ATP-activated cation conductance in a *Xenopus* renal epithelial cell line

Masahiro Mori, Tomoyuki Nishizaki, Katsumasa Kawahara* and Yasuhiro Okada

*Department of Physiology, Kobe University, School of Medicine, Kobe 650, Japan and *Second Department of Physiology, Chiba University, School of Medicine, Chiba 260, Japan*

1. Using a whole-cell voltage-clamp technique and fura-2 fluorescence measurements, the actions of extracellular adenosine 5′-triphosphate (ATP) in single cells of an epithelial cell line (A6), were investigated.

2. ATP (0·1–1000 μM) induced currents in cells held under voltage clamp. The sequence of purinergic agonist potency in activating the currents (2-methylthio ATP > adenosine 5′-diphosphate (ADP) > ATP > α,β-methylene ATP) was consistent with that of P_{2y} receptors.

3. Reversal potentials (E_{rev}) of the currents under various ionic conditions suggest that potassium channels and non-selective cation channels were responsible for the ATP-activated conductance, which was permeable to calcium.

4. ATP activated the currents in a calcium-free extracellular solution. In the presence of extracellular calcium, the currents were completely inhibited with 10 mM EGTA in the pipette.

5. ATP (10 μM) increased the intracellular calcium concentration ($[Ca^{2+}]_i$) whether cells were bathed in a solution containing calcium or not.

6. These results indicate that ATP evoked a calcium-dependent cation conductance, permeable to calcium, through P_{2y} receptors by releasing calcium from intracellular stores in A6 cells.

Extracellular ATP has been reported to be a possible modulator of cellular responses in a variety of excitable and non-excitable cells. ATP is released with acetylcholine at the neuromuscular junction of the small intestine (Burnstock, 1972). It was proposed that ATP exerts its effect via a specific receptor, the purinergic (P_2) receptor, which is different from the adenosine (P_1) receptor (Burnstock, 1978, 1981). Since then, many studies have confirmed ATP-selective actions mediated via a P_2 receptor.

ATP directly activated ligand-gated channels in smooth muscle cells from rabbit ear arteries (Benham & Tsien 1987; Benham, Bolton, Byrne & Large, 1987), in smooth muscle cells from guinea-pig urinary bladder (Inoue & Brading, 1990; Schneider, Hopp & Isenberg, 1991), in rat and bullfrog sensory neurons (Bean, Williams & Ceelen, 1990), in cultured neurons from rat parasympathetic cardiac ganglia (Fieber & Adams, 1991), in rat pheochromocytoma cells (Sela, Ram & Atlas, 1991) and in rat lacrimal cells (Vincent, 1992). As described above, ATP-activated membrane conductance via ligand-gated channels is well characterized.

On the other hand, ATP has been reported to increase intracellular calcium concentration ($[Ca^{2+}]_i$). ATP increased $[Ca^{2+}]_i$ in a mouse macrophage cell line (Greenberg, Virgilio, Steinberg & Silverstein, 1988), in various neural cell lines (Hirano, Okajima, Tomura, Majid, Takeuchi & Kondo, 1991) and in rat ventricular myocytes (Christie, Sharma & Sheu, 1992). The increase in $[Ca^{2+}]_i$ probably activates calcium-dependent channels. ATP activated calcium-dependent potassium channels in mouse macrophages (Hara, Ichinose, Sawada, Imai & Maeno, 1990) and in rat megakaryocytes (Ueyama, Ueyama & Akaike, 1993).

We found that extracellularly applied ATP induced membrane cation conductance in single A6 cells (Kawahara & Mori, 1993; Mori, Okada & Kawahara, 1993). In the experiments described here, the actions of extracellular ATP on membrane conductance and $[Ca^{2+}]_i$ in single A6 cells were studied further using the whole-cell patch-clamp method accompanied by fura-2 fluorescence measurements. The data suggest that in A6 cells ATP did not activate ligand-gated channels but did activate calcium-dependent channels through release of calcium from intracellular

stores. The role of adenine nucleotides in renal tubules is discussed. Preliminary results have appeared in abstract form (Mori, Okada & Kawahara, 1994).

METHODS

Cell culture

A6 cells, derived from renal distal tubules of *Xenopus laevis* (Rafferty, 1969), were used at passage numbers 70–80. For amphibian cells, Dulbecco's modified Eagle's Medium (Gibco, Grand Island, NY, USA) was diluted to 85% with water. The medium was supplemented with 10% fetal bovine serum (Gibco, USA), 100 u ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Gibco, USA). The bicarbonate concentration of the medium was 6 mM. The cells were seeded at low density (10–20 cells mm^{-2}) and cultured at 26 °C. pH of the medium was maintained at 7·6 in an atmosphere of humidified air with 0·5% CO_2. Single cells cultured on glass coverslips for 3–6 days were used for the experiments at room temperature (22–24 °C).

Patch-clamp recording

ATP was applied with a pressure application pipette, positioned about 50 μm from the soma membrane to give maximal responses under control conditions. Positive pressure of 0·1 kg cm^{-2} was used and the duration of the application was indicated by horizontal bars in the figures. The ion composition of extracellular solutions and intracellular pipette solutions are shown in Table 1. ATP was not included in the pipette solution as any solution leaking out of the pipette before making a seal could desensitize the cell. The liquid junction potentials between the normal external solution (Solution 1) and each of the other solutions used (internal and external) were directly measured with a 3 M KCl electrode. The values are listed in Table 1 for each solution. The actual membrane potential was corrected for the liquid junction potential between the external and internal solutions and calculated from these values, on the assumption that the junction potential between the internal solution and the interior of the cell was negligible (Hagiwara & Ohmori, 1982). In this experiment, the external solution did not change between the zero-current potential measurement and the whole-cell current measurements under voltage clamp. A capillary filled with 120 mM NaCl–agar was used as a reference electrode. Whole-cell membrane currents were recorded by the standard patch-clamp method (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The tip resistance of the patch electrodes filled with 120 mM KCl was 3–6 MΩ. The whole-cell membrane currents and potentials were recorded with an Axopatch 200A patch-clamp amplifier (Axon Instruments). Data acquisition and analysis were controlled using the pCLAMP 6 system (Axon Instruments) with a 12-bit resolution Labmaster A/D converter (DigiData 1200, Axon Instruments) and an IBM PC/AT personal computer. Current traces were filtered at 5 kHz and stored on a hard disk for later analysis. The cell capacitance was measured by integrating the capacity transient with a 10 mV depolarizing voltage step and dividing by the amplitude of the step (10 mV). The peak amplitude of whole-cell currents was normalized by the cell capacitance as a current density (in pA pF^{-1}). The value of series resistance during the recording of whole-cell currents typically ranged between 6 and 15 MΩ (85–95% compensated). The seal resistance was in the range of 1–10 GΩ. To study current–voltage relationships for ATP-activated currents, voltage pulses for 50 ms (range, −120 to +60 or −120 to +120 mV in 20 mV increments) were applied from a holding potential at the moment when the currents peaked after application of ATP, monitoring the current level. These voltage pulses were completed in 0·65 s. The amplitudes of the currents were then measured at 49 ms of each voltage step and the current–voltage curves during ATP activation were constructed.

Fluorescence measurements

$[Ca^{2+}]_i$ was recorded by means of fura-2 fluorescence (Grynkiewicz, Poenie & Tsien, 1985). Single cells on glass coverslips coated with collagen were incubated in a standard Ringer solution (Solution 1) with 5 μM fura-2 AM (Molecular Probes, USA) for 60 min at 22–24 °C. Using a diffraction grating spectroscope (CAM 230, Hamamatsu Photonics K.K., Hamamatsu City, Shizuoka, Japan) attached to an inverted microscope (Diaphot TMD 300, Nikon, Tokyo, Japan), the fluorescence signals passing through an emission filter of 500 nm (bandwidth, 11 nm) were measured after alternating excitation wavelengths (bandwidth, 10 nm) between 340 and 380 nm every second. The background fluorescence from collagen coated on the glass coverslip was negligible. In cells not loaded with fura-2, application of ATP did not change the fluorescence intensity at each wavelength. *In vitro* calibration was made with different free calcium concentrations, ranging from 3·19 nM to 5·03 μM in the solution composed of (mM): 120 KCl, 20 piperazine-*N*,*N*′-bis(2-ethanesulphonic acid (Pipes) and appropriate amounts of $CaCl_2$ and EGTA, as well as 4 μM fura-2 free acids (Molecular Probes, USA).

All statistical results are given as the mean ± S.E.M.

Drugs

ATP, ADP, AMP and adenosine were purchased from Boehringer Mannheim GmbH (Germany). α,β-Methylene ATP, β,γ-methylene ATP and 2-methylthio ATP were from Research Biochemicals Inc. (Natick, MA, USA). Fura-2 AM and fura-2 free acid were from Molecular Probes. EGTA, Hepes and other chemicals were purchased from Nacalai Tesque (Kyoto, Japan).

RESULTS

ATP-activated current in single A6 cells

The cell capacitance of a single A6 cell was 31·1 ± 1·3 pF ($n = 50$) and the resting membrane potential was −14·1 ± 1·3 mV ($n = 50$) 1 min after the patched membrane was broken. The cells were bathed in a standard Ringer solution (Solution 1) and the intracellular pipette solution was Solution A (120 mM KCl). Figure 1*A* shows the current response of a single A6 cell to extracellularly applied ATP (10 μM, for 60 s). ATP activated a transient outward current at a holding potential of 0 mV. The latency was 5·1 ± 0·6 s ($n = 30$) and the time to peak was 1·6 ± 0·2 s ($n = 30$). The current density at a holding potential of 0 mV was 13·1 ± 1·9 pA pF^{-1} ($n = 13$). Desensitization developed within seconds. The current was inactivated with a half-decay time of 6·7 ± 0·4 s ($n = 30$). Because of the latency (5·1 s), the duration of ATP application was set for 5 s in this experiment. The response to 5 s application was the same as that to 60 s application of ATP. A current response to 10 μM ATP for 5 s is shown in Fig. 1*B*. A second application of ATP after a 2 min interval produced a smaller response (Fig. 1*C*). In some cells, no current was induced by the second application of ATP. Because of desensitization of the current responses to multiple

Table 1. Ion composition of solutions used

External solution	NaCl (mM)	KCl (mM)	$MgCl_2$ (mM)	$CaCl_2$ (mM)	$BaCl_2$ (mM)	Na-Glu (mM)	EGTA (mM)	E_j (mV)	Osmolality (mosm $(kg\ H_2O)^{-1}$)	pH
1	120	3	1	0·5	—	—	—	0	250	7·6*
2	120	3	1	0·5	5	—	—	0·3	256	7·6*
3	24	3	1	0·5	—	96	—	−5·9	243	7·6*
4	120	3	1	—	—	—	0·1	0	248	7·6*
5	125	—	—	—	—	—	0·1	0	252	7·6*
6	123·5	—	—	1	—	—	—	0·1	250	7·6*
7	117·5	—	—	5	—	—	—	0·3	249	7·6*
8	110	—	—	10	—	—	—	0·7	248	7·6*
9	—	—	—	85	—	—	—	5·1	241	7·6†
Internal solution	**NaCl (mM)**	**KCl (mM)**	**CsCl (mM)**	**LiCl (mM)**		**K-Glu (mM)**	**EGTA (mM)**	**E_j (mV)**	**Osmolality (mosm $(kg\ H_2O)^{-1}$)**	**pH**
A	—	120	—	—		—	1	−4·1	240	7·4‡
B	—	24	—	—		96	1	−10·1	239	7·4‡
C	120	—	—	—		—	0·1	−0·2	240	7·4*
D	120	—	—	—		—	1	−0·3	241	7·4*
E	120	—	—	—		—	5	−0·5	245	7·4*
F	120	—	—	—		—	10	−0·9	250	7·4*
G	—	—	120	—		—	1	−4·5	240	7·4§
H	—	—	—	120		—	1	1·5	237	7·4‖

All external solutions contained 10 mM Hepes and 5·5 mM glucose. All internal solutions contained 10 mM Hepes. E_j is the liquid junction potential between each solution and the standard external solution (Solution 1); the sign is positive when the external side is negative. Na-Glu, sodium gluconate; K-Glu, potassium gluconate. pH adjusted with NaOH (*), $Ca(OH)_2$ (†), KOH (‡), CsOH (§) or LiOH (‖).

applications of ATP, data presented were obtained after a single application of ATP. Increasing the extracellular concentration of calcium (> 10 mM) or bathing in a standard Ringer solution for longer than 90 min resulted in rounding of the cells and caused detachment from the collagen-coated glass coverslip. Application of ATP, however, had no such effect. This suggests that ATP may not affect the viability of the cells.

Purinergic receptor subtype

The peak current density–concentration relationships for the currents activated at a holding potential of 0 mV by pressure applications of ATP or purinergic agonists are shown in Fig. 2. From the half-maximal concentration (EC_{50}) values, the order of agonist potency for activating the currents was the P_{2y} receptor agonist 2-methylthio ATP (44 nM) > ADP (66 nM) > ATP (600 nM). The potency of other agonists was compared with the concentration that caused an increase in current density of 3 pA pF^{-1}. The potency order of these agonists was the P_{2x} receptor agonist α,β-methylene ATP (67 μM) > adenosine (100 μM) > AMP (350 μM) > the non-hydrolysable analogue β,γ-methylene ATP (2·6 mM). The overall sequence of agonist potency for activating the currents was 2-methylthio ATP > ADP > ATP > α,β-methylene ATP > adenosine > AMP > β,γ-methylene ATP. This is characteristic of a P_{2y} receptor.

Ionic mechanism of the ATP-activated current

Figure 3*A* shows an example of ATP-activated currents in a single A6 cell at different voltages. Voltage pulses for 50 ms (range, −120 to +60 mV in 20 mV increments) are given from a holding potential of 0 mV. The extracellular solution was Solution 1 and the intracellular pipette solution was Solution A (120 mM KCl). The peak current density–voltage relationship for the ATP-activated currents exhibited a slight outward rectification with a reversal potential (E_{rev}) of $-64{\cdot}4 \pm 3{\cdot}7$ mV ($n = 7$) (Fig. 3*B*, Table 2). This E_{rev} does not correspond to the equilibrium potentials of any of the ions used. Under these ionic conditions, potassium was the only ionic species with a negative equilibrium potential (−95 mV). Therefore, ATP activated channels selectively permeable to potassium, i.e. potassium channels, and also activated other channels with positive E_{rev} values. When potassium in the internal solution was replaced with caesium (Solution G), sodium (Solution D) or lithium (Solution H) on an isomolar basis, ATP induced currents which reversed at $-1{\cdot}4 \pm 1{\cdot}2$ mV ($n = 7$), $-8{\cdot}0 \pm 1{\cdot}4$ mV ($n = 10$) and $-13{\cdot}1 \pm 1{\cdot}5$ mV ($n = 5$), respectively (Fig. 3*C* and Table 2). Under each ionic condition, E_{rev} was near 0 mV, consistent with that of non-selective cation channels. ATP probably activated two channels, a potassium channel and a non-selective cation channel. To address this possibility, the current–voltage

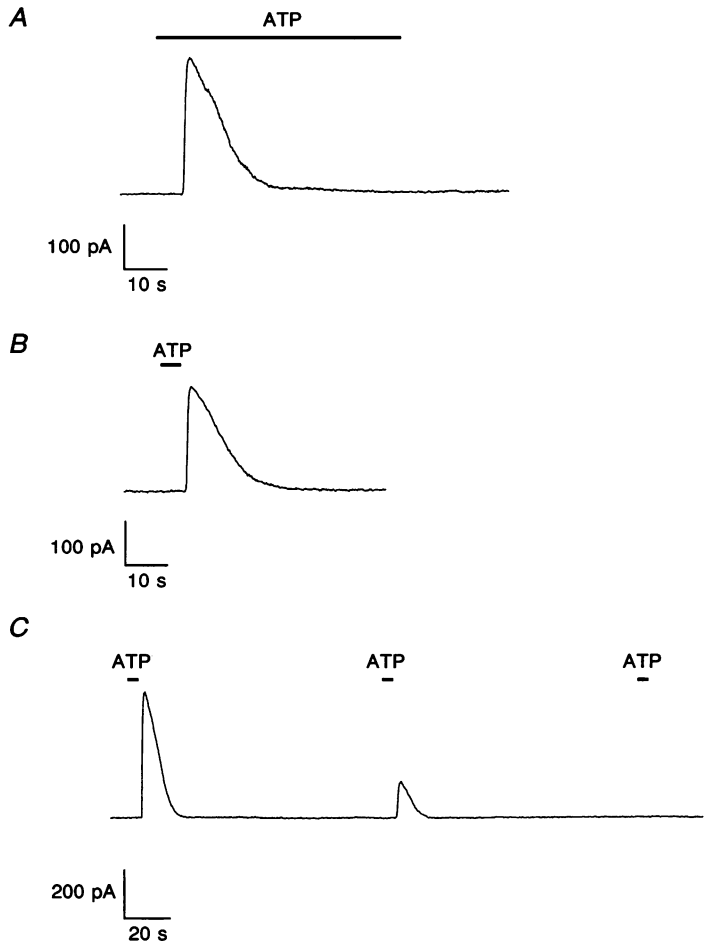


Figure 1. Response of membrane current in A6 cells to pressure application of ATP

ATP (10 μM) was applied during the period indicated by the horizontal bar. The external solution was standard Ringer solution (Solution 1) and the pipette solution was Solution A (120 mM KCl). The holding potential was 0 mV. *A*, a typical time course of the current activated by application of ATP for 60 s. *B*, a typical example of the current activated by application of ATP for 5 s. *C* shows the desensitization of ATP-activated currents to multiple applications of ATP at 2 min intervals. The third application could not evoke the current.

relationship was studied using an external solution containing 5 mM barium (Solution 2) and the potassium internal solution (Solution A). Under these ionic conditions, ATP evoked currents. The current–voltage curve reversed at $-6{\cdot}0 \pm 1{\cdot}7$ mV ($n = 8$) (Fig. 3*D* and Table 2), suggesting that 5 mM barium blocked potassium channels and the non-selective cation channels remained activated.

When 75% of chloride in the KCl internal solution was replaced with isomolar gluconate (Solution B) and the external solution was a standard Ringer solution (Solution 1),

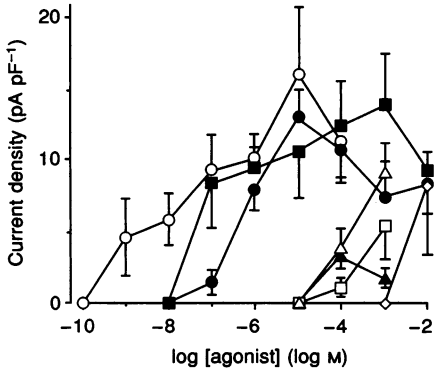


Figure 2. The influence of the concentration of ATP and purinergic agonists on the peak current density

The holding potential was 0 mV. The bath contained standard Ringer solution (Solution 1). For the pipette solution 120 mM KCl internal solution was used (Solution A). Points represent the mean of 5–16 cells from a total of 347 cells. Vertical lines show S.E.M. ATP (●); 2-methylthio ATP (○); ADP (■); AMP (□); adenosine (▲); α,β-methylene ATP (△); β,γ-methylene ATP (◇).

Table 2. Reversal potentials of the ATP-activated currents under various ionic conditions

Experiment	Internal and external solutions	E_{rev} (mV)	No. of experiments
K^+ intracellular solution	A and 1	−64·4 ± 3·7	7
Na^+ intracellular solution	D and 1	−8·0 ± 1·4	10
Cs^+ intracellular solution	G and 1	−1·4 ± 1·2	7
Li^+ intracellular solution	H and 1	−13·1 ± 1·5	5
Low intracellular Cl^- solution	B and 1	−66·0 ± 4·3	6
Low extracellular Cl^- solution	D and 3	−8·1 ± 4·1	4
Blocking K^+ channels with Ba^+	A and 2	−6·0 ± 1·7	8

See Table 1 for composition of internal and external solutions.

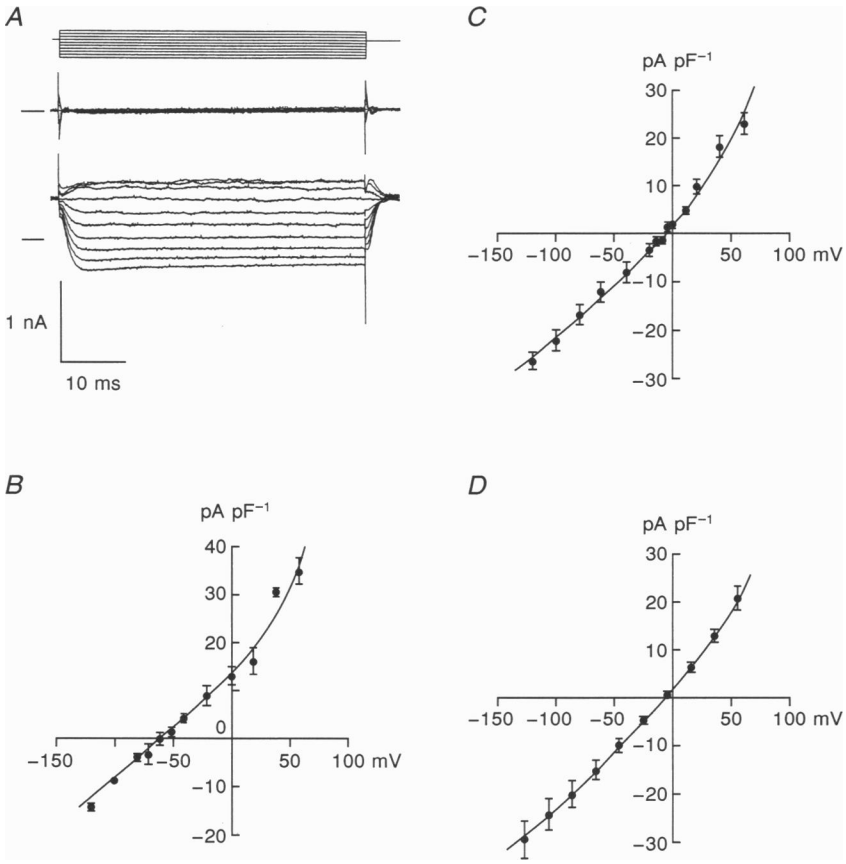


Figure 3. Whole-cell currents activated by ATP at different voltages

Voltage pulses for 50 ms were applied from a holding potential of 0 mV (range, −120 to +60 mV in 20 mV increments; holding potential, 0 mV; top inset in *A*). *A*, a typical example of whole-cell currents of a single A6 cell at different voltages obtained before application of ATP (middle trace) and at the moment when the currents peaked after application of 10 μM ATP (lower trace). The pipette solution was Solution A (120 mM KCl) and the external solution was standard Ringer solution (Solution 1). Left horizontal bars indicate zero current levels. *B*, current density–voltage relationship of ATP-activated currents under the same ionic conditions as described above. The amplitude of the currents was measured at 49 ms of each voltage step. Each point represents the mean of 7 cells. Vertical lines show S.E.M. *C*, current density–voltage relationship of ATP-activated currents with Solution D (120 mM NaCl) in the pipette and Solution 1 in the bath. Each point represents the mean of 10 cells. Vertical lines show S.E.M. *D*, current density–voltage relationship of ATP-activated currents with the external solution containing 5 mM $BaCl_2$ and the potassium internal solution (Solution A). Each point represents the mean of 8 cells. Vertical lines show S.E.M.

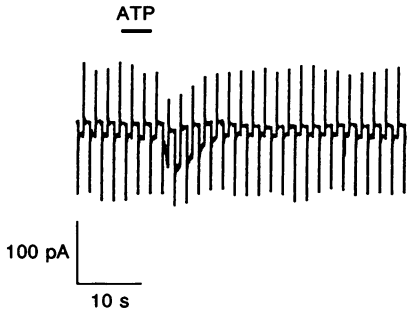


Figure 4. An inward current activated by ATP (10 μM) with an isotonic $CaCl_2$ external solution

Voltage pulses of −20 mV for 1 s were applied every other second from a holding potential of −60 mV. The internal solution was Solution D (120 mM NaCl). The external solution was Solution 9 (85 mM $CaCl_2$).

the reversal potential of ATP-activated current was −66·0 ± 4·3 mV ($n = 6$). Moreover, when 75% of chloride in the external solution (Solution 1) was replaced with isomolar gluconate and the internal solution was Solution D (120 mM NaCl), E_{rev} did not shift significantly either (Table 2). When Solution D as the internal chloride solution is replaced with gluconate (an impermeant anion), this changes the equilibrium potential of chloride from −1 to −42 mV, and the external chloride solution (Solution 1) substition with gluconate changes the equiliblium potential of chloride from −1 to +35 mV. Therefore, it is concluded that ATP did not significantly alter the membrane permeability to chloride.

To study whether ATP-activated conductance is permeable to calcium, all the extracellular cations were replaced with 85 mM calcium on an isotonic basis (Solution 9). In this condition, ATP activated an inward current at potentials of −20 and −60 mV, accompanied by an increase in membrane conductance (Fig. 4). Therefore, ATP activated the channels permeable to calcium. However, extracellular calcium was cytotoxic as the cells became rapidly round and finally detached from a glass coverslip in the presence of a high concentration of calcium. The extracellular calcium concentration was increased up to 10 mM by replacing NaCl in Solution 5 with $CaCl_2$ on an isotonic basis (EGTA not added). Figure 5 shows current density–voltage

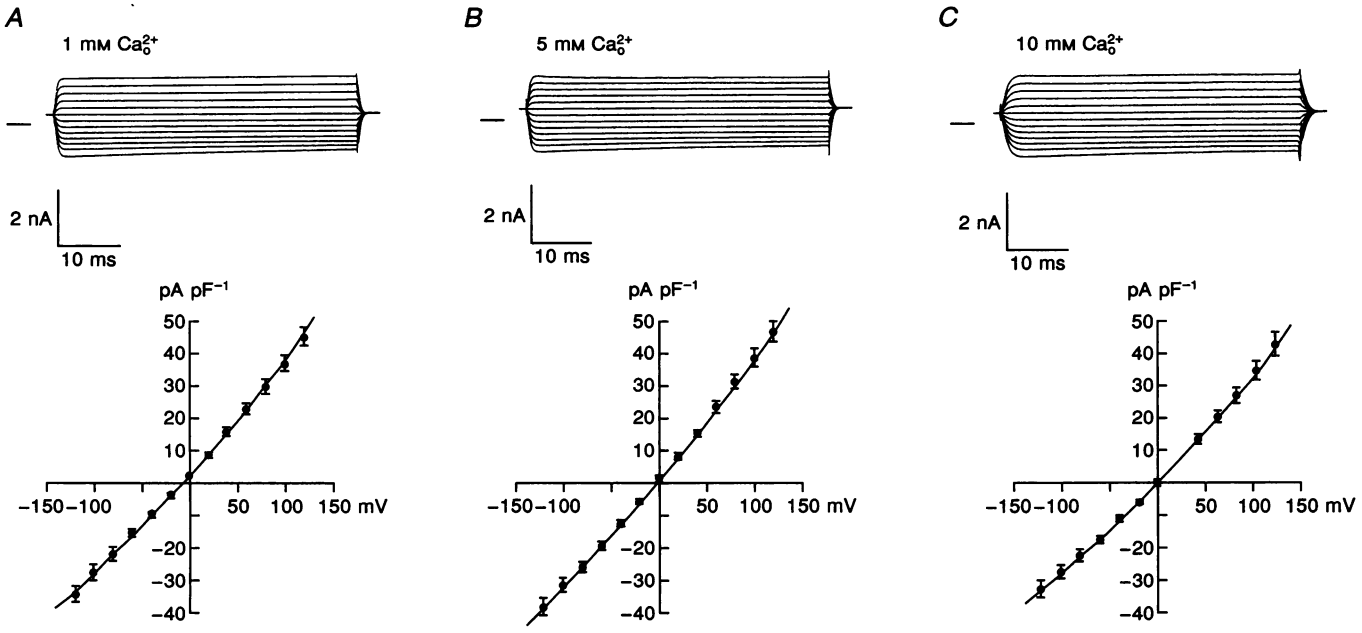


Figure 5. Current density–voltage relationships of ATP-activated currents with different concentrations of extracellular calcium

Both a typical current record of a single A6 cell evoked by 10 μM ATP at different voltages (upper) and current density–voltage relationship of ATP-activated currents (lower) were shown for each concentration of extracellular calcium. *A*, 1 mM $CaCl_2$ (Solution 6); *B*, 5 mM $CaCl_2$ (Solution 7); *C*, 10 mM $CaCl_2$ (Solution 8) in the bath. The internal solution was Solution D (120 mM NaCl). Voltage pulses for 50 ms (range, −120 to +120 mV in 20 mV increments) were applied from a holding potential of +20 mV. Left horizontal bars indicate zero current levels. The amplitude of the currents was measured at 49 ms of each voltage step. Each point represents the mean of 8 (*B* and *C*) or 9 (*A*) cells. Vertical lines show S.E.M.

Table 3. Reversal potentials of the ATP-activated currents with different concentrations of calcium in the bath

Extracellular solution	Internal and external solutions	E_{rev} (mV)	No. of experiments
1 mM Ca^{2+}	D and 6	$-7{\cdot}0 \pm 1{\cdot}4$	9
5 mM Ca^{2+}	D and 7	$-5{\cdot}6 \pm 2{\cdot}0$	8
10 mM Ca^{2+}	D and 8	$-3{\cdot}4 \pm 2{\cdot}4$	8

See Table 1 for composition of internal and external solutions. External NaCl was replaced with $CaCl_2$ on an isotonic basis.

Figure 6. The influence of internal or external concentration of EGTA on ATP-activated currents

Peak current density was recorded at a holding potential of +20 mV. The concentration of EGTA in the internal solution (120 mM NaCl) was increased from 0·1 to 10·0 mM (Solution C, D, E and F). The external solution was standard Ringer solution (Solution 1) except in the experiment with zero calcium in the bath (Solution 4, 0·1 mM EGTA added), indicated as $[Ca^{2+}]_o = 0$. Each column represents the mean of 7–10 cells. Vertical lines show S.E.M. * Statistical significance with P value < 0·01 from data in the experiment with Solution D (1·0 mM EGTA) in the pipette and standard Ringer solution in the bath (ANOVA with Fisher's least-significant difference test).

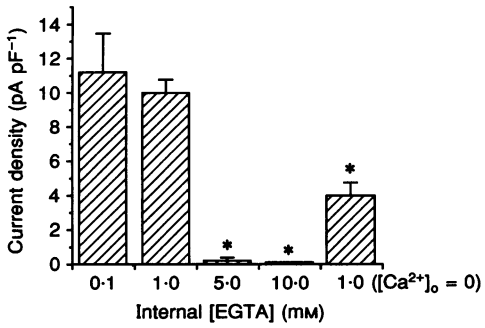
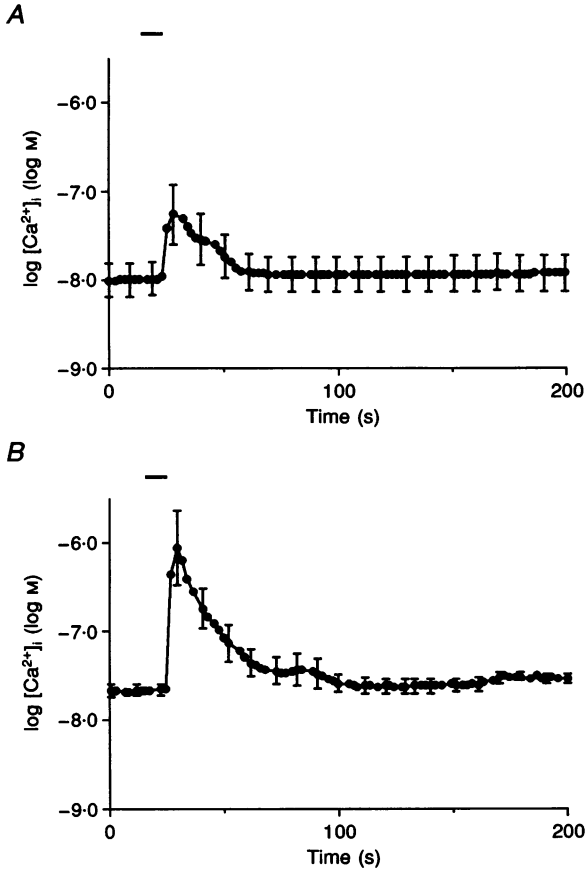


Figure 7. The increase in $[Ca^{2+}]_i$ by application of ATP (10 μM) in the absence or presence of extracellular calcium

A, extracellular calcium concentration was 1·0 mM (Solution 6). *B*, extracellular calcium concentration was 0 mM with 0·1 mM EGTA in the bath (Solution 5). Horizontal bars indicate the period of ATP application. Each point represents the mean of 5 cells. S.E.M. is shown every 10 s as a vertical line.



relationships for ATP-activated currents with different concentrations of calcium in the bath (Solution 6, 7 and 8). The E_{rev} values of the currents are shown in Table 3. As the extracellular calcium concentration increased, E_{rev} shifted in the positive direction.

Calcium dependence of the ATP-activated current

Considering the relatively long latency (5·1 s) of the activation of currents by ATP, it is possible that ATP activates the currents through signal transduction such as intracellular calcium mobilization. When the concentration of EGTA was increased from 0·1 to 10 mM (0·1, 1·0, 5·0 and 10·0 mM) in the internal solution containing 120 mM NaCl (Solution C, D, E and F), ATP-activated currents at a holding potential of +20 mV with 0·5 mM extracellular calcium in the bath were partially or completely inhibited (Fig. 6). This inhibition was consistent at all holding potentials from −120 to +60 mV. The inhibition of the currents by internal EGTA also occurred with 120 mM KCl in the pipette (data not shown), indicating that channels activated by ATP were dependent on $[Ca^{2+}]_i$ under both ionic conditions. ATP activated the currents even with calcium-free external solution (Solution 4), suggesting that ATP evoked the release of calcium from intracellular stores (Fig. 6).

$[Ca^{2+}]_i$ increase by ATP

To confirm the ATP-induced increase of $[Ca^{2+}]_i$ in single A6 cells, the fluorescence of the cells loaded with fura-2 was measured. With an external solution, containing 1·0 mM $CaCl_2$ (Solution 6), ATP increased $[Ca^{2+}]_i$ transiently (Fig. 7*A*). Multiple applications of ATP abolished the $[Ca^{2+}]_i$ increase. With a calcium-free external solution (Solution 5), ATP increased $[Ca^{2+}]_i$, but to a lesser extent than with 1·0 mM extracellular calcium in the bath (Fig. 7*B*). These $[Ca^{2+}]_i$ measurements suggest that ATP increased $[Ca^{2+}]_i$ by releasing calcium from intracellular stores and by increasing the membrane permeability to calcium.

DISCUSSION

Agonist potency for activating the current was 2-methylthio ATP > ADP > ATP > α,β-methylene ATP > adenosine > AMP > β,γ-methylene ATP. The P_{2y} receptor agonist 2-methylthio ATP was more potent than ATP, and the P_{2x} receptor agonist α,β-methylene ATP was much less potent than ATP. This indicates that ATP activated the currents through P_{2y} receptors in A6 cells.

The ATP-activated currents reversed at −64·4 mV when the pipette solution was Solution A (120 mM KCl) and the external solution was standard Ringer solution. Under these ionic conditions, potassium was the only ion with a negative equilibrium potential (−95 mV). Thus, it appears that ATP activated channels selectively permeable to potassium. However, if these channels were highly selective potassium channels, there should be no significant currents through the potassium channels if the internal potassium is replaced with other cations. When potassium in the internal solution was replaced with caesium, sodium or lithium, ATP-activated currents were also observed. Under each ionic condition, the E_{rev} values were near 0 mV, suggesting that the currents might be carried through non-selective cation channels. According to the constant field equation (Adams, Dwyer & Hille, 1980), the relative permeabilty ratios of these ions for these non-selective cation channels were determined ($Na^+ : Cs^+ : Li^+ = 1·0 : 0·8 : 1·2$). Moreover, adding 5 mM barium in standard Ringer solution shifted the reversal potential from −64·4 to −6·0 mV with the potassium internal solution. This suggests that 5 mM barium blocked the potassium channels but the cation channels, activated by ATP, remained unaffected. Thus ATP might activate both potassium channels and non-selective cation channels.

An inward current was activated by ATP at potentials of −20 and −60 mV when the pipette solution was Solution D (120 mM NaCl) and the external solution contained 85 mM $CaCl_2$, in which calcium was the only cation. As the extracellular calcium concentration increased, E_{rev} shifted in the positive direction. Using a modified constant field equation (Lewis, 1979), the permeability ratio of calcium to sodium can be calculated as 1·7 using the change of E_{rev} with different concentrations of $CaCl_2$ up to 10 mM, although there is only a difference between the reversal potentials with 1 mM external calcium and 10 mM external calcium ($P = 0·18$; ANOVA with Fisher's least-significant difference test). Reversal potentials at higher concentrations of extracellular calcium need to be measured for precise estimation of the permeability ratio of calcium to sodium; however, extracellular calcium is toxic to A6 cells at concentration > 10 mM (see Results).

It was reported that ATP activated chloride conductance in A6 cells that had reached a state of confluency on plastic culture wells (Middleton, Mangel, Basavappa & Fits, 1993). In our experiment, however, ATP-activated membrane conductance was not permeable to chloride in A6 cells, which were single cells on collagen-coated glass coverslips. This discrepancy may come from the difference in states of cell growth or culture conditions, as described above. The membrane potential increased from −14·1 mV to around −60 mV, as single A6 cells multiplied and became subconfluent.

In cultured renal epithelial cells from mouse kidneys, calcium currents activated by parathyroid hormone were inhibited by the voltage-dependent calcium channel blocker nifedipine (Bacskai & Friedman, 1990). It is possible that some calcium channels might be activated by ATP in single A6 cells. Nicardipine (10 μM) did not have any effect on ATP-activated currents in A6 cells (Kawahara & Mori, 1993). It has not yet been clarified if this membrane permeability to calcium is due to the non-selective cation channels mentioned above or calcium channels other than the L-type voltage-dependent calcium channels. Single

channel analysis on the ATP-activated current needs to be studied further.

As the concentration of the calcium chelator EGTA was increased in the pipette solutions, ATP-activated currents were diminished whether the major cation in the pipette was potassium or sodium. With 10 mM EGTA in the pipette, ATP failed to activate currents at any holding potential between −120 and +60 mV, suggesting that the channels activated by ATP might not be ligand-gated channels but instead might be calcium-dependent channels.

ATP increased the fura-2 fluorescence ratio in single A6 cells whether the extracellular solution contained calcium or not. However, the increased $[Ca^{2+}]_i$ level was much higher with 1·0 mM calcium than without calcium in the bath. These results suggest that the increased $[Ca^{2+}]_i$ might be both from intracellular stores and from the influx of extracellular calcium, and that the $[Ca^{2+}]_i$ increase from intracellular stores might subsequently activate the calcium-dependent channels. The ATP-activated channels permeable to calcium, presumably the non-selective cation channels, might accelerate the increase in $[Ca^{2+}]_i$.

Purinergic receptors have been reported to be coupled to G protein (O'Connor, Dainty & Leff, 1991; Barnard, Burnstock & Webb, 1994), which leads to the production of inositol 1,4,5-trisphosphate (IP_3) (Charest, Blackmore & Exton, 1985; Sasakawa, Nakaki, Yamamoto & Kato, 1989). ATP-induced phosphatidylinositol hydrolysis is mediated by G proteins and phospholipase C in post-mortem human brain membranes (Jope, Song & Powers, 1994). It is reported that in A6 cells, the apical membrane Na^+ channels are modulated by G_i protein (Cantiello, Patenaude & Ausiello, 1989). Thus after ATP binding to a P_{2y} receptor, the calcium release from intracellular stores might be induced through activation of G proteins with subsequent production of IP_3. The calcium release consequently leads to activation of calcium-dependent channels such as potassium channels and non-selective cation channels. In single A6 cells, addition of 100 μM GDPβS to the pipette solution inhibited the ATP-activated currents (Mori, Nishizaki, Okada & Kawahara, 1995) and adding IP_3 (1 to ~100 μM) in the pipette solution evoked currents without ATP application both in the presence and the abscence of calcium in the bath (M. Mori, T. Nishizaki, Y. Okada & K. Kawahara, unpublished observations).

A6 cells are derived from renal distal tubules of *Xenopus laevis* (Rafferty, 1969). If extracellularly released ATP reaches the distal tubule cells, the increase in $[Ca^{2+}]_i$ and membrane permeabilities to cations including calcium would be induced and, consequently, secretion of potassium and uptake of sodium and calcium would occur. ATP-induced cation conductance may have an important role in homeostatic regulation of ion transport in renal tubules. These ATP-evoked responses, including the increase in $[Ca^{2+}]_i$, may also trigger cellular signal transduction. In rat renal cortex, P_2 receptors mediated formation of IP_3 (Nanoff, Freissmuth, Tuisl & Schutz, 1990). In rat renal cortical slices, P_{2y} receptors stimulated renin secretion (Churchill & Ellis, 1993). In rat kidney, ATP modulated tubuloglomerular feedback responsiveness (Mitchell & Navar, 1993). Thus ATP-activated cationic conductance may have an important function in renal distal tubule cells.

Adams, D. J., Dwyer, T. M. & Hille, B. (1980). The permeability of endplate channels to monovalent and divalent metal cations. *Journal of General Physiology* **75**, 493–510.

Bacskai, B. J. & Friedman, P. A. (1990). Activation of latent Ca^{2+} channels in renal epithelial cells by parathyroid hormone. *Nature* **347**, 388–391.

Barnard, E. A., Burnstock, G. & Webb, T. A. (1994). G protein-coupled receptors for ATP and other nucleotides: a new receptor family. *Trends in Pharmacological Sciences* **15**, 67–70.

Bean, B. P., Williams, C. A. & Ceelen, P. W. (1990). ATP-activated channels in rat and bullfrog sensory neurons: current–voltage relation and single channel behavior. *Journal of Neuroscience* **10**, 11–19.

Benham, C. D., Bolton, T. B., Byrne, N. G. & Large, W. A. (1987). Action of externally applied adenosine triphosphate on single smooth muscle cells dispersed from rabbit ear artery. *Journal of Physiology* **387**, 473–488.

Benham, C. D. & Tsien, R. W. (1987). A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle. *Nature* **328**, 275–278.

Burnstock, G. (1972). Purinergic nerves. *Pharmacological Review* **24**, 509-581.

Burnstock, G. (1978). A basis for distinguishing two types of purinergic receptor. In *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach*, ed. Straub, R. W. & Bolis, L., pp. 107–118. Raven Press, New York.

Burnstock, G. (1981). Neurotransmitters and trophic factors in the autonomic nervous system. *Journal of Physiology* **313**, 1–35.

Cantiello, H. F., Patenaude, C. R. & Ausiello, D. A. (1989). G protein subunit, αi-3, activates a pertussis toxin-sensitive Na^+ channel from the epithelial cell line, A6. *Journal of Biological Chemistry* **264**, 20867–20870.

Charest, R., Blackmore, P. F. & Exton, J. H. (1985). Characterization of responses of isolated rat hepatocytes to ATP and ADP. *Journal of Biological Chemistry* **260**, 15789–15794.

Christie, A., Sharma, V. K. & Sheu, S.-S. (1992). Mechanism of extracellular ATP-induced increase of cytosolic Ca^{2+} concentration in isolated rat ventricular myocytes. *Journal of Physiology* **445**, 369–388.

Churchill, P. C. & Ellis, V. R. (1993). Purinergic P_{2y} receptors stimulate renin secretion by rat renal cortical slices. *Journal of Pharmacology and Experimental Therapeutics* **266**, 160–163.

Fieber, L. A. & Adams, D. J. (1991). Adenosine triphosphate-evoked currents in cultured neurons dissociated from rat parasympathetic cardiac ganglia. *Journal of Physiology* **434**, 239–256.

Greenberg, S., Virgilio, F. D., Steinberg, T. H. & Silverstein, S. C. (1988). Extracellular nucleotides mediate Ca^{2+} fluxes in J774 macrophages by two distinct mechanisms. *Journal of Biological Chemistry* **263**, 10337–10343.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440–3450.

HAGIWARA, S. & OHMORI, H. (1982). Studies of calcium channels in rat clonal pituitary cells with patch electrode voltage clamp. *Journal of Physiology* **331**, 231–252.

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 Archiv* **391**, 85–100.

HARA, N., ICHINOSE, M., SAWADA, M., IMAI, K. & MAENO, T. (1990). Activation of single Ca^{2+}-dependent K^+ channel by external ATP in mouse macrophages. *FEBS Letters* **267**, 281–284.

HIRANO, Y., OKAJIMA, F., TOMURA, H., MAJID, M. A., TAKEUCHI, T. & KONDO, Y. (1991). Change of intracellular calcium of neural cells induced by extracellular ATP. *FEBS Letters* **284**, 235–237.

INOUE, R. & BRADING, A. F. (1990). The properties of the ATP-induced depolarization and current in single cells isolated from the guinea-pig urinary bladder. *British Journal of Pharmacology* **100**, 619–625.

JOPE, R. S., SONG, L. & POWERS, R. (1994). [^{3}H]PtdIns hydrolysis in postmortem human brain membranes is mediated by the G-proteins $G_{q/11}$ and phospholipase C-β. *Biochemical Journal* **304**, 655–659.

KAWAHARA, K. & MORI, M. (1993). Activation of calcium permeable ion channel by extracellular ATP in cultured renal distal tubule cells (A6). *Japanese Journal of Physiology* **43**, S47.

LEWIS, C. A. (1979). Ion-concentration dependence of the reversal potential and the single channel conductance of ion channels at the frog neuromuscular junction. *Journal of Physiology* **286**, 417–445.

MIDDLETON, J. P., MANGEL, A. W., BASAVAPPA, S. & FITS, J. G. (1993). Nucleotide receptors regulate membrane ion transport in renal epithelial cells. *American Journal of Physiology* **264**, F867–873.

MITCHELL, K. D. & NAVAR, L. G. (1993). Modulation of tubulo-glomerular feed back responsiveness by extracellular ATP. *American Journal of Physiology* **264**, F458–466.

MORI, M., NISHIZAKI, T., OKADA, Y. & KAWAHARA, K. (1995). The action of extracellular ATP on intracellular calcium concentration in A6 cells. *Japanese Journal of Physiology* **45**, S35.

MORI, M., OKADA, Y. & KAWAHARA, K. (1993). Activation of potassium currents by extracellular ATP in cultured renal tubule cells (A6 cells). *Japanese Journal of Physiology* **43**, S47.

MORI, M., OKADA, Y. & KAWAHARA, K. (1994). Increase in intracellular calcium concentration by extracellular ATP in cultured renal tubule cells. *Japanese Journal of Physiology* **44**, S48.

NANOFF, C., FREISSMUTH, M., TUISL, E. & SCHUTZ, W. (1990). P_2-, but not P_1-purinoceptors mediate formation of 1,4,5-inositol trisphosphate and its metabolites via a pertussis toxin-insensitive pathway in the rat renal cortex. *British Journal of Pharmacology* **100**, 63–68.

O'CONNOR, S. E., DAINTY, I. A. & LEFF, P. (1991). Further subclassification of ATP receptors based on agonist studies. *Trends in Pharmacological Sciences* **12**, 137–141.

RAFFERTY, K. A. (1969). Mass culture of amphibian cells: Methods and observations concerning stability of cell type. In *Biology of Amphibian Tumors*, ed. MIZELL, M., pp. 52–81. Springer-Verlag, New York.

SASAKAWA, N., NAKAKI, T., YAMAMOTO, S. & KATO, R. (1989). Stimulation by ATP of inositol trisphosphate accumulation and calcium mobilization in cultured adrenal chromaffin cells. *Journal of Neurochemistry* **52**, 441–447.

SCHNEIDER, P., HOPP, H. H. & ISENBERG, G. (1991). Ca^{2+} influx through ATP-gated channels increments $[Ca^{2+}]_i$ and inactivates I_{Ca} in myocytes from guinea-pig urinary bladder. *Journal of Physiology* **440**, 479–496.

SELA, D., RAM, E. & ATLAS, D. (1991). ATP receptor. *Journal of Biological Chemistry* **266**, 17990–17994.

UEYAMA, H., UEYAMA, C. & AKAIKE, N. (1993). Intracellular mechanism of cytoplasmic Ca^{2+} oscillation in rat megakaryocyte. *Journal of Biological Chemistry* **268**, 168–174.

VINCENT, P. (1992). Cationic channels sensitive to extracellular ATP in rat lacrimal cells. *Journal of Physiology* **449**, 313–331.

Acknowledgements

We thank Dr Dermot Cox for careful reading of the manuscript and correcting the English. This work was supported by grants from the Ministry of Education, Science and Culture of Japan and was done during the tenure of a Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists.

Received 19 January 1995; accepted 14 September 1995.