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PEPTIDERGIC AND NITRERGIC INHIBITORY NEUROTRANSMISSIONS IN THE HAMSTER JEJUNUM: REGULATION OF VASOACTIVE INTESTINAL PEPTIDE RELEASE BY NITRIC OXIDE

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Abstract—Regulation of vasoactive intestinal peptide (VIP) release by nitric oxide (NO) was investigated in the hamster jejunum. Electrical field stimulation and applied NO (3–100 μM) evoked biphasic hyperpolarizations consisting of an initial transient hyperpolarizing component followed by a second more slowly developing component (late component). The NO synthase inhibitor N^G-nitro-L-arginine methyl ester (200 μM) abolished the biphasic inhibitory junction potential evoked by electrical field stimulation. The NO scavenger oxyhemoglobin (50 μM) and the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ; 10 μM) abolished both components of the inhibitory junction potentials and the NO-induced hyperpolarizations. VIP(6–28) (1 μM), which abolished VIP (3 μM)-induced hyperpolarizations, also inhibited the late components of the inhibitory junction potentials and the NO-induced hyperpolarizations. ODQ inhibited VIP release and cAMP production by electrical field stimulation and NO application. N^6-2,0-Dibutyryladenosine 3′,5′-cyclic monophosphate (0.1–3 mM) caused a membrane hyperpolarization.

These results suggest that NO may stimulate VIP release from enteric nerves in the hamster jejunum. In addition, we propose that NO and NO-stimulated VIP contribute to the early and late components of the inhibitory junction potentials, respectively, in the circular smooth muscle cells of the hamster jejunum.

Key words: cyclic nucleotide, enteric nerve, guanylate cyclase, inhibitory junction potential, non-adrenergic non-cholinergic, relaxation.

Nitric oxide synthase (NOS) and vasoactive intestinal peptide (VIP) are present in enteric nerves (Berezin et al., 1994; Costa et al., 1992; Grider and Jin, 1993; Lefebvre et al., 1995; Toole et al., 1998). With the use of a NOS inhibitor and a specific VIP radioimmunoassay, it has been demonstrated that nitric oxide (NO), which is derived from L-arginine by the catalytic activity of NOS, and VIP can be released from stomach, small intestine and colon by electrical or mechanical stimulation (Allescher et al., 1996; Grider, 1993; Jin et al., 1996). However, the relationships between NO and VIP in the inhibitory neurotransmission underlying inhibitory junction potentials (IJPs) and relaxations of the gastrointestinal tract are controversial.

In the pig gastric fundus (Lefebvre et al., 1995) and the hamster jejunum and ileum (Toole et al., 1998), NOS is colocalized with VIP in a portion of neurons in the myenteric plexus, favoring the idea that NO and VIP might be released from these nerves. Functional evidence in the canine gastric fundus suggests that both VIP and NO are released from enteric nerves and may serve as independent neurotransmitters to induce relaxation (Bayguinov et al., 1999). In the guinea-pig gastric fundus, at least a part of the relaxation by neuronally released VIP is dependent on NO release in the smooth muscle cells (Grider, 1993), suggesting that there is an interplay between VIP and NO. However, the interaction between VIP and NO in the inhibitory neurotransmission underlying the IJPs has not been fully understood. Furthermore, none of these studies investigated whether at least a part of the hyperpolarization and/or relaxation in the smooth muscle cells induced by neurally released NO depends on VIP release from the enteric nerves.

Some neurons in the myenteric plexus of the hamster small intestine show colocalization of NOS and VIP (Toole et al., 1998). Our previous study in the hamster small intestine has shown that the early components of the IJPs are evoked by the release of NO from myenteric plexus while the mediators of the late components are not known. Nevertheless, we have suspected that a neuronally released VIP might be involved in the generation of the late component of the IJPs (Matsuyama et al., 1999a, 2001). Therefore, the aim of the present study

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Abbreviations: DMSO, dimethyl sulfoxide; EC, early component; EDTA, ethylenediaminetetra-acetate; EFS, electrical field stimulation; HRP, horseradish peroxidase; IJP, inhibitory junction potential; LC, late component; L-NAME, N^G-nitro-L-arginine methyl ester; NOS, nitric oxide synthase; ODQ, 1H-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one; Oxy-Hb, oxyhemoglobin; PB, phosphate buffer; PDE, phosphodiesterase; PSS, physiological salt solution; TCA, trichloroacetic acid; VIP, vasoactive intestinal peptide.

was to investigate the hypothesis that the induction of VIP release by neuronally released and exogenously applied NO contributes to the late components of the IJPs and hyperpolarizations, respectively, in the hamster jejunum using intracellular microelectrode recording techniques and enzyme immunoassay method. We completed this study by measuring the levels of VIP, cAMP and cGMP in the hamster jejunum.

EXPERIMENTAL PROCEDURES

Tissue preparation

Male Syrian hamsters (80–120 g) were obtained from SLC (Japan) and were anesthetized with diethyl ether and exsanguinated via the carotid arteries. Tissue preparations and electrophysiological techniques were similar to those previously described (Matsuyama et al., 1999b). After the abdominal cavity was opened, a length of about 3–4 cm of jejunum 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 surgical procedures of the animals conformed to the guidelines of the Gifu University Animal Care and Use Committee in accordance with Japanese Department of Agriculture guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Electrophysiological recordings

Whole segments, 1 cm in length, were gently stretched and fixed to a rubber block in an experimental chamber with pins (diameter: 100 μm). The bath had a volume of 4 ml and was continuously perfused with PSS containing 0.5 μM atropine, 1 μM guanethidine and 0.2 μM nifedipine at a constant flow rate of about 3 ml/min. The PSS was previously oxygenated by bubbling with a 95% O_2:5% CO_2 gas mixture and maintained at 32 ± 0.5°C to decrease muscle contractions and displacement of the recording microelectrode. Tissues were allowed to equilibrate for approximately 45–60 min before experiments were undertaken.

Membrane potentials were recorded using conventional glass microelectrodes filled with 3 M KCl with resistances of 50–80 MΩ. The electrode insertions were made into the circular muscle cells of the deep layer from the serosal side (Takewaki and Ohashi, 1977). A successful insertion was confirmed when a sharp change in the voltage to a membrane potential negative to about −45 mV remained stable for at least 5 min. A pair of silver wire electrodes, one placed in the intestinal lumen, 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. IJPs were evoked by EFS of intramural nerves of the tissue with square-wave pulses (one to five 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-1100M, Nihon Kohden, Tokyo, Japan) for illustration and analysis.

Isometric tension recordings

The tissues were transferred to a dissecting dish containing oxygenated PSS at room temperature (20°C). One end of the tissue was anchored to a glass rod. The other end was connected to an isometric force transducer for continuous recording of the changes in isometric tension in the direction of the circular muscle layer. The preparations were immersed in organ bath containing PSS and bubbled with a mixture of 95% O_2 and 5% CO_2 at 35°C. Changes in isometric tension were monitored via the isometric force transducer and displayed on a thermal array recorder (RTA-1100M, Nihon Kohden) for illustration and analysis. The tissues were equilibrated at a resting tension of 1 g for 90–120 min in the continuous presence of atropine (0.5 μM) and guanethidine (1 μM), and gained tone without addition of exogenous agonists. Relaxation was induced by EFS, using a pair of silver wire electrodes connected to a stimulator (SEN-3301, Nihon Kohden). Pulses were delivered in 5-s trains at 10-min intervals. Stimulus strength was 15 V, pulse duration was 0.5 ms, and pulse frequency was 20 Hz. In some experiments, the tissues were first treated for 20 min with one of the following: N^G-nitro-L-arginine methyl ester (L-NAME), VIP(6–28), zaprinast or rolipram. In the parallel control strips, only the solvents of the tested drugs were added. The responses to EFS were reproducible in the control strips. At the end of each experiment a maximal relaxation was induced by addition of 10 μM sodium nitroprusside.

Measurement of VIP release

For measuring VIP from the jejunal smooth muscle strips, they were incubated in a 2-ml bath filled with oxygenated PSS solution containing 0.1% bovine serum albumin, 1000 U/ml aprotinin and 20 mM bacitracin. After a 1-h equilibration period, the muscle strips were incubated for 10 min in PSS, and the perfusate was collected for measuring the basal release of VIP. To examine the release of the VIP in response to EFS and applications of NO and 8-bromo-cGMP, the bath perfusates, were collected immediately after the termination of EFS (20 Hz for 30 s), (3 and 10 μM for 30 s) and 8-bromo-cGMP (3 mM for 30 s). The strips were allowed to stand for 45 min with intermittent washing before another 10-min sample was collected for the measurement of basal VIP release, which was followed by EFS, NO and 8-bromo-cGMP. Samples were also collected in the presence of L-NAME (200 μM) and ODQ (10 μM) for 30-min periods before the applications of EFS or NO, respectively. All the samples were collected in siliconized tubes and immediately stored at −80°C until used for the assay of VIP.

VIP was measured by a modified radioimmunoassay (Allescher et al., 1996; Pandian et al., 1982) with a porcine VIP antibody (Peninsula Laboratories). In brief, VIP antibody (100 μl) and standard (100 μl, porcine VIP, Sigma, St. Louis, MO, USA) or unknown samples (100 μl) and 100 μl of [^{125}I]VIP (1 fmol, Amersham) were incubated at 4°C for 96 h in 500 μl of 0.06 M phosphate–EDTA buffer (pH 7.2) containing 0.6% bovine serum albumin. After the incubation, bound and unbound tracers were separated by the addition of 500 μl dextran-coated charcoal (12 mg/ml, Sigma) suspended in assay buffer containing 0.25% gelatin.

Radioiodination of VIP was achieved at room temperature by using a modified chloramine-T method (Hunter and Greenwood, 1962). Ten microliters of VIP solutions, 10 μl of sodium phosphate buffer (PB) and 10 μl of chloramine-T were added to the glass ampoule containing 10 μl of 1 mCi Na^{125}I (Amersham). The solution was continuously stirred, and then the reaction was terminated by addition of 100 μl of sodium metabisulfite. The reaction medium composition were 0.25 M PB, pH 7.5, 0.5 mg/ml chloramine-T in 0.05 M PB, pH 7.5 and 0.17 mg/ml cysteine in 0.05 M PB, pH 7.5. The specific activity of the label was 2.26 Ci/μM. In a cold room at 4°C the gel filtration medium was equilibrated in 0.5 ml of phosphate-carrier protein buffer and packed into a Sephadex G-15 column, which was saturated with 100 mg/ml solution of bovine serum albumin. The iodination reaction mixture was quantitatively transferred to the prepared column and eluted with phosphate-carrier protein buffer. Fractions of 1 ml were collected into polystyrene tubes of the same dimensions as the iodination reaction tube. The column was run until both the protein and the [^{125}I]iodine peaks had been eluted, and the radioactivity in all the fractions was measured with an automatic gamma counter. Intra-assay and interassay variabilities were 5 and 10%, respectively.

Measurement of cyclic nucleotides

cGMP and cAMP levels were measured by a modified enzyme immunoassay (Mayer et al., 1974). Tissues were incubated for 30 min with zaprinast (100 μM), rolipram (40 μM), L-NAME (200 μM), ODQ (10 μM) or VIP(6–28) (1 μM). At the end of incubation, either EFS (20 Hz for 30 s) or NO (3, 10 and 30 μM for 30 s) was applied. Immediately after exposure of tissues to EFS or NO, the tissues were snap-frozen in liquid N_2. Each frozen tissue sample was homogenized in ice-cold 6% (w/v) trichloroacetic acid (TCA). Then the sample was centrifuged ($200\times g$ for 15 min) at 4°C. Protein level of the pellets was measured according to the method described by Lowry et al. (1951). The supernatants were separated from the precipitates, and TCA was extracted by washing the supernatant four times with diethyl ether saturated with H_2O. The remaining ether was removed from the extracts by heating in a water bath at 45°C for 15 min. These nucleotide levels were assayed by an enzyme immunoassay, in duplicate, using the cyclic nucleotide enzyme immunoassay kit from Amersham Pharmacia Biotech (Tokyo, Japan). Cyclic nucleotide levels in samples and standards were detected following competition between cyclic nucleotides and horseradish peroxidase (HRP)-linked cyclic nucleotides for specific antiserum binding sites. The antiserum complex, linked to HRP, was used to cleave tetramethylbenzidine, and absorbance was measured at 630 nm. Cyclic nucleotide levels of samples were determined from a standard curve constructed from determination of known amounts of cyclic nucleotide added to the plate. Levels of cyclic nucleotides are expressed as pmol nucleotides/mg protein. Duplicate and interassay variations in the cyclic nucleotide assay were less than 5%.

Physiological solutions and drugs

The PSS used in this study composed of the following (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.

L-NAME, rolipram, forskolin, guanethidine monosulfate, dibutyryl cAMP, 8-bromo-cGMP, sodium nitroprusside, ODQ, L-arginine, D-arginine, hemoglobin, zaprinast, VIP(6–28) and nifedipine were obtained from Sigma. VIP was purchased from Peptide Institute (Osaka, Japan).

Zaprinast, rolipram and ODQ were dissolved in dimethyl sulfoxide (DMSO), nifedipine in ethyl acetate, and all other drugs were dissolved in distilled water. Stock solutions were prepared at more than 100 times higher concentrations than those used for experiments. Final concentrations of DMSO, ethanol or distilled water in the bathing solution were less than 0.01% and had no effect on the membrane potential and tone of the tissues. The drug concentrations given in the text are final concentrations in the bathing solution. NO solutions were kept at 4°C and diluted to their final concentration in PSS containing atropine, guanethidine and nifedipine as indicated.

Preparation of NO solution

A stock solution of NO was prepared as described by Stark et al. (1991). NO gas was injected into PSS which was previously deoxygenated by gassing with helium for 2 h, to give stock solutions of NO ranging from 0.01 to 1.0% (v/v). The deoxygenated solution had no effect on membrane potential.

Preparation of oxyhemoglobin

Oxyhemoglobin (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 'hemoglobin' in distilled water. Excess $Na_2S_2O_4$ was then removed by dialyzing 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.

Data presentation and statistical analysis

The amplitudes of the early (EC) and late component (LC) of the IJPs and NO-induced hyperpolarizations were recorded at their maximal values of the EFS- and NO-induced hyperpolarization. Relaxations are expressed as a percentage of the sodium nitroprusside (10 μM)-induced relaxation. The cyclic nucleotide contents are expressed as pmol cyclic nucleotide/mg protein.

Data are expressed as mean ± S.E.M., and n represents the number of experiments performed using different tissue preparations from different hamsters. When recordings were carried out from more than one cell in an individual preparation, a mean value was calculated and used. Differences between the means were analyzed by either one-way analysis of variance, followed by Dunnett's test for multiple group comparisons, or Student's t-test (paired or unpaired) for comparison of two groups. A P value of less than 0.05 was considered significant.

RESULTS

Membrane potential responses to electrical field stimulation

The circular smooth muscle cells of the hamster jejunum displayed either electrical quiescence (in 74/248 cells) or spontaneous rhythmic potentials (in 174/248 cells), which represented slow wave activity. Quiescent cells had an average resting membrane potential of -44.2 ± 0.3 mV, while the most negative potential between the slow waves in unquiescent cells was -47.5 ± 0.2 mV. There was no significant difference between the average resting membrane potentials of the quiescent cells and most negative potentials (unpaired t-test; $P>0.05$; $n=60$). Slow waves when present occurred at 8.9 ± 0.2 cycles/min, with an amplitude of 2.5 ± 0.2 mV.

EFS (0.5 ms duration, 15 V) with five pulses at 20 Hz evoked IJPs. These IJPs comprised two components; the first component (EC) of the IJPs peaked about 2 s after the stimulus. The second more slowly developing hyperpolarizing component (LC) peaked approximately 10 s after and then slowly returned to the control membrane potential (Fig. 1). The duration of the IJPs was approximately 30 s.

Effects of L-NAME and oxyhemoglobin on inhibitory junction potential

Both the EC and LC of the IJPs were inhibited by L-NAME (200 μM for 30 min). The effects of L-NAME were reversed by the subsequent addition of L-arginine (5 mM), but not by its stereoisomer, D-arginine (5 mM) (Fig. 1A, C). Oxy-Hb (50 μM for 30 min) significantly inhibited the EC and LC of the nitrergic IJPs (Fig. 1B, C).

Application of NO (3–100 μM for 10 s) induced a transient hyperpolarization (EC), which peaked after approximately 3.3 ± 0.4 s, followed by sustained hyperpolarization (LC) that returned to the control membrane potential after about 50 s (Fig. 2). The addition of Oxy-Hb (50 μM) markedly inhibited both EC and LC of the NO (10 μM)-induced hyperpolarizations. In these experi-

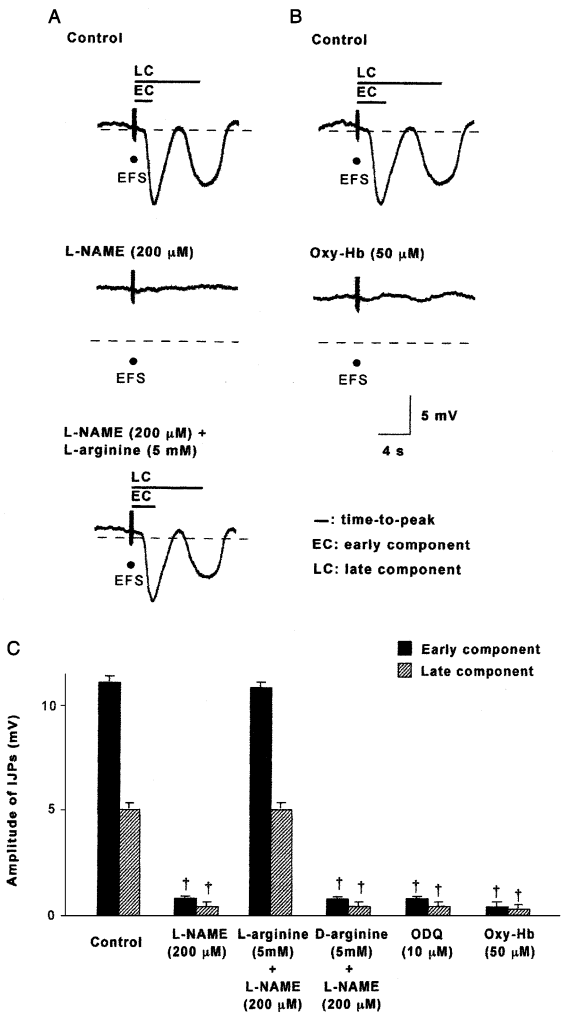

Fig. 1. Effects of L-NAME, L-arginine, D-arginine, Oxy-Hb and ODQ on inhibitory junction potentials. (A) Response to EFS (delivered at dots) was characterized by EC followed by LC of IJPs. (B) Responses in the same cell to EFS after exposure of muscle strips to L-NAME. The times to peak of EC and LC of the IJPs from the stimulus are shown at the time indicated by the bar under EC and LC, respectively. Horizontal dotted line indicates the most negative membrane potential before application of L-NAME. (C) Summary plot of the amplitude of the IJP. Each column and bar represents the mean and S.E.M. of eight observations. $^{\dagger}P<0.01$; significantly different from control (Dunnett's test).

ments, the amplitudes of the EC and LC were 0.4 ± 0.2 mV and 0.2 ± 0.1 mV after the addition of Oxy-Hb (10.2 ± 1.7 mV and 3.5 ± 1.2 mV, respectively, in control; unpaired t-test; $P<0.01$; $n=8$). L-NAME (200 μM) failed to affect either component of the NO-induced hyperpolarization.

Effect of ODQ on inhibitory junction potential and no-induced hyperpolarization

A specific soluble guanylate cyclase inhibitor, ODQ (10 μM for 30 min), did not have a significant effect on the membrane potential (Table 1). ODQ (10 μM) strongly reduced the amplitude of both components of

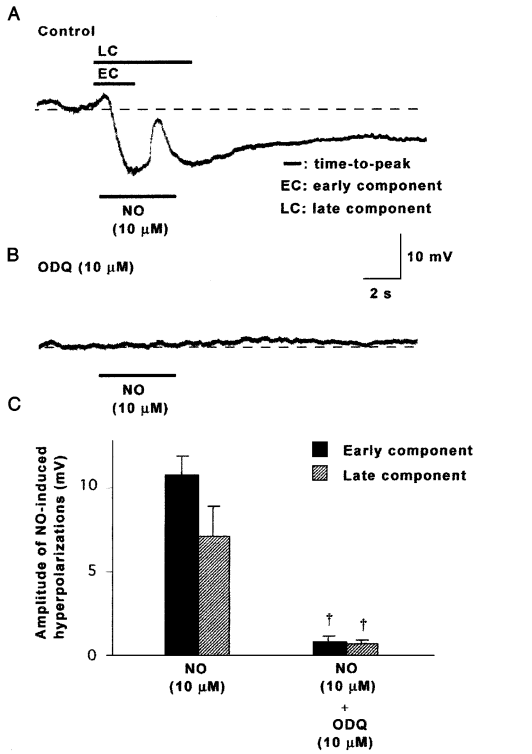

Fig. 2. Effects of ODQ on NO-induced hyperpolarization. (A, B) NO-induced hyperpolarizations recorded before and after the application of ODQ, respectively. The time-to-peak of EC and LC of IJPs and NO-induced hyperpolarizations from the stimulus are shown at the time indicated by the bar under EC and LC, respectively. NO was applied at the time indicated by the bar in trace. Horizontal dotted line indicates the resting membrane potential before applications of EFS and NO. (C) Summary plot of the amplitude of the NO-induced hyperpolarizations. Each column and bar represents the mean and S.E.M. of eight observations. $^{\dagger}P<0.01$; significantly different from control (unpaired t-test).

the nitrergic IJPs and NO-induced hyperpolarizations (Figs. 1C and 2).

Effect of VIP(6–28) on inhibitory junction potential, NO- and VIP-induced hyperpolarization

To further explore whether the nitrergic IJPs and NO-induced hyperpolarization occur via VIP release, the effect of the potent VIP receptor antagonist VIP(6–28) was examined on the nitrergic IJPs and NO-induced hyperpolarizations. When applied for 20 min, VIP(6–

Table 1. Effects of drugs on the most negative membrane potential

	n	Most negative potential (mV)
Control	21	-48.5 ± 1.6
L-NAME (200 μM)	8	-40.1 ± 0.8*
Oxy-Hb (50 μM)	8	-39.1 ± 0.7*
ODQ (10 μM)	8	-47.0 ± 0.8
VIP(6–28) (1 μM)	8	-47.7 ± 0.7
Rolipram (40 μM)	7	-51.0 ± 2.7
Zaprinast (100 μM)	7	-51.2 ± 2.4

Values are means ± S.E.M.; n, number of preparations. *$P<0.05$, compared with control.

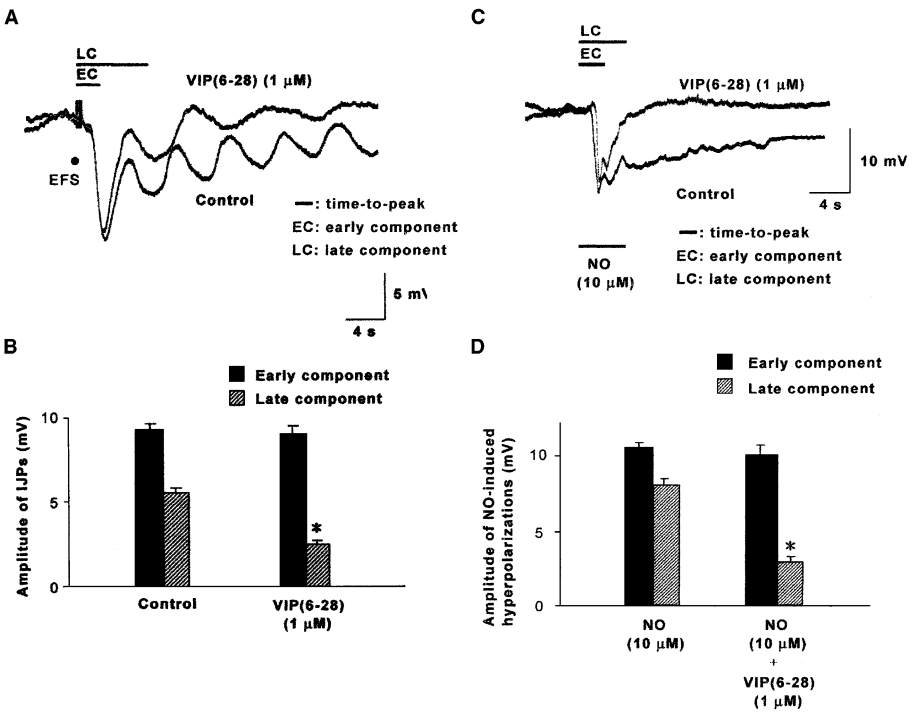

Fig. 3. Effects of VIP(6–28) on IJP and NO-induced hyperpolarization. (A, C) Overlays of pairs of IJPs and NO-induced hyperpolarizations recorded before and after the application of VIP(6–28), respectively. (B, D) Summary plots of the amplitudes of the IJPs and NO-induced hyperpolarizations, respectively. Each column and bar represents the mean and S.E.M. of nine observations. $*P < 0.05$; significantly different from control (unpaired t-test).

28) (1 μM) significantly inhibited the VIP (3 μM)-induced hyperpolarization, but it did not alter the membrane potential. The VIP receptor antagonist (1 μM) inhibited the late component of the nitrergic IJPs and the NO-induced hyperpolarizations, without significantly affecting the early component of the nitrergic IJPs or the NO-induced hyperpolarizations (Fig. 3).

Effects of L-NAME and ODQ on VIP-induced hyperpolarization

To investigate whether VIP stimulates NO production, we examined the effect of L-NAME on the VIP-induced hyperpolarization. VIP (1 μM)-induced hyperpolarization was not affected by L-NAME (up to 200 μM) and ODQ (10 μM) (Fig. 4). These results suggest that the effect of VIP may be independent of NO release.

Effects of electrical field stimulation, NO and 8-bromo-cGMP on VIP release

Table 2 shows the levels of VIP measured in hamster jejunal preparations exposed to either EFS (20 Hz for 30 s) or NO (3 and 10 μM for 30 s). Application of EFS resulted in a significant increase in VIP release. Preincubation of muscle strips with L-NAME (200 mM) inhibited VIP release stimulated by EFS. The inhibitory effect of L-NAME implied that VIP release was dependent on NO release. Consistent with this notion, the addition of NO (10 μM) or 8-bromo-cGMP (3 mM) to the medium also significantly increased VIP release. The increases in VIP release induced by EFS and NO were suppressed by ODQ (10 μM).

Effects of VIP(6–28), ODQ and rolipram on cAMP

Figure 5 shows the levels of cAMP measured in hamster jejunal preparations applied to either EFS or NO. VIP(6–28) (1 μM), ODQ (10 μM) and the specific cAMP phosphodiesterase (PDE) type IV inhibitor rolipram (40 μM) failed to alter basal levels of cAMP. In contrast, EFS, NO (10 μM), VIP (1 μM) or forskolin (3 μM) induced 2.0–2.5-fold increases in cAMP levels. These increases in cAMP produced by EFS or NO (10 μM) were prevented by VIP(6–28) (1 μM) and ODQ (10 μM). Addition of rolipram significantly increased cAMP levels triggered by EFS and NO, while the increases in cAMP produced by VIP (1 μM) were not altered by ODQ.

Effect of cAMP on membrane potential

Application of the membrane-permeable cAMP analogue dibutyryl cAMP (0.1–1 mM) caused membrane hyperpolarizations in a concentration-dependent manner (from 1.5 ± 0.8 mV to 12.3 ± 1.5 mV; $n = 10$).

Effects of zaprinast, ODQ and L-NAME on cGMP levels

EFS and NO induced a 1.5–4.0-fold increase in cGMP level. Neither the cGMP PDE type V inhibitor zaprinast (100 μM) nor ODQ (10 μM) influenced the basal levels

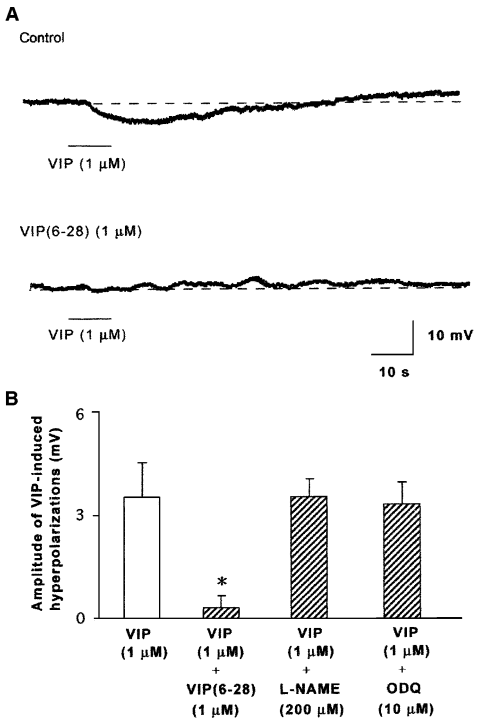

Fig. 4. Effects of VIP(6–28) and L-NAME on VIP-induced hyperpolarization. (A) VIP (1 μM)-induced hyperpolarizations in the absence and presence of L-NAME. (B) Summary plots of the amplitude of VIP-induced hyperpolarizations. Each column and bar represents the mean and S.E.M. of four to eight observations. $*P < 0.05$; significantly different from respective controls (Dunnett's test).

of cGMP. In the presence of ODQ (10 μM), EFS and NO did not change the cGMP level, while cGMP accumulation evoked by EFS and NO (3 μM) was enhanced by zaprinast (Fig. 6).

In a separate series of experiments, the effect of applied VIP on cGMP level was investigated. Although the application of VIP at a concentration of 1 μM did not influence the cGMP level of the tissue, the exposure to VIP (10 μM) caused a significant accumulation of cGMP. Pretreatment with L-NAME (200 μM) significantly reduced the accumulation of cGMP evoked by VIP (10 μM) without affecting the basal cGMP level.

Effects of rolipram and zaprinast on inhibitory junction potential and NO-induced hyperpolarization

In order to ascertain any contribution of cAMP and cGMP in the generation of the IJP, the effects of rolipram and zaprinast were tested. Rolipram (40 μM for 20 min) did not significantly alter the membrane potential (Table 1). Rolipram enhanced the amplitude of the LC, but did not affect the EC of the nitrergic IJPs and NO-induced hyperpolarizations (Fig. 7). Rolipram also prolonged the durations of the IJPs and the NO-induced hyperpolarizations from 20.6 ± 1.0 s and 50.0 ± 1.5 s to 47.1 ± 0.8 s and 70.0 ± 0.9 s, respectively ($n = 8$).

The membrane potential after exposure to zaprinast (100 μM for 20 min) was not significantly different from control. Zaprinast enhanced the amplitudes of the EC and LC of the IJPs and NO-induced hyperpolarizations. The durations of the IJPs and NO-induced hyperpolarizations were made longer by addition of the drug. In these experiments, zaprinast (100 μM) enhanced the duration of the nitrergic IJPs and NO-induced hyperpolarizations from 20.0 ± 0.8 s and 48.7 ± 1.7 s to 43.7 ± 1.5 s and 65.8 ± 1.8 s, respectively ($n = 7$).

Effects of L-NAME, VIP(6–28), zaprinast and rolipram on electrical field stimulation-evoked relaxations

EFS at 20 Hz evoked contraction followed by relaxation. Preincubation of hamster jejunal preparations with L-NAME (200 μM) caused an increase in tone ($55 \pm 5\%$ increase; $P < 0.01$), whereas VIP(6–28) (1 μM), rolipram (40 μM) and zaprinast (100 μM) had no effect on resting tension ($n = 10$) ($P > 0.05$). EFS-induced relaxation was strongly inhibited by L-NAME. However, VIP(6–28) significantly decreased the amplitude of relaxation. On the other hand, rolipram and zaprinast enhanced the amplitude of the EFS-induced relaxation (Fig. 8).

DISCUSSION

The results of the present study suggest that the induction of VIP release by NO contributes to the late components of the IJPs and hyperpolarizations in the hamster jejunum. These conclusions are supported by the following observations: (i) the late components of the nitrergic IJPs and the NO-induced hyperpolarizations were inhibited by VIP(6–28) and ODQ; (ii) EFS-stimulated VIP release was inhibited by L-NAME; (iii) VIP release produced by EFS and NO application was abolished by ODQ; and (iv) 8-bromo-cGMP increased VIP release.

Interaction between VIP and NO in inhibitory neurotransmission

In the present experiments, we have demonstrated that there is an interaction between VIP and NO in inhibitory neurotransmission in producing the late component of the IJPs in the hamster jejunum. We have also found that the EFS-stimulated VIP release and the VIP-medi-

Table 2. Effects of L-NAME, ODQ and cGMP on VIP release

	n	VIP release (fmol/mg wet wt)
Basal	15	3.1 ± 0.3
L-NAME (200 μM)	7	3.5 ± 0.2
EFS	7	10.2 ± 0.3*
EFS+L-NAME (200 μM)	7	3.2 ± 0.2
ODQ (10 μM)	7	2.7 ± 0.4
EFS+ODQ (10 μM)	7	3.7 ± 0.2
NO (3 μM)	6	4.3 ± 0.3*
NO (10 μM)	7	10.5 ± 0.2*
NO (10 μM)+ODQ (10 μM)	7	3.7 ± 0.4
8-bromo-cGMP (3 mM)	7	5.3 ± 0.2*

Values are means ± S.E.M.; *n*, number of preparations. $*P < 0.05$, compared with control.

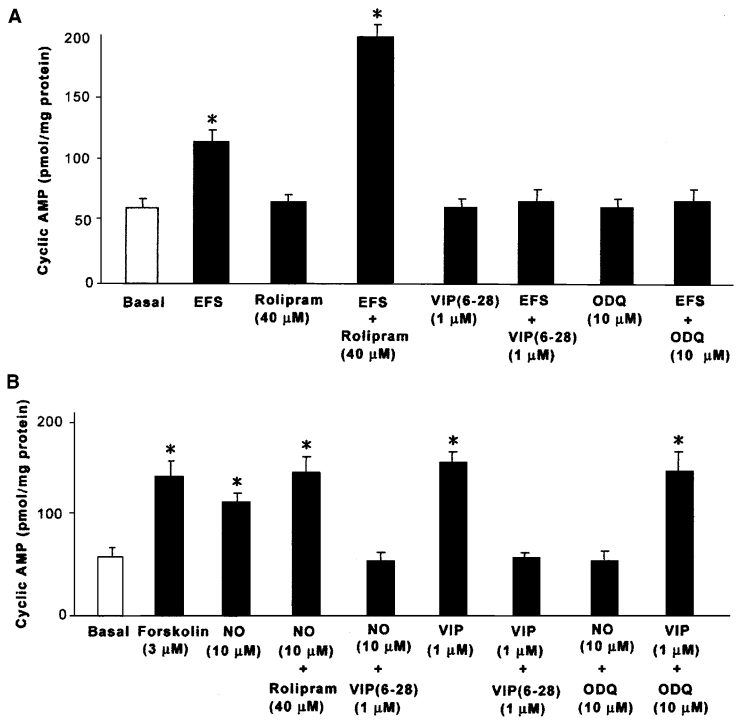

Fig. 5. Effects of rolipram, ODQ and VIP(6–28) on cAMP levels. (A, B) The increases in cAMP levels produced by EFS, NO and VIP. Each column and bar represents the mean and S.E.M. of six or seven observations. $*P < 0.05$; significantly different from respective controls (Dunnett's test).

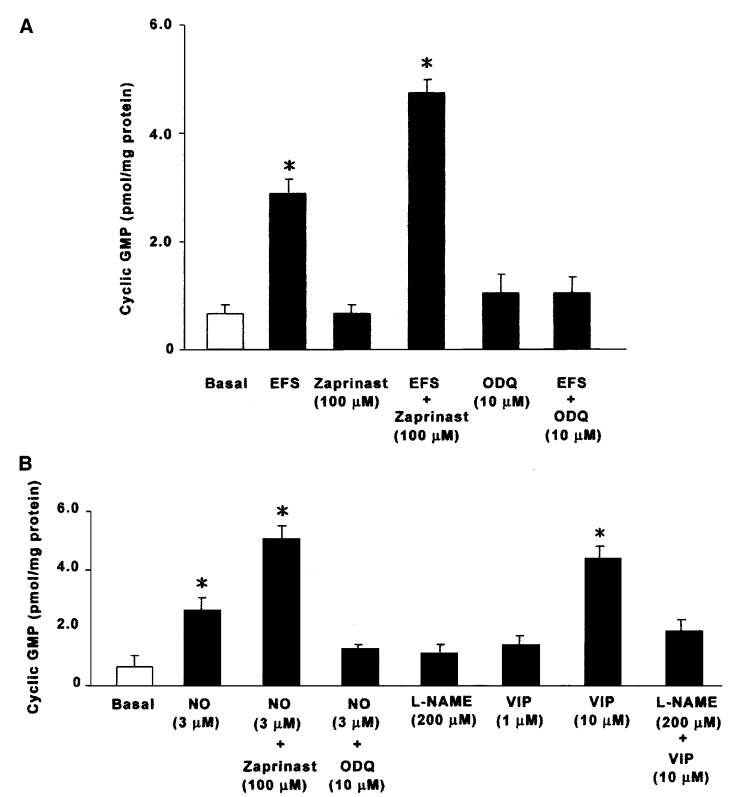

Fig. 6. Effects of zaprinast, ODQ and L-NAME on cGMP levels. (A, B) The increases in cGMP levels produced by EFS, NO and VIP. Each column and bar represents the mean and S.E.M. of seven to 10 observations. $*P < 0.05$; significantly different from respective controls (Dunnett's test).

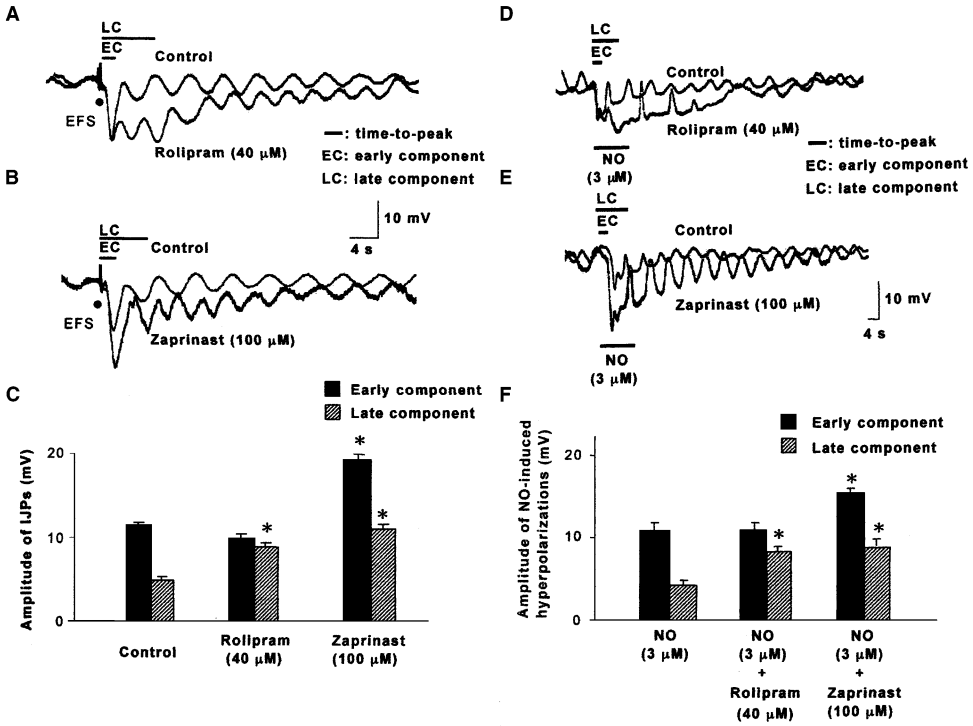

Fig. 7. Effects of rolipram and zaprinast on IJP and NO-induced hyperpolarization. (A, B, D, E) Overlays of pairs of IJPs and NO-induced hyperpolarizations recorded before and after the applications of rolipram and zaprinast. (C, F) Summary plots of the amplitudes of IJPs evoked by EFS and NO-induced hyperpolarizations, respectively. Each column and bar represents the mean and S.E.M. of seven or eight observations. $*P < 0.05$; significantly different from control (Dunnett's test).

ated component of the nitrergic IJPs cannot occur in the absence of NO and that NO stimulates VIP release in the hamster jejunum. Furthermore, 1 μM VIP-induced hyperpolarization was not affected by L-NAME in the hamster jejunum, suggesting that the VIP effect is independent of NO. Endogenous and exogenous NO-induced hyperpolarizations were inhibited by VIP(6–28) in the hamster jejunum. In addition, we have demonstrated that endogenous and exogenous NO stimulated VIP release via cGMP production in the hamster jejunum. The latter views are consistent with the following observations. In the ganglia isolated from the myenteric plexus of the guinea-pig small intestine, VIP released in response to either nerve stimulation or NO application is inhibited by an inhibitor of cGMP-dependent protein kinase activity and an inhibitor of soluble guanylate cyclase (Grider and Jin, 1993). Similar data were reported from the isolated enteric synaptosomes of the rat small intestine, where VIP release was stimulated by a NO donor and cGMP analogue (Allescher et al., 1996). Taken together, the present results suggest that the NO-stimulated VIP release partly underlies the nitrergic IJPs.

However, we have also found that 10 μM of VIP (but not less than 3 μM) evoked the accumulation of cGMP in the hamster jejunum, suggesting that NO could be produced in muscle cells or neurons by the action of the high concentration (more than 10 μM) of VIP. Previous studies have shown that NOS localizes in the smooth muscle cells of the canine small intestine (Berezin et al., 1994). VIP stimulated NO release in isolated rabbit gastric muscle strips (Jin et al., 1996). The release of NO in response to VIP may be involved in the relaxation caused by EFS in the opossum internal anal sphincter (Chakder and Rattan, 1996). Thus, we cannot exclude the possibility that NO formation could be stimulated in target muscle cells or neurons by a high concentration of VIP released into the synaptic cleft.

Involvement of cAMP in nitrergic inhibitory junction potentials

Few studies have demonstrated that endogenous NO increases the levels of cAMP and hyperpolarizes the membrane potential in the mammalian gastrointestinal tract. In the present study, the late component of the nitrergic IJPs and NO-induced hyperpolarizations was influenced by VIP(6–28) and an inhibitor of specific cAMP PDE, rolipram. Furthermore, the present experiments have also confirmed that VIP(6–28) inhibited the increases in cAMP levels induced by EFS and NO application, and that the cAMP analogue caused a membrane hyperpolarization in the hamster jejunum. This idea is supported by the observation that cAMP is involved in the NO-induced relaxation in the opossum internal anal sphincter (Chakder and Rattan, 1993). In addition, VIP(6–28) inhibited the increase in cAMP levels induced by NO and the cAMP-mediated component of the IJPs and NO-induced hyperpolarizations. Thus, we propose that endogenous and exogenous NO could produce cAMP through stimulating VIP release in the hamster jejunum. It has been demonstrated that the cAMP-dependent pathway mediates the effect of VIP in the rat

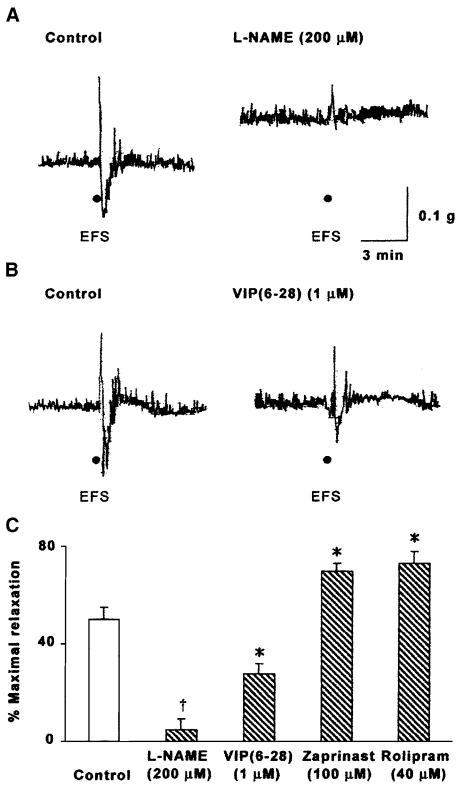

Fig. 8. Effects of L-NAME, VIP(6–28), rolipram and zaprinast on EFS-induced relaxation. (A, B) Representative traces showing the effects of L-NAME and VIP(6–28) on responses to EFS (16 V, 0.1 ms duration, at 20 Hz, 5-s trains). (C) Summary plot of the amplitude of EFS-induced relaxations in 10 preparations before (control) and after L-NAME, VIP(6–28), rolipram and zaprinast. Data in C are means ± S.E.M. $^{*}P < 0.05$, $^{\dagger}P < 0.01$; significantly different from control (Dunnett's test).

colon (Kishi et al., 2000) and opossum internal anal sphincter (Chakder and Rattan, 1993). However, it is not known whether protein kinase is involved in the VIP-mediated nitrergic IJPs. Additional studies are required to investigate the possible role of protein kinase in mediating the nitrergic IJPs and NO-induced hyperpolarizations.

Temporal differences

To our knowledge, this is the first report proposing that nitrergic IJPs and NO-induced hyperpolarizations can be classified into early and VIP-mediated late IJPs of the gastrointestinal tract. Two hypotheses can be considered in support of this observation. First, as the production and diffusion of cGMP and cAMP, along with these cyclic nucleotide binding sites on ion channels, are different from each other, there may well be temporal differences between early and late components of the IJPs. Indeed, the early and late components of the nitrergic IJPs and NO-induced hyperpolarizations occur via the increase in cGMP and cAMP productions, respectively, in the hamster jejunum. The time course of the cGMP-mediated relaxation has been faster than that mediated by cAMP in the cat trachea (Imoto et al., 1998). Therefore, we speculate that the time course of the hyperpolarizations mediated by cGMP may be more fast-tracked than those mediated by cAMP in the hamster jejunum. However, the actual features of each transduction process remain to be explored in this tissue. The second hypothesis is based on the finding that VIP release did not occur without NO release in the hamster jejunum. Thus, NO may act as a presynaptic modulator of VIP release, which in turn causes the late component of the nitrergic IJPs and NO-induced hyperpolarizations via a cAMP-dependent pathway. However, the early component of the nitrergic IJPs and NO-induced hyperpolarizations may be produced by NO via cGMP. Therefore, the time course of the NO–cGMP-dependent pathway might be faster than that of the VIP–cAMP-dependent pathway in the hamster jejunum.

Functional implications

The present study was the first to examine the involvement of cGMP- and cAMP-dependent pathways in both the nitrergic IJPs and relaxation in the hamster jejunum. However, cGMP has been suggested to cause relaxation of smooth muscle by lowering the intracellular calcium concentration via either K^+ channel-independent or K^+ channel-dependent mechanisms leading to hyperpolarization and subsequent reduction of Ca^{2+} influx through voltage-operated Ca^{2+} channels (Cayabyab and Daniel, 1996; Selemidis and Cocks, 2000). In our previous study, cGMP-associated nitrergic IJPs and NO-induced hyperpolarizations were inhibited by the small conductance Ca^{2+}-activated K^+ channel blocker apamin in the hamster ileum (Matsuyama et al., 1999b). These results indicate that a K^+ channel may mediate the cGMP- and cAMP-associated nitrergic IJPs and NO-induced hyperpolarizations in the hamster jejunum.

CONCLUSION

In conclusion, our studies suggest that NO and NO-stimulated VIP are involved in the early and late components of the nitrergic IJPs, respectively, in the hamster jejunum. Furthermore, we propose that endogenous and exogenous NO cause membrane hyperpolarization not only via production of cGMP but also by VIP released from enteric nerves which in turn produces cAMP in the hamster jejunum.

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