Br. J. Pharmacol. (1993), **110**, 1413–1420

Different muscarinic receptor subtypes mediating the phasic activity and basal tone of pig isolated intravesical ureter

[1]M. Hernández, U. Simonsen, D. Prieto, L. Rivera, P. García, E. Ordaz & A. García-Sacristán

Departamento de Fisiología, Facultad de Veterinaria, Universidad Complutense, Madrid, Spain

1 We have studied the effects of muscarinic cholinoceptor agonists and specific antagonists on both phasic activity and basal tone of the isolated intravesical ureter of the pig by means of isometric techniques *in vitro*.

2 Acetylcholine in the presence and absence of physostigmine increased both phasic activity and basal tone of ureteral strips in a concentration-dependent manner. Moreover carbachol, methacholine and oxotremorine-M increased both contractile parameters while bethanechol and McN-A-343 evoked only increases in tone without affecting the frequency of the phasic contractions.

3 The nicotinic receptor blocker, hexamethonium (10^{-6}–10^{-4} M), failed to modify the contractions evoked by a single dose of carbachol (10^{-5} M), whilst the muscarinic antagonist, atropine inhibited both phasic and tonic responses.

4 The muscarinic M_1 (pirenzepine), M_2 (AF-DX 116 and methoctramine), M_3 (4-DAMP, HHSiD and *p*-F-HHSiD), and putative M_4 receptor (tropicamide) antagonists significantly reversed increases in both frequency of phasic activity and baseline tone induced by a submaximal dose of carbachol (10^{-5} M). The pIC_{50} values for inhibition of the induced phasic activity were: atropine (10.16) > 4-DAMP (9.12) > HHSiD (8.22) = methoctramine (7.98) = *p*-F-HHSiD (7.88 > tropicamide (7.62) = pirenzepine (7.53) = AF-DX 116 (7.45) and for inhibition of basal tone were: atropine (10.73) > 4-DAMP (9.32) > HHSiD (8.65) = pirenzepine (8.43) = *p*-F-HHSiD (8.38) > methoctramine (7.79) > tropicamide (7.53) > AF-DX 116 (7.04).

5 The antagonist profile indicates that an M_1 receptor mediates the tonic response while the phasic activity could involve either both M_2 and M_3 or an M_4 muscarinic receptor. These results suggest that different muscarinic receptor subtypes mediate the phasic and tonic contractile activity induced by a submaximal concentration of carbachol in the porcine intravesical ureter.

Keywords: Intravesical ureter of pig; muscarinic receptors; phasic activity; tone

Introduction

Autonomic receptors play an important role in the regulation of distal ureteral function. Support for the contention that the autonomic nervous system exerts an influence on the ureter can be derived from the demonstration of a rich supply of tyrosine hydroxylase (TH) and acetylcholinesterase-positive nerve fibres, which are distributed throughout the distal ureter forming dense neuromuscular, subepithelial and perivascular plexuses (Schulman, 1985; Prieto *et al.*, 1989; 1990), in contrast with proximal portions of ureter and kidney pelvis where evidence for cholinergic innervation is scarce (Barajas & Wang, 1983; Prieto *et al.*, 1990).

Furthermore, *in vitro* studies of the sheep ureterovesical junction have demonstrated the presence of functionally active adrenoceptors (Rivera *et al.*, 1992a) and we have recently demonstrated that noradrenaline modulates both phasic and tonic contractile activity of the pig intravesical ureter, through specific populations of adrenoceptors belonging to α_1-, β_1- and β_2-subtypes, with a possible involvement of α_2-receptors in the maintenance of ureteral tonus (Hernández *et al.*, 1992). Also acetylcholine induced contractions of the sheep ureterovesical junction (Rivera *et al.*, 1992b) and evoked increases in both phasic activity and basal tone of the isolated ureteral component of the ureterovesical junction. However, the role of cholinergic innervation in the control of motor activity of the ureter at the ureterovesical junction and its functional significance in the regulation of the bladder filling and emptying at micturition is not well understood. It is also largely unknown which postjunctional muscarinic cholinoceptor subtypes are involved in the modulation of the activity.

Five unique genes coding for muscarinic receptors have been cloned and denoted m1, m2, m3, m4 and m5 (Bonner *et al.*, 1987; Peralta *et al.*, 1987). Moreover radioligand studies of cloned receptors show a close correlation between m1, m2, m3 gene products and the pharmacologically defined M_1, M_2 and M_3 muscarinic receptor subtypes (Buckley *et al.*, 1989; Hulme *et al.*, 1990).

The different antagonists now available make it possible to determine the muscarinic receptor subtypes present in tissues. The antagonist pirenzepine shows high affinity for M_1 receptors (Hammer & Giachetti, 1982; Eglen & Whiting, 1986; Giachetti *et al.*, 1986; Doods *et al.*, 1987), low affinity for M_2 (or cardiac receptors) and intermediate affinity for M_3 (ileal or smooth muscle receptors) (Eglen & Whiting, 1986; Giachetti *et al.*, 1986; Doods *et al.*, 1987). Thus, AF-DX 116 (11-[(2-{(diethylamino)-methyl]-1-piperidinyl)acetyl]-5, 11-dihydro-6H-pyrido-[2,3,6] [1,4]-benzodiazepine-6-one) (Eglen & Whiting, 1986; Giachetti *et al.*, 1986) and methoctramine (Melchiorre, 1988) are selective for M_2, and 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidinemethiodide) (Barlow *et al.*, 1976; Eglen & Whiting, 1986), hexahydrosiladiphenidol (Fuder *et al.*, 1985) and *p*-F-hexahydrosiladiphenidol (Lambrecht *et al.*, 1988; Duckles, 1990; Eglen *et al.*, 1990) for M_3-receptors. Recently, both tropicamide and himbacine have been used as M_4-receptor antagonists (Lazareno *et al.*, 1990; 1993).

Histochemical studies have demonstrated a rich supply of cholinergic nerves in the intravesical ureter (Prieto *et al.*, 1990), in sharp contrast to the proximal ureter. Therefore, the aim of the present study was to characterize the postjunctional muscarinic cholinoceptors involved in the modulation of the pig intravesical ureteral smooth muscle activity.

[1] Author for correspondence.

Methods

Urinary bladders with attached ureters were removed from adult pigs of either sex with no lesions in their urinary tract, selected at the local slaughterhouse immediately after the animals were killed. The bladders were placed in chilled physiological saline solution (PSS) at 4°C. The adjacent connective and fatty tissues were removed with care and longitudinal preparations (4–6 mm long and 2–3 mm wide) of the intravesical ureter were isolated from the bladder by dissection as described earlier (Hernández et al., 1992). The strips were suspended vertically in 30 ml organ baths containing PSS maintained at 37°C and gassed with 5% CO_2 in O_2, pH = 7.4. The distal end of a preparation was attached to a metal hook and the other end connected to an isometric transducer (Grass FT03C) with the signal continuously recorded on a polygraph (Grass 79E). Passive tension of 2 g was applied to the preparations and they were allowed to equilibrate for 60 min.

Experimental procedure

The contractile capacity of the preparations was challenged by exposing the preparations to 120 mM potassium-rich physiological saline solution (K^+PSS). Induced phasic activity described by frequency (number of contractions min^{-1}) and amplitude (g) of rhythmic contractions and increases in basal tone (g) were examined by single application of increasing concentrations of cholinoceptor agonists such as carbachol, methacholine, oxotremorine-M, bethanechol, acetylcholine and McN-A-343. Carbachol, acetylcholine and methacholine concentration-response curves were generated in the presence of physostigmine (10^{-6} M) to block acetylcholinesterase activity. The ureteral strips were stimulated with a single concentration of the muscarinic agonists and potassium-rich Krebs during a period of 3 and 4 min, respectively.

Due to the development of a strong tachyphylaxis of the tissue to the agonists, two consecutive concentration-response curves could not be constructed in the same preparation. However, the response to a single submaximal concentration (10^{-5} M) of carbachol was reproducible during repetitive exposures. Therefore, it was used to determine the effect of the muscarinic antagonists: atropine, pirenzepine, methoctramine, AF-DX 116, 4-DAMP, HHSiD, *p*-F-HHSiD and tropicamide. First, a control response to carbachol (10^{-5} M) in absence of antagonist was obtained. The preparations were then incubated with the antagonist for 30 min before carbachol was added. An inhibition curve of single concentrations of antagonist was constructed in a single strip. Control preparations without antagonist incubation were run in parallel to correct for tissue fatigue and time-induced changes (Kenakin, 1984).

Drugs and solutions

The composition of physiological saline solution (PSS) was (mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 24.9, glucose 11, KH_2PO_4 1.2, EDTA (ethylene diamine tetraacetic acid) 0.027. The K^+PSS was PSS with KCl exchanged for NaCl on an equimolar basis. Stock solutions were prepared daily in distilled water.

The following drugs were used: AF-DX 116 (11-(2-((diethyl-amino)methyl)-1-piperidinylacetyl)-5,-11-dihydro-6H-pyrido(2,3-b)-(1,4)-benzodiazepine-6-one); pirenzepine HCl (Dr Karl Thomae GmbH, Germany); atropine sulphate (Merck, Germany); bethanechol (Sigma, U.S.A.); carbamoylcholine HCl (carbachol) (Sigma, U.S.A.); 4-DAMP (4-diphenylacetoxy-N-methyl piperidine methiodide) (courtesy of Dr R.B. Barlow, Bristol, U.K.); hexamethonium bromide (Serva); McN-A-343 (4-hydroxy-2-butynyl)-1-trimethylammonium *m*-chloro carbimilate chloride, Sigma); methacholine (acetyl-β-methylcholine, Sigma); methoctramine (Sigma, U.S.A.); oxotremorine-M (ICN Biochemicals); HHSiD (hexahydrosiladiphenidol, Research Biochemicals Incorporated, U.K.); *p*-F-HHSiD (*para* fluoro hexa-hydrosiladiphenidol, Research Biochemicals Incorporated, U.K.); tropicamide (Sigma, U.S.A.).

Physostigmine and *p*-F-HHSiD were dissolved in ethanol while tropicamide was dissolved in 0.1 N HCl and further diluted in distilled water. The other drugs were dissolved in distilled water. Previous experiments showed that the solvents had no effect on the preparation.

Calculations

For each concentration-response curve, the concentration required to give half-maximal response (EC_{50}) was determined by computerized iteration, fitting the responses and logarithmic concentrations to the Hill equation (Graph Pad software 3.0, San Diego, Calif., U.S.A.). Sensitivities to drugs are expressed in terms of pD_2 values, where $pD_2 = -\log EC_{50}$, the EC_{50} being the agonist concentration needed to produce half-maximal response. pIC_{50} values for the muscarinic antagonists were also calculated, as $pIC_{50} = -\log IC_{50}$, where IC_{50} is the concentration of antagonist required to cause half-maximal inhibition of the response induced by a single dose (10^{-5} M) of the agonist (Skärby & Larsson, 1987). IC_{50} values were converted to K_b values using the equation of Leff & Dougall (1993):

$$K_b = \frac{[IC_{50}]}{\left(2+\left(\frac{[A_f]}{[EC_{50}]}\right)^b\right)^{1/b} - 1}$$

A_f and b are the fixed concentration (10^{-5} M) and the slope factor of single increasing dose-response curves to carbachol, respectively.

Statistics

The results are expressed as mean ± s.e.mean. Statistical differences were calculated by Student's *t* test and one-way analysis of variance (ANOVA) with *a posteriori* Bonferroni test (Wallestein et al., 1980). Differences were considered significant with a probability level of $P < 0.05$.

Results

Agonist study

Acetylcholine (10^{-8}–3×10^{-4} M) and methacholine (10^{-8}–10^{-4} M) in the presence and absence of physostigmine (10^{-6} M) evoked increases in both phasic and tonic contractile activity, respectively (see Table 1). There was a significant difference ($P < 0.05$, paired *t* test) between the pD_2 values of tone induced by acetylcholine in the presence and absence of physostigmine (Figure 1). Moreover carbachol, methacholine and oxotremorine-M induced concentration-dependent increases in both frequency of phasic activity and basal tone of porcine intravesical ureteral strips. However, bethanechol and the putative M_1 agonist, McN-A-343 evoked only increases in the tone of ureteral preparations (Figures 2 and 3, Table 1). The concentration-response curve to McN-A-343 was bimodal with a $pD_2 = 7.21 \pm 0.14$ and $E_{max} = 0.81 \pm 0.17$ g for the first phase and $pD_2 = 5.26 \pm 0.16$ and $E_{max} = 2.02 \pm 0.22$ g for the second phase.

Table 1 shows pD_2 and E_{max} values for the different muscarinic cholinoceptor agonists, for both phasic activity and tone. The rank order of potency for the increase in tone was: McN-A-343 (first phase) > acetylcholine = oxotremorine-M > carbachol = methacholine = bethanechol = McN-A-343 (second phase), while for induced phasic activity it was: oxotremorine-M > carbachol = acetylcholine = methacholine.

Concentration-response curves for agonists could not be

Table 1 Effects of cholinoceptor agonists on porcine intravesical ureter

		Tone			*Phasic activity*		
Agonist	*n*	pD_2	E_{max}	Slope	pD_2	E_{max}	Slope
– Physostigmine							
Acetylcholine	8	5.58 ± 0.15	1.50 ± 0.08	0.70 ± 0.08	5.52 ± 0.11	12.57 ± 0.90	1.12 ± 0.06
Methacholine	6	5.46 ± 0.16	1.14 ± 0.27*	1.11 ± 0.10	5.47 ± 0.04	14.61 ± 2.33	1.36 ± 0.09
Carbachol	12	5.60 ± 0.28	1.81 ± 0.29	1.57 ± 0.28[a]	5.88 ± 0.16	14.27 ± 1.39	1.22 ± 0.17
+ Physostigmine							
Acetylcholine	8	6.72 ± 0.14*	1.61 ± 0.25	1.42 ± 0.12[a]	5.57 ± 0.15	13.91 ± 3.08	0.99 ± 0.15
Methacholine	6	5.63 ± 0.19	1.17 ± 0.18*	1.26 ± 0.06	5.53 ± 0.06	14.75 ± 4.55	1.07 ± 0.02
Carbachol	8	5.64 ± 0.35	1.83 ± 0.24	1.52 ± 0.23[a]	5.79 ± 0.21	15.11 ± 1.17	1.19 ± 0.12
Oxotremorine-M	8	6.62 ± 0.12*	2.16 ± 0.44	2.23 ± 0.23[a]	6.59 ± 0.05*	15.01 ± 3.49	1.95 ± 0.69[a]
Bethanechol	6	5.37 ± 0.13	1.76 ± 0.28	1.03 ± 0.04	–	–	–
McN-A-343	8	5.69 ± 0.16	2.02 ± 0.22	0.84 ± 0.14	–	–	–

n, number of ureters. $pD_2 = -\log EC_{50}$; EC_{50} is the effective concentration which induced 50% of the maximal response. E_{max} is the maximum effect in case of ureteral tonus (g) or phasic activity (number contractions min^{-1}). Results are expressed in absolute values as mean ± s.e.mean.

[a]Slope factor significantly different from unity.

*Significantly different parameter compared to carbachol ($P<0.05$, *a posteriori*, Bonferroni).

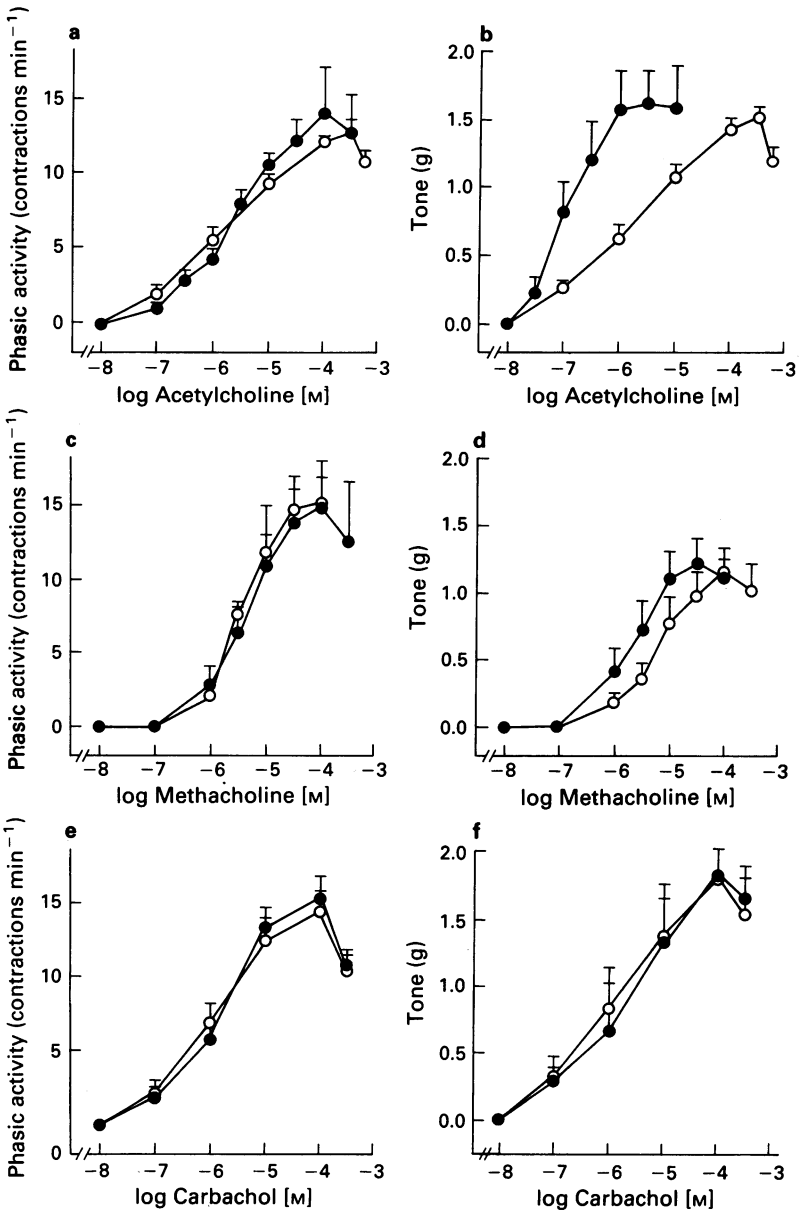

Figure 1 Concentration-response curves of porcine intravesical ureteral preparations to increasing single doses of acetylcholine (a, b), methacholine (c, d) and carbachol (e, f) in absence (○) and presence (●) of physostigmine (10^{-6} M). (a, c and e) Phasic activity, and (b, d and f) tone of the preparations. Each point represents mean (± s.e.mean, vertical bar) of 6–8 preparations. Results are expressed as absolute values.

repeated on the same preparation due to the development of strong tachyphylaxis. Thus, in a first concentration-response curve, the pD_2 and E_{max} values for carbachol on basal tone were 5.60 ± 0.28 and 1.81 ± 0.29 ($n = 12$), respectively, while in a second curve the pD_2 and E_{max} values were 5.24 ± 0.21 and 1.37 ± 0.44 ($P < 0.05$, paired t test). However, increases in both phasic activity and basal tone induced by a submaximal dose (10^{-5} M) of carbachol were reproducible during repetitive exposures. The phasic activity and tone evoked by a single dose (10^{-5} M) of carbachol on the first exposure were 12.36 ± 1.54 contractions min^{-1} and 1.36 ± 0.38 g respectively, compared to 11.21 ± 1.72 contractions min^{-1} and 1.32 ± 0.29 g respectively to the fifth exposure ($n = 24$).

Antagonist study

Treatment of ureteral strips with the selective nicotinic cholinoceptor antagonist, hexamethonium (10^{-6}–10^{-4} M) did not alter the response to 10^{-5} M carbachol. The tone was 1.42 ± 0.21 g and phasic activity 10.18 ± 1.33 contractions min^{-1} before, and tone 1.37 ± 0.39 g and phasic activity 9.43 ± 1.52 contractions min^{-1} after incubation with hexamethonium (10^{-4} M) ($n = 8$).

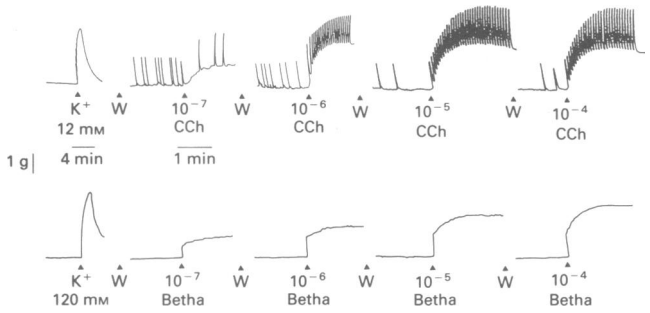

Figure 2 Traces showing the response of intravesical ureteral preparations of the pig to 120 mM K^+-PSS and increasing concentrations of carbachol (CCh, 10^{-7}–10^{-4} M) and bethanechol (Betha, 10^{-7}–10^{-4} M) added in single doses with washout (W) between each response. Numbers indicate molar concentration in the bath.

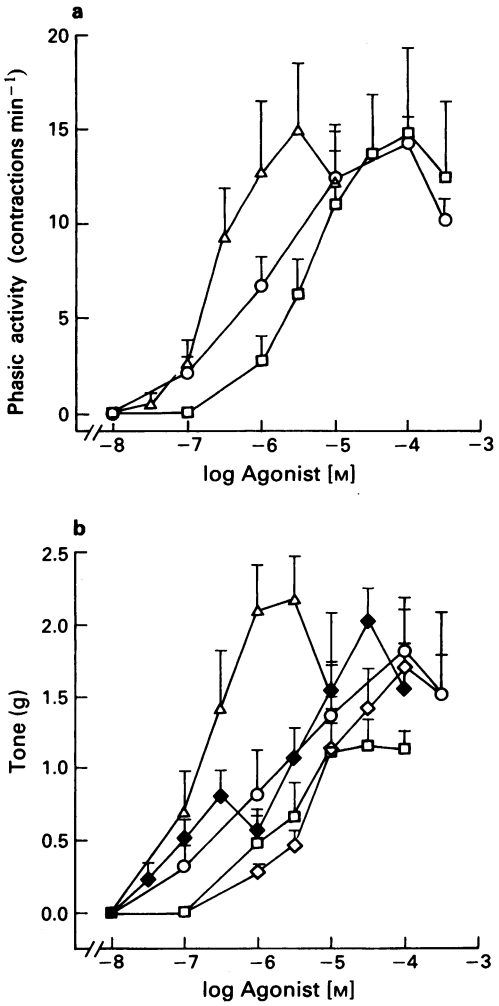

Figure 3 Concentration-response curves of porcine intravesical ureteral preparations to increasing single doses of carbachol (○), methacholine (□), oxotremorine-M (△), bethanechol (◇), and McN-A343 (◆). (a) Phasic activity, and (b) tone of the preparations. Each point represents mean (± s.e.mean, vertical bar) of 6–12 preparations. Results are expressed as absolute values.

Table 2 Effects of muscarinic cholinoceptor antagonists on tone and phasic activity induced by carbachol (10^{-5} M) in porcine intravesical ureter

		Tone			*Phasic activity*		
Antagonist	*n*	pIC_{50}	Slope	pK_B	pIC_{50}	Slope	pK_B
Atropine	8	10.73 ± 0.13	1.43 ± 0.21	10.81	10.16 ± 0.06*	1.72 ± 0.17	10.62[a]
Pirenzepine	8	8.43 ± 0.18	0.83 ± 0.09	8.60	7.53 ± 0.18*	0.86 ± 0.08	7.86
AF-DX 116	6	7.04 ± 0.22	0.77 ± 0.10	6.91	7.45 ± 0.16	1.33 ± 0.11	7.77
Methoctramine	6	7.79 ± 0.24	0.82 ± 0.06	8.12	7.98 ± 0.20	0.76 ± 0.10	8.35
4-DAMP	6	9.32 ± 0.06	0.93 ± 0.06	9.44	9.12 ± 0.09	1.14 ± 0.08	9.58
HHSiD	6	8.41 ± 0.05	1.18 ± 0.33	8.55	8.30 ± 0.12	1.26 ± 0.19	8.76
p-F-HHSiD	6	8.38 ± 0.13	1.12 ± 0.02	8.47	7.88 ± 0.15*	1.83 ± 0.18	8.28
Tropicamide	10	7.53 ± 0.09	0.68 ± 0.11	7.70	7.62 ± 0.11	0.72 ± 0.13	8.11[a]

n, number of ureters. pIC_{50} defined as the negative logarithm of the antagonist concentration that causes a 50% inhibition of the contraction induced by carbachol (10^{-5} M). Results are expressed in absolute values as mean ± s.e.mean.
*Significantly different parameter compared to pIC_{50} values for tone.
[a]Significantly different parameter compared to pIC_{50} and pK_b values of antagonist inhibiting carbachol-induced phasic activity in the same preparation ($P < 0.05$, paired t test). pK_b defined as the negative logarithm of K_b; K_b is the dissociation constant. IC_{50} values were converted to K_b values by using the equation of Leff & Dougall (1993):

$$\text{Equation of Leff \& Dougall} \quad K_b = \frac{[IC_{50}]}{\left(2 + \left(\frac{[A_f]}{[EC_{50}]}\right)^b\right)^{1/b} - 1}$$

A_f and b are the fixed concentration (10^{-5} M) and the slope factor of single increasing dose-response of carbachol, respectively. For abbreviations, see text.

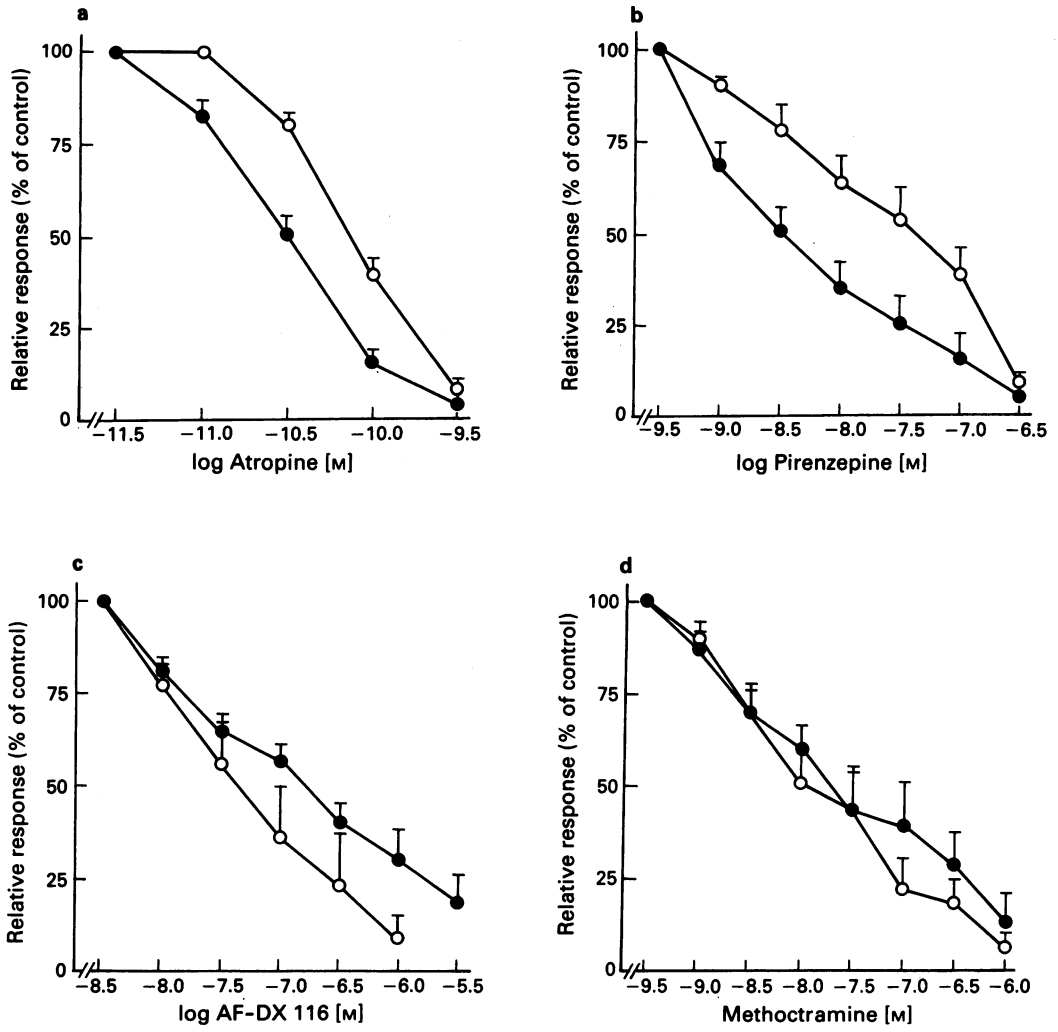

Figure 4 Effects of atropine (a), pirenzepine (b), AF-DX 116 (c) and methoctramine (d) on phasic activity (○) and tone (●) induced by carbachol (10^{-5} M). Each curve represents mean (± s.e.mean) of 6–8 strips. Results show the relative response to carbachol after incubation with increasing concentrations of atropine, pirenzepine, AF-DX 116 and methoctramine.

All the antagonists significantly inhibited both tone and phasic activity induced by a submaximal dose of carbachol (10^{-5} M). The effects of atropine, the selective M_1 antagonist, pirenzepine, and the selective M_2 antagonists AF-DX 116 and methoctramine are shown in Table 2 and Figure 4. The effects of the M_3 selective antagonists 4-DAMP, HHSiD and *p*-F-HHSiD and the M_4 selective antagonist, tropicamide are shown in Table 2 and Figure 5. Atropine and both the preferential M_1 and M_3 muscarinic receptor antagonists, pirenzepine and *p*-F-HHSiD, respectively, showed a higher inhibitory effect on tonic than phasic contractile activity while the selective M_4 antagonist, tropicamide blocked both contractile parameters induced by a submaximal dose of carbachol (Table 2). This indicates different profiles of the muscarinic receptor antagonists in inhibiting tone and frequency of contractions to 10^{-5} M carbachol in the porcine intravesical ureter.

Discussion

Functional studies have shown that autonomic neurotransmitters can affect the rate of urine transport at physiological flow rates through the canine ureter by modulating not only the peristaltic frequency but also the urine bolus volume. Thus, acetylcholine increased the peristaltic frequency, reduced the bolus volume and decreased the fluid transport (Morita *et al.*, 1987). In our study, acetylcholine in the presence of physostigmine evoked increases in both phasic activity and basal tone of pig intravesical ureteral preparations. However, preincubation of ureteral strips with physostigmine only enhanced the effect of acetylcholine on the tonic contraction of the ureter, suggesting that the muscarinic receptors involved in this response are in a different location from those enhancing the phasic activity, and are protected from bath-applied acetylcholine by acetylcholinesterase. In contrast, the neuronal uptake blocker of noradrenaline increased the phasic activity without affecting the basal tone (Hernández *et al.*, 1992), although exogenously applied noradrenaline was shown to modulate both phasic and tonic contractile activity. Although the effects of intrinsic nerve stimulation have not been studied, these results suggest that both sympathetic and parasympathetic nerves may control the transport of urine into the bladder, the sympathetics enhancing urine flow by increasing phasic activity, and the parasympathetics producing sustained contraction to prevent reflux during evacuation of the bladder as suggested earlier (Rivera *et al.*, 1992b).

Agonist study

The present study was performed in order to characterize the muscarinic receptors involved in the phasic and tonic contractile activity of the pig isolated intravesical ureter. For this

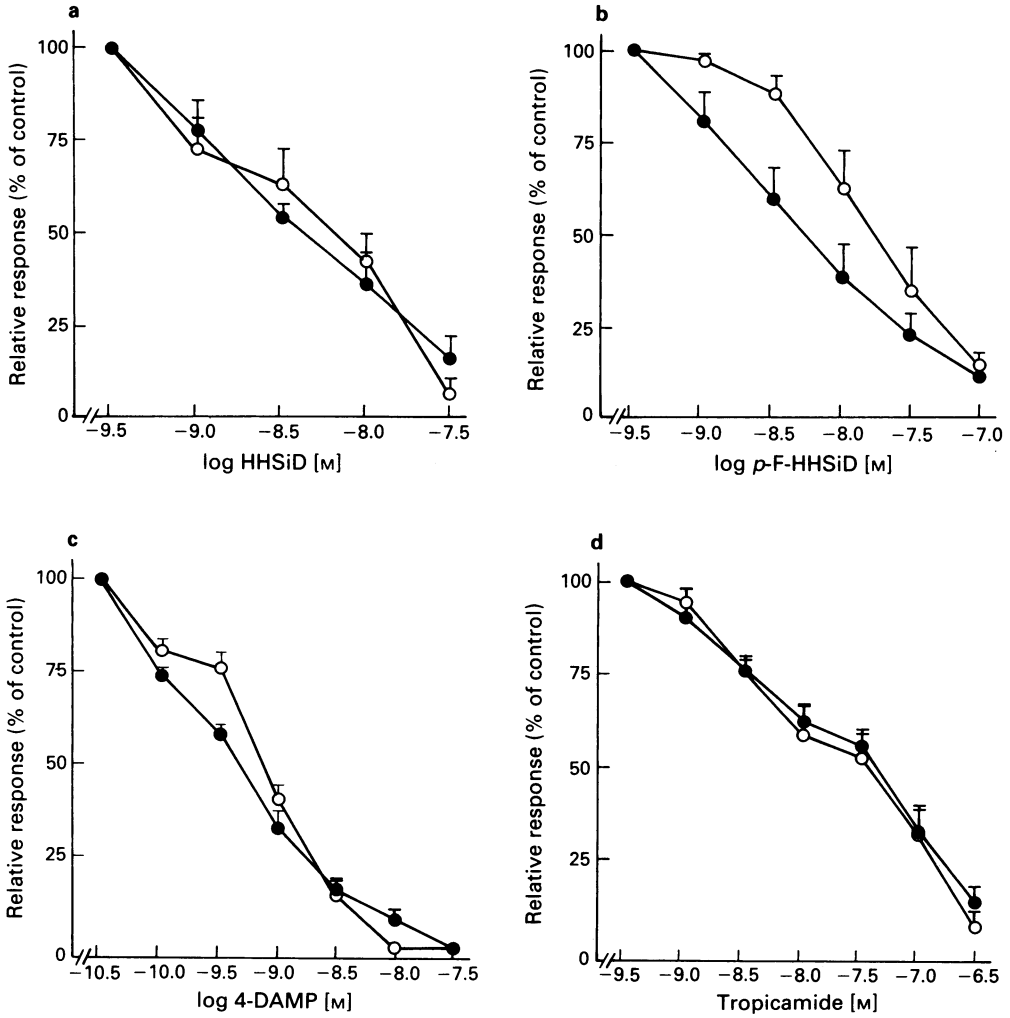

Figure 5 Effects of HHSiD (a), *p*-F-HHSiD (b), 4-DAMP (c) and tropicamide (d) on phasic activity (○) and tone (●) induced by carbachol (10^{-5} M). Each point represents mean (± s.e.mean) of 6–10 strips. Results show the relative response to carbachol after incubation with increasing concentrations of HHSiD, *p*-F-HHSiD, 4-DAMP and tropicamide. For abbreviations, see text.

purpose we used selective agonists and antagonists to determine the muscarinic receptor subtypes that mediate the phasic contractile activity and increases of basal tone of the isolated intravesical ureteral strips to carbachol.

The potency order of agonists which enhanced the phasic contractions in our preparations is similar to that obtained in the rat bladder (Grana *et al.*, 1987) and rabbit vas deferens (Eltze, 1988), both of which possess M_2-receptors. The putative M_1 (Hammer & Giachetti, 1982) and M_3 (Rattan & Goyal, 1984) agonists, McN-A-343 and bethanechol respectively, only increased the tone without producing phasic activity.

Our agonist study suggests that both M_1 and M_3 muscarinic receptor subtypes mediate the increases in the basal tone, while M_2-receptors are involved in the increases in the frequency of phasic contractions in the porcine isolated intravesical ureter in response to cholinoceptor agonists, although the selectivity of the muscarinic agonists is very low.

Antagonist study

The potencies of the antagonists are expressed as pIC_{50} values and are compared with pA_2 values obtained in other tissues. We also obtained pK_b values using the equation of Leff & Dougall (1993) with the aim of comparing these values with the pIC_{50} values and evaluating the validity of this equation for application in functional studies of ureteral strips as in other tissues which manifested tachyphylactic activity. The pK_b values obtained were not significantly different from the pIC_{50} values, which suggests that the equation of Leff & Dougall (1993) could be a good tool for antagonist study in pig isolated intravesical ureter.

Hexamethonium, a ganglionic nicotinic receptor blocker failed to modify the carbachol effect, suggesting that activation of nicotinic receptors is not involved in the response. The lack of activity of hexamethonium could be related to the absence of AChE-positive ganglion-cells in the porcine isolated intravesical ureter in contrast to the body of the urinary bladder and the sheep ureter vesical junction (Rivera *et al.*, 1991; 1992b), where there were numerous autonomic intramural ganglia mostly composed of AChE-positive neurones.

In contrast to hexamethonium, atropine inhibited with high affinity both the phasic activity and basal tone induced by carbachol in intravesical ureteral strips, suggesting that these contractile responses are mediated through muscarinic receptors.

The clearest evidence for postulating different muscarinic receptor subtypes has been obtained with the selective M_1-antagonist, pirenzepine, which differentiates muscarinic receptor subtypes through its markedly higher affinity for the M_1-receptor (Hammer *et al.*, 1980; Watson *et al.*, 1982). The pIC_{50} values obtained for this drug on both phasic activity (7.53) and tone (8.45) of the pig intravesical ureter seem to

indicate that tonic but not phasic activity could be mediated through M_1 receptors. This is supported by the range of selectivity of pirenzepine for M_1-receptors (Eglen & Whiting, 1986; Mitchelson *et al.*, 1989). Moreover, pirenzepine had a significantly higher affinity for inhibition of the tone than the phasic activity which could indicate that M_1 muscarinic receptor activation is implicated in the constriction of the ureter.

On the other hand, the selective M_2 antagonist, AF-DX 116 (Giachetti *et al.*, 1986) exhibited similar pIC_{50} values on both tone (7.04) and phasic activity (7.45) which seems to correspond with the stimulation of M_2 receptors. These results are consistent with pA_2 values found in the vas deferens (6.85–7.89) (Eltze, 1988). In order to confirm these findings we used methoctramine and obtained a pIC_{50} of 7.79 and 7.98 on tone and phasic activity, respectively, which is similar to the pA_2 value (7.8) reported for M_2 in the porcine basilar artery (Eglen & Whiting, 1990).

The high pIC_{50} values obtained for 4-DAMP on both phasic activity and basal tone (9.12 and 9.32, respectively), are very close to those reported for M_3 receptors (9.04–9.50) (Clague *et al.*, 1985; Grider *et al.*, 1987; Kurtel *et al.*, 1990). Moreover, these results are similar to those found in the guinea-pig muscularis mucosae (9.32) (Barocelli *et al.*, 1990), common bile duct (8.99) (Karahan *et al.*, 1991), in the chick and guinea-pig ileum (9.2 and 9.1 respectively) (Choo *et al.*, 1988), and in the sheep detrusor muscle and ureterovesical junction (9.26 and 9.41 respectively) (Rivera *et al.*, 1991; 1992b). However, since 4-DAMP does not clearly differentiate between M_1 and M_3 receptors (Delmendo *et al.*, 1989; Hulme *et al.*, 1990), we used *p*-F-HHSiD and HHSiD in our study. These antagonists exhibit high affinity for the M_3 receptor subtype (Lambrecht *et al.*, 1988; Duckles, 1990; Eglen *et al.*, 1990). In the present study, the pIC_{50} values (8.38 and 7.88) obtained with *p*-F-HHSiD on tone and phasic activity, respectively, seem to correspond to M_3 receptors. *p*-F-HHSiD was more potent in inhibiting the tone than phasic activity, indicating a possible involvement of M_3 receptors in contraction of the ureteral smooth muscle. These results are consistent with pA_2 values for M_3-receptors (7.50–8.22) obtained in different vascular preparations, human SH-SY5Y cells, guinea-pig ileum, and oesophageal muscularis mucosae (Duckles, 1990; Eglen *et al.*, 1990). HHSiD showed high affinity in inhibition of both phasic activity and basal tone ($pIC_{50} = 8.22$ and 8.65, respectively) consistent with the activation of M_3 receptors, such as found in the guinea-pig ileum ($pA_2 = 8.08–8.40$) (Fuder *et al.*, 1985), in the sheep detrusor muscle (8.49) (Rivera *et al.*, 1991) and ureterovesical junction (8.66) (Rivera *et al.*, 1992b).

Finally, with a muscarinic antagonist reported to have modest affinity for the M_4-receptors (Lazareno *et al.*, 1993), we obtained a pK_b value for inhibition of the phasic contractions induced with carbachol similar to the pK_b value obtained in rabbit lung (Lazareno *et al.*, 1990). This indicates that the phasic contractions induced by the cholinoceptor agonists in the pig intravesical ureter might be mediated through M_4-receptors.

The present investigation demonstrates that M_1, M_2 and M_3 muscarinic receptors could be involved in both phasic and tonic contractile activity of the pig intravesical ureter smooth muscle. These results are similar to those found in the gastrointestinal tract (Goyal, 1988), in the human lung (MacLagen & Barnes, 1990) and in the sheep ureterovesical junction (Rivera *et al.*, 1992b), where the contractile responses of muscarinic agonists are mediated through three pharmacologically defined muscarinic subtypes.

Moreover, we suggest the presence of an M_4 muscarinic receptor subtype possibly involved in the phasic activity, due to the high affinity exhibited for methoctramine, 4-DAMP, HHSiD and tropicamide in porcine ureteral strips. This antagonist profile is similar to that previously reported for M_4 receptors characterized in binding studies of NG108-15 cells and rat forebrain (Michel *et al.*, 1989; Waelbroeck *et al.*, 1990).

In summary, some of the cholinoceptor agonists evoked increases in the phasic contractions while all induced tone in the pig intravesical ureter; as different antagonist profiles were obtained in inhibition of the phasic activity and tone, the present study suggests that different muscarinic receptor subtypes mediate the phasic and tonic contractile activity induced by a submaximal concentration of carbachol. This might be of importance for both the urine bolus transport during bladder filling and the parasympathetic constriction of the ureter preventing the vesicoureteral reflux during the emptying phase of the urinary bladder.

This work was supported by project PM 88-0035 (DGYCIT). The authors thank Dr Barlow, Bristol, U.K., for the gift of 4-DAMP, Dr Karl Thomae, GmbH, for the gift of AF-DX 116, and the Madrid Municipal Slaughterhouse for kindly donating the ureters.

References

BARAJAS, L. & WANG, P. (1983). Simultaneous ultrastructural visualization of acetylcholinesterase activity and tritiated norepinephrine uptake in renal nerves. *Anat. Rec.*, **205**, 185–195.

BARLOW, R.B., BERRY, K.J., GLENTON, P.A.M., NIKOLAOU, N.M. & SOH, K.S. (1976). A comparison of affinity constants for muscarine-sensitive acetylcholine receptors in guinea-pig atrial pacemaker cells at 29°C and 37°C. *Br. J. Pharmacol.*, **58**, 613–620.

BAROCELLI, E., CHIAVARINI, M., MORINI, G., BALLABENI, V., GENTILE, A. & IMPICCIATORE, M. (1990). Pharmacological evidence that muscarinic M_1-receptors are not involved in the contractile activity of guinea-pig oesophageal muscularis mucosae. XIth International Congress of Pharmacology. Amsterdam, The Netherlands. *Eur. J. Pharmacol.*, **183**, 1725–1732.

BONNER, T.I., BUCKLEY, N.J., YOUNG, A.C. & BRANN, M.R. (1987). Identification of a family of muscarinic acetylcholine receptor genes. *Science*, **237**, 527–532.

BUCKLEY, N.J., BONNER, T.I., BUCKLEY, C.M. & BRANN, M.R. (1989). Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol. Pharmacol.*, **35**, 469–476.

CHOO, L.K., MITCHELSON, F. & NAPIER, P. (1988). Difference in antagonist affinities at muscarinic receptors in chick and guinea-pig. *J. Auton. Pharmacol.*, **8**, 259–266.

CLAGUE, R.U., EGLEN, R.M., STRACHEN, A.C. & WHITING, R.L. (1985). Action of agonists and antagonists at muscarinic receptors present on ileum and atria *in vitro*. *Br. J. Pharmacol.*, **86**, 163–170.

DELMENDO, R.E., MICHEL, A.D. & WHITING, R.L. (1989). Affinity of muscarinic receptor antagonists for three putative muscarinic receptor binding sites. *Br. J. Pharmacol.*, **96**, 457–464.

DOODS, H.N., MATHY, M.J., DAVESKO, D., VAN CHARLDORP, K.J., DE JONGE, A. & VAN ZWEITEN, P.A. (1987). Selectivity of muscarinic agonists in radioligand and in vivo experiments for the putative M_1, M_2 and M_3 receptors. *J. Pharmacol. Exp. Ther.*, **242**, 257–262.

DUCKLES, S.P. (1990). p-Fluoro-hexahydro-sila-difenidol: affinity for vascular muscarinic receptors. *Eur. J. Pharmacol.*, **185**, 227–230.

EGLEN, R.M., MICHEL, A.D., MONTGOMERY, W.W., KUNYSZ, E.A., MACHADO, C.A. & WHITING, R.L. (1990). The interaction of parafluorohexahydrosiladiphenidol at muscarinic receptors *in vitro*. *Br. J. Pharmacol.*, **99**, 637–642.

EGLEN, R.M. & WHITING, R.L. (1986). Muscarinic receptor subtypes a critique of the current classification and a proposal for a working nomenclature. *J. Auton. Pharmacol.*, **5**, 323–346.

EGLEN, R.M. & WHITING, R.L. (1990). Heterogeneity of vascular muscarinic receptors. *J. Auton. Pharmacol.*, **19**, 233–245.

ELTZE, M. (1988). Muscarinic M_1- and M_2-receptors mediating opposite effects on neuromuscular transmission in rabbit vas deferens. *Eur. J. Pharmacol.*, **151**, 205–221.

FUDER, H., KILBINGER, H. & MÜLLER, H. (1985). Organ selectivity of hexahydrosiladifenidol in blocking pre- and postjunctional muscarinic receptors studied in guinea-pig ileum and rat heart. *Eur. J. Pharmacol.*, **113**, 125–127.

GIACHETTI, A., MICHELETTI, R. & MONTAGNA, E. (1986). A cardioselective profile of AF-DX 116, a muscarine M_2 receptor antagonist. *Life Sci.*, **38**, 1663–1672.

GOYAL, R.K. (1988). Identification, localization and classification of muscarinic receptor subtypes in the gut. *Life Sci.*, **43**, 2209–2220.

GRANA, E., LUCCHELLI, A., ZONTA, F. & BOSELLI, C. (1987). Determination of dissociation constants and relative efficacies of some potent muscarinic agonists at postjunctional muscarinic receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **335**, 8–15.

GRIDER, J.R., BITAR, K.N. & MAKHLOUF, G.M. (1987). Identification of muscarinic M_2 receptors on single muscle cells of the human and guinea-pig intestine. *Gastroenterology*, **93**, 951–957.

HAMMER, R., BERRIE, C.P., BIRDSALL, N.J., BURGEN, A.S.V. & HULME, E.C. (1980). Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature*, **283**, 90–94.

HAMMER, R. & GIACHETTI, A. (1982). Muscarinic receptor subtypes M_1 and M_2. Biochemical and functional characterization. *Life Sci.*, **31**, 2991–2994.

HERNANDEZ, M., PRIETO, D., SIMONSEN, U., RIVERA, L., BARAHONA, M.V. & GARCIA-SACRISTAN, A. (1992). Noradrenaline modulates smooth muscle activity of the isolated intravesical ureter of the pig through different types of adrenoceptors. *Br. J. Pharmacol.*, **107**, 924–931.

HULME, E.C., BIRDSALL, N.J.M. & BUCKLEY, N.J. (1990). Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 633–673.

KARAHAN, F., ALICAN, I., OZKUTLU, U., ONAT, F., YEGEN, B.C., ULUSOY, B. & OKTAY, S. (1991). Muscarinic receptor subtypes of guinea-pig common bile duct. *Arch. Int. Pharmacodyn.*, **312**, 140–145.

KENAKIN, T.P. (1984). The classifications of drugs and drug receptors in isolated tissues. *Pharmacol. Rev.*, **36**, 165–222.

KURTEL, H., DEDEOGLU, A., YEGEN, B.C., ULUSOY, N.B. & OKTAY, S. (1990). Muscarinic receptor subtypes of guinea-pig gallbladder smooth muscle. *Arch. Int. Pharmacodyn.*, **308**, 39–46.

LAMBRECHT, G., FEIFEL, R., FORTH, B., STROHMANN, C., TACKE, R. & MUTSCHLER, E. (1988). p-Fluoro-hexahydro-sila-difenidol: the first $M_{2\beta}$-selective muscarinic antagonist. *Eur. J. Pharmacol.*, **152**, 193–194.

LAZARENO, S., BUCKLEY, N.L. & ROBERTS, F.F. (1990). Characterization of muscarinic M_4 binding sites in rabbit lung, chicken heart, and NG 108-15 cells. *Mol. Pharmacol.*, **38**, 805–815.

LAZARENO, S., FARRIES, T. & BIRDSALL, N.J.M. (1993). Pharmacological characterization of guanine nucleotide exchange reactions in membranes from CHO cells stably transfected with human muscarinic receptors M_1–M_4. *Life Sci.*, **52**, 449–456.

LEFF, P. & DOUGALL, I.G. (1993). Further concerns over Cheng-Prusoff analysis. *Trends Pharmacol. Sci.*, **14** (Suppl.), 110–112.

MACLAGAN, J. & BARNES, P.J. (1990). Muscarinic pharmacology of the airways. *Trends Pharmacol. Sci.*, **10** (Suppl.), 88–92.

MELCHIORRE, C. (1988). Polymethylene tetramines: a new generation of selective muscarinic antagonists. *Trends Pharmacol. Sci.*, **9**, 216–220.

MICHEL, A.D., DELMENDO, E., STEFANICH & WHITING, R.L. (1989). Binding characteristics of the muscarinic receptor subtype of the NG108-15 cell line. *Naunyn-Schmied. Arch. Pharmacol.*, **340**, 62–71.

MITCHELSON, F., CHOO, L.K. & DARROCH, S. (1989). Muscarinic receptor differentiation. *Clin. Exp. Pharmacol. Physiol.*, **16**, 523–528.

MORITA, T., WADA, I., SAEKI, H., TSUCHIDA, S. & WEISS, R.M. (1987). Ureteral urine transport: changes in bolus volume, peristaltic frequency, intraluminal pressure and volume of flow resulting from autonomic drugs. *J. Urol.*, **137**, 132–135.

PERALTA, E.G., WINSLOW, J.W., PETERSON, G.L., SMITH, D.H., ASHKENAZI, A., RAMACHANDRAN, J., SCHIMERLIK, M.I. & CAPON, D.J. (1987). Primary structure and biochemical properties of an M_2 muscarinic receptor. *Science*, **236**, 600–605.

PRIETO, D., BENEDITO, S., RIVERA, L., HERNANDEZ, M. & GARCIA-SACRISTAN, A. (1990). Autonomic innervation of the equine urinary bladder. *Anat. Histol. Embryol.*, **19**, 276–285.

PRIETO, D., BENEDITO, S., RODRIGO, J., MARTINEZ-MURILLO, R. & GARCIA-SACRISTAN, A. (1989). Distribution and density of neuropeptide Y-immunoreactive nerve fibers and cells in the horse urinary bladder. *J. Auton. Nerv. Syst.*, **27**, 173–180.

RATTAN, S. & GOYAL, R.K. (1984). Identification of M_1 and M_2 muscarinic receptor subtypes in the control of the lower esophageal sphincter in the opossum. *Trends Pharmacol. Sci.*, **5** (Suppl.), 78–81.

RIVERA, L., HERNANDEZ, M., BENEDITO, S., PRIETO, D. & GARCIA-SACRISTAN, A. (1992a). Mediation of contraction and relaxation by alpha and beta-adrenoceptors in the ureterovesical junction of the sheep. *Res. Vet. Sci.*, **52**, 57–61.

RIVERA, L., HERNANDEZ, M., BENEDITO, S., PRIETO, D. & GARCIA-SACRISTAN, A. (1992b). Mediation of contraction by cholinergic muscarinic receptors in the ureterovesical junction. *J. Auton. Pharmacol.*, **12**, 175–182.

RIVERA, L., PRIETO, D., HERNANDEZ, M., BENEDITO, S. & GARCIA-SACRISTAN, A. (1991). Distribution and function of cholinergic receptors in the sheep detrusor muscle. *J. Auton. Nerv. Syst.*, **34**, 95–102.

SCHULMAN, C.C. (1985). Innervation of the ureter: a histochemical and ultrastructural study. In *Urodynamics, Upper and Lower Urinary Tract*, II. ed. Lutzeyer, W. & Hannappel, ?. pp. 292–361. Berlin: Springer-Verlag.

SKARBY, T.V. & LARSSON, B. (1987). Theoretical and functional studies on $alpha_1$ and $alpha_2$-adrenoceptors: and examination using the Schild plot. *J. Auton. Pharmacol.*, **7**, 185–198.

WAELBROECK, M., TASTENOY, M., CAMUS, J. & CHRISTOPHE, J. (1990). Binding of selective antagonists to four muscarinic receptors (M_1–M_4) in rat forebrain. *Mol. Pharmacol.*, **38**, 267–274.

WALLESTEIN, S., ZUCKER, G.L. & FLEISS, J.L. (1980). Some statistical methods useful in circulation research. *Circ. Res.*, **47**, 1–9.

WATSON, M., ROESKE, N.R. & YAMAMURA, H.I. (1982). [^{3}H]-pirenzepine selectively identifies a high affinity population of muscarinic cholinergic receptors in the rat cerebral cortex. *Life Sci.*, **31**, 2019–2023.

(Received March 5, 1993
Revised August 4, 1993
Accepted August 13, 1993)