Mechanism of inhibition of VIP-induced LES relaxation by heme oxygenase inhibitor zinc protoporphyrin IX

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Rattan, Satish, Ya-Ping Fan, and Sushanta Chakder. Mechanism of inhibition of VIP-induced LES relaxation by heme oxygenase inhibitor zinc protoporphyrin IX. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G138–G145, 1999.—The putative heme oxygenase inhibitor zinc protoporphyrin IX (ZnPP IX) is known to exert diverse actions, including inhibitory action on smooth muscle relaxation by vasoactive intestinal polypeptide (VIP). The studies were performed in the opossum lower esophageal sphincter (LES) smooth muscle to determine the site of the inhibitory action of ZnPP IX in the smooth muscle relaxation by VIP. We also examined the effect of a direct Gi protein activator, cholera toxin (CTX), known to stimulate adenylate cyclase (AC). CTX caused relaxation of the LES smooth muscle by its action directly at the smooth muscle cells. The convergence of the common mechanisms of actions of VIP and CTX on AC was directly at the smooth muscle cells. The suppression of their effects by the AC inhibitor and CTX desensitization. ZnPP IX caused attenuation of the LES smooth muscle relaxation by VIP but not by CTX. ZnPP IX but not zinc deuteroporphyrin IX caused significant inhibition of VIP binding to the membrane receptor. We conclude that ZnPP IX attenuates VIP-induced LES smooth muscle relaxation by inhibition of VIP binding to G protein-coupled receptors linked to AC at a point proximal to G protein activation.

Vasoactive intestinal polypeptide; lower esophageal sphincter; inhibitory neurotransmission; nitric oxide synthase; G protein coupled.

MOST OF THE NEUROHUMORAL receptor interactions leading to a physiological action occur in the following steps. The binding of an agonist to the specific receptor causes change in the receptor conformation, followed by activation of the specific Gi protein, interaction with the specific enzyme associated with second messenger, interaction with ion channels or other target protein, and finally the specific action. The specific action in the case of the gastrointestinal smooth muscle may either be a relaxation or contraction. The neurohumoral substance examined in the present study is vasoactive intestinal polypeptide (VIP), which causes relaxation of the lower esophageal sphincter (LES) by the activation of receptors located on the smooth muscle cell membranes.

Carbon monoxide (CO) may be produced endogenously from heme by its interaction with heme oxygenase (HO) (26). Two types of HO have been recognized, HO-2 primarily in neural tissues and HO-1 in nonneural tissues. Zinc protoporphyrin IX (ZnPP IX) has been suggested to be a selective inhibitor of HO in a number of systems (26). Although the exact role of CO in the gastrointestinal smooth muscle is not known, it has been shown to cause a direct relaxation in a number of smooth muscle preparations (16, 31, 43), including the LES (28) and the internal anal sphincter (IAS) (31). Progress on the role of the HO pathway in inhibitory neurotransmission has been limited by the lack of selective HO inhibitors.

The putative HO inhibitor ZnPP IX has been recognized to have multiple actions especially in different smooth muscles, in addition to HO inhibition (14, 21). Because of this, caution should be exercised while using this agent primarily to determine the role of the HO pathway. Among other actions, ZnPP IX has been suggested to cause the blockade of VIP-induced relaxation of the IAS (31) as well as LES (28) and other smooth muscles. The exact site of action of ZnPP IX in blocking VIP response has not been investigated. To analyze this issue, specific tools to block or activate different steps along VIP receptor interaction, direct activator of Gi protein (cholera toxin or CTX), inhibitors of adenylate cyclase (AC), and CTX desensitization were employed. There is substantial evidence to suggest that VIP-induced smooth muscle relaxation is primarily mediated by the G protein-coupled receptor stimulation of AC (4, 35).

The main purpose of the present investigation therefore was to determine the mechanism of the inhibitory action of ZnPP on the LES smooth muscle relaxation caused by VIP. In the process, direct receptor binding studies with VIP before and after ZnPP IX were also performed.

MATERIALS AND METHODS
Preparation of smooth muscle strips. The LES smooth muscle strips from opossums (Didelphis virginiana) of either sex were prepared for the recording of isometric tension as described previously (32). Briefly, after receiving anesthesia with pentobarbital (40–50 mg/kg ip) the animals were killed by exsanguination, and the LES along with a section of the esophagus and stomach was isolated and transferred to oxygenated (95% oxygen plus 5% carbon dioxide) Krebs physiological solution of the following composition (in mM): 118.07 NaCl, 4.69 KCl, 2.52 CaCl2, 1.16 MgSO4, 1.01 NaH2PO4, 25 NaHCO3, and 11.10 glucose. The LES was carefully freed of all extraneous tissues, including the large blood vessels, opened, and pinned flat with the mucosal side up on a dissecting tray containing oxygenated Krebs solution. The mucosal and submucosal layers were removed by sharp dissection, and LES circular smooth muscle strips (1 × 10 mm) were prepared as described previously (32).
Measurement of isometric tension. The smooth muscle strips were tied at both ends with silk sutures (6-0 Ethicon, Sommerville, NJ) and transferred to 2-ml muscle baths containing oxygenated Krebs solution (37°C). One end of the muscle strip was anchored at the bottom of the muscle bath and the other end was attached to a force transducer (model FTO3; Grass Instruments, Quincy, MA) for the measurement of isometric tension on a Dynograph recorder (model R411; Beckman Instruments, Schiller Park, IL). The muscle strips were stretched initially at 1 g of tension and then allowed to equilibrate for at least 1 h with regular washings at 20-min intervals. Only the strips that developed spontaneous steady tension and relaxed in response to electrical field stimulation (EFS) were used. The optimal length and the baseline of the smooth muscle strips were determined as described previously (27).

NANC nerve stimulation with EFS. EFS was delivered from a Grass stimulator (model S88; Quincy, MA) connected in series to a Stimu-Splitter II (Med-Lab Instruments, Loveland, CO). The Stimu-Splitter served an important purpose to amplify and measure the stimulus intensity using the optimal stimulus parameters for the neural stimulation (12 V, 0.5-ms pulse duration, 200–400 mA, 4-s train) at varying frequencies of 0.5 to 20 Hz. The electrodes used for the EFS consisted of a pair of platinum wires fixed at both sides of the smooth muscle strip. The parameters of EFS stated above are known to selectively cause relaxation of the LES smooth muscle via the activation of nonadrenergic noncholinergic (NANC) myenteric neurons.

Drugs and chemicals. The following chemicals were used in the study: ZnPP IX, isoproterenol hydrochloride, and N-ethylmaleimide (NEM) (Aldrich Chemical, Milwaukee, WI); zinc deuteroporphyrin IX 2,4 bis-ethylene glycol (ZnDP IX) (Porphin Products, Logan, UT); N⁶-nitro-l-arginine (l-NNA) and sodium nitroprusside (SNP) (Sigma Chemical, St. Louis, MO); and VIP (Bachem Bioscience, Torrance, CA); and bacitracin (1 mg/ml), and EDTA (2 mM) in a final volume of 0.2 ml. The membranes were diluted in the buffer (50 µg/ml final concentration) and incubated with radiolabeled VIP (45 pM) for 10 min at 30°C with or without unlabeled VIP. For examining the effects of ZnPP IX or ZnDP IX on VIP binding to the LES smooth muscle membranes, the membranes were first incubated with 1 × 10⁻⁴ M ZnPP IX or ZnDP IX for 10 min before performing the binding studies with 125I-VIP in the presence or absence of different concentrations of unlabeled VIP. The incubation was stopped by adding 1 ml of ice-cold Tris buffer to the incubates, and the membrane-bound radioactivity was separated from the unbound radioactivity by centrifugation at 16,000 g for 5 min. The pellets were washed again with the addition of 1 ml Tris buffer and recentrifugation. The radioactivity in the pellets were counted using a gamma counter (Genesis, Laboratory Technology, Schaumburg, IL). The specific binding was determined by subtracting the radioactivity remaining in the presence of 1 × 10⁻³ M unlabeled VIP from the total radioactivity. The nonspecific binding was ~25% of the total binding.

Drug responses. Pretreatment with ZnPP IX and ZnDP IX (1 × 10⁻⁴ M) for 10 min was used to examine their effects on the basal LES tone and changes in response to different agonists. The dose was chosen based on previous data that showed that ZnPP IX in this dose was maximally effective in blocking NO activity in the gastrointestinal smooth muscle preparations (28). To examine the influence of AC inhibitors, the smooth muscle strips were pretreated with SQ-22536 (1 × 10⁻³ to 3 × 10⁻⁴ M) or NEM (1 × 10⁻⁴ M) for 10 min before testing the effects of EFS, VIP, and CTX. The concentrations of different antagonists were selected in view of the earlier literature and our own experiments. The doses examined were relatively selective against the intended effects.

In some experiments the effect of VIP was examined in the presence of CTX desensitization. CTX desensitization was achieved by the repeated administration of the toxin (1 µg/ml) until its effect was substantially reduced.

All experiments involving ZnPP IX and ZnDP IX were carried out in the dark. All the agonists were given in a cumulative fashion. Once the concentration-response curve to an agent was determined, the smooth muscle strips were washed at least six times and the resting tension was allowed to recover to the preinjection levels.

Data analysis. The results are expressed as means ± SE of different experiments. The fall of the resting LES tension is expressed as the percentage of maximal potential response (100%) to a supramaximal concentration (5 mM) of EDTA. Statistical significance between different groups was determined by using paired or unpaired t-test where applicable, and P < 0.05 was considered statistically significant.
RESULTS

Influence of HO inhibitors ZnPP IX and ZnDP IX on LES smooth muscle relaxation by VIP. The data in Fig. 1 show that ZnPP IX caused a marked and significant \((P < 0.05, n = 5)\) shift in the dose-response curve of VIP in causing the LES relaxation. ZnDP IX on the other hand had a relatively limited effect. The values of the fall in the basal tension of LES with 3 \(\times 10^{-7}\) M VIP before and after ZnPP IX \((1 \times 10^{-4} \text{ M})\) were 75.5 \(\pm\) 7.8 and 25.1 \(\pm\) 3.9\%, respectively \((P < 0.05, n = 5)\), and the corresponding values before and after ZnDP IX \((1 \times 10^{-4} \text{ M})\) were 72.2 \(\pm\) 5.5 and 70.0 \(\pm\) 3.8\%, respectively \((P > 0.05, n = 5)\). However, ZnDP IX did cause a significant suppression of the LES smooth muscle relaxation in response to lower concentrations of VIP \((1 \times 10^{-8} \text{ to } 1 \times 10^{-7} \text{ M})\). This suppression by ZnDP IX was significantly less compared with that by ZnPP IX. ZnPP IX as well as ZnDP IX had no significant effect on the basal tone of the LES. The basal LES tone before and after ZnPP IX was 1.8 \(\pm\) 0.1 and 1.8 \(\pm\) 0.1 g, and before and after ZnDP IX it was 2.2 \(\pm\) 0.1 and 2.2 \(\pm\) 0.1 g, respectively \((P > 0.05, n = 5)\).

We have substantial data to show that in contrast to its actions on VIP, ZnPP IX had no significant effect on the LES smooth muscle relaxation caused by forskolin, SNP, nitric oxide (NO), and CO (14). Furthermore, the LES smooth muscle contraction caused by bethanechol was also not modified by ZnPP IX.

Influence of ZnPP IX and ZnDP IX on LES smooth muscle relaxation by NANC nerve stimulation with EFS. Neither ZnPP IX nor ZnDP IX had any significant effect on the LES smooth muscle relaxation by the NANC nerve stimulation with EFS (Fig. 2; \(P > 0.05, n = 9\)). The fall in the basal LES tension with 5 and 10 Hz of EFS in these experiments was 71.5 \(\pm\) 2.7 and 76.5 \(\pm\) 2.7\%, and in the presence of ZnPP IX and ZnDP IX these values were 68.8 \(\pm\) 4.6, 75.7 \(\pm\) 3.3, 70.7 \(\pm\) 3.9, and 74.3 \(\pm\) 3.3\%, respectively.

To determine the relationship between the actions of VIP and CTX, the effects and the site of action of CTX in causing the LES smooth muscle relaxation were investigated first.

Effects and site of action of CTX on basal LES tone: influences of l-NNA, TTX, and \(\omega\)-conotoxin. The role of \(G_\text{s}\) protein in the VIP-induced relaxation of the LES smooth muscle and the influence of ZnPP IX were examined by the use of CTX, which causes direct activation of \(G_\text{s}\) protein. CTX caused a concentration-dependent fall in the basal tension of the LES (Fig. 3). A maximal fall in the basal LES tension was observed with 2 \(\mu\text{g/ml}\) CTX in the muscle bath. The percent fall in the basal tension of the LES with 2 \(\mu\text{g/ml}\) CTX was 79.6 \(\pm\) 2.0. The responses of CTX on the LES were not modified by the NO synthase (NOS) inhibitor l-NNA, the neurotransmitter TTX \((1 \times 10^{-6} \text{ M})\), and the combination of TTX and the neuronal calcium channel blocker \(\omega\)-conotoxin GVIA \((1 \times 10^{-6} \text{ M})\) \((P > 0.05, n = 4; \text{Fig. } 4)\).

Influence of ZnPP IX on LES smooth muscle relaxation by CTX. The dose-response curves for CTX showing the percent fall in the basal tension of the LES,
obtained before and after ZnPP IX were found not to be significantly different ($P > 0.05$, $n = 5$). The percent fall in the basal LES tension with 0.5, 1.0, and 2.0 µg/ml in control experiments was 65.2 ± 3.7, 72.9 ± 3.4, and 79.6 ± 2.0, respectively. These values in the presence of ZnPP IX were 67.5 ± 2.5, 75.4 ± 3.0, and 81.7 ± 3.0%, respectively.

Influence of AC inhibitors on LES smooth muscle relaxation by VIP and CTX. The commonly used AC inhibitor SQ-22536 was found to be relatively nonselective and ineffective in blocking AC in the LES smooth muscle. In a different range of concentrations ($1 \times 10^{-5}$ to $3 \times 10^{-4}$ M), previously reported to be specific for the purpose, SQ-22536 caused a significant fall in the basal tone of the LES. Additionally, SQ-22536 had no significant effect on the LES relaxation caused by CTX, known to activate AC. SQ-22536 ($1 \times 10^{-4}$ M and $3 \times 10^{-4}$ M) caused a fall in the basal tension of the LES from 2.2 ± 0.3 to 1.5 ± 0.2, and 1.1 ± 0.3 g, respectively ($P < 0.05$, $n = 5$).

For this reason, studies to examine the role of AC in the LES smooth muscle relaxation were performed using NEM, previously reported to cause a significant blockade of the AC pathway (11, 27, 32, 38). NEM ($1 \times 10^{-2}$ M) caused a significant suppression of the fall in LES tension by EFS (Fig. 5B) and isoproterenol (Fig. 5C) ($P < 0.05$, $n = 5$) but had no significant effect on the relaxant actions of SNP ($P > 0.05$, $n = 5$; Fig. 5A). NEM ($1 \times 10^{-4}$ M) also caused a significant blockade of the effect of different concentrations of CTX in causing the LES smooth muscle relaxation ($P < 0.05$, $n = 5$; Fig. 6). Likewise, the fall in the basal LES tension by different concentrations of VIP was also significantly blocked by NEM ($P < 0.05$, $n = 5$; Fig. 7).

The data reported suggest that the actions of both VIP and CTX in causing the LES smooth muscle relaxation are mediated via the activation of G protein that is linked to the AC. To further establish the common pathway for their relaxation, the influence of CTX desensitization on VIP-induced relaxation of the LES was examined next.

Influence of CTX desensitization on VIP-induced relaxation of LES. The frequent administration of CTX caused a significant reduction in its responses. Furthermore, CTX desensitization also caused a significant reduction in VIP responses in the LES ($P < 0.05$, $n = 4$; Fig. 8), suggesting that the LES relaxation by CTX and VIP follows the same biochemical pathway.

The site of action of ZnPP IX in suppressing the LES relaxation by VIP appears to be at a point above the activation of the G protein since the fall in the LES tension by CTX was not affected by ZnPP IX. The next focus therefore was to examine the possibility of inhibition of VIP binding by ZnPP IX at the membrane receptor.

Influence of ZnPP IX on binding of VIP to its receptors in LES. We compared the effects of different concentrations of unlabeled VIP on $^{125}$I-VIP binding to the LES smooth muscle membranes before and after ZnPP IX and ZnDP IX ($1 \times 10^{-4}$ M). The data given in Fig. 9 show that in control experiments, VIP caused a significant and concentration-dependent displacement of the bound $^{125}$I-VIP. Although ZnDP IX had no significant effect on this control VIP receptor binding curve, ZnPP IX caused a significant inhibition of the displacement curve. These data suggest that ZnPP IX interferes with the binding of VIP to the receptor. Furthermore, the data correspond to the functional data in Fig. 1 that
show the suppression of the VIP-induced fall in the basal LES tension in the presence of ZnPP IX.

DISCUSSION

Multiple actions of ZnPP IX are well recognized. In the LES the protoporphyrin caused blockade of the smooth muscle AC and guanylate cyclase (GC) stimulation by VIP and atrial natriuretic factor or peptide, respectively (14, 29). Although the nonspecificity of actions of ZnPP IX may be widespread, in the present study we focused primarily on the mechanism of its inhibitory action on VIP.

The studies show that ZnPP IX caused significant attenuation of the G protein-coupled receptor activation by VIP. It is well known that the major part of the relaxant action of VIP in the LES is mediated by direct action at the VIP receptor on the smooth muscle cells via the activation of AC (1, 9, 32, 36). To determine the site of action of ZnPP IX in blocking VIP-induced relaxation of the LES smooth muscle, we systematically investigated the influence of ZnPP IX on agents that work along different steps to produce VIP receptor-mediated smooth muscle relaxation. This included the examination of the actions of VIP on the basal LES tension.

Fig. 5. Influence of adenylate cyclase inhibitor N-ethylmaleimide (NEM) on percent fall in LES tension caused by sodium nitroprusside (SNP) (A), EFS (B), and isoproterenol (C). Note that NEM caused significant attenuation of fall in LES tension induced by EFS and isoproterenol (*P < 0.05, n = 5) but not by SNP (P > 0.05, n = 5).

Fig. 6. Data show that fall in LES tension caused by CTX was significantly blocked by adenylate cyclase inhibitor NEM (1 × 10⁻⁴ M) (*P < 0.05, n = 5).

Fig. 7. Data show significant blockade of effect of VIP on LES by adenylate cyclase inhibitor NEM (1 × 10⁻⁴ M) (*P < 0.05, n = 5).
tone, VIP receptor binding, and comparison of the actions of VIP vs. CTX before and after ZnPP IX.

CTX is known to cause direct activation of G protein associated with AC (5, 41). The present studies in the LES showed for the first time that CTX causes a concentration-dependent fall in the basal tension by its action directly at the smooth muscle cells since it was not modified by neuronal blockade. The convergence of VIP and CTX on AC activation to produce LES relaxation was verified by their similar attenuation by the AC inhibitor NEM. The convergence of the actions of VIP and CTX on a similar intracellular pathway was further confirmed by the blockade of the inhibitory action of VIP on the LES smooth muscle by CTX desensitization.

The data suggest that the fall in the basal tone in the LES induced by VIP and CTX follows the same final biochemical pathway, i.e., the stimulation of AC. Along this final pathway, however, the original sites of action of VIP and CTX are different in causing the LES smooth muscle relaxation. The action of VIP is mediated via activation of G protein-coupled receptors and that of CTX is downstream, bypassing the receptor interaction, and being exerted directly at the level of G protein activation. This difference in the locus of action of these agents in the present study played an important role in the determination of the site of action of ZnPP IX in blocking the action of VIP in the LES.

The inhibitory action of isoproterenol in the LES especially in the lower concentrations was also attenuated by ZnPP IX (13). The suppressant effect of ZnPP IX on isoproterenol-induced smooth muscle relaxation may involve its interaction with the β-adrenoceptors. The data show that ZnPP IX blocks the action of VIP but has no significant effect on the fall in the LES tension caused by CTX. This suggests that the site of action of ZnPP IX in inhibiting the action of VIP lies at a point between the receptor interaction and G protein activation. The VIP binding experiments further confirmed that the major mechanism of action of ZnPP IX in inhibiting VIP action is due to inhibition of the coupling of VIP with the receptor. In separate studies (14) we have shown that ZnPP IX causes no significant modification of the LES relaxation by forskolin, a direct stimulator of AC that bypasses G protein activation.

As stated above, ZnPP IX exerts multiple actions in the smooth muscle. However, the inhibitory action of ZnPP IX on the G protein-coupled receptor activation leading to LES smooth muscle relaxation cannot be explained simply on the basis of complete nonselectivity for the following reasons. The actions of the muscarinic agonist bethanechol that stimulate a specific G protein-coupled receptor (10, 12, 19), causing an increase in the basal tone of the LES (17), and the fall in the LES tone induced by CTX (a polypeptide), SNP, and forskolin were not modified. Furthermore, in the LES, unlike the IAS, the NANC nerve stimulation-induced relaxation of the sphincteric smooth muscle was also not modified by the HO inhibitor. We suggest that in the LES the predominant pathway for the NANC nerve-induced relaxation is NOS. The predominance of the NOS pathway in the LES relaxation is evident from the previously published data that show the NOS inhibitor nearly abolishes the relaxation by NANC nerve stimulation (30, 40).

In light of the strong evidence in favor of the VIP as an inhibitory neurotransmitter (3, 20), it is rather...
surprising that ZnPP IX caused near obliteration of VIP response but had no effect on the NANC relaxation. One of the plausible explanations may be NOS upregulation. A leftward shift in the EFS-frequency response curve in the feline LES in the presence of ZnPP IX plus L-NNA compared with L-NNA alone (28) and release of NO by ZnPP IX in the rabbit IAS (7) may lend support to this speculation. This may have important pathophysiological implications in the counterregulation between HO and NOS pathways and in protecting the tissues against the deleterious effects of overproduction of NO. The colocalization of HO and NOS, as recently demonstrated in brain neurons (39) and in the feline LES (28), further suggests this possibility.

Despite multiple and nonselective actions of ZnPP IX in different smooth muscles, in the IAS the interaction between VIP and HO was found to be relatively defined since the actions of a closely related peptide, PHI, were not modified by the HO inhibitor (31). The concentrations of ZnPP IX used in the present studies were similar to those found to be effective in inhibiting HO activity in the LES (28). Furthermore, ZnDP IX, which is known to block HO activity with a greater potency than ZnPP IX, had a limited effect on the VIP-induced relaxation of the LES as well as on VIP receptor binding. The data suggest a lack of correlation between the inhibition of HO activity by the porphyrins and their ability to block the responses to VIP.

Because of the nonselectivity of action of ZnPP IX, the exact role of the HO pathway in the LES relaxation by NANC nerve stimulation cannot be determined at the present time. The direct action of CO on the smooth muscle via direct activation of GC, the presence of basal HO activity, its increase by NANC nerve stimulation, and inhibition by ZnPP IX in certain gastrointestinal tissues (28, 31), the presence of HO-2 immunoreactivity in the myenteric plexuses (6, 6, 28, 34); the electrophysiological correlation between CO and NANC relaxation (15); the colocalization of HO with NOS and VIP immunoreactivities (2, 28, 34); and the inhibition of NANC nerve-mediated relaxation by the selective knock-out of the HO-2 gene in certain gastrointestinal tissues (31, 37, 42) suggest participation of the HO pathway in some way in the gastrointestinal motility.

In conclusion, ZnPP IX causes blockade of the action of VIP at the LES smooth muscle membrane receptor that is G protein coupled to AC. The site of action of ZnPP IX is above the level of activation of G protein since the effects of direct G protein activation by CTX were not modified by ZnPP IX. The failure of ZnPP IX to modify NANC nerve stimulation-induced relaxation of the LES may be explained by the possibility that the smooth muscle relaxation by the NO released on NANC nerve-mediated stimulation uses a unique receptor activation that bypasses the G protein coupling to GC. The data further suggest that ZnPP IX, especially at high concentrations, may not be a specific HO inhibitor and that there is a need for a more selective HO inhibitor. The discovery of such an agent may facilitate investigations of the role of the HO pathway in gastrointestinal motility.

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