Inhibitory Effect of Zinc Protoporphyrin IX on Lower Esophageal Sphincter Smooth Muscle Relaxation by Vasoactive Intestinal Polypeptide and Other Receptor Agonists

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ABSTRACT
This study was performed in the opossum lower esophageal sphincter (LES) smooth muscle strips to determine the action of the heme oxygenase inhibitor zinc protoporphyrin IX (ZnPP IX) on the relaxant effect of vasoactive intestinal polypeptide and isoproterenol, which are known to stimulate adenylate cyclase (AC) via G protein coupling, and of the direct activator of AC catalytic subunit forskolin. To investigate the cGMP pathway, we examined the effect of atrial natriuretic factor known to activate the receptor linked to the particulate guanylate cyclase via G protein coupling and that of sodium nitroprusside [nitric oxide (NO) donor], authentic NO and carbon monoxide, which stimulate the intracellular soluble fraction of GC. The smooth muscle relaxation caused by nonadrenergic noncholinergic (NANC) nerve stimulation also was investigated. ZnPP IX caused concentration-dependent attenuation of the relaxant effect of vasoactive intestinal polypeptide, isoproterenol and atrial natriuretic factor without any effect on that of forskolin, sodium nitroprusside, NO and CO. Interestingly, ZnPP IX had no significant effect on the LES relaxation caused by NANC nerve stimulation and the smooth muscle contraction by betahanechol. From these results, we conclude that ZnPP IX attenuates the LES smooth muscle relaxation caused by the stimulation of G protein-coupled receptors to particulate AC and guanylate cyclase. The lack of effect of ZnPP IX on the NANC nerve-mediated LES relaxation suggests either lack of a role of heme oxygenase pathway in the response or an up-regulation of NOS leading to normal LES relaxation.

CO has been shown to be a direct smooth muscle relaxant in a number of smooth muscle preparations (Furchgott and Jothianandan, 1991; Rattan and Chakder, 1993; Zygmun et al., 1994; Ny et al., 1995), including the LES (Ny et al., 1996) and IAS (Rattan and Chakder, 1993). CO is known to be produced endogenously by the interaction between heme and HO (Maines, 1988, 1997). Furthermore, two types of HO have been recognized (Maines, 1988, 1997), HO-2, found primarily in the neural tissues, and HO-1, found in the non-neural tissues. ZnPP IX has been suggested to be a selective inhibitor of HO in a number of systems (Maines, 1981; Luo and Vincent, 1994).

The exact physiological role of CO has not been determined. One of the major obstacles in the process is the lack of a selective HO inhibitor in different systems. For example, ZnPP IX, in addition to inhibiting HO activity, caused an attenuation of the smooth muscle relaxation induced by NANC nerve stimulation and VIP in the IAS (Rattan and Chakder, 1993; Tottrup et al., 1995) and by VIP in the LES (Ny et al., 1996). The observations raised the possibility of involvement of HO pathway in the NANC nerve-mediated smooth muscle relaxation and an interaction between VIP and HO pathway. The other possibility is that ZnPP IX is nonselective in blocking the smooth muscle relaxation in response to NANC nerve stimulation and VIP. There are distinct resemblances between the studies that deal with the HO pathway in the opossum and rabbit IAS (Rattan and Chakder, 1993; Tottrup et al., 1995) and feline LES (Ny et al., 1996). The resemblances include the direct inhibitory action of CO in the smooth muscles via the activation of cGMP, the...
presence of HO activity in the basal state and suppression of VIP-induced smooth muscle relaxation and preservation of the inhibitory effect of NO or the NO donor SNP in the presence of the HO inhibitor. The striking difference, however, was the lack of the effect of ZnPP IX on the NANC nerve-mediated relaxation of the feline LES smooth muscle. Recent studies in the rabbit IAS have shown that in addition to the above actions, ZnPP IX also may have NOS-stimulating effects (Chakder et al., 1996). The role of HO pathway in the opossum LES (an important animal model for the inhibitory neurotransmission) is not known.

The purpose of the present investigation was to systematically examine the effects of ZnPP IX on the opossum LES relaxation caused by NANC nerve stimulation and by agonists that act at different sites of the AC and GC pathways. Both AC and GC are well documented to play a significant role in the relaxation of opossum LES smooth muscle (Torphy et al., 1986; Rattan and Moummi, 1989; Szewczak et al., 1990; Chakder and Rattan, 1993).

Materials and Methods

Preparation of smooth muscle strips. The opossum LES smooth muscle strips were prepared for the recording of isometric tension as described previously (Rattan and Moummi, 1989). Briefly, the animals were anesthetized with pentobarbital (40 to 50 mg/kg i.p.), and the LES along with a section of the esophagus and stomach was isolated and transferred to oxygenated (95% O 2/5% CO 2) Krebs’ physiological solution of the following composition (in mM): NaCl, 118.07; KCl, 4.69; CaCl 2, 2.52; MgSO 4, 1.16; NaH 2PO 4, 1.01; NaHCO 3, 25; glucose, 11.10. 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 (Rattan and Moummi, 1989).

Measurement of isometric tension. The smooth muscle strips were secured at both ends with silk sutures 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 measurement of isometric tension on a Dynograph recorder (model R411; Beckman Instruments, Quincy, MA). CO and NO were from Matheson Gas (Bridgeport, NJ). VIP and isoproterenol are known to activate AC via G protein coupling and forskolin directly on the catalytic subunit of AC (Grundemar and Ny, 1997). Conversely, ANF causes the smooth muscle relaxation via the activation of particulate GC via G protein coupling (Rattan et al., 1991; Anand-Srivastava and Trachte, 1993; Wright et al., 1996) and SNP, NO and CO via activation of intracellular soluble fraction of GC (Chakder and Rattan, 1993; Stone and Marletta, 1984; Lincoln et al., 1996; McDonald and Murad, 1996; Murad, 1996). All chemicals except ZnPP IX and forskolin were dissolved and diluted in Krebs’ solution and prepared fresh on the day of the experiment.

Stock solutions of ZnPP IX and SnPP IX were prepared and kept in the dark. ZnPP IX was dissolved in 0.2 N sodium hydroxide and diluted with Krebs’ solution. The pH of ZnPP IX solution was adjusted to 7.4 using 0.2 N HCl. Forskolin was dissolved in ethanol and diluted subsequently in Krebs’ solution. The final dilutions of sodium hydroxide and ethanol used for ZnPP IX and forskolin, respectively, produced no significant effect on the basal LES tone.

Saturated solutions of NO and CO were prepared at room temperature by bubbling the gases into deoxygenated Krebs’ physiological solution in a sealed vial for 20 min at a pressure slightly higher than the atmospheric pressure and mixing well. The saturated solutions of NO and CO were considered to be 3 (Shikano et al., 1987) and 1 (Suematsu et al., 1985) mM, respectively. Different volumes of the stock solution of NO and CO were added to the muscle bath to achieve the desired concentrations. The corresponding volumes of deoxygenated Krebs’ physiological solution that served as a control produced no significant effect on the resting tone of the LES. The stock solutions of NO and CO thus prepared have been previously used in our laboratory (Rattan and Chakder, 1992; Rattan and Chakder, 1993). These solutions were used within 1 hr of preparation.

The vials and pipette tips were siliconized while the muscle baths were treated with 2.5% bovine serum albumin to reduce the binding of peptides to the surface of glass or plastic.

Drug responses. Pretreatment with two concentrations of ZnPP IX (1 × 10^{-6} and 3 × 10^{-4} M) and SnPP IX (1 × 10^{-4} M) for 10 min was used to examine their effects on the basal LES tone and its changes in response to different agonists.

All experiments were carried out in the dark and in the presence of guanethidine (3 × 10^{-6} M) and atropine (1 × 10^{-5} M) with the exception of betahistine experiments, for which atropine was excluded. All the agonists except NO and CO were given in a cumulative fashion. The LES relaxation in response to NO and CO were rapid and brief, making it difficult to determine their effects when given in a cumulative manner. 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 mean ± S.E. of different experiments. The fall of the resting IAS tension is expressed as the percent of E max (100%) in response to supramaximal concentration (5 mM) of EDTA. Statistical significance between different groups was determined by using paired or unpaired t test where applicable, and a value of P < .05 was considered statistically significant.

Results

Influence of ZnPP IX on LES relaxation induced by VIP, isoproterenol and forskolin. The influence of ZnPP IX on the LES relaxation by VIP, isoproterenol and forskolin
is shown in figures 1, 2 and 3, respectively. The data show that the fall in the basal LES tension caused by all concentrations of VIP and lower concentrations of isoproterenol were significantly suppressed by ZnPP IX in a concentration-dependent manner (P < .05; n = 9; figs. 1 and 2). However, the fall in the LES tension caused by forskolin was not modified by any of the concentrations of ZnPP IX (P > .05; n = 7; fig. 3). Furthermore, the fall in the basal LES tone by PHI tested in the maximal effective concentration (3 × 10^{-6} M) also was not modified by ZnPP IX (data not shown).

In all of these experiments, two concentrations of ZnPP IX (1 × 10^{-4} and 3 × 10^{-4} M) were used that have previously been shown to inhibit EFS-induced relaxation in the opossum IAS (Rattan and Chakder, 1993) and HO activity in the feline LES (Ny et al., 1996).

**Influence of ZnPP IX on the LES relaxation by NANC nerve stimulation.** NANC nerve stimulation using the appropriate stimulus parameters caused a frequency-dependent fall in the basal LES tension. In contrast to the inhibitory effects of VIP, isoproterenol and ANF, the fall in the LES tension by NANC nerve stimulation was not modified by the HO inhibitor (P < .05; n = 12; fig. 4) at any of the frequencies examined.

**Influence of ZnPP IX on LES relaxation induced by ANF, SNP, NO and CO.** ANF and SNP caused concentration-dependent fall in the basal tone of the LES. As shown in figure 6, the inhibitory effect of ANF on the LES tension was significantly attenuated by the heme oxygenase inhibitor ZnPP IX in both the concentrations (P < .05; n = 7; fig. 6). However, the fall in LES tension caused by SNP was not significantly modified by ZnPP IX (P > .05; n = 9; fig. 5).

NO and CO also produced a concentration-dependent relaxation of the LES that was not modified by ZnPP IX (n = 6; Fig. 7). Calculated on the bases of EC_{50} values (the concentration that caused 50% fall in the basal LES tone), NO was found to be ~150 times more potent than CO. EC_{50} values for NO and CO were ~1 and ~150 μM, respectively. The falls in LES tension by NANC nerve stimulation, forskolin, SNP, NO and CO also were not modified by another porphyrin analog, SnPP IX (1 × 10^{-4} M) (not shown).

In a separate series of experiments, it was determined that repetitions of dose-response curves constructed with VIP, isoproterenol, forskolin, ANF or SNP in different LES smooth muscle strips at 2-hr intervals after appropriate washing were not significantly different. This suggests the lack of tachyphylaxis with these agents under the experimental conditions of the present studies.

**Influence of ZnPP IX on LES contraction in response to bethanechol.** ZnPP IX in either of the concentrations tested caused no significant change in the fall in the LES tension by forskolin (P > .05; n = 7).
found to have no significant effect on the rise in the basal LES tone in response to the muscarinic agonist bethanechol (P < .05; n = 5; fig. 8).

**Influence of ZnPP IX on the basal LES tone.** ZnPP IX had apparently no significant adverse effect on the basal tone of the opossum LES. The basal LES tone was 2.2 ± 0.1 g in control experiments and 2.1 ± 0.1 and 1.9 ± 0.3 g in the presence of 1 × 10⁻⁴ and 3 × 10⁻⁴ M ZnPP IX, respectively (P > .05; n = 7).

**Influence of the NOS inhibitor L-NA on the LES relaxation by NANC nerve stimulation.** In contrast to the effect of ZnPP IX, the NOS inhibitor L-NA caused significant and concentration-dependent suppression of the NANC nerve-mediated LES relaxation by all the frequencies of EFS examined (P < .05; n = 8; fig. 9). However, although the effect of the lower frequencies of EFS (0.5–2 Hz) was nearly obliterated, a significant fall in the LES tension with the higher frequencies could still be observed.

**Discussion**

The results of the study suggest that ZnPP IX inhibits the LES smooth muscle relaxation caused by the agonists that activate the specific membrane bound G protein-coupled receptors to AC or GC. cAMP and cGMP play a significant role in the relaxation of LES smooth muscle in response to a
number of agonists and stimuli (Torphy et al., 1986; Rattan and Moummi, 1989). To investigate the site of action of ZnPP IX on the cAMP pathway, we compared the effects of VIP, isoproterenol and forskolin. VIP and isoproterenol are well known to cause the activation of their specific membrane receptors coupled to G protein for the stimulation of AC and to increase in cAMP (Benovic et al., 1988; Anand-Srivastava and Trachte, 1993). Forskolin, on the other hand, bypasses the G protein and works via the direct activation of the intracellular catalytic subunit of AC (Seamon and Daly, 1986; Anand-Srivastava and Trachte, 1993). To determine the possible site of action of ZnPP IX in the inhibition of cGMP pathway, we investigated the actions of the particulate GC stimulant ANF, whose receptor is coupled with G protein (Rattan et al., 1991; Anand-Srivastava and Trachte, 1993), and direct stimulants of the soluble fraction of GC, SNP, NO and CO (Murad, 1996). The data show that although the relaxant effects of VIP, isoproterenol and ANF in the LES were significantly impaired, the inhibitory effects of forskolin, SNP, NO and CO remained unaffected by any of the concentrations of ZnPP IX used.

For LES smooth muscle relaxation in response to VIP, isoproterenol and ANF, we conceptualize the occurrence of the following sequential steps: receptor binding of the agonists at the plasma membrane, coupling to specific G protein, production of cyclic nucleotides (cAMP or cGMP), activation of specific protein kinase, a number of intermediary steps such as dephosphorylation of myosin light chain and finally the smooth muscle relaxation. From the forskolin and SNP experiments, it is quite clear that the effects of ZnPP IX are upstream of the actions of cyclic nucleotides on protein kinases and the subsequent effects on the myofilaments. The exact site of action of ZnPP IX in suppressing the smooth muscle relaxation may therefore lie between its inhibition of the receptor binding and of GC or AC activation that involves G protein coupling.

To test whether ZnPP IX is nonselective in blocking the actions of different agonists that work via the activation of membrane receptors coupled with G protein, we examined the effect of bethanechol and PHI. Bethanechol causes LES smooth muscle contraction (Gilbert et al., 1984) through the activation of the muscarinic receptor coupled to G protein (Goyal, 1989; Eglen et al., 1996). PHI is another neuropeptide closely related to VIP and also is known to cause the smooth muscle relaxation via activation of G protein-coupled receptor activation. The effects of bethanechol were not at all modified, negating the possibility that ZnPP IX was nonselective to the extent that it blocked the actions of all the agonists that work via G protein coupling. In addition, unlike VIP, the inhibitory effect of PHI tested in the maximal concentration was not modified by ZnPP IX. Furthermore, ZnPP IX had no effect on the basal LES smooth muscle tone in the opossum. The data in the LES are similar to those in the IAS, in which the actions of PHI were not modified by the HO inhibitor (Rattan and Chakder, 1993). Considering that the HO inhibitor blocked the smooth muscle relaxation by diverse agonists without modifying the NANC relaxation of the opossum LES may in fact suggest relative selectivity of action of ZnPP IX.

The exact role of HO pathway in the LES relaxation by NANC nerve stimulation remains to be determined. However, it appears that if HO is involved, it is not sensitive to either of the currently available HO inhibitors, ZnPP IX and SnPP IX, according to the present and an earlier study (Ny et al., 1996). Whether the blockade of the action of VIP, isoproterenol and ANF in the opossum LES is dependent on the inhibitory effect of ZnPP IX on HO also remains to be determined; this will require systematic stoichiometric studies with monitoring of HO activity and smooth muscle relaxation in response to different agonists.

cGMP and cAMP serve as the second messengers for the NANC nerve-mediated relaxation of the LES and other smooth
muscles in response to the inhibitory neurotransmitters NO and VIP, respectively. It was interesting that ZnPP IX attenuated the relaxation in response to the activation of both cGMP and cAMP pathways but did not modify the LES relaxation by NANC nerve stimulation. The data may not be interpreted to imply that these pathways and VIP are not involved in the NANC nerve-mediated smooth muscle relaxation. On the contrary, we speculate that the NANC nerve-mediated LES smooth muscle relaxation is unique and offers a challenge in the understanding of gastrointestinal smooth muscle pathophysiology. This may be so because of diverse back-up mechanisms for the multiplicity of inhibitory neurotransmitters and the smooth muscle relaxation.

The lack of effect of ZnPP IX on the NANC nerve-mediated LES relaxation suggests the absence of the role of HO in the opossum LES relaxation. Alternatively, when VIP pathway is blocked by the HO inhibitor, the NOS pathway may be up-regulated and compensates for the inhibitory neurotransmission in response to the NANC nerve stimulation. The present data in the LES and earlier data in the IAS (Rattan and Chakder, 1993) showing that the smooth muscle relaxations by SNP, NO and CO are unaffected by ZnPP IX are consistent with the hypothesis because they activate soluble GC directly, bypassing the conventional receptor activation that involves G protein coupling. There are two types of data that appear to support the NO up-regulation hypothesis. First, studies by Ny et al. (1996) showed results similar to those of our study; the HO inhibitor, unlike the NOS inhibitor (that caused a dramatic attenuation of the relaxation), had no significant effect on the NANC nerve-mediated relaxation of the feline LES. Interestingly, when the NOS inhibitor was combined with the HO inhibitor, the attenuation of the LES relaxation by the NOS inhibition was significantly less. Second, studies in the rabbit IAS (Chakder et al., 1996) showed the direct stimulation of NOS by the HO inhibitor. The data show a facilitatory modulation of NOS by HO in inhibitory or inhibitory modulation by endogenous CO, the end product of HO pathway.

Further support for the hypothesis of counter regulation of NO by HO comes from the observations in the primary cultures of cerebellar granule cells (Ingi et al., 1996) and some other systems (White and Marletta, 1992; Meffert et al., 1994) monitoring CO production, NOS activity and cGMP levels. The counterregulation between HO and NOS pathways may prevent the tissues from deleterious effects by overproduction of NO. Interestingly, colocalization of HO and NOS, as recently shown in brain neurons (Vincent et al., 1994) and of the gut in the feline LES (Ny et al., 1996), may facilitate the counterregulation.

Why the HO inhibitor by itself causes smooth muscle relaxation in one system (Chakder et al., 1996), suppression of the NANC nerve-mediated relaxation in some (Tottrup et al., 1995; Rattan and Chakder, 1993) and virtually no effect in the other is not known. The possible explanation may lie in the fact that the pathways for neurotransmission and the intracellular mechanisms of basal tone and relaxation are tissue and species specific.

From these data, we conclude that ZnPP IX causes inhibition of AC and GC via specific G protein-coupled receptor activation by VIP, isoproterenol and ANF. Because NANC nerve-mediated relaxation in the LES predominantly uses the NOS pathway and released NO uses a unique receptor activation that bypasses the G protein coupling, it is not surprising that the NANC nerve-mediated relaxation of LES smooth muscle is not modified by ZnPP IX. The lack of effect of ZnPP IX on the NANC-mediated LES relaxation may be due to either the absence of the role of HO in the relaxation or the up-regulation of the NOS when the VIP pathway is blocked.

References
Tottrup A, Kruusen MA, Sorensen PH and Glavind EB (1995) Pharmacological...

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