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Pharmacological characterization of muscarinic receptors in rabbit isolated iris sphincter muscle and urinary bladder smooth muscle

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1 The pharmacological characteristics of muscarinic receptors in the rabbit iris sphincter muscle were studied and compared to M_3 receptors in rabbit urinary bladder smooth muscle.

2 (+)-*Cis*-dioxolane induced concentration-dependent contractions of the iris sphincter muscle ($pEC_{50} = 6.41 \pm 0.10$, $E_{max} = 181 \pm 17$ mg, $n = 38$) and urinary bladder smooth muscle ($pEC_{50} = 6.97 \pm 0.04$, $E_{max} = 4.28 \pm 0.25$ g, $n = 54$). These contractions were competitively antagonized by a range of muscarinic receptor antagonists (pK_B values are given for the iris sphincter muscle and the bladder smooth muscle, respectively): atropine (9.30 ± 0.07 and 9.40 ± 0.04), AQ-RA 741 (6.35 ± 0.04 and 6.88 ± 0.03), darifenacin (9.56 ± 0.05 and 9.12 ± 0.05), methoctramine (5.75 ± 0.07 and 5.81 ± 0.06), oxybutynin (8.10 ± 0.09 and 8.59 ± 0.06), pirenzepine (6.79 ± 0.05 and 6.89 ± 0.04), secoverine (7.54 ± 0.05 and 7.66 ± 0.05), p-F-HHSiD (7.55 ± 0.09 and 7.50 ± 0.05) and zamifenacin (8.69 ± 0.10 and 8.36 ± 0.06). A significant correlation between the pK_B values in the bladder and the pK_B values in the iris was obtained.

3 In both tissues, the pK_B values correlated most favorably with pK_i values for these compounds at human recombinant muscarinic m3 receptors. A reasonable correlation was also noted at human recombinant muscarinic m5 receptors given the poor discriminative ability of ligands between m3 and m5 receptors.

4 Overall, the data from this study suggest that the muscarinic receptors mediating contraction of the rabbit iris sphincter muscle and urinary bladder smooth muscle are similar and equate most closely with the pharmacologically-defined muscarinic M_3 receptor.

Keywords: Muscarinic receptors; M_3-receptor; iris sphincter muscle; urinary bladder smooth muscle

Introduction

Muscarinic receptors are operationally divided into four subtypes, M_1, M_2, M_3 and M_4 which equate with four of the known muscarinic receptor gene products (m1, m2, m3 and m4) (Hulme *et al*., 1990; Caufield, 1993; Eglen *et al*., 1996 for reviews). A fifth gene has been identified, m5, for which no functional correlate has yet been identified (Bonner *et al*., 1987; 1988).

Being widely distributed in gastrointestinal, genitourinary, vascular and ocular smooth muscle tissues, muscarinic receptors play a key physiological role in peripheral organs. For example, the iris of the eye, which controls the size of the pupil and thus regulates the amount of light admitted, is endowed with muscarinic receptors that are innervated by parasympathetic nerves. Agonism of muscarinic receptors in the iris sphincter causes smooth muscle contraction (miosis). Conversely, antagonists cause mydriasis, a side-effect seen with most nonselective anti-cholinergics.

In vivo studies have shown that the postjunctional muscarinic receptor in the rat iris sphincter muscle may be M_3 in nature, according to the mydriatic potency of selective muscarinic antagonists: 4-DAMP > pirenzepine > AF DX 116 (Hagan *et al*., 1988). The pharmacological identity of contractile muscarinic receptors in the iris sphincter, particularly that of the rabbit, has been the subject of extensive investigations. Akhtar *et al*. (1987) concluded that M_2 receptors were involved based on the low affinity of pirenzepine in this tissue. Subsequent studies, which used a range of antagonists, obtained an affinity profile (pK_B values of 6.43, 6.22, 7.23, 5.34 and 6.84 for p-F-HHSiD, AQ-RA-741, (**R**)-trihexyphenidyl, **S**-trihexyphenidyl and secoverine, respectively) which was incompatible with the involvement of M_1, M_2, M_3 and M_4 receptors (Fuder *et al*., 1989; Bognar *et al*., 1992). A drawback of the previous studies was that the ligands used had only limited selectivity for any one subtype, particularly the M_3 and M_4 subtypes. The objective of the present study was therefore to re-examine the pharmacological characteristics by including some novel and more selective ligands including darifenacin (M_3-selective), zamifenacin (M_3-selective) and Mamba toxin 3 (MTx3, M_4-selective) and correlate the affinities of these and other ligands with binding affinity estimates at the five recombinant muscarinic receptors expressed in Chinese hamster ovary (CHO) cells (Dörje *et al*., 1991; Nilvebrant *et al*., 1996; Eglen *et al*., 1997; Hegde *et al*., 1997). Furthermore, we performed parallel pharmacological studies in the rabbit urinary bladder for comparison since contraction of this tissue has been shown to be mediated by a homogeneous population of M_3 muscarinic receptors (Tobin, 1995).

A preliminary account of the findings has been presented previously to the British Pharmacological Society (Choppin *et al*., 1997).

Methods

In vitro *contractile studies*

Female New Zealand white rabbits (2.5–3 kg) were killed by CO_2 asphyxiation. The eyes were removed and placed in

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oxygenated Tyrode solution (composition in mM: NaCl 137.0, KCl 2.7, $CaCl_2$ 1.8, $MgCl.6H_2O$ 1.0, KH_2PO_4 0.4, $NaHCO_3$ 11.9 and dextrose 5.6). The urinary bladder was isolated, cleared of adhering adipose tissue and placed in oxygenated Krebs solution (composition in mM: NaCl 118.2, KCl 4.6, $CaCl_2$ 2.5, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.8 and dextrose 10.0). Both physiological solutions contained indomethacin (10 μM) in order to reduce prostaglandin-induced spontaneous activity of the tissues. Two strips of iris sphincter muscle were cut from each eye (ciliary margin removed) and four strips of urinary bladder smooth muscle were cut from the supratrigonal portion of the bladder (longitudinal section, mucosa removed). The tissues were mounted in 10 ml organ baths containing Tyrode or Krebs solution, maintained at 37°C and constantly aerated with 95% O_2/5% CO_2 (pH = 7.4). Grass FT03 transducers were used to measure changes in isometric tension of the tissue which were displayed on a Grass 7E polygraph. The tissues were maintained at a resting tension of 150 mg and 2 g for the iris and the bladder, respectively, during an equilibration period of 60 min. Tension adjustments were made as necessary. The tissues were washed every 15 min.

The viability of each tissue was assessed by determining the contractile response to KCl (30 mM) at the start of the experimental protocol. After washing, tissues were re-equilibrated for 10 min and allowed to regain baseline tension. Cumulative concentration-effect curves to (+)-*cis*-dioxolane, a non-selective muscarinic agonist, (1 nM – 0.3 mM) were then constructed in each tissue. Thereafter, tissues were equilibrated in either the absence (time control) or presence of antagonist for a 90 min period during which tissues were washed every 10 min. Subsequently, a second concentration-effect curve to (+)-*cis*-dioxolane was constructed.

Data analysis

Contractions were recorded as changes in tension from baseline and expressed as a percentage of the maximum response of the first agonist concentration-effect curve. Agonist concentration-response curves were fitted using a nonlinear iterative fitting programme (Origin, Microcal Software, Inc., Northampton, MA, U.S.A.) using the relationship of Parker and Waud (1971). Agonist potencies and maximum response are expressed as pEC_{50} (− logarithm of the molar concentration of agonist producing 50% of the maximum response) and E_{max}, respectively. Concentration-ratios (CRs) were determined from EC_{50} values in the presence and absence of antagonist. Antagonist affinity estimates (pA_2 values) were determined by the method of Arunlakshana and Schild (1959) using at least three concentrations of the antagonist. All data are expressed as mean ± s.e.mean. In cases where the slope of the linear regression was not significantly different from unity, the slope was constrained to 1 and the data expressed as the pK_B value. Pearson correlation coefficients (r) and associated P values were calculated using the method described by Dixon and Massey (1983). The sum of squares of differences in affinity estimates for each plot ($\Sigma(y\text{-}x)^2$, noted ssq) defines the proximity of the data points to the line of identity ($y = x$).

Drugs

Atropine sulphate, indomethacin and oxybutynin chloride were obtained from Sigma Chemical Co (MO, U.S.A.). (+)-*Cis*-dioxolane, pirenzepine dihydrochloride, methoctramine hydrochloride and *para* fluoro hexahydrosiladifenidol (p-F-HHSiD) hydrochloride were obtained from Research Biochemicals Inc. (MA, U.S.A.). Darifenacin hydrobromide and zamifenacin fumerate were generously provided by Pfizer Central Research (Sandwich, Kent, U.K.). AQ-RA 741 (11-({4-[4-(diethylamino)butyl]-1-piperidinyl}acetyl)-5,11-dihydro-6H-pyrido(2,3-b)(1,4)benzodiazepine-6-one) was donated by Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT, U.S.A.). Secoverine hydrochloride was synthesized at Roche Bioscience (Palo Alto, U.S.A.). Mamba Toxin 3 (MTx 3) was generously provided by Dr Evert Karlsson (Uppsala, Sweden).

Results

Characterization of muscarinic receptors mediating contractions of the rabbit isolated iris sphincter muscle

(+)-*cis*-Dioxolane induced concentration-dependent contractions of the rabbit iris sphincter muscle ($pEC_{50} = 6.41 \pm 0.10$; $E_{max} = 181 \pm 17$ mg, $n = 38$). Time-control experiments showed that two consecutive concentration-effect curves to this agonist could be constructed in the same tissue without any significant temporal change in the agonist potency and maximum response ($pEC_{50} = 6.41 \pm 0.10$; $E_{max} = 181 \pm 17$ mg and $pEC_{50} = 6.55 \pm 0.06$; $E_{max} = 186 \pm 18$ mg for the first and second agonist curves respectively, $n = 38$).

Several antagonists (atropine, AQ-RA 741, darifenacin, methoctramine, oxybutynin, pirenzepine, secoverine, p-F-HHSiD and zamifenacin) were tested for their ability to inhibit (+)-*cis*-dioxolane-induced responses and their functional affinity estimates (pA_2/pK_B) are summarized in Table 1. Cumulative agonist concentration-response curves were

Table 1 Affinity estimates for muscarinic antagonists in rabbit iris sphincter muscle and urinary bladder smooth muscle

	Iris		*Bladder*	
Antagonist	pA_2	pK_B	pA_2	pK_B
Atropine	9.24 ± 0.25	9.30 ± 0.07	9.63 ± 0.21	9.40 ± 0.04
Pirenzepine	6.80 ± 0.10	6.95*	6.79 ± 0.05	7.02 ± 0.12	6.89 ± 0.04
Methoctramine	5.80 ± 0.17	5.75 ± 0.07	5.85 ± 0.15	5.81 ± 0.06
p-F-HHSiD	7.42 ± 0.21	6.43*	7.55 ± 0.09	7.71 ± 0.20	7.50 ± 0.05
Darifenacin	9.42 ± 0.09	9.56 ± 0.05	9.09 ± 0.07	9.12 ± 0.05
Zamifenacin	8.62 ± 0.26	8.69 ± 0.10	8.19 ± 0.11	8.36 ± 0.06
Secoverine	7.49 ± 0.12	6.43*	7.54 ± 0.05	7.78 ± 0.16	7.66 ± 0.05
Oxybutynin	7.88 ± 0.15	8.10 ± 0.09	8.29 ± 0.14	8.59 ± 0.06
AQ-RA 741	6.25 ± 0.05	6.22*	6.35 ± 0.04	6.96 ± 0.11	6.88 ± 0.03
MTx 3		< 7.0		< 7.0

Values shown are means ± s.e.mean, $n = 3-5$ (5–30 determinations). All Schild slopes were not significantly different from unity. pK_B values were determined after imposing the unity constraint. *pA_2 values in iris taken from Bognar *et al*. (1992).

surmountably antagonized by these compounds in a concentration-dependent fashion, with parallel rightward displacements (Figure 1a shows representative data with p-F-HHSiD). The slopes of the Schild plots were not significantly different from unity for all these antagonists, suggesting that the antagonism was competitive in nature. The rank order of antagonist affinities (pK_B) was: darifenacin (9.56 ± 0.05) > atropine (9.30 ± 0.07) > zamifenacin (8.69 ± 0.10) > oxybutynin (8.10 ± 0.09) > p-F-HHSiD (7.55 ± 0.09) > secoverine (7.54 ± 0.05) > pirenzepine (6.79 ± 0.05) > AQ-RA 741 (6.35 ± 0.04) > methoctramine (5.75 ± 0.07).

Comparison of functional data for rabbit iris sphincter muscle with binding data at human recombinant muscarinic receptors

Correlation analysis between the affinities of the antagonists at muscarinic receptors in the rabbit iris sphincter muscle and the

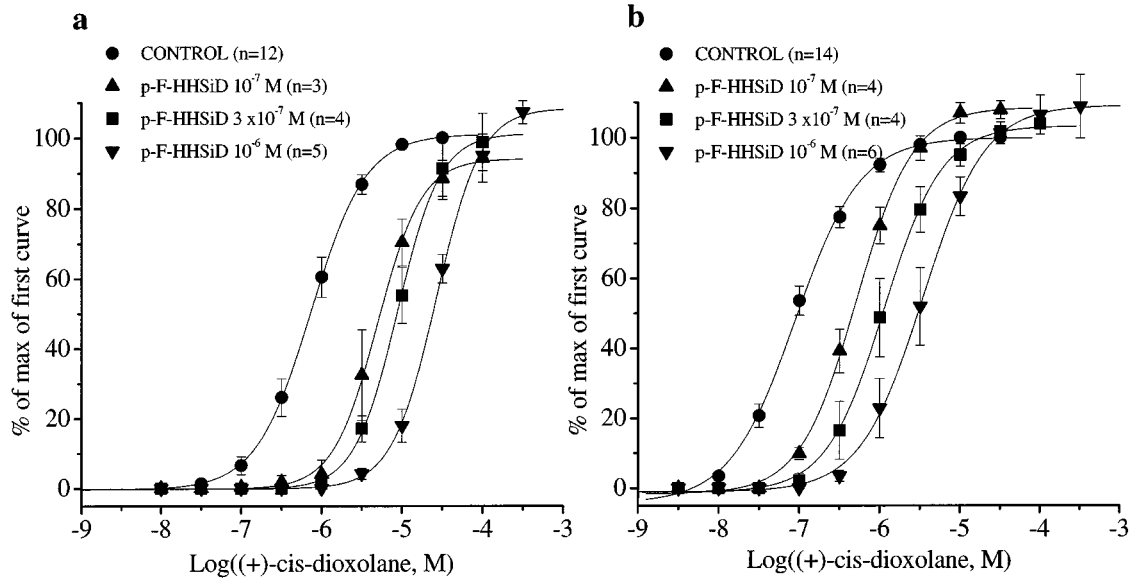

Figure 1 Effects of p-F-HHSiD on the cumulative concentration-response curves of (+)-*cis*-dioxolane (a) on the rabbit iris sphincter muscle and (b) on the rabbit urinary bladder smooth muscle. Contractile effects were expressed as percentages of the maximum response of the control curve. The values shown are means and vertical lines s.e.mean, $n = 3-5$ animals (5–14 determinations). A single concentration of antagonist was applied to each tissue.

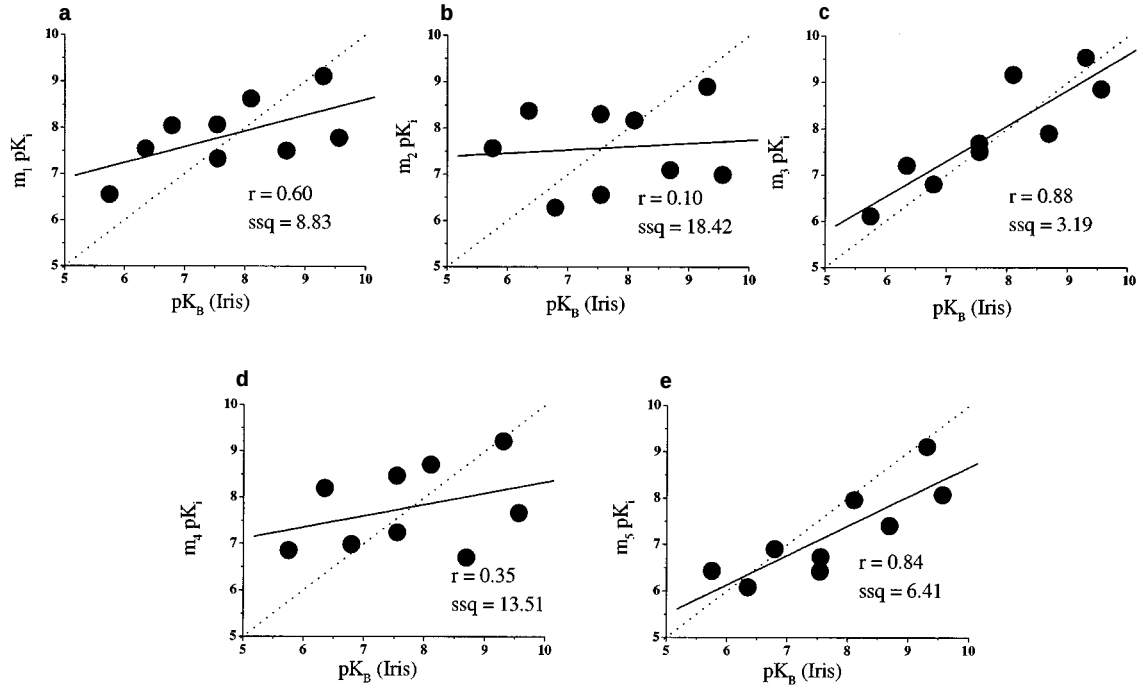

Figure 2 Correlation between the functional affinities (pK_B values) of muscarinic antagonists at muscarinic receptor in rabbit isolated iris sphincter muscle and binding affinities (pK_i values) at human recombinant muscarinic receptors (m1–m5; a–e, respectively). The binding data were taken from Dörje *et al.* (1991), Eglen *et al.* (1997) Hegde *et al.* (1997) and Nilvebrant *et al* (1996). The broken line is the line of identity (x = y) while the solid line is the correlation plot (the inserts give the correlation factors (*r*) and the sum of squares values (ssq)).

affinities at human recombinant muscarinic receptors showed a significant correlation ($r=0.88$, $P=0.002$, ssq = 3.19) at m3 but also at m5 receptors ($r=0.84$, $P=0.005$, ssq = 6.41). In contrast, poor correlations were observed at m1, m2 and m4 ($r=0.60$, ssq = 8.83; $r=0.10$, ssq = 18.42; $r=0.35$, ssq = 13.51, respectively) (Figure 2).

Characterization of muscarinic receptors mediating contractions of the rabbit urinary bladder smooth muscle

(+)-*Cis*-dioxolane produced concentration-dependent contractions of the rat urinary bladder smooth muscle ($pEC_{50}=6.97\pm0.04$; $E_{max}=4.28\pm0.25$ g, $n=54$). No time-dependent changes in agonist sensitivity were observed during the construction of the second curve ($pEC_{50}=6.97\pm0.04$; $E_{max}=4.28\pm0.25$ g and $pEC_{50}=6.85\pm0.05$; $E_{max}=4.76\pm0.27$ g for the first and second agonist curves, respectively, $n=54$). Pharmacological characterization of the muscarinic receptor involved was done by determination of antagonist affinities. Concentration-effect curves to (+)-cis-dioxolane were surmountably antagonized by atropine, darifenacin, oxybutynin, zamifenacin, secoverine, p-F-HHSiD, pirenzepine, AQ-RA 741 and methoctramine (pA_2/pK_B are summarized in Table 1). As an example, the effect of p-F-HHSiD on the cumulative concentration-response curve to (+)-cis-dioxolane on the rabbit urinary bladder smooth muscle is shown in Figure 1b.

Comparison of functional data for rabbit urinary bladder smooth muscle with binding data at human recombinant muscarinic receptors

The best correlation between the affinities of antagonists at the muscarinic receptor in rabbit urinary bladder smooth muscle and the affinities at human recombinant receptors was obtained at m3 receptors ($r=0.96$, $P<0.0001$, ssq = 0.84). However, a significant correlation was also found with the m5 receptor ($r=0.87$, $P=0.003$, ssq = 5.66). In contrast, the correlation was less favorable at the other subtypes ($r=0.74$, ssq = 5.11; $r=0.27$, ssq = 13.46; $r=0.53$, ssq = 8.45 at m1, m2 and m4, respectively (Figure 3)).

Comparison between functional data in the rabbit iris sphincter muscle and urinary bladder smooth muscle

When the affinities of antagonists (Table 1) in the rabbit iris sphincter muscle were compared with the affinities in the rabbit

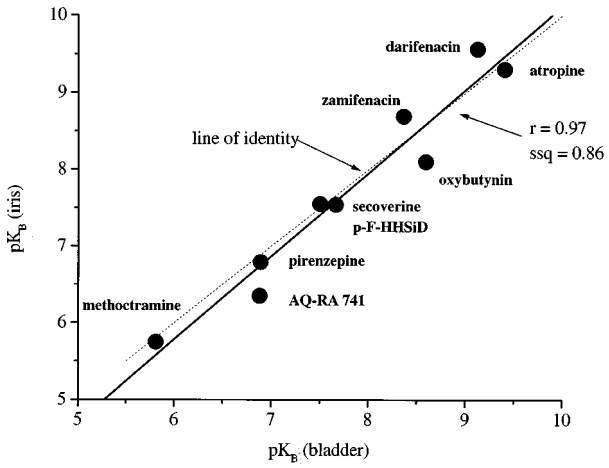

Figure 4 Comparison of the pK_B values of muscarinic receptor antagonists at receptors in rabbit isolated urinary bladder smooth muscle and in isolated iris sphincter muscle. The broken line is the line of identity (x = y) and the solid line is the correlation plot (the correlation factors (r) and the sum of squares values (ssq) are indicated).

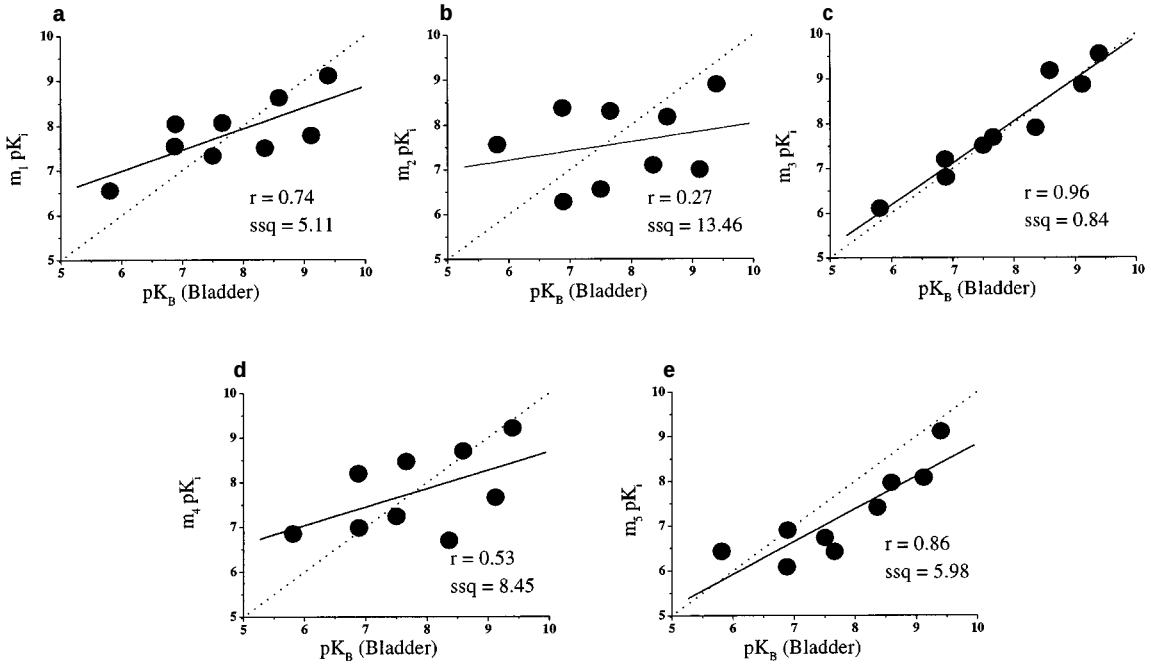

Figure 3 Correlation between the functional affinities (pK_B values) of muscarinic antagonists at muscarinic receptor in rabbit isolated urinary bladder smooth muscle and binding affinities (pK_i values) at human recombinant muscarinic receptors (m1–m5; a–e, respectively). Binding data were taken from Dörje *et al.* (1991), Eglen *et al.* (1997), Hegde *et al.* (1997), Nilvebrant *et al.* (1996). The broken line is the line of identity (x = y) while the solid line is the correlation plot (the inserts give the correlation factors (r) and the sum of squares values (ssq)).

urinary bladder smooth muscle, a highly significant correlation ($r=0.97$, $P<0.0001$) was obtained (Figure 4), close to the line of identity (ssq = 0.86).

Discussion

The muscarinic receptor subtype mediating contraction of the rabbit isolated iris sphincter has been previously characterized as being atypical (i.e. non M_1, M_2, M_3 and M_4) (Bognar *et al*., 1989; 1992; Fuder *et al*., 1989). The present study has re-examined the pharmacological characteristics of this tissue and compares it with another smooth muscle tissue from the same species, the rabbit urinary bladder.

Rabbit urinary bladder smooth muscle

(+)-Cis-dioxolane produced concentration-dependent contractions which were blocked in a concentration-dependent and competitive fashion by muscarinic antagonists. The apparent affinity estimates of these antagonists correlated most strikingly with the binding affinities of the antagonists at m3 recombinant muscarinic receptors (pK_i are: atropine 9.54; AQ-RA 741, 7.20; darifenacin 8.86; methoctramine 6.11; oxybutynin 9.17; pirenzepine 6.80; secoverine 7.69; p-F-HHSid 7.51 and zamifenacin 7.90; $r=0.95$, ssq = 1.14; Dörje *et al*., 1991; Nilvebrant *et al*., 1996; Eglen *et al*., 1997; Hegde *et al*., 1997) and are consistent with the involvement of M_3 muscarinic receptors in the contractile response to muscarinic agonists. This is in accordance with the findings in the rabbit (Tobin, 1995; Tobin & Sjogren, 1995), rat (Longhurst *et al*., 1995; Hegde *et al*., 1997) and human (Newgreen & Naylor, 1996) bladder. Interestingly, darifenacin, which has been shown to behave unsurmountably in the rat bladder (Hegde *et al*., 1997), was shown to produce surmountable antagonism in the rabbit bladder. This could be due to either species differences or the different range of concentrations studied. However, it should be noted that a reasonably good correlation ($r=0.87$, ssq = 5.66) was also obtained with the binding affinities of the antagonists at m5 recombinant muscarinic receptors (pK_i are: atropine 9.11; AQ-RA 741, 6.08; darifenacin 8.07; methoctramine 6.43; oxybutynin 7.96; pirenzepine 6.90; secoverine 6.42; p-F-HHSiD 6.73 and zamifenacin 7.40; Dörje *et al*., 1991; Nilvebrant *et al*., 1996; Eglen *et al*., 1997; Hegde *et al*., 1997). This is not surprising given that most ligands discriminate poorly between M_3 and m5 receptors, and highlights the difficulty of excluding a role for the latter in M_3-mediated responses.

It is interesting that M_3 receptors mediate contraction of this tissue directly despite a predominance of m2 over m3 mRNA in the rat, pig (Maeda *et al*., 1988) and human (Yamaguchi *et al*., 1996) bladder (shown by Northern blot hybridization analysis), as well as in human, rat, rabbit and guinea-pig bladder membranes (Wang *et al*., 1995) (shown by immunoprecipitation). Functional studies in the rat bladder have demonstrated an indirect contractile role for M_2 receptors (only after M_3 depletion), whereas M_3 receptor activation causes direct bladder contraction (Hegde *et al*., 1997). It is conceivable that M_2 receptors in the rabbit bladder have a similar role.

Rabbit iris sphincter muscle

Data from previous pharmacological studies (Bognar *et al*., 1992) have suggested that a muscarinic receptor different from the M_1, M_2, M_3 and M_4 subtypes mediates the contraction of the rabbit isolated iris sphincter, whereas other studies have implicated the M_3 receptor (Akhtar *et al*., 1987; Honkanen & Abdel-Latif, 1988; Bognar *et al*., 1989). Specifically, the intermediate affinity of pirenzepine (6.95; Bognar *et al*., 1989) is compatible with either an M_4 or M_5 receptor, but the low affinities of himbacine and secoverine ($pA_2=6.36$ and 6.43, respectively; Bognar *et al*., 1989), methoctramine ($pA_2=5.93$; Fuder *et al*., 1989) and AQ-RA 741 ($pA_2=6.22$; Bognar *et al*., 1989) would argue against the involvement of an M_4 receptor. Also, the low affinity of M_1/M_3 selective antagonist p-F-HHSiD (6.43; Bognar *et al*., 1992) and secoverine ($pA_2=6.43$; Bognar *et al*., 1989) raised doubts about the identity of this receptor with M_3.

A discrepancy was found in the affinity (pA_2s) of p-F-HHSiD (7.42 ± 0.21) and secoverine (7.49 ± 0.12) in the present study with that of Bognar *et al*. (1989; 1992). Thus, we were unable to confirm the low affinities reported for these compounds ($pA_2=6.43$ and 6.43, respectively; Bognar *et al*., 1989; 1992). Indeed, these estimates were highly consistent with an interaction at a muscarinic M_3 receptor. Some experimental differences may account for these differences, including the agonist: methacholine (Bognar *et al*., 1992) versus (+)-*cis*-dioxolane (this study). However, we do not believe this to be the reason for the disparate results since we obtained similar results using methacholine as the agonist (unpublished data).

Among the five recombinant muscarinic receptors, the best correlation of the functional pK_B estimates in the rabbit iris was obtained with affinities at the recombinant human m3 receptor. This conclusion is supported by the highly significant correlation of the affinity values at muscarinic M_3 receptors in rabbit isolated bladder smooth muscle. Collectively, the muscarinic receptors mediating contraction of the rabbit iris sphincter muscle equate most closely with $m3/M_3$ receptors. However, as stated above, the poor discriminative ability of most ligands between M_3 and m5 receptors highlights the difficulty of unambiguous classification. It should be noted that the human iris is one of the few examples of the localization of the m5 subtype outside the CNS, the others being in human macrophages and ciliary muscle (Ferrari-Dileo & Flynn, 1995; Zhang *et al*., 1995). Immunoprecipitation of muscarinic receptors from human iris has confirmed that the m3 subtype is the predominant muscarinic receptor ($59.1\pm7.8\%$; Gil *et al*., 1997), but also indicated its coexistence with all four other subtypes: 7.4% m1, $7.8\pm5.5\%$ m2, $11.4\pm4.1\%$ m4 and $5.4\pm3.1\%$ m5.

Conclusions

The present study has shown that the pharmacological antagonist profiles of the rabbit iris and bladder are similar to each other and equate most closely with the m3 muscarinic receptor. These results therefore do not support an earlier proposal for the involvement of a muscarinic receptor different from the M_1, M_2, M_3 and M_4 subtypes in the rabbit iris sphincter muscle (Bognar *et al*., 1992). However, the involvement of m5 receptors in both tissues cannot be ruled out entirely until ligands which discriminate between this and the m3 receptor become available. Indeed, the presence of functional m5 receptors in peripheral tissues may have been overlooked, given its similarity in pharmacology to the M_3 receptor.

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