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Two Types of Cation Channel Activated by Stimulation of Muscarinic Receptors in Guinea-Pig Urinary Bladder Smooth Muscle

Masayuki Yamamoto[1], Toshihiro Unno[1,2], Hayato Matsuyama[2], Masanobu Kohda[3], Noriyuki Masuda[3], Masakazu Nishimura[1], Toshiaki Ishii[1], and Seiichi Komori[1,2,*]

[1]*Department of Pathogenic Veterinary Science, United Graduate School of Veterinary Science, Gifu University, Gifu 501-1193, Japan*
[2]*Laboratory of Pharmacology, Department of Veterinary Medicine, Faculty of Applied Biological Science, Gifu University, Gifu 501-1193, Japan*
[3]*Pharmacology Research Labs., Drug Discovery Research, Astellas Pharma Inc., Ibaraki 305-8585, Japan*

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Abstract. The present study, aiming to elucidate ion channel mechanisms underlying muscarinic receptor–induced depolarization, has characterized membrane currents induced by carbachol in single guinea-pig urinary bladder myocytes. Application of carbachol to cells that were voltage-clamped at −50 mV produced an atropine-sensitive, biphasic inward current consisting of an initial peak followed by a smaller sustained phase. Replacing the extracellular Na^+ and intracellular Cl^- with impermeable $tris^+$ and $glutamate^-$, respectively, demonstrated that the biphasic current is entirely composed of cation currents. Its initial peak phase was abolished by buffering intracellular Ca^{2+} to a constant level of 100 nM or depleting intracellular Ca^{2+} stores, and it was mimicked by the Ca^{2+} releaser caffeine. Ca^{2+} entry evoked by voltage steps in the sustained phase induced no noticeable change, indicating that this phase of cation current is insensitive to a rise of $[Ca^{2+}]_i$. These results demonstrate that muscarinic receptor stimulation invokes the openings of two types of cation channel, a Ca^{2+}-activated and a receptor-operated type; the former channels are gated by a rise in $[Ca^{2+}]_i$ upon intracellular Ca^{2+} release, and the latter are gated through other muscarinic receptor–coupled signal transduction mechanisms.

Keywords: cation channel, muscarinic receptor, carbachol, bladder smooth muscle, guinea pig

Introduction

The urinary bladder smooth muscle is densely innervated by cholinergic nerve fibers derived from the lumbosacral parasympathetic nerves. The cholinergic neurotransmitter acetylcholine causes its contraction by activating muscarinic receptors on the muscle cell membrane, which provides a substantial rise in intravesical pressure for micturition (1). Although M_2 and M_3 muscarinic receptors are both expressed in bladder muscle (2), the contractile responses to acetylcholine and other muscarinic agonists are believed to be mediated exclusively by the M_3 subtype (3, 4). The use of mutant mice lacking the M_2 or M_3 receptors has supported this idea (5, 6).

In various smooth muscles including those of the urinary bladder, contraction induced by stimulation of muscarinic receptors has been known to be accompanied by a depolarization that may trigger Ca^{2+} entry into the cell through voltage-dependent Ca^{2+} channels. Actually, muscarinic agonist-evoked contractions are abolished or markedly reduced by Ca^{2+}-entry blockers (7 – 9). Also, depolarization in response to muscarinic receptor stimulation has been demonstrated by the intracellular microelectrode method in the urinary bladder (10 – 12) and many other smooth muscle tissues (see ref. 13). Membrane current studies using patch-clamp techniques have attempted to characterize or identify ion channels responsible for muscarinic receptor–induced depolarization. In many smooth muscles studied, cation channels with low ion selectivity are found to be activated through a metabotropic signal transduction mechanism operated

*Corresponding author. skomori@gifu-u.ac.jp
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by muscarinic receptor stimulation, termed 'receptor-operated' cation channels, but in some of them, cation channels of another type, termed 'Ca^{2+}-activated' cation channels, are also gated, probably as a secondary effect of intracellular Ca^{2+} release (for rat ileum, see ref. 14; for canine gastric fundus, ref. 15). In the urinary bladder, however, no direct evidence has been provided for cation channel activation by muscarinic receptor stimulation; although there have been some positive results from analysis of contractions, the contractile responses produced by muscarinic receptor stimulation were significantly inhibited by replacing extracellular Na^+ with impermeable $NMDG^+$ or by blocking non-selective cation channels after drug treatment (8, 16). A patch-clamp study in pig urinary bladder myocytes has shown that muscarinic receptor stimulation causes Ca^{2+}-activated Cl^- channels to open upon intracellular Ca^{2+} release and suggested that this type of Cl^- channel is responsible for muscarinic receptor–induced depolarization (17).

Aiming to see whether muscarinic receptor stimulation causes cation channels to open in the urinary bladder smooth muscle, we have studied whole-cell membrane currents evoked by carbachol in single smooth muscle cells from a guinea-pig urinary bladder. The most extensively studied cation currents are found in guinea-pig ileal smooth muscle (18). Therefore, we chose guinea-pigs for the present study to compare the properties of cation channels in ileal and bladder myocytes. Our data clearly demonstrate that muscarinic receptor stimulation invokes the opening of two types of cation channel in bladder myocytes, the Ca^{2+}-activated and the receptor-operated type, through mechanisms associated with intracellular Ca^{2+} release and those independent of it, respectively. The characteristics of the latter type of cation channel seem to differ from those in ileal myocytes in terms of the sensitivity to intracellular Ca^{2+} and voltage dependency.

Materials and Methods

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

Cell preparation

Male guinea-pigs weighing 300 – 400 g were euthanized by a sudden blow to the head followed by cervical exsanguination. The urinary bladder was removed, placed in a Petri dish filled with a physiological salt solution (PSS, see below for composition), freed of the connective tissues, and then cut open in the ventral wall. After the tissue was freed of the mucosa, part of it was cut into small pieces (1 – 2-mm cubes) and incubated in nominally Ca^{2+}-free PSS containing collagenase type II (250 U/ml) and type XI (1300 U/ml), papain (11 U/ml), and bovine serum albumin (1 mg/ml) for 30 min at 34°C. The digested tissues were then transferred to fresh PSS containing 120 μM $CaCl_2$, and single smooth muscle cells were harvested by tissue agitation and subsequent centrifugation. Afterwards, they were re-suspended in PSS containing 500 μM $CaCl_2$, placed on cover slips in small aliquots, and stored at 4°C until use on the same day.

Membrane current recordings

Membrane currents were recorded at room temperature (23°C – 26°C) with patch pipettes (4 – 6 MΩ in tip resistance) using conventional whole-cell patch clamp techniques. Current signals were detected via an EPC7 patch-clamp amplifier (List, Darmstadt, Germany), filtered at 1 kHz and captured at a sampling rate of 4 kHz using an analog-digital converter (Digidata1320; Axon Instruments, Foster City, CA, USA) interfaced to a computer running with the pCLAMP program (version 9, Axon Instruments).

A cover slip with cells on it was placed on the bottom of a 0.5-ml organ bath, which was filled with 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). In experiments where carbachol- or caffeine-induced membrane currents, except for the Ca^{2+}-activated K^+ current ($I_{K\text{-}Ca}$), were recorded, PSS or a Cs^+-rich solution was used as the extracellular solution bathing the cells. The Cs^+-rich solution had either the same composition as PSS except for Cs^+ substituting for Na, or had the following composition: 120 mM CsCl, 12 mM glucose, and 10 mM HEPES (pH was adjusted to 7.4 with CsOH). The latter Cs^+-rich solution lacking the divalent cations Ca^{2+} and Mg^{2+} (Ca^{2+}/Mg^{2+}-free Cs^+-rich solution) has been used to magnify muscarinic receptor–operated cation currents in intestinal myocytes (19). In some experiments, the Na^+ in PSS was entirely replaced with the impermeable cation $tris^+$ ($tris^+$-rich solution). Pipette solution, with which the cells were intracellularly dialyzed, generally had the following composition: 134 mM CsCl, 1.2 mM $MgCl_2$, 10 mM MgATP, 1.0 mM Mg_2GTP, 0.05 mM EGTA, 14 mM glucose, and 10.5 mM HEPES (adjusted to pH 7.2 with CsOH). If necessary, Cl^- in the pipette solution was entirely replaced with the impermeable anion glutamate. In order for $[Ca^{2+}]_i$ to be buffered at a constant level, the following Cs^+-rich solution containing a BAPTA/calcium mixture was used as the pipette solution: 80 mM CsCl, 1.0 mM MgATP, 1.0 mM

Mg_2GTP, 5 mM creatine, 20 mM glucose, 10 mM HEPES, 10 mM BAPTA, and 4.6 mM $CaCl_2$ (adjusted to pH 7.4 with CsOH; Cs^+ = 144 mM in total, calculated free-ionized calcium = approximately 100 nM). All the Cs^+-rich pipette solutions served to prevent generation of a K^+ current.

When $I_{K\text{-}Ca}$ evoked by carbachol or caffeine was measured, cells were bathed in PSS and dialyzed with a pipette solution consisting of 134 mM KCl, 1.2 mM $MgCl_2$, 1.0 mM MgATP, 0.1 mM Mg_2GTP, 0.05 mM EGTA, 14 mM glucose, and 10.5 mM HEPES (adjusted to pH 7.2 with KOH) (20).

Membrane potential recordings

Changes in membrane potential of single myocytes were measured at room temperature using the nystatin-perforated patch-clamp technique (21). Cells were bathed in PSS and held under a current clamp using patch pipettes (3 – 5 MΩ) filled with a solution containing 134 mM KCl, 10.5 mM HEPES, and 0.2 mg/ml nystatin (adjusted to pH 7.4 with KOH). Membrane potential changes were amplified, filtered, and captured using the same equipment as described for membrane current recording.

Chemicals

The enzymes used for tissue digestion (papain and collagenase type II and type XI) were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Nystatin, atropine, carbachol, and thapsigargin were also from Sigma, and caffeine obtained from Wako Pure Chemical Industries, Ltd. (Osaka). All other chemicals were of reagent grade.

Thapsigargin was dissolved in dimethyl sulfoxide (DMSO) at more than 1000 times higher concentrations than that used in the experiments. Nystatin was initially dissolved in DMSO at 10 mg/ml, and then the stock solution was diluted with pipette solution to a final concentration of 0.2 mg/ml just before use.

Data analyses

The concentration-response curves for carbachol-evoked currents were measured by a cumulative application protocol and fitted with the logistic function $I = I_{max}\{1 + ([EC_{50}] / [A])^h\}^{-1}$, where I is the current amplitude, I_{max} is the maximal amplitude, EC_{50} is the concentration required for the agonist to produce half of I_{max}, and h is the Hill slope. Current-voltage (I-V) relationships for the carbachol-induced currents were investigated with a negative-going ramp from +50 to −120 mV over 5 s. In the construction of I-V curves, the leak current evoked by the ramp pulse in the absence of carbachol was subtracted from the current evoked by the same pulse in its presence, and net current amplitudes at different potentials were normalized by a net current at −50 mV. Activation curves obtained by dividing the amplitudes of the carbachol-induced current by the driving forces were fitted by the Boltzmann equation in the following form: $G = G_{max} / \{1 + e^{(V-V_{0.5})/k}\}$, where G is the membrane conductance at potential V, G_{max} is the maximal membrane conductance, $V_{0.5}$ is the potential at which $G = 0.5G_{max}$, and k is the slope factor of the activation curve.

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

Results

Inward currents

Single myocytes isolated from a guinea-pig urinary bladder were bathed in PSS (a Na^+-rich solution), dialyzed with Cs^+-rich pipette solution, and held under a voltage clamp at −50 mV close to their resting membrane potential (E_{rest} = approximately −48 mV, see below). Under these conditions, application of 1 μM carbachol [around EC_{50} in producing contraction in guinea-pig detrusor muscles (22)] evoked no detectable inward current or induced a slight sustained inward current accompanied by high-frequency noise (Fig. 1A). The sustained current amplitude varied from almost null to 13 pA among different cells with a mean of 3.6 ± 1.7 pA (n = 8) when measured just before the washout of carbachol. Applied at the higher concentration of 10 μM [supra-maximally effective in producing contraction (22)], carbachol induced an inward current that rapidly reached a peak followed by a smaller sustained phase, as shown in Fig. 1B. The initial current lasted 5 to 10 s and had a peak amplitude of 28.0 ± 5.2 pA (n = 9, Fig. 1G). The subsequent sustained current was accompanied by a marked increase in noise and sometimes declined with time even in the continued presence of carbachol. The mean amplitude of the sustained inward current was 3.1 ± 0.3 pA (n = 9, Fig. 1G). When atropine (1 μM) was applied 1 min before and after, carbachol (10 μM) was without effect at all (n = 3, Fig. 1C), indicating that the carbachol-induced inward currents are totally mediated by muscarinic receptors.

In general, compared with Na^+, Cs^+ is relatively more permeable to non-selective cation channels in the various smooth muscles studied (23). Therefore, we examined the effects of substituting Cs^+ for the extracellular Na^+. As exemplified in Fig. 1D, the biphasic inward current response induced by carbachol was made

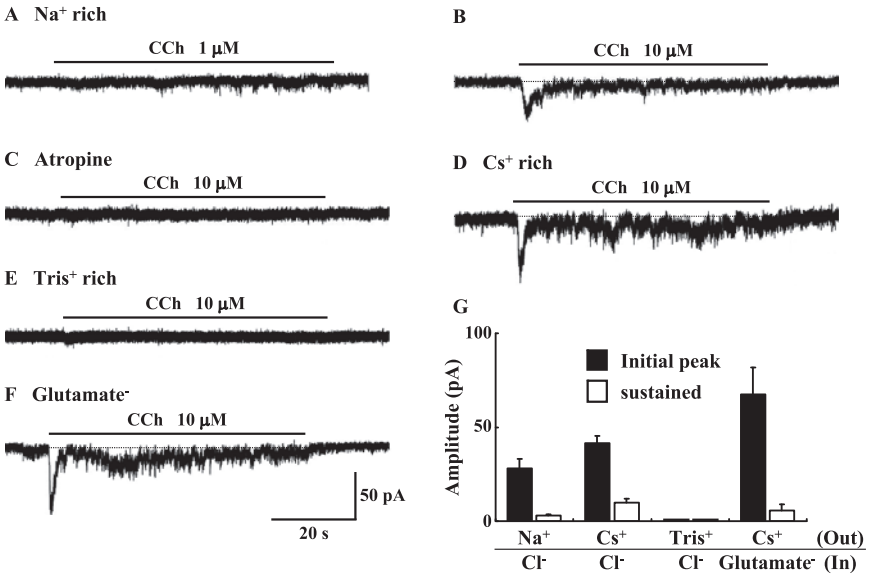

Fig. 1. Inward current responses to carbachol (CCh) in single guinea-pig urinary bladder myocytes. Cells were voltage-clamped at −50 mV using patch pipettes filled with a Cs^+-rich solution (A – E) or the same solution with glutamate$^-$ substituted for Cl^- (F). A – F: typical examples of current responses to CCh (1 or 10 μM) in cells bathed in normal PSS (A – C), Cs^+-rich solution (D and F), or $tris^+$-rich solution (E). In C, atropine (1 μM) was added as pretreatment 1 min before CCh application. The dotted lines in each trace in this and subsequent figures indicate the initial current level before drug application. G: mean amplitudes of initial peak and subsequent sustained currents evoked by 10 μM CCh under ionic conditions as in B and D – F. A – F: from different cells. Note that the results indicate the CCh-induced currents to be totally composed of cation currents. See text for details.

more prominent, so that the initial peak and subsequent sustained currents were significantly increased to 41.5 ± 3.8 and 9.8 ± 2.1 pA (n = 14), respectively (Fig. 1G). When extracellular Na^+ or Cs^+ was substituted with $tris^+$, an impermeable cation, the holding current at −50 mV was decreased by 5 – 10 pA, suggesting that the background current constitutively flows through the cation channel at the holding potential. In the presence of extracellular $tris^+$, carbachol evoked no appreciable inward current (n = 2) or slightly induced only the initial component (n = 4, Fig. 1E). We further examined the influence of substituting the Cl^- in the pipette solution with the impermeable anion glutamate, where Cs^+-rich solution was used as a bath solution, as in Fig. 1D. As shown in Fig. 1F, carbachol (10 μM) induced a biphasic inward current that was similar in form or amplitude to such a control response as in Fig. 1D. Actually, the amplitudes of the initial peak and subsequent sustained currents (60.7 ± 12.4 and 5.6 ± 3.2 pA, respectively; n = 4) (Fig. 1G) resembled the respective control values (41.5 ± 3.8 and 9.8 ± 2.1 pA, respectively).

The results clearly demonstrate that stimulation of muscarinic receptors by carbachol produces cation currents in guinea-pig urinary bladder myocytes.

Ca^{2+} dependence of muscarinic receptor–operated cation currents

Experiments were carried out in near-symmetrical Cs^+ solutions, as described for the experiment in Fig. 1D, unless otherwise stated. Infusion of the Ca^{2+}-chelating agent EGTA (20 mM) into the cell via patch pipettes resulted in abolition of the initial peak of the biphasic current responses to 10 μM carbachol and inhibition of the sustained inward current (Fig. 2B). The remaining sustained current evoked had a considerably smaller amplitude of 1.5 ± 0.7 pA (n = 5) than the control value shown in Fig. 1G (9.8 ± 2.1 pA). Under conditions of $[Ca^{2+}]_i$ buffered to 100 nM with a BAPTA/$CaCl_2$ buffer system (see Methods), the initial peak was eliminated again, but a slow, sustained current was elicited with an amplitude of 9.3 ± 2.0 pA (n = 15) (Fig. 2C), which was comparable to the control value (9.8 ± 2.1 pA). Similar results were obtained in cells treated with thapsigargin (1 μM), which is known to cause depletion of intra-

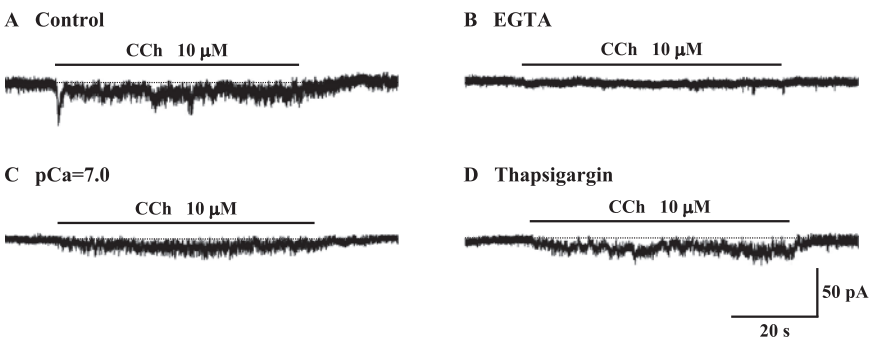

Fig. 2. Ca^{2+} dependency of the CCh-evoked cation currents. Cells were bathed in Cs^+-rich solution; held at −50 mV; and dialyzed with Cs^+-rich solution (A and D), Cs^+-rich EGTA solution (B), or Cs^+-rich pCa 7.0 solution (C). In D, thapsigargin (1 μM) was applied as pretreatment 1 min before CCh application. A – D: from different cells.

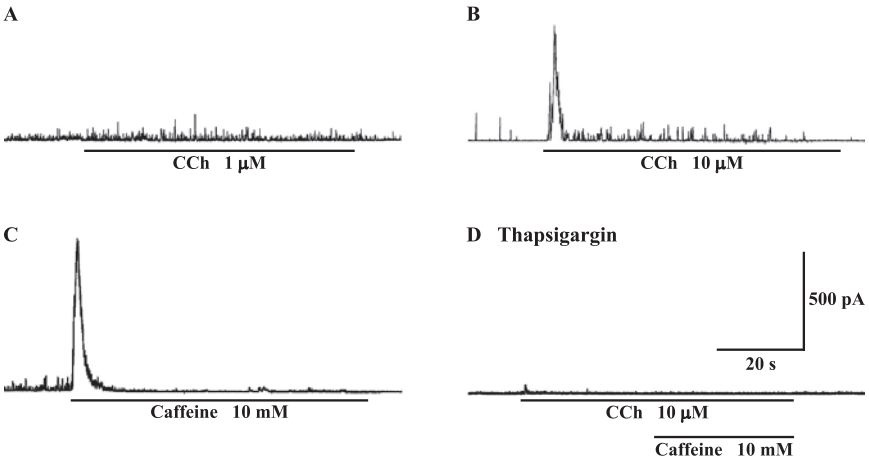

Fig. 3. Ca^{2+}-activated K^+ currents ($I_{K\text{-}Ca}$) evoked by 1 μM (A) or 10 μM (B and D) CCh and 10 mM caffeine (C and D) in cells bathed in PSS, dialyzed with a K^+-rich solution, and held at 0 mV. In D, thapsigargin (1 μM) was pretreated 1 min before CCh application. A – D: from different cells.

cellular Ca^{2+} stores (Fig. 2D and see also Fig. 3D). Sustained currents evoked by carbachol in these cells had an amplitude of 5.9 ± 1.1 pA (n = 5), which was again comparable to the control value. These findings strongly suggested that the initial peak of the biphasic current response is closely associated with intracellular Ca^{2+} release.

So we examined the Ca^{2+}-releasing activity of carbachol in these myocytes by measuring the Ca^{2+}-activated K^+ current ($I_{K\text{-}Ca}$), which represents the massive release of stored Ca^{2+} within the cell (24). Cells were bathed in PSS, filled with K^+ via patch pipettes, and held at 0 mV close to the reversal potential for cation currents (see Fig. 7A). Upon application of 1 μM carbachol, no prominent $I_{K\text{-}Ca}$ was evoked in two out of six cells. However, in the remaining four cells, brief, transient outward $I_{K\text{-}Ca}$ occurred sporadically (Fig. 3A). When carbachol was applied at 10 μM, a rapid and massive $I_{K\text{-}Ca}$ of 647 ± 77 pA (n = 7) lasting some 5 s was initially evoked, and then small, brief $I_{K\text{-}Ca}$ occurred in frequent bursts. Thus, the initial outward $I_{K\text{-}Ca}$ was a mirror image of the initial peak of the biphasic inward current (c.f. Fig. 3B and Fig. 1). A similar outward $I_{K\text{-}Ca}$ (mean amplitude: 2866 ± 414 pA, n = 5) was also evoked by a potent Ca^{2+}-store releaser, caffeine (10 mM), which was capable of depleting stored Ca^{2+} in guinea-pig ileal myocytes (20). After the initial outward $I_{K\text{-}Ca}$ evoked by caffeine, brief, spontaneous outward $I_{K\text{-}Ca}$ no longer occurred, indicating depletion of internal Ca^{2+} stores. As expected, the carbachol- or caffeine-evoked $I_{K\text{-}Ca}$ was blocked by thapsigargin treatment (Fig. 3D). These results corroborated the idea that the initial peak cation current is closely associated with intracellular Ca^{2+} release.

Since caffeine also evoked a massive $I_{K\text{-}Ca}$, we examined whether caffeine mimics carbachol's effect in eliciting a brief, rapid cation current. In symmetrical

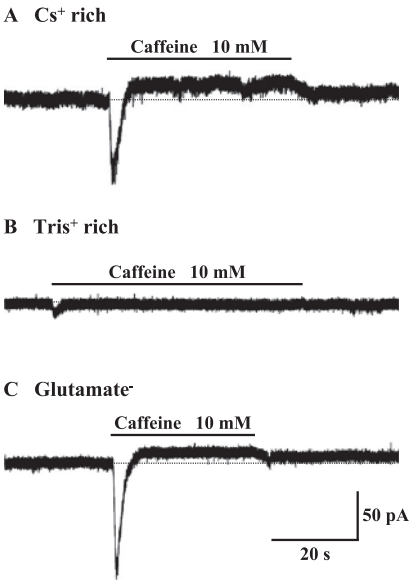

Fig. 4. Inward current responses to caffeine (10 mM) in cells bathed in Cs^+-rich solution (A and C) or $Tris^+$-rich solution (B), dialyzed with a Cs^+-rich solution (A and B) or Cs^+-rich $glutamate^-$ solution (C). Cells were held at −50 mV. A–C: from different cells.

Cs^+ solutions, 10 mM caffeine induced an initial rapid inward current lasting some 5 s and having an amplitude of 37.7 ± 7.8 pA (n = 16). The inward current was followed by a smaller, sustained outward current of 4.7 ± 1.0 pA (n = 16), as shown in Fig. 4A. The biphasic current response to caffeine was almost totally blocked by replacement of the extracellular Cs^+ with $tris^+$, although in a few cells, an initial inward current was still evoked with smaller amplitude (Fig. 4B). On the other hand, the caffeine response was normally seen when $glutamate^-$ was substituted for intracellular Cl^- (Fig. 4C). The initial inward and subsequent outward currents in $glutamate^-$-filled cells had respective amplitudes of 32.1 ± 8.6 and 4.4 ± 1.0 pA (n = 6). These values were

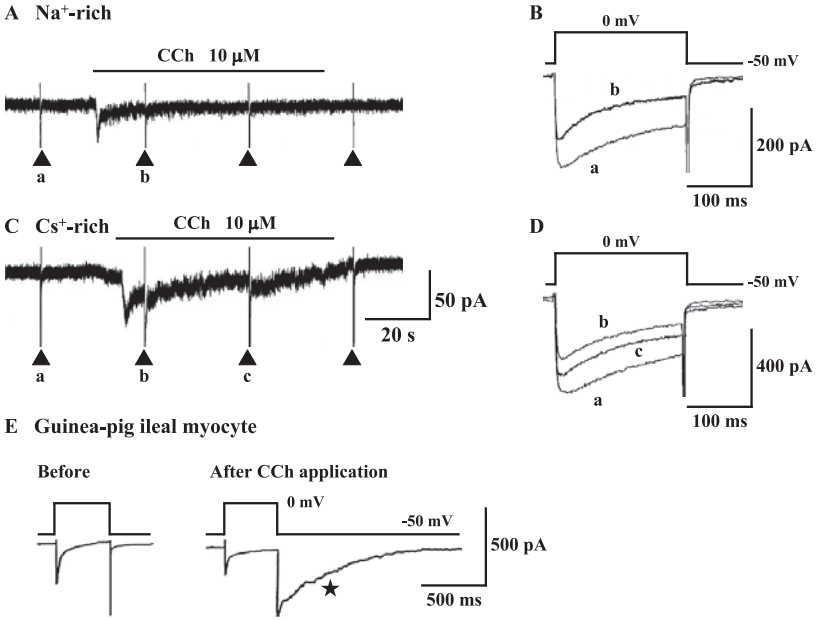

Fig. 5. Effects of voltage-gated Ca^{2+} entry on CCh-evoked sustained inward currents. Cells were dialyzed with Cs^{+}-rich solution and bathed in PSS (A) or Cs^{+}-rich solution (C). Voltage steps from −50 to 0 mV (duration: 200 ms) were applied (closed triangles) to evoke voltage-gated Ca^{2+} currents in the absence and presence of CCh (10 μM). The time-expanded traces of Ca^{2+} currents in the absence (a) and presence of CCh (b and c) in A and C are shown in B and D, respectively. E: in guinea-pig ileal myocytes, the Ca^{2+} current evoked in the presence of CCh was followed by a great inward current at the end of the voltage step, as marked by the closed star.

not significantly different from the corresponding values in Cl^{-}-filled cells. These findings indicated that caffeine can mimic carbachol's effect in terms of the initial inward current component. Regarding the sustained component of the caffeine-induced current, it is explained by assuming that the sustained outward current may not be due to the occurrence of a "net outward current", but may result from reductions of the background cation current constitutively flowing inwardly at the holding potential of −50 mV. Caffeine is known to block various ion channels including those responsible for background conductance of the cell membrane (25 – 27). Such an inhibitory effect of caffeine may disappear if background cation conductances are already blocked by replacement with $tris^{+}$.

To see whether the carbachol-induced sustained cation currents are potentiated by a rise in $[Ca^{2+}]_i$, Ca^{2+} currents were activated by a 0.2-s voltage step to 0 mV to increase $[Ca^{2+}]_i$ in the presence of carbachol (10 μM). Experiments were done in PSS or a Cs^{+}-rich solution (see Fig. 5: A and C). In either case, the Ca^{2+} currents evoked were not followed by any additional inward current at cessation of the voltage step, as seen from the time-expanded recording traces (Fig. 5: B and D). We performed similar experiments using guinea-pig gut myocytes. As exemplified in Fig. 5E, the Ca^{2+} current evoked in the presence of carbachol was followed by a great inward current at the end of the voltage step, as previously reported (28). Thus, the sustained cation currents induced by carbachol in urinary bladder myocytes seems insensitive to a rise in $[Ca^{2+}]_i$.

The overall findings suggest that the initial peak phase of the carbachol-induced biphasic inward current is due to Ca^{2+}-activated cation channels gated by a rise in $[Ca^{2+}]_i$ upon intracellular Ca^{2+} release, while the smaller sustained phase is due to receptor-operated cation channels through other signal transduction mechanisms coupled with muscarinic receptor.

Muscarinic receptor–operated cation currents ($_mI_{cat}$)

The carbachol-induced sustained cation current or $_mI_{cat}$ was further characterized under conditions optimal for activation and isolation of $_mI_{cat}$ (nearly symmetrical Cs^{+} solutions with both Ca^{2+} and Mg^{2+} omitted in the bath solution and $[Ca^{2+}]_i$ buffered to 100 nM) (19, 23).

Figure 6A shows a typical example of $_mI_{cat}$ evoked by cumulative applications of the ascending series of carbachol concentrations (1 – 300 μM). The current response increased as the agonist concentration was increased, and the maximum response was attained at 10 or 30 μM. By curve fitting of data from individual cells, the I_{max}, EC_{50}, and Hill slope were estimated to be 33.3 ± 4.5 pA, 2.4 ± 0.7 μM and 1.3 ± 0.4 on average (n = 4), respectively (Fig. 6B).

A negative-going ramp pulse from 50 mV up to −120 mV over 5 s was applied before and during 10 μM carbachol application (see the inset in Fig. 7A), and the I-V relationship for carbachol-induced $_mI_{cat}$ was constructed with the leakage subtraction method. Figure 7A shows an averaged I-V relationship obtained from 4 different cells, in each of which net amplitudes of the $_mI_{cat}$ at different potentials were normalized by the current amplitude at −50 mV. Current polarity was reversed at a potential close to 0 mV. From the reversal potential 0 to −50 mV, the current increased in proportion to the increase in the electromotive force, but further

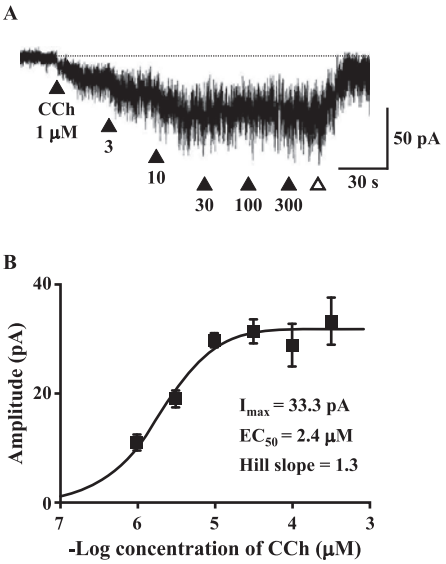

Fig. 6. Concentration dependency of muscarinic receptor–operated, sustained cation currents ($_mI_{cat}$). Cells were bathed in Ca^{2+}/Mg^{2+}-free, Cs^+-rich solution, dialyzed with Cs^+-rich pCa 7.0 solution, and held at −50 mV. A: a typical example of the $_mI_{cat}$ response to CCh applied at ascending concentrations (1 – 300 μM) as indicated by the closed triangles. The open triangle indicates the point of the wash-away of CCh. B: the averaged concentration-response relationship of CCh-evoked $_mI_{cat}$. Each point indicates the mean ± S.E.M. of measurements in 4 cells and is fitted by the Hill equation. The maximal response (I_{max}), EC_{50}, and the Hill slope estimated by curve fitting are shown.

increase in negativity no longer caused a prominent increase in the current.

Figure 7B shows the conductance activation curve obtained from the averaged I-V relationship in Fig. 7A. With curve fitting, the G_{max}, $V_{0.5}$, and slope factor for the curve were estimated to be 1.4 nS, −88.9 mV, and 40.3 mV, respectively.

Membrane potential responses

To assess the physiological relevance of the carbachol-induced cation currents, we observed changes in the membrane potential produced by the agonist, using the nystatin-perforated patch-clamp technique (21). Cells were bathed in PSS, dialyzed with a K^+-rich pipette solution, and held under current-clamp mode. Under such equi-physiological conditions, the cells had an E_{rest} of −47.7 ± 1.9 mV (n = 22), and many of them (70%) spontaneously generated action potentials at a frequency of 0.55 ± 0.11 Hz (n = 15).

Application of 1 μM carbachol resulted in the acceleration or generation of spike activity and a sustained depolarization that considerably varied in size from almost null to 30 – 40 mV among different cells (n = 12) (Fig. 8: A and B). Especially, in cells responding with a large depolarization, the membrane potential reached a level close to the equilibrium potential (about −10 mV) reported for muscarinic receptor–induced depolarization in physiological ionic gradients (29, 30), and the spike activity was soon arrested (open triangle in Fig. 8B). Such a large depolarization was more often evoked by 10 μM carbachol (Fig. 8C) and the size of depolarizations evoked in 10 cells tested was 30.7 ± 2.0 mV on average. The depolarizations evoked by 10 μM peaked more rapidly than those evoked by 1 μM, but they were also more frequently interrupted by brief hyperpolarizations (closed triangles in Fig. 8C). These observations are consistent with the idea that carbachol-induced cation currents are relevant in producing depolarization in urinary bladder myocytes.

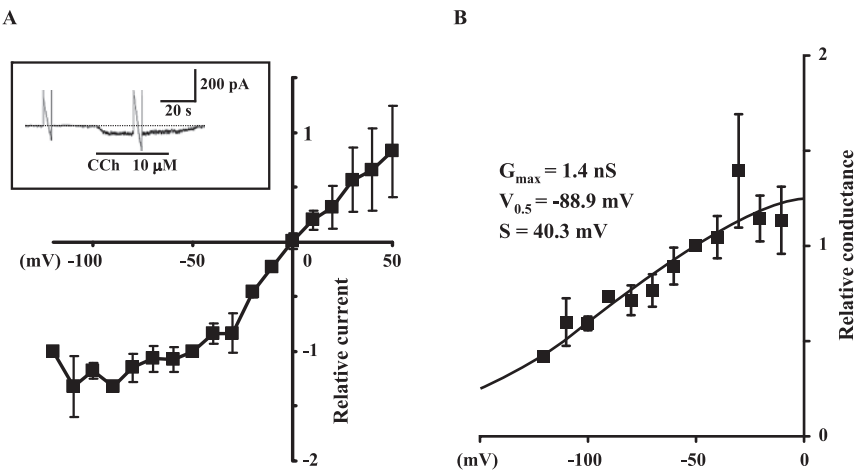

Fig. 7. Voltage dependency of CCh-evoked $_mI_{cat}$. A: the averaged current-voltage (I-V) relationship for $_mI_{cat}$ evoked by CCh (10 μM). Each point indicates the mean ± S.E.M. of measurements in 4 cells. The current amplitude is expressed relative to the value at −50 mV, which was taken as −1 in each cell. In the inset, a typical example of CCh-evoked $_mI_{cat}$, recorded under the same conditions as in Fig. 6 but with the application of a negative-going ramp pulse from 50 mV up to −120 mV over 5 s before and during the generation of $_mI_{cat}$, is shown. B: the activation curve derived from the I-V relationship shown in A and fitted by the Boltzmann equation (superimposed smooth line). The conductance is expressed relative to the value at −50 mV, which was taken as +1 in each cell. The maximal membrane conductance (G_{max}), potential of half-maximal activation ($V_{0.5}$), and slope factor (S) estimated by the curve fitting are shown.

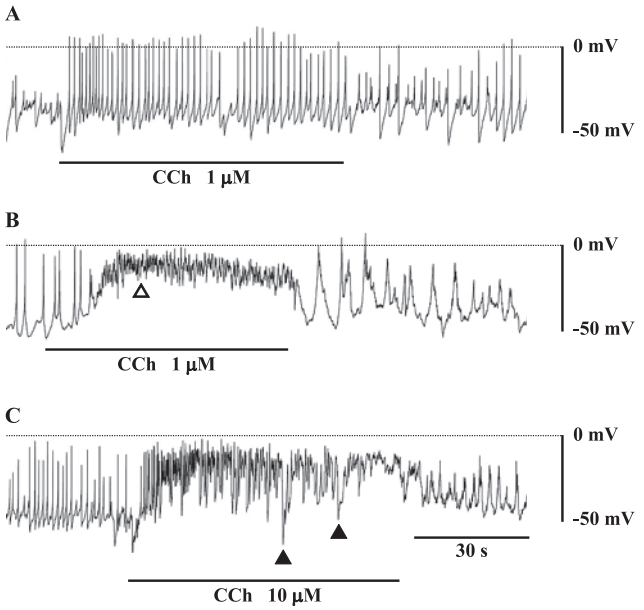

Fig. 8. The effects of CCh on the electrical membrane activity in single bladder myocytes. Changes in the membrane potential were measured by the nystatin-perforated patch-clamp technique. CCh (1 or 10 μM) produced an increased frequency of action potential discharge with a slight sustained depolarization (A) or with a strong depolarization that caused a depolarization block of the spike discharge (open triangle, B and C). In C, the depolarization was frequently interrupted by brief hyperpolarizations (closed triangles). A – C: from different cells. See text for details.

Discussion

Both M_2 and M_3 muscarinic receptors are found in urinary bladder smooth muscle, but contractions by muscarinic agonists including carbachol are regarded as exclusively mediated by the M_3 receptor (3, 4). Furthermore, evidence has suggested that M_3-mediated contractions are brought about through the signal transduction pathways involving Gq-type G proteins and phospholipase C_β (PLC_β), leading to the formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (2, 4). The IP_3 formed serves to mobilize intracellular Ca^{2+} by releasing stored Ca^{2+} within the cell, while the DAG phosphorylates various proteins, and their actions result in excitation or depolarization and contraction of the smooth muscle.

The present whole-cell voltage clamp experiments have revealed that stimulation of muscarinic receptors by carbachol produces inward cation currents in guinea-pig urinary bladder myocytes (Fig. 1). Application of 10 μM carbachol evoked an atropine-sensitive inward current with its form consisting of an initial peak followed by a smaller sustained phase. The current response was totally abolished by substitution of extracellular Na^+ with the impermeable cation $tris^+$, but was not significantly affected by the substitution of intracellular Cl^- with the impermeable anion $glutamate^-$. Hence, it is highly probable that the biphasic inward current occurs mostly because of the opening of cation channels. To the best of our knowledge, this is the first demonstration that cation channels are activated by muscarinic receptor stimulation in urinary bladder smooth muscle.

Further analyses of the biphasic inward currents induced by carbachol using EGTA, a mixture of BAPTA and calcium, or thapsigargin provided evidence that their initial peak phase is caused by a rise in $[Ca^{2+}]_i$ upon intracellular Ca^{2+} release. Actually, in the present study 10 μM carbachol evoked a massive release of stored Ca^{2+} in these myocytes from $I_{K\text{-}Ca}$ (Fig. 3). The present data also demonstrated that caffeine shares an effect with carbachol: namely, eliciting an initial, brief cation current accompanied with release of stored Ca^{2+}. The sustained component of the carbachol-induced biphasic response was not abolished by the BAPTA/Ca^{2+} mixture or thapsigargin. Moreover, it was not noticeably modulated by Ca^{2+} entering the cell via voltage-gated Ca^{2+} channels (Fig. 5). This may make it unlikely that the initial peak phase is a consequence of the potentiation of the sustained inward current by Ca^{2+} released from stores. The sustained inward current was markedly reduced by treatment with EGTA, suggesting that the sustained phase requires a certain level of intracellular Ca^{2+} for its generation. Taken together, we conclude that the initial peak and sustained inward currents are mediated by two different types of cation channels. The former current occurs because of the opening of Ca^{2+}-activated cation channels by a rise in $[Ca^{2+}]_i$ upon release of stored Ca^{2+}, probably via the M_3/Gq/PLC_β/IP_3 signaling system. Ca^{2+}-activated cation currents have been observed in rat ileal and canine gastric myocytes exposed to carbachol (14, 15). The latter current occurs because of the opening of receptor-operated cation channels of the type originally envisaged (18, 31).

The sustained cation currents or $_mI_{cat}$ evoked by carbachol in urinary bladder myocytes were almost insensitive to a rise in $[Ca^{2+}]_i$ upon voltage-gated Ca^{2+} entry. This is different from $_mI_{cat}$ in guinea-pig and mouse intestinal myocytes. Because of its high Ca^{2+} sensitivity, intestinal $_mI_{cat}$ is strongly potentiated upon voltage-gated Ca^{2+} entry as well as intracellular Ca^{2+} release (refs. 18 and 19; also see Fig. 5E in the present paper). Our present analysis of the concentration–response curves for $_mI_{cat}$ activation by carbachol provided mean values of 2.4 μM for EC_{50}, 1.3 for the Hill slope, and 33.3 pA for I_{max}; the former two values resemble those reported for the intestinal $_mI_{cat}$, whereas the latter one is as small as 5% – 10% of the I_{max} of the

intestinal $_mI_{cat}$ (19). This is also true of the G_{max} value estimated from the conductance activation curves for $_mI_{cat}$ (32). The difference in the I_{max} or the G_{max} does not involve cell size, as the whole-cell membrane capacitance is not so different between the two types of myocytes [54.4 ± 2.9 pF, n = 13 for the urinary bladder; 53.7 ± 1.2 pF for the ileum (33)]. As for I-V curves, the intestinal $_mI_{cat}$ displays a U-shaped I-V curve with a clear trough at −50 mV (19, 32), whereas the present $_mI_{cat}$ had no prominent trough, declining gradually in the potential range from −50 to −120 mV (Fig. 7A). The difference in these $_mI_{cat}$ profiles makes it likely that the signal transduction pathways and/or the biophysical properties of cation channels involved in the generation of $_mI_{cat}$ are different in the two types of smooth muscle.

Extensive studies of the intestinal $_mI_{cat}$ have suggested that it is brought about through a signaling pathway that absolutely requires both M_2 and M_3 receptors (19, 20, 34) and that its major part is mediated by cation channels of 60 – 70 pS unitary conductances (33, 35, 36). Our recent study in M_2-knockout mouse intestinal myocytes has found another intestinal $_mI_{cat}$ that is mediated by M_3 receptors alone (36). Interestingly, the M_3-mediated $_mI_{cat}$ has only a small maximum amplitude of some 24 pA in symmetrical Cs^+ solutions, is insensitive to voltage-gated Ca^{2+} entry, and displays an I-V curve that is not U-shaped but rather roughly linear (36). Furthermore, this current is suggested to be mediated by both 60-pS and 120-pS cation channels activated via the M_3/Gq/PLCβ/DAG signaling system (36). Our present suggestion is that the urinary bladder $_mI_{cat}$ involves a similar signaling system and cation channels in its generation.

The primary role of cation channels activated by muscarinic receptor stimulation is to depolarize the membrane. The present current-clamp experiments on single myocytes revealed that carbachol produced a significant depolarization; if it is too strong, spike discharges cease because of depolarization block. A full depolarization (30 – 40 mV in size) produced by 10 μM carbachol reached a peak more rapidly than that by 1 μM did. The faster depolarization at 10 μM is at least partly caused by the initiation of a rapid, brief cation current by carbachol at this concentration. Membrane depolarizations produced by 10 μM carbachol were often interrupted by brief hyperpolarizations (closed triangles in Fig. 8C). This might reflect the activation of Ca^{2+}-activated K^+ channels brought about by sporadic Ca^{2+} release from internal stores (see Fig. 3B), since a localized, small amount of released Ca^{2+} (Ca^{2+} sparks) is capable of activating this type of K^+ channels and thereby hyperpolarizing the membrane in several types of smooth muscle cells (37, 38).

With substitution of $tris^+$ for Na^+ in the bath solution, a brief inward current, very small in size, was still occasionally evoked by carbachol or caffeine (see Fig. 1E and Fig. 4B). This current might be a Ca^{2+}-activated Cl^- current. Actually, Kajioka et al. (17) have demonstrated that carbachol or caffeine evokes Ca^{2+}-activated Cl^- currents by activating intracellular Ca^{2+} release in pig urinary bladder myocytes. Therefore, it seems likely that the Ca^{2+}-activated Cl^- current, as well as $_mI_{cat}$ and the Ca^{2+}-activated cation current, contributes to the muscarinic receptor–induced depolarization observed in single cells (the present study) and whole tissues of urinary bladder smooth muscle (10, 11).

In summary, we have demonstrated that guinea-pig urinary bladder myocytes are endowed with two types of cation channel that are activated by muscarinic receptor stimulation. They are of the Ca^{2+}-activated type and of the receptor-operated type. The former-type channel is gated by a rise in $[Ca^{2+}]_i$ upon intracellular Ca^{2+} release, probably mediated by IP_3 formed at that time, while the latter type is activated through some other mechanism(s) not involving the IP_3-induced Ca^{2+} release. The present findings provide new insights into the signal transduction mechanisms underlying the excitation and contraction produced by muscarinic receptor stimulation in urinary bladder smooth muscle. Further studies are needed to identify the molecular basis of muscarinic receptor–operated cation channels and to elucidate their activation mechanisms in more detail.

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