Experimental Physiology

Inhibition of carbachol-evoked oscillatory currents by the NO donor sodium nitroprusside in guinea-pig ileal myocytes

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The effect of sodium nitroprusside (SNP) on carbachol (CCh)-evoked inward cationic current (I_{cat}) oscillations in guinea-pig ileal longitudinal myocytes was investigated using the whole-cell patch-clamp technique and permeabilized longitudinal muscle strips. SNP (10 μM) completely inhibited I_{cat} oscillations evoked by 1 μM CCh. 1H-(1,2,4) Oxadiazole [4,3-a] quinoxaline-1-one (ODQ; 1 μM) almost completely prevented the inhibitory effect of SNP on I_{cat} oscillations. 8-Bromo-guanosine 3′,5′-cyclic monophosphate (8-Br-cGMP; 30 μM) in the pipette solution completely abolished I_{cat} oscillations. However, a pipette solution containing Rp-8-Br-cGMP (30 μM) almost completely abolished the inhibitory effect of SNP on I_{cat} oscillations. When the intracellular calcium concentration ($[Ca^{2+}]_i$) was held at a resting level using BAPTA (10 mM) and Ca^{2+} (4.6 μM) in the pipette solution, CCh (1 μM) evoked only the sustained component of I_{cat} without any oscillations and SNP did not affect the current. A high concentration of inositol 1,4,5-trisphosphate (IP_3; 30 μM) in the patch pipette solutions significantly reduced the inhibitory effect of SNP (10 μM) on I_{cat} oscillations. SNP significantly inhibited the Ca^{2+} release evoked by either CCh or IP_3 but not by caffeine in permeabilized preparations of longitudinal muscle strips. These results suggest that the inhibitory effects of SNP on I_{cat} oscillations are mediated, in part, by functional modulation of the IP_3 receptor, and not by the inhibition of cationic channels themselves or by muscarinic receptors in the plasma membrane. This inhibition seems to be mediated by an increased cGMP concentration in a protein kinase G-dependent manner.

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Many different cell types exhibit oscillating changes in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). This usually occurs in response to hormones and neurotransmitters, and can sometimes occur spontaneously. Because of the general occurrence of $[Ca^{2+}]_i$ oscillations and the existence of cellular functions that are mediated by an increase in $[Ca^{2+}]_i$, many possible roles for the $[Ca^{2+}]_i$ oscillations have been suggested (Tsien & Tsien, 1990).

Oscillations of $[Ca^{2+}]_i$ evoked by acetylcholine (ACh) or carbachol (CCh) were observed in a single intestinal smooth muscle cell (Pacaud & Bolton, 1991; Komori *et al.* 1992, 1993). According to data previously reported, activation of a G protein by muscarinic stimulation results in the opening of non-selective cationic channels, which is further potentiated by increases in $[Ca^{2+}]_i$ (Benham *et al.* 1985; Inoue & Isenberg, 1990*a*,*b*). Stimulation of muscarinic receptors also causes Ca^{2+} release from internal stores by inositol 1,4,5-trisphosphate (IP_3) formed through phosphatidylinositol breakdown (Komori & Bolton, 1990, 1991). During muscarinic stimulation, Ca^{2+} inhibition of IP_3-induced Ca^{2+} release (IICR) at some critical level of $[Ca^{2+}]_i$ allows the Ca^{2+} stores to refill, which leads to a fall in $[Ca^{2+}]_i$ to a level at which IP_3 can release Ca^{2+} from stores again. In these ways, oscillatory changes in $[Ca^{2+}]_i$ occur in response to muscarinic stimulation (Zholos *et al.* 1994), which results in oscillation of the

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inward cationic current (I_{cat}; Kohda *et al.* 1998). Although the physiological relevance of I_{cat} oscillation remains to be elucidated, evidence has suggested that it may play a role in stimulating and maintaining intestinal contractility in response to muscarinic agonists.

Nitric oxide (NO) and NO-liberating compounds exert a relaxing effect in various smooth muscles, including those of the intestine (Lincoln, 1989; Kuriyama *et al.* 1995). They also cause activation of soluble guanylate cyclase with a subsequent increase in cyclic 3,5-guanosine monophosphate (cGMP) levels (Katsuki *et al.* 1977), in turn activating protein kinase G (PKG; Wahler & Dollinger, 1995), which results in a reduction of $[Ca^{2+}]_i$ through poorly understood mechanisms (Lincoln *et al.* 1994). Kwon *et al.* (2000) reported that the NO donor sodium nitroprusside (SNP) inhibits the contractile response to CCh of gastrointestinal smooth muscle by decreasing $[Ca^{2+}]_i$ through voltage-dependent inward Ca^{2+} current, I_{cat} inhibition, and Ca^{2+}-activated K^+ current activation. Therefore, it is possible that SNP also affects oscillatory changes in I_{cat} as well as oscillations in $[Ca^{2+}]_i$. However, this possibility remains to be tested. Therefore, in the present work, we used patch-clamp techniques to examine the effect of SNP on I_{cat} oscillations evoked by CCh in single longitudinal smooth muscle cells from guinea-pig ileum. Furthermore, the effect of SNP on the release of Ca^{2+} from intracellular stores evoked by caffeine or IP_3 was examined in chemically permeabilized ileal longitudinal muscle strips.

Methods

Isolation of the longitudinal smooth muscle layer from guinea-pig ileum

All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee. The longitudinal smooth muscle layer from guinea-pig ileum was isolated by a previously described method (Komori *et al.* 1993; Zholos *et al.* 1994). Briefly, guinea-pigs of both sexes, weighing about 300–350 g, were exsanguinated after being stunned. The ileum was isolated and cut into segments 3–4 cm in length and then placed in a physiological salt solution (PSS; composition given below). The longitudinal muscle layer of the intestinal segments was peeled from the underlying circular muscle and washed in PSS.

Preparation of cells

Ileal smooth muscle cells were enzymatically dissociated with some modification to the method described previously (Komori *et al.* 1993; Zholos *et al.* 1994). Briefly, the longitudinal muscle layer from the ileum was cut into small pieces and placed into Ca^{2+}-free PSS. Ca^{2+}-free PSS was then replaced with PSS containing 30 μM Ca^{2+} (low-Ca^{2+} PSS) and 30 min incubation at 37°C were carried out in fresh, low-Ca^{2+} PSS that contained collagenase (0.3 mg ml^{-1}), papain (0.6 mg ml^{-1}) and bovine serum albumin (1 mg ml^{-1}). After enzyme digestion, tissue fragments were suspended in fresh 120 μM Ca^{2+}-containing PSS and gently agitated. The resulting suspension was centrifuged at 600*g* for 2 min, and the cells were resuspended in 0.5 mM Ca^{2+}-containing PSS. Aliquots (~2–3 drops) of the cell suspension were placed into 12 mm cover glasses and stored in a humidified atmosphere at 4°C. Experiments were carried out at 22–24°C within 12 h of harvesting.

Whole-cell voltage clamp

Whole-cell membrane currents were recorded at room temperature using standard patch-clamp techniques. The patch pipette had a resistance of 3–6 MΩ when filled with pipette solution. Membrane currents were measured with an Axoclamp 200A voltage-clamp amplifier (Axon Instruments, Foster City, CA, USA). Command pulses were applied using pCLAMP (version 6.0) software and an IBM-compatible computer. The data were filtered at 5 kHz and displayed on an oscilloscope, computer monitor and pen recorder.

In these experiments, the oscillatory inward I_{cat} was evoked by CCh (1 μM) in cells voltage-clamped at −60 mV (Komori *et al.* 1993). The agonist was applied at least 3 min after the break-through.

Permeabilized longitudinal muscle cell preparation

A muscle strip, 4–6 mm in length and 0.2–0.3 mm in width, was prepared from the longitudinal muscle layer of the ileum. The strip was mounted horizontally in a 1 ml organ chamber; one of its cut ends was fixed to the chamber and the other attached to an isometric force transducer. The organ chamber was filled with PSS kept at 23°C and the muscle strip was equilibrated under a tension of 150–180 mg for 30–60 min. Permeabilization of cell membranes was then performed by incubating the muscle strip with *Staphylococcus aureus* α-toxin (10 μg protein ml^{-1}) in a Ca^{2+}-containing solution (pCa 6) for 30–60 min until the gradual rise in tension became a steady plateau. After permeabilization, the muscle strip was bathed in a relaxing solution containing 2 mM EGTA (RI solution; composition given below).

In control experiments, intracellular Ca^{2+} stores of the permeabilized tissue were loaded with Ca^{2+} by replacing the bath medium (RI solution) with Ca^{2+}-containing solution (pCa 5) for 10 min. Then the relaxing solution (RI solution) was reintroduced for 5 min, followed by application of caffeine or IP_3 for 1–1.5 min by replacing the RI solution with another relaxing solution (RII solution; composition given below) containing the drug. The series of procedures from Ca^{2+} loading to drug application was repeated at an interval of 20 min. However, in the second

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experiment, SNP was added to the RI solution during its reintroduction and the application of caffeine or IP_3. GTP (100 μM) was present during the application of caffeine or IP_3 (Takemura *et al.* 1989).

Solutions

The PSS used for cell isolation and for recording of CCh-evoked I_{cat} had the same composition as previously described (Komori *et al.* 1993) and is as follows (mM): 126 NaCl, 6 KCl, 2 $CaCl_2$, 1.2 $MgCl_2$, 14 glucose and 10.5 Hepes (titrated to pH 7.4 with NaOH).

The Ca^{2+}-free PSS was prepared by omitting $CaCl_2$ from the PSS. The patch pipette solution for oscillatory I_{cat} recording had the following composition (mM): 134 CsCl, 1.2 $MgCl_2$, 4 MgATP, 0.3 Na_2GTP, 0.05 EGTA, 10 phosphocreatine, 10 glucose and 10 Hepes (titrated to pH 7.2 with CsOH; Komori *et al.* 1993). In some experiments, to hold $[Ca^{2+}]_i$ close to a resting value typical for intestinal smooth muscle and to minimize the influence of changes in $[Ca^{2+}]_i$ on I_{cat}, a mixture of 10 mM BAPTA and 4.6 mM Ca^{2+} was used instead of 0.05 mM EGTA, since BAPTA is superior to EGTA in buffering $[Ca^{2+}]_i$ to an almost constant level (calculated $[Ca^{2+}]_i \approx 100$ nM; Zholos *et al.* 2000). The relaxing solution for cell membrane permeabilization had the following composition (mM): 130 potassium propionate, 4 $MgCl_2$, 5 Na_2ATP, 2 creatine phosphate, 10 creatine phosphokinase, 20 Tris-maleate, and 2 EGTA (for RI solution) or 0.05 EGTA (for RII solution) (pH 6.8), to which two agents were added, i.e. the mitochodrial inhibitor carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone (1 μM) and the protease inhibitor E-64 (1 g ml^{-1}). Ca^{2+} concentrations were changed by adding an appropriate amount of $CaCl_2$. The apparent binding constant of EGTA for Ca^{2+} was considered to be 1 M at pH 6.8 and 20°C.

Chemicals

Sodium nitroprusside (SNP), EGTA, carbachol (CCh), caffeine, guanosine triphosphate (sodium salt; Na_2GTP), adenosine triphosphate (magnesium salt; MgATP), Hepes, BAPTA, 8-bromo-guanosine 3′,5′-cyclic monophosphate (8-Br-cGMP), Rp-8-bromo-cyclic guanosine 3′,5′-cyclic monophosphate (Rp-8-Br-cGMP), creatine phosphokinase, nifedipine, heparin, *Staphylococcus aureus* α-toxin, E-64, D-myo-inositol-1,4,5-trisphosphate (D-myo-IP_3), and 1H-(1,2,4) oxadiazole [4,3-a] quinoxaline-1-one (ODQ) were purchased from Sigma. All other chemicals were of the highest grade commercially available.

Statistics

All results are expressed as means ± S.E.M. The statistical significance of differences between given sets of data was evaluated by Student's unpaired *t* test. A *P* value less than 0.05 was considered significant.

Results

Activation of I_{cat}, which is very sensitive to changes in $[Ca^{2+}]_i$ (Pacaud & Bolton, 1991; Komori *et al.* 1993), was recorded to detect oscillations in $[Ca^{2+}]_i$ induced by CCh in single guinea-pig ileal cells.

In most cells (32 of 35 cells), application of 1 μM CCh at −60 mV produced an oscillatory I_{cat} response (Fig. 1). The oscillatory changes arose from a small sustained I_{cat} component with a more or less regular frequency or without the development of a noticeable sustained current. The current oscillation persisted for the early period or the entire application of CCh (2–10 min) as previously described (Komori *et al.* 1993). The oscillation frequency varied among different cells from 0.05 to 0.47 Hz, giving a mean value of 0.17 ± 0.02 Hz ($n = 32$).

Effect of SNP on I_{cat} oscillations

Figure 1 demonstrates a typical example of the SNP inhibitory effect on CCh-induced I_{cat} oscillations. Application of SNP (10 μM) during on-going oscillations in I_{cat} resulted in their complete cessation in all cells tested ($n = 6$). This effect was reversible; oscillations

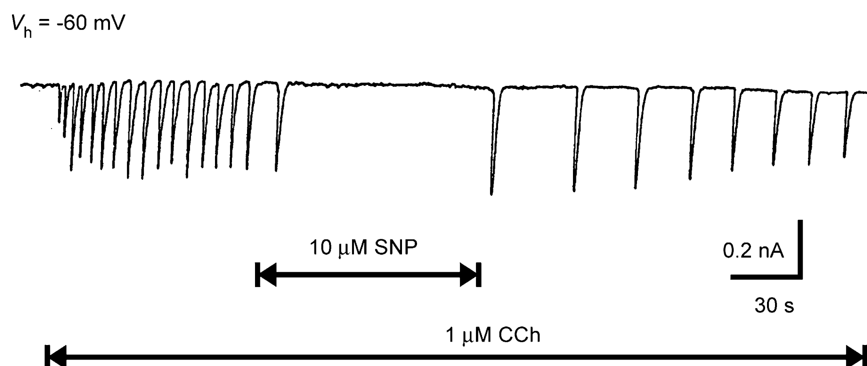

Figure 1. Carbachol (CCh)-induced inward cationic current (I_{cat}) oscillations and the effects of SNP on I_{cat} oscillations at a holding potential (V_h) of −60 mV in guinea-pig ileal smooth muscle cells
I_{cat} oscillations evoked by 1 μM CCh were completely inhibited by 10 μM SNP.

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reappeared after the wash-out of SNP with a frequency of 0.07 ± 0.01 Hz ($n = 6$).

Effects of ODQ and 8-Br-cGMP on the SNP-induced inhibition of I_{cat} osillations

Treatment of cells with 1 μM ODQ, a soluble guanylate cyclase inhibitor (Kwon *et al.* 2000), for about 5 min did not significantly affect the CCh-evoked I_{cat} oscillations (0.18 ± 0.03, $n = 8$). However, the on-going oscillations remained unchanged after application of 1 μM SNP (Fig. 2*A*). Intracellular application of 8-Br-cGMP (30 μM), a membrane-permeable analogue of cGMP (Rapoport *et al.* 1982), via patch pipettes completely prevented the generation of I_{cat} oscillations in response to CCh ($n = 6$; Fig. 2*B*). These results suggest that the SNP-induced inhibition of I_{cat} oscillations involves an increased intracellular level of cGMP.

Effect of Rp-8-Br-cGMP on the SNP-induced inhibition of I_{cat} oscillations

The cellular effects of cGMP are generally regarded as being mediated by PKG, which phosphorylates a variety of functional proteins, including ion channels, and thereby alters their function (McDonald & Murad, 1996). We tested the possible involvement of cGMP in the SNP-induced inhibition of I_{cat} oscillations.

Rp-8-Br-cGMP, the Rp-diastereoisomer of cGMP, is a highly specific PKG antagonist (Butt *et al.* 1994;

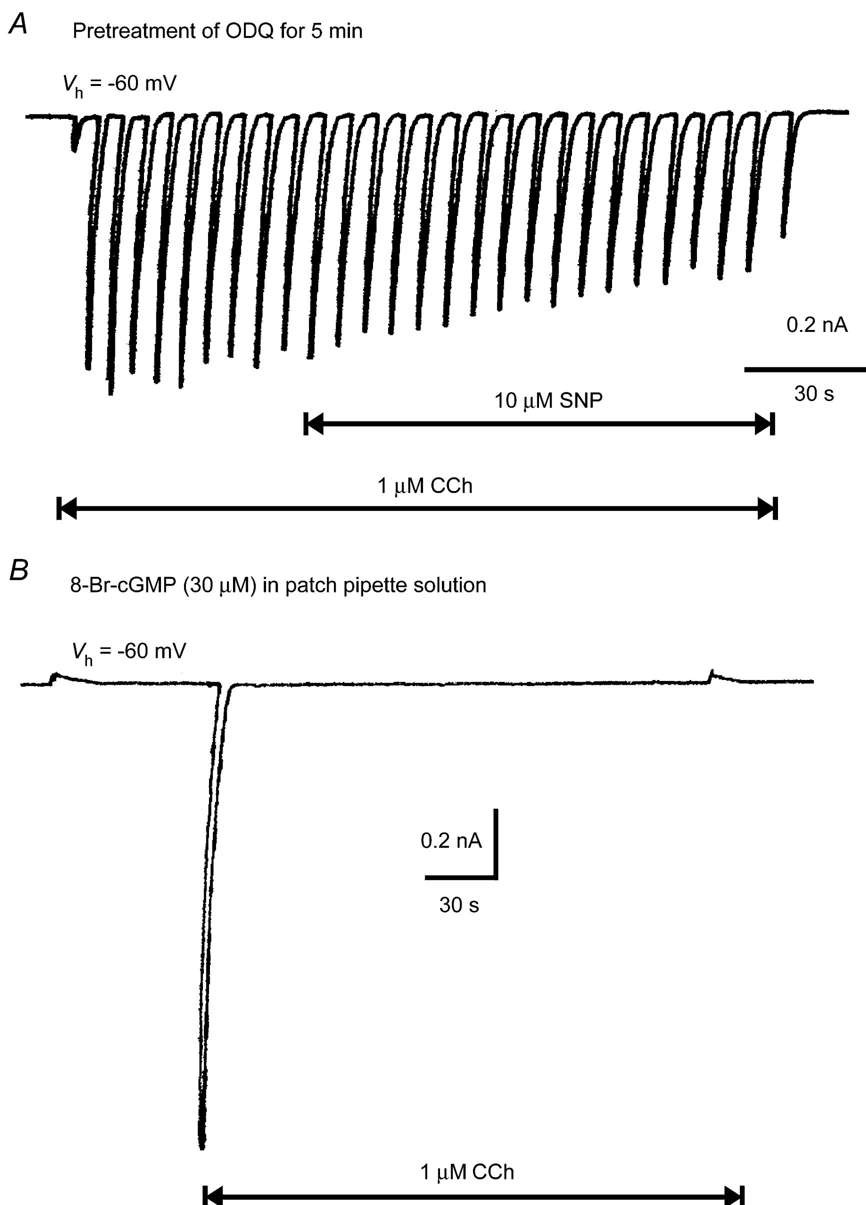

Figure 2. Influences of ODQ and 8-Br-cGMP on the inhibitory effect of SNP on I_{cat} oscillations
A, the current record from a cell pretreated with ODQ (1 μM). CCh (1 μM) applied after a 5 min pretreatment still evoked I_{cat} oscillations (0.18 ± 0.03 Hz, $n = 8$). When SNP was applied in the presence of ODQ and CCh, the inhibitory effect of SNP on I_{cat} oscillations was almost completely prevented. *B*, the current record from a cell dialysed intracellularly with 8-Br-cGMP (30 μM) for about 3 min. CCh (1 μM) did not evoke any oscillatory current in the presence of 8-Br-cGMP in the pipette solution.

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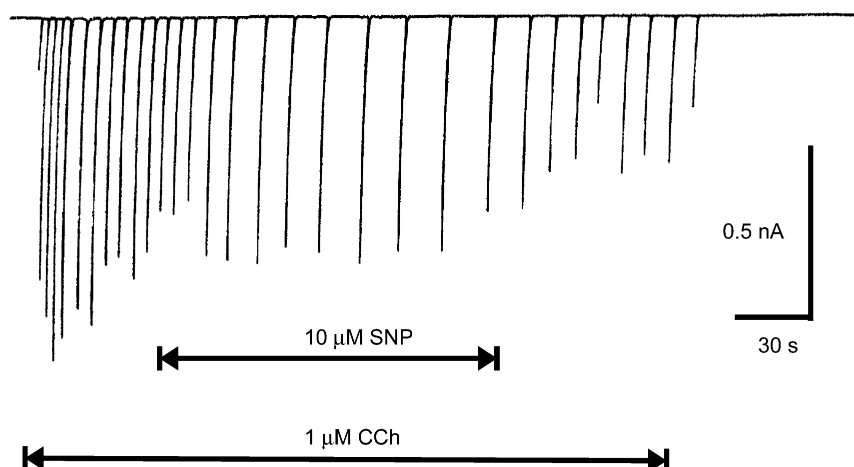

Figure 3. Influence of Rp-8-Br-cGMP on the inhibitory effect of SNP on I_{cat} oscillations
The current record from a cell dialysed intracellularly with Rp-8-Br-cGMP (30 μM) for about 3 min. CCh (1 μM) still evoked I_{cat} oscillations (0.17 $\pm$ 0.03 Hz, $n = 9$). When applied in the presence of Rp-8-Br-cGMP in pipette solution, 10 μM SNP did not stop the I_{cat} oscillations, but reduced the oscillation frequency (0.08 $\pm$ 0.02 Hz).

Carvajal *et al.* 2001). Applied intracellularly via patch pipettes, Rp-8-Br-cGMP (30 μM) had little effect on CCh-evoked I_{cat} oscillations (0.17 $\pm$ 0.03 Hz, $n = 9$). In the intracellular presence of Rp-8-Br-cGMP, application of SNP caused a significant decrease in the frequency of on-going oscillations (0.08 $\pm$ 0.02 Hz), but not cessation of them (Fig. 3). Therefore, SNP-induced inhibition of I_{cat} oscillations was suggested to involve a cGMP/PKG-dependent mechanism.

Effect of SNP on a sustained I_{cat}

It is possible that the cGMP/PKG-dependent mechanism responsible for the SNP-induced inhibition of I_{cat} oscillations affects the function of muscarinic receptors, cationic channels, or their accessory proteins. To test this possibility, $[Ca^{2+}]_i$ was held to a certain level with 10 mM BAPTA and 4.6 mM Ca^{2+} ($[Ca^{2+}]_i \approx 100$ nM, $n = 8$; Fig. 4), which prevented changes in $[Ca^{2+}]_i$ from altering I_{cat}. Under such conditions, CCh evoked a sustained component of I_{cat} without any oscillations, as previously described (Komori *et al.* 1993; $n = 8$). Application of SNP (10 μM) did not significantly affect the on-going sustained I_{cat}, as shown in Fig. 4. Thus, some functional process other than those of muscarinic receptors, cationic channels and their accessory proteins may be targeted by the cGMP/PKG-dependent mechanism.

Effect of IP_3 on the SNP-induced inhibition of I_{cat} oscillations

IICR may play an essential role in I_{cat} oscillations (Komori *et al.* 1993; Zholos *et al.* 1994), so it is possible that SNP stops the current oscillation by blocking IICR by reducing IP_3 receptor sensitivity or IP_3 generation. We investigated this possibility by applying a maximally effective concentration of IP_3 (30 μM) intracellularly via patch pipettes. This concentration of IP_3 is high enough to

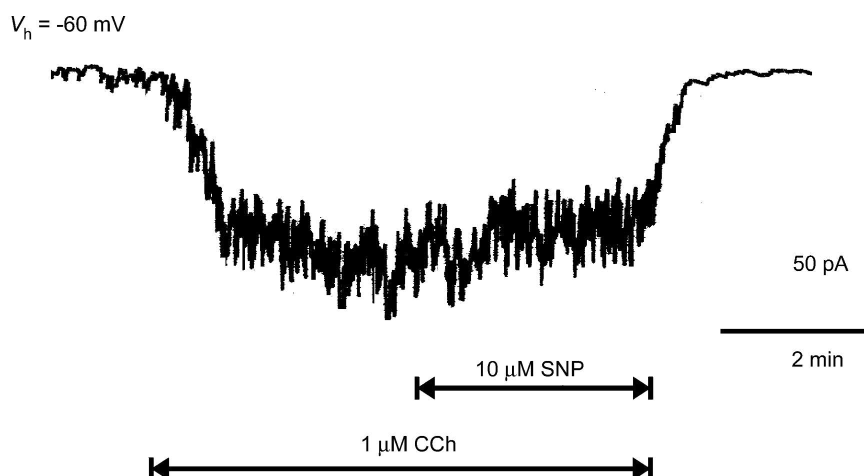

Figure 4. The effect of BAPTA- and Ca^{2+}-containing patch pipette solution on I_{cat} oscillations and the effect of SNP on sustained current evoked by CCh (1 μm)
In the presence of 10 mM BAPTA and 4.6 mM Ca^{2+}, I_{cat} oscillations disappeared, leaving a small sustained current. SNP did not show any inhibitory effect on the sustained current.

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release a maximal amount of Ca^{2+} from stores at a maximal rate regardless of the amount of IP_3 produced by 1 μM CCh (Somlyo *et al.* 1992). In addition, PKG is reported to inhibit IICR in competition with IP_3 (Murthy & Zhou, 2003). Thus, if the inhibitory effect of SNP on I_{cat} oscillation is due to functional modulation of the IP_3 receptor by activation of PKG, SNP is less effective in preventing I_{cat} oscillations at higher intracellular levels of IP_3. In the present experiments, CCh still evoked I_{cat} oscillations with a frequency of 0.35 ± 0.02 Hz ($n = 5$) in cells recorded with IP_3 (30 μM) contained in the pipette. The oscillation frequency was higher than that of I_{cat} oscillations evoked in control cells. Application of SNP (10 μM) during the on-going I_{cat} oscillations reduced the oscillation frequency to 17 ± 0.01 Hz ($n = 5$), but failed to stop the oscillation (Fig. 5). When the IP_3 concentration was increased to 300 μM, SNP did not change the oscillation frequency (data not shown).

Effects of SNP on IP_3-induced tension developments in permeabilized muscle

To determine whether SNP inhibits IICR, we tested its effect on IP_3-induced tension developments and, for comparison, on caffeine-induced tension effects in α-toxin-permeabilized muscle strips.

During the period of Ca^{2+} loading (see Methods), a rise in tension occurred, which reached a plateau within 3 min. The peak tension remained almost unchanged or declined gradually by less than 30%, as previously described (Komori *et al.* 1995). Caffeine (10 mM), applied 5 min after reintroduction of the relaxing solution (RI solution) following Ca^{2+} loading, produced a transient rise in tension due to the release of stored Ca^{2+}. The caffeine responses reached a peak within 1 min and then declined to the initial tension level before caffeine application. The second application of caffeine evoked a reproducible tension increase corresponding to $96.8 \pm 2.4\%$ ($n = 4$) of the first response (Fig. 6*A*). This reproducibility held true when the second application of caffeine was made in the presence of SNP. Indeed, the tension increase evoked was $94.1 \pm 3.0\%$ ($n = 4$) of the first response in the absence of SNP (Fig. 6*B*). IP_3 (30 μM), applied in the same way as the caffeine, also elicited a transient rise in tension, which was generally smaller in size and slower in time course compared with the caffeine response. This small and slow tension response to IP_3 is mainly due to a rapid breakdown of IP_3 by endogenous phosphatase activity during the diffusion of IP_3 into the permeabilized strip (Walker *et al.* 1987; Ozaki *et al.* 2002). The second application of IP_3 evoked a reproducible tension increase ($93.3 \pm 6.67\%$ of the first response, $n = 4$; Fig. 7*A*). The second response to IP_3 was significantly attenuated in the presence of SNP ($48.9 \pm 7.8\%$ of the first control response, $n = 5$; Fig. 7*B*).

Discussion

This study shows that SNP, a NO donor, prevents the oscillatory change in I_{cat}, and suggests that its effect involves a cGMP/PKG-dependent process. It is also suggested that inhibition of IICR, but not of muscarinic receptor or cationic channels, may account, at least in part, for the inhibitory effect of SNP.

In the gastrointestinal enteric nervous system, the non-adrenergic, non-cholinergic (NANC) inhibitory nerves play a crucial role in smooth muscle regulation. Evidence

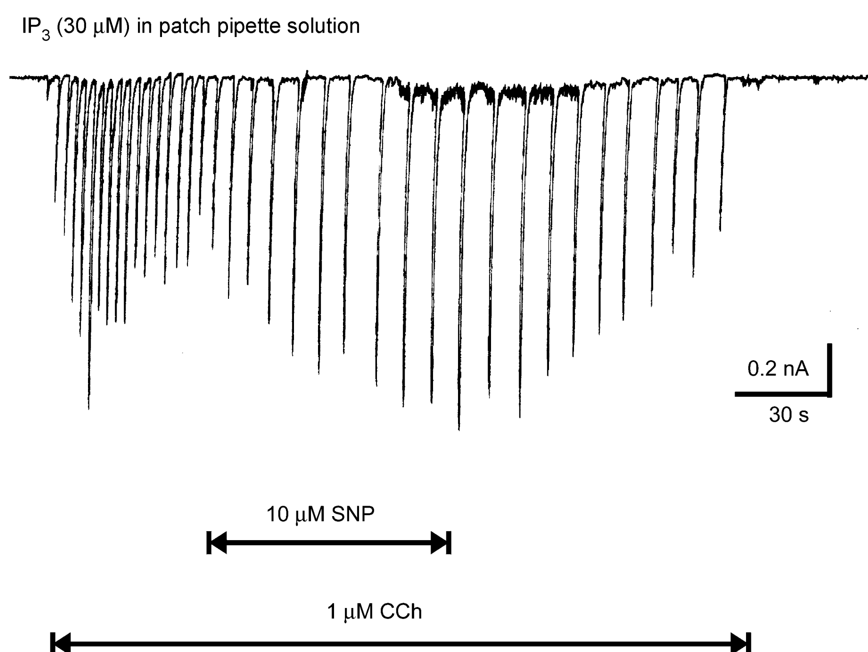

Figure 5. The effect of IP_3-containing patch pipette solution on the SNP inhibitory effect on I_{cat} oscillations
In the presence of a patch pipette solution containing 30 μM IP_3, CCh still evoked I_{cat} oscillations with an increased frequency. SNP (10 μM) did not abolish the I_{cat} oscillations, but reduced their frequency ($n = 5$).

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indicates that NO or a related NO-donating substance are the major candidates for NANC inhibitory transmitters (Lefebvre *et al.* 1991; Stark *et al.* 1991). Because NO is an unstable gaseous agent, NO donors, such as glyceryl trinitrate, SNP and 3-morpholinosydnonimine (SIN-1), have been widely used used as a tool for studying the effects of NO (Hirata & Murad, 1994). NO is known to activate soluble guanylate cylcase with a subsequent increase in cGMP level. Increased cGMP triggers relaxation of smooth muscle by activating PKG, which in turn phosphorylates a variety of functional proteins, including ion channels, to alter their functions (Lincoln *et al.* 1994; McDonald & Murad, 1996). In the present study, SNP (10 μM) completely inhibited the oscillatory change in I_{cat} induced by 1 μM CCh in all cells tested (Fig. 1), and ODQ (1 μM), a soluble guanylate cylcase inhibitor, prevented the inhibitory effect of SNP (Fig. 2*A*). The cGMP analogue 8-Br-cGMP (30 μM) itself blocked the generation of I_{cat} oscillations in response to CCh (Fig. 2*B*). Furthermore, Rp-8-Br-cGMP, the specific PKG antagonist, significantly attenuated the inhibitory effect of SNP (Fig. 3). These results suggest that the inhibitory effect of SNP on I_{cat} oscillations arises via PKG activation as a result of increasing intracellular cGMP levels.

In general, the smooth muscle-relaxing action of NO donors and cGMP-increasing agents is thought to result from modification of various functional proteins involved in Ca^{2+} homeostasis as well as those directly associated with the contractile event (Lincoln, 1989; Kuriyama *et al.* 1995). Increased cGMP may reduce $[Ca^{2+}]_i$ through activation of PKG, which causes phosphorylation of some proteins and leads to activation of Ca^{2+}-activated K^+ channels (Yamakage *et al.* 1996; Zhou *et al.* 1996), inhibition of voltage-dependent Ca^{2+} channels (Horowitz *et al.* 1996; Kwon *et al.* 2000), inhibition of IP_3 receptors

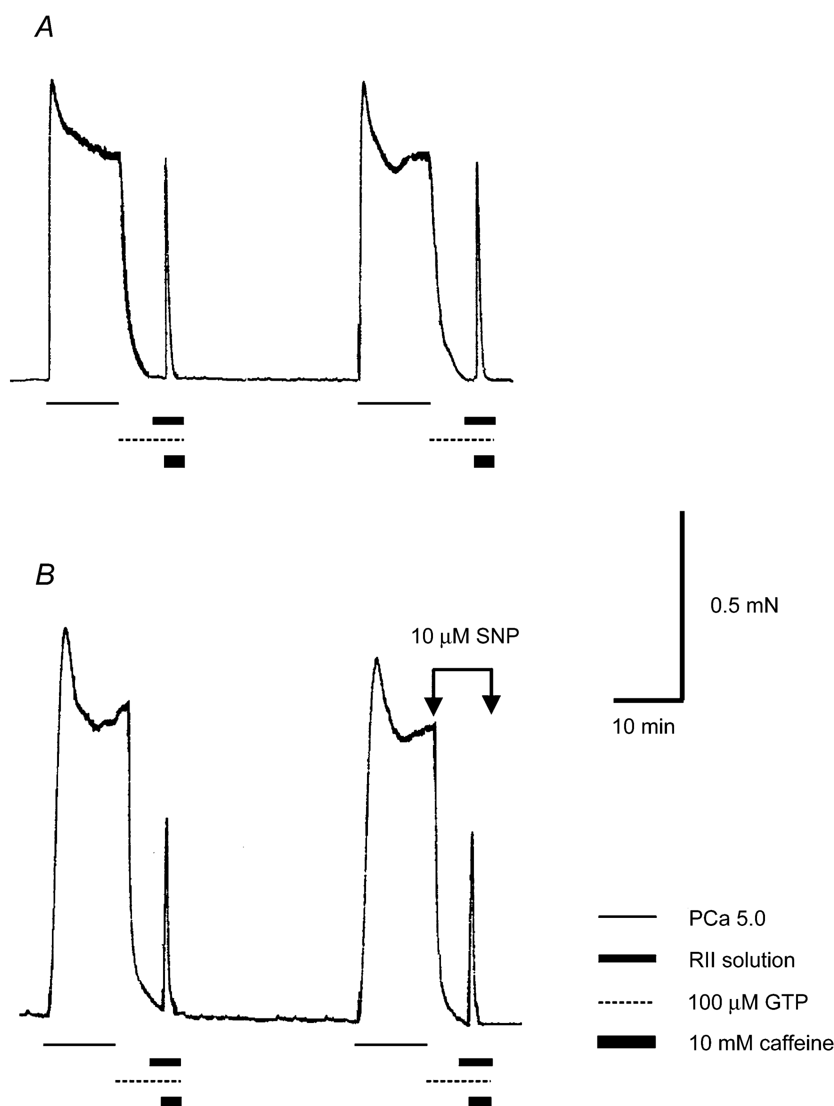

Figure 6. The effect of SNP on tension responses due to the application of caffeine in α-toxin-skinned ileal muscle
A, the record of tension response to caffeine in the control experiment. The responses to caffeine reached a peak within 1 min then declined to the initial level before caffeine application. The second application of caffeine evoked tension with an amplitude very similar to that of first application (96.8 ± 2.4%, $n = 4$). *B*, the record of tension response to caffeine in the SNP experiment. The tension increase resulting from the second application of caffeine in the presence of SNP was almost the same as that from the first application of caffeine in the absence of SNP (94.1 ± 3.0%, $n = 4$). See text for details.

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(Komalavilas & Lincoln, 1994, 1996) and reduction of IP_3 (Hirata *et al.* 1990). These result in the reduction of $[Ca^{2+}]_i$ and relaxation of smooth muscle. In the present study, CCh-evoked I_{cat} oscillations were measured at a holding potential of −60 mV in 130 mM Cs-filled cells, in which voltage-dependent Ca^{2+} channels are deactivated and various K^+ channels, including those activated by Ca^{2+}, are totally blocked. Thus the inhibitory effect of SNP on I_{cat} oscillations is unlikely to involve inhibition of these channels.

As mentioned in the Introduction, it has been suggested that IICR plays an essential role in sustaining I_{cat} oscillation (Komori *et al.* 1993; Zholos *et al.* 1994). IP_3-gated Ca^{2+} release channels are under a dual regulation by $[Ca^{2+}]_i$; their opening is accelerated as $[Ca^{2+}]_i$ is increased to a certain level, but inhibited when $[Ca^{2+}]_i$ rises higher than this level. Ca^{2+} inhibition of IICR at some critical level of $[Ca^{2+}]_i$ allows Ca^{2+} stores to refill and leads to a fall in $[Ca^{2+}]_i$, thus contributing to the I_{cat} oscillations. Therefore, even in the presence of a constant level of intracellular IP_3, Ca^{2+}-dependent inhibition of the IP_3-gated Ca^{2+}-release channel can also play an important role as a negative feedback control in giving rise to I_{cat} oscillation. These circumstances raise at least two possible mechanisms that might be responsible for the SNP-induced inhibition of I_{cat} oscillations: (1) functional modulation of CCh-operated cationic channels, their accessory proteins, or muscarinic receptors; and (2) reduction of $[Ca^{2+}]_i$ by inhibition of IICR. As shown in Fig. 4, CCh-evoked sustained I_{cat} was not affected by SNP, which may exclude the first possible mechanism. Evidence suggests that phosphorylation of IP_3 receptors by PKG causes a reduction in their channel activity in response to IP_3, resulting in inhibition of IICR and smooth muscle relaxation (Komalavilas & Lincoln, 1994, 1996; Murthy & Zhou, 2003). Furthermore, the inhibitory effect of PKG on IICR caused by IP_3 receptor phosphorylation is in competition with the intracellular level of IP_3 (Murthy & Zhou, 2003); that is, the higher the intracellular concentration of IP_3, the less potent is the

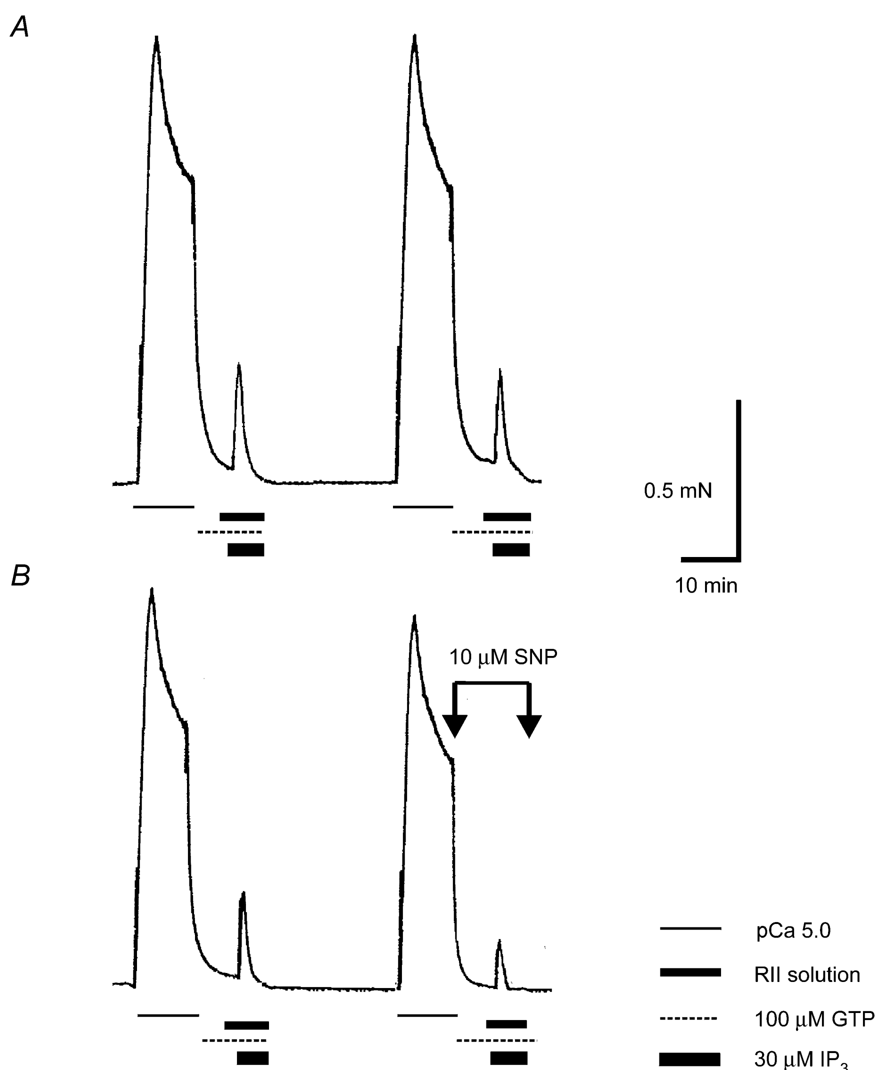

Figure 7. The effect of SNP on tension responses due to the application of IP_3 in α-toxin-skinned ileal muscle
A, the record of tension response to IP_3 (30 μM) in the control experiment. The second application of IP_3 evoked tension with an amplitude very similar to the first application (to 93.3 ± 6.67%, $n = 4$). *B*, the record of the effect of SNP on the tension response resulting from IP_3 application. The amplitudes of tension in response to the second application of IP_3 in the presence of SNP were significantly attenuated compared to that of the first application in the absence of SNP (to 48.9 ± 7.8%, $n = 5$). See text for details.

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inhibitory effect of PKG on IICR. Moreover, the maximum rate of Ca^{2+} release is increased as a function of IP_3 concentration and is saturated at 4 μM (Somlyo *et al.* 1992). So it is possible that 30 μM IP_3 in the patch pipette solutions can release a maximal amount of Ca^{2+} from the stores at a maximal rate, and may effectively prevent the PKG effect on IICR. In the present experiments, SNP (10 μM) failed to prevent I_{cat} oscillation in the intracellular presence of 30 μM IP_3, although it reduced the oscillation frequency (Fig. 5). Increasing the IP_3 concentration to 300 μM prevented the oscillation frequency effect of SNP. Tension experiments on α-toxin-permeabilized muscle strips showed that SNP reduces the increase in tension produced by IP_3, but not by caffeine (Figs 6 and 7). Taken together, these results suggest that the inhibitory effect of SNP on I_{cat} oscillation is brought about, at least in part, by inhibition of IICR via functional modulation of the IP_3 receptor. In addition, another possibility, that SNP-induced inhibition of I_{cat} oscillation involves reduced IP_3 production, cannot be excluded.

In conclusion, this study has demonstrated that SNP may inhibit CCh-induced Ca^{2+} release and I_{cat} oscillations, and suggests that the effect of SNP involves functional modulation of IP_3 receptors, but not cationic channels or muscrinic receptors. SNP regulation may also arise through a cGMP/PKG-dependent mechanism. These results provide a more comprehensive mechanism for the inhibitory action of NO on the cholinergic stimulation of intestinal motility.

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