British Journal of Pharmacology (1995) 114, 1507-1517

In vitro characterization of tripitramine, a polymethylene tetraamine displaying high selectivity and affinity for muscarinic M_2 receptors

A. Chiarini, R. Budriesi, M.L. Bolognesi, A. Minarini & [1]C. Melchiorre

Department of Pharmaceutical Sciences, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy

1 The antimuscarinic effects of tripitramine were investigated *in vitro* in isolated driven left (force) and spontaneously beating right (force and rate) atria as well as in the ileum of guinea-pig and rat and in the trachea and lung strip of guinea-pig and compared with the effects of methoctramine.

2 Tripitramine was a potent competitive antagonist of muscarinic M_2 receptors in right and left atria. The pA_2 values ranged from 9.14 to 9.85. However, in the guinea-pig and rat left atria but not in guinea-pig right atria, tripitramine at lower concentrations (3–10 nM) produced a less than proportional displacement to the right of agonist-induced responses owing to the presence of a possible saturable removal process.

3 Tripitramine was about three orders of magnitude less potent in ileal and tracheal than in atrial preparations (pA_2 values ranging from 6.34 to 6.81) which makes it more potent and more selective than methoctramine.

4 Another intriguing finding was the observation that the pA_2 value of 7.91 observed for tripitramine in guinea-pig lung does not correlate with that found at both muscarinic M_2 and M_3 receptor subtypes, which clearly indicates that the contraction of guinea-pig lung strip is not mediated by these muscarinic receptor subtypes.

5 A combination of tripitramine with atropine resulted in addition of the dose-ratios for left atria as required for two antagonists interacting competitively with the same receptor site, whereas the same combination gave a supra-additive antagonism on guinea-pig ileum which suggests that tripitramine interacts with a second interdependent site.

6 Tripitramine was more specific than methoctramine since, in addition to muscarinic receptors, it inhibited only frog rectus abdominis muscular (pIC_{50} value of 6.14) and rat duodenum neuronal (pIC_{50} value of 4.87) nicotinic receptors among receptor systems investigated, namely α_1-, α_2-, and β_1-adrenoceptors, H_1- and H_2-histamine receptors, and muscular and neuronal nicotinic receptors.

Keywords: Tripitramine; methoctramine; polymethylene tetraamines; muscarinic M_2 receptors; muscarinic receptor subtypes; saturable removal process

Introduction

Molecular biology studies have clearly established that muscarinic receptors are heterogeneous and are comprised of at least five subtypes (m_1 to m_5) (Bonner, 1989; Hulme *et al.*, 1990; Caulfield, 1993). Four of them have been pharmacologically characterized and are designated as M_1, M_2, M_3 and M_4 according to their affinity for selective antagonists (Hulme *et al.*, 1990; Caulfield, 1993; Friebe *et al.*, 1993; Wess, 1993). For example, muscarinic M_1 receptors are characterized by high affinity for pirenzepine and are found in high density in neuronal tissues and autonomic ganglia (Hammer *et al.*, 1980); muscarinic M_2 receptors have low affinity for pirenzepine and high affinity for methoctramine (Melchiorre *et al.*, 1987b), AF-DX 116 (11[[2-[(diethylamino)-methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b]-[1,4]benzodiazepin-6-one) (Hammer *et al.*, 1986) and himbacine (Anwar-ul *et al.*, 1986), and are located mainly in cardiac tissue, whereas muscarinic M_3 receptors display low affinity for pirenzepine and high affinity for 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide) (Barlow *et al.*, 1976) and *p*-fluoro-hexahydrosiladiphenidol (Lambrecht *et al.*, 1988), and are found in smooth muscles and glands. At present, no selective antagonist is available for the muscarinic M_4 receptor subtype which has been characterized, however, in rat striatum (Waelbroeck *et al.*, 1991). Furthermore, this receptor subtype has been found in NG 108-15 cells (Michel *et al.*, 1989; Lazareno *et al.*, 1990; Caulfield & Brown, 1991), chicken heart, rabbit lung (Lazareno *et al.*, 1990) and guinea-pig uterus (Dörje *et al.*, 1990). However, Eglen *et al.* (1989) and Bognar *et al.* (1992) have argued that guinea-pig uterus contains muscarinic M_2 rather than M_4 receptors. Recently, Bognar *et al.* (1992) have suggested the existence in rabbit iris sphincter of a muscarinic receptor subtype differing from muscarinic M_1–M_4 receptors.

Among available muscarinic receptor antagonists, methoctramine, although its discovery dates from only 1987, has contributed significantly to muscarinic receptor subtype characterization and classification owing to its high affinity for muscarinic M_2 receptors, low affinity for muscarinic M_3 receptors and intermediate affinity for muscarinic M_1 receptors (Melchiorre *et al.*, 1987b; 1989; Melchiorre, 1988; 1990). In spite of these properties, methoctramine lacks receptor specificity since it inhibits, albeit with modest potency, other receptor systems, such as α_1- (Melchiorre *et al.*, 1987b) and β_1-adrenoceptors (Eglen *et al.*, 1988) and muscular (Melchiorre *et al.*, 1987b) and neuronal nicotinic receptors (Watson *et al.*, 1992). In an effort to improve affinity and selectivity of polymethylene tetraamines we started a study by performing structural modifications on methoctramine such as to incorporate in its tetraamine backbone a 11-acetyl-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one moiety of AQ-RA 741 (11-[[4-[4-(diethylamino)-butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one) (Eberlein *et al.*, 1989; 1992), an analogue of pirenzepine

[1] Author for correspondence.

which is claimed to display high selectivity for muscarinic M_2 receptors. This investigation has led, very recently, to the discovery of tripitramine (Figure 1) that, to the best of our knowledge, represents the most potent and the most selective muscarinic receptor antagonist known until now in both functional (M_2 and M_3) and binding (M_1 to M_4 and m_1 to m_5) assays (Melchiorre *et al.*, 1993; Maggio *et al.*, 1994).

We now describe further *in vitro* experiments with tripitramine in comparison to methoctramine in atrial, tracheal and ileal preparations derived from guinea-pig and rat and with different agonists. The guinea-pig lung strip preparation was also included in this study to verify whether the contraction of this tissue is mediated by muscarinic M_2-like receptors (Roffel *et al.*, 1993). Furthermore, antagonist combination experiments with atropine (Clark & Mitchelson, 1976; Stockton *et al.*, 1983) were performed to verify the mechanism of action of tripitramine. In addition, receptor specificity was assessed by investigating tripitramine in comparison to methoctramine on other receptor systems, namely rat vas deferens α_1- and α_2-adrenoceptors, guinea-pig atrial β_1-adrenoceptors, guinea-pig right atrial H_2-histamine receptors, guinea-pig ileal H_1-histamine receptors, rat duodenum neuronal nicotinic receptors and frog rectus abdominis muscular nicotinic receptors. The data were analysed quantitatively according to well established pharmacological methods, namely Schild analysis (Arunlakshana & Schild, 1959; Tallarida *et al.*, 1979; Tallarida & Murray, 1991) and assessment of interaction between antagonists according to the dose-ratio method (Paton & Rang, 1965).

Methods

Guinea-pigs (200–400 g), rats (150–200 g) or frogs (10–20 g) were killed by cervical dislocation under ketamine anaesthesia and the organs required were set up rapidly under a suitable resting tension in 15 ml organ baths containing physiological salt solution kept at appropriate temperature (see below) and aerated with 5% CO_2:95% O_2 at pH 7.4. Dose-response curves were constructed by cumulative addition of the agonist (Van Rossum, 1963). The concentration of agonist in the organ bath was increased approximately five fold at each step, with the exception of guinea-pig lung strip and frog rectus abdominis preparations, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady. Contractions were recorded by means of a force displacement transducer (FT.03 Grass and 7003 Basile) connected to a four-channel pen recorder (Battaglia-Rangoni KV 380). In all cases, parallel experiments in which tissues did not receive any antagonist were run in order to check any variation in sensitivity. It was always verified that the EC_{50} values for the agonist in tissues receiving only the solvent were not significantly different ($P > 0.05$) from control values. In all other cases experiments were discarded.

Guinea-pig left atria

The hearts of guinea-pigs of either sex were rapidly removed, washed by perfusion through the aorta with oxygenated physiological salt solution and right and left atria were separated out. The left atria were mounted under 0.2–0.3 g tension at 35°C in Tyrode solution of the following composition (mM): NaCl 136.9, KCl 5.4, $MgSO_4 \cdot 7H_2O$ 1.0, $CaCl_2$ 2.52, NaH_2PO_4 0.4, $NaHCO_3$ 11.9 and glucose 5.5. Tissues were stimulated through platinum electrodes by square-wave pulses (0.6–0.8 ms, 1 Hz, 1–5 V). Inotropic activity was recorded isometrically. Tissues were equilibrated for 1h, and cumulative dose-response curves to carbachol (0.01–1 μM), oxotremorine (1–100 nM), methoctramine (10–500 μM) or tripitramine (10–500 μM) were constructed. Following incubation with the antagonist for 30–180 min, a new dose-response curve to carbachol or oxotremorine was obtained. In combination experiments (simultaneous administration) of tripitramine (0.1–0.5 μM) with atropine (0.5–1 μM), the contact time for antagonists was always 60 min. The end of the tripitramine effect was determined by the following procedure. A control dose-response curve to carbachol was obtained followed by a curve in the presence of tripitramine (50 nM). The antagonist was then removed from the bath and a dose-response curve to carbachol was constructed after a washing period of 60 min. The start of tripitramine effect was investigated by exposing the atria to tripitramine (10, 50 and 100 nM) for 30, 60, 120 and 180 min, respectively, before a second dose-response curve to carbachol was obtained. To verify whether a polyamine oxidase is involved in tripitramine degradation, a few experiments were also performed in the presence of N,N′-*bis*(2,3-butadienyl)-1,4-butanediamine dihydrochloride (10 μM). When determining methoctramine- and tripitramine-induced positive inotropic responses in atrial tissue isolated from guinea-pigs pretreated with reserpine, reserpine was injected i.p., at single doses of 2 mg kg^{-1} 48 and 24 h before the animals were killed in order to eliminate the influence of noradrenaline, which might be released from sympathetic nerve terminals (Temma *et al.*, 1977).

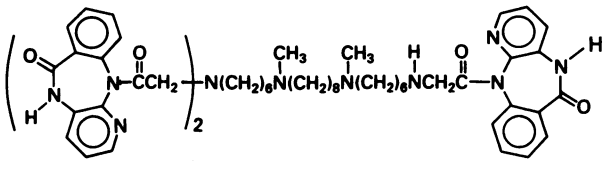

Figure 1 Structural formula of tripitramine.

Rat left atria

The heart of male rats was rapidly removed and right and left atria were separated out. The left atria were mounted under 0.1–0.2 g tension and stimulated as described for guinea-pigs. Inotropic activity was recorded isometrically. Tissues were equilibrated for 1 h, and cumulative dose-response curves to carbachol (0.01–1 μM) were constructed. Following incubation with the antagonist for 60 (> 10 nM) or 120 min (≤ 10 nM), a new dose-response curve to carbachol was obtained.

Spontaneously beating guinea-pig right atria

Spontaneously beating right atria were suspended under 0.15–0.20 g tension at 35°C in Tyrode solution as for left atria. Heart rate and size of contractions were recorded isometrically with carbachol (0.01–1 μM), isoprenaline (1–100 μM) or histamine (0.1–50 μM) as agonists. The contact time for antagonists was 60 min except when determining antimuscarinic activity of tripitramine. In this case, the contact time was 120 min for concentrations ≤ 10 nM.

Guinea-pig ileum

The terminal portion of the ileum was excised after discarding the 4–6 cm nearest to the ileo-caecal junction. The tissue was cleaned and segments of approximately 2 cm were set up under 1 g tension at 37°C in organ baths containing Tyrode solution of the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, $MgSO_4$ 1.2, KH_2PO_4 1.19, $NaHCO_3$ 25 and glucose 11. Tension changes were recorded isotonically. Tissues were allowed to equilibrate for at least 30 min during which time the bathing solution was changed every 10 min. Dose-response curves to carbachol (0.01–0.5 μM), oxotremorine (0.01–1 μM) or histamine (0.01–5 μM) were obtained at 30 min intervals, the first one being discarded and the second one taken as control. Following incubation with the antagonist for 60 min, a new dose-response curve to the agonist was obtained.

Rat ileum

The procedure described for guinea-pig ileum was followed with carbachol (0.1–5 μM) used as the agonist. Following incubation with the antagonist for 60 min, a new dose-response curve to the agonist was obtained.

Frog rectus abdominis

The rectus abdominis muscle was set up at 25°C in an organ bath containing Clark frog Ringer solution of the following composition (mM): NaCl 111, KCl 1.88, $CaCl_2$ 1.08, NaH_2PO_4 0.08, $NaHCO_3$ 2.38 and glucose 11.1. Dose-response curves to carbachol (1–100 μM) were obtained at 30 min intervals, the first one being discarded and the second one taken as control. Following incubation with the antagonist for 60 min, a new dose-response curve to the agonist was obtained. The end of the tripitramine effect was determined by the following procedure. A control dose-response curve to carbachol was obtained followed by a curve in the presence of tripitramine (3 μM). The antagonist was then removed from the bath and a dose-response curve to carbachol was constructed after a washing period of 60 min.

Rat duodenum

The procedure described by Irie *et al.* (1994) was followed. Briefly, duodenum of adult rats, 9–11 weeks old, was dissected out and segments of approximately 2.2 cm of the proximal portion but not the bulb were set up under 1 g tension at 35°C in organ baths containing modified Locke solution of the following composition (mM): NaCl 154, KCl 4.02, $CaCl_2$ 1.36, $MgCl_2$ 0.9, $NaHCO_3$ 2.97 and glucose 5.56. Tension changes were recorded isotonically. Tissues were equilibrated for 1 h, and the effect of a single dose of 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP, 100 μM), taken as control, was recorded. Following 30-min recovery and incubation with the antagonist for 60 min, a new response to a single dose of DMPP (100 μM) was obtained.

Rat vas deferens

Both vasa deferentia from male albino rats were isolated, freed from adhering connective tissue and transversely bisected. Prostatic, 12 mm in length, and epididymal portions, 14 mm in length, were prepared and mounted individually at 37°C in organ baths containing Krebs solution of the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.52, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.1. $MgSO_4$ concentration was reduced to 0.6 mM when the twitch response to field stimulation was studied. The loading tension used to assess α_1- or α_2-blocking activities was 0.4 g or 0.5–0.8 g, respectively. The tissues were allowed to equilibrate for at least 1 h before addition of any drug. Field stimulation of the tissue was carried out by means of two platinum electrodes, placed near the top and bottom of the vas deferens, at 0.1 Hz with square pulses of 3 ms duration at voltage of 10–35 V. The stimulation voltage was fixed throughout the experiments. Propranolol hydrochloride (1 μM) and desipramine hydrochloride (10 nM) were present in the Krebs solution throughout the experiments outlined below to block β-adrenoceptors and neuronal uptake mechanisms, respectively. The α_1-adrenoceptor blocking activity was determined on the epididymal portion of the vas deferens. Noradrenaline (0.01–5 μM) dose-response curves were obtained cumulatively, the first one being discarded and the second one taken as control. After incubation with the antagonist for 60 min, a third dose-response curve was obtained. The α_2-adrenoceptor blocking activity was assessed on the prostatic portion of the vas deferens by antagonism to clonidine. A first clonidine (1–100 nM) dose-response curve, taken as control, was obtained cumulatively avoiding the inhibition of more than 90% of twitch responses, while the concentration of clonidine causing 100% inhibition was deduced from the second dose-response curve obtained from parallel experiments. Under these conditions it was possible to obtain a second dose-response curve which was not significantly different from the first one. Thus, after incubation with antagonist for 60 min a dose-response curve was obtained.

Guinea-pig trachea

The trachea was cut transversally between the segment of cartilage and four groups of tracheal segments, each one made up of three rings, were tied together and mounted under a tension of 1 g at 37°C in organ baths containing Krebs-Ringer solution of the following composition (mM): NaCl 95, KCl 4.7, $CaCl_2$ 2.50, $MgSO_4$ 1.0, KH_2PO_4 1.17, $NaHCO_3$ 25 and glucose 10.6. The tissues were allowed to stabilize for 90 min. The tension was recorded isometrically. Cumulative dose-response curves to carbachol (0.01–1 μM) were constructed. Following incubation with the antagonist for 60 min, a new dose-response curve to the agonist was obtained.

Guinea-pig lung

The procedure described by Roffel *et al.* (1993) was followed. Briefly, strips of peripheral lung tissue, approximately 15 × 2 × 2 mm, were cut either from the body of a lower lobe with the longitudinal axis of the strip parallel to the bronchus or from the peripheral margin of the lobe and set up under 0.3 g tension at 37°C in organ baths containing Krebs-Henseleit buffer solution of the following composition (mM): NaCl 118.78, KCl 4.32, $CaCl_2$ 2.52, $MgSO_4$ 1.18, KH_2PO_4 1.28, $NaHCO_3$ 25 and glucose 5.5. Tension changes were recorded isotonically. After a 60 min equilibration period the strips were precontracted twice by cumulative administration of carbachol (0.1, 1, 10 and 0.1, 1, 10, 100 μM with washing periods of 45 min) before determination of the control dose-response curve. After a 45-min washing period, a consecutive dose-response curve was made in the presence of antagonist. The contact time for antagonists was 60 min.

Statistical and data analysis

Dose ratios (DR) at the EC_{50} values of the agonists were calculated at one to six antagonist concentrations and each concentration was tested from four to eight times. Dissociation constants (pA_2 values) were estimated by Schild plots (Arunlakshana & Schild, 1959) constrained to slope −1.0, as required by the theory (Tallarida *et al.*, 1979). When applying this method, it was always verified that the experimental data generated a line whose derived slope was not significantly different from unity ($P > 0.05$). An apparent dissociation constant (K_B) for the antagonists at a single concentration was derived from the equation K_B = [antagonist]/(DR − 1) (Furchgott, 1972). pIC_{50} values represent the negative logarithm of that dose of antagonist which inhibits 50% of the maximum response to an agonist. Data are presented as means ± s.e. of n experiments. Differences between mean values were tested for significance by Student's t test.

Drugs

All drugs used, carbachol (carbamoylcholine chloride, EGA-Chemie), atropine sulphate (Merck), (±)-ketamine hydrochloride, (±)-isoprenaline hydrochloride, clonidine hydrochloride and desipramine hydrochloride (Sigma), histamine dihydrochloride (C. Erba), R-(−)-noradrenaline hydrochloride, (±)-propranolol hydrochloride, oxotremorine (1-[4-[1-pyrrolidinyl]-2-butynyl]-2-pyrrolidinone sesquifumarate), DMPP (1,1-dimethyl-4-phenylpiperazinium iodide) and tyramine hydrochloride (Aldrich-Chemie), N,N′-*bis*(2,3-butadi-

enyl)-1,4-butanediamine dihydrochloride, tripitramine (1,1,24-tris[[5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]methyl]-8,17-dimethyl-1,8,17,24-tetraazatetracosane tetraoxalate) and methoctramine tetrahydrochloride (synthesized in our laboratory), were dissolved in twice distilled water with the exception of reserpine (Fluka) which was dissolved in 50% ethanol containing 0.8 mg of ascorbic acid for 2 mg of reserpine.

Results

Affinity of tripitramine at muscarinic receptor subtypes

Tripitramine (0.015–3 μM) antagonized potently the negative inotropic effects induced by carbachol or oxotremorine at muscarinic M_2 receptors in electrically stimulated left atria from rat and guinea-pig. There was a dose-dependent parallel shift to the right of the agonist dose-response curves without either basal tension or maximum response being affected (shown for carbachol in guinea-pig left atria in Figure 2). However, tripitramine at concentrations lower than 15 nM in the range 3–10 nM gave a parallel shift to the right of the agonist dose-response curves but the shift was not proportional to the concentration being markedly lower than the expected value (Figure 2). Schild analysis of the results gave linear plots in the range 0.015–3 μM with slopes which were not significantly different from unity (Table 1). However, in guinea-pig left atrial preparations and in the concentration-range 3–10 nM, nonlinear Schild plots were obtained and the slopes were much higher than unity (3.68 ± 0.03 and 3.53 ± 0.05 for carbachol and oxotremorine as agonists, respectively). Similar results were obtained in rat left atria (Figure 3).

In guinea-pig left atria, the maximum effect of tripitramine was reached within 60 min for concentrations > 10 nM and within 120 min for concentrations ≤ 10 nM (Figure 4). Experiments performed in the presence of N,N'-*bis*(2,3-butadienyl)-1,4-butanediamine (10 μM), which is known to block polyamine oxidase (Bey *et al.*, 1985), did not modify the maximum effect of tripitramine (10 nM) (results not shown).

As for methoctramine (Melchiorre *et al.*, 1987a), tripitramine antagonism was easily reversible in guinea-pig left atria, since 60 min washing of the tissues after incubation for 60 min with tripitramine (50 nM) brought the EC_{50} value (± 95% C.F.) for carbachol [81 (75–89) nM ($n = 5$)] to the control value [73 (65–82) nM ($n = 5$)]. The carbachol- or oxotremorine-induced responses in left atria were antagonized by tripitramine in the range 0.015–3 μM with pA_2 values (Table 1) that closely resembled the pK_i value

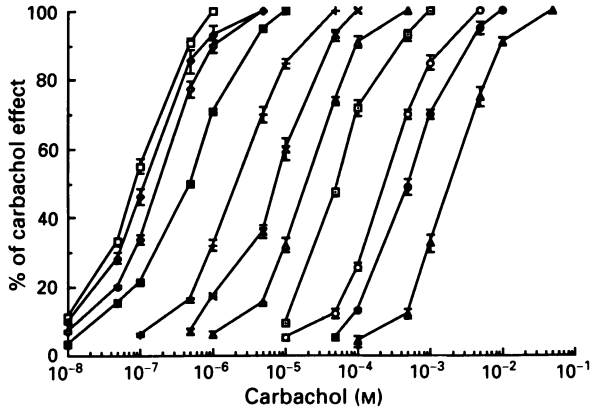

Figure 2 Antagonism of carbachol-induced negative inotropic effect at muscarinic M_2 receptors in guinea-pig left atria. Dose-response curves for carbachol were obtained before (□) and after exposure to 3 (◆), 5 (◇), 7 (■), 10 (+), 15 (×), 50 (△), 100 (⊡), 500 (○), 1000 (●) and 3000 nM (▲) tripitramine for 120 (3–10 nM) or 60 min (15–3000 nM). Each point is the mean (± s.e.mean) of five to eight observations.

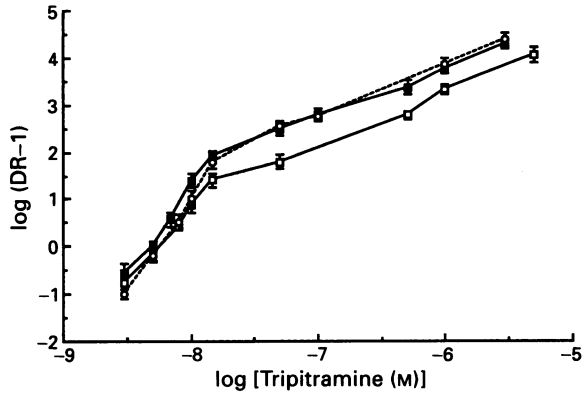

Figure 3 Schild plot for the antagonism between carbachol or oxotremorine and tripitramine at cardiac muscarinic M_2 receptors in guinea-pig (carbachol, ■; oxotremorine, ○) and rat (carbachol, □) left atrium. Each point is the mean (± s.e.mean) of five to eight observations.

Table 1 Antagonist affinities at guinea-pig atria, ileum, trachea and lung, and rat ileum and left atrium muscarinic receptors

Preparation[a]	Concentration (μM)	*Tripitramine* pA_2[b]	Slope[c]	*Methoctramine* pA_2[b]	Slope[c]
Guinea-pig					
Left atrium	0.015, 0.05, 0.1, 0.5, 1, 3	9.69 ± 0.02	1.01 ± 0.04	7.78 ± 0.01	0.99 ± 0.02
Left atrium[d]	0.015, 0.05, 0.1, 1, 3	9.85 ± 0.04	1.07 ± 0.04	7.89 ± 0.04	1.01 ± 0.03
Right atrium					
(force)	0.003, 0.005, 0.01, 0.1, 1	9.20 ± 0.12	1.02 ± 0.05	7.56 ± 0.08	1.06 ± 0.03
(rate)	0.003, 0.005, 0.01, 0.1	9.34 ± 0.02	0.99 ± 0.01	7.47 ± 0.10	1.09 ± 0.07
Ileum	5, 10, 50, 100	6.50 ± 0.05	1.00 ± 0.02	6.28 ± 0.06	1.04 ± 0.02
Ileum[d]	10, 50, 100	6.81 ± 0.02	1.03 ± 0.03	6.35 ± 0.07	1.02 ± 0.04
Trachea	10, 50, 100	6.34 ± 0.04	1.03 ± 0.06	6.30 ± 0.02	1.06 ± 0.07
Lung	0.05, 0.1, 1, 10	7.91 ± 0.03	1.02 ± 0.02	6.97 ± 0.07	1.05 ± 0.04
Rat					
Left atrium	0.015, 0.05, 0.5, 1, 5	9.14 ± 0.09	1.08 ± 0.04	7.80 ± 0.08	1.03 ± 0.04
Ileum	3, 5, 50	6.45 ± 0.06	1.05 ± 0.02	5.99 ± 0.03	1.02 ± 0.02

[a]The agonist was carbachol unless otherwise indicated. [b]Each pA_2 value was obtained from 12 to 36 dose-ratios at the indicated concentrations for tripitramine and at 0.05, 0.5 and 5 μM (atria) or 5, 10 and 50 μM (ileum) concentrations for methoctramine and determined by the method of constrained plot (Tallarida *et al.*, 1979; Tallarida & Murray, 1991). Results are presented as the mean ± s.e. [c]Slopes were estimated by Schild plots (Arunlakshana & Schild, 1959) and were not significantly different ($P > 0.05$) from unity. [d]The agonist was oxotremorine.

(9.54 ± 0.08) obtained in heart membranes (Melchiorre *et al.*, 1993). Under the same experimental conditions methoctramine was about two orders of magnitude less potent than tripitramine (Table 1).

In spontaneously beating right atria, tripitramine (0.003–1 μM, force; 0.003–0.1 μM, rate) caused a dose-dependent inhibition of the negative chronotropic and inotropic effects induced by carbachol without modifying basal rate or tension. The Schild plots, unlike the left atria, were linear over all the range of concentrations investigated and the slopes of the regression lines were not significantly different from unity (Figure 5, Table 1). pA_2 values of 9.20 ± 0.12 and 9.34 ± 0.02 were found at muscarinic M_2 receptors in atrial tissue and sinus atrial pacemaker cells, respectively, which were not significantly different and slightly lower than the pA_2 value obtained in guinea-pig left atria (Table 1).

Tripitramine (5–100 μM) caused a dose-dependent parallel shift to the right of carbachol dose-response curves of guinea-pig and rat ileum with no changes in the maximum response (shown for guinea-pig ileum in Figure 6). Similarly, tripitramine (10–100 μM) antagonized in a dose-dependent fashion oxotremorine-induced contractions in guinea-pig ileum without affecting the maximum response (not shown). The Schild plots were linear and the slopes of the regression lines were not significantly different from unity pointing to competitive antagonism (Figure 5, Table 1). Irrespective of agonist and species used, the pA_2 values were similar, ranging from 6.45 ± 0.06 to 6.81 ± 0.02. Methoctramine was slightly less potent than tripitramine, the pA_2 values ranging from 5.99 ± 0.03 to 6.35 ± 0.07.

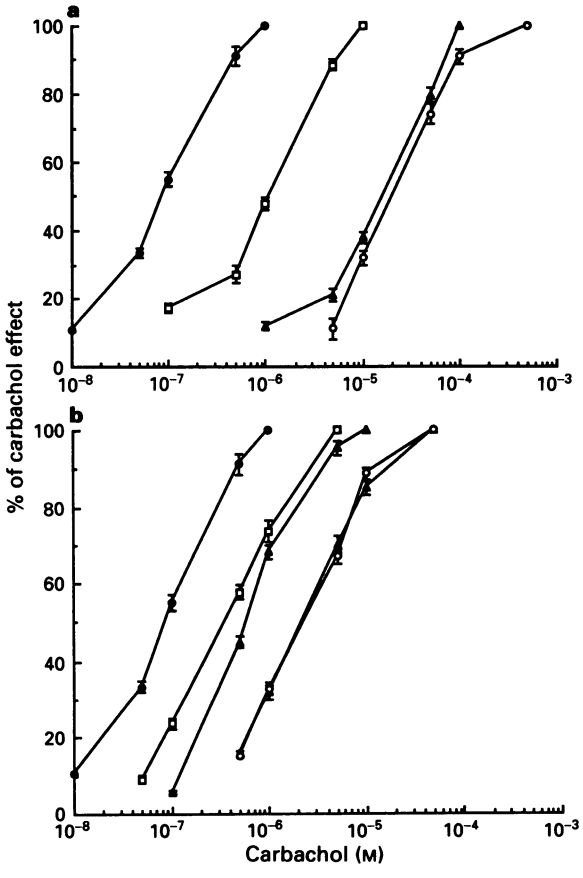

Figure 4 Time course of tripitramine effect on carbachol-induced negative inotropic effect at muscarinic M_2 receptors in guinea-pig left atria. Dose-response curves for carbachol were obtained before (●) and after exposure (a) to 50 nM tripitramine for 30 (□), 60 (▲) and 120 min (○), and (b) to 10 nM tripitramine for 30 (□), 60 (△), 120 (○) and 180 min (▲). Each point is the mean (± s.e.mean) of four to eight observations.

Tripitramine (0.05–10 μM) caused a dose-dependent inhibition of carbachol-induced contractions of guinea-pig lung strip. The agonist dose-response curves were displaced in a parallel fashion with no changes in the maximum response (Figure 7). The data derived from Schild analysis point to competitive antagonism (Figure 5, Table 1). A pA_2 value of 7.91 ± 0.01 was found at muscarinic receptors in guinea-pig lung strip, which was markedly lower than the value obtained at muscarinic M_2 receptors in atria while being significantly higher than that at muscarinic M_2 receptors in smooth muscle (ileum and trachea). This finding clearly suggests that muscarinic receptors mediating contraction in guinea-pig lung strip can be classified neither as M_2 nor as M_3 subtypes. Under the same experimental conditions methoctramine gave a pA_2 value of 6.97 ± 0.07 which is similar to the value (7.30 ± 0.06) obtained by Roffel *et al.* (1993).

In guinea-pig tracheal rings, tripitramine (10–100 μM) caused a dose-dependent inhibition of carbachol-induced contraction with no changes in the maximum response (not shown). The Schild plot was linear and the slope of the regression line was not significantly different from unity pointing to an apparent competitive antagonism (Table 1; Figure 5). The pA_2 value (6.43 ± 0.04) was similar to that found in the ileum. Methoctramine was slightly less potent than tripitramine, the pA_2 value amounting to 6.30 ± 0.02.

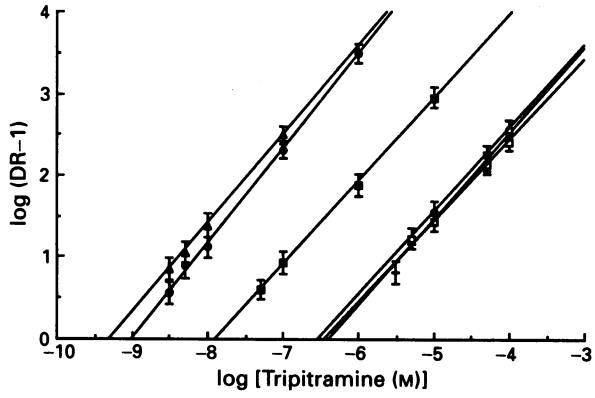

Figure 5 Schild plot for the antagonism between carbachol and tripitramine at cardiac (M_2) and smooth muscle (M_3) muscarinic receptors in guinea-pig right atrium (rate, ▲; force, ●), lung strip (■), trachea (□) and ileum (○), and rat ileum (+). Each point is the mean (± s.e.mean) of four to seven observations.

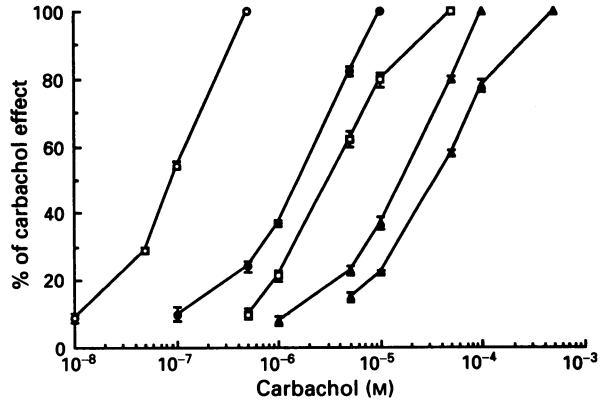

Figure 6 Antagonism of carbachol-induced contractions at muscarinic M_3 receptors in guinea-pig ileum. Dose-response curves for carbachol were obtained before (○) and after exposure to 5 (●), 10 (□), 50 (▲) and 100 μM (△) tripitramine for 60 min. Each point is the mean (± s.e.mean) of four to seven observations.

Antagonism at muscarinic M_2 and M_3 receptors by tripitramine and atropine in combination

The mutual competition between tripitramine and atropine was investigated on the basis of dose-ratio analysis (Paton & Rang, 1965).

In the guinea-pig left atria, the experimental dose-ratios of the combination of tripitramine (0.5 and 0.1 μM) with atropine (1 and 0.5 μM) amounted to 6329 ± 433 and 2073 ± 189, respectively, and were not significantly different from those expected for a combination of two competitive antagonists (6184 and 1897) (Table 2).

Guinea-pig ileal preparations were exposed simultaneously to tripitramine (5 and 100 μM) and atropine (0.005 and 0.1 μM). The experimental dose-ratios of the combination resulted 132 ± 7.5 and 5926 ± 160, respectively, and were significantly higher than those expected for a combination of two competitive antagonists (36 and 635) (Table 3).

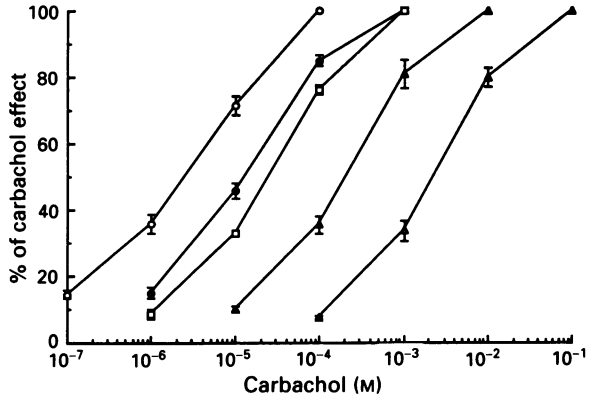

Figure 7 Antagonism of carbachol-induced contractions at muscarinic receptors in guinea-pig lung strip. Dose-response curves for carbachol were obtained before (○) and after exposure to 0.05 (●), 0.1 (□), 1 (▲) and 10 μM (△) tripitramine for 60 min. Each point is the mean (± s.e.mean) of four to six observations.

Specificity and selectivity of tripitramine vs methoctramine

Table 4 shows the selectivity ratios for tripitramine and methoctramine. It is evident that tripitramine is more than one order of magnitude more selective than methoctramine for muscarinic M_2 receptors. Furthermore, tripitramine is two orders of magnitude more potent than methoctramine. It should be noted, however, that the selectivity ratios (atria vs. ileum and trachea) may be inaccurate owing to the finding that tripitramine did not behave as a competitive antagonist in the ileum in combination experiments with atropine. For this reason, the M_2/M_3 selectivity ratios reported in Table 4 are only apparent values.

Table 5 shows the receptor specificity of tripitramine in comparison to methoctramine. Methoctramine (10–500 μM) induced a concentration-dependent positive inotropic response at β_1-adrenoceptors in guinea-pig left atrial preparations. At the maximum concentration employed (500 μM), a percentage of 209 ± 3.1 ($n = 5$) in developed tension was observed. The EC_{50} value was not calculated since no maximum response was attained (Figure 8). Under the same conditions, tripitramine (10–500 μM) did not give a significant increase in developed tension. In tissues isolated from animals pretreated with reserpine, noradrenaline depletion was determined by exposing isolated atria to a single dose of tyramine (10 μg ml^{-1}) before starting experiments (Muskus, 1962). Responses to tyramine were abolished. The EC_{50} value (± 95% C.F.) for tyramine control was 11.5 (10.8–12.4) μM ($n = 4$). In pretreated tissues, the response to methoctramine was lower than that observed in tissues from untreated animals, whereas tripitramine was completely devoid of activity (Figure 8).

Tripitramine and methoctramine (10 μM) did not antagonize isoprenaline- or histamine positive chronotropic responses at β_1-adrenoceptors or H_2-histamine receptors in sinus atrial pacemaker cells from guinea-pig right atria, respectively. The EC_{50} values (± 95% C.F.) for isoprenaline in the presence of tripitramine [2.11 (1.50–2.90) nM] ($n = 4$) or methoctramine [1.92 (1.60–2.40) nM] ($n = 4$) were not significantly different from that of control [3.25 (2.50–3.90) nM] ($n = 8$). Similarly, the EC_{50} values for histamine in the

Table 2 Dose-ratios obtained with tripitramine and atropine alone and in combination on muscarinic receptors of guinea-pig left atrium with carbachol as agonist

Tripitramine concentration (μM)	*Tripitramine dose-ratio* (DR_1)	*Atropine concentration* (μM)	*Atropine dose-ratio* (DR_2)	*Experimental combination dose-ratio*	*Expected combination dose-ratio* ($DR_1 + DR_2 - 1$)
0.5	3344 ± 315 ($n = 5$)	1	2841 ± 263 ($n = 5$)	6329 ± 433[a] ($n = 7$)	6184
0.1	890.4 ± 102 ($n = 5$)	0.5	1008 ± 35 ($n = 5$)	2073 ± 189[a] ($n = 6$)	1897

Each dose-ratio (DR) value is the mean ± s.e. and the number of observations is given in parentheses.
[a]Not significantly different ($P > 0.05$) from expected combination dose-ratio for two competitive antagonists.

Table 3 Dose-ratios obtained with tripitramine and atropine alone and in combination on muscarinic receptors of guinea-pig ileum with carbachol as agonist

Tripitramine concentration (μM)	*Tripitramine dose-ratio* (DR_1)	*Atropine concentration* (μM)	*Atropine dose-ratio* (DR_2)	*Experimental combination dose-ratio*	*Expected combination dose-ratio* ($DR_1 + DR_2 - 1$)
100	356 ± 42 ($n = 4$)	0.1	280 ± 37 ($n = 5$)	5926 ± 160[a] ($n = 7$)	635
5	30 ± 2.5 ($n = 4$)	0.005	6.3 ± 2.7 ($n = 5$)	132 ± 7.5[a] ($n = 6$)	36

[a]Significantly different ($P < 0.05$) from expected combination dose-ratio for two competitive antagonists.

absence [0.66 (0.40–0.90) μM] ($n = 8$) and in the presence of 10 μM methoctramine [0.74 (0.60–1.0) μM] ($n = 4$) or tripitramine [0.66 (0.50–0.90) μM] ($n = 4$) were not significantly different.

Tripitramine and methoctramine (10 μM) did not antagonize histamine-induced responses at H_1-histamine receptors of guinea-pig ileum. The EC_{50} values (± 95% C.F.) for histamine in the absence [0.21 (0.17–0.27) μM] ($n = 10$) and in the presence of tripitramine [0.15 (0.12–0.19) μM] ($n = 5$) or methoctramine [0.18 (0.14–0.23) μM] ($n = 5$) were not significantly different.

Tripitramine (0.3–3 μM) caused a dose-dependent inhibition of the maximum contraction induced by carbachol at muscular nicotinic receptors in frog rectus abdominis with a pIC_{50} value of 6.14 ± 0.07 (Figure 9). This antagonism was reversible, since 60 min washing of the tissues after incubation with tripitramine (1 μM) brought the EC_{50} value (± 95% C.F.) for carbachol [7.03 (6.49–7.90) μM ($n = 5$)] back to the control value [6.85 (6.19–7.60) μM ($n = 5$)]. Under the same experimental conditions methoctramine behaved similarly, the pIC_{50} value was 6.52 ± 0.05 in agreement with results previously reported (Melchiorre *et al.*, 1987b).

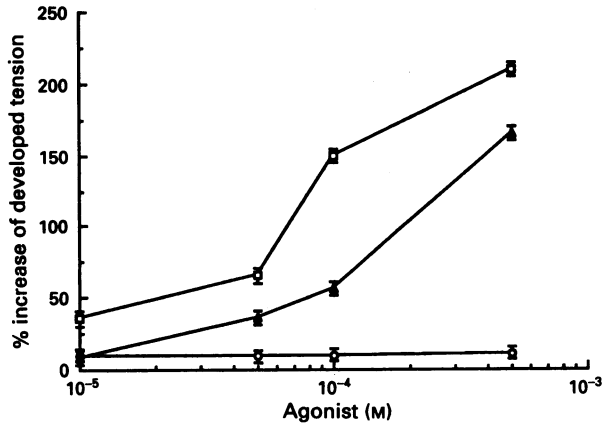

Figure 8 Increase in isometric tension developed by methoctramine (□, △) and tripitramine (○) at β_1-adrenoceptors in electrically driven guinea-pig left atrium. Data from reserpine-pretreated animals are shown only for methoctramine (△) because tripitramine was devoid of activity as in unpretreated animals. Each point is the mean (± s.e.mean) of five observations.

Table 4 Selectivity ratios of tripitramine and methoctramine expressed as the antilog of the difference between the pA_2 values for atrial, tracheal and ileal muscarinic receptors with carbachol used as agonist unless otherwise indicated

Preparation[a]	*Selectivity ratio* *Tripitramine*	*Methoctramine*
Left atrium/ileum	1549	32
Left atrium/ileum[b]	1096	35
Left atrium/trachea	2239	30
Right atrium (force)/ileum	501	19
Right atrium (force)/trachea	724	18
Right atrium (rate)/ileum	692	15
Right atrium (rate)/trachea	1000	15
Right atrium (rate)/right atrium (force)	1	1
Left atrium/lung	60	6
Right atrium (force)/lung	19	4
Right atrium (rate)/lung	27	3
Lung/ileum	26	5
Lung/trachea	37	5
Ileum/trachea	1	1
Rat left atrium/rat ileum	490	65

[a]Tissues were from guinea-pigs unless otherwise indicated. [b]The agonist was oxotremorine in both preparations.

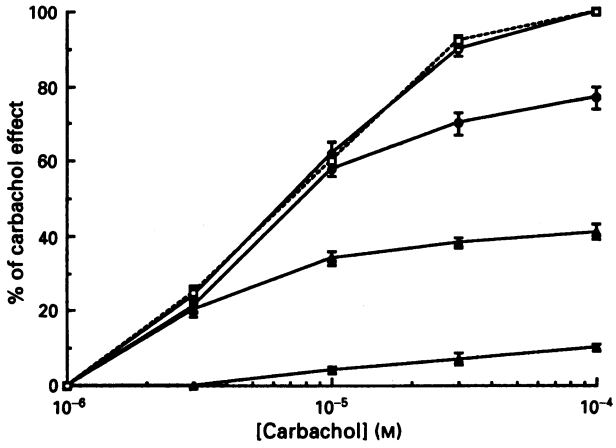

Figure 9 Noncompetitive antagonism of carbachol-induced contractions at muscular nicotinic receptors in frog rectus abdominis. Dose-response curves for carbachol were obtained before (○) and after exposure to 0.3 (●), 1 (▲) and 3 μM (△) tripitramine for 60 min, and after exposure to 1 μM (□) tripitramine for 60 min followed by 60 min washing. Each point is the mean (± s.e.mean) of four to six observations.

Table 5 Receptor systems affected by tripitramine and methoctramine

Receptor gene or subtype	*Tripitramine*	*Methoctramine*
m_1-muscarinic	yes (pK_i, 8.80[a])	yes (pK_i, 7.30[a])
M_1-muscarinic	yes (pK_i, 7.63[b])	yes (pK_i, 7.43[b])
m_2-muscarinic	yes (pK_i, 9.57[a])	yes (pK_i, 7.84[a])
M_2-muscarinic	yes (pA_2, 9.14–9.85; pK_i, 9.54[b])	yes (pA_2, 7.47–7.89; pK_i, 7.84[b])
m_3-muscarinic	yes (pK_i, 7.42[a])	yes (pK_i, 6.56[a])
M_3-muscarinic	yes (pA_2, 6.45–6.81; pK_i, 6.19[b])	yes (pA_2, 5.99–6.35; pK_i, 5.96[b])
m_4-muscarinic	yes (pK_i, 8.19[a])	yes (pK_i, 7.42[a])
M_4-muscarinic	yes (pK_i, 7.93[b])	yes (pK_i, 7.58[b])
m_5-muscarinic	yes (pK_i, 7.47[a])	yes (pK_i, 6.50[a])
α_1-adrenoceptor	no	yes (pK_B, 5.66)
α_2-adrenoceptor	no	no
β_1-adrenoceptor	no	yes[c]
H_1-histamine	no	no
H_2-histamine	no	no
muscular nicotinic	yes (pIC_{50}, 6.14)	yes (pIC_{50}, 6.52)
neuronal nicotinic	yes (pIC_{50}, 4.87)	yes (pIC_{50}, 4.96)

Data from this study and [a]Maggio *et al.* (1994), [b]Melchiorre *et al.* (1993). [c]Methoctramine behaved as a weak agonist. The EC_{50} value was not calculated since no maximum response was attained. At the maximum concentration used (500 μM), an increase in developed tension of 209% was obtained.

Tripitramine (5, 10 and 30 μM) inhibited the maximum relaxation induced at neuronal nicotinic receptors in rat duodenum by 100 μM DMPP with a pIC_{50} value of 4.87 ± 0.08. Under the same experimental conditions methoctramine behaved similarly, the pIC_{50} value amounting to 4.96 ± 0.05.

Tripitramine (10 μM) did not antagonize noradrenaline-induced contraction or clonidine-induced inhibition of twitch responses at α_1- and α_2-adrenoceptors in epididymal and prostatic portions of rat vas deferens, respectively. The EC_{50} values (± 95% C.F.) for noradrenaline or clonidine in the absence [0.18 (0.14–0.23) μM ($n = 5$) and 4.12 (3.50–4.72) nM ($n = 4$), respectively] and in the presence of tripitramine [0.19 (0.12–0.28) nM ($n = 5$) and 2.83 (2.30–3.63) nM ($n = 4$), respectively] were not significantly different. Under the same experimental conditions methoctramine (10 μM) did not affect clonidine-induced responses whereas it caused a parallel shift to the right of noradrenaline dose-response curves without affecting the maximum response. The EC_{50} values (± 95% C.F.) for noradrenaline in the absence and presence of methoctramine were [0.18 (0.14–0.23) μM ($n = 5$)] and [1.01 (0.94–1.10) μM ($n = 4$)] which allowed calculations of a pK_B value of 5.66 ± 0.03.

Discussion

The heterogeneity of muscarinic receptors is well documented and multiple receptor subtypes have been characterized (Caulfield, 1993). For this reason in the last decade there has been a resurgence of interest in muscarinic receptors owing to the observation that multiple subtypes may exist both in the central nervous system and periphery, which may represent potential targets for therapeutically useful drugs (Hulme *et al.*, 1990; McKinney & Coyle, 1991). Our effort to determine the structural elements of polymethylene tetraamines which confer selectivity toward a muscarinic receptor subtype, rather than to another (Melchiorre *et al.*, 1987b; 1989; Melchiorre, 1990) led to the design of tripitramine (Melchiorre *et al.*, 1993). This new tetraamine incorporates (Figure 1) the structural features of not only methoctramine but also pirenzepine, a selective muscarinic M_1 receptor antagonist (Hammer *et al.*, 1980), and of its analogues AF-DX 116 and AQ-RA 741, selective muscarinic M_2 receptor antagonists (Eberlein *et al.*, 1992; Hammer *et al.*, 1986). Preliminary results showed that tripitramine is not only more potent but also significantly more selective than the two prototypes methoctramine and AQ-RA 741 for muscarinic M_2 receptors (Melchiorre *et al.*, 1993). Furthermore, tripitramine displayed a unique binding profile for muscarinic receptor subtypes: $M_2 > M_4 \geq M_1 > M_3$. Clearly, it was able to distinguish among M_2 and all other muscarinic receptor subtypes investigated, M_2 to M_4, as well as between M_1 and M_3, and M_4 and M_3 subtypes (Melchiorre *et al.*, 1993). Furthermore, tripitramine displayed a similar binding profile ($m_2 > m_1 > m_4 > m_3 = m_5$) at human cloned muscarinic receptors (Table 5) (Maggio *et al.*, 1994). Thus, it was demonstrated that tripitramine discriminates markedly between both native and cloned muscarinic M_2 and M_4 receptor subtypes with a selectivity ratio value ranging from 24 to 41 (Melchiorre *et al.*, 1993; Maggio *et al.*, 1994). This finding may have relevance since all selective muscarinic M_2 receptor antagonists available to date, bind to muscarinic M_2 and M_4 receptors with similar affinities. Methoctramine displayed only 2 fold higher affinity for muscarinic m_2 compared to m_4 receptors. Similarly, Dörje *et al.* (1991) found that methoctramine, himbacine, AF-DX 250, AF-DX 384 and AQ-RA 741, all classified as selective muscarinic M_2 receptor antagonists, possess similar affinities at both muscarinic m_2 and m_4 receptor subtypes. We have now investigated further the antimuscarinic effects of tripitramine in comparison to methoctramine in atrial, tracheal, lung strip and ileal preparations derived from different species and with different agonists. Furthermore, antagonist combination experiments were carried out with atropine in order to verify the mechanism of action of tripitramine. In addition, tripitramine effects were determined also on other receptors, namely α_1-, α_2-, and β_1-adrenoceptors, H_1- and H_2-histamine receptors, and muscular and neuronal nicotinic receptors to assess its receptor specificity.

Tripitramine was an apparently simple competitive muscarinic receptor antagonist in all preparations investigated, namely right and left atria, ileum, trachea and lung strip, since it produced antagonism that was reversible and surmountable over a wide range of concentrations (Figures 2, 6 and 7). Schild analysis of the data yielded slopes that were not significantly different from unity (Table 1). It is known that the slope of a Schild regression is of paramount importance to assess the type of antagonism to an agonist response in a tissue. Competitive antagonism requires linear Schild regressions with a slope equal to unity. It is not unusual, however, that Schild regressions for competitive antagonists are not linear or have slopes significantly different from unity and several factors may be accounted for. Irrespective of agonist and species used, tripitramine failed to produce a linear Schild regression over the concentration-range investigated in left atria (Figure 3). At higher concentrations (0.015–3 μM) tripitramine gave linear plots with slopes not significantly different from unity as required for a competitive antagonist (Table 1). However, at lower concentrations (< 0.015 μM) a deviation from linearity was observed and in the 3–10 nM range the Schild plots had a slope of the regression line significantly higher than unity. Slopes greater than unity may be obtained when (a) there are nonequilibrium steady states or (b) a saturable removal mechanism for the antagonist is present in the tissue (Kenakin, 1993). In the first hypothesis, low K_B estimates were obtained and this phenomenon is particularly true for low concentrations which may require longer time of onset of equilibrium than high concentrations. It was observed indeed that the maximum effect of tripitramine was reached within 60 min for concentrations > 10 nM and 120 min for concentrations ≤ 10 nM (Figure 4). Although the tissues were incubated for the time necessary to reach the maximum effect of tripitramine, a nonlinear Schild plot (Figure 3) was obtained for lower concentrations. It appears that the low K_B estimates for tripitramine (≤ 10 nM) can hardly be ascribed to nonequilibrium steady states. As a consequence, it may well be that guinea-pig and rat left atria have a saturable removal mechanism for tripitramine. In this case the concentration of tripitramine in the receptor compartment will be considerably lower than that in the organ bath because of the presence of a removal site for the antagonist thus accounting for the low K_B estimates until the concentration of tripitramine reaches a value (> 10 nM) sufficient to saturate the removal process. If confirmed, this phenomenon would not be surprising since it has already been observed for atropine in rabbit ileum (Kenakin, 1993; Kenakin & Beek, 1987) and mouse urinary bladder as well as for N-methylatropine in mouse urinary bladder (Durant *et al.*, 1991). These tissues possess an esterase enzyme that degrades atropine leading to nonlinear Schild regression with a slope greater than unity and an anomalous pA_2 value. The regression of the line was reduced to unity by blocking the enzyme with an excess of 4-methylbutyrate which acted as an alternative substrate preventing atropine degradation. Interestingly, atropinesterase was not found in guinea-pig ileum (Kenakin, 1993; Kenakin & Beek, 1987). Similarly, the presence of a possible saturable removal mechanism for tripitramine in guinea-pig left atria as well as in rat left atria was shown but not in guinea-pig right atria. However, atropinesterase cannot be the enzyme responsible for tripitramine degradation since tripitramine lacks an ester function. Considering the structure of tripitramine, two functionalities might be the target of enzymes, that is four amine functions and six amide groups. Since polyamines such as spermidine and spermine are

biodegraded by the enzyme polyamine oxidase, we investigated whether this enzyme might also be responsible for tripitramine degradation. However, experiments performed in the presence of N,N'-*bis*(2,3-butanedienyl)-1,4-butanediamine, an irreversible inhibitor of polyamine oxidase (Bey *et al.*, 1985), ruled out this possibility since its presence in the bath did not modify the effect of tripitramine at low concentrations. Further experiments are needed to clarify the mechanism underlying tripitramine removal from receptor compartment. However, we can safely exclude the possibility that tripitramine removal is due to an artefact, such as binding to glassware used in the experiments at low concentrations of tripitramine, because the phenomenon was not observed in guinea-pig right atria with the same glassware and, what is more important, the same low concentrations (up to 3 nM). Whatever the reason is, either a nonequilibrium steady state or a saturable removal process, it can be assumed that the pA_2 values, ranging from 9.14 ± 0.09 to 9.85 ± 0.04, calculated at high concentrations (0.015–3 μM) may represent the true dissociation constant of tripitramine in rat and guinea-pig left atria.

Clearly, the affinity of tripitramine at muscarinic M_2 receptors is about three orders of magnitude greater than that at muscarinic M_3 receptors in ileal and tracheal preparations (Table 1). Tripitramine displayed a selectivity ratio ranging from 490 to 1820 (Table 4), which is in agreement with results previously reported (Melchiorre *et al.*, 1993). Thus, the M_2/M_3 selectivity profile of tripitramine is qualitatively analogous to that of methoctramine, but it is evident that the cardioselectivity of tripitramine is much greater (Table 4). Furthermore, a comparison between tripitramine and AQ-RA 741, another muscarinic receptor antagonist classified as M_2-selective, reveals that tripitramine has a better affinity profile since AQ-RA 741 displayed a M_2/M_3 selectivity ratio value of about 80 and a pA_2 value around 8 for cardiac muscarinic M_2 receptors (Eberlein *et al.*, 1989) which are markedly lower than those of tripitramine.

One most intriguing finding of the present investigation was the result obtained in guinea-pig lung strip preparation. Using a number of subtype selective muscarinic receptor antagonists, Roffel *et al.* (1993) suggested that cholinergic contraction of the guinea-pig lung strip is mediated by muscarinic M_2-like receptors. The affinity of tripitramine for muscarinic receptor subtypes in guinea-pig lung does not appear, however, in agreement with the conclusion reached by Roffel *et al.* (1993). The pA_2 values of 7.91 ± 0.03 for tripitramine in guinea-pig lung does not correlate with the affinity found for both muscarinic M_2 and M_3 receptor subtypes in atria and smooth muscles (ileum and trachea) where pA_2 values of 9.14–9.85 and 6.45–6.81 were obtained, respectively (Table 1). In parallel experiments in guinea-pig lung strips, methoctramine displayed an affinity value of 6.97 ± 0.07 which is similar to that (pA_2, 7.30 ± 0.06) reported by Roffel *et al.* (1993). An analysis of the results shown in Table 5 reveals that the functional affinity of tripitramine in guinea-pig lung correlates well with the affinity at either native or cloned muscarinic receptors only for the M_4 subtype. However, the affinity of tripitramine for cloned muscarinic m_3 and m_5 receptors is only 3 fold lower than that in guinea-pig lung strip. Nevertheless, on the basis of tripitramine affinity profile, it can be suggested that the contraction of guinea-pig lung strip is not mediated by muscarinic M_2 receptors. It remains to be established, however, whether the M_4 subtype or, as suggested by Roffel *et al.* (1993), a novel subtype or a mixture of muscarinic receptor subtypes is involved in the contractile process of guinea-pig lung strip.

The mutual competition between tripitramine and atropine was investigated in electrically stimulated left atria and in ileum according to the dose-ratio method (Paton & Rang, 1965). As shown in Table 2, in the atria a combination of tripitramine and atropine resulted in dose-ratios equal to the sum of the dose-ratios produced by the two antagonists when tested alone. This suggests that tripitramine and atropine compete for the same receptor site. These results are very similar to those reported for a combination of methoctramine and atropine (Melchiorre *et al.*, 1987a).

Experiments performed in guinea-pig ileum with a combination of tripitramine and atropine gave results which were not consistent with a competitive mode of action of tripitramine (Table 3). In fact, the combination dose-ratios were somewhat greater than expected, but these results are not consistent with the multiplication of dose-ratios which would be expected if two antagonists interacted exclusively at independent sites. It follows that tripitramine and atropine compete for two distinct but interdependent sites. Thus tripitramine may interact with muscarinic M_3 receptors in an allosteric fashion and, consequently, its dissociation constants derived in guinea-pig and rat ileum and guinea-pig trachea may be incorrect and should be considered as apparent pA_2 values. Again these results are very similar to those reported for a combination of methoctramine and atropine (Melchiorre *et al.*, 1987a).

Since methoctramine was shown to antagonize rat vas deferens α_1-adrenoceptors (Melchiorre *et al.*, 1987b), muscular (Melchiorre *et al.*, 1987b) and neuronal nicotinic receptors (Watson *et al.*, 1992), and to display positive inotropic activity at β_1-adrenoceptors in guinea-pig left atria (Eglen *et al.*, 1988), tripitramine was investigated also in other receptor preparations to assess its receptor specificity in comparison to methoctramine. Experiments performed in electrically stimulated guinea-pig left atria revealed that tripitramine, unlike methoctramine, did not induce positive inotropic responses (Figure 8). Methoctramine elicited a modest positive inotropic activity which was more pronounced in tissues from unpretreated than from reserpine-pretreated animals in contrast with results previously reported (Eglen *et al.*, 1988). However, it should be noted that, though the chronic pretreatment with reserpine increases sensitivity to β-adrenoceptor agonists, reserpine induced changes may be species-dependent since guinea-pigs failed to reveal a significant receptor increase, whereas rats did (Chess-Williams *et al.*, 1986). In addition, the time course and dosage of reserpine may be of relevance, since an increase in receptor number in rat cardiac tissue was found after seven days but not after one or three days of treatment (Chess-Williams *et al.*, 1986).

Experiments performed on frog rectus abdominis showed that tripitramine, like methoctramine (present results and Melchiorre *et al.*, 1987b), inhibited muscular nicotinic receptors with a noncompetitive mechanism because the maximum response to carbachol was depressed (Figure 9). Furthermore, this blockade was reversed after tissue washing. Tripitramine displayed a pIC_{50} value of 6.14 ± 0.07 that was slightly lower than that obtained for methoctramine (6.52 ± 0.05). Watson *et al.* (1992) reported that methoctramine has antagonistic activity in the micromolar range at ganglionic nicotinic receptors. Very recently, Irie *et al.* (1994) showed that the action at neuronal nicotinic receptors can be easily investigated in rat isolated duodenum. It turned out indeed that both tripitramine and methoctramine inhibited with modest potency neuronal nicotinic receptors with pIC_{50} values of 4.87 ± 0.08 and 4.96 ± 0.05, respectively. In all other experiments performed tripitramine did not antagonize α_1-, α_2-, and β_1-adrenoceptors, and H_1- and H_2-histamine receptors (Table 5). Methoctramine gave similar results but it antagonized noradrenaline-induced responses in rat vas deferens α_1-adrenoceptors with a pK_B value of 5.66 ± 0.03, which is in agreement with results previously reported (Melchiorre *et al.*, 1987b).

As shown in Table 5, it is evident that tripitramine has not only an outstanding affinity and selectivity for M_2 muscarinic receptors but also a superior receptor specificity compared with methoctramine. Furthermore, in guinea-pig lung strip, tripitramine displayed a marked lower potency than that observed at cardiac muscarinic M_2 receptors allowing the

conclusion that the contraction in the guinea-pig lung strip is not mediated by muscarinic M_2-like receptors. Beside muscarinic receptors, tripitramine antagonized with a modest potency, among receptor systems investigated so far, only muscular and neuronal nicotinic receptors whereas methoctramine affected, in addition, α_1- and β_1-adrenoceptors.

In conclusion, tripitramine represents the most potent and the most selective muscarinic M_2 receptor antagonist at present available. It may serve as a powerful tool for the characterization of muscarinic receptor subtypes.

Supported by MURST (Rome).

References

ANWAR-UL, S., GILANI, H. & COBBIN, L.B. (1986). The cardioselectivity of himbacine: a muscarine receptor antagonist. *Naunyn-Schmied. Arch. Pharmacol.*, **332**, 16–20.

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

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

BEY, P., BOLKENIUS, F.N., SEILER, N. & CASARSA, P. (1985). N-2,3-butadienyl-1,4-butanediamine derivatives: potent irreversible inactivators of mammalian polyamine oxidase. *J. Med. Chem.*, **28**, 1–2.

BOGNAR, I.T., ALTER, U., BEINHAUER, C., KESSLER, I. & FUDER, H. (1992). A muscarinic receptor different from M_1, M_2, M_3 and M_4 subtypes mediates the contraction of the rabbit iris sphincter. *Naunyn-Schmied. Arch. Pharmacol.*, **345**, 611–618.

BONNER, T.I. (1989). The molecular basis of muscarinic receptor diversity. *Trends Neurosci.*, **12**, 148–151.

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

CAULFIELD, M.P. & BROWN, D.A. (1991). Pharmacology of the putative M_4 muscarinic receptor mediating Ca-current inhibition in neuroblastoma glioma (NG 108-15) cells. *Br. J. Pharmacol.*, **104**, 39–45.

CHESS-WILLIAMS, R.G., BROADLEY, K.J. & SHERIDAN, D.J. (1986). Calculated and actual changes in β-adrenoceptor number associated with increases in rat and guinea pig cardiac sensitivity. *J. Pharm. Pharmacol.*, **38**, 902–906.

CLARK, A.L. & MITCHELSON, F. (1976). The inhibitory effect of gallamine on muscarinic receptors. *Br. J. Pharmacol.*, **58**, 323–331.

DÖRJE, F., FRIEBE, T., TACKE, R., MUTSCHLER, E. & LAMBRECHT, G. (1990). Novel pharmacological profile of muscarinic receptors mediating contraction of the guinea pig uterus. *Naunyn-Schmied. Arch. Pharmacol.*, **342**, 284–289.

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.

DURANT, P.A.C., SHANKLEY, N.P., WELSH, N.J. & BLACK, J.W. (1991). Pharmacological analysis of agonist-antagonist interactions at acetylcholine muscarinic receptors in a new urinary bladder assay. *Br. J. Pharmacol.*, **104**, 145–150.

EBERLEIN, W.G., ENGEL, W., HASSELBACH, K.M., MAYER, N., MIHM, G., RUDOLF, K. & DOODS, H. (1992). Tricyclic compounds as selective muscarinic antagonists: structure activity relationships and therapeutic implications. In *Trends in Receptor Research*. ed. Angeli, P., Gulini, U. & Quaglia, W. pp. 231–249. Amsterdam: Elsevier Science Publishers B.V.

EBERLEIN, W.G., ENGEL, W., MIHM, G., RUDOLF, K., WETZEL, B., ENTZEROTH, M., MAYER, N. & DOODS, H. (1989). Structure-activity relationships and pharmacological profile of selective tricyclic antimuscarinics. *Trends Pharmacol. Sci.*, **10** (Suppl.), 50–54.

EGLEN, R.M., MICHEL, A.D. & WHITING, R.L. (1989). Characterization of the muscarinic receptor subtype mediating contractions of the guinea-pig uterus. *Br. J. Pharmacol.*, **96**, 497–499.

EGLEN, R.M., MONTGOMERY, W.W., DAINTY, I.A., DUBUQUE, L.K. & WHITING, R.L. (1988). The interaction of methoctramine and himbacine at atrial, smooth muscle and endothelial muscarinic receptors *in vivo*. *Br. J. Pharmacol.*, **95**, 1031–1038.

FRIEBE, T.P., MUTSCHLER, E. & LAMBRECHT, G. (1993). Muscarinic receptor subtypes: pharmacological characterization, molecular structure, location, function and receptor-effector coupling. *Pharm. Ztg. Wiss.*, **138**, 3–11.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Handbook of Experimental Pharmacology, Catecholamines*, vol. 33. ed. Blashko, H. & Muscholl, E. pp. 283–335. New York: Springer-Verlag.

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

HAMMER, R., GIRALDO, E., SCHIAVI, G.B., MONFERINI, E. & LADINSKY, H. (1986). Binding profile of a novel cardioselective muscarine receptor antagonist, AF-DX 116, to membranes of peripheral tissues and brain in the rat. *Life Sci.*, **38**, 1653–1662.

HULME, E.C., BIRDSALL, N.J.M. & BUCKLEY, N.J. (1980). Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 633–673.

IRIE, K., FURUKAWA, K., NOMOTO, T., FUJII, E. & MURAKI, T. (1994). Developmental changes in the response of rat isolated duodenum to nicotine. *Eur. J. Pharmacol.*, **251**, 75–81.

KENAKIN, T.P. (1993). Competitive antagonism. In *Pharmacologic Analysis of Drug-Receptor Interaction*. pp. 278–322. New York: Raven Press.

KENAKIN, T.P. & BEEK, D. (1987). The effects on Schild regressions of antagonist removal from the receptor compartment by a saturable process. *Naunyn-Schmied. Arch. Pharmacol.*, **335**, 103–108.

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

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

MAGGIO, R., BARBIER, P., BOLOGNESI, M.L., MINARINI, A., TEDESCHI, D. & MELCHIORRE, C. (1994). Binding profile of the selective muscarinic receptor antagonist tripitramine. *Eur. J. Pharmacol. Mol. Pharmacol. Sect.*, **268**, 459–462.

McKINNEY, M. & COYLE, J.T. (1991). The potential for muscarinic receptor subtype-specific pharmacotherapy for Alzheimer's disease. *Mayo Clin. Proc.*, **66**, 1225–1237.

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

MELCHIORRE, C. (1990). Polymethylene tetraamines: a novel class of cardioselective M_2-antagonists. *Med. Res. Rev.*, **10**, 327–349.

MELCHIORRE, C., ANGELI, P., LAMBRECHT, G., MUTSCHLER, E., PICCHIO, M.T. & WESS, J. (1987a). Antimuscarinic action of methoctramine, a new cardioselective M-2 muscarinic receptor antagonist, alone and in combination with atropine and gallamine. *Eur. J. Pharmacol.*, **144**, 117–124.

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. (1987b). 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., QUAGLIA, W., PICCHIO, M.T., GIARDINA', D., BRASILI, L. & ANGELI, P. (1989). Structure-activity relationships among methoctramine-related polymethylene tetraamines. Chain-length and substituent effects on M-2 muscarinic receptor blocking activity. *J. Med. Chem.*, **32**, 79–84.

MICHEL, A.D., DELMONDO, R., STEFANICH, E. & WHITING, R.L. (1989). Binding characteristics of the muscarinic receptor subtype of the NG 105-18 cell line. *Naunyn-Schmied. Arch. Pharmacol.*, **340**, 62–67.

MUSKUS, A.J. (1962). Effect of pretreatment with reserpine analogs on the response of isolated guinea-pig atria to tyramine. *J. Pharmacol. Exp. Ther.*, **138**, 296–300.

PATON, W.D.M. & RANG, H.P. (1965). The uptake of atropine and related drugs by intestinal smooth muscle of the guinea pig in relation to acetylcholine receptors. *Proc. R. Soc. B.*, **163**, 1–44.

ROFFEL, A.D., ELZINGA, C.R.S. & ZAAGSMA, J. (1993). Cholinergic contraction of the guinea pig lung strip is mediated by muscarinic M_2-like receptors. *Eur. J. Pharmacol.*, **250**, 267–279.

STOCKTON, J.M., BIRDSALL, N.J.M., BURGEN, A.V.S. & HULME, E.C. (1983). Modification of the binding properties of muscarinic receptors by gallamine. *Mol. Pharmacol.*, **23**, 551–557.

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.

TALLARIDA, R.J. & MURRAY, R.B. (1991). pA_2 analysis I: Schild plot. In *Manual of Pharmacologic Calculations With Computer Programs*, Version 4.2. pp. 53–56. New York: Springer-Verlag.

TEMMA, K., AKERA, T., BRODY, T. & MANNIAN, A.A. (1977). Hydroxylated chloropromazine metabolite: positive inotropic action and the release of catecholamines. *Mol. Pharmacol.*, **13**, 1076–1085.

VAN ROSSUM, J.M. (1963). Cumulative dose-response curves. Techniques for the making of dose-response curves in isolated organs and the evaluation of drug parameters. *Arch. Int. Pharmacodyn. Ther.*, **143**, 299–330.

WAELBROECK, M., CAMUS, J., TASTENOY, M., MUTSCHLER, E., STROHMANN, C., TACKE, R., LAMBRECHT, G. & CHRISTOPHE, J. (1991). Binding affinities of hexahydro-difenidol and hexahydro-sila-difenidol analogues at four muscarinic receptor subtypes: constitutional and stereochemical aspects. *Eur. J. Pharmacol. Mol. Pharmacol. Sect.*, **206**, 95–103.

WATSON, N., BARNES, P.J. & MACLAGAN, J. (1992). Actions of methoctramine, a muscarinic M_2 receptor antagonist, on muscarinic and nicotinic cholinoceptors in guinea-pig airways *in vivo* and *in vitro*. *Br. J. Pharmacol.*, **105**, 107–112.

WESS, J. (1993). Molecular basis of muscarinic acetylcholine receptor function. *Trends Pharmacol. Sci.*, **14**, 308–313.

(Received September 9, 1994
Revised December 13, 1994
Accepted December 15, 1994)