Nitric Oxide Modulates Neuropeptide Y Regulation of Ion Transport in Mouse Ileum

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Accepted for publication March 1, 1996

ABSTRACT

The possible involvement of nitric oxide in the regulation of intestinal ion transport induced by neuropeptide Y (NPY) was investigated by evaluating the effects of N\textsuperscript{G}-methyl-L-arginine (L-NMA), L-arginine and S-nitroso-N-acetylpenicillamine (SNAP) on NPY activity in mouse ileum mounted in Ussing chambers in vitro. Serosal NPY (10 nM) produced a sustained decrease in basal transmural short circuit current (I\textsubscript{sc}) and potential difference without altering the tissue conductance. Pretreatment of tissues with L-arginine (3 mM), but not D-arginine (10 mM), blocked the NPY-mediated changes in I\textsubscript{sc}. This L-arginine effect on NPY activity was reversed by L-NMA (3 mM), and not by N\textsuperscript{G}-methyl-\textsuperscript{d}-arginine (10 mM). The L-arginine effect on NPY activity was concentration-related with an A\textsubscript{50} (95% CL) value of 1.6 (0.9-2.3) mM. In contrast to L-arginine, L-NMA (1 mM) pretreatment of tissues produced an enhancement of NPY activity, resulting in a 3.8-fold leftward displacement of the NPY concentration-response curve; N\textsuperscript{G}-methyl-\textsuperscript{d}-arginine was without effect. The effect of L-NMA on NPY activity was concentration-related with an A\textsubscript{50} (95% CL) value of 45.3 (23.2-68.8) \mu M. Serosal application of SNAP, a nitric oxide donor, produced a concentration-related decrease in basal I\textsubscript{sc} and potential difference without altering tissue conductance with an A\textsubscript{50} (95% CL) value of 22.5 (11.1-40.5) \mu M. Pretreatment of tissue with SNAP (100 \mu M) reduced the NPY activity with rightward displacement of NPY concentration-response curve. Pretreatment of tissues with L-arginine also blocked the reduction of I\textsubscript{sc} by [\textsuperscript{d}-Pen\textsuperscript{2},\textsuperscript{d}-Pen\textsuperscript{5}]enkephalin (10-30 nM), H\textsubscript{2}N-Tyr-\textsuperscript{d}-Ala-Phe-Glu-Val-Val-Gly-NH\textsubscript{2} (10-30 nM) and somatostatin (0.3-1.0 \mu M), but had no effect on norepinephrine (0.1-0.3 \mu M)-induced decrease in mouse ileal I\textsubscript{sc}. These results show that {\textsuperscript{fgc}}l-arginine and SNAP block NPY-mediated changes in ion transport, suggesting that nitric oxide may play a role in the regulation of NPY-mediated ion transport in the mouse ileum.

NPY, a peptide of 36 amino acids has been detected in the small intestine of a number of species, and located predominantly in the enteric nerves innervating the mucosa and submucosa (Lundberg et al., 1984; Feher and Burnstock, 1986). In the guinea pig small intestine, NPY neurons represent approximately 25% of all submucosal neurons (Furness et al., 1984). Studies both in vivo and in vitro have demonstrated that NPY has a significant influence on the intestinal ion transport. Central application of NPY produced a sustained decrease in I\textsubscript{sc} of the guinea pig, rat and rabbit small intestine (Friel et al., 1986; Hubel and Renquist, 1986; Cox et al., 1988). Such a decrease in I\textsubscript{sc} by NPY was shown to be a result of increased net absorption of chloride in the porcine distal jejunum and rabbit ileum. Our previous studies have shown that NPY (luminal or contraluminal application) produced a sustained decrease in the I\textsubscript{sc} and PD also in the mouse jejunum mounted to Ussing chambers in vitro (Riviere et al., 1990, 1993). This effect of NPY was mediated by the activity of enteric neurons as it was blocked completely by tetrodotoxin, a neural conductance blocker, and chlorisondamine, a ganglionic blocker.

More recent studies have also demonstrated that release of NO from the enteric nervous system tonically regulates ion transport in the mouse ileum (Rao et al., 1994). Administration of L-NMA, an inhibitor of NO-synthase, produced a sustained increase of I\textsubscript{sc} and PD in the mouse ileum mounted to Ussing chambers, and this effect of L-NMA was reversed by L-arginine. Therefore, endogenously released NO may influence the activity of different neurons in the enteric nervous system. In order to determine whether there is an involvement of NO release in the activity of NPY in intestinal ion transport, we studied the effects of L-arginine, L-NMA and SNAP on NPY-induced changes of I\textsubscript{sc} in the mouse ileum.

Received for publication September 11, 1995.

\textsuperscript{1} This work was supported by Grant DK36289 (National Institute of Health) and Jouvenial Laboratories.

ABBREVIATIONS: NPY, neuropeptide Y; I\textsubscript{sc}, short circuit current; PD, potential difference; NO, nitric oxide; L-NMA, N\textsuperscript{G}-methyl-L-arginine; SNAP, S-nitroso-N-acetylpenicillamine; DPDPE, [\textsuperscript{d}-Pen\textsuperscript{2},\textsuperscript{d}-Pen\textsuperscript{5}]enkephalin; [\textsuperscript{d}-Ala\textsuperscript{5},Glu\textsuperscript{5}]deltorphin, H\textsubscript{2}N-Tyr-\textsuperscript{d}-Ala-Phe-Glu-Val-Val-Gly-NH\textsubscript{2}; G\textsubscript{i}, tissue conductance; D-NMA, N\textsuperscript{G}-methyl-\textsuperscript{d}-arginine; L-NNA, N\textsuperscript{G}-nitro-L-arginine.
Methods
Preparation of ileal tissues. Male ICR mice (Holtzman, Madison, WI) (35-40 g) were sacrificed by cervical dislocation and the small intestine was excised at pyloric and ileocecal junctions. The distal third (ileum) of the small intestine was cut along the mesenteric border, rinsed free of luminal contents and placed in oxygenated Krebs-Ringer buffer. Ileal sheets (2-3 cm length) were mounted into Ussing chambers (0.65 cm² exposed surface area) as described previously (Sheldon et al., 1989). The main reason for using the intact tissue in this study was to have ileal sheets with intact neural plexuses. This was important, as our previous studies have demonstrated that regulation of intestinal ion transport by NPY and L-NMA was mediated neurally; NPY had no effect on ileal ion transport. Administration of drugs such as theophylline, carbobol and bombesin produces immediate changes of Iₑ of the unstripped ileum without a lag period, suggesting that the penetration of drugs through the tissue was not a problem. Furthermore, under our experimental conditions, tissues could be viably maintained for at least 6 hr, indicating a proper delivery of glucose to the epithelium.

Bioelectric measurements. Ileal sheets mounted in Ussing chambers were bathed on both serosal and mucosal surfaces with Krebs-Ringer bicarbonate buffer containing 20 mM of either d-glucose (serosal buffer) or mannitol (mucosal buffer) and gassed constantly with 95% O₂-5% CO₂ at 37°C. Tissues were equilibrated under short circuit conditions for 30 min. Transmural Iₑ and PD, indicators of net ion transport, were measured as described previously (Sheldon et al., 1989). Open circuit PD was measured by unclamping tissues for approximately 5 sec. Gₑ was calculated by dividing the Iₑ (microamperes per squared centimeters) by the open circuit PD (millivolts), yielding Gₑ in millisiemens per squared centimeter. All compounds were added to the medium bathing on the serosal surface and the maximum change in Iₑ and PD were recorded. Concentration-effect curves were constructed noncumulatively whereby each tissue was exposed to only one concentration of compound. Modulators (L-NMA, D-NMA, L-arginine, D-arginine and SNAP) were applied to the serosal medium 10 min before the addition of NPY. DPDPE, [d-Ala²,Glu⁴]deltorphin, somatostatin and norepinephrine were also applied to the serosal medium. At all concentrations, arginine and NMA were applied to both serosal and mucosal buffers to compensate for changes in tonicity. In experiments in which L-arginine and L-NMA were evaluated in combination, arginine and NMA were administered concurrently as serosal + mucosal pretreatments, 10 min before NPY application. All tissues were challenged with 1 mM theophylline at the end of the experiment as a measure of tissue viability; data from tissues that responded with less than 50 μA/cm² increase in Iₑ were discarded (usually less than 5% of tissues).

Chemicals. The following compounds were purchased: L-NMA, D-NMA, L-arginine, D-arginine, somatostatin-14 and theophylline (Sigma Chemical Co., St Louis, MO). NPY was purchased from Bachem California (Torrence, CA). DPDPE and [d-Ala²,Glu⁴]deltorphin were synthesized as described previously (Misicka et al., 1991; Mosberg et al., 1983). SNAP was purchased from Calbiochem (La Jolla, CA).

Statistics. Two adjacent segments of ileum from each mouse were used in these studies. Values under each group are derived from different mice. Concentrations of compounds eliciting 50% of the maximal response (Aₜ₀) at 95% CL and relative potency values were obtained from least-square analysis of the linear portions of the concentration-response curves by using a computer program (Tallarida and Murray, 1987). Comparison between two groups was made by the Student’s t tests for grouped data, and comparisons of two values from an individual tissue was made by a Student’s t test for paired data. The significance in all tests was derived at the 95% or greater CL.

Results
Effect of L-arginine pretreatment on NPY-induced decrease in ileal Iₑ. Serosal application of NPY (10 nM) produced a sustained decrease in Iₑ and PD without altering the Gₑ in mouse ileal sheets mounted into Ussing flux chambers (fig. 1). The activity of NPY on ileal ion transport was concentration-related with Aₜ₀ (95% CL) values of 4.3 (2.2-6.3) nM. L-Arginine (3 mM) alone produced no effect on basal Iₑ, whereas pretreatment with this dose of L-arginine reduced NPY-mediated decrease in mouse ileal Iₑ (fig. 1); pretreatment with D-arginine (10 mM) produced no significant effect on basal Iₑ, or it altered the NPY-mediated decrease in ileal Iₑ. The inhibitory effect of L-argine on NPY activity was concentration-related (fig. 2) with Aₜ₀ (95% CL) values of 1.6 (0.9-2.3) nM; at concentrations greater than 3 mM, L-arginine produced a sustained decrease in Iₑ. The L-arginine (3 mM) blockade of NPY was reversed by the coadministration of L-NMA (3 mM), but not by D-NMA (10 mM) (fig. 2). L-Arginine up to 3 mM produced no significant effect on theophylline-induced increase in Iₑ (data not shown).

Effect of L-NMA pretreatment on NPY-induced decrease in ileal Iₑ. Serosal application of L-NMA (1 mM) produced a sustained increase in mouse ileal Iₑ and PD without affecting Gₑ. Although pretreatment of tissue with L-NMA (1 mM) had no significant effect on higher doses of NPY (i.e., 10 nM), it significantly increased the activity of lower doses of NPY on ileal Iₑ (fig. 3). Pretreatment with L-NMA produced 3.8-fold leftward, nonparallel, shift of NPY concentration-effect curve (fig. 3), by reducing the Aₜ₀ (95% CL) values for NPY to 1.0 (0.5-2.1) nM. This potentiation of NPY activity by L-NMA was concentration-related (fig. 4) with Aₜ₀ (95% CL) values of 45.3 (23.2-66.8) μM. Pretreatment of tissue with D-NMA showed no significant effect on NPY activity. L-NMA up to 1 mM produced no significant effect on theophylline-induced increase in Iₑ (data not shown).

![Fig. 1. Representative traces of decrease in Iₑ after application of NPY in a control tissue (A) or in tissues pretreated with either L-arginine (B) or D-arginine (C).](image-url)
The present study demonstrates a modulation of NPY-mediated regulation of intestinal ion transport by L-arginine, the substrate for NO-synthase, and SNAP, a NO donor, and suggests that NO may negatively modulate NPY activity in the mouse ileum. It has been demonstrated previously (Friel et al., 1986; Hubel and Renquist, 1986; Cox et al., 1988; Riviere et al., 1990, 1993) that NPY produced a proabsorptive effect on the mammalian small intestine. This activity of NPY on small intestinal ion transport involved increased transepithelial absorption of chloride ions. NPY-induced
changes in the intestinal ion transport appears to involve enteric neurons at preganglionic levels as indicated by a complete attenuation of this activity by treatment of the mouse jejunum with tetrodotoxin, a neural conductance blocker, and chlorisondamine, a ganglionic blocker (Riviere et al., 1993). Furthermore, NPY-induced changes in mouse jejunal ion transport was demonstrated to be sensitive to haloperidol (Riviere et al., 1993), suggesting a possible involvement of putative sigma receptors in this activity of NPY. The mechanism of regulation of intestinal ion transport by NPY, however, is not known.

A number of previous studies have demonstrated both prosecretory and proabsorptive effects of NO in the small and large intestine. NO produced a prosecretory effect in rat (Tamai and Gaginella, 1993) and guinea pig (MacNaughton 1993) colon in vitro, and mediates interleukin 1β-induced colonic secretion (Eutamene et al., 1995) and sodium choleate-induced diarrhea in rats (Mascolo et al., 1994) in vivo. However, in dogs, rabbits and rats, NO produced proabsorptive effects in the small intestine in vivo (Barry et al., 1994; Maher et al., 1995; Schirgi-Degen and Beubler, 1995). Although it produced no effect in rat ileal L_{sc} in vitro (Li et al., 1994), our previous studies (Rao et al., 1994) have shown that NO produces proabsorptive effect in the mouse ileum in vitro. Therefore, the type of response by NO may depend on the segment of intestine studied, animal species involved and other experimental conditions (such as in vivo and in vitro conditions and intact and stripped tissues). Our studies have shown that tonic release of NO limits L_{sc} in mouse ileum in vitro (Rao et al., 1994). Treatments with L-NMA or L-NNA, competitive inhibitors of NO-synthase, produced sustained increases in transmural L_{sc} and PD in the mouse ileum, whereas application of acidified sodium nitrite (which generates NO) produced a transient decrease in L_{sc} and PD (Rao et al., 1994). The activity of L-NMA in altering ileal L_{sc} was reversed by L-arginine, the substrate for NO-synthase in a concentration-dependent manner. Ion replacement studies suggested that NO may increase the absorption of chloride in the mouse ileum. Therefore, NO may function as a transmitter for drugs and neuropeptides that alter intestinal ion transport or smooth muscle relaxation. The activities of vasoactive intestinal peptide in the relaxation of gastric smooth muscle (Grider and Jin, 1993) and cholecystokinin in relaxation of arterial smooth muscle (Zawadzki et al., 1983) have been shown previously to be mediated, at least in part, by NO release. The possibility of stimulated synthesis and release of NO as a mediator of proabsorptive agents can be tested by evaluating the effect of L-NMA on the activities of proabsorptive agents. In the present study, however, L-NMA pretreatment failed to suppress the activity of NPY at a dose (10 nM) that produced nearly 90% maximal effect, suggesting that the activity of NPY is not mediated by the release of NO in the mouse ileum. In the case of drugs that produce proabsorptive effect, it might be expected that they would increase the release of NO, and hence these activities are blocked by L-NMA pretreatment. The present observation that net proabsorptive effect of NPY on mouse ileal ion transport was not blocked by L-NMA suggests that the activity of NPY was not mediated by NO.

Interestingly enough, the present study shows that L-arginine, the substrate for NO-synthase, reduces the effect of NPY on ileal L_{sc} in a concentration-related manner. This effect of L-arginine was reversed by coadministration of L-NMA, and not by d-NMA. d-Arginine, the inactive isomer, failed to alter NPY activity. These results suggest that synthesis and release of NO from exogenously added L-arginine

![Fig. 6. Effect of pretreatment with SNAP (100 μM) on NPY (3 or 10 nM)-induced decrease in mouse ileal L_{sc}. Data are the mean (and S.E.M.) of four to six tissues for each point.](image)

![Fig. 7. Effect of L-arginine on decrease in L_{sc} produced by DPDPE (10 or 30 nM) and [D-Ala²,Glu⁸]deltorphin (10 or 30 nM). Data are the mean (and S.E.M.) of four tissues for each group. *Values that are significantly (P < .05) different from value for corresponding "None" group.](image)

![Fig. 8. Effect of L-arginine on decrease in L_{sc} produced by somatostatin (0.1 or 1.0 μM) and norepinephrine (0.1 or 1.0 μM). Data are the mean (and S.E.M.) of four tissues for each group. *Values that are significantly (P < .05) different from value for corresponding "None" group.](image)
may exert an inhibitory influence on NPY-induced changes in intestinal ion transport. This interpretation depends on the assumption that NO-synthase is under subsaturation condition with respect to endogenous L-arginine concentration, and the administration of exogenous L-arginine elevates the levels of NO in the tissue. The latter observation is supported by our previous report that L-arginine at concentrations greater than 3 mM produces a sustained decrease in ileal \( I_{ac} \), and this effect can be reversed by hemoglobin, which is known to sequester NO and reduce free NO levels (Rao et al., 1994). As described above, we have demonstrated previously that endogenous NO has a neurally mediated tonic proabsorptive/prosecretory influence in the mouse ileum in vitro. It is not clear how NO, a proabsorptive agent, can block the activity of NPY, another proabsorptive agent. Due to the complexity of the neural network in the intestine and the fact that NO-synthase is present in neurons, smooth muscle and mucosal immune cells (Costa et al., 1991; Shuttleworth et al., 1991; Furness et al., 1991), it is difficult to assign a specific mechanism for this action of NO at this time. As discussed above, both prosecretory and proabsorptive effects of NO were recorded in the small and large intestine of mammals under different experimental conditions. Therefore, under physiological conditions, NO may produce an effect that is different from its activity in response to pharmacological or pathophysiological conditions, and these differences may depend on the specific type of neural pathway involved. One possible explanation is that both NO and NPY utilize a common pathway to produce the proabsorptive effect and therefore the NPY effect is suppressed by NO. Alternatively, depending on the rate of uptake of L-Arg or SNAP, certain cell types may be influenced differently from the other. NO produced at different sites (blood vessels, smooth muscle and immunocytes) in the intestinal wall may play different roles.

Another possibility is that L-arginine blocks NPY activity by a mechanism other than release of NO. Such a non-NO pathway has been suggested to be involved in the gastric mucosal protective activity of L-arginine (Brozozowski et al., 1995). Protection of gastric mucosa from ethanol-induced damage by L-arginine was unaffected by the administration of L-NMA, suggesting that L-arginine produced its effect by an unknown mechanism which does not involve release of NO. To test this possibility, we evaluated further the effect of SNAP, an NO releasing agent, on NPY activity. SNAP by itself produced a concentration-related decrease in \( I_{ac} \) in the mouse ileum, which is consistent with our previous reports that NO produced a proabsorptive effect in the mouse ileum (Rao et al., 1994). Similar to L-arginine, pretreatment with SNAP also reduced the NPY-induced decrease in \( I_{ac} \) in a concentration-related manner. This effect of SNAP may support the suggestion that the effect of L-arginine on NPY-induced changes in ileal ion transport was indeed due to the release of NO in this tissue rather than a non-NO pathway.

The main proabsorptive/antisecretory mechanisms of regulation of the intestinal ion transport include opioid, somatostatin and adrenergic receptors (Brown and Miller, 1991). The agonists of opioid delta receptors have been shown previously to produce sustained decrease in \( I_{ac} \) and PD without altering \( G_{j} \) (Sheldon et al., 1990). Similarly, somatostatin and norepinephrine also produced proabsorptive changes in intestinal ion transport (Brown and Miller, 1991). Our present studies confirm these effects of DPDPE, [D-Ala\(^2\),Glu\(^4\)Deltorphin, somatostatin and norepinephrine on \( I_{ac} \) in the mouse ileum. Similar to NPY activity, the activities of DPDPE, [D-Ala\(^2\),Glu\(^4\)Deltorphin and somatostatin were also reduced significantly by pretreatment of tissues with L-arginine; however, activity of norepinephrine was unaffected. These results indicate that the effect of L-arginine is not selective to NPY, but may affect the actions of different proabsorptive agonists that involve a common converging pathway to regulate intestinal ion transport. On the basis of these observations, one can speculate that the effect of NO is a generalized effect on neurally mediated absorptive processes. It has been demonstrated previously that the proabsorptive effects of DPDPE, [D-Ala\(^2\),Glu\(^4\)Deltorphin and somatostatin are neurally mediated, and those of norepinephrine involve direct interaction with enterocytes (Brown and Miller, 1991). Additionally, our present study also observed that L-arginine or L-NMA produced no significant effect on theophylline-induced increase in \( I_{ac} \).

The present study also shows that L-NMA significantly enhances the activity of NPY at lower concentrations. This effect of L-NMA is complimentary to the above discussed effects of L-arginine and suggests that endogenously released NO may tonically suppress the activity of NPY on ion transport in the mouse ileum. Such enhancing actions of L-NMA or L-NNA have been shown recently in substance P-induced stimulation of the rat colonic muscle tone (Scheurer et al., 1993) and in endothelin-1-induced secretory response of the guinea pig distal colon (Reddix et al., 1993). The mechanism involved in the NO modulation of NPY activity is not clear at the present time. NO released from certain specific sites may exert an inhibitory effect on enteric neurons either at the NPY binding site or at the level of transmitter release.

These studies thus demonstrate that L-arginine and SNAP modulate NPY-induced changes in intestinal ion transport in the mouse ileum in vitro, suggesting that NO modulates NPY activity in the mouse ileum in vitro. Further studies are needed to understand the mechanism involved in this modulation of NPY activity.

References


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