Br. J. Pharmacol. (1990), **100**, 269–276

The actions of adenosine 5′-triphosphate on guinea-pig intracardiac neurones in culture

[1]T.G.J. Allen & G. Burnstock

Department of Anatomy and Developmental Biology and Centre for Neuroscience, University College London, Gower Street, London WC1E 6BT

1 The actions of adenosine 5′-triphosphate (ATP) and related nucleotides and nucleosides on the membrane ion conductances of M and AH type intracardiac neurones cultured from ganglia within the atria and interatrial septum of newborn guinea-pig heart were studied with intracellular current- and voltage-clamp techniques.

2 Approximately 74% (120 out of 161) of AH type cells and 41% (5 out of 12) M cells responded to direct application of ATP (500 μM) onto their soma.

3 In 41% of M and 43% of AH type cells, focal application of ATP (500 μM) evoked rapid depolarization with an increase in conductance which frequently elicited action potential discharge. The underlying inward current had a null potential of −11.2 mV and was reduced in solutions containing low extracellular sodium and calcium but unaffected by reduced chloride-containing solutions.

4 In a further 31% of AH type cells, ATP evoked a multi-component response consisting of an initial depolarization followed by a hyperpolarization and a slow prolonged depolarization. The current underlying the initial depolarization resulted from an increase in conductance and had a null potential of −19.1 mV. The current was increased in low chloride-containing solutions and was only slightly reduced in low sodium- and calcium-containing solutions. The subsequent hyperpolarization and outward current resulted from an increase in membrane conductance and had a null potential of −88.5 mV, which was close to the potassium equilibrium potential in these cells. The slow depolarization and inward current was not associated with change in membrane conductance.

5 In less than 2% of AH cells, ATP evoked a second type of slow depolarization. This was associated with a fall in conductance and had a null potential of −90.7 mV.

6 In 40% of AH cells, adenosine (10–100 μM) inhibited the calcium-sensitive potassium current responsible for the after-hyperpolarization. The action of adenosine was antagonized by the P_1-purinoceptor antagonist 8-phenyltheophylline (1–10 μM).

7 The potency order of agonists for all of the ATP-evoked responses, except the slow depolarization associated with a fall in conductance was ATP > ADP with AMP and adenosine being ineffective.

8 Responses to ATP were only weakly desensitized by α,β-methylene ATP (3×10^{-6} M) and the potency order of analogues was 2-methylthio ATP $\geqslant$ ATP > α,β-methylene ATP, indicating the involvement of receptors similar to P_{2Y} purinoceptors.

Introduction

There is now considerable evidence to suggest that purine nucleotides and nucleosides act as neurotransmitters and neuromodulators in a variety of different tissues (Burnstock, 1972; 1985; 1986; Phillis & Wu, 1981; Stone, 1981; Su, 1983; Gordon, 1986). In addition, exogenously applied purines have also been shown to act directly on a number of peripheral and central neurones (for example see Jahr & Jessell, 1983; Krishtal *et al.*, 1983; Salt & Hill, 1983; Fyffe & Perl, 1984; Akasu & Koketsu, 1985; Dolphin *et al.*, 1986; Palmer *et al.*, 1987; Williams, 1987; Katayama & Morita, 1989).

The division of purinoceptors into two subtypes was first proposed by Burnstock (1978). According to this classification, P_1-purinoceptors, which are most sensitive to adenosine, produce changes in the levels of adenosine 3′:5′-cyclic-monophosphate (cyclic AMP) and are competitively inhibited by methylxanthines. In contrast P_2-purinoceptors recognize ATP, are not associated with changes in intracellular levels of cyclic AMP and are not antagonized by methylxanthines. Subdivisions of both these receptor subtypes have subsequently been proposed. P_1-purinoceptors consist of two subtypes; A_1/R_i and A_2/R_a (Van Calker *et al.*, 1979; Londos *et al.*, 1980), while P_2-purinoceptors have been subdivided into P_{2t}, P_{2X}, P_{2Y} and P_{2Z} receptors (Burnstock & Kennedy, 1985; Gordon, 1986).

For many years it has been known that exogenous ATP and adenosine exert a powerful influence upon the mammalian heart (Drury & Szent-Györgyi, 1929). Their effects include negative chronotrophic and dromotrophic actions upon the sino-atrial and atrio-ventricular nodes, as well as potent vasodilatation of coronary blood vessels (for review, see Burnstock, 1980). The possibility that there might be purinergic innervation of the heart is supported by the observation of nerve fibres and intramural neurones in guinea-pig and rabbit atria showing positive reactions to quinacrine, a fluorescent compound that binds strongly to ATP (Irvin & Irvin 1954; Da Prada *et al.*, 1978; Crowe & Burnstock, 1982). At present, the projections of the intramural ganglia are largely unknown; however, many of the ganglia are concentrated around the sino-atrial node and in the interatrial septum and, as such, are ideally placed to influence both nodal and conducting tissues (King & Coakley, 1958).

We have been utilizing a dissociated mixed cell culture preparation of newborn guinea-pig atria and interatrial septum (Hassall & Burnstock, 1986) to study the electrophysiological and neurochemical properties of intracardiac neurones (Allen & Burnstock, 1987; 1990). In a previous electrophysiological study of the properties of guinea-pig intracardiac neurones in culture, we distinguished two main cell types which were termed AH and M cells (Allen & Burnstock, 1987). M cells displayed non-accommodating tonic firing characteristics when stimulated by intrasomal current injection, whilst AH type neurones were highly refractory and displayed pronounced calcium-dependent after-

[1] Author for correspondence at: Department of Pharmacology, University College London, Gower Street, London WC1E 6BT.

hyperpolarizations. In the present study we have investigated the actions of exogenously applied ATP and related nucleotides and nucleosides on these neurones using single electrode current- and voltage-clamp techniques.

Methods

Experiments were carried out on intracardiac neurones, dissociated from the atria and interatrial septum of newborn guinea-pigs. The mixed cell cultures containing these neurones were prepared and maintained for between 5 and 14 days by use of the methods developed by Hassall & Burnstock (1986). Prior to starting experiments, the culture chamber was dismantled and the coverslip bearing the cultured cells was gently rinsed in oxygenated Krebs solution. The coverslip was then sealed to the underside of a Perspex recording bath with paraffin wax, so that it formed the base of the chamber. The combination of bath and coverslip were then clamped to the modified stage of an inverted microscope (Zeiss invertoscope D), equipped with conventional phase-contrast optics.

The preparation was perfused at a rate of $6\,ml\,min^{-1}$ with oxygenated Krebs solution, warmed to 36–37°C with a remote thermostatically controlled heating coil. The Krebs solution had the following composition (mM): NaCl 117, KCl 4.7, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11 and was gassed with 95% O_2/5% CO_2.

Impalements were made with electrodes having d.c. resistance between 90 and 130 MΩ, containing 2 M potassium citrate solution (pH 7.3). Sylgard (Dow Corning) was used to coat the outside of electrodes to within 100 μm of their tips in order to reduce capacitance and therefore increase the frequency response of the electrodes. Discontinuous single electrode voltage- and current-clamp recordings were made with an amplifier that had a 30% current duty cycle (Axoclamp-2A) with sampling frequencies of 3–5 kHz.

Prior to impalement, electrode resistance and tip offset potentials were nulled to permit estimations of input resistance and membrane potential to be made during the recording. These were checked at the end of each experiment by withdrawing the electrode and passing currents of similar magnitude to those used during the impalement. The fractional increase in input conductance during drug-induced responses was calculated as $(R/R^1) - 1$ where R was the input resistance at resting membrane potential and R^1 that during the drug-mediated response.

To study the actions of adenosine on the post-spike calcium-dependent potassium current, a 'hybrid' voltage-clamp technique was employed. This entailed switching the recording mode of the amplifier briefly from voltage-clamp into current-clamp. In current-clamp, a short train of action potentials was evoked with a train of intrasomal current pulses (30 Hz/1.5 s). At the end of this train the amplifier was then automatically switched back into the voltage-clamp recording mode to enable measurement of the evoked current.

The whole-cell patch-clamp recording technique (Hamill *et al.*, 1981) was used to make recordings from a small sample of M type intracardiac neurones, which due to their small size were otherwise difficult to record from by conventional intracellular microelectrodes. A few AH type cells were also studied by this technique, but the presence of glial cells over these neurones (see ultrastructural study by Kobayashi *et al.*, 1986) prevented the use of this technique to study the majority of cells. The patch electrode solution had the following composition (mM): KCl 110, HEPES 40, $MgCl_2$ 3 and EGTA 3; pH 7.2.

Drugs were applied either by local pressure ejection or by bath perfusion. Pressure application was carried out with blunt microelectrodes (3–10 μm diameter tip) containing drugs diluted in Krebs solution. Electrodes placed 100–500 μm away from the cell surface and the drugs ejected under pressures of 50–100 kPa.

Data were either stored on tape for future analysis (Racal store 4DS) or displayed using a Tektronix storage oscilloscope (model D13) and a Gould pressure ink recorder (model 2200S). Numerical data are expressed as mean ± s.e.mean.

Ionic substitution

Low chloride-containing solutions were made by substituting sodium chloride with sodium gluconate. Low sodium-containing solutions were made by substituting sodium chloride with choline chloride. Reduced calcium-containing solutions were made by substitution with magnesium.

Drugs

Adenosine, adenosine 5'monophosphate (AMP), adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), α,β-methylene ATP, 2-methylthio ATP, indomethacin, 8-phenyltheophylline, reactive blue 2 and tetrodotoxin were obtained from Sigma.

Results

The actions of exogenously applied ATP, adenosine and related purine nucleotides upon guinea-pig intracardiac neurones in culture were studied with single electrode current- and voltage-clamp techniques. The majority of recordings were made from the larger AH type cells, with a few additional studies being made on M type cells by use of the whole-cell patch-clamp technique.

With the exception of depolarization-evoked firing, all of the observed actions of ATP were unaffected by superfusion with tetrodotoxin (0.3 μM). Approximately 74% (120 out of 161) of AH type and 41% of M type cells (5 out of 12) responded to direct application of ATP on to their soma. Three different responses were observed. In 43% of AH type cells and all ATP-responsive M cells, ATP elicited a monophasic depolarization (amplitude 5–30 mV) of short latency (18–55 ms) which was associated with an increase in membrane conductance that frequently resulted in action potential discharge (see Figure 1a). In a further 31% of AH cells, ATP evoked a multi-component response. This second type of response consisted of an initial transient depolarization, followed by a small hyperpolarization and a slow prolonged depolarization (see Figure 1b). In a number of cells, the hyperpolarizing outward current phase of the response was observed to be very small or absent. The initial inward current in all cells resulted from an increase in membrane conductance (see Figure 1bii). The subsequent hyperpolarization and outward current also resulted from a small increase in membrane conductance (see Figure 1bii). However, the slow membrane depolarization/inward current following these initial components was never seen to be associated with any measurable change in input resistance. The third type of response to ATP, seen in only two of the cells studied (both AH type), consisted of a slow depolarization lasting for up to 2 min which was associated with a fall in resting membrane conductance (see Figure 1c).

The rapid depolarization and inward current evoked by ATP in neurones which displayed only a monophasic response differed in a number of respects from the initial depolarization seen in cells exhibiting multi-phase responses. Although both these events resulted in an increase in membrane conductance, the latency and rate of rise of the rapid monophasic depolarization, observed in the majority of cells, was considerably shorter than the initial depolarization seen in cells displaying multi-component responses. The latency of this response in 'monophasic' cells was between 18 and 55 ms (mean 39.4 ± 2.2 ms, $n = 20$) and the mean time to peak was 304 ± 22.4 ms ($n = 17$), compared with a latency of 90 to 210 ms (mean 155.4 ± 15.3 ms, $n = 11$) and a mean time to peak of 662 ± 35 ms ($n = 18$) for the response in cells displaying multi-component responses.

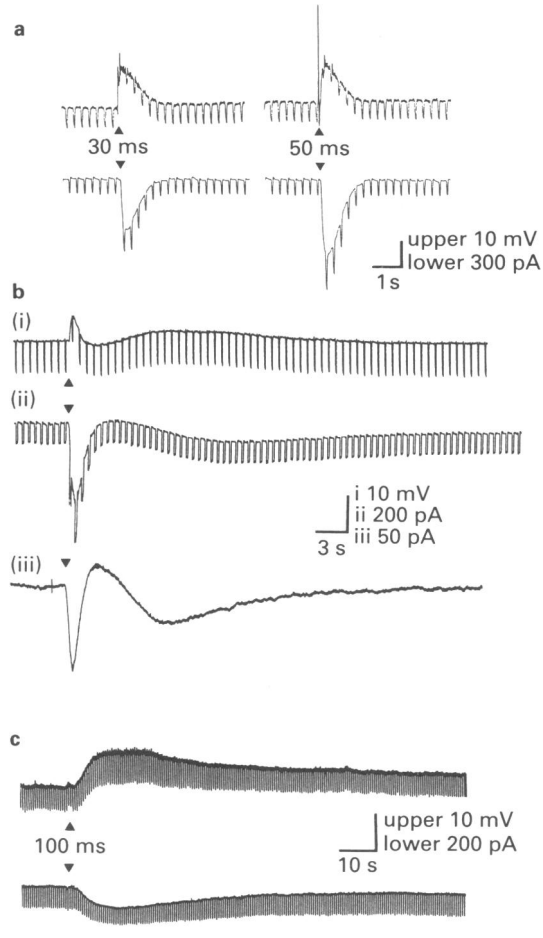

Figure 1 The actions of exogenously applied ATP on AH type intracardiac neurones cultured from guinea-pig heart. (a) Responses from an AH type cell to focally applied ATP (500 μM) for the period indicated by the arrows (membrane potential −64 mV). Upper records were obtained under current-clamp and the lower records with voltage-clamp. Downward deflections on current- and voltage-clamp records were responses to 110 pA/−10 mV pulses of 50 ms duration which were used to monitor changes in input resistance. (b) Multi-component responses to ATP evoked from AH type cells. Upper trace (i) a current-clamp recording showing a three component response to ATP consisting of an initial transient depolarization followed by a small hyperpolarization and a prolonged depolarization. Membrane −64 mV, downward deflections were membrane voltage changes to 150 pA/50 ms intrasomal current pulses. Traces (ii) and (iii), voltage-clamp records from two cells displaying multi-component responses to ATP (membrane potentials were −60 and −52 mV respectively). Downward deflections in (ii) were membrane currents evoked by passing −10 mV/100 ms duration voltage steps. (c) ATP-induced membrane depolarization and fall in membrane conductance in an AH type cell evoked by application of ATP (500 μM) for 100 ms at point indicated by arrows. Upper record: current-clamp. Lower record: voltage-clamp. Membrane potential was −53 mV, downward deflections were the result of 100 pA/50 ms and −9 mV/50 ms duration current/voltage steps used to monitor changes in input resistance.

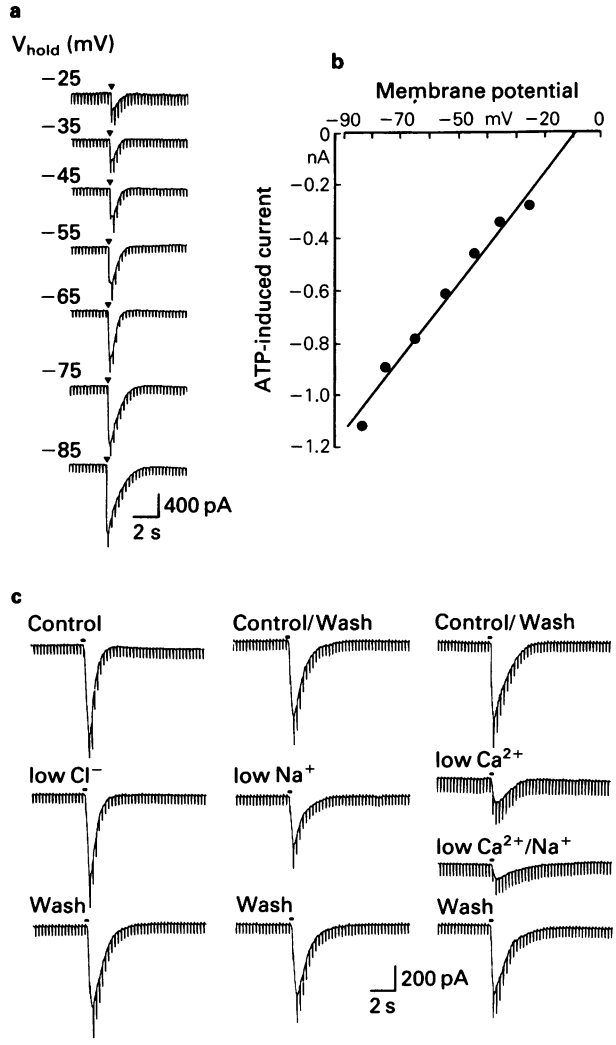

Figure 2 The ionic- and voltage-dependence of the ATP-induced rapid transient inward current and increase in conductance in an AH type intracardiac neurone. (a) The inward current evoked by focal application of ATP (500 μM/50 ms; as indicated by the arrows) at different holding potentials. Downward deflections were the currents evoked by −10 mV, 50 ms duration voltage steps used to monitor changes in membrane conductance. (b) A plot of evoked current against membrane potential for the cell shown in (a). The amplitude of the ATP-induced current was linearly related to membrane potential (correlation coefficient = 0.998) and had a null/equilibrium potential of −9.8 mV. (c) The ionic-dependence of the ATP-induced transient inward current in an AH type intracardiac neurone. ATP (500 μM) was applied by focal pressure ejection for the period indicated by the bar above each trace. Left panel, superfusion with low (9 mM) chloride-containing solution had no effect on the ATP-induced inward current or conductance change. Middle panel, superfusion with low (26.2 mM) sodium-containing solutions reduced the amplitude of the ATP-induced inward current by approximately 34%, but had little effect upon the time course of the underlying conductance change. Right panel, superfusion with low (0.25 mM) calcium-containing solutions increased resting membrane conductance and greatly reduced (approximately 68%) the amplitude of the inward current. Subsequent superfusion with a low calcium- and low sodium-containing solution (0.25 and 26.2 mM respectively) produced a further reduction in the amplitude of the inward current (approximately 82%). Holding potential throughout the recording was −70 mV.

Ionic- and voltage-dependence

ATP-indiced transient depolarizations The amplitude of the ATP-induced transient depolarization in monophasically responsive M and AH type cells at resting membrane potential was between 5 and 30 mV (mean 16.6 ± 1.27 mV, $n = 22$). Under voltage-clamp, the increase in input conductance calculated as a fractional increase (see methods) was 2.45 ± 0.28 ($n = 12$) and resulted in a large inward current, mean 788.8 ± 86.2 pA ($n = 21$). The inward current was linearly related to membrane potential and increased with hyperpolarization (see Figure 2a and b). Extrapolation of the plot of ATP-induced current against membrane potential, indicated a mean null/equilibrium potential for this current of −11.2 ± 1.45 mV ($n = 10$). The amplitude of the ATP-induced inward current was reduced when the cell was superfused with low sodium- (26.2 mM) and/or calcium- (0.25 mM) containing solutions, whereas reducing extracellular chloride concentration (9 mM) had no effect on either the evoked current or the underlying conductance change ($n = 3$; see Figure 2c).

The longer latency, initial transient depolarization in AH cells displaying multi-component responses to ATP was generally smaller than that seen in monophasically responsive M and AH cells and never elicited action potential discharge. The average amplitude of the response was 11.2 ± 1.15 mV ($n = 18$), whilst the underlying fractional increase in conductance and evoked currents were also smaller, having mean values of 0.46 ± 0.08 ($n = 15$) and 203.7 ± 22.4 pA ($n = 30$) respectively. The inward current increased with membrane hyperpolarization and was linearly related to membrane potential between -75 and -45 mV. The predicted null/equilibrium potential of this response, determined by extrapolation was -19.1 ± 1.1 mV ($n = 2$), which was slightly more negative than the value for the fast inward current in other M and AH cells (see Figure 3a and b). Low extracellular chloride-containing solutions (9 mM) increased the amplitude of the current (mean increase $58 \pm 8.1\%$, $n = 3$), whilst superfusion with low sodium- or low calcium-containing solutions only slightly reduced the current, mean reduction $15.7 \pm 1.45\%$ and $9.3 \pm 3.71\%$ ($n = 3$) respectively (see Figure 3d).

Slow outward current/hyperpolarization This response was generally small, range -1 to -3.5 mV (mean -2.0 ± 0.2 mV, $n = 10$) and resulted from a small increase in membrane conductance, mean fractional increase 0.11 ± 0.025 ($n = 6$). Under voltage clamp, the ATP-induced outward current at resting membrane potential was 41.1 ± 5.6 pA ($n = 13$). In all cells studied, the current decreased with membrane hyperpolarization (see Figure 3a and c). The current was linearly related to membrane potential and extrapolation of the plot of evoked current against membrane potential gave a predicted mean null/equilibrium potential of -88.5 mV ($n = 2$), indicating that the current may have resulted from an efflux of potassium ions.

Slow inward current with no associated change in conductance This current was the most commonly observed slow inward current in AH cells which displayed multi-component responses. The response had a slow rate of rise and the time to peak was 10.8 ± 0.37 s ($n = 23$) following focal application of ATP (500 μM). The mean inward current associated with this ATP-induced current was 107.1 ± 13 pA ($n = 28$), and the average observed depolarization was 4.5 ± 0.64 mV ($n = 9$). However, no significant change in membrane conductance was observed in any of the cells studied. Furthermore, it was not possible to determine ionic- or voltage-dependence of this current because of difficulties encountered in reproducibly eliciting the response. However, it was generally observed to increase with membrane hyperpolarization (see Figure 3a).

Slow inward current associated with a fall in conductance This ATP-induced slow inward current was only observed in two cells. It was distinct from the more commonly observed slow inward current that was seen in cells displaying multiphase responses, in that it resulted from a clear decrease in membrane conductance. In one experiment the outward current was reduced by membrane hyperpolarization, and was linearly related to membrane potential between -40 and -70 mV (correlation coefficient $r = 0.989$). Extrapolation of the least squares fit of the raw data gave an equilibrium potential for the response of -90.7 mV.

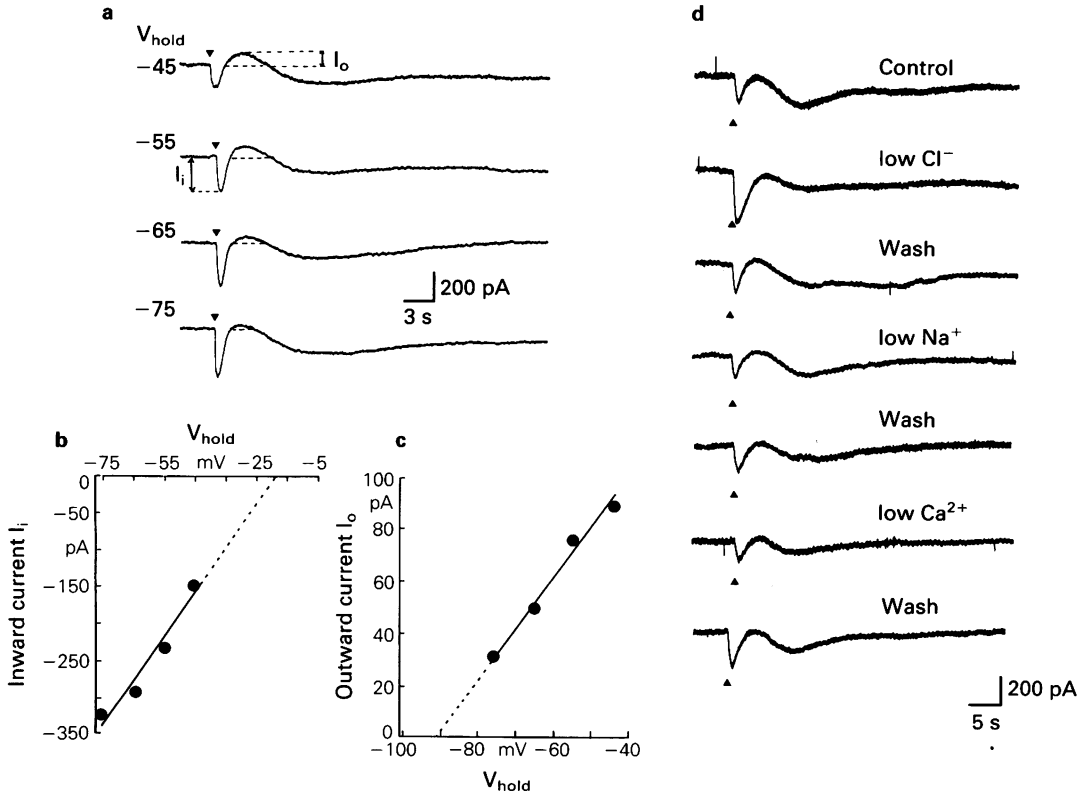

Figure 3 The ionic- and voltage-dependence of the initial transient inward and slow outward current in AH type neurones cultured from the guinea-pig heart. (a) The voltage-dependence of a typical three-component response to brief (50 ms) focal application of ATP (500 μM) to the soma of the cell (see arrows). (b and c) Plots of the amplitude of the initial inward (I_i) and subsequent outward currents (I_o) as a function of membrane potential. In both cases the evoked currents were linearly related to membrane potential between -45 and -75 mV (correlation coefficients, 0.987 and 0.978 respectively). Extrapolation from the obtained data gave a predicted null/equilibrium potential for the inward current of -18 mV and a value of -90.8 mV for the outward current. (d) The ionic-dependence of the transient inward current in an AH type cell displaying a multi-component response to ATP. In low extracellular chloride- (9 mM) containing solutions the current was enhanced by approximately 88%. Superfusion with low sodium- (26.2 mM) or low calcium- (0.25 mM) containing solutions produced only a small reduction in the amplitude of the evoked current. Holding potential -57 mV.

Other purine compounds

2-Methylthio ATP, which potently stimulates P_2-purinoceptors, mimicked all the observed actions of ATP and was generally found to be slightly more potent than ATP (see Figure 4a and b). In general α,β-methylene ATP, which has been shown to stimulate potently P_{2X}-purinoceptors, only poorly mimicked the ATP-evoked rapid transient depolarization (see Figure 4a). In most cells, α,β-methylene ATP evoked only a very small depolarization/current. In a few cells, however, ($n = 4$) it was seen to elicit a depolarization of similar amplitude to that produced by ATP. In these cells, the peak increase in conductance and current examined under voltage-clamp was always smaller than that evoked by ATP and the rate of rise of the depolarization produced was notably slower. When applied to cells that produced a multi-component response to ATP, α,β-methylene ATP weakly mimicked the initial transient inward current, but was never observed to evoke the subsequent slow outward and inward currents (see Figure 4b).

ADP only very weakly mimicked the actions of ATP, whilst AMP and adenosine were ineffective in mimicking any of the actions of ATP. However, adenosine (10–100 μM) was found to reduce the calcium-dependent after-hyperpolarization that followed a train of action potentials (see Figure 5). Both the amplitude and duration of the potassium current and the underlying conductance increase recorded by use of a hybrid voltage-clamp technique (see methods) were reduced by adenosine in a concentration-dependent manner. This effect was antagonized by 8-phenyltheophylline (10 μM; see Figure 5).

Antagonism of the actions of ATP

Superfusion with the putative P_{2Y} receptor antagonist, reactive blue 2, at concentrations up to 3×10^{-5} M initially enhanced the fast transient inward current evoked by ATP by up to 100% (mean 40.4 ± 15.1%, $n = 5$). With continuous application (>3 min) the duration of responses became considerably prolonged. The mean increase in duration was 203 ± 23% ($n = 7$). In addition, there was generally a fall in input resistance and an increase in the overall level of current noise, which reflected a general reactive blue 2-induced deterioration in the cell (see Figure 6c and d). With longer periods of application (up to 25 min) responses to ATP slowly declined in all cells studied ($n = 14$). Complete washout of the effects of reactive blue 2 were never achieved even after prolonged periods (up to 45 min). Reactive blue 2 also strongly depressed $GABA_A$ receptor-mediated chloride currents (mean reduction 78.7 ± 7.2%, $n = 3$) and reduced, though to a lesser extent, the amplitude of the acetylcholine-induced nicotinic responses (mean reduction 26 ± 2.6%, $n = 3$).

Superfusion with α,β-methylene ATP (3×10^{-6} M) only partially reduced the amplitude of the ATP-induced brief latency inward current observed in M and AH type cells (see Figure 6a). The maximum observed reduction in this inward current ranged between 24 and 60% (mean 44.8 ± 6.3%, $n = 6$). When applied to neurones exhibiting multi-component responses to ATP, a similar 19–59% reduction in the initial transient current (mean 35.6 ± 7.2%, $n = 5$) was observed (see Figure 6b).

Inhibition of prostaglandin synthesis with indomethacin (5×10^{-5} M) had no effect upon any of the ATP-induced currents ($n = 7$). Similarly the P_1-purinoceptor antagonist 8-phenyltheophylline (10 μM) was also without effect ($n = 11$).

Discussion

The present study shows that a large population of guinea-pig intracardiac neurones in culture responded to exogenous application of ATP. Three different responses to ATP were observed. The first was a rapid transient depolarization, exhibited by all ATP-responsive M cells (41%) and approximately 43% of AH type neurones. This response displayed a similar agonist potency (ATP > ADP with AMP and adenosine ineffective) and brief latency to the ATP-induced responses seen in mammalian dorsal horn and sensory ganglion neurones (Jahr & Jessell, 1983; Krishtal *et al.*, 1983). In all these cell types, ATP produced a transient increase in mem-

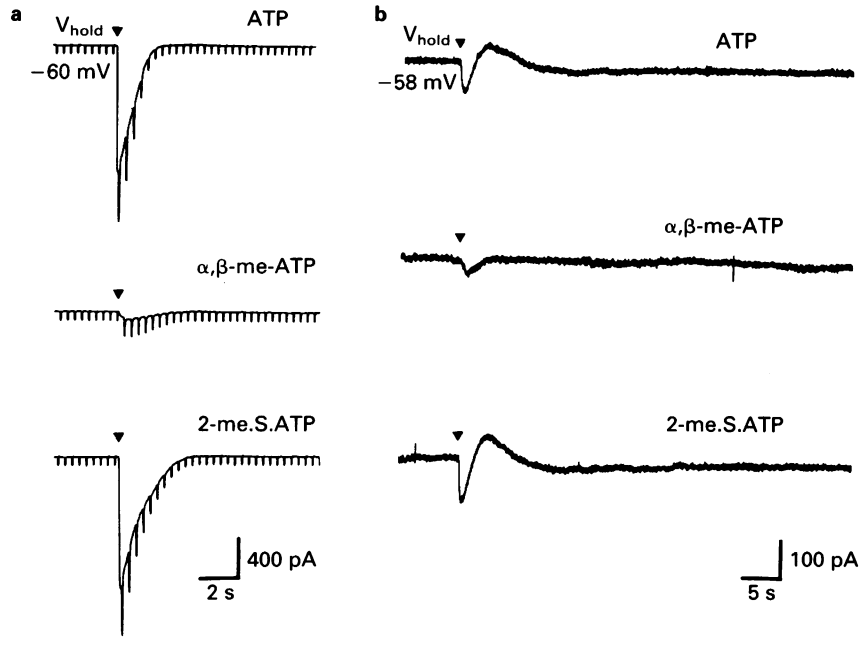

Figure 4 (a) and (b) The actions of ATP, α,β-methylene ATP (α,β-me-ATP) and 2-methylthio ATP (2-me.S.ATP) on intracardiac neurones cultured from the guinea-pig heart. (a) The rapid monophasic inward current evoked by focal pressure application of ATP (500 μM/50 ms). α,β-me-ATP (500 μM/50 ms) only weakly mimicked this response, whereas 2-me.S.ATP (500 μM/50 ms), potently mimicked this action of ATP. Downward deflections were evoked by −10 mV hyperpolarizing voltage steps of 50 ms duration used to monitor changes in input resistance. Holding potential −60 mV. (b) An AH type cell exhibiting a three-component response to exogenous application of ATP. α,β-me-ATP weakly mimicked the initial transient inward current but did not evoke the subsequent slow outward and inward components of the response. 2-me-S.ATP mimicked ATP and evoked a similar three-component response which was slightly larger than that produced by ATP. Holding potential −58 mV.

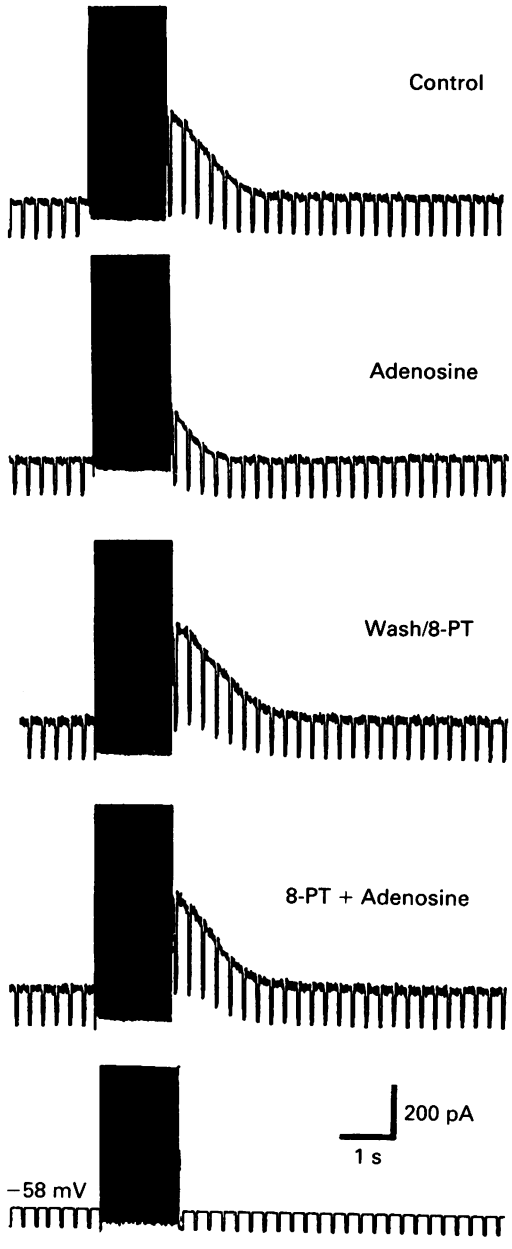

Figure 5 Inhibition of the post-spike calcium-activated potassium current in an AH type intracardiac neurone cultured from guinea-pig heart. The recording was carried out with a hybrid voltage-clamp technique (see methods). Under voltage-clamp at a membrane potential of −58 mV the cell was briefly switched into current-clamp and a train of action potentials was evoked by passing a train of intrasomal current pulses (30 Hz/1.5 s). At the end of this train, the cell was then switched back to voltage-clamp and the evoked outward current and conductance change monitored. Downward deflections were evoked by −10 mV negative command pulses of 50 ms duration used to monitor changes in membrane conductance. Adenosine (50 μM) reduced the outward current and conductance that underlies the after-hyperpolarization. In the presence of 8-phenyltheophylline (8-PT; 10 μM) this inhibitory action of adenosine was inhibited.

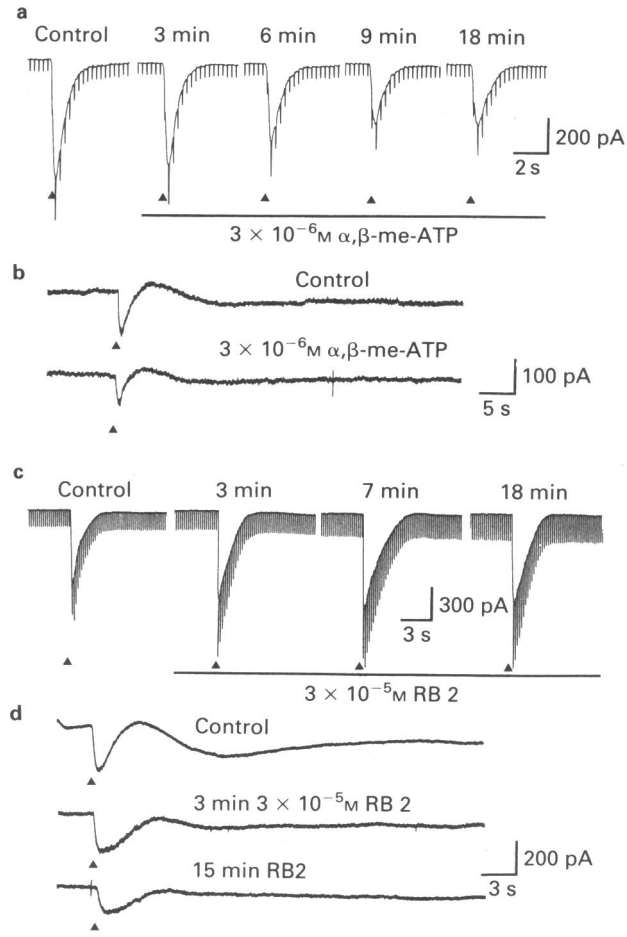

Figure 6 The actions of α,β-methylene ATP (α,β-me-ATP) and reactive blue 2 (RB2) on AH and M type intracardiac neurones. (a) Prolonged superfusion with α,β-me-ATP (3×10^{-6} M) reduced the amplitude of the fast transient inward current evoked by focal application of ATP (500 μM/70 ms) in an M type cell. The ATP-evoked current was maximally inhibited after 9 min superfusion, at which point the current was reduced by approximately 50%. Holding potential −68 mV. A similar reduction in the ATP-induced currents was also seen in cells (all AH type) displaying multi-component responses to ATP (see (b); holding potential −58 mV). (c and d) Show the effect of prolonged superfusion with RB2 (3×10^{-5} M). In cells displaying only a single transient inward current in response to ATP, RB2 produced an initial increase in the evoked current and a slight increase in resting membrane conductance. During prolonged application input resistance continued to decline and the duration of the response was increased. Continued superfusion (up to 30 min) subsequently led to a slow decline in the amplitude of the current and further reduction in input resistance. Washout of RB2 back to control values was never obtained in any cell studied. Holding potential −62 mV. (d) Superfusion with RB2 on to cells displaying multi-component responses produced a similar prolongation of the initial transient inward current and fall in resting membrane conductance. With prolonged exposure to RB2 all of the different component currents were substantially reduced and input resistance continued to decline. Holding potential −61 mV.

brane conductance resulting in a large inward current. In rat dorsal horn and cat vesical parasympathetic ganglia (Jahr & Jessell, 1983; Akasu *et al.*, 1984) as in the present study, application of ATP frequently evoked action potential discharge. The underlying ionic conductance changes observed were similar, in that a large proportion of the current was carried by sodium ions. In dorsal horn and dorsal root ganglion cells, sodium appears to be the sole charge carrier, whilst in sensory, vesical parasympathetic and intracardiac neurones the response to ATP appeared to be mediated via non-selective cationic channels (Krishtal *et al.*, 1983; Akasu *et al.*, 1984). Interestingly, the inward current in AH and M cells giving single component responses to exogenous application of ATP, differed from the initial inward current displayed by AH cells exhibiting multi-component responses. In the latter type of cell, the latency of the response was approximately 100 ms slower and was never observed to evoke action potential discharge. In addition, the ionic- and voltage-dependence of the underlying current was also different. The null/equilibrium potential was 7–8 mV more negative and the current was only slightly reduced in low extracellular calcium- and sodium-containing solutions, but was greatly enhanced when the chloride concentration was reduced, indicating that

this response may have resulted primarily from an increase in chloride conductance.

The inhibitory response which was frequently observed following the initial inward current in AH type cells which displayed multi-component responses to ATP, resulted from an increase in membrane conductance and had a null potential close to the potassium equilibrium potential (E_K; Allen & Burnstock, 1987), which suggests that it may result from an increase in potassium conductance. Similar biphasic responses to ATP, consisting of an initial depolarization followed by hyperpolarization, have also been reported in cat vesical parasympathetic neurones and in chick skeletal muscle cells (Akasu *et al.*, 1984; Hume & Thomas, 1988). As in intracardiac neurones, the inhibitor response in these cells also appeared to be the result of an increase in potassium conductance. In vesical parasympathetic neurones, this response was mimicked by adenosine, indicating that it was mediated via P_1-purinoceptors. Adenosine-induced hyperpolarization resulting from an increase in potassium conductance has also been reported in rat hippocampal, mouse striatal and guinea-pig myenteric plexus neurones (Segal, 1982; Haas & Greene, 1984; Trussell & Jackson, 1985; Palmer *et al.*, 1987). In the current study of intracardiac neurones, however, adenosine was never observed to mimic the ATP-induced hyperpolarization which indicates that this response was not mediated by P_1-purinoceptors.

Two different types of ATP-induced slow depolarizations were observed in AH type cells but were never observed in M type cells. In the majority of cells, the ATP-evoked depolarization and inward current was not associated with any measurable change in membrane conductance. Furthermore, it was not antagonized by indomethacin or the P_1-purinoceptor antagonist 8-phenyltheophylline, suggesting that it did not arise as a result of increased prostaglandin synthesis or from the breakdown of ATP to adenosine. Unlike the most commonly observed slow depolarization, the second type of ATP-induced slow depolarization was associated with a distinct decrease in membrane conductance and was not preceded by a transient depolarization or hyperpolarization. This current most probably resulted from inhibition of a tonically active potassium conductance since it displayed a null potential close to E_K (Allen & Burnstock, 1987). ATP and adenosine have similar actions on a variety of different neurones (Akasu *et al.*, 1983; Morita *et al.*, 1984; Katayama & Morita, 1989).

Both P_1- and P_2-purinoceptor subtypes are present in guinea-pig intracardiac neurones. All of the observed actions of ATP appeared to result from the direct action of ATP on P_2-purinoceptors rather than as a consequence of its breakdown to ADP, AMP or adenosine. Exogenous application of adenosine was never observed to mimic any of the actions of ATP. However, (although the actions of adenosine were not studied in detail in the current report), adenosine, acting on P_1-purinoceptors, was observed to inhibit the outward post-spike calcium-sensitive potassium current in a significant population of AH type cells.

In a number of tissues, α,β-methylene ATP, a slowly degraded analogue of ATP has been shown to act potently (>ATP) and selectively on P_{2X}-purinoceptors. Whilst 2-methylthio ATP acts most potently ($\geqslant$ATP) upon P_{2Y}-purinoceptors (for review see Burnstock & Kennedy, 1985). In the current study, α,β-methylene ATP only weakly mimicked the initial transient inward currents evoked by ATP and was never seen to mimic the slow outward or inward currents. On the other hand, 2-methylthio ATP mimicked all the observed actions of ATP, but was only slightly more potent than ATP. Thus the order of potency for these purines on intracardiac neurones was 2-methylthio ATP $\geqslant$ ATP > α,β-methylene ATP. According to the original classification proposed by Burnstock & Kennedy (1985), α,β-methylene ATP also selectively desensitizes P_{2X} receptors. Prolonged exposure to α,β-methylene ATP generally reduced, but never abolished the response to ATP, indicating that these responses to ATP were not mediated via P_{2X}-purinoceptors. Reactive blue 2, an anthraquinone sulphoric acid derivative, has been shown to display a degree of selectivity in antagonizing P_{2Y}-purinoceptors in a variety of tissues (Kerr & Krantis, 1979; Manzini *et al.*, 1986; Burnstock & Warland, 1987; Hopwood & Burnstock, 1987; Houston *et al.*, 1987). In the current study, reactive blue 2 initially increased and prolonged the actions of ATP, whilst prolonged exposure generally reduced the amplitude of the ATP-induced responses and also slowly reduced the input resistance of all the cells studied. Furthermore, reactive blue 2 (10 μM) also reduced responses to exogenously applied GABA and acetylcholine, which indicates a non-selective action.

The presence of receptors for ATP and adenosine raises the possibility that in the heart they may act pre- or post-junctionally to modulate the transmitter release from the intramural neurones and thereby regulate the activity of the effector tissue. In addition, the presence of quinacrine-positive intramural neurones and nerve fibres in guinea-pig atria suggests that some intracardiac neurones may be purinergic (Crowe & Burnstock, 1982). ATP and adenosine are known to produce potent vasodilatation of coronary vessels and also to have pronounced effects upon heart muscle, particularly in the atrium and the sino-atrial node (Drury & Szent-Györgyi, 1929; Yatani *et al.*, 1978; Berne, 1980; West & Bellardinelli, 1985). Therefore, it is possible that release of ATP from intracardiac neurones may be responsible, at least in part, for mediating some of these actions. Furthermore, the present finding that a considerable proportion of the intracardiac neurones were responsive to ATP and adenosine, raises the possibility that ATP may be released from one population of intracardiac neurones to modulate the excitability of other intracardiac neurones through local reflex pathways.

This work was supported by grants from the British Heart Foundation and the Medical Research Council. The authors wish to thank Miss D. Bailey, Miss M. Windsor and Dr C.J.S. Hassall for growing the cultures used in these experiments and Mrs P.J. Charatan and Dr F.A. Cribbin for editorial assistance in the preparation of this manuscript.

References

AKASU, T., HIRAI, K. & KOKETSU, K. (1983). Modulatory actions of ATP on membrane potentials of bullfrog sympathetic ganglion cells. *Brain Res.*, **258**, 313–317.

AKASU, T. & KOKETSU, K. (1985). Effect of adenosine triphosphate on the sensitivity of the nicotinic acetylcholine-receptor in the bullfrog sympathetic ganglion cell. *Br. J. Pharmacol.*, **84**, 525–531.

AKASU, T., SHINNICK-GALLAGHER, P. & GALLAGHER, J.P. (1984). Adenosine mediates a slow hyperpolarizing synaptic potential in autonomic neurones. *Nature*, **311**, 62–65.

ALLEN, T.G.J. & BURNSTOCK, G. (1987). Intracellular studies of the electrophysiological properties of cultured intracardiac neurones of the guinea-pig. *J. Physiol.*, **388**, 349–366.

ALLEN, T.G.J. & BURNSTOCK, G. (1990). M_1 and M_2 muscarinic receptors mediate excitation and inhibition of guinea-pig intracardiac neurones in culture. *J. Physiol.*, **422**, 463–480.

BERNE, R.M. (1980). The role of adenosine in the regulation of coronary blood flow. *Circulation Res.*, **47**, 807–813.

BURNSTOCK, G. (1972). Purinergic nerves. *Pharmacol. Rev.*, **24**, 509–581.

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

BURNSTOCK, G. (1980). Purinergic receptors in the heart. Supplement I, *Circulation Res.*, **46**, 1175–1182.

BURNSTOCK, G. (1985). Purinergic mechanisms broaden their sphere of influence. *TINS*, **8**, 5–6.

BURNSTOCK, G. (1986). Purines as cotransmitters in adrenergic and cholinergic neurones. In *Progress in Brain Research*, 68, Chapter 13, ed. Hökfelt, T., Fuxe, K. & Pernow, B. Amsterdam: Elsevier.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

BURNSTOCK, G. & WARLAND, J.J.I. (1987). P_2-purinoceptors of two subtypes in the rabbit mesenteric artery: reactive blue 2 selectively inhibits responses mediated via the P_{2y}- but not P_{2x}-purinoceptor. *Br. J. Pharmacol.*, **90**, 383–391.

CROWE, R. & BURNSTOCK, G. (1982). Fluorescent histochemical localization of quinacrine-positive neurones in the guinea-pig and rabbit atrium. *Cardiovascular Res.*, **16**, 384–390.

DA PRADA, M., RICHARDS, J.G. & LOREZ, H.P. (1978). Blood platelets and biogenic monoamines: Biochemical, pharmacological, and morphological studies. In *Platelets: A Multidisciplinary Approach*, ed. de Gaetano, G. & Garattini, S. pp. 331–353. New York: Raven Press.

DOLPHIN, A.C., FORDA, S.R. & SCOTT, R.H. (1986). Calcium-dependent currents in cultured rat dorsal root ganglion neurones are inhibited by an adenosine analogue. *J. Physiol.*, **373**, 47–61.

DRURY, A.N. & SZENT-GYÖRGYI, A. (1929). The physiological activity of adenine compounds with special reference to their action upon mammalian heart. *J. Physiol.*, **68**, 213–237.

FYFFE, R.E.W. & PERL, E.R. (1984). Is ATP a central synaptic mediator for certain primary afferent fibers from mammalian skin? *Proc. Natl. Acad. Sci., U.S.A.*, **81**, 6890–6893.

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

HAAS, H.L. & GREENE, R.W. (1984). Adenosine enhances after-hyperpolarization and accommodation in hippocampal pyramidal cells. *Pflügers Arch.*, **402**, 244–247.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. (1981). Improved patch-clamp techniques for high resistance recording from cells and cell free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HASSALL, C.J.S. & BURNSTOCK, G. (1986). Intrinsic neurones and associated cells of the guinea-pig heart in culture. *Brain Res.*, **364**, 102–113.

HOPWOOD, A.M. & BURNSTOCK, G. (1987). ATP mediates coronary vasoconstriction via P_{2x}-purinoceptors and coronary vasodilatation via P_{2y}-purinoceptors in isolated perfused rat heart. *Eur. J. Pharmacol.*, **136**, 49–54.

HOUSTON, D.A., BURNSTOCK, G. & VANHOUTTE, P.M. (1987). Different P_2-purinergic receptor subtypes of endothelium and smooth muscle in canine blood vessels. *J. Pharmacol. Exp. Ther.*, **241**, 501–506.

HUME, R.I. & THOMAS, S.A. (1988). Multiple actions of adenosine 5'-triphosphate on chick skeletal muscle. *J. Physiol.*, **406**, 503–524.

IRVIN, J.L. & IRVIN, E.M. (1954). The interaction of quinacrine with adenine nucleotides. *J. Biol. Chem.*, **210**, 45–56.

JAHR, C.E. & JESSELL, T.M. (1983). ATP excites a subpopulation of rat dorsal horn neurones. *Nature*, **304**, 730–733.

KATAYAMA, K. & MORITA, K. (1989). Adenosine 5'-triphosphate modulates membrane potassium conductance in guinea-pig myenteric neurones. *J. Physiol.*, **408**, 373–390.

KERR, D.I.B. & KRANTIS, A. (1979). A new class of ATP antagonist. *Proc. Aust. Physiol. Pharmacol. Soc.*, **10**, 156P.

KING, T.S. & COAKLEY, J.B. (1958). The intrinsic nerve cells of the cardiac atria of mammals and man. *J. Anat.*, **92**, 353–375.

KOBAYASHI, Y., HASSALL, C.J.S. & BURNSTOCK, G. (1986). Culture of intramural cardiac ganglia of the newborn guinea-pig. I. Neuronal elements. *Cell and Tissue Res.*, **244**, 595–604.

KRISHTAL, O.A., MARCHENKO, S.M. & PIDOPLICHKO, V.I. (1983). Receptors for ATP in the membrane of mammalian sensory neurones. *Neurosci. Lett.*, **35**, 41–45.

LONDOS, C., COOPER, D.M.F. & WOOLF, J. (1980). Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 2551–2554.

MANZINI, S., HOYLE, C.H.V. & BURNSTOCK, G. (1986). An electrophysiological analysis of the effect of reactive blue 2, a putative P_2-purinoceptor antagonist, on inhibitory junction potentials of rat caecum. *Eur. J. Pharmacol.*, **127**, 197–204.

MORITA, K., KATAYAMA, Y., KOKETSU, K. & AKASU, T. (1984). Actions of ATP on the soma of bullfrog primary afferent neurons and its modulatory action on the GABA-induced response. *Brain Res.*, **293**, 360–363.

PALMER, J.M., WOOD, J.D. & ZAFIROV, D.H. (1987). Purinergic inhibition in the small intestinal myenteric plexus of the guinea-pig. *J. Physiol.*, **387**, 357–369.

PHILLIS, J.W. & WU, P.H. (1981). The role of adenosine and its nucleotides in central synaptic transmission. *Prog. Neurobiol.*, **16**, 187–239.

SALT, T.E. & HILL, R.G. (1983). Excitation of single sensory neurones in the rat caudal trigeminal nucleus by ionophoretically applied adenosine 5'-triphosphate. *Neurosci. Lett.*, **35**, 53–57.

SEGAL, M. (1982). Intracellular analysis of a postsynaptic action of adenosine in the rat hippocampus. *Eur. J. Pharmacol.*, **79**, 193–199.

STONE, T.W. (1981). Physiological roles for adenosine and adenosine 5'-triphosphate in the nervous system. *Neuroscience*, **6**, 523–555.

SU, C. (1983). Purinergic neurotransmission and neuromodulation. *Ann. Rev. Pharmacol. Toxicol.*, **23**, 397–411.

TRUSSELL, L.O. & JACKSON, M.B. (1985). Adenosine activated potassium conductance in cultured striatal neurones. *Proc. Natl. Acad. Sci., U.S.A.*, **182**, 4857–4861.

VAN CALKER, D., MÜLLER, M. & HAMPRECHT, B. (1979). Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurosci.*, **33**, 999–1005.

WEST, G.A. & BELLARDINELLI, L. (1985). Correlation of sinus slowing and pacemaker shift caused by adenosine in rabbit SA node. *Pflügers Arch.*, **403**, 66–74.

WILLIAMS, M. (1987). Purinergic receptors and central nervous system function. In *Psychopharmacology: The Third Generation of Progress*. ed. Meltzer, Y. pp. 289–301. New York: Raven Press.

YATANI, A., GOTO, M. & TSUDA, Y. (1978). Nature of catecholamine-like actions of ATP and other energy rich nucleotides on the bullfrog atrial muscle. *Jpn. J. Physiol.*, **28**, 47–61.

(Received December 8, 1989
Revised January 29, 1990
Accepted February 9, 1990)