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Phospholipase C Involvement in Activation of the Muscarinic Receptor-Operated Cationic Current in Guinea Pig Ileal Smooth Muscle Cells

Hiroyuki Okamoto[1], Toshihiro Unno[2], Daisuke Arima[2], Maki Suzuki[2], Hai-Dun Yan[2], Hayato Matsuyama[2], Masakazu Nishimura[1], and Seiichi Komori[2,*]

[1]*United Graduate School of Veterinary Science, Gifu University, 1-1 Yanagido, Gifu City, Gifu 501-1193, Japan*
[2]*Laboratory of Pharmacology, Department of Veterinary Medicine, Faculty of Applied Biological Science, Gifu University, 1-1 Yanagido, Gifu City, Gifu 501-1193, Japan*

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Abstract. In guinea pig single ileal smooth muscle cells held under voltage-clamp, the role of phospholipase C (PLC) in activation of the muscarinic receptor-operated cationic current (I_{cat}) was studied. U73122, a PLC inhibitor, prevented the generation of I_{cat} by the muscarinic agonist carbachol. The effect did not involve muscarinic receptor block since it also blocked I_{cat} which was evoked by GTPγS applied intracellularly to activate G proteins bypassing muscarinic receptors. Also, neither cationic channel block nor other possible nonspecific actions seemed to be involved since its analogue (U73343), structurally close but deficient of the PLC-inhibiting activity, did not significantly affect carbachol- or GTPγS-evoked I_{cat}. Antibodies against the α subunits of G_q/G_{11} proteins ($G\alpha_q/G\alpha_{11}$-antibody) blocked only the small component of carbachol-evoked I_{cat}, which was associated with an increase in $[Ca^{2+}]_i$ linked to an increase in $G_{q/11}$ protein-regulated PLC activity. 1-Oleoyl-2-acetyl-*sn*-glycerol (OAG), an analogue of diacylglycerol (DAG) produced via PLC-catalyzed metabolism, produced no or only a small current by itself, with the carbachol-evoked I_{cat} remaining unchanged. These results provide evidence for the importance of PLC in I_{cat} generation, and they also strongly suggest that the activity of PLC involved in the primary activation of I_{cat} is neither under regulation by $G_{q/11}$ proteins nor dependent on the action of DAG.

Keywords: muscarinic receptor, nonselective cationic current, phospholipase C, diacylgrycerol, smooth muscle

Introduction

Activation of muscarinic receptors in gastrointestinal smooth muscle cells produces nonselective cationic current (I_{cat}) (1, 2). The evoked I_{cat}, via causing membrane depolarization, leads to sustained increases in the open probability of voltage-gated Ca^{2+} channels and/or to increased discharges of Ca^{2+} action potentials, providing substantial Ca^{2+} influx for contraction (3, 4). In various gastrointestinal smooth muscles, two major subtypes of the muscarinic receptor, M2 and M3, are found. Pharmacological analyses of muscarinic agonist-evoked I_{cat} in guinea pig gastric and ileal smooth muscle cells have revealed the primary role of the M2 subtype in I_{cat} generation (5 – 7). In these cell types, the I_{cat} response is abolished by pertussis toxin (PTX) which selectively uncouples G_i/G_o proteins from the associated receptors (2, 7 – 10). With the use of antibodies, the G protein involved in the signal transduction was identified as G_o protein (11, 12). Based on these results and the general thought that M2 receptors preferencially couple PTX-sensitive G proteins (8), the M2 subtype is supposed to mediate cationic channel opening through G_o proteins.

Nonetheless, evidence has suggested that the M3 subtype also plays a crucial role in I_{cat} generation. The M3 receptors link via $G_{q/11}$ proteins to PLC activation which results in hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) and thus production of inositol-1,4,5-trisphosphate ($InsP_3$) and 1,2-diacyl-*sn*-glycerol

*Corresponding author. FAX: +81-58-293-2942
E-mail: skomori@cc.gifu-u.ac.jp

(DAG). The $InsP_3$ then acts to release Ca^{2+} from intracellular stores and causes $[Ca^{2+}]_i$ to rise. This rise can potentiate I_{cat} generation because of high Ca^{2+} sensitivity of the cationic channel opening (13 – 15). However, bisides the rise in $[Ca^{2+}]_i$ linked to the $G_{q/11}$/PLC/$InsP_3$ system, another synergistic signal for M2-initiated channel opening may also be provided by M3 receptors. This is supported by the observation that M2-receptor antagonists inhibited carbachol-evoked I_{cat} in a competitive manner, while M3-receptor antagonists severely depressed its maximum response, under conditions where $[Ca^{2+}]_i$ was buffered to a certain level (5). Under the same conditions, significant correlations of agonist potencies between for I_{cat} activation and M3-receptor stimulation have also been found (16). However, the mechanisms underlying the synergistic activation of I_{cat} by M2 and M3 receptors remain to be elucidated.

PLC-mediated signaling pathways have been suggested to play a pivotal role in G protein-coupled receptor-induced activation of nonselective cationic channels in various cell types including smooth muscle cells of the rabbit portal vein (17) and mouse gastric myocyte (18). Therefore, our aim in the present work is to test possible involvement of PLC in activation of the muscarinic receptor-operated cationic channels in guinea pig ileal smooth muscle cells, using a PLC inhibitor (U73122) and $G\alpha_q$/$G\alpha_{11}$-antibody. We also examined whether the PIP_2-hydrolysed product DAG participates in cationic channel activation, using its membrane-permeable analogue, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG). Our results show the absolute importance of PLC in the activation of I_{cat}, and they suggest that the role of PLC is neither under $G_{q/11}$ protein regulation nor mediated by DAG, which provides relevant insights into elucidation of the M2/M3 synergistic signaling mechanism for I_{cat} generation in response to muscarinic receptor agonists.

Materials and Methods

Cell preparation

Single intestinal smooth muscle cells were prepared as previously described (16). Briefly, male guinea pigs weighing 350 – 450 g were killed by a stunning blow followed by immediate exsanguination, from which the ileum of 15 – 20 cm in length was removed and the longitudinal muscle layer peeled from the underlying tissue. Single smooth muscle cells were isolated from the muscle layers after incubation with a combination of collagenase (0.3 – 0.6 mg/ml) and papain (0.2 – 0.3 mg/ml) and then stored at 4°C until use on the same day.

Current recordings

Whole-cell membrane current was recorded at room temperature with patch pipettes (4 – 7 MΩ in tip resistance) and CEZ-2300 voltage-clamp amplifier (Nihon Kohden, Tokyo). Current signals were filtered at 1 kHz and displayed on an oscilloscope (VC9, Nihon Kohden) and a thermal array recorder (RTA-1100, Nihon Kohden). They were also saved on a digital tape with a recorder (RD-111T; TEAC, Tokyo) for later analysis and illustration.

Unless otherwise stated, measurements of I_{cat} were made usually in the extracellular presence of a Cs^+-rich solution consisting of 120 mM CsCl, 12 mM glucose, 10 mM HEPES, pH adjusted to 7.2 with CsOH. This solution was introduced into the recording chamber (0.5 ml) 30 – 60 s before I_{cat} recording by exchange with normal physiological salt solution (PSS) that consisted of 126 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 14 mM glucose, 10.5 mM HEPES, pH adjusted to 7.2 with NaOH. The pipette solution with which the cells were intracellularly dialyzed had the following composition: 80 mM CsCl, 1 mM MgATP, 1 mM Na_2GTP, 5 mM creatine, 20 mM glucose, 10 mM HEPES, 10 mM BAPTA (1,2-*bis*(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid), 4.6 mM $CaCl_2$ (calculated $[Ca^{2+}]_i$ = 100 nM), pH adjusted to 7.2 with CsOH (Cs^+ = 140 mM in total). Such ionic environments inside and outside the cell served to block generation of any K^+ current and prevent the modulation of I_{cat} by changes in $[Ca^{2+}]_i$ (19). In some experiments, I_{cat} was measured using PSS as the extracellular solution and a BAPTA/$CaCl_2$ buffer-omitted solution as the pipette solution. This pipette solution had the following composition: 134 mM CsCl, 1.2 mM $MgCl_2$, 1 mM MgATP, 0.1 mM Na_2GTP, 14 mM glucose, 10.5 mM HEPES, 0.05 mM EGTA, pH adjusted to 7.2 with CsOH. The use of these solutions allowed I_{cat} to oscillate in response to changes in $[Ca^{2+}]_i$ associated with Ca^{2+} release from internal stores (20, 21).

When Ca^{2+}-activated K^+ current ($I_{K\text{-}Ca}$) was measured, cells were immersed in PSS and dialyzed with a pipette solution consisting of 134 mM KCl, 1.2 mM $MgCl_2$, 1 mM MgATP, 0.1 mM Na_2GTP, 0.05 mM EGTA, 14 mM glucose, 10.5 mM HEPES, pH adjusted to 7.4 with KOH. The membrane potential was held at 0 mV, close to the reversal potential of I_{cat} in these ionic conditions (1), so that $I_{K\text{-}Ca}$ was not contaminated by I_{cat} (8).

Chemicals

Drugs used were OAG, guanosine 5'-*O*-(3-thiotriphosphate) tetralithium salt (GTPγS) (Sigma Chemical Co., St. Louis, MO, USA); carbamylcholine chloride (carbachol), 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-

17yl]amino]-1*H*-pyrrole-2,5-dione (U73122), 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17yl]amino]-2-5-pyrrolidine-dione (U73343), caffeine, quinidine sulfate (Wako, Tokyo). Antibodies against α subunits of G_q and G_{11} proteins ($G\alpha_q/G\alpha_{11}$-antibody) were obtained from Perkin-Elmer Life Sciences (NEN) (Boston, MA, USA); each antibody can recognize a specific sequence of the C-terminal of the specific α subunit (12). All other chemicals were obtained from Sigma or Wako.

U73122, U73343, and OAG were dissolved in dimethyl sulfoxide (DMSO) at more than 100 times higher concentrations than used in the experiments, stocked at −20°C, and diluted with the bath solution to the desired concentrations. DMSO (up to 0.3%), the highest concentration exposed to cells in the present experiments had no effect on the basal current or carbachol-evoked $I_{K\text{-}Ca}$ or I_{cat}. GTPγS and $G\alpha_q/G\alpha_{11}$-antibody were diluted with pipette solution to the respective concentrations 400 μM and 1:100 v/v, included into patch pipettes and intracellularly applied after breakthrough of the patched membrane, as described previously (8, 12). Drug concentrations in the text and figures are expressed as final concentrations applied to cells.

Data analysis

The carbachol concentration-effect curves for I_{cat} activation were analyzed using computer software (DeltaGraph 4.0; SPSS Inc., Chicago, IL, USA) that fits the data directly with a logistic function, providing the EC_{50} value (the concentration required for the agonist to produce a half-maximal response), the maximum current response (I_{max}), and the slope factor for the curve. Curve fitting anaysis was also made for estimation of the IC_{50} value of U73122 (the concentration required to inhibit the I_{max} by 50%).

Values in the text are given as means ± S.E.M. with the number of measurements (n). Student's unpaired *t*-test was used for statistical comparison, and when $P<0.05$, differences were considered significant.

Results

Effectiveness of U73122 and $G\alpha_q/G\alpha_{11}$-antibody in inhibiting PLC

In the present study, we first examined the ability of a PLC inhibitor, U73122 (22 – 24), as well as $G\alpha_q/G\alpha_{11}$-antibody to inhibit PLC activity in ileal muscle cells. For these tests, $I_{K\text{-}Ca}$ evoked by carbachol was used as an indicator, because the current response has been shown to represent Ca^{2+}-store release mediated by the $G_{q/11}$/PLC/$InsP_3$ system (6, 25). Cells were immersed in PSS, dialyzed with K^+-rich pipette solution, and held at 0 mV (see Materials and Methods).

In control cells for the test of U73122, carbachol was applied at a maximally effective concentration (100 μM) after 5- to 6-min cell dialysis. Upon its application, $I_{K\text{-}Ca}$ lasting 3 – 10 s was elicited, as described (6, 16) (Fig. 1A), whose amplitude amounted to 3.90 ± 0.63 nA on the average (n = 6). In the presence of carbachol, 30 – 40-s later, caffeine (10 mM), which shares the releasable Ca^{2+} stores with carbachol (6, 16), was applied. The caffeine produced no or only a small $I_{K\text{-}Ca}$ (Fig. 1A), implying the previous depletion of Ca^{2+} stores by carbachol. Treatment with 1 μM U73122 applied 3 min beforehand affected carbachol's effect with cell-to-cell variations, where carbachol evoked no noticeable current in one cell, but a sizable $I_{K\text{-}Ca}$ in another (null to 4.1 nA, n = 6). Similarly, subsequent caffeine evoked variable amplitudes of $I_{K\text{-}Ca}$ (0.10 to 2.60 nA, n = 6). The overall averaged sizes of $I_{K\text{-}Ca}$ for carbachol and caffeine were 1.33 ± 0.64 and 1.23 ± 0.45 nA (Fig. 1C), respectively, significantly different from the corresponding control values ($P<0.05$). With U73122 treatment at 3 μM, carbachol failed at all to evoke $I_{K\text{-}Ca}$ (n = 6), followed by generation of sizable $I_{K\text{-}Ca}$ (3.40 ± 0.63 nA, n = 6) by caffeine (Fig. 1: A and C).

Similar tests were made for $G\alpha_q/G\alpha_{11}$-antibody, where cells were dialyzed with the antibody (1:100 v/v) for 18 – 20 min in order to allow it to diffuse adequately into the cell (12). With such antibody treatment, carbachol (100 μM) evoked no noticeable $I_{K\text{-}Ca}$, whereas subsequent caffeine did evoke sizable $I_{K\text{-}Ca}$ (3.68 ± 0.64 nA, n = 6) (Fig. 1B). In time-matched control cells dialyzed without the antibody, carbachol evoked an $I_{K\text{-}Ca}$ of 3.58 ± 0.55 nA (n = 6), following which caffeine evoked no or only a small $I_{K\text{-}Ca}$ (0.70 ± 0.55 nA, n = 6) (Fig. 1: B and C).

These results indicated that U73122 and $G\alpha_q/G\alpha_{11}$-antibody blocked the $I_{K\text{-}Ca}$ mediated by the $G_{q/11}$/PLC/$InsP_3$ system with no considerable change in Ca^{2+} store function or Ca^{2+}-activated K^+ channel activity and thus supported their usefulness for characterizing other G protein-regulated, PLC-mediated responses in this cell type.

Effects of U73122 on carbachol-evoked I_{cat}

Carbachol-evoked I_{cat} was measured under conditions where membrane potential was held at −40 mV and $[Ca^{2+}]_i$ buffered to 100 nM with the combination of BAPTA/$CaCl_2$ (26). Figure 2A shows a typical example of I_{cat} evoked by ascending concentrations of carbachol (1 – 300 μM). The current response increased in a graded manner after each increase in agonist concentration and reached the maximum when the concentration

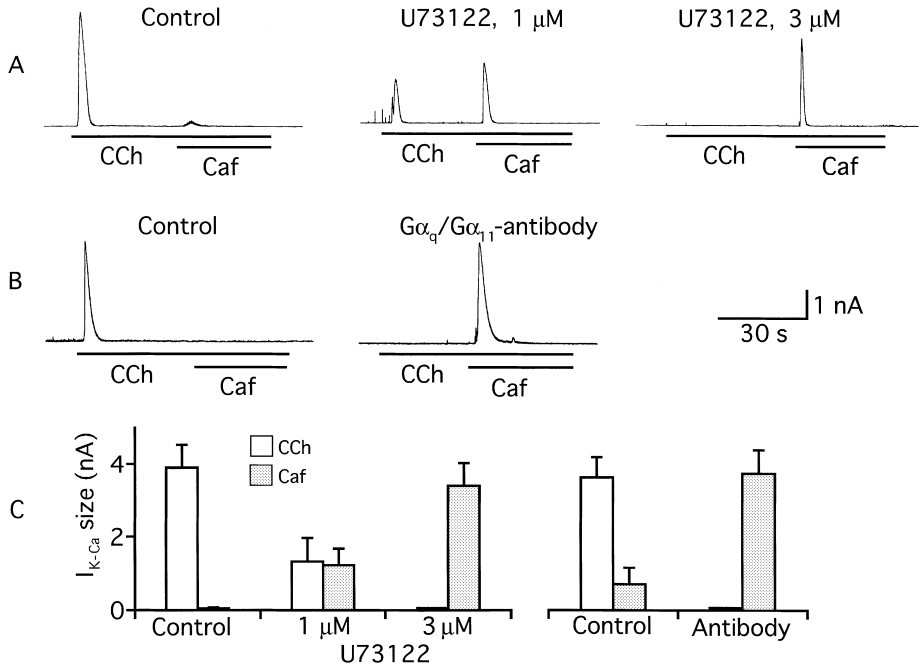

Fig. 1. Effect of U73122 and $G\alpha_q/G\alpha_{11}$-antibody on the generation of Ca^{2+}-activated K^+ current ($I_{K\text{-}Ca}$) by carbachol (CCh). Cells were immersed in normal PSS and voltage-clamped at 0 mV using patch pipettes filled with a K^+-rich solution, to which CCh (100 μM) and then caffeine (Caf, 10 mM) were applied as indicated by the lines. A: current traces from a control cell (left) and test cells treated with U73122 at 1 μM (middle) and 3 μM (right). Five minutes after whole-cell configuration, U73122 was applied 3 min before CCh application. B: similar traces from cells dialyzed intracellularly without (left; control) and with $G\alpha_q/G\alpha_{11}$ antibody (1:100 v/v) (right) over an 18- to 20-min period. C: quantitative summary of the effects of U73122 and $G\alpha_q/G\alpha_{11}$-antibody on CCh- and Caf-evoked $I_{K\text{-}Ca}$. The individual columns indicate the mean ± S.E.M. of measurements in 6 cells, respectively.

increased to 30 or 100 μM. Occasionally, at 100 and/or 300 μM, some decrease in I_{cat}, or desensitization, was seen. The averaged relationship between I_{cat} amplitude and carbachol concentration is shown in Fig. 3 (see closed squares). The I_{max}, EC_{50}, and slope factor were estimated by curve fitting of data from the individual cells, for which the mean values were 713 ± 45 pA, 4.9 ± 1.5 μM, and 1.7 ± 0.2 (n = 10), respectively. These values were consistent with those reported previously (12).

The carbachol concentration-effect curve for I_{cat} activation was also measured in the presence of U73122 applied 3 min beforehand. U73122 (0.1 – 1 μM) did not affect the holding current; however, with U73122 treatment, the I_{cat} response to carbachol was inhibited, especially in terms of the maximum response (Fig. 2: B and C). The mean values of the I_{max}, EC_{50}, and slope factor after U73122 treatments at 0.1 and 0.3 μM were 425 ± 62 pA, 4.2 ± 1.5 μM, and 1.4 ± 0.1 (n = 6) and 216 ± 83 pA, 4.0 ± 1.3 μM, and 1.8 ± 0.6 (n = 5), respectively (Fig. 3A). Of these mean values, those for the I_{max} alone differed significantly from the control (P<0.01). With 1-μM treatment, I_{cat} was too small for curve fitting analysis, so the I_{max} alone was determined from data at 300 μM carbachol. The mean value was 24 ± 8 pA (n = 7). Relationship between the mean I_{max} value and U73122 concentration gave the IC_{50} value of U73122 as 0.18 μM, similar to that reported for the inhibition of noradrenaline-activated I_{cat} in rabbit portal vein (17).

Since the PLC inihibitor U73122 has been reported to affect various cellular events through its nonspecific actions (27), we also examined U73343 that is structurally close to it but inactive in inhibiting PLC activity (22, 28). Treatment with U73343 at 0.3 or 1 μM did not noticeably affect the I_{cat} response to carbachol (Fig. 2: D and E), but with 1 μM treatment, the concentration-effect curve was shifted to the right with some 3-fold increase in the EC_{50} value (Fig. 3B). The mean values of I_{max}, EC_{50}, and slope factor were 624 ± 78 pA, 4.3 ± 1.2 μM, and 1.5 ± 0.3 (n = 5) for 0.3 μM treatment, and 588 ± 85 pA, 13.6 ± 2.0 μM, and 1.4 ± 0.1 (n = 6) for 1 μM treatment (Fig. 3B). The EC_{50} value for 1 μM treatment alone differed significantly from the control (P<0.01).

To see if U73122 affects the voltage-dependency of

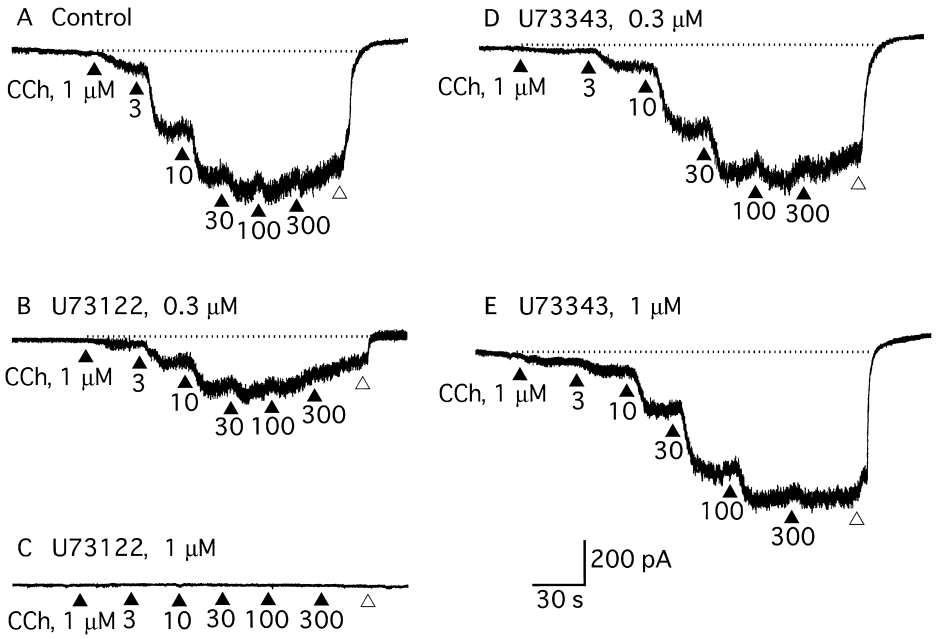

Fig. 2. Effects of U73122 and the analogue U73343 on the nonselective cationic current (I_{cat}) evoked by CCh. Cells were immersed in a Cs^+-rich solution and voltage-clamped at −40 mV using patch pipettes filled with another Cs^+-rich solution to which a BAPTA/$CaCl_2$ buffer was added to clamp $[Ca^{2+}]_i$ at 100 nM. In these coditions, CCh was applied at ascending concentrations (1 to 300 μM) as indicated by the closed triangles. A – E: typical examples of the I_{cat} response to CCh in the absence (A, control) and presence of U73122 (B, C) and of U73343 (D, E). These compounds were applied 3 min beforehand at the indicated concentrations. The open triangles indicate the point of the wash-away of CCh with PSS, and the dotted lines, an initial level before CCh application, which was used as a baseline to determine I_{cat} amplitude. A – E: from different cells.

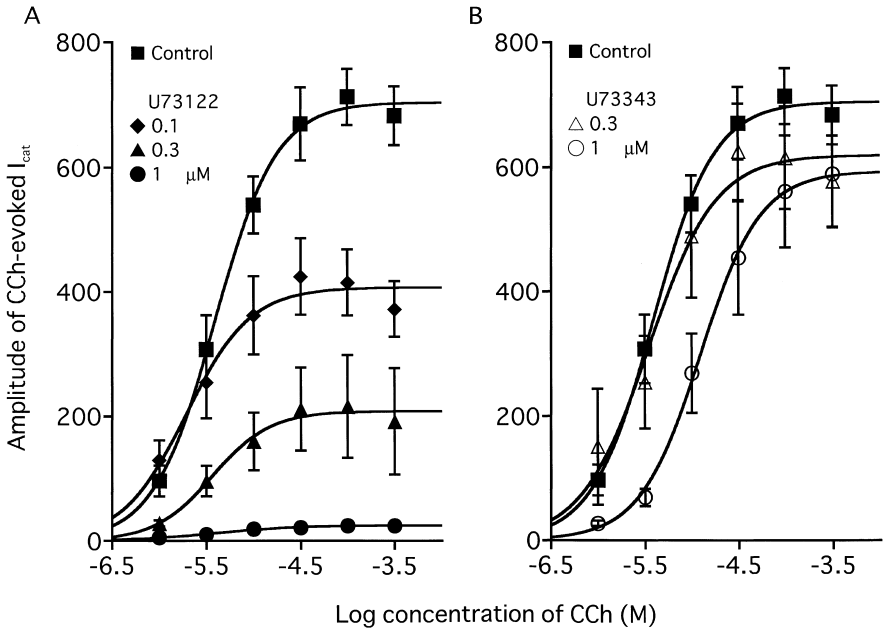

Fig. 3. Averaged concentration-effect curves of CCh-evoked I_{cat} in the presence of U73122 (A) and U73343 (B). The curves were obtained by curve fitting of the data from similar experiments to those in Fig. 2. Control curves in A and B are common. Each point indicates the mean ± S.E.M. of measurements in 5 – 10 cells.

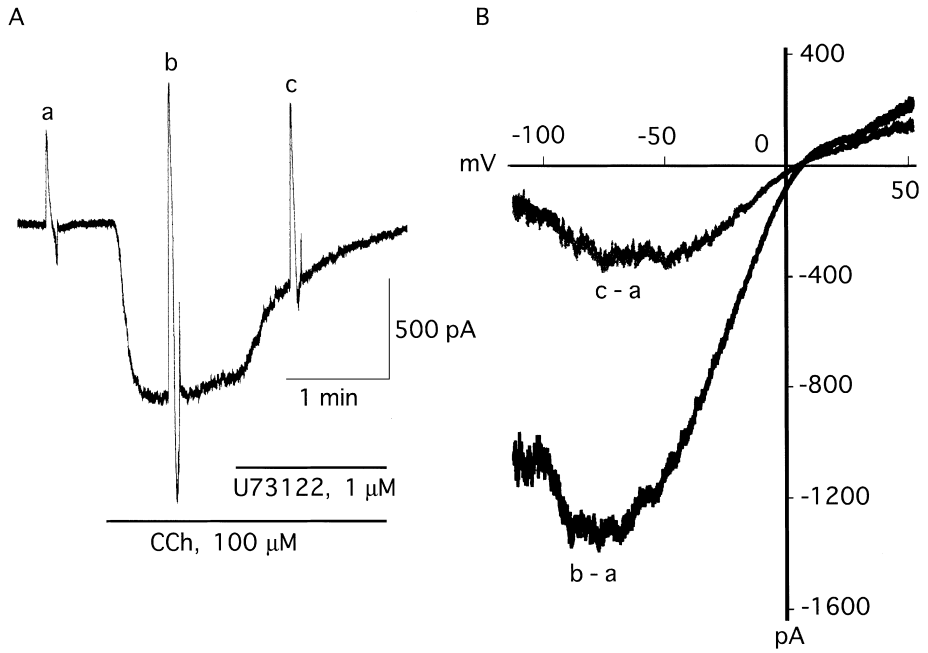

Fig. 4. Current-voltage (I-V) curves for CCh-activated I_{cat} before and after application of U73122. A: in the same current recording conditions as in Fig. 2, a negative going ramp pulse from 60 mV to −120 mV over 5 s was applied before (a) and after application of 10 μM CCh (b) and after additional application of 1 μM U73122 (c), as indicated. B: I-V curves obtained from the above experiment, which were constructed by leakage subtraction (b – a, control; c – a, U73122). Note that the I-V relation retained its characteristic U-shape in the presence of U73122. See text for details.

I_{cat} activation, we compared the current-voltage (I-V) curves for carbachol (10 μM)-evoked I_{cat} before and after application of 1 μM U73122. As exemplified in Fig. 4A, a negative-going ramp pulse from 60 to −120 mV over 5 s was applied at times during the generation of carbachol-evoked I_{cat}. A pair of I-V curves constructed after leakage subtraction are shown in Fig. 4B; one is before and the other after U73122 addition. Although the maximum voltage for I_{cat} activation was shifted toward the positive direction by 10 to 15 mV after U73122 application, the I-V relation retained its characteristic U-shape of the I-V curve (29) (Fig. 4B). Similar results were obtained in two other cells. Thus, U73122 seemed unlikely to impair the voltage-dependent gating mechanism of cationic channels.

Effects of U73122 on GTPγS-evoked I_{cat}

In this cell type, the G protein activator GTPγS produces the same type of I_{cat} as activated by muscarinic agonists (8). We compared effects of U73122 and U73343 on GTPγS-evoked I_{cat}.

Intracellular injection of GTPγS (400 μM) via patch pipettes produced I_{cat} which reached a peak 3 to 6 min after breakthrough to the whole-cell voltage clamp at −40 mV (closed star in Fig. 5). After a steady progress of the current response was confirmed for 2 – 3 min, U73122 (1 μM) was applied, which resulted in a roughly exponential decline of the ongoing I_{cat} with time (Fig. 5A). The amplitude of I_{cat} immediately before U73122 application (492 ± 151 pA, n = 9) decreased to the value of 84 ± 12 pA (n = 9) at a time of 7 min after its application, with the percent reduction of 77 ± 4% (n = 9). The remaining current at that time was abolished upon application of 10 μM quinidine, a potent I_{cat} blocker (30). The inhibitory effect of U73122 was not reversed at least for 5 min after it was washed away.

When U73343 (1 μM) was instead used, it had no considerable effect on the steady ongoing I_{cat} in GTPγS-injected cells (Fig. 5B). The percent reduction of I_{cat} amplitude estimated 7 min after U73343 application was only the value of 14 ± 2% (n = 4), ranging within normal rundown limits.

The results presented above argue that the muscarinic receptor-mediated activation of I_{cat} as well as $I_{K\text{-}Ca}$ involves PLC as a major signaling factor.

Effects of U73122 and Gα_q/Gα_{11}-antibody on the oscillatory type of I_{cat}

To determine whether the PLC involved in I_{cat} activation is the same or similar to the one involved in $I_{K\text{-}Ca}$ activation, we compared effects of U73122 and

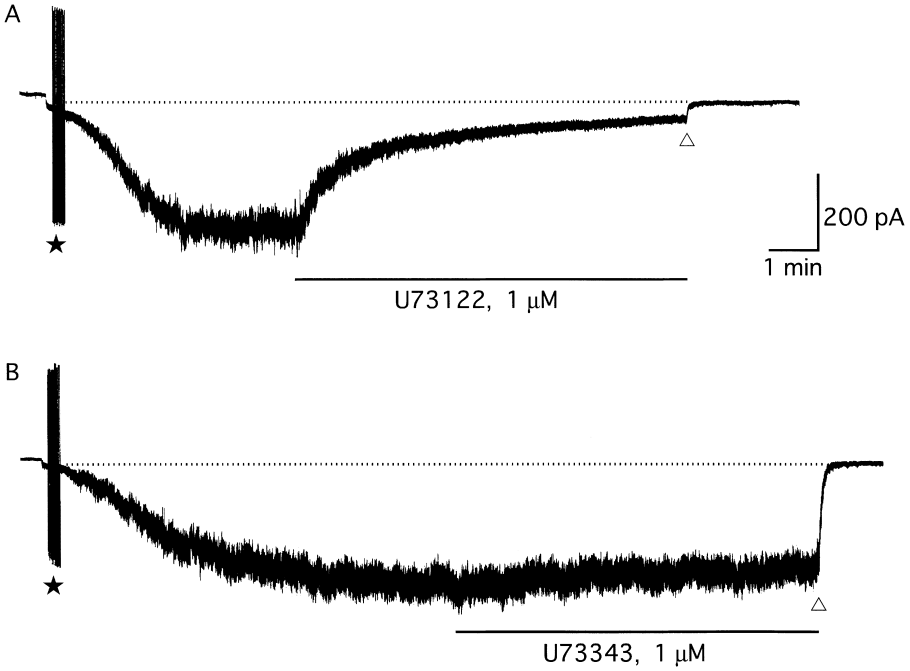

Fig. 5. Inhibition by U73122, but not the analogue U73343, of GTPγS-activated I_{cat}. In the same current recording conditions as in Fig. 2, GTPγS (400 μM) was applied intracellularly via patch pipettes following the breakthrough of patched membrane (the stars). After GTPγS-induced I_{cat} reached a steady peak, U73122 (A) or U73343 (B) was applied as indicated by the lines below the current traces, which was followed by additional application of the potent I_{cat} blocker quinidine (10 μM), (open triangles). The dotted lines, indicating current levels carried by quinidine, were used as a baseline to determine GTPγS-evoked current amplitudes.

$G\alpha_q/G\alpha_{11}$-antibody on I_{cat} evoked by carbachol in cells whose $[Ca^{2+}]_i$ was not buffered by means of the BAPTA/$CaCl_2$ combination (see Materials and Methods). Such cells, stimulated by carbachol at sub-maximal concentrations, respond with I_{cat} that oscillates following $[Ca^{2+}]_i$ oscillations mediated by the $G_{q/11}$ /PLC/$InsP_3$ system (20).

In time-matched control cells for $G\alpha_q/G\alpha_{11}$-antibody tests (see above), application of 10 μM carbachol at −50 mV produced an oscillatory I_{cat} consisting of a sustained component on which repetitive transient components were superimposed at a frequency of 0.33 ± 0.03 Hz (n = 11) (Fig. 6A). The transient and sustained components were measured by taking an initial level before agonist application as a baseline, where the latter component was depicted by inter-polating current levels from which the transients arose. The averaged amplitudes of the greatest transient and the apparent sustained I_{cat} were 352 ± 52 and 122 ± 16 pA (n = 11), respectively (Fig. 6: A and C). Two other cells exhibited only a biphasic I_{cat} consisting of an initial transient followed by a sustained component. In cells treated with $G\alpha_q/G\alpha_{11}$-antibody (1:100 v/v), only a sustained I_{cat} was elicited in the 7 of 8 cells (Fig. 6A). The averaged amplitude of 211 ± 42 pA (n = 7) was not significantly different from the control (Fig. 6: A and C). One remaining cell responded with a biphasic I_{cat} as described above.

In control cells used for U73122 tests, application of 10 μM carbachol after 5 – 6-min cell dialysis also evoked an oscillatory I_{cat}, in which the oscillation frequency was estimated to be 0.37 ± 0.06 Hz, the greatest transient component to be 738 ± 124 pA, and the apparent sustained component to be 360 ± 87 pA (n = 5) (Fig. 6: B and C). Treatment with 1 μM U73122 for 3 min inhibited almost completly of both I_{cat} components (Fig. 6B); the averaged amplitudes of the greatest transient and the apparent sustained components were 32 ± 25 and 30 ± 30 pA (n = 5), respectively (Fig. 6C).

The results indicated that PLC activity involved in the primary activation of I_{cat} is not under regulation by $G_{q/11}$ proteins.

Activity of DAG in producing I_{cat}

Activation of PLC hydrolyzes PIP_2 to produce two principal second messengers, $InsP_3$ and DAG (31, 32). Since $InsP_3$ is unlikely to be involved as a direct mediator in I_{cat} activation in this cell type (33), we examined the possible involvement of the counterpart

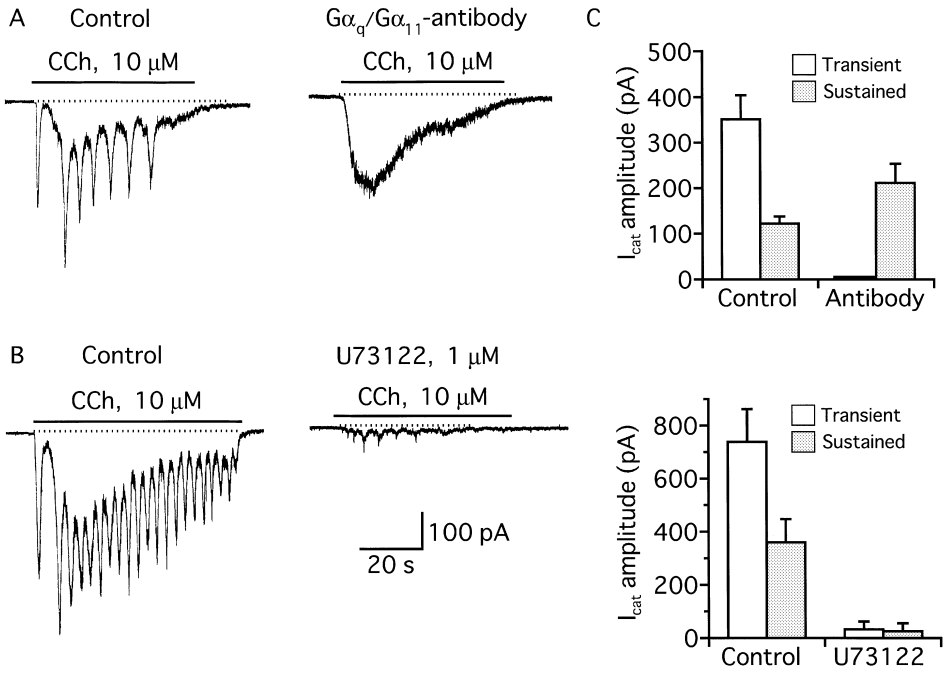

Fig. 6. Comparison of the effects of U73122 and Gα_q/Gα_{11}-antibody on the oscillatory type of I_{cat} response to CCh. Cells were immersed in PSS and voltage-clamped at −50 mV using patch pipettes filled with a Cs^+-rich solution in which the BTPTA/calcium buffer was not added. A: I_{cat} traces were obtained from cells dialyzed intracellularly without (left, control) and with Gα_q/Gα_{11} antibody (1:100 v/v, right) over 18 to 20 min. Thereafter, CCh was applied as indicated by the lines. B: Five minutes after whole-cell configuration, similar current traces were obtained from cells untreated (left, control) and treated with U73122 (right) applied 3 min before CCh application. C: quantitative summary of effects of the antibody and U73122 on the oscillatory I_{cat} response; the amplitudes of the greatest transient and the sustained component in each I_{cat} response were determined by taking the initial level before CCh application as a baseline, in which the latter component was depicted by interpolation of current levels from which the transients arose. Each column indicates the mean ± S.E.M. of measurements in 7 cells for the Gα_q/Gα_{11}-antibody test and those in 5 cells for the U73122 test. Note that the antibody blocked the oscillatory but not the sustained component.

DAG, using the membrane permeable DAG analogue OAG.

Under conditions of $[Ca^{2+}]_i$ strongly buffered, that is, the same as those where the carbachol concentration-effect curve for I_{cat} activation was measured (see Fig. 2), application of OAG at 10 μM produced a noisy inward current as small as 23 ± 3 pA (n = 3) (Fig. 7A), and at 100 μM, 15 ± 3 pA (n = 3). Application of 10 μM carbachol in the continued presence of OAG at 10 and 100 μM, produced I_{cat} of 290 ± 95 (n = 3) and 487 ± 100 pA (n = 3), respectively (Fig. 7A). These mean values did not significantly differ from that for 10 μM carbachol in the concentration-effect curve (540 ± 46 pA; see the control curve in Fig. 3A).

In the same conditions as used for measurement of the oscillatory I_{cat}, namely, where $[Ca^{2+}]_i$ was not buffered (see Fig. 6), OAG (10 or 30 μM) produced no detectable current (Fig. 7B, n = 13) or only a small inward current of 15 pA or so (n = 2). Since the muscarinic receptor-operated I_{cat} is strongly potentiated upon voltage-gated Ca^{2+} influx (13, 14), 200-ms step pulses to 0 mV were applied repeatedly at 0.1 Hz throughout the presence of OAG. However, the procedure failed to manifest the potential of OAG for producing inward current (Fig. 7B). Carbachol (10 μM), if applied in the continued presence of OAG, normally evoked oscillatory I_{cat}, as shown in Fig. 7B. Furthermore, the oscillatory current response was additionally potentiated upon application of the depolarizing step pulses, as indicated by the asterisk in the figure.

The results provided no evidence for supporting a major role of the second messenger DAG in I_{cat} generation.

Discussion

In the present study, we examined possible involvement of PLC in the activation of muscarinic receptor-operated I_{cat} in guinea pig ileal muscle cells, using a putative PLC inhibitor of U73122 and Gα_q/Gα_{11}-antibody.

Under conditions of $[Ca^{2+}]_i$ either buffered or not

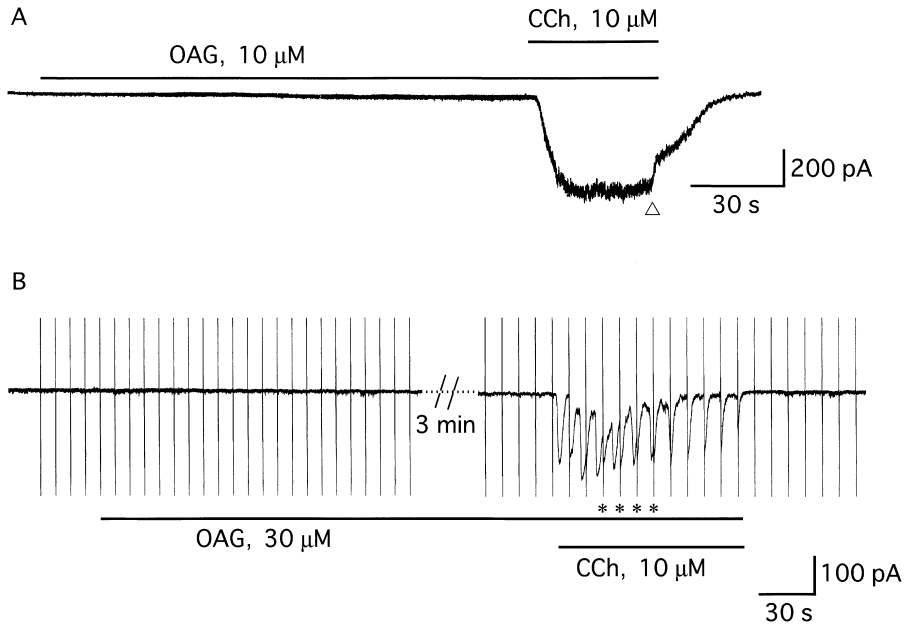

Fig. 7. The activity of OAG in inducing current and its effect on CCh-evoked I_{cat}. A: in the same current recording conditions as in Fig. 2, application of OAG (10 μM) as indicated produced only a small inward current, and subsequent CCh normally evoked I_{cat} in the continued presence of OAG. B: in the same current recording conditions as in Fig. 6, OAG (10 μM) was applied throughout a period during which voltage-gated Ca^{2+} influxes were activated repeatedly at 0.1 Hz by 200-ms step pulses to 0 mV, but no detectable current was elicited. Subsequently, CCh (10 μM) was applied as indicated evoked normally an oscillatory I_{cat} response that was potentiated upon the step pulse (see asterisk). The individual sharp deflections in the current trace indicate a mixture of the capacitative and the inward Ca^{2+} current evoked by the step pulse.

buffered, the I_{cat} response to carbachol was profoundly inhibited by U73122. The inhibition did not involve muscarinic receptor block or cationic channel block or other possible nonspecific actions, as judged from the following results. U73122 blocked GTPγS-evoked I_{cat} as well, which is bypassing muscarinic receptors but carried via the same type of channels as activated by muscarinic agonists (8). To the contrary, U73343, which is structurally close to U73122 but deficient of the PLC-inhibiting activity (22, 28), had no considerable effect on the carbachol- or GTPγS-induced I_{cat}, but somewhat decreased the sensitivity of the I_{cat} response to carbachol (Fig. 3B). The U-shape of I-V curves representing the unique voltage-dependency of cationic channel gating was retained after U73122 treatment (Fig. 4B). In addition, the specificity of U73122 (at least up to 3 μM) for PLC inhibition was also supported by blockade of carbachol-evoked $I_{K\text{-}Ca}$ (Fig. 1), a cellular event mediated by the $G_{q/11}$/PLC/$InsP_3$ system (6, 25). Therefore, the observed inhibition by U73122 of carbachol-evoked I_{cat} is considered to result from inactivation of PLC activity, indicating the involvement of PLC in the current activation.

The present study also shows that the PLC involved in the primary activation of I_{cat} is not under regulation by $G_{q/11}$ proteins. This is based on the different effects of U73122 and Gα_q/Gα_{11}-antibody on the oscillatory type of I_{cat} response; the former abolished both oscillatory and sustained components, whereas the latter abolished only oscillatory component (Fig. 6). This component has been shown to occur because cationic channel opening is potentiated cyclycally by oscillations in $[Ca^{2+}]_i$ mediated by the $G_{q/11}$/PLC/$InsP_3$ system (20), which readily accounts for the effect of Gα_q/Gα_{11}-antibody. If I_{cat} generation depends entirely on $G_{q/11}$ protein-coupled PLC activity, the antibody should have abolished not only the oscillatory component but also the sustained component, as did U73122 (Fig. 6). The partial effect of the antibody is not caused by inadequate inactivation of the PLC isozyme, since it completely blocked the $I_{K\text{-}Ca}$ response to maximally effective carbachol (Fig. 1). Our previous study showed that without changes in cationic channel opening by Ca^{2+} released from the store, the I_{cat} response to carbachol was unaltered by Gα_q/Gα_{11}-antibody (12). Overall, the results support the hypothesis that some PLC other than one coupled to $G_{q/11}$ proteins is involved as a major mediator in the primary activation of the muscarinic receptor cationic channels.

This raises the question about how the $G_{q/11}$-

independent PLC isozyme is activated upon muscarinic receptor stimulation. It has been suggested that M2 receptors can couple to PLC activation via $\beta\gamma$ subunits of G_i/G_o proteins in intestinal smooth muscle (34). However, Yan et al. (12) observed in this cell type that neither G$\beta\gamma$ subunits nor Gβ-antibody applied intracellularly exerted any significant effect on carbachol-evoked I_{cat}, which may argue against the $G_{i/o}\beta\gamma$ activation of the $G_{q/11}$-independent PLC isozyme. Another possibility is the activation by α subunits of the G_i protein family via tyrosine kinase-dependent pathways (35), which is consistent with blockade of the I_{cat} response by PTX (8), Gα_o-antibody (12), or a tyrosine kinase inhibitor (36).

Regardless of the way of how it occurs, PLC activation results in production of two principle second messengers, $InsP_3$ and DAG. In our previous studies using a photo-sensitive caged $InsP_3$ in ileal cells, it was found that during muscarinic receptor activation, photo-released $InsP_3$ exerted no more effect than potentiating I_{cat} via releasing stored Ca^{2+} (25). In the present experiments, OAG (a DAG analogue) produced no or only small inward current in different recording conditions where carbachol normally evoked I_{cat}. There was no considerable alteration by OAG of carbachol-evoked I_{cat} (Fig. 7A). These observations may indicate that the importance of PLC in I_{cat} activation is not related to the second messenger or DAG or $InsP_3$, in contrast to the case for I_{cat} activation by α_1 adrenoceptor stimulation in rabbit portal vein (17) and muscarinic receptor stimulation in mouse gastric myocytes (18).

Studies of photo-signal transduction in *Drosophila* have suggested that PLC isozymes are organized with G proteins, ionic channels, and other signaling molecules into supramolecular functional units by certain scaffolding proteins (37). It should be noted that in ileal muscle cells, pharmacological inactivation of PLC (the present study) produced a similar effect to that of M3 receptors (5) or G_o proteins (12); all these procedures resulted in severe depression of the I_{max} of carbachol-evoked I_{cat} with no noticeable change in the EC_{50} value. There is also evidence that the M2 receptors primarily mediate the I_{cat} response (6). Therefore, by analogy with *Drosophila* photo-signal transduction, it is plausible that the $G_{q/11}$-independent PLC isozymes, together with M2 and M3 receptors, G_o proteins, cationic channels, and others, which may form supramolecular functional units in which these molecules mutually interact for opening and modulating the channels. Such functional units might be responsible for the M2/M3 synergistic activation of cationic channels (see Introduction). Consistent with this, we most recently using M2- or M3-receptor subtype-knockout mice found that both M2 and M3 receptors are essential for the generation of I_{cat} by carbachol in intestinal smooth muscle cells (38).

In conclusion, the present study provides evidence for the hypothesis that PLC idependent of $G_{q/11}$ proteins regulation plays a pivotal role in activation of the muscarinic receptor-operated I_{cat} in ileal muscle cells. It is also suggested that the role of PLC does not depend on the second messenger DAG, as well as $InsP_3$, derived from PLC-catalyzed PIP_2 hydrolysis. The present demonstration of the importance of the $G_{q/11}$-independent PLC isozyme in I_{cat} generation may provide relevant insights into understanding of the mechanisms underlying the M2/M3 synergistic activation of cationic channels in the gastrointestinal and other smooth muscles. It remains to be clarified how the $G_{q/11}$-independent PLC isozyme is activated and participates in the opening of cationic channels.

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