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Contrasting effects of carbachol, McN-A-343 and AHR-602 on Ca^{2+}-mobilization and Ca^{2+}-influx pathways in taenia caeci

[1]Shigeru Hishinuma, [2]Ikuko Hongo, [3]Yukio Matsumoto, [4]Fumihiro Narita & [5]Masanori Kurokawa

Department of Pharmacology, Meiji College of Pharmacy at Tanashi, Yato 1-22-1, Tanashi, Tokyo 188, Japan

1 We compared the binding profiles and contractile mechanisms of putative muscarinic M_1 agonists McN-A-343 and AHR-602 with those of carbachol in smooth muscle of guinea-pig taenia caeci.

2 McN-A-343 and AHR-602, as well as carbachol, completely displaced the atropine-sensitive binding of [^{3}H]-quinuclidinyl benzilate to muscarinic receptors present in the membrane preparation. The potency order for the affinity of these agents for muscarinic receptors was carbachol > McN-A-343 > > AHR-602.

3 In the presence of 2.2 mM extracellular Ca^{2+}, McN-A-343 and AHR-602 induced contraction corresponding to 79 and 85%, respectively, of the maximal contraction to 0.1 mM carbachol. Contractions induced by these agents were mediated via activation of the muscarinic receptor subtype that had a high affinity for 4-DAMP (M_3 selective) but a low affinity for pirenzepine (M_1 selective) and AF-DX 116 (M_2 selective). These contractions were inhibited by an L-type Ca^{2+} channel blocker, verapamil.

4 In Ca^{2+}-free solution containing 2 mM EGTA, carbachol elicited a transient contraction whereas no contraction was observed in response to McN-A-343 and AHR-602. Application of McN-A-343 or AHR-602 inhibited the carbachol-induced contraction in Ca^{2+}-free solution, and this inhibition was surmounted by a higher concentration of carbachol.

5 The EC_{50} value for carbachol-induced contraction in the presence of extracellular Ca^{2+} was approximately 175 times lower than that in the absence of Ca^{2+}. After treatment with propylbenzilylcholine mustard, carbachol induced contraction only in the presence of extracellular Ca^{2+}.

6 The results suggest that in the taenia caeci there is a greater receptor reserve for muscarinic M_3 receptor-mediated Ca^{2+} influx than for M_3 mediated Ca^{2+} release. The compounds McN-A-343 and AHR-602 are agonists of the Ca^{2+} influx pathway, but do not appear to stimulate the Ca^{2+} release pathway.

Keywords: McN-A-343; AHR-602; muscarinic M_3 receptor; Ca^{2+} signalling; Ca^{2+} influx; Ca^{2+} release; agonism; receptor reserve; signal transduction; smooth muscle

Introduction

Muscarinic receptors have been divided into five subtypes (M_1-M_5) (Wess, 1993): M_1, M_3 and M_5 receptors couple with phospholipase C-β via the G_q family of G proteins and thus stimulate phosphoinositide metabolism. M_2 and M_4 receptors inhibit adenylyl cyclase activity via activation of G_i proteins and hence inhibit adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production. Activation of M_3 receptors mediates smooth muscle contraction by the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce the second messenger inositol 1,4,5-trisphosphate (IP_3). IP_3 then releases intracellularly stored Ca^{2+} through specific IP_3-receptor-Ca^{2+}-channels (Iino, 1990; Berridge, 1993; Mikoshiba, 1993). However, intracellularly stored Ca^{2+} can also be mobilized through Ca^{2+}-channels which are sensitive to the plant alkaloid ryanodine. These ryanodine receptor-Ca^{2+} channels may be activated endogenously by cyclic ADP-ribose as well as Ca^{2+} itself (Iino, 1989; Sorrentino & Volpe, 1993; Galione *et al*., 1993; Meszaros *et al*., 1993). This implies that intracellularly stored Ca^{2+} may be also mobilized via mechanisms other than stimulation of phosphoinositide metabolism. Activation of M_3 receptors may also induce contraction in smooth muscle via stimulation of Ca^{2+} influx from the extracellular space through plasma membrane CA^{2+} channels (Bolton, 1979; Brading, 1981; Fasolato *et al*., 1994), although mechanisms behind this are still unclear. A large proportion of M_2 receptors may be co-expressed with M_3 receptors in smooth muscle and a role for M_2 receptors in smooth muscle contraction has been recently suggested (Eglen *et al*., 1994).

Muscarinic agents AHR-602 (N-benzyl-3-pyrrolidyl acetate methobromide) and McN-A-343 (4-[[[(3-chlorophenyl)amino]carbonyl]oxy]-N,N,N,-trimethyl-2-butyn-1-aminium chloride) were originally introduced as M_1 agonists (Franko *et al*., 1963; Jones, 1963), which acted preferentially on the muscarinic receptors of the sympathetic ganglia. These agents have little effect on phosphoinositide metabolism in the brain (Fisher, 1986) and in the heart (Brown *et al*., 1985). Moreover, in smooth muscle of guinea-pig taenia caeci, McN-A-343 and AHR-602 induce contraction without causing any stimulation of phosphoinositide metabolism (Gardner *et al*., 1988a). In this study, we investigated the muscarinic receptor subtypes and the Ca^{2+} signalling mechanisms involved in the McN-A-343- and AHR-602-induced contraction in guinea-pig taenia caeci, comparing the responses with carbachol-induced contractions. Our findings indicate that activation of muscarinic M_3 receptors by McN-A-343 and AHR-602 leads to contraction by stimulation of a Ca^{2+} influx pathway only. Possible mechanisms for the muscarinic receptor-mediated Ca^{2+} signalling by these agents are discussed.

[1] Author for correspondence.
Present addresses: [2] Cellular Biochemistry Laboratory, Biomolecular Engineering Department, National Institute of Bioscience and Human-Technology, Higashi 1-1, Tsukuba, Ibaraki 305, Japan. [3] Lead Optimization Research Laboratory, Tanabe Seiyaku Co., Ltd., Kawagishi 2-2-50, Toda, Saitama 335, Japan. [4] Development Department III, Pharmaceutical Development Division, Takeda Chemical Industries Ltd., Nihonbashi 2-12-10, Chuo-ku, Tokyo 103, Japan. [5] The Tokyo Institute of Psychiatry, Kamikitazawa 2-1-8, Setagaya, Tokyo 156, Japan.

Methods

Preparation of guinea-pig taenia caeci membrane fraction

The membrane fraction of guinea-pig taenia caeci was prepared as described previously (Hishinuma *et al*., 1993). The experimental protocols of the present research were approved by the Institutional Ethics Committee for Animal Research, Meiji College of Pharmacy. Briefly, strips of taenia caeci, isolated from guinea-pigs of either sex, weighing 250–400 g, were cut into small pieces and homogenized three times in 20 volumes of 20 mM MOPS buffer (pH 7.4 at 30°C) containing 250 mM sucrose (MOPS buffer A) with Polytron PT-10 at a power setting of 6. All the procedures were carried out at 0–4°C. The homogenate was centrifuged at 12,000 *g* for 20 min and the supernatant was recentrifuged at 100,000 *g* for 60 min. The precipitate obtained from the second centrifugation was suspended in 10 volumes of 20 mM MOPS buffer (pH 7.4 at 30°C) containing NaCl 154 mM, KCl 5.6 mM, $CaCl_2$ 2.2 mM and $MgCl_2$ 2.1 mM (MOPS buffer B) as the membrane preparation. The membrane preparations were stored at −85°C and used within one week, during which time the binding characteristics of the muscarinic receptors did not change.

Binding analysis of muscarinic receptors

For the measurement of the dissociation constant (K_d) and the maximal binding (B_{max}) for [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB), the membrane preparation was incubated with various concentrations of [^{3}H]-QNB (0.05–0.8 nM) in the presence and absence of 10^{-5} M atropine in MOPS buffer B for 60 min at 30°C. The specific binding was determined as the 10^{-5} M atropine-sensitive binding of [^{3}H]-QNB. For measuring the K_d values for agonists (carbachol, McN-A-343 and AHR-602), the membrane preparation was incubated with 0.6 nM [^{3}H]-QNB containing carbachol (10^{-8}–10^{-1} M), McN-A-343 (10^{-8}–10^{-2} M) or AHR-602 (10^{-8}–10^{-1} M) in MOPS buffer B for 60 min at 30°C. For measuring the K_d values of competitive muscarinic antagonists, atropine (subtype-non-selective), pirenzepine (M_1-selective), 11-({2-[(diethylamino)-methyl]-1-piperidyl}acetyl)-5,11-dihydro-6H-[2,3-b][1,4]benzodiazepine-6-one (AF-DX 116) (M_2-selective) and 4-diphenylacetoxy-N-methyl-piperidine methiodide (4-DAMP) (M_3-selective), the membrane preparation was incubated with 0.6 nM [^{3}H]-QNB containing atropine (10^{-11}–10^{-6} M), pirenzepine (10^{-9}–10^{-3} M), AF-DX 116 (10^{-9}–10^{-4} M) or 4-DAMP (10^{-9}–10^{-4} M) in MOPS buffer B for 60 min at 30°C. The reaction was terminated by filtration through Whatman GF/B glass microfibre filters under vacuum with a 24-place cell harvester (Brandel, Gaithersburg, MD, U.S.A.), and radioactivity trapped on the filter was counted by use of liquid scintillation spectrophotometry. Determinations were made in duplicate or in quadruplicate. The protein concentration was determined by the method of Lowry *et al*. (1951) with bovine serum albumin as an internal standard. The reaction mixture (1 ml, approximately 50 μg protein) contained approximately 60 fmol of muscarinic receptors.

Contraction in the presence of Ca^{2+}

The isolated strips of taenia caeci were maintained in normal Locke-Ringer solution (normal solution in mM: NaCl 154, KCl 5.6, $CaCl_2$ 2.2, $MgCl_2$ 2.1, $NaHCO_3$ 6 and glucose 5.6), and contractions were recorded isotonically with a load of 0.5 g as described in a previous paper (Hishinuma & Uchida, 1987). For comparison of dose-response curves for McN-A-343 or AHR-602 with those for carbachol, contractions were cumulatively induced by McN-A-343 (10^{-8}–10^{-3} M) or AHR-602 (10^{-9}–10^{-3} M) after the control cumulative response for carbachol (10^{-9}–10^{-4} M) had been obtained. Contractions induced by 10^{-4} M carbachol, 10^{-3} M McN-A-343 or 10^{-3} M AHR-602 were also compared by use of a single dose method. To obtain the pA_2 values for the competitive antagonists, contractions induced by carbachol, McN-A-343 and AHR-602 were measured in the presence and absence of various concentrations of atropine (3×10^{-9}–10^{-7} M), pirenzepine (3×10^{-8}–3×10^{-6} M), AF-DX 116 (10^{-7}–10^{-5} M) or 4-DAMP (10^{-9}–10^{-7} M). Muscles were pretreated with these antagonists for 5 min before the measurement of the test contraction was started. For measuring effects of an L-type Ca^{2+} channel inhibitor, verapamil, muscle strips were pretreated with 3×10^{-6} M verapamil for 10 min before contractions were measured in the continued presence of verapamil. For measuring effects of the irreversible muscarinic antagonist, propylbenzilylcholine mustard (PrBCM) (Burgen *et al*., 1974), the contractions were measured before and 10 min after treatment with 3×10^{-6} M PrBCM for indicated time periods (10–90 min). During the PrBCM treatment, the bathing solution was changed to the fresh normal solution containing PrBCM every 10 min. Contractions were expressed as percentages of the maximal contraction of the control response, taking 0% as the baseline at the resting state measured just before the addition of agonists for the control response.

Contraction in Ca^{2+}-free solution

Contraction of isolated strips of taenia caeci was measured according to the methods of Ohashi *et al*. (1974; 1975) and Casteels & Raeymaekers (1979) with a minor modification (Hishinuma Uchida, 1988; 1991). The isolated muscle strips were equilibrated in normal solution for 1 h with a resting tension of 0.5 g, then incubated in Ca^{2+}-free Locke-Ringer solution (Ca^{2+}-free solution: $CaCl_2$ was omitted from the normal solution and EGTA was added at a final concentration of 2 mM) for 1 h at a resting tension of 0.05 g. The relatively long incubation time with Ca^{2+}-free solution was due to the original protocol that had been set out to perform drug treatment in Ca^{2+}-free conditions (Hishinuma & Uchida, 1988; 1991). The muscle strips were then soaked in isotonic 42 mM high K^+ solution (high K^+ solution in mM: NaCl 118, KCl 42, $CaCl_2$ 2.2, $MgCl_2$ 2.1, $NaHCO_3$ 6 and glucose 5.6) for 10 min to load Ca^{2+} into intracellular Ca^{2+} stores, replaced in Ca^{2+}-free solution, and exactly 2 min later exposed to carbachol at a final concentration of 10^{-4} M. The Ca^{2+} loading procedure with high K^+ solution, which is known to induce an enlarged contraction in Ca^{2+}-free solution (Ohashi *et al*., 1974; 1975; Casteels & Raeymaekers, 1979), was designed to detect a very small contraction. The muscle was re-suspended in the normal solution for about 45 min before the next cycle of testing, which included various concentrations of carbachol, McN-A-343 or AHR-602 in Ca^{2+}-free solution. By use of this protocol, the contraction elicited by 10^{-4} M carbachol was reproducible even after 4 exposures (Hishinuma & Uchida, 1988), thus allowing a direct comparison between agonists in the same muscle preparation. Antagonistic effects of McN-A-343 and AHR-602 on carbachol-induced contraction in Ca^{2+}-free solution were measured according to the same procedure described above, except that 10^{-3} M McN-A-343 or 10^{-3} M AHR-602 was simultaneously applied with either 10^{-4} M or 10^{-2} M carbachol at the second test response. For measuring effects of PrBCM on carbachol-induced contraction, one test cycle consisted of 60 min incubation in normal solution, 10 min incubation with high K^+ solution and then 2 min incubation with Ca^{2+}-free solution before addition of carbachol in Ca^{2+}-free solution. Muscles were then treated with 3×10^{-6} M PrBCM for 10 or 90 min in normal solution before being soaked in high K^+ solution at the second cycle. Contraction during the second test response in Ca^{2+}-free solution was induced either by 10^{-4} M carbachol or by 10^{-1} M carbachol. The test contractions during the second trial were expressed as percentages of the control response to 10^{-4} M carbachol.

Analysis of data

In the binding experiments, the K_d value and the B_{max} value for [^{3}H]-QNB were obtained by the Scatchard analysis (Scatchard, 1952), from the following equation: $B/F = (B_{max}\text{-}B)/K_{QNB}$. The K_d value and the Hill coefficient for a displacer against [^{3}H]-QNB binding was calculated from the following relationship: $B/B_{max} = L^{n_H}/(L^{n_H} + IC_{50}{}^{n_H})$ and $K_{dis} = IC_{50}/(F/K_{QNB} + 1)$. B represents specific binding of [^{3}H]-QNB, F the concentration of [^{3}H]-QNB used, B_{max} the maximal specific binding of [^{3}H]-QNB, K_{QNB} the K_d value for [^{3}H]-QNB, L the concentration of the displacer, n_H the Hill coefficient, IC_{50} the concentration of the displacer giving half maximal inhibition of [^{3}H]-QNB binding, and K_{dis} the K_d value for the displacer.

In the contraction experiments, the EC_{50} value was evaluated by the following relationship: $E/E_{max} = A^n/(A^n + EC_{50}{}^n)$. E represents contractile response, E_{max} the maximal response to the agonist, A the concentration of the agonist, n the slope factor and EC_{50} the concentration of the agonist giving half its maximal contraction. The pA_2 values for the competitive antagonists were evaluated by the Schild analysis (Arunlaskshana & Schild, 1959), from the following equation: $\log(DR-1) = \log X + pA_2$, where DR represents the dose-ratio of the concentrations of agonist giving half maximal contractions in the presence and absence of the antagonist, X is the concentration of the antagonist used. The slope of the line obtained by linear regression of least squares was constrained to one, since competitive antagonists were used. Statistical significance was evaluated by Student's t test with $P < 0.05$.

Drugs

We purchased carbachol and pirenzepine dihydrochloride from Sigma, McN-A-343 and 4-DAMP methiodide from Research Biochemicals Inc., atropine sulphate, verapamil hydrochloride, and 3-(N-morpholino)propanesulphonic acid (MOPS) from Wako Pure Chemicals Industries, Ltd., [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) and propylbenzilylcholine mustard (PrBCM) from New England Nuclear and oxotremorine from Aldrich. AHR-602 and AF-DX 116 were generous gifts from Wyeth-Ayerst Research and Boehringer Ingerheim, respectively.

Results

Binding properties of muscarinic agents for the membrane preparation

The K_d and B_{max} values of the atropine-sensitive [^{3}H]-QNB binding to the membrane preparation of guinea-pig taenia caeci, as defined by Scatchard analysis, were 96.9 ± 6.5 pM and 1.22 ± 0.11 pmol mg^{-1} protein (mean $\pm$ s.e.mean, $n = 4$), respectively.

Carbachol, McN-A-343 or AHR-602 completely displaced the atropine-sensitive [^{3}H]-QNB binding to muscarinic receptors (Figure 1a). The K_d values and Hill slopes were 2.8 ± 0.9 μM and 0.48 ± 0.01 for carbachol ($n = 3$), 6.3 ± 0.9 μM and 0.78 ± 0.01 for McN-A-343 ($n = 3$) and 58 ± 8 μM and 1.00 ± 0.03 for AHR-602 ($n = 3$), respectively. The rank order of potency of these agents in inhibiting [^{3}H]-QNB binding was carbachol > McN-A-343 $\gg$ AHR-602.

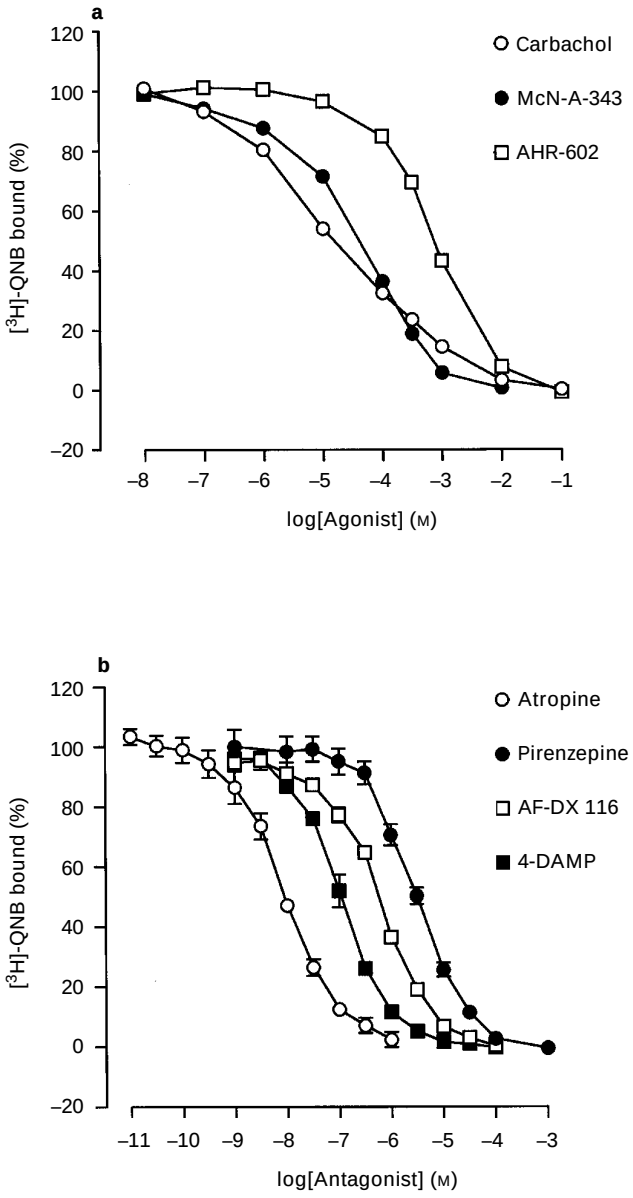

Figure 1 Displacement of [^{3}H]-QNB binding by (a) agonists and (b) antagonists in guinea-pig taenia caeci membranes. [^{3}H]-QNB binding in the presence and absence of 10^{-5} M atropine was taken as 0% and 100% binding, respectively. (a) The points represent mean and s.e.mean from quadruplicate determinations within a single experiment, which was repeated twice further with similar results. (b) The points represent mean, and vertical lines show s.e.mean, of duplicate determinations from 4 experiments.

Table 1 Inhibition by atropine, pirenzepine, AF-DX 116 and 4-DAMP of [^{3}H]-QNB binding and of contractions induced by carbachol, McN-A-343 and AHR-602

Agonist	*pK_d value from [^{3}H]-QNB binding*	*pA_2 value from contraction induced by*		
		Carbachol	*McN-A-343*	*AHR-602*
Atropine	8.94 ± 0.02 (4)	9.00 ± 0.09 (21)	9.04 ± 0.19 (15)	9.33 ± 0.24 (8)
Pirenzepine	6.44 ± 0.04 (4)	7.01 ± 0.09 (15)	6.95 ± 0.20 (8)	6.87 ± 0.15 (8)
AF-DX 116	6.99 ± 0.08 (4)	6.65 ± 0.10 (16)	6.16 ± 0.17 (8)	6.49 ± 0.05 (8)
4-DAMP	7.90 ± 0.06 (4)	9.67 ± 0.11 (15)	9.04 ± 0.20 (9)	9.38 ± 0.26 (9)

The pK_d values and the pA_2 values were calculated from the binding and contractile experiments, respectively, as described in Methods. Values are mean ± s.e.mean of the number of experiments in parentheses.

Pirenzepine, AF-DX 116 and 4-DAMP completely displaced the atropine-sensitive [^{3}H]-QNB binding to muscarinic receptors (Figure 1b). The pK_d values for these antagonists were 8.94±0.02 for atropine (n=4), 6.44±0.04 for pirenzepine (n=4), 6.99±0.08 for AF-DX 116 (n=4) and 7.90±0.06 for 4-DAMP (n=4) (Table 1). The rank order of potency of the antagonists in inhibiting [^{3}H]-QNB binding was atropine > 4-DAMP > AF-DX 116 > pirenzepine. The Hill coefficients for these antagonists were not significantly different from unity.

Contraction in the presence of extracellular Ca^{2+}

In the presence of extracellular Ca^{2+}, carbachol, McN-A-343 and AHR-602 induced a large contraction in guinea-pig taenia caeci (Figure 2). Contractions induced by 10^{-3} M McN-A-343 and 10^{-3} M AHR-602 corresponded to 79.3±2.8% (n=15) and 85.4±1.3% (n=18) of the maximal contraction induced by 10^{-4} M carbachol, respectively (Figure 3). The EC_{50} values were 48.6±3.1 nM for carbachol (n=33), 2.1±0.3 μM for McN-A-343 (n=15) and 9.5±1.4 μM for AHR-602 (n=18).

Table 1 shows the pA_2 values for antagonists in inhibiting contractions induced by carbachol, McN-A-343 and AHR-602, as defined by Schild analysis. The rank order of potency for the antagonists in inhibiting these contractions was 4-DAMP ≥ atropine > pirenzepine > AF-DX 116, which differs from that evaluated from the displacement of [^{3}H]-QNB binding.

Contractions induced by these agonists were inhibited by verapamil (Figure 4). Maximal contractions induced by these agonists were differentially affected by 3 μM verapamil, resulting in reduction of 32.8±4.1% for carbachol (n=4), 78.4±4.3% for McN-A-343 (n=4) and 77.5±2% for AHR-602 (n=4) (Figure 4).

Contraction in the absence of extracellular Ca^{2+}

In Ca^{2+}-free solution, 10^{-4} M carbachol elicited a large and transient contraction (Figure 5). In contrast, neither 10^{-3} M McN-A-343 nor 10^{-3} M AHR-602 elicited detectable contraction, although subsequent application of 10^{-4} M carbachol, 1 min after wash out of McN-A-343 or AHR-602 with Ca^{2+}-free solution, induced contraction corresponding to 66.4±4.7% (n=4) and 91.9±4.4% (n=3) of its control response, respectively (Figure 6, right traces).

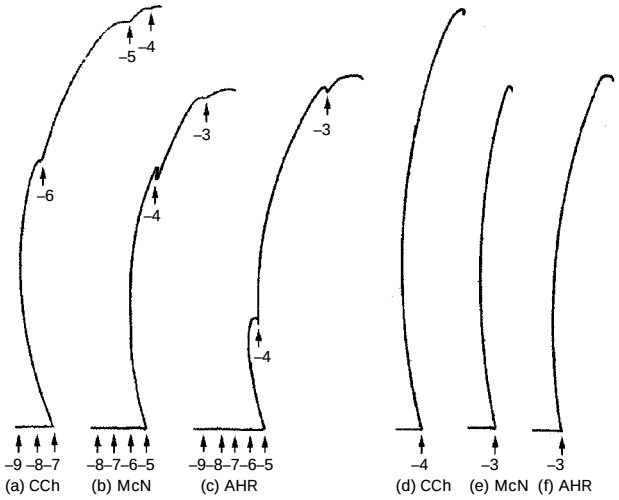

Figure 2 Traces of contraction induced by (a,d) carbachol (CCh), (b,e) McN-A-343 (McN) or (c,f) AHR-602 (AHR) in the presence of extracellular Ca^{2+}. (a–c) Cumulative contractions or (d–f) contractions induced by a single dose method were induced with these agonists. Arrows indicate application of various concentrations of agonists and the numbers are log[agonist concentration] (M).

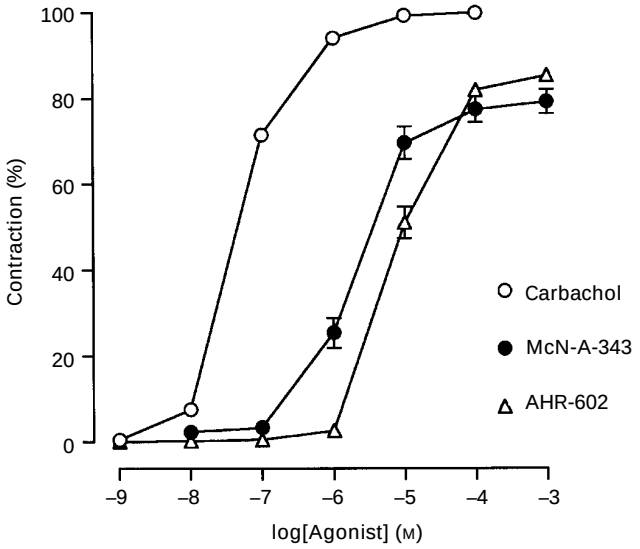

Figure 3 Contraction induced by carbachol, McN-A-343 or AHR-602 in the presence of extracellular Ca^{2+}. Cumulative contraction was induced by McN-A-343 (n=15) or AHR-602 (n=18) after the control contraction to carbachol had been obtained. The base line just before measurement of the control contraction was taken as 0% and the maximal contraction induced by 10^{-4} M carbachol was taken as 100%. Values represent mean and s.e.mean (vertical lines).

The EC_{50} value for carbachol of 8.5±1.0 μM (Figure 7a) was approximately 175 times higher than that for carbachol-induced contraction in the presence of extracellular Ca^{2+}. The EC_{50} values for McN-A-343 and AHR-602 could not be calculated due to the absence of significant contraction to these agents up to 10^{-1} M (Figure 7a). When 10^{-3} M McN-A-343 or 10^{-3} M AHR-602 was simultaneously applied with 10^{-4} M carbachol, McN-A-343 and AHR-602 inhibited carbachol-induced contraction in Ca^{2+}-free solution to 39.6±3.2% (n=4) and 70.2±2.2% (n=4) of control, respectively (Figure 7b). Inhibition by 10^{-3} M McN-A-343 or 10^{-3} M AHR-602 of 10^{-4} M carbachol-induced contraction in Ca^{2+}-free solution was surmounted by the use of a higher concentration of carbachol (10^{-2} M), resulting in no significant differences between 10^{-2} M carbachol-induced contractions in the presence and absence of these agents (Figure 7b).

Effects of PrBCM pretreatment on carbachol-, McN-A-343- and AHR-602-induced contractions

Pretreatment of guinea-pig taenia caeci strips with 3×10^{-6} M PrBCM for 10 to 30 min shifted the dose-response curve for carbachol-induced contractions in the presence of extracellular Ca^{2+} to the right and reduced the maximum response (Figure 8a). The treatment with PrBCM for longer time periods (up to 90 min) did not induce further changes in the dose-response curves for carbachol (Figure 8a). The maximum achievable inhibition was 42.5±7.5% of control response (n=4). In contrast, contraction induced by McN-A-343 or AHR-602 was abolished by 10 min PrBCM pretreatment (Figure 8b and c).

Contraction induced by 10^{-4} M carbachol in Ca^{2+}-free solution was reduced to 3.1±0.9% of control (n=4) by pretreatment with PrBCM for 90 min (Figure 9). Even when a higher concentration of carbachol (10^{-1} M) was applied at the test response, the contraction was again reduced to 3.7±0.3% of control (n=4) by treatment with PrBCM for a shorter time period (10 min) (Figure 9).

Discussion

The rank orders of potency of subtype selective antagonists evaluated from contraction and [^{3}H]-QNB binding experiments were not identical: 4-DAMP $\geqslant$ atropine > pirenzepine > AF-DX 116 for contractions and atropine > 4-DAMP > AF-DX 116 > pirenzepine for [^{3}H]-QNB binding, suggesting the presence of muscarinic receptor subtype(s) that may not be involved in the contractile response. Comparison of the pA_2 values for subtype selective antagonists obtained in this paper with those described in others (Gardner *et al*., 1988b; Ladinsky *et al*., 1988; Fukuda *et al*., 1989; Eglen *et al*., 1994) indicated that contractions induced by carbachol, McN-A-343 and AHR-602 were mediated by the muscarinic receptor subtype that had high affinity for 4-DAMP (>9) and low affinity for both pirenzepine (<7) and AF-DX 116 (<7) i.e. the M_3 receptor subtype. However, pK_d values for the subtype selective antagonists showed the existence of muscarinic receptor subtypes that had a high affinity for AF-DX 116 ($\geqslant$7) and a low affinity for pirenzepine (<7) and 4-DAMP (<8). This suggests that M_2 receptors are also present (Gardner *et al*., 1988b; Ladinsky *et al*., 1988; Fukuda *et al*., 1989; Eglen *et al*., 1994). The relatively low pK_d value for 4-DAMP compared with its high pA_2 value also suggests the presence of M_2 receptors. Displacement curves for the subtype selective antagonists were best-fitted to a one-site model with Hill coefficients not different from unity. This was probably due to a high expression ratio of M_2 compared to M_3 receptors in this tissue, as has been previously obtained in smooth muscle (M_2:M_3 ratio 80:20; Maeda *et al*., 1988; Eglen *et al*., 1994). Carbachol, McN-A-343 and AHR-602 completely displaced the atropine-sensitive component of [^{3}H]-QNB binding to muscarinic receptors, indicating that these compounds bind to all of the muscarinic receptors present in smooth muscle of guinea-pig taenia caeci.

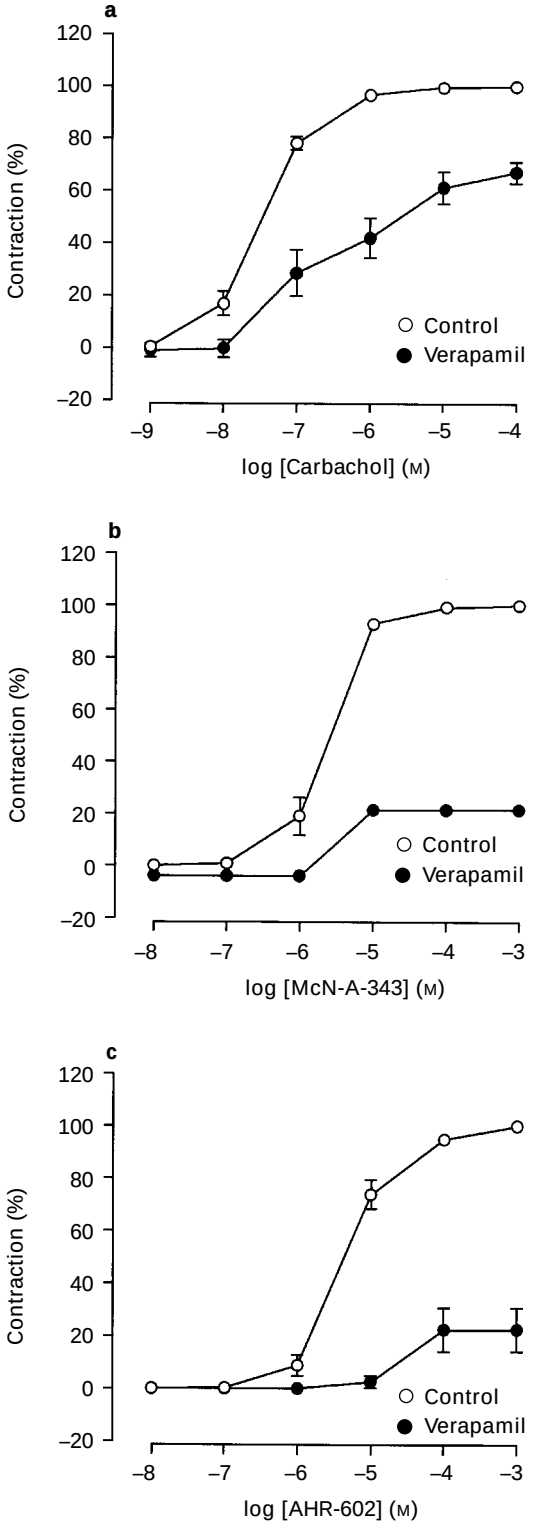

Figure 4 Effects of verapamil on contraction induced by (a) carbachol, (b) McN-A-343 or (c) AHR−602 in the presence of extracellular Ca^{2+}. Cumulative contraction was induced by these agonists in the presence of 3 μM verapamil after the control contraction had been obtained. The base line just before measurement of the control contraction was taken as 0% and the maximal contraction in the control response induced by each agonist was taken as 100%. Values represent mean and s.e.mean (vertical lines) of 4 experiments.

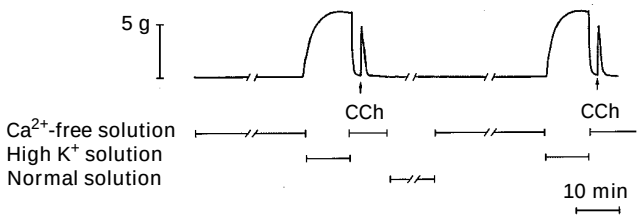

Figure 5 Repetitive responses induced by carbachol (CCh) in Ca^{2+}-free solution. Traces show contraction in the first (control) and second test cycle elicited by 10^{-4} M carbachol (arrows) after the Ca^{2+}-loading procedure with high K^+ as described in Methods.

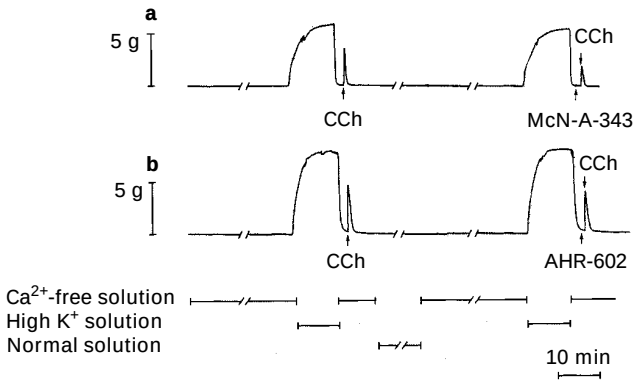

Figure 6 Lack of effects of (a) McN-A-343 and (b) AHR-602 in Ca^{2+}-free solution. Responses to carbachol (CCh, left) and those to carbachol after application and a subsequent washing out of 10^{-3} M McH-A-343 or 10^{-3} M AHR-602 (right) are shown. The subsequently applied carbachol after McN-A-343 or AHR-602 induced contraction under conditions where McN-A-343 or AHR-602 induced no contraction.

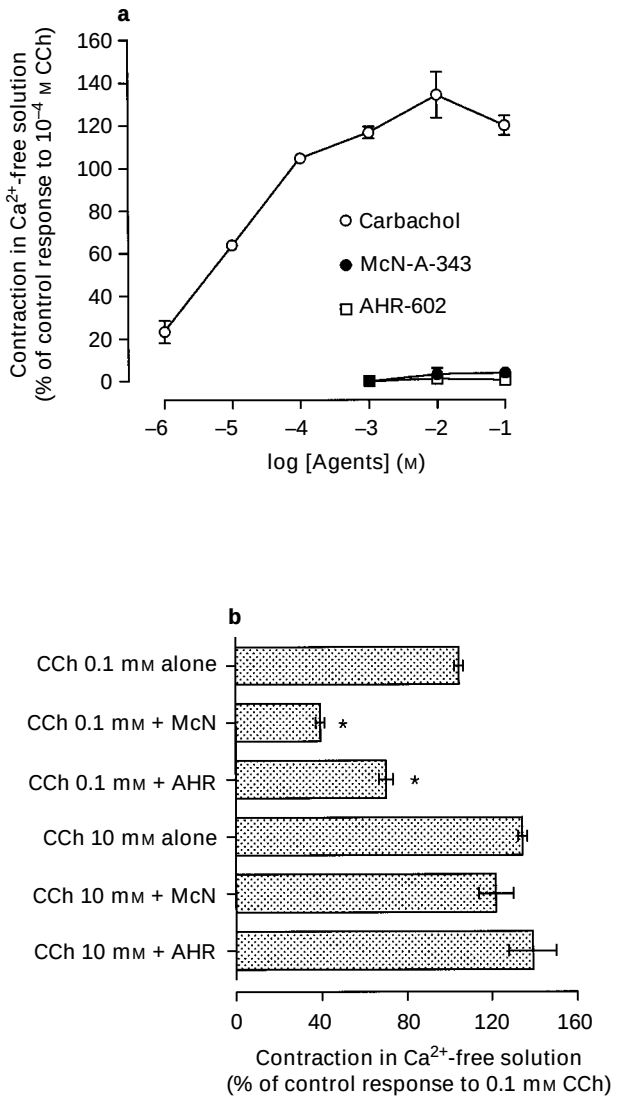

Figure 7 Contraction induced by carbachol in Ca^{2+}-free solution and its inhibition by McN-A-343 and AHR-602. (a) Contractions elicited by various concentrations of these agents are expressed as percentages of control response to 10^{-4} M carbachol. In contrast to carbachol, neither McN-A-343 nor AHR-602 induced significant contraction. (b) Contractions elicited by 10^{-4} M or 10^{-2} M carbachol (CCh) in the presence and absence of 10^{-3} M McN-A-343 or 10^{-3} M AHR-602 are expressed as percentages of control response to 10^{-4} M carbachol. Contraction induced by 10^{-4} M carbachol, but not by 10^{-2} M carbachol, was inhibited by 10^{-3} M McN-A-343 or 10^{-3} M AHR-602. $*P<0.001$.

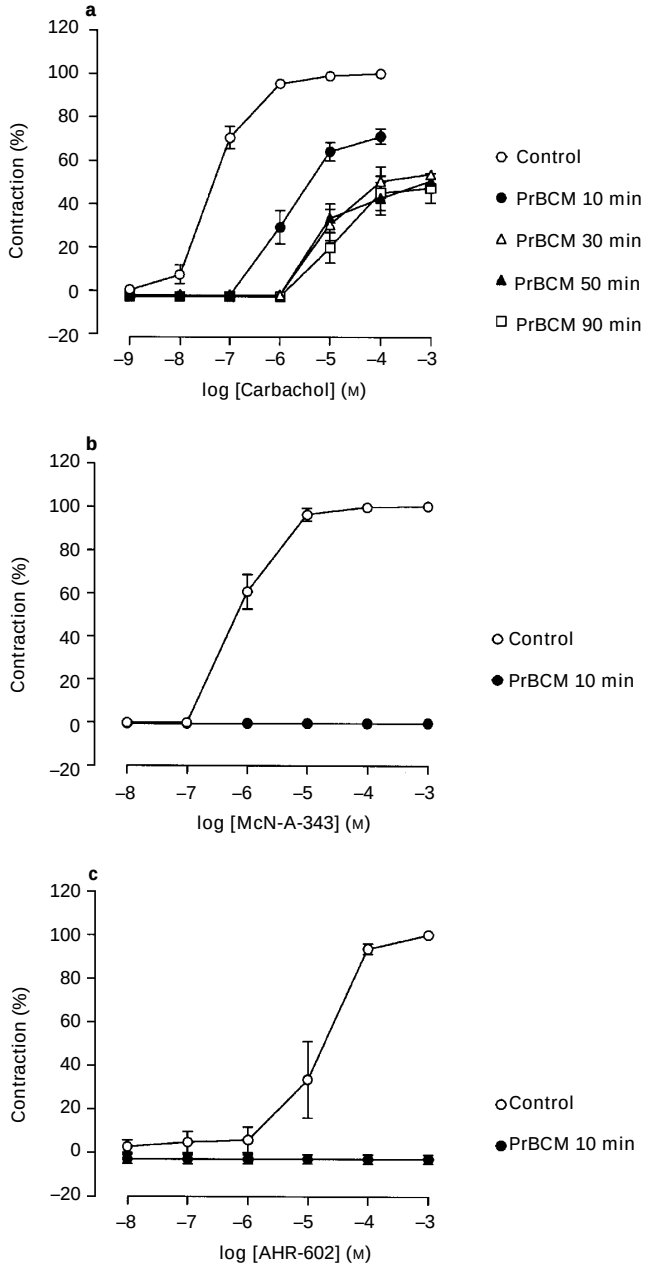

Figure 8 Effects of PrBCM on contractions induced by (a) carbachol, (b) McN-A-343 and (c) AHR-602 in the presence of extracellular Ca^{2+}. (a) Cumulative contractions were induced by carbachol before (control) and 10 min after treatment with 3×10^{-6} M PrBCM for 10 min, 30 min, 50 min and 90 min. The base line just before measurement of the control contraction was taken as 0% and the maximal contraction induced by 10^{-4} M carbachol in the control response was taken as 100%. (b) Cumulative contractions were induced by McN-A-343 before (control) and 10 min after treatment with 3×10^{-6} M PrBCM for 10 min. The base line just before measurement of the control contraction was taken as 0% and the maximal contraction induced by 10^{-3} M McN-A-343 in the control response was taken as 100%. (c) Conditions were as in (b) except that AHR-602 was used instead of McN-A-343. In (a), (b) and (c), values represent mean and s.e.mean (vertical lines) of 4 experiments. Only carbachol could induce a contraction after PrBCM treatment.

The rise in cytoplasmic Ca^{2+} concentration via activation of muscarinic M_3 receptors could be via a Ca^{2+} release pathway from intracellular stores and/or a Ca^{2+} influx pathway from the extracellular space (Brading & Sneddon, 1980; Parekh & Brading, 1992; Wang *et al*., 1992). In the absence of extracellular Ca^{2+}, and in contrast to the effect of carbachol, neither McN-A-343 nor AHR-602 induced a contractile response. Moreover, McN-A-343 and AHR-602 competitively inhibited carbachol-induced contraction in Ca^{2+}-free solution. The antagonistic potency of McN-A-343 was greater than that of AHR-602, presumably due to the higher affinity of muscarinic receptors for McN-A-343 than for AHR-602. Thus, even though McN-A-343 and AHR-602 bind to and activate the M_3 subtype, they do not appear to stimulate intracellular Ca^{2+} release (as measured by a contractile response in the absence of extracellular Ca^{2+}). Indeed, neither McN-A-343 nor AHR-602 appear to stimulate phosphoinositide hydrolysis in this preparation (Gardner *et al*., 1988a).

In the presence of extracellular Ca^{2+}, McN-A-343 or AHR-602 induced contractions corresponding to 80–85% of the maximal response to carbachol, indicating that McN-A-343 and AHR-602 stimulate contractions via a Ca^{2+} influx pathway. The contractions by McN-A-343 and AHR-602 were sensitive to the L-type Ca^{2+} channel blocker, verapamil, suggesting the involvement of voltage-dependent Ca^{2+} channels (Bolton, 1979; Brading, 1981). However, M_2 subtype activa-

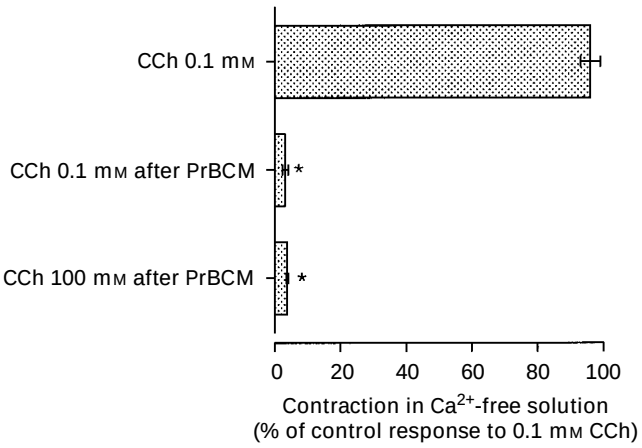

Figure 9 Effects of PrBCM on contractions induced by carbachol (CCh) in Ca^{2+}-free solution. Carbachol of 10^{-4} M and 10^{-1} M hardly induced contraction after treatment with 3×10^{-6} M PrBCM for 90 min and 10 min, respectively. Values represent mean ± s.e.mean of 4 experiments. $*P < 0.001$.

tion may also depolarize the plasma membrane due to stimulation of non-selective cation channels (via activation of pertussis toxin-sensitive G proteins, most probably G_i) (Inoue & Isenberg, 1990; Eglen *et al*., 1994). Although guinea-pig taenia caeci appears to have a large proportion of M_2 receptors, it is the M_3 subtype that is involved in contractions induced by carbachol, McN-A-343 and AHR-602 (see above, and Table 1). Furthermore, treatment with pertussis toxin, 1 μg ml^{-1} for 5 h, failed to affect carbachol-induced contractions either in the presence or absence of extracellular Ca^{2+} (data not shown). It is, therefore, most likely that Ca^{2+} entry induced by carbachol, McN-A-343 and AHR-602 is via activation of M_3 receptors, although the coupling mechanism between M_3 receptors with verapamil-sensitive (voltage-dependent) Ca^{2+} channels is still unclear.

The EC_{50} value for carbachol-induced contractions in the presence of extracellular Ca^{2+} was much lower than that in the absence of Ca^{2+}, whilst McN-A-343 and AHR-602 were able to induce contractions only in the presence of extracellular Ca^{2+}. Furthermore, PrBCM treatment prevented carbachol from stimulating the Ca^{2+} release pathway but only partially blocked the carbachol-mediated Ca^{2+} influx pathway. This indicates that there is a greater receptor reserve for the Ca^{2+} influx pathway than for the Ca^{2+} release pathway, and hence, Ca^{2+} release will be more sensitive than Ca^{2+} influx to a reduction in receptor number. Such a theory is supported by the observation that carbachol-stimulated phosphoinositide metabolism is markedly reduced by PrBCM treatment in this tissue (Gardner *et al*., 1988a). The lower maximum responses to McN-A-343 and AHR-602 compared with carbachol, combined with an increased sensitivity to PrBCM indicates that these compounds act as partial agonists at the M_3 receptor. In support of this, both McN-A-343 and AHR-602 competitively inhibit the contractile response to carbachol.

It is unclear, at present, why there is apparently a greater receptor reserve for M_3-mediated Ca^{2+} influx than for M_3-mediated Ca^{2+} release. One possibility is that M_3 receptors are functionally divided into two subclasses that couple to either Ca^{2+} influx or Ca^{2+} release, as has been suggested for α_1-adrenoceptors (Han *et al*., 1987; Suzuki *et al*., 1990), and that McN-A-343 and AHR-602 activate the Ca^{2+} influx-coupled M_3 receptors, but block the Ca^{2+} release-coupled subtype. Another possibility is that conformational changes in single M_3 receptor molecules induced by carbachol mediate both Ca^{2+} influx and Ca^{2+} release, but McN-A-343 or AHR-602 only activate the Ca^{2+} influx pathway. Such multifunctional signalling by G protein-coupled receptors mediated via multiple conformational states has been previously discussed (Milligan, 1993; Lefkowitz *et al*., 1993; Gudermann *et al*., 1996). Gardner (1995) has also suggested that agonists may determine receptor conformation and hence G protein affinity. It may be possible that conformational states of the receptors brought about by stimulation with McN-A-343 or AHR-602 were different from those with carbachol, resulting in selective coupling to the Ca^{2+} influx pathway. The apparent differences in efficacy of response to carbachol, McN-A-343 and AHR-602 between the two different pathways may also be a result of different levels of post-receptor amplification of response.

As mentioned above, McN-A-343 and AHR-602 have a unique feature in that, in this study, they activate the muscarinic M_3 receptor-mediated Ca^{2+} influx pathway but not the Ca^{2+} release pathway. It would be of interest to examine how other full and partial agonists affect the influx and mobilization phases of the Ca^{2+} response. Recent observations show that oxotremorine has intermediate characteristics between carbachol and McN-A-343 or AHR-602: oxotremorine 10^{-4} M, which induced 100.5 ± 3.7% of contraction compared with maximal contraction induced by 10^{-4} M carbachol in the presence of Ca^{2+} ($n = 4$), induced only 43.1 ± 2.4% of contraction in Ca^{2+}-free solution compared with 10^{-4} M carbachol-induced contraction in Ca^{2+}-free solution ($n = 7$). Such differential activation of the Ca^{2+} signal between agonists may possibly determine characteristics of cellular responsiveness to the agonists.

In conclusion, further investigations are needed to clarify molecular mechanisms for differential activation of influx- and mobilization-mediated Ca^{2+} signalling via G protein-coupled receptors. In this respect, McN-A-343 and AHR-602 may provide crucial information on muscarinic receptor-mediated Ca^{2+} signalling mechanisms in smooth muscle.

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