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Muscarinic receptor subtypes controlling the cationic current in guinea-pig ileal smooth muscle

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1 The effects of muscarinic antagonists on cationic current evoked by activating muscarinic receptors with the stable agonist carbachol were studied by use of patch-clamp recording techniques in guinea-pig single ileal smooth muscle cells.

2 Ascending concentrations of carbachol (3–300 μM) activated the cationic conductance in a concentration-dependent manner with conductance at a maximally effective carbachol concentration (G_{max}) of 27.4±1.4 nS and a mean $-\log EC_{50}$ of 5.12±0.03 (mean±s.e.mean) (n=114).

3 Muscarinic antagonists with higher affinity for the M_2 receptor, methoctramine, himbacine and tripitramine, produced a parallel shift of the carbachol concentration-effect curve to the right in a concentration-dependent manner with pA_2 values of 8.1, 8.0 and 9.1, respectively.

4 All M_3 selective muscarinic antagonists tested, 4-DAMP, *p*-F-HHSiD and zamifenacin, reduced the maximal response in a concentration-dependent and non-competitive manner. This effect could be observed even at concentrations which did not produce any increase in the EC_{50} for carbachol. At higher concentrations M_3 antagonists shifted the agonist curve to the right, increasing the EC_{50}, and depressed the maximum conductance response. Atropine, a non-selective antagonist, produced both reduction in G_{max} (M_3 effect) and significant increase in the EC_{50} (M_2 effect) in the same concentration range.

5 The depression of the conductance by 4-DAMP, zamifenacin and atropine could not be explained by channel block as cationic current evoked by adding GTPγS to the pipette (without application of carbachol) was unaffected.

6 The results support the hypothesis that carbachol activates M_2 muscarinic receptors so initiating the opening of cationic channels which cause depolarization; this effect is potentiated by an unknown mechanism when carbachol activates M_3 receptors. As an increasing fraction of M_3 receptors are blocked by an antagonist, the effects on cationic current of an increasing proportion of activated M_2 receptors are disabled.

Keywords: Gastrointestinal smooth muscle; M_2 and M_3 muscarinic receptors; carbachol; cationic current; muscarinic antagonists

Introduction

Acetylcholine is the major neurotransmitter causing contraction of various smooth muscles by activating muscarinic receptors. In various gastrointestinal smooth muscles two muscarinic receptor subtypes, M_2 and M_3, are found with no measurable quantities of M_1 or M_4 receptors. Binding studies indicate that the number of M_2 receptors is much greater than the number of M_3 receptors (4:1 to 5:1) (Giraldo *et al*., 1987; 1988; Michel & Whiting, 1988; 1990; Candell *et al*., 1990; Ford *et al*., 1991; Zhang *et al*., 1991; Cuq *et al*., 1994a,b). However, functional studies have shown that the contractile response is mediated by the M_3 receptor subtype and the functional role of M_2 receptors remains unclear (Clague *et al*., 1985; Michel & Whiting, 1988; Candell *et al*., 1990; Ford *et al*., 1991; Eglen & Harris, 1993; Cuq *et al*., 1994a; Kerr *et al*., 1995). The presence of two different muscarinic receptor subtypes suggests that both would be activated by acetylcholine and would contribute to the contractile response but the signal transduction pathways (G proteins and effectors) involved are likely to be different. M_3 receptor activation produces contraction by phospholipase C activation resulting in $InsP_3$ formation and intracellular Ca^{2+} release (Candell *et al*., 1990; Prestwich & Bolton, 1991; 1995a; Zhang & Buxton, 1991; Cuq *et al*., 1994a, b; Phillippe & Basa, 1997). The functional role of the major population of M_2 receptors is generally believed to be indirect. This effect involves inhibition of adenylate cyclase activity mediated by a Pertussis toxin-sensitive G protein resulting in the inhibition of the relaxation produced by activation of other receptors (Candell *et al*., 1990; Zhang & Buxton, 1991; Griffin & Ehlert, 1992; Thomas *et al*., 1993; Cuq *et al*., 1994b; Thomas & Ehlert, 1994; Ehlert & Thomas, 1995; Prestwich & Bolton, 1995b; Reddy *et al*., 1995).

Apart from releasing intracellular Ca^{2+} via the phospholipase C/$InsP_3$ system, muscarinic agonists also produce membrane depolarization which triggers action potential generation (Bülbring, 1954; Burnstock, 1958; Bülbring & Kuriyama, 1963; Bolton, 1972; 1977). This membrane depolarization results from muscarinic receptor cationic channel activation (Benham *et al*., 1985). Thus, cationic channels, which are not measurably permeable to Ca^{2+}, may nonetheless allow Ca^{2+} to enter the cell by indirectly activating voltage-dependent Ca^{2+} channels via membrane depolarization so causing contraction. However, despite this important physiological function there are no pharmacological data characterizing the muscarinic receptor subtype mediating cationic channel opening. The aim of the present study was therefore to establish the identity of this receptor by use of selective muscarinic antagonists.

A preliminary communication of some of these results has been published (Bolton & Zholos, 1997).

Methods

Experimental procedures were generally the same as those already described (Zholos & Bolton, 1996a). Male adult guinea-pigs (300–400 g) were killed by dislocation of the neck followed by immediate exsanguination. Experiments were performed at room temperature on single ileal smooth muscle cells

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from the longitudinal muscle layer obtained after collagenase treatment (1 mg ml^{-1}) at 36°C for about 25 min.

Electrical recordings

Whole cell membrane current was recorded with low-resistance borosilicate patch pipettes (1 to 3 MΩ) and Axopatch 200A (Axon Instruments Inc., Foster City, CA, U.S.A.) voltage-clamp amplifier. Series resistance was compensated by about 80%. Recordings were sampled at 48 kHz and stored on a digital tape recorder (DTR-1204, Biologic Science Instruments, Claix, France). Background conductance was less than 4% of that activated by carbachol and was digitally subtracted off-line.

Solutions

Pipettes were filled with the following solution (in mM): CsCl 80, MgATP 1, creatine 5, NaGTP 1, glucose 20, HEPES 10, BAPTA 10, $CaCl_2$ 4.6 (calculated $[Ca^{2+}]_i = 100$ nM), pH adjusted to 7.4 with CsOH (total Cs^+ 124 mM). The use of Cs^+ internally effectively abolishes K currents as K channels have a low permeability to this cation. However, Cs^+ readily permeates the cationic channel activated by muscarinic receptor activation (Zholos & Bolton, 1996b). The presence of 1 mM GTP in this solution reduces desensitization to a minimum (Zholos & Bolton, 1996a). In some experiments carbachol was not applied to the cell in the bathing solution, instead GTP in the pipette solution was replaced with 0.2 mM GTPγS to generate cationic current directly (Zholos & Bolton, 1994; 1996a). The basic external solution in which I_{cat} was recorded consisted of (in mM): CsCl 120, glucose 12, HEPES 10, pH adjusted to 7.4 with CsOH (total Cs^+ 124 mM). Also, under these conditions I_{cat} modulation by both intracellular Ca^{2+} (Inoue & Isenberg, 1990; Pacaud & Bolton, 1991) and external divalent cations (Zholos & Bolton, 1995) was prevented. Under physiological conditions membrane depolarization is produced by Na^+ influx through cationic channels. Thus, in a separate series of experiments, Cs^+ was replaced by Na^+ (both in external and internal solutions) and 2.5 mM $CaCl_2$ and 1.2 mM $MgCl_2$ were added to the external solution. The cells after separation were kept in the following solution before the experiment (mM): NaCl 120, KCl 6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, glucose 12, HEPES 10, pH adjusted to 7.4 with NaOH; this solution was generally used to wash the cell after carbachol application.

Up to 11 different solutions with various concentrations of carbachol and muscarinic antagonists could be applied to the same cell. Complete exchange of the external solution was achieved within about one second as described previously (Zholos & Bolton, 1995).

Measurement and data analysis

Concentration-effect curves were constructed by plotting cationic conductance vs carbachol concentration. Muscarinic receptor cationic current *I*-V relationship is U-shaped at negative potentials (inward current plotted downwards) and correspondingly the cationic conductance activation curve is sigmoidal. Moreover, its position on the voltage axis depends on the agonist concentration (Zholos & Bolton, 1994). Thus, in the present experiments maximal conductance activated usually at potentials less negative than −40 mV was used as the agonist-sensitive parameter. Membrane potential was held at −40 mV and the effect of each concentration of carbachol was assessed after the cationic current had reached its steady-state by applying a slow voltage ramp from 0 to −40 mV (1.2 s duration). The speed of the ramp was sufficiently slow to reach a steady-state activation at each potential (Zholos & Bolton, 1996a). The linear portion of the *I*-V curve visualized by eye was fitted by a linear regression to obtain maximal chord conductance. Concentration-effect curves were fitted by a logistic function in the following form:

$$G/G_{max} = \{1 + ([EC_{50}]/[A])^b\}^{-1}$$

where G is the maximal cationic conductance activated at a given carbachol concentration, G_{max} is the cationic conductance at a maximally effective carbachol concentration, EC_{50} is the agonist concentration ([A]) when G was 50% of G_{max} and b is the slope factor of the agonist curve. The EC_{50} value in the presence of a fixed antagonist concentration was divided by that in the absence of antagonist to obtain the dose-ratio. Apparent antagonist affinities (and from these the pA_2) were obtained by Schild regression analysis (Arunlakshana & Schild, 1959). Each cell was exposed to antagonist at only one fixed concentration. In separate experiments the concentration-effect curves were obtained several times without antagonist to examine the effects of desensitization.

Data were analysed and plotted by use of MicroCal Origin software (MicroCal Software, Inc., Northampton, MA, U.S.A.) which uses the Levenberg-Marquardt nonlinear least square curve fitting algorithm. Values are given as the means ± s.e.mean except for the slope of Schild plot where s.d. is given. Student's *t* test was used for statistical comparison and differences were judged to be statistically significant when $P < 0.05$.

Chemicals used

Collagenase (type 1A), adenosine 5′ triphosphate (ATP, magnesium salt), guanosine 5′-triphosphate (GTP, sodium salt), guanosine 5′-O-(3-thiotriphosphate) (GTP-γS, tetralithium salt), creatine, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), 1,2-*bis*(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA), carbamylcholine chloride (carbachol) and atropine were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Methoctramine (N,N′-bis[6-[[(2-methoxyphenyl) methyl]amino] hexyl] - 1,8 - octanediamine tetrahydrochloride), tripitramine tetraoxalate, 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide), *p*-F-HHSiD (*para*fluoro-hexahydro-sila-difenidol hydrochloride) were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). Himbacine was obtained from Calbiochem-Novabiochem (U.K.) Ltd. (Beeston, Nottingham, U.K.). Zamifenacin was generously provided by Dr V. Alabaster of Pfizer Ltd. (Sandwich, Kent, U.K.).

Results

Responses to carbachol

Inward current was evoked by carbachol applied in the bathing solution to cells voltage-clamped at −40 mV; ascending concentrations, usually four, were applied (Figure 1a). When the current was steady, the current-voltage relationship was estimated with a ramp from 0 mV to −40 mV (Figure 1b). Close to zero potential the conductance was constant but tended to decline in some cells as −40 mV was approached; in such cases conductance was estimated from the linear portion of the current-voltage relationship (as shown by the dotted lines in Figure 1b) after subtraction of the current-voltage relationship obtained with a similar ramp applied before carbachol application. A maximally effective concentration of carbachol produced a conductance of 27.4 ± 1.4 nS; mean −log EC_{50} was 5.12 ± 0.03 ($n = 114$). Unless carbachol was washed off the cell with calcium-containing solution, inward current declined very slowly usually leaving a residual inward current; application of a sodium-based, calcium- and magnesium-containing solution resulted in the rapid disappearance of inward current. This solution was applied before calcium-free caesium-based solution was added and a second or third series of carbachol concentrations applied. The moments when this latter solution was applied and removed are indicated in Figure 1a; note that Ca,

Mg-free Cs^+-based solution application resulted in the appearance of a small inward current (cf. Figure 3a,b).

Changes in G_{max}, EC_{50} and slope of the logistic function (b) of the agonist curve with repeat applications of carbachol (e.g. Figure 1) were measured in nine cells to estimate the extent to which desensitization might complicate our results with muscarinic antagonists. These findings can be summarized as follows: (i) there was no change of the slope (1.5 ± 0.1 for the first and 1.5 ± 0.2 for the second agonist curve; $n = 9$); (ii) G_{max} was decreased from 24.7 ± 4.8 nS (first agonist curve) to 20.4 ± 4.0 nS (second agonist curve), or by about 17%. By use of each cell as its own control and so normalizing the first G_{max} to 100% a similar reduction of the second G_{max} value to $82.0 \pm 8.2\%$ ($n = 9$) was calculated. These changes were found not to be significant by use of paired two-tailed t test ($P > 0.05$). However, on the assumption that desensitization always reduces G_{max} it was found to change significantly during a second carbachol application by a paired one-tailed t test ($P < 0.05$). Thus, these changes were taken into account (e.g. Figure 6 as shown by the dotted line); (iii) there was negligible effect of desensitization on the EC_{50} value which is the most important parameter used in the quantitative analyses below. If actual averaged values were compared it increased by only about 4% from 11.2 ± 3.2 μM (first agonist curve) to 11.7 ± 2.0 μM (second agonist curve), or expressed as $-\log EC_{50}$ the change was from 5.06 ± 0.10 to 4.98 ± 0.07. We noted that the EC_{50} values of a large number of cells tested ($n = 114$) showed normal distribution when plotted on a logarithmic scale (not illustrated). With each cell as its own control the second EC_{50} value was increased to $135.3 \pm 22.9\%$ ($n = 9$) compared to the first EC_{50} value normalized as 100%. Neither absolute nor relative changes were statistically significant ($P > 0.05$) no matter whether a paired two-tailed t test or a paired one-tailed t test (on the assumption that desensitization always decreases the sensitivity to carbachol) was applied. Any effect of desensitization if it occurred on the $(DR - 1)$ value was very small compared to values in the presence of antagonists (see below) and, moreover, it is unclear whether desensitization is simply additive to the effect produced by an antagonist, or inhibits or potentiates it.

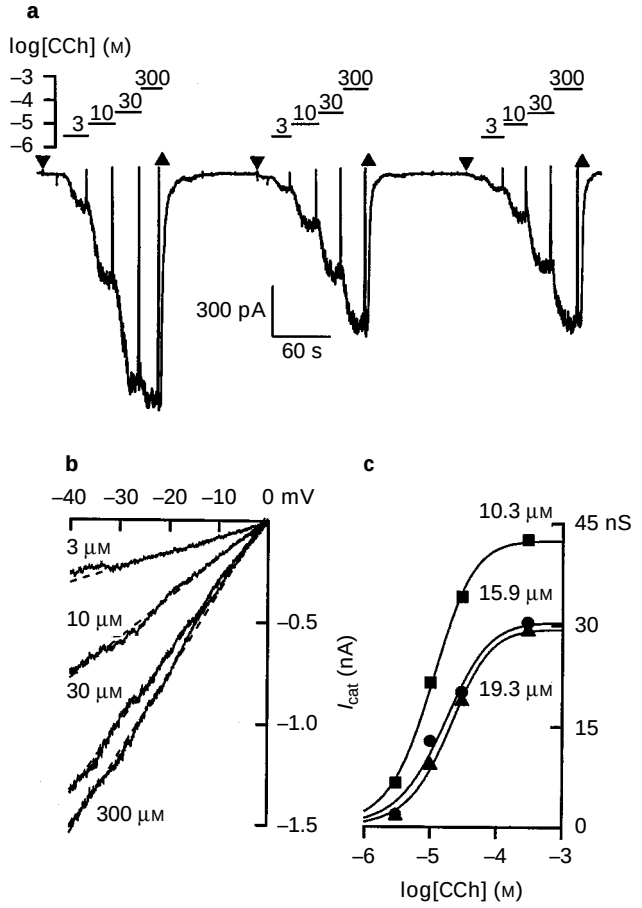

Figure 1 Effects of repeat application of carbachol on the concentration–cationic conductance curve. (a) Inward cationic current measured at the holding potential of -40 mV upon repetitive applications of ascending concentrations of carbachol (CCh). Horizontal bars above the trace in this and all subsequent figures indicate duration of each carbachol concentration application whereas vertical deflections on the current trace indicate slow voltage ramp application from 0 to -40 mV to estimate maximal conductance as described in Methods. The horizontal lines above the current trace indicate the application of various concentrations of carbachol given in μM above the lines; these are indicated by the vertical position of the line as measured on the logarithmic scale. Down and up triangles indicate the moments of Ca^{2+}, Mg^{2+}-free Cs^+-containing external solution application and wash-out, respectively. (b) I-V relationships for I_{cat} obtained by ramps measured during the first cumulative-carbachol application. Carbachol concentration is indicated near each trace. Current before carbachol has been subtracted here and elsewhere. The traces were fitted by linear regression (superimposed dotted lines; for all fits $r > 0.996$). (c) Concentration-effect curves for the experiment illustrated in (a). In this and all subsequent figures data points show cationic conductance corresponding to the slope of the linear portion of the I-V curve plotted against carbachol concentration used on a logarithmic scale. Data points were fitted by the logistic function with the EC_{50} values indicated near each trace. Pipette solution contained 1 mM GTP. In this experiment the EC_{50} increased slightly but in a series of similar experiments no significant change was found.

The time interval between carbachol applications was not crucial for the amount of desensitization observed. In many cases responses of similar size could be obtained with very short intervals (20–30 s) but if desensitization did develop even prolonged periods of washing (5–10 min) failed to restore the initial response (data not shown). The average time interval from wash-out after measuring one concentration-effect curve to the beginning of the next application of carbachol in control experiments was 100 ± 6 s ($n = 9$) and slightly longer than in the muscarinic receptor antagonist experiments (76 ± 5 s, $n = 107$) but it can be seen in Figure 3a that even a 40 s interval was sufficient to allow a maximal response to be obtained to a second series of carbachol applications. However, in Figure 1a 110 s wash-out interval between the first and second series of concentrations of carbachol resulted in a larger desensitization.

To test whether muscarinic antagonists achieved equilibrium in these experiments, they were applied in the presence of 50 μM carbachol (Figure 2). Rapid inhibition of the cationic current was produced by different muscarinic antagonists such as atropine (non-selective), methoctramine, tripitramine and himbacine (M_2-selective) and 4-DAMP (M_3-selective). The onset of inhibition could be approximated by a single exponential function (superimposed dotted lines) with time constants in the range 3–8 s. It should be noted that this experimental design gives an upper limit for the rates of binding because cationic current may outlast agonist dissociation from the receptor. Also, by occupying a proportion of the receptors the agonist may reduce the rate of association of the antagonist. We found that decreasing carbachol concentration from 50 to 5 μM resulted in a 1.7 fold increase in the rate of onset of atropine blockade ($P < 0.04$; unpaired two-tailed t test). Repeated applications of carbachol and himbacine in the presence of carbachol, showed that the rates of cationic current activation and deactivation upon carbachol application and wash out were comparable to the rates of offset and onset of himbacine blockade (Figure 2b). In the experiments which follow, antagonists were applied on average 27 ± 1 s ($n = 119$) before the second agonist concentration-effect curve in the presence of antagonist was measured. Since antagonist binding in the absence of agonist was facilitated even higher rates were expected, which thus would be sufficient to reach equilibrium with the receptors within this period. The rates observed on these naked, collagenase-treated cells, are

comparable to those observed in experiments where antagonists and agonists were iontophoresed onto the surface of muscle strips (Bolton, 1977) and are much faster than the rates normally seen in whole tissues, where diffusional barriers and uptake of lipid-soluble molecules significantly slow kinetics.

M_2 antagonists

Methoctramine, himbacine and tripitramine were tested as examples of antagonists having a higher affinity at M_2 compared to M_3 receptors. Methoctramine applied at 200 nM ($n=7$) produced a parallel shift in the carbachol concentration-effect curve; the slope factor, b, was not significantly different nor was there any significant reduction in the maximum response (Figure 3a and b, see also Figure 6). The increase in the EC_{50} in the example shown was about 27 fold. A lower concentration, 50 nM produced a lesser parallel shift in the carbachol concentration-effect curve (the dose ratio was 3.6 ± 1.8, $n=4$) and its slope factor was slightly and significantly increased. However, no change in the slope factor occurred with 1 μM methoctramine, but the maximum carbachol response was slightly, but significantly, reduced (Figure 6). When a Schild plot was constructed the slope was not significantly different from unity and when constrained to unity the intercept gave an equilibrium dissociation constant of 7.8×10^{-9} M corresponding to a pA_2 of 8.11 (Figure 4). These results with methoctramine at three concentrations are consistent with competitive antagonism at an M_2 receptor.

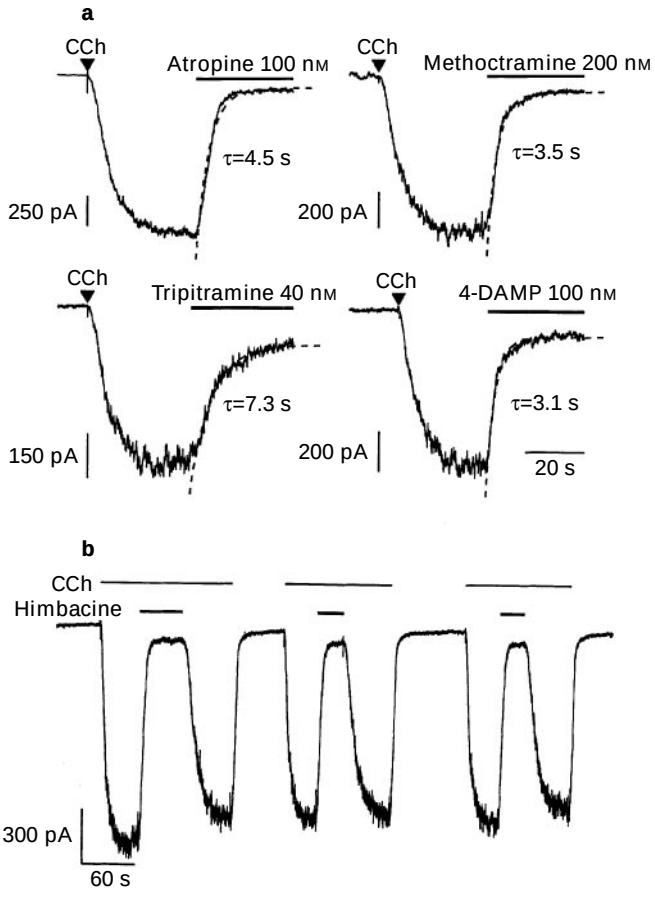

Figure 2 Rapid inhibition of the cationic current produced by different muscarinic antagonists in the continuing presence of carbachol. (a) Cationic current was activated by 50 μM carbachol (CCh) application (triangles). Typical examples to illustrate the effects of several muscarinic antagonists such as non-selective (atropine), M_2-selective (methoctramine and tripitramine) and M_3-selective (4-DAMP) applied at the concentrations indicated after the current reached its peak value. The inhibition was approximated by a single exponential function (superimposed dotted lines) with the time constant shown near each trace. (b) Effects of 100 nM himbacine applied several times to the same cell during repeated 50 μM carbachol applications as indicated by the horizontal lines.

Himbacine also behaved as a competitive antagonist of carbachol (Figure 3c and d). The increase in the EC_{50} in the example shown with 100 nM himbacine was about 11 fold. Three concentrations were applied (50 nM, 100 nM and 200 nM); none of these significantly reduced the maximum carbachol response and the slope factor was unchanged. A Schild plot had a slope not significantly different from unity and when constrained to unity gave a pA_2 of 7.97 consistent with an action at M_2 receptors (Figure 4). This antagonist was also tested under more physiological conditions where sodium replaced caesium (124 mM) in the internal and external solutions; calcium and magnesium were also added to the bathing solution. Smaller carbachol-induced currents were evoked decreasing the signal to noise ratio. However, essentially similar results were obtained to those in caesium-containing, divalent cation-free solution: the dose-ratio was 9.8 ± 3.2 ($n=5$) compared to 10.9 ± 1.5 ($n=9$) with 100 nM himbacine in the latter solution. The results are consistent with himbacine acting as a competitive antagonist at the M_2 receptor.

Three concentrations of tripitramine were used (10 nM, 40 nM and 100 nM). At low concentrations (10 and 40 nM) it also behaved as a competitive antagonist at the M_2 receptor. The highest concentration (100 nM) significantly depressed the maximum conductance change to carbachol (Figure 6). However, the slope factor, b, was not significantly changed. A Schild plot had a slope of 0.38 ± 0.13 ($\pm$s.d.) which gave a pA_2 of 9.08 (Figure 4).

M_3 antagonists

Three antagonists, 4-DAMP, *p*-F-HHSiD and zamifenacin, selective for M_3 receptors, and the non-selective antagonist, atropine, were tested. At lower concentrations the selective M_3 antagonists severely depressed the maximum conductance which could be achieved with carbachol with little or no increase in the EC_{50} (Figures 5 and 6). Higher concentrations also increased the EC_{50} while depressing the maximum response further; the slope factor, b, was not generally altered. The antagonist, 4-DAMP, was also tested in sodium-based solutions with calcium and magnesium in the bathing solution; at a concentration of 50 nM the dose ratio was 8.2 ± 0.2 ($n=5$) compared to 6.5 ± 2.2 ($n=5$) in the caesium-based divalent cation-free solution; this was not significantly ($P>0.06$) different. Thus, the M_3 selective antagonists at higher concentrations showed a non-competitive antagonism of the conductance change produced by carbachol. Atropine would be expected to bind to both M_2 and M_3 receptors with high affinity. It produced both a depression of the maximum conductance even at concentrations as low as 10–20 nM and an increase in the EC_{50} (Figure 5f).

The reduction in the maximum conductance change to carbachol by M_3 antagonists and atropine was further investigated as it was possible that some form of channel block might be involved. This seemed not to be the case. Cationic inward current was evoked in single cells voltage-clamped at -40 mV by including 0.2 mM GTPγS (instead of GTP) in the pipette solution; this evoked a large inward current (Zholos & Bolton 1995; 1996a) without the external application of carbachol, presumably by activating a G-protein and opening the cationic channels. The GTPγS-evoked current was not inhibited by 4-DAMP, or tripitramine at the highest concentrations used in the antagonism of carbachol (Figure 6). Atropine and zamifenacin were also without effect, but *p*-F-HHSiD produced about 25% reduction in the current at 1 μM so that some of its inhibitory action on the maximum carbachol conductance at 1 μM and 10 μM (Figures 5 and 6) could be ascribed to channel block.

Analysis of this pattern of antagonism is uncertain as the Schild assumption that equal responses result from equal

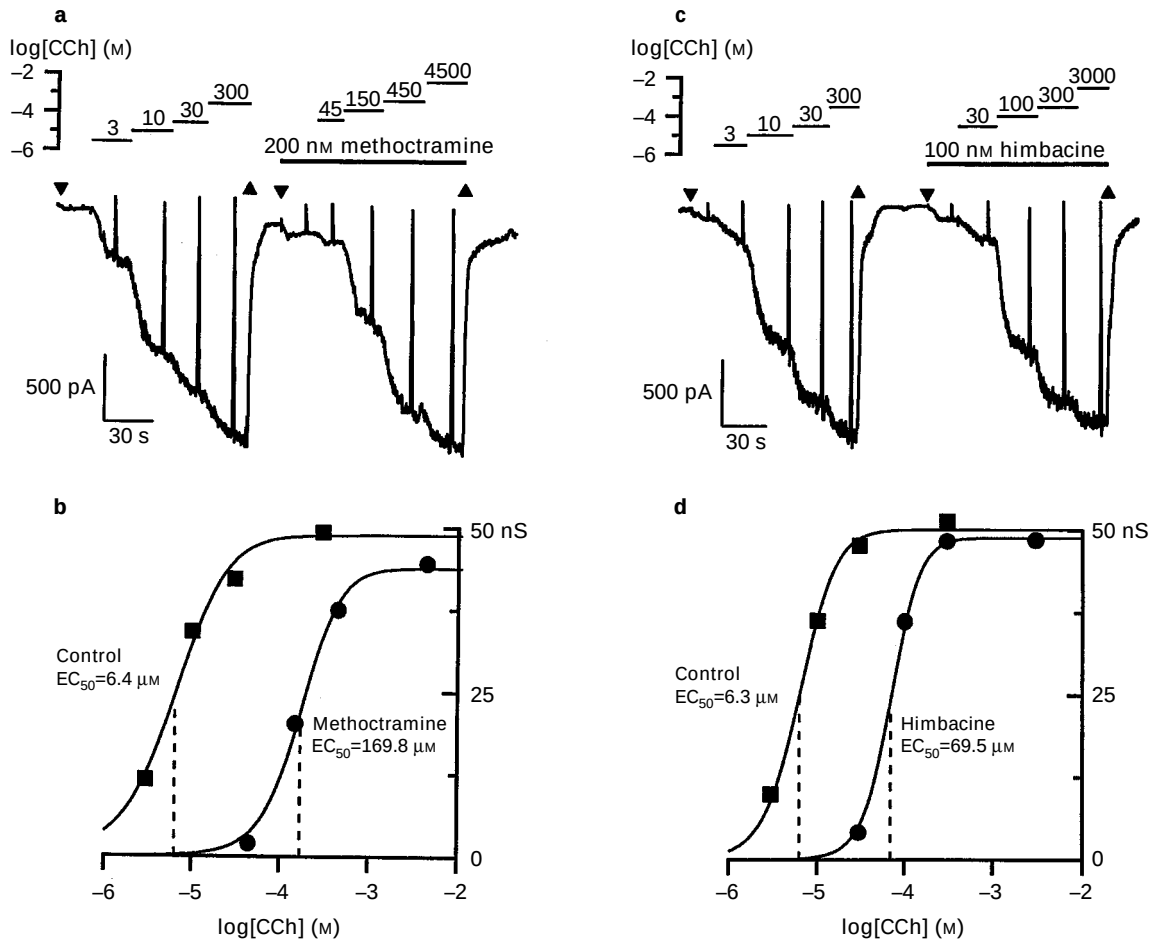

Figure 3 Effects of the M_2-selective antagonists methoctramine and himbacine on concentration-effect curves of carbachol-activated cationic conductance. (a) and (c). Cationic current activated by ascending carbachol concentrations before and in the presence of 200 nM methoctramine (a) or 100 nM himbacine (c) as indicated. Carbachol concentrations are indicated in μM above the traces; note that agonist concentrations were higher in the presence of antagonist. Down and up triangles indicate the moments of Ca^{2+}, Mg^{2+}-free Cs^{+}-containing external solution application and wash-out, respectively. It is not certain whether methoctramine or himbacine induced a small inward current because it was applied simultaneously with Ca^{2+}, Mg^{2+}-free solution which could also induce a similar current (compare Figure 1a). (b) and (d). Concentration-effect curves in control (squares) and in the presence of antagonist (circles) for the experiments shown in (a) and (c), respectively. Dotted lines indicate the EC_{50} values in each case.

fractional receptor occupancies cannot be applied. All four M_3 antagonists severely depressed the maximum whereas of the M_2 antagonists only tripitramine at the highest concentration used significantly depressed it. It is extremely unusual for atropine to behave as if it were a non-competitive antagonist of muscarinic responses. In view of this, and since there was generally no interference with cationic channel function, the possibility was considered that depression of the maximum response represented an M_3 effect. This hypothesis is strongly supported by the results shown in Figure 5. Indeed, if the rightward shift of the carbachol concentration-effect curve occurs due to M_2 receptor occupancy by the M_2 antagonist, then depression of the maximum probably represents an M_3 effect since this was generally not observed with M_2-selective antagonists. The data shown in Figure 5a and c are of particular interest because they show depression of the maximum without any change in the EC_{50} values. This result is consistent with the above hypothesis because under these conditions fractional occupancy of M_2 receptors by the M_3-selective antagonists will be low ($[B]/K_{b(M2)} = 0.13 - 0.19$) (hence no shift of the EC_{50}) but in contrast a significant proportion of the M_3 receptors will be occupied ($[B]/K_{b(M3)}$ are 31.7 and 6.3 for zamifenacin and *p*-F-HHSiD, respectively; K_b values taken from Table 1); the difference in fractional occupancy at the two receptor subtypes is 33–250 fold. Atropine, a non-selective high-affinity muscarinic antagonist, produced a combination of a shift of EC_{50} and depression of maximal response even at low concentrations (Figure 5f), which is also consistent with the above hypothesis because its fractional occupanices of the two subtypes would be expected to be about equal and to be appreciable.

Discussion

It is well established that in various gastrointestinal smooth muscles two different muscarinic receptors are present. They are defined pharmacologically as M_2 and M_3 corresponding to the genetically defined m_2 and m_3 subtypes. Evidence for this was obtained initially by radioligand binding studies (Giraldo *et al.*, 1987; 1988) and confirmed by Northern blot analysis (Maeda *et al.*, 1988; Ford *et al.*, 1991) and immunological techniques (Wall *et al.*, 1991). In many smooth muscles including guinea-pig ileum the M_2 receptor is the most abundant subtype (75% and more) with a minor contribution of the M_3 subtype. However, pharmacological functional studies indicated that the contractile response is mediated by this small M_3 fraction, whereas the functional role of the majority M_2 receptors remains unclear and has been suggested to be indirect (Clague *et al.*, 1985; Michel & Whiting, 1988; Candell *et al.*,

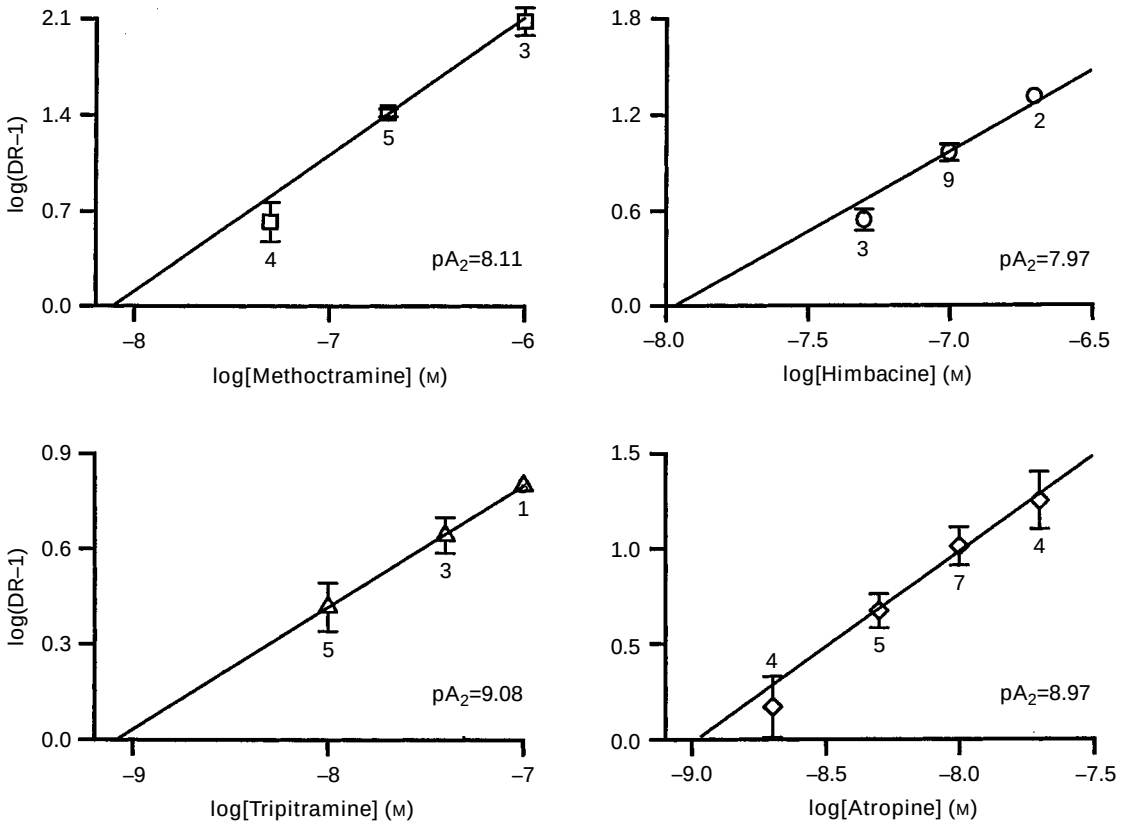

Figure 4 Schild regression analysis for three M_2-selective antagonists and atropine. Best-fit Schild slopes obtained by the method of least squares were as follows (mean ± s.d.): methoctramine, 1.05 ± 0.12; himbacine, 1.25 ± 0.12; tripitramine, 0.38 ± 0.09; atropine, 1.09 ± 0.20. Except for tripitramine they were not significantly different from unity and were constrained to 1.0 to obtain the intercept and from this the pA_2 value. The number of cells upon which each dose-ratio was measured (mean ± s.e.mean) is shown.

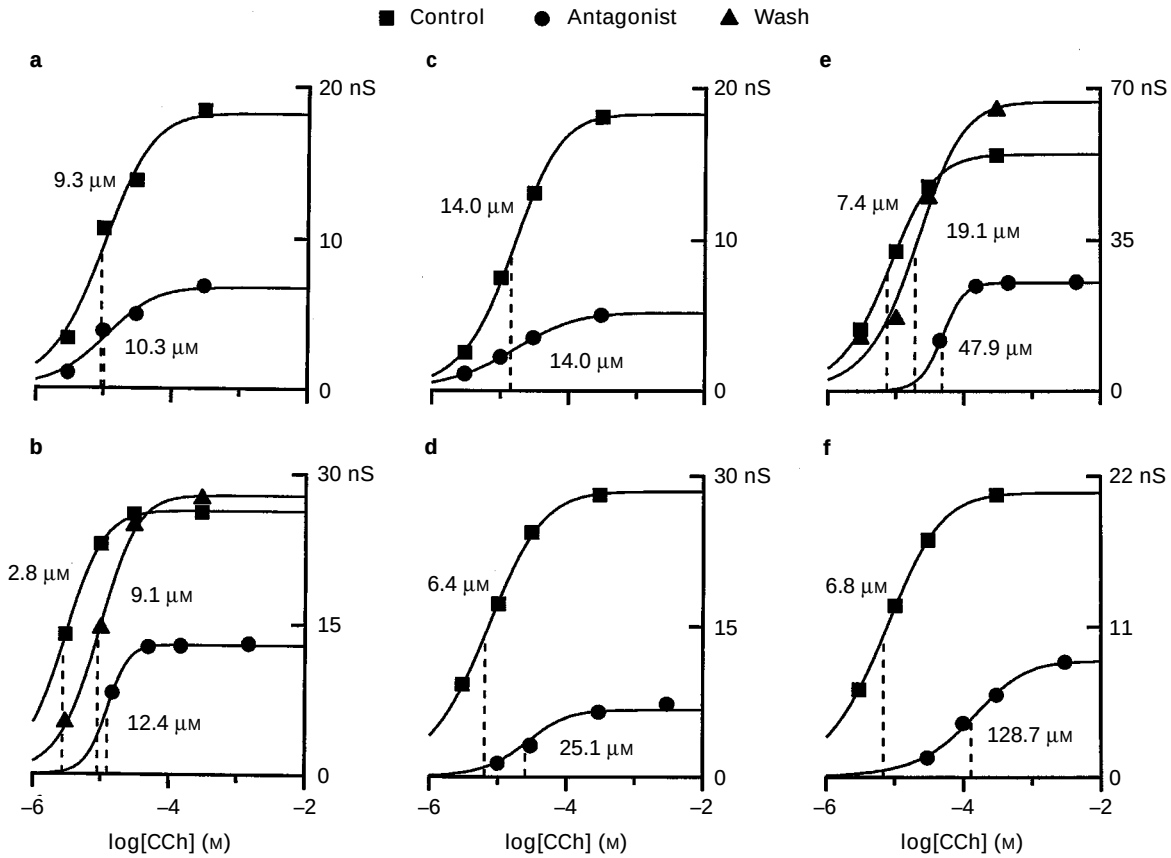

Figure 5 The effects of M_3-selective muscarinic antagonists *p*-F-HHSiD (a, 100 nM; b, 1 μM), zamifenacin (c, 20 nM; d, 50 nM) and 4-DAMP (50 nM, e) and the non-selective antagonist atropine (10 nM, f) on the concentration-effect curves for carbachol. Dotted lines indicate the EC_{50} values. Note that low concentrations of M_3 antagonists reduced the maximum conductance without change in EC_{50}; higher concentrations increased the EC_{50} value.

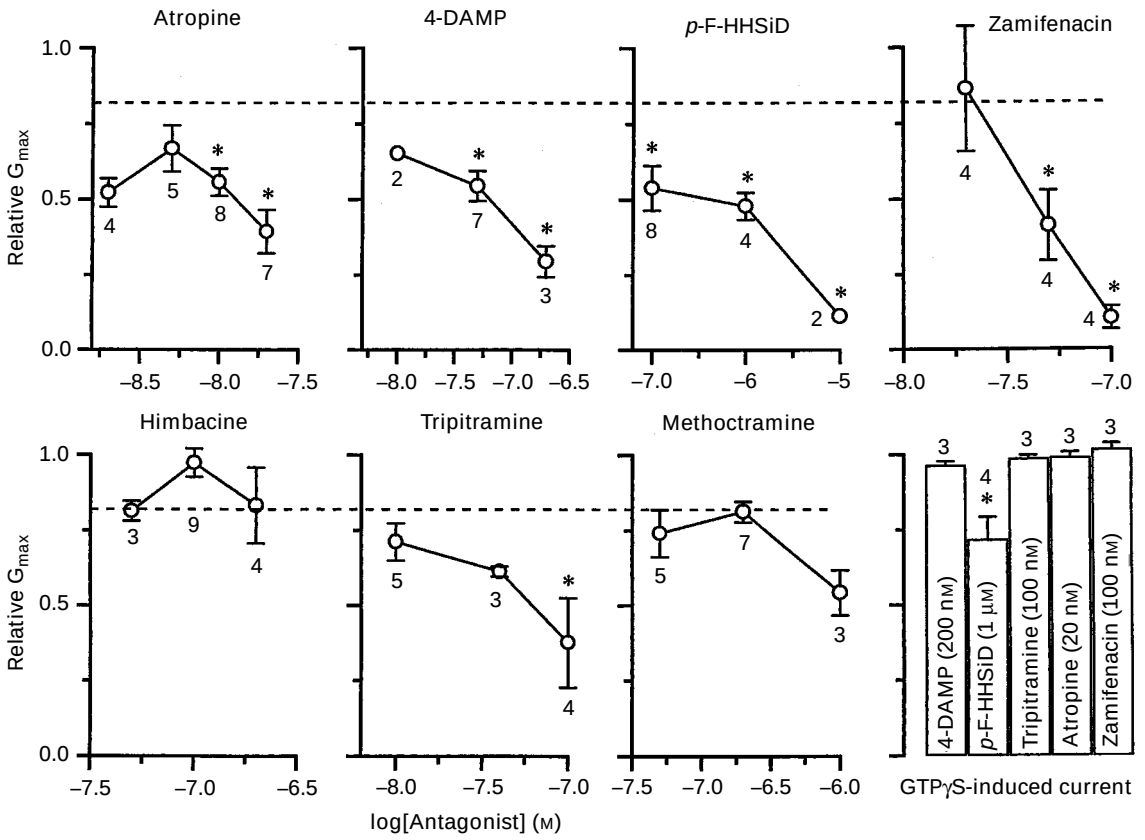

Figure 6 Reduction by various muscarinic antagonists of the response to a maximally-effective carbachol concentration (G_{max}). The number of observations is indicated near the data points. Horizontal dotted line shows relative G_{max} expected in an average cell during a second cumulative carbachol application under control conditions due to desensitization (0.82 ± 0.08; $n=9$). Right bottom panel shows the effects of several muscarinic antagonists on GTP-γS activated cationic current (no carbachol applied). See text for more details. $^*P<0.05$; unpaired two-tailed t test.

1990; Ford *et al.*, 1991; Eglen & Harris, 1993; Cuq *et al.*, 1994a; Kerr *et al.*, 1995). Indeed, guinea-pig ileum has been employed for many years as a convenient bioassay for M_3 receptor function (e.g. Wallis *et al.*, 1993).

In this study we establish for the first time that the M_2 muscarinic receptor subtype has a major functional role to open cationic channels producing membrane depolarization in this tissue. Our conclusion is based on the effects of selective muscarinic M_2 antagonists on the cationic current activated by carbachol in single guinea-pig ileal cells. Muscarinic antagonists used in the present study were chosen to allow discrimination between M_2 and M_3 receptors, as other subtypes are not present in these cells in measurable quantities. However, none of the available antagonists is specific for one receptor subtype. In the literature, antagonist affinity values vary considerably between preparations and even for the same receptor studied under different experimental conditions. Caulfield (1993) suggested that 'M_2 receptors are usefully defined by high affinity for methoctramine (7.9–8.3) and low affinity for pirenzepine (6.3–6.7), 4-DAMP (8.2–8.4) and *p*-F-HHSiD (6.0–6.9)....M_3 receptors have high affinity for 4-DAMP (8.9–9.3) and *p*-F-HHSiD (7.8–7.9).'

The unexpected action of M_3 receptor antagonists was to depress the maximum response by some mechanism which did not involve cationic channel block since they (except for *p*-F-HHSiD) did not depress GTPγS-evoked current. Thus, this depression of cationic current is brought about by an action at an earlier point in the transduction process, most likely by an action at the receptor. At low antagonist concentrations the depression occurred without any change in the EC_{50} values (Figure 5a, c) providing strong support for the hypothesis that M_3, but not M_2, receptor occupancy mediates this effect.

Table 1 Apparent antagonist affinities (pA_2)

	Present study	*M_2 receptor*	*M_3 receptor*
Methoctramine	8.1	7.8	6.2
Himbacine	8.0	8.2	7.3
Tripitramine	9.1	9.5	6.7
4-DAMP	(8.0)	7.9	9.0
p-F-HHSiD	(6.7)	6.3	7.8
Zamifenacin	(7.9)	6.8	9.2
Atropine	(9.0)	9.0	9.1

Antagonist affinities based on the dose-ratios measured from the EC_{50}s obtained in the present study (M_2 effect) (first column). Values in parentheses were obtained from concentration-effect curves where the maximum was depressed. The next two columns are the affinities of these antagonists at M_2 and M_3 receptors extracted from the literature: in most cases several values obtained in functional smooth muscle studies, or binding studies were averaged to obtain the values shown. Sources were: Arunlakshana & Schild (1959); Barlow *et al.* (1976); Michel & Whiting (1988, 1990); Lambrecht *et al.* (1989); Waelbroeck *et al.* (1989); Hulme *et al.* (1990); Wallis *et al.* (1993); Watson & Eglen (1994); Chiarini *et al.* (1995); Reddy *et al.* (1995); Tobin & Sjögren (1995); Watson *et al.* (1995). Though the present experiments were made at room temperature, whereas most of the published values refer to 37°C it should be noted that lowering the temperature by 8°C for a range of muscarinic antagonists tested resulted in an average pK_b increase of 0.2 corresponding to a 1.6 fold increase in the affinity (Barlow *et al.*, 1976). However, antagonist affinity values for any one receptor vary more (by about 3 fold) when measured at the same temperature but in different systems and in experiments by different groups (Caulfield, 1993).

Further evidence comes from the action of atropine which also, as would be predicted from its M_3 receptor blocking action, reduced the maximum cationic current.

If the depression of the maximum is an M_3 effect, then the rightward shift of the concentration-effect curves and the increase in the EC_{50} are likely to represent an M_2 receptor effect, especially since the M_3-selective antagonists displayed this effect at concentrations higher than those which depressed the maximum response. Analysis of the rightward shift produced by M_3 antagonists is quite speculative; as the slope factor was unchanged one approach is to assume that the M_3 effect does not alter the relationship between fractional receptor occupancy of the M_2 subtype and fractional response (i.e. the depression of the M_2-receptor evoked current is constant over the carbachol concentration range) in which case a Schild analysis with dose-ratios calculated from the shift in the EC_{50} can be applied. Atropine was subject to this analysis and provided support for this approach as, despite the severe depression of the maximum it produced, the Schild plot had a slope of unity and the pA_2 value was 9.0 (Figure 4). For 4-DAMP, *p*-F-HHSiD and zamifenacin the slopes of Schild plots were 1.22 ± 0.31 ($n=10$), 0.67 ± 0.18 ($n=12$) and 0.54 ± 0.20 ($n=6$) and pA_2 values were 7.97, 6.74 and 7.94, respectively. For 4-DAMP and *p*-F-HHSiD the pA_2 values were close to those found by others at the M_2 receptor (Table 1); for zamifenacin the value was midway between published values at M_2 and M_3 receptors. The rightward shift of the concentration-effect curves for 4-DAMP and *p*-F-HHSiD could be attributed to an effect on M_2 receptors (Table 1).

These data highlight the mystery surrounding the function of the dominant M_2 receptor subtype in smooth muscle. It has been known for about 50 years that in intestinal smooth muscle muscarinic agonists cause membrane depolarization and an increase in the spike frequency with associated increase in tension or frequency of contraction (reviewed by Bolton, 1979). At low agonist concentrations, burst-type action potential discharge occurs without a noticeable depolarization (Bolton, 1972). With increasing concentration action potentials are reduced in size and prolonged in duration and are eventually abolished with strong depolarization; in some smooth muscles slow waves may occur. The depolarizing action is thought to be mediated by muscarinic receptor cationic current which has a reversal potential of about -10 mV. Membrane depolarization, particularly when it triggers calcium spikes due to L-type Ca^{2+} channel activation, would contribute to smooth muscle contraction by providing substantial Ca^{2+} influx. Action potential discharge is controlled by a balance of inward and outward currents. Thus, it is plausible that even without notable depolarization, as seen at low agonist concentrations, small muscarinic receptor cationic current may alter this balance in favour of net inward current thus accelerating action potential generation. In this connection there is an important property of the cationic conductance which increases at low fractional receptor occupancies as it is activated by membrane depolarization (Benham *et al.*, 1985; Zholos & Bolton, 1994) potentially creating a regenerative system.

Of course, this sequence of events in which according to our present results M_2 receptor subtype plays a crucial role does not question the importance of the well-established Ca^{2+}-releasing effect mediated by the M_3/PLC/$InsP_3$ system. Instead, it raises the possibility that M_2 receptor function could be more direct than previously thought. In discussing this possibility all available data should be carefully considered, because there is an obvious discrepancy between binding studies, numerous pharmacological functional studies indicating that M_3 receptor mediates contraction, and strong electrophysiological evidence for muscarinic excitation (depolarization and action potential discharge) mediated by M_2 receptors according to the present findings. Considering this paradox, first and foremost the possible interaction between these two receptors in coupling to their effectors should be taken into account. Our present results suggest that there is a message generated by M_3 receptor activation which strongly potentiates, cationic channel openings initiated by the M_2 subtype. Thus, M_2 function may become ineffective when M_3 receptors are inactivated. One obvious candidate for such interaction is cytoplasmic Ca^{2+} which is known to potentiate strongly cationic channel opening (Inoue & Isenberg, 1990; Pacaud & Bolton, 1991). However, this would seem an unlikely possibility in these experiments where $[Ca^{2+}]_i$ was 'clamped' to 10^{-7} M by 10 mM BAPTA. If there was close juxtaposition of M_2 and M_3 receptors, and the SR Ca-release system releasing Ca into a submicron cleft between SR and plasma membrane, the possibility that the $[Ca^{2+}]_i$ 'clamp' may be inefficient in the restricted junctional space between SR and sarcolemma due to diffusional limitations cannot be excluded.

Another possibility is that hetero-oligomers of M_2 and M_3 receptors exist and that blockade of M_3 receptors inactivates the whole complex, such that activation of M_2 receptors in that complex is without effect. Only complexes where M_3 activation can occur simultaneously with activation of M_2 receptors would be effective in opening cationic channels.

A further possibility is that different muscarinic receptors may provide different contributions to the contraction depending on the agonist concentration. Their relative contributions to the rapid phasic, and tonic, contractions may also be different. Acetylcholine or carbachol produce contraction of the guinea-pig ileum with EC_{50} values in the nanomolar range and it is this component that is a subject of functional pharmacological studies. It is possible that the M_3/PLC/$InsP_3$ system is relatively more important at these concentrations because membrane depolarization in this tissue requires somewhat higher agonist concentration (Bolton, 1972). A steep increase in the membrane conductance in muscle strips was seen over the range 1.4 to 55 μM (Bolton, 1972), corresponding well to the EC_{50} of about 8 μM in the present study on single cells.

In conclusion, the results support the hypothesis that muscarinic receptor cationic channel opening in smooth muscle is gated primarily by M_2 receptor subtype activation and that blockade of the M_3 receptor subtype strongly reduces the effects of M_2 receptor activation equally over a range of fractional receptor occupancy. This gives an important functional role to the M_2 subtype. However, further evaluation of this hypothesis will require investigation to discover the mechanisms responsible for the interactions of receptor signal pathways between M_2 and M_3 subtypes.

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