Activation of the muscarinic K^+ channel by P_2-purinoceptors via pertussis toxin-sensitive G proteins in guinea-pig atrial cells

Hiroshi Matsuura, Masayuki Sakaguchi, Yoshikazu Tsuruhara and Tsuguhisa Ehara

Department of Physiology, Saga Medical School, Saga 849, Japan

1. Whole-cell voltage clamp and cell-attached patch-clamp techniques were applied to single atrial myocytes enzymatically dissociated from adult guinea-pig hearts.

2. In whole-cell clamp conditions, external application of ATP activated the muscarinic K^+ (K_{ACh}) current, identified by its inward rectification, its reversal potential near the calculated K^+ equilibrium potential (E_K) and its relaxation properties during step changes of whole-cell membrane potential. Theophylline, an antagonist for P_1-purinoceptors, did not affect the action of ATP on the K_{ACh} current, indicating that the response was evoked through P_2-purinoceptors.

3. The concentration–response relationship for ATP was well described by a Hill equation with a half-maximal concentration of 1·84 μM and a Hill coefficient of 0·94. ATP (100 μM) produced a maximal increase of the K_{ACh} current to 10·92 $\mu A\ \mu F^{-1}$, which corresponds to 44·9 and 80·9% of the maximal increases evoked by ACh (10 μM) and adenosine (100 μM), respectively.

4. The ATP-induced K_{ACh} current gradually declined to a steady level despite the continuous presence of ATP (desensitization). Recovery from the desensitization was relatively rapid with a half-time of approximately 1·5 min.

5. The activation of K_{ACh} current by ATP was completely abolished by pre-incubating myocytes with pertussis toxin (PTX, 5 $\mu g\ ml^{-1}$), indicating that P_2-purinoceptors are coupled to PTX-sensitive G proteins to activate the K_{ACh} channel.

6. In the cell-attached patch recording, ATP (5 μM) applied to the pipette solution enhanced the activity of a channel with single-channel conductance of 52·7 $\pm$ 0·9 pS (mean $\pm$ S.E.M., $n = 10$), reversal potential near E_K and mean open time of 1·1 $\pm$ 0·1 ms. These conductance and kinetic properties are identical to those of the K_{ACh} channel in the heart. In contrast, ATP applied to the bath solution did not significantly affect the basal activity of K_{ACh} channel openings. These observations suggest that the mechanism coupling the P_2-purinoceptor to the activation of the K_{ACh} channel involves membrane-delimited component(s) rather than soluble second messenger(s).

7. These results strongly suggest a direct coupling of the P_2-purinoceptor to the K_{ACh} channel through PTX-sensitive G proteins, analogous to the coupling mechanism of the muscarinic ACh receptor and P_1-purinoceptor to this channel.

Activation of the muscarinic K^+ (K_{ACh}) channel by acetylcholine (ACh) is involved in a slowing of spontaneous pacemaker activity in sino-atrial (SA) and atrioventricular (AV) nodal cells associated with the negative chronotropic and dromotropic effects of vagal activity, respectively (for review see Hartzell, 1988). In atrial tissue, the K_{ACh} channel shortens the action potential duration and causes membrane hyperpolarization (Belardinelli & Isenberg, 1983). The muscarinic ACh receptor and P_1-purinoceptor have been clearly demonstrated to be directly coupled to the K_{ACh} channel via a pertussis toxin (PTX)-sensitive G protein (G_K) in atrial cell membranes (Pfaffinger, Martin, Hunter, Nathanson & Hille, 1985; Breitwieser & Szabo, 1985; Kurachi, Nakajima & Sugimoto, 1986). In addition to the regulation by muscarinic ACh and P_1-purinergic receptors, the K_{ACh} channel has recently been found to receive control from other types of receptors, including α_1-adrenergic receptors (Kurachi, Ito, Sugimoto, Shimizu,

Miki & Ui, 1989*b*) and receptors for calcitonin gene-related peptide (Kim, 1991*a*), endothelin (Kim, 1991*b*; Ono, Tsujimoto, Sakamoto, Eto, Masaki, Ozaki & Satake, 1994), somatostatin (Lewis & Clapham, 1989), serum factor (Banach, Hüser, Lipp, Wellner & Pott, 1993) and platelet-activating factor (Nakajima, Sugimoto & Kurachi, 1991; Ramos-Franco, Lo & Breitwieser, 1993). The activation of the K_{ACh} channel through these receptors is also mediated by G_K.

In guinea-pig atrial cells extracellular application of ATP has been demonstrated to shorten action potential duration, and this effect is thought to be mediated preferentially through the stimulation of P_1-purinoceptors by adenosine formed on ATP degradation by ecto-5′-nucleotidase on the extracellular membrane surface of cardiac cells (Ragazzi, Wu, Shryock & Belardinelli, 1991). The direct effects of ATP on the K_{ACh} channel have, until recently, been regarded as relatively unimportant, and little information has been available on the regulation of the cardiac K_{ACh} channel by P_2-purinoceptors. Friel & Bean (1990) have shown that, in whole-cell and cell-attached patch recordings on bovine atrial cells, ATP (100 μM) activates a population of K^+ channels with conductance and kinetic properties similar to those elicited by carbachol (100 μM), and suggested that both agonists regulate the same type of K^+ channel, i.e. the K_{ACh} channel. However, the signal transduction system associated with activation of the K_{ACh} channel by P_2-purinoceptors is not well understood. In the present work we examined whether P_2-purinoceptor stimulation can activate the K_{ACh} channel in guinea-pig atrial cell membranes. Our results provide evidence favouring a direct coupling of P_2-purinoceptors to the K_{ACh} channel through G_K, analogous to the mechanism of muscarinic and P_1-purinergic activation of this channel.

METHODS

Cell preparation

Single atrial cells were obtained from guinea-pig hearts using an enzymatic dissociation procedure (Powell, Terrar & Twist, 1980). The full details of the cell dissociation procedure are described in the preceding paper (Matsuura, Tsuruhara, Sakaguchi & Ehara, 1996). Spindle-like quiescent atrial cells with clear striations were used in the experiments.

Voltage-clamp technique

Membrane currents were recorded in 'whole-cell' and 'cell-attached' configurations of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) with a List 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 electrode tips were then fire-polished with a microforge. Electrode resistance ranged from 1·2 to 2·0 MΩ for whole-cell recording and from 5·0 to 8·0 MΩ for single-channel recording, when filled with the respective pipette solutions. Cell membrane capacitance, measured by the integral of the charging transient in response to a 5 or 10 mV step pulse in the voltage-clamp condition, was 60·1 ± 2·7 pF (mean ± S.E.M.; $n = 53$).

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). In the whole-cell recording, $CdCl_2$ (0·1 mM) was added to the normal Tyrode solution to block the L-type Ca^{2+} channel. The pipette solution for the whole-cell recording 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). Since the amount of KOH required for titration was 24 mM on average, the total K^+ concentration in the pipette solution was 154 mM. In cell-attached patch-clamp conditions, the pipette solution contained (mM): KCl, 140; $CaCl_2$, 1·8; $MgCl_2$, 1·0; and Hepes, 5 (pH adjusted to 7·4 with KOH). The KB medium (Isenberg & Klöckner, 1982) used 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). Pertussis toxin (200 μg ml^{-1}; Seikagaku Kogyo, Tokyo) was dissolved in KB solution for cell incubation at a final concentration of 5 μg ml^{-1} (Hwang, Horie, Nairn & Gadsby, 1992). The experiments reported here were carried out at 34–36 °C.

Data analysis

Current and voltage signals were recorded on digital audiotape (DT-120; SONY, Tokyo) using a PCM data recorder (RD101T; TEAC, Tokyo) for subsequent computer analysis (PC98 RL; NEC, Tokyo). Current records were fed to the computer every 1 or 2 ms for the analysis of whole-cell currents and every 0·1 or 0·2 ms for that of single-channel currents, through a low-pass filter (48 dB per octave; E-3201A; NF, Tokyo) at an appropriate cut-off frequency. Data are expressed as mean values ± S.E.M.

RESULTS

Activation of the K_{ACh} current through P_2-purinoceptors

In the presence of 500 μM theophylline, a saturating concentration (100 μM) of adenosine failed to activate the K_{ACh} current in guinea-pig atrial myocytes (data not shown, refer to Banach *et al.* 1993), indicating that 500 μM theophylline was sufficient to block P_1-purinoceptors. In the experiments shown in the present study, theophylline was, therefore, included in the external solution to block P_1-purinoceptors before the application of ATP. Bath application of 1 μM ATP induced an outward shift of the holding current, followed by a gradual decline despite the continuous presence of ATP (see Fig. 3). In the preceding paper (Matsuura *et al.* 1996), we demonstrated that P_2-purinoceptor stimulation potentiates the delayed rectifier K^+ current (I_K), which is activated at potentials positive to −30 mV. In the present study, we examined the properties of the ATP-induced membrane current activated at a holding potential of −40 mV and during hyperpolarizing steps applied from −40 mV, where the activation of I_K was absent. Figure 1*A* shows the current traces in

response to hyperpolarizing command pulses to potentials between −50 and −120 mV in 10 mV steps in control conditions (upper row) and during exposure to ATP (middle row), as well as the ATP-induced current obtained by digital subtraction at each test potential (lower row). The ATP-induced current gradually increased during strong hyperpolarizing pulses (e.g. −100, −110 or −120 mV) and decayed with time to steady current levels upon return to a holding potential of −40 mV (Fig. 1*A*, lower row). This relaxation property was identical to that of the K_{ACh} current activated by ACh or adenosine in rabbit SA nodal cells (Noma & Trautwein, 1978) and guinea-pig atrial cells (Kurachi *et al.* 1986). Figure 1*B* demonstrates the current–voltage (*I–V*) relationships of late current recorded under control conditions (○) and during exposure to ATP (●). The *I–V* relationship of the ATP-induced

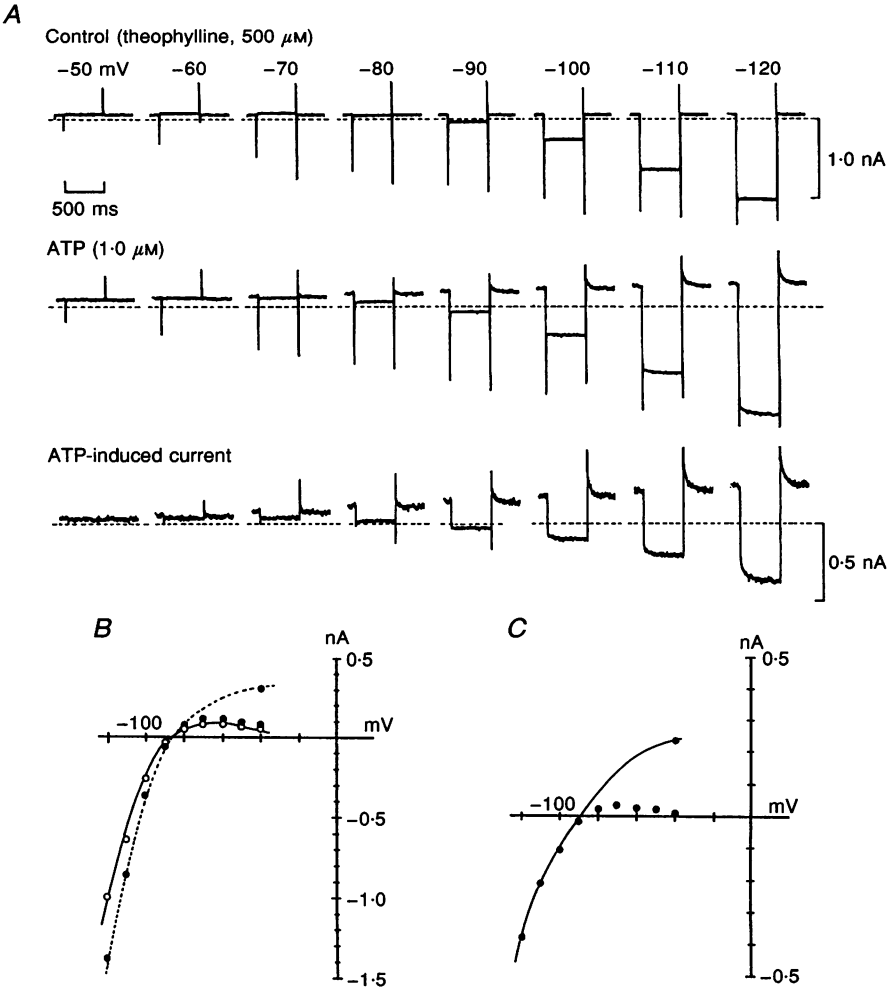

Figure 1. Characterization of external ATP (1 μM)-induced current

A, current traces in response to 500 ms hyperpolarizing test pulses from a holding potential of −40 mV under control conditions (upper row) and during exposure to ATP (middle row), and ATP-induced current (lower row) obtained by digital subtraction of the current traces recorded under control conditions from those recorded in the presence of ATP. Test potentials are indicated above the control traces. Zero current level is indicated by dashed lines in each trace. Note differences in the holding current levels upon the imposition of test pulses observed both in the current recorded in the presence of ATP (middle row) and in the ATP-induced current (lower row), which were associated with the gradual decline of the holding current despite the continuous presence of ATP. *B*, *I–V* relationships of the late current from the records in *A*, under control conditions (○) and in the presence of ATP (●). Late current levels were measured near the end of each pulse. At the −40 mV holding potential, both the maximal and minimal values are plotted for the current recorded in the presence of ATP. Continuous and dotted lines through data points were fitted by eye for control and ATP (using the maximal value at −40 mV), respectively. *C*, *I–V* relationship of the ATP-induced current, obtained from *B*. At the −40 mV holding potential, both the maximal and minimal values are plotted. Continuous line through data points was fitted by eye using the maximal value at −40 mV.

current (Fig. 1*C*) exhibited an inward-going rectification and crossed the voltage axis at −87 mV, which is close to the calculated equilibrium potential of K^+ ($E_K = -88{\cdot}2$ mV), given a bath $[K^+]$ of 5·4 mM and a pipette $[K^+]$ of 154 mM, indicating that this current was predominantly carried by K^+. Based on these electrophysiological properties (high K^+ selectivity, inward rectifying *I–V* relationship and relaxing properties on voltage jumps), this ATP-induced current during hyperpolarizing pulses can be identified as the K_{ACh} current.

External application of ADP (>1 μM) and adenosine-5′-*O*-(3-thiotriphosphate) (ATP-γ-S; >1 μM) were also effective in activating the same type of K^+ current (see Fig. 4 in Matsuura *et al.* 1996). These results indicate that stimulation of P_2-purinoceptors results in an activation of the K_{ACh} current.

Figure 2 shows the concentration–response relationship for the activation of the K_{ACh} current by ATP. The ATP-induced increase in outward current at −40 mV associated with the activation of K_{ACh} current was normalized with reference to the cell membrane capacitance. The relationship between the current density of the K_{ACh} current thus obtained and ATP concentration was well fitted by a Hill equation with a half-maximal concentration ($K_{½}$) of 1·84 μM and a Hill coefficient of 0·94. ATP (100 μM) produced a maximal activation of the K_{ACh} current of 10·92 ± 1·14 μA μF^{-1} ($n = 12$), which corresponds to 44·9 ± 4·5 and 80·9 ± 4·4% of the maximal increases evoked by ACh (10 μM, $n = 6$) and adenosine (100 μM, $n = 4$), respectively. In bovine atrial myocytes the $K_{½}$ of ATP for the activation of the K_{ACh} current was reported to be 9·5 μM (Friel & Bean, 1990), which is about five times greater than that in guinea-pig atrial myocytes, indicating that ATP is more effective in guinea-pig atrium than in bovine atrium in activating the K_{ACh} current.

Desensitization of ATP-induced K_{ACh} current

K_{ACh} current induced by high concentrations of ACh or adenosine was demonstrated to exhibit two phases of desensitization, i.e. a rapid phase appearing within the first 20 s and a secondary slower phase continuing over a few minutes, in atrial cells (Kurachi, Nakajima & Sugimoto, 1987; Kim, 1991*c*) and Purkinje fibres (Carmeliet & Mubagwa, 1986). In guinea-pig atrial myocytes adenosine was reported to increase the K_{ACh} current in a concentration-dependent manner with a $K_{½}$ of 0·8 μM (Kurachi *et al.* 1986) or 3·1 μM (Ragazzi *et al.* 1991) and Hill coefficient of 1·0 (Kurachi *et al.* 1986; Ragazzi *et al.* 1991), values which are similar to those for ATP, suggesting that the concentration–response relationship for adenosine is compatible with that for ATP. Since the maximal response induced by ATP (100 μM) was 80·9% of that evoked by adenosine (100 μM), the amplitude of the K_{ACh} current activated by ATP at a given concentration is expected to be nearly similar (about 80%) to that evoked by an equimolar concentration of adenosine. The desensitization process of the ATP-induced K_{ACh} current was, therefore, compared with that of adenosine-induced current at an equimolar concentration. Figure 3 shows a representative result from such a comparison at a concentration of 1 μM. The cell was initially exposed to 1 μM adenosine and subsequently to 1 μM ATP, with a 5 min wash-out period, which was shown to be sufficient to allow recovery from the desensitization in the adenosine response (Kurachi *et al.* 1987). Whereas adenosine and ATP caused outward shifts of the holding current, associated with the activation of the K_{ACh} current, of 0·33 and 0·42 nA, respectively, there was a significant difference in the time course of the response during the continuous presence of the agonists. Adenosine (1 μM)-induced outward current gradually declined to approximately 80% of its peak value by the end of a 2 min exposure. An acute

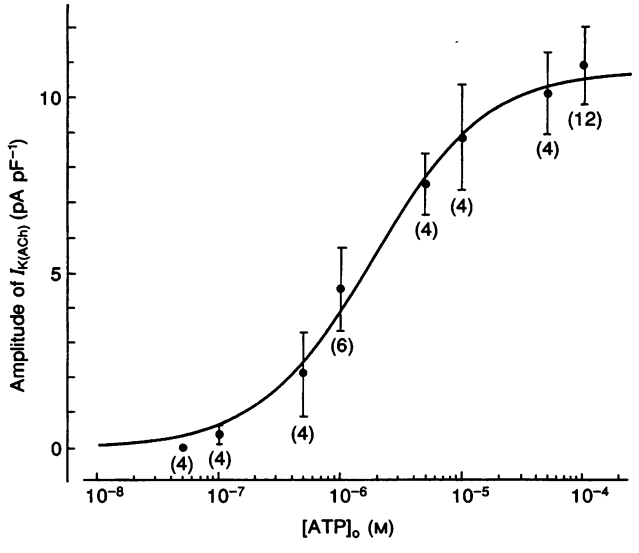

Figure 2. Concentration–response relationship for activation of K_{ACh} current by ATP

The maximal amplitude of ATP-induced K_{ACh} current was measured at a holding potential of −40 mV at each concentration of ATP and was normalized with reference to the cell membrane capacitance. Data are expressed as mean values ± S.E.M. (number of measurements). The curve was drawn by a least-squares fit of the Hill equation: $I = I_{max}/\{1 + (K_{½}/[Agonist])^{n_H}\}$, with I_{max} (maximal response) = 10·92, $K_{½}$ (half-maximal concentration of agonist) = 1·84 μM and n_H (Hill coefficient) = 0·94. Only one concentration of ATP was examined in a given cell to exclude the influence of desensitization. In order to compare the maximal response of K_{ACh} current induced by ATP with that induced by ACh or adenosine, the cell was initially exposed to 100 μM ATP and then to 10 μM ACh or 100 μM adenosine with a wash-out period of about 5 min.

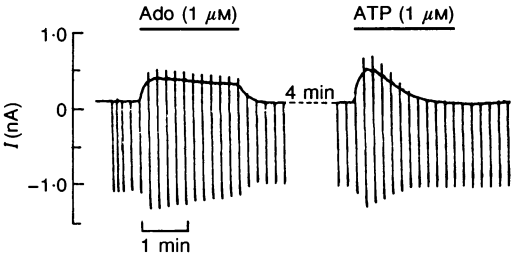

Figure 3. Desensitization of adenosine- and ATP-induced K_{ACh} currents

Chart record of whole-cell current at the −40 mV holding potential and in response to 500 ms step pulse to −110 mV applied every 10 s. The cell was successively exposed to adenosine (Ado; 1 μM) and ATP (1 μM) for 2 min each, as indicated by bars over the chart record. Theophylline (500 μM) was present during the application of ATP.

desensitization process (Kurachi *et al.* 1987) was not evident in this decline of the K_{ACh} current, possibly due to the low concentration (1 μM) of adenosine used here. In contrast, the ATP-activated outward current decayed rapidly to control levels during a 2 min exposure. The K_{ACh} currents activated by 0·5, 1 and 10 μM ATP at a holding potential of −40 mV declined to 35·1 ± 4·8% ($n = 4$), 26·9 ± 3·7% ($n = 12$) and 3·3 ± 2·3% ($n = 6$) of their peak amplitudes during a 1 min exposure, respectively, showing that the extent of desensitization became more pronounced with increasing ATP concentration. On the other hand, 1 and 10 μM adenosine-induced K_{ACh} currents decreased to 91·5 ± 1·6% ($n = 6$) and 75·5 ± 1·9% ($n = 4$) of their peak amplitude during a 1 min exposure, respectively. Thus, the ATP-induced K_{ACh} current showed much more rapid and more prominent desensitization than the adenosine-induced current, when compared at an equimolar concentration.

We then examined the time course of the recovery from desensitization of ATP-induced K_{ACh} current by monitoring the outward K^+ current responses during twin-pulse application of ATP to the cell, the first application producing a steady-state desensitization and the second application measuring the amount of recovery from desensitization that occurred during the wash-out period. Examples of such experiments are shown in Fig. 4. To induce the conditioning desensitization, cells were exposed to 1 μM ATP for 70–85 s. With a 1 min wash-out period (Fig. 4*A*, upper trace), the peak amplitude (I_2) of the outward current during the second application of ATP was only about 30% of that (I_1) during its first application, while with a 3 min wash-out period (Fig. 4*A*, lower trace), I_2 was almost equal to I_1. The ratio of I_2 to I_1 was plotted against the duration of the interval (recovery time) between the end of the first application and the beginning of the second one (Fig. 4*B*).

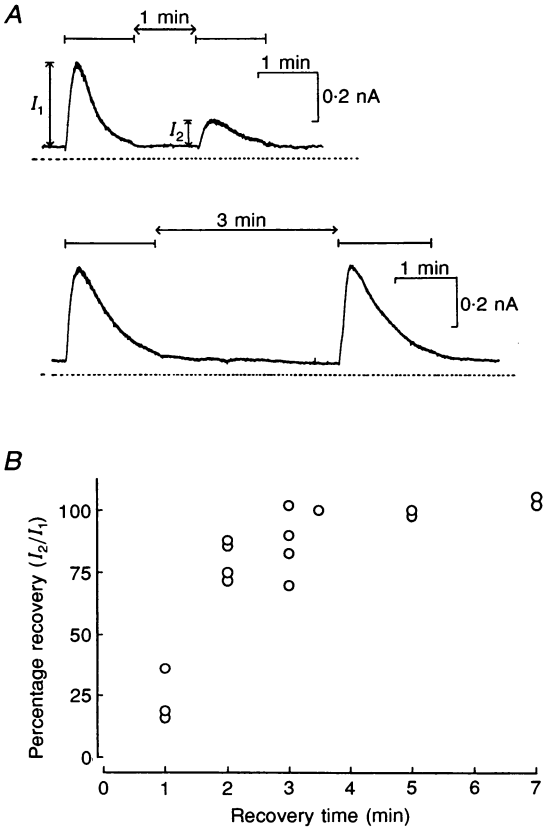

Figure 4. Recovery from desensitization by ATP

A, original current traces in response to twin-pulse applications of ATP (1 μM) to a cell continuously voltage clamped to −40 mV. The intervals between the first and second applications of ATP were 1 min in the upper trace and 3 min in the lower trace. The periods of application of ATP are denoted by the horizontal bars above the current records. I_1 and I_2 shown in the original trace indicate the peak amplitudes of the outward currents evoked by the two applications of ATP (1 μM). Zero current level is indicated by the dotted lines. *B*, time course of recovery from desensitization. The ratio of I_2 to I_1 (percentage recovery) was plotted against the duration of the interval between the end of the first application and the beginning of the second application (recovery time).

With a 4–5 min wash-out period, the ratio (I_2/I_1) returned to approximately 1, indicating that the desensitization produced by 1 μM ATP was almost completely removed within these time periods. Half-recovery from the desensitization took place with a wash-out period of about 1·5 min.

Involvement of PTX-sensitive G proteins in the ATP-induced activation of K_{ACh} current

We carried out experiments investigating the signal transduction mechanisms mediating the P_2-purinergic activation of the K_{ACh} current. Since G proteins sensitive to PTX were shown to couple both muscarinic ACh and P_1-purinergic receptors to the K_{ACh} channels (Pfaffinger *et al.* 1985; Breitwieser & Szabo, 1985; Kurachi *et al.* 1986), we examined the role of PTX-sensitive G proteins in the signal transduction from P_2-purinoceptors to the K_{ACh} channel. Atrial myocytes were pretreated with 5 μg ml^{-1} PTX for 2 h at 32 °C (Hwang *et al.* 1992) and the inhibition of the PTX-sensitive G proteins by this procedure was confirmed by the observation that 1 μM ACh could not activate the K_{ACh} channel in the atrial myocytes pretreated with PTX in this way (data not shown). As shown in Fig. 5, application of 5 μM ATP to a PTX-treated cell failed to produce both the outward shift of the holding current and the increase in membrane currents during hyperpolarizing pulses (Fig. 5*A* and *B*), indicating that a PTX-sensitive G protein was indeed involved in the ATP-induced activation of the K_{ACh} current. On the contrary, the time-dependent outward current (I_K) during depolarizing pulses was still enhanced by exposure to ATP in PTX-treated cells (Fig. 5*C*), suggesting that PTX-sensitive G proteins did not act as transducers of the signal from the P_2-purinoceptor to the delayed rectifier K^+ channel. Thus, two kinds of cardiac K^+ channels (delayed rectifier K^+ and K_{ACh} channels) seem to be modulated by the P_2-purinoceptor through two distinct signal transduction pathways.

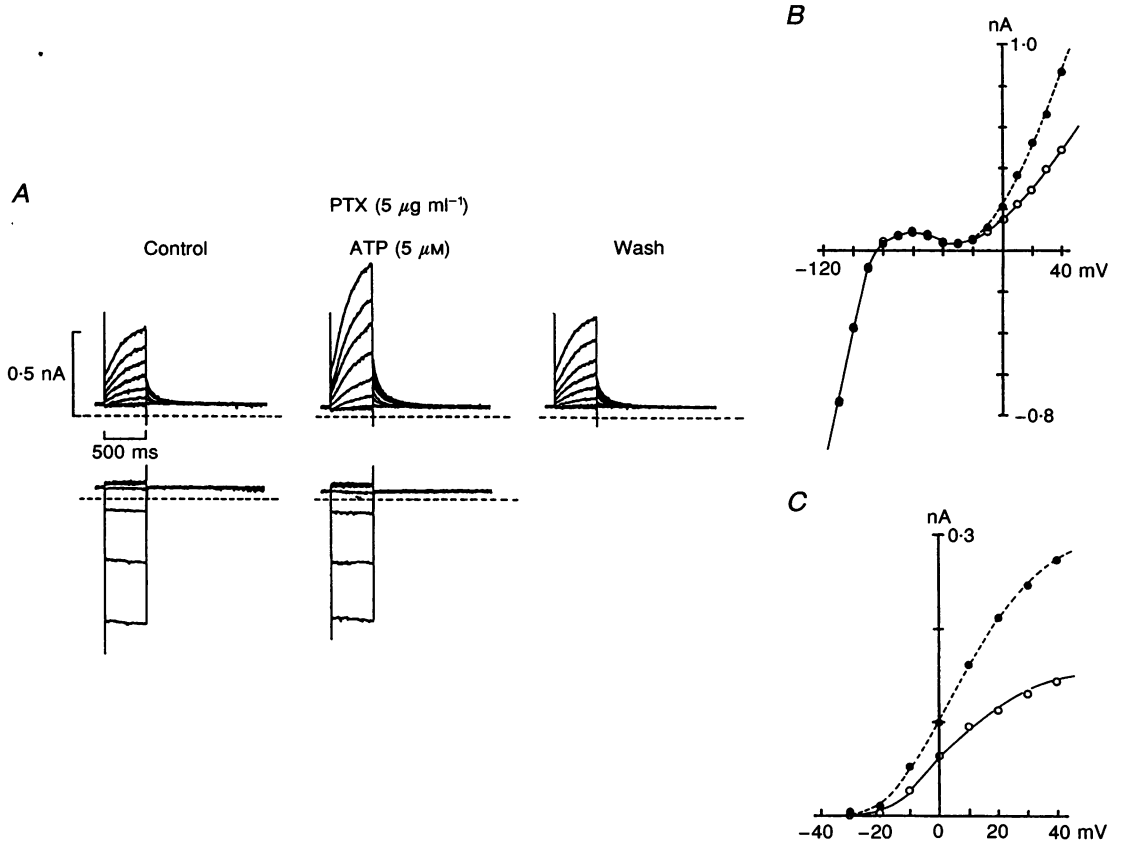

Figure 5. Involvement of PTX-sensitive G proteins in the signal transduction from the P_2-purinoceptor to the K_{ACh} channel

The cell was previously incubated for 2 h at 32 °C in KB solution containing 5 μg ml^{-1} PTX. *A*, current traces during 500 ms voltage steps to membrane potentials of −30 to +40 mV (upper traces) and −50 to −110 mV (lower traces) from a holding potential of −40 mV recorded under control conditions (left), during exposure to ATP (middle) and after washing out (right). Zero current level is indicated by dashed lines. *B*, *I–V* relationships of the late current measured near the end of each clamp pulse, obtained from the data in *A*, under control conditions (○) and during exposure to ATP (●). Continuous and dashed lines through data points were fitted by eye for control and ATP, respectively. *C*, *I–V* relationship of I_K tail current elicited on return to the holding potential of −40 mV after 500 ms voltage pulses to various test potentials, obtained from *A*, for control conditions (○) and ATP (●). Continuous and dashed lines through data points were fitted by eye for controls and ATP, respectively.

Properties of ATP-induced K_{ACh} currents at the single-channel level

Properties of single K_{ACh} channel currents activated by ACh and adenosine have been examined in pacemaker (Sakmann, Noma & Trautwein, 1983) and atrial cells (Soejima & Noma, 1984; Kurachi *et al.* 1986), and they are characterized by a single-channel conductance of about 45–55 pS and a mean open time of the order of 1 ms. Single-channel current recordings were made to obtain conclusive evidence for P_2-purinergic activation of the K_{ACh} channel. With the control pipette solution (140 mM KCl), a cell-attached patch recording on an atrial cell in

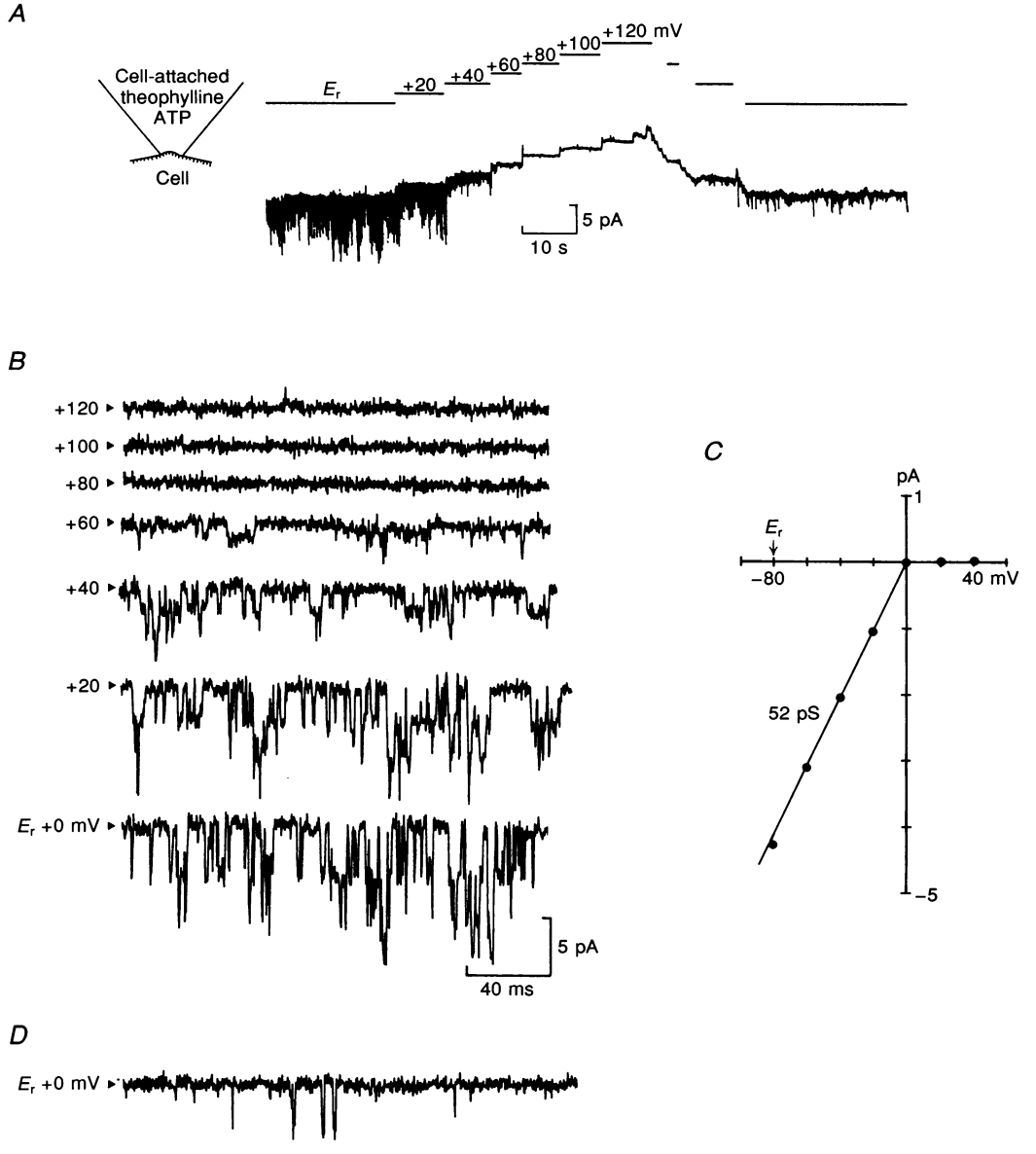

Figure 6. ATP-induced K_{ACh} channel current in the cell-attached patch

Inward channel currents are recorded as downward deflections. *A*, chart-recording of single-channel currents from a cell-attached patch exposed to 5 μM ATP in a pipette. Theophylline (100 μM) was also included in the pipette to block P_1-purinoceptors. Pipette potential was stepped to the levels indicated above the current trace. E_r, resting membrane potential. The gigaohm seal was established approximately 10 s before the onset of this record. *B*, expanded current traces at various patch membrane potentials in *A*. Zero current level is indicated by the closed triangle beside each trace and the membrane potential, expressed as the voltage deviation from E_r, is indicated to the left. The current was sampled at 5 kHz through a low-pass filter of 2 kHz. *C*, *I–V* relationship of ATP-induced K^+ channel currents obtained from the traces shown in *B*. Unitary amplitude of channel current at each test potential was plotted against the membrane potential, and E_r was assumed to be −80 mV. *D*, channel current at E_r recorded 2 min after the establishment of the gigaohm seal. Note that the channel activities were dramatically reduced and that unit amplitude at E_r was not changed.

Tyrode solution revealed sporadic channel openings (open probability, 0·01 ± 0·002) with unitary amplitude of 4·10 ± 0·07 pA ($n = 8$) at the resting membrane potential (E_r) (see Fig. 7), which is referred to as the basal activity of the K_{ACh} channel (Sakmann *et al.* 1983; Soejima & Noma, 1984). Figure 6 demonstrates typical examples of channel currents recorded in the cell-attached patch when 5 μM ATP was added to the control pipette solution. Since external ATP might directly stimulate the P_1-purinoceptor, or be degradated to adenosine and then stimulate the P_1-purinoceptor, 100 μM theophylline was also included in the pipette solution to block the P_1-purinoceptor (Kurachi

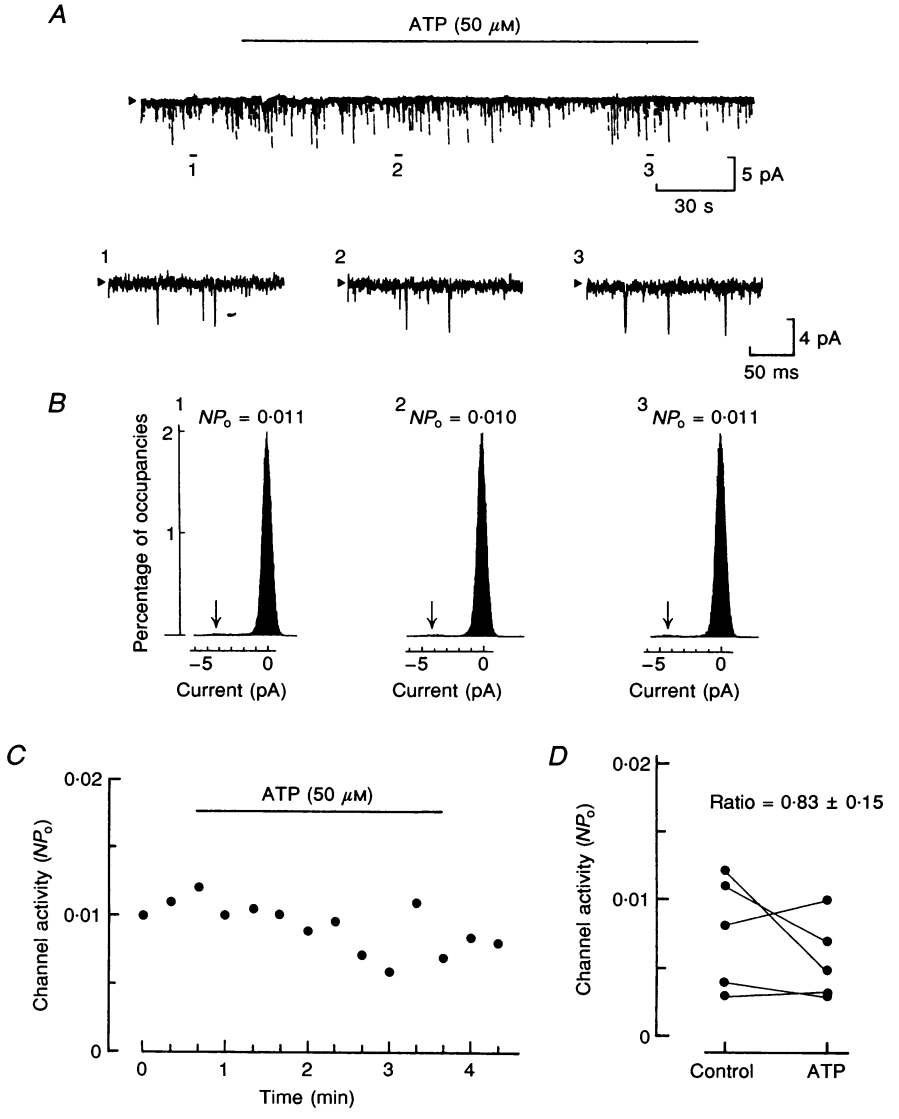

Figure 7. Effects of ATP added to the bath on activity of the K_{ACh} channel

A, chart-recording of single-channel currents from a cell-attached patch before and during the application of 50 μM ATP to the bath solution (normal Tyrode solution). The recording was made at E_r (assumed to be −80 mV). Expanded current traces in lower row were recorded at points marked 1, 2 and 3 on the chart-recording. Zero current level is indicated by closed triangle on the left of each row. *B*, histograms of current amplitude constructed from 40 sequential current traces (8·192 s in duration) at points identified by numerals (1–3) on the chart-recording in *A*. The mean patch currents, obtained by integrating the amplitude histogram, at points marked 1, 2 and 3 were −0·046, −0·042 and −0·047 pA, respectively. The unit amplitude of the channel current, measured as the interval between the closed level and the open level (denoted by arrows), at points marked 1, 2 and 3 were −4·18, −4·17 and −4·25 pA, respectively. NP_o, calculated by dividing the mean patch current by the unit amplitude of the channel current, is shown for each point (1–3). *C*, plot of channel activity, represented as mean NP_o during 8·192 s periods, *versus* time of perfusion with ATP in the experiment shown in *A*. *D*, changes in channel activities (mean NP_o) induced by 3 min exposure to 50 μM ATP in 5 experiments.

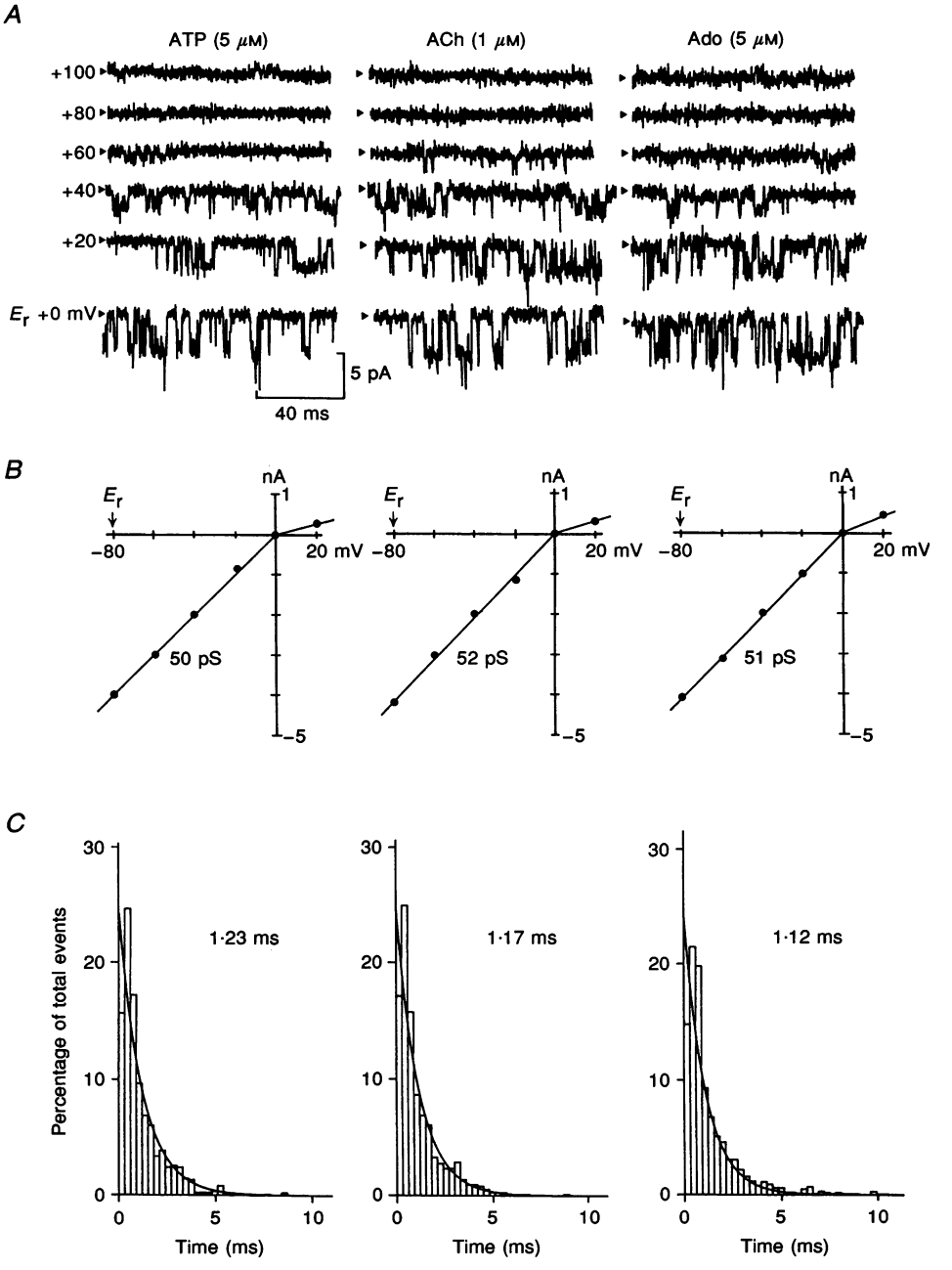

Figure 8. Conductance and kinetic properties of channel currents activated by ATP, ACh and adenosine

A, typical examples of single-channel currents activated by 5 μM ATP (left traces), 1 μM ACh (middle) and 5 μM adenosine (Ado, right) in the pipette solution. The ATP-induced single-channel currents were measured within the first 2 min after the establishment of a gigaohm seal. The membrane potential, expressed as voltage deviation from E_r, is indicated beside each trace on the left. The closed triangle beside each trace represents zero current level. All records were sampled at 10 kHz through a low-pass filter of 2 kHz. *B*, *I–V* relationship of ATP- (left), ACh- (middle) and adenosine-induced (right) channel currents obtained from the records in *A*. E_r was estimated to be −80 mV on the voltage axis. The slope conductances of unitary inward currents activated by ATP, ACh and adenosine were 50, 52 and 51 pS, respectively. The lines through the data were fitted by eye. *C*, dwell-time histograms in the open-state for ATP-, ACh- and ATP-induced channel currents at E_r. A threshold level for discrimination of open and closed states was set at around half of the main open level. Each histogram was formed with a bin of 0·3 ms and was fitted by a single-exponential function using a least-squares algorithm with the time constant indicated. The number of events was 830 in ATP, 718 in ACh and 1022 in adenosine.

et al. 1986). The high-density trace in the chart-record of the cell-attached patch currents indicates the ATP-induced channel openings at a high frequency (Fig. 6*A*). Figure 6*B* demonstrates examples of the channel currents at various test potentials. In this patch, at least three channels were included and the unitary current amplitude increased with hyperpolarization. The step size of channel current became zero at around E_K (assumed to be E_r +80 mV). With further depolarization of the patch membrane, outward channel currents were rarely detected. Unitary amplitude of ATP-induced channel current was plotted against the membrane potential (Fig. 6*C*). The *I–V* relationship exhibits an inward rectification in the potential range positive to E_K, and slope conductance of inward channel currents is 52 pS (Fig. 6*C*), which is similar to those reported for K_{ACh} channels in nodal (Sakmann *et al.* 1983) and atrial cell membranes (Soejima & Noma, 1984; Kurachi *et al.* 1986). The channel activities gradually declined during exposure of the patch membrane to 5 μM ATP, becoming only sporadic by 2 min (Fig. 6*D*), which is very similar to the pattern of the whole-cell current desensitization (Fig. 3). To quantify the channel activity shown in Fig. 6, the mean patch current ($I = NP_o i$), obtained by integrating the amplitude histogram (not shown), was divided by the unit amplitude of the single-channel current (i) to yield NP_o (I/i), where N is the total number of channels active within the patch and P_o is the probability of the channel being open. The values of NP_o thus measured at 20 s and 2 min after exposure to ATP were 0·32 and 0·02, respectively.

We then examined whether ATP applied outside the patch membrane affects the activity of the K_{ACh} channel. Figure 7*A* shows a continuous recording of single-channel currents from a cell-attached patch before and during the application of 50 μM ATP to the bath solution. Sporadic and brief openings of K_{ACh} channels were observed throughout the recordings. The amplitude histogram of the current traces was measured every 20 s to calculate the mean patch current (Fig. 7*B*). The mean patch currents at points marked 1 (control), 2 (1 min exposure to ATP) and 3 (2 min 40 s exposure) were −0·046, −0·042 and −0·047 pA, respectively. The amplitude histogram also shows that the unit amplitude of the channel current, measured as the interval between the first and the second peak, was not significantly affected by adding ATP to the bath (Fig. 7*B*). The values of NP_o, obtained by dividing the mean patch current by unit amplitude, at points marked 1, 2 and 3 were 0·011, 0·010 and 0·011, respectively. Figure 7*C* illustrates changes in the channel activity, calculated as NP_o, during ATP application in the experiment shown in *A*. Although the channel activity was associated with temporal variation (Bünemann & Pott, 1993), it is evident that ATP did not increase the channel activity. Figure 7*D* summarizes the effects of ATP added to the bath on the activities of the K_{ACh} channel in five experiments. The ratio of NP_o measured 3 min after ATP application to that in control conditions was 0·83 ± 0·15 ($n = 5$), showing that the activities of the K_{ACh} channel were not significantly affected by ATP applied outside the patch. Thus, K_{ACh} channels in the cell-attached patch were not activated by ATP added to the bath, suggesting that the signal transduction mechanism does not involve a diffusible second messenger(s).

In Fig. 8, single-channel currents activated by ATP in the pipette solution are compared with those activated by ACh and adenosine. Figure 8*A* demonstrates examples of single-channel currents activated by 5 μM ATP (left traces), 1 μM ACh (middle) and 5 μM adenosine (right) in the pipette solution at various test potentials. The slope conductances of unitary inward currents activated by ATP, ACh and adenosine were 50, 52 and 51 pS, respectively (Fig. 8*B*). Open-time histograms of channel currents activated by ATP (Fig. 8*C*, left histogram), ACh (middle) and adenosine (right) could be fitted with a single exponential function with time constants of 1·23, 1·17 and 1·12 ms, respectively. Thus, conductance and kinetic properties of the K^+ channel activated by ATP were almost identical to those of the K^+ channels activated by ACh or adenosine, providing evidence that extracellular ATP activates the K_{ACh} channel in guinea-pig atrial cell membranes.

DISCUSSION

The major findings of the present investigation are: (1) ATP (>0·5 μM) activates the K_{ACh} channel through stimulation of P_2-purinoceptors in atrial myocytes and this activation can be completely blocked by pretreatment of the myocytes with PTX; (2) ATP activates the K_{ACh} channel via a membrane-delimited pathway in analogy to its activation by ACh and adenosine; and (3) the K_{ACh} channel activated by ATP exhibits a marked and rapid desensitization, which is associated with a relatively rapid recovery.

In mammalian heart, the K_{ACh} channel is expressed in supraventricular tissues and is characterized by a slope conductance of 45–55 pS and a mean open time of about 1 ms. In bovine atrial myocytes ATP was shown to activate a class of K^+ channel with a conductance property similar to that activated by carbachol (Friel & Bean, 1990), suggesting that stimulation of the P_2-purinoceptor causes activation of the K_{ACh} channel. The signal transduction pathway involved in the regulation of the K_{ACh} channel by the P_2-purinoceptor, however, remains unknown. Bath application of ATP, which is expected to elevate the intracellular levels of potential second messengers, had no effect on the basal activity of the K_{ACh} channel (Fig. 7), whereas ATP applied to the pipette solution markedly increased the activities of the channel in the cell-attached membrane patch (Fig. 6). These results suggest that the signal transduction system involves a membrane-delimited component rather than a cytosolic second messenger. The failure of ATP to activate the K_{ACh} channel in the PTX-treated

atrial myocytes (Fig. 5) indicates that P_2-purinoceptors are coupled to PTX-sensitive G proteins. Based on these experimental results, we suggest a direct coupling of the P_2-purinoceptor to the K_{ACh} channel through G_K in guinea-pig atrial cell membrane, which is analogous to the coupling mechanism of the muscarinic ACh and P_1-purinoceptors to this channel (Pfaffinger *et al.* 1985; Breitwieser & Szabo, 1985; Kurachi *et al.* 1986). Activation of the K_{ACh} channel by other agonists, such as somatostatin, endothelin, calcitonin gene-related peptide and serum factor, are also inhibited by PTX treatment, indicating that their respective receptors are also coupled through G_K to activate the K_{ACh} channel. The stimulation of these receptors causes the activation of G_K by GDP–GTP exchange, which results in the dissociation of heterotrimer G_K into α- and $\beta\gamma$-subunits. There is evidence that both α- (Codina, Yatani, Grenet, Brown & Birnbaumer, 1987; Cerbai, Klöckner & Isenberg, 1988) and $\beta\gamma$-subunits (Logothetis, Kurachi, Galper, Neer & Clapham, 1987) affect K_{ACh} channel activities. There is apparently still some controversy over whether K_{ACh} channels are activated by α- or $\beta\gamma$-subunits, or both, under physiological conditions.

Arachidonic acid metabolites produced through the 5-lipoxygenase pathway, such as 5-hydroperoxyeicosatetraenoic acid (5-HPETE), leukotriene A_4 and leukotriene C_4, have been demonstrated to activate the K_{ACh} channel by directly stimulating GDP–GTP exchange of G_K in atrial myocytes (Kurachi, Ito, Sugimoto, Shimizu, Miki & Ui, 1989*a*; Kim, Lewis, Graziadei, Neer, Bar-Sagi & Clapham, 1989). Activation of the K_{ACh} channel through stimulation of α-adrenoceptors (Kurachi *et al.* 1989*b*) and receptors for platelet-activating factor (Nakajima *et al.* 1991) was shown to be caused by 5-lipoxygenase metabolite-induced stimulation of G_K. On the other hand, P_2-purinergic stimulation has been suggested to induce arachidonic acid metabolism through cyclo-oxygenase and, thereby, produce prostaglandins in rabbit heart (Schwartzman, Pinkas & Raz, 1981; Takikawa, Kurachi, Mashima & Sugimoto, 1990). While it is presently unclear whether 5-lipoxygenase pathway metabolites are also produced by the stimulation of P_2-purinoceptors, the findings from the cell-attached patch recording in this study may rule out the possible involvement of diffusible second messengers, including 5-lipoxygenase metabolites, in the P_2-purinergic activation of the K_{ACh} channel in guinea-pig atrial cells.

The ATP-induced K_{ACh} current exhibited much more rapid and marked desensitization than the adenosine-induced current (Fig. 3). In several cardiac tissues a high concentration of ACh- (>1 μM) or adenosine (>100 μM)-induced K_{ACh} current has been described as showing biphasic desensitization (Carmeliet & Mubagwa, 1986; Kurachi *et al.* 1987; Kim, 1991*c*). Although several factors, such as G_K (Kurachi *et al.* 1987) or dephosphorylation of the channel protein, possibly by Ca^{2+}–calmodulin-dependent phosphatase (Kim, 1991*c*), have been implicated in the desensitization process of the ACh- and adenosine-induced K_{ACh} current in atrial myocytes, the precise mechanism is not totally elucidated. Provided that the coupling mechanism of the P_2-purinoceptor to the K_{ACh} channel is similar to that of the P_1-purinoceptor (i.e. direct coupling to the K_{ACh} channel through the same class of G proteins, G_K), it seems reasonable to propose that the difference in the desensitization process between ATP- and adenosine-induced K_{ACh} currents (Fig. 3) occurred at the level preceding the activation of G_K, i.e. the receptor (agonist–receptor interaction) and/or the receptor–G_K coupling. The rapid onset of and rapid recovery (half-time of about 1·5 min) from the desensitization of the ATP-induced K_{ACh} current (Fig. 4) may indicate that P_2-purinoceptors did not undergo a downregulation process during the time period, since the onset and offset of the downregulation of the receptors usually occur more slowly. Although the present study provides little information on the molecular mechanism involved in the P_2-purinoceptor-induced desensitization process, modification of receptor proteins which causes functional uncoupling of the receptor from its associated G proteins (G_K), such as phosphorylation, might be a cause of desensitization. In turkey erythrocyte membrane, desensitization of P_{2Y}-purinoceptor-regulated phospholipase C, which developed with a half-time of 0·5–2·0 min, was suggested to be caused by phosphorylation of the receptor and the resultant uncoupling from its associated G proteins (Martin & Harden, 1989).

ATP has been suggested to be released, along with principal neurotransmitters, from the autonomic nerve endings upon nerve stimulation. In addition, aggregating platelets, vascular endothelial cells, smooth muscle cells (for review see Gordon, 1986) and even hypoxic myocardium (Clemens & Forrester, 1980) can contribute to the local release of ATP into the plasma. The extracellular ATP concentration has been reported to reach biologically effective levels, i.e. more than 10^{-6} M (Born & Kratzer, 1984) from these sources. It is, therefore, possible that ATP released extracellularly may activate the K_{ACh} channel through P_2-purinoceptors under physiological and pathophysiological conditions.

Intravenous administration of ATP has been shown to terminate rapidly more than 90% of episodes of supraventricular tachycardia in humans (Belhassen, Glick & Laniado, 1988), while adenosine (not ATP), produced by the rapid hydrolysis of ATP by ecto-5′-nucleotidase in plasma, has been assumed to mediate these effects (Belardinelli, Shryock, West, Clemo, DiMarco & Berne, 1984) by activating the K_{ACh} channel through P_1-purinoceptors. Our results, however, raise the possibility that ATP itself directly contributes, at least in part, to slow conduction through the AV nodal region by opening the K_{ACh} channel via P_2-purinoceptors, although quantitative data are not available on how rapidly intravenously administrated ATP is converted to adenosine.

BANACH, K., HÜSER, J., LIPP, P., WELLNER, M.-C. & POTT, L. (1993). Activation of muscarinic K^+ current in guinea-pig atrial myocytes by serum factor. *Journal of Physiology* **461**, 263–281.

BELARDINELLI, L. & ISENBERG, G. (1983). Isolated atrial myocytes: adenosine and acetylcholine increase potassium conductance. *American Journal of Physiology* **244**, H734–737.

BELARDINELLI, L., SHRYOCK, J., WEST, G. A., CLEMO, H. F., DIMARCO, J. P. & BERNE, R. M. (1984). Effects of adenosine and adenosine nucleotides on the atrioventricular node of isolated guinea pig hearts. *Circulation* **70**, 1083–1091.

BELHASSEN, B., GLICK, A. & LANIADO, S. (1988). Comparative clinical and electrophysiologic effects of adenosine triphosphate and verapamil on paroxysmal reciprocating junctional tachycardia. *Circulation* **77**, 795–805.

BORN, G. V. R. & KRATZER, M. A. A. (1984). Source and concentration of extracellular adenosine triphosphate during haemostasis in rats, rabbits and man. *Journal of Physiology* **354**, 419–429.

BREITWIESER, G. E. & SZABO, G. (1985). Uncoupling of cardiac muscarinic and β-adrenergic receptors from ion channels by a guanine nucleotide analogue. *Nature* **317**, 538–540.

BÜNEMANN, M. & POTT, L. (1993). Membrane-delimited activation of muscarinic K current by an albumin-associated factor in guinea-pig atrial myocytes. *Pflügers Archiv* **425**, 329–334.

CARMELIET, E. & MUBAGWA, K. (1986). Desensitization of the acetylcholine-induced increase of potassium conductance in rabbit cardiac Purkinje fibres. *Journal of Physiology* **371**, 239–255.

CERBAI, E., KLÖCKNER, U. & ISENBERG, G. (1988). The α subunit of the GTP binding protein activates muscarinic potassium channels of the atrium. *Science* **240**, 1782–1783.

CLEMENS, M. G. & FORRESTER, T. (1980). Appearance of adenosine triphosphate in the coronary sinus effluent from isolated working rat heart in response to hypoxia. *Journal of Physiology* **312**, 143–158.

CODINA, J., YATANI, A., GRENET, D., BROWN, A. M. & BIRNBAUMER, L. (1987). The α-subunit of the GTP binding protein G_K opens atrial potassium channels. *Science* **236**, 442–445.

FRIEL, D. D. & BEAN, B. P. (1990). Dual control by ATP and acetylcholine of inwardly rectifying K^+ channels in bovine atrial cells. *Pflügers Archiv* **415**, 651–657.

GORDON, J. L. (1986). Extracellular ATP: effects, sources and fate. *Biochemical Journal* **233**, 309–319.

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.

HARTZELL, H. C. (1988). Regulation of cardiac ion channels by catecholamines, acetylcholine and second messenger systems. *Progress in Biophysics and Molecular Biology* **52**, 165–247.

HWANG, T.-C., HORIE, M., NAIRN, A. G. & GADSBY, D. C. (1992). Role of GTP-binding proteins in the regulation of mammalian cardiac chloride conductance. *Journal of General Physiology* **99**, 465–489.

ISENBERG, G. & KLÖCKNER, U. (1982). Calcium tolerant ventricular myocytes prepared by preincubation in a 'KB Medium'. *Pflügers Archiv* **395**, 6–18.

KIM, D. (1991*a*). Calcitonin-gene-related peptide activates the muscarinic-gated K^+ current in atrial cells. *Pflügers Archiv* **418**, 338–345.

KIM, D. (1991*b*). Endothelin activation of an inwardly rectifying K^+ current in atrial cells. *Circulation Research* **69**, 250–255.

KIM, D. (1991*c*). Modulation of acetylcholine-activated K^+ channel function in rat atrial cells by phosphorylation. *Journal of Physiology* **437**, 133–155.

KIM, D., LEWIS, D. L., GRAZIADEI, L., NEER, E. J., BAR-SAGI, D. & CLAPHAM, D. E. (1989). G-protein $\beta\gamma$-subunits activate the cardiac muscarinic K^+-channel via phospholipase A_2. *Nature* **337**, 557–559.

KURACHI, Y., ITO, H., SUGIMOTO, T., SHIMIZU, T., MIKI, I. & UI, M. (1989*a*). Arachidonic acid metabolites as intracellular modulators of the G protein-gated cardiac K^+ channel. *Nature* **337**, 555–557.

KURACHI, Y., ITO, H., SUGIMOTO, T., SHIMIZU, T., MIKI, I. & UI, M. (1989*b*). α-Adrenergic activation of the muscarinic K^+ channel is mediated by arachidonic acid metabolites. *Pflügers Archiv* **414**, 102–104.

KURACHI, Y., NAKAJIMA, T. & SUGIMOTO, T. (1986). On the mechanism of activation of muscarinic K^+ channels by adenosine in isolated atrial cells: involvement of GTP-binding proteins. *Pflügers Archiv* **407**, 264–274.

KURACHI, Y., NAKAJIMA, T. & SUGIMOTO, T. (1987). Short-term desensitization of muscarinic K^+ channel current in isolated atrial myocytes and possible role of GTP-binding proteins. *Pflügers Archiv* **410**, 227–233.

LEWIS, D. L. & CLAPHAM, D. E. (1989). Somatostatin activates an inwardly rectifying K^+ channel in neonatal rat atrial cells. *Pflügers Archiv* **414**, 492–494.

LOGOTHETIS, D. E., KURACHI, Y., GALPER, J., NEER, E. J. & CLAPHAM, D. E. (1987). The $\beta\gamma$ subunits of GTP-binding proteins activate the muscarinic K^+ channel in heart. *Nature* **325**, 321–326.

MARTIN, M. W. & HARDEN, T. K. (1989). Agonist-induced desensitization of a P_{2Y}-purinergic recptor-regulated phospholipase C. *Journal of Biological Chemistry* **264**, 19535–19539.

MATSUURA, H., TSURUHARA, T., SAKAGUCHI, M. & EHARA, T. (1996). Enhancement of delayed rectifier K^+ current by P_2-purinoceptor stimulation in guinea-pig atrial cells. *Journal of Physiology* **490**, 647–658.

NAKAJIMA, T., SUGIMOTO, T. & KURACHI, Y. (1991). Platelet-activating factor activates cardiac G_K via arachidonic acid metabolites. *FEBS Letters* **289**, 239–243.

NOMA, A. & TRAUTWEIN, W. (1978). Relaxation of the ACh-induced potassium current in the rabbit sinoatrial node cell. *Pflügers Archiv* **377**, 193–200.

ONO, K., TSUJIMOTO, G., SAKAMOTO, A., ETO, K., MASAKI, T., OZAKI, Y. & SATAKE, M. (1994). Endothelin-A receptor mediates cardiac inhibition by regulating calcium and potassium currents. *Nature* **370**, 301–304.

PFAFFINGER, P. J., MARTIN, J. M., HUNTER, D. D., NATHANSON, N. M. & HILLE, B. (1985). GTP-binding proteins couple cardiac muscarinic receptors to a K channel. *Nature* **317**, 536–538.

POWELL, T., TERRAR, D. A. & TWIST, V. W. (1980). Electrical properties of individual cells isolated from adult rat ventricular myocardium. *Journal of Physiology* **302**, 131–153.

RAGAZZI, E., WU, S.-N., SHRYOCK, J. & BELARDINELLI, L. (1991). Electrophysiological and receptor binding studies to assess activation of the cardiac adenosine receptor by adenine nucleotides. *Circulation Research* **68**, 1035–1044.

RAMOS-FRANCO, J., LO, C. F. & BREITWIESER, G. E. (1993). Platelet-activating factor receptor-dependent activation of the muscarinic K^+ current in bullfrog atrial myocytes. *Circulation Research* **72**, 786–794.

SAKMANN, B., NOMA, A. & TRAUTWEIN, W. (1983). Acetylcholine activation of single muscarinic K^+ channels in isolated pacemaker cells of the mammalian heart. *Nature* **303**, 250–253.

SCHWARTZMAN, M., PINKAS, R. & RAZ, A. (1981). Evidence for different purinergic receptors for ATP and ADP in rabbit kidney and heart. *European Journal of Pharmacology* **74**, 167–173.

SOEJIMA, M. & NOMA, A. (1984). Mode of regulation of the ACh-sensitive K-channel by the muscarinic receptor in rabbit atrial cells. *Pflügers Archiv* **400**, 424–431.

TAKIKAWA, R., KURACHI, Y., MASHIMA, S. & SUGIMOTO, T. (1990). Adenosine-5′-triphosphate-induced sinus tachycardia mediated by prostaglandin synthesis via phospholipase C in the rabbit heart. *Pflügers Archiv* **417**, 13–20.

Acknowledgements

We would like to thank Dr T. Shioya for his comments on the manuscript. This work was supported by grants from the Ministry of Education, Science and Culture of Japan (No. 04770077 and 05670048) and from the Naito Foundation (No. 93–147). The secretarial assistance of Ms M. Fuchigami is also greatly appreciated.

Received 5 January 1995; accepted 4 August 1995.