Membrane current responses to externally-applied ATP in the longitudinal muscle of the chicken rectum

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1 Membrane current responses to ATP in enzymically-dispersed single smooth muscle cells from the chicken rectum were investigated by the whole-cell voltage clamp technique.

2 In cells dialysed with a KCl-rich solution under voltage clamp at a holding potential of -40 mV, ATP ($10\text{ }\mu\text{M}$) produced an inward current followed by an outward current. When the holding potential was changed to 0 mV and -80 mV, the biphasic current response to ATP was converted to an outward current alone and an inward current alone, respectively.

3 External application of tetraethylammonium (TEA, 5 mM), intracellular dialysis with a CsCl-rich solution, or inclusion of EGTA (10 mM) in the pipette abolished the outward current response to ATP.

4 Neither depletion of Ca^{2+} store with caffeine (10 mM) nor block of voltage-gated Ca^{2+} channels with nifedipine ($10\text{ }\mu\text{M}$) affected the biphasic current response to ATP. After removal of the extracellular Ca^{2+} the outward current response to ATP was abolished.

5 α,β -methylene ATP ($100\text{ }\mu\text{M}$) elicited a current similar to the ATP-induced current. In the presence of α,β -methylene ATP ($100\text{ }\mu\text{M}$), application of ATP ($100\text{ }\mu\text{M}$) was without effect.

6 In CsCl-filled cells, ATP analogues elicited an inward current and the order of potency was $\text{ATP} \approx \alpha,\beta\text{-methylene ATP} > \text{ADP} \gg \text{AMP}$.

7 Inclusion of GTP $\gamma\text{ S}$ (0.2 mM) or GDP $\beta\text{ S}$ (2 mM) in the pipette did not affect the ATP-induced inward current in CsCl-filled cells. The reversal potential of the ATP-induced inward current was about 0 mV and was completely inhibited after replacement of the cations in the bath solution by Tris. The reversal potential remained almost unchanged after replacement of Na^+ in the bath solution with 110 mM Ca^{2+} , but shifted in the negative direction after replacement of Na^+ or both Na^+ and Ca^{2+} with glucosamine.

8 The results suggest that ATP acts on P_2 purinoceptors to cause activation of cation channels with selectivity for Ca^{2+} over Na^+ . Moreover, it appears that no G-protein-mediated mechanism is involved and increased Ca^{2+} entry through the cation channels causes activation of Ca^{2+} -activated K^+ channels.

Keywords: Smooth muscle; ATP; membrane current; chicken rectum; cationic channel; purinoceptor; voltage-clamp; G-protein

Introduction

Non-adrenergic, non-cholinergic (NANC) neurotransmission occurs in artery (Sneddon & Burnstock, 1985; Suzuki, 1985), vas deferens (Sneddon *et al.*, 1982; Sneddon & Westfall, 1984) and urinary bladder (Hoyle & Burnstock, 1985; Fujii, 1988; Brading & Mostwin, 1989) of several species of mammals. There is considerable evidence that suggests that ATP serves as a transmitter, mediating fast excitatory junction potentials (e.j.ps) in these preparations. Recent studies with the whole-cell voltage clamp technique in enzymatically isolated cells revealed that ATP causes activation of non-selective cation channels leading to membrane depolarization with the necessary properties of the fast excitatory neuromuscular transmitter (ear artery: Benham *et al.*, 1987; Benham & Tsien, 1987; vas deferens: Nakazawa & Matsuki, 1987; Friel, 1988; urinary bladder: Inoue & Brading, 1990).

In the rectum of the fowl, the presence of NANC innervation has been reported (Takewaki & Ohashi, 1977; Komori & Ohashi, 1988a) and the e.j.p. evoked by stimulation of the NANC nerves is very similar in temporal and spatial properties to the fast e.j.p. recorded from the smooth muscle of mammals (Komori & Ohashi, 1982). ATP, when applied locally by pressure ejection from a micropipette, also causes the membrane of the smooth muscle of the chicken rectum to depolarize and the membrane depolarization can mimic the NANC e.j.p. in some respects. However, there are some unfavourable observations for the hypothesis that ATP

serves as a transmitter for NANC neuromuscular transmission. A general criterion used to characterize the transmitter is desensitization. In chicken rectum, e.j.ps can be recorded without any noticeable change in amplitude from cells in which desensitization to the depolarizing action of ATP has developed with repetitive application of ATP (Komori & Ohashi, 1988b). Furthermore, a non-hydrolysable ATP analogue, α,β -methylene ATP, which has been shown to activate and then desensitize P_2 purinoceptors in many different cell types of mammals, does not cause selective desensitization of P_2 purinoceptors and therefore does not represent a useful pharmacological tool for the study of the transmitter in NANC neurones in the rectal muscle of the chicken (Komori *et al.*, 1988).

In the present study, membrane currents in response to ATP were measured by use of the whole-cell voltage clamp technique in single smooth muscle cells isolated from the longitudinal muscle layer of the chicken rectum. Some characteristics and intracellular transduction mechanisms of current responses to ATP were investigated for comparison with those in mammalian smooth muscle in which ATP acts on P_2 purinoceptors as an excitatory transmitter.

Methods

Preparation of cells

White Leghorn chickens of either sex, aged 3–6 months (0.8 – 2.0 kg) were stunned and bled. The rectal region of the

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intestine was removed and flushed clean with a physiological salt solution (PSS; composition given in Table 1). The isolated rectum was sectioned lengthwise and pinned serosal side up on a rubber board. Ten to fifteen pieces of muscle strips (about 1 mm in width and 10 mm in length) were dissected from the longitudinal muscle layer of the intestine. The strips were cut into small pieces, about 1 mm square, incubated in a Ca^{2+} -free PSS for 10 min at 37°C and then re-incubated in a mixture of collagenase (1.25 mg ml^{-1}) and papain (4.5 mg ml^{-1}) in a low Ca^{2+} ($30 \mu\text{M}$) containing-PSS for 60 min at 37°C . After the enzymic digestion, tissue pieces were placed in a fresh $120 \mu\text{M}$ Ca^{2+} -containing PSS and gently agitated by drawing them in and out of a blunt glass pipette 50–60 times. The resulting suspension was filtered through a fine nylon mesh and centrifuged at 700 r.p.m. for 2 min and the cells were resuspended in 3–5 ml PSS containing 0.5 mM Ca^{2+} . Small aliquots of cell suspension were placed on 10 to 20 cover-glasses and kept in a moist atmosphere at 4°C until use. The isolated cells were used for the experiments on the day they were prepared.

Recording of membrane currents

A cover-glass with cells was placed in a small bath (1.5 ml) on the stage of a microscope (Olympus: CK-2). The organ bath was filled with PSS. Whole-cell current recordings were made at room temperature with standard patch-clamp techniques (Hamill *et al.*, 1981). Patch pipettes had resistances of 3–7 $\text{M}\Omega$ when filled with pipette solution. Current recordings were made through an amplifier (List; EPC-7) and were displayed on an oscilloscope and stored on FM tape with a recorder (Sony; FFR-3215W) and then replayed onto a thermal array chart recorder (Nihon Kohden: RTA-1100M) for illustration and analysis.

To measure the reversal potential of the ATP-induced current in CsCl-filled cells, the membrane potential was held at -40 mV and step pulses (200–300 ms in duration) were applied during the application of ATP. For a precise current-voltage relationship, current amplitudes at the test potentials should be measured at a steady conductance level. In practice this was not possible because of the transient nature of the ATP-induced current. The peak amplitude of the ATP-induced current at -40 mV ($I_{\text{ATP-40}}$) was measured and the amplitudes of ATP-induced currents at the two test potentials were estimated by an extrapolation method using the decay time constant for $I_{\text{ATP-40}}$.

The experimental values in the text are expressed as the mean $\pm$ s.e.mean. Statistical significance was tested by Student's unpaired *t* test and differences were considered significant when $P < 0.05$.

Application of drugs

Extracellular application of a drug was made by replacing the bath solution with the drug-containing solution. Intracel-

lular application of a drug was made by allowing it to diffuse into the cell from patch pipettes filled with the drug-containing pipette solution.

Solutions and drugs

The ionic compositions of salt solutions are present in Table 1.

A KCl-rich patch pipette solution had the following composition (mM): KCl 134, ATP 1, EGTA 0.05, glucose 14, HEPES 10.5 (titrated to pH 7.2 with NaOH). A CsCl-rich pipette solution was prepared by equimolar substitution of KCl with CsCl and adjusted to pH 7.2 by titration with Tris.

Drugs used were adenosine 5'-triphosphate magnesium salt (ATP; Sigma), adenosine 5'-disphosphate sodium salt (ADP; Sigma), adenosine 5'-monophosphate sodium salt (AMP; Sigma), α,β -methylene adenosine 5'-triphosphate lithium salt (α,β -methylene ATP; Sigma), carbachol chloride (Tokyo Kasei), caffeine (Wako), guanosine 5'-O-(3-thiotriphosphate) (GTP γ S; Sigma), guanosine 5'-O-(2-thiodiphosphate) (GDP β S; Sigma), glycol etherdiamine tetraacetic acid (EGTA; Wako), D-(+)-glucosamine hydrochloride (Glu; Wako), nifedipine (Tokyo Kasei) and tetraethylammonium chloride (TEA; Wako). All other chemicals used were of reagent grade.

Results

Current responses to externally applied ATP

Single smooth muscle cells from chicken rectum were bathed in PSS and held under voltage-clamp at a holding potential of -40 mV using pipettes filled with a KCl-rich solution. In a fraction of the cells, spontaneous transient outward currents (STOCs; Benham & Bolton, 1986) were observed.

ATP ($10 \mu\text{M}$), when applied extracellularly, produced an inward current followed by an outward current. The initial inward current component of the biphasic current response to ATP developed rapidly to reach a peak amplitude within 1 s and was then converted to an outward current (Figure 1). Such a rapid transition from inward to outward current apparently determined the peak amplitude of the inward current which varied from a barely detectable level (about 20 pA) to 0.5 nA ($n = 26$). The outward current reached a peak amplitude of 0.1 to 2.0 nA 0.4 to 2.4 s after the peak of the preceding inward current and then declined to the basal current level within 10 s in the continued presence of ATP. ATP also increased the frequency of STOCs. When application of ATP ($10 \mu\text{M}$) was repeated at an interval of 3 to 5 min, desensitization to ATP developed and the third or fourth application of ATP produced no detectable current response.

Differentiation between the inward and outward current components of the biphasic ATP response

In cells held at 0 mV, ATP ($10 \mu\text{M}$) evoked an outward current lasting 4 to 10 s, and the peak amplitude was between 0.2 and 1.6 nA ($n = 5$) (Figure 2a). The frequency and amplitude of STOCs increased during the current response to ATP. When the holding potential was -80 mV , close to K^+ equilibrium potential, ATP ($10 \mu\text{M}$) evoked an inward current with a peak amplitude of 100 to 420 pA ($n = 6$) which lasted for about 15 s. Figure 2b shows current responses of a cell to ATP ($10 \mu\text{M}$) obtained at holding potentials of -80 mV and -40 mV . The cell responded with a single inward current at -80 mV but with a biphasic current at -40 mV .

When a potassium channel blocker, TEA (5 mM), was added to the bath solution, no outward current was elicited

Table 1 Ionic composition of the bathing solutions

Bath solution	Concentration (mM)				
	NaCl	KCl	MgCl ₂	CaCl ₂	Glucosamine HCl
PSS	126	6	1.2	2	—
130-Na	130	—	—	—	—
110-Ca	—	—	—	110	—
2-Ca/135-Glu	—	—	—	2	135
0-Ca/135-Glu	—	—	—	—	135

All solutions contained 14 mM glucose and 10.5 mM HEPES. The PSS was adjusted to pH 7.2 with NaOH and the other solutions to pH 7.2 by titration with Tris-(hydroxymethyl)-aminomethane. At this pH, glucosamine ($\text{pK}_a = 8.6$) is 96% ionized.

Figure 1 Biphasic current responses to ATP ($10\ \mu\text{M}$) in two different cells held under voltage-clamp at a holding potential of $-40\ \text{mV}$ (HP $-40\ \text{mV}$). The cells were dialysed with a KCl-rich pipette solution. (a) A transient small inward current was followed by a relatively large outward current; (b) initial inward and subsequent outward currents with a similar amplitude. In all 26 cells, ATP ($10\ \mu\text{M}$) evoked a biphasic current response although both inward and outward current components varied in shape and amplitude from one cell to another. See text for details.

by ATP ($10\ \mu\text{M}$) in cells held at $-40\ \text{mV}$. After removal of TEA from the bath solution, ATP produced a biphasic current response (Figure 3a). When a CsCl-rich pipette solution to block K^+ current was used, ATP ($10\ \mu\text{M}$) elicited a brief inward current (see Figure 6b and Figure 7). Furthermore, the outward component of the biphasic current to ATP was not observed with $10\ \text{mM}$ EGTA in a KCl-rich pipette solution (Figure 3b).

These results strongly suggest that the outward current component of the biphasic ATP response results from activation of K^+ channels which is brought about by a rise in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and the inward current component results from activation of other ion channels which are not regulated by $[\text{Ca}^{2+}]_i$.

Figure 2 Current responses to ATP ($10\ \mu\text{M}$) at three different holding potentials. KCl-rich pipette solution was used. (a) ATP-induced current response at $0\ \text{mV}$; (b) current responses to ATP at $-80\ \text{mV}$ (left), and at $-40\ \text{mV}$ (right). Current records in (a) and (b) are from different cells. Note that ATP-induced current response was biphasic at $-40\ \text{mV}$, but simply inward at $-80\ \text{mV}$ or outward at $0\ \text{mV}$.

Calcium sources responsible for the ATP-induced K^+ current

Release of Ca^{2+} from intracellular stores produced by bath application of caffeine ($10\ \text{mM}$) (Benham & Bolton, 1986) produced a brief outward current with a peak amplitude of $0.1\text{--}2.7\ \text{nA}$ ($n = 5$) which resulted from opening of Ca^{2+} -activated K^+ channels. STOCs were also abolished by caffeine. In the continued presence of caffeine, application of ATP ($10\ \mu\text{M}$) produced a biphasic current response similar to that seen in normal PSS (Figure 4a). After removal of Ca^{2+} (with addition of $0.5\ \text{mM}$ EGTA) from the bath solution, ATP activated only an inward current whereas caffeine ($10\ \text{mM}$), applied in the presence of ATP, produced a large outward current (Figure 4b). Readmission of Ca^{2+} to the bath solution resulted in restoration of a biphasic current response to ATP (Figure 4c). A voltage-dependent Ca^{2+} channel blocker, nifedipine ($10\ \mu\text{M}$), when applied in the bath solution, had no noticeable effect on the biphasic ATP response.

These results suggest that a rise in $[\text{Ca}^{2+}]_i$ responsible for the ATP-induced outward K^+ current may result from Ca^{2+} influx through a nifedipine-insensitive pathway rather than release of Ca^{2+} from internal Ca^{2+} stores.

Effects of α,β -methylene ATP on the ATP-induced current

Bath application of α,β -methylene ATP ($100\ \mu\text{M}$) induced a biphasic current response resembling the ATP-induced current. A transient inward current was followed by a relatively long-lasting outward current (Figure 5a) and the outward current component was absent in CsCl-filled cells (Figure 5b). Application of ATP (10 or $100\ \mu\text{M}$) in the presence of α,β -methylene ATP did not evoke a current (Figure 5a,b).

Figure 3 The effect of external TEA and intracellular EGTA on the outward current component of the ATP-induced biphasic current. The cells were voltage-clamped at -40 mV (HP -40 mV) and a KCl-rich pipette solution was used. (a) An inward current alone in response to ATP ($10\text{ }\mu\text{M}$) with extracellular application of TEA (5 mM) (left) and a biphasic current response after removal of TEA (right). (b) An inward current response to ATP ($10\text{ }\mu\text{M}$) with inclusion of EGTA (10 mM) in the pipette solution. Current records in (a) and (b) are from different cells.

Concentration-dependence of the ATP-induced inward current and the effects of ATP analogues

The shape and amplitude of the ATP-induced inward current were less variable in CsCl-filled cells than in cells with KCl-filled pipettes. Thus, the concentration-dependence of the ATP-induced inward current was examined in cells with CsCl-filled pipettes held at -40 mV. Current responses to ATP at various concentrations were obtained from different cells in order to avoid underestimation that might arise from desensitization following repetitive exposure to ATP. ATP at a concentration of $0.1\text{ }\mu\text{M}$ was without effect ($n = 4$), but at concentrations greater than $1\text{ }\mu\text{M}$, elicited a brief inward current. The peak amplitude of current responses increased in a concentration-dependent manner. The maximum amplitude of $292 \pm 28\text{ pA}$, ($n = 3$) was attained with $100\text{ }\mu\text{M}$ ATP. The concentration required for half the maximum amplitude could not be determined precisely but was in the micromolar range (see Figure 6a).

The effective concentrations of α,β -methylene ATP were also in the micromolar range and the amplitude of peak inward currents induced by $100\text{ }\mu\text{M}$ α,β -methylene ATP was $315 \pm 90\text{ pA}$ ($n = 5$), which was not significantly different from that of ATP ($100\text{--}200\text{ }\mu\text{M}$)-induced currents. Another ATP derivative, ADP, at $100\text{ }\mu\text{M}$ also produced an inward current with amplitudes of 10 to 40 pA ($n = 3$), but AMP ($100\text{ }\mu\text{M}$) produced no detectable current. Thus, the order of potency in producing the inward current was $\text{ATP} \approx \alpha,\beta\text{-methylene ATP} > \text{ADP} \gg \text{AMP}$, suggesting that the current response is mediated by P_2 purinoceptors (Burnstock, 1978).

Figure 4 ATP-induced current responses after depletion of the intracellular Ca^{2+} store and in the absence of extracellular Ca^{2+} . The cells were voltage-clamped at -40 mV and a KCl-rich pipette solution was used. (a) A biphasic current response to ATP ($10\text{ }\mu\text{M}$) in the continued presence of caffeine (10 mM) which depleted the intracellular Ca^{2+} stores (see a transient outward current on application of caffeine). (b) An inward current response to ATP ($10\text{ }\mu\text{M}$) and an outward current response to caffeine (10 mM) applied in the continued presence of ATP in a cell bathed in Ca^{2+} -free, with 0.5 mM EGTA-added, PSS. (c) A biphasic current response to ATP ($10\text{ }\mu\text{M}$) after readmission of Ca^{2+} in the bath solution. Current records in (b) and (c) are from the same cell.

Effects of GTP γ S and GDP β S on the ATP-induced inward current

Involvement of a GTP-binding protein in the ATP-induced inward current was tested with a non-hydrolysable GTP analogue, GTP γ S, and a non-hydrolysable GDP analogue, GDP β S. These agents are known to affect muscarinic receptor-activated cation channels in intestinal smooth muscle cells (Inoue & Isenberg, 1990; Komori *et al.*, 1992a). With GTP γ S (0.2 mM) in the patch pipette solution, there were small and irregular inward currents for several minutes after rupturing the cell membrane. This was not observed with GDP β S. Neither GTP γ S (0.2 mM) nor GDP β S (2 mM) affected the ATP ($10\text{ }\mu\text{M}$)-induced inward current and its shape and amplitude were very similar to those in normal cells (Figure 6b): The mean amplitude of the ATP-induced current was $253 \pm 53\text{ pA}$ ($n = 11$) in GTP γ S-treated cells and $273 \pm 64\text{ pA}$ ($n = 10$) in GDP β S-treated cells. Each of the mean values did not significantly differ from the control value of $221 \pm 43\text{ pA}$ ($n = 15$) (see Figure 6a).

Figure 5 Currents to α,β -methylene ATP and its effect on the response to ATP. The cells were voltage-clamped at -40 mV. (a) A biphasic current to α,β -methylene ATP ($100\ \mu\text{M}$) in a cell dialysed with KCl-rich pipette solution which blocked the response to ATP ($100\ \mu\text{M}$). (b) An inward current to α,β -methylene ATP ($100\ \mu\text{M}$) which blocked the response to ATP ($100\ \mu\text{M}$) in a cell dialysed with CsCl-rich pipette solution. Note that α,β -methylene ATP induced similar currents to ATP shown in Figures 1 and 6b and rendered the cells insensitive to ATP.

Figure 6 Concentration-dependence and lack of effect of intracellularly-applied GTP γ S and GDP β S on the ATP-induced inward current. A CsCl-rich pipette solution was used and cells were voltage-clamped at -40 mV. Each measurement of ATP-induced currents was made in a different cell. (a) Plot of the peak amplitude of ATP-induced current against ATP concentration in control cells (○). The amplitudes of current responses to $10\ \mu\text{M}$ ATP in GTP γ S ($0.2\ \text{mM}$)-treated cells (●) and GDP β ($2\ \text{mM}$)-treated cells (▲), are also presented. Each point represents the mean $\pm$ s.e.mean. The number of measurements is indicated by numbers close to the points. The concentration-response relationship shows that the concentration required for half maximum response of the ATP-induced inward current is in the micromolar range. (b) Inward current responses to ATP ($10\ \mu\text{M}$) in normal (left), GTP γ S ($0.2\ \text{mM}$)-treated (middle) and GDP β S ($2\ \text{mM}$)-treated cells (right). The GTP analogues were included in the patch pipette solution.

Ionic basis of the ATP-activated current

In an attempt to evaluate the ionic selectivity of the ATP-induced inward current, the reversal potential of the current to ATP ($10\ \mu\text{M}$) was measured in cells dialysed with a CsCl-rich pipette solution and bathed in various extracellular solutions (see Table 1).

Substitution of external cations with Tris, normally a less permeant cation through cationic channels, resulted in abolition of the ATP ($10\ \mu\text{M}$)-induced inward current. After returning to normal PSS ATP evoked an inward current (Figure 7).

Cells were bathed in 130-Na solution (see Table 1), and the membrane potential was stepped from the holding potential of -40 mV to test potentials of -10 and $+10$ mV during application of ATP ($10\ \mu\text{M}$). Figure 8a shows a typical recording of an ATP-induced current from a cell bathed in 130-Na solution. The ATP-induced current was inward at -40 and -10 mV but outward at $+10$ mV. To estimate the reversal potential of the ATP-induced current, the apparent peak amplitudes of ATP-induced currents at the three different potentials were obtained by an extrapolation method (see Methods). Figure 8c shows a plot of the peak current amplitudes obtained from the recording in Figure 8a against the membrane potential (closed triangles). The current-voltage relationship gave a reversal potential in this cell of $+0.3$ mV. In seven cells, the mean reversal potential of the current was 0.4 ± 1.0 mV. According to a modified Goldman-Hodgkin-Katz equation (Fatt & Ginsborg, 1958), the mean reversal potential corresponded to a relative permeability for Na^+ over Cs^+ ($P_{\text{Na}}/P_{\text{Cs}}$) of 1.2 (see Table 2). Similar experiments were carried out in cells bathed in PSS. In eight different cells, measurement of the reversal potential of the current gave a mean value of $+4.2 \pm 3.0$ mV.

With $110\ \text{mM}$ CaCl_2 in the bath solution (110-Ca solution: Table 1), ATP produced an inward current at the holding potential of -40 mV, and the peak amplitude was comparable with that evoked in PSS or 130-Na solution. The ATP-induced current was also inward at -40 and -10 mV and outward at $+10$ mV (Figure 8b and c, closed circles). In four different cells, the mean reversal potential of the current was $+8.0 \pm 2.5$ mV ($n=4$) which gives a ratio of permeability of Ca^{2+} to Cs^+ ($P_{\text{Ca}}/P_{\text{Cs}}$) of 2.9 (Table 2). With a reduction of the external Ca^{2+} concentration to $2\ \text{mM}$ (2-Ca/135-Glu solution; Table 1), ATP still induced an inward current at the holding potential of -40 mV, but the current amplitude became much smaller than that observed in 110-Ca solution. As illustrated in Figure 9a (also see closed circles in Figure 9c), the ATP-induced current obtained in 2-Ca/135-Glu solution was inward at -40 mV and outward at -20 mV. The mean reversal potential was -31.7 ± 0.4 mV ($n=7$) which corresponds to a relative

Figure 7 Abolition of ATP-induced inward current by substitution of the external cations with Tris. The cell was held at -40 mV and CsCl-rich pipette solution was used. No response to bath-applied ATP ($10\ \mu\text{M}$) was observed in the presence of Tris (left) but after returning to normal PSS, ATP elicited an inward current (right).

Figure 8 Relationship between the amplitude of the ATP-induced currents and the clamp potential. The cells were dialysed with a CsCl-rich pipette solution and held at -40 mV. (a and b) Current responses to ATP ($10 \mu\text{M}$) in cells bathed in 130-Na solution (a) and 110-Ca solution (b) (see Table 1). Test step potentials were -10 and $+10$ mV. Upper traces, voltage step protocol; lower traces, current record. (c) Plot of the amplitude of ATP-induced currents against clamp potential: (▲) from the recording in (a); (●) from that in (b). The current amplitudes at -10 mV and 10 mV were estimated by an extrapolation method using the decay time constant for the ATP-induced current at -40 mV (700 ms in (a) and 1250 ms in (b). (see Methods). Lines were drawn by the least-squares method.

Table 2 Reversal potentials of the ATP-induced current

Bath solution	Reversal potential (mean $\pm$ s.e.mean) (mV)	Permeability ratio
PSS	$+4.2 \pm 3.0$ ($n = 8$)	—
130-Na	$+0.4 \pm 1.0$ ($n = 7$)	PNa/PCs 1.2
110-Ca	$+8.0 \pm 2.5$ ($n = 4$)	PCa/PCs 2.9
2-Ca/135-Glu	-31.7 ± 0.4 ($n = 7$)	PCa/PCs 7.1
0-Ca/135-Glu	-46.6 ± 1.5 ($n = 3$)	PGlu/PCs 0.15

CsCl-rich pipette solution (containing 130 mM CsCl) was used. Permeability ratios were calculated using the mean values of the reversal potential according to a modified Goldman-Hodgkin-Katz equation (Fatt & Ginsborg, 1958). Monovalent ion activities were taken as 0.75 and divalent ion activities as 0.25 (110-Ca solution) or 0.3 (2-Ca/135-Glu solution) (Benham & Tsien, 1987).

permeability for Ca^{2+} over Cs^+ ($\text{P}_{\text{Ca}}/\text{P}_{\text{Cs}}$) of 7.1, if $\text{P}_{\text{Glu}}/\text{P}_{\text{Cs}}$ is taken as 0.15 (see below). Further, with removal of external 2 mM CaCl_2 (0-Ca/135-Glu solution: Table 1), the ATP-induced current was outward at -40 and -20 mV, as shown in Figure 9b and c (closed triangles). With these solutions the mean reversal potential of the current was -46.6 ± 1.5 mV ($n = 3$), which was significantly ($P < 0.05$) different from the value (-31.7 ± 0.4 mV) obtained in 2-Ca/135-Glu solution. The mean reversal potential obtained in 0-Ca/135-Glu solution gives a relative permeability for glucosamine- H^+ over Cs^+ ($\text{P}_{\text{Glu}}/\text{P}_{\text{Cs}}$) of 0.15 (Table 2).

These results suggest that the ATP-induced inward current may be carried by Ca^{2+} -permeable cation channels.

Figure 9 Relationship between the amplitude of the ATP-induced current and the clamp potential. The cells were dialysed with CsCl-rich pipette solution and held at -40 mV. (a and b) Current responses to ATP ($10 \mu\text{M}$) in two different cells bathed in 2-Ca/135-Glu (a) and 0-Ca/135-Glu (b) (see Table 1). During application of ATP, the membrane potential was stepped from -40 mV to test potentials of -30 and -20 mV in (a) and to -50 and -20 mV in (b). Upper traces, voltage step protocol; lower traces, current record. (c) Plot of the amplitude of ATP-induced currents against clamp potential: (●) and (▲) are from the recordings in (a) and (b) respectively. Lines were drawn by the least-squares method.

Discussion

The present study shows that in enzyme-dispersed single smooth muscle cells from the longitudinal muscle layer of chicken rectum, ATP induces two types of membrane current. One type appears to flow through cation selective channels and its reversal potential is close to 0 mV in PSS, and the other type passes through K^+ channels which are activated by intracellular Ca^{2+} . Since activation of the cation channels is expected to depolarize the membrane of smooth muscle cells, the inward current described in this paper may underlie the depolarization evoked by ATP in tissue preparations from chicken rectum (Komori & Ohashi, 1988b).

Properties of the ATP-induced outward current

The outward current component of the biphasic response to ATP was blocked by TEA in the bath solution and was not observed at the potassium equilibrium potential (-80 mV) or if potassium was not present in the pipette and bathing solution. Thus, the outward current to ATP appears to be due to activation of K^+ channels. Since inclusion of EGTA

(10 mM) in the pipette blocked the outward current, the link between ATP receptor and the opening of K^+ channels may be an increase in Ca^{2+} concentration in the cytoplasm. Elevation of $[Ca^{2+}]_i$ caused by increased Ca^{2+} entry across the membrane may play a major role for the activation of K^+ channels, since the ATP-induced outward current was elicited after depletion of intracellular Ca^{2+} stores by caffeine. The outward current was abolished after removal of the extracellular Ca^{2+} with intact caffeine-releasable intracellular Ca^{2+} stores. The Ca^{2+} influx pathway is not the voltage-dependent Ca^{2+} channel because of insensitivity of the Ca^{2+} -activated K^+ current to nifedipine.

In ear artery cells from the rabbit, it has been demonstrated, with a fluorescent Ca^{2+} indicator (Indo-1), that ATP activates Ca^{2+} -permeable nonselective channels, which results in a rise in $[Ca^{2+}]_i$ due to Ca^{2+} entry (Benham, 1989). In the present study, it was shown that the ATP-induced inward current could be carried by calcium ions. In the present work single channel activity was not recorded but it seems likely that the ATP-induced current is carried by Ca^{2+} -permeable cation channels. Ca^{2+} entry then leads to activation of Ca^{2+} -activated K^+ channels. This view is compatible with the finding that the outward current component of the biphasic response to ATP was always preceded by the inward current component. In rabbit jejunal smooth muscle cells, carbachol induces an inward current due to activation of cation channels almost impermeable to Ca^{2+} as well as an outward current due to activation of Ca^{2+} -activated K^+ channels brought about by release of Ca^{2+} from internal stores (Komori & Bolton, 1990). Thus, there is apparently no causal relationship between inward and outward currents. It is worth noting that the outward current is not necessarily preceded by the inward current in this type of smooth muscle cell (Komori & Bolton, 1990).

ATP, unlike caffeine, did not reduce or abolish the activity of STOCs (see Figure 2a) which are considered to represent the sporadic release of Ca^{2+} from intracellular stores (Benham & Bolton, 1986; Bolton & Lim, 1989), suggesting that ATP may not release Ca^{2+} from internal stores in chicken rectal cells. Application of caffeine subsequent to ATP elicited an outward current due to activation of Ca^{2+} -activated K^+ channels which was comparable to that elicited by caffeine alone. Similarly, it has been reported that ATP does not release Ca^{2+} stores in smooth muscle cells dispersed from rabbit ear artery (Benham *et al.*, 1987; Benham, 1989). On the other hand, ATP produces release of Ca^{2+} stores in cultured smooth muscle cells from rat and pig aorta (Tawada *et al.*, 1987; Phaneuf *et al.*, 1987; Droogmans *et al.*, 1991). The existence of a subtype of ATP receptor (P_{2y} purinoceptor), which is coupled to phospholipase C leading to the formation of inositol 1,4,5-trisphosphate mediating the release of Ca^{2+} , has been suggested in turkey erythrocytes (Boyer *et al.*, 1989) and in rat renal cortex (Nanoff *et al.*, 1990). The different effects of ATP on Ca^{2+} stores in many types of cells may be largely due to the existence of two different subtypes of P_2 purinoceptor (P_{2x} and P_{2y}).

Properties of the ATP-induced inward current

The ATP-induced inward current in chicken rectal cells is similar in many properties to that in a variety of smooth muscle cells (rabbit ear artery, Benham *et al.*, 1987; Benham & Tsien, 1987; rat vas deferens, Nakazawa & Matsuki, 1987; Friel, 1988; guinea-pig urinary bladder, Inoue & Brading, 1990). First, there is rapid desensitization of the current. Secondly, the current does not depend on intracellular Ca^{2+} . Thirdly, activation of ion channels responsible for the current is mediated via P_2 purinoceptors and the effective concentration of ATP is in the micromolar range. Fourthly, the current reverses near 0 mV in PSS (with Na^+ as a main cation)

and it is carried by cations. Some of these are the properties usually linked with the P_{2x} receptor subtype. In different cell types, however, α,β -methylene ATP is not as potent an agonist as ATP and may or may not block the response to ATP. After exposure to α,β -methylene ATP, ATP is completely ineffective in chicken rectum muscle and guinea-pig urinary bladder muscle (Inoue & Brading, 1990), but it is still effective in rat vas deferens muscle (Friel, 1988). α,β -methylene ATP was as potent as ATP in activating the inward current in smooth muscle cells of chicken rectum and rabbit ear artery, but less potent than ATP in guinea-pig urinary bladder and rat vas deferens. In the present study, permeabilities of Ca^{2+} relative to Na^+ (P_{Ca}/P_{Na}) in the ATP-activated cation channels were estimated to be 2.4 and 5.9 with 110 mM Ca^{2+} and 2 mM Ca^{2+} in the external medium, respectively (see Table 2), which are similar to the values described in rabbit ear artery (Benham & Tsien, 1987). Based on these facts, the ATP receptor subtype in chicken rectum muscle seems closely related to that in rabbit ear artery muscle.

GTP γ S and GDP β S, applied in the same way and at the same concentration as in our previous study, where these agents markedly affected the activation of ion channels following stimulation of G-protein-coupled receptors in guinea-pig ileal muscle cells (Komori *et al.*, 1992a), did not modify the ATP-induced inward current. This result suggests no involvement of any G-protein-mediated mechanisms in activation of the cation channels. It seems likely that binding of ATP to the receptor could activate directly ion channels, as described for the nicotinic ACh receptor (Hille, 1984). Benham & Tsien (1987) suggested a direct coupling between ATP binding and channel opening, based on their observations in rabbit ear artery muscle that ATP activates unitary inward currents in outside-out membrane patches but not in cell-attached patches.

A physiological role of ATP

ATP is considered to act as a non-adrenergic, non-cholinergic (NANC) excitatory neuromuscular transmitter in blood vessels, vas deferens and the urinary bladder of some species of mammals (artery, Suzuki & Kou, 1983; Suzuki, 1985; vas deferens, Sneddon & Westfall, 1984; urinary bladder, Fujii, 1988).

The presence of NANC excitatory innervation to the avian rectum has been also reported (Ohashi *et al.*, 1977; Takewaki & Ohashi, 1977; Komori & Ohashi, 1982). The neurotransmitter has not been identified, but ATP and neurotensin have been proposed as candidates (Meldrum & Burnstock, 1985; Komori *et al.*, 1986). Local application of either ATP or neurotensin by means of pressure ejection from micro-pipettes evokes a brief, monophasic membrane depolarization similar to the e.j.p. (Komori & Ohashi, 1988b; Komori *et al.*, 1992b). Further, patch clamp experiments revealed that both ATP and neurotensin are channel activators with specific membrane receptors. Both agents activate current with a reversal potential close to 0 mV in a quasi-physiological ionic environment (Komori *et al.*, 1992b; the present study). Activation of such currents leads to membrane depolarization. The e.j.p. has been shown to be associated with an increase of membrane conductance with a reversal potential of about -15 mV (Komori & Ohashi, 1988a). Further experiments are required to elucidate the identity of the excitatory transmitter in the chicken rectum.

We would like to thank Dr C.D. Benham (SmithKline Beecham Pharmaceuticals, U.K.) for his helpful suggestions.

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(Received April 4, 1993)

Revised April 19, 1993

Accepted April 21, 1993