Enhancement of delayed rectifier K^+ current by P_2-purinoceptor stimulation in guinea-pig atrial cells

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1. We studied the effects of P_2-purinoceptor stimulation on the delayed rectifier K^+ current (I_K) in guinea-pig atrial myocytes using a whole-cell voltage-clamp technique.

2. External application of ATP increased I_K, evoked by a 500 ms depolarizing pulse from a holding potential of −40 mV, under conditions in which the L-type Ca^{2+} channel was blocked; the effect was dose dependent with a half-maximal concentration ($K_{1/2}$) of 0·95 μM. ATP (50 μM) produced a maximal increase of I_K of about a factor of 2.

3. External ADP also enhanced I_K in a dose-dependent manner with a $K_{1/2}$ of 3·65 μM, whereas adenosine (100 μM) failed to evoke this response. Theophylline (500 μM), a blocker of the P_1-purinoceptor, did not antagonize the stimulating action of ATP on I_K. These results indicate that I_K was enhanced via P_2-purinoceptors.

4. External ATP or ADP did not produce a significant change in the current kinetics of I_K.

5. Pre-incubation of the atrial myocytes with pertussis toxin (PTX, 5 μg ml^{-1}) did not affect the stimulating action of ATP on I_K, indicating that PTX-sensitive G proteins did not mediate the ATP action.

6. The enhancement of I_K by ATP developed slowly; the effects usually reached a maximum approximately 30–60 s after the application of ATP. This suggests the involvement of a diffusible cytosolic second messenger(s) in the response. ATP could further increase I_K after maximal enhancement by isoprenaline (0·5–1·0 μM), suggesting that the intermediate steps were independent of cyclic AMP-dependent protein kinase (protein kinase A).

7. Potentiation of I_K by ATP was not attenuated by either (i) pretreatment of the cells with 5 μM 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine dihydrochloride (H-7) or (ii) intracellular perfusion of 20 mM 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), suggesting that protein kinase C and intracellular Ca^{2+} did not mediate the response.

8. It is concluded that the activation of P_2-purinoceptors increases I_K through intracellular mechanisms independent of protein kinase A, protein kinase C or intracellular free Ca^{2+} in guinea-pig atrial myocytes.

ATP is stored in secretory granules of sympathetic or parasympathetic nerve terminals and is probably co-released together with noradrenaline or acetylcholine (ACh) in response to nerve stimulation (for review see Gordon, 1986; Burnstock, 1986). ATP has, therefore, been implicated in autonomic neurotransmission in various tissues (for review see Burnstock, 1986). In addition, several cell types, such as platelet, vascular endothelial and smooth muscle cells, can release ATP into the plasma under certain conditions (for review see Gordon, 1986). Extracellular ATP elicits diverse cellular responses, in various types of cells, such as smooth muscle contraction, neural excitation and pancreatic insulin secretion (for review see Gordon, 1986). In the heart, depending upon the animal species, region and ATP concentration, ATP has intricate effects, including positive inotropy (Legssyer, Poggioli, Renard & Vassort, 1988), positive chronotropy (Takikawa, Kurachi, Mashima & Sugimoto, 1990), negative inotropy (Burnstock & Meghji, 1983) and negative chronotropy (Burnstock & Meghji, 1983).

It has recently been demonstrated in mammalian cardiac muscle that activation of P_2-purinoceptors by extracellular ATP or ADP modulates several kinds of ionic channels or exchangers. These are the L-type Ca^{2+} channel (Scamps, Legssyer, Mayoux & Vassort, 1990; Scamps, Rybin, Puceat,

Tkachuk & Vassort, 1992; Zheng, Christie, Levy & Scarpa, 1993), the Cl^- channel (Matsuura & Ehara, 1992), the muscarinic K^+ channel (Friel & Bean, 1990; Matsuura, Sakaguchi, Tsuruhara & Ehara, 1996), the non-selective cation channel (Hirano, Abe, Sawanobori & Hiraoka, 1991; Matsuura & Ehara, 1992; Zheng *et al.* 1993) and the chloride–bicarbonate exchanger (Scamps & Vassort, 1990; Pucéat, Clément & Vassort, 1991). The modulation of these ionic channels through P_2-purinoceptors seems to underlie the diverse actions of ATP on cardiac electrical activities (see above).

The delayed rectifier K^+ current (I_K), which is slowly activated during the plateau of the action potential and facilitates the repolarization by providing outward current, plays an important role in determining the action potential duration and, thereby, Ca^{2+} influx in atrial and ventricular tissues. In pacemaker cells the deactivation of I_K has been regarded as one of the major factors contributing to the development of pacemaker depolarization (for review see Irisawa, 1978). This important K^+ current system has been shown to be regulated by several mechanisms, such as protein kinase A (for references see Yazawa & Kameyama, 1990), protein kinase C (Tohse, Kameyama & Irisawa, 1987; Walsh & Kass, 1988) and intracellular Ca^{2+} (Tohse *et al.* 1987; Tohse, 1990). Here we report that stimulation of P_2-purinoceptors in atrial cells potentiates I_K through intracellular steps that appear to be independent of protein kinase A, protein kinase C and intracellular Ca^{2+}.

METHODS

Cell preparation

Atrial cells were obtained from guinea-pig hearts by an enzymatic dissociation procedure similar to that previously described (Powell, Terrar & Twist, 1980). Briefly, guinea-pigs (250–400 g body weight) were killed by an overdose of sodium pentobarbitone (120 mg kg^{-1} I.P.) and a thoracotomy was performed. Hearts were excised, cannulated via the ascending aorta and retrogradely perfused, initially with normal Tyrode solution containing 3 U l^{-1} heparin, then for 5 min with nominally Ca^{2+}-free Tyrode solution (approximately 1–2 μM free Ca^{2+}), and then for 8–12 min with nominally Ca^{2+}-free Tyrode solution containing 0·06 mg ml^{-1} collagenase (Yakult, Tokyo). The collagenase was then washed out of the heart with a high-K^+, low-Cl^- 'KB' solution (Isenberg & Klöckner, 1982). The temperature of all perfusates was kept at 36–37 °C during coronary perfusion. Finally, the digested atrium was cut into small pieces and single myocytes were dispersed by gentle agitation. The cells were kept at 4 °C in KB solution until use. Spindle-like quiescent atrial cells with clear striations were used in the experiments.

Voltage-clamp technique

Single atrial cells were voltage clamped using the whole-cell configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) with an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). Patch electrodes were made from glass capillaries (o.d. 1·5 mm, i.d. 1·0 mm; Narishige Scientific Instrument Laboratories, Tokyo) using a horizontal microelectrode puller (P-80/PC; Sutter Instrument Co., Novato, CA, USA) and the tips were then fire-polished with a microforge. Electrodes fabricated in this way had a resistance of 1·2–2·0 MΩ when filled with the standard pipette solution. Current and voltage signals were stored on digital audiotape (DT-120; Sony, Tokyo) using a PCM data recorder (RD-101T; TEAC, Tokyo) for subsequent computer analysis (PC98RL; NEC, Tokyo). Current records were fed to the computer every 2 ms through a low-pass filter (48 dB per octave; E-3201A; NF, Tokyo) at a cut-off frequency of 3 kHz, unless otherwise stated. Data are given as mean values ± S.E.M. Statistical comparisons were made using Student's t test, and differences were considered significant at $P < 0·05$.

Solutions

Normal Tyrode solution contained (mM): NaCl, 140; NaH_2PO_4, 0·33; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 0·5; glucose, 5·5; and Hepes, 5·0 (pH adjusted to 7·4 with NaOH). The nominally Ca^{2+}-free Tyrode solution used for the cell isolation procedure was prepared by omitting $CaCl_2$ from the normal Tyrode solution. The external solution for the measurement of I_K was normal Tyrode solution plus 0·1 mM $CdCl_2$. ATP (disodium salt), adenosine-5′-*O*-(3-thiotriphosphate), (ATP-γ-S, tetralithium salt), ADP (sodium salt) or adenosine (sodium salt) was added to normal Tyrode solution containing 0·1 mM $CdCl_2$. The control pipette solution contained (mM): potassium aspartate, 70; KCl, 50; KH_2PO_4, 10; $MgSO_4$, 1; Na_2-ATP, 3; Li_2-GTP, 0·1; EGTA, 5; and Hepes, 5 (pH adjusted to 7·2 with KOH). The amount of KOH required for titration, measured in several experiments, was found to average 24 mM. Therefore, the total K^+ concentration in the control pipette solution was 154 mM. The internal free Ca^{2+} concentration was estimated to be approximately $6·0 \times 10^{-11}$ M (pCa 10·2) with 5 mM EGTA in the pipette solution (Fabiato & Fabiato, 1979; Tsien & Rink, 1980). In some experiments, 5 mM EGTA in the pipette solution was replaced with 20 mM 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA). To inhibit protein kinase A or protein kinase C, *N*-(2-(methylamino)ethyl)-5-isoquinoline sulphonamide dihydrochloride (H-8) or 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine dihydrochloride (H-7) was added to the external solution. The KB medium for cell preservation contained (mM): potassium glutamate, 70; KCl, 30; KH_2PO_4, 10; $MgCl_2$, 1; taurine, 20; EGTA, 0·3; glucose, 10; and Hepes, 10 (pH adjusted to 7·2 with KOH). The experiments reported here were carried out at 34–36 °C.

RESULTS

Effects of external ATP on whole-cell K^+ currents

The effects of various extracellular adenine nucleotides on whole-cell K^+ currents were investigated in guinea-pig atrial myocytes, under conditions in which Na^+ channels were inactivated by holding the membrane potential at −40 mV and L-type Ca^{2+} channels were blocked by adding 0·1 mM Cd^{2+} to the normal Tyrode solution. Figure 1*A* shows superimposed current traces in response to depolarizing (upper traces) and hyperpolarizing voltage-clamp steps (lower traces) applied from a holding potential of −40 mV under control conditions (left), during exposure to 0·1 μM ATP (middle) and after washing out the ATP (right). External ATP enhanced the slowly activating

outward current during the depolarizing voltage step and the tail current developing upon return to −40 mV (Fig. 1*A*, upper traces). External ATP also increased the membrane currents in response to the hyperpolarizing steps as well as the holding current (Fig. 1*A*, lower traces). These effects were fully reversed upon washing the ATP out of the bath (Fig. 1*A*, right). The current–voltage (*I–V*) relationship of the late current recorded during exposure to ATP (Fig. 1*B*; ●) crossed the control current (○) at −83 mV, close to the calculated equilibrium potential of K^+ ($E_K = -88{\cdot}2$ mV), indicating that external ATP affected K^+ currents.

Characterization of the ATP-induced currents

The external ATP-induced currents were obtained by computer subtraction at each test potential. Figure 2*A* shows superimposed current traces during 500 ms voltage steps to +40 mV (upper traces) and −120 mV (lower traces) recorded under control conditions (○) and during exposure to ATP (●), during the experiment shown in Fig. 1. Figure 2*B* demonstrates the ATP-induced currents in response to voltage steps to +40 mV (upper trace) and −120 mV (lower trace) obtained by digital subtraction of the corresponding traces in Fig. 2*A*. The ATP-induced current in response to a voltage step to +40 mV exhibited a time-dependent increase in the outward direction during the pulse and a slow decay to the steady current level on return to the −40 mV holding potential. Similar voltage- and time-dependent changes were observed in the ATP-induced current during the other depolarizing test pulses. Based on these characteristics, the ATP-induced current elicited on depolarization can be identified as I_K. The peak amplitude of the I_K tail current, measured on return to the holding potential, is plotted against the test potential in Fig. 2*D*. ATP (0·1 μM) increased the amplitude of the I_K tail current by about 40% at each test potential.

On the other hand, the ATP-induced current in response to a voltage step to −120 mV gradually increased during the pulse and declined with time to the steady level on return to the holding potential (Fig. 2*B*, lower trace). Since I_K is activated at potentials positive to −30 mV (Fig. 2*D*), the membrane current activated at a holding potential of −40 mV and during hyperpolarizing steps should be independent of I_K. As shown in Fig. 2*C*, this current reversed its polarity at −83 mV, close to the calculated E_K,

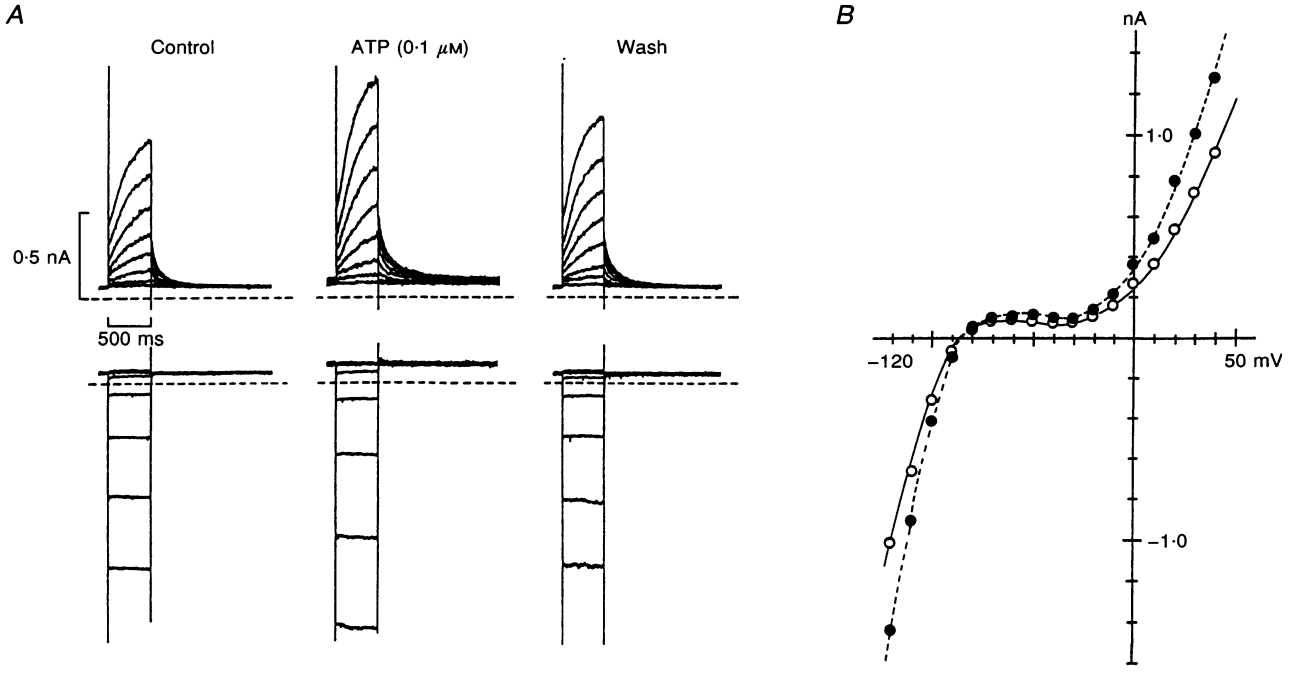

Figure 1. Effects of external ATP on whole-cell currents in an isolated guinea-pig atrial myocyte

A, current recordings obtained during 500 ms voltage-clamp steps to membrane potentials of −30 to +40 mV (upper traces) and −50 to −120 mV (lower traces) in 10 mV steps applied from a holding potential of −40 mV under control conditions (left), during exposure to 0·1 μM ATP (middle) and after washing out the ATP (right). Zero current level is indicated by dashed line. *B*, *I–V* relationships of the late currents from the data in *A*, under control conditions (○) and during exposure to ATP (●). Late current levels were measured near the end of each clamp pulse of 500 ms duration. Continuous and dashed lines through data points were fitted by eye for control and ATP, respectively.

indicating that ATP activated another kind of K^+ current. The time-dependent changes of this K^+ current induced by voltage jumps are very similar to the relaxation properties of the muscarinic K^+ current in supraventricular tissues (Kurachi, Nakajima & Sugimoto, 1986). The nature of this ATP-induced K^+ current activated at potentials negative to −40 mV will be separately analysed in the accompanying paper (Matsuura *et al.* 1996).

Figure 3 shows semilogarithmic plots of the amplitude of the I_K tail current elicited on return to −40 mV after a 500 ms depolarization to +40 mV in control conditions (*A*) and during exposure to 0·1 μM ATP (*B*). The deactivation time course of I_K could be fitted with the sum of two exponentials both in control conditions and during exposure to ATP. The magnitudes of these fast and slow components of the I_K tail current, estimated by extrapolating the fitted line to time zero, were increased almost equally, i.e. 1·4-fold, by 0·1 μM ATP. The time constants of the fast and slow components of I_K decay under control conditions were 46 and 227 ms, and those during exposure to ATP were 52 and 237 ms, respectively. Thus, external ATP potentiated I_K without significantly affecting its kinetic properties.

Effects of other adenine nucleotides on I_K

To identify the purinoceptor type involved, we tested the effects of ADP on I_K. Bath application of 5 μM ADP

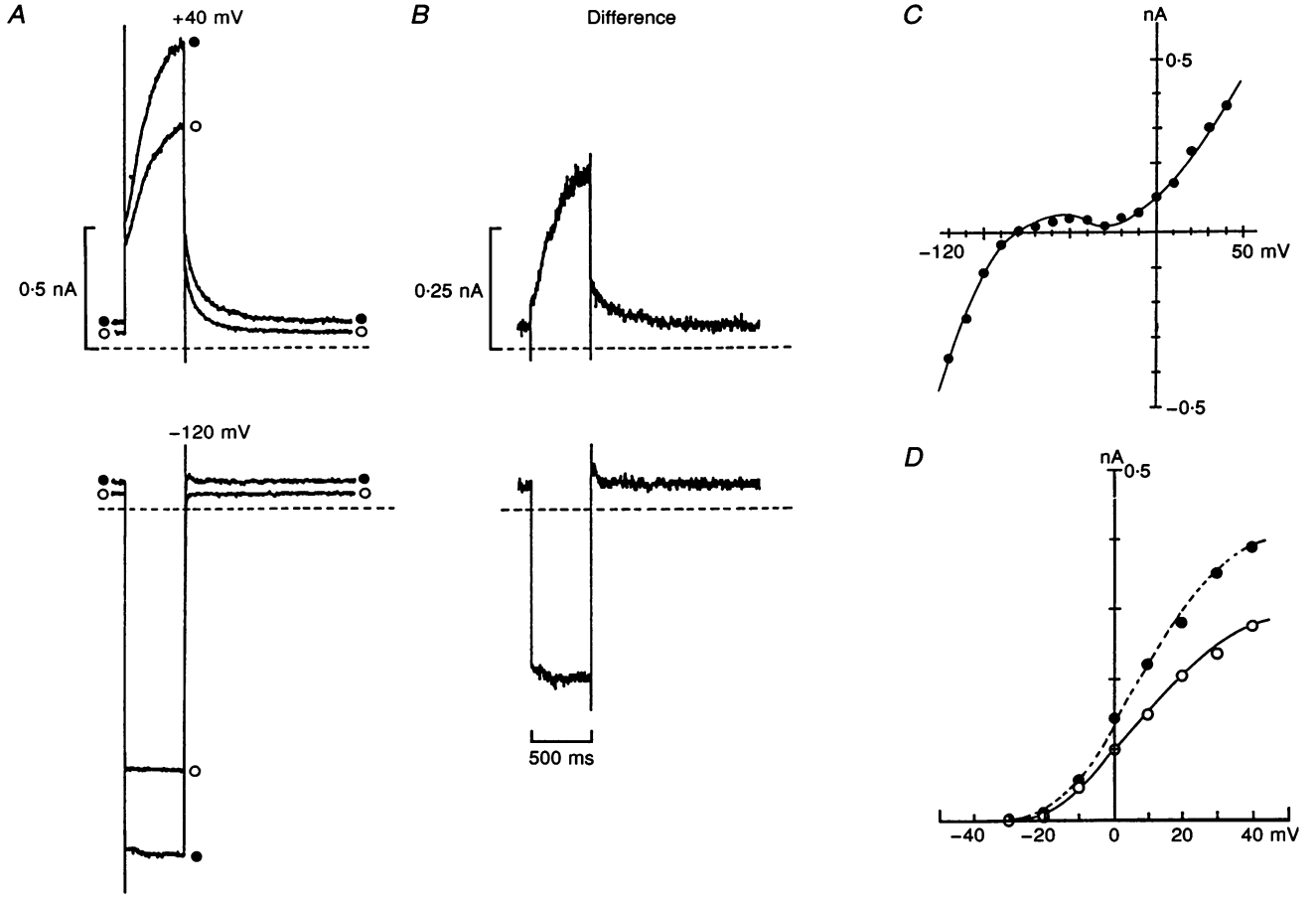

Figure 2. Characterization of the ATP-induced currents from the experiment shown in Fig. 1

A, superimposed current traces in response to 500 ms voltage-clamp steps to +40 mV (upper traces) and −120 mV (lower traces) from a holding potential of −40 mV, before (○) and during exposure to 0·1 μM ATP (●). *B*, the ATP-induced currents stepped to +40 mV (upper trace) and −120 mV (lower trace) obtained by subtracting the currents recorded under control conditions from those recorded during exposure to ATP, shown in *A*. *C*, *I–V* relationship of the ATP-induced current from the records in *B*, measured near the end of the 500 ms clamp pulses. Continuous line through data points was fitted by eye. *D*, *I–V* relationships for I_K tail currents measured on return to a holding potential of −40 mV after 500 ms voltage pulse to the various test potentials shown in Fig. 1*A*, under control conditions (○) and during exposure to ATP (●). Continuous and dashed lines through the data points were fitted by eye for control and ATP, respectively. Note that this figure shows an example of the largest response of I_K to 0·1 μM ATP (1·4-fold increase) among the six cells examined.

increased I_K (Fig. 4*A*, upper traces) as well as a kind of membrane current characterized by the relaxation property upon step changes of membrane potential (Fig. 4*A*, lower traces) and the reversal potential near E_K (Fig. 4*B*). These effects on membrane currents were similar to those of ATP. ADP enhanced the peak amplitude of the I_K tail current by about 1·8-fold at each test potential (Fig. 4*C*). The outward tail current on repolarization to −40 mV after a 500 ms depolarizing pulse was well fitted with the sum of two exponentials both in control conditions and during exposure to ADP with time constants of 64 and 242 ms, and 54 and 217 ms, respectively (not shown). Thus, external ADP also increased I_K without significantly changing its current kinetics. The ADP-induced K^+ current during hyperpolarizing steps appears to have similar voltage- and time-dependent properties to that activated by ATP, and the nature of this current is investigated in the accompanying paper (Matsuura *et al.* 1996).

We then examined the influences of adenosine on I_K using the same voltage-clamp protocols. The adenosine (>0·1 μM)-intduced outward current decayed with time on depolarization and gradually increased on return to a holding potential of −40 mV, which appears to reflect deactivation and activation of the muscarinic K^+ current, respectively (Kurachi *et al.* 1986). We could not detect any enhancement of I_K with up to 100 μM adenosine, as judged from the current behaviour during the depolarizing steps as well as on return to the holding potential (data not shown).

Hydrolysis of external ATP by ecto-MgATPase was shown to result in the translocation of ions across the cell membrane associated with changes in membrane conductance in rat ventricular myocytes (Scamps & Vassort, 1990). We therefore examined whether a slowly hydrolysable ATP analogue, ATP-γ-S, is also effective in enhancing I_K. External application of ATP-γ-S produced an increase in I_K in a concentration-dependent manner without significantly changing the current kinetics (data not shown). Figure 5 demonstrates the concentration–response relationship for the potentiation of I_K by ATP, ADP and ATP-γ-S. The data were well described by a Hill equation with the following parameters: $K_{½} = 0·95$ μM, $n_H = 0·85$ and $I_{max} = 1·93$ for ATP; $K_{½} = 3·65$ μM, $n_H = 0·96$ and $I_{max} = 1·91$ for ADP; and $K_{½} = 1·44$ μM, $n_H = 0·87$ and $I_{max} = 1·91$ for ATP-γ-S, where I_{max} is the maximal response and n_H is the Hill coefficient. Whereas the $K_{½}$ for ATP-γ-S (1·44 μM) was slightly higher than that for ATP (0·95 μM), ATP-γ-S increased I_K to an extent similar (statistically insignificant) to ATP at each concentration tested (0·05, 0·5, 1·0, 5 and 50 μM). This result can be interpreted to indicate that the hydrolysis of ATP was not primarily involved in the I_K response; rather, the relatively higher affinity of the cardiac P_2-purinoceptor for ATP over ATP-γ-S may have caused the difference, as in bullfrog atrial cells (Friel & Bean, 1988). The potency order for the enhancement of I_K (ATP ≥ ATP-γ-S > ADP ≫ adenosine) indicates that the P_2-purinoceptor was involved in the I_K response.

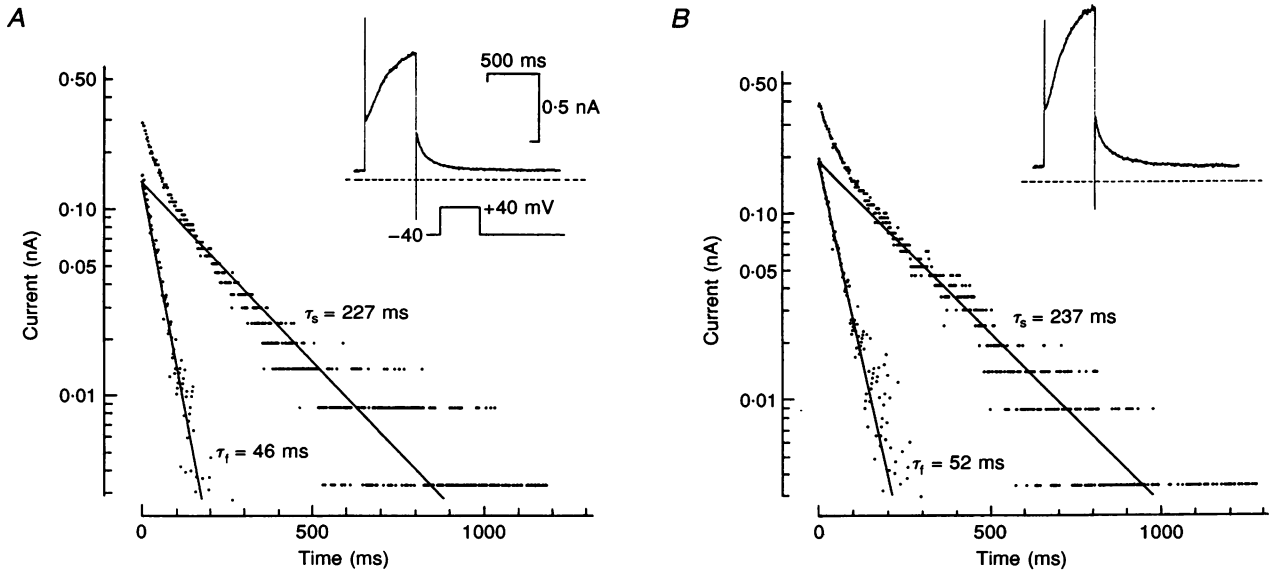

Figure 3. Effect of external ATP on the deactivation kinetics of I_K

Semilogarithmic plot of an I_K tail current elicited on repolarization to −40 mV after a 500 ms voltage step to +40 mV in control conditions (*A*) and during exposure to 0·1 μM ATP (*B*). The decay of the I_K tail current recorded in control conditions and during exposure to ATP was fitted with two exponentials with the time constants indicated in each panel; τ_f and τ_s represent time constants of the fast and slow components, respectively. The inset shows the original current traces used for the analysis, which are obtained from the experiment shown in Fig. 1.

Signal transduction pathways involved in the P_2-purinoceptor-induced I_K enhancement

Is a PTX-sensitive G protein involved? External application of 5 μM ATP increased the I_K tail current elicited upon repolarization to −40 mV from +40 mV by 62·0 ± 11·7% ($n = 4$) even in atrial cells pretreated with PTX (5 μg ml^{-1}) for 2 h at 32 °C (see Fig. 5 in Matsuura *et al.* 1996). This value is not significantly different from the potentiation of I_K observed in control cells (68·7 ± 7·2%, $n = 4$), indicating that G proteins sensitive to PTX were not involved in the signal transduction from the P_2-purinoceptor to I_K.

Is protein kinase A involved? The maximum response of I_K occurred approximately 30–60 s after the application of ATP (data not shown). This relatively slow response suggests that the signal transduction pathway may involve a diffusible cytosolic second messenger(s). β-Adrenergic agonists are known to enhance I_K through protein kinase A in various cardiac cells (for references see Yazawa & Kameyama, 1990). To determine whether protein kinase A mediates the enhancement of I_K through P_2-purinoceptors, we tested whether the stimulating effects of isoprenaline and ATP on I_K were additive. In guinea-pig atrial myocytes, increasing the concentration of isoprenaline above 1 μM produced no further increase in the amplitude of I_K, showing that 1·0 μM isoprenaline produced a maximal enhancement of I_K (data not shown), which is in good agreement with the observation in guinea-pig ventricular myocytes (Yazawa & Kameyama, 1990). In the experiment shown in Fig. 6, the atrial cell was initially exposed to 1·0 μM isoprenaline, which dramatically enhanced the I_K

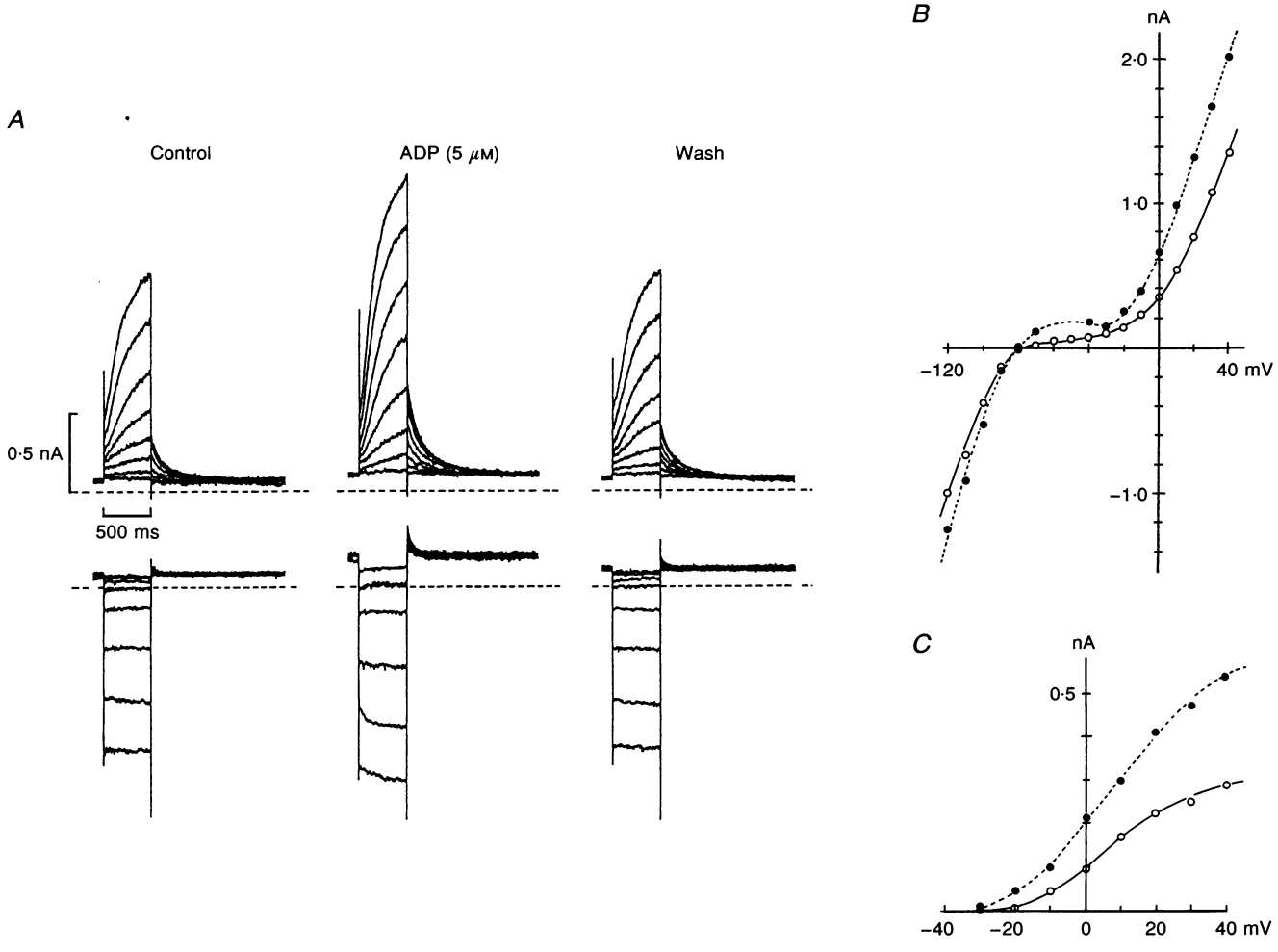

Figure 4. Effects of external ADP (5 μM) on whole-cell currents

A, current recordings during 500 ms voltage-clamp steps to membrane potentials from −30 to +40 mV (upper traces), and −70 to −120 mV (lower traces) applied from a holding potential of −40 mV under control conditions (left), during exposure to ADP (middle) and after washing out (right). Note that the effects were reversed to control levels with wash-out of ADP. *B*, *I–V* relationships of the late current from the records in *A*, under control conditions (○) and during exposure to ADP (●). *C*, *I–V* relationships of the I_K tail current, obtained from *A*. Continuous and dashed lines through data points were fitted by eye for control (○) and ADP (●), respectively.

tail current from 0·16 to 0·45 nA. This degree of enhancement (2·8-fold) of I_K by isoprenaline in atrial cells is compatible with a maximal response of I_K (2·5- to 3-fold) elicited by 1 μM isoprenaline in guinea-pig ventricular cells (Yazawa & Kameyama, 1990). After the response to isoprenaline reached a steady state, 5 μM ATP was then added. Even in the presence of 1 μM isoprenaline, 5 μM ATP produced a further increase in the I_K tail current to 0·64 nA, i.e. the effects of isoprenaline and ATP were additive, indicating that the β-adrenoceptor and P_2-purinoceptor do not share common signal transduction pathways to potentiate I_K. In a total of four atrial cells, subsequent application of 5 μM ATP in the presence of 0·5–1·0 μM isoprenaline produced an additional increase of I_K of 36·8 ± 12·3%. In addition, the pretreatment of atrial myocytes with 5 μM H-8, a non-specific protein kinase inhibitor, did not significantly affect the enhancement of I_K by ATP (data not shown). These results strongly suggest that protein kinase A did not mediate the enhancement of I_K through P_2-purinoceptors.

Is intracellular Ca^{2+} or protein kinase C involved? In rabbit heart, P_2-purinoceptor is suggested to be coupled to the activation of phospholipase C (Takikawa *et al.* 1990), which may lead to the generation of two second messengers: inositol 1,4,5-triphosphate ($InsP_3$), which mobilizes Ca^{2+}, and diacylglycerol (DAG), which activates protein kinase C. Both an elevation in intracellular free Ca^{2+} (Tohse *et al.* 1987; Tohse, 1990) and an activation of protein kinase C (Tohse *et al.* 1987; Walsh & Kass, 1988) have been associated with an enhancement of I_K in guinea-pig ventricular myocytes. Our finding that extracellular ATP potentiates I_K in cells dialysed with 5 mM EGTA suggests that intracellular Ca^{2+} does not play an essential role in mediating the stimulatory effects of ATP on I_K. This idea was further tested by replacing EGTA with BAPTA (Tsien, 1980) in a pipette solution, which should provide more rapid and more efficient Ca^{2+}-buffering conditions inside the cells. As shown in Fig. 7, 1 μM ATP increased I_K even in the cell loaded with 20 mM BAPTA. In a total of four myocytes loaded intracellularly with 20 mM BAPTA, 1 μM ATP increased

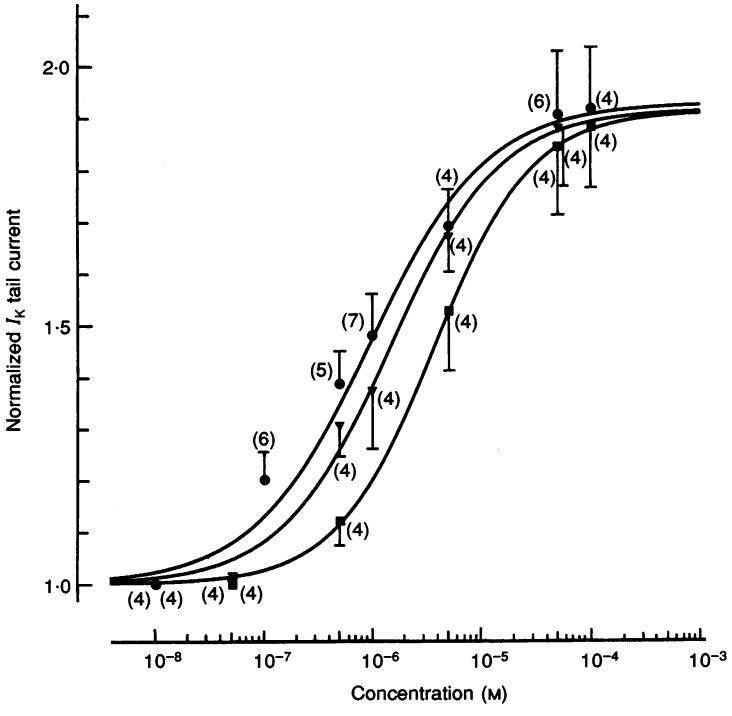

Figure 5. Concentration–response relationship for the enhancement of I_K by ATP, ATP-γ-S and ADP

The peak amplitude of the I_K tail current was measured on return to a holding potential of −40 mV after a 500 ms voltage step to +40 mV with various concentrations of ATP (●), ADP (■) and ATP-γ-S (▼), and was normalized with reference to the control value. Mean values (± S.E.M.) of normalized I_K tail current were plotted against agonist concentration semilogarithmically, and the smooth curve through the data points shows the least-squares fit of the Hill equation, $I = I_{max}/\{1 + (K_{½}/[\text{Agonist}])^{n_H}\}$, where I_{max} is the maximal response, $K_{½}$ the half-maximal concentration of agonist and n_H the Hill coefficient. For ATP, $K_{½} = 0{\cdot}95$ μM, $n_H = 0{\cdot}85$ and $I_{max} = 1{\cdot}93$; for ADP, $K_{½} = 3{\cdot}65$ μM, $n_H = 0{\cdot}96$ and $I_{max} = 1{\cdot}91$; and for ATP-γ-S, $K_{½} = 1{\cdot}44$ μM, $n_H = 0{\cdot}87$ and $I_{max} = 1{\cdot}91$. The number of measurements at each concentration is shown in parentheses. To exclude the influence of desensitization, only one concentration was tested in a given cell.

the I_K tail current elicited on return to the holding potential (−40 mV) from +40 mV by 39·6 ± 8·9%, which was similar to the enhancement observed in cells loaded with 5 mM EGTA (48·0 ± 7·9%, $n = 7$), showing that the stimulatory effect of ATP on I_K was not affected by an increased Ca^{2+}-buffering capacity achieved by BAPTA. This observation can be interpreted to indicate that ATP exerted its stimulatory effect on I_K through an intracellular mechanism independent of intracellular Ca^{2+}.

To test whether protein kinase C mediated the I_K response to external ATP, we investigated the effects of ATP on I_K in cells pretreated with H-7, which is known to completely abolish the enhancement of I_K produced through the activation of protein kinase C (Tohse *et al.* 1987). Figure 8 shows a representative result of such experiments with 10 μM H-7. External application of ATP increased I_K even after pretreatment with H-7. In a total of four atrial myocytes pretreated with 10 μM H-7 for 5–10 min, subsequent application of 5 μM ATP enhanced the I_K tail current elicited upon return to a holding potential of −40 mV after a voltage step to +40 mV by 58·6 ± 14·3%. This value does not differ significantly from the control value obtained in the absence of H-7 (68·7 ± 7·2%, $n = 4$), suggesting that protein kinase C was not involved in the ATP-induced enhancement of I_K.

DISCUSSION

The present study demonstrates that the stimulation of P_2-purinoceptors increases the amplitude of I_K without significant changes in current kinetics in guinea-pig atrial myocytes. In various types of cardiac cells, I_K has been demonstrated to be regulated by protein kinase A (see Introduction in Yazawa & Kameyama, 1990), protein kinase C (Tohse *et al.* 1987; Walsh & Kass, 1988) and intracellular Ca^{2+} ($>10^{-8}$ M) (Tohse *et al.* 1987; Tohse, 1990). In our study, the stimulatory effect of extracellular ATP on I_K was found to be additive to that of maximally effective concentrations (0·5–1·0 μM) of isoprenaline (Fig. 6), suggesting that extracellular ATP enhanced I_K through a mechanism independent of protein kinase A. Scamps *et al.* (1990, 1992) also demonstrated, using a standard radioimmunoassay technique in rat ventricular myocytes, that the stimulatory effects of ATP and isoprenaline on the L-type Ca^{2+} current are additive, and that external application of ATP does not significantly increase intracellular cyclic AMP levels. All these observations indicate that P_2-purinoceptor stimulation is not associated with the increase in activity of protein kinase A.

We have suggested that the P_2-purinoceptor is coupled to a PTX-sensitive G protein, G_K, and then to the muscarinic K^+ channel in guinea-pig atrial cell membrane (Matsuura

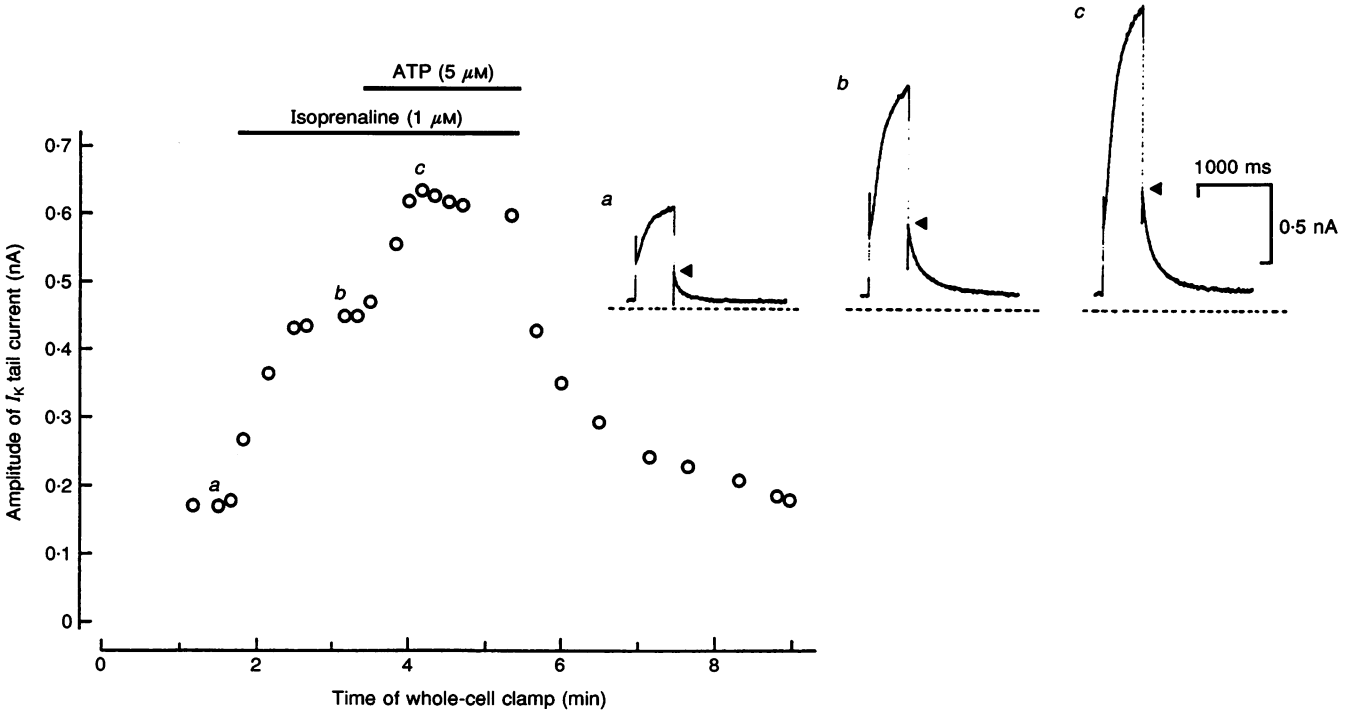

Figure 6. Effect of ATP on I_K in the presence of isoprenaline

Time course of response of the I_K tail current in response to isoprenaline (1 μM) and ATP (5 μM). The cell was initially exposed to isoprenaline (1 μM) and subsequently to isoprenaline plus ATP (5 μM). The amplitude of the I_K tail current was measured upon return to a holding potential of −40 mV after a 500 ms voltage step to +40 mV. Periods of exposure to isoprenaline and ATP are denoted by horizontal bars. The inset shows examples of the original current traces recorded at the times indicated on the graph (*a–c*). Dotted lines indicate zero current levels.

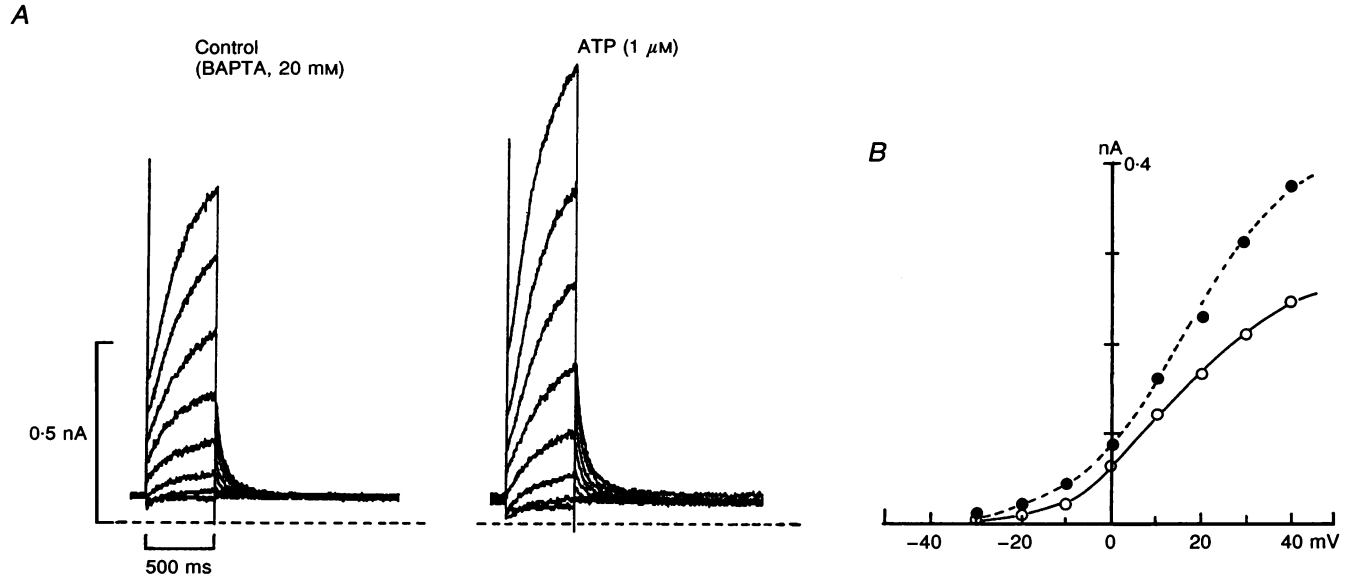

Figure 7. Effects of internal application of BAPTA on the ATP-induced enhancement of I_K

After the patch membrane was ruptured, a pipette solution containing 20 mM BAPTA was allowed to dialyse the cell interior for approximately 8 min before conducting current measurements. The concentration of intracellular free Ca^{2+} was estimated to be approximately $2{\cdot}0 \times 10^{-11}$ M (pCa 10·7) with 20 mM BAPTA in the pipette solution (Fabiato & Fabiato, 1979; Tsien & Rink, 1980). *A*, superimposed current traces in response to 500 ms voltage steps to membrane potentials of −30 to +40 mV in 10 mV steps applied from a holding potential of −40 mV. The currents on the left were obtained under control conditions (loaded with 20 mM BAPTA) and those on the right, during exposure to 1 μM ATP. The zero current level is indicated by the dashed line. *B*, *I–V* relationships for I_K tail currents obtained from the data in *A*, before (○) and during application of ATP (●).

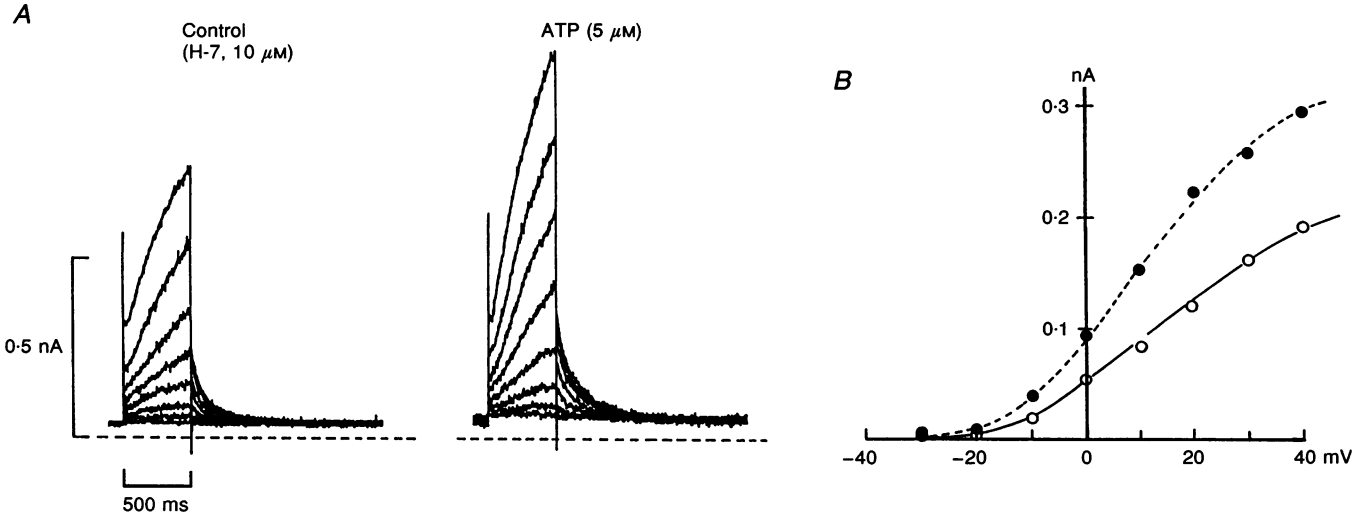

Figure 8. Enhancement of I_K by 5 μM ATP in the presence of protein kinase C inhibitor, H-7 (10 μM)

The cell was initially exposed to H-7 (10 μM) for 8 min to inhibit protein kinase C and then to H-7 plus ATP (5 μM). *A*, superimposed current traces recorded after 8 min exposure to H-7 (left) and 2 min after subsequent addition of ATP (right) using the same voltage-clamp protocol as in Fig. 7. Zero current level is indicated by the dashed lines. *B*, *I–V* relationships for I_K tail currents obtained from the records in *A*, before (○) and during application of ATP (●).

et al. 1996), which is analogous to the coupling mechanism of the muscarinic ACh receptor or P_1-purinoceptor for the activation of the muscarinic K^+ channel (Pfaffinger, Martin, Hunter, Nathanson & Hille, 1985; Breitwieser & Szabo, 1985; Kurachi *et al.* 1986). The question then arises as to whether P_2-purinoceptors also link with a PTX-sensitive inhibitory G protein (G_i) which counteracts the β-adrenergic stimulation of adenylate cyclase, as suggested for the muscarinic ACh receptor (Hescheler, Kameyama & Trautwein, 1986; Hwang, Horie, Nairn & Gadsby, 1992) and P_1-purinoceptor (Isenberg, Cerbai & Klöckner, 1987). Our finding that ATP further increased the isoprenaline-stimulated I_K (Fig. 6) suggests that P_2-purinoceptors do not effectively interact with G_i in guinea-pig atrial cell membrane. In contrast, in guinea-pig ventricular myocytes, extracellular ATP was shown to antagonize the isoprenaline-induced increases in the L-type Ca^{2+} and the Cl^- currents (Rankin, Sitsapesan & Kane, 1990), although the signal transduction pathway involved remains unknown. Further studies are necessary in both atrial and ventricular tissues to elucidate whether P_2-purinoceptor couples to G_i.

Regarding the possible role of protein kinase C, ATP potentiated I_K, even in cells pretreated with 10 μM H-7, to an extent similar to the controls (Fig. 8), indicating that protein kinase C does not mediate the enhancement of I_K via P_2-purinoceptors. It has been demonstrated in isolated resting cardiac myocytes that extracellular ATP increases intracellular free Ca^{2+} through the production of InsP_3 (Legssyer *et al.* 1988) or a membrane depolarization induced by activation of a non-selective cation channel (Hirano *et al.* 1991; Christie, Sharma & Sheu, 1992). However, our results show that external ATP potentiated I_K under the strong and efficient Ca^{2+}-buffering conditions achieved by 5 mM EGTA or 20 mM BAPTA (Fig. 7), indicating that the stimulatory effect of ATP on I_K was not obligatorily dependent upon intracellular Ca^{2+}. The deactivation time course of I_K has been shown to be slowed by the elevation of intracellular Ca^{2+} (Tohse *et al.* 1987; Tohse, 1990), whereas the present investigation demonstrates that extracellular ATP or ADP increased I_K without significantly changing the current kinetics (Figs 3 and 4). This finding may also support our view that intracellular Ca^{2+} does not mediate the I_K response to ATP. In guinea-pig ventricular myocytes, I_K is known to be regulated by intracellular Ca^{2+} concentrations greater than 10^{-8} M (Tohse *et al.* 1987; Tohse, 1990). Under the experimental conditions employed here, however, this Ca^{2+}-dependent I_K regulation should already be abolished by the 5 mM EGTA included in the pipette solution. Whether ATP potentiates the Ca^{2+}-sensitive I_K should be elucidated by using a reduced Ca^{2+}-buffering capacity inside the cell. The present experiments have shown that ATP enhances I_K via pathways that appear to be independent of protein kinase A, protein kinase C and intracellular Ca^{2+}. In the rabbit heart, P_2-purinoceptor stimulation has been demonstrated to stimulate arachidonic acid metabolism through cyclo-oxygenase and, thereby, produce prostaglandins (Takikawa *et al.* 1990). It remains to be elucidated whether such pathways are involved in the intracellular mechanism mediating the P_2-purinergic regulation of I_K.

Pre-incubation of the myocytes with PTX did not affect the enhancement of I_K by ATP, whereas PTX pretreatment abolished the ATP-induced activation of the muscarinic K^+ channel (see Fig. 5 in Matsuura *et al.* 1996). This clearly indicates that different signal transduction pathways are activated following P_2-purinoceptor stimulation to regulate the I_K and the muscarinic K^+ channel differentially in guinea-pig atrial myocytes, although it is unclear at present whether the same or distinct P_2-purinoceptors are coupled to mechanisms regulating I_K and the muscarinic K^+ channel. Nevertheless, a variety of postreceptor processes can be a characteristic feature of P_2-purinergic regulation of cellular functions. Indeed, in addition to the cellular reactions already mentioned (see above), several signal transduction patterns have been suggested for P_2-purinergic regulation of ionic channels. Direct coupling of the P_2-purinoceptor to the ionic channels is shown in the Ca^{2+}-permeable channels in rat aortic smooth muscle cells (Benham & Tsien, 1987). An isoform of a stimulatory guanine nucleotide regulatory protein (G_s) not coupled to adenylate cyclase has been implicated as a signal transducer for the P_2-purinergic regulation of the L-type Ca^{2+} channels in rat ventricular myocytes (Scamps *et al.* 1992). In this respect, the P_2-purinoceptor forms a striking contrast to the β-adrenoceptor, where protein kinase A is exclusively involved in the response.

ATP is supposed to act as a co-neurotransmitter with catecholamines in the heart (Hoyle & Burnstock, 1986). Our results raise the possibility that, in addition to β-adrenoceptors (See Introduction in Yazawa & Kameyama, 1990) and α-adrenoceptors (Tohse, Nakaya & Kanno, 1992), P_2-purinoceptors may also be involved in the autonomic regulation of I_K in the heart. Since β-adrenoceptors, α-adrenoceptors and P_2-purinoceptors do not share the same intracellular signal transduction pathways for the regulation of I_K, ATP and catecholamines seem to co-operate in potentiating the response of I_K effectively. In addition, platelets, vascular endothelial cells, smooth muscle cells (Gordon, 1986) and even hypoxic myocardium (Forrester & Williams, 1977; Borst & Schrader, 1991) can contribute to the local release of ATP into the plasma. From these sources, the blood concentration of ATP has been reported to rise to micromolar levels (Born & Kratzer, 1984), which are compatible with the activation range for the enhancement of I_K. Thus, under physiological and pathophysiological conditions, cardiac I_K may be affected by P_2-purinoceptors stimulated by extracellular ATP.

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