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# Rat duodenum nitrergic-induced relaxations are cGMP-independent and apamin-sensitive

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**Abstract**

The effects of the $K^+$ channel blockers, apamin, tetraethylammonium and 4-aminopyridine, upon the relaxations of the isolated rat proximal duodenum induced by nitrergic nerve ac tivation, nitric oxide (NO), the NO donor 3-morpholinosydnonimine (SIN-1) and Br-cyclic GMP were determined. The effects of the guanylate cyclase inhibitors, cystamine and *N*-methylhydroxylamine, on NO-, SIN-1- and nitrergic nerve-induced responses were also investigated. Apamin inhibited nitrergic nerve-, NO-and SIN-1-induced relaxations but did not affect those induced by Br-cGMP. Tetraethylammonium and 4-aminopyridine as well as cystamine and *N*-methylhydroxylamine failed to affect the relaxations caused by any of the agents tested. These findings indicate that, in the rat proximal duodenum, nitrergic nerve activation as well as exogenous nitric oxide cause relaxation through a cGMP-independent, apamin sensitive mechanism.

*Keywords:* Proximal duodenum, rat; Nitric oxide (NO); NANC (non-adrenergic, non-cholinergic) nerve; Smooth muscle relaxation; Apamin; $K^+$ channel; cGMP

## 1. Introduction

In the gastrointestinal tract nitric oxide (NO) or a NO-related compound has recently been proposed as one of the inhibitory non-adrenergic non-cholinergic (NANC) neurotransmitters (Bult et al., 1990; Sanders and Ward, 1992). As it was postulated that the relaxation of vascular smooth muscle by NO is mediated by cyclic GMP (Ignarro, 1990), it has been assumed that cGMP also mediates NANC-induced relaxations of gastrointestinal smooth muscle. However, recent evidence obtained with the opossum lower esophageal sphincter indicates that NANC nerves and NO cause relaxation in a cGMP-independent manner (Knudsen et al., 1992; Murray et al., 1992). We have recently described that NO or a related compound could also be involved in the NANC nerve-induced relaxations of the longitudinal muscle of the rat proximal duodenum (Martins et al., 1993).

NANC nerve-induced relaxations and inhibitory junction potentials in some smooth muscles from the gastrointestinal tract including the rat duodenum, have been shown to be blocked by apamin, a toxin isolated from bee venom (Maas and Den Hertog, 1979; Shuba and Vladimirova, 1980; Muller and Baer, 1980; Ferrero et al., 1980; Maas, 1981; Bywater et al., 1981; Bauer and Kuriyama, 1982; Costa et al., 1986). Since the apamin effect results from the blockade of a subset of $Ca^{2+}$-activated $K^+$ channels (Blatz and Magleby, 1986) the objective of the present work was to determine whether nitrergic nerve and NO-induced relaxations of the rat duodenum involve the activation of apamin-sensitive $K^+$ channels and whether this effect is dependent on cGMP.

## 2. Materials and methods

Male Wistar rats weighing 250–300 g were killed by decapitation. A 2–2.5-cm long whole segment of the proximal portion of the duodenum was removed and placed under 2 g of resting tension in a 10-ml organ bath containing calcium-free Tyrode solution (composi-

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tion in mmol/l: NaCl 137, KCl 3, $MgCl_2$ 1, $NaHCO_3$ 12, $NaHPO_4$ 0.4 and glucose 5.5) maintained at 32°C and continuously bubbled with air. Longitudinal muscle tension was recorded in a polygraph by means of an isometric force transducer. Electrical field stimulation was applied through two ring electrodes placed below and above the segment and consisted of square wave pulses of 32 V, 0.25 ms, 1 Hz for 5 s. The duodenal segments were washed every 15 min for up to 120 min with $Ca^{2+}$-free Tyrode solution. Tension was then induced by adding $CaCl_2$ to the bath (1 mM final concentration); under these conditions the tonus induced was stable and the spontaneous activity had a low amplitude. The effect of the different drugs was determined in the presence of atropine (1 $\mu$M) and guanethidine (3 $\mu$M). The effect of the different inhibitors was determined in the same preparation after obtaining consistent control responses; separate preparations were employed for testing each inhibitor which was incubated for a 20-min period.

### 2.1. Drugs

Atropine sulphate, guanethidine, 8-bromo cGMP sodium salt, 8-bromo cAMP sodium salt, cystamine, *N*-methylhydroxylamine, L-arginine and D-arginine were obtained from Sigma Chemical Company. Tetraethylammonium, 4-aminopyridine and apamin were obtained from Research Biochemicals. 3-Morpholinosydnonimine (SIN-1) was kindly provided by Cassella AG, Frankfurt/Main. NO was obtained by diluting sodium nitrite in de-aerated distilled water acidified to pH 2 with HCl (Cocks and Angus, 1990). NO concentrations were estimated assuming complete transformation of sodium nitrite to NO.

### 2.2. Statistical analysis

Responses were expressed as percentages of the reduction in tension and are presented as means ± S.E.M. Statistical significance was determined with Student's *t*-test; *P* values less than 0.05 were considered significant.

## 3. Results

### 3.1. Effect of cystamine and N-methylhydroxylamine on nitrergic nerve-induced NANC relaxations

As we have previously shown (Martins et al., 1993) activation of NANC nerves by electrical field stimulation (1 Hz) of the rat proximal duodenum produces a 60 ± 4.5% ($n = 6$) decrease in tension which is blocked by $N^G$-nitro-L-arginine methyl ester in an L-arginine, but not D-arginine, preventable manner (Fig. 1), confirming that NO or a related compound formed from L-arginine is involved in this response. Since it has been shown that NO increases the activity of guanylate cyclase in several cells and tissues we tested the hypothesis that cGMP is the intracellular mediator of nitrergic nerve induced relaxations. The relaxation of the rat proximal duodenum induced by electrical field stimulation was not affected by the guanylate cyclase inhibitors, cystamine (1 mM) and *N*-methylhydroxylamine (1 mM) (Fig. 2a).

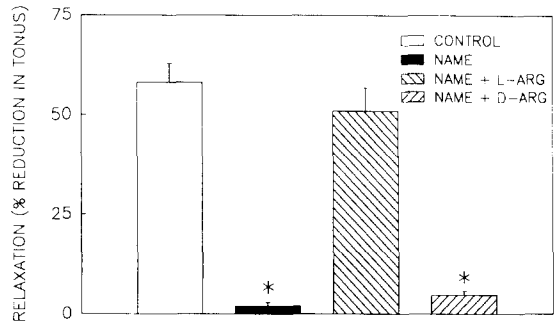


Fig. 1. Effect of $N^G$-nitro-L-arginine (100 $\mu$M) in the absence or in the presence of either L-arginine or D-arginine (1 mM) upon NANC nerve-induced relaxations of the isolated rat duodenum. Values shown represent the means ± S.E.M. of six experiments. * $P < 0.05$.

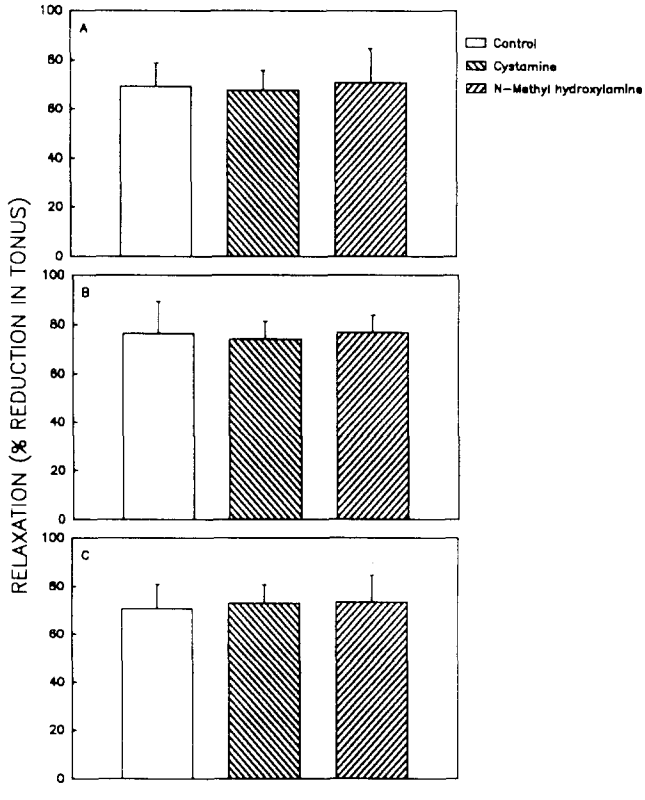


Fig. 2. Lack of effect of cystamine (1 mM) and *N*-methylhydroxylamine (1 mM) upon relaxations induced by NANC nerve stimulation (A), nitric oxide (5 $\mu$M) (B) and SIN-1 (10 $\mu$M) (C) of the isolated rat duodenum.

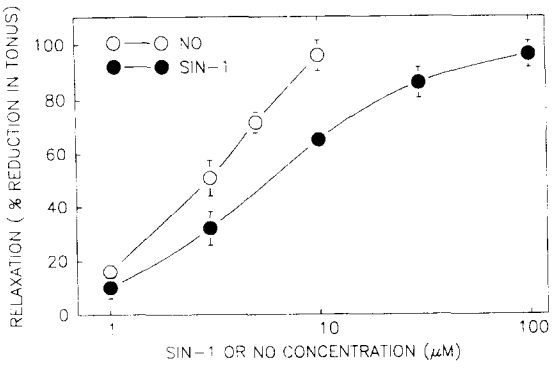


Fig. 3. Concentration-response curves for SIN-1 and nitric oxide (NO) in the isolated rat duodenum. Values shown represent the means ± S.E.M. for four to six experiments.

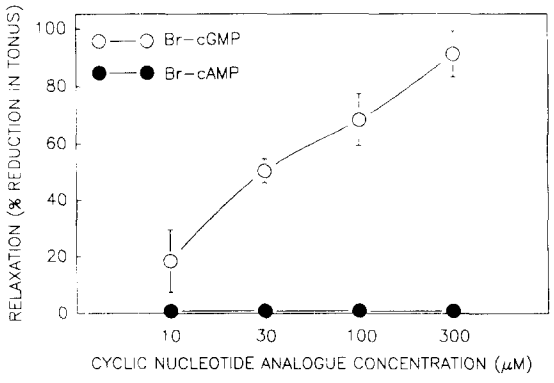


Fig. 4. Concentration-response curves for Br-cGMP and Br-cAMP in the isolated rat duodenum. Note that Br-cAMP did not cause any relaxation. Values shown represent the means ± S.E.M. of six experiments.

### 3.2. Effect of cystamine and N-methylhydroxylamine upon NO and SIN-1-induced relaxations

NO (1–10 μM) and SIN-1 (1–100 μM) caused a concentration-dependent relaxation of the rat proximal duodenum (Fig. 3). The relaxations induced by NO (10 μM) or SIN-1 (30 μM) were not influenced by tetrodotoxin (3 μM) or $N^G$-nitro-L-arginine methyl ester (100 μM) (data not shown). Cystamine (1 mM) and *N*-methylhydroxylamine (1 mM) failed to alter the relaxations induced by SIN-1 or NO (Fig. 2b,c).

### 3.3. Effect of Br-cGMP and Br-cAMP

The membrane permeant analogue of cGMP, Br-cGMP (10–300 μM) produced a concentration-dependent relaxation of the rat proximal duodenum which was not modified by tetrodotoxin (3 μM) or $N^G$-nitro-L-arginine methyl ester (100 μM) (Fig. 4). Br-cAMP (10–300 μM) did not relax the rat proximal duodenum (Fig. 4). Cystamine and *N*-methylhydroxylamine did not affect the relaxations induced by Br-cGMP (data not shown).

### 3.4. Effect of apamin, tetraethylammonium and 4-aminopyridine

Apamin (1 μM) abolished the relaxations of the rat proximal duodenum caused by nitrergic nerve stimulation (electrical field stimulation, 1 Hz) and significantly

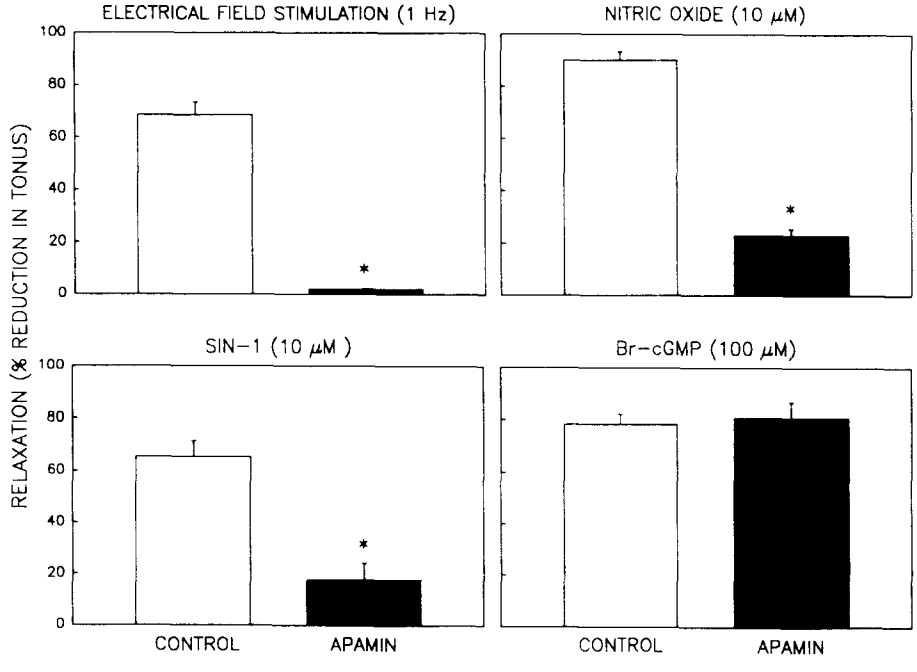


Fig. 5. Effect of apamin (1 μM) upon the relaxations of the rat proximal duodenum induced by NANC nerve stimulation, nitric oxide (10 μM), SIN-1 (10 μM) or Br-cGMP (100 μM). Values shown represent the means ± S.E.M. of four to six experiments. * $P < 0.05$.

Table 1
Effect of tetraethylammonium (TEA) and 4-aminopyridine (4-AP) on on relaxations of the rat duodenum induced by electrical field stimulation (EFS), nitric oxide (NO) and SIN-1

| Relaxations induced by | Control ($n=6$) | TEA (100 $\mu$M) ($n=6$) | 4-AP (10 $\mu$M) ($n=6$) |
|---|---|---|---|
| EFS (1 Hz) | 59.3 ± 5.3 | 60.9 ± 7.8 | 58.5 ± 6.8 |
| NO (10 $\mu$M) | 92.2 ± 1.8 | 98.0 ± 3.4 | 96.0 ± 2.4 |
| SIN-1 (30 $\mu$M) | 70.0 ± 7.1 | 72.0 ± 4.2 | 68.7 ± 3.4 |

Values are the means ± S.E.M.

reduced SIN-1-and NO-induced responses but did not affect the relaxations induced by Br-cGMP (Fig. 5). Tetraethylammonium (0.1–1.0 mM) and 4-aminopyridine (0.1–1.0 mM) did not modify the relaxations caused by electrical field stimulation, NO or SIN-1 (Table 1). Apamin also abolished the contraction observed after cessation the electrical field stimulation. Basal tension was not affected by apamin and tetraethylammonium while 4-aminopyridine induced a slight increase.

## 4. Discussion

In the rat proximal duodenum, NO or a related compound derived from L-arginine appears to be involved in NANC nerve-induced relaxations elicited by low frequency (less than 2 Hz) electrical stimulation (Martins et al., 1993). The present findings showed that nitrergic nerve- as well as NO-induced relaxations of the rat duodenum are sensitive to apamin but not to tetraethylammonium and 4-aminopyridine. Apamin has been shown to inhibit nitrergic nerve-induced inhibitory junction potentials in the canine small intestine (Christinck et al., 1991) as well as nerve- and sodium nitroprusside-induced relaxations of the guinea pig ileal longitudinal muscle (Osthaus and Galligan, 1992) and *S*-nitrosocysteine-induced hyperpolarization of rat gastric fundus (Kitamura et al., 1993). Since tetraethylammonium and 4-aminopyridine, which block most $K^+$ channels excepting small conductance $K_{Ca}$ (Cook, 1988), did not affect nitrergic-induced relaxations whereas apamin, a selective small conductance $K_{Ca}$ channel blocker (Blatz and Magleby, 1986; Cook, 1988), could reduce nitrergic-induced relaxations, the present findings suggest that NO or a related molecule derived from L-arginine stimulates the opening of such channels to cause relaxation. It is worth noting that opening of apamin-sensitive $K^+$ channels is not involved in every nitrergic nerve-induced relaxation of gastrointestinal muscles. Indeed, in the opossum esophagus circular muscle, nitrergic nerve-induced relaxations as well as inhibitory junction potentials are apamin-insensitive (Juri et al., 1985; Ballejo et al., 1992). Taken together these observations indicate that at least two pharmacologically different $K^+$ channels are involved in the relaxation or hyperpolarization of gastrointestinal smooth muscle induced by nitrergic nerve activation.

cGMP has been proposed to mediate the relaxations induced by NO in vascular and visceral smooth muscle (Ignarro, 1990) and by NANC nerve stimulation in the opossum lower esophageal sphincter (Torphy et al., 1986; Barnette et al., 1989), canine internal anal sphincter (Grous et al., 1991) and human lower esophageal sphincter (Barnette et al., 1991). In addition, it has been proposed that the effect of NO on the membrane potential in canine ileocolonic circular smooth muscle cells is mediated by cGMP since M&B 22948, a selective inhibitor of cGMP phosphodiesterase, was able to hyperpolarize the membrane and prolong the inhibitory junctional potentials elicited by NANC nerve stimulation (Ward et al., 1992a). The present findings, however, indicate that this appears not to be the case in the rat proximal duodenum. First, *N*-methylhydroxylamine and cystamine, two compounds that have been reported as inhibitors of soluble guanylate cyclase activity (Deguchi et al., 1978; Rapoport and Murad, 1988; Murray et al., 1992) failed to inhibit nitrergic nerve-and NO-induced relaxations. Second, apamin at concentrations which abolished nitrergic nerve-induced relaxations failed to affect the relaxation caused by a permeant analogue of cGMP. Consistent with our findings, neither NO-induced hyperpolarization nor NANC nerve-induced inhibitory junction potentials of canine colonic cells were influenced by methylene blue or Ly 83,583 (Ward et al., 1992b). Similarly, NO-Cys-and 8-Br-cGMP-induced hyperpolarizations of the rat gastric fundus were unaffected by methylene blue and apamin respectively (Kitamura et al., 1993). These findings suggest that NO induces hyperpolarization in some gastrointestinal muscles, most likely through $K^+$ channel activation, in a cGMP-independent manner. Also, the findings are consistent with the present observations in the rat proximal duodenum, showing that cGMP does not appear to mediate the effect of NO or nitrergic nerve stimulation on apamin-sensitive relaxations. The fact that methylene blue and cystamine failed to affect nitrergic nerve-induced relaxations of the opossum lower esophageal sphincter (Knudsen et al., 1992; Murray et al., 1992) further questions the universal role of cGMP as a mediator of NO actions in gastrointestinal muscle cells. Although the former authors observed that methylene blue was able to block NO-induced relaxations, it is unlikely that this effect resulted from guanylate cyclase inhibition since methylene blue did not affect relaxation induced by sodium nitroprusside or *S*-nitrosocysteine. Furthermore, it is worth remarking that there is a recent report showing that NO can activate a $K^+$ channel in vascular smooth muscle in a

cGMP-independent manner (Bolotina et al., 1994) as well as other reports indicating that NO can act through cGMP-independent mechanisms. For instance, NO-containing compounds inhibit cell proliferation and decrease cytosolic free calcium concentration in BALB/c 3T3 fibroblasts which lack soluble guanylate cyclase (Garg and Hassid, 1991) and induce protein ADP-ribosylation in several cells through a cGMP-independent process (Brune and Lapetina, 1989).

In summary, our results suggest that, in the longitudinal muscle of the rat proximal duodenum, NO or a related compound formed from L-arginine is released by NANC nerve stimulation and causes relaxation through a cGMP-independent mechanism which involves the activation of apamin-sensitive potassium channels.

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