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### Research Article

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## Neuronal Constitutive Nitric Oxide Synthase Is Involved in Murine Enteric Inhibitory Neurotransmission

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### Abstract

Mice lacking neuronal nitric oxide synthase gene (*ncNOS*) were used to determine the enzymatic source of nitric oxide (NO) and its relationship with other putative inhibitory neurotransmitters. Inhibitory junction potentials (IJP) of circular smooth muscle of gastric fundus were studied. The IJP in the wild-type mice consists of overlapping components, the fast and slow IJPs. NOS inhibitor L-NA or VIP receptor antagonist VIP<sub>10-28</sub>, blocks the slow IJP but not the fast IJP. The fast IJP is blocked by  $\alpha$ - $\beta$  methylene ATP tachyphylaxis, by reactive blue 2, and by apamin. The IJP in the *ncNOS*-deficient [*ncNOS*(-)] mutant is of short duration and is abolished by blockers of the fast IJP, but is unaffected by blockers of the slow IJP. Exogenous VIP produces membrane hyperpolarization in strips from wild-type but not *ncNOS*(-) mice. The hyperpolarizing action of VIP is resistant to nifedipine but is sensitive to  $\omega$ -conotoxin GVIA. In conclusion: (a) NO derived from *ncNOS* is an inhibitory neurotransmitter rather than a postjunctional mediator; (b) VIP is a prejunctional neurotransmitter that causes release of evanescent NO; and (c) ATP acts in parallel with the VIP/NO pathway. (*J. Clin. Invest.* 1996. 98:8-13.) Key words: VIP • ATP • inhibitory junction potential • smooth muscle • transgenic

### Introduction

Nitric oxide (NO)<sup>1</sup> is implicated in a variety of biological functions. Several lines of evidence support that NO is a neurotransmitter of nonadrenergic noncholinergic (NANC)<sup>1</sup> nerves, playing a key role in the autonomic control of smooth muscle throughout the body, including blood vessels, bronchial tree,

urinary bladder, and gastrointestinal tract (1-5). Nitric oxide synthase (NOS), an enzyme that synthesizes NO from the amino acid L-arginine, is localized to neurons and nerve endings at the neuromuscular junction (6, 7); authentic NO or NO donors mimic the actions of the endogenously-released mediator (2, 4, 5); NO is released upon electrical or chemical stimulation of NANC nerves (8, 9); and chemical inhibitors of NOS suppress both the nerve-stimulated release of NO and the inhibition of the target smooth muscles (4, 5, 8). However, these observations can also be explained if NO were not a true antegrade neurotransmitter, but a mediator produced from NOS in the postjunctional smooth muscle cells by the action of other putative inhibitory neurotransmitters, such as ATP and vasoactive intestinal peptide (VIP) (5, 8, 10).

There are several different molecular forms of NOS, including two constitutive forms, "neuronal" NOS (*ncNOS*) and "endothelial" NOS (*ecNOS*), and inducible "macrophage" NOS (*iNOS*), named for their initial localization primarily in neurons, vascular endothelial cells, and macrophages, respectively (1, 3). Both *ncNOS* and *ecNOS* are calcium and calmodulin dependent. However, *ncNOS* is a soluble cytosolic enzyme whereas *ecNOS* is particulate and membrane bound. At the smooth muscle neuromuscular junction in the gut, *ncNOS* is primarily localized to nerve endings and *ecNOS* is primarily in the smooth muscle cells (11). These isoforms cannot be distinguished using pharmacologic agents, but they are products of distinct genes which can be selectively disrupted by homologous recombination (8, 11-15).

*ncNOS*-deficient mice were studied by intracellular microelectrode recordings of gastric fundus circular smooth muscle to determine whether *ncNOS* is the enzymatic source of NO involved in the inhibitory neurotransmission. Studying the smooth muscle membrane potential provides a sensitive method to elucidate neurotransmitters that act by electromechanical coupling (16). Such mediators hyperpolarize the membrane, suppress calcium entry via the voltage-dependent calcium channels, and thereby produce smooth muscle relaxation. Our studies demonstrate that *ncNOS* is the source of NO which acts as an antegrade inhibitory neurotransmitter. VIP is an intermediary mediator acting prejunctionally to release evanescent NO on demand.

### Methods

**Mice and tissues.** Adult C57Bl/6J and 129/J strain mice were used as wild-type mice, and *ncNOS*(-) mice were generated from embryonic stem cells with targeted disruption by homologous recombination, as we have described previously (13). Briefly, clones of embryonic stem cells with the targeted disruption of *ncNOS* were injected into donor

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1. Abbreviations used in this paper: *ecNOS*, endothelial NOS; EFS, electrical field stimulation; IJP, inhibitory junction potential; NANC, nonadrenergic noncholinergic; *ncNOS*, neuronal NOS; NO, nitric oxide; NOS, nitric oxide synthase; RB2, reactive blue 2; SP, substance P; TTX, tetrodotoxin; VIP, vasoactive intestinal peptide.

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blastocysts to produce chimeras, which were crossed to 129/J strain mice to produce heterozygous mice. Heterozygous mice were crossed to each other to produce homozygous mutant mice lacking ncNOS, which show complete absence of NADPH diaphorase reactivity in the myenteric neurons. Adult mice weighing between 25 and 35 grams were sacrificed by carbon dioxide narcosis and gastric fundus tissue was prepared for intracellular recording and bathed in KRB solution essentially as described previously (17).

**Drugs.** Drugs used in this study included apamin, D-arginine, L-arginine, atropine,  $\alpha,\beta$ -methylene adenosine 5'-triphosphate (MeATP, a stable analogue of ATP), guanethidine, N<sup>o</sup>-nitro L-arginine (L-NA), nifedipine, reactive blue 2 (RB2), substance P (SP), tetrodotoxin (TTX), vasoactive intestinal peptide (VIP); and VIP receptor antagonist VIP<sub>10-28</sub>, all were obtained from Sigma Chemical Co. (St. Louis, MO).  $\omega$ -conotoxin GVIA was obtained from Peninsula Lab., Belmont, CA. All drugs were made up fresh on the day of the experiment. KRB solution and authentic NO were prepared as described elsewhere (18). The following drug concentrations were used: apamin (0.3  $\mu$ M), D-arginine (1 mM), L-arginine (1 mM), atropine (1  $\mu$ M), MeATP (33  $\mu$ M),  $\omega$ -conotoxin (0.1  $\mu$ M), guanethidine (5  $\mu$ M), nifedipine (0.1  $\mu$ M), NO (1 mM), RB2 (10  $\mu$ M), SP (1  $\mu$ M), TTX (1  $\mu$ M), VIP (100  $\mu$ M), and VIP<sub>10-28</sub> (10  $\mu$ M). Solution containing L-NA (200  $\mu$ M) was perfused for 15 min before study of its effect.

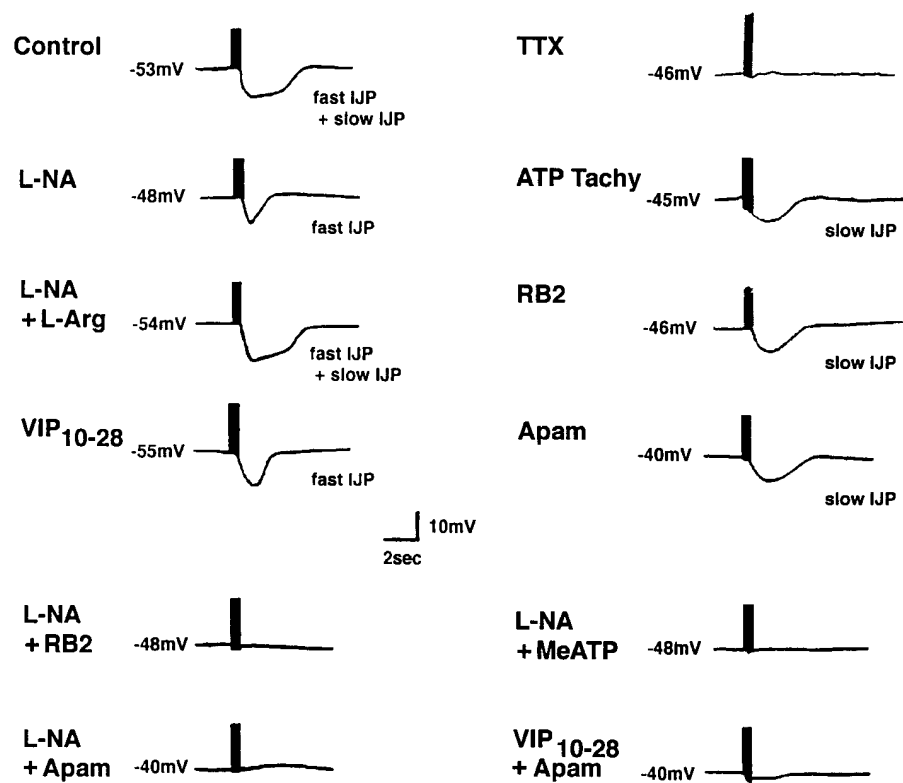
**Smooth muscle recordings.** Intracellular membrane potentials were recorded from smooth muscle cells of circular muscle strips obtained from the fundic region of the stomach using standard techniques (2, 17). Atropine, guanethidine, and desensitizing concentrations of SP were always present in the perfusate. Guanethidine and atropine suppress adrenergic and cholinergic responses respectively and provide the expression of NANC neurotransmission. The NANC transmission includes an excitatory junction potential mediated by SP and an inhibitory junction potential (IJP). Desensitizing SP receptors allows full expression of this IJP and the hyperpolarizing action of VIP (17, 18). Electrical field stimulation (EFS) was provided using silver chloride electrodes (120 V, 2 ms duration square pulses, 10 pulses delivered over 0.5 s) as described earlier (17). Effects of chemical antago-

nists were studied in the same cell whenever possible. However, when electrode displacement made it impossible, recordings were made from adjacent cells.

**Statistical analysis.** Statistical comparisons were made using Student's standard paired and unpaired *t* statistics, and all data are expressed as means  $\pm$  SEM.

## Results

**IJP in wild-type mice.** In the wild-type normal mice, the resting membrane potential of gastric fundic circular muscle strips treated with atropine, guanethidine and desensitizing concentrations of SP was  $52.4 \pm 2.1$  mV. Standard EFS produced a TTX-sensitive hyperpolarizing response, showing that this response is mediated by substances released by nerves, and is therefore called IJP. This IJP (Fig. 1) was  $9.2 \pm 0.4$  mV in amplitude and  $3.8 \pm 0.1$  s in duration ( $n = 22$  cells from five animals). The NOS inhibitor L-NA caused a significant decrease in the duration of IJP from  $3.8 \pm 0.1$  s to  $2.1 \pm 0.1$  s ( $P < 0.05$ ), without affecting the onset, time to peak response, or the amplitude ( $9.9 \pm 0.8$  mV vs.  $9.2 \pm 0.4$  mV,  $P < 0.05$ ). Interestingly, the effects of the VIP receptor antagonist VIP<sub>10-28</sub> on the IJP were similar to those of L-NA. In the case of VIP<sub>10-28</sub>, the duration of IJPs before and after VIP<sub>10-28</sub> were  $3.8 \pm 0.1$  s and  $2.2 \pm 0.1$  s, respectively ( $P < 0.05$ ). The early portion of the control IJP that is resistant to L-NA and to VIP<sub>10-28</sub> is designated the fast IJP. The fast IJP measured approximately 2 s in duration and 10 mV in amplitude in response to standard EFS. The fast IJP is blocked by ATP receptor desensitization with MeATP, suggesting that it is due to neuronally-released ATP or a related compound. It is also blocked by RB2 which is known to block P<sub>2</sub> receptors, and by apamin, which blocks a species of small conductance calcium-dependent potassium



**Figure 1.** Inhibitory junction potentials in the stomach fundus of wild-type mice: effects of various chemical treatments. Note that the control IJP of wild-type mice is TTX-sensitive and consists of two overlapping components. The slow component is blocked by L-NA and is reversed by L-arginine but not by D-arginine (not shown). It is also blocked by VIP<sub>10-28</sub>. L-NA and VIP<sub>10-28</sub> treatments leave the fast IJP unaffected. The fast component is blocked by ATP tachyphylaxis, RB2, or apamin, leaving the slow IJP unaffected. Simultaneous treatments with L-NA and RB2, L-NA and apamin, L-NA and MeATP, or VIP<sub>10-28</sub> and apamin abolish the entire IJP. This shows that in wild-type mice the control IJP consists of two overlapping components: a fast IJP mediated by ATP acting on apamin-sensitive channels and a slow IJP mediated by VIP and NO.

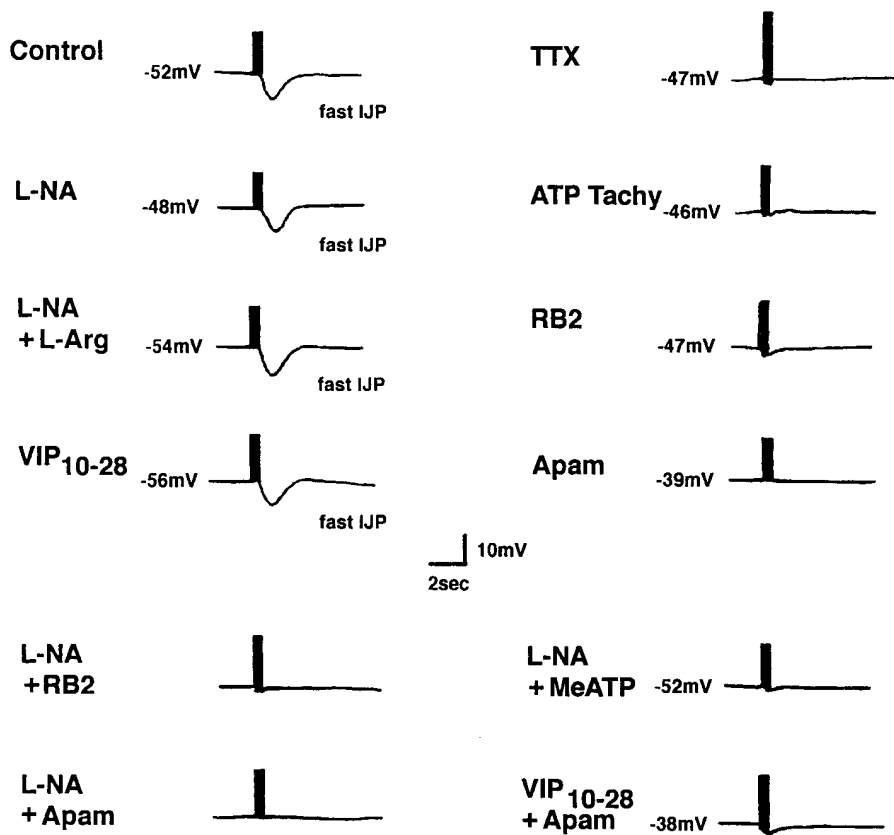


Figure 2. Inhibitory junction potentials in the stomach fundus of *ncNOS(-)* mice: effect of various chemical treatments. Note that under NANC conditions, the control IJP of the mutant mice lack the slow IJP, but the resulting hyperpolarization is abolished by TTX, ATP tachyphylaxis, RB2, or apamin. The hyperpolarization response is not altered by L-NA, VIP<sub>10-28</sub> or simultaneous treatment with L-NA and L-arginine, L-NA and RB2, L-NA and MeATP, L-NA and apamin, or VIP<sub>10-28</sub> and apamin. This establishes that the enzymatic source of NO mediating the slow IJP is *ncNOS*.

channels. These observations suggest that ATP acting on P<sub>2</sub> receptors, opens apamin-sensitive potassium channels to produce the fast IJP.

Treatment of the muscle strips with apamin alone unmasks the slow IJP. Upon standard EFS, this hyperpolarization is slower to reach its nadir, but is similar in onset, duration ( $3.7 \pm 0.2$  s), and amplitude ( $9.3 \pm 0.5$  mV) to the control IJP. The apamin-resistant slow IJP is abolished by L-NA or VIP<sub>10-28</sub>. The slow component of the control IJP that is blocked by L-NA is reversed ( $3.8 \pm 0.0$  s duration,  $n = 14$  cells from three animals) by L-arginine but not by D-arginine.

**IJP in the *ncNOS(-)* mice.** Standard EFS of gastric smooth muscle strips from *ncNOS(-)* mice (Fig. 2) also produced a TTX-sensitive IJP that is  $2.1 \pm 0.1$  s in duration and  $8.2 \pm 0.4$  mV in amplitude ( $n = 22$  cells from five animals). The IJP in the mutant mice is unaffected by NOS inhibitor L-NA or by excess amounts of L-arginine. After L-NA treatment, the IJP is  $2.1 \pm 0.1$  s in duration and  $7.6 \pm 2$  mV in amplitude and after L-arginine treatment it is  $2.4 \pm 0.0$  s in duration and  $9.4 \pm 0.3$  mV in amplitude ( $n = 14$  cells from three animals). This IJP is also not affected by VIP antagonist VIP<sub>10-28</sub>. These observations demonstrate that the IJP in *ncNOS(-)* mice does not involve NO or VIP. MeATP tachyphylaxis obliterates the IJP in the *ncNOS(-)* mice. Moreover, this IJP is also abolished by RB2 ( $n = 14$  cells from two animals) and apamin ( $n = 9$  cells from three animals). These observations indicate that in *ncNOS(-)* mice, the ATP-mediated fast IJP but not the VIP/NO-mediated slow IJP is generated postjunctionally.

**Interaction between NO and VIP** The above studies suggest that VIP and NO act serially to produce the slow IJP, but they do not distinguish whether NO releases VIP or VIP re-

leases NO. Therefore we examined the effect of exogenous VIP on the smooth muscle membrane potential (Fig. 3). In the wild-type mice, VIP produces hyperpolarization of  $9.2 \pm 0.4$  mV ( $n = 5$  cells from three animals) which is abolished by pretreatment with L-NA, suggesting that VIP stimulates a form of constitutive NOS. In gastric muscle strips of *ncNOS(-)* mutant mice, however, VIP fails to produce any hyperpolarization ( $n = 4$  cells from three animals). These observations indicate that NO is derived from *ncNOS* acting downstream of VIP. Authentic NO produces a similar degree of hyperpolarization in the wild-type ( $11 \pm 0.5$  mV,  $n = 11$  cells from three animals) and *ncNOS(-)* mice ( $10.5 \pm 1.3$  mV,  $n = 11$  cells from three animals). The action of NO is not suppressed by L-NA or VIP receptor antagonist. These observations therefore demonstrate that VIP exerts its inhibitory effect through NO and not vice versa. The hyperpolarizing action of VIP in the wild-type mice is not suppressed by TTX which blocks sodium-dependent axonal action potentials. The data suggest that VIP causes IJP by its action below the site of action of TTX that includes the nerve cell body and axon of NANC neurons, i.e., the nerve terminals.

**Influence of nifedipine and  $\omega$ -conotoxin GVIA.** Nifedipine blocks voltage-dependent L-type calcium channels which play an important role in calcium entry in the smooth muscle cells. VIP has been reported to stimulate eNOS in the smooth muscle cells by stimulating L-type calcium channels. On the other hand,  $\omega$ -conotoxin GVIA blocks N-type calcium channels which are present on nerve terminals and their activation causes neurotransmitter release. To examine whether VIP acts prejunctionally on nerve terminals or postjunctionally on smooth muscle cells to elicit the hyperpolarization, we exam-

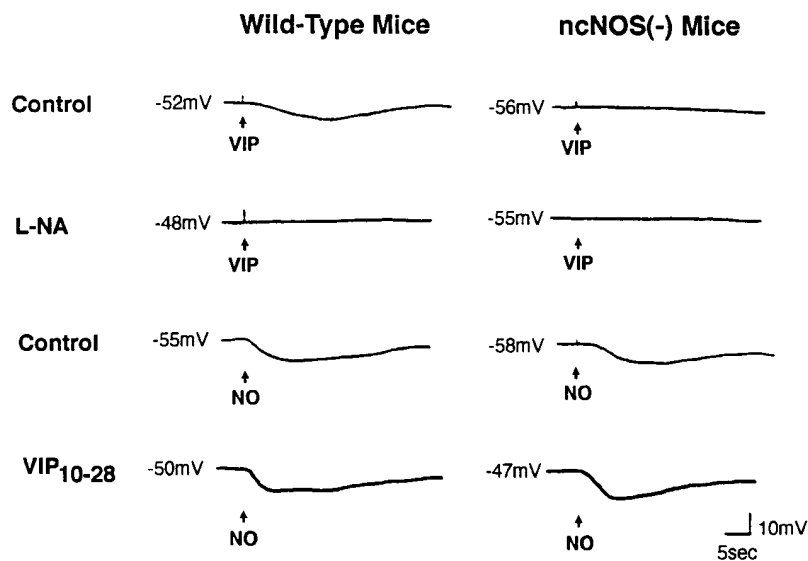


Figure 3. Effect of VIP and NO on gastric smooth muscle cell membrane potential in wild-type and ncNOS(-) mice. Note that VIP produces hyperpolarization in wild-type mice, but not in ncNOS(-) mice or tissues pre-treated with L-NA. Exogenously applied NO produces hyperpolarization in both wild-type and ncNOS(-) smooth muscle cells which is unaffected by VIP<sub>10-28</sub>. This demonstrates that VIP acts by releasing NO.

ined the effects of the two calcium channel blockers (Fig. 4). Nifedipine had no effect on the IJP or the hyperpolarizing action of VIP. However,  $\omega$ -conotoxin GVIA blocked the hyperpolarizing action of VIP, suggesting that VIP acts prejunctionally.

### Discussion

These studies show that in the gastric fundus circular smooth muscle of normal mice, EFS (in the presence of blockade of adrenergic, cholinergic, and SP responses) produces an IJP consisting of two components: the fast IJP mediated by ATP and the slow IJP mediated by NO and VIP. In contrast, the ncNOS(-) mice exhibit only the ATP-mediated fast IJP and lack the NO- and VIP-associated slow IJP. In the wild-type mice, exogenous VIP causes smooth muscle membrane hyperpolarization that is blocked by the NOS inhibitor, L-NA. In ncNOS(-) mice, however, exogenous VIP failed to produce

hyperpolarization. Moreover, the hyperpolarizing action of VIP was blocked by the N-type calcium channel blocker  $\omega$ -conotoxin GVIA, but not by the L-type calcium channel blocker nifedipine.

The serial action of both VIP and NO to effect the slow IJP in circular smooth muscle of murine stomach is similar to that reported in guinea pig ileum (18). Our findings are also consistent with mechanical studies showing that both VIP and NO are involved in EFS-induced relaxation of fundic circular smooth muscle strips (19). While some studies suggest that NO releases VIP from the myenteric plexus (20), others report that it is VIP which releases NO from myenteric neurons (21). Studies in intrinsically innervated smooth muscle preparations suggest that VIP acts by releasing NO (22). However, these studies used chemical blockers of NOS that indiscriminately suppress all forms of NOS cannot identify the enzymatic source of NO involved in inhibitory neurotransmission.

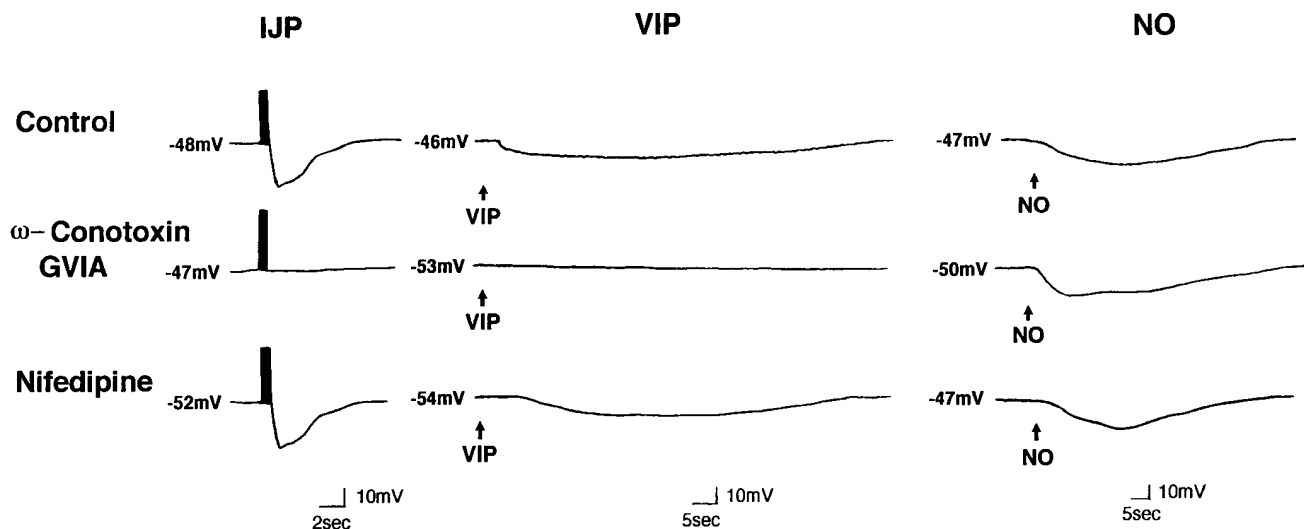


Figure 4. Effect of calcium channel blockers on the IJP and on VIP- and NO-associated hyperpolarization of gastric smooth muscle cells from wild-type mice. Note that the hyperpolarization caused by VIP is inhibited by  $\omega$ -conotoxin GVIA but not by nifedipine. The hyperpolarization caused by exogenously applied NO is unaffected by either calcium channel blockers. This suggests that VIP-induced hyperpolarization is due to the action of VIP on nerve endings rather than on smooth muscle.

Recent biochemical studies show that ecNOS is the source of NO that is produced by the action of VIP. These studies suggest that VIP stimulates VIP receptors on smooth muscle cells to cause membrane depolarization and calcium influx (20) into the smooth muscle cells. This calcium influx then activates a particulate, membrane-bound, calcium- and calmodulin-dependent constitutive isoform of NOS resembling ecNOS (10). The activation of ecNOS in the smooth muscle produces NO which causes elevation of intracellular cGMP and smooth muscle relaxation (11). By this formulation, VIP is the inhibitory neurotransmitter and NO acts as an intracellular mediator of VIP (20). The present studies, however, demonstrate that ncNOS rather than ecNOS is the source of NO that is involved in the slow IJP and the hyperpolarizing action of VIP.

Recent electron microscopic immunocytochemical studies have shown that although ncNOS is consistently and abundantly present in nerve terminals, this enzyme is also occasionally found in smooth muscle cells (23). Therefore, it is formally possible that the inhibitory neurotransmitter, VIP, exerts its effect by stimulating ncNOS in the smooth muscle cells rather than prejunctionally in the nerve endings. Arguing mostly for a VIP action prejunctionally are the observations that: (a) the inhibitory action of VIP is not blocked by TTX, indicating that VIP at least does not act by stimulating these neurons to generate sodium-dependent conducted action potentials; (b) VIP receptors have been localized on prejunctional nerve terminals (23, 24); (c) The IJP and the inhibitory action of VIP is suppressed by  $\omega$ -conotoxin GVIA but not by nifedipine. Therefore, this indicates that VIP acts on the nerve terminal to release NO. Calcium entry in the nerve terminals occur via N-type voltage-sensitive channels whereas L-type voltage-sensitive channels are the main channels of calcium entry in smooth muscle cells. VIP-induced calcium entry may stimulate calcium-calmodulin-dependent ncNOS in the nerve terminals (4). The IJP and the hyperpolarizing action of VIP is antagonized by oxyhemoglobin, supporting a role for NO as a neurotransmitter (4, 25). Oxyhemoglobin scavenges NO in the extracellular space and thereby blocks the action of NO released into the neuromuscular junction.

The present studies also support the role of ATP as one of the inhibitory motor neurotransmitters in the gastric fundus acting independent of VIP or NO. This has been debated since Okwuasaba et al. (27) reported that EFS-induced relaxation of guinea pig fundic muscle strip is suppressed by desensitization to ATP. Apamin, a toxin that blocks small conductance calcium-activated potassium channels, also suppresses the IJP and blocks the hyperpolarizing action of ATP (28) but not of VIP (29). Apamin reportedly decreases VIP release from prejunctional nerves (20), and some investigators have concluded that VIP, and not ATP, is the inhibitory neurotransmitter (30, 31). Others suggest that both ATP and VIP are involved in inhibitory neurotransmission (17, 18, 32, 33). Still others propose that ATP, like VIP, releases NO which is the final common inhibitory neurotransmitter (34, 35). Our studies with ncNOS(-) mice clearly demonstrate that ATP is involved, independent of VIP or NO, in the inhibitory neurotransmission.

Based on the findings of this study, we propose the following model for inhibitory smooth muscle neuromuscular transmission (Fig. 5). There are two parallel and overlapping inhibitory pathways. The neurotransmitter, ATP, acts on  $P_2$  receptors and opens apamin-sensitive potassium channels to produce the fast IJP. This pathway is in parallel with the VIP/

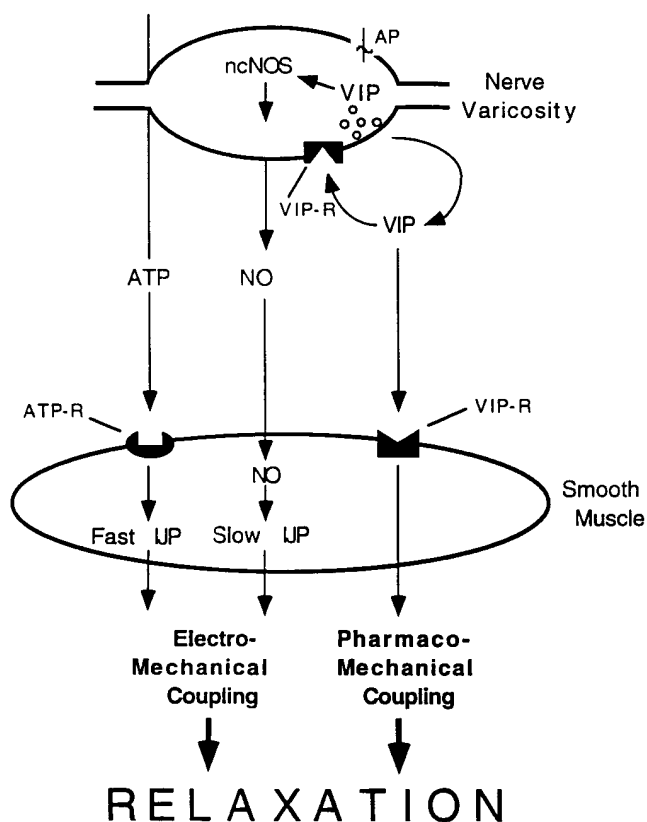


Figure 5. Model of NANC inhibitory neurotransmission at smooth muscle neuromuscular junction. ATP acts as an independent inhibitory neurotransmitter that is responsible for the fast IJP. VIP and NO interact with each other to provide a second inhibitory pathway, the slow IJP. VIP is the neurotransmitter responsible for the slow IJP. A physiological action potential (AP) at the nerve ending or EFS first initiates VIP release from neurotransmitter vesicles at the nerve varicosity. The released VIP acts prejunctionally on the same nerve ending (autocrine) or on a neighboring varicosity (paracrine) to stimulate ncNOS to produce and release on demand NO from the NANC nerve terminal. The neurotransmitter NO then acts on the postjunctional smooth muscle cells to produce the slow IJP. The fast and slow IJPs produce smooth muscle relaxation by electromechanical coupling involving suppression of calcium entry via L-type calcium channels due to membrane hyperpolarization. This model shows that VIP may also cause smooth muscle relaxation by voltage-independent pharmacomechanical coupling.

NO pathway in which VIP that is stored in the secretory granules in the nerve terminals is first released by stimulation of nerve endings. The released VIP then acts prejunctionally on VIP receptor to cause calcium entry through the N-type calcium channels. The influx of calcium causes activation of calcium-calmodulin-dependent cytosolic ncNOS to produce NO, which produces the IJP. Thus, peptide VIP acts to generate NO on demand.

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