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Characterisation of the prejunctional inhibitory muscarinic receptor on cholinergic nerves in the rat urinary bladder

Albert Shen *, Fred Mitchelson

Department of Pharmaceutical Biology and Pharmacology, Victorian College of Pharmacy (Monash University), 381 Royal Parade, Parkville, 3052, Victoria, Australia

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Abstract

The nature of the prejunctional inhibitory muscarinic receptor on cholinergic nerve endings in the rat urinary bladder was investigated by measuring stimulated endogenous acetylcholine release via high pressure liquid chromatography (HPLC), in the presence of various selective muscarinic antagonists. The rank order of potencies for the antagonists used was: atropine (− log concentration = 7.8) > 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine) (7.6) > tripitramine (7.3) = HHD (hexahydrodifenidol) (7.3) > pFHHSiD (*p*-fluorohexahydrosiladifenidol hydrochloride) (7.0) > himbacine (6.5) > methoctramine (5.9) ≥ pirenzepine (5.8) > gallamine (4.3). A comparison of the antagonist potencies obtained, with affinity constants at muscarinic M_1 to M_5 receptors, suggests that the prejunctional inhibitory muscarinic receptor is of the M_4 receptor subtype. © 2001 Published by Elsevier Science B.V.

Keywords: Muscarinic receptor, prejunctional; Cholinergic nerve; Urinary bladder, rat

1. Introduction

The urinary bladder is innervated by both the sympathetic and parasympathetic nervous system with the parasympathetic nervous system providing the main excitatory innervation to the detrusor smooth muscle (De Groat, 1993). Stimulation of parasympathetic postganglionic nerves releases acetylcholine, and a non-adrenergic non-cholinergic transmitter, possibly ATP (see Andersson, 1993), causing a contraction and thus voiding, in both animals and humans. Muscarinic receptors are present in bladder smooth muscle (detrusor), as well as on parasympathetic and sympathetic nerve endings (Andersson, 1993).

While the muscarinic M_2 receptor appears to be the predominant muscarinic receptor present in the smooth muscle of the bladder in most species (see Eglen et al., 1996), it is the muscarinic M_3 receptor which is responsible for smooth muscle contraction. Pharmacological antagonist characterisation of the subtype involved in contraction of the bladder in the rat (Longhurst et al., 1995; D'Agostino et al., 1997; Tong et al., 1997; Braverman et al., 1998), guinea pig (Noronha-Blob et al., 1989), mouse (Durant et al., 1991), rabbit (Tobin and Sjogren, 1995; Choppin et al., 1998), monkey (Lai et al., 1998) and human (Newgreen and Naylor, 1996) suggests muscarinic M_3 receptor involvement. The role of the muscarinic M_2 receptor population is speculative. It has recently been observed that in the rat bladder, muscarinic M_2 receptors may be involved indirectly in reversing β-adrenoceptor induced cAMP-mediated relaxation (Hegde et al., 1997; Braverman and Ruggieri, 1999; Hegde and Eglen, 1999). Thus, in bladder emptying, the muscarinic receptors would directly contract the smooth muscle and the activation of muscarinic M_2 receptors would reverse the relaxation mediated by the sympathetic nervous system.

In the rat bladder, prejunctional inhibitory muscarinic receptors were first noted following the observation that carbachol and muscarine inhibited electrical field stimulated-induced [^{3}H]acetylcholine overflow (D'Agostino et al., 1986, 1989). Somogyi and De Groat (1992) characterised the inhibitory receptor as the muscarinic M_2 receptor subtype. This was supported by Braverman et al. (1998) who observed an increase in contractions induced by electrical field stimulation in the presence of methoctramine. However, D'Agostino et al. (1997) characterised this receptor using a large number of muscarinic antago-

* Corresponding author. Tel.: +61-3-9903-9575; fax: +61-3-9903-9638.

E-mail address: Albert.Shen@vcp.monash.edu.au (A. Shen).

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nists and concluded the involvement of muscarinic M_4 receptors. Interestingly, Braverman et al. (1998) detected the presence of muscarinic M_1, M_2, M_3 and M_4 receptor mRNA via reverse transcriptase-polymerase chain reaction, indicating that muscarinic M_4 receptors are indeed present in the rat bladder. Similarly, in the guinea pig bladder, Tobin and Sjogren (1995, 1998) characterised the prejunctional inhibitory receptor as belonging to the muscarinic M_2 receptor subtype but by using an extensive number of antagonists, Alberts (1995) concluded that this receptor was of the muscarinic M_4 receptor subtype. The rank order of muscarinic antagonists which enhanced electrical field stimulation-induced [^{3}H]acetylcholine release, correlated best for this receptor. The prejunctional inhibitory muscarinic receptor in the human bladder was recently characterised as the muscarinic M_4 receptor (D'Agostino et al., 2000).

Somogyi et al. (1994) observed facilitation of acetylcholine release following continuous stimulation with trains of 100 shocks, and that facilitation was completely blocked by atropine or pirenzepine, concluding the involvement of prejunctional facilitatory muscarinic M_1 receptors. This muscarinic M_1 receptor facilitation was also observed by Braverman et al. (1998). However, unlike Somogyi et al. (Somogyi and De Groat, 1992; Somogyi et al., 1994) who noted facilitation only at high frequencies and inhibition at lower frequencies, Braverman et al. observed facilitation and inhibition at both low and high frequencies. In human tissue, muscarinic M_1 receptors also appear to mediate facilitation (see Somogyi and De Groat, 1999). It is possible that these facilitatory receptors assist in the act of voiding, however, the functional role of these receptors requires further investigation.

The aim of this paper was to investigate the nature of the prejunctional muscarinic receptor(s) present in the rat urinary bladder by utilising an acetylcholine high pressure liquid chromatography (HPLC) assay (see Shen et al., 1995). The advantage of this technique over the [^{3}H]choline method is that it measures changes in endogenous acetylcholine release as opposed to [^{3}H]acetylcholine following incubation with radiolabelled choline. The contribution of [^{3}H]choline to total acetylcholine release depends on the ultimate location of [^{3}H]acetylcholine in the nerve terminal in reserve vesicles, recycling vesicles or the cytoplasm (see Whittaker, 1986).

2. Materials and methods

2.1. Isolated tissue preparations

Hooded–Wistar rats of either sex were killed by a sharp blow to the back of the neck and exsanguinated from the carotid arteries. The whole urinary bladder plus both ureters were dissected out and placed in Krebs solution. The bladder was tied to a tissue hook, then attached to an isotonic transducer and suspended in a 3-ml organ bath containing Krebs solution bubbled with carbogen (95%O_2:5% CO_2). Physostigmine (10 μM) was added to prevent acetylcholine breakdown by cholinesterases in the tissue.

A ring electrode was placed around both ureters as close to the base of the bladder as possible. Previous studies have shown electrical stimulation to give rise to a contraction of the preparation which is abolished by tetrodotoxin (Choo and Mitchelson, 1980) and reduced by atropine (Vanov, 1965). A 'slit' was made from the base to the apex of the bladder to facilitate transmitter diffusion from both sides of the bladder wall. The bath solution was collected after a 15-min resting period to act as the control. The bladder was then stimulated for 15 min at 10 Hz, 1 ms duration at 80 V and the solution collected (S_1). This procedure was then repeated after a 60-min rest period (S_2). In some experiments, a third 15-min stimulation (S_3) was elicited after a further 60-min rest period. All release studies were corrected for S_2/S_1 and S_3/S_1 ratios in control experiments.

Antagonists were added to the bath and allowed to equilibrate for 45 min before nerve stimulation was elicited for a further 15 min. Bath solutions following the 15-min stimulation periods were collected and assayed for acetylcholine and choline.

In order to investigate the facilitatory muscarinic receptor, a series of experiments were conducted where bladder preparations were stimulated for 5 min at 10 Hz, 0.25 ms duration at 100 V before collection of the bath solution. These parameters were used to simulate similar conditions to experiments that have shown the presence of facilitatory receptors (Somogyi and De Groat, 1999).

2.2. Acetylcholine extraction

Acetylcholine and choline, released from isolated tissues following nerve stimulation, were extracted by the

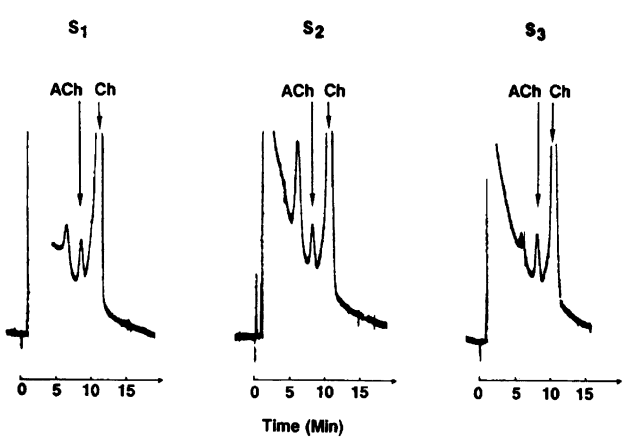

Fig. 1. Chromatogram showing the amount of acetylcholine released from the rat isolated urinary bladder following three periods of stimulation given 60 min apart. Choline peak in each case is off-scale with the sensitivity range used (recording retouched).

method of Shen et al. (1995). Briefly, 3 ml samples of eserinised physiological solution were added to 1.5 ml of tetraphenylboron in 3-heptanone (10 mg/ml). Following vigorous shaking for 2 min, vortexing and a brief centrifu-

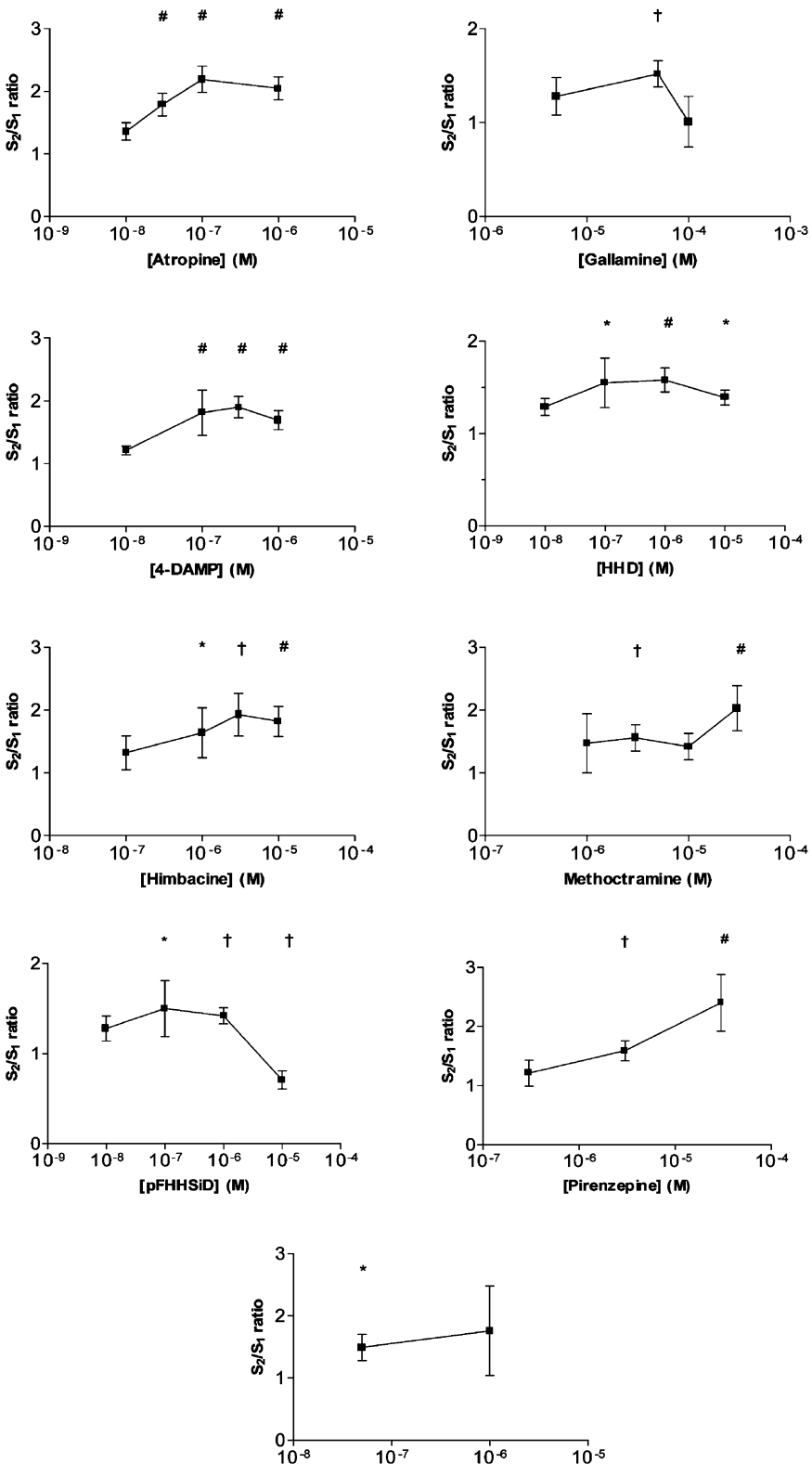

Fig. 2. Effect of antagonists on acetylcholine release in the rat bladder. Data expressed as mean ± S.E.M. of 3–8 experiments ($^{*}P < 0.05$; †$P < 0.01$; #$P < 0.001$; significantly different from control S_2/S_1).

gation, a 500-μl aliquot was mixed with 500 μl of 0.4 N hydrochloric acid. After further shaking, vortexing and centrifugation, 400 μl of the acid phase was evaporated to dryness by evaporative centrifugation and if necessary stored at −20°C overnight. The residue was then reconstituted in 125 μl of HPLC mobile phase before assay for acetylcholine.

2.3. HPLC assay for acetylcholine

The HPLC apparatus consisted of a BAS CC4 liquid chromatograph with a BAS MF-8910 acetylcholine/choline assay kit containing two cartridge columns which consisted of a polymeric analytical column followed by an immobilised enzyme reactor column. A BAS polymer physostigmine guard column (15 × 3.2 mm) was also attached. The columns were stationed in an oven maintained at 28°C by a BAS LC-22A temperature controller. The apparatus was coupled to a BAS PM-60 pump, set at a flow rate of 1 ml/min. Samples (50 μl) were injected onto the column via a Rheodyne 7125 injection valve fitted with a 20-μl sample loop. The mobile phase consisted of 0.025 M Na_2HPO_4 in HPLC-grade water at pH 8.5 with Kathon (5 ml/l) added as a bacteriostatic. Before use it was filtered through a 0.45-μm Millipore cellulose filter.

A BAS LC-4B amperometric detector containing a Ag/AgCl reference electrode and platinum working electrode set at +500 mV was used. Output was recorded on a BAS RYT chart recorder with a chart speed of 2 mm/min.

2.4. Data analysis

Antagonist potency values were determined by measuring the negative log of the antagonist concentration resulting in an increase in acetylcholine levels at an S_2/S_1 ratio of 1.5. Nonlinear curves were plotted with the program

Table 1
Correlation of −log concentration values obtained for acetylcholine release in the rat urinary bladder at an S_2/S_1 ratio of 1.5 with −log dissociation constants for cloned and native muscarinic receptors in separate published studies where several muscarinic antagonists were used

Correlation coefficient (r)					Slope					Constant	Antagonist	Reference
M_1	M_2	M_3	M_4	M_5	M_1	M_2	M_3	M_4	M_5			
0.542	0.676	0.853	0.994[a]		0.485	0.500	1.09	0.818		pK_D	Pirenzepine 4-DAMP Methoctramine Himbacine	Waelbroeck et al. (1990)
0.946[a]	0.432	0.940[a]	0.981[b]	0.961[a]	0.555	0.315	0.740	0.721	0.932	pK_i pK_B	Pirenzepine 4-DAMP Methotramine Tripitramine Gallamine	Brauner-Osborne and Brann (1996) Cembala et al. (1998) Maggio et al. (1994)
0.861[c]	0.706	0.949[a]	0.948[a]		0.793	0.530	1.10	0.886		pK_i	Pirenzepine 4-DAMP Atropine Methoctramine pFHHSiD Himbacine Gallamine	Doods et al. (1993)
0.912	0.130	0.954[c]	0.953[a]		1.02	0.05	1.34	0.719		pK_i	4-DAMP Atropine Methoctramine Himbacine	Lazareno et al. (1990)
0.800	0.400	0.924	0.805	0.797	0.443	1.076	0.751	0.847	1.005	pK_i	Pirenzepine HHD Atropine Methoctramine	Buckley et al. (1989) Hulme et al. (1990)
0.521	0.540	0.927[c]	0.809	0.698	0.511	0.454	1.094	0.809	0.698	pK_i	Pirenzepine 4-DAMP Methoctramine pFHHSiD Himbacine	Dorje et al. (1991)
0.668	0.884	0.911	0.939		0838	0.903	0.996	1.09		pK_B	Pirenzepine Methoctramine Himbacine Gallamine	Lazareno and Birdsall (1993)

[a] $P < 0.01$.
[b] $P < 0.001$.
[c] $P < 0.05$.

GraphPad Prism. Statistical evaluation was performed using Student's *t*-test with significance at the 5% level.

2.5. Drugs used

The following drugs were used: acetylcholine chloride (Sigma, St. Louis, MO, USA), atropine sulphate (Sigma), 4-DAMP methiodide (4-diphenylacetoxy-*N*-methylpiperidine) (gift from Dr. Richard Barlow, Bristol, UK), eserine hemisulphate (physostigmine) (Sigma), gallamine triethiodide (Sigma), HHD (hexahydrodifenidol) (gift courtesy Profs. E. Mutschler and G. Lambrecht, Frankfurt, FRG), 3-heptanone (Sigma), kathon (BAS, Indiana, USA), methoctramine tetrachloride (gift from Dr. Carlo Melchiorre, Bologna, Italy), *p*-chloromercuribenzoate (Sigma), pFHHSiD (*p*-fluoro-hexahydrosiladifenidol hydrochloride) (Research Biochemicals, Natick, MA, USA), pirenzepine hydrochloride (Thomae, Biberach an der Riss, Germany), tetraphenylboron (Sigma), tripitramine (gift from Dr. Carlo Melchiorre).

3. Results

3.1. Effects of antagonists on acetylcholine release

In control experiments, acetylcholine release during nerve stimulation was constant over the three 15-min stimulation periods, with an S_2/S_1 ratio of 1.00 ± 0.02 $(n = 7)$ and S_3/S_1 ratio of 1.20 ± 0.06 $(n = 7)$ being observed. The S_3/S_1 ratio did not differ significantly from S_2/S_1 $(P < 0.05)$ indicating that transmitter output was maintained over 3 stimulation periods (Fig. 1). After correction for recovery, the amount of acetylcholine spontaneously released in the bladder preparation was 0.91 ± 0.07 nmol/g of bladder over a 15-min period. This was increased to 2.99 ± 1.15 pmol/g bladder following nerve stimulation.

All of the muscarinic antagonists added to the preparation significantly elevated acetylcholine levels above control levels (see Fig. 2). The maximum S_2/S_1 ratio observed was 2.4 ± 0.48 $(n = 6)$ with pirenzepine (30 μM). A 50% increase in acetylcholine release at an S_2/S_1 ratio

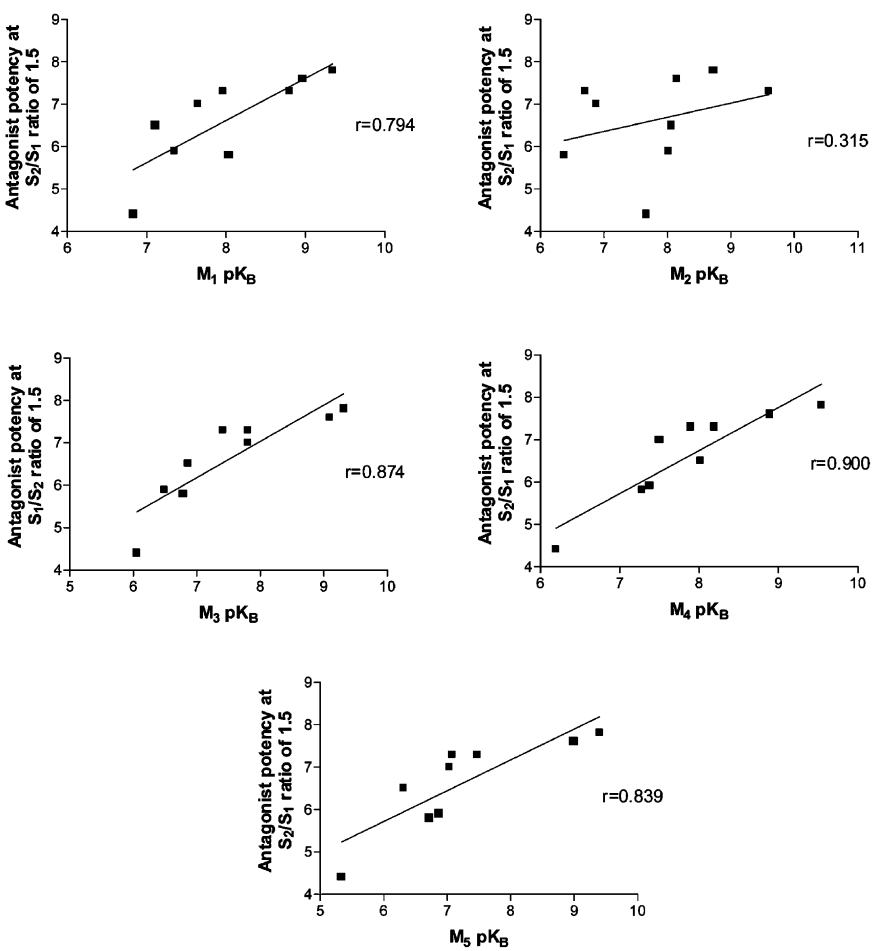

Fig. 3. Correlation between potency values obtained for acetylcholine release at an S_2/S_1 ratio of 1.5 in the rat urinary bladder with $-\log$ dissociation constants for native and cloned muscarinic M_1, M_2, M_3, M_4 and M_5 receptors for atropine, pirenzepine, 4-DAMP, methoctramine, himbacine, HHD, pFHHSiD, tripitramine and gallamine. Constants obtained from references listed in Table 1. The value of the correlation coefficient (r) is also shown.

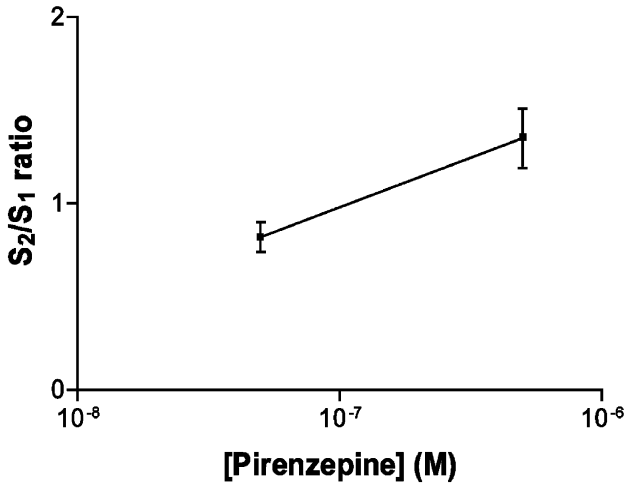

Fig. 4. Effect of pirenzepine (50–500 nM) on acetylcholine release following 5-min stimulation periods at 10 Hz, 0.25 ms, 100 V. Data expressed as mean ± S.E.M. for 6–7 experiments.

of 1.5 was used to determine a potency value for each antagonist with the rank order of: atropine (−log concentration = 7.8) > 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine) (7.6) > tripitramine (7.3) = HHD (hexahydrodifenidol) (7.3) > pFHHSiD (*p*-fluoro-hexahydrosiladifenidol) (7.0) > himbacine (6.5) > methoctramine (5.9) ≥ pirenzepine (5.8) > gallamine (4.3).

A comparison of the correlation between antagonist potency values obtained in the rat bladder with affinity values for antagonists at muscarinic M_1 to M_5 receptors from various published studies are shown in Table 1. The correlation between the values obtained in the rat bladder with the mean affinity values from previous published studies is shown in Fig. 3.

3.2. Effect of pirenzepine on acetylcholine release during a 5-min stimulation period

When preparations were stimulated for 5 min at 10 Hz, 0.25 ms duration at 100 V, pirenzepine (50–500 nM) caused no significant decrease in acetylcholine release in comparison to that of control experiments (Fig. 4). The S_2/S_1 ratio in control experiments was 0.96 ± 0.04 ($n = 3$) and the S_3/S_1 ratio, 1.19 ± 0.24 ($n = 3$).

4. Discussion

Many of the studies to date investigating neural release of acetylcholine from the bladder of different species, utilise radiolabelled choline. Furthermore, few investigators separate the secreted [^{3}H]acetylcholine from [^{3}H]choline (for example, see Alberts, 1995). A disadvantage of this method is that the [^{3}H]choline may not label all neural stores of releasable acetylcholine and thus any changes in tritium levels may not accurately reflect changes in actual acetylcholine release. An advantage of the acetylcholine extraction method and HPLC utilising electrochemical detection (HPLC-EC) is that acetylcholine can be measured separate from choline levels, it is sensitive to nanomolar levels of acetylcholine and with extraction of acetylcholine from the bath fluid, the life of the column is extended with little or no loss in sensitivity or reproducibility. HPLC-EC has recently been used to measure acetylcholine release from the rabbit bladder (Inadome et al., 1998). It was necessary to have physostigmine present to obtain measurable levels of acetylcholine. This may have led to increased activation of prejunctional muscarinic autoreceptors, which is a limitation of this technique.

The aim of this study was to characterise the prejunctional muscarinic autoreceptor present in the rat urinary bladder by measuring changes in acetylcholine release following electrical stimulation of cholinergic nerves. In order to determine potency values, the negative log of the antagonist concentration resulting in an increase in acetylcholine levels at an S_2/S_1 ratio of 1.5 was measured. When the values obtained in the bladder were compared to the mean antagonist affinity values from published studies, the muscarinic M_4 receptor showed the best correlation ($r = 0.900$) (Fig. 3).

When these values were compared with published measures of −log dissociation constants of the antagonists for native and cloned muscarinic receptor subtypes in individual studies, the best correlation was observed for the muscarinic M_4 receptor ($P < 0.001$) although the muscarinic M_3 receptor also showed a high correlation (Table 1). This was also seen in studies by D'Agostino et al. (1997) and Alberts (1995). Overall, these results agree with those of D'Agostino et al. (1997) in the rat urinary bladder, D'Agostino et al. (2000) in the human bladder and Alberts (1995) in the guinea pig bladder, all of who used a large number of antagonists in order to determine that the prejunctional muscarinic receptor involved the muscarinic M_4 receptor subtype.

Studies by Somogyi and De Groat (1992) and Braverman et al. (1998) in the rat and Tobin and Sjogren (1995, 1998) in the rabbit have characterised the prejunctional receptor as being of the muscarinic M_2 receptor subtype. This may be due to the limited number of antagonists used in these studies where, for example, only the effects of pirenzepine (as muscarinic M_1 receptor selective) and methoctramine (as muscarinic M_2 receptor selective) were studied. Braverman et al. (1998) has shown via reverse transcriptase-polymerase chain reaction, the existence of muscarinic M_1, M_2, M_3 and M_4 receptor mRNA in the rat urinary bladder, although the mRNA could not be localised to prejunctional sites.

It was not possible to obtain concentration–response curves for inhibition by acetylcholine at prejunctional receptors, and thus it was not possible to obtain pA_2 values,

so the observed antagonist potencies cannot be equated with dissociation constants directly. However, if a line with a slope of 1 is fitted through the data for the muscarinic M_4 receptor, it would appear that the obtained values are uniformly of the order of 1.3 log units, or 20-fold lower than published pK_D values for the muscarinic M_4 receptor (Fig. 5).

The presence of facilitatory muscarinic M_1 receptors has been observed in rat (Somogyi et al., 1994; Braverman et al., 1998), rabbit (Tobin and Sjogren, 1995, 1998) and human bladder (see Somogyi and De Groat, 1999). Somogyi and De Groat (1992) suggested that the presence of physostigmine is necessary to observe facilitation by muscarinic M_1 receptors following continuous stimulation consisting of a train of 360 shocks at 10 Hz (100 V, 0.25 ms duration) as opposed to intermittent field stimulation of 10 shocks. This facilitation was abolished upon addition of atropine and pirenzepine.

Several studies have shown the facilitatory muscarinic M_1 receptors to be activated by higher frequencies of stimulation (10 Hz), whereas the inhibitory receptors operate at lower frequencies (0.5–5 Hz) (D'Agostino et al., 1986, 1989; Somogyi and De Groat, 1992; Somogyi et al., 1994; Alberts, 1995; D'Agostino et al., 1997; Tobin and Sjogren, 1998). For example, in guinea pig bladder stimulated at 5 Hz, atropine and pirenzepine only caused an increase in [^{3}H]acetylcholine release (Alberts, 1995). Similarly, in the rat bladder, at a frequency of 3 Hz, facilitation did not occur (D'Agostino et al., 1997). However, in the rabbit bladder stimulated at 10 Hz, Tobin and Sjogren (1995) observed pirenzepine to decrease [^{3}H]acetylcholine efflux, whereas atropine, 4-DAMP and methoctramine elevated release, suggesting that both inhibitory and facilitatory mechanisms were operating when preparations were stimulated at 10 Hz. This was also the case in a study by Braverman et al. (1998) where both facilitation and inhibition by muscarinic antagonists were observed at both low and high frequencies. However, preparations were stimulated submaximally at 8 V compared to other studies where 100 V was used. It has been suggested that these mechanisms in the bladder are plastic and can operate in one mode or the other (that is, facilitatory or inhibitory) depending on acetylcholine levels (Somogyi and De Groat, 1999).

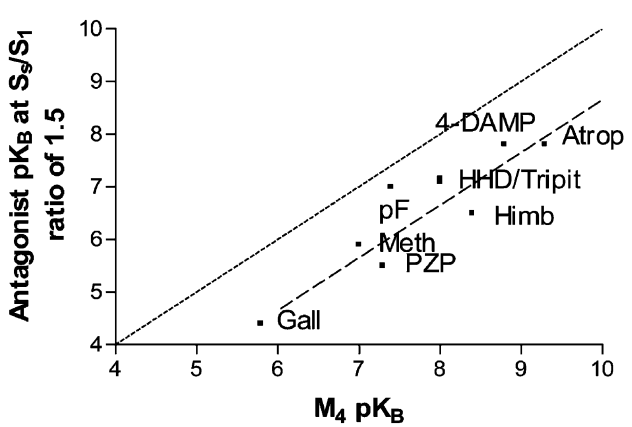

Fig. 5. Relationship between potency values obtained for acetylcholine release at an S_2/S_1 ratio of 1.5 in the rat urinary bladder with $-\log$ dissociation constants at the muscarinic M_4 receptor. A dashed line with a slope of unity has been fitted through the data points. Constants obtained from references listed in Table 1. The dotted line shows a line of equivalence.

In the present study, facilitation by muscarinic M_1 receptors did not occur, despite the presence of physostigmine, as both atropine and pirenzepine only caused a significant increase in acetylcholine levels suggesting that negative feedback was occurring. This is presumably due to the greater number of shocks utilised in this study (9000 shocks). Experimental conditions were thus altered to simulate the parameters used in the studies of Somogyi and De Groat (1999) showing the presence of facilitatory muscarinic M_1 receptors, that is, continuous stimulation of 100–360 shocks. Although changes in acetylcholine levels could be detected, the absolute levels were not high enough to determine a predicted significant decrease in the presence of pirenzepine. That is, if facilitatory muscarinic M_1 receptors were present and functioning, the presence of pirenzepine would be expected to cause a decrease in acetylcholine levels. However, the short stimulation periods produced only a small increase in acetylcholine, and a reduction caused by pirenzepine would be difficult to quantitate. Stimulation periods were thus altered to 5 min where measurable acetylcholine peaks were obtained. In two out of six experiments, acetylcholine levels were observed to be markedly reduced, however overall, pirenzepine failed to significantly reduce acetylcholine levels compared to that of control experiments (see Fig. 4). Thus, in agreement with the findings of Somogyi et al. (1994) and Braverman et al. (1998), it would appear that once stimulation parameters exceed 360 shocks, consistent demonstration of the presence of facilitatory prejunctional receptors is not possible. It has been suggested that the facilitation is only seen at the lower range of stimulation as it mimics the physiological firing rate of the parasympathetic nerves in the bladder (De Groat et al., 1982).

It is worth noting that a significant decrease in acetylcholine release was observed upon addition of a high concentration (30 μM) of pFHHSiD. It is possible that blockade of the prejunctional facilitatory receptors occurred, resulting in a decrease in acetylcholine release. However, as such an effect was not observed with the other antagonists having similar or greater affinity for the muscarinic M_1 receptor, this would not appear to be the case. Also, pFHHSiD has similar affinity at muscarinic M_1 and M_4 receptors, and blockade of the facilitatory and inhibitory mechanisms would be expected to oppose each other to some extent. However, pFHHSiD does have high affinity for the muscarinic M_3 receptor and would thus inhibit smooth muscle contraction, as was observed. A

possible explanation for the lower acetylcholine levels observed at 30 μM may be that contraction of the bladder smooth muscle aids in facilitating the flow of released acetylcholine from within the bladder to the bath fluid. If contractions are markedly inhibited, less acetylcholine would be able to be transferred to the bath fluid and decreased levels would be extracted. Interestingly, acetylcholine release appears to peak then decrease at the higher concentrations of other antagonists that also have high affinity for muscarinic M_3 receptors (4-DAMP, HHD) (Fig. 2).

In summary, the acetylcholine extraction procedure and detection via HPLC-EC provides a convenient method for examining acetylcholine release within the rat urinary bladder. Based on the present results, it would appear that the prejunctional inhibitory muscarinic receptor in rat urinary bladder is the muscarinic M_4 receptor subtype, in agreement with the findings of D'Agostino et al. (1997) in the rat bladder, D'Agostino et al. (2000) in the human bladder and Alberts (1995) in the guinea pig bladder.

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