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Muscarinic receptor subtypes in the rat prostate gland

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Abstract

Muscarinic receptor subtypes mediating carbachol-induced contractions of the rat prostatic smooth muscle were determined. The rank order of potency of muscarinic receptor antagonists in blocking the effects of carbachol was (mean pK_B estimates in parentheses): atropine (8.90) ≫ *para*-fluorohexahydrosiladifenidol (7.75) ≥ hexahydrosiladifenidol (7.62) > methoctramine (6.89) ≥ pirenzepine (6.68) ≥ himbacine (6.67). The specific binding of [^{3}H]quinuclidinyl benzilate to the rat prostatic homogenates was competitively inhibited by (mean pK_i values in parentheses): atropine (8.89) ≫ hexahydrosiladifenidol (7.86) > *para*-fluorohexahydrosiladifenidol (7.28) ≥ himbacine (7.22) > pirenzepine (6.63) ≥ methoctramine (6.38). These profiles, whilst different, indicate the probable involvement of muscarinic M_3 receptors in the carbachol-induced contraction. © 1998 Elsevier Science B.V.

Keywords: Smooth muscle, prostate; Muscarinic M_3 receptor; Carbachol; Radioligand binding; 1-Quinuclidinyl [phenyl-4-^{3}H]benzilate

1. Introduction

The prostate gland of the rat has been used extensively as an animal model in prostate research. As in other species, the sympathetic noradrenergic innervation is believed to play a major role in the regulation of prostatic smooth muscle contractility to expel secreted fluid into the urethra during ejaculation (Vaalasti and Hervonen, 1979). The α-adrenoceptor-mediated contraction of the rat prostatic smooth muscle has been well documented (Testa et al., 1993; Yazawa and Honda, 1993b). In contrast, the role played by the cholinergic nerves supplying the prostate gland is unclear. Sympathetic cholinergic nerves are thought to innervate the glandular epithelium and to stimulate prostatic secretion (Farnsworth and Lawrence, 1965; Wang et al., 1991). Radioligand binding techniques have indicated a predominance of muscarinic M_3 receptors in the rat prostate gland (Latifpour et al., 1991; Yazawa and Honda, 1993a). Although the rat prostatic smooth muscle has been shown to contract in response to acetylcholine and carbachol (Cohen and Drey, 1989; Raz et al., 1973), to date there have been no functional studies to determine the muscarinic receptor subtypes mediating cholinergic effects on the prostatic smooth muscle tone. The present study was, therefore, undertaken to characterise functional muscarinic receptor subtypes mediating contraction of the rat prostatic smooth muscle. A parallel series of radioligand binding experiments was also conducted using a number of subtype-preferring muscarinic receptor antagonists.

2. Methods

2.1. Animals

Adult male Sprague–Dawley rats (250–420 g) were housed at 22°C, with a 12:12 h light:dark cycle. Rodent chow and water were provided ad libitum. Prior approval for animal experimentation was obtained from the Monash University Standing Committee on Ethics in Animal Experimentation (SCEAE Approval No. 94/149). Rats were killed by stunning and exsanguination.

2.2. Functional studies

Preparations of the ventral prostates were mounted in 5 ml-organ baths containing Krebs–Henseleit solution (pH 7.4, maintained at 37°C and bubbled with 5% CO_2 in O_2)

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of the following composition (mM): NaCl 118.1, KCl 4.87, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.7, $MgSO_4.7H_2O$ 0.5, $CaCl_2.2H_2O$ 2.5. Tissues were allowed to equilibrate for 60 min under a resting force of 0.5 g. Contractions were recorded with Grass FT03C force-displacement transducers connected to a MacLab data acquisition system (Chart 3.3) interfaced with a Macintosh LC575 computer.

Log concentration–response curves to carbachol (0.1 μM–1 mM), administered in one log unit increments, were constructed cumulatively with a contact time of 60 s for each concentration. A 'priming' concentration–response curve was first constructed. Log concentration–response curves to carbachol were then constructed in the absence and presence of atropine or subtype-preferring muscarinic receptor antagonists following a preincubation period of 20–30 min. Parallel control experiments using preparations without antagonist incubation were conducted to correct for any changes due to time and/or vehicle (bath concentration of up to 0.01% ethanol). In a separate series of experiments the effects of tetrodotoxin (1 μM) and hexamethonium (0.1 mM) on responses to carbachol were determined by obtaining constant responses to a single concentration of carbachol (10 μM), exposing the preparation to the antagonist for 30 min, and then repeating the application of carbachol.

Responses to carbachol in the absence and presence of the muscarinic receptor antagonists, tetrodotoxin and hexamethonium were determined by measuring the peak force developed (in g) to each concentration of carbachol.

Using the GraphPad PRISM software program, linear regression analyses of the linear portion (approximately 15–85% of maximum response) of each log concentration–response curve to carbachol were undertaken to determine parallelism and coincidence of the curves in the absence and presence of antagonists. When this analysis indicated parallelism, non-linear, least squares, regression analysis of individual experiments was then conducted to fit log concentration curves to each data set. Mean estimates of apparent dissociation constants (K_B) for each concentration of each antagonist were obtained by calculating concentration ratios for each experiment using the equation from Furchgott (1972): K_B = (antagonist concentration)/(concentration ratio − 1). K_B values were converted to the negative logarithm and expressed as pK_B.

2.3. Radioligand binding studies

The whole prostate gland was dissected out and placed in ice-cold Na_2HPO_4 buffer (50 mM, pH 7.4). The prostatic tissues were blotted dry, weighed (mean weight = 0.47 ± 0.03 g, $n = 23$), finely minced with scissors and homogenised twice for 30 s in 10 ml of ice-cold buffer with a 2 min interval on ice using an Ultra-Turrax at 80% maximal speed. The homogenates were then centrifuged at 1000 × g for 12 min at 0°C.

For the binding assay 150 μl of the homogenate was added to tubes containing a final volume of 1 ml (protein concentration of approximately 380 μg/ml), determined by the method of Lowry et al. (1951). In saturation experiments, eight concentrations of [^{3}H]quinuclidinyl benzilate (0.02–4 nM) were used. For competition experiments, fourteen concentrations of each competing ligand were used with [^{3}H]quinuclidinyl benzilate (0.2 nM). Non-specific binding was determined in the presence of atropine (10 μM). All assays were performed in triplicates and incubation was at 37°C for 60 min, following a preincubation of 10 min. The binding reaction was terminated by rapid filtration through Whatman GF/B glass fibre filters using a Brandel M-48 cell harvester. The filters had been presoaked overnight in polyethyleneimine (0.1%) and atropine (10 μM) to prevent binding to filters. After washing twice with 5 ml of ice-cold buffer and partial drying under vacuum, tissue-bound radioactivity was extracted from the filters in 4 ml of Ecolite scintillant. The radioactivity was determined by the United Technologies Packard Tri-carb 4000 series liquid scintillation counter with an efficiency of 45–50%.

Binding data were analysed using software programs EBDA and LIGAND by McPherson (1985). Data are presented as mean values ± standard error of the means (S.E.M.).

2.4. Drugs

The following drugs were used: carbachol (carbamylcholine chloride), atropine sulphate, and tetrodotoxin were from Sigma, hexahydrosiladifenidol hydrochloride, *para*-fluorohexahydrosiladifenidol hydrochloride and methoctramine tetrahydrochloride were from Research

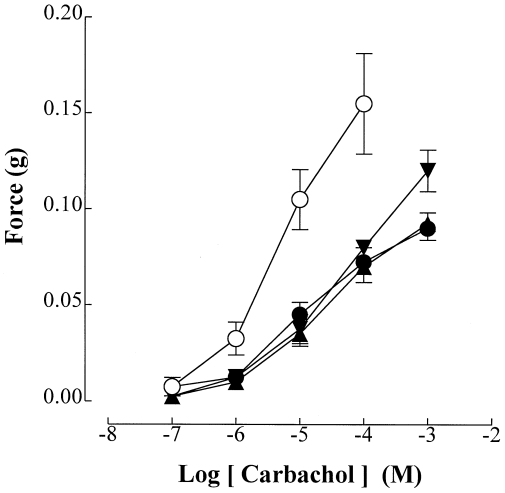

Fig. 1. The effects of time (○: $t = 0$; ●: $t = 35$ min; ▲: $t = 70$ min; ▼: $t = 105$ min) on the mean log concentration–response curves to carbachol in the rat ventral prostate ($n = 3$–4).

Biochemicals International and hexamethonium tartrate was from May and Baker. Pirenzepine dihydrochloride and himbacine hydrochloride were gifts from Dr. K. Thomae of Boehringer Ingelheim and Professor W.C. Taylor of University of Sydney, Australia, respectively. 1-Quinuclidinyl [phenyl-4-^{3}H]benzilate (specific activity 47 Ci/mmol) was from Amersham. All drugs except hexahydrosiladifenidol and *para*-fluorohexahydrosiladifenidol were dissolved in distilled water. Stock concentrations of hexahydrosiladifenidol (1 mM) and *para*-fluoro-

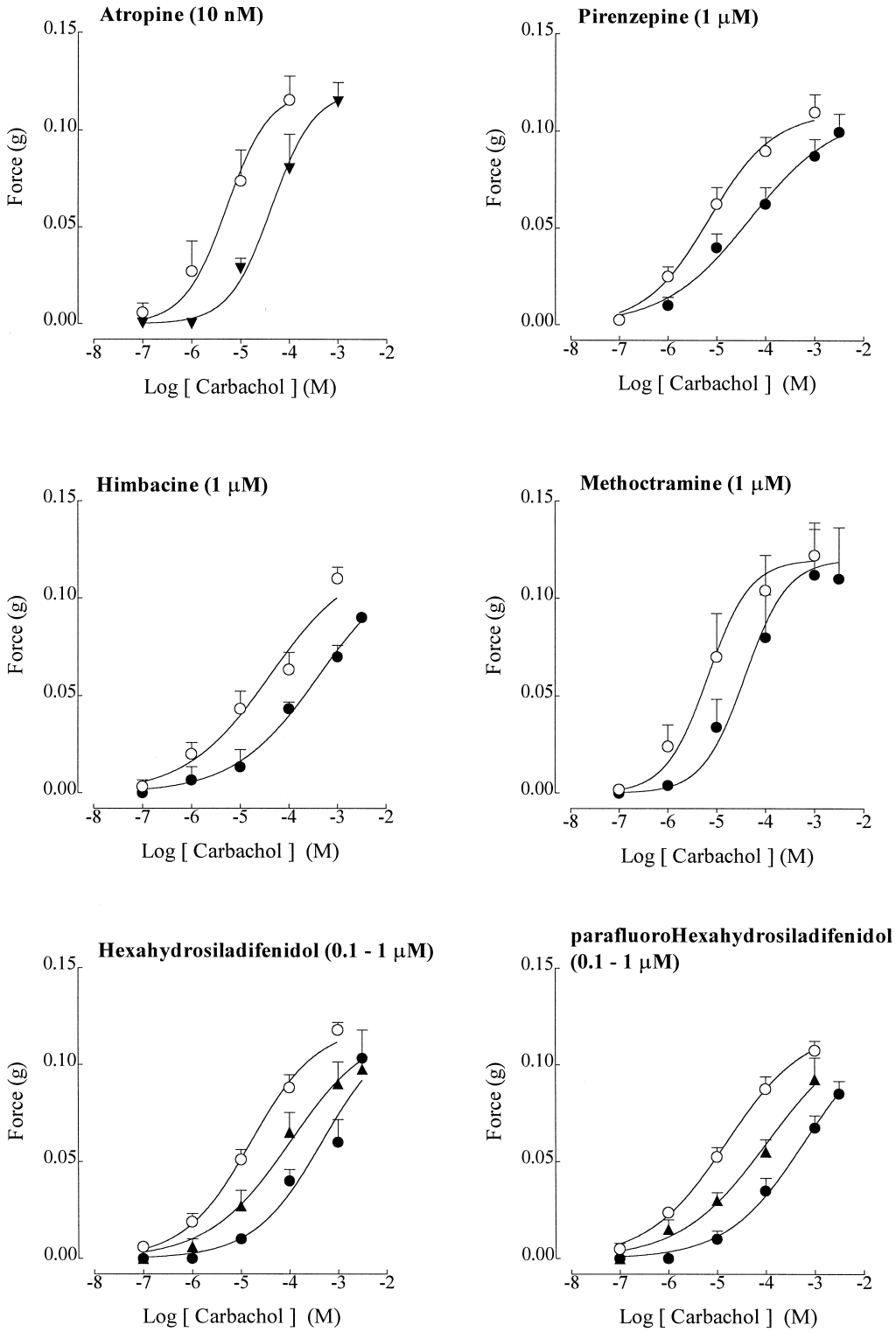

Fig. 2. Mean log concentration–response curves to carbachol in the absence (○) and in the presence of muscarinic receptor antagonists (▼: 10 nM; ▲: 0.1 μM; ●: 1 μM) in the rat ventral prostate ($n = 3–5$).

hexahydrosiladifenidol (1 mM) were prepared in absolute ethanol. Dilutions to working concentrations were made in Krebs–Henseleit and Na_2HPO_4 buffer solution in functional and binding experiments, respectively.

3. Results

3.1. Functional studies

Cumulative addition of carbachol (0.1 μM–1 mM) evoked concentration-dependent contractions of the rat prostatic smooth muscle. The initial concentration–response or priming curve to carbachol had a higher maximum than did subsequent curves which were highly reproducible (Fig. 1). Antagonist vehicle (bath concentration of up to 0.01% ethanol; $n = 3$) had negligible effect on responses to carbachol; the latter curves were reproducible. Log concentration–response curves to carbachol were shifted significantly to the right in a parallel fashion in the presence of atropine (10 nM), methoctramine (1 μM), himbacine (1 μM), hexahydrosiladifenidol (0.1–1 μM), *para*-fluorohexahydrosiladifenidol (0.1–1 μM) (Fig. 2). The shifts in the presence of pirenzepine (1 μM) were, however, not parallel. The rank order of antagonist potency was atropine ≫ *para*-fluorohexahydrosiladifenidol ≥ hexahydrosiladifenidol > methoctramine ≥ pirenzepine ≥ himbacine. Their respective antagonist apparent dissociation constants (expressed as pK_B estimates) are listed in Table 1.

Neither tetrodotoxin (1 μM; $n = 3$) nor hexamethonium (0.1 mM; $n = 4$) significantly affected the mean magnitude of responses to a single concentration of carbachol (10 μM; $P > 0.05$, Student's unpaired *t*-test). In the absence of these agents the mean response to carbachol was 54 ± 6 mg; in the presence of tetrodotoxin and hexamethonium, responses were 57 ± 6 and 50 ± 7 mg, respectively.

Table 1
Mean estimates of pK_B values of muscarinic receptor antagonists in inhibiting carbachol-induced contractions of prostatic smooth muscle of the rat

Antagonist	*n*	$pK_B \pm$S.E.M.
Atropine	4	8.90 ± 0.28
Pirenzepine	4	6.68 ± 0.09[a]
Methoctramine	5	6.89 ± 0.32
Himbacine	3	6.67 ± 0.10
Hexahydrosiladifenidol	5	7.62 ± 0.10
Para-fluoroHexahydrosiladifenidol	4	7.75 ± 0.09

n = number of experiments.
[a]Log concentration–response curves in the absence and presence of pirenzepine were not parallel; apparent pK_B values were estimated after constraining Hill slopes to 1.

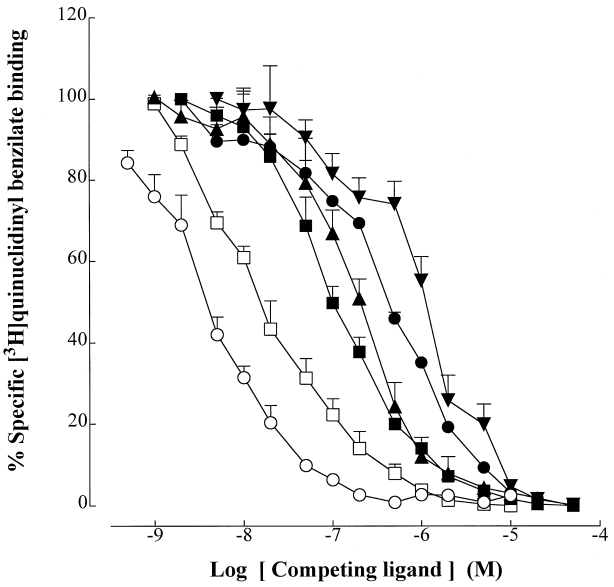

Fig. 3. Competition curves for atropine (○, $n = 4$), pirenzepine (●, $n = 3$), himbacine (▲, $n = 4$), methoctramine (▼, $n = 3$), hexahydrosiladifenidol (□, $n = 5$) and *para*-fluorohexahydrosiladifenidol (■, $n = 4$) on [^{3}H]quinuclidinyl benzilate binding sites in the rat prostatic homogenates.

3.2. Radioligand binding studies

Saturation studies using [^{3}H]quinuclidinyl benzilate (0.02–4 nM) indicated the presence of a high affinity binding site in the rat prostatic homogenates. This showed an equilibrium dissociation constant (K_d) of 120 ± 42 pM, a maximum number of binding sites (B_{max}) of 69 ± 12 fmol/mg protein or 463 ± 73 fmol/g tissue and a Hill coefficient (n_H) of 0.94 ± 0.02 ($n = 4$). At higher concentrations (2–4 nM) of the radioligand further non-saturable binding occurred, this was not investigated further.

In competition studies the specific binding of [^{3}H]quinuclidinyl benzilate (0.2 nM) to the rat prostatic homogenates was inhibited competitively by muscarinic receptor antagonists with the following rank order of potency: atropine ≫ hexahydrosiladifenidol > *para*-fluorohexahydrosiladifenidol ≥ himbacine > pirenzepine ≥ methoctramine (Fig. 3). Mean values of the negative loga-

Table 2
Mean values of the negative logarithm of dissociation constants (pK_i) and slopes of the Hill plot (n_H) for the muscarinic receptor antagonist competition with [^{3}H]quinuclidinyl benzilate binding sites in the rat prostatic homogenates

Antagonist	*n*	$pK_i \pm$S.E.M.	$n_H \pm$S.E.M.
Atropine	4	8.89 ± 0.13	0.96 ± 0.03
Pirenzepine	3	6.63 ± 0.01	0.95 ± 0.03
Himbacine	4	7.22 ± 0.09	1.18 ± 0.09
Methoctramine	3	6.38 ± 0.07	1.05 ± 0.04
Hexahydrosiladifenidol	5	7.86 ± 0.1	0.86 ± 0.05
Para-fluorohexahydrosiladifenidol	4	7.28 ± 0.04	0.84 ± 0.08

n = number of experiments.

rithm of dissociation constants (pK_i) and slopes of the Hill plot (n_H) from estimates of individual experiments are shown in Table 2.

4. Discussion

The nature of muscarinic receptor subtypes mediating the contractile responses to carbachol in the rat ventral prostate was determined using the subtype-preferring muscarinic receptor antagonists: pirenzepine (M_1), methoctramine (M_2), himbacine (M_2/M_4), hexahydrosiladifenidol (M_1/M_3) and *para*-fluorohexahydrosiladifenidol (M_3/M_1) (Caulfield, 1993, for a review). A set of experiments using the ganglion blocking drug, hexamethonium, and tetrodotoxin, as well as atropine, indicated that carbachol exerted its contractile effect directly on rat prostate smooth muscle by activation of muscarinic receptors, rather than by activation of receptors located on the nerves supplying the tissue.

The initial log concentration–response curves to carbachol were much more sensitive than the second and subsequent curves (see Fig. 1). It may be that the rat prostate, in response to carbachol, releases secretions which in turn may enhance responses to carbachol and that subsequent washouts remove any secretory substances from the rat prostatic preparations. The muscarinic receptor antagonists, except pirenzepine, caused parallel rightward shifts of the log concentration–response curves to carbachol. The rank order of potencies of the subtype-preferring muscarinic receptor antagonists was *para*-fluorohexahydrosiladifenidol > hexahydrosiladifenidol > methoctramine ≥ pirenzepine ≥ himbacine.

The apparent pK_B estimate for *para*-fluorohexahydrosiladifenidol obtained in the present study (7.75) is comparable to that reported for the interactions with muscarinic M_3 receptors in the guinea-pig ileum (pA_2 value of 7.8; Eglen et al., 1990). It is too high to indicate action at muscarinic M_2 receptors, and is over 0.5 log unit higher than its pA_2 value reported for M_1 receptors (7.2; Table 2 in Eglen et al., 1996). Hexahydrosiladifenidol has a high affinity for both muscarinic M_1 and M_3 receptors (Lambrecht et al., 1989) and relatively low potency at muscarinic M_2 receptors. It has been shown to display differential apparent affinity constants in various muscarinic M_3 receptor preparations, varying from 7.1 in the human bronchial smooth muscle (Watson et al., 1995) to 8.1 in the guinea-pig ileal circular muscle (Dietrich and Kilbinger, 1995). The pK_B estimate for hexahydrosiladifenidol obtained in the present study (7.62) lies within this range. The low pK_B estimate we obtained for pirenzepine (6.68) suggested that muscarinic M_1 receptors are not involved in the contractile response to carbachol. In addition, the pK_B value for himbacine (6.67) was approximately ten times less than that of *para*-fluorohexahydrosiladifenidol; suggesting that neither muscarinic M_2 nor M_4 receptors are important in mediating contractile responses to carbachol in the rat prostatic smooth muscle. Additionally, the muscarinic M_2-receptor preferring antagonist, methoctramine had a low potency at the receptor. Taken together, the potency profiles of the muscarinic receptor antagonists in inhibiting responses to carbachol best approximate those determined at pharmacologically characterised muscarinic M_3 receptors (Lambrecht et al., 1989; Eglen et al., 1990; Dörje et al., 1991).

Analysis of binding data using LIGAND indicated that [^{3}H]quinuclidinyl benzilate binding over the range (0.02–2 nM) to the rat prostatic homogenates involved a single affinity binding site, with mean K_d value and B_{max} similar to those reported by Latifpour et al. (1991) using this tissue. At higher concentrations (2–4 nM), there was further binding that was non-saturable and may be non-specific. Choo et al. (1985) have, however, reported that [^{3}H]quinuclidinyl benzilate binding in homogenates of the guinea-pig ileal longitudinal muscle showed the presence of two binding sites. Binding studies by Latifpour et al. (1991) and by Yazawa and Honda (1993a) indicated that muscarinic receptors present in the rat prostate gland were mainly of the M_3-subtype. [^{3}H]quinuclidinyl benzilate competition studies in the homogenates of the rat prostate gave the rank order of pK_i values of atropine ≫ hexahydrosiladifenidol > *para*-fluorohexahydrosiladifenidol ≥ himbacine > pirenzepine ≥ methoctramine. The rank order that we obtained for *para*-fluorohexahydrosiladifenidol, pirenzepine and methoctramine was identical to that obtained by Yazawa and Honda (1993a), who also used these antagonists, but was not identical to that in our functional studies.

Using the classification scheme by Dörje et al. (1991) and Eglen et al. (1994), based on the antagonist binding profiles of the five cloned human muscarinic receptor subtypes (m1–m5), the low pK_i value we obtained for himbacine suggests the relative unimportance of muscarinic M_2 and M_4 receptors in the rat prostate gland. The low pK_i value we obtained for methoctramine also suggests that muscarinic M_2 receptors are not prominent. The muscarinic M_1-receptor preferring antagonist, pirenzepine also yielded a fairly low pK_i value, suggesting a lack of muscarinic M_1 receptors.

Although the rank order of antagonist potency in the present study indicates the predominance of muscarinic M_3 receptors in the rat prostatic homogenates, hexahydrosiladifenidol and particularly *para*-fluorohexahydrosiladifenidol, exhibited slightly lower pK_i values when compared with those reported in other M_3 systems. The slopes of the Hill plot (n_H) for hexahydrosiladifenidol and *para*-fluorohexahydrosiladifenidol were less than one, consistent with heterogeneity of muscarinic receptors in the rat prostate gland; analyses of binding data for these two antagonists, however, showed better fits (F-test: $P < 0.05$) for one-site rather than two-site models. While the use of hypotonic buffers in binding assays may have influenced the esti-

mates of antagonist dissociation constants, (Pedder et al., 1991; Eglen et al., 1996), we did not employ gallamine or AF DX-116, which are the two antagonists most affected by the use of such buffers.

The rat prostate contains a relatively high proportion of glandular tissue relative to smooth muscle (Lepor et al., 1994). Our study has confirmed those of previous workers indicating that the predominant muscarinic binding sites present in the rat prostate gland are that of the M_3 subtype, a finding not surprising for a predominantly glandular rather than muscular tissue, in which M_2 binding sites often predominate (Eglen et al., 1996). The rat prostate differs from that of the human, which has been reported to express muscarinic receptors of the M_1-subtype (Ruggieri et al., 1995).

We have in this study also examined, for the first time, the nature of the muscarinic receptor subtype mediating smooth muscle contraction in the rat ventral prostate. The profiles estimated indicate that as for most smooth muscle (see Eglen et al., 1996), muscarinic M_3 receptors are likely to mediate carbachol-induced contractions of the rat ventral prostate.

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