Characterization of Muscarinic Receptor-Mediated Cationic Currents in Longitudinal Smooth Muscle Cells of Mouse Small Intestine

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Abstract. In mouse intestinal smooth muscle cells held at −50 mV, carbachol evoked an atropine-sensitive inward current in the intracellular presence of Cs⁺. The current response consisted of an initial peak followed by a smaller plateau component on which oscillatory currents frequently arose. Results from various experimental procedures indicated that the inward current is a muscarinic receptor-operated cationic current (mI_cat) sensitive to cytosolic Ca²⁺ concentration ([Ca²⁺]_i) and that the initial peak and oscillatory components are contaminated by Ca²⁺-activated Cl⁻ currents. Under conditions of [Ca²⁺]_i buffered to 100 nM, the mI_cat response to cumulative carbachol applications was inhibited competitively by an M₂-selective antagonist but non-competitively by an M₃-selective one. Also it was severely reduced by pertussis toxin (PTX) treatment or a phospholipase C (PLC) inhibitor. Comparative analysis of mI_cat in mouse and guinea-pig intestinal myocytes indicated that the underlying channels resemble between those myocytes in agonist sensitivity, current-voltage relationship, and unitary conductance. The results suggest that in mouse intestinal myocytes, mI_cat arises mainly via an M₂/M₃ synergistic mechanism involving PTX-sensitive G-proteins and PLC activity in the absence of current modulation by [Ca²⁺]_i changes, as described for guinea-pig ileal mI_cat. The channels underlying mI_cat are also indistinguishable in gating properties between both types of myocytes.

Keywords: muscarinic receptor, receptor-operated cation channel, pertussis toxin, phospholipase C, intestinal smooth muscle

Introduction

In visceral smooth muscles including those of the small intestine, M₂ and M₃ muscarinic receptors co-exist as the target for the neurotransmitter acetylcholine (1, 2). Their activation produces depolarization and an increase in voltage-dependent Ca²⁺ entry into the cell, which results in contraction (3). The depolarization has been attributed mainly to the opening of cationic channels that are less selective for Na⁺ and K⁺ (4, 5). Although signal transduction mechanisms leading to activation of the muscarinic cationic channels have been explored in various types of smooth muscles (see refs. 6 and 7), the most extensive study is performed in guinea-pig ileum, a model for intestinal smooth muscle research. Based on the pharmacological analysis of muscarinic receptor-mediated cationic current (mI_cat) in single ileal myocytes, a unique signaling system for the channel activation has been proposed; M₂ receptors primarily activate cationic channels via coupling with the PTX-sensitive G-protein G₉, but M₃ receptors also play a crucial role by providing a permissive signal upon channel gating independently of the G₉ protein/phospholipase C (PLC) pathway (8 – 12). However, M₂ and M₃ roles in such an M₂/M₃ synergistic system remain to be exactly understood and vigorous experimental tests are needed to establish this system as the ubiquitous one.
in intestinal smooth muscle. One of the promising approaches to do this is supposed at present to be the use of mutant mice lacking each subtype of muscarinic receptor and other gene modified mice (13, also see ref. 14).

However, information on signal transductions and channel gating properties underlying \( m_{I_{cat}} \) is very limited in mouse intestinal smooth muscle. Furthermore, it is uncertain that the signaling pathways for \( m_{I_{cat}} \) generation are shared between the guinea-pig and mouse intestines, as deduced from the different results obtained in their stomach smooth muscle. Indeed, \( m_{I_{cat}} \) in gastric myocytes arises via \( M_{I/G} \), whereas via \( M_{I/G} \) in the mouse (15). Therefore, in the present study, we pharmacologically characterized \( m_{I_{cat}} \) in longitudinal smooth muscle cells from the small intestine of mouse, but also directly compared the mouse and guinea-pig intestinal myocytes with respect to some certain properties of channels responsible for \( m_{I_{cat}} \).

Our present data indicates that the main signaling pathway for \( m_{I_{cat}} \) generation in the mouse intestinal myocyte is substantially similar to that suggested for the guinea-pig ileal \( m_{I_{cat}} \) and that the underlying cationic channels are also not distinctive between both species in the unitary conductance, voltage-dependent gating property, and agonist sensitivity.

**Materials and Methods**

All procedures described below were performed according to the guideline approved by a local animal ethics committee of the Faculty of Applied Biological Sciences, Gifu University.

**Cell preparation**

Male mice, weighing 30 – 40 g, were sacrificed by cervical dislocation, and a 15-cm segment of the small intestine except the terminal 3 cm was cut off without distinction of the jejunum and ileum. A male guinea pig (300 – 400 g) was stunned, followed by immediate exsanguination, and a 10-cm segment of the ileum except the terminal 10 cm was excised. Either intestinal segment was 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); cut into 1.5- to 2.0-cm segments; and then the longitudinal muscle layer was carefully peeled from the underlying tissue and cut into small pieces. The tissue pieces from either species were then subjected to a 10- to 11-min digestion successively twice each with a 2-ml solution containing collagenase (15.2 U/ml), papain (0.22 – 0.24 mg/ml), and bovine serum albumin (0.75 mg/ml). After the digestions, single myocytes were harvested by tissue agitation and subsequent centrifugation, and then they re-suspended in a solution containing 0.5 mM Ca\(^{2+}\), placed on coverslips in small aliquots, and stored at 4°C until use on the same day.

**Current recordings**

Whole-cell membrane currents were recorded at room temperature (22°C – 26°C) using patch pipettes (3 – 7 MΩ in tip resistance) and a voltage-clamp amplifier (CEZ-2300; Nihon Kohden, Tokyo). Current signals were filtered at 1 kHz and captured at a sampling rate of 4 kHz using an analog-digital converter (DIGIDATA 1322A; Axon Instruments, Foster City, CA, USA) interfaced to a computer (IMC-P642400; Inter Medical Co., Nagoya) running with the pCLAMP program (version 9, Axon Instruments). The current signals were also stored on a digital tape (DM120D; Hitachi Maxell, Tokyo) with a digital tape recorder (RO-101; TEAC, Tokyo) for later analysis and illustration.

For recording of \( m_{I_{cat}} \), cells were bathed in PSS (compositions mentioned above) and dialyzed with a Cs\(^+\)-rich pipette solution composed of 134 mM CsCl, 1.2 mM MgCl\(_2\), 1.0 mM MgATP, 1.0 mM Na\(_2\)GTP, 0.05 mM EGTA, 14 mM glucose, and 10.5 mM HEPES (pH adjusted to 7.2 with CsOH). Alternatively, they were bathed in a Cs\(^+\)-rich solution consisting of 120 mM CsCl, 12 mM glucose, and 10 mM HEPES (pH adjusted to 7.4 with CsOH) and dialyzed with another Cs\(^+\)-rich solution consisting of 80 mM CsCl, 1.0 mM MgATP, 1.0 mM Na\(_2\)GTP, 5 mM creatine, 20 mM glucose, 10 mM HEPES, 10 mM BAPTA, and 4.6 mM CaCl\(_2\) (calculated free-ionized calcium = approximately 100 nM; pH adjusted to 7.4 with CsOH; Cs\(^+\) = 144 mM in total). Cell dialysis with the Cs\(^+\)-rich solutions prevented generation of K\(^+\) current by muscarinic agonists (17, 18). The use of the latter pair of bathing and pipette solutions prevented \( m_{I_{cat}} \) modulation by changes in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) and magnified \( m_{I_{cat}} \) because Cs\(^+\) is more permeable than Na\(^+\) (8, 19). If necessary, chloride in the pipette solutions was replaced with the impermeable anion glutamate or sodium in the bathing solution was replaced with the impermeable cation N-methyl-d-glucamine (NMDG).

When Ca\(^{2+}\)-activated K\(^+\) current was recorded, cells were bathed in PSS and dialyzed with a pipette solution composed of 134 mM KCl, 1.2 mM MgCl\(_2\), 1.0 mM MgATP, 0.1 mM Na\(_2\)GTP, 0.05 mM EGTA, 14 mM glucose, and 10.5 mM HEPES (pH adjusted to 7.2 with KOH), as described previously (20).

**Pertussis toxin (PTX) treatment**

PTX was injected i.p. into mice at a dose of 100 μg/kg
body weight, and 70–74 h later, the cell preparation was made as described above. Such PTX treatment has been shown to inhibit carbachol-induced contractions in mouse ileal longitudinal muscle (13).

Data analyses

Current-voltage (I-V) relations for mI\text{cat} were investigated with a negative-going ramp pulse from 40 up to −120 mV over 5 s. In the construction of I-V curves, a leak current obtained by the pulse before carbachol application was subtracted from a current evoked by it during the generation of mI\text{cat}.

The unitary conductance of ion channels responsible for mI\text{cat} was estimated by means of non-stationary noise analysis (21, 22). A carbachol-induced mI\text{cat} was filtered through a 0.4 – 800 Hz band-pass filter (−3 dB) and a 0.4 Hz low-pass filter (−3 dB) using an 8-pole Butterworth digital filter operated by pCLAMP software. Signals from the respective filtrations were then split into 1-s blocks each containing 4000 samples, and a calculation was made for every block to determine current variance (δ²) from the former filtered signal and mean current (µ) from the latter, using pCLAMP software. Relationships between the δ² and µ were analyzed using the graph software Origin 7.0 (Origin Lab., Northampton, MA, USA) that fits data points directly by either a linear equation of δ² = iµ or a quadratic equation of δ² = iµ − (µ²/N) (22), where i is the amplitude of single channel current and N is the number of functional channels. The unitary channel conductance (γ) was given by dividing an estimated i by the driving force for mI\text{cat} (50 mV, see Results).

Carbachol concentration-response curves were measured by a cumulative application protocol and fitted with a logistic function of \( I / I_{max} = (1 + ([EC_{50}]/[A]))^{-1} \), where \( I \) \ is an mI\text{cat} amplitude, \( I_{max} \) \ is the maximal mI\text{cat}, \( EC_{50} \) \ is the concentration required for the agonist to produce half of \( I_{max} \), and \( h \) \ is the Hill slope.

The \( pA_2 \) \ value of muscarinic receptor antagonists was calculated from the following equation: \( pA_2 = \log(DR-1) − \log[B] \), where \( [B] \) \ denotes the concentration of the antagonist, and \( DR \) \ is the ratio of the mean \( EC_{50} \) value of carbachol estimated in the absence of the antagonist divided by that estimated in the presence of the antagonist (23).

Values in the text are given as the mean ± S.E.M. with the number of measurements. Statistical significance between two groups was assessed using Student's unpaired t-test. Differences were considered as significant when \( P<0.05 \).

Chemicals

Papain and collagenase (type 2) used for tissue digestion were from Sigma Chemical Co. (St. Louis, MO, USA) and Worthington Biochemical Co. (Lakewood, NJ, USA), respectively. Charbachol, methoctramine, 1,2-bis(2-aminophenoxy)-ethan-N,N,N',N'-tetraacetic acid (BAPTA), PTX, and thapsigargin were all purchased from Sigma; 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17y]amino]hexyl]-1H-pyrido-le,2,5-dione (U73122), 1-[6-[[17β-3-meth-oxyestra-1,3,5(10)-trien-17y]amino]hexyl]-2-5- pyrrolidinedione (U73343), caffeine, and atropine were from Wako (Tokyo); 4-diphenylacetoxycarbamoyl-N-methyl-piperidine methiodide (4-DAMP) was from Tocris (Ballwin, MO, USA). All other chemicals were of reagent grade.

U73122, U73343, and 4-DAMP were each dissolved in dimethyl sulfoxide (DMSO) and prepared as stock solution of more than 1000-fold the concentrations used for experiments. Exposure of the cells to DMSO at up to 0.1% in the present experiments had no effect on the holding current or carbachol-evoked mI\text{cat}. Drug concentrations in the text and figures are expressed as the final concentration applied to cells.

Results

Carbachol-induced inward currents

Mouse intestinal myocytes were bathed in PSS and dialyzed with Cs⁺-rich pipette solution (see Materials and Methods) and held under voltage-clamp at −50 mV. Applications of a maximally effective concentration of carbachol (100 µM) induced an inward current with a form showing an initial peak followed by a smaller plateau. Following the initial peak, smaller oscillations frequently occurred on the plateau throughout the agonist application for 60–90 s (Fig. 1A) or for an early period (Fig. 1B). The oscillation was usually constant in periodicity, and its frequency was 0.46 ± 0.01 Hz (n = 12) with cell-to-cell variations of 0.15 to 1.15 Hz. The amplitudes of the initial peak and the latter sustained inward current were 249.6 ± 43.1 and 23.9 ± 5.1 pA (n = 22), respectively (Fig. 1E). When atropine (1 µM) was applied 1 min beforehand, carbachol had no effect (n = 3), indicating that the carbachol-evoked currents are all mediated by muscarinic receptors.

Infusion of the Ca²⁺-chelating agent EGTA (20 mM) into the cell via the patch pipette almost blocked the generation of currents by carbachol (n = 10) (Fig. 1: C and E). Treatment with the Ca²⁺-ATPase inhibitor thapsigargin (1 µM) causing depletion of intracellular Ca²⁺ stores (24) prevented the generation of both the initial peak and subsequent current oscillation, so that a plain, sustained inward current occurred upon carbachol application (Fig. 1D). The current amplitude was 34.9 ± 5.5 pA (n = 10), not significantly different from
the control value $(23.9 \pm 5.1 \text{ pA})$ (Fig. 1E). Neither EGTA nor thapsigargin had any effects on the holding current.

The current responses to carbachol were somewhat affected by replacing most of the chloride in the pipette solution with the impermeable anion glutamate. In glutamate-filled myocytes, carbachol $(100 \mu M)$ evoked a similar form of inward current to that seen in control myocytes (Fig. 2: A and B), but current oscillation persisting throughout the agonist application was not seen, nor was an initial peak current seen in some cells. On overall average, the initial peak decreased to $119.6 \pm 69.5 \text{ pA}$ (n = 7), but the difference from the control value was insignificant. The sustained inward current $(30.0 \pm 7.7 \text{ pA}, \text{n} = 7)$ did not significantly differ from the corresponding control (Fig. 1E).

When extracellular Na$^+$ in bath solution was totally replaced with the impermeable cation NMDG, carbachol $(100 \mu M)$ evoked a small initial peak current, but a sustained inward current was not seen (Fig. 2C). The average current amplitude of the initial peak current was $29 \pm 14.7 \text{ pA}$ (n = 4), being significantly smaller than the corresponding control value (Fig. 1E).

Figure 2D shows a typical experiment on glutamate-filled cells (n = 8), where inward Ca$^{2+}$ currents were evoked by a 0.2-s voltage step to 0 mV before and during the application of carbachol. In the latter case, cessation of the voltage step was immediately followed by a prominent inward current of approximately 500 pA that gradually declined and terminated to an on-going sustained current. The generation of such tail currents following Ca$^{2+}$ injection has been attributed to Ca$^{2+}$-induced increase in open probability of cationic channels activated by carbachol (25).

The overall findings suggest that the inward current evoked by carbachol is mostly a muscarinic receptor-operated cationic current ($mI_{\text{cat}}$) sensitive to changes in [Ca$^{2+}$], but Ca$^{2+}$-activated Cl$^-$ current is also partially involved, especially in the initial peak currents.

In the following experiments, to measure pure and
Magnified mLcat, we used another recording condition in which the external Na\(^+\) was substituted with Cs\(^+\) and \([\text{Ca}^{2+}]\), was clamped at 100 nM with a BAPTA/\text{Ca}^{2+} buffer system (see Materials and Methods), unless otherwise mentioned.

**M\(_2\)- or M\(_3\)-receptor antagonist**

Figure 3A shows a typical example of mLcat evoked by cumulative applications of carbachol at a series of ascending concentrations (1 – 300 µM). The current response increased with increasing agonist concentrations, and the maximum response (I\(_{\text{max}}\)) was attained at 30 or 100 µM (Fig. 3A), although occasionally it somewhat declined (desensitization) at higher concentrations. Mean values for the I\(_{\text{max}}\), EC\(_{50}\), and Hill slope estimated by curve fitting of data from each myocyte were 232.6 ± 27.9 pA, 3.9 ± 0.7 µM, and 1.2 ± 0.2 (n = 12), respectively (Fig. 3D). Desensitization of mLcat evoked by higher concentration of carbachol may have resulted in an underestimation of the I\(_{\text{max}}\) value. Therefore, we compared amplitude of mLcat evoked by a single application of carbachol with those obtained by the cumulative application method. Single application of carbachol at 3 µM (near EC\(_{50}\)) and 100 µM (maximally effective concentration) evoked a sustained mLcat whose amplitude increased to reach a peak and then declined somewhat (ex. Figs. 5A and 8A). The mean peak amplitude at 3 and 100 µM were 78.3 ± 27.8 (n = 5) and 251 ± 38.6 pA (n = 4), respectively, which were not significantly different from the corresponding values obtained by the cumulative application method (77.9 ± 14.8 and 232.6 ± 27.9 pA, n = 12). Thus, the measurement of I\(_{\text{max}}\) value in the cumulative application protocol seemed to be seriously unaffected by the mLcat desensitization.

In the presence of a muscarinic antagonist added in the bathing solution 30 – 60 s beforehand, carbachol concentration-response curves were measured using the cumulative application protocol. The M\(_2\)-preferring antagonist methoctramine (300 nM) shifted the agonist curve to the right in a parallel manner (Fig. 3: B and D). The I\(_{\text{max}}\), EC\(_{50}\), and Hill slope values in the presence of methoctramine were 269.9 ± 152.1 pA, 39.9 ± 12.7 µM, and 1.6 ± 0.2 (n = 8), respectively, and the EC\(_{50}\) value...
alone significantly differed from the control (3.9 ± 0.7 µM), implying a competitive antagonism. Calculation made from a 10.2-fold increase in the EC\textsubscript{50} by 300 nM methoctramine gave a pA\textsubscript{2} value of 7.5 for the antagonist, which was close to its published dissociation constant for the M\textsubscript{2} receptor (7.6, from ref. 2). Similar application of the M\textsubscript{3}-preferring antagonist 4-DAMP (30 nM) severely reduced the I\textsubscript{max} value to 60.8 ± 9.0 pA (n = 7) with no significant change in the EC\textsubscript{50} (4.3 ± 1.6 µM, n = 7) (Fig. 3: C and D).

The results suggest that M\textsubscript{2} and M\textsubscript{3} receptors are both involved in mI\textsubscript{cat} generation, but in different manners.

**PTX and U73122**

Cells isolated from PTX-treated mice were much less sensitive to carbachol than control cells as shown in Fig. 4A. The cumulative mI\textsubscript{cat} responses to the agonist in the PTX-treated cells had only an I\textsubscript{max} value to 60.8 ± 9.0 pA (n = 7) with no significant change in the EC\textsubscript{50} (4.3 ± 1.6 µM, n = 7) (Fig. 3: C and D).

The results suggest that M\textsubscript{2} and M\textsubscript{3} receptors are both involved in mI\textsubscript{cat} generation, but in different manners.

**Fig. 3.** Effects of the muscarinic antagonists, methoctramine, and 4-DAMP on CCh-evoked cationic channel currents (mI\textsubscript{cat}). Cells were immersed in a Cs\textsuperscript{+}-rich bath solution and voltage-clamped at −50 mV using patch pipettes filled with the Cs\textsuperscript{+}-based pCa 7.0 pipette solution in which a BAPTA/CaCl\textsubscript{2} buffer was added to clamp [Ca\textsuperscript{2+}]\text sub at 100 nM. A – C: typical examples of the mI\textsubscript{cat} response to CCh (1 – 300 µM) in control (A), methoctramine-treated (B), and 4-DAMP-treated (C) cells. Open triangles indicate the moment of wash-out of CCh. Methoctramine (300 nM) or 4-DAMP (30 nM) was applied 30 – 60 s before the beginning of CCh application. D: the averaged concentration-response curves of mI\textsubscript{cat} in control (closed circle), methoctramine-treated (closed square), and 4-DAMP-treated (closed triangle) cells. Each point in D indicates the mean ± S.E.M. of measurements in 7 – 12 cells. The M\textsubscript{2}-preferring antagonist methoctramine inhibited the mI\textsubscript{cat} response in a competitive manner, but the M\textsubscript{3}-preferring antagonist 4-DAMP did so in a non-competitive manner.
The results suggest that \( m_{\text{L}_{\text{cat}}} \) generation depends largely on both PTX-sensitive G proteins and PLC activity.

**Comparison of \( m_{\text{L}_{\text{cat}}} \) in mouse and guinea-pig intestinal myocytes**

Comparative analysis of \( m_{\text{L}_{\text{cat}}} \) was made between mouse and guinea-pig intestinal myocytes. These cells were prepared and handled as similarly as possible.

**Dependence on agonist concentration:** As exemplified in Fig. 6, the \( I_{\text{max}} \) value for \( m_{\text{L}_{\text{cat}}} \) evoked by cumulative carbachol applications was clearly smaller in the mouse myocyte. As \( m_{\text{L}_{\text{cat}}} \) size was converted into increased conductance, the maximum conductance (\( G_{\text{max}} \)) in the mouse myocyte was 4.7 ± 0.6 nS (n = 12), no more than 0.3 relative to the guinea-pig \( G_{\text{max}} \) (15 ± 3.5 nS, n = 8) (Fig. 6C). The whole-cell membrane capacitance, estimated from capacitative currents upon a hyperpolarizing 10-mV step, was similar between the mouse (38.3 ± 2.3 pF, n = 7) and guinea-pig myocytes (34.5 ± 5.0 pF, n = 5), implying that the surface area or size of the cell is irrelevant to the difference in \( G_{\text{max}} \). There was no significant difference in the EC\(_{50}\) or Hill slope (4.5 ± 0.8 \( \mu \)M and 1.4 ± 0.2 (n = 8) for the guinea pig and 3.9 ± 0.7 \( \mu \)M and 1.2 ± 0.2 (n = 12) for the mouse).
Current-voltage (I-V) relations: A negative-going voltage ramp from 40 mV up to −120 mV over 5 s was applied before and during the mI_{cat} response to 100 µM carbachol (Fig. 7: A and C). As seen from Fig. 7, B and D, in the myocytes from either species, the net mI_{cat} had a U-shaped I-V relationship: from 0 mV to about −60 mV, the current increased in proportion to the increase in the electromotive force, but as negativity was further increased, the current progressively declined. The polarity of mI_{cat} was reversed at 2.6 ± 2.1 mV (n = 7) in the mouse myocytes and at 3.8 ± 0.8 mV (n = 7) in the guinea-pig myocytes. There was no significant difference between the two mean values, and each mean value did not significantly differ from 0 mV.

Single channel conductance (γ): We performed non-stationary noise analysis to estimate the conductance of single channels underlying mI_{cat}. As exemplified in Fig. 8, mI_{cat} evoked by 100 µM carbachol was subjected to a band-pass (0.4 – 800 Hz) and a low-pass (0.4 Hz) filter, and noise variances (δ²) obtained from the former filtered signal were plotted against mean currents (µ) obtained from the latter (Fig. 8: B and D). In the mouse myocyte, the µ-δ² relationships could be well fitted with either a single straight line or a single quadratic curve, giving a single channel amplitude (i) of 2.0 ± 0.2 pA (n = 6) for the former case and of 1.8 ± 0.2 pA (n = 4) for the latter. Mean γ values for the respective cases were estimated to be 40.4 ± 5.0 (n = 6) and 36.0 ± 4.8 pS (n = 4). There was no significant difference between each pair of mean values, and the overall averaged values for i and γ were 1.9 ± 0.2 pA and 38.6 ± 3.5 pS (n = 10), respectively. In the guinea-pig myocyte, µ-δ² relationships were all fitted with single quadratic curves (Fig. 8B), having a mean value of 1.7 ± 0.1 pA for i (n = 7) and 33.1 ± 2.4 pS (n = 7) for γ. Each mean value did not significantly differ from the corresponding mouse value.

An attempt was made to analyze mI_{cat}, which was evoked by cumulative carbachol applications in PTX-treated mouse myocytes. In 3 of the 6 myocytes examined, the µ-δ² relationships could not be fitted with either straight lines or quadratic curves, but rather done with a pair of straight lines having an i value (slope) between 1.9 and 2.7 pA and another i value between 0.5 and 0.7 pA. In the three remaining myocytes, the µ-δ² relationships were fitted with single straight lines, giving an i value of 1.6 pA in one cell and smaller value of 0.5 and 0.8 pA in the other two. The overall i values obtained could be classified into the relatively large (1.6 to 2.7 pA) and small values (0.5 to 0.8 pA), with the respective mean values of 2.1 ± 0.2 (n = 4) and 0.7 ± 0.1 pA (n = 5). The respective mean γ values were 41.9 ± 4.8 (n = 4) and 13.1 ± 1.3 pS (n = 5). The mean i and γ values in the large group were similar to those obtained in intact myocytes.

Discussion

In single longitudinal smooth muscle cells from mouse small intestine, carbachol evoked an atropine-sensitive inward current. The form of the current response showed an initial peak followed by a smaller plateau on which oscillations were often superimposed (Fig. 1). The results obtained with EGTA and thapsigargin indicate that the current responses require Ca^{2+}...
inside the cell for their generation and the initial peak and oscillatory currents are both associated with intracellular Ca\(^{2+}\) release. Replacement of intracellular Cl\(^{-}\) with the impermeable anion glutamate did not affect the sustained inward current, while the initial peak current was partly reduced, and the long-lasting oscillation became unseen. Furthermore, substitution of extracellular Na\(^{+}\) with the impermeable cation NMDG decreased significantly the amplitude of initial peak current and abolished the sustained inward current. These observations indicate that the carbachol-evoked inward current in mouse intestinal myocytes is mostly an mI\(_{\text{cat}}\) which can be potentiated by a rise in [Ca\(^{2+}\)]\(_i\), as described for that in guinea-pig ileal myocytes (25). However, it seems likely that a Ca\(^{2+}\)-activated Cl\(^{-}\) current is partly involved, especially in generation of the initial peak and oscillatory currents. The present demonstration of mI\(_{\text{cat}}\) and Ca\(^{2+}\)-activated Cl\(^{-}\) current implies that those currents, via causing depolarization, underlie the muscarinic contraction of mouse small intestinal muscles, especially the component sensitive to voltage-dependent Ca\(^{2+}\) channel blockers (13).

The present results from the experiments carried out to investigate signal pathways for mI\(_{\text{cat}}\) generation in the mouse intestinal myocytes almost overlapped those reported for mI\(_{\text{cat}}\) in guinea-pig ileal myocytes (5, 8, 9, 12, 20, 25). Actually, as found in the guinea-pig myocytes, the M\(_2\)-preferring antagonist methoctramine caused a rightward parallel shift of the carbachol concentration-response curve, while the M\(_3\)-preferring antagonist 4-DAMP severely depressed the maximum response without a notable change in the agonist EC\(_{50}\) value. The depression of the maximum response by 4-DAMP is likely due to blockade of M\(_3\) receptors but not due to blockade of cationic channels because the agent does not suppress mI\(_{\text{cat}}\) evoked by the direct G protein activator GTP\(_\gamma\)S in guinea-pig ileal smooth muscle cells (8). Either PTX or U73122 treatment selectively blocked the mI\(_{\text{cat}}\) response to carbachol. On the basis of these and other results, it has been suggested in the guinea-pig ileum that M\(_2\) and M\(_3\) receptors provide concurrent, but different signals for mI\(_{\text{cat}}\) generation, the M\(_2\) signal primarily activates cationic channels via PTX-sensitive G-protein G\(_\text{o}\), while the M\(_3\) signal exerts a permissive effect on channel gating via some G\(_\text{q}\)-independent PLC but without involvement of IP\(_3\) DAG or changes in [Ca\(^{2+}\)]\(_i\) (8 – 12). Hence, it is highly probable that such an M\(_2\)/M\(_3\) synergistic signal pathway
Evidence is accumulating for supporting that receptor-operated cationic channels in various visceral smooth muscles are homo- or heteromultimers of transient receptor potential (TRP) proteins, and cDNAs coding different TRP proteins are cloned from one type of smooth muscle tissue or cell (16, 27). In the present study, we made a comparative analysis of mI$_{cat}$ in mouse and guinea-pig intestinal myocytes. Between these myocytes, the agonist curves for mI$_{cat}$ activation resemble each other with respect to the EC$_{50}$ or Hill slope, and the both I-V curves exhibited a similar U-shape form. Also similar was observed in the single channel conductance estimated from non-stationary noise analysis, as being 39 pS for the mouse and 33 pS for the guinea pig. These results may reflect the similarity in the channel gating property that varies depending on different molecular basis of the channel. Therefore, it is plausible that if indeed the cationic channels activated via the M$_2$/M$_3$ synergistic pathway belong to the TRP channel family, those in the mouse and guinea-pig intestinal myocytes are of an identical subtype of TRP channel. However, G$_{max}$ activated by carbachol in the mouse myocyte (4.7 nS) was no more than one third of the guinea-pig G$_{max}$ (15 nS) despite the similarity in the cell size, reversal potential, or unitary channel conductance. Hence, the difference in G$_{max}$ might reflect different densities of the channel distribution.

Dresvyannikov et al. (28), using single channel recording techniques under symmetric Cs$^+$ solutions inside and outside the cell, detected two types of muscarinic cationic channel, 70 and 17 pS, in mouse ileal myocytes. Similarly, Zholos et al. (29) detected the three types (130, 57, and 10 pS) in guinea-pig ileal myocytes. The 70- and 57-pS channels were also demonstrated to contribute most to the whole-cell mI$_{cat}$ in the respective myocytes. Non-stationary noise analysis has a tendency to underestimate γ values of channels underlying a whole-cell current (30). Taking this into account, the 33- and 39-pS channels we detected by such analysis may correspond to the respective channels of 57 and 70 pS detected by single channel analysis.

It is of interest to note that non-stationary noise
Muscarinic $I_{\text{cat}}$ in Mouse Smooth Muscle

analysis of $I_{\text{cat}}$ in PTX-treated mouse myocytes detected two types of muscarinic cationic channel; one had a unitary conductance of 42 pS, probably corresponding to the 39-pS channel, and the other had a smaller value of 13 pS, which failed to be detected in intact myocytes. The reason for this would be explained by assuming that the 13-pS channels are activated via a PTX-insensitive pathway, but the resultant $I_{\text{cat}}$ is relatively so small that it can be unmasked only when activation of the 39-pS channels are markedly reduced.

In mouse gastric myocytes, the $M_3/G_q/PLC$ system has been suggested to induce $I_{\text{cat}}$ via the second messenger DAG (16). During the course of the present study, we observed that OAG, an analogue of DAG, produced a small sustained cationic current in mouse intestinal myocytes, and non-stationary noise analysis estimates the unitary conductance of OAG-gated channels to be about 10 pS, a similar value for muscarinic cationic channels (13 pS) detected in PTX-treated cells. Therefore, it is possible that not only does the $M_2/M_3$ synergistic system but the $M_3/G_q/PLC/DAG$ system also participates in $I_{\text{cat}}$ generation in the intestinal myocytes, although the relative contribution of the latter system is weak at least under conditions of $[Ca^{2+}]_i$ clamped at certain levels.

In conclusion, the present study suggests that the major signaling pathway for $I_{\text{cat}}$ generation in mouse intestinal smooth muscle cells is eventually the same as that suggested for guinea-pig ileum and that the channels responsible for $I_{\text{cat}}$ are indistinguishable in gating properties examined between the intestinal myocytes of both species. These results may promote further study to assess and analyze the possible $M_2/M_3$ synergistic system in gut smooth muscles using gene modified mice including those lacking $M_2$ or $M_3$ receptor. Our recent study using such knock-out mice has found an $I_{\text{cat}}$-generating system that absolutely requires both the $M_2$ and $M_3$ for its operation (31).

Fig. 8. Non-stationary noise analysis of CCh-evoked $I_{\text{cat}}$. A and C: CCh (100 µM)-evoked $I_{\text{cat}}$ in mouse (A) and guinea-pig (C) intestinal myocytes (upper trace) and their 0.4 Hz low-pass filtered (middle trace) and 0.4 – 800 Hz band-pass filtered (lower trace) signals; the filtered signals were used to calculate the mean current ($\bar{I}$) and the variance ($\delta^2$), respectively. B and D: the relationship between $\delta^2$ and $\bar{I}$ obtained from data shown in A and C, respectively. Each data point was fitted by a linear regression or quadratic function to estimate the unitary amplitude ($i$). The unitary channel conductance ($\gamma$) was then calculated by dividing an estimated $i$ by the driving force for $I_{\text{cat}}$ (see Materials and Methods).
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References