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DIMETHYLPHENYLPIPERAZINIUM (DMPP)-INDUCED RELAXATION AND ELEVATION OF CYCLIC GMP CONTENT IN CANINE LOWER ESOPHAGEAL SPHINCTER (LES)*

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Abstract—Cyclic GMP has been proposed as an intracellular mediator of neuronally-induced relaxation in lower esophageal sphincter (LES) smooth muscle. If cyclic GMP is indeed an intracellular messenger, then agents that activate enteric neurons of the sphincter [e.g. the ganglionic nicotinic receptor agonist dimethylphenylpiperazinium (DMPP)] also should cause a relaxation that is associated with an increase in cyclic GMP content. In isolated smooth muscle strips of canine LES, DMPP produced a rapid relaxation that was accompanied by a significant ($P < 0.05$) increase in cyclic GMP content with no change in cyclic AMP content. Pretreatment of tissues with either tetrodotoxin or hexamethonium antagonized both the DMPP-induced relaxation and the associated increase in cyclic GMP. The combination of phentolamine and meclofenamic acid also antagonized both the relaxation and the elevation of cyclic GMP produced by DMPP. Electrical field stimulation (EFS)-induced relaxation and elevation in cyclic GMP was unaltered by meclofenamic acid and phentolamine. In conclusion, DMPP (like neuronal electrical activation) relaxed isolated canine LES through an enteric neuronal inhibitory pathway. The presence of phenotolamine and meclofenamic acid did not affect EFS-induced effects, but blocked both the relaxation and the increase in cyclic GMP produced by DMPP, suggesting a more complicated pathway for DMPP in the release of inhibitory transmitter.

The lower esophageal sphincter (LES) provides the barrier that prevents reflux of gastric contents into the esophagus [1]. Transient or prolonged abolition of this high pressure zone is associated with reflux in both asymptomatic individuals and in persons with gastroesophageal reflux disease (GERD) [2–4]. Therefore, a greater understanding of the pharmacological and biochemical regulation of LES relaxation may provide insight into the development of novel therapeutic agents to treat individuals with GERD.

LES pressure is neurally regulated. Stimulation of the preganglionic fibers, present in the vagus, produces relaxation [5, 6]. These preganglionic neurons are believed to synapse with the intrinsic inhibitory neurons [6, 7] present in the myenteric plexus. Electrical field stimulation (EFS) of these neurons relaxes isolated LES smooth muscle. Furthermore, pharmacological activation of this system by ganglionic stimulants such as 1,1-dimethyl-4-phenylpiperazinium (DMPP) also relaxes the LES [6].

More recently, we have shown that LES relaxation is associated with an elevation of either cyclic AMP or cyclic GMP content [8–12]. Electrical stimulation of the myenteric plexus produces an increase in cyclic GMP content but not in cyclic AMP [8, 9, 11, 12]. If cyclic GMP is indeed an intracellular messenger, then in addition to EFS, agents that activate receptors to stimulate inhibitory neurons should also cause LES relaxation associated with increased cyclic GMP. In this study, we evaluated the functional and biochemical effects of activation of these inhibitory neurons by DMPP, the ganglionic nicotinic receptor agonist.

METHODS

Functional studies. Mongrel dogs of either sex were given an overdose of sodium pentobarbitol (100 mg/kg, i.v.) or anesthetized dogs received a super-saturated solution of potassium chloride (i.v.). The intra-abdominal esophagus and gastric fundus were removed and placed in buffered Krebs solution containing the following composition (mM): NaCl, 117.9; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.18; $MgSO_4$, 1.18; $NaHCO_3$, 25.0; dextrose, 11.1. The tissue was cut along the lesser curvature and pinned to a dissecting board. The mucosa was removed, and strips of circular smooth muscle, approximately 4×15 mm, were cut from the LES at the gastroesophageal junction. Each strip was tied at one end to a glass rod positioned between two silver electrodes mounted approximately 1.5 cm apart. The other end was tied to a Grass force transducer (FT-03) to measure isometric contractions or relaxations. The electrode and tissue strips were suspended in 10-mL water-jacketed, quick-release tissue baths containing Krebs buffer oxygenated with 95% O_2–5% CO_2 and maintained at 37°. An initial tension of 2 g was applied to each muscle strip, which was then

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equilibrated for a minimum of 1 hr. After equilibration, an initial relaxation to EFS was determined. Only tissues that gained tone spontaneously during equilibration and relaxed to electrical field stimulation (EFS; 65 V, 1 msec, 4 Hz) were considered to be LES. All other tissues were eliminated. All data are expressed as a percentage of the initial relaxation response. Antagonist pretreatments were for 30–50 min prior to EFS- or DMPP-induced relaxations. Muscle strips were frozen during peak relaxations to EFS (30 sec) or DMPP (10–20 sec) and stored frozen at −80° until assayed for cyclic nucleotide content by radioimmunoassay (RIA).

Biochemical studies. Frozen tissues were cut (10–15 mg) and homogenized in 10% trichloroacetic acid (TCA) with [^{3}H]cyclic AMP (4000 cpm) added to monitor recovery. The homogenate was centrifuged at 3000 *g* for 15–20 min to remove precipitated protein. TCA was removed from the supernatant fraction by washing four times with water-saturated ether. Using the acetylation method of Brooker *et al.* [13], cyclic AMP and cyclic GMP content in the supernatant fraction was measured by radioimmunoassay with commercially available kits from New England Nuclear, Boston, MA. Protein content was determined by the method of Lowry *et al.* [14] using bovine serum albumin as the standard. The percentage of recovery (85–95%) was used to correct the cyclic nucleotide content, which was expressed as femtomoles (cyclic GMP) or picomoles (cyclic AMP) of cyclic nucleotide per milligram of protein.

The results are expressed as means ± SE. Significant differences were determined for the functional data by using Student's *t*-test. The cyclic nucleotide data were analyzed for statistical significance by Dunnett's *t*-test. All changes were considered as significant when $P < 0.05$.

Drugs. The following drugs were used: 1,1-dimethyl-4-phenylpiperazinium iodide (Park Davis & Co.), hexamethonium Bromide (ICN Pharmaceuticals), phentolamine HCl (CIBA), meclofenamic acid (Warner Lambert Co.), and tetrodotoxin (Sigma).

RESULTS

Relaxation by DMPP or EFS. Addition of DMPP produced a concentration-dependent relaxation of isolated canine LES smooth muscle (Fig. 1A). This relaxation was transient with peak relaxations occurring 10–20 sec after the addition of DMPP. The maximum response obtained to DMPP was 5.4 ± 0.5 g and the EC_{50} value was 13.7 μM. In subsequent experiments, 10 or 100 μM DMPP was used to investigate the effect on cyclic nucleotide content.

EFS-induced relaxations occurred in a frequency-dependent manner (Fig. 1B). Maximum relaxations measured 4.3 ± 1.03 g at 2 Hz and occurred within 30 sec after the onset of stimulation. In subsequent studies, frequencies of 1 and 4 Hz were chosen to determine cyclic nucleotide accumulation.

Effects of tetrodotoxin (TTX) on relaxation. Neuronal blockade with TTX (1 μM) for 30–35 min inhibited DMPP-induced relaxations by 98.8 ± 1.2% (10^{-5} M) and 96.1 ± 2.9% (10^{-4} M) respectively (Fig. 2A). EFS-induced relaxations also were

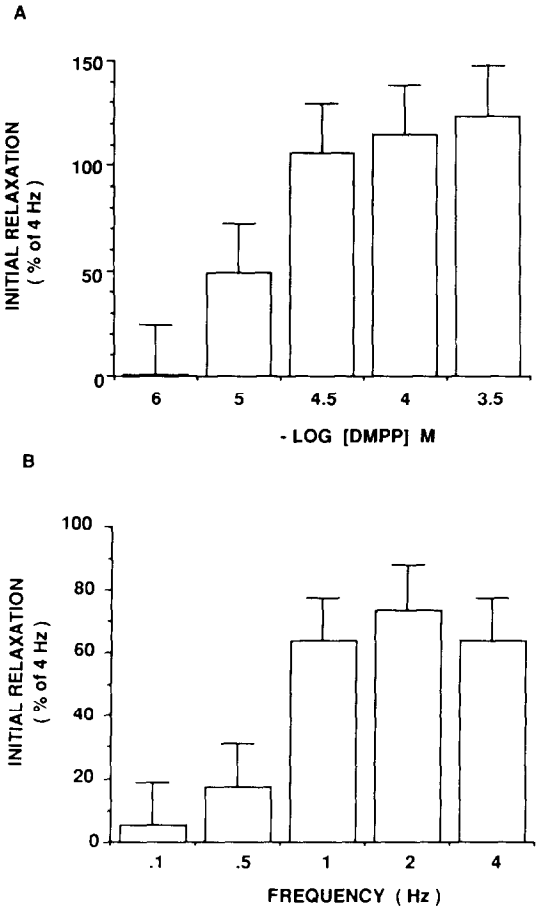

Fig. 1. DMPP-induced (A) and EFS-induced (B) relaxation of isolated canine lower esophageal sphincter. Results are the means ± SE of 3–4 experiments.

inhibited in the presence of TTX by 100% (1 Hz) and 83.3 ± 3.9% (4 Hz) respectively (Fig. 2B). TTX alone caused a variable increase in LES tone ranging from 0.8 to 4.8 g.

Changes in cyclic nucleotide levels associated with DMPP- and EFS-induced relaxations in the absence and presence of tetrodotoxin. In the absence of TTX, both DMPP- and EFS-induced relaxations were accompanied by concentration or frequency, respectively, related elevations in cyclic GMP content (Table 1), whereas cyclic AMP levels were not altered significantly. Pretreatment with TTX abolished both the EFS- and DMPP-induced increases in cyclic GMP content. Furthermore, TTX reduced the basal content of cyclic GMP without altering cyclic AMP content (Table 1A and B).

Effects of hexamethonium on relaxation. Pretreatment of tissues with the ganglionic blocker hexamethonium (5 μM) for 30–40 min significantly inhibited DMPP-induced relaxations by 94.1 + 2.8 (10^{-5} M) and 63.8 ± 10.6 (10^{-4} M) % (Fig. 3A). Hexamethonium did not antagonize EFS-induced relaxation significantly. A small inhibition of EFS-induced relaxation (1.0 Hz) was noted: however, this was not statistically significant (Fig. 3B).

Effects of hexamethonium on cyclic nucleotides. In

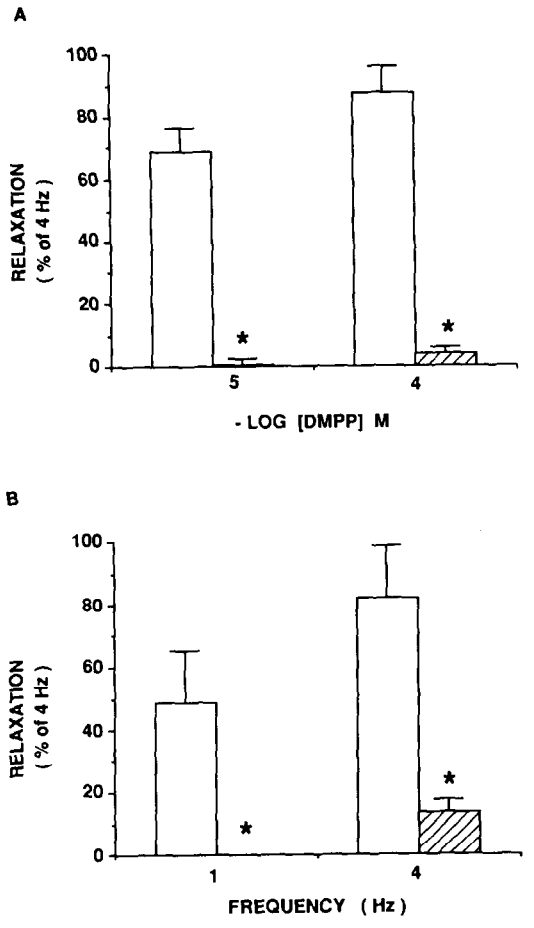

Fig. 2. Effects of tetrodotoxin (TTX) on DMPP-induced (A) or EFS-induced (B) relaxations. Tissues were pretreated for approximately 30–35 min in the absence (□) or presence (▨) of TTX (1 μM) prior to addition of DMPP. Twenty seconds after the addition of DMPP, tissues were freeze-clamped, and the cyclic nucleotide content was measured by RIA. Results are the means ± SE of 6 experiments. Key: (*) Significantly decreased from control ($P < 0.05$, Student's *t*-test).

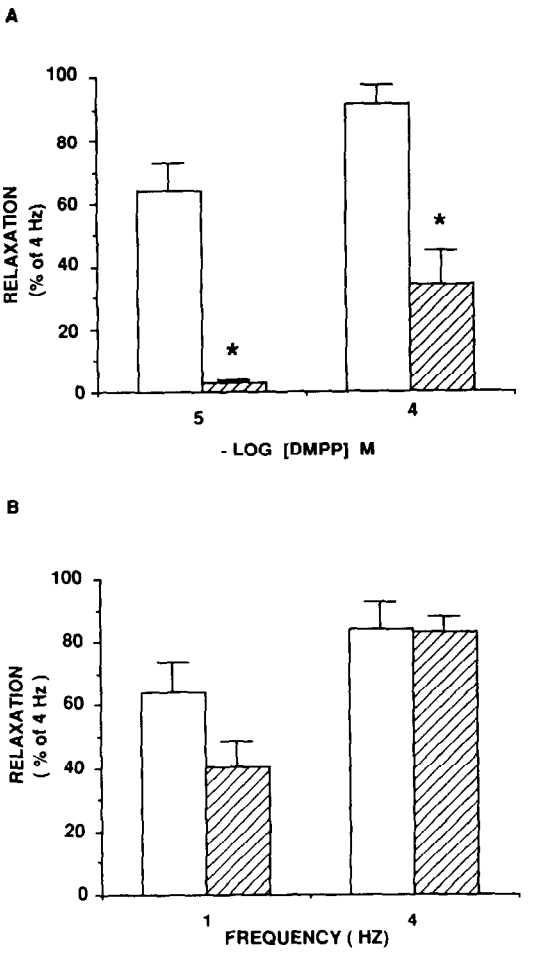

Fig. 3. Effects of hexamethonium (HEX) on DMPP-induced (A) and EFS-induced (B) relaxation of isolated canine lower esophageal sphincter. Tissues were pretreated for approximately 30 min in the absence (□) or presence (▨) of HEX (5 μM) prior to the addition of DMPP. Twenty seconds after the addition of DMPP, tissues were freeze-clamped and the cyclic nucleotide content was measured by RIA. Results are the means ± SE of 7–8 experiments. Key: (*) Significantly decreased from control ($P < 0.05$, Student's *t*-test).

Table 1. Effect of TTX (1 μM) on DMPP or EFS induced changes in cyclic nucleotide content of the lower esophageal sphincter

		cGMP (fmol/mg protein)		cAMP (pmol/mg protein)	
	Relaxant	Controls	TTX (1 μM)	Controls	TTX (1 μM)
(A)	None (basal)	239.0 ± 30.9	91.6 ± 19.9	6.0 ± 0.4	5.1 ± 0.3
	10^{-5} M DMPP	408.6 ± 60.2	80.9 ± 10.8	5.8 ± 0.7	5.2 ± 0.4
	10^{-4} M DMPP	739.0 ± 106.8*	110.5 ± 14.2	5.9 ± 0.4	5.4 ± 0.4
(B)	None (basal)	361.7 ± 87.1	108.3 ± 33.8	6.9 ± 0.5	5.7 ± 0.5
	EFS 1 Hz	417.9 ± 73.2	77.4 ± 15.1	7.6 ± 0.7	6.1 ± 0.3
	EFS 4 Hz	875.3 ± 257.7*	64.0 ± 9.5	7.6 ± 0.5	6.1 ± 0.6

Abbreviations: TTX, tetrodotoxin; DMPP, dimethylphenyl piperazinium; and EFS, electrical field stimulation. Results are the means ± SE of 6 experiments.

* Significantly different from basal levels ($P < 0.05$, Dunnett's *t*-test).

Table 2. Effect of hexamethonium (HEX) on DMPP-induced (A) or EFS-induced (B) changes in cyclic nucleotide content of the lower esophageal sphincter

		cGMP (fmol/mg protein)		cAMP (pmol/mg protein)	
	Relaxant	Controls	HEX (5 μM)	Controls	HEX (5 μM)
(A)	None (basal)	443.4 ± 69.0	380.7 ± 107.1	7.3 ± 0.9	6.4 ± 0.9
	10^{-5} M DMPP	518.8 ± 117.3	369.1 ± 98.5	7.4 ± 1.1	6.3 ± 0.7
	10^{-4} M DMPP	922.4 ± 238.6*	430.8 ± 67.3	7.0 ± 0.9	7.2 ± 0.8
(B)	None (basal)	217.6 ± 67.7	242.4 ± 44.7	5.6 ± 0.3	5.8 ± 0.4
	1 Hz EFS	270.6 ± 40.6	278.1 ± 53.0	5.3 ± 0.2	5.8 ± 0.3
	4 Hz EFS	747.9 ± 108.1*	778.1 ± 121.4*	6.8 ± 0.4*	6.7 ± 0.3

Results are the means ± SE of 6–8 experiments.
* Significantly different from basal levels ($P < 0.05$, Dunnett's t-test).

a similar manner, pretreatment with hexamethonium inhibited the DMPP-induced elevation of cyclic GMP (Table 2A), whereas it did not alter significantly the EFS-induced increase in cyclic GMP content (Table 2B). Furthermore, DMPP in the absence or presence of hexamethonium did not increase cyclic AMP content (Table 2A). However, in this study, EFS (4 Hz) increased cyclic AMP content.

Effects of phentolamine and/or meclofenamic acid on relaxation. In an additional study, the effects of stimulation of intrinsic inhibitory neurons were examined. The tissues were first pretreated with both adrenergic and cholinergic antagonists as well as a cyclooxygenase inhibitor. These drugs included: phentolamine (10 μM), atropine (1 μM), propranolol (1 μM) and meclofenamic acid (1 μM). In the presence of these antagonists, DMPP-induced relaxations were inhibited (data not shown). In later experiments we demonstrated that it was the combination of phentolamine and meclofenamic acid that inhibited the DMPP response. Pretreatment of the tissues for approximately 30–50 min with phentolamine (10 μM) alone or meclofenamic acid (1 μM) alone inhibited the response to DMPP (100 μM) by 23.7 ± 7.0 and 16.0 ± 6.0% respectively (data not shown). This inhibition appeared to be additive since pretreatment of the tissues with the combination of phentolamine (10 μM) and meclofenamic acid (1 μM) for approximately 30–50 min inhibited the response to DMPP (100 μM) by 68.7 ± 15.4% (Fig. 4A). In another experiment, tissues were pretreated with prazosin (0.1 μM), a selective α-adrenergic antagonist, and the response to DMPP (100 μM) was reduced by 31.9 ± 16.1% (data not shown). The degree of inhibition with prazosin was similar to that of phentolamine. On the other hand, EFS-induced relaxations were potentiated by 34.1 ± 7.2% (4 Hz) in the presence of phentolamine and meclofenamic acid (Fig. 4B).

Effects of phentolamine and meclofenamic acid on cyclic nucleotides. Cyclic GMP levels for DMPP-induced relaxations (100 μM) were reduced significantly from controls in the presence of phentolamine (10 μM) and meclofenamic acid (1 μM) by 66.1% (Table 3A). Basal levels of cGMP were unaffected in the presence of phentolamine plus meclofenamic acid. On the other hand, cyclic GMP levels

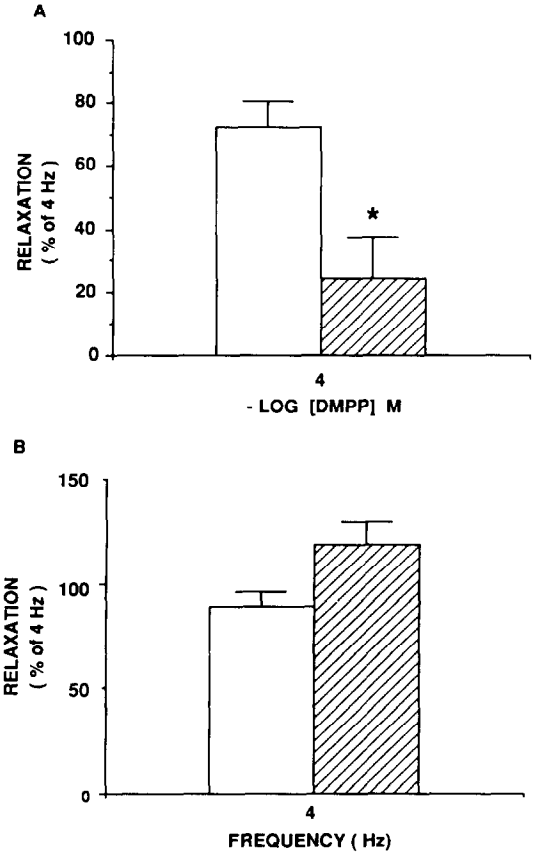

Fig. 4. Effect of phentolamine and meclofenamic acid on DMPP-induced (A) and EFS-induced (B) relaxation of isolated canine lower esophageal sphincter. Tissues were pretreated for approximately 30 min in the absence (□) or presence (▨) of phentolamine (10.0 μM) and meclofenamic acid (1 μM) prior to the addition of DMPP. Twenty seconds after the addition of DMPP, tissues were freeze-clamped and the cyclic nucleotide content was measured by RIA. Results are the means ± SE of 5 experiments. Key: (*) Significantly decreased from control ($P < 0.05$, Student's t-test).

for EFS-induced relaxations (4 Hz) in the presence of phentolamine plus meclofenamic acid showed a 36.2% reduction from controls (Table 3B). However, the basal level of cyclic GMP for this group

Table 3. Effect of phentolamine and meclofenamic acid (P + M) on DMPP-induced (A) or EFS-induced (B) changes in cyclic nucleotide content of the lower esophageal sphincter

		cGMP (fmol/mg protein)		cAMP (pmol/mg protein)	
	Relaxant	Control	P (10 μM) + M (1 μM)	Control	P (10 μM) + M (1 μM)
(A)	None (basal	112.9 ± 30.5	114.5 ± 21.5	4.3 ± 0.8	4.0 ± 0.3
	10^{-4} DMPP	1177.3 ± 215.9*	399.5 ± 145.4	5.6 ± 0.5	4.7 ± 0.2
(B)	None (basal)	341.8 ± 86.4	188.7 ± 40.8	5.3 ± 0.4	4.0 ± 0.3
	4 Hz	1027.3 ± 142.2*	655.4 ± 203.8*	6.6 ± 0.7	4.8 ± 0.4

Results are the means ± SE of 4–6 experiments.
* Significantly different from basal levels ($P < 0.05$, Dunnett's t-test).

of tissues also decreased by 44.8% of the control. This represents a 3- and 3.5-fold increase over the basal levels for controls and phentolamine plus meclofenamic acid treated tissues respectively. Cyclic AMP levels for DMPP- or EFS-induced relaxations in controls or in the presence of phentolamine plus meclofenamic acid were not significantly different (Table 3, A and B).

DISCUSSION

Previous studies in isolated strips of opossum and canine LES smooth muscle have shown that electrical stimulation of the intrinsic neurons produces a relaxation and an increase in cyclic GMP content but not cyclic AMP [8, 9, 11, 12]. Both the relaxation and the elevation in cyclic GMP are blocked by TTX, suggesting that both are neuronally mediated. However, since EFS should stimulate all neurons in the myenteric plexus of muscle strips, we made an attempt to activate more selectively the inhibitory pathway by examining both the mechanical and biochemical changes produced by the classical ganglionic stimulant DMPP.

In isolated strips of canine LES, DMPP produced a concentration-dependent relaxation and elevation in cyclic GMP without a change in cyclic AMP. Pretreatment with TTX antagonized both the relaxation and the elevation in cyclic GMP content produced by DMPP. These findings suggest that the *in vitro* relaxation produced by DMPP is neuronally mediated. Furthermore, in a parallel study, TTX blocked the EFS-induced relaxation and elevation of cyclic GMP content without a change in cyclic AMP. Together these data indicate that both DMPP and EFS appear to activate the intrinsic inhibitory neurons, and in both cases an associated increase in cyclic GMP content also was observed.

Since DMPP stimulates neurons by activating a nicotinic receptor, we examined the ability of hexamethonium to antagonize both the relaxation and the elevation in cyclic GMP content produced by DMPP. Hexamethonium inhibited both the relaxation and the elevation of cyclic GMP content produced by DMPP and although hexamethonium produced some inhibition (albeit not significant) of EFS-induced relaxation at 1 Hz it did not inhibit either the relaxation or the increase in cyclic GMP content produced by the higher frequency. These results are consistent with those obtained in the anesthetized opossum with electrical stimulation of the vagus [6]. In anesthetized opossum, intravenous administration of DMPP produces a dose-dependent fall in LES pressure that was antagonized by hexamethonium [6]. Furthermore, hexamethonium also antagonizes the decrease in LES pressure elicited by low frequencies of vagal stimulation, suggesting the presence of a nicotinic vagal input [6].

As mentioned earlier, previous studies have shown that electrical stimulation of the intrinsic inhibitory neurons produces a relaxation and an increase in cyclic GMP content, but not cyclic AMP [8, 9, 11, 12]. These studies were conducted in the presence of various receptor antagonists and cyclooxygenase inhibitors. However, in the present study, no receptor antagonists were used and EFS unexpectedly tended to increase cyclic AMP levels (by 10–25% at 4 Hz) although this increase usually was not statistically significant (Tables 1–3). One possible explanation for this elevation may be the absence of β-adrenergic antagonists in this study. It is known that there are sympathetic fibers present in the LES [15]; therefore, EFS could be acting to release norepinephrine. Norepinephrine acting on the β-adrenergic receptors present in the LES may have produced this small elevation in cyclic AMP content.

Also, unexpectedly, in the present study when we attempted to examine the effects of DMPP-induced relaxations in the presence of antagonists, we found its effects were reduced markedly (data not shown). Preliminary studies suggested that phentolamine, a non-selective α-adrenergic antagonist, prazosin, a selective α-adrenergic antagonist, or meclofenamic acid, a cyclooxygenase inhibitor, were all capable of inhibiting DMPP-induced relaxations. The inhibition appeared to be additive, since pretreatment with the combination of phentolamine and meclofenamic acid blocked the DMPP-induced relaxations. However, these drugs did not inhibit EFS-induced relaxations significantly. This serendipitous finding allowed us to ask the question: When DMPP-induced relaxations were inhibited, was the increase in cyclic GMP also blocked under conditions where EFS-induced relaxations were unaltered? We found that when DMPP-induced relaxations were reduced, there also was a corresponding reduction in the elevation of cyclic GMP content. These findings support the hypothesis that cyclic GMP is an important intracellular

mediator of relaxation in the LES. In addition, these results suggest that DMPP and EFS activate the intrinsic inhibitory neurons differentially. It is difficult to speculate why phentolamine and meclofenamic acid antagonize DMPP effects. In a number of other issues, the effects of nicotinic agonists are dependent on the release of catecholamines [16–18]. Since sympathetic fibers are present in the myenteric plexus [19], it is possible that part of the effect of DMPP in the LES may be mediated through the release of norepinephrine and its subsequent activation of the effect on the intrinsic inhibitory neurons. Furthermore, prostaglandins have been implicated in the action of nicotine on gut smooth muscle [20, 21]. However, EFS-induced responses were not altered with this antagonism. These results suggest that DMPP does not directly activate the intrinsic inhibitory neurons but may act in a more complex manner to stimulate some other fibers and/or interneurons. However, additional experiments to define the neuronal circuitry are needed.

In conclusion, we have demonstrated that both pharmacological and electrical stimulation of the enteric neurons produced relaxation of isolated canine LES and, more importantly, that this relaxation was accompanied by an elevation in cyclic GMP content. These data provided additional confirmation to our initial observation in the opossum, dog and human LES and support the hypothesis that cyclic GMP is an important intracellular mediator of neuronally-induced LES relaxation.

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