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Muscarinic agonist potencies at three different effector systems linked to the M_2 or M_3 receptor in longitudinal smooth muscle of guinea-pig small intestine

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1 The abilities of muscarinic agonists (arecoline, bethanechol, carbachol, McN-A343, methacholine, pilocarpine) to inhibit isoprenaline-induced cyclic AMP production in chopped fragments (*via* M_2 receptors), and to evoke cationic current (I_{cat}) (*via* M_2 receptors) or calcium store release (*via* M3 receptors) in enzyme-dispersed, single voltage-clamped cells from longitudinal smooth muscle of the guinea-pig small intestine were examined.

2 All muscarinic agonists (1–300 μM) examined inhibited isoprenaline (1 μM)-induced accumulation of cyclic AMP, the IC_{50} varying from 52 to 248 μM. However, their relative potencies to evoke this M_2 effect were not significantly correlated with their ability to evoke I_{cat}, also a M_2 effect, whether or not calcium stores were depleted; pilocarpine and McN-A343 inhibited the I_{cat} response to carbachol.

3 Muscarinic agonists (concentration 300 or 1000 μM), except pilocarpine and McN-A343 which were ineffective, evoked Ca^{2+}-activated K^+ current ($I_{K\text{-}Ca}$) resulting from Ca^{2+} store release (M_3 effect). Their effectiveness was tested by estimating residual stored calcium by subsequent application of caffeine (10 mM). The relative potencies to evoke Ca^{2+} store release (M_3) and for I_{cat} activation (M_2) were closely correlated ($P<0.001$).

4 These data might be explained if M_2-mediated adenylyl cyclase inhibition and I_{cat} activation involve different G proteins, or involve different populations of M_2 receptors. The observed correlation of agonist potency between I_{cat} activation and Ca^{2+} store release supports the proposal (Zholos & Bolton, 1997) that M_3 activation can potentiate M_2-cationic channel coupling through Ca^{2+}-independent mechanisms.

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Abbreviations: BAPTA, 1, 2-bis (2-aminophenoxy) ethan-N,N′ N′-tetraacetic acid; $[Ca^{2+}]_i$, intracellular calcium concentration; D600, methoxyverapamil; EGTA, ethyleneglycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; G protein, GTP binding protein; IBMX, isobutylmethylxanthine; I_{cat}, nonselective cationic current; IC_{50}, concentration required to produce 50% inhibition; $I_{K\text{-}Ca}$, Ca^{2+}-activated K^+ current; I_{max}, maximum current; $InsP_3$, inositol-1,4,5-triphosphate; McN-A343, 4-(N-[3-chlorophenyl]-carbamoyloxy)-2-butynyl-trimethylammonium chloride; OXA-22, cis-2-methyl-5-trimethylammoniummethyl-1,3-oxathiolane iodide; PLC, phospholipase C; PTX, petrussis toxin

Introduction

In various gastrointestinal smooth muscles, two different muscarinic receptor types are present as the target for the neurotransmitter acetylcholine. They are defined pharmacologically as M_2 and M_3 corresponding to the genetically defined m_2 and m_3 subtypes (Hulme *et al*., 1990; Eglen *et al*., 1996). Evidence for this was obtained initially by radioligand binding studies (Doods *et al*., 1987; Giraldo *et al*., 1987; Gomez *et al*., 1992; Zhang *et al*., 1991; Michel & Whiting, 1988; 1990) and confirmed by mRNA hybridization (Maeda *et al*., 1988; Ford *et al*., 1991) and immunoprecipitation studies (Wall *et al*., 1991). The number of M_2 receptors is greater than that of M_3 receptors (4 : 1 to 5 : 1). The co-existence of M_2 and M_3 subtypes suggests that both would be activated by acetylcholine or other muscarinic stimulants, and would contribute to the contractile response, but the intracellular signal transduction pathways (G proteins and effectors) involved are likely to be different.

The M_2 receptor subtype in intestinal smooth muscle is well documented to couple *via* pertussis toxin (PTX)-sensitive G proteins to inhibition of adenylyl cyclase activity. Activation of M_2 receptors, therefore, results in inhibition of the increase in cyclic AMP levels elicited by forskolin, isoprenaline and other compounds that stimulate adenylyl cyclase activity (Peralta *et al*., 1988; Candell *et al*., 1990; Griffin & Ehlert, 1992; Reddy *et al*., 1995). M_2 receptor activation has also been shown to evoke a cationic current (I_{cat}) (Benham *et al*., 1985; Bolton & Zholos, 1997; Zholos & Bolton, 1997; Komori *et al*., 1998). The current response is blocked by PTX (Inoue & Isenberg, 1990a; Komori *et al*., 1992), but it is not secondary to changes in cytosolic cyclic

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AMP levels (Komori *et al*., 1998). Therefore, the M_2 receptor is believed to link to adenylyl cyclase and to cationic channels *via* separate signalling pathways that involve PTX-sensitive family G proteins. However, the exact nature of the M_2-linked signal transduction mechanisms is still elusive; for example it is unclear whether the G proteins involved in these systems are identical or not.

The M_3 receptors in intestinal smooth muscle are well known to couple *via* PTX-insensitive G proteins to stimulation of phospholipase C (PLC) leading to formation of inositol-1,4,5-trisphosphate ($InsP_3$) and in turn to the release of Ca^{2+} from intracellular stores (Prestwich & Bolton, 1995a; Komori & Bolton, 1991). There is abundant evidence to show that Ca^{2+} mobilized from internal stores is an intermediate link between M_2 and M_3 subtypes, whereby M_3 activation can potentiate M_2-mediated I_{cat} because of the high Ca^{2+} sensitivity of cationic channel opening (Pacaud & Bolton, 1991; Komori *et al*., 1993, 1996; Kohda *et al*., 1998). Recently, the existence of another link between both the subtypes was proposed, in which M_3 activation generates a Ca^{2+}-independent message potentiating I_{cat} at the level of receptor or G protein (Bolton & Zholos, 1997; Zholos & Bolton, 1997). The underlying mechanism is still unknown, but such functional linkage implies that without the Ca^{2+}-induced facilitation of I_{cat}, the ability of muscarinic agonist to evoke I_{cat} *via* M_2 receptors depends also on its ability to stimulate the M_3 receptor (perhaps *via* a conformational change in the receptor or activation of associated G proteins). The hypothesis deserves to be tested, in order to validate the proposed functional link between M_2 and M_3 subtypes.

In this report, we have examined the ability of various muscarinic agonists to elicit a M_3-mediated Ca^{2+}-activated K^+ current ($I_{K\text{-}Ca}$) that occurs as a result of Ca^{2+} store release, and two M_2-mediated responses, namely the inhibition of isoprenaline-stimulated cyclic AMP accumulation and the activation of I_{cat}, in longitudinal smooth muscle of guinea-pig small intestine. The $I_{K\text{-}Ca}$ response is used to assess the M_3-stimulating activity. Our data indicated that there is no significant correlation of agonist potency between the two M_2-mediated responses, whereas there was a significant correlation between I_{cat} activation and M_3 receptor stimulation.

Methods

Male guinea-pigs (300–400 g) were killed by either stunning or cervical dislocation, followed by immediate exsanguination. The small intestine was removed and exposed in a physiological medium. The longitudinal muscle layer in the ileal region or the whole length of the small intestine except the terminal 5 cm was carefully peeled from the underlying tissue, and then subjected to procedures for the use in whole-cell current recordings or cyclic AMP measurements.

Whole-cell current recordings

Experiments were performed on single smooth muscle cells from the ileal longitudinal muscle layer obtained after treatment with a combination of collagenase (0.3–0.6 mg^{-1}) and papain (0.2–0.3 mg^{-1}) at 37°C for 30 min, as described previously (Komori *et al*., 1998).

Whole cell membrane current was recorded at room temperature with patch pipettes (5–7 MΩ in tip resistance) and CEZ-2300 voltage-clamp amplifier (Nihon Kohden, Tokyo, Japan). Current signals were filtered at 1 KHz and then displayed on an oscilloscope (Nihon Kohden, VC9) and a thermal array recorder (Nihon Kohden, RTA-1100). They were also saved on a digital tape using a recorder (TEAC, RD-111T, Tokyo) for later analysis and illustration.

For recording of $I_{K\text{-}Ca}$, cells were bathed in a conventional physiological salt solution (PSS) that had the following composition (mM): NaCl 126, KCl 6, $CaCl_2$, 2, $MgCl_2$ 1.2, glucose 14, HEPES 10.5, pH adjusted to 7.2 with NaOH. Patch pipettes were filled with a solution consisting of (mM): KCl 134, $MgCl_2$ 1.2, MgATP 1, Na_2GTP 0.1, EGTA 0.05, glucose 14, HEPES 10.5, pH adjusted to 7.4 with KOH. Membrane potential was clamped at a level of 0 mV, close to the reversal potential for I_{cat} in these ionic conditions, so that the $I_{K\text{-}Ca}$ response was not contaminated by I_{cat} activated at the same time (Komori *et al*., 1992). The I_{cat} response was recorded in an external solution consisting of (mM): CsCl 120, glucose 12, HEPES 10, pH adjusted to 7.4 with CsOH. This solution was introduced to the bath (0.5 ml) 30–40 s before the start of I_{cat} recordings by exchange with PSS. The pipette solution had the following composition (mM): CsCl 80, MgATP 1, Na_2GTP 1, creatine 5, glucose 20, HEPES 10, BAPTA 10, $CaCl_2$ 4.6 (calculated $[Ca^{2+}]_i = 100$ nM), pH adjusted to 7.4 with CsOH. Under these conditions, the I_{cat} response was neither contaminated by any K^+ currents including $I_{K\text{-}Ca}$, nor modulated by intracellular Ca^{2+} or external divalent cations (Pacaud & Bolton, 1991; Zholos & Bolton, 1995, 1997). Unless otherwise stated, I_{cat} was measured at a holding potential of −40 mV which is suitable for receptor-cationic channel coupling (Zholos & Bolton, 1994). In some experiments, I_{cat} was recorded where $[Ca^{2+}]_i$ was non-clamped, by using PSS as the bath solution and pipettes filled with the following solution (mM): CsCl 134, $MgCl_2$ 1.2, MgATP 1, NaGTP 0.1, glucose 14, HEPES 10.5, EGTA 0.05, pH adjusted to 7.2 with CsOH (Komori *et al*., 1996).

Concentration-effect curves of muscarinic agonists for I_{cat} activation were obtained by their application in an ascending series of six different concentrations (1–300 μM), with some exceptional occasions. The curves from individual cells were analysed using computor software (SPSS Inc., DeltaGraph 4.0) that fits the data directly with a logistic function, providing the EC_{50} value, the maximal current (I_{max}) and the value (h) of the Hill slope.

Cyclic AMP determination

Smooth muscle fragments were prepared and incubated as described previously (Prestwich & Bolton, 1995b). In brief, the longitudinal muscle layers from the whole length of the small intestine were chopped to 350 × 350 μm fragments with a McIlwain tissue chopper and placed in Krebs Ringer bicarbonate (KRB) consisting of the following composition (mM): NaCl 120, KCl 5.9, $NaHCO_3$ 15.4, NaH_2PO_4, 1.2, glucose 11.5, $MgCl_2$, 1.2; $CaCl_2$, 2.5, pH adjusted to 7.25 with NaOH. After incubation under aeration with a mixture of 95% O_2 and 5% CO_2 at 37°C for 15–20 min, the fragments were transferred to fresh KRB containing IBMX (1 mM), a phosphodiesterase inhibitor, and then divided into 300 μl aliquots in microfuge tubes and placed in a water bath at

37°C for 15 min. If necessary, both thapsigargin (1 μM), a sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor and D600 (10 μM), a voltage-dependent Ca^{2+} channel blocker were added to the incubation buffers when IBMX was added.

To investigate the effects, if any, of muscarinic agonists on isoprenaline-induced increases in cyclic AMP levels, the fragments were incubated for 10 min in the presence or absence of each muscarinic agonist (1–300 μM). After this incubation, isoprenaline was added at desired concentrations (0.1–100 μM), followed by further 5 min incubation. The reaction was then stopped with 10% (final concentration) trichloroacetic acid (TCA). The fragments were incubated for 15 min on ice, centrifuged for 20 min at 13,000 × *g*, and the supernatant removed. The pellet was stored ready for protein determination (the average protein concentration was 1.10 ± 0.02 mg/tube, $n = 20$). TCA in the supernatant was removed by diethyl ether extraction, and then TCA-free samples were neutralized using 1 mM NaOH, and aliquots removed for cyclic AMP determination by a competition binding assay using a [^{3}H]-cyclic AMP-labelled kit (Amersham International Ltd), as described previously (Prestwich & Bolton, 1995b).

Chemicals

Ten muscarinic agonists were used; acetylcholine chloride, McN-A343 (4-(N-[3-chlorophenyl]-carbamoyloxy)-2-butynyl-trimethylammonium chloride), oxotremorine, methacholine chloride, pilocarpine hydrochloride (purchased from Sigma, St Louis, MO, U.S.A.), carbachol chloride (from WAKO, Tokyo, Japan), arecoline HBr, bethanechol chloride, OXA-22 (cis-2-methyl-5-trimethylammoniummethyl-1,3-oxathiolane iodide), and oxotremorine-M (oxotremorine methiodide) (from Research Biochemicals, Natick, MA, U.S.A.). All the muscarinic agonists were dissolved in distilled water and stocked at −20°C in concentrations 100 times or more higher than those used in experiments.

Guanosine 5′ triphosphate (GTP, sodium salt), isobutylmethylxanthine (IBMX), thapsigargin, methoxyverapamil (D600), and 1,2-bis(2-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid (BAPTA) were obtained from Sigma, and caffeine from WAKO. All other chemicals were obtained from Sigma or WAKO.

Data analysis

Values in the text are given as means ± s.e.mean of the number of experiments with smooth muscle fragments or cells (n). Student's paired or unpaired t-test was used for statistical comparison and when $P < 0.05$, differences were considered significant. Correlationships between agonist potencies at different responses were analysed with a Pearson's correlation coefficient test.

Results

Inhibition of cyclic AMP accumulation

The ability of muscarinic agonists to inhibit isoprenaline-stimulated cyclic AMP accumulation was examined in chopped fragments of longitudinal intestinal smooth muscle. These experiments were carried out to ascertain a suitable concentration of isoprenaline such that application of muscarinic agonist might decrease or increase the cyclic AMP response. Basal cyclic AMP levels were estimated to be 22.0 ± 4.9 pmol mg^{-1} protein ($n = 5$). Incubation with isoprenaline (0.1 to 100 μM) increased cyclic AMP levels above the basal level in a concentration-dependent manner (Figure 1). The level of cyclic AMP reached in 100 μM isoprenaline was 108.0 ± 12.6 pmol mg^{-1} protein ($n = 4$), and by 1 μM, 54.8 ± 6.3 pmol mg^{-1} protein ($n = 4$), about half of the former mean value.

Six muscarinic agonists (carbachol, arecoline, bethanechol, methacholine, pilocarpine and McN-A343) were tested; none of these up to 100 μM affected basal cyclic AMP levels. However, they all (at 100 μM) inhibited the increase of cyclic AMP elicited by isoprenaline (1 to 100 μM), although there were variations in the extent of inhibition (Figure 1). For example, the level of cyclic AMP reached by 100 μM isoprenaline (108.0 ± 12.6 pmol mg^{-1} protein) was decreased to 44.5 ± 11.7 and 73.0 ± 12.8 pmol mg^{-1} protein (each $n = 4$) by carbachol and bethanechol, respectively, and to intermediate values by other agonists. The maximum concentration used (300 μM) may not have achieved a maximum response but 1 mM or more may have had non-specific effects.

The ability of muscarinic agonists to inhibit the rise in cyclic AMP levels produced by 1 μM isoprenaline was studied. Carbachol inhibited the accumulation of cyclic

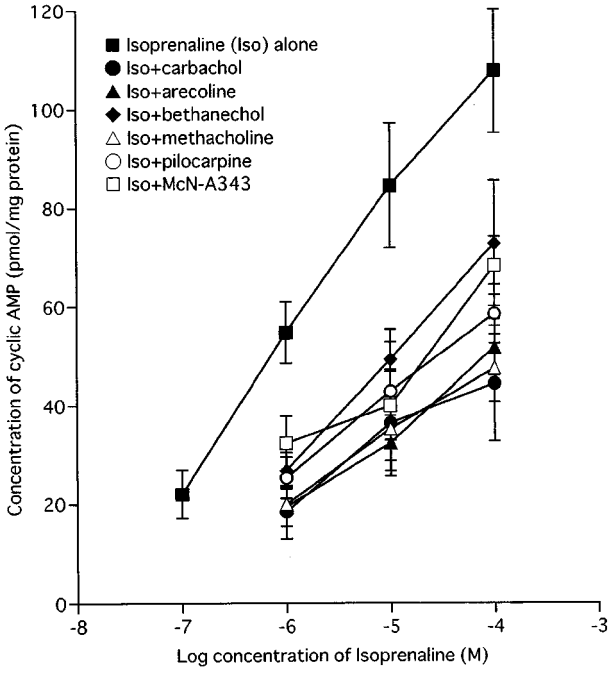

Figure 1 The effect of muscarinic agonists on the concentration-effect curve for isoprenaline-induced increases in cyclic AMP levels. Smooth muscle fragments were incubated with isoprenaline (0.1–100 μM) for 5 min in the absence or presence of each indicated muscarinic agonist (100 μM) applied 10 min before. Changes in cyclic AMP were measured in the continuous presence of 1 mM IBMX, a phosphodiesterase inhibitor, using a ^{3}H-cyclic AMP labelled kit. Results represent the mean ± s.e.mean of four experiments performed on separate occasions and are expressed as absolute pmol mg^{-1} protein.

AMP in a concentration-dependent manner, with an average 72.2±2.7% ($n=4$) inhibition at 300 μM; 52 μM carbachol (by interpolation) reduced the response to 1 μM isoprenaline to half (see open circles in Figure 2a). The other five agonists also behaved similarly (Figure 2b–f); the per cent inhibitions at 300 μM and the concentrations producing 50% inhibition of the 1 μM isoprenaline response were 64.8±7.1% and 103 μM for methacholine, 62.7±4.4% and 117 μM for arecoline, 58.5±2.0% and 127 μM for bethanechol, 66.4±5.7% and 65 μM for pilocarpine and 53.1±3.3% and 248 μM for McN-A343 (each, $n=4$). The potencies at 300 μM relative to carbachol ranged between 0.73 (McN-A343) and 0.92 (pilocarpine) and the rank order was carbachol > pilocarpine > methacholine > arecoline > bethanechol > McN-A343 (see Figure 7). The rank order for 50% inhibition was the same.

Similar experiments were carried out under conditions where internal Ca^{2+} stores were depleted by pretreatment with thapsigargin (1 μM) and voltage-dependent Ca^{2+} channel activity blocked by D600 (10 μM). Under these conditions, all six agonists were also effective in inhibiting the accumulation of cyclic AMP (see closed circles in Figure 2). For carbachol, methacholine and arecoline, the per cent inhibitions at 300 μM and concentrations reducing the 1 μM isoprenaline response to 50% were 71.0±1.6% and 36 μM, 66.0±1.9% and 58 μM and 61.5±2.8% and 91 μM, respectively (all, $n=4$), values which were similar to the corresponding values obtained in the absence of thapsigargin and D600. For pilocarpine, bethanechol and McN-A343, however, the per cent inhibition at 300 μM was not as great as in the absence of thapsigargin and D600, being 51.8±3.1, 36.8±7.0 and 29.1±7.7% (all, $n=4$), respectively. Consequently, 253 μM pilocarpine inhibited the response to isoprenaline by 50%, while neither bethanechol nor McN-A343 achieved 50% inhibition. The relative potencies of the five agonists at 300 μM relative to carbachol fell between 0.41 (McN-A343) and 0.93 (methacholine) and the apparent rank order was carbachol > methacholine > arecoline > pilocarpine > bethanechol > McN-A343 (see Figure 7). It was noted that the position of pilocarpine in the rank order shifted from the second to the fourth when thapsigargin and D600 were present.

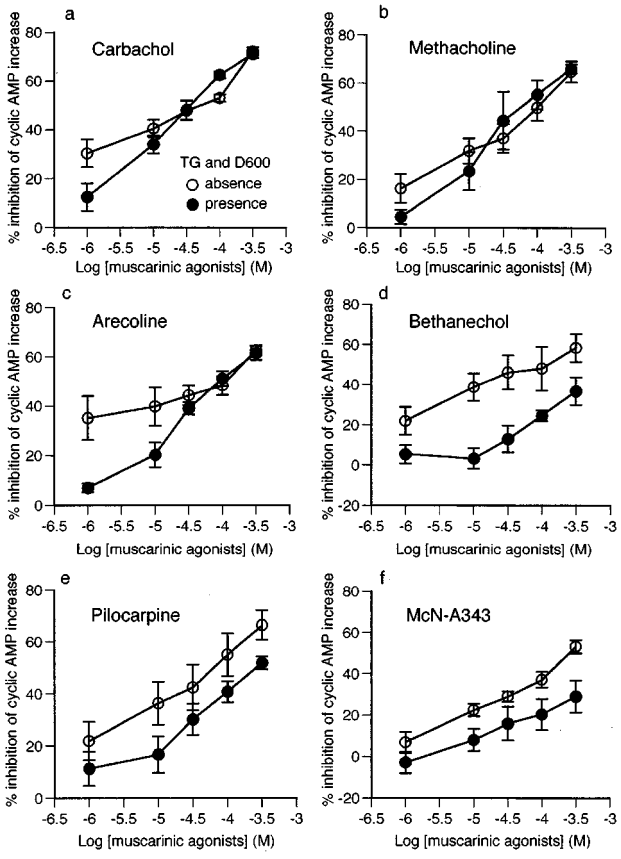

Figure 2 Concentration-effect curves of six muscarinic agonists (a–f) for inhibition of 1 μM isoprenaline-stimulated cyclic AMP accumulation in the absence or presence of 1 μM thapsigargin and 10 μM D600. Cyclic AMP levels were determined as described in Figure 1. Results represent the mean±s.e.mean of four experiments performed on separate occasions and are expressed as the per cent inhibition of the increase in cyclic AMP compared to control.

Activation of nonselective cationic current, I_{cat}

The above six muscarinic agonists and four others (acetylcholine, oxotremorine-M, oxotremorine and OXA-22) were studied for their ability to evoke I_{cat}. Intracellular calcium concentration $[Ca^{2+}]_i$ was clamped at 100 nM by the use of a BAPTA/$CaCl_2$ combination in order to prevent I_{cat} being modulated by changes in $[Ca^{2+}]_i$ during muscarinic stimulation (Pacaud & Bolton, 1991; Komori *et al*., 1993). Five to 6 min after establishing whole cell recording mode, the solution bathing the cells was changed from PSS to Cs^+-based solution in order to block K^+ currents (see closed triangles in Figure 3), which resulted in the generation of an inward current, probably carried by Cs^+ ion (Zholos & Bolton, 1995). The Cs^+ current gradually developed to reach a steady level (up to 50 pA in size) in 20 s or so, and then each muscarinic agonist was applied in ascending concentrations of 1, 3, 10, 30, 100 and 300 μM, unless otherwise stated.

Figure 3a shows a typical I_{cat} response to application of the ascending series of carbachol concentrations. The current response developed in a graded manner and it reached a new sustained level in 10–20 s after the increase in agonist concentration; maximum current was usually achieved at 100 μM. The averaged relationship between the amplitude of I_{cat} and carbachol concentration ($n=14$) is shown in Figure 4a. I_{cat} amplitudes were determined by taking the initial sustained level of current in Cs^+ solution as a base line. Mean values for the I_{max}, EC_{50} and slope of the curve (h) estimated by curve fitting of a series of data from the individual cells were 1028.6±123.1 pA, 7.5±1.6 μM and 1.2±0.1 ($n=14$, Table 1), respectively. The EC_{50} value was close to that (7.6 μM) reported by Zholos & Bolton (1997).

Other agonists except pilocarpine and McN-A343 were also effective in eliciting I_{cat}. Figure 3b–e shows typical examples of I_{cat} responses to methacholine, OXA-22, bethanechol and arecoline. The I_{cat} response to OXA-22 often decreased when the agonist concentration was increased to 100 or 300 μM (Figure 3c). As seen from the averaged concentration-effect curves in Figure 4, all the effective agonists, except bethanechol, produced a maximal response at 30 to 300 μM. Bethanechol often required a higher concentration of 1000 μM to do so. The mean values of I_{max} for the seven effective agonists were all smaller than the corresponding value for carbachol, and this was statistically

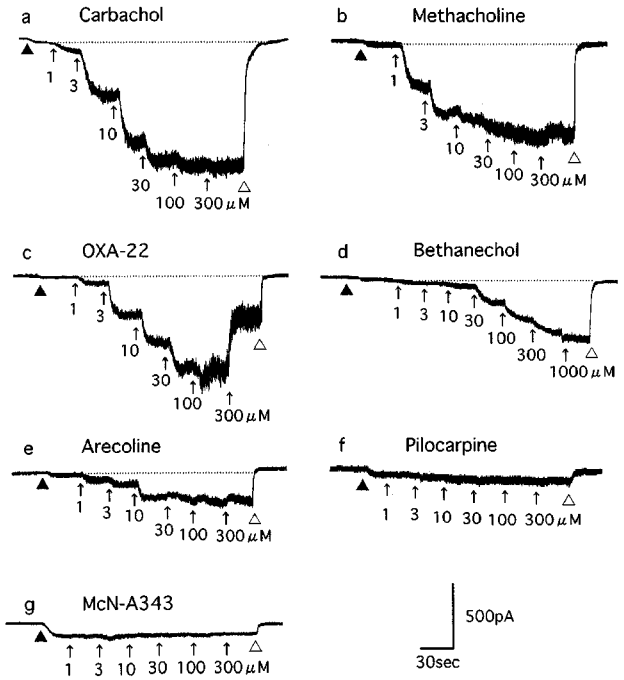

Figure 3 Cationic current (I_{cat}) induced by muscarinic agonists (a–g) in single ileal smooth muscle cells. The current response was recorded at a holding potential of −40 mV and under conditions where $[Ca^{2+}]_i$ was clampled at 100 nM with a BAPTA/$CaCl_2$ combination. Five to 6 min after establishment of whole-cell recording mode, a physiological salt solution (PSS) bathing the cells was replaced with a Cs^+-rich solution (at closed triangle) and then each indicated agonist was applied at ascending concentrations (1 to 300 μM) as indicated by the arrows. The agonists applied were washed away with PSS (at open triangle). The dotted lines indicate the levels of holding current in the Cs^+-rich solution, which were used as base lines to determine the amplitude of I_{cat} (see Figure 4). (a–g) are from different cells.

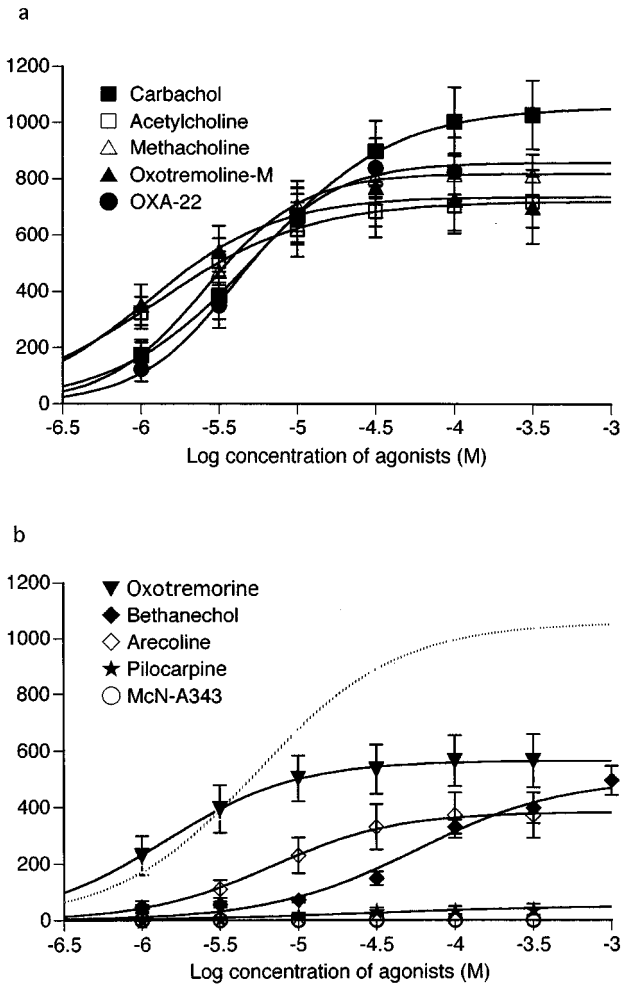

Figure 4 Averaged concentration-effect curves of muscarinic agonists for I_{cat} activation. The curves were obtained from experiments as described in Figure 3. The data points for each agonist were fitted with a logistic function. The curves, depending on whether significantly different from carbachol's curve in the maximum response, are classified into the two groups; (a), not significant and (b), significant ($P<0.05$). The dotted curve in (b) is for carbachol. Each point indicates the mean±s.e.mean of measurements in 5–14 cells.

significant ($P<0.05$) for oxotremorine, bethanechol and arecoline (Table 1). The mean EC_{50} values for acetylcholine, oxotremorine-M and oxotremorine were 3.5 to 4.5 times lower ($P<0.05$) and bethanechol nine times higher ($P<0.05$), than carbachol. The mean h values for all agonists were not significantly differed from carbachol.

Pilocarpine had little or no effect in eliciting I_{cat} (Figures 3f and 4b); however, when applied at 300 μM, it produced a small, noisy inward current (up to 120 pA) in five of 11 cells. The current amplitude averaged 23.5±15.1 pA ($n=11$), only 2% of the I_{max} value for carbachol (Table 1). McN-A343 was completely ineffective in all seven cells (Figures 3g and 4b). Further characterization of pilocarpine and McN-A343 was performed in different experimental conditions. Their effects on the carbachol concentration-effect curve for I_{cat} activation were investigated. Both agonists (100 μM) shifted the curve to the right with depression of the maximum response (Figure 5); the EC_{50} value for carbachol was increased from 7.5±1.6 μM (Table 1) to 178.6±65.4 μM ($n=5$) by pilocarpine and to 40.8±6.5 μM ($n=5$) by McN-A343 (both $P<0.05$) and the I_{max} value decreased from 1028.6±123.1 pA (Table 1) to 635.5±101.1 pA ($n=5$) and 487.2±76.1 pA ($n=5$) by the respective agonists (both $P<0.05$). Their ability to induce I_{cat} was examined over a broader range of membrane potential using a negative going ramp pulse from 40 to −80 mV over 0.5 s (Figure 6a). Current-voltage (I-V) relationships in the presence of either agonist were obtained after leakage subtraction. In two of six cells, the I-V curve for pilocarpine (300 μM) lay on the voltage axis, indicating no activation of I_{cat} in these cells. In the remaining four cells, a detectable, but slight, current activation occurred in the voltage range between −20 and −60 mV with a maximum at some −40 mV. The I-V curve with the most clear activation is shown in Figure 6b. In this curve, current amplitude at any voltage was less than 5% of that at the corresponding voltage in a typical I-V curve for 300 μM carbachol ($n=5$; Figure 6a,b). The I-V curve for 300 μM McN-A343 overlapped the voltage axis in all five cells tested (Figure 6b). Finally, their I_{cat}-eliciting activity was examined in cells immersed in PSS and internally dialyzed with a pipette solution without the BAPTA/$CaCl_2$-buffer (Komori *et al.*, 1996). At a holding potential of −40 or −50 mV, neither pilocarpine nor McN-A343 (100–1000 μM)

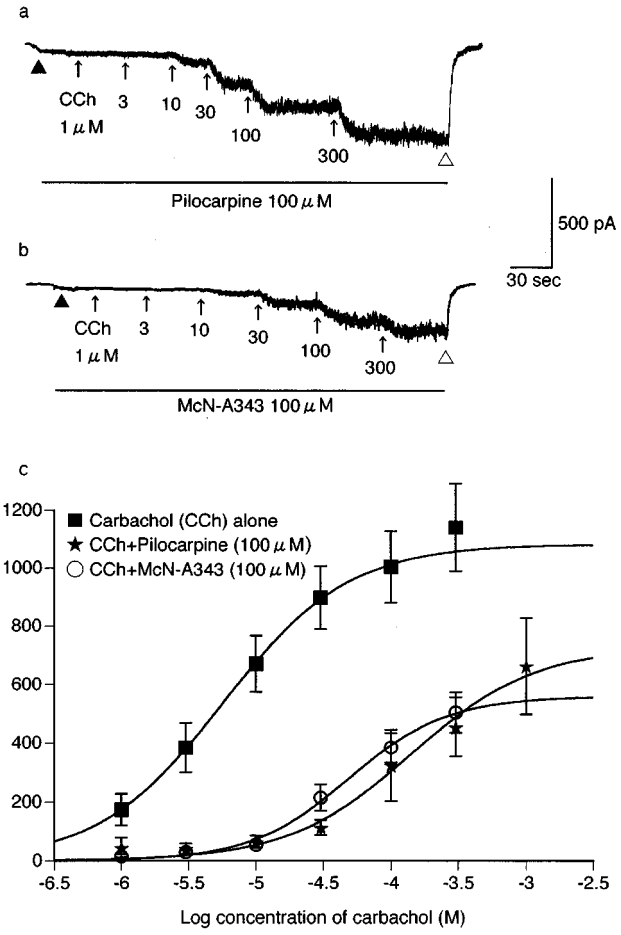

Figure 5 The effect of pilocarpine and McN-A343 on the carbachol concentration-effect curve for I_{cat} activation. The current response to carbachol was recorded as described in Figure 1, except for the presence of 100 μM pilocarpine (a) or McN-A343 (b) in the bath solution as indicated by the lines. (c) The averaged curves for carbachol in the presence of pilcarpine or McN-A343 and the control curve repeated from Figure 4a. Each point for the former two curves indicates the mean$\pm$s.e.mean of measurements in five cells.

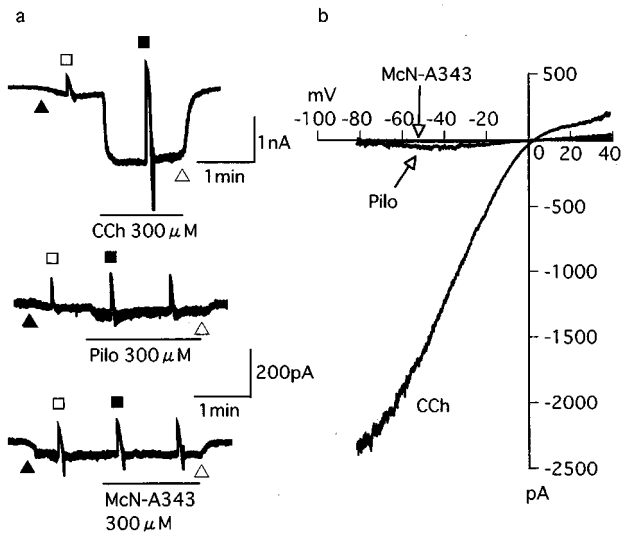

Figure 6 The ability of pilocarpine, McN-A343 and carbachol to activate I_{cat} over a broad range of membrane potential. (a) Current traces from three different cells held at -40 mV to which a negative going ramp pulse from 40 to -80 mV over 0.5 s was applied before (open sqaure) and during application (closed square) of 300 μM carbachol (CCh), pilocarpine (Pilo) or McN-A343. (b) Current-voltage (I-V) curves for CCh, Pilo and McN-A343 from the respective cells in (a), which were constructed by subtracting the I-V curve before application of the agonists from that obtained during their application. See text for details.

Table 1 Maximum amplitude (I_{max}), half-effective concentration (EC_{50}), and slope factor (h) in the concentration-effect curves for muscarinic agonist-evoked cationic current (I_{cat})

	I_{max} (pA)	EC_{50} (μM)	h	n
Carbachol	1028.6$\pm$123.1	7.5$\pm$1.6	1.2$\pm$0.1	14
OXA-22	827$\pm$119.4	4.0$\pm$0.9	1.7$\pm$0.2	8
Methacholine	814.3$\pm$73.4	3.2$\pm$0.9	1.5$\pm$0.2	7
Acetylcholine	720.0$\pm$90.6	1.7$\pm$0.3*	1.1$\pm$0.1	7
Oxotremorine-M	702.0$\pm$132.2	1.9$\pm$0.4*	1.1$\pm$0.2	5
Oxotremorine	564.3$\pm$94.1*	2.2$\pm$0.7*	1.5$\pm$0.2	6
Bethanechol	494.8$\pm$53.2*	67.8$\pm$10.2*	1.0$\pm$0.1	6
Arecoline	371.1$\pm$80.2*	8.3$\pm$1.2	1.2$\pm$0.1	7
Pilocarpine	23.5$\pm$15.1*	ND	ND	11
McN-A343	ND	ND	ND	7

The values for I_{max}, EC_{50} and h indicate mean$\pm$s.e.mean, and n, the number of cells used for measurements. ND, not determined because I_{cat} was too small or absent. *Significantly different from the corresponding value for carbachol ($P<0.05$).

produced an appreciable I_{cat} ($n=12$ and 9, respectively), whereas in cells which had been insensitive to these agonists, carbachol (3–100 μM) invariably elicited a sustained or oscillatory pattern of I_{cat} ($n=8$; data not shown). These results show that pilocarpine and McN-A343 have binding affinity but little or no potency at receptors mediating I_{cat}.

Relationship between agonist potencies at the two M_2-mediated responses

Using the data from the group of six agonists whose activity was examined for both cyclic AMP inhibition and I_{cat} activation, we investigated mutual relation of potencies for both responses. Potencies of the individual agonists for the cyclic AMP response and I_{cat} activation, expressed by taking potency of carbachol as unity, are summarized in Figure 7. The relative potencies for the cyclic AMP response were estimated from the inhibition attained at 300 μM of cyclic AMP accumulation in the absence and presence of thapsigargin and D600 (Figure 2) and those for I_{cat} activation from the maximum I_{cat} response (I_{max} value in Table 1). As seen from Figure 7, the relative potency in evoking the two responses varied considerably among the different agonists, regardless of the use of thapsigargin and D600 in cyclic AMP assays; for example, arecoline and pilocarpine were as potent as methacholine for the cyclic AMP response, but obviously less potent for the I_{cat} response. The relation between the relative potencies for both responses was statistically tested. The calculated correlation coefficient had a value of 0.582 when cyclic AMP was assayed without thapsigargin and D600, and a value of 0.679 when assayed with these drugs present. These values were smaller than the value (0.729, when $n=6$) for a significant correlation ($P<0.05$), suggesting

a lack of a parallelism of agonist potency between the two effector systems linked to the M_2 subtype receptor.

Activation of Ca^{2+}-activated K^+ current, $I_{K\text{-}Ca}$

M_3 receptor activation in this type of cell induces $I_{K\text{-}Ca}$ *via* Ca^{2+} release from the stores (Komori & Bolton, 1991; Komori *et al*., 1998). To assess potency for Ca^{2+} store release, we examined the ability to elicit $I_{K\text{-}Ca}$ of the same group of 10 agonists as tested for I_{cat} activation. The individual agonists were applied at a maximally effective concentrations of 300 or 1000 μM and 30–40 s later, 10 mM caffeine, a potent Ca^{2+}-store releaser, was routinely applied to check if releasable Ca^{2+} remained in the stores (Komori *et al*., 1998).

Application of carbachol evoked $I_{K\text{-}Ca}$ lasting 3–10 s (Figure 8a), as previously reported (Komori *et al*., 1998), the peak amplitude of which amounted to 3.2 ± 0.7 nA on average ($n=6$). Subsequent application of caffeine in the presence of carbachol was usually without effect (Figure 8a; Table 2). All other agonists, except bethanechol, pilocarpine and McN-A343, also evoked a sizable $I_{K\text{-}Ca}$ (Figure 8b–d). The mean peak amplitude varied from 2.0 to 3.1 nA among the six agonists; acetylcholine, oxotremorine-M, arecoline, OXA-22, methacholine and oxotremorine (Table 2). Subsequent caffeine was without effect or evoked $I_{K\text{-}Ca}$ with varied amplitude, depending on the previously applied agonist and different cells stimulated with the same agonist (Table 2).

The ability of bethanechol (300 μM) varied too extensively among different cells to be reliably assessed. However, when used at a higher concentration of 1000 μM, the agonist invariably evoked $I_{K\text{-}Ca}$ with a peak amplitude of 1.6 ± 0.4 nA ($n=7$) but subsequent caffeine also invariably evoked $I_{K\text{-}Ca}$ which averaged 1.5 ± 0.6 nA ($n=7$) (Figure 8e). Neither pilocarpine nor McN-A343 was able to evoke an appreciable $I_{K\text{-}Ca}$, but caffeine applied following the respective agonists evoked $I_{K\text{-}Ca}$ of 3.7 ± 1.2 nA ($n=15$) and 2.2 ± 0.5 nA ($n=11$). Even at 1000 μM, both agonists were without effect ($n=3$, respectively). When pilocarpine (100 μM) was previously added in the bath solution, carbachol evoked only a small $I_{K\text{-}Ca}$ of 0.1–0.2 nA followed by subsequent caffeine-evoked $I_{K\text{-}Ca}$ of some 2.0–3.0 nA ($n=3$). Substantially similar results were obtained when carbachol was applied in the presence of McN-A343 (100 μM). Therefore, pilocarpine and McN-A343 seemed to behave as antagonists rather than agonists at M_3 receptors.

When the amplitude of $I_{K\text{-}Ca}$ evoked by a given agonist was compared with the summed amplitude of this and the subsequent caffeine current, there was a significant ($P<0.05$) difference for bethanechol, arecoline and oxotremoline, as well as pilocarpine and McN-A343, but not for carbachol, acetylcholine, OXA-22, methacholine and oxotremoline-M (Table 2). The results indicated that these agonists have different potencies for Ca^{2+} store release.

Relationship between agonist potency for Ca^{2+} store release and for I_{cat} activation

We compared the relationship of agonist potencies for Ca^{2+} store release and for I_{cat} activation, using the data obtained so far. The potency of the former was estimated from the proportional size of agonist-evoked $I_{K\text{-}Ca}$ to the sum of this and subsequent caffeine current and normalized by the value of 0.97 for carbachol-evoked $I_{K\text{-}Ca}$ (see Agonist/Sum. in Table 2). The potency for the latter determined and normalized on the basis the I_{max} value for carbachol. Figure 9 illustrates plots of the relative potency at I_{cat} activation against that at Ca^{2+} store release for the 10 muscarinic agonists. The data points are scattered along or near the solid

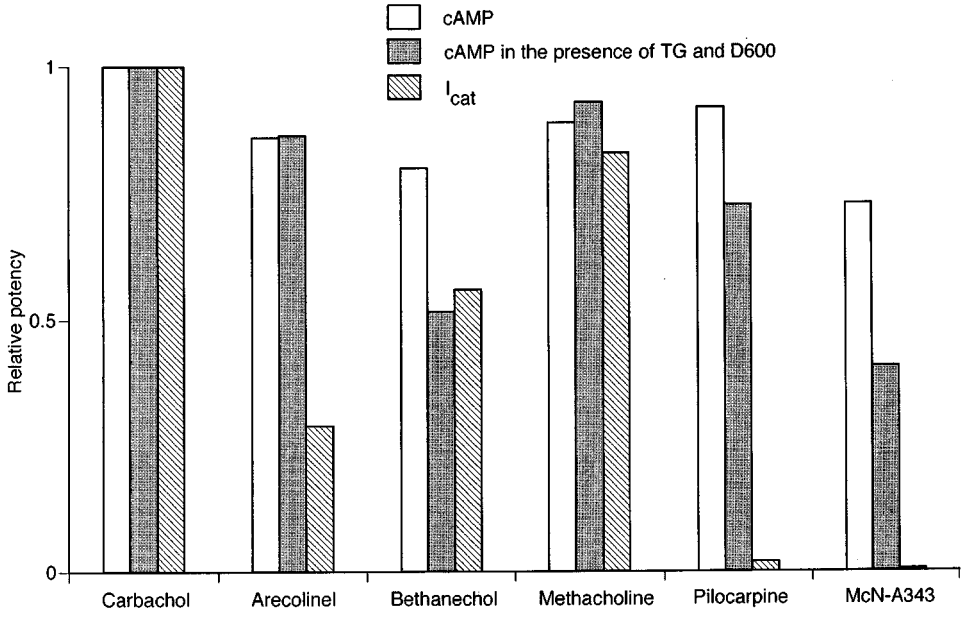

Figure 7 Summary of muscarinic agonist potency for cyclic AMP and I_{cat} responses. The former potency was defined by the per cent inhibition (at 300 μM) of the isoprenaline-stimulated cyclic AMP accumulation (Figure 2) and the latter by the maximum response of I_{cat} (the I_{max} in Table 1). These respective potencies were normalized by the value for carbachol. The relative potencies are shown for the cyclic AMP responses in the absence and presence of both 1 μM thapsigargin and 10 μM D600 and for the I_{cat} response of the indicated individual agonists.

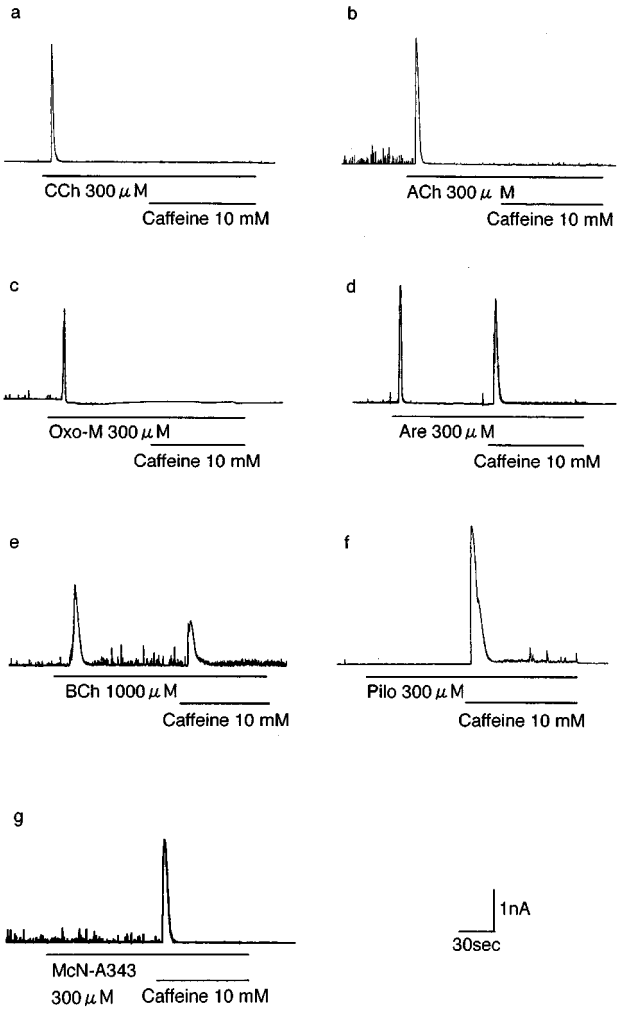

Figure 8 Examples of Ca^{2+}-activated K^+ current ($I_{K\text{-}Ca}$) evoked by muscarinic agonists. (a–g) Current responses of different cells to (a) carbachol, (b) acetylcholine (ACh), (c) oxotremoline-M (Oxo-M), (d) arecoline (Are), (e) bethanechol (BCh), (f) pilocarpine (Pilo) and (g) McN-A343 applied at a maximally effective concentration (300 or 1000 μM) and subsequent application of 10 mM caffeine as indicated by the lines. The cells were held at 0 mV, a level close to the reversal potential for I_{cat} and exposed to the drugs 5 min after establishment of whole-cell recording mode. Mean values for the amplitudes of agonist-evoked $I_{K\text{-}Ca}$, subsequent caffeine-evoked $I_{K\text{-}Ca}$ and the sum of both currents are given in Table 2.

line drawn to indicate a positive correlation of potency between both of them. The calculated correlation coefficient of 0.923 was greater than the value (0.872, when $n=10$) expected for a significant correlation ($P<0.001$).

Discussion

Muscarinic receptors in intestinal smooth muscle mediate multiple responses including adenylyl cyclase inhibition, I_{cat} activation, Ca^{2+} store release and contraction. The former two responses are mediated by M_2 subtype receptors coupling to PTX-sensitive G proteins (Candell *et al*., 1990; Griffin & Ehlert, 1992; Zholos & Bolton, 1997; Komori *et al*., 1998), and the latter two by M_3 subtype receptors coupling to PTX-insensitive G proteins (Komori *et al*., 1992, 1998; Eglen *et al*., 1996).

In the present study, the six agonists of carbachol, methacholine, bethanechol, arecoline, pilocarpine and McN-A343, all inhibited the isoprenaline-evoked cyclic AMP response believed to be mediated *via* M_2 receptors. However pilocarpine and McN-A343 were virtually without ability to evoke I_{cat} which is also believed to be evoked *via* M_2 receptors. Moreover, the relationship of relative potencies for the two responses varied considerably among the different agonists (Figure 7) and statistical tests indicated no significant correlation between them. If M_2 receptors utilize one multifunctional G protein to mediate both cyclic AMP and I_{cat} responses, the individual agonists tested might be expected to display a parallelism in potency for the two responses. Therefore, it is possible that the G proteins involved in the individual responses are different. Based on this, it is postulated that pilocarpine and McN-A343, if they occupy M_2 receptors, cannot or poorly induce the required conformational change in the receptor to activate the G protein linked to the cationic channel system. In agreement with this, both agonists acted as antagonists on the I_{cat} response to carbachol. However, if a receptor subtype is predisposed to couple preferentially to one G protein/effector system (see Gudermann *et al*., 1996), then another possibility is that different populations of M_2 receptors exist, one coupling to a G protein that affects adenylyl cyclase and a second to another G protein that affects cationic channels. In this case pilocarpine and McN-

Table 2 Ca^{2+}-activated K^+ current ($I_{K\text{-}Ca}$) evoked by muscarinic agonists at maximally effective concentrations and by subsequent application of caffeine

	$I_{K\text{-}Ca}$ *amplitude* (nA)				
	Agonist	*Caffeine*	*The sum*	n	*Agonist/Sum*
Carbachol	3.2±0.7	0.2±0.2	3.3±0.6	6	0.97
OXA-22	2.3±0.4	0.1±0.1	2.4±0.3	5	0.96
Methacholine	2.8±0.4	1.2±0.6	4.0±0.4	5	0.70
Acetylcholine	3.1±0.7	0	3.1±0.7	3	1.0
Oxotremorine-M	2.4±0.6	0.4±0.4	2.8±0.9	6	0.86
Oxotremorine	2.2±1.1*	4.1±1.7	6.3±1.6	5	0.35
Arecoline	2.0±0.7*	1.9±0.6	3.9±1.1	7	0.51
Bethanechol	1.6±0.4*	1.5±0.6	3.1±0.5	7	0.52
Pilocarpine	0	3.7±1.2	3.7±1.2	15	0
McN-A343	0	2.2±0.5	2.2±0.5	11	0

$I_{K\text{-}Ca}$ responses to the muscarinic agonists (300 or 1000 μM) and caffeine (10 mM) were obtained from experiments in Figure 8. Each value indicates the mean±s.e.mean of measurements in the number of cells indicated by *n*. Pilocarpine and McN-A343 produced no detectable $I_{K\text{-}Ca}$. The values for Agonist/Sum were obtained by dividing the mean value for the agonist-evoked $I_{K\text{-}Ca}$ by that for the sum of it and subsequent caffeine-evoked $I_{K\text{-}Ca}$. *Significantly different ($P<0.05$) from the corresponding summed amplitude.

A343 might behave as an agonist at the former receptor, and as an antagonist at the latter.

In the present study, the two M_2-mediated responses were recorded under considerably different experimental conditions. The inhibition of the cyclic AMP response was measured in chopped fragments of smooth muscle without control of $[Ca^{2+}]_i$ and membrane potential (hence GTP was not added to the bathing solution), whereas the I_{cat} response was recorded in single isolated cells whose $[Ca^{2+}]_i$ and membrane potential were both clamped. These differences would mean some difficulty in comparing agonist potencies for both responses. However, when thapsigargin and D600 were used to block intracellular Ca^{2+} mobilization as well as electrical membrane activity in cyclic AMP assays, in an attempt to mimic the conditions used for I_{cat} recording, the same conclusion that the agonist potencies for cyclic AMP and I_{cat} responses did not correlate was reached. Conversely, to mimic the cyclic AMP assay system, we tried I_{cat} recording conditions both in a physiologically relevant range of membrane potential (−80 to 40 mV) and without clamping $[Ca^{2+}]_i$ as well as the presence of extracellular Ca^{2+}. However, McN-A343 and pilocarpine still had little or no effect in eliciting I_{cat}. These results confirmed the comparison of agonist potency, nevertheless measurement of cyclic AMP or adenylyl cyclase activity in conditions similar to those used for I_{cat} recording would be of interest.

The presence of thapsigargin and D600 in assay systems resulted in reduced abilities to elicit a cyclic AMP response. This suggests the action of internal Ca^{2+} mobilized by Ca^{2+} store release and/or by an increase in voltage-dependent Ca^{2+} channel activity may affect the adenylyl cyclase response. In other words, the M_2/adenylyl cyclase system might be under a Ca^{2+}-dependent regulation by the M_3/PLC/$InsP_3$ system and the M_2/cationic channel system which causes voltage-dependent Ca^{2+} channels to open by depolarizing the cell membrane. These possible interactions would be expected to have important functional implications *in vivo*. However, the extent to which agonist ability was affected by thapsigargin and D600 varied from one agonist to another; a relatively severe change in the ability was seen for bethanechol, pilocarpine and McN-A343 which are rather weak in their Ca^{2+}-mobilizing activity, as judged from their weak ability to evoke $I_{K\text{-}Ca}$. Therefore, beside the inhibition of intracellular Ca^{2+} mobilization, some non-specific actions of thapsigargin and/or D600 (Kobayashi *et al*., 1991) might be involved.

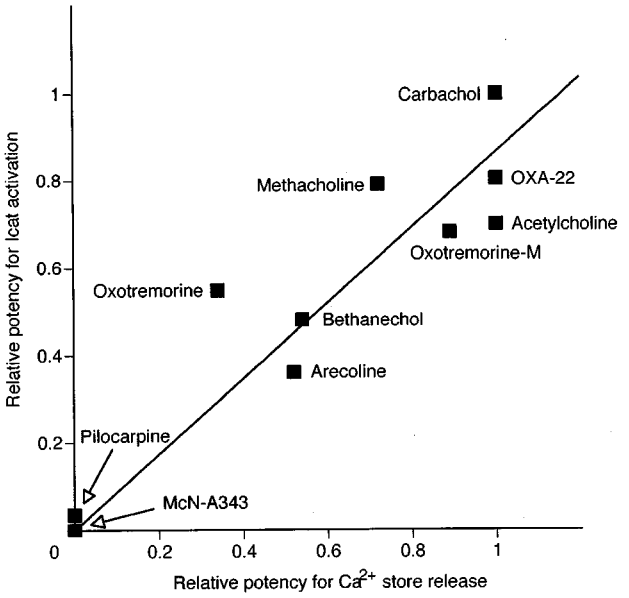

Figure 9 Correlation between muscarinic agonist poptency for I_{cat} activation and for Ca^{2+} store release. The former potency was defined by the I_{max} values in Table 1 and the latter, by the proportional size of agonist-evoked $I_{K\text{-}Ca}$ to the sum of this and subsequent caffeine current in Table 2 (see column Sum/Agonist), and these respective potencies were normalized by the value for carbachol and plotted for Ca^{2+} store release on the X axis and for I_{cat} activation on the Y axis. The solid line indicates a regression line calculated using the data points from the 10 agonists, as expressed by $Y = 0.865X$. The obtained Pearson's correlation-coefficient of 0.923 was greater than the expected value for a significant correlation (0.872, when $n = 10$ and $P < 0.001$).

The 10 muscarinic agonists showed a significant correlation for their abilities to evoke $I_{K\text{-}Ca}$ and I_{cat} indicating a relationship between their ability to release Ca^{2+} stores and to evoke I_{cat} activation (Figure 9). It is well known that the I_{cat} response is strongly potentiated by a rise in $[Ca^{2+}]_i$ due to Ca^{2+} store release (Pacaud & Bolton, 1991; Inoue & Isenberg, 1990b). However, this mechanism seems unlikely to be involved in the observed correlation of agonist potency, because I_{cat} was recorded under conditions where $[Ca^{2+}]_i$ was clamped at 100 nM with the Ca^{2+} buffer (10 mM BAPTA/4.6 mM $CaCl_2$). Therefore, some other mechanism, independent of Ca^{2+}, seems to underlie it. In this context, an idea has been proposed that M_3 activation, as well as inducing Ca^{2+} store release, can potentiate I_{cat} through an action at the level of receptor or G protein (Bolton & Zholos, 1997; Zholos & Bolton, 1997). This hypothesis implies that independently of I_{cat} modulation by $[Ca^{2+}]_i$ the ability of an agonist to elicit I_{cat} depends on its ability to stimulate M_3 receptors leading to a conformational change in receptor or activation of associated G proteins. The present observation, i.e. the close correlation of agonist potencies, substantiates this implication, corroborating the possible existence of a Ca^{2+}-independent functional link between M_2 and M_3 receptors (Bolton & Zholos, 1997; Zholos & Bolton, 1997).

In studies using several muscarinic agonists there was a close correlation between their abilities to evoke contraction of longitudinal muscle from isolated guinea pig ileum and phosphoinositide hydrolysis in CHO cells with expressed m_3 receptor (Ehlert *et al*., 1999). The rank orders of potencies for the agonists to cause contraction or phosphoinositide hydrolysis were very similar to the results presented in this study supporting a link between M_3 receptor activation, Ca store release and contraction. However Mc-N-343 and pilocarpine were antagonists or ineefective in our studies on single cells whereas they were weakly effective in the experiements of Ehlert *et al*. (1999).

The present and other results lead us to suggest possible arrangements of the functional expression of muscarinic receptors in intestinal smooth muscle. One possible relationship consists of an M_2 and an M_3 subtype receptor, the former coupling to two different PTX-sensitive G proteins that affect either adenylyl cyclase or the cationic channel (see above), and the latter coupling to PTX-insensitive G proteins, potentially G_q/G_{11}, linked to the PLC/$InsP_3$ system. In this

scheme, it would be predicted that the Ca^{2+}-independent potentiation of I_{cat} by M_3 activation would arise through associated G proteins or some downstream factors other than Ca^{2+}. The prediction, however, contradicts our recent observation that anti-G_q/G_{11} protein antibodies had no significant effect on the I_{cat} response to carbachol, but blocked the agonist-evoked $I_{K\text{-}Ca}$ (Yan *et al*., 2000). Thus, the M_3 effect on M_2 activation is likely to be upstream of the G_q/G_{11} protein or a separate system.

Another possibility is the existence of two different types of M_2 receptors, a population of independent M_2 receptors and a second population of M_2/M_3 receptor complexes (Zholos & Bolton, 1997) which would be consistent with the fact that M_2 binding sites are much more abundant than M_3 binding sites (4 : 1 to 5 : 1; Giraldo *et al*., 1987). The discrete M_2 receptor couples *via* a PTX-sensitive G protein to the adenylyl cyclase system, while the M_2 part of the M_2/M_3 complex couples *via* another PTX-sensitive G protein to a cationic channel system, and the M_3 part, *via* G_q/G_{11} proteins, to a PLC/$InsP_3$ system. When an agonist occupies the M_2/M_3 complex, the resulting conformational change in the M_3 receptor generates a Ca^{2+}/G protein-independent message to potentiate M_2-mediated I_{cat}, and also activates the PLC/$InsP_3$ system *via* G_q/G_{11}, leading to Ca^{2+} store release and in turn Ca^{2+}-induced potentiation of I_{cat}. Our recent experiments using antibodies against various G proteins provided evidence favorable for involvement of G_o proteins in the activation of I_{cat} (Yan, Zholos, Unno, Komori & Bolton; unpublished data), as suggested in guinea-pig gastric myocytes (Kim *et al*., 1998). Together with the general belief that adenylyl cyclase inhibition involves G_i proteins, it may be speculated that the major G proteins coupling to the discrete and complex-type M_2 receptors are a G_i and a G_o G-protein, respectively. The model discussed here is a working hypothesis amenable to various molecular biological and pharmacological tests such as have been made in the opioid system, where it has been demonstrated that G protein-coupled opioid δ and κ subtypes dimerize to form a functional receptor (Jordan & Devi, 1999).

In the present study, pilocarpine and McN-A343 failed to evoke $I_{K\text{-}Ca}$, but were able to inhibit the $I_{K\text{-}Ca}$-evoking activity of carbachol, indicating their antagonist behaviour at M_3 receptors. Such properties are also indicated from other studies in which their effects on phosphoinositide turnover and Ca^{2+} store-dependent changes in $[Ca^{2+}]_i$ or tension were examined (Gardner & Mitchelson, 1988; Hishinuma *et al*., 1997; Morel *et al*., 1997; Wang *et al*., 1992). Both agonists also shifted the carbachol concentration-effect curve for I_{cat} activation to the right with depression of the maximum response (Figure 5). Similar changes in the carbachol curve are seen with certain muscarinic antagonists, and it is suggested that the rightward shift is due to M_2 blockade and depression of the maximum, due to M_3 blockade (Zholos & Bolton, 1997). These properties of pilocarpine and McN-A343 combined with their obvious potency for the cyclic AMP response can be readily explained by the expression pattern we postulate for the M_2 receptor and M_2/M_3 complexes.

In conclusion, the present study shows that in longitudinal smooth muscle of guinea-pig small intestine, there was no significant correlation of muscarinic agonist potency between the M_2/adenylyl cyclase and the M_2/I_{cat} system, while a significant correlation exists between the potencies of the M_2/I_{cat} and M_3/PLC/$InsP_3$ systems. These results combined with others provide a hypothetical model for the functional expression pattern of muscarinic receptors. However, further evaluation of this model will require investigation to clarify the precise muscarinic signalling mechanisms involved.

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