British Journal of Pharmacology (2001) 132, 1009–1016

www.nature.com/bjp

Analysis of the muscarinic receptor subtype mediating inhibition of the neurogenic contractions in rabbit isolated vas deferens by a series of polymethylene tetra-amines

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1 The pharmacological characteristics of the presynaptic muscarinic receptor subtype, which mediates inhibition of the neurogenic contractions in the prostatic portion of rabbit vas deferens, have been investigated by using a series of polymethylene tetra-amines, which were selected for their ability to differentiate among muscarinic receptor subtypes.

2 It was found that all tetra-amines antagonized McN-A-343-induced inhibition in electrically stimulated rabbit vas deferens in a competitive manner and with affinity values (pA_2) ranging between 6.27 ± 0.09 (spirotramine) and 8.51 ± 0.02 (AM170).

3 Competition radioligand binding studies, using native muscarinic receptors from rat tissues (M_1, cortex; M_2, heart; M_3, submaxillary gland) or from NG 108-15 cells (M_4) and human cloned muscarinic M_1-M_4 receptors expressed in CHO-K1 cells, were undertaken with the same tetra-amines employed in functional assays. All antagonists indicated a one-site fit.

4 The affinity estimates (pK_i) of tetra-amines calculated in binding assays using native receptors were similar to those obtained using cloned receptors. Among these compounds some displayed selectivity between muscarinic receptor subtypes, indicating that they may be valuable tools in receptor characterization. Spirotramine was selective for M_1 receptors versus all other subtypes (pK_i native: M_1, 7.32 ± 0.10; M_2, 6.50 ± 0.11; M_3, 6.02 ± 0.13; M_4, 6.28 ± 0.16; pK_i cloned: M_1, 7.69 ± 0.08; M_2, 6.22 ± 0.14; M_3, 6.11 ± 0.16; 6.35 ± 0.11) whereas CC8 is highly selective for M_2 receptors versus the other subtypes (pK_i native: M_1, 7.50 ± 0.04; M_2, 9.01 ± 0.12; M_3, 6.70 ± 0.08; M_4, 7.56 ± 0.04; pK_i cloned: M_1, 7.90 ± 0.20; M_2, 9.04 ± 0.08; M_3, 6.40 ± 0.07; M_4, 7.40 ± 0.04). Furthermore, particularly relevant for this investigation were tetra-amines dipitramine and AM172 for their ability to significantly differentiate M_1 and M_4 receptors.

5 The apparent affinity values (pA_2) obtained for tetra-amines in functional studies using the prostatic portion of rabbit vas deferens correlated most closely with the values (pK_i) obtained at either native or human recombinant muscarinic M_4 receptors. This supports the view that the muscarinic receptor mediating inhibition of neurogenic contractions of rabbit vas deferens may not belong to the M_1 type but rather appears to be of the M_4 subtype.

British Journal of Pharmacology (2001) **132,** 1009–1016

Keywords: Muscarinic receptors; muscarinic M_1 receptors; muscarinic M_4 receptors; tetra-amines; rabbit vas deferens

Abbreviations: AM170, *N*1,*N*22-Bis [[(5,10-dihydro-11-oxo-11*H*-dibenzo [*b,e*][1,4] diazepin-5-yl) carbonyl] methyl]-7,16-dimethyl-7,16-diaza-1,22-docosanediamine tetraoxalate; AM172, *N*1,*N*1,*N*22-Tris[[(5,10-dihydro-11-oxo-11*H*-dibenzo [*b,e*][1,4]diazepin-5-yl)carbonyl]methyl]-7,16-dimethyl-7,16-diaza-1,22-docosanediamine tetraoxalate; CC7, *N*1, *N*22-Bis[[(6,11-dihydro-5-oxo-5*H*-pyrido[2,3-*b*][1,4]benzodiazepin-11-yl) carbonyl]methyl]-7,16-dimethyl-7,16-diaza-1,22-docosanediamine tetraoxalate; CC8, *N*1,*N*1,*N*22-Tris[[(6,11-dihydro-5-oxo-5*H*-pyrido[2,3-*b*[1,4]benzodiazepin-11-yl)carbonyl]methyl]-7,16-dimethyl-7,16-diaza-1,22-docosanediamine tetraoxalate; CC9, *N*1,*N*1,*N*22, *N*22-Tetrakis [[(6,11-dihydro-5-oxo-5*H*-pyrido[2, 3-*b*][1, 4]benzodiazepin-11-yl) carbonyl]methyl]-7,16-dimethyl-7, 16-diaza-1,22-docosanediamine tetraoxalate; CHO-K1 cells, Chinese hamster ovary cells; [^{3}H]-NMS, [^{3}H]-N-methyl scopolamine

Introduction

Muscarinic receptors belong to the G-protein coupled receptor family and, like other members of this family, comprise multiple subtypes. Based on existing knowledge, it has been recommended that M_1, M_2, M_3, M_4, and M_5 should be used to describe the five muscarinic receptor subtypes which have been cloned and pharmacologically characterized (Caulfield & Birdsall, 1998).

The characterization of the receptor subtype which is present in a given tissue has represented the basis for the development of relevant structure-activity relationships for both agonists and antagonists. Although several selective

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ligands are emerging from these studies, none of them is able yet to target only one receptor subtype while not affecting the others. Clearly, the lack of selectivity makes difficult the unequivocal characterization of receptor subtypes and also the development of therapeutically useful muscarinic drugs (Hulme *et al*., 1990; Caulfield, 1993; Eglen *et al*., 1996; Widzowski *et al*., 1997). Besides the inherent difficulty to discover novel selective drugs, another aspect may further complicate the issue, that is the availability of simple and reliable functional assays for drug characterization.

Historically, the guinea-pig atria and ileum have played a prominent role in muscarinic receptor research, because of their ease of preparation and the clarity of the response to agonists. It has been clearly demonstrated that these tissues give a response consistent with the activation of muscarinic M_2 and M_3 receptor subtypes, respectively. The situation appears less clear for the assessment of the affinity of drugs at muscarinic M_1 receptors (Caulfield & Birdsall, 1998). The rabbit vas deferens has been one of the most used preparation for the putative muscarinic M_1 receptor (Eltze, 1988; Eltze & Figala, 1988). However, emerging evidence indicates that the prejunctional rabbit vas deferens muscarinic receptors mediating inhibition of the neurogenic contractions may not belong to the M_1 subtype (Caulfield & Brown, 1991; Caulfield, 1993; Sagrada *et al*., 1994; Waelbroeck *et al*., 1996). A possible explanation for the confusion arising in receptor classification may be that putative muscarinic M_1 receptors have long been identified as the sites which bind pirenzepine with high affinity (Hammer *et al*., 1980). However, it has been clearly shown that this antagonist is not able to distinguish between cloned muscarinic M_1 and M_4 receptors, indicating that the identification in functional assays of muscarinic M_1 receptors on the basis of pirenzepine affinity might be misleading. In addition, most of the available antagonists are not able to discriminate between cloned M_1 and M_4 receptor subtypes thus preventing from an unambiguous classification of these receptors in functional experiments.

Since we have developed a series of polymethylene tetra-amines, several of which display differing selectivity at the muscarinic receptor subtypes, we thought it of interest to evaluate selected tetra-amines on rabbit vas deferens and to compare their functional affinity with that obtained in radioligand binding studies on either native and human cloned muscarinic receptor subtypes. The tetra-amines used in the present investigation are shown in Figure 1. They were selected for their ability to distinguish among muscarinic receptor subtypes (Barbier *et al*., 1998; Bolognesi *et al*., 1998).

Methods

Electrical field stimulation (EFS)-induced contractions of the prostatic half of rabbit vas deferens

This preparation was set up according to Eltze (1988). Briefly, New Zealand white rabbits (3.0–3.5 kg) were killed by cervical dislocation. Vasa deferentia were carefully dissected free of surrounding tissue and were divided into four segments, two prostatic portions of 1 cm and two epididymal portions of approximately 1.5 cm length. Prostatic portions were set up rapidly under 1 g of tension in 15 ml organ baths containing physiological salt solution with the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.52, $MgSO_4$ 0.6, KH_2PO_4 1.18, $NaHCO_3$ 25, glucose 11.1. Idazoxan (1 μM) was added to block α_2-adrenoceptors. The solution was maintained at 30°C, aerated with 5% CO_2-95% O_2 and tissues were stimulated through platinum electrodes by square-wave pulses (0.1 ms, 2 Hz, 10–15 V). Contractions were measured isometrically after tissues were equilibrated for 1 h. A concentration-response curve was constructed by cumulative addition of the reference agonist, McN-A-343. The concentration of agonist in the organ bath was increased approximately 5 fold at each step, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady. Following 30 min of washing, a new concentration-response curve, taken as control, to the agonist was obtained.

Antagonist affinity estimates were obtained by Schild regression analysis (Arunlakshana & Schild, 1959) of McN-A-343-induced inhibition of electrically stimulated rabbit vas deferens. Each concentration of antagonist was tested at least four times, and was equilibrated with the tissue for 2 h. This period was sufficient to allow equilibration of the antagonist, as no further antagonism was observed when the incubation was increased to 3 h for each antagonist (data not shown).

In all cases, control experiments were run in parallel in order to check any variation in sensitivity.

CHO-K1 cells expressing human muscarinic receptors: preparation of membrane homogenate

Cloned human M_1–M_4 muscarinic receptors inserted in pCD vector (Okayama & Berg, 1983) were obtained from the National Institutes of Health (a kind gift of Dr T.I. Bonner). CHO-K1 cells were grown in Ham F12 medium supplemented with 10% foetal calf serum, 100 u ml^{-1} penicillin G and streptomycin in a humidified environment of 95% air and 5% CO_2 at 37°C. Cells were transfected using a modified calcium phosphate procedure (Gibco, Life Technologies). Cells were plated in 100 mm dishes. 3 h before transfection, medium was changed with fresh whole medium. A calcium solution containing 20 μg of DNA (10 μg of plasmid DNA and 10 μg of carrier salmon sperm DNA) was mixed with a phosphate solution in 1 × HBS solution and let sit at 25°C for 20 min. The suspension was subsequently added to cells and incubated at 37°C in humidified air containing 5% CO_2 for 24 h. Following incubation, medium was changed with fresh whole medium. 48 h after, medium was changed and selection started with 400 μg ml^{-1} G418. Selection with G418 continued for about three weeks. During the selection the medium was changed every 3 days. Clonal cell lines were assayed with [^{3}H]-N-methyl-scopolamine ([^{3}H]-NMS). Cells were grown to about 80% confluence, washed with phosphate buffered saline, scraped into ice-cold binding buffer and homogenized using a Thomas teflon pestle tissue homogenizer 2 ml (10 strokes). Membranes were centrifuged at 35,000 × *g* 15 min 4°C. The pellet was suspended in 5 ml ice-cold binding buffer, the membranes were homogenized a second time and aliquots were stored at −80°C until used.

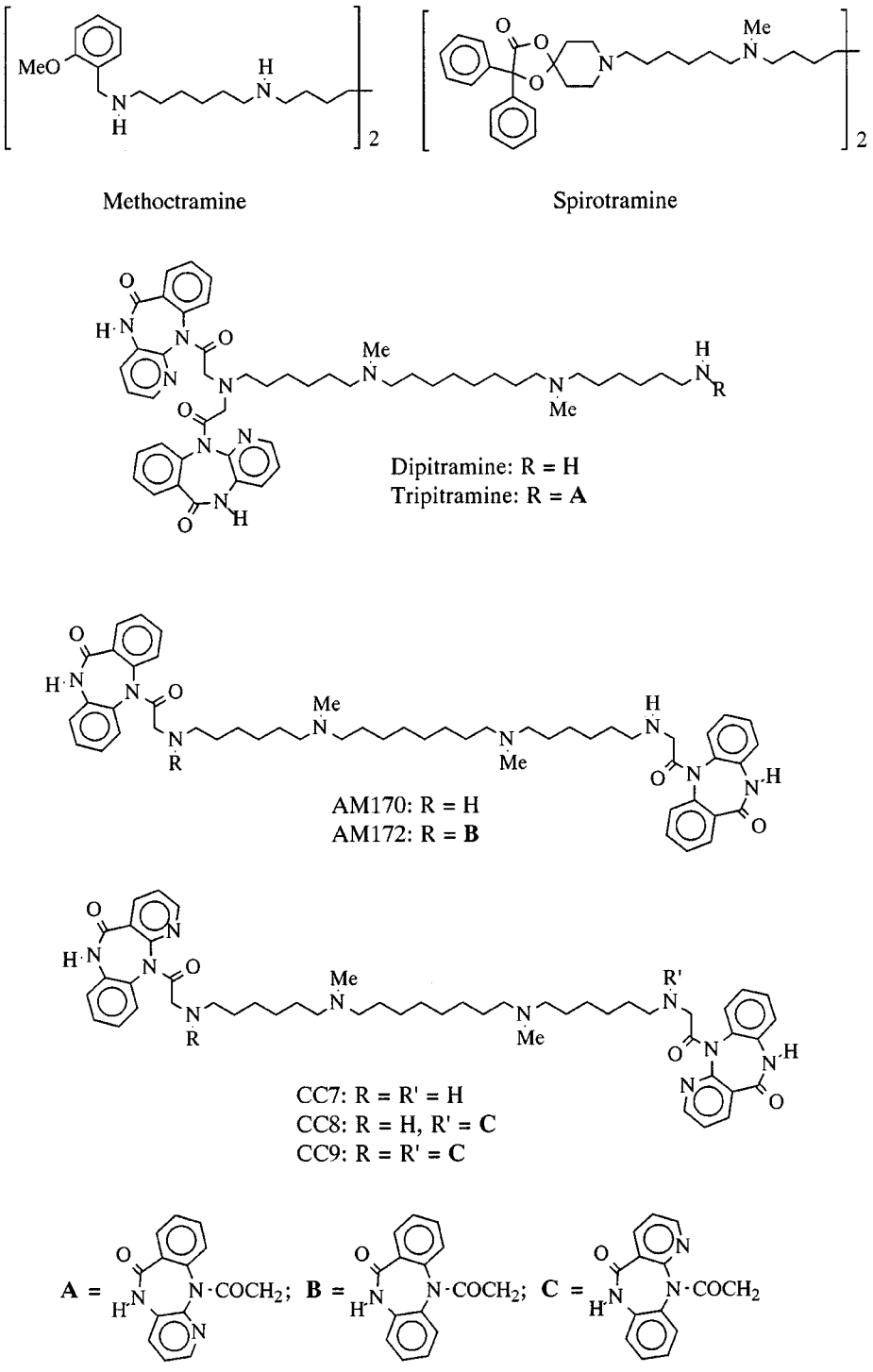

Figure 1 Chemical structures of tetra-amines used in present study.

Preparation of the homogenates or crude membranes of native muscarinic receptors

Muscarinic M_1 receptors (rat cortex) Wistar albino rats were sacrificed, cortex was rapidly removed and weighed. Cortex was suspended in 20 volumes of homogenizing buffer (50 mM Tris, 5 mM EDTA, pH 7.4, 4°C) and homogenized using a Wheaton tissue homogenizer B 40 ml (10 strokes). The suspension was centrifuged at 45,000 × *g* 4°C for 12 min, and washed with the same amount of buffer. Following centrifugation at 45,000 × *g* 4°C for 12 min, the pellet was suspended in 20 volumes of assay buffer (50 mM Tris, 0.5 mM EDTA, pH 7.4, 4°C) centrifuged at 45,000 × *g* 4°C. The pellet was washed a second time with 20 volumes of assay buffer and centrifuged as before. After the second suspension an aliquot was retained to determine protein content (Lowry *et al.*, 1951). Membranes were used on the same day.

Muscarinic M_2 receptors (rat heart)

Wistar albino rats were sacrificed, heart was rapidly removed and weighed. Hearts were minced in five volumes of homogenizing buffer (50 mM Tris, 4 mM EDTA, pH 7.4, 4°C) using Ultraturrax and homogenized with a Wheaton tissue homogenizer B 40 ml (10 strokes). The suspension was filtered through gauze and centrifuged at $1000 \times g$ 4°C for 10 min. The supernatant was kept on ice while the pellet was washed with two volumes of homogenizing buffer and centrifuged as before. The two supernatants were merged and centrifuged at $45{,}000 \times g$ 4°C for 12 min. The pellet was suspended in five volumes of assay buffer (50 mM Tris, 0.5 mM EDTA, pH 7.4, 4°C) centrifuged at $45{,}000 \times g$ 4°C. The pellet was washed a second time with five volumes of assay buffer and centrifuged as before. After the second wash an aliquot of the suspension was retained to determine protein content (Lowry *et al*., 1951). Pellet was stored at −80°C until used.

Muscarinic M_3 receptors (rat submaxillary gland)

Wistar albino rats were sacrificed, submaxillary glands were rapidly removed and weighed. Submaxillary glands were minced in 10 volumes of homogenizing buffer (50 mM Tris, 5 mM EDTA, pH 7.4, 4°C) using Ultraturrax and homogenized with a Wheaton tissue homogenizer B 40 ml (10 strokes). The suspension was filtered through gauze and centrifuged at $1000 \times g$ 4°C for 10 min. The supernatant was kept on ice while the pellet was washed with two volumes of homogenizing buffer and centrifuged as before. The two supernatants were merged and centrifuged at $45{,}000 \times g$ 4°C for 12 min. The pellet was suspended in 10 volumes of assay buffer (50 mM Tris, 0.5 mM EDTA, pH 7.4, 4°C) and centrifuged at $45{,}000 \times g$. The pellet was washed a second time with 10 volumes of assay buffer and centrifuged as before. After the second suspension an aliquot was retained to determine protein content (Lowry *et al*., 1951). The pellet was stored at −80°C until used.

Muscarinic M_4 receptors (NG 108-15 cells)

NG 108-15 cells were grown in Dulbecco's modified Eagle medium (Gibco Life Technology), containing 10% foetal bovine serum (Gibco Life Technology), 0.1 μM hypooxanthine, 10 μM aminopterin and 17 μM thymidine in a humidified environment of 95% air and 5% CO_2 at 37°C (Ammer *et al*., 1996). Cells were grown in 75 ml flasks to about 80% confluence, washed with phosphate buffered saline and scraped. The content of each flask was transferred in 15 ml sterile polypropylene tubes and centrifuged at $1200 \times g$ for 3 min. The pellet was suspended in 1 ml tube of homogenizing buffer (25 mM HEPES, 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, pH 7.4, 4°C) and homogenized using a Thomas teflon pestle tissue homogenizer 2 ml (10 strokes). The homogenate was centrifuged at $30{,}000 \times g$ 4°C for 15 min and suspended again with an equal volume of homogenizing buffer. The suspension was centrifuged as before (an aliquot was retained to determine protein content, Lowry *et al*., 1951) and the pellet saved at −80°C until used.

Binding assay

Competition binding studies were performed using homogenates of the indicated cells or rat tissues in incubation buffer (Tris 50 mM, EDTA 0.5 mM and $MgCl_2$ 5 mM, pH 7.4, 25°C, for submaxillary glands and NG 108-15 (Michel & Whiting, 1988; Waelbroeck *et al*., 1990); 25 mM phosphate buffer, pH 7.4, 25°C, enriched with 2 mM $MgCl_2$ for heart, CHO-K1 and the same with 1% bovine serum albumin for cortex). Homogenates (200 μg of protein for cortex, 500 μg of protein for heart and submaxillary gland, 600 μg of protein for NG 108-15 cells and 10 μg of protein for CHO-K1) were incubated for 2 h at 25°C in 1 ml of incubation buffer with the following concentrations of tracers: 5 nM [^{3}H]-pirenzepine for cortex, 0.8 nM [^{3}H]-NMS for heart, submaxillary gland and NG 108-15 and 0.2 nM [^{3}H]-NMS for CHO-K1. Non specific binding was determined with 10 μM atropine. Binding assays were terminated by filtration on Whatman GF/C (cortex, NG 108-15 and CHO-K1) or GF/B (heart and submaxillary glands) glass-fibre filters previously soaked in 0.1% polyethylenimine and then rinsed four times with 5 ml of ice-cold incubation buffer. Saturation studies were performed as indicated above, in presence of [^{3}H]-NMS (25–4000 pM) or [^{3}H]-pirenzepine (0.08–20 nM) in the cortex, in the absence or presence of atropine. The results were analysed according to the method of Scatchard (McPherson, 1985; Bolognesi *et al*., 1998).

Data analysis

In functional experiments, dose ratios at the EC_{50} values of the agonist were calculated at different antagonist concentrations, and each concentration was tested at least four times. The results are expressed as pA_2 values (Arunlakshana & Schild, 1959; Tallarida *et al*., 1979). Data are presented as means ± s.e.mean of n experiments. Differences between mean values were tested for significance by Student's t-test.

Binding data were analysed using LIGAND (Munson & Rodbard, 1980). Differences in the slope of the curves were determined by the test of parallelism as described by Tallarida and Murray (1991) and were not different ($P > 0.05$). Scatchard plots were linear or almost linear in all preparations data (Dörje *et al*., 1991, Michel *et al*., 1989). All Hill numbers (n_H) were not significantly different from unity ($P > 0.05$). Equilibrium dissociation constants (K_i) were derived from the Cheng–Prusoff equation (Cheng & Prusoff, 1973), $K_i = IC_{50}/(1 + L/K_d)$, where L and K_d are the concentration and the equilibrium dissociation constant of [^{3}H]-NMS or [^{3}H]-pirenzepine, respectively. pK_i values are the mean ± s.e.mean of three separate experiments performed in triplicate.

Pearson correlation coefficients (r) and the sum of squares (ss) were calculated using GraphPad Prism (GraphPad Software Inc., version 3.0a for Macintosh, San Diego CA, U.S.A.). The sum of squares defines the proximity of the data points to the line of identity (x = y) and is the sum of the vertical distances of the points from the line.

Drugs

[^{3}H]-N-methylscopolamine chloride ([^{3}H]-NMS, 79.5 Ci mmol^{-1}) and [^{3}H]-pirenzepine dihydrochloride (86.2 Ci m-

mol^{-1}) were obtained from NEN. Methoctramine (Melchiorre *et al*., 1987), spirotramine (Melchiorre *et al*., 1995), dipitramine and tripitramine (Melchiorre *et al*., 1993; Minarini *et al*., 1994), AM172, AM170, CC7, CC8 and CC9 (Bolognesi *et al*., 1998) were synthesized in the Department of Pharmaceutical Sciences of the University of Bologna. Pirenzepine dihydrochloride and McN-A-343 were synthesized according to the literature (Engle *et al*., 1989; Nilsson *et al*., 1992).

Results

Antagonism of polymethylene tetraamines of EFS-induced contractions of the prostatic half of rabbit vas deferens

McN-A-343 induced concentration-dependent inhibition of EFS-induced contractions of the prostatic half of rabbit vas deferens with a $-\log EC_{50} = 6.36 \pm 0.05$ ($n = 120$). 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 ($n = 10$, $P \geqslant 0.05$).

Polymethylene tetra-amines (Figure 1) antagonized the inhibition induced by McN-A-343 at the muscarinic receptor subtype in electrically stimulated rabbit vas deferens with an affinity (pA_2) ranging between 6.27 ± 0.09 and 8.51 ± 0.02 (Table 1). There was a concentration-dependent parallel shift to the right of McN-A-343 concentration-response curves, without either basal tension or maximum response being affected, for all polymethylene tetra-amines investigated in this study (shown for tripitramine and AM170 in Figure 2).

Schild analysis of the results gave linear plots (shown for tripitramine and AM170 in Figure 3) over the range of concentrations investigated with slopes which were not significantly different from unity (Table 1).

The maximum effect of polymethylene tetra-amines was assumed to be reached within 2 h, since the effect observed following 3 h incubation with tripitramine, spirotramine, CC7, CC9 or AM170 was not increased (results not shown).

Radioligand binding studies

The following K_d and B_{max} were determined: cortex (M_1) $K_d = 2.1 \pm 0.2$ nM, $B_{max} = 1.9 \pm 0.13$ fmol mg^{-1} protein; heart (M_2) $K_d = 0.32 \pm 0.04$ nM, $B_{max} = 77.8 \pm 15.3$ fmol mg^{-1} protein; submaxillary glands (M_3) $K_d = 0.48 \pm 0.03$ nM, $B_{max} = 1102 \pm 85$ fmol mg^{-1} protein; NG108-15 (M_4) $K_d = 0.54 \pm 0.03$ nM, $B_{max} = 190 \pm 4$ fmol mg^{-1} protein; CHO-K1 M_1 $K_d = 0.054 \pm 0.001$ nM, $B_{max} = 2518 \pm 125$ fmol mg^{-1} protein; CHO-K1 M_2 $K_d = 0.083 \pm 0.004$ nM, $B_{max} = 747 \pm 21$ fmol mg^{-1} protein; CHO-K1 M_3 $K_d = 0.052 \pm 0.002$ nM, $B_{max} = 830 \pm 40$ fmol mg^{-1} protein; CHO-K1 M_4 $K_d = 0.026 \pm 0.005$ nM, $B_{max} = 1778 \pm 182$ fmol mg^{-1} protein.

Table 1 Antagonist affinities (pA_2) in rabbit vas deferens for several muscarinic antagonists ($n = 4$ for each antagonist concentration)

Compound	*Concentration* (μM)	pA_2	*Slope*
Methoctramine	0.5, 1, 10	6.45 ± 0.09	1.02 ± 0.04
Spirotramine	10, 50, 100	6.27 ± 0.09	0.98 ± 0.03
Dipitramine	1, 5, 50	6.79 ± 0.07	1.02 ± 0.02
Tripitramine	0.01, 1, 10	7.95 ± 0.07	1.08 ± 0.01
AM170	0.05, 1, 10	8.51 ± 0.02	1.05 ± 0.06
AM172	0.1, 1, 10	7.33 ± 0.04	1.01 ± 0.02
CC7	1, 5, 10	7.39 ± 0.08	0.99 ± 0.03
CC8	0.1, 1, 10	7.83 ± 0.05	1.08 ± 0.01
CC9	5, 10, 50	6.69 ± 0.03	1.02 ± 0.08
Pirenzepine	0.1, 1, 10	7.95 ± 0.08	1.01 ± 0.04

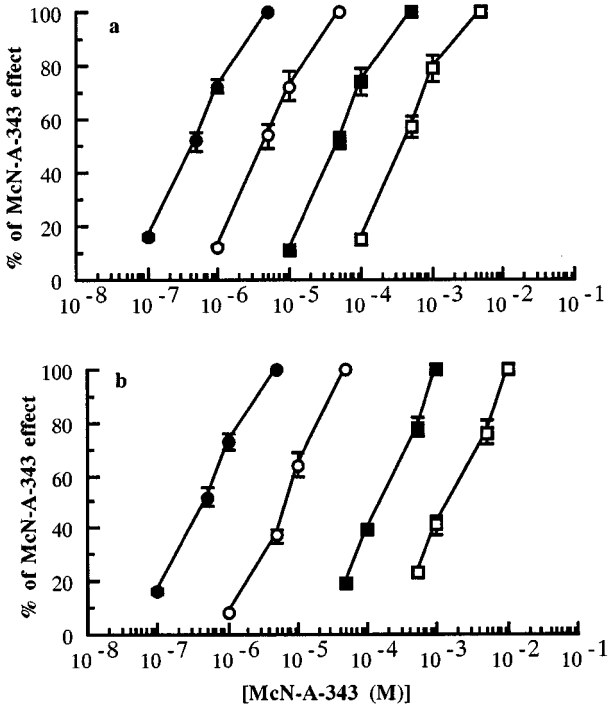

Figure 2 Antagonism of McN-A-343-induced inhibition of electrical field stimulation-induced contractions of the prostatic half of rabbit vas deferens. Concentration-response curves for McN-A-343 were obtained before (●) and after exposure (a) to 0.1 (○), 1 (■) and 10 μM (□) tripitramine, and (b) to 0.05 (○), 1 (■) and 10 μM (□) AM170 for 120 min. Each point is the mean ± s.e.mean of at least four observations.

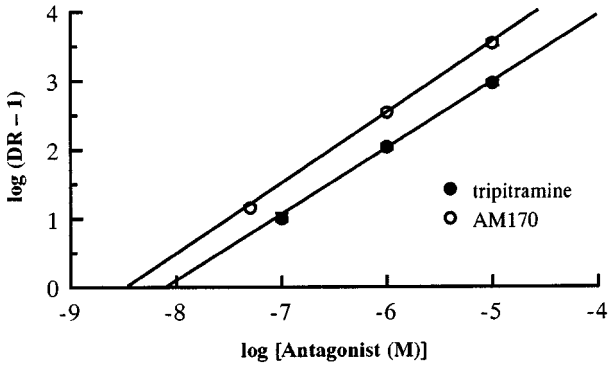

Figure 3 Schild plot for the antagonism between McN-A-343 and tripitramine and AM170 at muscarinic receptors in the prostatic half of rabbit vas deferens. Each point is the mean ± s.e.mean of four observations.

All the competition curves obtained with polyamines were compatible with competitive inhibition of tracer binding. All compounds were tested for affinity versus muscarinic receptors M_1-M_3 expressed in rat tissues or M_4 in NG 108-15 cells (Table 2), and in human M_1-M_4 stable transfectants of CHO-K1 cells (Table 3). Our results are in agreement with literature data (Dörje *et al*., 1991) and show a good correlation between data obtained in assays carried out on native receptors and on transfected CHO-K1 homogenates. Among the compounds tested, the order of affinity versus M_1 receptor present in rat cortex was: AM172 > tripitramine ≅ dipitramine ≅ pirenzepine > CC8 ≅ CC7 ≅ AM170 ≅ spirotramine ≅ CC9 > methoctramine. Similarly, in CHO-K1 M_1 transfectant the order was AM172 > tripitramine ≅ pirenzepine > dipitramine ≅ AM170 > CC7 ≅ CC8 ≅ spirotramine ≅ CC9 > methoctramine. The order of affinity for both M_2 native and cloned receptor was tripitramine > AM172 > AM170 > CC8 > CC9 > dipitramine ≅ methoctramine ≅ CC7 > pirenzepine > spirotramine. All compounds bind with relatively low affinity both cloned and native M_3 receptor. AM170 and tripitramine show the highest affinity for M_4 receptors.

Dipitramine shows selectivity for muscarinic M_1 receptors expressed in rat cortex and in transfected CHO-K1 membranes ($M_1 > M_2 > M_4 >> M_3$), while CC8 and CC9 are selective for M_2 receptor ($M_2 >> M_1 > M_4 > M_3$). AM 170 is a selective ligand for M_2 receptor, although maintaining high affinity for M_4 receptors ($M_2 > M_4 > M_1 > M_3$). AM172, on the other hand, was not able to distinguish clearly between M_1 an M_2, for which it displayed higher affinity ($M_2 \cong M_1 > M_4 > M_3$).

Table 2 Affinity estimates, expressed as pK_i values, in rat cortex (M_1), heart (M_2), and submaxillary gland (M_3) and NG 108-15 cell (M_4) muscarinic receptor subtypes (mean of three experiments performed in triplicate)

Compound	*M_1*	*M_2*	*M_3*	*M_4*
Methoctramine	7.08±0.12	7.75±0.08	6.04±0.14	7.08±0.15
Spirotramine	7.69±0.08	6.22±0.14	6.11±0.16	6.35±0.11
Dipitramine	8.36±0.09	7.84±0.12	6.01±0.18	7.16±0.36
Tripitramine	8.45±0.11	9.52±0.07	6.83±0.16	7.94±0.12
AM170*	7.83±0.15	9.25±0.10	6.94±0.06	8.55±0.16
AM172*	9.02±0.20	9.36±0.08	6.96±0.04	7.38±0.08
CC7*	7.84±0.32	7.68±0.22	5.59±0.10	7.44±0.08
CC8*	7.90±0.20	9.04±0.08	6.40±0.07	7.40±0.04
CC9*	7.60±0.07	8.03±0.12	5.60±0.06	6.80±0.08
Pirenzepine	8.22±0.08	6.31±0.09	6.98±0.10	7.66±0.17

*Data taken from Bolognesi *et al*., (1998)

Table 3 Affinity estimates, expressed as pK_i values, obtained in CHO-K1 cells expressing the human muscarinic M_1-M_4 receptors (mean of three experiments performed in triplicate)

Compound	*M_1*	*M_2*	*M_3*	*M_4*
Methoctramine	6.94±0.13	7.91±0.07	6.54±0.10	7.01±0.15
Spirotramine	7.32±0.10	6.50±0.11	6.02±0.13	6.28±0.16
Dipitramine	8.29±0.11	7.95±0.14	5.90±0.14	7.05±0.20
Tripitramine	8.60±0.10	9.59±0.07	7.32±0.12	8.11±0.12
AM170	8.06±0.09	9.01±0.15	6.42±0.07	8.30±0.12
AM172	9.04±0.16	9.27±0.08	6.76±0.07	7.55±0.07
CC7	7.50±0.09	7.82±0.13	5.82±0.16	7.62±0.07
CC8	7.50±0.04	9.01±0.12	6.70±0.08	7.56±0.04
CC9	7.30±0.06	8.12±0.06	5.90±0.07	6.72±0.10
Pirenzepine	8.58±0.10	6.73±0.11	7.39±0.09	7.85±0.13

Comparison of antagonist data for rabbit vas deferens with binding affinities at native or cloned muscarinic M_1-M_4 receptors

When the affinities of tetra-amines (pA_2, Table 1) in the rabbit vas deferens were compared with the affinity estimates (pK_i) in either native and cloned muscarinic receptor homogenates, a good correlation was obtained for the muscarinic M_4 receptor (cloned: $r=0.948$ and ss = 0.636; native: $r=0.925$ and ss = 0.828). By contrast, poor correlations were observed at M_1 (cloned: $r=0.525$ and ss = 8.032; native: $r=0.376$ and ss = 9.555), M_2 (cloned: $r=0.505$ and ss = 15.34; native: $r=0.504$ and ss = 15.96), and M_3 (cloned: $r=0.569$ and ss = 10.63; native: $r=0.687$ and ss = 12.07) subtypes (Figure 4). Interestingly, the binding affinity estimates at cloned muscarinic M_5 receptors reported for tripitramine and pirenzepine ($pK_i = 7.3$ and 6.9, respectively (Eglen *et al*., 1996)) are rather different from the functional affinities calculated in rabbit vas deferens, suggesting that this receptor subtype may hardly mediate inhibition of neurogenic contractions of rabbit vas deferens.

Discussion

The pharmacological characterization of the muscarinic receptor subtype that mediates contraction in rabbit vas deferens tissue is still under debate (Caulfield & Birdsall, 1998), as for a long time it has been classified as M_1-type (Eltze, 1988; Eltze & Figala, 1988), whereas more recently has been advanced that it might correspond to the M_4 subtype (Caulfield & Brown, 1991; Caulfield, 1993; Sagrada *et al*., 1994; Waelbroeck *et al*., 1996).

Since misleading results in receptor characterization may often be due to the small amount of functional affinity data and to the lack of subtype specific muscarinic receptor antagonists, we thought it would be of interest to investigate in radioligand binding and functional assays a series of polyamines synthesized in our laboratory, which are structurally related to methoctramine and tripitramine (Figure 1). These polyamines were selected for their selectivity for different muscarinic receptor subtypes (Barbier *et al*., 1998; Bolognesi *et al*., 1998). Consequently, these profiles make these compounds useful in characterizing the receptor subtype that is responsible for the functional response in a given tissue.

In the present investigation, we have used the polyamines shown in Figure 1 to further characterize the muscarinic receptor subtype that mediates inhibition of the electrical field stimulation-induced contractions of the prostatic half of rabbit vas deferens. All polyamines were apparently simple competitive antagonists since they produced antagonism that was reversible and surmountable over the range of investigated concentrations as shown in Figure 2 for tripitramine and AM170. Schild analysis of the data yielded slopes that were not significantly different from unity (Figure 3, Table 1). In rabbit vas deferens muscarinic receptor preparation AM170 was two to 174 times more potent than

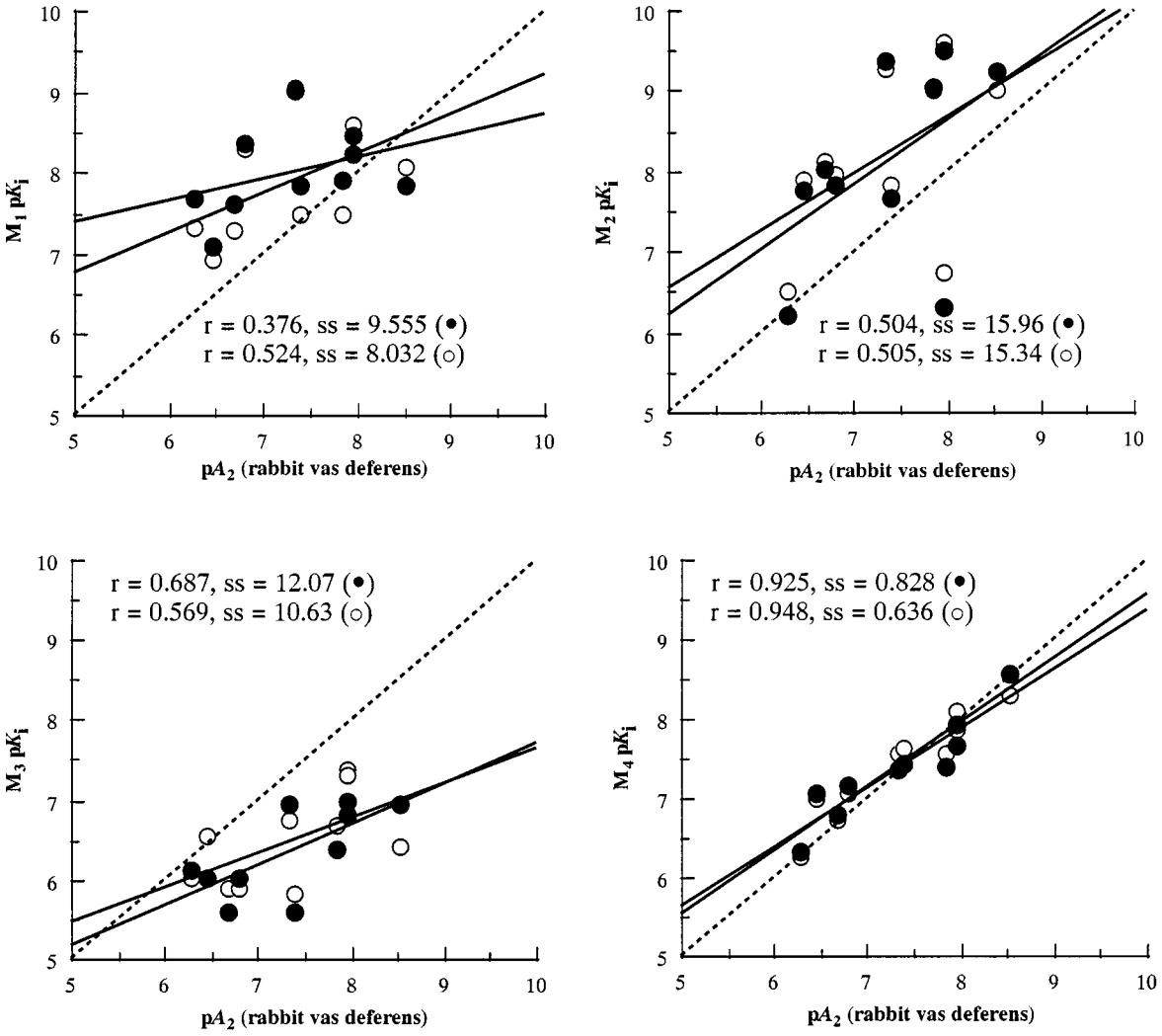

Figure 4 Relationship between the functional affinities (pA_2) determined in rabbit vas deferens and the binding affinities (pK_i) calculated at native (closed circles) or human recombinant (open circles) muscarinic M_1-M_4 receptors of the antagonists listed in Tables 1–3. For receptor identity ($pK_i = pA_2$, x = y), the normal regression line of the experimental data points (solid) should not deviate significantly from the depicted theoretical equality line (dotted). The inserts give the correlation factors (r) and the sum of squares values (ss).

the other polyamines as revealed by a comparison of pA_2 values shown in Table 1.

The aforementioned polyamines were also investigated in binding assays to make relevant comparison of the pK_i values obtained at native and cloned muscarinic M_1-M_4 receptors with the pA_2 values calculated from functional experiments in rabbit vas deferens.

The results obtained using native muscarinic receptors are qualitatively similar to those observed at cloned muscarinic receptors as revealed by an analysis of the affinity estimates reported in Tables 2 and 3. The difference between pK_i values calculated at native and cloned muscarinic receptors was not greater than 0.5 log unit. Several polyamines were able to significantly differentiate among muscarinic receptor subtypes. For example, spirotramine was significantly more potent at muscarinic M_1 receptors than at the other receptor subtypes; dipitramine was slightly selective for the M_1 subtype relative to the M_2 type, while being markedly less potent at M_3 and M_4 subtypes. Tripitramine, AM170, AM172, CC8 and CC9 were selective M_2 antagonists. None of investigated polyamines was more potent at either muscarinic M_3 and M_4 receptor subtypes relative to the other subtypes.

A comparison of pA_2 values obtained in rabbit vas deferens preparation with pK_i values calculated at native or cloned muscarinic M_1-M_4 receptors was then performed. Figure 4 clearly shows that the best correlation was found between the muscarinic M_4 receptor and the rabbit vas deferens muscarinic receptor. A very weak correlation was observed between the rabbit vas deferens site and the muscarinic M_1 receptor subtype, indicating that the two muscarinic receptors may hardly be the same. Similarly, no correlation was found comparing the antagonist potencies in vas deferens with the pK_i affinity values at native or cloned muscarinic M_2 and M_3 receptors. Based on these considerations we can conclude that the muscarinic receptors of the prostatic half of rabbit vas deferens seem to be pharmacological similar to the muscarinic M_4 subtype, considering that

the pA_2 values for a series of different antagonists best match with the correspondent pK_i values found at this muscarinic receptor subtype. This finding is in agreement with that obtained recently by Akbulut *et al*. (1999) in rat duodenum.

This research was supported by grants from University of Bologna, European Community (BMH4-CT97-2395), and MURST.

References

AKBULUT, H., GÖREN, Z., ISKENDER, E., ERASLAN, A., ÖZDEMIR, O. & OKTAY, S. (1999). Subtypes of muscarinic receptors in rat duodenum. A comparison with rabbit vas deferens, rat atria, guinea-pig ileum and gallbladder by using imperialine. *Gen. Pharmacol.,* **32,** 505 – 511.

AMMER, H. & SCHULZ, R., (1996). Chronic exposure of NG 108-15 cells to inhibitory acting drugs reduces stimulatory prostaglandin E_1 receptor number. *Eur. J. Pharmacol.,* **302,** 199 – 205.

ARUNLAKSHANA, D. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol.,* **14,** 48 – 58.

BARBIER, P., COLELLI, A., BOLOGNESI, M.L., MINARINI, A., TUMIATTI, V., CORSINI, G.U., MELCHIORRE, C. & MAGGIO, R. (1998). Antagonist binding profile of the split chimeric muscarinic m2-trunc/m3-tail receptor. *Eur. J. Pharmacol.,* **355,** 267 – 274.

BOLOGNESI, M.L., MINARINI, A., BUDRIESI, R., CACCIAGUERRA, S., CHIARINI, A., SPAMPINATO, S., TUMIATTI, V. & MELCHIORRE, C. (1998). Universal template approach to drug design: polyamines as selective muscarinic receptor antagonists. *J. Med. Chem.,* **41,** 4150 – 4160.

CAULFIELD, M.P. & BIRDSALL, N.J.M. (1998). International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol. Rev.,* **50,** 279 – 290.

CAULFIELD, M.P. & BROWN, D.A. (1991). Pharmacology of the putative M4 muscarinic receptor mediating Ca-current inhibition in neuroblastoma × glioma hybrid (NG 108-15) cells. *Br. J. Pharmacol.,* **104,** 39 – 44.

CAULFIELD, M.P. (1993). Muscarinic receptors - Characterization, coupling and function. *Pharmacol. Ther.,* **58,** 319 – 379.

CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.,* **22,** 3099 – 3108.

DÖRJE, F., WESS, J., LAMBRECHT, G., TACKE, R., MUTSCHLER, E. & BRANN, M.R. (1991). Antagonist binding profiles of five cloned human muscarinic receptor subtypes. *J. Pharmacol. Exp. Ther.,* **256,** 727 – 733.

EGLEN, R.M., HEDGE, S.S. & WATSON, N. (1996). Muscarinic receptor subtypes and smooth muscle function. *Pharmacol. Rev.,* **48,** 531 – 565.

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.

ELTZE, M. & FIGALA, V. (1988). Affinity and selectivity of biperiden enantiomers for muscarinic receptor subtypes. *Eur. J. Pharmacol.,* **158,** 11 – 19.

ENGEL, W.W., EBERLEIN, W.G., MIHM, G., HAMMER, R. & TRUMMLITZ, G. (1989). Tricyclic compounds as selective muscarinic receptor antagonists. 3. Structure-selectivity relationships in a series of cardioselective (M_2) antimuscarinics. *J. Med. Chem.,* **32,** 1718 – 1724.

HAMMER, R., BERRIE, C.P., BIRDSALL, N.J.M., BURGEN, A.S.V. & HULME, E.C. (1980). Pirenzepine distinguishes between subclasses of muscarinic receptors. *Nature,* **283,** 90 – 92.

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

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193,** 265 – 275.

MCPHERSON, G.A. (1985). Analysis of radioligand binding experiments. A colleection of computer programs for the IBM PC. *J. Pharmacol. Methods,* **14,** 213 – 228.

MELCHIORRE, C., BOLOGNESI, M.L., CHIARINI, A., MINARINI, A. & SPAMPINATO, S. (1993). Synthesis and biological activity of some methoctramine-related tetraamines bearing a 11-acetyl-5,11-dihydro-6*H*-pyrido[2,3-*b*[1,4]-benzodiazepin-6-one moiety as antimuscarinics: a second generation of highly selective M_2 muscarinic receptor antagonists. *J. Med. Chem.,* **36,** 3734 – 3737.

MELCHIORRE, C., CASSINELLI, A. & QUAGLIA, W. (1987). Differential blockade of muscarinic receptor subtypes by polymethylene tetraamines. Novel class of selective antagonists of cardiac M-2 muscarinic receptors. *J. Med. Chem.,* **30,** 201 – 204.

MELCHIORRE, C., MINARINI, A., SPAMPINATO, S. & TUMIATTI, V. (1995). Design, synthesis and biological activity of some tetraamines related to methoctramine and 4-DAMP. *Bioorg. Med. Chem. Lett.,* **5,** 785 – 790.

MICHEL, A.D. & WHITING, R.L. (1988). Methoctramine, a polymethylene tetraamine, differentiates three subtypes of muscarinic receptors in direct binding studies. *Eur. J. Pharmacol.,* **145,** 61 – 66.

MICHEL, A.D., DELMONDO, R., STEFANICH, E. & WHITING, R.L. (1989). Binding characteristics of the muscarinic receptor subtype of the NG 108-15 cell line. *Naunyn-Schmied. Arch. Pharmacol.,* **340,** 62 – 67.

MINARINI, A., BOLOGNESI, M.L., BUDRIESI, R., CANOSSA, M., CHIARINI, A., SPAMPINATO, S. & MELCHIORRE, C. (1994). Design, synthesis, and biological activity of methoctramine-related tetraamines bearing an 11-acetyl-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one moiety: structural requirements for optimum occupancy of muscarinic receptor subtypes as revealed by symmetrical and unsymmetrical polyamines. *J. Med. Chem.,* **37,** 3363 – 3372.

MUNSON, P.J. & RODBARD, D. (1980). LIGAND: a versatile computerised approach for the characterization of ligand binding systems. *Anal. Biochem.,* **107,** 220 – 239.

NILSSON, B.M., VARGAS, H.M. & HACKSELL, U. (1992). Amide, urea, and carbamate analogues of the muscarinic agent [4-[[*N*-(3-chlorophenyl)carbamoyl]oxy]-2-butynyl]trimethylammonium chloride. *J. Med. Chem.,* **35,** 2787 – 2798.

OKAYAMA, H. & BERG, P. (1983). A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. *Mol. Cell. Biol.,* **3,** 280 – 289.

SAGRADA, A., DURANTI, P., GIUDICI, L. & SCHIAVONE, A. (1994). Himbacine discriminates between putative muscarinic M_1 receptor-mediated responses. *Life Sci.,* **54,** PL 305 – 310.

TALLARIDA, R.J. & MURRAY, R.B. (1991). *Manual of Pharmacological Calculations with Computer Programs*. Version 4.2*, New York: Springer-Verlag*.

TALLARIDA, R.J., COWAN, A. & ADLER, M.W. (1979). pA_2 and receptor differentiation: a statistical analysis of competitive antagonism. *Life Sci.,* **25,** 637 – 654.

WAELBROECK, M., TASTENOY, M., CAMUS, J. & CHRISTOPHE, J. (1990). Binding of selective antagonists for four muscarinic receptors (M_1 to M_4) in rat forebrain. *Mol. Pharmacol.,* **38,** 267 – 273.

WAELBROECK, M., LAZARENO, S., PFAFF, O., FRIEBE, T., TASTENOY, M., MUTSCHLER, E. & LAMBRECHT, G. (1996). Stereoselective recognition of the enantiomers of phenglutarimide and of six related compounds by four muscarinic receptor subtypes. *Br. J. Pharmacol.,* **119,** 1319 – 1330.

WIDZOWSKI, D., HELANDER, H.F. & WU, E.S.C. (1997). Selective muscarinic M_1 antagonists: drug design and discovery. *Drug Discovery Today,* **2,** 341 – 350.

(Received September 14, 2000
Revised November 23, 2000
Accepted December 8, 2000)