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Dual actions of S-nitrosylated derivative of vasoactive intestinal peptide as a vasoactive intestinal peptide-like mediator and a nitric oxide carrier

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Abstract

Vasoactive intestinal peptide (VIP) has been postulated as a non-adrenergic non-cholinergic (NANC) transmitter in the relaxation of vascular and non-vascular systems. In order to synergize the vasoactivities of VIP with nitric oxide (NO), we synthesized a S-nitrosylated derivative of VIP, VIP-Gly-Cys-NO (VIPGC-NO). On aortic rings, VIPGC-NO exhibited a dose-dependent vasorelaxation similar to *S*-nitrosoglutathione (GSNO), and both induced complete vasorelaxation at 1 μM, whereas, VIP at 1 μM only produced 19% relaxation. The degree of vasorelaxation was proportional to the increases in cyclic GMP with no significant enhancement in cyclic AMP (cAMP) level. On precontracted tracheal rings, VIP, VIPGC-NO, VIPGC and GSNO produced relaxation with EC_{50} of 74 ± 5, 32 ± 6, 59 ± 9, and 251 ± 32 nM, respectively, which was consistent with increases in cyclic GMP (cGMP). A marked increase in cAMP was observed from the tracheal rings pretreated with VIP, VIPGC-NO and its parent VIP-Gly-Cys (VIPGC) as well as isoproterenol. Propranolol only blocked the airway relaxation induced by isoproterenol, but did not antagonize the relaxation induced by VIP, VIPGC and VIPGC-NO. On rabbit sphincter of Oddi, VIP, VIPGC-NO and VIPGC inhibited both basic and acetylcholine-induced contraction frequency and amplitude, whereas, GSNO was less potent than VIP and its derivatives over a range of 2 log units in this respect. On rat gastric fundus, these compounds inhibited contraction amplitude and frequency induced with 5-hydroxytryptamine (5-HT) in the order of inhibitory potency VIP > VIPGC-NO > VIPGC > isoproterenol > GSNO. Our data suggest that: (1) NO is selective in relaxing vascular smooth muscle via the cGMP pathway, whereas VIP is selective in relaxing non-vascular smooth muscles via the activation of both cGMP and cAMP pathways; (2) VIPGC-NO preserves the intrinsic function of VIP but acquires NO-like vasoactivities.

Keywords: VIP (vasoactive intestinal polypeptide); S-nitrosylated-VIP; Nitric oxide (NO); *S*-nitrosoglutathione (GSNO); NANC (non-adrenergic non-cholinergic); Smooth muscle; cAMP; cGMP

1. Introduction

Vasoactive intestinal polypeptide (VIP), a 28 amino acid peptide, is widely distributed in the central and peripheral nervous system as an inhibitory neurotransmitter (Stretton et al., 1991), and in the most vascular beds where it functions as a vasodilator. In addition, VIP relaxes tracheobronchial and gastrointestinal smooth muscle. Recent studies indicate that VIP may be a putative neurotransmitter of the non-adrenergic non-cholinergic (NANC) inhibitory neurons, and further document a link between NANC relaxation and nitric oxide (NO) activity (D'Amato et al., 1992; Belvisi et al., 1993). There is evidence that VIP and NO may be co-released and act as functional antagonists of cholinergic bronchoconstriction (Barnes et al., 1991). For example, a VIP antibody only partially reduced the responses to NANC stimulation, and the residual responses were further reduced by a NO synthase inhibitor in the presence of VIP antibody (Li and Rand, 1990).

S-nitrosylation of amino acids (Jia and Blantz, 1998), peptides and proteins such as hemoglobin, albumin and tissue-type plasminogen activator (Jia et al., 1996; Simon et al., 1996; Stamler et al., 1997) has been reported to confer NO-like biological activities on the thiol-containing parent molecules under physiological conditions and appears to have regulatory consequences. The fact that NO, a split product of *S*-nitrosothiols, has led to the suggestion

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that the biological activities of *S*-nitrosothiols are a direct consequence of releasing NO to the target tissues. Although the decomposition of most of *S*-nitrosothiols has not been characterized in detail, it probably occurs by homolytic cleavage of the S–N bond under physiological condition, resulting in the production of NO and the corresponding R group with either SH or respective disulfide attached (Kharitonov et al., 1995; Singh et al., 1996; Keshive et al., 1996). This may consequently provide us new compounds with dual actions, one resulting from the parent RS (where R can be any one of a large range of chemical entities), and another from split NO. Indeed, *S*-nitrosocaptopril crystals have been found possessing the abilities of both an angiotensin converting enzyme inhibitor and an NO donor (Jia and Blantz, 1998). Both NO and VIP regulate smooth muscle tone, but neither has been emphasized, in part due to either the limited half-life or potency. One strategic approach to improve efficacy of each may be to achieve synergic effects of the two compounds. Therefore, it is of great interest to synthesize a prototypic VIP derivative with a cysteine as an N-terminal residue for covalent attachment of the NO group to sulfhydryl in cysteine, which may exhibit both direct VIP-like properties and NO-like vasodilation, resulting in synergic effects of VIP and NO. In this study, we synthesized a S-nitrosylated derivative of VIP, i.e., VIP-Gly-Cys-NO (VIPGC-NO), and investigated whether the intrinsic bioactivities of VIP were influenced by the presence of the NO group in the various isolated tissues, and the results were compared to those obtained with VIP, VIP derivative VIP-Gly-Cys (VIPGC) and a long acting NO donor, namely *S*-nitrosoglutathione (GSNO).

2. Methods and materials

2.1. Preparations of rabbit aortic rings and tension recording

The preparation of rabbit aortic rings was similar to that described previously (Jia and Furchgott, 1993). Briefly, male New Zealand rabbits weighing 1.5 to 2.0 kg were anesthetized by an intravenous injection of sodium pentobarbital (40 mg/kg) into a marginal ear vein. The descending thoracic aorta was quickly removed and placed in ice-cold Krebs' solution containing the following composition (mM): NaCl, 118; KCl, 4.8; $MgSO_4$, 1.2; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 11; Na_2EDTA, 0.03. The thoracic aorta was first trimmed free of most adipose and connective tissue. It was then cut into transverse rings by placing it at a right angle across five parallel razor blades mounted at 2.5 mm intervals in a plastic holder and then rolling a large polypropylene pipette tip over it (simultaneously producing four rings of equal size). The aortic rings and other tissues (see the following) used in this study, were mounted on pairs of L-shaped hooks and suspended in Krebs' solution (gassed with 95% O_2/5% CO_2, 37°C) in 20-ml organ chambers unless stated otherwise.

Tension was measured isometrically using Grass FT03C transducers, and was displayed on model 7 Grass polygraphs (Grass Instruments, Quincy, MA). Rings and other tissues used (see the following) in this study were allowed to equilibrate for at least 60 min with four times rinse before experiments were begun unless stated otherwise. Basal tension was maintained at approximately 2 g. Most experiments were carried out on sets of four rings from the same aorta. To allow studies on relaxation, each ring was precontracted submaximally (30–70% of maximum tone) by addition of 200 nM phenylephine to the bathing solution. Results are expressed in percentage of relaxation of phenylephine-induced tone.

2.2. Preparation of airways

Male Hartley guinea pigs (300–400 g) were anesthetized by use of intraperitoneal injection of pentobartital sodium (40 mg/kg) to achieve a deep plane of anesthesia. The tracheas were dissected out, transferred to cold Krebs' solution. Tracheas were then dissected free from surrounding fat and connective tissue and cut transversely between the segments of cartilage, so as to give tracheal rings about 2 mm thick. The rings were suspended between stainless-steel hooks in the 20-ml organ baths containing the above-mentioned Krebs' solution at 37°C. The hooks were connected to the FT03C transducers. Rings were equilibrated at a basal tension of 1 g as described above and then primed twice with methacholine (100 nM). The rings were rinsed throughout after each priming exposure. Tracheal rings were not used if they could not sustain at least 1 g tension when exposed to 100 nM methacholine. For relaxation studies, methacholine (10 μM) was used to contract the tracheal rings and results are expressed in percentage of relaxation by VIP, VIPGC, VIPGC-NO, GSNO and isoproterenol of methacholine-induced tone.

2.3. Preparation of rabbit sphincters of Oddi

The preparation of rabbit sphincters of Oddi was similar to that described previously (Slivka et al., 1994) with minor modifications. Briefly, the rabbit abdomen was cut open at the midline. The gall bladder, bile duct, gastric antrum and contiguous duodenum were isolated, removed en bloc, and transferred to ice cold Krebs' solution at pH 7.4. The duodenum was opened along the anti-mesenteric border as was the adjacent pylorus and gastric antrum. From the luminal surface, the ampulla of Vater was identified and the sphincters of Oddi isolated on ice under an illuminated magnifier by sharp dissection.

The sphincters of Oddi were mounted on pairs of the hooks and suspended in organ chambers containing the Krebs' solution at 37°C. Resting tension was adjusted to

1 g, and circular contractions were monitored by the FT03C force transducer and recorded on the model 7 Grass polygraphs. The sphincters of Oddi were allowed to equilibrate as described above. To investigate the direct effects of VIP and VIPGC-NO on the contraction frequency and amplitude of sphincters of Oddi, varying concentrations of the two compounds as well as VIPGC and GSNO were added to organ baths. Some experiments were performed by using acetylcholine to intensify contractility of sphincters of Oddi, and then the effects of VIP, VIPGC-NO, VIPGC and GSNO on contractility of sphincter of Oddi. After each experimental observation was complete, the sphincters of Oddi were washed at least three times with fresh Krebs' solution.

2.4. Preparation of rat gastric fundus

Male and female Sprague–Dawley rats weighing 200–300 g were anesthetized with 40 mg/kg sodium pentobarbital given intraperitoneally. After deep anesthesia was obtained, exsanguination was accomplished by severing both the jugular vein and common carotid artery. The abdomen was cut open at the midline and the stomach was removed, and transferred to the ice cold oxygenated Krebs' solution at pH 7.4. The stomach was dissected out and the pink pyloric end cut away from the grey fundal end. The fundal end was split open so as to form a sheet, the contents were washed away. Longitudinal muscle strips (3×20 mm) of the gastric fundus were prepared and mounted under a 1-g resting tension inside the organ baths containing 20 ml of the gassed Krebs' solution. The Krebs' solution also contained atropine (1 μM) and guanethidine (4 μM) to block cholinergic and adrenergic involvement and 5-hydroxytryptamine (5-HT) (5–10 μM) to raise the tone of the smooth muscle. Indomethacin (10 μM) was present in Krebs' to avoid the influence of endogenous prostaglandins. Tissues were allowed to equilibrate for 1 h with changes of Krebs' solution every 10 min. After the equilibration period, relaxations of smooth muscle were elicited by VIP, VIPGC, VIPGC-NO, isoproterenol and GSNO.

2.5. Measurement of cyclic nucleotide

In order to assess the mechanism of relaxation induced by VIPGC-NO and its analogs, tissues exposed to these compounds to the period of the peak relaxation (10 min required for vassel relaxation, 5 min and 20 min required for tracheal relaxation induced by isoproterenol and VIPGC-NO and its analogs, respectively) were immediately frozen in liquid nitrogen. Frozen tissues were homogenized in ice-cold 6% trichloroacetic acid to give approximately a 10% (w/v) homogenate. The homogenates were centrifuged at $2000 \times g$ for 15 min at 4°C. The supernatant fractions were decanted off the pellets and washed four times with 5 volumes of water-saturated ethyl ether, and the aqueous extracts remaining were saved for assay for cyclic GMP (cGMP) and cyclic AMP (cAMP) using enzyme-immunoassay kits (Amersham Life Science, Arlington Heights, IL).

2.6. Materials

VIPGC (amide) was synthesized by Analytical Biotechnology Services (Boston, MA). The purity of VIPGC was checked using nuclear magnetic resonance, infrared spectra and high performance liquid chromatography. VIPGC-NO was prepared by reaction of VIPGC (0.5 mM in 0.5 N HCl) with equimolar $NaNO_2$ at room temperature. The formation of VIPGC-NO in the mixture was directly determined by using the modified colorimetric assay of Saville (Todd and Gronow, 1969; Jia et al., 1996). The molar ratio for *S*-NO/VIPGC was determined by Saville assay for *S*-NO formation using GSNO as the *S*-NO standard and by Coomassie Plus protein assay (Pierce Chemical, Rockford, IL) for peptide amount using bovine serum albumin as the peptide standard, respectively. To avoid photo-effects on VIPGC-NO stability, VIPGC-NO was sealed in eppendorf test tubes wrapped in the aluminum foil when an experiment was going on. Low molecular weight GSNO was synthesized using established methods with minor modification (Jia et al., 1996). All other chemicals were purchased from Sigma (St. Louis, MO).

2.7. Statistics

All results are presented as mean ± S.E.M. Only one S.E.M. is shown either above or below the mean to improve figure clarity. Unless otherwise noted, paired samples were compared by Student's *t*-test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Effects of VIP, VIPGC, VIPGC-NO and GSNO on rabbit aortic rings

In intact aortic rings precontracted with phenylephrine (100 nM), the addition of VIPGC-NO produced relaxation in a dose-dependent manner, which was also observed in the rings exposed to GSNO in a dose range from 10 nM to 1 μM. However, in time-matched aortic rings from same rabbit, successively cumulative additions of VIP and VIPGC produced certain degree of relaxation only at 1 μM (Fig. 1).

On aortic rings of rabbits, single doses (1 μM) of VIPGC-NO induced complete vasorelaxation that was of rapid onset and sustained duration (Fig. 2). In comparison with VIPGC-NO, the same doses of VIP only produced 19 ± 4% ($n = 3$) relaxation.

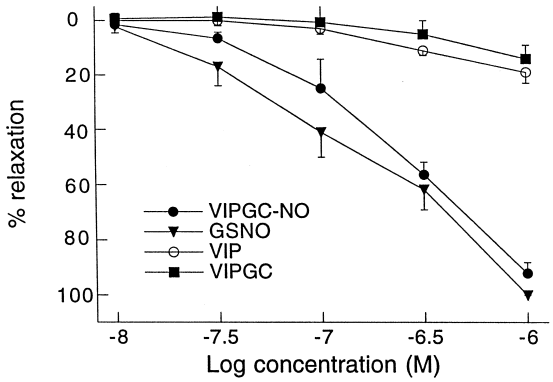

Fig. 1. Effects of VIPGC-NO, VIP, VIPGC and GSNO on rabbit aortic rings precontracted with phenylephrine. Each point represents mean ± S.E.M. of five to seven experiments.

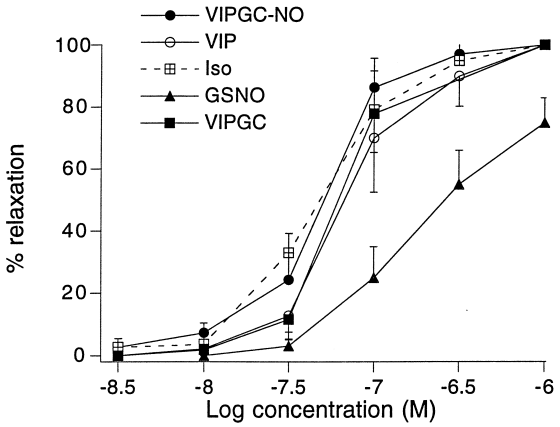

Fig. 3. Comparison of relaxation effects of VIPGC-NO, VIP, VIPGC, GSNO and isoproterenol on tracheal rings of guinea pig precontracted with 10 μM methacholine. Results are presented as mean ± S.E.M. of five separate experiments.

3.2. Effects of VIP, VIPGC, VIPGC-NO and GSNO on guinea-pig trachea

To compare the potency of VIP and its nitrosylated analogue VIPGC-NO in relaxing airway, dose–response curves for VIP, VIPGC-NO and VIPGC as well as GSNO in the isolated guinea-pig trachea precontracted with 10 μM methacholine were first made (Fig. 3). VIP, VIPGC-NO, VIPGC and GSNO produced relaxation of tracheal rings with EC_{50} of 74 ± 5, 32 ± 6, 59 ± 9, and 251 ± 32 nM (all $n = 4$), respectively. In contrast, isoproterenol caused relaxation of the tracheal rings with EC_{50} of 47 ± 3 nM. The relaxation of guinea-pig trachea by VIP, VIPGC-NO and VIPGC (all 100 nM) reached a plateau in about 20 min. Isoproterenol (100 nM) reached a plateau of tracheal relaxation in about 5 min. Pretreatment of the tracheal rings with 100 nM propranolol blocked the airway relaxation induced by cumulative additions of isoproterenol in a dose range 10–320 nM. However, propranolol did not antagonize the relaxation induced by VIP, VIPGC, and VIPGC-NO in the same dose range.

3.3. Effects of VIP, VIPGC, VIPGC-NO and GSNO on sphincters of Oddi

After being equilibrated in oxygenated Krebs at 37°C for about 30 min, all isolated rabbit sphincters of Oddi recovered intrinsic rhythmic contractility. The average contraction frequency and amplitude were 8.0 ± 0.6/min and 540 ± 60 mg ($n = 20$), respectively. Cumulative addition of VIP, VIPGC-NO and VIPGC in a dose range of 1 nM–1 μM inhibited both basic contraction frequency and

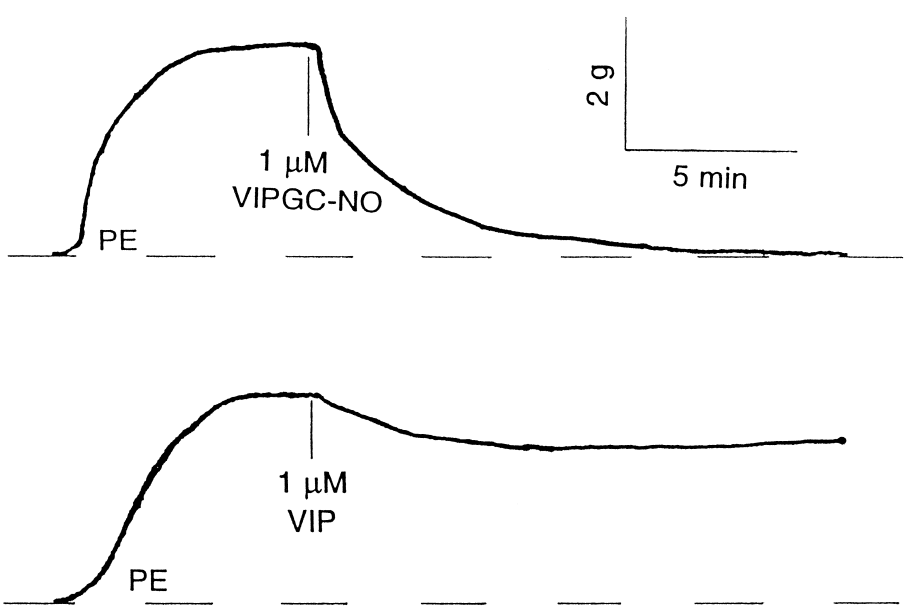

Fig. 2. Comparison of relaxation effects of VIPGC-NO and VIP on rabbit aortic rings precontracted with phenylephrine (PE). Tracings are typical of three separate experiments.

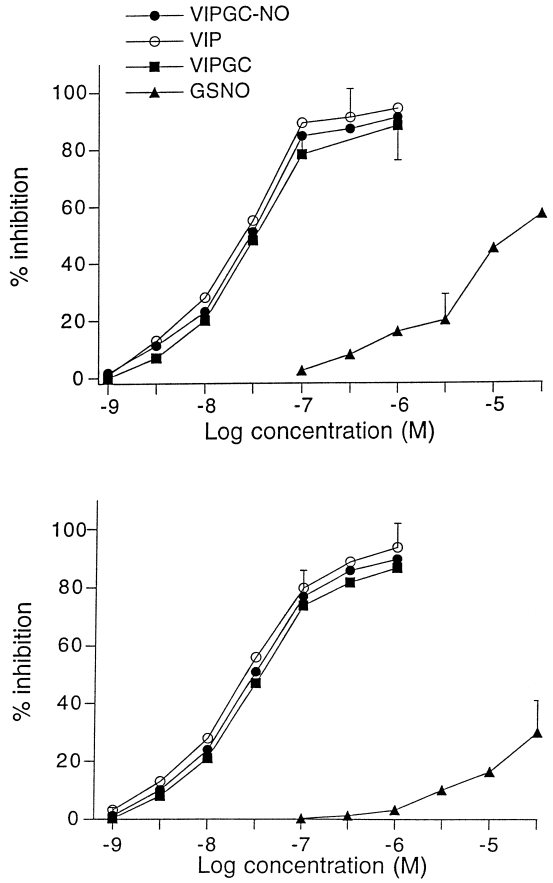

Fig. 4. Inhibitory effects of VIPGC-NO, VIP, VIPGC and GSNO on spontaneous contraction frequency (upper panel) and amplitude (lower panel) of rabbit sphincter of Oddi. Each point represents the mean of four preparations.

amplitude dose-dependently (Fig. 4). Effects of these compounds were observed within 1 min after addition of them to the organ chambers and persisted at least 90 min. At 1 μM level, VIP, VIPGC-NO and VIPGC inhibited the contraction frequency by 94 ± 16 ($n = 6$), 91 ± 10 ($n = 7$) and 88 ± 12% ($n = 4$), respectively, whereas GSNO was observed to be significantly less potent: at 100 μM, GSNO inhibited the basic contraction frequency of sphincters of Oddi by 91 ± 9% ($n = 3$, no figures shown at this concentration). The potency of GSNO in inhibiting the contractility of sphincters of Oddi was less than VIP and its derivatives over a range of at least 2 log units. All four compounds exhibited inhibition of contraction amplitude of the tissue similar to that of frequency (Fig. 4).

Addition of acetylcholine produced a dose-dependent increase in basal pressure. Final concentration of acetylcholine (10 μM) resulted in 69 ± 15% acceleration in contraction frequency and 73 ± 12% elevations in contraction amplitude ($n = 12$). On rabbit sphincters of Oddi pretreated with acetylcholine (10 μM), cumulative addition of VIP, VIPGC-NO, VIPGC (1 nM–1 μM) and GSNO (0.1–100 μM) gradually attenuated the acetylcholine-induced contractions of sphincters of Oddi. VIP, VIPGC-NO and VIPGC (1 μM) as well as GSNO (100 μM) inhibited acetylcholine-enhanced (10 μM) phasic contractions of sphincter of Oddi by 93 ± 7, 90 ± 3, 72 ± 22 and 89 ± 5% ($n = 4$ per group), respectively.

In order to compare the potency of inhibition by VIP with VIPGC-NO on phasic contraction of sphincter of Oddi, the tissues were incubated with single doses (0.1 μM) of VIP or VIPGC-NO for 30 min (Fig. 5). During the period of the incubation, the irregular and incomplete contractions of the sphincter of Oddi were noted more frequently (40 ± 11, $n = 5$) in the presence of 0.1 μM VIP than in the presence of 0.1 μM VIPGC-NO (26 ± 13, $n = 5$, $P < 0.05$).

3.4. Effects of VIP, VIPGC, VIPGC-NO and GSNO on gastric fundus of rat

Gastric fundus strips of rats, when incubated in oxygenated Krebs at 37°C, developed intrinsic rhythmic contractility within 30 min. The addition of 5-HT (10 μM)

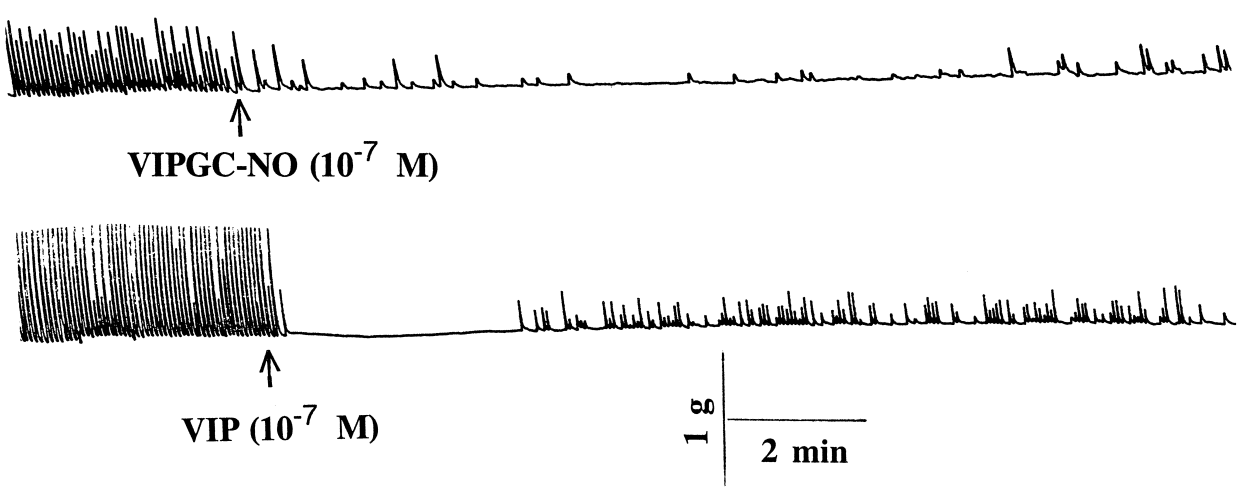

Fig. 5. Representative traces showing the effects of VIPGC-NO and VIP on spontaneous contraction of rabbit sphincter of Oddi. Addition of VIPGC-NO and VIP abolished the spontaneous contraction, and the inhibition lasted for at least 1 h. Tracings are typical of three separate experiments.

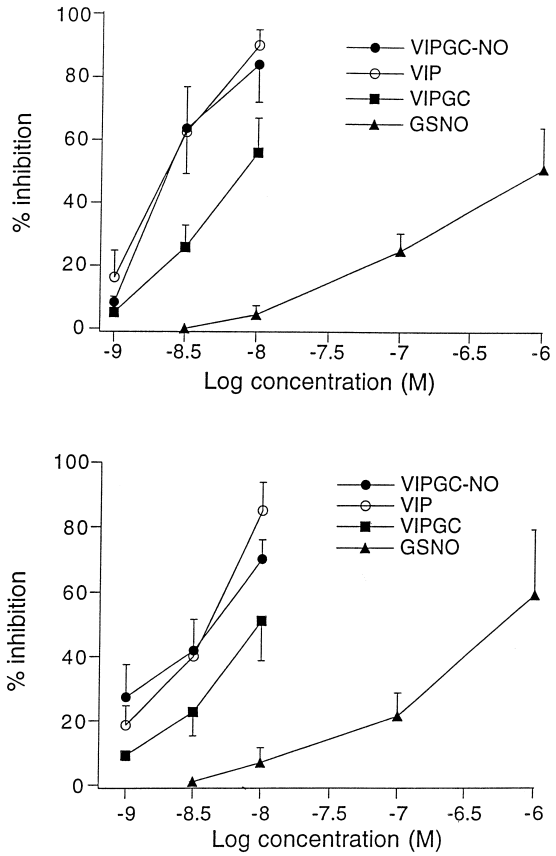

Fig. 6. Inhibitory effects of VIPGC-NO, VIP, VIPGC and GSNO on intrinsic contraction frequency (upper panel) and amplitude (lower panel) of longitudinal muscle strips from the rat gastric fundus. Each point represents mean ± S.E.M. of four preparations.

produced a significant increase in contractions of the strips. The average contraction amplitude and frequency of the strips were 1.1 ± 0.2 g and $24 \pm 2/10$ min ($n = 17$). All four compounds relaxed the rat gastric fundus in a concentration-dependent manner. VIPGC appeared less potent than VIP ($P < 0.05$ by ANOVA); however, the further potentiation of effects of parent VIP by using VIPGC-NO was not observed. GSNO was the least inhibitor in this respect (Fig. 6). The relaxant effects of these compounds were sustained with no significant difference between amplitude and frequency inhibition produced by them.

3.5. Effects of VIP, VIPGC, VIPGC-NO and GSNO on the cGMP and cAMP content of aortic and tracheal rings

Pretreatment of rabbit aortic rings with 1 μM VIPGC-NO and GSNO resulted in a significant increase in cGMP concentrations to 341 ± 45, 321 ± 19 (pmol/g tissue; both $P < 0.01$, vs. control) when these rings reached a maximum relaxation. In time-matched aortic rings from same rabbits, incubation of aortic rings with VIP or VIPGC produced increase in cGMP level to 131 ± 17, 128 ± 29 (pmol/g tissue; both $P < 0.05$, vs. control) as well; however, the increase in cGMP content induced by VIP or VIPGC was less than that of VIPGC-NO or GSNO (Fig. 7, upper panel). There was no significant increase in cAMP content when simultaneous determination of both cAMP and cGMP was made on the same tissue pretreated with VIP, VIPGC, VIPGC-NO or GSNO (all 1 μM; Fig. 7, upper panel).

When tracheal rings of guinea pigs reached a plateau of relaxation induced by VIPGC-NO, VIP, VIPGC and GSNO (all 1 μM, $n = 4$), a significant increase in the content of cGMP was observed. cGMP values for VIP, VIPGC-NO, VIPGC and GSNO were 263 ± 38, 278 ± 30, 245 ± 50 (all $P < 0.001$) and 173 ± 19 ($P < 0.05$, vs. control; pmol/g tissue), respectively. In addition, there were concomitantly significant increases in the cAMP level of those tissues pretreated with VIP, VIPGC-NO and VIPGC. VIP, VIPGC and VIPGC-NO induced an increase in cAMP level compared to the control ($P < 0.05$; Fig. 7, lower

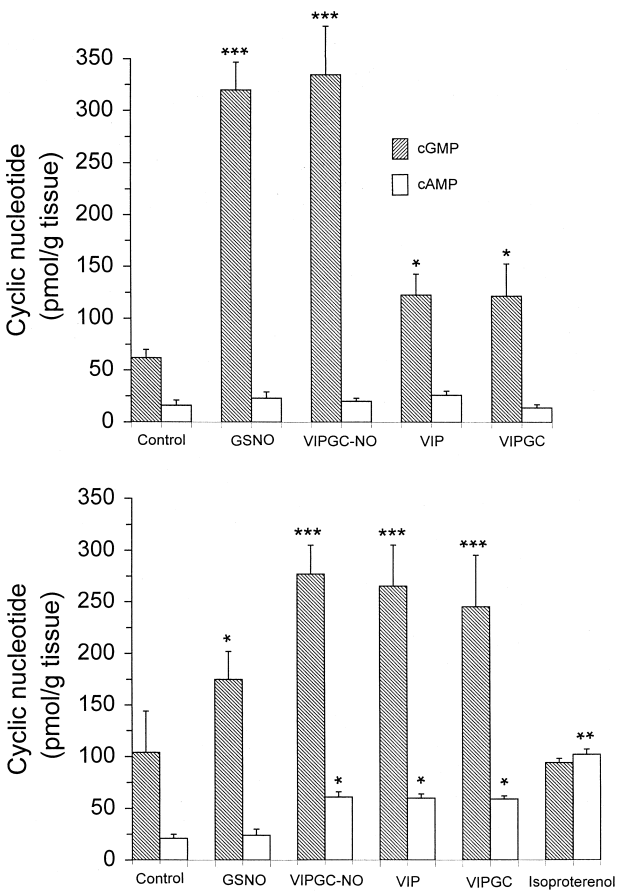

Fig. 7. Cyclic nucleotide determinations. Rabbit aortic rings (upper panel) incubated with 1 μM GSNO, VIP and its respective derivatives for 10 min exhibited increases in cyclic nucleotide content compared to control preparations pretreated with 200 nM phenylephrine. Tracheal rings of guinea pig (lower panel) incubated with isoproterenol (for 5 min), GSNO, VIP and its respective derivatives (all 100 nM) for 20 min also exhibited increases in cyclic nucleotide content in comparison to control preparations pretreated 100 nM methacholine. Each bar represents the mean ± S.E.M. of four to six experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control.

panel). Isoproterenol (100 nM) produced a significant increase in the cAMP level ($P < 0.01$, $n = 4$) with no appreciable changes in the cGMP content compared to the time-matched controls (Fig. 7, lower panel).

4. Discussion

The synthetic VIPGC-NO was tested in comparison with VIP, VIPGC and GSNO for biological activity in three isolated smooth muscle preparations and a sphincter of Oddi. The differences in the effects of these compounds on the tested tissues are of great interest for several reasons. First, though all the compounds relaxed all preparations tested, the relative sensitivity of these preparations to VIP and its nitroso derivatives varied. In general, the aorta was most sensitive to the relaxant effect of the *S*-nitroso compounds. Both VIPGC-NO and GSNO induced a more profound relaxation in the rabbit aortic rings than VIP. The greater effect of VIPGC-NO in this respect is likely due to the biological activity of *S*-nitrosothiols that can release NO upon decomposition of the parent *S*-nitrosothiols (Kharitonov et al., 1995; Singh et al., 1996; Keshive et al., 1996), instead of VIP activity, because the starting VIP and VIPGC were less active in the assay, and GSNO containing the same *S*-NO moiety as VIPGC-NO had vasoactivity profiles similar to VIPGC-NO (Figs. 1 and 2). The common mechanism by which NO relaxes vascular smooth muscle is believed to be due to stimulation by NO of soluble guanylate cyclase (Furchgott and Jothianandan, 1991; Jia et al., 1997), and the elevated cGMP levels may regulate tone in smooth muscle. Although the ability of intact *S*-nitrosothiols to cross membranes is not known, VIPGC-NO and GSNO would not be expected to be able to enter cells easily. VIPGC-NO was found to be almost equipotent to the lower molecular weight GSNO in the rabbit aorta assay, suggesting that transport of intact *S*-nitrosothiols across cell membranes does not appear to be required for activity. The possible mechanisms for stimulation of soluble guanylate cyclase include activation by NO released from the *S*-nitrosothiols either outside the cell or at the cell surface enzymatically or non-enzymatically. The lipophilic NO can readily cross cell membranes to activate guanylate cyclase perhaps by transnitrosation of the active site heme moiety to form catalytically active NO-heme (Ignarro, 1990). Regardless of the mechanism for the greater effect of VIPGC-NO upon vascular smooth muscle in vitro, this finding suggests that VIPGC-NO may preferentially induce vasodilation in vivo.

Second, the data presented here show that *S*-nitrosylation of the thiol group of VIP derivative does not alter parent VIP's ability to relax the non-vascular smooth muscle and sphincter of Oddi, because VIPGC-NO was no less potent than VIP in this respect. However, all these non-vascular preparations were more sensitive to the relaxant effect of VIP and its derivatives than that of GSNO (Figs. 3–7), indicating that a mechanism by which VIPGC-NO and VIP-induced non-vascular relaxation is not dependent on NO-signaling pathway, but a direct effect of parent VIP on smooth muscle. Taken with an additive modulation of VIP by attaching an NO group to VIP, the similarities in potency between VIP and VIPGC-NO to relax non-vascular smooth muscles suggest that *S*-nitrosylation of VIP confers NO-like vasoactivity to the peptide, but does not necessarily attenuate its original activity. The ability of NO groups to modify protein activity will critically depend on both the reactivity of the constituent amino acids and their relative importance in determining protein structure and function. As the addition of a cysteine as an N-terminal residue to VIP did not quash VIP-like activity, *S*-nitrosylation may synergize the effects of VIP by extending its signal pathways to both cGMP and cAMP systems (Fig. 7).

Some types of smooth muscle relaxants such as β-adrenoceptor agonists interact with extracellular receptors to elicit cAMP-dependent relaxation (Benovic et al., 1988). Other types of relaxants including NO and nitrovasodilators produce dilation by activating the guanylate cyclase pathway, leading to an increase in cGMP concentration and relaxation. To investigate the differences in mechanisms of action among VIP, NO and β-adrenoceptor agonists, we compared the effects of VIP, VIPGC-NO and isoproterenol. VIP and isoproterenol are well known to cause the activation of their specific membrane receptors coupled to G protein for the stimulation of adenylate cyclase and to increase in cAMP (Altiere and Diamond, 1984). The present studies showed that although the mechanisms of both VIP and isoproterenol are involved in the cAMP pathway (Fig. 7), VIP does not function via β-adrenoceptor stimulation because in our tracheal preparations propranolol only blocked the airway relaxation induced by isoproterenol without attenuating the effects of VIP and VIPGC-NO. Evidence that VIP-induced relaxation is associated with the coexistence of parallel cAMP and cGMP pathways has been demonstrated in both vascular and non-vascular smooth muscles as well as sphincters (Ignarro et al., 1987; Chakder and Rattans, 1993; Ward et al., 1995). Our data (Fig. 7) suggest that, in addition to the previously described VIP-initiated cAMP-dependent relaxation pathway confirmed in isolated smooth muscles, a physiologically relevant cGMP-dependent pathway for VIP relaxant activity is concomitantly present as well. Undoubtedly, the potentiation by VIPGC-NO of vasorelaxation must be attributable to the profound activation by VIPGC-NO of guanylate cyclase, which was comparable to the effect of GSNO (Fig. 7, upper panel).

Steps in the pathway activated by VIPGC-NO may be conceptualized as the following sequence: NO released from VIPGC-NO diffuses into intracellular compartments, activates soluble guanylate cyclase directly to produce cGMP. The resulting activation of cGMP-dependent pro-

tein kinase initiates a number of intermediary steps such as dephosphorylation of myosin light chain, changes in intracellular [Ca^{2+}], and finally the smooth muscle relaxation. It is noteworthy that the degree of relaxation produced by VIPGC-NO in non-vascular smooth muscles is almost the same as that by VIP. The data may be interpreted to imply that the conventional receptor activation and G protein coupling may also be involved in the above sequential steps.

Taken together, our data demonstrate that chemical modification of VIP by *S*-nitrosylation leads to a specific potentiation of VIP's vasoactivity without affecting the profound inhibition produced by parent VIP on non-vascular smooth muscle and sphincter of Oddi.

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