Biochemical Pharmacology, Vol. 46, No. 6, pp. 933–1003, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

THE INTERACTION OF McN-A-343 WITH MUSCARINE RECEPTORS IN CARDIAC AND SMOOTH MUSCLE

A. ELNATAN and F. MITCHELSON*

School of Pharmacology, Victorian College of Pharmacy (Monash University), 381 Royal Parade, Parkville, Australia 3052

(*Received* 25 *February* 1993; *accepted* 7 *June* 1993)

Abstract—The interaction of the muscarine receptor partial agonist (4-*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride (McN-A-343) was investigated at muscarine receptors in the atria and taenia caeci of the guinea-pig to compare its interaction at the muscarine M_2 receptor in the two tissues. In the smooth muscle, the muscarine M_3 receptor subtype is responsible for the contractile response but the major subtype detected in binding or antibody experiments is the M_2 subtype. In guinea pig atria the dissociation constant of McN-A-343 at muscarine receptors was 15.2 μM determined in functional experiments on left atria in McEwen's solution or 14.8 μM in binding experiments with [^{3}H]-(−)-quinuclidinyl benzilate ([^{3}H]QNB) in the same medium containing 5′-guanylylimidodiphosphate (50 μM). In the taenia caeci, the dissociation constant estimated for McN-A-343 at the M_3 receptor from functional experiments based on the contractile response to the agonist in McEwen's solution was 4.6 μM. This value was similar to the dissociation constant (6.2 μM) estimated from binding studies versus [^{3}H]QNB conducted in the same medium although studies with 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine 6-one (AF-DX 116) versus [^{3}H]-(−)-*N*-methylscopolamine suggested that 70% of the receptors were the M_2 subtype. The presence of the M_2 subtype in the taenia caeci was also confirmed by the ability of oxotremorine to inhibit the increase in cAMP produced by isoprenaline (10 μM) since apparent pK_B values for AF-DX 116 and hexahydrosiladiphenidol were 6.95 and 6.75, respectively. McN-A-343 (100 μM) failed to inhibit the response to isoprenaline and did not antagonize the inhibitory response to oxotremorine. It is concluded that the apparent affinity of McN-A-343 for muscarine M_2 receptors in the atria and the taenia caeci differs and a number of explanations are discussed.

Smooth muscle in the intestinal tract contains a mixture of M_2 and M_3 muscarine receptors, the bulk of the receptors being the M_2 subtype although the M_3 receptor is the subtype responsible for the contractile response to muscarine receptor agonists [1–3].

As a consequence, 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*]-[1,4]benzodiazepine 6-one (AF-DX 116†) and other muscarinic receptor antagonists with higher affinity for M_2 than for M_3 receptors, appear to bind to two sites in displacing (−)-*N*-methylscopolamine [^{3}H] NMS from ileal smooth muscle. The high affinity site, constituting *ca.* 70–80% of the total sites, corresponds to the M_2 receptor, while the lower affinity site (20–30%) appears to correspond to the muscarine M_3 receptor site that activates contraction of the smooth muscle or salivary gland secretion [1–4].

In support, studies using *in situ* hybridization have detected mRNA for the M_2 receptor in ileal smooth muscle [5] and investigations with antibodies raised against cloned receptors have demonstrated that 65–70% of the muscarine receptors in the ileum are of the M_2 subtype [6, 7]. Some inconsistencies persist; in many cases the absolute affinities of antagonists for the binding sites in the studies with [^{3}H]NMS do not agree with the affinity determined in functional studies [1, 4] but this may be due to the variations in the composition of the incubation mediums used in binding and functional studies [8, 9]. Also, immunological studies have determined that the proportion of M_3 receptors in the ileum is $<4\%$ [6, 7], a far lower estimate than that determined in binding studies. Stable GTP analogues are known to produce a greater decrease in agonist affinity in binding studies in cardiac tissue than in intestinal muscle [10], a finding which suggests that the M_2 receptor in the two tissues may be coupled differently. Further, the muscarine receptor antagonist UH-AH 37 shows high affinity for M_3 receptors in functional studies in ileum [11, 12] and in binding studies on cloned m3 receptors [12] but low affinity for glandular M_3 receptors [11] suggesting that M_3 receptors in ileum and glandular tissue may not be identical.

Recently, displacement studies with the partial agonist (4-*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride (McN-A-343) versus (−)-quinuclidinyl benzilate ([^{3}H]QNB) binding detected only a single binding site for the agonist in smooth muscle from the guinea pig taenia caeci and

* Corresponding author.

† Abbreviations: AF-DX 116, 11-[[2-[(diethylamino)-methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine 6-one; EGTA, ethylene glycol-bis-(β-aminoethylether)*N,N′*-tetra acetic acid; Gpp(NH)p, 5′-guanylylimidodiphosphate; HHSiD, hexahydrosiladiphenidol; IBMX, 3-isobutyl-1-methyl-xanthine; McN-A-343, (4-*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride; NMS, (−)-*N*-methylscopolamine; QNB, (−)-quinuclidinyl benzilate; PSS, physiological salt solution; AUFS, absorbance units full scale.

the K_i value was of a similar order to the K_A or K_B values found in functional experiments on this tissue with McN-A-343 [13]. Consequently a more detailed investigation of the affinity of the agonist in cardiac and smooth muscle was undertaken to determine whether studies with an agonist would support the hypothesis, based on studies with antagonists, that a major population of M_2 muscarine receptors exists in intestinal smooth muscle similar to M_2 receptors in cardiac muscle.

MATERIALS AND METHODS

Materials

Materials were obtained from the following suppliers: McN-A-343, Research Biochemicals Inc. (Natick, MA, U.S.A.); [^{3}H]QNB and [^{3}H]NMS, Amersham (Sydney, Australia); AF-DX 116, Thomae (Biberach an der Riss, Germany); hexahydrosiladiphenidol (HHSiD), Profs E. Mutschler and G. Lambrecht (Frankfurt, Germany); carbon tetrachloride, British Drug House (Poole, U.K.); tetrodotoxin, Calbiochem (La Jolla, CA, U.S.A.); methanol (HPLC grade), Mallinckrodt (Paris, TX, U.S.A.); oxotremorine, ICN Pharmaceuticals (Plainview, NY, U.S.A.); atropine sulphate, carbamylcholine chloride (carbachol), 5'-guanylyl-imidodiphosphate (Gpp(NH)p), polyethylenimine, 3-isobutyl-1-methyl-xanthine (IBMX), isoprenaline, tri-*N*-octylamine, and ethylene glycol-bis-(β-aminoethylether)*N,N'*-tetra acetic acid (EGTA), the Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Isolated tissue experiments

Left atria. Guinea pig left atria were suspended between two platinum wire electrodes (approx. 5 mm apart) under 1 g tension in a 10 mL organ bath containing McEwen's solution [14], gassed with 5% CO_2, 95% O_2, at 32° and stimulated at a frequency of 3 Hz, a duration of 2 msec, and 1.5 × the voltage producing a maximal contractile response. Responses were recorded using an isometric transducer (Neomedix Systems) connected to a Neotrace Model NEO200ZEF recorder. After a 30–45 min equilibration period a concentration–response curve for the negative inotropic effect of carbachol (50–400 nM) was obtained. Each single dose was given a 90–120 sec contact time to establish the response fully followed by a 5 min rest between washout and addition of the next concentration.

After a 15 min incubation with McN-A-343 (50, 100 or 300 μM) the concentration–response curve to carbachol was re-established, McN-A-343 being replaced in the organ bath after each washout.

Analysis of results. The K_B for McN-A-343 in the atria was estimated by the method described by Kaumann and Blinks [15], which utilizes the inhibitory action of a partial agonist on the responses to a full agonist. Equiactive concentrations of carbachol in the absence and presence of McN-A-343 were plotted as the ordinate and abscissa, respectively to give a linear plot. The slope of the plot was then used to estimate K_B according to the equation:

$$K_B = \frac{[A]}{1 - \text{slope}} - [A] \qquad (1)$$

where [A] denotes the concentration of McN-A-343 used, and "slope" is the slope of the regression line fitted through the data points of the plot of the equiactive concentrations of carbachol in the absence versus the presence of McN-A-343. In this method the responses to all concentrations of carbachol were expressed as a per cent inhibition of the maximum inotropic response (contraction) of the tissue with no drug present. All lines of best fit through the data points were obtained using the computer program *Fig. P.* (Elsevier-Biosoft).

A second method of estimating the K_B value was also employed which ignored the negative inotropic effect of McN-A-343. Each response to carbachol was expressed as a per cent inhibition of the inotropic response immediately prior to its addition. The resulting dose-ratios from the parallel shifts in the concentration–response curves were used for Arunlakshana–Schild (A–S) plots [16] of log (dose-ratio − 1) vs log [McN-A-343] with concentrations of McN-A-343 in the range of 50–300 μM. The pA_2 value was calculated from the regression line together with a K_B estimate when the slope of the plot was constrained to unity.

Taenia caeci. Lengths of guinea pig taenia caeci (2–3 cm) were dissected from the medial section of the caecum and suspended under 0.5 g tension in a 10 mL organ bath containing McEwen's or Tyrode's solution gassed with 5% CO_2, 95% O_2 at 32°. Contractile activity was recorded on a Neotrace Model NEO200ZEF recorder via an isotonic transducer (Ugo Basile 7006). After an equilibration period of 30–60 min, carbachol (1 nM to 5 μM) and McN-A-343 (0.1 μM to 0.1 mM) were administered as single concentrations with an interval of 10–20 min between each washout and addition.

Analysis of results. Data point obtained from the concentration–response relationship were fitted to a logistic function [17] by the method of least squares. The affinity of McN-A-343 (K_P) was estimated from a modified double reciprocal plot [18] using the equation:

$$[A] = \frac{K_A}{(\varepsilon_A/\varepsilon_P) - 1} + \frac{K_P}{(\varepsilon_P/\varepsilon_A) - 1} \cdot \frac{[A]}{[P]} \qquad (2)$$

where [A] and [P] are equiactive concentrations, ε_A and ε_P are intrinsic efficacies, and K_A and K_P are equilibrium dissociation constants of carbachol and McN-A-343, respectively.

K_P was estimated from the slope of the regression line of the plot [A] vs $[A]\cdot[P]^{-1}$ since:

$$K_P = \text{slope}\left(\frac{\varepsilon_P}{\varepsilon_A} - 1\right). \qquad (3)$$

The correction factor $\varepsilon_P/\varepsilon_A$ was estimated using the method devised by Furchgott [19] by plotting responses to agonists against receptor occupancy. The relative intrinsic efficacy was estimated from the relationship:

$$\frac{\varepsilon_P}{\varepsilon_A} = \frac{P_A}{P_P} \qquad (4)$$

where $\varepsilon_P/\varepsilon_A$ is the relative intrinsic efficacy of McN-A-343 to carbachol and P_A/P_P is the relative receptor occupancy of carbachol to McN-A-343.

Radioligand binding experiments

Radioligand binding experiments were carried out using McEwen's solution pre-gassed with 5% CO_2, 95% O_2 prior to experiments.

Dissected atria and taenia caeci were homogenized separately in 35 and 100 vol. (w/v) of ice-cold McEwen's solution, respectively, using an Ultra Turrax homogenizer (Medium setting, 2 × 30 sec) and centrifuged at 1000 *g* for 10 min, at 0°. The resulting supernatant was decanted and used for the radioligand binding assay.

Competition assays between [^{3}H]QNB (60 pM) and McN-A-343 (20 nM to 1 mM) were performed in duplicate at 32° for 3 hr in the presence and absence of Gpp(NH)p (50 μM) in a total incubation volume of 1 mL (using 50 μL of tissue aliquot (equivalent to *ca.* 75 μg protein in atria, 25 μg protein in taenia). Incubations were terminated with the addition of 5 mL ice-cold McEwen's solution, followed by rapid aspiration through glass fibre filters (Whatman GF/B) using a Brandel cell harvester. Filters were washed with two additional 5 mL of McEwen's solution, dried by suction, and following addition of 5 mL Filtercount (Packard), binding of the [^{3}H]QNB to the filter membranes was determined by liquid scintillation counting. Non-specific binding (*ca.* 5% in both tissues) was determined using atropine (10 μM). All filters used in the assay were presoaked for at least 15 hr in a solution containing polyethylenimine (0.5%,v/v) and atropine (10 μM) prior to the assay. In some experiments 50 mM Na-phosphate buffer (pH 7.4) was used in place of McEwen's solution.

The binding of AF-DX 116 was investigated in the taenia caeci using both [^{3}H]NMS and [^{3}H]QNB as the ligand in McEwen's solution with a 60- and 180-min incubation period, respectively. Non-specific binding with [^{3}H]NMS was *ca.* 15%; other details as above.

All data were analysed using the computer program EBDA [20] to obtain initial equilibrium dissociation parameter estimates followed by LIGAND [21] for final estimates based on the [^{3}H]-QNB K_D value of 41.1 pM or the [^{3}H]NMS K_D value of 0.28 nM.

EBDA converted the dpm output data of the liquid scintillation counter into per cent binding and into a file compatible with the input format of LIGAND. Initial equilibrium dissociation parameters of single or multiple binding sites were estimated by EBDA based on the Eadie–Hofstee plot. Using a non-linear iterative curve fitting technique and the initial estimates obtained from EBDA, LIGAND estimated final equilibrium dissociation constants for single or multiple binding sites. The appropriateness of a one or two site model fit was compared by a one way analysis of variance of the sum of squares of the two models. $P < 0.05$ was deemed significant.

Statistical analysis

Comparison of group geometric mean values was undertaken by the program MULTCOMP based on a multiple comparison test [22].

Cyclic AMP accumulation

Assays were carried out at 32° in physiological salt solution (PSS) gassed with 5% CO_2 and 95% O_2. Pieces of taenia caeci (10–25 mg) were incubated with IBMX (0.25 mM) and where appropriate, an antagonist or McN-A-343, for 30 min prior to addition of isoprenaline (10 μM) or isoprenaline plus oxotremorine (3 nM to 3 μM) for 5 min. In some experiments McN-A-343 (10 nM to 3 mM) was tested as an agonist in place of oxotremorine. To terminate the incubation, the tissue was frozen rapidly in a clamp, cooled in liquid nitrogen. The tissue was then homogenized in 9 vol. of ice-cold 5% trichloroacetic acid, and centrifuged at 10,000 *g* for 10 min at 0°. The acidic supernatant was neutralized using a mixture of tri-*N*-octylamine in carbon tetrachloride (1:3), before injection (50 μL) into the HPLC column.

The HPLC instrument from Bioanalytical Systems (BAS) consisted of a PM-60 pump, an LC-22A temperature controller, a UV-116 ultraviolet/visible light detector, a Rheodyne 7125 injector mounted within a CC-4 column heater cabinet, and a Phase-II ODS 3 μm (100 × 3.2 mm) reverse phase type column. The mobile phase consisted of 1–2% methanol in 100 mM sodium phosphate buffer (pH 6.8). Peaks were monitored at 254 nm wavelength (0.001 AUFS). Using a pump flow rate of 1.0 mL/min retention time was maintained around 12 min as the column aged by varying the temperature of the column from 32° to 38°. Apparent pK_B values for antagonists were calculated from induced shifts of the concentration–response curve for oxotremorine (dose-ratios) using the relationship:

$$\text{Apparent } pK_B = -\log [B/(\text{dose-ratio} - 1)] \quad (5)$$

where B is the concentration of antagonist.

Solutions (mM)

McEwen's solution: NaCl 130; KCl 5.6; $CaCl_2$ 2.2; NaH_2PO_4 1.0; $NaHCO_3$ 25; glucose 11.1; sucrose 13.1.

Tyrode's solution: NaCl 137; KCl 2.7; $CaCl_2$ 1.8; $MgCl_2$ 1.0; NaH_2PO_4 0.4; $NaHCO_3$ 11.9; glucose 5.6.

PSS: NaCl 116; KCl 5.7; $CaCl_2$ 1.26; $MgSO_4$ 2.33; NaH_2PO_4 1.17; $NaHCO_3$ 25; glucose 11.0; EGTA 0.03.

Sodium phosphate buffer: Na_2HPO_4 50; pH 7.4 (HCl acid).

RESULTS

McN-A-343 in the atria

In McEwen's solution, McN-A-343 (50–300 μM) produced a negative inotropic response which did not always reach 50% inhibition of maximum contraction over the concentration range studied. The geometric mean EC_{30} was 71 μM (43–120, N = 7) (95% confidence limits) (Fig. 1). The response to McN-A-343 was inhibited by atropine (10–50 nM) but not by tetrodotoxin (0.2 μM).

Responses to carbachol were inhibited by McN-A-343 (50–300 μM) (Fig. 2) and the resulting K_B value for McN-A-343, determined by the method of

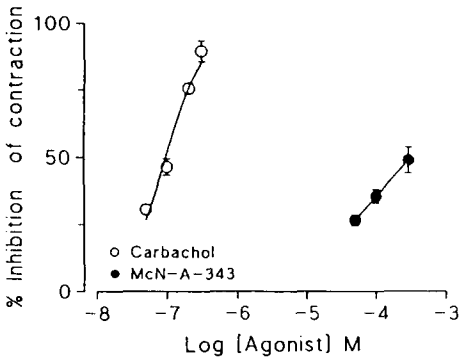


Fig. 1. Concentration–response curve to carbachol (○) and McN-A-343 (●) in electrically stimulated guinea pig left atria. Ordinate: response to the two agonists expressed as per cent inhibition of the maximal contraction to carbachol. Abscissa: Logarithm of molar concentration of agonist. Data points represent the mean ± SEM from seven experiments.

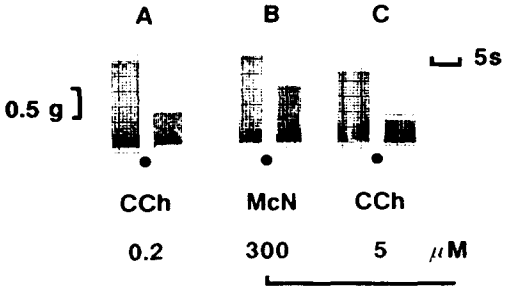
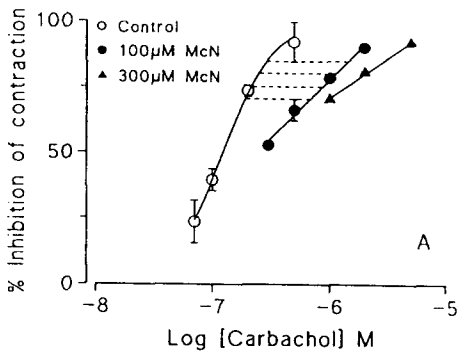


Fig. 2. Trace recording showing the effect of carbachol (CCh) and McN-A-343 (McN) in an electrically stimulated guinea pig left atrium 60–90 sec after addition (●) of drug. (A) Carbachol (0.2 μM) produced a 63% inhibition of contraction. (B) McN-A-343 (300 μM) caused 34% inhibition of contraction initially. (C) In the continued presence of McN-A-343, which after 15 min produced only 19% inhibition, the concentration of carbachol was raised to 5 μM to produce a comparable response (74%) to that in (A). Vertical scale: 0.5 g tension, time marker: 5 sec.



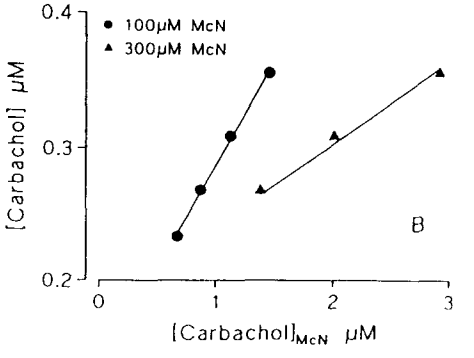


Fig. 3. Estimation of K_B for McN-A-343 in atria using the method described by Kaumann and Blinks [15]. (A) Effect of McN-A-343 on the negative inotropic response to carbachol. Ordinate: response to carbachol expressed as per cent inhibition of maximal contraction. Abscissa: logarithm of molar concentration of carbachol. Horizontal dotted lines (---) show the equiactive concentrations used to estimate K_B. Data points represent the mean ± SEM from a single representative experiment. (B) Plot of the equiactive concentration of carbachol in the absence (ordinate) vs presence (abscissa) of McN-A-343 100 μM (●) and 300 μM (▲). The slope of the curve is used to estimate K_B according to equation 1 (see Materials and Methods).

Kaumann and Blinks [15] was 15.20 μM (12.28–18.81, N = 16) (Fig. 3).

The atria showed a partial recovery for the peak negative inotropic response to McN-A-343 over the course of 15 min (Fig. 2). The percentage inhibition produced by McN-A-343 (50, 100 or 300 μM), just prior to addition of carbachol was 5% (N = 2), 17 ± 4% (N = 6), 33 ± 8% (N = 6), respectively. The pK_B was also estimated by a second method which ignored the residual negative inotropic response of McN-A-343 (Fig. 4 and Materials and Methods). With this technique, the concentration–response curve to carbachol in the presence of McN-A-343 appeared parallel to the control. The Schild plot constructed from the dose-ratios obtained gave a pA_2 value of 4.99 and had a slope of 0.90 ± 0.10 (N = 16) which was not significantly different from unity (P > 0.05). The K_B value obtained from the constrained plot was 13.30 μM (9.33–18.94, N = 16).

McN-A-343 in the taenia caeci

The geometric mean EC_{50} for the contraction produced by McN-A-343 in McEwen's solution was 1.21 μM (0.97–1.52, N = 4) and the maximum response was 72.3 ± 4.0% of that produced by carbachol indicating that McN-A-343 was a partial agonist. The K_A value for McN-A-343, estimated by comparing equiactive responses of McN-A-343 to those of the full agonist, carbachol (Fig. 5 and Materials and Methods) was estimated as 4.57 μM (3.79–5.51, N = 4). This value was not corrected for the relative intrinsic efficacy of McN-A-343 to carbachol ($\varepsilon_P/\varepsilon_A$) (see Materials and Methods) as it was only 0.009 ± 0.002, N = 4 (Fig. 6) and thus could be ignored. Experiments conducted in Tyrode's

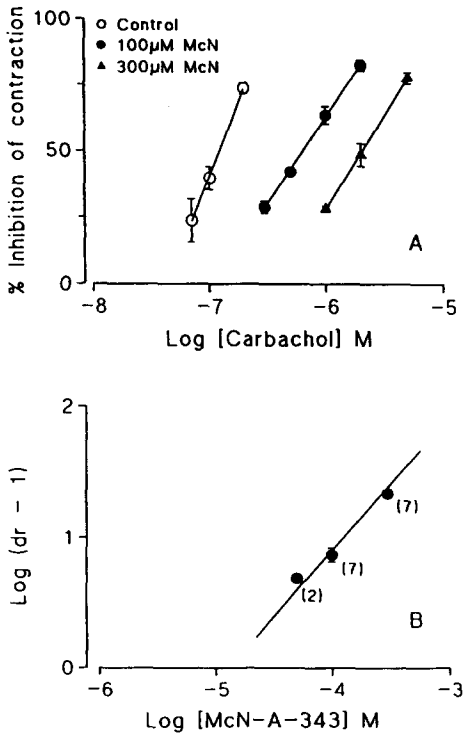


Fig. 4. Estimation of K_B for McN-A-343 in atria based on the parallel displacements of the negative inotropic response to carbachol. (A) Effect of McN-A-343 on the negative inotropic response to carbachol. Ordinate: response to carbachol expressed as per cent inhibition of the inotropic response immediately prior to its addition. Abscissa: Logarithm of molar concentration of carbachol. Data points represent mean ± SEM from a single representative experiment. (B) Arunlakshana–Schild plot. Ordinate: logarithm of dose-ratio minus one (dr − 1). Abscissa: Logarithm of molar concentration of McN-A-343. The number of experiments associated with each data point are shown inside brackets. The resulting slope has a value of 0.90 ± 0.10 and was not significantly different from unity ($P > 0.05$).

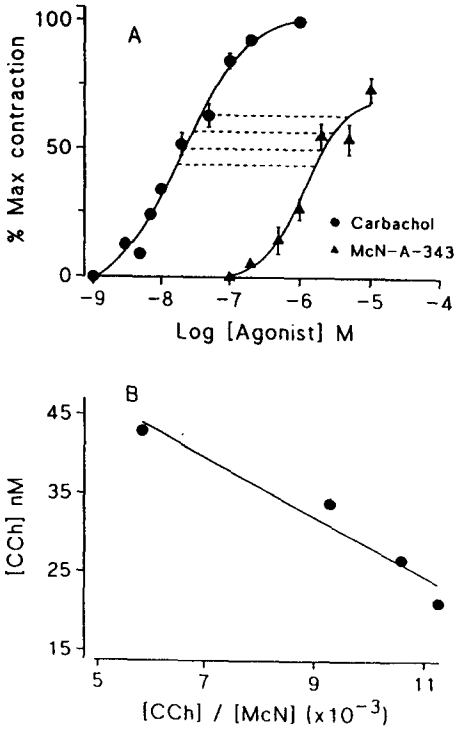


Fig. 5. Estimation of K_A for McN-A-343 in taenia caeci by comparison of equiactive concentrations against carbachol. (A) Concentration–response curve to carbachol (●) and McN-A-343 (▲). Ordinate: response to both agonists expressed as a per cent of the maximal response to carbachol. Abscissa: logarithm of molar concentration of agonist. Horizontal dotted lines (---) show the equiactive concentrations used to estimate K_A. Data points represent the mean ± SEM from a single representative experiment. (B) Plot of the equiactive concentrations according to equation 2 (see Materials and Methods). Ordinate: concentration of carbachol. Abscissa: ratio of equiactive concentrations of carbachol to McN-A-343. The resulting slope of the regression line is used to estimate K_A of McN-A-343 according to equation 3 (see Materials and Methods).

solution gave a similar K_A value of 5.85 μM (3.68–9.28, N = 7).

Binding experiments

Atria. Displacement experiments (Fig. 7) conducted in McEwen's solution containing Gpp(NH)p (50 μM) with [^{3}H]QNB as the ligand gave a K_I for McN-A-343 of 14.8 μM (10.81–20.31, N = 6). In the absence of Gpp(NH)p the K_I was slightly lower (Table 1). In phosphate buffer (50 mM) the K_I values for McN-A-343 changed little on addition of Gpp(NH)p (Table 1).

Taenia caeci. The K_I values obtained with the taenia did not alter irrespective of the incubation medium and whether Gpp(NH)p was present or not (Table 1). Simultaneous comparative analysis of the K_I values obtained in binding experiments showed the K_I value of McN-A-343 in the presence of Gpp(NH)p in McEwen's solution in atria was significantly different to all other K_I values obtained in the atria or taenia in the presence of Gpp(NH)p ($P < 0.01$) (Table 1).

Displacement experiments with AF-DX 116 (20 pM to 50 μM) vs [^{3}H]NMS (400 pM) in the presence of Gpp(NH)p (50 μM) gave a two site fit with a K_H value of 17.1 nM and a K_L value of 245.5 nM. Respective receptor population fractions for the two sites were 0.70 and 0.30 (Table 2). With [^{3}H]QNB as the ligand, AF-DX 116 displaced from only one site with a K_I of 75.1 nM (Table 2).

Cyclic AMP accumulation

Oxotremorine (0.1–3 μM) produced little inhibition of the increase in cyclic AMP induced by isoprenaline (10 μM) when experiments were conducted in McEwen's solution (Fig. 8). For this reason experiments involving the effect of oxotremorine on adenylyl cyclase in taenia caeci were conducted in PSS (see Materials and Methods).

In taenia caeci oxotremorine (3 nM to 3 μM) (Fig.

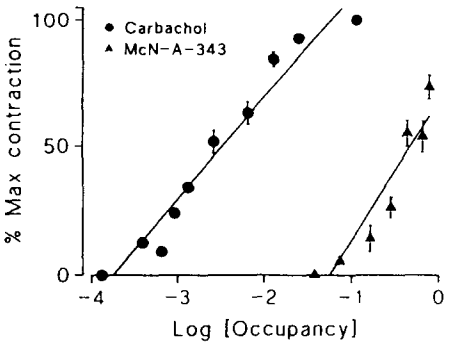


Fig. 6. Occupancy–response curve for estimating the relative intrinsic efficacy of McN-A-343 to carbachol. Ordinate: Response to carbachol (●) and McN-A-343 (▲) expressed as per cent of the maximal response to carbachol. Abscissa: Logarithm of receptor occupancy. Data points represent mean ± SEM from a single representative experiment. Independently obtained K_A values used to calculate receptor occupancy were: 7.6 μM (carbachol) and 2.5 μM (McN-A-343) [13]; both values were determined after irreversible inactivation of receptors with phenoxybenzamine. Using linear regression curves fitted through data points, the relative intrinsic efficacy was calculated from the occupancy of agonists producing 50% of the maximal contraction to carbachol (see Materials and Methods).

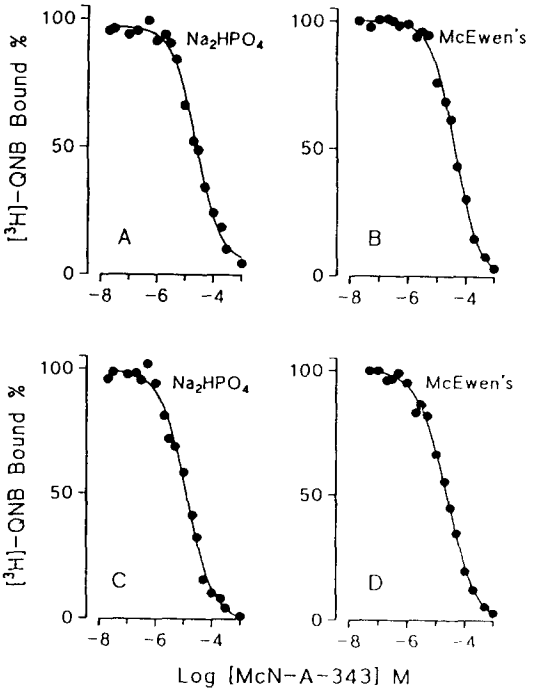


Fig. 7. Competitive inhibition of specific [^{3}H]QNB binding by McN-A-343 in atria (A and B) and taenia caeci (C and D) in the presence of Gpp(NH)p 50 μM using phosphate buffer 50 mM or McEwen's solution. Data points in each panel are from single representative experiments.

8) and carbachol (0.1 μM to 0.1 mM) (not shown) produced a similar maximal inhibition (*ca.* 45%) of the isoprenaline-induced increase in cyclic AMP. For carbachol, this effect was maximal in the range of 0.3–1 μM, higher concentrations being less effective. McN-A-343 (10 nM to 3 mM) had little or no effect on the response to isoprenaline, only at a concentration of 3 mM was there a significant inhibition of the response to isoprenaline (Fig. 8). Similar findings with McN-A-343 (10 μM to 3 mM) were obtained in McEwen's solution (Fig. 8). McN-A-343 (100 μM) had no effect on the response to oxotremorine (Fig. 9). Increasing the concentration of McN-A-343 to 300 μM (1 experiment) or 1 mM (1 experiment) did not further antagonize responses to oxotremorine (not shown). AF-DX 116 (1–3 μM) and HHSiD (1–3 μM) antagonized oxotremorine induced inhibition of adenylyl cyclase with apparent pK_B values of 6.95 and 6.75, respectively (Table 3).

DISCUSSION

In both guinea pig atria and taenia caeci, McN-A-343 was a partial agonist. In atria McN-A-343

Table 1. Displacement of [^{3}H]QNB binding in atria and taenia caeci by McN-A-343 in McEwen's solution [14] and phosphate buffer (50 mM) with and without Gpp(NH)p (50 μM)

		Atria		Taenia	
	Gpp(NH)p (50 μM)	n_H (± SEM)	K_I‡ (μM)	n_H (± SEM)	K_I‡ (μM)
McEwen's	+	0.97 (0.05)	14.8 (6) (10.8–20.3)*	0.91 (0.05)	6.22 (6) (5.21–7.43)
	−	0.98 (0.04)	10.9 (3) (8.48–14.0)	0.92 (0.08)	7.24 (3) (5.89–8.95)
Na_2HPO_4	+	0.92 (0.06)	7.01 (4) (5.52–8.92)	0.96 (0.06)	5.69 (3) (3.76–8.59)
	−	0.83 (0.09)	6.97 (3) (4.47–10.9)†	1.00 (0.03)	6.74 (3) (4.07–11.2)
			K_H 0.30 (0.001–6.64) 27%†		
			K_L 10.3 (3.68–28.8) 73%		

* $P < 0.01$; value significant compared with other K_I values in the presence of Gpp(NH) p.

† The two site model was a better fit over the one site model ($P < 0.05$).

‡ First values in parentheses represent the number of experiments (N), the range of values in parentheses are 95% confidence limits.

Table 2. Displacement of [^{3}H]NMS or [^{3}H]QNB by AF-DX 116 from taenia caeci in McEwen's solution [14] at 32° with Gpp(NH)p (50 μM)

Ligand	n_H (± SEM)	K_I (nM) (95% C.L.) N = 3* One site	Two site
[^{3}H]NMS	0.73 (0.03)	46.8 (31.0–70.7)	K_H 17.1 (0.25–114) (70%)† K_L 245.5 (12.5–4163) (30%)
[^{3}H]QNB	0.95 (0.04)	75.1 (62.2–90.6)	—

* K_I values shown are the mean from pooled data taken from three experiments. 95% C.L., 95% confidence limits.

† The two site model resulted in a more significant fit than the one site model ($P < 0.01$).

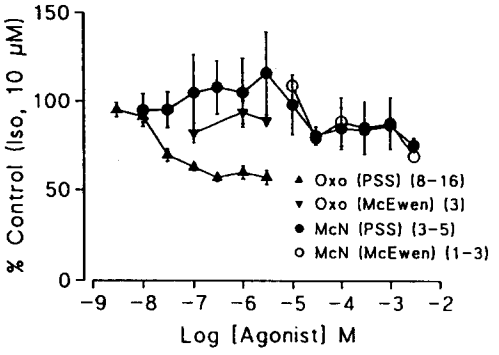


Fig. 8. Level of cyclic AMP accumulated over a period of 5 min in guinea pig taenia caeci in response to oxotremorine and McN-A-343 in two different solutions as indicated. Cyclic AMP production was induced by isoprenaline (10 μM) in the presence of IBMX (250 μM). Ordinate: cyclic AMP level as per cent of the response to isoprenaline (10 μM) alone. Abscissa: logarithm of molar concentration of agonist. Data points represent mean ± SEM; the number of experiments associated with each curve is shown in brackets.

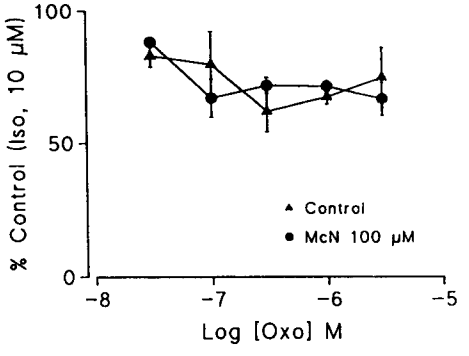


Fig. 9. Level of cyclic AMP accumulated over a period of 5 min in guinea pig taenia caeci in response to oxotremorine in absence (▲) and presence (●) of McN-A-343 (100 μM 30 min). Cyclic AMP production was induced by isoprenaline (10 μM) in the presence of IBMX (250 μM). Ordinate: cyclic AMP level as per cent of the response to isoprenaline (10 μM) alone. Abscissa: logarithm of molar concentration of oxotremorine. Data points represent mean ± SEM from three experiments.

Table 3. Apparent pK_B values of AF-DX 116 and HHSiD in antagonizing oxotremorine induced adenylyl cyclase inhibition

Antagonist	Concn (μM)	pK_B (95% C.L., N)*
AF-DX 116	1–3	6.95 (6.67–7.23, 5)
HHSiD	1–3	6.75 (6.41–7.08, 4)

* Values in parentheses represent 95% confidence limits and the number of experiments (N).

produced an atropine-sensitive, negative inotropic response only in high concentrations; the EC_{30} value being 71 μM. Muscarine M_1 receptors from vagal ganglia were not involved as the response was unaffected by tetrodotoxin. Low potency of McN-A-343 on muscarine receptors in guinea pig atria has been reported previously [23–25] and Pappano and Rembish [26] found McN-A-343 produced a weak, atropine-sensitive, negative chronotropic and inotropic effect in spontaneously beating guinea pig right atria.

In the taenia caeci McN-A-343 was more potent than in the atria; the EC_{50} was 1.2 μM and the maximum response to McN-A-343 reached only 72% of that to carbachol, in agreement with previous findings that McN-A-343 was a partial agonist in this tissue [13, 27]. The action of McN-A-343 was mediated via muscarine M_3 receptors since the contraction has been found to be unaffected by tetrodotoxin, with a pK_B value of inhibition by pirenzepine of 6.8 [13] and an apparent pK_B value for AF-DX 116 of 5.8 (Darroch, unpublished). Similar pK_B values have been obtained in the taenia with carbachol as the agonist [28].

In left atria, the ability of McN-A-343 to act as an antagonist to carbachol was exploited to obtain an affinity constant using two methods. The first, described by Kaumann and Blinks [15], took into account the weak partial agonist activity of McN-A-343 and gave a K_B value of 15.2 μM. A similar value was obtained with an Arunlakshana–Schild analysis for competitive antagonism [16].

In taenia caeci, because the agonist activity of McN-A-343 was greater than in atria, a direct measure of the dissociation constant (K_A) was chosen to estimate the interaction of McN-A-343 with the

Table 4. Summary of estimates of the dissociation constants for McN-A-343 obtained in guinea pig taenia caeci or left atria in McEwen's solution [14]

Parameter	Technique	Taenia (μM) (95% C.L.)*	Atria (μM) (95% C.L.)*
K_A	Comparison of agonist/partial agonist	4.6 (3.8–5.5)	—
K_B	Antagonism of carbachol	—	15.2 (12.3–18.8)
K_I	[^{3}H]QNB, Gpp(NH)p	6.2 (5.2–7.4)	14.8 (10.8–20.3)

* 95% confidence limits.

M_3 receptor. The plot, [A] vs $[A]\cdot[P]^{-1}$, suggested by Kenakin [18], was used to estimate K_A and gave a value of 4.57 μM. This value was similar to the apparent K_B value of 3.35 μM obtained for McN-A-343 in the taenia at 18° in Tyrode's solution [13]. At 18°, the response to McN-A-343 was abolished and the drug could be used as an antagonist to carbachol. The results obtained from the functional studies in McEwen's solution indicate the ability of McN-A-343 to discriminate between muscarine M_2 and M_3 subtypes from guinea pig atria and taenia caeci, respectively. Although the difference in affinity was small (*ca.* 3-fold) it was highly significant ($P < 0.01$). Previous estimates of the affinity of McN-A-343 for muscarine M_2 and M_3 receptors have not revealed this difference, possibly because of the various media employed in different experiments [25, 28].

The difference between the dissociation constants for McN-A-343 obtained in atria and taenia caeci was also detected in binding studies conducted in McEwen's solution in the presence of Gpp(NH)p ($P < 0.01$) but was not apparent when binding experiments were conducted in phosphate buffer (Table 1). Using McEwen's solution, a higher ionic strength medium, in place of the phosphate buffer the affinity of McN-A-343 for cardiac muscarine receptors was decreased by 2–3-fold but the affinity of McN-A-343 for binding sites in the taenia caeci was unaffected. Watson *et al.* [29] noted that changing the binding medium from phosphate buffer to a Krebs solution had a similar effect with McN-A-343 in rat heart to that observed in the present experiments; in the presence of Gpp(NH)p the $K_{0.5}$ of McN-A-343 was increased from 2.9 μM in phosphate buffer to 14 μM (indicating a decrease in affinity) when modified Krebs solution was used.

Thus the K_I values of McN-A-343 in atria and the taenia caeci obtained from binding studies in McEwen's solution containing Gpp(NH)p corresponded to the respective dissociation constants obtained functionally in the same medium (Table 4). This result appeared inconsistent with other findings that suggested the majority of muscarine receptors in ileal smooth muscle to be the M_2 subtype (see the introduction). Failure to see two site binding with McN-A-343 in the presence of Gpp(NH)p was considered due to the small difference (*ca.* 3-fold) in the dissociation constant of McN-A-343 for M_2 vs M_3 receptors since ligands must have a 6–10-fold selectivity to resolve a mixture of receptors where the minor component accounts for at least 20% of the mixture [1, 30]. Consequently, McN-A-343 may not be a suitable ligand for the detection of two site binding because of its low selectivity for M_3 over M_2 receptors.

Nevertheless, the higher affinity shown by McN-A-343 in the taenia caeci appeared to be opposite to that expected if the majority (*ca.* 70%) of sites were of the M_2 subtype and this was confirmed by computer simulation of the binding experiments using a dissociation constant of 5 μM for the high affinity site and 15 μM for the low affinity site (Table 5). These values corresponded to the dissociation constants for McN-A-343 obtained experimentally for the M_3 and M_2 receptors, respectively. The simulation showed that the expected K_I value for McN-A-343 would be 11.4 μM for a tissue with a 30:70 ratio of M_3:M_2 receptors. The experimentally obtained K_I of 6.2 μM would only be obtained when M_3 receptors were in the majority (80%). With the introduction of random errors, ranging from 1 to 8%, comparable to that obtained experimentally, LIGAND was unable to resolve the simulated experimental data into two site binding but the estimated K_I was still 11.0 μM.

It was considered unlikely that the receptor population in the guinea pig taenia caeci differed from that in ileal longitudinal muscle but recently rat colon has been reported to contain 61% of M_3 receptors [31], the converse of that reported for rat ileum (18%) [1]. Binding studies with AF-DX 116 in the taenia confirmed a similar heterogeneity to that reported in rat ileum. Using [^{3}H]NMS as the binding ligand for comparison with data from ileal longitudinal muscle [1], AF-DX 116 bound to two sites in displacing [^{3}H]NMS, 70% of the sites exhibiting high affinity (pK_I 7.76). With [^{3}H]QNB only a single high affinity binding site for AF-DX 116 was detected (K_I 75.1 nM) suggesting the majority of the receptors in the taenia caeci were the M_2 subtype. The proportion of M_3 subtype would appear too low to be detected with [^{3}H]QNB.

Binding of full agonists to cardiac muscarine receptors is strongly influenced by the presence of Gpp(NH)p, suggesting that a large proportion of the M_2 receptors are present in a high affinity state

Table 5. EBDA/LIGAND computer analysis of affinity constants for McN-A-343 based on theoretical competition binding curves

	Proportion of binding sites (high/low affinity) K_I (μM)							
	0/100	10/90	20/80	30/70	40/60	50/50	80/20	100/0
*No variance								
1 site	15.2	13.9	12.6	11.4	10.3	9.25	6.51	5.06
2 sites								
K_H(%)		3.47 (4)	4.70 (15)	5.07 (27)	5.23 (39)	5.32 (51)	5.38 (85)	
K_L(%)		14.5 (96)	14.5 (85)	14.6 (73)	14.7 (61)	14.9 (49)	16.6 (15)	
†1–8%								
1 site		13.6	12.3	11.0	10.2	8.89	6.63	
2 sites								
K_H(%)		14.2 (34)	10.1 (64)	10.9 (23)	4.43 (20)	6.89 (42)	5.74 (92)	
K_L(%)		13.3 (66)	17.0 (36)	11.1 (77)	12.1 (80)	10.5 (58)	26.1 (8)	

The above data show affinity constants for various proportions of a mixed receptor population with theoretical dissociation constants of 5 and 15 μM for the high and low affinity sites, respectively.

* The upper part of the table shows estimates based on simulated data generated without any variance about the data points. All two site fits derived from the analysis were significant over the one site fit ($P < 0.01$).

† The lower part of the table shows estimates when an error of 1–8% is introduced randomly to each data point. All LIGAND calculations were based on pooled data taken from three simulated experiments. The two site models, where resolvable, are shown but none were significant over the one site model ($P > 0.05$).

and this is converted to a lower affinity state in the presence of the stable GTP analogue [29, 32–34]. In binding studies in rat heart, the $K_{0.5}$ for oxotremorine was increased 10-fold in phosphate buffer (10 mM) in the presence of Gpp(NH)p [29]. Partial agonists were affected to a much smaller extent; the $K_{0.5}$ for McN-A-343 increased only 2.4-fold. In a modified Krebs solution, the corresponding changes were 25-fold for oxotremorine and 5-fold for McN-A-343 [29]. In the present experiments, the binding of McN-A-343 in guinea pig atria was converted from two site binding in phosphate buffer to binding at a single site with the addition of Gpp(NH)p (50 μM). In McEwen's solution the binding curve was little affected by the presence of Gpp(NH)p and could be described by one site binding. The K_I value 14.8 μM agreed well with the value obtained in functional experiments but was significantly different to that obtained in the low ionic strength phosphate buffer in the presence of Gpp(NH)p, demonstrating the importance of performing functional and binding studies in the same medium whenever possible. Other workers have noted the importance of the bath medium for determining the activity of McN-A-343 in functional [35] or binding studies [29]. It should be noted that the K_I values for McN-A-343 in binding experiments conducted in the taenia caeci were not influenced by either Gpp(NH)p or by the change in medium indicating a further difference from atrial tissue.

The affinity of agonists is dependent on the extent of receptor coupling with G proteins. The muscarine M_2 receptor subtype in cardiac tissue appears to be capable of interacting with more than one G protein or transduction system [36–39]. The possibility of different proportions of various G proteins being associated with M_2 receptors in the taenia would not account for the different affinity values obtained from the binding experiments with atria and taenia caeci conducted in McEwen's solution as the presence of Gpp(NH)p would have minimized association of the receptor to G proteins [40].

In cardiac tissue one of the second messenger systems negatively coupled to the muscarine M_2 receptor is the adenylyl cyclase-cyclic AMP pathway. In the taenia caeci the M_2 receptor also appeared to be coupled negatively to adenylyl cyclase; the apparent pK_B values obtained for AF-DX 116 and hexahydrosiladiphenidol against oxotremorine-induced inhibition of cyclic AMP production were within the ranges reported for interaction at an M_2 receptor in functional studies on cardiac muscarinic receptors [4, 41]; or in binding studies on cloned m2 receptors [42]. Recently Candell *et al.* [43] have suggested that the muscarine receptor negatively coupled to adenylyl cyclase in rat ileal longitudinal muscle is the M_2 subtype.

McN-A-343 (≤ 100 μM) failed to exhibit any inhibition of the increase in cyclic AMP produced by isoprenaline and was not effective at antagonizing the inhibitory action of oxotremorine. These experiments were conducted in a different medium to the binding studies, as oxotremorine had little effect on the action of isoprenaline in McEwen's solution but a similar lack of effectiveness of McN-A-343 has been reported for cardiac tissue [44]. In rat ventricle oxotremorine-induced inhibition of forskolin-stimulated adenylyl cyclase activity was found to be unaffected by McN-A-343 except at high concentrations (≥ 0.3 mM). Thus the present findings suggest that M_2 receptors are present in the taenia caeci and are coupled to adenylyl cyclase as in cardiac tissue. However, the apparent affinity of McN-A-343 for the M_2 receptors in the taenia differs slightly from that found in cardiac tissue.

It is possible that M_2 receptors in cardiac tissue are not homogeneous. Mizushima *et al.* [45] have suggested that there are two M_2 binding sites in

cardiac muscle, designated $M_{2\alpha}$ and $M_{2\beta}$ and muscarine M_2 receptors in heart that were GTP-insensitive have been detected as well as GTP-sensitive receptors [46, 47]. Recently Ford *et al.* [48] have suggested a possible heterogeneity of cardiac muscarine receptors based on the dissociation constants found for antagonists blocking the increase in phosphatidyl inositol produced by muscarinic agonists in the heart. Another possibility is that the M_2 receptor agonist complex may exist in two states, activated and non-activated [49], and this proportion varies in the two tissues. This could influence the apparent affinity estimated in binding studies although the K_A values of McN-A-343 for the two states are identical in the two tissues.

Alternatively, since McN-A-343 is a muscarinic agonist that has been suggested to act allosterically with cardiac M_2 muscarine receptors [50], it is possible that binding to an allosteric site on the M_2 receptor may be influenced by the lipid environment of the membrane and differences between the microenvironment of ileal and cardiac tissue rather than the M_2 receptor *per se* could account for the findings. Muscarine receptor binding may be influenced by lipids [51] or the general microenvironment of the receptor [52].

In summary, the findings in this paper re-emphasize that the activity of McN-A-343 both in functional studies [24, 35] and in binding studies [29] appears to be dependent on the medium employed. Nevertheless, when the same medium was employed in both functional and binding studies there was good agreement between estimates of its affinity for muscarine M_2 receptors in cardiac tissue. A small but significant difference in its affinity for the putative muscarine M_2 receptor in smooth muscle suggests the possibility of at least functional M_2 receptor heterogeneity that may be amenable to therapeutic exploitation.

Acknowledgements—Gifts of drugs from Prof. E. Mutschler and G. Lambrecht (HHSiD) and from Dr Karl Thomae GmbH (AF-DX 116) are gratefully acknowledged.

REFERENCES

1. Ladinsky H, Hiraldo E, Monferini E, Schiavi GB, Vigano MA, De Conti L, Micheletti R and Hammer R, Muscarinic receptor heterogeneity in smooth muscle: binding and functional studies with AF-DX 116. *Trends Pharmacol Sci* **2** (Suppl): 44–48, 1988.
2. Michel AD and Whiting RL, Methoctramine reveals heterogeneity of M_2 muscarinic receptors in longitudinal ileal smooth muscle membranes. *Eur J Pharmacol* **145**: 305–311, 1988.
3. Giraldo E, Vigano MA, Hammer R and Ladinsky H, Characterization of muscarinic receptors in guinea pig ileum longitudinal smooth muscle. *Mol Pharmacol* **33**: 617–625, 1988.
4. Lazareno S and Roberts FF, Functional and binding studies with muscarinic M_2-subtype selective antagonists. *Br J Pharmacol* **98**: 309–317, 1989.
5. Maeda A, Kubo T, Mishina M and Numa S, Tissue distribution of mRNAs encoding muscarinic acetylcholine receptor subtype. *FEBS Lett* **239**: 339–342, 1988.
6. Dörje F, Levey AI and Brann MR, Immunological detection of muscarinic receptor subtype proteins (m1–m5) in rabbit peripheral tissues. *Mol Pharmacol* **40**: 459–462, 1991.
7. Wall SJ, Yasuda RP, Li M and Wolfe BB, Development of an antiserum against m3 muscarinic receptors: Distribution of m3 receptors in rat tissues and clonal cell lines. *Mol Pharmacol* **40**: 783–789, 1991.
8. Delmendo RE, Michel AD and Whiting RL, Affinity of muscarinic antagonists for three putative muscarine receptor binding sites. *Br J Pharmacol* **96**: 457–464, 1989.
9. Pedder EK, Eveleigh P, Pyner D, Hulme EC and Birdsall NJM, Modulation of the structure-binding relationships of antagonists for muscarinic acetylcholine receptor subtypes. *Br J Pharmacol* **103**: 1561–1567, 1991.
10. Ehlert FJ, Roeske WR and Yamamura HI, The nature of muscarinic receptor binding. In: *Handbook of Psychopharmacology, Vol. 17, Biochemical studies of CNS receptors* (Eds. Iverson LL, Iverson SD and Snyder SH), pp. 241–283. Plenum Press, New York, 1983.
11. Doods HN and Mayer N, UH-AH 37, an ileal-selective muscarinic antagonist that does not discriminate between M_2 and M_3 binding sites. *Eur J Pharmacol* **161**: 215–218, 1989.
12. Wess J, Lambrecht G, Mutschler E, Braun MR and Dörje F, Selectivity profile of the novel muscarinic antagonist UH-AH 37 determined by the use of cloned receptors and isolated tissue preparations. *Br J Pharmacol* **102**: 246–250, 1991.
13. Darroch SA, Gardner AL, Choo LK and Mitchelson F, Effect of temperature reduction on responsiveness to cholinomimetics in the taenia caeci of the guinea-pig. *J Auton Pharmacol* **11**: 109–119, 1991.
14. McEwen LM, The effect on the isolated rabbit heart of vagal stimulation and its modification by cocaine, hexamethonium and ouabain. *J Physiol (Lond)* **131**: 678–689, 1956.
15. Kaumann AJ and Blinks JR, β-Adrenoceptor blocking agents as partial agonists in isolated heart muscle: Dissociation of stimulation and blockade. *Naunyn Schmiedebergs Arch Pharmacol* **311**: 237–248, 1980.
16. Arunlakshana O and Schild HO, Some quantitative uses of drug antagonists. *Br J Pharmacol* **14**: 48–58, 1959.
17. Parker RB and Waud DR, Pharmacological estimation of drug receptor dissociation constants. Statistical evaluation. 1. Agonists. *J Pharmacol Exp Ther* **177**: 1–12, 1971.
18. Kenakin TP, *Pharmacologic Analysis of Drug–Receptor Interaction*. Raven Press, New York, 1987.
19. Furchgott RF, The use of β-haloalkylamines in the determination of dissociation constants of receptor–agonist complexes. *Adv Drug Res* **3**: 21–55, 1966.
20. McPherson GA, A practical computer-based approach to the analysis of radioligand binding experiments. *Comput Methods Programs Biomed* **17**: 107–114, 1983.
21. Munson PJ and Rodbard D, LIGAND. A versatile computerized approach for the characterization of ligand binding systems. *Anal Biochem* **107**: 220–239, 1980.
22. Miller RG, *Simultaneous Statistical Inference*. McGraw-Hill, New York, 1966.
23. Roszkowski AP, An unusual type of sympathetic ganglionic stimulant. *J Pharmacol Exp Ther* **132**: 156–170, 1961.
24. Eglen RM, Kenny BA, Michel AD and Whiting RL, Muscarinic activity of McN-A-343 and its value in muscarinic receptor classification. *Br J Pharmacol* **90**: 693–700, 1987.
25. Kromer W, Baron E, Beinborn M, Boer R and Eltze M, Characterization of the muscarine receptor type on paracrine cells activated by McN-A-343 in the

mouse isolated stomach. *Naunyn Schmiedebergs Arch Pharmacol* **341**: 165–170, 1990.
26. Pappano AJ and Rembish RA, Negative chronotropic effects of McN-A-343 and nicotine in isolated guinea-pig atria: insensitivity to blockade by tetrodotoxin. *J Pharmacol Exp Ther* **177**: 40–47, 1971.
27. Gardner AL, Choo LK and Mitchelson F, Comparison of the effects of some muscarinic agonists on smooth muscle function and phosphatidylinositol turnover in the guinea-pig taenia caeci. *Br J Pharmacol* **94**: 199–211, 1988.
28. Takayanagi I, Hisayama T, Kiuchi Y and Sudo H, Propylbenzilylcholine mustard discriminates between two subtypes of muscarinic cholinoceptors in guinea-pig taenia caecum. *Arch Int Pharmacodyn Ther* **298**: 210–219, 1989.
29. Watson M, Yamamura HI and Roeske WR, [^{3}H]-Pirenzepine and (−)-[^{3}H]quinuclidinyl benzilate binding to rat cerebral cortical and cardiac muscarinic cholinergic sites. I. Characterization and regulation of agonist binding to putative muscarinic subtypes. *J Pharmacol Exp Ther* **237**: 411–418, 1986.
30. De Lean A, Hancock AA and Lefkowitz RJ, Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol Pharmacol* **21**: 5–16, 1982.
31. Gomez A, Martos F, Bellido I, Marquez E, Garcia AJ, Pavia J and De La Cuesta FS, Muscarinic receptor subtypes in human and rat colon smooth muscle. *Biochem Pharmacol* **43**: 2413–2419, 1992.
32. Berrie CO, Birdsall NJM, Burgen ASV and Hulme EC, Guanine nucleotides modulate muscarinic receptor binding in the heart. *Biochem Biophys Res Commun* **87**: 1000–1005, 1979.
33. McMahon KK and Hosey MM, Agonist interactions with cardiac muscarinic receptors. Effects of Mg^{2+}, guanine nucleotides, and monovalent cations. *Mol Pharmacol* **28**: 400–409, 1985.
34. Burgen ASV, The effect of ionic strength on cardiac muscarinic receptors. *Br J Pharmacol* **88**: 451–455, 1986.
35. Rubinstein R and Cohen S, Lack of agonistic activity in McN-A-343 may be circumstantial. *J Auton Pharmacol* **12**: 1–4, 1992.
36. Matesic DF, Manning DR and Luthin GR, Tissue-dependent association of muscarine acetylcholine receptors with guanine nucleotide-binding regulatory proteins. *Mol Pharmacol* **40**: 349–353, 1991.
37. Lai J, Waite SL, Bloom JW, Yamamura HI and Roeske WR, The m2 muscarinic acetylcholine receptors are coupled to multiple signaling pathways via pertussis toxin-sensitive guanine nucleotide regulatory proteins. *J Pharmacol Exp Ther* **258**: 938–944, 1991.
38. Matsumoto K and Pappano AJ, Carbachol activates a novel sodium current in isolated guinea pig ventricular myocytes via M_2 muscarinic receptors. *Mol Pharmacol* **39**: 359–463, 1991.
39. Kenakin TP and Boselli C, Biphasic dose–response curves to arecoline in rat atria-mediation by a single promiscuous receptor or two receptor subtypes? *Naunyn Schmiedebergs Arch Pharmacol* **344**: 201–205, 1991.
40. Matesic DF, Manning DR, Wolfe BB and Luthin GR, Pharmacological and biochemical characterization of complexes of muscarinic acetylcholine receptor and guanine nucleotide-binding proteins. *J Biol Chem* **264**: 21638–21645, 1989.
41. Ehlert FJ, Delen FM, Yun SH, Freedman DJ and Self DW, Coupling of subtypes of the muscarinic receptor to adenylate cyclase in the corpus striatum and heart. *J Pharmacol Exp Ther* **251**: 660–670, 1989.
42. Buckley NJ, Bonner TI, Buckley CM and Brann MR, Antagonist binding properties of five cloned muscarinic receptors expressed in CHO K1 cells. *Mol Pharmacol* **35**: 469–476, 1989.
43. Candell LM, Yun SH, Tran LLP and Ehlert FJ, Differential coupling of subtypes of the muscarinic receptor to adenylate cyclase and phosphoinositide hydrolysis in the longitudinal muscle of the rat ileum. *Mol Pharmacol* **38**: 689–697, 1990.
44. Gil DW and Wolfe BB, Pirenzepine distinguishes between muscarinic receptor-mediated phosphoinositide breakdown and inhibition of adenylate cyclase. *J Pharmacol Exp Ther* **232**: 608–616, 1985.
45. Mizushima A, Uchida S, Zhou X, Kagiya T and Yoshida H, Cardiac M_2 receptors consist of two different types, both regulated by GTP. *Eur J Pharmacol* **135**: 403–409, 1987.
46. Burgen ASV, The effect of agonists on the components of the cardiac muscarinic receptor. *Br J Pharmacol* **92**: 327–332, 1987.
47. Potter LT, Balleseros LA, Bichajian LH, Ferrendelli CA, Fisher A, Hanchett HE and Zhang R, Evidence for paired M2 muscarinic receptors. *Mol Pharmacol* **39**: 211–221, 1991.
48. Ford APDW, Eglen RM and Whiting RL, Analysis of muscarinic cholinoceptors mediating phosphoinositide hydrolysis in guinea pig cardiac muscle. *Eur J Pharmacol* **225**: 105–112, 1992.
49. del Castillo J and Katz B, Interaction at end plate receptors between different choline derivatives. *Proc R Soc Lond [Biol]* **146**: 369–381, 1957.
50. Birdsall NJM, Burgen ASV, Hulme EC, Stockton JM and Zigmond MJ, The effect of McN-A-343 on muscarinic receptors in the cerebral cortex and heart. *Br J Pharmacol* **78**: 257–259, 1983.
51. Aronstam RS, Abood LG and Baumgold J, Role of phospholipids in muscarinic binding by neural membranes. *Biochem Pharmacol* **26**: 1689–1695, 1977.
52. Sulakhe PV, Jagadeesh G and Phan NT, Divalent cation-sensitive antagonist binding to muscarinic receptors. *Gen Pharmacol* **22**: 375–379, 1991.