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Dissociation constants and relative efficacies of acetylcholine, (+)- and (−)-methacholine at muscarinic receptors in the guinea-pig ileum

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1 Dissociation constants (K_A) and relative efficacies of acetylcholine, (+)-methacholine and (−)-methacholine at muscarinic receptors in the guinea-pig isolated ileum were determined in the absence and presence of the cholinesterase inhibitor diisopropylfluorophosphate. The method used involved analysis of dose-response data before and after fractional inactivation of receptors with propylbenzilylcholine mustard.

2 The K_A values, estimated after cholinesterase inhibition, of acetylcholine, (+)- and (−)-methacholine were 1.7, 2.0 and 620 μM, respectively.

3 The large (730 fold) difference in spasmogenic activity between (+)- and (−)-methacholine is due primarily to a difference in affinity for ileal muscarinic receptors although differences in efficacy (2 to 4 fold) also contribute.

4 It is suggested that the methyl group at the chiral centre of (+)-methacholine has no apparent effect on the binding to muscarinic receptors, whereas the corresponding methyl group of (−)-methacholine interferes with binding, presumably by stabilizing a conformation of the drug which does not fit the receptor very well.

Introduction

Methyl substitution at the β-carbon atom of acetylcholine (ACh) gives rise to the specific muscarinic agonist methacholine (MCh) (Major & Cline, 1932; Major & Bonnett, 1935). In the guinea-pig and rat isolated ileum, (S)-(+)-MCh is equiactive with ACh as a spasmogen, whereas (R)-(−)-MCh is several hundred fold less active (Ellenbroek & van Rossum, 1960; Beckett *et al.*, 1963; Triggle, 1984). Activity measures (EC_{50} values), however, reflect a complex series of events, including binding to the receptor and subsequent receptor activation, and are often poor estimates of agonist affinity. This is especially true in tissues such as the guinea-pig ileum where many agonists need to occupy only a small fraction of the receptors to produce a maximum response (Burgen & Spero, 1968; Ringdahl, 1984a,b; 1985). In order to define closer the effect of methyl substitution in ACh on the binding to muscarinic receptors, we have estimated the affinities (as reflected by the dissociation constant, K_A, of the agonist-receptor complex) of ACh, (+)-MCh and (−)-MCh for muscarinic receptors in the guinea-pig isolated ileum using the method of Furchgott & Bursztyn (1967).

Methods

Guinea-pigs (male, English short hair, 350–400 g) were killed by a blow to the head and bled. Segments of the ileum (about 2 cm long) were removed and suspended in a 10 ml organ bath containing Tyrode solution at 37°C and continuously gassed with 5% CO_2 plus 95% O_2. The Tyrode solution had the following composition (mM): NaCl 137, $NaHCO_3$ 12, glucose 5.0, KCl 2.7, $MgSO_4$ 1.0, NaH_2PO_4 0.4, $CaCl_2$ 1.8 (pH 7.4). Hexamethonium (300 μM) was always included in the Tyrode solution. Contractions were recorded isotonically at 1 g tension, with an electromechanical displacement transducer and a potentiometric recorder. The ileal strips were allowed to equilibrate for at least 1 h before drug addition. Concentration-response curves were constructed by the cumulative dose-response technique by increasing stepwise the concentration of agonist in the organ bath by a factor of 2.15

Dissociation constants (K_A) and relative efficacies of ACh and (+)-MCh at muscarinic receptors of guinea-pig isolated ileum were estimated by the method of Furchgott & Bursztyn (1967) as modified by Ringdahl (1984a). Corresponding parameters of (−)-MCh were obtained in the same experiment by

comparing equieffective concentrations of (−)-MCh and (+)-MCh (Mackay, 1966). On each preparation, control concentration-response curves were obtained to the three agonists. The curves were arranged in such a way that the concentration-response curves to (+)-MCh and (−)-MCh each was preceded by a concentration-response curve to ACh. The ileum was then treated with propylbenzilylcholine mustard (PrBCM), 6–8 μM, for 15 min and washed for 45 min. This treatment reduced the maximal responses to ACh and (+)-MCh by 30–70%. Several equieffective concentrations of each agonist before, [A], and after, [A'], treatment with PrBCM were estimated graphically. A hyperbolic function (1) was then fitted by nonlinear regression analysis to pairs of concentrations [A] and [A'] to give best estimates of the dissociation constant of the agonist-receptor complex (K_A) and of the fraction (y) of receptors inactivated (Parker & Waud,

$$[A] = \frac{[A']}{[A'] + K_A/y} \cdot K_A \cdot \frac{1-y}{y} \qquad (1)$$

The efficacy of (+)-MCh relative to that of ACh was calculated from the respective K_A and EC_{50} values as described previously (Ringdahl & Jenden, 1983). Our supply of (−)-MCh was not sufficient to allow K_A determination by this method. Instead, the concentration-response curves of (−)-MCh and (+)-MCh, recorded before treatment of the ileum with PrBCM, were compared by the procedure of Mackay (1966). Several equieffective concentrations of (+)-MCh ([A]) and (−)-MCh ([B]) were determined graphically. The hyperbolic function (2), derived from an equation of Mackay (1966), was then fitted to the data points by nonlinear regression analysis.

$$[A] = \frac{[B]}{[B] + K_B \frac{e_A}{e_A - e_B}} \cdot K_A \cdot \frac{e_B}{e_A - e_B} \qquad (2)$$

Since the dissociation constant of (+)-MCh (K_A) was known (above), Equation 2 provided estimates of the efficacy ratio of (+)- and (−)-MCh (e_A/e_B) and of the dissociation constant of (−)-MCh (K_B). The accuracy of the method was greatly enhanced by always recording the concentration-response curves to (+)- and (−)-MCh after an intervening concentration-response curve to ACh (see above) and by using a K_A value of (+)-MCh that had been determined on the same piece of tissue.

Dissociation constants and relative efficacies of ACh, (+)-MCh and (−)-MCh were also estimated in tissue treated with an irreversible cholinesterase inhibitor essentially as described by Furchgott & Bursztyn (1967). Thus, the ileum was treated with diisopropylfluorophosphate (DFP) 50 μM for 20 min. This treatment produced a marked contraction of the muscle which persisted after washout of DFP. To block this contraction, PrBCM (0.15 μM) was added to the organ bath after the DFP had been washed out. It was left in the bath long enough (about 15 min) to cause relaxation of the muscle to the original basal tension. The receptors remaining active after this treatment were capable of producing full responses to ACh, (+)-MCh and (−)-MCh. However, even after prolonged washing, it was not possible to obtain reproducible control concentration-response curves in the DFP-PrBCM-pretreated tissue. Rather, an apparent increase in potency of the three agonists occurred with time as evidenced by a parallel leftward shift of their concentration-response curves. Therefore, concentration-response curves to ACh were always recorded before and after the concentration-response curves to (+)-MCh and (−)-MCh. Potencies of (+)-MCh and (−)-MCh relative to that of ACh were calculated from their EC_{50} values and the mean of the two EC_{50} values obtained from the preceding and succeeding ACh curve. The tissue was then treated with PrBCM (5 μM for 15 min) to reduce the maximal responses to the three agonists. Their K_A values were determined from the depressed concentration-response curves and the control curves as described above. The efficacies of (+)-MCh and (−)-MCh relative to that of ACh were calculated from the K_A values thus obtained and from the EC_{50} values normalized to the EC_{50} value of ACh.

Unpaired Student's t test was used to assess the statistical significance ($P < 0.05$) of differences between means.

The following drugs were used: acetylcholine perchlorate (synthesized in this laboratory), (S)-(+)- and (R)-(−)-methacholine iodide (synthesized by Dr K.A. Scott and kindly provided by Dr J.M. Young, Cambridge, U.K.), hexamethonium chloride (K & K Laboratories), propylbenzilylcholine mustard (kindly provided by Dr N.J.M. Birdsall, London) which was cyclized to the corresponding aziridinium ion in 10 mM phosphate buffer (pH 7.4) for 45 min at room temperature.

Results

In the naive ileum, the potency (EC_{50}) of (+)-MCh in eliciting contractions was not significantly ($P > 0.05$) different from that of ACh (Table 1). The maximum response to (+)-MCh, however, was greater than that observed for ACh (Figure 1). With ACh, maximal and near maximal responses were not always well main-

Table 1 Parameters characterizing the muscarinic activity of acetylcholine (ACh), (+)- and (−)-methacholine in the guinea-pig ileum

Compound	*EC_{50}* (μM)	*E_{max}*†	K_A (μM)	*Relative efficacy*	*% occupancy for 50% response*‡
Acetylcholine	0.036 ± 0.004 (10)	1.00	12.0* ± 1.3 (8)	1.00	0.30
(+)-Methacholine	0.043 ± 0.004 (8)	1.17 ± 0.02 (8)	4.1* ± 0.48 (4)	0.40 ± 0.09 (4)	1.0
(−)-Methacholine	31.5 ± 4.0 (8)	1.17 ± 0.01 (8)	805** ± 173 (7)	0.11 ± 0.029 (7)	3.7

Values are means ± s.e.
†Maximum response relative to the maximum for ACh which equals 1.00.
*Determined according to Furchgott & Bursztyn (1967).
**Determined according to Mackay (1966).
‡Calculated as $100 \times EC_{50} / (K_A + EC_{50})$ (Besse & Furchgott, 1976).

tained at a steady level, but gradually declined. (−)-MCh produced the same maximum response as (+)-MCh, but was 730 fold less potent than the latter. The K_A value of ACh, estimated after fractional inactivation of receptors with PrBCM and without cholinesterase inhibition, was 2.9 fold greater ($P < 0.005$) than that similarly estimated for (+)-MCh. As a result, ACh had greater efficacy than (+)-MCh under these conditions (Table 1). The K_A value of (−)-MCh, estimated in naive ileum by the method of Mackay (1966), exceeded the K_A value of (+)-MCh by almost 200 fold. (+)-MCh had a 3.6 fold greater ($P < 0.005$) efficacy than (−)-MCh.

Because of sustained contraction of the ileum, presumably caused by accumulated endogenous ACh, it was not possible to determine EC_{50} values of the three agonists after inhibition of cholinesterases with DFP. Treatment of the ileum with PrBCM (0.15 μM for 15 min) antagonized completely this contraction

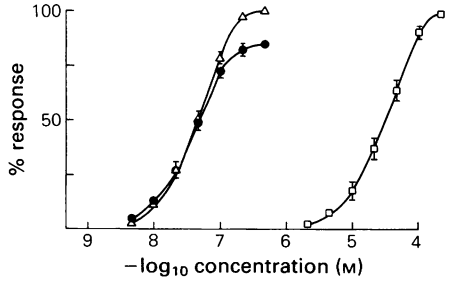

Figure 1 Concentration-response curves in the guinea-pig isolated ileum of acetylcholine (●), (+)-methacholine (△) and (−)-methacholine (□). Responses are expressed as a percentage of the maximum contraction elicited by (+)-methacholine. Each point is the mean response and the vertical lines show s.e. Eight to ten preparations were used.

by reducing the concentration of active receptors. (−)-MCh is poorly hydrolyzed by acetylcholinesterase (Beckett *et al.*, 1963; Cocolas & Robinson, 1970). It may therefore be assumed that its EC_{50} value is not much affected by DFP treatment. From the rightward shift (about 6 fold) in the (−)-MCh concentration-response curve observed after pretreatment with DFP and PrBCM, it may be calculated that the initial PrBCM treatment inactivated about 85% of the receptors. In the DFP-PrBCM-treated ileum, ACh was significantly more potent than (+)-MCh (Table 2) although the compounds were equipotent in naive ileum (Table 1). These results are in agreement with the observation that ACh is a better substrate than (+)-MCh for acetylcholinesterase (Beckett *et al.*, 1963; Cocolas & Robinson, 1970). Furthermore, in contrast to untreated ileum, the three compounds investigated produced the same maximum response in DFP-PrBCM-treated tissue (Table 2).

The results of a typical experiment for the determination of the K_A value of (+)-MCh in DFP-PrBCM-pretreated muscle are shown in Figure 2. Dose-response curves were normally recorded 45 min after the second PrBCM-incubation (5 μM for 15 min). As noted previously with other agonists (Ringdahl, 1984a; 1985), some recovery of responses was observed after prolonged washout time for PrBCM. However, K_A values obtained after prolonged washing (90 min) were not significantly different from those measured after 45 min. (+)-MCh had a 2 fold lower K_A value in DFP-treated tissue as compared to tissue in which cholinesterases were not inhibited, whereas the K_A value of (−)-MCh was not significantly ($P > 0.05$) different under the two conditions. The K_A value of ACh decreased 7 fold after DFP-treatment and was similar to that observed for (+)-MCh. The efficacy of (−)-MCh in DFP-treated tissue was 2 fold lower than that of (+)-MCh which had about half the efficacy of ACh (Table 2).

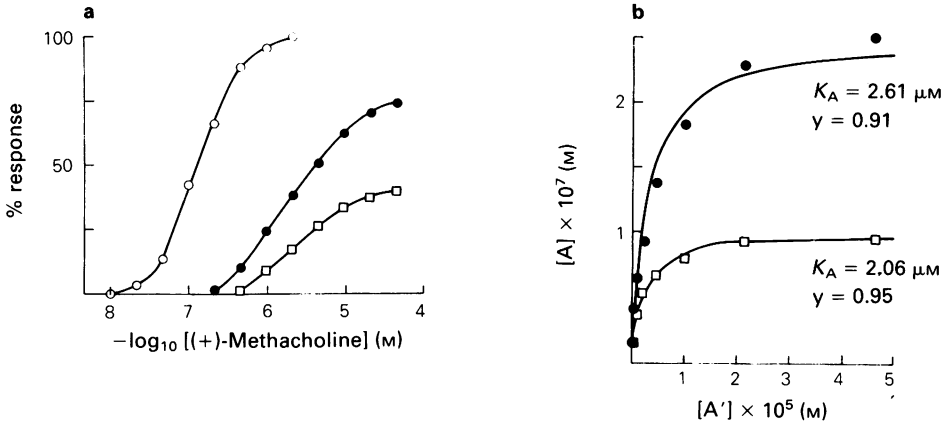

Figure 2 Concentration-response curves in the guinea-pig isolated ileum of (+)-methacholine (a) and plot of [A] vs. [A'] (b). The ileum was pretreated with diisopropylfluorophosphate and propylbenzilylcholine mustard (PrBCM) to inactivate cholinesterases and to block the response to accumulated endogenous acetylcholine as described in Methods. (a) Control concentration-response curve (○), concentration-response curve after incubation with 5 μM PrBCM for 15 min and after 45 (□) and 90 min (●) wash. (b) Values for [A] and [A'] were obtained from the control concentration-response curve and the plotted points after incubation with PrBCM (a). The fraction of receptors inactivated (γ) is given relative to the receptor concentration after the initial treatment with PrBCM which reduced the original receptor concentration by about 85%. The theoretical curves represent the best fit to Equation 1 in Methods.

Table 2 Parameters characterizing the muscarinic activity of acetylcholine (ACh), (+)- and (−)-methacholine in diisopropylfluorophosphate-treated guinea-pig ileum

Compound	n	*e.p.m.r.*†	E_{max}*	K_A** (μM)	*Relative efficacy*
Acetylcholine	8	1.00	1.00	1.7 ± 0.18	1.00
(+)-Methacholine	5	1.64 ± 0.20	0.99 ± 0.01	2.0 ± 0.14	0.60 ± 0.071
(−)-Methacholine	4	1238 ± 170	0.96 ± 0.02	620 ± 69	0.28 ± 0.046

Values are means ± s.e. n = number of preparations used.
†Equipotent molar ratio relative to ACh.
*Maximum response relative to the maximum for ACh.
**K_A values were determined according to Furchgott & Bursztyn (1967).

Discussion

The potencies of (+)- and (−)-MCh in eliciting contractions of intestinal smooth muscle have been extensively studied (Triggle, 1984). Beckett *et al.* (1963) showed that (+)-MCh was equiactive with ACh and 240 fold more active than (−)-MCh in whole ileum of the guinea-pig, whereas Chang & Triggle (1973) and Ward & Young (1977) found enantiomeric potency ratios of 1000 and 470, respectively, in the longitudinal muscle of the guinea-pig ileum. All of these results were obtained in the absence of a cholinesterase inhibitor. The relative potencies of ACh, (+)-MCh and (−)-MCh measured in the present study agreed reasonably well with those reported previously. Cholinesterase inhibition had no apparent effect on the potency ratio of the MCh enantiomers although it increased the relative potency of ACh.

Fuder & Jung (1985) recently determined the K_A values of (+)- and (−)-MCh at presynaptic muscarinic receptors mediating inhibition of noradrenaline release in rat heart. The K_A values were determined in the absence of a cholinesterase inhibitor as physostigmine had no effect on the inhibitory potency of (+)-MCh. By applying the method of Furchgott & Bursztyn (1967) and using phenoxybenzamine as an irreversible antagonist, they obtained a K_A value of

2.5 μM for (+)-MCh. This value is somewhat smaller than that (4.1 μM) determined for (+)-MCh at postsynaptic muscarinic receptors of the guinea-pig ileum by the same method but with PrBCM as irreversible antagonist (Table 1). However, Fuder & Jung (1985) noted that the K_A value of (±)-MCh was slightly higher when PrBCM was used instead of phenoxybenzamine. It therefore appears that (+)-MCh has similar affinity for presynaptic muscarinic receptors in the rat heart and postsynaptic receptors in the guinea-pig ileum. Fuder & Jung (1985), using the method of Mackay (1966), obtained a K_A value of 440 μM for (−)-MCh. Their value was slightly, but not significantly lower than that similarly determined at ileal muscarinic receptors (Table 1). Furthermore, the efficacy ratio (+/−) of the enantiomers of MCh was similar in the rat heart (4.4) and in the guinea-pig ileum (3.6). However, the degree of receptor occupancy required for a given response differed in the two tissues. Thus, (+)-MCh and (−)-MCh had to occupy 4 and 16%, respectively, of the receptors to inhibit by 50% the stimulation-evoked noradrenaline release in the rat heart (Fuder & Jung, 1985). In the guinea-pig ileum, 1 and 3.7% receptor occupancy by (+)-MCh and (−)-MCh, respectively, was sufficient to induce a half-maximal response (Table 1). These results suggest a larger receptor reserve for (+)- and (−)-MCh with respect to contractile responses in the guinea-pig ileum than with respect to muscarinic inhibition of noradrenaline release in the rat heart.

Although the K_A value of ACh measured in the absence of cholinesterase inhibition (Table 1) clearly underestimates its affinity for the receptor, it appears that its relative efficacy can be satisfactorily estimated under these conditions. Thus, the relative efficacies of ACh, (+)-MCh and (−)-MCh were similar in untreated and in DFP-treated ileum. Furthermore, the percentage receptor occupation required by ACh for 50% response (Table 1) agreed with that (0.48%) estimated for carbachol under similar conditions (Ringdahl, 1984a). ACh and carbachol have been shown previously to have identical efficacy at postsynaptic muscarinic receptors of rabbit stomach muscle (Furchgott & Bursztyn, 1967).

The K_A values of ACh and (+)-MCh estimated in DFP-treated ileum were similar (Table 2). The value for ACh agreed with those determined previously in the longitudinal muscle of the guinea-pig ileum (Sastry & Cheng, 1972) and in rabbit stomach muscle (Furchgott & Bursztyn, 1967). The K_A value of (+)-MCh does not appear to have been estimated before at postsynaptic muscarinic receptors. Furchgott & Bursztyn (1967), however, found a K_A value of 2.48 μM for (±)-MCh at muscarinic receptors in rabbit stomach muscle.

ACh and the enantiomers of MCh have been extensively investigated in radioligand displacement experiments (Birdsall *et al.*, 1978; 1980; Ehlert *et al.*, 1980), but only a few of these have been carried out in the guinea-pig ileum (Yamamura & Snyder, 1974; Ward & Young, 1977; Aronstam *et al.*, 1979). The concentrations (IC_{50}) of ACh (2–4 μM) and (±)-MCh (2–3 μM) required to inhibit by 50% the binding of [^{3}H]-3-quinuclidinyl benzilate to a membrane fraction of guinea-pig ileal smooth muscle (Yamamura & Snyder, 1974) agree well with the functional K_A values of ACh and (+)-MCh estimated in the present study (Table 2). On the other hand, the inhibition constants (K_i) similarly obtained for (+)-MCh and (−)-MCh by Aronstam *et al.* (1979) were about 10 fold lower than the K_A values in Table 2. K_A values of muscarinic agonists often agree with low affinity dissociation constants (K_L) obtained from binding experiments (Birdsall *et al.*, 1978; Ringdahl, 1985). As noted by Fuder & Jung (1985), it is difficult, however, to relate the K_A values of (+)-MCh and (−)-MCh to affinity for any of the sub-sites (superhigh, high and low affinity) detected in binding studies.

In the present study, the enantiomers of MCh differed 200–300 fold in affinity for muscarinic receptors. It should be pointed out, however, that both methods used to estimate the K_A value of (−)-MCh may be subject to a certain error which may affect the enantiomeric affinity ratio. Thus, in the analysis according to Furchgott & Bursztyn (1967), very high concentrations of (−)-MCh had to be used after fractional receptor inactivation. In the K_A determination according to Mackay (1966), the low ratio (2–4) of intrinsic efficacies of (+)- and (−)-MCh may increase the error (Fuder & Jung, 1985). Nevertheless, our enantiomeric affinity ratio agreed reasonably well with that (200) obtained from inhibition of [^{3}H]-PrBCM binding to intact muscle strips of guinea-pig intestine (Ward & Young, 1977) and with that (180) determined in functional studies by Fuder & Jung (1985). Consequently, the large difference in spasmogenic activity between (+)- and (−)-MCh in the guinea-pig ileum is determined mainly by a difference in affinity for muscarinic receptors. Similar observations have been made previously with analogues of oxotremorine (Ringdahl, 1984a,b) and with lactoylcholines (Sastry & Cheng, 1972) which showed little or no stereoselectivity with respect to their receptor activating abilities (efficacy).

The similar K_A values of ACh and (+)-MCh (Table 2) indicate that the methyl group at the chiral centre of (+)-MCh does not enhance binding to the muscarinic receptor. This observation contrasts with some recent results obtained with optically active analogues of oxotremorine in which a methyl group may substantially reinforce binding (Amstutz *et al.*, 1985). In (−)-MCh, the methyl group clearly interferes with binding. ACh and MCh are believed to bind to muscarinic receptors in conformations similar to those depicted in

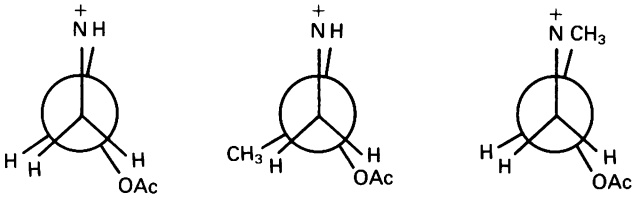

Figure 3 Anticlinal conformations of acetylcholine (ACh), (+)-methacholine ((+)-MCh) and (−)-methacholine.

Figure 3 in which the N-C-C-O grouping has an anticlinal arrangement (Casy, 1975). The present results suggest that the methyl group of (+)-MCh does not significantly affect the ability to attain the anticlinal conformation as compared to ACh. In (−)-MCh, however, the methyl group may destabilize the anticlinal conformer, presumably because it requires eclipsed methyl and trimethylammonium groups (Figure 3). According to this view, the 300 fold lower affinity of (−)-MCh as compared to (+)-MCh is due to the difficulty with which the former assumes the anticlinal conformation necessary for binding to muscarinic receptors.

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