M2 and M3 Muscarinic Receptors Couple, Respectively, With Activation of Nonselective Cationic Channels and Potassium Channels in Intestinal Smooth Muscle Cells

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ABSTRACT—Smooth muscle cells of guinea pig ileum express both M2 and M3 subtypes of muscarinic receptors. Under voltage clamp, activation of the muscarinic receptors with carbachol (CCh) induces Ca^{2+}-activated K^+ current ($I_{K\text{-}Ca}$) and nonselective cationic current (I_{cat}). Receptor subtypes mediating the current responses were characterized by using pirenzepine, AF-DX116, 4-DAMP and atropine, which have different profiles of the affinity constants for muscarinic receptor subtypes. The muscarinic antagonists inhibited either CCh-evoked $I_{K\text{-}Ca}$ or I_{cat} with different potencies. Their relative potencies for $I_{K\text{-}Ca}$ and I_{cat} inhibition resembled the relative affinity constants for M3 and M2 subtypes, respectively. Thus, the $I_{K\text{-}Ca}$ is mediated via the M3 subtype and the I_{cat} via the M2 subtype.

Keywords: Carbachol, Muscarinic receptor, Smooth muscle, Ca^{2+}-activated K^+ current, Nonselective cationic current

Activation of muscarinic receptors causes contraction of many types of smooth muscle. Isolated guinea pig and rat ileums have offered models for the subclassification and analytical research on the function of muscarinc receptors in intestinal smooth muscle. Radioligand-binding studies revealed the existence of M2 and M3 subtypes, but not M1 and M4 subtypes, of muscarinic receptors with a predominant population of the M2 subtype (1, 2). Such co-existence of M2 and M3 subtypes was comfirmed by Northern blot analyses of receptor mRNA (3, 4). However, the contractions evoked by muscarinic agonists are regarded as mediated almost exclusively via the M3 subtype (5–8). Thus, as yet, what role the M2 subtype plays in the muscarinic contractile response is not understood (1).

The muscarinic contractile response of intestinal smooth muscle is mediated by an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which results from an influx of Ca^{2+} from the extracellular fluid through membrane depolarization and release of Ca^{2+} from intracellular stores (9). Voltage-clamp studies showed that acetylcholine and carbachol (CCh) elicit the nonselective cationic current (I_{cat}) underlying the membrane depolarization and Ca^{2+}-activated K^+ current ($I_{K\text{-}Ca}$) resulting from Ca^{2+} store release (10–13).

The present study was designed to characterize muscarinic receptors mediating the CCh-evoked I_{cat} and $I_{K\text{-}Ca}$ using muscarinic antagonists of pirenzepine, AF-DX116, 4-DAMP and atropine, which have different profiles of the affinity constants for muscarinic receptor subtypes (14). A preliminary account of the findings has been presented to The Japanese Pharmacological Society (15).

MATERIALS AND METHODS

Male guinea pigs, weighing 350–450 g, were stunned and killed by exanguination. A 10- to 15-cm length of the ileum was removed and divided into 3-cm segments. The longitudinal muscle layer of the segments was peeled from the underlying circular muscle and washed in physiological salt solution (PSS, composition given below). Smooth muscle cells of the muscle layers were dispersed by a combination of collagenase (0.2–0.6 mg/ml) and papain (0.3–0.6 mg/ml), as described previously (13). The cells were suspended in PSS containing 0.5 mM Ca^{2+}, and 0.1-ml aliquots were placed on glass coverslips (15 mm in diameter) and kept in a moist atmosphere at 4℃ until use on the same day.

Single isolated ileal muscle cells were voltage-clamped with patch pipettes filled with a solution (13). Patch

pipettes had a tip resistance of 4–6 ΩM. Whole-cell membrane current was recorded at room temperature (20–25℃) by the conventional patch-clamp technique. A voltage-clamp amplifier (SEZ-2300; Nihon Kohden, Tokyo) was used for current recording, and membrane currents were stored on FM tape and replayed onto a thermal array recorder (RTA-1100M, Nihon Kohden) for illustrations and analysis.

CCh-evoked $I_{K\text{-}Ca}$ was recorded from cells bathed in PSS of the following composition: 126 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 14 mM glucose and 10.5 mM HEPES (titrated to pH 7.2 with NaOH), using patch pipettes filled with a solution of the following composition: 134 mM KCl, 1.2 mM $MgCl_2$, 1 mM MgATP, 0.1 mM Na_2GTP, 0.05 mM EGTA, 14 mM glucose and 10.5 mM HEPES (titrated to pH 7.2 with KOH), at a holding potential of 0 mV. At 0 mV, the potassium current can be recorded with almost no contamination of CCh-evoked I_{cat} (13).

For recordings of CCh-evoked I_{cat}, a Cs-based solution with a Ca^{2+}-buffering system was used as a pipette solution, which had the following composition: 80 mM CsCl, 2.5 mM $MgCl_2$, 1 mM MgATP, 0.1 mM Na_2GTP, 14 mM glucose, 10 mM HEPES, 20 mM BAPTA and 8 mM $CaCl_2$ (titrated to pH 7.2 with CsOH). In this solution, an approximate concentration of free Ca^{2+} was 100 nM. The pipette solution served to block potassium currents including $I_{K\text{-}Ca}$ and to eliminate a potentiating effect of Ca^{2+} store release on the CCh-evoked I_{cat} by keeping $[Ca^{2+}]_i$ constant (16). Bathing solution used in the experiments had the following composition: 120 mM CsCl, 14 mM glucose and 10.5 mM HEPES (titrated to pH 7.2 with CsOH). The relationship between the cationic conductance activated by CCh and the membrane potential is sigmoidal, and the activation curve is shifted on the voltage axis in the negative direction as CCh concentration (3–300 μM), i.e., fractional receptor occupancy, is increased (17). Since the activation curve in 3 μM CCh reaches a roughly maximal level at −60 mV (see Fig. 2C in ref. 17), the I_{cat} was recorded at −60 mV in the present experiments.

CCh and muscarinic antagonists were applied by changing the solution bathing cells to one containing them. As a rule, application of CCh to one cell to record either $I_{K\text{-}Ca}$ or I_{cat} was not repeated because of the run-down of the current response with repeated applications of a concentration of CCh. Recordings of CCh-evoked currents were made approximately 5 min after establishment of whole-cell voltage-clamp configuration. When treated with a muscarinic antagonist, cells were exposed to the antagonist for 3–4 min before and throughout CCh application.

The data are expressed as the mean±S.E.M. The statistical significance was assessed by Student's unpaired *t*-test. Values of $P<0.05$ were considered significant. The concentration required for muscarinic antagonists to produce 50% inhibition of the CCh effect (IC_{50}) was determined by Hill plot analysis with computer software (Delta Graph Software; Polaroid Computing Inc., Tokyo).

Drugs used were carbachol chloride (CCh) (Tokyo Kasei, Tokyo), caffeine, atropine sulphate (Wako, Tokyo), pirenzepine (Sigma, St. Louis, MO, USA), 4-diphenyl acetoxy *N*-methyl piperidine (4-DAMP) (Research Biochemical International, Natick, MA, USA) and 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dyhydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine-6-one (AF-DX116) (kindly given to us as a gift from Boehringer Ingelheim, Ingelheim am Rhein, Germany).

RESULTS

CCh-evoked $I_{K\text{-}Ca}$

Application of CCh induced $I_{K\text{-}Ca}$ as a brief outward current that reached a peak some 1 sec after its onset and then decayed to the basal level within several seconds even in the continued presence of CCh (Fig. 1A). The current response increased in a concentration-dependent manner with the concentrations of 30 μM and 1 μM required to produce the maximum effect and half the maximum effect (EC_{50}), respectively (see closed circles in Fig. 1B). When 10 mM caffeine, a potent Ca^{2+} releaser from storage sites in this type of cells (13), was applied in the presence of, and 45–60 sec after, CCh, a brief outward current was elicited (Fig. 1A). The current response to caffeine decreased in size inversely as the previously-applied CCh concentration increased (open circles in Fig. 1B), but the sum of the sizes of the caffeine- and CCh-evoked currents was roughly constant at any CCh concentration (open triangles in Fig. 1B). The complementary relationship indicated that CCh-evoked $I_{K\text{-}Ca}$ is due to Ca^{2+} release from intracellular stores.

Treatment of cells with pirenzepine, AF-DX116, 4-DAMP or atropine resulted in a concentration-dependent inhibition of 10 μM CCh-evoked $I_{K\text{-}Ca}$ (95% of the maximum effect) (Fig. 2: A and B). The muscarinic antagonists exerted the action with different potencies. As shown in Fig. 2B, a significant inhibition of the current ($P<0.05$ or $P<0.01$) was produced with 1,000 nM pirenzepine, 10,000 nM AF-DX116, 10 nM 4-DAMP or 1 nM atropine. The concentration eliciting IC_{50} determined by Hill plot analysis of data points was 0.6 nM for atropine, 2.4 nM for 4-DAMP, 314.9 nM for pirenzepine and 2194.2 nM for AF-DX116. Taking the IC_{50} for atropine as unity, those for 4-DAMP, pirenzepine and AF-DX116 were 4, 525 and 3657, respectively (Table 1). The relative affin-

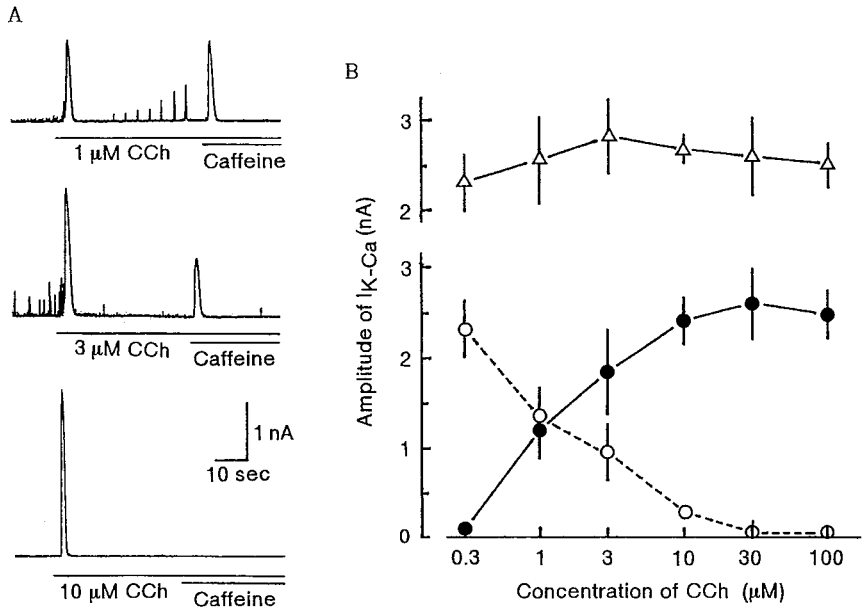

Fig. 1. Ca^{2+}-activated K^+ current ($I_{K\text{-}Ca}$) in response to carbachol (CCh) and caffeine in single smooth muscle cells of guinea pig ileum held under voltage clamp at 0 mV. A: $I_{K\text{-}Ca}$ responses to 1, 3 and 10 μM CCh and to subsequent 10 mM caffeine in three different cells. The drugs were applied as indicated by bars below the current recording traces. B: relationships between CCh concentration and the amplitude of CCh-evoked $I_{K\text{-}Ca}$ (●) or subsequent caffeine (10 mM)-evoked $I_{K\text{-}Ca}$ (○) or their summed amplitude (△). Each point indicates the mean±S.E.M. (vertical bars) of measurements in 5 to 12 cells.

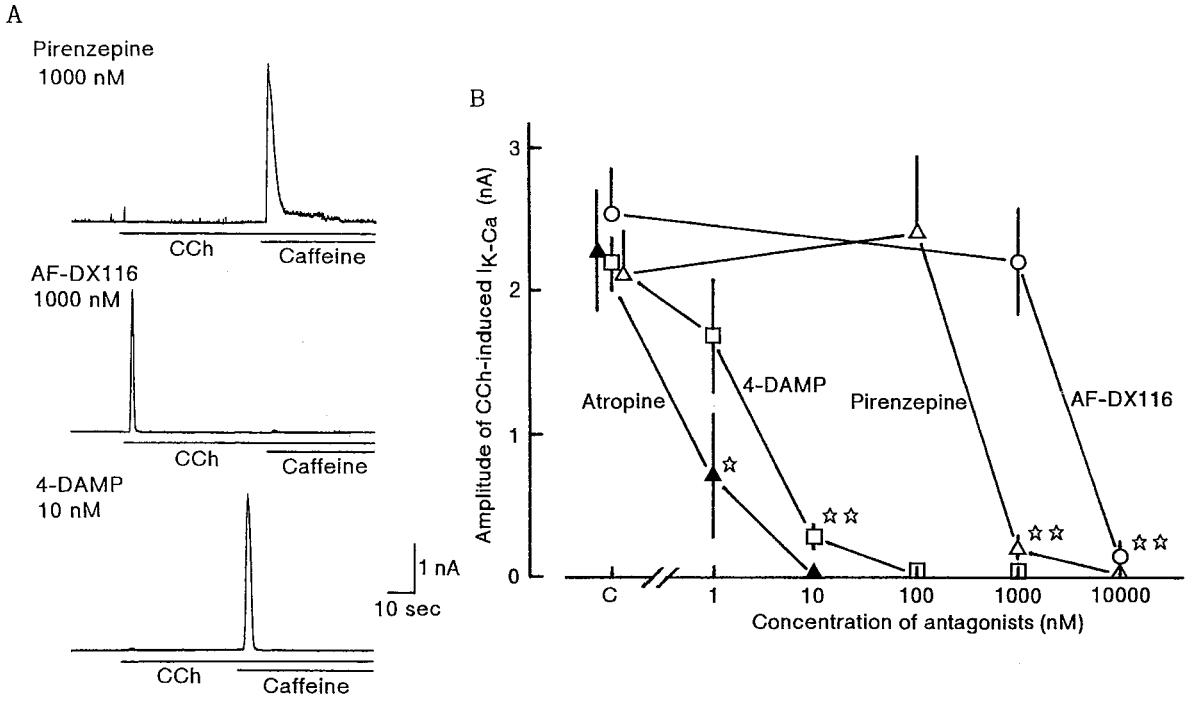

Fig. 2. Effects of muscarinic antagonists, pirenzepine, AF-DX116, 4-DAMP and atropine on carbachol (CCh)-evoked $I_{K\text{-}Ca}$. The current was recorded at 0 mV. Each antagonist was allowed to act for 3–4 min before and throughout CCh application. A: $I_{K\text{-}Ca}$ responses to 10 μM CCh and subsequent 10 mM caffeine in cells treated with pirenzepine, AF-DX116 and 4-DAMP at the indicated concentrations, respectively. B: relationships between antagonist concentration and the mean maximum amplitude of 10 μM CCh-evoked $I_{K\text{-}Ca}$. Data points for each antagonist were obtained by measuring CCh-evoked currents recorded alternately from cells untreated (control, C) or treated with the antagonist at various concentrations. Each point indicates the mean±S.E.M. (vertical bars) of measurements in 12–27 control cells or 4–9 antagonist-treated cells. $^{☆☆}P<0.01$, $^{☆}P<0.05$; significantly different from the corresponding control value. See text for details.

ities of atropine, 4-DAMP, pirenzepine and AF-DX116 for M1, M2 and M3 subtypes of muscarinic receptors are also presented in Table 1, which come from the affinity constants obtained from radioligand-binding studies (see Table 3 in ref. 14). The profile of the relative IC_{50} values resembles that of the relative affinity constants for the M3 subtype, suggesting that CCh-evoked $I_{K\text{-}Ca}$ is mediated via the M3 subtype.

CCh-evoked I_{cat}

CCh evoked I_{cat} as an inward current that persisted until CCh application for 45–60 sec was terminated. With increasing CCh concentration up to 300 μM, the current response was shortened in the time to peak and increased in size (Fig. 3A). The maximum current response was attained at 100 μM, and EC_{50} was estimated to be about 20 μM (Fig. 3B).

Table 1. IC_{50} values of muscarinic antagonists for inhibition of Ca^{2+}-activated K^+ current ($I_{K\text{-}Ca}$) and nonselective cationic current (I_{cat}) evoked by carbachol (CCh) in guinea pig ileal smooth muscle cells

Antagonist	$I_{K\text{-}Ca}$		I_{cat}		Ratio of K_d*		
	IC_{50}	ratio	IC_{50}	ratio	M1	M2	M3
Atropine	0.6 nM	1	0.8 nM	1	1	1	1
4-DAMP	2.4	4	6.1	8	4	13	3
Pirenzepine	314.9	525	253.8	317	40	631	397
AF-DX116	2194.2	3657	64.3	80	1260	100	3166

IC_{50}s were determined using data points in Figs. 2B and 4B. The data points were normalized by expressing them as a percentage of the corresponding control value and subjected to Hill plot analysis by computer software. Ratios of the IC_{50}s are expressed by taking the IC_{50} of atropine as unity. *Dissociation constants (K_d) of the antagonists for M1, M2 and M3 subtypes of muscarinic receptors are obtained from their affinity constants determined by radioligand-binding studies (see Table 3 in ref. 14) and expressed as ratios to the K_d of atropine. Note the similarity in ratio profile between IC_{50} for $I_{K\text{-}Ca}$ and K_d for the M3 subtype and between IC_{50} for I_{cat} and K_d for the M2 subtype.

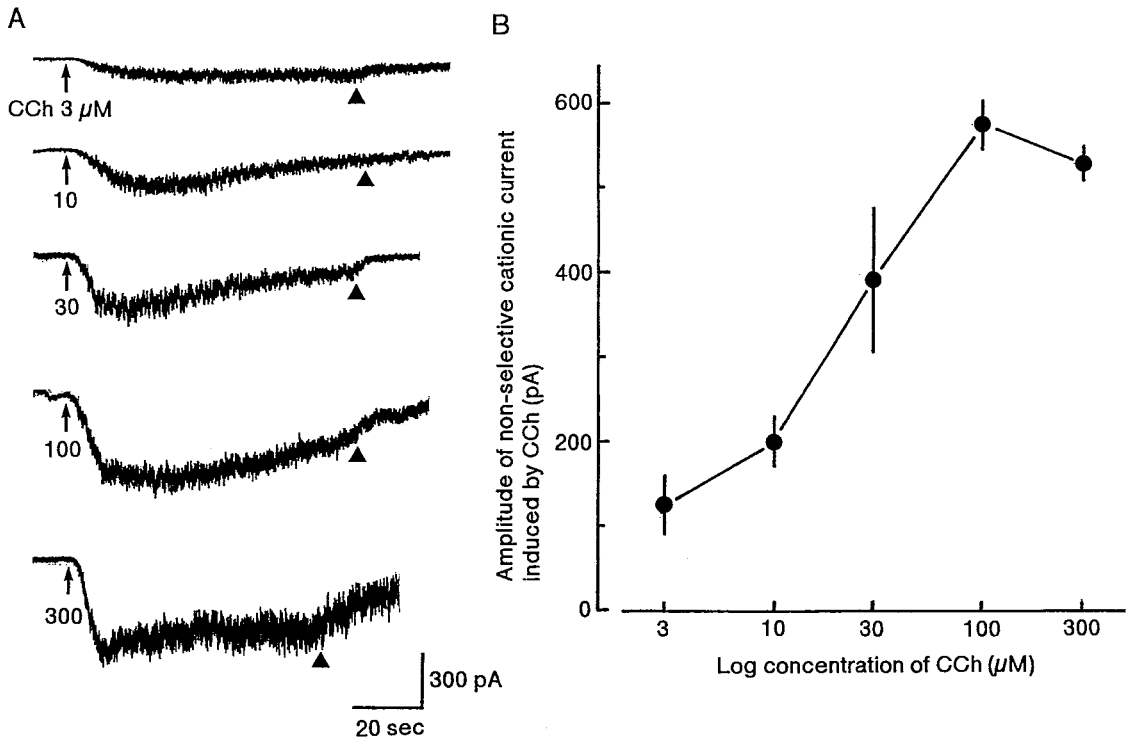

Fig. 3. Nonselective cationic current (I_{cat}) evoked by carbachol (CCh) in single ileal muscle cells held under voltage clamp at −60 mV. A: I_{cat} responses to CCh at concentrations of 3 to 300 μM in different cells. CCh was applied at the arrows and removed at the closed triangles. B: relationship between CCh concentration and the mean maximum amplitude of I_{cat}. Each point indicates the mean±S.E.M. (vertical bars) of measurements in 7–38 cells.

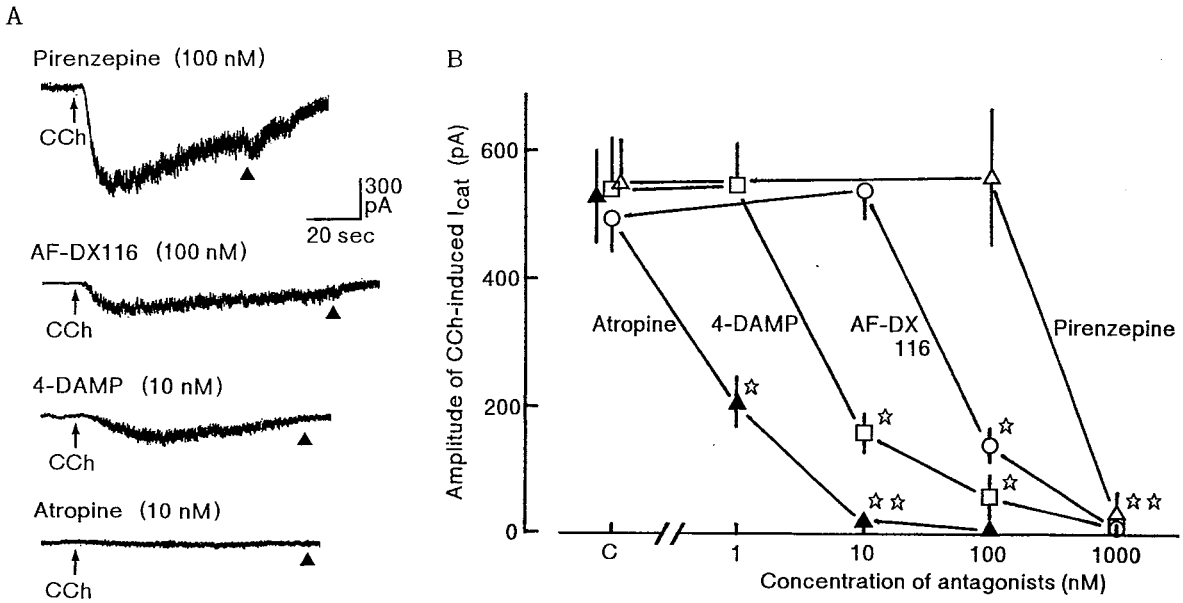

Fig. 4. Effects of muscarinic antagonists on carbachol (CCh)-evoked I_{cat}. A: I_{cat} responses to 100 μM CCh in different cells treated with pirenzepine, AE-DX116, 4-DAMP and atropine at the indicated concentrations, respectively. Each antagonist was allowed to act for 3–4 min before and throughout CCh application. The points of additon and removal of CCh are indicated by arrows and closed triangles, respectively. B: relationships between antagonist concentration and the mean maximum amplitude of 100 μM CCh-evoked I_{cat}. Data points for each antagonist were obtained by using a similar protocol to that described in Fig. 2. Each point indicates the mean±S.E.M. (vertical bars) of measurements in 12–19 control cells or 4–7 antagonist-treated cells. $^{☆☆}P<0.01$, $^{☆}P<0.05$, significantly different from the corresponding control value (C).

The muscarinic antagonists inhibited 100 μM CCh-evoked I_{cat} with different potencies (Figs. 4: A and B). A significant inhibition of the current ($P<0.05$ or $P<0.01$) was produced with 1,000 nM pirenzepine, 100 nM AF-DX116, 10 nM 4-DAMP and 1 nM atropine. IC_{50} was determined to be 0.8 nM for atropine, 6.1 nM for 4-DAMP, 64.3 nM for AF-DX116 and 253.8 nM for pirenzepine in a similar way to that for $I_{K\text{-}Ca}$, giving the relative values of 1, 8, 80 and 317, respectively (Table 1).

The voltage-dependent curve for the activation of the cationic conductance induced by CCh varies with CCh concentration (17). This property could affect the potency of antagonists for inhibition of CCh-evoked I_{cat}. Therefore, effects of antagonists on I_{cat} in response to 10 μM CCh were further investigated. It was found that the same order of potency as described above was held for 10 μM CCh-evoked I_{cat}. In fact, IC_{50} values for 4-DAMP, AF-DX116 and pirenzepine were estimated to be 4.4 nM, 45.2 nM and 192.0 nM, respectively. The order of AF-DX116 and pirenzepine was opposite to the order for the action to inhibit $I_{K\text{-}Ca}$. Therefore, it seems likely that the I_{cat} is mediated via subtypes other than the M3 subtype. The similar profile between the relative IC_{50} for I_{cat} inhibition and the relative affinity constant for M2 subtype (see Table 1) suggests mediation of I_{cat} via the M2 subtype.

DISCUSSION

The results suggest that in smooth muscle cells of guinea pig ileum, CCh-evoked $I_{K\text{-}Ca}$ is mediated via the M3 subtype and CCh-evoked I_{cat} via the M2 subtype. Activation of the M3 subtype by CCh is suggested to stimulate inositol phospholipid hydrolysis, leading to formation of inositol 1,4,5-trisphosphate ($InsP_3$) in the same cell type (8). $InsP_3$ is established to mediate CCh-evoked $I_{K\text{-}Ca}$ as well as Ca^{2+}-store release (12, 13). The effect of CCh in forming $InsP_3$ is only partially inhibited by pertussis toxin (PTX) (18, 19). It is, therefore, possible that the M3 subtype links via PTX-sensitive and -insensitive G proteins to activation of phospholipase C, leading to $InsP_3$ formation.

The idea of M2 subtype mediation of CCh-evoked I_{cat} is supported by the result described only recently by Bolton and Zholos (20). They found in the same cell type as used here that himbacine, methoctramine and tripitramine, each a relatively selective M2 antagonist, inhibit CCh-evoked I_{cat} with affinities typical of antagonism at a M2 muscarinic receptor subtype.

The CCh-evoked I_{cat} is completely inhibited by PTX (11,13, 21), and the accumulated evidence suggests that the M2 subtype couples to inhibition of adenylate cyclase via a PTX-sensitive G protein (1, 2). Therefore, it seems

likely that the M2 subtype couples to multiple intracellular signals via an identical G protein, presumably Gi or Go, to produce both activation of nonselective cationic channels and inhibition of adenylate cyclase. However, the former response is unlikely to have a causual relation to the latter response resulting in a decrease in intracellular levels of cyclic AMP, since CCh evoked I_{cat} with no appreciable change even after intracellular application of 1 mM cyclic AMP (unpublished data; S. Komori, T. Unno and H. Ohashi).

M2-subtype muscarinic receptors on ileal smooth muscle, when activated with agonists, stimulate Ca^{2+} entry via voltage-dependent Ca^{2+} channels through membrane depolarization associated with I_{cat} and produce an increase in $[Ca^{2+}]_i$ available for contraction. CCh-evoked I_{cat} is strongly potentiated by a rise in $[Ca^{2+}]_i$, so that it becomes very small when Ca^{2+} store release is blocked (16, 22). Namely, the M2 subtype-stimulated Ca^{2+} influx is highly dependent on simultaneous activation of the M3 subtype that mediates Ca^{2+} store release. Such a cooperative relation between M2 and M3 subtypes might account partly for the failure of pharmacological analyses of the contractile response to demonstrate the M2 subtype (1). The cellular function of the M2 subtype may be elucidated by further study to quantify the extent to which the M2 subtype contributes to the total increase in $[Ca^{2+}]_i$ following muscarinic receptor activation and to determine which subtype, M2 or M3, mediates the muscarinic agonist-induced increase in Ca^{2+} sensitvity of the contractile proteins (23, 24).

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