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Autoinhibition of endothelial nitric oxide synthase (eNOS) in gut smooth muscle by nitric oxide

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Abstract

Nitric oxide in the gut is produced by nNOS in enteric neurons and by eNOS in smooth muscle cells. The eNOS in smooth muscle is activated by vasoactive intestinal peptide (VIP) released from enteric neurons. In the present study, we examined the effect of nitric oxide on VIP-induced eNOS activation in smooth muscle cells isolated from human intestine and rabbit stomach. NOS activity was measured as formation of the 1:1 co-product, L-citrulline from L-arginine. VIP caused an increase in L-citrulline production that was inhibited by NO in a concentration dependent manner ($IC_{50} \sim 25 \mu M$; maximal inhibition 72% at $100 \mu M$ NO). Basal L-citrulline production, however, was unaffected by NO. The effect was not mediated by cGMP/PKG since the PKG inhibitor KT5823 had no effect on eNOS autoinhibition. The autoinhibition was selective for NO since the co-product L-citrulline had no effect on VIP-induced NOS activation. Similar effects were obtained in rabbit gastric and human intestinal smooth muscle cells. The results suggest that NO produced in smooth muscle cells as a result of the activation of eNOS by VIP exerts an autoinhibitory restraint on eNOS thereby regulating the balance of the VIP/cAMP/PKA and NO/cGMP/PKG pathways that regulate the relaxation of gut smooth muscle.

Keywords

Vasoactive intestinal peptide; Relaxation; Human intestine; Rabbit stomach; L-citrulline

1. Introduction

Depending on the species and region of the gut, relaxation of the smooth muscle is mediated by vasoactive intestinal peptide (VIP) and related peptides, nitric oxide (NO), and adenosine triphosphate (ATP). VIP is synthesized solely in enteric neurons whereas NO is synthesized in neural and non-neural tissues by three isoforms of nitric oxide synthase (NOS): neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). There is extensive evidence to suggest that there is an important co-dependent stimulatory interplay between VIP and NO in the gut. Activation of nNOS in enteric neurons causes the release of VIP from these neurons [1,2]. VIP, in turn, activates receptors on the postsynaptic membrane to activate eNOS in gut smooth muscle [3–5]. Relaxation thus results from the combined VIP-

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induced activation of adenylate cyclase and production of cAMP, and NO-induced activation of soluble guanylate cyclase and production of cGMP[5].

A contrary inhibitory autoregulation of NOS occurs in several tissues and serves to limit the role of NO although it is not clear if this autoinhibition is mediated by NO and/or another component [6–12]. This seems to be particularly true of eNOS, the NOS isoform present in gut smooth muscle [5,15]. In the gut, autoinhibition of nNOS by NO has been suggested by functional studies of NO mediated neurotransmission [12,13]. Direct measurements of NOS activity in rat intestinal enteric synaptosomal preparations suggest that NO autoregulation of enteric nNOS may be mediated by the formation of a nitrosothiol intermediate [11].

In contrast to the evidence for autoregulation of nNOS in the gut, this relationship has not been examined with respect to the regulation of eNOS in gut smooth muscle. The fact that VIP activates eNOS in preparations of isolated gut smooth muscle cells, which are free of other cell types (enteric neurons, ICC, mast cells etc.) that contain other forms of NOS [5], provides the unique opportunity to examine the effects of exogenous NO on VIP-stimulated eNOS activity.

2. Materials and methods

2.1. Preparation of dispersed muscle cells from rabbit gastric antrum

Experimental protocols using rabbits were approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University. Muscle cells were isolated from rabbit gastric antrum as described previously [3,5]. Briefly, muscle strips were cut from the circular muscle layer and incubated for 20 min at 31 °C in a 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) medium containing 0.1% collagenase type II and 0.01% soybean trypsin inhibitor. The medium consisted of 115 mM NaCl, 5.8 mM KCl, 2.1 mM KH₂PO₄, 2 mM CaCl₂, 0.6 mM MgCl₂, 25 mM HEPES, 14 mM glucose and 2.1% essential amino acid mixture (pH 7.4). The partly digested tissues were washed with 50 ml of enzyme-free medium, and the muscle cells were allowed to disperse spontaneously for 30 min. The cells were harvested by filtration through 500 µm Nitex and centrifuged twice at 350 g for 10 min to eliminate cell debris.

2.2. Preparation of dispersed muscle cells from human intestine

Normal human jejunal segments (~ 5 cm long) were obtained from patients undergoing bypass surgery for morbid obesity according to protocols approved by the Committee on the Conduct of Human Research at Virginia Commonwealth University. Muscle cells were isolated from these segments as described previously [5] and above. After removal of the mucosa and serosa, the circular muscle layer was separated from the longitudinal layer by slicing in a Stadie-Riggs tissue slicer. The strips were digested in collagenase and HEPES medium and cells harvested as described above.

2.3. Stimulation and measurement of NO formation in isolated muscle cells

Nitric oxide production was measured in cells preloaded with L-[³H]arginine and expressed as the amount of L-[³H]citrulline which is produced as a 1:1 product of NOS with NO.

Muscle cells were incubated for 5 min in a medium containing 3 $\mu\text{Ci/ml}$ of L-[^3H]arginine and the tissue was sampled for L-[^3H]citrulline at the end of 5 min as described previously [3].

The activation of eNOS in smooth muscle cells was initiated by addition of 1 μM VIP alone in the presence of exogenous NO (1–200 μM in rabbit gastric muscle or 100 μM in human intestinal muscle). To determine the role of the cGMP–PKG signaling pathway in mediating the effect of NO, the experiment was repeated in the presence of 1 μM KT-5823 an inhibitor of PKG. In some studies, the other product of NOS, L-citrulline (1 μM), was added alone and in the presence of VIP to determine if it also might affect NO production. As ATP also participates in relaxation of gut smooth muscle, the effects of ATP (0.1 mM) on VIP induced NO production by isolated muscle cells were also tested in some studies.

2.4. Materials

VIP was purchased from Bachem (Torrance, CA), and KT-5823 from Kamiya Biomedical (Thousand Oaks, CA). NO solutions were prepared from 99% NO gas obtained from MG Industries (Valley Forge, PA) as described previously [3,4]. L-[^3H]arginine was purchased from New England Nuclear (Boston, MA).

L-citrulline and all other chemicals were purchased from Sigma (St. Louis, MO).

2.5. Statistical analysis

Data were calculated as means $\pm$ SE of n experiments using cell suspensions from n rabbit or human samples. Statistical significance was tested using ANOVA and Student's t test.

3. Results

3.1. NO production by rabbit gastric smooth muscle cells

VIP caused a significant increase in NO production, measured as an increase in the 1:1 co-product L-[^3H]citrulline in cells loaded with L-[^3H]arginine. The basal level of L-[^3H]citrulline was 1815 ± 215 dpm/ 10^6 cells; addition of NO in the range of 10–200 μM had no significant effect on basal L-[^3H]citrulline production (Fig. 1). VIP (1 μM) increased L-[^3H]citrulline production by $134 \pm 15\%$. Addition of NO in the presence of VIP inhibited the ability of VIP to induce L-[^3H]citrulline production in a concentration-dependent manner (Fig. 2). The IC_{50} was 25 ± 4 μM ; the maximal inhibition was $72 \pm 7\%$ ($38 \pm 5\%$ increase above basal L-[^3H]citrulline production) and was obtained in the presence of 1 μM VIP plus 100 μM NO.

The role of the cGMP–PKG signaling pathway in mediating the autoinhibition by NO was tested with the PKG inhibitor, KT-5823 (1 μM). This inhibitor had no effect on the VIP-induced increase in L-[^3H]citrulline ($128 \pm 31\%$ increase above basal) and did not abate the ability of NO to inhibit VIP-induced L-[^3H]citrulline production ($82 \pm 7\%$ inhibition of VIP induced L-[^3H]citrulline production) (Fig. 3).

L-citrulline (10 μM), the other product generated from L-arginine by the action of NOS had no effect on VIP-induced L-[^3H]citrulline production ($117 \pm 7\%$ increase above basal; data

not shown). Similarly, ATP had no effect on the VIP-induced increase in L-[³H]citrulline production ($130 \pm 10\%$ increase above basal; data not shown).

3.2. NO production by human intestinal smooth muscle cells

Similar to the rabbit gastric smooth muscle, addition of $1 \mu\text{M}$ VIP to isolated human intestinal smooth muscle cells caused a significant $65 \pm 5\%$ increase above basal L-[³H]citrulline production (basal: $2754 \pm 222 \text{ dpm}/10^6 \text{ cells}$) (Fig. 4). Addition of exogenous NO ($100 \mu\text{M}$) caused an $89 \pm 6\%$ inhibition of VIP-induced L-[³H]citrulline production ($8 \pm 2\%$ above basal) (Fig. 5) but had no effect on basal L-[³H]citrulline production (Fig. 4). As with the rabbit gastric muscle cells, the inhibitor of the cGMP–PKG signaling pathway, KT 5823 ($1 \mu\text{M}$) had no effect on the ability of NO to inhibit VIP-induced L-[³H]citrulline production. In the presence of KT 5823, $100 \mu\text{M}$ NO caused a $90 \pm 2\%$ inhibition of the VIP-induced L-[³H]citrulline production ($5 \pm 3\%$ increase above basal) (Fig. 5). Also similar to rabbit gastric smooth muscle, neither L-citrulline nor ATP had an effect on the VIP-induced increase in L-[³H]citrulline production ($73 \pm 5\%$ and $62 \pm 7\%$ increase above basal, respectively; data not shown).

4. Discussion

The results of the present study demonstrate that NO itself causes a concentration-dependent autoinhibition of VIP-activated eNOS in isolated smooth muscle cells. The results are similar in muscle cells isolated from rabbit stomach and human intestine, although the limited amounts of human intestine prevented full concentration–response curves from being constructed. To our knowledge, this is the first demonstration of NO autoinhibition and its role in regulating VIP-mediated relaxation in gut smooth muscle of human or any other species. It is noteworthy also that in the present study autoinhibition is demonstrated in an intact cell system in which NOS is stimulated by a neuropeptide. The majority of earlier studies on autoinhibition of NOS by NO have been conducted in tissue homogenates, purified enzyme, or computer simulations. The presence of the other intact signaling pathways in the native cell that are activated by VIP provides a more physiological setting in which to interpret NO autoregulation than would be provided in a cell homogenate or purified enzyme system.

Consistent with the findings in tissue homogenates and in purified NOS preparations, the results in intact gut smooth muscle cells from human and rabbit further suggest that the co-product of eNOS activation, L-citrulline, has no autoinhibitory property in either region or species examined. This is consistent with the failure of L-citrulline to inhibit nNOS in enteric neurons of rat [2] and in cerebellar neurons [8].

The present study did not identify the mechanism by which NO exerts its autoinhibition. In gut smooth muscle, NO produces relaxation mainly via activation of the cGMP–PKG pathway [4]. PKG has been shown to exert an inhibitory feedback regulation of soluble guanylate cyclase and the NPR-C receptor activated as part of this VIP pathway [14] so it seemed possible that PKG might also mediate the autoinhibition of eNOS activated as a part of the VIP relaxation pathway. We tested this notion by using the PKG inhibitor KT-5823. The failure of this agent to inhibit autoinhibition suggests that the cGMP–PKG pathway is

not involved. This is consistent with other systems such as mitochondrial respiratory enzymes in rat hepatocytes where NO has been found to be inhibitory via a cGMP-independent mechanism [12]. Several regulatory mechanisms have been proposed for NOS including the presence of inter- and intradomain electron transfer control elements, redox modulation of active site cysteines, and binding of NO to the heme group in NOS [6,7,9]. It is also possible that multiple reactive species participate in NO autoregulation. In two studies of nNOS from pig cerebellum [6,8], authentic NO was found to be the only agent to be able to completely inhibit NOS activity; $(\text{NO}_2)^-$ and $(\text{NO}_3)^-$ were ineffective, and ONOO^- produced only a 39% inhibition of NOS activity. Similarly, in an alveolar macrophage cell line, neither $(\text{NO}_2)^-$ nor $(\text{NO}_3)^-$ had any effect on NOS activity [13]. In contrast, in rat enteric synaptosomes, NO has been postulated to act via a nitrosothiol intermediate [11]. Thus, it is possible that in our system, some of the effects of added NO could be attributed to oxidation products generated downstream from NO. We did not investigate this possibility since the ultimate physiological effect is the same, namely, inhibition of NO generation.

It is also noteworthy that the NOS isoform activated by VIP in gut smooth muscle is of the eNOS subtype [5]. Our previous studies have demonstrated that the VIP-induced generation of NO in isolated smooth muscle cells in response to VIP is solely due to VIP-induced activation of NOS because VIP-induced L-[^3H]citrulline production is abolished by the NOS inhibitor L-NNA and the VIP antagonist, VIP10-28 [4,15]. This notion is also supported by studies in smooth muscle from guinea pig tenia coli where eNOS is absent and the VIP receptor is a unique VIP-specific receptor [5,16]. In this case there is no VIP-induced generation of L-[^3H]citrulline. Studies of various isoforms of NOS and differing autoregulatory mechanisms indicate that the eNOS isoform has much lower maximal enzymatic activity than nNOS or iNOS [9]. This may be due to the presence of an autoinhibitory amino acid loop in the reductase domain of eNOS that impairs electron transfer [7,9]. Additionally, it is interesting to note that the constitutive forms of NOS require a much lower concentration of NO to elicit autoinhibition than the iNOS isoform (10 vs 100 μM) [13]. This is consistent with the IC_{50} of about 24 μM for NO autoinhibition of eNOS in gut smooth muscle as noted in the present study. The 10–200 μM range of concentrations used in the present study is similar to those used in other studies of autoinhibition of NOS in cells and homogenates [6–8,13]. It should be noted that the concentration is the calculated solution concentration based on addition of NO to the medium; however, since NO is rapidly oxidized, the actual final concentrations are unknown. In the study by Rengasamy and Johns [6], NO added to a calculated final concentration of 100, 200 and 400 μM actually resulted in measured concentrations of 0.16, 0.28, and 0.56 μM . It is likely that considering that in our preparation, NO must traverse the cell membrane and diffuse to its site of action inside the smooth muscle cell, the actual concentrations are likely to be much less than calculated. It is also likely that in the physiological condition of generation of NO within the cell, NO concentrations may be at or above the effective concentrations for autoinhibition determined in the present study.

Although addition of NO significantly inhibited the VIP-induced L-[^3H]citrulline production at all concentrations, it had no effect on the basal level of L-[^3H]citrulline production both in rabbit gastric or human intestinal smooth muscle cells. This might be explained in two ways: the eNOS is not susceptible to autoinhibition at low levels of activity such as present in the

basal state or NO might be acting at a point upstream between the NPR-C receptor that is activated by VIP and activation of eNOS itself. Studies of the purified NOS enzyme indicate that it partitions between a productive cycle that generates NO and a nonproductive cycle that does not generate NO [17–19]. The latter is dependent on the formation of a heme–NO complex and results in autoinhibition. The formation of the heme–NO complex is dependent on the concentration of NO and since eNOS is particularly slow and does not generate high levels of NO to induce autoinhibition [17], it is likely that the low level of activity of eNOS in the basal state is insufficient to form the heme–NO complex. It might be expected that with the addition of NO to the bathing solution, higher levels of NO might lead to formation of the heme–NO complex and activation of the nonproductive cycle. This might not be the case if the solution NO is rapidly oxidized and NO concentrations rapidly fall as noted above for solutions [6]. If one considers that in the present study, NO must additionally enter the cell where it is exposed to further oxidation, it is not surprising that there is no autoinhibition of basal L-[³H]citrulline production during the 5 minute study period. In contrast, VIP-induced activation of the eNOS within the cell is likely to generate high local intracellular concentrations in the vicinity of eNOS. In this case, addition of solution NO likely adds to endogenous NO and enhances formation of the heme–NO complex leading to nonproductive cycling and autoinhibition. The second possibility is that solution NO is acting upstream of eNOS to prevent VIP binding to the NPR-C receptor, Ca²⁺ influx, or binding of the Ca²⁺–calmodulin complex to eNOS. The notion that these sites of action might explain the difference in autoinhibition between basal and VIP-stimulated states of the enzyme seems less likely. We have previously tested the effect of NO and L-NNA on VIP binding to isolated muscle cells and found no effect (unpublished observations). Most studies on autoinhibition of NOS by NO have been done in tissue homogenates or pure enzyme preparation where Ca²⁺ and calmodulin are added in excess; thus, if NO autoinhibition was to occur through regulation of these intermediates, it would not occur in these systems. Although we cannot rule out this latter possibility, it is most likely that NO acts by directly binding to the heme moiety of eNOS as indicated in Fig. 6.

The notion that NO can act in an autoinhibitory manner has significant implications in the regulation of gut function. VIP, NO and ATP are the main transmitters mediating relaxation of the gut. In the present study, ATP did not affect the ability of VIP to induce NO production. VIP and NO, however, are intertwined at the postsynaptic level as shown in the present study as well as at the presynaptic level as illustrated in Fig. 6. Thus, NO stimulates VIP release from enteric neurons [1,2] and VIP in turn generates NO in postjunctional smooth muscle by activating eNOS ([3–5], reviewed in Ref. [14]). Studies in innervated preparations of gut in which non-adrenergic, non-cholinergic relaxation, which is mediated by both NO and VIP, was elicited suggested that the relaxation is limited by an autoinhibitory effect of NO [20,21]. Studies of enteric synaptosomes from rat suggested that this autoinhibitory effect occurs as an interaction between NO and nNOS in the nerve terminal [2,11]. The present study demonstrates additionally, that the autoinhibitory effect of NO is exerted on the second source of NO mediating relaxation, namely the VIP-activated eNOS of the smooth muscle cell. It is noteworthy that in this latter case, the limiting effect of autoinhibition is directly on the relaxation initiated by the regulatory neuropeptide VIP rather than on the relaxation initiated by NO released from the enteric inhibitory neuron.

Thus, this restraining mechanism must be considered in combination with other negative feed back pathways that regulate the level of VIP-induced relaxation such as the feedback regulation of adenylate cyclase by PKA, and the feedback regulation of guanylate cyclase and NPR-C by PKG (see Ref. [14] for review). The combination of these autoregulatory and feedback pathways serve to regulate the amount of VIP and NO-mediated relaxation that occurs as part of receptive or adaptive relaxation in the stomach and as part of the descending relaxation component of the peristaltic reflex in intestine and colon. The degree of NO autoinhibition can also shift the balance between the VIP/cAMP/PKA pathway and the NO/cGMP/PKG pathway, both of which mediate gut smooth muscle relaxation [4,14]. This becomes important in examining which pathway is responsible for relaxation at different levels of stimulation. At low concentrations, the relaxation induced by VIP is largely mediated by NO–GMP–PKG pathway in smooth muscle cells whereas with increasing concentrations of VIP, the role of the cAMP–PKA pathway becomes more important [4,15]. Thus at low levels of stimulation of inhibitory nerves, the role of NO autoinhibition would significantly affect relaxation whereas with increasing stimulation, NO autoinhibition would play less of a role in determining the degree of relaxation. Physiologically, this may play a role in matching relaxation levels to the need for descending relaxation during peristalsis in intestine and during gastric receptive relaxation and accommodation.

In summary, the present study demonstrates that in human and rabbit gut smooth muscle, NO generated as a result of VIP binding to NPR-C receptors and activation of VIP-dependent eNOS exerts an autoregulatory effect on eNOS activity and thereby attenuates VIP-induced relaxation. The effect is unique to NO and is not shared by the co-product L-citrulline. Combined with the similar autoregulatory regulation of nNOS in enteric neurons, this may shift the balance between the relative roles of NO and other relaxant transmitters in mediating propulsive motility.

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References

- [1]. Grider JR, Jin JG. Vasoactive intestinal peptide release and l-citrulline production from isolated ganglia of the myenteric plexus: evidence for regulation of vasoactive intestinal peptide release by nitric oxide. *Neurosci.* 1993; 54:521–6.
- [2]. Allescher HD, Kurjak M, Huber A, Trudrung P, Schusdziarra V. Regulation of VIP release from rat enteric nerve terminals: evidence for a stimulatory effect of NO. *Am J Physiol.* 1996; 271:G568–74. [PubMed: 8897874]
- [3]. Grider JR, Murthy KS, Jin JG, Makhlof GM. Stimulation of nitric oxide from muscle cells by VIP: prejunctional enhancement of VIP release. *Am J Physiol.* 1992; 262:G774–8. [PubMed: 1566853]
- [4]. Jin JG, Murthy KS, Makhlof GM. Stoichiometry of neurally induced VIP release, NO formation, and relaxation in rabbit and rat gastric muscle. *Am J Physiol.* 1996; 271:G357–69. [PubMed: 8770052]

- [5]. Teng BQ, Murthy KS, Kuemmerle JF, Grider JR, Sase K, Michel T, Makhoul GM. Expression of endothelial nitric oxide synthase in human and rabbit gastrointestinal smooth muscle. *Am J Physiol.* 1998; 275:G342–51. [PubMed: 9688662]
- [6]. Rengasamy A, Johns RA. Regulation of nitric oxide synthase by nitric oxide. *Molec Pharmacol.* 1993; 44:124–8. [PubMed: 7688067]
- [7]. Hu H, Xin M, Balayev LL, Zhang J, Block ER, Patel JM. Autoinhibitory domain fragment of endothelial NOS enhances pulmonary artery vasodilation by the NO–cGMP pathway. *Am J Physiol.* 2004; 286:L1066–74.
- [8]. Kotsonis P, Frey A, Frohlich LG, Hofmann H, Reif A, Wink DA, Feelisch M, Schmidt HHW. Autoinhibition of neuronal nitric oxide synthase: distinct effects of reactive nitrogen and oxygen species on enzyme activity. *Biochem J.* 1999; 340:745–52. [PubMed: 10359660]
- [9]. Nishida CR, Montellano PR. Autoinhibition of endothelial nitric-oxide synthase. *J Biol Chem.* 1999; 274:14692–8. [PubMed: 10329664]
- [10]. Gorren AC, Schrammel A, Riethmuller C, Schmidt K, Koesling D, Werner ER, Mayer B. Nitric oxide-induced autoinhibition of neuronal nitric oxide synthase in the presence of the autooxidation-resistant pteridine 5-methyltetrahydrobiopterin. *Biochem J.* 2000; 347:475–84. [PubMed: 10749677]
- [11]. Kurjak M, Koppitz P, Schusdziarra V, Allescher HD. Evidence for a feedback inhibition of NO synthesis in enteric synaptosomes via a nitrosothiol intermediate. *Am J Physiol.* 1999; 277:G875–84. [PubMed: 10516155]
- [12]. Stadler J, Billiar TR, Curran DD, Stuehr DJ, Ochoa JB, Simmons RL. Effect of exogenous and endogenous nitric oxide on mitochondrial respiration of rat hepatocytes. *Am J Physiol.* 1991; 260:C910–6. [PubMed: 1903597]
- [13]. Griscavage JM, Rogers NE, Sherman MP, Ignarro IJ. Inducible nitric oxide synthase from a rat alveolar macrophage cell line is inhibited by nitric oxide. *J Immunol.* 1993; 151:6329–37. [PubMed: 7504017]
- [14]. Murthy KS. Signaling for contraction and relaxation in smooth muscle of the gut. *Annu Rev Physiol.* 2006; 68:345–74. [PubMed: 16460276]
- [15]. Murthy KA, Zhang KM, Jin JG, Grider JR, Makhoul GM. VIP-mediated G protein-coupled Ca^{2+} influx activates a constitutive NOS in dispersed gastric muscle cells. *Am J Physiol.* 1993; 265:G660–71. [PubMed: 7694477]
- [16]. Zhou H, Huang J, Murthy K. Molecular cloning and functional expression of a VIP-specific receptor. *Am J Physiol.* 2006; 291:G728–34.
- [17]. Abu-Soud HM, Ichimori K, Presta A, Stuehr DJ. Electron transfer, oxygen binding, and nitric oxide feedback inhibition in endothelial nitric-oxide synthase. *J Biol Chem.* 2000; 275:17349–57. [PubMed: 10749853]
- [18]. Santolini J, Meade AL, Stuehr DJ. Differences in three kinetic parameters underpin the unique catalytic profiles of nitric-oxide synthases I, II, and III. *J Biol Chem.* 2001; 276:48887–98. [PubMed: 11684690]
- [19]. Stuehr DJ, Santolini J, Wang ZQ, Wei CC, Adak S. Update on mechanism and catalytic regulation in the NO synthases. *J Biol Chem.* 2004; 279:36167–70. [PubMed: 15133020]
- [20]. De Man JG, Boeckstaens GE, De Winter BY, Moreels TG, Herman AG, Pelckmans PA. Inhibition of non-adrenergic, non-cholinergic relaxations by nitric oxide donors. *Eur J Pharmacol.* 1995; 285:269–74. [PubMed: 8575513]
- [21]. Hosoda K, Takahashi T, Fujino M, Owyang C. Inhibitory effects of nitric oxide donors on nitric oxide synthase in rat gastric myenteric plexus. *J Pharmacol Exp Ther.* 1998; 286:1222–30. [PubMed: 9732382]

Fig. 1. Concentration-dependent effect of exogenous NO (10–200 μM) on basal L-[³H] citrulline production in suspensions of smooth muscle cells isolated from rabbit stomach. Data are expressed as dpm/10⁶ cells. Values are means ± SEM of 4–6 experiments.

Fig. 2.

Concentration-dependent effect of exogenous NO (10–200 μ M) on L-[³H]citrulline production induced by addition of 1 μ M VIP to suspensions of smooth muscle cells isolated from rabbit stomach. Data are expressed as % increase in L-[³H]citrulline production above basal levels of 1815 ± 215 dpm/ 10^6 cells. Values are means $\pm$ SEM of 4–6 experiments. * = $p < 0.05$; ** = $p < 0.01$.

Fig. 3.

Lack of effect of the PKG inhibitor, KT-5823 (1 μ M) on the autoinhibition of VIP (1 μ M)-induced L-[³H]citrulline produced by NO (100 μ M) in suspensions of smooth muscle cells isolated from rabbit stomach. Data are expressed as % increase in L-[³H]citrulline production above basal levels. Values are means $\pm$ SEM of 4–6 experiments. ** = $p < 0.01$.

Fig. 4. Effect of exogenous NO (100 μM) on basal L-[³H]citrulline production in suspensions of smooth muscle cells isolated from human intestine. Data are expressed as dpm/10⁶ cells. Values are means ± SEM of 3 experiments.

Fig. 5.

Effect of exogenous NO (100 μM) in the absence and presence of the PKG inhibitor, KT-5823 (1 μM), on L-[³H]citrulline production induced by addition of 1 μM VIP to suspensions of smooth muscle cells isolated from human intestine. Data are expressed as % increase in L-[³H]citrulline production above basal levels of 2754 ± 222 dpm/ 10^6 cells. Values are means $\pm$ SEM of 3 experiments. ** = $p < 0.01$.

Fig. 6.

Model of the interplay between VIP and NO and autoinhibition by NO. Activation of nNOS produces NO which stimulates the release of VIP from enteric neurons [1,2] but which also causes autoinhibition of nNOS in the presynaptic varicosity [2,11]. VIP activation of the NPR-C receptor on the smooth muscle cell causes Ca^{2+} influx which activates a membrane bound eNOS [14,15]. NO activates the cGMP signaling pathway leading to relaxation; however neither this pathway nor the co-product of eNOS activation, L-citrulline, appears to mediate the autoinhibition. It is possible that NO generated by nNOS in the enteric inhibitory neuron and which diffuses into the smooth muscle cell may also participate in autoinhibition of eNOS. Autoinhibition is depicted as occurring at the site of the membrane bound eNOS enzyme although an additional action upstream of eNOS cannot be completely ruled out.