Effects of excitatory neurotransmitters on Ca^{2+} channel current in smooth muscle cells isolated from guinea-pig urinary bladder

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- 1 A whole-cell voltage clamp technique was used to examine the effects of purinoceptor and muscarinic receptor agonists on voltage-sensitive Ca^{2+} channels in guinea-pig isolated urinary bladder cells.
- 2 When the cell membrane was clamped at the holding potential, rapid application of ATP elicited a large inward current in normal solution containing 2.5 mM Ca^{2+} , and reduced the subsequent Ca^{2+} channel current evoked by a depolarizing pulse (0 mV). Carbachol (CCh) elicited little membrane current, but similarly reduced the Ca^{2+} current.
- 3 When purinoceptor agonists were rapidly applied during conditioning depolarizations at +80 mV, an outward current was elicited, and the Ca^{2+} channel current evoked by the subsequent test potential of 0 mV was not affected. Application of CCh at +80 mV also elicited an outward current, but it reduced the subsequently evoked Ca^{2+} current.
- 4 The inhibitory effect of muscarinic agonists on the Ca^{2+} channel current was attenuated by caffeine (10 mM).
- 5 In Ca^{2+} -free, low- Mg^{2+} solution, a Na^{+} current flowing through voltage-dependent Ca^{2+} channels was evoked by depolarization. This current was not reduced by bath application of purinoceptor agonists (ATP and α,β -methylene ATP).
- 6 These results suggest that the main effect of purinoceptor stimulation is opening of non-selective cation channels, and that muscarinic stimulation triggers Ca^{2+} release from intracellular stores. Voltage-sensitive Ca^{2+} channels are inactivated through an increase in intracellular Ca^{2+} induced by either activation of purinoceptor or muscarinic receptors.

Keywords: Smooth muscle; urinary bladder; Ca^{2+} channels; non-selective cation channels; purinoceptors; muscarinic receptors

Introduction

In the urinary bladder of most mammals, contraction elicited by nerve stimulation is diminished, but not completely abolished by atropine, a muscarinic antagonist (Langley & Anderson, 1885; Henderson & Roepke, 1934). In the guinea-pig, the response to local application of adenosine 5'-triphosphate (ATP) mimics that to non-cholinergic nerve stimulation (Burnstock *et al.*, 1972), and ATP is released from intramural nerves (Burnstock *et al.*, 1978). Thus, in urinary bladders of various species both ATP and acetylcholine (ACh) have been proposed as excitatory neurotransmitters (Fujii, 1988). However, the underlying mechanism involved in the action of these neurotransmitters seems to be quite different. Purinoceptor agonists depolarize the cell membrane (Fujii, 1988), whereas muscarinic agonists produce little or no change in membrane potential (Creed, 1971; Creed *et al.*, 1983).

Recently, in smooth muscle cells isolated from guinea-pig urinary bladder, patch clamp techniques have revealed a transient activation of non-selective cation channels by ATP (Inoue & Brading, 1990). Inward current through these channels is carried by Ca^{2+} ions as well as other monovalent cations. ACh is also known to activate a non-selective cation conductance in many mammalian smooth muscle cells (Benham *et al.*, 1985). In guinea-pig ileum, the ACh-induced cation conductance is facilitated by depolarization and intracellular Ca^{2+} (Inoue & Isenberg, 1990a,b). In canine gastric smooth muscle cells (Sims, 1992), and in canine and guinea-pig tracheal smooth muscle cells (Janssen & Sims, 1992), it has recently been suggested that ACh-induced Ca^{2+} release from intracellular stores triggers activation of the non-selective cation current. However, in guinea-pig detrusor cells, an ACh-activated conductance has not yet been clearly demonstrated.

Voltage-dependent Ca^{2+} channels are also known to play an important role in excitation-contraction coupling. Previously, we described the activation and inactivation properties of the Ca^{2+} channels in guinea-pig detrusor cells (Nakayama & Brading, 1993a,b). In the present study, the membrane currents evoked by these two excitatory neurotransmitters, and their interaction with the voltage-dependent Ca^{2+} channels have been examined. The experiments were carried out with only a low concentration of Ca^{2+} chelator (0.1 mM EGTA) in the pipette, to allow the effects of changes in $[\text{Ca}^{2+}]_i$ on membrane currents to be observed. To investigate the involvement of Ca^{2+} entry in the responses, in some experiments the agonists were applied in the absence of extracellular Ca^{2+} , or during large depolarizations (+80 mV) to reduce the driving force for Ca^{2+} entry.

Methods

Single detrusor cells

Male guinea-pigs (450–750 g) were killed either by stunning, or cervical dislocation, or decapitation after anaesthesia by halothane. The urinary bladder was immediately dissected. Muscle strips (total approximately 0.1 g wet weight), from which the epithelium had been removed, were incubated in a nominally Ca^{2+} -free solution for 60 min (at 35°C). Subsequently, the strips were digested in an enzyme-containing (0.05% collagenase and 0.1% pronase) Ca^{2+} -free solution for 10–20 min, and then agitated with a glass pipette. Some of the cell suspension was stored at 5°C and used for up to 6 h.

Whole-cell voltage clamp

A conventional whole-cell configuration of the patch clamp method was used (Hamill *et al.*, 1981). Single smooth muscle

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cells were allowed to settle in a recording bath, and continuously superfused with physiological saline. The resistance of the patch pipette used was in the region of 5 M Ω . The membrane potential was clamped with a List amplifier (EPC 7, Germany). Unless otherwise stated, the membrane potential was clamped at -60 mV (holding potential). An AD/DA converter (DT 2801A, Data Translation, U.K.) was used for voltage step generation and on-line data acquisition. Data were collected on an IBM compatible personal computer using the Quick Basic software package. The membrane current was also monitored by a digital storage oscilloscope (DSO1602, Gould, U.K.) and a brush pen recorder (Gould). A cut-off frequency of 10 kHz (3 pole Bessel filter) was applied to reduce the noise.

Most smooth muscle cells used had a membrane capacitance between 40 and 70 pF. The initial sealing between the patch pipette and cell was performed in Ca²⁺-free solution, to avoid contraction of the cell induced by ATP contained in the pipette. Subsequently, in normal solution, high seal resistance (= 10 G Ω) was obtained by repeated gentle suction. After rupture of the cell membrane, the series resistance was less than 10 M Ω . The capacitive surge was electrically compensated. In most experiments, particularly when large inward tail currents were evoked by application of extremely high (+80 mV) conditioning potentials, the series resistance was partially compensated (by 50–70%). All experiments were performed at room temperature (24–26°C).

Application of agonists

A pressure ejector (Picospritzer II, General Valve, U.S.A.) was used to apply rapidly purinoceptor and muscarinic receptor agonists, except in Ca²⁺-free, low-Mg²⁺ solution. Glass pipettes filled with the agonist had resistance of around 10 M Ω . The tip of the pipette was positioned approx. 100 to 200 μ m from the cell so that it was against the flow of the bathing solution. Upon application of the agonists, an air pressure of 4000 kPa was charged for several hundred ms.

When effects of purinoceptor stimulation were examined in Ca²⁺-free, low-Mg²⁺ solution, which allows Na⁺ current to flow through the voltage-dependent Ca²⁺ channels, the agonists were applied to the bathing solution. This application method was chosen, because the stability of the membrane seal resistance is poor during exposure to solutions containing low concentration of divalent cations, and the mechanical shock upon pressure ejection can break the seal.

Data analysis and statistics

Curve fitting of the decay of the purinoceptor agonist-activated membrane currents was by fitting the digital data points iteratively with single or multiple exponential terms using a modified 'simplex' programme. The total residual current were used as a criterion for the convergence. At convergence, the mean residual currents were usually less than 8 pA or 2% of the peak inward current. When the decay was fitted by two exponential terms, the faster time constant (150 to 230 ms) was nearly the same as that obtained by single exponential fitting. The second exponential had a time constant of 3 to 6 s, and the amplitude was small and its sign was opposite to that of the first exponential term. Thus, the single exponential was judged to be appropriate in the present experiments.

Current traces of slow time base were drawn using an X–Y plotter (7470A, Hewlett-Packard, U.S.A.)

Numerical data were expressed as means $\pm$ standard deviation (s.d.)

Solutions

Normal (bathing) solution for superfusion of smooth muscle cells had the following composition (mM): NaCl 125, KCl 5.9, CaCl₂ 2.5, MgCl₂ 1.2, glucose 11.8 and HEPES (N-2-

hydroxyethyl)piperidine-N-2-ethanesulphonic acid), 11.8. The pH of the solution was adjusted to 7.4 (25°C) with Tris base. Modification of the solution was made by iso-osmotic substitution of NaCl. When monovalent cation (Na⁺) currents through Ca²⁺ channels were measured, Ca²⁺ was removed and Mg²⁺ was reduced to 0.1 mM by iso-osmotically substituting with Na⁺ and 1 mM EGTA (ethyleneglycol-bis-(β -aminoethylether) N, N, N', N'-tetraacetic acid) was added.

The composition of the pipette solution was (mM): CsCl 141, MgCl₂ 1.4, ATP 1, GTP (guanosine 5'-triphosphate 0.1, EGTA 0.1, HEPES/Tris 10 (pH 7.2 at 25°C). The pipette solutions were kept frozen before use.

In preliminary experiments, when a relatively high concentration (2 mM) of Ca²⁺ chelator (EGTA) was used in the patch pipette, although application of ATP elicited a large inward current, which involved Ca²⁺ influx (Inoue & Brading, 1990), the subsequently evoked Ca²⁺ channel current was not reduced in size. Thus, in the present experiments, the pipette solution contained only 0.1 mM EGTA in order to demonstrate the effects of changes in [Ca²⁺]_i more clearly.

Drugs and chemicals

The following chemicals were used: ATP (disodium salt), α,β -methylene ATP (lithium salt), guanosine 5'-triphosphate (GTP) (sodium salt), carbamylcholine chloride (carbachol, CCh), caffeine, collagenase (type 1, all from Sigma) and pronase (Fluka).

Results

Effects of excitatory agonist application at the holding potential

Figure 1 shows an example of the effect of ATP on the Ca²⁺ channel current. Single smooth muscle cells were continuously superfused with normal solution containing 2.5 mM Ca²⁺. The tip of a glass pipette containing 0.1 mM ATP was set close to a detrusor cell which was voltage-clamped. While the membrane potential of the recording cell was held at -60 mV, ATP was rapidly applied by pressure ejection. The application of ATP transiently elicited a large inward current (arrow in a) (1.6 ± 0.9 nA, $n = 7$).

In the same experiment, voltage-dependent Ca²⁺ channel currents were evoked every 30 s by depolarizing the cell membrane to 0 mV. The peak amplitude of the Ca²⁺ channel current evoked approx. 1.5 s after application of ATP was decreased by 21% (ii) but after 30 s, the current had recovered (iii). Similar effects of ATP were observed in three other cells.

It has been shown that the ATP-activated inward current flowing through non-selective cation channels involves Ca²⁺ influx as well as monovalent cations (Inoue & Brading, 1990). As voltage-dependent Ca²⁺ channels are known to inactivate through increases in [Ca²⁺]_i (Ca²⁺-dependent inactivation), the reduction of the Ca²⁺ current (ii) could be due to this mechanism (Schneider *et al.*, 1991).

In the present experiments, the patch pipette contained only 0.1 mM EGTA in order to demonstrate effects of changes in [Ca²⁺]_i clearly. When 2 mM EGTA was used in some cells, application of purinoceptor agonists similarly elicited a transient, large inward current, but caused little changes in the subsequent Ca²⁺ channel current. In some cells there was a small reduction of the Ca²⁺ current, however it failed to recover upon washout (data not shown).

In Figure 2, carbachol (CCh, 1 mM in the pressure ejection pipette) was rapidly applied. In five cells, application of CCh (arrow in a) elicited a very small (<50 pA) or no inward current at -60 mV. However, there was a marked reduction in the subsequent Ca²⁺ channel current (Figure 2a(ii)) (by 20 to 45%, on average $30.3 \pm 12.2\%$, $n = 5$). Recovery of the

Figure 1 Effects of ATP on membrane current at the holding potential and on depolarization-activated Ca^{2+} channel current. The membrane potential was clamped at -60 mV, and a transient depolarizing pulse to 0 mV (200 ms) was repeated at 30 s intervals. ATP was applied by a pressure ejector for 500 ms just before (approx. 1.5 s) the Ca^{2+} channel current (ii) was evoked. The glass pipette for pressure ejection was filled with a normal solution containing 0.1 mM ATP. Pen recording of the membrane current is shown in (a). The voltage-dependent Ca^{2+} channel currents (i) to (iii) are shown expanded in (b). Zero current level is indicated by the dotted line. Note the transient inward current elicited by ATP (in a) and the reduction of the subsequently evoked Ca^{2+} channel current ((ii) in a and b).

Ca^{2+} current was slow after CCh application, and sometimes incomplete. Since there is no detectable Ca^{2+} influx observed upon application of muscarinic agonists, if the reduction of Ca^{2+} current is due to Ca^{2+} -dependent inactivation, the source of Ca^{2+} must be intracellular stores.

Purinoreceptor stimulation in the absence of extracellular Ca^{2+}

Purinoreceptor agonists were applied in the absence of Ca^{2+} , in order to address whether inactivation of voltage-dependent Ca^{2+} channels is due to Ca^{2+} influx upon purinoreceptor stimulation. After observing Ca^{2+} channel current evoked by

depolarization to 0 mV in normal solution (2.5 mM Ca^{2+}), extracellular Ca^{2+} was removed from the perfusate. In nominally Ca^{2+} -free solution (1.2 mM Mg^{2+}), the Ca^{2+} channel current was completely eliminated. However, addition of 1 mM EGTA and reduction of Mg^{2+} to 0.1 mM yielded an inward current upon depolarization of the cell membrane. During exposure to solutions containing low concentrations of divalent cations, the voltage-dependent Ca^{2+} channels become permeable to monovalent cations (Fukushima & Hagiwara, 1985). Therefore in this case, inward currents evoked by depolarizations are carried mainly by Na^+ . When the holding potential was -60 mV, the voltage-dependent Ca^{2+} channel current was gradually reduced with time, pos-

Figure 2 Effects of carbachol (CCh) application at the holding potential (-60 mV). The cell membrane was transiently (100 ms) depolarized to 0 mV every 30 s. Pressure ejection (900 ms) of CCh was applied approx. 8 s before (ii). The glass pipette for pressure ejection was filled with a normal solution containing 1 mM CCh. (a) Pen recording; (b) (i) to (iii) shown expanded.

Figure 3 Effects of purinoceptor stimulation in the absence of extracellular Ca^{2+} . In low- Mg^{2+} (0.1 mM), EGTA-containing (1 mM) solution, the cell membrane was transiently depolarized to -10 mV (200 ms) every 30 s. The holding potential was -70 mV. ATP (30 μM : a, b) or α, β -methylene ATP (10 μM : c, d) was added to the bathing solution for approx. 5 min. The Ca^{2+} channel currents (i) and (ii) in (a) are shown expanded in (b). The same for (c) and (d).

sibly due to changes in surface charge (being identical to the effects of mild depolarization of the cell membrane). Thus, in the following experiments, a more negative holding potential (-70 mV) was applied.

Figure 3 shows effect of purinoceptor agonist application in the absence of Ca^{2+} (low- Mg^{2+} , EGTA-containing solution). Voltage-dependent Ca^{2+} channel current was evoked every 30 s by depolarizing the membrane to -10 mV. Bath application of 30 μM ATP still elicited a large inward current in the absence of external Ca^{2+} (Figure 3a) (the peak amplitude varied from 150 to 900 pA, 409 ± 308 pA, $n = 5$). This inward current decayed during application of ATP, but the time course was much slower than that elicited by rapid application. However, reduction of the Ca^{2+} channel current was not observed after application of ATP (Figure 3b). In three cells, similar results were reproduced by application of α, β -methylene ATP (10 μM) (Figure 3c,d). These results suggest that the main effect of purinoceptor stimulation is opening of non-selective cation channels in the plasma membrane. In these experiments, the lack of inactivation of the Ca^{2+} channel current upon application of purinoceptor agonists could be attributed to the fact that there was no Ca^{2+} entry.

When ATP (30 μM) was applied in the absence of Ca^{2+} (low- Mg^{2+} , EGTA-containing solution), the amplitude of the Na^{+} current through the voltage-dependent Ca^{2+} channels slightly increased (Figure 3b, by $19 \pm 9\%$, $n = 5$) throughout the 5 min application, and was reversed on washout (data not shown). This small increase induced by ATP application was also observed after purinoceptor desensitization with α, β -methylene ATP (10 μM), despite the fact that the ATP-induced current was eliminated at the holding potential (data not shown). The persistent enhancement of the Ca^{2+} channel current and the lack of effect of desensitization by α, β -methylene ATP suggest that this effect of ATP is not through purinoceptor stimulation, but probably through chelation of extracellular Mg^{2+} (divalent cation block of Ca^{2+} channel current carried by monovalent cations: Fukushima & Hagiwara, 1985). In rabbit portal vein, a similar phenomenon has been reported (Xiong *et al.*, 1991).

Figure 4 Purinoceptor stimulation during large depolarizations. The cell membrane was clamped at $+80$ mV for 5 s, then switched to 0 mV (100 ms) and -60 mV. This step pulse protocol was repeated at 2 min intervals. During the large depolarization in (ii), α, β -methylene ATP was applied by pressure ejection (400 ms). (a) Slow time base (pen recording) of current. In (b), the membrane currents evoked by repolarizations are shown expanded. The last 25 ms of the $+80$ mV step is shown.

Figure 5 Muscarinic stimulation during large depolarizations. The same experimental protocol as Figure 4 was applied, but carbachol (CCh, 1 mM in the pressure ejection pipette, 500 ms) was used as the agonist. Note the reduction of inward current evoked by returning the membrane potential to 0 mV after application of CCh.

Figure 6 Effects of caffeine on carbachol (CCh)-induced effects. In (a), step depolarization (0 mV, 100 ms) was repeated at 30 s intervals. The holding potential was -60 mV. In the presence of caffeine (10 mM, bath application), pressure ejection (900 ms) of CCh (1 mM) was applied. The Ca^{2+} channel currents (i) to (iii) in (a) are shown expanded in (b). In (c), the same voltage steps shown in Figure 4 (conditioning step: +80 mV, 5 s; test step: 0 mV, 100 ms) were repeated at 2 min intervals. The last 25 ms of the conditioning step is shown. After observing control membrane currents (i), caffeine (10 mM) was applied to the bathing solution; (ii) was obtained approx. 1 min after the caffeine application. In the continuous presence of caffeine, CCh (1 mM) was applied by pressure ejection (900 ms), when the membrane was clamped at +80 mV (iii).

Agonist application during large depolarizations

With large depolarizations of the cell membrane, there is little inward driving force for Ca^{2+} . Therefore, we can neglect Ca^{2+} influx during such depolarizing steps ($> +80$ mV), if agonists open non-selective cation channels during large depolarizations. Figure 4 shows the effect of purinoceptor stimulation during a large depolarization. The cell membrane was clamped at $+80$ mV for 5 s, during which α, β -methylene ATP (0.1 mM in the pressure ejection pipette) was rapidly applied by pressure ejection (Figure 4a(ii)). The membrane potential was then switched to 0 mV (100 ms) and subsequently to -60 mV (holding potential). In Figure 4b, the membrane currents evoked at 0 and -60 mV steps are shown expanded. The voltage-dependent Ca^{2+} channel currents show little inactivation after large depolarizations (Nakayama & Brading, 1993b). Application of α, β -methylene ATP immediately elicited a large outward, rapidly declining current at $+80$ mV. The time course was very similar to that observed on application of ATP at the holding potential (Figure 1a). However, the subsequently evoked Ca^{2+} channel current was not reduced.

The same experiments were repeated using CCh as the agonist (Figure 5). Pressure ejection of CCh (1 mM) at $+80$ mV similarly elicited a large outward current, but its rising time course was much slower and the membrane current lasted longer (Figure 5a(ii)). The Ca^{2+} channel current evoked by returning the membrane potential to 0 mV (from the preconditioning step at 80 mV) was reduced by approx. 30% after CCh application. Similar reduction of the Ca^{2+} current was observed in two other cells (on average 28%).

Effects of caffeine on muscarinic receptor stimulation

In order to assess whether intracellular Ca^{2+} stores were involved in particular functions, the effects of caffeine on responses induced by stimulation of muscarinic cholinergic receptors were examined (Figure 6). After observing control Ca^{2+} current evoked by depolarizations to 0 mV, 10 mM caffeine was added to the bathing solution (Figure 6a). The bath application of caffeine elicited a small (20 to 30 pA) inward current at the holding potential, and reduced the Ca^{2+} current (Figure 6a and b(ii)) (by 24%). In the presence of caffeine, a pressure ejection of CCh (1 mM) failed to reduce the subsequently evoked Ca^{2+} current (Figure 6a and b(iii)). Caffeine also inhibited further reduction of Ca^{2+} channel current after application of CCh at $+80$ mV (Figure 6c).

Agonist-induced membrane currents at different potentials

Membrane currents elicited by purinoceptor or muscarinic receptor stimulation at different potentials are shown in Figure 7. In (a), α, β -methylene ATP (0.1 mM, in the pipette) was applied by pressure ejection (500 ms). Outward and inward currents were immediately elicited at the membrane potentials of $+80$ mV and -60 mV, respectively. Since the α, β -methylene ATP-activated current became smaller on repeated application, it was difficult to quantify the difference in current recorded at these two potentials. However, when the purinoceptor-operated current was alternately elicited at the potentials, the amplitude at -60 mV was consistently, considerably larger than that at $+80$ mV. The decay time constants were similar at both of the membrane potentials. In the same cell, α, β -methylene ATP was also applied at -40 and -80 mV (data not shown). The decay time courses of the α, β -methylene ATP-activated current were well fitted by a single exponential ($\tau = 150 \sim 210$ ms), and the time constant was not systematically changed by changing the membrane potential.

Pressure ejection (500 ms) of CCh (1 mM) was applied in Figure 7b. At -60 mV, application of CCh caused no detectable change in membrane current. In contrast, when the

Figure 7 Membrane currents elicited by either pressure ejection (500 ms) of purinoceptor (a) or muscarinic receptor agonists (b) at potentials of $+80$ (a) and -60 mV (ii). The pressure ejection pipette contained 0.1 mM α, β -methylene ATP or 1 mM carbachol (CCh). The membrane currents shown in (a) and (b) were obtained from the same cells. Dotted line corresponds to zero current level. Note, in (a), each current trace has its own scale. In (b), the zero level for the current (i) is almost identical to the level of the current (ii).

membrane was held at $+80$ mV, CCh elicited a large outward current (1.5 ± 0.5 nA) which was activated very slowly (time to peak, 2.5 ± 0.4 s, $n = 5$) and had a long duration. Figure 8 shows the effect of depolarization after CCh application. CCh was applied approx. 2 s before depolarization to $+80$ mV. Rapid application of CCh did not induce any

Figure 8 The effects of large depolarizations after carbachol (CCh) application (1 mM, 900 ms) at the holding potential (-60 mV). After a control depolarization to $+80$ mV (2 s) was observed in normal solution (a), CCh was applied approx. 2 s before the second depolarization (b). Depolarization 1 min after application of 10 mM caffeine (c). CCh was applied in the continuous presence of caffeine (d). The depolarizing pulse (a) to (d) was repeated at 2 min intervals. Note, the outward current induced by CCh was attenuated in the presence of caffeine (10 mM).

membrane current while the cell was clamped at the holding potential, but the subsequent depolarization elicited a large outward current (b). Caffeine resulted in a small increase in outward current at +80 mV (c), but attenuated the CCh response (d).

Discussion

Application of purinoceptor agonists (ATP and α,β -methylene ATP) by pressure ejection activated inward and outward currents at negative (−60 mV) and positive membrane potentials (+80 mV), respectively. The membrane currents developed immediately, and decayed rapidly during application. These properties were very similar to those described by Inoue & Brading (1990), although the application methods and ionic composition of the pipette solutions were different. In the present experiments, the decay time constants observed were similar over a wide voltage-range. At the holding potential (−60 mV), Ca^{2+} entry is involved in the purinoceptor-induced current, but does not occur during large depolarizations (+80 mV), because there is little inward driving force for Ca^{2+} ions. These results suggest that the ATP-induced current is not modulated by intracellular Ca^{2+} . This deduction is also consistent with the previous observation that intracellular perfusion with high concentration of Ca^{2+} chelator or pretreatment with caffeine did not significantly change the ATP-induced current (Inoue & Brading, 1990). These authors also defined the decay time course of the (α,β -methylene) ATP-activated non-selective cation channel current as the sum of two exponential terms. In the present study, however, the decay of the purinoceptor-induced current is well fitted by a single exponential term, the time constant corresponding to the faster of the two exponential terms obtained previously. The discrepancy could be due to the shorter exposure time of the agonists used in the present study.

Bath application of ATP elicited a large inward current at the holding potential in Ca^{2+} -free, low- Mg^{2+} solution. However, the rising and the decay time courses were much slower compared to pressure ejection. In normal solution, similar inward currents were observed (data not shown). Since this inward current was not elicited after prior desensitization with α,β -methylene ATP, it is presumably identical to the non-selective cation channel current activated by pressure ejection of purinergic agonists. The slow decay time course may correspond to the second exponential obtained using the concentration jump methods (Inoue & Brading, 1990).

Pressure ejection of muscarinic agonists caused little change in membrane current at the holding potential. On the other hand, CCh elicited a large outward current during a depolarizing step to +80 mV. However, the development and the decay of the outward membrane current were much slower than those of the ATP-activated current, implying involvement of different processes. CCh application at both −60 and +80 mV reduced the subsequently evoked Ca^{2+} channel current. From these results, we can deduce that the reduction of the Ca^{2+} current is due to Ca^{2+} -dependent inactivation, and the source of Ca^{2+} is the intracellular stores. The slow rise time of the outward current may reflect the increase in $[\text{Ca}^{2+}]_i$. Application of caffeine, which is known to release Ca^{2+} from the sarcoplasmic reticulum (SR) (Ebashi, 1976; Endo, 1977; Karaki & Weiss, 1988) attenuated all of the CCh-induced responses, supporting the hypothesis that the main source of Ca^{2+} upon muscarinic receptor stimulation is the intracellular stores. The release of Ca^{2+} from the intracellular store (the SR) could be through production of inositol trisphosphate (IP_3), because flash photolysis of caged IP_3 , which had been introduced through the patch pipette, produced similar results to those obtained by CCh application (A. Zholos & S. Nakayama, unpublished observation).

If depolarization to +80 mV was preceded by CCh appli-

cation, the depolarization resulted in a large outward current. However, it was not elicited by either procedure alone. This suggests that the large outward current observed is due to ion channels which possess both Ca^{2+} - and voltage-sensitivities, e.g. Ca^{2+} -activated Cl^- -channels (Amédée *et al.*, 1990; Evans & Marty, 1986), Ca^{2+} -activated non-selective cation channels (Inoue & Isenberg, 1990a,b; Loirand *et al.*, 1991). Outward Cs^+ current through Ca^{2+} -activated K^+ channels may also have some contribution. The CCh-induced current was quite small at negative potentials compared to the ATP-induced current, and would therefore be much less effective for depolarization of the cell membrane. However, it is possible that ion channels activated through muscarinic transduction may be depressed by perfusion of the pipette solution due to changes in intracellular Ca^{2+} -buffering power etc (Amédée *et al.*, 1990; Wang & Large, 1991). Further identification of the CCh-induced current was not undertaken in the present study.

Two activation mechanisms have been proposed for voltage-dependent Ca^{2+} channels (voltage-dependent and Ca^{2+} -dependent inactivation: reviewed by Pelzer *et al.*, 1990). Previously, we described Ca^{2+} channels in guinea-pig detrusor cells that were inactivated through both of the inactivation mechanisms (Nakayama & Brading, 1993b). In the same smooth muscle, Schneider *et al.* (1990) have shown that bath application of ATP induced an inward current and an increase in $[\text{Ca}^{2+}]_i$. The authors suggested that the reduction of the Ca^{2+} channel current during application is due to Ca^{2+} -dependent inactivation. The present results obtained by pressure ejection of purinoceptor agonists at the holding potential agree well with their results. However, in arterial smooth muscle cells, it has also been reported that ATP triggered Ca^{2+} release from the sarcoplasmic reticulum (SR), and subsequently activated Cl^- channels (Droogmans *et al.*, 1991). One would suspect that Ca^{2+} released from the SR would also inactivate the voltage-dependent Ca^{2+} channels upon purinoceptor stimulation in detrusor cells. In the present study, there are two pieces of evidence which exclude this possibility. Firstly, in Ca^{2+} -free, low- Mg^{2+} solution, the Na^+ current through Ca^{2+} channels was not reduced by application of ATP. Secondly, in normal solution (2.5 mM Ca^{2+}), application of purinoceptor agonists during a +80 mV conditioning depolarization hardly affected the Ca^{2+} current subsequently evoked by the test potential (0 mV). Although in the former case, intracellular Ca^{2+} stores might have been depleted during the exposure to Ca^{2+} -free solution before agonist application, in the latter case, such a depletion is unlikely. Therefore, the fact that Ca^{2+} channel current was not inactivated is attributed solely to lack of Ca^{2+} influx due to the absence of a significant inward driving force.

Thus, we can infer that the main effect of purinoceptor activation is opening non-selective cation channels in the plasma membrane. Recently, Ganitkevich & Isenberg (1992) have reported that depolarization-mediated Ca^{2+} channel current triggers Ca^{2+} release from the SR through a Ca^{2+} -induced Ca^{2+} release mechanism. Similarly, it is possible that Ca^{2+} influx upon purinoceptor stimulation may induce Ca^{2+} release. A possibility that Ca^{2+} -induced Ca^{2+} release also contributed to inactivation of the Ca^{2+} channel still remains. On the other hand, the Ca^{2+} channel current was reduced by muscarinic agonist application at both −60 mV and +80 mV. Caffeine also reduced the Ca^{2+} channel current; however, it attenuated further reduction of the Ca^{2+} current following CCh application. It is likely that upon muscarinic receptor stimulation, Ca^{2+} released from intracellular stores, which can be depleted by caffeine, inactivates the Ca^{2+} channels through a Ca^{2+} -dependent inactivation mechanism. The caffeine-induced reduction of the Ca^{2+} current may also involve direct block of voltage-dependent Ca^{2+} channels (Martin *et al.*, 1989; Hughes *et al.*, 1990; Zholos *et al.*, 1991).

Muscarinic receptor activation triggers Ca^{2+} release from the SR probably through the IP_3 pathway. However, the present experiments do not imply that the IP_3 -sensitive Ca^{2+}

store is also caffeine sensitive, because it has been reported that Ca^{2+} release from this store is potentiated by a small increase in $[\text{Ca}^{2+}]_i$, (biphasic Ca^{2+} dependence of IP_3 -induced Ca^{2+} release: $[\text{Ca}^{2+}]_i > 300 \text{ nM}$ inhibits the Ca^{2+} release: Iino, 1990). In the presence of caffeine, a small reduction of the Ca^{2+} channel current was observed 30 s after CCh application (data not shown). This may be due to slower IP_3 -induced Ca^{2+} release in the presence of a greater Ca^{2+} concentration (Iino, 1990) or other mechanisms induced by muscarinic receptor activation (e.g. possible inhibition of the Ca^{2+} current through activation of protein kinase C: Ozaki *et al.*, 1992).

Previously, we showed lack of inactivation of the Ca^{2+} current after a depolarization at +80 mV for 5 s, a duration often used to achieve a steady state (Nakayama & Brading, 1993a,b). This was explained by lack of voltage-dependent inactivation of a long open state induced by large depolarizations, as well as little Ca^{2+} influx at +80 mV. In the present study, application of α,β -methylene ATP at +80 mV little affected, while CCh reduced the subsequent Ca^{2+} channel current, although both applications elicited large outward currents during the +80 mV step. The contrast of the effects between purinoceptor and muscarinic agonists on the subsequently evoked Ca^{2+} channel current supports the hypothesis that the sources of Ca^{2+} are different for these two agonists. The persistent effect of muscarinic agonists can be explained since the Ca^{2+} release mechanism triggered by muscarinic stimulation is not affected by the membrane potential. This effect could also be evidence that Ca^{2+} channels in the long open state can be inactivated by a Ca^{2+} -dependent inactivation mechanism, and also support the notion that voltage-dependent and Ca^{2+} -dependent inactivation mechanisms operate separately (Hadley & Lederer, 1991). After a large depolarization, tail currents were evoked by returning the membrane potential to -60 mV. When the patch pipette contained a high concentration (2 mM) of EGTA, the tail current and the inward current evoked at 0 mV were reduced

by dihydropyridine Ca^{2+} antagonists in the same manner, suggesting that the tail current was also flowing through L-type Ca^{2+} channels (Nakayama & Brading, 1993a). However, in the present experiments, since EGTA was reduced to 0.1 mM to demonstrate clearly Ca^{2+} -dependent inactivation, the tail current may be contaminated by other Ca^{2+} -dependent conductances.

In microelectrode recording of the membrane potential, guinea-pig urinary bladder smooth muscle shows spontaneous action potential generation. Purinoceptor agonists evoke depolarizations, while muscarinic agonists cause only small changes in membrane potential (Fujii, 1988). The results shown in the present study (a large inward current upon purinoceptor activation; little membrane current induced by muscarinic agonists) are consistent with the microelectrode recordings. Although action potential frequency is increased by application of purinoceptor and muscarinic receptor agonists, the amplitude is often reduced (Personal communication from N. Bramich). The reduction of action potential amplitude can be explained by Ca^{2+} -dependent inactivation of voltage-dependent Ca^{2+} channels.

In conclusion, activation of purinoceptors results in opening of non-selective cation channels in the plasma membrane. Stimulation of muscarinic receptors triggers Ca^{2+} release from intracellular stores. Voltage-operated Ca^{2+} channels are inactivated by an increase in intracellular Ca^{2+} , irrespective of the channel state. Activation of either purinoceptors or muscarinic receptors can contribute to Ca^{2+} -dependent inactivation.

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References

- AMÉDÉE, T., LARGE, W.A. & WANG, Q. (1990). Characteristics of chloride current activated by noradrenaline in rabbit ear artery cells. *J. Physiol.*, **428**, 501–516.
- BENHAM, C.D., BOLTON, T.B. & LANG, R.J. (1985). Acetylcholine activates an inward current in single mammalian smooth muscle cells. *Nature*, **316**, 345–347.
- BURNSTOCK, G., COCKS, T., CROWE, R. & KASAKOV, L. (1978). Purinergic innervation of the guinea-pig bladder. *Br. J. Pharmacol.*, **63**, 125–138.
- BURNSTOCK, G., DUMSDAY, B.H. & SMYTHE, A. (1972). Atropine resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide. *Br. J. Pharmacol.*, **44**, 451–467.
- CREED, K.E. (1971). Effects of ions and drugs on the smooth muscle cell membrane of the guinea-pig urinary bladder. *Pflügers Arch.*, **326**, 127–141.
- CREED, K.E., ISHIKAWA, S. & ITO, Y. (1983). Electrical and mechanical activity recorded from rabbit urinary bladder in response to nerve stimulation. *J. Physiol.*, **338**, 149–164.
- EBASHI, S. (1976). Excitation-contraction coupling. *Annu. Rev. Physiol.*, **38**, 293–311.
- ENDO, M. (1977). Calcium release from sarcoplasmic reticulum. *Physiol. Rev.*, **57**, 71–108.
- EVANS, M.G. & MARTY, A. (1986). Calcium-dependent chloride currents in isolated cells from rat lacrimal glands. *J. Physiol.*, **378**, 437–460.
- DROOGMANS, G., GALLEWAERT, G., DECLERCK, I. & CASTEELS, R. (1991). ATP-induced Ca^{2+} release and Cl^- current in cultured smooth muscle cells from pig aorta. *J. Physiol.*, **440**, 623–634.
- FUJII, K. (1988). Evidence for adenosine triphosphate as an excitatory transmitter in guinea-pig, rabbit and pig urinary bladder. *J. Physiol.*, **404**, 39–52.
- FUKUSHIMA, Y. & HAGIWARA, S. (1985). Current carried by monovalent cations through calcium channels in mouse neoplastic B lymphocytes. *J. Physiol.*, **358**, 255–284.
- GANITKEVICH, V.YA & ISENBERG, G. (1992). Contribution of Ca^{2+} -induced Ca^{2+} release to the $[\text{Ca}^{2+}]_i$ transients in myocytes from guinea-pig urinary bladder. *J. Physiol.*, **458**, 119–137.
- HADLEY, R.W. & LEDERER, W.J. (1991). Ca^{2+} and voltage inactivate Ca^{2+} channels in guinea-pig ventricular myocytes through independent mechanisms. *J. Physiol.*, **444**, 257–268.
- HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.
- HENDERSON, V.E. & ROEPKE, M.H. (1934). The role of acetylcholine in bladder contractile mechanisms and in parasympathetic ganglia. *J. Pharmacol. Exp. Ther.*, **51**, 97–111.
- HUGHES, A.D., HERING, S. & BOLTON, T.B. (1990). The action of caffeine on inward barium current through voltage-dependent calcium channels in single rabbit ear artery cells. *Pflügers Arch.*, **416**, 462–466.
- IINO, M. (1990). Biphasic Ca^{2+} dependence of inositol 1,4,5-trisphosphate-induced Ca^{2+} release in smooth muscle cells of the guinea-pig taenia caeci. *J. Gen. Physiol.*, **95**, 1103–1122.
- INOUE, R. & BRADING, A.F. (1990). The properties of the ATP-induced depolarization and current in single cells isolated from guinea-pig urinary bladder. *Br. J. Pharmacol.*, **100**, 619–625.
- INOUE, R. & ISENBERG, G. (1990a). Effect of membrane potential on acetylcholine-induced inward current in guinea-pig ileum. *J. Physiol.*, **424**, 57–71.
- INOUE, R. & ISENBERG, G. (1990b). Intracellular calcium ions modulate acetylcholine-induced inward current in guinea-pig ileum. *J. Physiol.*, **424**, 73–92.
- JANSSEN, L.J. & SIMS, S.M. (1992). Acetylcholine activates non-selective cation and chloride conductances in canine and guinea-pig tracheal cells. *J. Physiol.*, **453**, 197–218.
- KARAKI, H. & WEISS, G.B. (1988). Calcium release in smooth muscle. *Life Sci.*, **42**, 111–122.

- LANGLEY, K.N. & ANDERSON, H.K. (1885). The innervation of the pelvic and adjoining viscera. Part II. The bladder. *J. Physiol.*, **19**, 71–84.
- LOIRAND, G., PACAUD, P., BARON, A., MIRONNEAU, C. & MIRONNEAU, J. (1991). Large conductance calcium-activated non-selective cation channel in smooth muscle cells isolated from rat portal vein. *J. Physiol.*, **437**, 461–475.
- MARTIN, C., DACQUET, C., MIRONNEAU, C. & MIRONNEAU, J. (1989). Caffeine-induced inhibition of calcium channel current in cultured smooth muscle cells from pregnant rat myometrium. *Br. J. Pharmacol.*, **98**, 493–498.
- NAKAYAMA, S. & BRADING, A.F. (1993a). Evidence for multiple open states of the Ca^{2+} channels in smooth muscle cells isolated from the guinea-pig detrusor. *J. Physiol.*, (in press).
- NAKAYAMA, S. & BRADING, A.F. (1993b). Inactivation of the voltage-dependent Ca^{2+} channel current in smooth muscle cells isolated from the guinea-pig detrusor. *J. Physiol.*, (in press).
- OZAKI, H., ZHANG, L., BUXTON, I.L.O., SANDERS, K.M. & PUBLICOVER, N.G. (1992). Negative-feedback regulation of excitation-contraction coupling in gastric smooth muscle. *Am. J. Physiol.*, **263**, C1160–1171.
- PELZER, D., PELZER, S. & McDONALD, T.F. (1990). Properties and regulation of calcium channels in muscle cells. *Rev. Physiol. Biochem. Pharmacol.*, **114**, 107–207.
- SCHNEIDER, P., HOPP, H.H. & ISENBERG, G. (1991). Ca^{2+} influx through ATP-gated channels increments $[\text{Ca}^{2+}]_i$ and inactivates I_{Ca} in myocytes from guinea-pig urinary bladder. *J. Physiol.*, **440**, 479–496.
- SIMS, S.M. (1992). Cholinergic activation of a non-selective cation current in canine gastric smooth muscle is associated with contraction. *J. Physiol.*, **449**, 377–398.
- WANG, Q. & LARGE, W.A. (1991). Noradrenaline-evoked cation conductance recorded with the nystatin whole-cell method in rabbit portal vein cells. *J. Physiol.*, **435**, 21–39.
- XIONG, Z., KITAMURA, K. & KURIYAMA, H. (1991). ATP activates cationic currents and modulates the calcium current through GTP-binding protein in rabbit portal vein. *J. Physiol.*, **440**, 143–165.
- ZHOLOS, A.V., BAIDAN, L.V. & SHUBA, M.F. (1991). The inhibitory action of caffeine on calcium currents in isolated intestinal smooth muscle cells. *Pflügers Arch.*, **419**, 267–273.

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