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Cyclic GMP-associated apamin-sensitive nitrergic slow inhibitory junction potential in the hamster ileum

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1 The mediators of non-adrenergic, non-cholinergic (NANC) inhibitory junction potentials (i.j.ps) in the circular smooth muscle cells of the hamster ileum were studied.

2 Electrical field stimulation (EFS; 0.5 ms duration, 15 V) of the intramural nerves with a train of five pulses at 20 Hz evoked a rapidly developing hyperpolarization (fast i.j.p.) followed by a sustained hyperpolarization (slow i.j.p.).

3 N^G-nitro-L-arginine methyl ester (L-NAME; 50–200 μM) and N^G-nitro-L-arginine (L-NNA; 50–200 μM), NO synthase inhibitors, inhibited or abolished the EFS-induced fast and slow NANC i.j.ps. The effects of these NO synthase inhibitors were reversed by L-arginine (5 mM) but not by D-arginine (5 mM).

4 Exogenously applied nitric oxide (NO; 1–100 μM) induced concentration-dependent hyperpolarizations.

5 Oxyhaemoglobin (5–50 μM), NO scavenger, inhibited only the slow i.j.p., and the NO-induced hyperpolarization.

6 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ; 10 μM) and cystamine (10 mM), guanylate cyclase inhibitors, inhibited only the slow i.j.p. Zaprinast (100 μM), a phosphodiesterase type V inhibitor, enhanced the amplitude and duration of the slow i.j.p.

7 Apamin (100 nM), a small conductance Ca^{2+}-activated K^+ channel blocker, inhibited only the slow i.j.p., and NO-induced hyperpolarization. A high concentration of 8-bromoguanosine 3′:5′-cyclic monophosphate (8-bromo-cGMP; 1 mM)-induced membrane hyperpolarization which was blocked by apamin.

8 These results suggest that NO, or a related compound, may be the inhibitory transmitter underlying the apamin-sensitive NANC slow i.j.p. and cyclic GMP mediates the slow i.j.p. in the hamster ileum. It is also likely that NO, without involvement of guanylate cyclase is associated with the fast i.j.p.

Keywords: Hamster ileum; nitric oxide; cyclic GMP; apamin-sensitive NANC slow i.j.p.

Abbreviations: 8-Bromo-cGMP, 8-bromoguanosine 3′:5′-cyclic monophosphate; CYSNO, S-nitroso-L-cysteine; EFS, electrical field stimulation; e.j.p. excitatory junction potential; GC, guanylate cyclase; HQ, hydroquinone, i.j.p.; inhibitory junction potential, L-NAME, N^G-nitro-L-arginine methyl ester; L-NNA, N^G-nitro-L-arginine; NANC, non-adrenergic, non-cholinergic; NO, nitric oxide; NOS, NO synthase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one; Oxy-Hb, oxyhaemoglobin; PDE V, phosphodiesterase type V; PSS, physiological salt solution; SK, small conductance Ca^{2+}-activated K^+

Introduction

Mechanical and electrical activation of a certain class of enteric nerves originating from the myenteric plexus evoke non-adrenergic, non-cholinergic (NANC) inhibitory junction potentials (i.j.ps) in the circular muscle of the mammalian gastrointestinal tract (Hirst & McKirdy, 1974; Christinck *et al*., 1991; Smith *et al*., 1991; Kishi *et al*., 1996; Watson *et al*., 1996b). Apamin is a blocker of small conductance Ca^{2+}-activated K^+ (SK) channels (Blatz & Magelby, 1986). The composite i.j.ps are made up of a fast component (apamin-sensitive) and a slow component (apamin-insensitive) in the guinea-pig gastric fundus (Ohno *et al*., 1996), ileum (Niel *et al*., 1983) and colon (Blatz & Magelby, 1986). It has been reported that the apamin-sensitive fast i.j.p. is mediated by ATP, and the apamin-insensitive slow i.j.p. by VIP and NO in the guinea-pig ileum (He & Goyal, 1993) and colon (Zagorodnyuk & Maggi, 1994). However, PACAP and VIP are involved in both the apamin-sensitive fast and the apamin-insensitive slow i.j.p., respectively, in the rat distal colon (Kishi *et al*., 1996). In contrast, neither ATP nor VIP is the main transmitter responsible for NANC i.j.ps in the opossum oesophagus (Daniel *et al*., 1983) or guinea-pig gastric fundus (Ohno *et al*., 1996). These results indicated that the nature of the inhibitory transmitter released responsible for the i.j.ps evoked in gastrointestinal smooth muscle differs between species and regions.

More recently, there is increasing evidence that NO is an important NANC inhibitory transmitter in the mammalian gastrointestinal tract (Smits & Lefebvre, 1996; Watson *et al*., 1996b; Iversen *et al*., 1997). However, the nature of the inhibitory transmitter of nitrergic nerves, which utilize NO synthase for the generation of their neurotransmitter (Rand & Li, 1995), is the subject of considerable debate. Except for the rat gastric fundus (De Man *et al*., 1996), actual nitrergic NANC neurotransmitter is probably not free NO but a NO-donating compound (Gillespie & Sheng, 1990). NO, released from exogenously-applied NO-donors or upon NANC nerve stimulation, induces an apamin-insensitive membrane hyperpolarization which is mediated by cyclic GMP in the canine ileocolonic sphincter (Ward *et al*., 1992b) and opossum

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esophagus (Cayabyab & Daniel, 1995). These results suggest that NO does not activate SK channels. On the other hand, the hyperpolarization induced by the NO-donor in the rat gastric fundus (Kitamura *et al*., 1993) and guinea-pig colon (Watson *et al*., 1996a,b) was inhibited by apamin, but not reduced upon the blockade of guanylate cyclase activity, suggesting that NO-donor directly activates SK channels. Thus, it is not clear whether the hyperpolarization induced by neurally-released NO is sensitive or insensitive to apamin or mediated by cyclic GMP.

The purpose of the present study was to investigate the involvement of NO in the NANC i.j.p. and to determine whether apamin distinguishes different types of NANC i.j.ps in the hamster ileum, using intracellular microelectrode recording techniques. This was attempted, by examining the following: (i) the effects of two NOS inhibitors on the NANC i.j.ps; (ii) the effect of authentic NO on membrane potential; (iii) the effects of two NO scavengers on the NANC i.j.ps and exogenous NO; (iv) the effect of apamin on the NANC i.j.ps. We further determined the mechanism involved in the NANC slow i.j.p., by examining the effects of two guanylate cyclase inhibitors, a cyclic GMP phosphodiesterase inhibitor and apamin on the NANC slow i.j.p., and those of apamin on cyclic GMP analogue-induced hyperpolarization. Preliminary accounts of some of these results have appeared in abstract form (Matsuyama *et al*., 1998).

Methods

Tissue preparation

Male Syrian hamsters of 5–8 weeks-old were anaesthesized with diethyl ether and exsanguinated *via* the carotid arteries. After the abdominal cavity was opened, a length of about 3–4 cm of the middle part of the ileum was removed and immediately immersed in physiological salt solution (PSS; see below) at room temperature. The contents of the excised segment were flushed with a small cannula containing PSS. The segment was gently stretched and fixed to a rubber block in an experimental chamber with a pin (diameter: 100 μm). The bath had a volume of 4 ml and was continuously perfused with PSS containing atropine 0.5 (in μM):, guanethidine 5 and nifedipine 5 at a constant flow rate of about 3 ml min^{-1}. The bath fluid was oxygenated by bubbling with a 95% O_2:5% CO_2 gas mixture and maintained at 32 ± 0.5°C so as to decrease muscle contractions and displacement of the recording microelectrode. Tissues were allowed to equilibrate for approximately 45–60 min before experiments were undertaken.

Electrophysiological recordings

Membrane potentials were recorded with a conventional glass microelectrode filled with 3 M KCl with a resistance of 50–80 MΩ. The electrode insertions were made into the circular muscle cells of the deep layer from the serosal side (Takewaki & Ohashi, 1977). A successful insertion was confirmed when a sharp change in voltage to a membrane potential negative to about −40 mV was observed between spontaneous activity and the resting membrane potential remained stable for at least 5 min. A pair of silver wire electrodes, one placed in the intestinal wall, the other in the organ bath, were used for electrical field stimulation (EFS) of intramural nerves of the preparation. To record membrane potential responses to EFS, a microelectrode was inserted into a smooth muscle cell located within 2 mm of the stimulating electrode. I.j.ps were evoked by EFS of intramural nerves of the tissue with square-wave pulses (1–5 pulses) of 0.5 ms duration at 15 V. Membrane potential changes were displayed on an oscilloscope (CS 4025, Kenwood, Tokyo, Japan). Analogue electrical signals were recorded on a thermal-array recorder (RTA-1100 M, Nihon Kohden, Tokyo, Japan) for illustration and analysis.

Physiological solutions and drugs

The physiological salt solution (PSS) used in this study had the following compositions (mM): NaCl 137, KCl 4.0, NaH_2PO_4 0.5, $NaHCO_3$ 11.9, $CaCl_2$ 2.0, $MgCl_2$ 1.0 and glucose 5.6.

Apamin, N^G-nitro-L-arginine (L-NNA), N^G-nitro-L-arginine methyl ester (L-NAME), tetrodotoxin, guanethidine sulphate, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), D-arginine monohydrochloride, haemoglobin, zaprinast, 8-bromoguanosine 3′:5′-cyclic monophosphate (8-bromo-cyclic GMP), and hydroquinone (HQ) were obtained from Sigma Chemical Co., St. Louis, U.S.A. Nifedipine, L-arginine hydrochloride, atropine sulphate monohydrate, cystamine dihydrochloride and dimethyl sulphoxide (DMSO) were purchased from Wako Pure Chem., Osaka, Japan.

L-NNA was dissolved in 0.1 N HCl solution. Zaprinast and ODQ were dissolved in DMSO. Nifedipine was dissolved in ethanol. All other drugs were dissolved in distilled water. Stock solutions were at 100 or more than 100 times higher concentrations than those used for experiments, and further dilutions were made in PSS. Final concentrations of HCl, DMSO, ethanol or distilled water in the bathing solution were less than 0.01% and had no effect on the membrane potential. The drug concentrations given in the test were final concentrations in the bathing solution. Nitric oxide (NO) solutions were kept at 4°C and diluted to their final concentration in PSS containing atropine, guanethidine and nifedipine as indicated.

Preparation of nitric oxide solution

A stock solution of NO was prepared by a modification of the method of Stark *et al*. (1991). Briefly, NO gas was injected into PSS which was previously deoxygenated by gassing with He for 2 h, to give stock solutions of NO ranging from 0.01–1.0% (v v^{-1}). The deoxygenated solution had no effect on membrane potential of the same cell.

Preparation of oxyhaemoglobin

Oxyhaemoglobin (Oxy-Hb) was prepared by a modification of the method of Martin *et al*. (1984) as follows: a 10 fold molar excess of sodium dithionite ($Na_2S_2O_4$), a reducing agent was added to a 1 mM solution of purchased 'haemoglobin' in distilled water. Excess $Na_2S_2O_4$ was then removed by dialysing three times against 100 volumes of distilled water at 4°C. The solutions were frozen in aliquots at −20°C and stored for up to 14 days.

Statistical analysis

Data are expressed as mean ± s.e.mean, and n represents the number of experiments performed using different tissue preparations from different hamsters. When, in one preparation, recordings were carried out more than one cell, a mean value was calculated and used. Differences between the means were analysed by one-way analysis of variance, followed by the Dunnett's or Tukey-Kramer tests for multiple group compar-

isons, or Student's *t*-test for comparison of two groups. *P* value of less than 0.05 was considered significant.

Results

Membrane potential responses to electrical field stimulation

In the presence of atropine (0.5 μM), guanethidine (5 μM) and nifedipine (5 μM), circular smooth muscle cells had an average resting membrane potential of -41.3 ± 0.5 mV (284 cells in 156 preparations) and displayed either electrically quiescence (30%) or spontaneous electrical rhythmic potentials (70%), which represented slow wave activity. The slow waves occurred at 9.6 ± 0.1 cycles min^{-1}, and the amplitude was 3.5 ± 0.2 mV ($n=30$).

Electrical field stimulation (EFS; 0.5 ms duration, 15 V) of the intramural nerves with single pulse evoked a rapidly developing hyperpolarization (fast inhibitory junction potential (fast i.j.p.)) followed by a sustained hyperpolarization (slow i.j.p). Table 1 summarizes the latency and time-to-peak of the fast, the slow and the composite i.j.ps from the stimulus. The fast i.j.p. had a duration of approximately 2 s and the slow i.j.p. lasted for 4.5 s in 75% of all preparations, but 15 s in the remaining preparation. EFS with trains of 2–5 pulses at 20 Hz evoked composite i.j.ps in 77% of all preparations (Figure 1(i)). In 11% of all preparations, EFS with single pulse evoked only a fast i.j.p. When the number of stimuli was increased, the repetitive stimuli evoked composite i.j.ps. In contrast, in 23% of all the preparations, EFS with the single pulse evoked only the slow i.j.p. In these cells, increasing the number of stimuli did not evoke the composite i.j.ps. The averaged amplitude of the fast and slow i.j.p. evoked by trains of stimuli (five pulses) was 4.6 ± 0.5 mV and 15.1 ± 1.1 mV ($n=53$), respectively.

Effects of N^G-nitro-L-arginine methyl ester and N^G-nitro-L-arginine on the composite i.j.ps

The addition of N^G-nitro-L-arginine methyl ester (L-NAME; 200 μM) and N^G-nitro-L-arginine (L-NNA; 200 μM) induced a significant depolarization of the membrane potential from -43.4 ± 1.5 mV and -42.5 ± 1.2 mV to -33.1 ± 2.4 mV and -33.5 ± 2.2 mV, respectively (unpaired *t*-test; $P<0.01$; $n=12$), and reduced the amplitude of slow waves by more than 80% ($n=12$). L-NAME reduced the amplitude of the composite i.j.ps evoked by EFS (five pulses at 20 Hz) in a concentration-dependent manner (10–200 μM; $n=9-13$) (Figure 1b). L-NAME (200 μM) abolished the composite i.j.ps but unmasked excitatory junction potentials (e.j.ps; $n=12$), which had a time course overlapping that of the composite i.j.ps. The fast i.j.ps were less sensitive to L-NAME (100 μM) compared to the slow i.j.ps ($P<0.05$; $n=9$). The abolition by L-NAME of composite i.j.ps, was selectively reversed by the subsequent addition of L-arginine (5 mM) but not by its stereoisomer D-arginine (5 mM) (data not shown) ($n=5$). In these experiments, the amplitude of the fast and slow i.j.p. after the addition of L-arginine was 4.0 ± 0.8 mV and 12.0 ± 1.1 mV ($n=12$), respectively. L-NNA (100–200 μM) also abolished the composite i.j.ps and unmasked e.j.ps. (4.1 ± 1.8 mV; $n=9$).

Table 1 Parameters of fast, slow and composite i.j.ps

i.j.p.	n	*Latency (ms)*	*Time-to-peak (fast i.j.p.) (ms)*	*Time-to-peak (slow i.j.p) (ms)*
Only fast	6	243 ± 17	763 ± 33	
Only slow	12	695 ± 27**††		2120 ± 61
Composite	41	250 ± 24	822 ± 75	1942 ± 138

Values are means$\pm$s.e.means. **$P<0.01$ and ††$P<0.01$ denotes significant difference from values for only fast and composite i.j.ps, respectively evoked by EFS (Tukey-Kramer test).

Effects of oxyhaemoglobin and hydroquinone on i.j.ps and NO-induced hyperpolarizations

The NO scavengers, oxyhaemoglobin (Oxy-Hb; 5–50 μM) and hydroquinone (HQ; 50–500 μM), were used to investigate the nature of the neurotransmitter underlying the i.j.ps. The addition of Oxy-Hb (50 μM) for 30 min induced a depolarization of the membrane potential from -50.4 ± 1.4 mV to -37.1 ± 1.5 mV (unpaired *t*-test; $P<0.01$; $n=7$). Treatment of HQ (500 μM) for 10 min had a non-significant effect on the membrane potential from -47.0 ± 2.9 mV to -43.9 ± 2.3 mV (unpaired *t*-test $P>0.05$; $n=7$). Oxy-Hb reduced the amplitude of the slow i.j.ps evoked by EFS with five pulses at 20 Hz in a

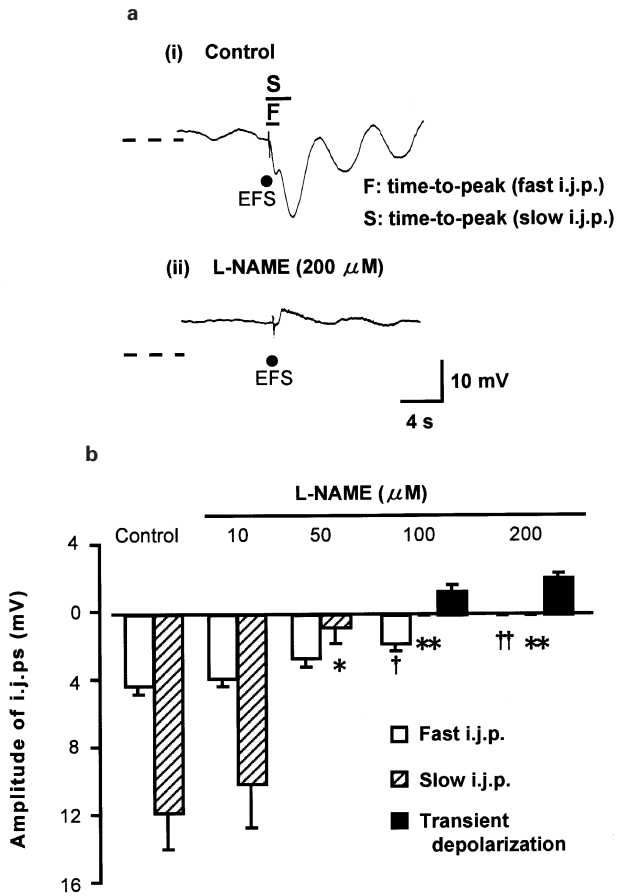

Figure 1 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) and L-arginine on the composite i.j.ps evoked by electrical field stimulation (EFS; five pulses at 20 Hz). (a(i)) Representative composite i.j.p. in the control solution is shown. (a(ii)) Note that treatment with L-NAME (200 μM) for 15 min abolished the composite i.j.p. but unmasked an excitatory junction potential which had a time course overlapping that of the composite i.j.p. Broken lines indicate resting membrane potential. (b) Shows a summary plot of the amplitude of the composite i.j.ps evoked by EFS (five pulses at 20 Hz). Each column and bar represents mean and s.e.mean of 9–13 observations. †$P<0.05$, ††$P<0.01$, *$P<0.05$, **$P<0.01$ compared to the control (Dunnett's test).

concentration-dependent manner (Dunnett's test; $P<0.01$; $n=7$) (Figure 2) but had no effect on the fast component of the i.j.p. HQ had no effect on either the fast or slow i.j.ps; being 3.8 ± 0.5 mV and 15.0 ± 0.8 mV in control and 3.7 ± 0.3 mV and 14.0 ± 1.0 mV, respectively, after 10 min exposure to HQ (both paired t-test; $P>0.05$; $n=7$).

NO (3–10 μM for 10 s) induced a transient hyperpolarization which decayed with a slow time course back to the control membrane potential (Figure 3a(i),b(i)). The fast component had a time-to-peak of about 3 s and the slow component was measured as the amplitude of the response 10 s after the onset of the application of NO. The hyperpolarizing responses to NO (1–100 μM) were concentration dependent. Exposure of the ileum to HQ (100 μM) reduced the fast and slow components of the 3 μM NO-induced hyperpolarizations from 8.2 ± 2.1 mV and 3.0 ± 0.5 mV to 1.1 ± 0.4 mV and 0.8 ± 0.4 mV (unpaired t-test; $P<0.05$; $n=6$), respectively (Figure 3a(ii)). Exposure of the ileum to Oxy-Hb (50 μM) reduced the fast and slow components of the NO (10 μM)-induced hyperpolarizations from 13.0 ± 1.2 mV and 6.3 ± 1.0 mV to 1.5 ± 0.6 mV and 0.4 ± 0.1 mV (unpaired t-test; $P<0.01$; $n=7$), respectively (Figure 3b(ii)).

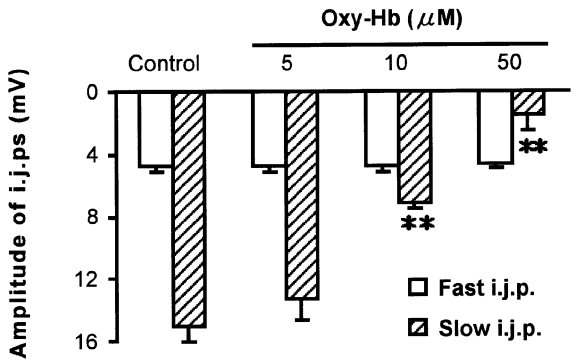

Figure 2 Effects of oxyhaemoglobin (Oxy-Hb) on the composite i.j.ps evoked by EFS (five pulses at 20 Hz). Treatment with Oxy-Hb (5–50 μM) for 30 min reduced the amplitude of the slow i.j.ps, but fast i.j.ps were unaffected. $^{**}P<0.01$ compared to the control (Dunnett's test).

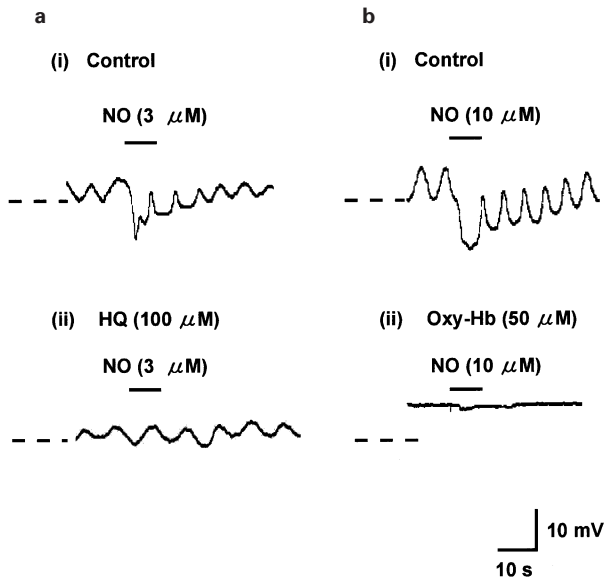

Figure 3 Effects of hydroquinone (HQ) and oxyhaemoglobin (Oxy-Hb) on the hyperpolarizations induced by nitric oxide (NO). (a(i), b(i)) NO-induced hyperpolarization consisted of an initial transient hyperpolarization which subsided to a less hyperpolarized potential. NO was applied at time indicated by bar in trace. (a(ii)) HQ (100 μM) and (b(ii)) Oxy-Hb (50 μM) inhibited the NO-induced hyperpolarizations. Broken lines indicate resting membrane potential.

Effects of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one and cystamine on i.j.ps

We tested the effects of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM) and cystamine (10 mM), specific inhibitors of soluble guanylate cyclase (GC), on the ileal smooth muscle cells to investigate whether the slow i.j.p. was mediated *via* the activation of cellular soluble GC. ODQ and cystamine did not have any significant effect on the membrane potential, changing from -46.3 ± 3.1 mV and -47.5 ± 3.7 mV to 48.8 ± 3.5 mV and -49.0 ± 2.7 mV, respectively before or in the presence of GC inhibitors (unpaired t-test; $P>0.05$; $n=10-12$). On the other hand, ODQ and cystamine reduced the amplitude of the slow i.j.ps (unpaired t-test; $P<0.05$; $n=6-7$) but not the fast i.j.ps (unpaired t-test; $P>0.05$; $n=6-7$) (Figure 4).

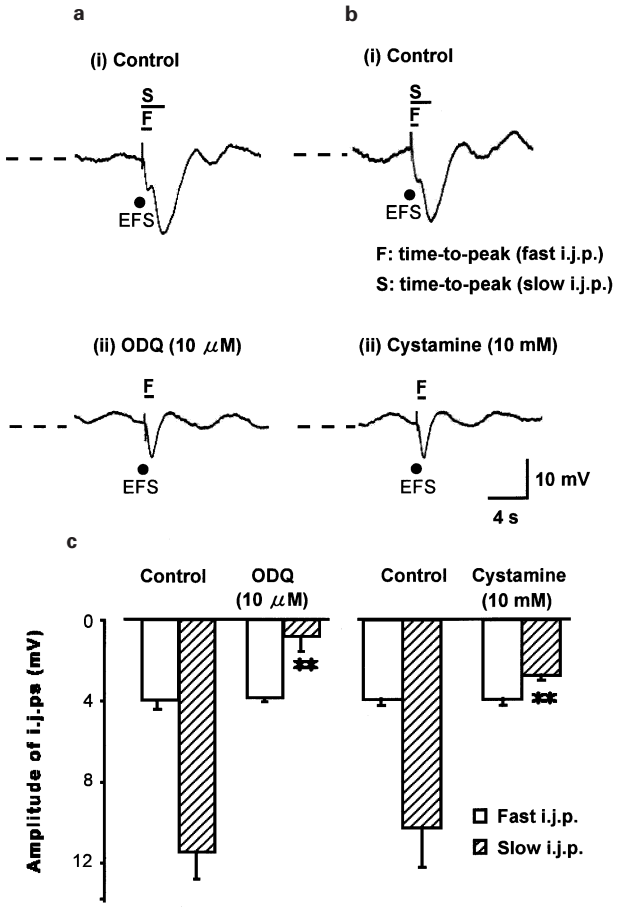

Figure 4 Effects of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. (ODQ) and cystamine on the composite i.j.ps evoked by EFS (five pulses at 20 Hz). (a(i), b(i)) Example of the composite i.j.ps in the control solution. Treatment with (a(ii)) ODQ (10 μM) and (b(ii)) cystamine (10 mM) for 30 and 60 min, respectively, reduced the amplitude of only the slow i.j.ps. Broken lines indicate resting membrane potential. (c) Shows a summary plot of the amplitude of the composite i.j.ps evoked by EFS (five pulses at 20 Hz). Each column and bar represents the mean and s.e.mean of 6–9 observations. $^{**}P<0.01$ compared to the control (unpaired t-test).

Effects of zaprinast on i.j.ps

Zaprinast, a selective phosphodiesterase type V inhibitor, was used to investigate whether cyclic GMP participates in the generation of the slow i.j.p. Zaprinast (100 μM) did not have any significant effect on the membrane potential, being -43.9 ± 2.2 mV and -45.9 ± 2.0 mV, respectively in the absence and presence of the drug (unpaired *t*-test; $P>0.05$; $n=7$). Zaprinast enhanced the amplitude of the slow i.j.ps from 10.8 ± 0.8 mV to 19.0 ± 1.2 mV (unpaired *t*-test; $P<0.01$; $n=7$), but had no appreciable effect on the fast i.j.ps from 3.2 ± 0.8 mV to 2.7 ± 1.0 mV (unpaired *t*-test; $P>0.05$; $n=7$). Zaprinast also enhanced the total duration of the composite i.j.ps from 4.5 ± 0.5 s to 11.1 ± 0.8 s (unpaired *t*-test; $P<0.01$; $n=7$).

Effects of apamin on NO- and 8-bromoguanosine 3′:5′-cyclic monophosphate-induced hyperpolarizations and i.j.ps

Apamin (100 nM), a small conductance Ca^{2+}-activated K^+ channel blocker, was used to clarify the nature of the smooth muscle membrane hyperpolarization evoked upon intramural nerve stimulation. Apamin induced a significant depolarization from -42.1 ± 1.2 mV to -38.6 ± 0.7 mV (unpaired *t*-test; $P<0.05$; $n=9$). Apamin significantly reduced the amplitude of the slow i.j.ps from 16.0 ± 1.5 mV to 1.1 ± 0.5 mV (unpaired *t*-test; $P<0.01$; $n=9$) (Figure 5a), and the fast and slow components of the NO (3 μM)-induced hyperpolarizations from 8.0 ± 1.0 mV and 3.8 ± 1.1 mV to 0 mV, respectively (unpaired *t*-test; $P<0.01$; $n=8$) (Figure 5b), but had no effect on the amplitude of the fast i.j.ps from 3.5 ± 0.5 mV to 3.3 ± 0.5 mV (unpaired *t*-test; $P>0.05$; $n=5$). 8-Bromo-cGMP (1 mM) hyperpolarized the membrane from -42.1 ± 1.0 mV to -49.6 ± 1.5 mV (unpaired *t*-test; $P<0.05$; $n=8$) and simultaneously induced spontaneous electrical oscillations. Exposure of the ileum to apamin significantly reduced the amplitude of the 8-bromo-cGMP-induced hyperpolarizations from 7.5 ± 1.1 mV to 1.1 ± 0.7 mV (unpaired *t*-test; $P<0.01$; $n=8$) (Figure 5c).

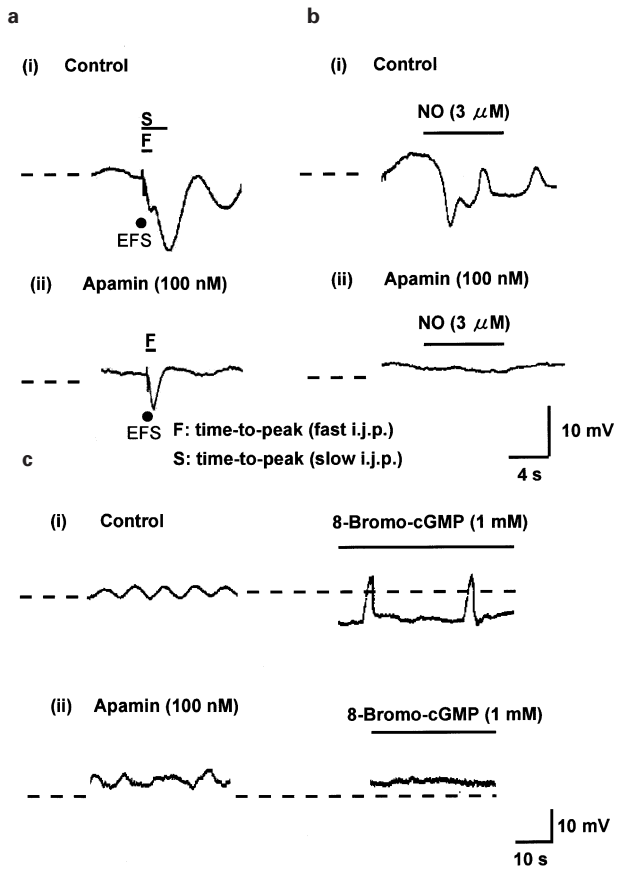

Figure 5 Effects of apamin on the composite i.j.p. evoked by EFS (five pulses at 20 Hz), NO- and 8-bromo-cGMP-induced hyperpolarizations. (b(i)) Representative example of the NO (3 μM)-induced hyperpolarization in the control solution. NO was applied at the time indicated by bars in the traces. (c(i)) 8-bromo-cGMP (1 mM) induced a large hyperpolarization upon which spontaneous oscillations of potential were recorded. Treatment with apamin (100 nM) for 10 min inhibited only the slow i.j.p. (a(ii)) and abolished the hyperpolarization induced by NO (3 μM) (b(ii)) and 8-bromo-cGMP (1 mM) (c(ii)).

Discussion

Our findings indicate that NO, or a related compound, is likely to be the inhibitory transmitter underlying the apamin-sensitive slow i.j.p. The following observations are consistent with this view: (i) the slow i.j.p. was inhibited by L-NAME and L-NNA, both NO synthase (NOS) inhibitors; (ii) the effects of these NOS inhibitors were reversed in the presence of excess L-arginine, but not by D-arginine; (iii) the slow i.j.p. was inhibited by oxyhaemoglobin (Oxy-Hb), a NO scavenger; (iv) exogenously applied NO induced a membrane hyperpolarization; and (v) both the slow i.j.p. and NO-induced hyperpolarization were inhibited by apamin. These findings fulfil some of the criteria necessary for NO, or a related compound, to be considered as a neurotransmitter responsible for the slow i.j.p. In contrast, it has been reported that slow i.j.p. is not inhibited by apamin in the guinea-pig ileum (He & Goyal, 1993), gastric fundus (Ohno *et al*., 1996), colon (Watson *et al*., 1996a), canine ileocolonic sphincter (Ward *et al*., 1992b) and rat caecum (Serio *et al*., 1996), and that the hyperpolarization induced by NO from NO donating compound (NO-donor) is partially inhibited by apamin in the rat gastric fundus (Kitamura *et al*., 1993) and guinea-pig colon (Watson *et al*., 1996a). Therefore, our observations that both the exogenous NO-induced hyperpolarization and the slow i.j.p. are completely inhibited by apamin are unusual.

In the present studies, the NO-induced hyperpolarization was inhibited by Oxy-Hb and hydroquinone (HQ), NO scavengers. Only the slow i.j.p. was inhibited by Oxy-Hb, but unaffected by HQ. There are two possible reasons for the lack of effectiveness of HQ on the slow i.j.p. First, Oxy-Hb can scavenge not only free NO but also NO from a number of NO-donors (Gibson *et al*., 1992). However, HQ is known to inhibit the responses to radical NO and S-nitroso-L-cysteine (CYSNO), one of the NO-donors previously used (Kitamura *et al*., 1993; Thornbury *et al*., 1991; Watson *et al*., 1996b), but does not affect the responses to other NO-donors (Gibson *et al*., 1992). Therefore, NANC nerve may release a NO-donor other than CYSNO in the hamster ileum. Second, the transmitter released from the nerves may indeed be free NO, but it is protected in the tissue by the presence of endogenous Cu/Zn SOD, that interacts with the NO scavengers but not free NO (Paisley & Martin, 1996). In the present study, other than NO, CYSNO induced a membrane hyperpolarization (Matsuyama *et al*. unpublished data) ($n=6$). Therefore, it is logical to suggest that NO, or NO-donors including CYSNO, may be released from nitrergic nerves. Additional studies are required to further investigate whether free NO, or a NO

donating compound, is involved in the nitrergic neurotransmission of the hamster ileum.

So far, no studies have demonstrated that neurally released NO, or a related compound, activates the SK channel *via* the generation of cyclic GMP. For example, in the canine ileocolonic sphincter (Ward *et al*., 1992b), the slow i.j.p. was inhibited by a NOS inhibitor, enhanced by cyclic GMP phosphodiesterase (PDE V) inhibitor, but was unaffected by apamin. NO-induced hyperpolarization was associated with an increase in the internal levels of cyclic GMP in the dog colon (Ward *et al*., 1992a), and was not inhibited by apamin in the human colon (Keef *et al*., 1993). 8-Bromo-cGMP, a membrane permeable cyclic GMP analogue, induced a hyperpolarization which was not inhibited by apamin in the rat gastric fundus (Kitamura *et al*., 1993). NO-donor-induced hyperpolarization was partially inhibited by apamin, but unaffected by a guanylate cyclase inhibitor in the guinea-pig colon (Watson *et al*., 1996a) and rat gastric fundus (Kitamura *et al*., 1993). In the present studies, the apamin-sensitive slow i.j.p. was inhibited by ODQ and cystamine (soluble guanylate cyclase inhibitors), enhanced by zaprinast, a selective PDE V inhibitor, and mimicked by low concentration of 8-bromo-cGMP (Matsuyama *et al*. unpublished data) ($n=6$). Only the slow i.j.p., NO- and 8-bromo-cGMP-induced hyperpolarization were completely inhibited by apamin. For the reason given above, NO, or a related compound, appears to activate an apamin-sensitive K^+ channel *via* the generation of cyclic GMP. Furthermore, it is also possible that hamsters have channels other than normal SK channels that are affected by apamin.

In the present studies, neither Oxy-Hb nor HQ affected the fast i.j.p. It is established that NO synthase converts L-arginine to equimolar amounts of NO and L-citrulline (Palmer & Moncada, 1989). However, L-citrulline had no effect on the membrane potentials of the circular smooth muscle cells of the hamster ileum (Matsuyama *et al*. unpublished data) ($n=5$) and therefore, is unlikely to be the neurotransmitter underlying the fast i.j.p. On the other hand, NOS inhibitors abolished the fast i.j.ps; an effect which was reversed in the presence of excess L-arginine. A possible explanation is that NO may be necessary for the fast i.j.p., but the effects of Oxy-Hb are not consistent with this. It appears that either NO is released in two forms with differential sensitivity to Oxy-Hb or that the burst of NO momentarily overwhelms the ability of Oxy-Hb to scavenge it. This hypothesis may be supported by the observations that the fast i.j.p. was less sensitive to L-NAME compared to the slow i.j.p. More sustained levels of NO may be needed for the slower responses which are thus more easily reduced by scavengers. The lack of effect of apamin on the fast i.j.p. is compatible with this involving a channel other than SK, and not involving cyclic GMP pathway. Another possible explanation is that the fast i.j.p. is mediated by metabolites of NO such as peroxynitrite, nitrogen dioxide and hydroxyl radical. It has been reported that NO and superoxide are metabolized to peroxynitrite. Peroxynitrite and protonation are metabolized to nitrogen dioxide and hydroxyl radical (Pryor & Squadrito, 1995). In addition, peroxynitrite and nitrogen dioxide induced a relaxation (Wu *et al*., 1994; Davidson *et al*., 1996). Therefore, it seems reasonable to suppose that these metabolites could be involved in the fast i.j.p. Thus, we speculate that NO, or a related compound including metabolites of NO, is likely to be associated with the generation of the fast i.j.p. Further studies on the fast i.j.p. are needed to determine the neurotransmitters in the hamster ileum.

Blockade of NO synthesis or scavenging of NO evoked membrane depolarization in circular smooth muscle cells in the absence of electrical stimulation. Other preparations, such as the canine colon (Ward *et al*., 1992a,b), mouse colon (Lyster *et al*., 1995) and guinea-pig colon (Watson *et al*., 1996a), also appear to be influenced by the tonic release of NO. Furthermore, previous studies reported the existence of bursting neurones in the enteric nervous system (Wood, 1994). These results suggest that the constant release of NO maintains the membrane potential at a more negative level than would occur in the absence of NO. If a constant release of NO stimulates production of cyclic GMP, then inhibiting generation of or metabolism of cyclic GMP should induce a similar membrane depolarization or hyperpolarization, respectively. However, ODQ, cystamine and zaprinast did not change the membrane potential. Therefore, we conclude that the constant release of NO does not stimulate production of cyclic GMP. We also speculate that a constant release of NO inhibits excitatory neurones. The following observations are consistent with this possibility: (i) NOS inhibitors enhanced contractile responses to nerve stimulation, but not modify the responses to application of acetylcholine or substance P in the guinea-pig ileum (Gustafsson *et al*., 1990; Wiklund *et al*., 1993); (ii) NOS inhibitors unmasked the e.j.p. in the circular smooth muscle cells of the hamster ileum.

In conclusion, our results demonstrated that NO or a related compound acts as an NANC inhibitory neurotransmitter underlying the apamin-sensitive slow i.j.p., which depends on the generation of cyclic GMP, in the circular smooth muscle cells of the hamster ileum. In addition, we also found that a NO-related compound is associated with the generation of the fast i.j.p.

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