traces before (A) and after application of 5 mM 4-AP (B) and after application of 10 M SNP in the presence of 5 mM 4-AP in a cell (C). D: current-voltage relationships show 4-AP reduces sustained outward K^+ currents from control levels and blocks effect of SNP (means±SEM, n=5, * Significantly different at P<0.05). ●; Control, ■; 4-AP presence, ▲; 4-AP plus SNP presence.

cells of the same population. This result indicates that approximately 37% of the sustained K^+ outward current in this tissue was Ca^{2+} dependent. After blockade of the outward currents using a pipette containing 10 mM EGTA, SNP did not increased the outward current (Fig. 5B).

Effect of ODQ on the outward K^+ currents

To determine if SNP activates outward K^+ currents directly or by the activation of protein kinase G signaling pathway, we explored the effects of ODQ, a specific inhibitor of soluble guanylate cyclase, on the current activated by SNP. ODQ (1 μM) itself did not inhibit the basal outward currents. As shown in Fig. 6, the current amplitude at the last step voltage (from −60 mV to +50 mV) increased from 2185±270 to 2845±317 pA (SEM, n=4) with the addition

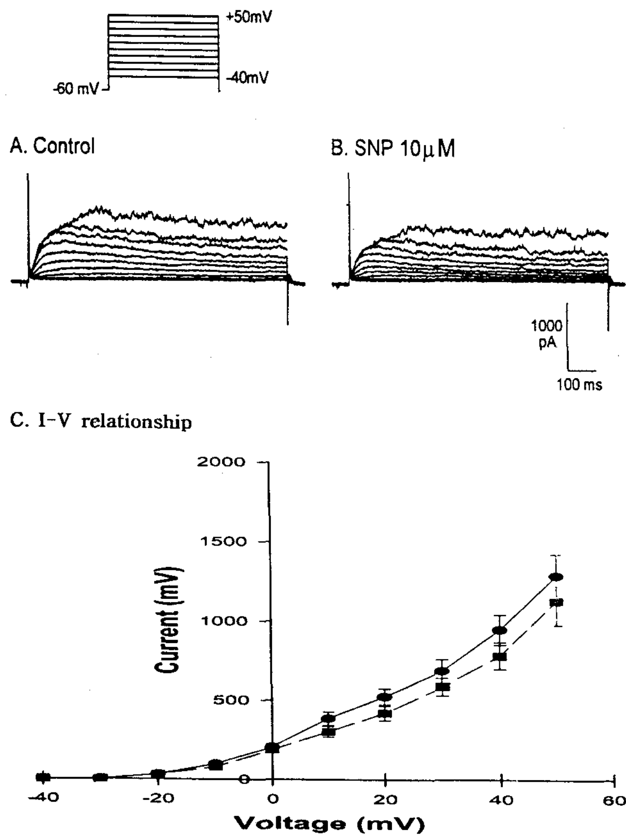


Fig. 5. Effects of SNP on outward K^+ currents in the intracellular presence of high EGTA. Currents are recorded with a pipette containing 10 mM EGTA, and therefore basal outward currents are reduced in amplitude. A: control. B: decrease in outward currents recorded from same cells after addition of 10 μM SNP to the bath. C: the current-voltage relationships for K^+ currents under control conditions (●) and in the presence of SNP (■).

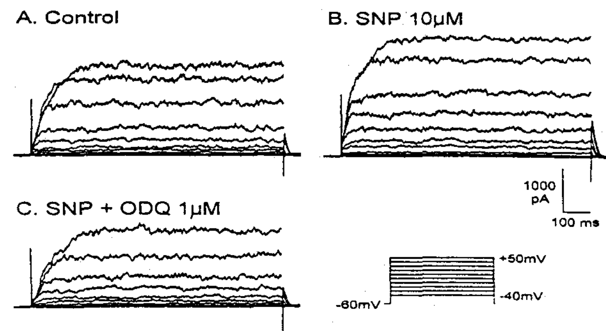


Fig. 6. Effects of SNP and ODQ on outward K^+ currents. Currents are recorded with a pipette containing 0.05 mM EGTA. A: control. B: enhancement of outward K^+ current by 10 μM SNP. C: ODQ (1 μM) almost completely blocked the SNP-enhanced outward K^+ currents.

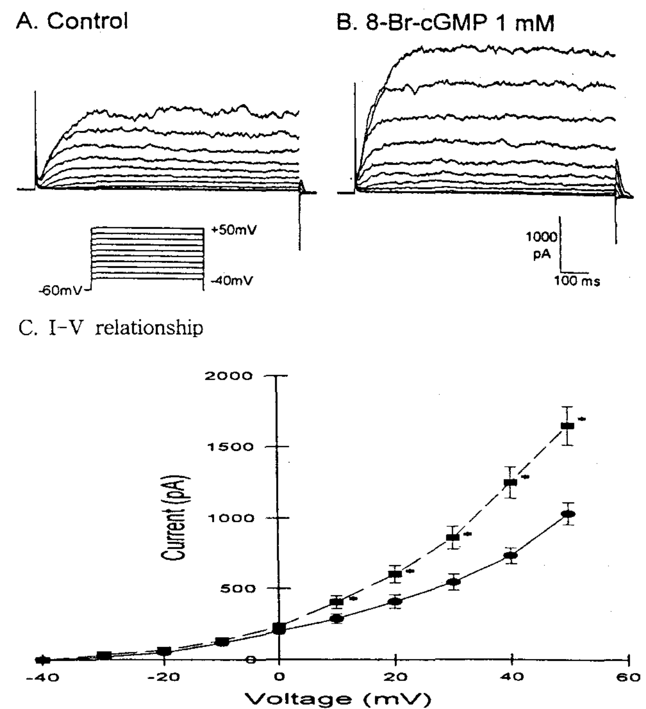


Fig. 7. Effects of 8-Br-cGMP on outward K^+ currents. Application of 1 mM 8-Br-cGMP to the purfusate increased outward K^+ currents. A: control currents under control conditions. B: increase in outward currents recorded from same cell after addition of 1 mM 8-Br-cGMP to the bath. C: current-voltage relationships showing effects of 8-Br-cGMP on sustained outward K^+ currents recorded from 5 cells (means±SEM, *$P<0.05$). ●; Control, ■; 8-Br-cGMP presence.

of SNP to the bath and then decreased to 1867±193 pA (SEM, n=4) in the presence of ODQ.

Effect of 8-Br-cGMP on the outward currents

In many smooth muscles, responses to NO are thought to be mediated by an increase in cGMP. To determine whether SNP also acts through the increase in intracellular cyclic GMP, we investigated the effect of 8-bromoguanosine 3,5-cyclic monophosphate (8-Br-cGMP), a membrane permeable cGMP analogue, on the outward K^+ currents. 8-Br-cGMP significantly increased the net outward currents at potentials more positive than −30 mV (Fig. 7). The characteristics of the 8-Br-cGMP-enhanced outward K^+ currents were similar to those of the current activated by SNP.

DISCUSSION

We have shown that outward currents carried by K^+ are induced by depolarization of isolated longitudinal smooth muscle cells from the guinea-pig ileum. The outward K^+ currents during whole cell recording from a holding potential of −60 mV were shown to have one component: sustained noninactivating current. The sustained outward current was in part Ca^{2+} dependent, since current was reduced when recorded with a pipette containing high levels of EGTA.

Recent evidence supports the thought that NO may be the primary mediator of the nonadrenergic noncholinergic inhibitory response in many gastrointestinal smooth muscles (Selemidis et al, 1997). Earlier studies in canine small intestine have shown that the K^+ currents activated during the IJP are unusual in which K^+ blocking agents including apamin had no effect on the IJP (Stark et al, 1991). However the K^+ channel blocker, charybdotoxin, did reduce the IJP. Two different NO releasing agents, SNP and SNAP, produced hyperpolarization of guinea-pig ileum when measured with the microelectrode or sucrose gap techniques (He & Goyal, 1993). This study, using single cells isolated from the guinea-pig ileum, showed an increase in outward current when SNP or 8-Br-cGMP was added to the bath solution.

NO donors that release NO activate K^+ channels, which are sensitive to TEA but insensitive to 4-AP, apamin, and glibenclamide. The finding of this study is consistent with that of a recent study (Carayab & Daniel, 1995). This study therefore supports the hypothesis that NO released from nerves or compounds such as SNP causes hyperpolarization of the smooth muscle membrane by increasing K^+ channel conductance. However, this study does not exclude the possibility that stimulation of the enteric nerves releases other mediators besides NO or that NO can activate other channels. Though the mechanism by which NO activates the Ca^{2+}-activated K^+ current has not been explained, it does not appear that it is a direct effect on the Ca^{2+}-activated K^+ channels, as seen in rabbit aortic cells (Bolotina et al, 1994). The addition of high levels of EGTA in the pipette should not have abolished the NO effects if NO was acting directly on the channel. The possibility of guanosine 3,5-cyclic monophosphate involvement in the mechanism of NO action has been established (Archer et al, 1994).

Since high levels of EGTA in the pipette prevented the effects of SNP, it appears that the K^+ channels that are activated by NO release are Ca^{2+} dependent. The Ca^{2+} required for their activation seems to be dependent on release of Ca^{2+} from the internal stores (Jury et al, 1996). This study does not establish that the opening of Ca^{2+}-activated K^+ channels by NO causes hyperpolarization in muscle strips, since appreciable increases in outward currents were observed only at depolarizations above −30 mV. However, in our experiments, 0.05 mM EGTA in pipettes may have limited increases in Ca^{2+} near the plasma membrane Ca^{2+}-activated K^+ channels are opened at more depolarized potentials when higher Ca^{2+} is present. Our study did show that Ca^{2+}-activated K^+ channels are activated by NO-releasing agents.

These studies demonstrate that the NO releasing agents, SNP and SNAP, and 8-Br-cGMP, both activate an outward current in guinea-pig ileum. These findings agree with other reports which showed that NO activates K_{Ca} channels in isolated vascular and intestinal smooth muscle (Botolina et al, 1994; Dalziel et al, 1991). In addition, blockers of K_{Ca} channels can inhibit NO-induced relaxations of vascular and intestinal smooth muscle (Khan et al, 1993; Thornbury et al, 1991). ODQ is a recently reported inhibitor of NO-sensitive guanylate cyclase. ODQ is selective for soluble guanylate cyclase, being without effect on particulate guanylate cyclase or on adenylate cyclase (Garthwaite et al, 1995). Because the effect of SNP on the outward current was mimicked by a cGMP analogue and was antagonized by the inhibitor of soluble guanylyl cyclase, ODQ, it is likely that

guanylyl cyclase is involved in the signal transduction pathway coupling of NO to the activation of K_{Ca} channels, as has been shown in vascular smooth muscle cells (Archer et al, 1994; Murray et al, 1995). This contrasts with a report that NO activates the Ca^{2+}-activated K^+ channel directly (Botolina et al, 1994). Our studies do not preclude a direct action of NO on K_{Ca} channels, but different techniques will be required to confirm this possibility.

The central role of cGMP in K_{Ca} channels is proven by the ability of 8-Br-cGMP to increase the outward currents. ODQ's inhibition of NO-induced K^+ channel activation argues against a direct effect of NO on the K^+ channel. There is a report that GMP rather than cGMP increases the activity of K_{Ca} channels. However, phosphodiesterase inhibition minimizes GMP production while augmenting cGMP levels and yet phosphodiesterase inhibitors cause relaxation and increase outward K^+ currents (Archer et al, 1994). This implies that cGMP is more important than GMP in augmenting outward K^+ currents.

In summary, this study shows that cells of guinea-pig ileum longitudinal muscle have multiple outward currents elicited by depolarization. One of these is a stable outward K^+ current, dependent on internal Ca^{2+}. This or a similar current is enhanced by NO donors, and this enhancement is abolished by chelating intracellular Ca^{2+} and by TEA, and also blocked by ODQ. We concluded that NO-donors-induced inhibitory effects may be mediated by cGMP and this cGMP also contributes to the intestinal smooth muscle relaxation, and these effects are related to the activation of K^+ currents, especially large-conductance Ca^{2+}-activated K^+ currents.

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