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RESEARCH PAPER

Multiple muscarinic pathways mediate the suppression of voltage-gated Ca^{2+} channels in mouse intestinal smooth muscle cells

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Background and purpose: Stimulation of muscarinic receptors in intestinal smooth muscle cells results in suppression of voltage-gated Ca^{2+} channel currents (I_{Ca}). However, little is known about which receptor subtype(s) mediate this effect.
Experimental approach: The effect of carbachol on I_{Ca} was studied in single intestinal myocytes from M_2 or M_3 muscarinic receptor knockout (KO) and wild-type (WT) mice.
Key results: In M_2KO cells, carbachol (100 μM) induced a sustained I_{Ca} suppression as seen in WT cells. However, this suppression was significantly smaller than that seen in WT cells. Carbachol also suppressed I_{Ca} in M_3KO cells, but with a phasic time course. In M_2/M_3-double KO cells, carbachol had no effect on I_{Ca}. The extent of the suppression in WT cells was greater than the sum of the I_{Ca} suppressions in M_2KO and M_3KO cells, indicating that it is not a simple mixture of M_2 and M_3 receptor responses. The $G_{i/o}$ inhibitor, *Pertussis* toxin, abolished the I_{Ca} suppression in M_3KO cells, but not in M_2KO cells. In contrast, the $G_{q/11}$ inhibitor YM-254890 strongly inhibited only the I_{Ca} suppression in M_2KO cells. Suppression of I_{Ca} in WT cells was markedly reduced by either *Pertussis* toxin or YM-254890.
Conclusion and implications: In intestinal myocytes, M_2 receptors mediate a phasic I_{Ca} suppression via $G_{i/o}$ proteins, while M_3 receptors mediate a sustained I_{Ca} suppression via $G_{q/11}$ proteins. In addition, another pathway that requires both M_2/$G_{i/o}$ and M_3/$G_{q/11}$ systems may be operative in inducing a sustained I_{Ca} suppression.
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Keywords: M_2 receptors; M_3 receptors; knockout mice; intestinal smooth muscle; calcium channel; *Pertussis* toxin; YM-254890

Abbreviations: I_{Ca}, voltage-gated Ca^{2+} channel currents; KO mice, knockout mice; PTX, *Pertussis* toxin; WT mice, wild-type mice

Introduction

In gastrointestinal smooth muscle, stimulation of muscarinic receptors by the parasympathetic neurotransmitter, acetylcholine (ACh) or other muscarinic agonists produces a rise in the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$), followed by a contractile response (Unno *et al.*, 2003). The muscarinic mobilization of cytosolic Ca^{2+} is generally believed to arise mainly via intracellular Ca^{2+} released by the second messenger, inositol-1,4,5-trisphosphate (IP_3). Additionally, Ca^{2+} entry into the cell through the L-type voltage-gated Ca^{2+} channels ($Ca_v1.2$; nomenclature follows Alexander *et al.*, 2008) is achieved by depolarization due to activation of the muscarinic cationic channels (Inoue and Isenberg, 1990; Komori *et al.*, 1992; Yan *et al.*, 2003). Previous work suggests that muscarinic receptor stimulation also produces inhibition of currents through L-type Ca^{2+} channels (I_{Ca}) (Unno *et al.*, 1995). This inhibitory effect is supposed to represent a negative feedback mechanism to prevent cytosolic Ca^{2+} overload induced by the muscarinic Ca^{2+} mobilization.

In guinea pig ileal smooth muscle cells, carbachol (CCh) suppressed I_{Ca} in a biphasic manner where an initial rapid suppression lasting 10 s was followed by a more sustained suppression lasting until CCh removal (Unno *et al.*, 1995). The initial transient I_{Ca} suppression is known to involve

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IP_3-induced Ca^{2+} release that occurs upon muscarinic stimulation (Unno *et al.*, 1995). Evidence indicates that Ca^{2+} release directly inhibits the activity of L-type Ca^{2+} channels through a Ca^{2+}-induced Ca^{2+} channel inactivation mechanism, which is dependent upon the binding of Ca^{2+} to the inactivation site of the Ca^{2+} channels (Ganitkevich *et al.*, 1987). However, the mechanisms underlying the later sustained suppression remain to be elucidated. Unno *et al.* (1995), studying CCh-induced I_{Ca} suppression in guinea pig ileal myocytes, reported that both the initial transient and the subsequent sustained I_{Ca} suppression were insensitive to *Pertussis* toxin (PTX), an agent known to inactivate $G_{i/o}$ G proteins. Conversely, Pucovsky *et al.* (1998) (who investigated the effect of PTX under conditions involving constant $[Ca^{2+}]_i$ levels in the same type of smooth muscle cells) concluded that the CCh-induced sustained suppression of I_{Ca} involves $G_{i/o}$ proteins. These contradictory results may have arisen from the different experimental conditions under which the CCh-induced I_{Ca} suppression was observed.

In the present study, we used M_2 and M_3 muscarinic receptor single knockout (KO) and M_2/M_3 receptor double KO mice as novel experimental tools. Specifically, we investigated the effect of CCh on I_{Ca} in longitudinal smooth muscle cells isolated from the small intestine. We also used YM-254890, a selective inhibitor of $G_{q/11}$ G proteins, as well as the $G_{i/o}$ inhibitor PTX. Our results suggest the existence of three distinct pathways linked to muscarinic receptor-mediated I_{Ca} suppression in these cells that are in addition to the M_3-mediated transient I_{Ca} suppression involving the Ca^{2+}-induced Ca^{2+} channel inactivation mechanisms. We propose that an $M_2/G_{i/o}$ pathway mediates phasic I_{Ca} suppression, that an $M_3/G_{q/11}$ pathway leads to sustained suppression and that a third pathway mediates sustained suppression requiring both $M_2/G_{i/o}$ and $M_3/G_{q/11}$ systems for its operation.

Methods

Experimental animals

All animal care and experimental procedures described below complied with the guidelines approved by the local animal ethics committee of the Faculty of Applied Biological Science, Gifu University.

The generation of the M_2 and M_3 muscarinic receptor single KO and M_2/M_3-double KO mice has been described previously (Gomeza *et al.*, 1999; Yamada *et al.*, 2001; Struckmann *et al.*, 2003). The genomic background of the mice used in the present study was 129J1 (50%) × CF1 (50%) for the M_2KO and their corresponding wild-type (WT) mice, 129SvEv (50%) × CF1 (50%) for the M_3KO and their corresponding WT mice, and 129J1 (25%) × 129SvEv (25%) × CF1 (50%) for the M_2/M_3-double KO mice and their corresponding WT mice. Animals were housed in polycarbonate ventilated cages. The animal room was maintained at 22–25°C with a relative humidity of 40–60% and a daily light/dark cycle (07:00–19:00). Food (MF or CMF; Oriental Yeast Co., Itabashi Tokyo, Japan) and distilled water were supplied *ad libitum*. Cells obtained from the three WT strains showed similar functional properties. Data obtained with these cells were therefore pooled (Sakamoto *et al.*, 2007).

Cell preparation

Mice of either sex, aged more than 2 months and weighing 20–50 g were killed by cervical dislocation. A gut segment of 15 cm in length was then removed from a region over the jejunum and ileum (except for the terminal 2.0 cm portion) and placed in physiological salt solution (PSS), consisting of 126 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 14 mM glucose and 10.5 mM HEPES (pH was adjusted to 7.2 with NaOH). The gut segment was cut into 1.0–1.5 cm pieces, from each of which the longitudinal muscle layer was carefully peeled off from the underlying tissue. Single intestinal smooth muscle cells were isolated enzymatically from the longitudinal muscle layers, as described previously (Sakamoto *et al.*, 2006). The cells were suspended in PSS containing 0.5 mM $CaCl_2$, placed on the cover-glass as a small aliquot and stored at 4°C for 2–8 h until use on the same day.

Whole-cell current recording

Cells on a cover-glass were attached to a 1.4 mL chamber on the stage of an inverted microscope (IMT-2, Olympus, Tokyo, Japan). Whole-cell membrane current recordings were made at room temperature (21–25°C) using standard patch-clamp techniques (Hamill *et al.*, 1981) with patch pipettes of 2–7 MΩ. Current signals were amplified by a current amplifier (EPC8, HEKA, Lambrecht, Germany), filtered at 1 kHz and captured at a sampling rate of 4 kHz using an analogue-digital converter (DIGIDATA 1320A, Axon Instruments, Inc., Union city, CA, USA) interfaced to a computer (IMC PC-433, Inter medical Co., Nagoya, Japan) running with the pCLAMP program (version 9, Axon Instruments). The signals were also stored on a digital tape using a PCM data recorder (RD-130T, TEAC, Tokyo, Japan) for later analysis and illustration.

I_{Ca} recordings were made from cells bathed in PSS and intracellularly dialysed with a Cs^+-rich pipette solution composed of 134 mM CsCl, 1.0 mM Na_2GTP, 1.2 mM $MgCl_2$, 1.0 mM MgATP, 14 mM glucose, 0.05 mM EGTA and 10.5 mM HEPES (pH adjusted to 7.2 with CsOH). After being held under whole-cell voltage clamp mode at −60 mV, cells were continuously perfused with PSS at a rate of 5 mL·min^{-1} using a gravity perfusing device, unless otherwise stated, and 30 ms step pulses to 0 mV generated by the pCLAMP program were applied to the cell repeatedly every 10 s to elicit I_{Ca}. If necessary, 20 mM EGTA was included in the pipette solution (in this case CsCl was reduced to 80 mM). When guanosine 5′-(γ-thio) triphosphate tetralithium salt (GTP-γ-S) was added in the pipette solution, Na_2GTP was omitted from it.

When currents through Ca^{2+}-activated BK channels (I_{BK}) were recorded, a K^+-rich pipette solution of the following composition was used (mM): KCl, 134; Na_2GTP, 1.0; $MgCl_2$, 1.2; MgATP, 1.0; glucose, 14; EGTA, 0.05; HEPES, 10.5 (pH adjusted to 7.2). Cells were bathed in PSS and held at a holding potential of 0 mV.

PTX treatment

PTX was injected into mice at a dose of 100 μg·kg^{-1} body weight (i.p.) and, 70–74 h later the cells were prepared as described above. This protocol has been used previously for

effective blockade of M_2 muscarinic receptor-mediated contraction of mouse gut smooth muscle (Unno *et al.*, 2005; 2006).

Data analysis

The amplitude of I_{Ca} was measured as the difference between the peak current level and the current level obtained by the step pulse to 0 mV after I_{Ca} blockade by nicardipine (1 μM). Current density of I_{Ca} was calculated using the measured membrane capacitance obtained by applying hyperpolarizing pulses (10 mV). The extent of I_{Ca} inhibition by CCh or other agents was expressed as percentage reduction compared with the control I_{Ca} immediately before drug application. If the I_{Ca} rundown observed for a period of 1 min before CCh application was more than 10%, the experimental data were not used for evaluation of CCh effects. The amplitude of cationic current and I_{BK} evoked by CCh was measured as the difference from the holding current before application of CCh.

The values in the text are given as means ± SEM. Statistical significance between two groups was tested using a Student's unpaired or paired *t*-test. When more than three groups were compared, one-way analysis of variance (ANOVA) followed by a *post hoc* Bonferroni-type multiple comparison test was used. If the data of the multiple group had not equal variance and normality, Kruskal–Wallis (non-parametric ANOVA) followed by a *post hoc* Steel–Dwass-type multiple comparison test was used. Differences were considered significant when $P < 0.05$.

Materials

Drugs used were carbamylcholine chloride (CCh), GTP-γ-S, PTX, nicardipine hydrochloride (all from Sigma, St. Louis, MO, USA), atropine sulphate and caffeine (Wako, Osaka, Japan), and YM-254890 (kindly given from Astellas Pharm. Inc., Tokyo, Japan). YM-254890 was dissolved in dimethyl sulphoxide as a stock solution, stored at –20°C and diluted with the pipette solution to the desired final concentrations. Drug concentrations in the text and figures are expressed as final concentrations applied to the cells.

Drugs were applied extracellularly by perfusion of an external solution with drug-containing PSS. Calculations made from a perfusion rate of 5 mL·min^{-1} and a bath volume of 1.4 mL, gave a time constant of 16.8 s for solution exchange. Thus, about 1 min after the start of perfusion, the entire external solution was replaced with drug-containing PSS. For the recording of I_{BK}, cells were not perfused with PSS, but drugs were rapidly applied by replacing of the external solution with drug-containing PSS within 10 s using a pair of syringes connected to the chamber, one for injection and the other one for suction. EGTA, GTP-γ-S or YM-254890 was applied intracellularly via diffusion from the patch pipette into the cell, as previously described (Komori *et al.*, 1992).

Results

Effects of CCh on I_{Ca} in WT mice

Mouse intestinal smooth muscle cells prepared from WT mice were held under voltage clamp at –60 mV using patch pipettes filled with a Cs^+-based solution, followed by their continuous perfusion with PSS. Under these conditions, a 30 ms step pulse to 0 mV was repeatedly applied every 10 s in order to elicit I_{Ca}. The amplitude of I_{Ca} usually reached a steady level 3–4 min after the beginning of cell perfusion (see *Methods*).

In WT cells, application of CCh (100 μM) produced an initial transient inward current followed by a smaller sustained current due to activation of cationic channels I_{CCh} (Sakamoto *et al.*, 2006). As shown in Figure 1B, I_{Ca} was reduced in amplitude after CCh (100 μM) application. The I_{Ca} suppression developed progressively with time and reached a plateau about 2–3 min later. The mean suppression of I_{Ca} at

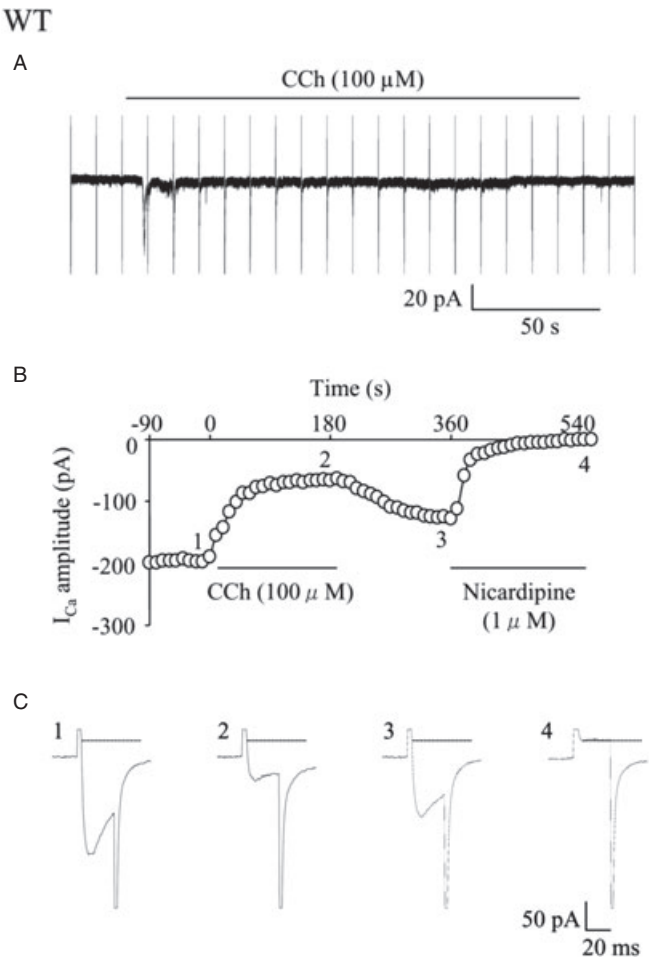

Figure 1 Effects of carbachol (CCh) on the holding current and the voltage-gated Ca^{2+} channel currents (I_{Ca}) in a single longitudinal smooth muscle cell isolated from the small intestine of a wild-type (WT) mouse. I_{Ca} was elicited by a 30 ms step pulse to 0 mV from the holding potential of –60 mV at a frequency of 0.1 Hz. CCh (100 μM) was applied extracellularly during application of the depolarizing pulses. (A) CCh induced a cationic inward current (I_{CCh}) consisting of an initial transient component followed by a sustained component. Every sharp current deflection shows overlapped I_{Ca} and the capacitive current. (B) Time course of the change in I_{Ca} amplitude (○) in the cell plotted against time (the beginning of CCh application was taken as zero). Points 1–4 in the graph correspond to actual I_{Ca} records (1–4) in (C). The amplitude of I_{Ca} was measured as the difference from the current level (interrupted line) obtained by the depolarizing pulse in the presence of nicardipine (1 μM). In the following figures, measurements and plots of I_{Ca} amplitude were made in the same way.

the end of a 3 min application of CCh (3 min point) was about 60% (Figure 3A and B). Removal of CCh from the perfusate allowed a partial recovery of I_{Ca} with a slow time course. Such sustained suppression of I_{Ca} was occasionally preceded by a transient suppression ($n = 5$; data not shown). This effect has previously been observed in guinea pig ileal smooth muscle cells (Unno *et al.*, 1995). The initial transient suppression (47.5 ± 8.5%, $n = 5$) was seen when the step pulse to 0 mV happened to be applied on the point of the initial transient I_{CCh} reaching a peak.

The effects of CCh on I_{Ca} were concentration-dependent. When 1 or 10 μM CCh was applied, the mean percentage suppression of I_{Ca} at the 3 min point was 18.3 ± 6.2% ($n = 3$) or 25.2 ± 6.6% ($n = 4$) respectively, which was significantly smaller than the inhibition observed after application of 100 μM CCh (59.2 ± 8.0%, $n = 11$). Because of the robust response observed with 100 μM CCh, this CCh concentration was used for subsequent experiments.

The I_{Ca} suppression and I_{CCh} generation observed after CCh application were abolished by 1 μM atropine ($n = 4$, data not shown), indicating that both responses were exclusively mediated via muscarinic receptors.

Muscarinic receptor subtypes involved in the I_{Ca} suppression

In order to identify the muscarinic receptor subtypes involved in the CCh-induced I_{Ca} suppression, we used cells isolated from M_2KO or M_3KO mice and M_2/M_3-double KO mice and compared data from these mutant mice with data from WT mice. The mean current densities of I_{Ca} measured just before CCh application were not significantly different among WT and the three mutant strains or any pair of them (WT: 4.3 ± 0.6 pA/pF, $n = 16$; M_2KO: 3.6 ± 0.7 pA/pF, $n = 15$; M_3KO: 4.3 ± 0.5 pA/pF, $n = 15$; M_2/M_3KO: 3.9 ± 1.8 pA/pF, $n = 12$).

In cells from M_2KO mice, CCh (100 μM) induced a suppression of I_{Ca} that persisted for the 3 min CCh application period, as seen in WT cells (Figure 2B). However, the mean I_{Ca} suppression at the 3 min point was significantly smaller than the corresponding value in WT cells (Figure 3B). In four out of 11 cells tested, the sustained I_{Ca} suppression was preceded by an initial, rapid suppression, as seen occasionally in WT cells. However, irrespective of the occurrence of the initial transient suppression, the percentage suppression of I_{Ca} in the sustained phase was again smaller than that in WT cells.

In M_3KO cells, CCh (100 μM) caused suppression of I_{Ca}, but the I_{Ca} suppression was observed only during the initial period of the 3 min application of CCh, as illustrated in Figure 2C. As can be seen from an average curve for percentage suppression of I_{Ca} (Figure 3A), the peak suppression of I_{Ca} was achieved 30 s after the beginning of CCh application (30 s point) and then the I_{Ca} amplitude gradually increased to recover from its suppression despite the continued presence of CCh. The mean values of I_{Ca} suppression at the 30 s and 3 min points are shown in Figure 3B. These two values were significantly different ($P < 0.05$, paired *t*-test) and the latter value was similar to the time-dependent rundown. Therefore, the CCh-induced I_{Ca} suppression in M_3KO cells was considered to be phasic, but not sustained in nature.

In cells from M_2/M_3-double KO mice, CCh (100 μM) had no effect on I_{Ca} (Figure 2D; mean values in Figure 3A and B).

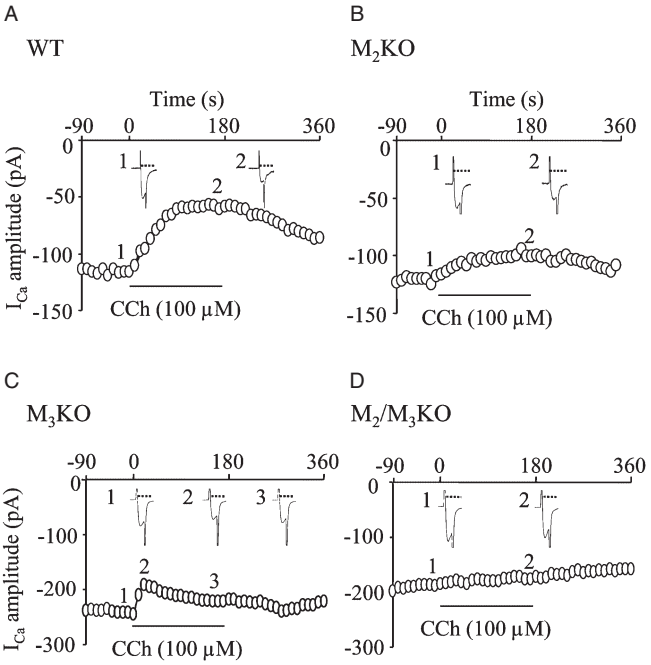

Figure 2 Effects of carbachol (CCh) on the voltage-gated Ca^{2+} channel currents (I_{Ca}) in cells isolated from muscarinic receptor knockout (KO) mice. (A–D) Time courses of the change in I_{Ca} amplitude in cells from wild-type (WT) (A), M_2KO (B), M_3KO (C) and M_2/M_3-double KO mice (D). Points 1–3 in each graph correspond to actual I_{Ca} records in insets 1–3.

Again, the I_{Ca} reduction was within the time-dependent rundown. These data indicated that no other muscarinic receptor subtypes, beside M_2 and M_3, participated in the CCh-induced I_{Ca} suppression.

Figure 3A and B shows summarized results of the CCh-induced I_{Ca} suppression in the individual mouse strains. The sum of the mean suppression at the 30 s point in M_2KO (7%) and M_3KO cells (15%) was 22%, a value comparable with the WT value at this time (25%). Therefore, the CCh-induced I_{Ca} suppression in WT cells at the 30 s point can be explained by a combined effect elicited by M_2 and M_3 receptors. Conversely, the sum of the mean suppression (26%) observed with M_2KO (20%) and M_3KO cells (6%) at the 3 min point was significantly smaller than the corresponding WT value (Figure 3B). If both the M_2- and M_3-mediated I_{Ca} suppression were subtracted from the WT curve (see Figure 3A), a large component of sustained I_{Ca} suppression still existed, as shown in Figure 3C. This component appeared about 30 s after CCh application, gradually developed, and reached a plateau level of about 33% inhibition within 2–3 min. Thus, the sustained I_{Ca} suppression evoked by CCh in WT cells is not a simple mixture of M_2 and M_3 receptor responses.

GTP-γ-S, a direct activator of GTP-binding proteins, has been shown to suppress I_{Ca} in guinea pig ileal myocytes (Beech, 1993; Unno *et al.*, 1995). We therefore also investigated the effects of GTP-γ-S on I_{Ca} in mouse intestinal smooth muscle cells. After intracellular application of GTP-γ-S (100 μM), the I_{Ca} amplitude progressively decreased with a much faster time course than that seen in untreated cells and

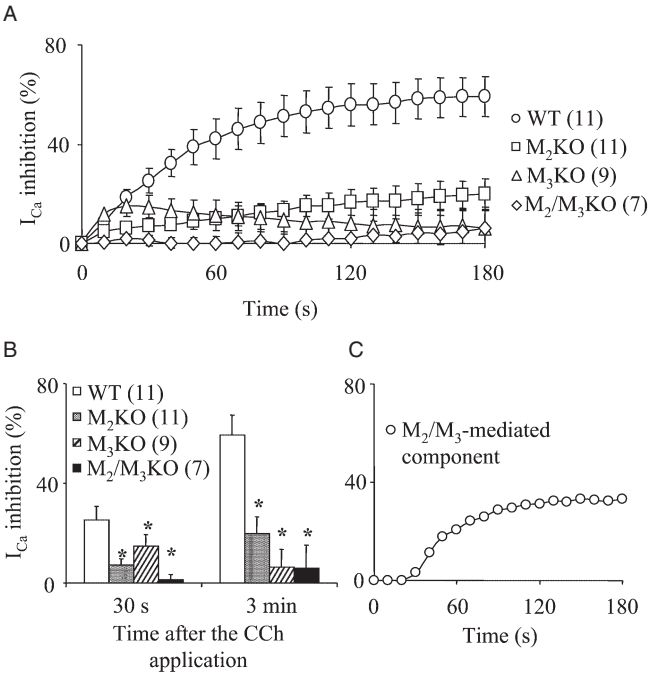

Figure 3 Comparison of the inhibitory effect of carbachol (CCh) on the voltage-gated Ca^{2+} channel currents (I_{Ca}) in cells from wild-type (WT) and muscarinic receptor knockout (KO) mice. (A) Time courses of the change in the mean percentage suppression of I_{Ca} [the beginning of CCh (100 μM) application was taken as zero]. Each point indicates mean ± SEM. (B) The mean percentage suppression of I_{Ca} measured 30 s (four left-hand columns) and 3 min (four right-hand columns) after CCh application. Each column shows the mean with one SEM. In (A and B), the numbers of cells used are presented in parentheses. Asterisks in (B) represent statistically significant differences from the corresponding value in WT cells ($P < 0.05$). (C) An assumed sustained component of I_{Ca} suppression mediated by M_2/M_3 pathway. Each point was estimated by subtracting the average percentage suppression curve of I_{Ca} in both M_2KO and M_3KO cells from that in WT cells shown in (A).

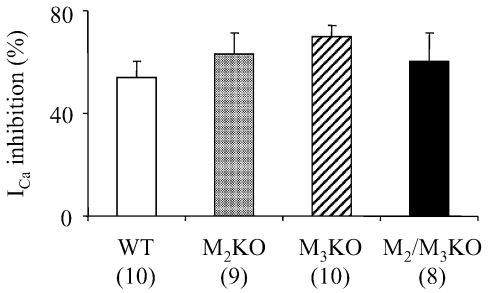

Figure 4 Effect of guanosine 5′-(γ-thio) triphosphate tetralithium salt (GTP-γ-S) on the voltage-gated Ca^{2+} channel currents (I_{Ca}) in cells from wild-type (WT) and muscarinic receptor knockout (KO) mice. GTP-γ-S (200 μM) was applied intracellularly via patch pipettes. The mean percentage suppression of I_{Ca} measured 10 min after the establishment of whole-cell clamp configuration are shown. Each column represents the mean with one SEM. The numbers of cells used are shown in parentheses.

reached a plateau about 4–10 min after breaking the patch membrane in cells from all mouse strains. As shown in Figure 4, the mean percentage suppression of I_{Ca} measured 10 min after breaking the patch membrane was not significantly different among WT and all other mutant strains or any pair of them. Therefore, all cells used showed preserved coupling between G proteins and I_{Ca} suppression.

Type of G proteins involved in the muscarinic pathways mediating I_{Ca} suppression

In order to identify the type of G proteins involved in the muscarinic I_{Ca} suppression, we used PTX (which is known to ADP-ribosylate the α-subunit of $G_{i/o}$) (Katada and Ui, 1982) and YM-254890 (which has been recently developed as a specific inhibitor of $G_{q/11}$) (Takasaki *et al.*, 2004).

Treatment of mice with PTX has been shown to prevent CCh from producing M_2-mediated contraction or cation channel currents in gut smooth muscle preparations (Unno *et al.*, 2005; 2006; Sakamoto *et al.*, 2006). So far, the ability of YM-254890 to inhibit $M_3/G_{q/11}$-mediated responses has not been tested in mouse intestinal smooth muscle cells. Therefore, we first investigated the effect of YM-254890 on the $M_3/G_{q/11}$-mediated Ca^{2+} release event, which could be detected as I_{BK} responses (Sakamoto *et al.*, 2007). Cells from M_2KO mice were dialysed with a K^+-rich pipette solution including dimethyl sulphoxide (0.3%) as control, or YM-254890 (1–10 μM) for 10 min after the establishment of whole-cell configuration at the holding potential of 0 mV (see *Material* and *Methods*). As shown in Figure 5A, CCh (100 μM) consistently evoked a brief I_{BK} in control cells (mean values in Figure 5C). Subsequent application of caffeine (10 mM), a potent releaser of Ca^{2+} from internal stores, had no effect or induced a small I_{BK} (mean values in Figure 5C), indicating the previous depletion of Ca^{2+} stores by CCh. In M_2KO cells treated with YM-254890 (1 μM), CCh induced no noticeable I_{BK} in one cell, but did induce a sizable I_{BK} of 2.1 ± 0.5 nA in three other cells. The overall size of I_{BK} induced by CCh (Figure 5C) was not significantly different from the control value. At higher concentrations of YM-254890 (3 or 10 μM), however, CCh evoked little or no I_{BK}, but subsequent application of 10 mM caffeine induced a sizable I_{BK}, as shown in Figure 5B. The average sizes of I_{BK} induced by CCh and caffeine in cells treated with 3 or 10 μM YM-2548790 are shown in Figure 5C and these values were significantly different from the corresponding control values (Figure 5C). Thus, YM-254890 at a concentration of 3 μM or higher was highly efficient in inhibiting the activity of $G_{q/11}$ proteins in mouse intestinal smooth muscle cells.

Treatment with PTX (100 $\mu g \cdot kg^{-1}$) or YM-254890 (3 μM) produced no noticeable effect on I_{Ca} in cells from WT, M_2KO and M_3KO mice. For example, PTX-treated and -untreated WT cells had similar current densities of I_{Ca} of 4.7 ± 1.0 pA/pF ($n = 12$) and 4.3 ± 0.6 pA/pF ($n = 16$) respectively. Similarly, YM-254890-treated and -untreated WT cells had mean values of 3.4 ± 0.7 ($n = 20$) and 3.5 ± 0.9 pA/pF ($n = 12$) respectively.

As shown in Figure 6A, PTX-treated M_2KO cells responded to 100 μM CCh with a sustained I_{Ca} suppression similar in the percent inhibition and time course to that seen in control cells. The mean suppression at the 3 min point was not different from the control values (Figure 6A and B). Conversely, YM-254890 (3 μM) treatment strongly attenuated the CCh-induced sustained suppression of I_{Ca}, as shown in Figure 6C (mean values in Figure 6D). These results suggested the involvement of $G_{q/11}$ proteins in the M_3-mediated sustained I_{Ca} suppression.

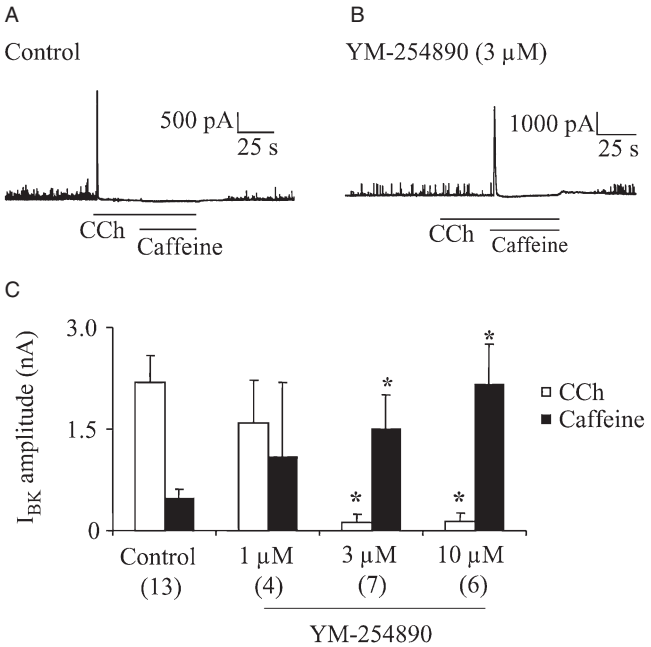

Figure 5 Effect of YM-254890 on BK current (I_{BK}) in cells isolated from M_2KO mice. CCh (100 μM)- or caffeine (10 mM)-induced Ca^{2+} release events were detected as I_{BK} at the holding potential of 0 mV in M_2KO cells. Cells were dialysed with a K^+-rich pipette solution containing 0.3% DMSO (control) or YM-254890 (1–10 μM). (A and B) Typical current traces from a control cell (A) and YM-254890 (3 μM)-dialysed cell (B). (C) Summary of CCh- and caffeine- induced I_{BK} in the presence or absence of YM-254890. The individual columns indicate the mean with one SEM. The numbers of cells used are shown in parentheses. Asterisks represent significant differences from the corresponding control values ($P < 0.05$). CCh, carbachol; DMSO, dimethyl sulphoxide; KO, knockout.

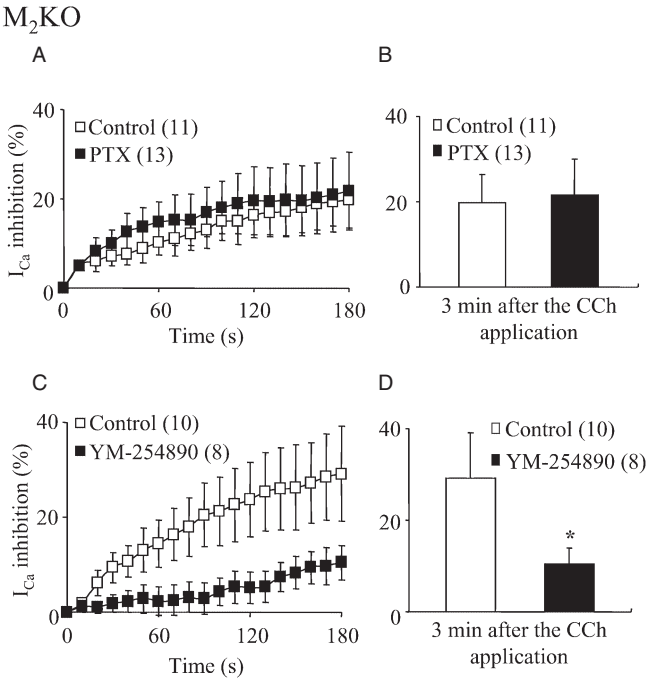

Figure 6 Effects of *Pertussis* toxin (PTX) and YM-254890 on the CCh-induced sustained I_{Ca} suppression in M_2KO cells. (A) Time courses of the change in the mean percentage suppressions of I_{Ca} mediated by CCh (100 μM) in PTX-untreated (control) and -treated cells (the beginning of CCh application was taken as zero). (C) Time courses of the change in the mean percentage suppressions of I_{Ca} in 0.3% DMSO-dialysed (control) and YM-254890 (3 μM)-dialysed cells. Each point indicates mean ± SEM. The numbers of used cells are shown in parentheses. (B and D) Summary of the mean percentage suppression of I_{Ca} measured 3 min after the CCh application in control, PTX-treated (B) or YM-254890-dialysed cells (D). Each column shows the mean with one SEM. The asterisk in (D) represents a significant difference from the corresponding control value ($P < 0.05$). PTX had no effect on the sustained I_{Ca} suppression, but YM-254890 markedly diminished it. CCh, carbachol; DMSO, dimethyl sulphoxide; I_{Ca}, voltage-gated Ca^{2+} channel currents; KO, knockout.

In contrast, PTX-treated M_3KO cells showed no suppression of I_{Ca} in response to CCh (Figure 7A; mean values in Figure 7B), but YM-254890 (3 μM) treatment did not affect the CCh-induced I_{Ca} suppression (Figure 7C and D). These results suggest the involvement of $G_{i/o}$ proteins in the M_2-mediated phasic I_{Ca} suppression.

In WT cells, PTX treatment strongly reduced the sustained I_{Ca} suppression (Figure 8A; mean values in Figure 8B). As shown in Figure 8A, the average I_{Ca} suppression curve against time after CCh application in PTX-treated WT cells closely resembled that in PTX-untreated M_2KO cells.

In WT cells treated with YM-254890 (3 μM), CCh induced a suppression that reached a peak inhibition at about 30 s (Figure 8C). The mean suppression values at the 30 s and 3 min points were significantly smaller than the corresponding control values (Figure 8D) but were the same as those in M_3KO cells (Figure 8C). The average I_{Ca} suppression curve in WT cells treated with YM-254890 (3 μM) overlapped that in M_3KO cells (see Figure 8C).

Ca^{2+} dependency of the muscarinic I_{Ca} suppression

In guinea pig ileal smooth muscle cells, a certain level of intracellular Ca^{2+} is required for the CCh-induced sustained suppression of I_{Ca} (Unno *et al.*, 1995). In order to test the Ca^{2+} dependency of M_2- or M_3-mediated I_{Ca} suppression, cells were dialysed with a pipette solution containing EGTA (20 mM). Intracellular application of EGTA itself caused I_{Ca} to increase by approximately two to fourfold in the different mouse strains used. For example, the I_{Ca} current density in WT cells was increased from 4.3 ± 1.1 pA/pF (n = 16) to 11.4 ± 2.7 pA/pF (n = 20) by EGTA treatment.

The intracellular application of EGTA had little or no effect on the average I_{Ca} suppression curve in M_2KO cells (Figure 9A and B) or in M_3KO cells (Figure 9C and D). In contrast, EGTA treatment of WT cells caused a marked reduction of the CCh-induced suppression of I_{Ca}. The I_{Ca} suppression in EGTA treated WT cells reached a peak within 1 min after the beginning of CCh application and then gradually recovered to some extent or maintained its decreased level during the presence of the agonist (Figure 9E). Thus the mean suppression at 30 s was not affected by EGTA but, at 3 min, EGTA significantly reduced the suppression of I_{Ca}. Interestingly, the averaged I_{Ca} suppression curve obtained in the EGTA-treated WT cells almost overlapped with a combined curve from the M_2-mediated suppression in M_3KO cells and the M_3-mediated suppression in M_2KO cells (see Figure 9E).

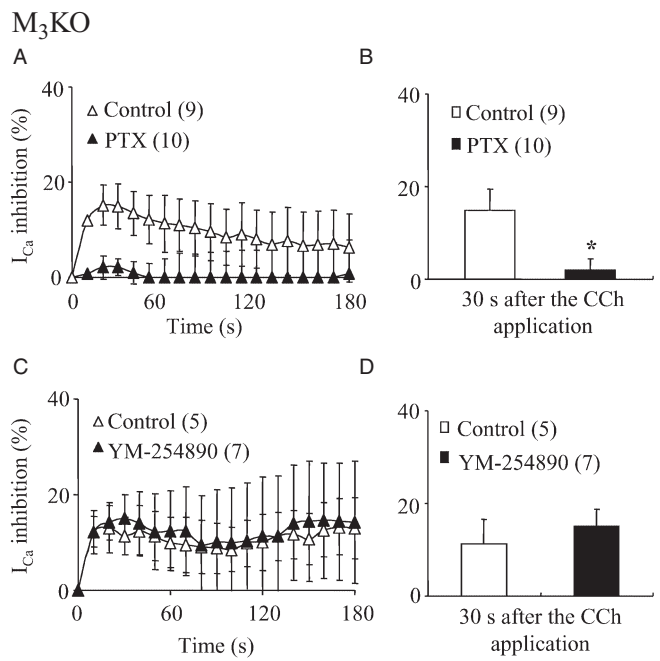

Figure 7 Effects of PTX and YM-254890 on the CCh-induced phasic I_{Ca} suppression in M_3KO cells. (A and C) Time courses of the change in the mean percentage suppression of I_{Ca} induced by CCh (100 μM) in PTX-treated (A) and YM-254890 (3 μM)-dialysed (C) M_3KO cells plotted in the same way as in Figure 6. (B and D) Summary of the mean percentage suppression of I_{Ca} measured 30 s after CCh application in PTX-treated (B) and YM-254890-dialysed (D) cells. Asterisk in (B) represents significant difference from the corresponding control value ($P < 0.05$). PTX abolished the phasic I_{Ca} suppression in M_3KO cells, but YM-254890 had no effect on this response. CCh, carbachol; I_{Ca}, voltage-gated Ca^{2+} channel currents; KO, knockout; PTX, *Pertussis* toxin.

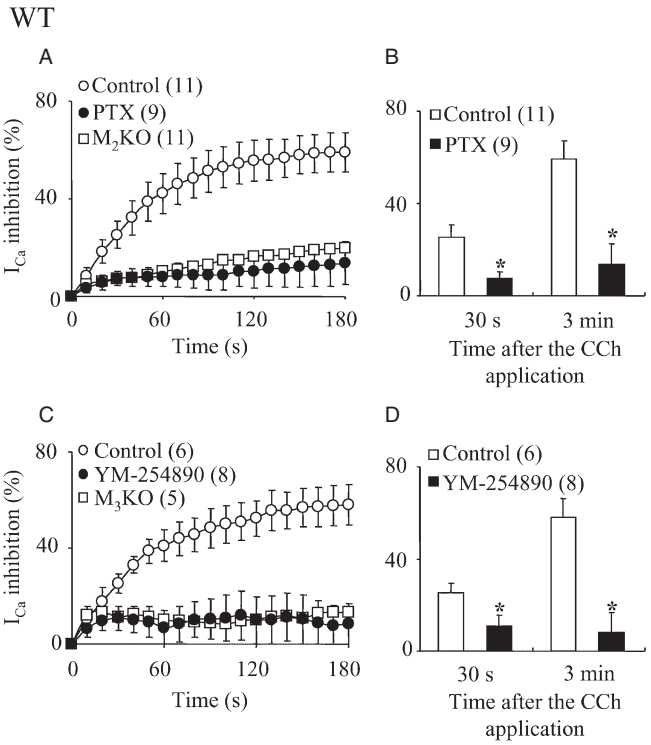

Figure 8 Effects of PTX and YM-254890 on the CCh-induced I_{Ca} suppression in WT cells. (A and C) Time courses of the change in the mean percentage suppression of I_{Ca} induced by CCh (100 μM) in PTX-treated (A) and YM-254890 (3 μM)-dialysed (C) WT cells. For comparison, the averaged I_{Ca} suppression curves against time after CCh application in PTX-untreated M_2KO cells (Figure 6) and YM-254890-untreated M_3KO cells (Figure 7) were superimposed in (A and C) respectively. (B and D) Summary of the mean percentage suppression of I_{Ca} measured 30 s (left-hand columns) and 3 min (right-hand columns) after CCh application in PTX-treated (B) and YM-254890-dialysed (D) WT cells. Asterisks represent significant differences from the corresponding control value ($P < 0.05$). CCh, carbachol; I_{Ca}, voltage-gated Ca^{2+} channel currents; KO, knockout; PTX, *Pertussis* toxin; WT, wild-type.

Discussion

The L-type voltage-gated Ca^{2+} channel represents one of the major pathways for Ca^{2+} influx into the cytosol and thereby plays an essential role in the regulation of $[Ca^{2+}]_i$ in smooth muscle cells. The channel activity is regulated by various neurotransmitters and hormones (see Imaizumi *et al*., 1991; Pacaud and Bolton, 1991; Beech, 1993; Han *et al*., 1995; Unno *et al*., 1995; Wade *et al*., 1996; Pucovsky *et al*., 1998), although the precise signal transduction mechanisms involved remains unclear. In the present study, the inhibitory effects of the muscarinic agonist CCh on I_{Ca} were investigated in order to elucidate the muscarinic receptor subtypes and possible signal transduction mechanisms involved in intestinal smooth muscle cells from WT mice and mutant mice lacking M_2 or M_3, or both subtypes of muscarinic receptors.

In WT cells, CCh (100 μM) induced either a sustained suppression of I_{Ca} or a biphasic suppression consisting of an early transient component followed by a slowly developing sustained component, as previously described in guinea pig ileal smooth muscle cells (Unno *et al*., 1995). No appreciable I_{Ca} suppression was observed upon CCh application in M_2/M_3-double KO cells, suggesting that only M_2 and M_3 receptors participate in the I_{Ca} suppression in WT cells. The early transient I_{Ca} suppression was detected during the initial transient component of cationic inward current, which reflects release of Ca^{2+} from intracellular stores (Komori *et al*., 1993; Unno *et al*., 1995). Such release of stored Ca^{2+} by CCh reflects M_3 subtype stimulation (Sakamoto *et al*., 2007), and the transient I_{Ca} suppression is likely to be mediated via the $M_3/G_{q/11}/PLC_\beta/IP_3/Ca^{2+}$ release pathway and Ca^{2+}-induced Ca^{2+} channel inactivation mechanisms (Figure 10).

In M_2KO cells, CCh elicited a sustained, yet smaller, I_{Ca} suppression than that seen in WT cells. In M_3KO cells, CCh induced a phasic I_{Ca} suppression, the time course of which was clearly different from that of the sustained suppression observed in WT cells. The I_{Ca} suppression in M_2KO and M_3KO cells are likely to be mediated through M_3 and M_2 receptors. The M_3-mediated sustained I_{Ca} suppression was inhibited by the $G_{q/11}$ inhibitor YM-254890, but was unaffected by the $G_{i/o}$ inhibitor PTX. In contrast, the M_2-mediated phasic I_{Ca} suppression was abolished by PTX, but remained unaffected by YM-254890. Also, intracellular application of EGTA (20 mM) had no effect on either M_2- or M_3-mediated suppression. These results suggest that stimulation of both M_2 and M_3 receptors induce the phasic and sustained I_{Ca} suppression via $G_{i/o}$ and $G_{q/11}$ type G proteins and that intracellular Ca^{2+} is unlikely to be involved (Figure 10).

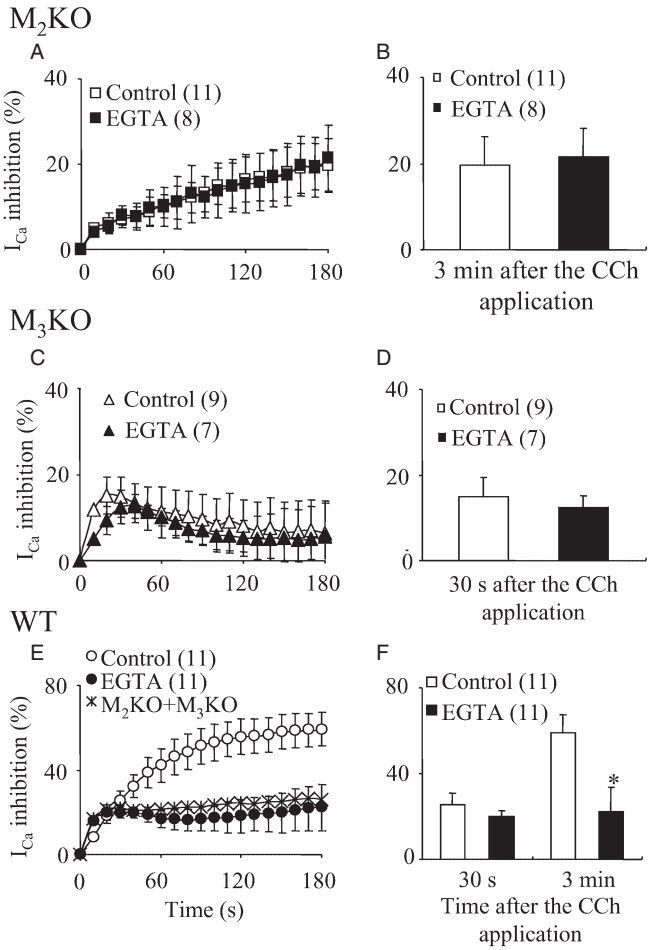

Figure 9 Effects of EGTA on the CCh-induced I_{Ca} suppressions in M_2KO, M_3KO and WT cells. (A, C and E) Time courses of the change in the mean percentage suppression of I_{Ca} induced by CCh (100 μM) in 0.05 mM EGTA (control)- and 20 mM EGTA-dialysed cells from M_2KO (A), M_3KO (C) and WT (E) mice. In (E), the sum of the average I_{Ca} suppression curve in M_2KO (A) and M_3KO (C) control cells is also shown. (B, D and F) Summary of the mean percentage suppression of I_{Ca} measured 30 s or 3 min after CCh application in M_2KO (B), M_3KO (D) and WT (F) cells. The asterisk in (F) represents a significant difference ($P < 0.05$) from the corresponding control value. CCh, carbachol; I_{Ca}, voltage-gated Ca^{2+} channel currents; KO, knockout; WT, wild-type.

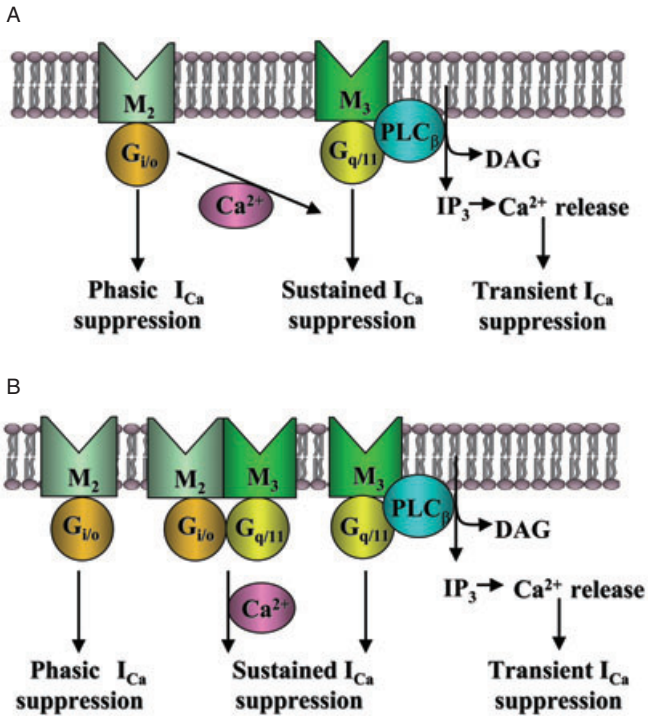

Figure 10 Assignment of muscarinic receptor subtypes and G proteins mediating the suppressions of L-type voltage-gated Ca^{2+} channels in intestinal smooth muscle cells. M_2 muscarinic receptors, via $G_{i/o}$ proteins, induce a phasic I_{Ca} suppression, while M_3 receptors, via $G_{q/11}$ proteins, induced a transient I_{Ca} suppression due to Ca^{2+} release from intracellular stores and a sustained I_{Ca} suppression. In addition, M_2 and M_3 receptors can cooperatively suppress I_{Ca}. The M_2/M_3-mediated pathway involves both $G_{i/o}$ and $G_{q/11}$ proteins and a Ca^{2+}-dependent process to induce a sustained I_{Ca} suppression. The present results are consistent with two possible models for the M_2/M_3-mediated pathway; one is a M_2/M_3-synergistic model in which the $M_3/G_{q/11}$-mediated sustained suppression is potentiated by the $M_2/G_{i/o}$-mediated, Ca^{2+}-dependent signal (A), and the other one is a M_2/M_3-complex model in which both the M_2 and M_3 receptor subtypes and the respective G proteins form a complex to operate a Ca^{2+}-dependent signal and induce a sustained I_{Ca} suppression (B), as suggested for the activation mechanisms of muscarinic cationic channels (Sakamoto *et al.*, 2007). I_{Ca}, voltage-gated Ca^{2+} channel currents.

In rat sympathetic neurons, substance P (SP) and somatostatin inhibited whole-cell N-type Ca^{2+} channel currents via PTX-insensitive and -sensitive G proteins (Shapiro and Hille, 1993). The SP-induced suppression developed slowly and then plateaued, while the somatostatin-induced suppression developed more rapidly, peaked and then gradually recovered even in the presence of the agonist. Furthermore, N-type Ca^{2+} channel suppression was resistant to BAPTA (20 mM). Thus, the characteristics of the SP- and somatostatin-induced suppression are similar to those of the M_3-mediated sustained and M_2-mediated phasic suppression. In these studies Shapiro and Hille (1993) suggested that both the SP- and somatostatin-induced suppression is not mediated by a diffusible cytosolic messenger, but by a membrane-delimited signal transduction pathway (Shapiro and Hille, 1993), possibly involving phosphatidylinositol 4,5-bisphosphate (PIP_2) (Michailidis *et al.*, 2007).

In rabbit colonic smooth muscle cells, Jin *et al.* (2002) demonstrated that M_2 receptor stimulation induced an enhancement of I_{Ca}, the effect was Ca^{2+}-independent and required G_i-dependent activation of the c-src kinase pathway. Moreover, in order to demonstrate M_2-mediated enhancement of I_{Ca}, Jin *et al.* (2002) had to block M_3-mediated signalling pathways by either treatment with 4-DAMP or by intracellular dialysis of an anti-$G\alpha_q$ antibody. Hence, we would have expected a similar result using M_3KO cells. However, we failed to detect any enhancement of I_{Ca} by muscarinic receptor stimulation, but detected a phasic *suppression* in M_3KO intestinal smooth muscle cells. This finding might be explained by the M_2-mediated phasic I_{Ca} suppression representing the sum of both an inhibitory and a facilitatory pathway operating in parallel.

In WT cells, some I_{Ca} suppression still occurred independent of the combined effect of M_2 and M_3 receptor responses (as demonstrated by data from M_2KO and M_3KO cells Figure 3C). While EGTA had no significant effect on the I_{Ca} suppression in

M_2KO or M_3KO cells, the Ca^{2+} chelator strongly inhibited the residual I_{Ca} suppression in WT cells (Figure 9E). These results suggest that the component of I_{Ca} suppression inhibited by EGTA in WT cells corresponds to a component that requires the simultaneous stimulation of both M_2 and M_3 receptors and that this M_2/M_3-mediated pathway may contribute to I_{Ca} suppression in WT cells.

The I_{Ca} suppression in WT cells was inhibited by either PTX or YM-254890. The CCh-induced I_{Ca} suppression curve in PTX-treated WT cells resembled that of M_3 receptor stimulation (Figure 8A). Similarly, the average I_{Ca} suppression curve in YM-254890-treated WT cells was similar to that of M_2 receptor stimulation (Figure 8C). These results suggest that PTX and YM-254890 not only inhibit the M_2-mediated and M_3-mediated suppression, but also the M_2/M_3-mediated sustained suppression. Taken together, these observations suggest that the M_2/M_3-mediated sustained suppression involves both $G_{i/o}$ and $G_{q/11}$ type G proteins and a Ca^{2+}-dependent process.

In our previous work using guinea pig ileal myocytes, we did not detect a PTX-sensitive component of the CCh-induced I_{Ca} suppression (Unno *et al.*, 1995). We reported that I_{Ca} suppression is Ca^{2+}-dependent and potentiated by an influx of Ca^{2+} through L-type Ca^{2+} channels (Unno *et al.*, 1995). However, in this previous study, we used Ba^{2+} as a charge carrier for the current recording, so that $[Ca^{2+}]_i$ was kept at a low level, which probably resulted in a reduced activity of the M_2/M_3-mediated pathway. As a result, only the PTX-insensitive, M_3-mediated sustained component of I_{Ca} suppression was detected. However, Pucovsky *et al.* (1998) did detect a PTX-sensitive component of CCh-induced I_{Ca} suppression in guinea pig ileal myocytes, and they used a pipette solution in which the Ca^{2+} concentration was kept constant at 100 nM. This concentration of Ca^{2+} may be sufficient to allow the operation of the M_2/M_3-mediated pathway.

Wade *et al.* (1996) reported that ACh decreased the open probability of L-type Ca^{2+} channels in guinea pig gastric myocytes under the cell-attached patch clamp mode. They postulated the involvement of a diffusible cytosolic messenger in the ACh-induced I_{Ca} suppression. A similar result was obtained on muscarinic suppression of N- and L-type Ca^{2+} channels in rat sympathetic neurons (Mathie *et al.*, 1992). This report also demonstrated that oxotremorine-M induced a sustained I_{Ca} suppression that was dependent upon intracellular Ca^{2+}. Hence, our findings suggest that the M_2/M_3-mediated I_{Ca} suppression involves a diffusible cytosolic messenger in the signal transduction pathway. Unno *et al.* (1999) suggested the involvement of polymerization of tubulin, a constituent of the microtubule cytoskeleton, in the sustained muscarinic I_{Ca} suppression in guinea pig ileal smooth muscle cells. As polymerization of tubulin is dynamically regulated by cytosolic Ca^{2+} (Dustin, 1984), this signalling molecule could also regulate the M_2/M_3-mediated I_{Ca} suppression.

Figure 10 depicts possible models for the assignment of muscarinic receptor subtypes and G proteins mediating the I_{Ca} suppression. M_2 receptors, via $G_{i/o}$ proteins, induce a phasic I_{Ca} suppression, while M_3 receptors, via $G_{q/11}$ proteins, induce a transient and a sustained I_{Ca} suppression. The third pathway (M_2/M_3) induces a sustained suppression when both M_2 and M_3 receptors are stimulated.

The M_3-mediated transient suppression is due to a Ca^{2+}-induced inactivation mechanism brought about by IP_3-induced Ca^{2+} release from intracellular stores. On the other hand, the M_2-mediated phasic and M_3-mediated sustained suppression probably occur through Ca^{2+}-independent mechanisms. For the M_2/M_3-mediated pathway, one possible model is depicted in Figure 10A. In this model, M_3 receptors mediate the sustained suppression through a Ca^{2+}-independent process, and this pathway is potentiated by an $M_2/G_{i/o}$-mediated, Ca^{2+}-dependent signal. Alternatively, the M_2/M_3-mediated pathway may arise from a receptor complex consisting of both M_2 and M_3 receptors possibly via the formation of M_2/M_3 heterodimers (Maggio *et al.*, 1999). Therefore, the M_2 and M_3 receptor subtypes may form a complex to activate two types of G proteins, $G_{i/o}$ and $G_{q/11}$, and the downstream signals may act to operate the sustained suppression through a Ca^{2+} dependent process, as shown in Figure 10B. This M_2/M_3 complex model is identical to that postulated for the activation mechanisms of muscarinic cationic channels in M_2 or M_3 KO mice (Sakamoto *et al.*, 2007).

Muscarinic receptor stimulation activates cation channels to produce membrane depolarization and thereby increases discharge rate of action potentials, which are accompanied by Ca^{2+} entry (Inoue and Isenberg, 1990; Komori *et al.*, 1992; Unno *et al.*, 2003). As action potential discharge is accelerated, operation of a process to suppress I_{Ca} is accelerated by the increased Ca^{2+} influx. Since the M_2/M_3-mediated pathway depends on cytosolic Ca^2, this pathway may detect the accumulation of $[Ca^{2+}]_i$ and then function to suppress the activity of L-type Ca^{2+} channels as a negative feedback system. The M_2/M_3-mediated pathway may involve a diffusible messenger and is sustained, allowing for prolonged inhibitory control of L-type Ca^{2+} channel activity. Using ileal smooth muscles isolated from muscarinic receptor KO mice, Griffin *et al.* (2004) reported that the heterologous desensitization induced by ACh is contingent upon activation of both M_2 and M_3 receptors and that activation of either receptor is insufficient to cause desensitization. Therefore, it is likely that the M_2/M_3-mediated suppression of L-type Ca^{2+} channel activity is responsible, at least partly, for the heterologous desensitization caused by muscarinic receptor stimulation.

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Conflicts of interest

The authors state no conflict of interest.

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