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In-vitro Effect of Pirenzepine on Motility of Canine Gall-bladder

M. J. POZO, M. D. SALIDO, J. A. MADRID AND G. M. SALIDO

Faculty of Veterinary Science, Department of Physiology, University of Extremadura, 10071-Cáceres, Spain

Abstract—The action of pirenzepine as an antimuscarinic drug has been investigated on motor responses of muscle strips in the canine gall-bladder. Pirenzepine was further used to examine whether gall-bladder motor responses to synthetic sulphated cholecystokinin octapeptide (CCK-8) are sensitive to pirenzepine. Pirenzepine (10^{-9}–10^{-5} M) antagonized muscle contractions in response to acetylcholine (10^{-9}–10^{-2} M) and CCK-8 (10^{-11}–10^{-6} M) in a significant manner. These findings indicate that pirenzepine is a potent antagonist of two substances that are the principal contractile mediators of gall-bladder contraction and suggest that long-term administration of pirenzepine could contribute to stasis of the gall-bladder.

Studying the actions of selective muscarinic agonists on the lower oesophageal sphincter, Goyal & Rattan (1978) identified two subclasses of muscarinic cholinergic receptors, which they labelled 'M1' and 'M2'. Differences between these receptor subtypes could not be demonstrated with atropine, the classical cholinergic receptor antagonist. However, their presence has been confirmed with the discovery of the M1-selective antagonist pirenzepine (Hammer et al 1980), a tricyclic drug with minimal central nervous system actions (Heathcote & Parry 1980). In that respect, some authors have found no difference between the effect of the drug on gastric secretion and its effect on lacrimal (Dobrilla et al 1983), salivary (Daly et al 1982; Dobrilla et al 1983) or pancreatic secretions (Madrid et al 1985). Muscarinic receptors showing high affinity for pirenzepine have been found in discrete areas of the brain and in peripheral ganglia. Low-affinity sites for pirenzepine have been localized in muscles of the heart and in the smooth muscle of the gastrointestinal tract and urinary bladder.

In a previous paper (Madrid et al 1988) we reported that pirenzepine, intravenously administered to anaesthetized dogs, significantly reduced the increase in intracholedochal pressure produced after the injection of acetylcholine (ACh), cholecystokinin (CCK) or vagal electrical stimulation. However, the mechanism by which pirenzepine exerts its inhibitory effect on the gall-bladder smooth muscle has not been elucidated.

Since gall-bladder stasis is a key factor in the pathogenesis of cholesterol gallstones (Pomerantz & Shaffer 1985) and some patients with peptic ulcers need long-term treatment with pirenzepine, the present study was undertaken to evaluate whether muscarinic receptors governing the in-vitro gall-bladder contraction in response to ACh and CCK are pirenzepine sensitive, since these two substances are the principal contractile mediators of the gall-bladder.

Materials and Methods

After induction of anaesthesia (sodium thiopentone 30 mg kg^{-1} i.v.) gall-bladders from male adult mongrel dogs, 10–15 kg, were removed through a midline incision and immediately placed in a tray filled with aerated Krebs-Ringer solution at 37°C (mM: NaCl 117; KCl 4·6; $MgCl_2.6H_2O$ 2·1; $CaCl_2.2H_2O$ 2·5; Na_2HPO_4 1·2; glucose 11·5). Each gall-bladder was opened along the longitudinal axis, and the mucosa removed by blunt dissection. A single muscle strip (3 × 10 mm) was obtained from the body of each gall-bladder by cutting parallel to the longitudinal axis. The strips were mounted vertically in a 10 mL organ bath containing Krebs-Ringer solution and bubbled with a mixture of 95% O_2–5% CO_2 and maintained at 37°C in a water bath. The contractile response was measured isometrically by a force-displacement transducer (Letica, Spain) mounted on a multichannel pen recorder (Poligraph Letica). The strips were adjusted to an initial tension of less than 2 g and, after a 60 min equilibration period, the length of each muscle strip was increased at the rate of 1 mm each time until the maximal active contractile response to ACh (10^{-5} M) stimulation was achieved. The muscle length corresponding to the optimal preload was maintained throughout the duration of the experiments.

The tissues were examined for their contractile response to ACh and the synthetic sulphated cholecystokinin octapeptide (CCK-8) alone and in the presence of atropine and pirenzepine. Additionally, to establish the specificity of this antagonist, the effects of pirenzepine on contractile response to histamine were also assayed.

Stock solutions of the agonists were diluted with buffer and added cumulatively to the tissue bath in μL amounts to achieve the final molar concentrations reported. Each muscle strip was used to evaluate the effect of not more than three concentrations of antagonists also diluted with buffer.

All the responses to the agonists tested were expressed as a percentage of the maximal response in the absence of antagonists.

ACh was purchased from C. Erba (Italy), CCK-8 and histamine from Sigma (USA), atropine from E. Merck (Germany) and pirenzepine from Boehringer Ingelheim (Germany).

Data analysis

As noted above, contraction was expressed as the increase in

Correspondence to: M. J. Pozo, Faculty of Veterinary Science, Department of Physiology, University of Extremadura, 10071-Cáceres, Spain.

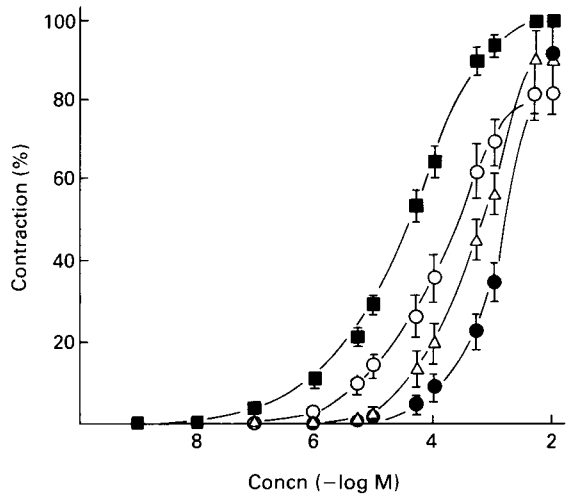

FIG. 1. Dose-response curve for ACh applied cumulatively to dog gall-bladder in absence (■) or in presence of $4{\cdot}2 \times 10^{-9}$ M (○), $4{\cdot}2 \times 10^{-8}$ M (△) and $4{\cdot}2 \times 10^{-7}$ M (●) atropine applied 10 min before the cumulative application of ACh. Each point represents the mean ±s.e.m. of 6 experiments.

strip force as a percentage of the maximal response in control. This normalized measurement was found to be more reproducible than the measurement of absolute values. The dose-response curves for each agonist were analysed for the maximal contractile response and the pA_2 value, defined according to Arunlakshana & Schild (1959). The equations for the Schild plots were derived by linear regression using the least squares method, and the s.e.m. for the y-intercept and slope of the equations were calculated by standard methods (Snedecor & Cochran 1967). The significance of the data was determined by ANOVA; $P < 0{\cdot}05$ was considered statistically significant.

Table 1. Effects of atropine and pirenzepine upon the contractile responses of dog gall-bladder strips to acetylcholine (n = 6).

Antagonist (M)	Max. response (%)	ED50 (−log [M])
Atropine		
0	100	4·44 ± 0·09
$4{\cdot}2 \times 10^{-9}$	81·67 ± 5·19	4·01 ± 0·12*
$4{\cdot}2 \times 10^{-8}$	90·24 ± 7·05	3·43 ± 0·25*
$4{\cdot}2 \times 10^{-7}$	91·13 ± 8·61	2·25 ± 0·52*
Pirenzepine (low doses)		
0	100	4·71 ± 0·19
10^{-9}	93·22 ± 3·96	4·46 ± 0·10*
10^{-8}	85·26 ± 5·46	4·22 ± 0·06*
10^{-7}	86·37 ± 5·60	3·76 ± 0·12*
Pirenzepine (high doses)		
0	100	4·41 ± 0·17
10^{-7}	97·52 ± 7·29	3·54 ± 0·14*
10^{-6}	96·46 ± 8·28	3·31 ± 0·09*
10^{-5}	100·06 ± 4·92	3·07 ± 0·11*

* $P < 0{\cdot}01$ compared with the value in the absence of antagonist.

Results

Effects of antagonists on ACh-stimulated gall-bladder contraction

When isometric contraction from the strips was studied as a function of the concentration of ACh, typical dose-response curves were generated. Strip contractions increased with increasing concentrations of ACh and peaked at 5×10^{-3} M. Higher concentrations of ACh resulted in a submaximal contraction. Fig. 1 presents the normalized data for ACh-stimulated gall-bladder contraction as a percentage of maximal response. Addition of atropine, the classical muscarinic receptor antagonist, at concentrations of $4{\cdot}2 \times 10^{-9}$ to $4{\cdot}2 \times 10^{-7}$ M caused a parallel rightward shift of the ACh dose-response curve. When pirenzepine, an M1 muscarinic receptor antagonist, was added, the dose-response curve to ACh was also shifted to the right in a parallel fashion without any significant decrease of the maximum response (Fig. 2).

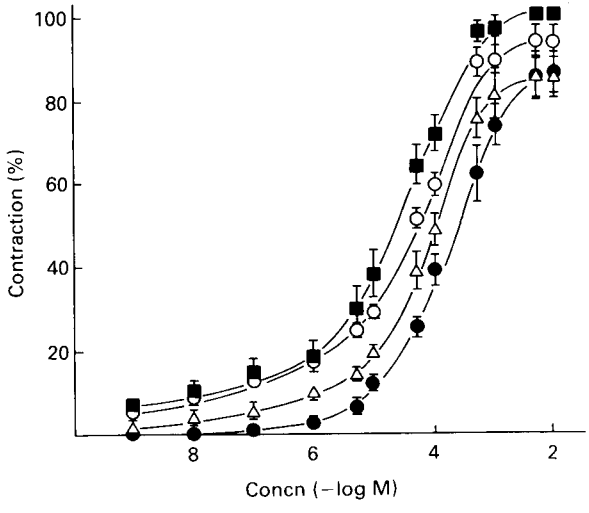

FIG. 2. Dose-response curve for ACh applied cumulatively to dog gall-bladder in absence (■) or presence of 10^{-9} M (○), 10^{-8} M (△) and 10^{-7} M (●) pirenzepine applied 10 min before the cumulative application of ACh. Each point represents the mean ± s.e.m. of 6 experiments.

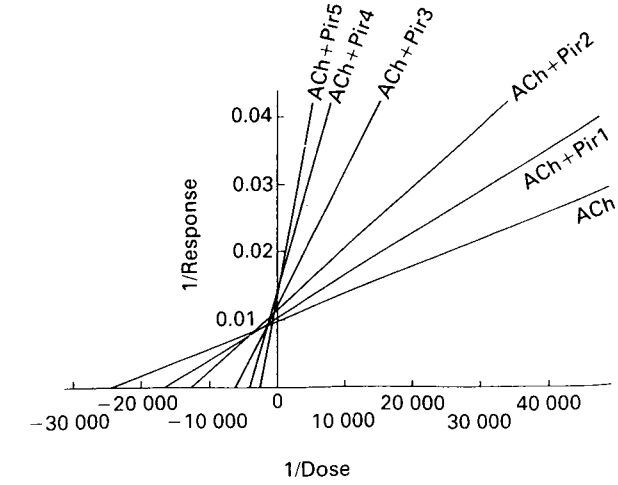

FIG. 3. Double reciprocal plot for the antagonism of ACh by pirenzepine (Pir $1 = 10^{-9}$ M; Pir $2 = 10^{-8}$ M; Pir $3 = 10^{-7}$ M; Pir $4 = 10^{-6}$ M; Pir $5 = 10^{-5}$ M) on dog gall-bladder. Each regression line was calculated from 6 data points.

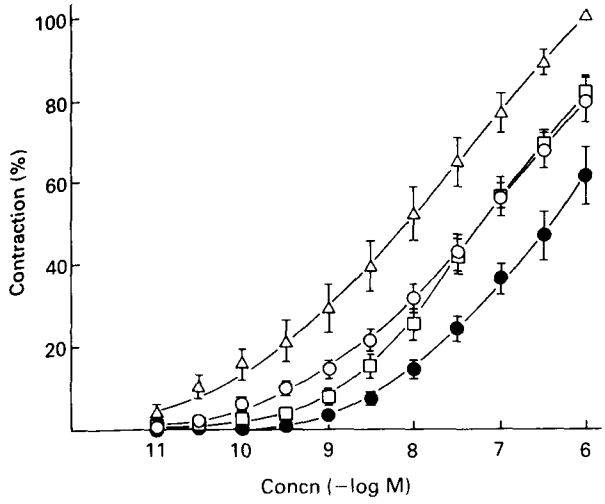

FIG. 4. Dose-response curve for CCK-8 applied cumulatively to dog gall-bladder in the absence (△) or in the presence of $4{\cdot}2 \times 10^{-9}$ M (○), $4{\cdot}2 \times 10^{-8}$ M (□) and $4{\cdot}2 \times 10^{-7}$ M (●) atropine applied 10 min before the cumulative application of CCK-8. Each point represents the mean ± s.e.m. of 6 experiments.

Table 2. Effects of atropine and pirenzepine upon the contractile responses of dog gall-bladder strips to CCK-8 (n = 6).

Antagonist (M)	Response to (10^{-6} M) CCK-8	ED50 (—log [M])
Atropine		
0	100	8·13 ± 0·26
$4{\cdot}2 \times 10^{-9}$	69·48 ± 5·49**	8·06 ± 0·29*
$4{\cdot}2 \times 10^{-8}$	81·83 ± 2·68**	7·63 ± 0·13*
$4{\cdot}2 \times 10^{-7}$	60·55 ± 6·91**	7·29 ± 0·09*
Pirenzepine (low doses)		
0	100	8·40 ± 0·19
10^{-9}	82·00 ± 6·10*	7·62 ± 0·13*
10^{-8}	76·50 ± 8·60*	7·58 ± 0·21*
10^{-7}	73·20 ± 7·50*	7·56 ± 0·22*
Pirenzepine (high doses)		
0	100	8·12 ± 0·09
10^{-7}	63·82 ± 8·03**	7·15 ± 0·09**
10^{-6}	46·04 ± 4·77**	6·99 ± 0·07**
10^{-5}	49·36 ± 2·53**	6·97 ± 0·05**

* $P < 0{\cdot}05$ compared with the value in the absence of the antagonist.
** $P < 0{\cdot}01$ compared with the value in the absence of the antagonist.

The increases in ED50 (agonist concentration that produced half the maximal response of the control curve) induced by both atropine and pirenzepine were statistically significant ($P < 0{\cdot}01$ and $P < 0{\cdot}01$, respectively; 23 d.f.) (Table 1).

The inhibition of ACh-stimulated gall-bladder contraction by atropine and pirenzepine was plotted according to Arunlakshana & Schild (1959). The data points were subjected to linear regression for calculation of the slope and x-intercept. The apparent pA_2 for atropine was 8·53 ± 0·32 (n = 6) and the slope of the line for atropine was not different from unity (−0·98 ± 0·22, n = 6), indicating competitive inhibition at a single receptor type system while for pirenzepine the slope was different from unity (−0·48 ± 0·08, n = 6 for low concentrations of pirenzepine and −0·26 ± 0·14, n = 6 for high concentrations of pirenzepine); this could be due to receptor heterogeneity. The double reciprocal plot (Fig. 3) of gall-bladder contractile response to ACh shows a competitive inhibition by pirenzepine (Segel 1975).

Effects of antagonists on CCK-8-stimulated gall-bladder contraction

The CCK-8-induced contractile responses of the gall-bladder were significantly inhibited in the presence of atropine. The dose response curve for CCK-8 was shifted in parallel to the right in the presence of $4{\cdot}2 \times 10^{-9}$ to $4{\cdot}2 \times 10^{-7}$ M of atropine (Fig. 4). The inhibitory effect of atropine on the contractile responses of gall-bladder to CCK-8 is summarized in Table 2. The ED50 obtained from the dose-response curves rises steadily and in a significant fashion ($P < 0{\cdot}05$, 23 d.f.) as a result of adding atropine; similarly, the response to the highest concentration of CCK-8 (10^{-6} M) assayed in the presence of increased concentrations of atropine decreases significantly ($P < 0{\cdot}01$, 23 d.f.). Analysis of the data according to Arunlakshana & Schild (1959) yielded a straight line whose slope (−0·23 ± 0·09, n = 6) was significantly different from unity.

Pirenzepine at doses of 10^{-9}, 10^{-8} and 10^{-7} M also shifted the concentration-response curve for CCK-8 to the right and depressed the contractile response to CCK-8 (10^{-6} M) by 18, 24 and 27%, respectively (Fig. 5, Table 2). In this case, too, the slope of the regression line obtained by means of Schild analysis was significantly different from unity (−0·10 ± 0·04, n = 6) and similar results were obtained with high concentrations of pirenzepine (slope = −0·09 ± 0·04, n = 6) (Table 2).

Effects of pirenzepine on histamine-stimulated gall-bladder contraction

As would be expected, pirenzepine (10^{-8} M) did not affect the sensitivity significantly ($P > 0{\cdot}1$, 11 d.f.) or the maximal

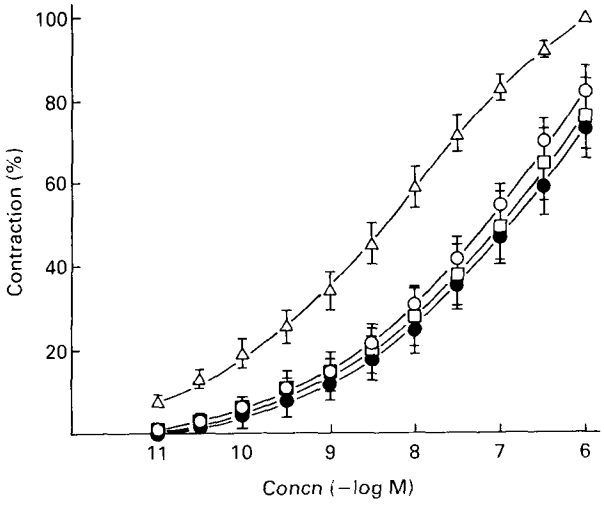

FIG. 5. Dose-response curve for CCK-8 applied cumulatively to dog gall-bladder in absence (△) or presence of 10^{-9} M (○), 10^{-8} M (■) and 10^{-7} M (●) pirenzepine applied 10 min before the cumulative application of CCK-8. Each point represents the mean ± s.e.m. of 6 experiments.

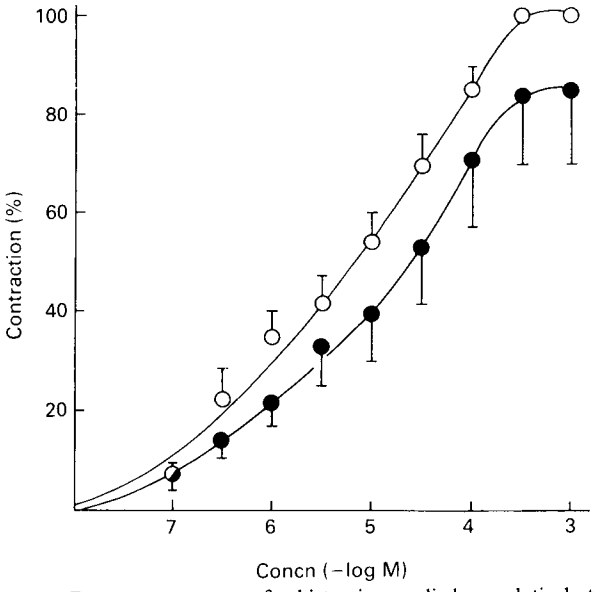

FIG. 6. Dose-response curve for histamine applied cumulatively to dog gall-bladder in the absence (○) or presence (●) of 10^{-8} M pirenzepine applied 10 min before the cumulative application of histamine. Each point represents the mean ± s.e.m. of 6 experiments.

response ($P > 0.1$, 11 d.f.) of gall-bladder strips to the contractile effects of histamine (Fig. 6).

Discussion

The antimuscarinic activity of pirenzepine has been confirmed under in-vitro conditions in dog gall-bladder. A competitive antagonism of atropine on muscarinic receptors is suggested by the parallel shift to the right of log concentration-response curves to ACh with no significant change in maximum response. A similar pattern for the ACh antagonism by pirenzepine has also been observed. However, the fact that the slope of the Schild plots found for pirenzepine on dog gall-bladder does not approach unity, i.e. the theoretical value for simple competitive inhibition at a single receptor type system, may suggest that muscarinic receptors on gall-bladder smooth muscle are not all M1 in type. This argument assumes that pirenzepine binds to gall-bladder muscarinic receptors in a simple competitive manner, which it does appear to do in the case of brain muscarinic receptors (M1 subtype) (El-Fakahany et al 1986). It is known that pirenzepine binds to the M1 receptor subtype with affinity while its affinity for the M2 receptor subtype is low (Hammer et al 1980). For a simple competitive inhibition the intersection of the dose-response lines should be on the 1/response axis; the shift observed (Fig. 3) of the intersection from y-axis may be due to a mixed affinity of pirenzepine for muscarinic receptors. Our results show no inhibitory effects of pirenzepine on gall-bladder contractile response to an ACh-non-related agonist, i.e. histamine.

Hong et al (1956) and Pellegrini et al (1985) reported that the gall-bladder response to exogenous CCK is not prevented by atropine, while our results indicate the opposite, that atropine reduces the contractions induced by CCK-8. Physiological and pharmacological findings suggest that CCK-8 acts on at least two sites in the gut, either on smooth muscle cells (Amer 1972; Morgan et al 1978; Yau et al 1973) or on cholinergic neurons, provoking a release of ACh (Vizi et al 1973; Yau et al 1974; Dockray & Hutchinson 1980). In the gall-bladder, CCK-8 acts not only on the smooth muscle but also on postganglionic cholinergic nerve terminals and both effects elicited contraction in the guinea-pig gall-bladder (Yamamura et al 1986). The partial inhibition of responses to CCK-8 by atropine that we observed may arise from competitive inhibition between atropine and ACh. However, another possibility could be that CCK receptors are adjacent to cholinergic receptors, and that the binding of atropine to the cholinergic receptor could interfere with the binding of CCK to its own receptor. This might explain why the inhibition of CCK-8 responses by atropine and pirenzepine did not follow a simple competitive-type model.

Acknowledgments

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