Mechanism of Gallbladder Relaxation in the Cat: Role of Norepinephrine¹,²

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Accepted for publication January 20, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

We investigated the mechanisms of neurally mediated relaxation of cat gallbladder muscle. Muscle strips from the gallbladder corpus placed in the muscle bath with oxygenated Krebs’ solution developed spontaneous active tension. Tension was measured with isometric force transducers, and muscle relaxation was expressed as percent decrease of active basal tension. Electrical field stimulation (EFS) evoked a tetrodotoxin-sensitive and hexamethonium-insensitive frequency-dependent relaxation with a maximal relaxation at 20 Hz. Gallbladder muscle strips also relaxed in response to increasing concentrations of vasoactive intestinal peptide (VIP), isoproterenol and, after pretreatment with phenolamine, norepinephrine. Nitric oxide synthase inhibitors N⁴-nitro-L-arginine and N⁴-nitro-L-arginine methyl ester at a concentration of 100 µM, which blocked EFS-induced relaxation in the lower esophageal sphincter, had no significant effect on EFS-induced gallbladder muscle relaxation. The VIP antagonists VIP10-28 and [D-Pro³, D-Phe⁶, Leu¹⁷]VIP at a concentration of 10 µM that blocked exogenous VIP-induced gallbladder relaxation also had no effect on the relaxation caused by EFS. In contrast, either propranolol or guanethidine at concentrations of ≥1 µM significantly reduced EFS-evoked gallbladder relaxation (P < .01, analysis of variance). It is concluded that norepinephrine utilizing beta adrenergic receptors mediates EFS-stimulating postganglionic intramural neurons in the cat gallbladder.

Gallbladder stores most of the hepatic bile during the interdigestive phase to make it available during the digestive phase for optimal fat digestion and absorption (Shaffer et al., 1980; Dodds et al., 1989). The mechanisms by which most of the hepatic bile flow is diverted toward the cystic duct and gallbladder, rather than flow through the sphincter of Oddi and empty into the duodenum, are poorly understood. It is believed that the sphincter of Oddi behaves like a resistor, with its tonic and phasic contractions preventing most of the bile from draining into the duodenum and diverting it toward the gallbladder (Tooili, 1984; Behar and Biancani, 1985; Dodds et al., 1989). It is unclear, however, whether gallbladder filling and distention is a purely passive process or whether it is in part mediated by an active neural reflex. In the cat, it has been shown in vivo that the gallbladder relaxes in response to distention of the common bile duct mediated by autonomic neural mechanisms (Thune et al., 1986). This finding is supported by the in vitro observation that EFS relaxes the human gallbladder strips by a TTX-sensitive mechanism (McKirdy et al., 1994). This neurally mediated gallbladder relaxation, however, does not appear to be present in all species; for instance, prairie dog and guinea pig gallbladders do not relax in response to similar stimuli (Li et al., 1994; Parkman et al., 1996).

The aim of this study was to determine the nature of the postganglionic neurotransmitters responsible for in vitro relaxation of cat gallbladder muscle strips.

Materials and Methods

Animals. Adult cats of either sex weighing 3 to 5 kg were purchased from Liberty Research Laboratory (Waverly, NY). Their use was approved by the Animal Welfare Committee of Rhode Island Hospital. The animals were fasted overnight and anesthetized initially with intramuscular ketamine hydrochloride (30 mg/kg) followed by intraperitoneal pentobarbital sodium (30 mg/kg). The gallbladder was exposed with midline incision, and the cystic duct was carefully clamped. Bile was removed from the gallbladder, and its cavity was rinsed with ice-cold, oxygenated Krebs’ physiological solution. The composition of the Krebs’ solution was as follows (in mM): NaCl 116.6, KCl 3.4, NaHCO₃ 21.9, NaH₂PO₄ 1.2, CaCl₂ 2.5, MgCl₂ 1.2 and glucose 5.4. The gallbladder was quickly removed from the liver bed and placed in ice-cold Krebs’ buffer continuously aerated with 95% O₂/5% CO₂.

Muscle strip preparation and measurement of isometric tension. Transverse rings, 2 mm in width, were obtained from the

ABBREVIATIONS: ANOVA, analysis of variance; EFS, electrical field stimulation; LES, lower esophageal sphincter; CCK, cholecystokinin; L-NA, N⁴-nitro-L-arginine; L-NAME, N⁴-nitro-L-arginine methyl ester; NE, norepinephrine; NO, nitric oxide; TTX, tetrodotoxin; VIP, vasoactive intestinal peptide.
gallbladder corpus by cutting with blades held in parallel in a metal block (Lee et al., 1989). Each ring was mounted in a separate 1-ml muscle bath in Krebs' buffer solution at pH 7.4 and 37°C. They were continuously aerated with 95% O2/5% CO2. The rings were stretched passively to an initial tension of 2.5 × g (at or close to optimal tension development) and equilibrated for 1 to 2 hr with occasional perfusion (Lee et al., 1989). Gallbladder muscle rings developed spontaneous active tension during the equilibration period. The experimental protocols were then carried out when the muscle rings developed steady active tension and perfusion had been stopped. Tension was measured with an isometric force transducer (Gould Statham UC2, Cleveland, OH) and recorded on a polygraph (model 7 PCM 12B, Grass Instruments, Quincy, MA). Basal active tension and change in tension were obtained after subtracting passive tension from all measurements. Passive tension was obtained at the end of the experiment after the administration of 20 mM EDTA. Muscle relaxation was expressed as percent reduction of the basal active tension after EFS or agonists.

EFS was delivered from a Grass S 48 stimulator. The electrodes consisted of a pair of platinum wires placed 4 mm apart parallel to each other on the either side of the muscle strips (Biancani et al., 1984). Neurolyl mediated muscle relaxation of gallbladder strips was measured in response to increasing frequencies (1–40 Hz) of EFS and using the parameters of 100 V and a 0.5-msec pulse duration for 20 sec. Relaxant agents and antagonists were delivered directly into muscle chambers in volumes of <0.1 ml. The antagonists were preincubated with muscle strips for 10 min before EFS or agonist stimulation.

**Drugs and chemicals.** TTX was purchased from Calbiochem-Behring (La Jolla, CA). VIP, VIP10–28 and [3Cl-D-Phe6,Leu17]VIP (VIP analog) were purchased from Bachem (Torrance, CA). Atropine sulfate, isoproterenol, propranolol, Cibacron blue 3GA (reactive blue 2), L-NA, L-NAME, norepinephrine and guanethidine were purchased from Sigma Chemical (St. Louis, MO).

**Statistical analysis.** All values are expressed as mean ± S.E. of three to six experiments from different animals. Statistical significance between different groups was determined by using two-factorial repeated measures ANOVA, and a value of P < .05 was considered statistically significant.

**Results**

Gallbladder muscle strips developed spontaneous and steady active tone after equilibration within the range of 2.5 to 3.5 × g. Electrical field stimulation (100 V, 0.5 msec, 20 sec) of gallbladder muscle strips evoked either a biphasic response with an initial small contraction followed by a relaxation or a relaxation alone (fig. 1). Relaxation was frequency dependent with a maximal relaxation of 71.2 ± 3.4% (mean ± S.E., n = 6) occurring at 20 Hz. Increasing the pulse duration of the stimulus from 0.2 to 1.0 msec did not result in any further increase in relaxation. The relaxation induced by EFS was not significantly affected by the cholinergic antagonists atropine (10 μM) and hexamethonium (10 μM). However, it was completely blocked by 10 μM tetrodotoxin (P < .001, by ANOVA) suggesting that the relaxation induced by EFS is neurally mediated.

We next examined the role of two inhibitory neurotransmitters NO and VIP, which have been shown to mediate the nonadrenergic noncholinergic inhibitory innervation of the gastrointestinal tract in all animals species studied (Biancani et al., 1984; Nurko et al., 1988; Bult et al., 1990; Tottrup et al., 1991). The effects were tested of two NO synthase inhibitors L-NA and L-NAME. There was no significant change in basal active tension after muscle strips were treated with either L-NA or L-NAME (100 μM). Neither L-NA nor L-NAME at a concentration of 100 μM blocked gallbladder relaxation induced by EFS with frequencies from 1 to 40 Hz (fig. 2). In contrast, the LES relaxation induced by EFS was almost abolished by the same concentration of L-NAME (fig. 3). Furthermore, the effects of two VIP antagonists VIP10–28 and [3Cl-D-Phe6,Leu17]VIP were also tested in the gallbladder muscle strips. Figure 4 shows that neither VIP10–28 nor [3Cl-D-Phe6,Leu17]VIP at 10 μM inhibited EFS-induced gallbladder muscle relaxation. In contrast, the gallbladder relaxation induced by exogenous VIP at concentrations of 100 nM to 10 μM was reduced by these two VIP antagonists (fig. 5).

Increasing concentration (1 nM to 1 mM) of isoproterenol, a beta adrenergic receptor agonist, caused a dose-dependent relaxation of the gallbladder muscle strips with a maximal relaxation of 87.1 ± 3.8 (mean ± S.E., n = 4) at 100 μM. Norepinephrine caused a weak gallbladder contraction or, most frequently, a prolonged relaxation after pretreatment of the muscle strips with phentolamine (10 μM) or TTX (10 μM); however, it consistently caused gallbladder relaxation. The dose-response curve with norepinephrine after phentolamine was similar to that observed with isoproterenol and unaffected by 10 μM TTX. The ED<sub>100</sub> values (100 μM) and ED<sub>50</sub> values (1 μM) were similar. Pretreatment of the muscle strips with the beta adrenergic receptor antagonist propranolol (10 μM) shifted the norepinephrine dose response to the

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**Fig. 1.** Relaxation of a gallbladder muscle strip in response to EFS with increasing frequencies (2–40 Hz), pulse duration of 0.5 msec and supramaximal voltage (100 V).
The affinity constant ($K_B$) of propranolol was 2.6 $\mu$M. To determine whether adrenergic neurotransmitters are involved in neurally mediated gallbladder relaxation, we examined the effect of propranolol, a beta adrenergic receptor antagonist, on gallbladder relaxation induced by EFS. Gallbladder muscle relaxation induced by EFS was significantly blocked by propranolol at a concentration of 10 $\mu$M ($P < .001$, by ANOVA) and abolished at 100 $\mu$M (fig. 7A). In contrast, propranolol had no effect on EFS-induced relaxation in the cat LES (Data not shown).

To further confirm this finding, the effect of another adrenergic innervation antagonist guanethidine that works via the inhibition of the release of norepinephrine was tested in the gallbladder muscle strips. As shown in figure 7B, the gallbladder relaxation evoked by EFS was also significantly reduced by pretreatment of the gallbladder muscle strips with 1 $\mu$M guanethidine ($P < .01$, by ANOVA) and abolished at 10 $\mu$M.

To further explore the possibility of other putative inhibitory neurotransmitter candidates, such as ATP, in gallbladder muscle relaxation, we examined the effect of the selective ATP antagonist reactive blue 2 on EFS-induced gallbladder muscle relaxation. Reactive blue 2 at 200 $\mu$M had no significant effect on EFS-induced gallbladder relaxation (fig. 8), suggesting purinergic neurotransmitters may not be involved in neurally mediated gallbladder relaxation.
the gastrointestinal tract (Lechin et al., 1997; Li et al., 1994; Parkman et al., 1984). Furthermore, conflicting results have been obtained between EFS and stimulation of the splanchnic nerves in cats and guinea pigs (Persson, 1973; Behar and Biancani, 1980; Doggrell and Scott, 1980). In some studies, relaxation was elicited only after stimulation with CCK.

The present results show that cat gallbladder muscle strips develop spontaneous steady tension and relax in response to EFS in a frequency-dependent manner. This relaxation was TTX sensitive but it was unaffected by cholinergic blockers. The muscle strips also relaxed in a dose-dependent manner in response to VIP and isoproterenol. The gallbladder response to exogenous norepinephrine alone could not be predicted because norepinephrine could induce contraction or relaxation, probably depending on the relative prevalence of alpha or beta adrenergic receptor populations (Persson, 1972); gallbladder muscle strips, however, invariably relaxed after pretreatment with the alpha adrenergic receptor blocker phentolamine.

Based on these findings, we investigated whether any of these inhibitory neurotransmitters participate in the gallbladder relaxation induced by EFS. Our findings suggest that norepinephrine may be a neurotransmitter of the adrenergic nerves responsible for this relaxation using beta adrenergic receptors. These conclusions are based on the following observations: (1) the gallbladder relaxation evoked by EFS was significantly reduced by the beta-adrenergic receptor antagonist propranolol but unaffected by cholinergic inhibitors hexamethonium or atropine; (2) the relaxation induced by isoproterenol and norepinephrine was TTX resistant, suggesting inhibitory neurons.

Thus, propranolol did not affect the ability of the muscle to respond to inhibition or excitation mediated through non-beta adrenergic receptors. Furthermore, the LES relaxation induced by EFS was unaffected by similar concentrations of propranolol.

Previous studies have shown that NO and VIP are the two leading candidates as inhibitory neurotransmitters of the noncholinergic nonadrenergic inhibitory innervation of the gastrointestinal tract (Biancani et al., 1984; Nurko et al., 1988; Dalziel et al., 1991; Tottrup et al., 1991). Both have been shown to be present by histochemical stain in the neuronal bodies and nerve terminals of the gallbladder (Bjorck et al., 1984; Talmage and Mawe, 1993) and are released from the gallbladder wall after vagal stimulation (Bjorck et al., 1986). VIP also causes relaxation by direct action on the gallbladder muscle because it is not antagonized by TTX (Ryan and Ryave, 1978; Feeley et al., 1984). However, VIP antagonists, known to block the relaxation induced by exogenous VIP and by EFS on gastrointestinal circular muscle (Grider and Rivier, 1990), had no effect on the gallbladder relaxation evoked by EFS. Furthermore, two NO synthase inhibitors, L-NA and L-NAME, which are known to block NO mediated relaxation in the gastrointestinal circular muscle, failed to antagonize the gallbladder relaxation induced by EFS. In contrast, and consistent with previous reports (Murray et al., 1991; Tottrup et al., 1991), these NO synthase inhibitors antagonized cat LES relaxation induced by EFS.

These findings suggest that neither VIP nor NO participates in neurally mediated gallbladder relaxation of spontaneous tone in the cat. These findings are in agreement with previous studies that show that NO synthase-positive neurons are confined to the cat gallbladder mucosa and that NO synthase inhibitors had no effect in the cat gallbladder motility (Thune et al., 1995). Thus, gallbladder relaxation may be different from the relaxation of gastrointestinal circular muscle that is mediated by NO and VIP. It is consistent with the adrenergic inhibitory innervation reported in the gallbladder (Persson, 1972; Persson, 1973). In human and guinea pig muscle strips stimulated with carbachol, CCK and histamine, EFS-induced relaxation was blocked by NO synthase inhibitors, which was reversed by L-arginine (McKirdy et al., 1994; Parkman et al., 1997). Furthermore, in guinea pigs, the CCK-induced contraction was enhanced by NO synthase inhibitors (Moure and Maw, 1993). Differences in species and experimental conditions may explain the discrepancies with our findings. It is also possible that the muscle strips may be under tonic inhibitory influence or that CCK may also stimulate inhibitory neurons.

In summary, the present results indicate the existence of a neural mechanism that evokes gallbladder relaxation supporting a physiological reflex relaxation. Adrenergic fibers using norepinephrine and acting on beta-adrenergic receptors...
on the gallbladder muscle appear to mediate EFS-stimulating postganglionic intramural neurons in the cat.

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