Journal of Physiology (1993), **471**, *pp.* 295–317
With 9 figures
Printed in Great Britain

MODULATION OF L-TYPE Ca^{2+} CURRENT BY EXTRACELLULAR ATP IN FERRET ISOLATED RIGHT VENTRICULAR MYOCYTES

BY YUSHENG QU*‡, DONALD L. CAMPBELL*§
AND HAROLD C. STRAUSS*†

*From the Departments of *Pharmacology and †Medicine, Duke University Medical Center, Durham, NC 27710, USA*

(*Received 5 January 1993*)

SUMMARY

1. The effects of extracellular adenosine triphosphate (ATP) on the basal L-type Ca^{2+} current (I_{Ca}) were investigated in ferret isolated right ventricular myocytes using the gigaohm seal voltage clamp in the whole-cell and cell-attached configurations.

2. Micromolar levels of extracellular ATP reversibly inhibited I_{Ca} in a concentration-dependent manner, without any significant changes in the voltage dependence of either the peak I_{Ca} I–V relationship or steady-state activation curve.

3. In contrast, micromolar levels of extracellular ATP did significantly alter the inactivation characteristics of I_{Ca}. Ten micromolar ATP: (i) increased the degree of steady-state inactivation of I_{Ca}; (ii) altered the time constants of I_{Ca} inactivation at 0 mV; and (iii) decreased the time constant of I_{Ca} recovery from inactivation at −70 mV.

4. The inhibitory effect of ATP on I_{Ca} was not blocked by atropine, a muscarinic cholinergic receptor antagonist, or CPDPX (8-cyclopentyl-3,4-dipropylxanthine), an A_1 adenosine receptor antagonist. In contrast, the inhibitory effect of 10 μM ATP could be nearly completely antagonized by 100 μM suramin, a purinergic P_2 receptor antagonist.

5. The potency order of ATP analogues in inhibiting I_{Ca} was 2-methyl-thio-ATP > ATP > α,β-methylene-ATP, indicating involvement of a P_{2Y}-type ATP receptor.

6. Pretreatment of cells with pertussis toxin (PTX) did not prevent the ATP-induced decrease in I_{Ca}. However, (i) ATP produced an irreversible decrease of I_{Ca} in the presence of intracellular GTPγS, and (ii) the inhibitory effect was significantly attenuated in the presence of intracellular GDPβS, indicating the involvement of a PTX-insensitive G protein in the P_{2Y} receptor-coupling process.

7. Neither (i) replacing extracellular Ca^{2+} with 1 mM Ba^{2+}, nor (ii) intracellular perfusion of 10 mM BAPTA for at least 30 min attenuated the inhibitory effect of ATP on the current through Ca^{2+} channels, suggesting that the inhibitory effect was not obligatorily dependent upon influx of Ca^{2+} or changes in $[Ca^{2+}]_i$.

‡ Present address: Department of Pharmacology, University of Washington, SJ-30, Seattle, WA 98195, USA.

§ To whom correspondence should be addressed.

MS 1998

8. Ensemble-average current behaviour constructed from cell-attached patch recordings of single L-type Ca^{2+} channels (110 mM $BaCl_2$) demonstrated that when 10 μM ATP was added to the superfusate on the outside of the patch electrode the inhibition of I_{Ca} was still observed, providing evidence for the involvement of intracellular diffusible second messenger(s).

9. Bath application of the protein kinase C (PKC) activator PMA (4β-phorbol-12-myristate-13-acetate) did not decrease I_{Ca}, and the PKC inhibitor staurosporine did not block the ATP-induced decrease in I_{Ca}, indicating that the inhibition was not mediated by activation of PKC.

10. Internal perfusion of 100 μM IP_3 + 7·7 μM IP_4 did not alter the inhibitory effect of ATP on I_{Ca}, indicating that inositol phosphates were not involved.

11. Neither (i) internal perfusion of a specific peptide inhibitor of protein kinase A (PKI) for 30 min, nor (ii) application of an intracellular 'cAMP clamp' (1 mM cAMP + 10 μM IBMX) changed the effect of ATP on I_{Ca}, indicating that the cAMP-dependent protein kinase A (PKA) pathway was not involved.

12. In ferret right ventricular myocytes inhibition of I_{Ca} by ATP occurs independently of adenosine A_1 receptors. ATP inhibits I_{Ca} without significantly affecting the voltage dependence of activation, but it does modify the inactivation gating properties. Inhibition occurs by extracellular ATP binding to a P_{2Y} receptor and subsequent activation of a PTX-insensitive G protein. Our results indicate that some of the conventional second messenger systems ($[Ca^{2+}]_i$, PKC, inositol phosphates, cAMP–PKA) are not essential for the inhibitory effect of extracellular ATP on I_{Ca}.

INTRODUCTION

The role of extracellular adenosine triphosphate (ATP) as either a primary neurotransmitter or modulator of ion channel activity is now recognized in a number of different tissues (e.g. Hoyle & Burnstock, 1986; Burnstock, 1990; Bean, 1992; Evans, Derkach & Surprenant, 1992; Edwards, Gibb & Colquhoun, 1992; El-Moatassim, Dornard & Mani, 1992). However, it is still generally considered that direct ATP-mediated effects on mammalian cardiac muscle are relatively unimportant (reviewed in Olsson & Pearson, 1990). A prevailing view is that ATP either has to be hydrolysed to adenosine or act via adenosine receptors to produce any pharmacological effects in mammalian cardiac muscle (e.g. Olsson & Pearson, 1990; Ragazzi, Wu, Shryock & Belardinelli, 1991). Studies on the effects of extracellular ATP on mammalian isolated cardiac myocytes are limited, and have to date been conducted almost exclusively on rat ventricular myocytes (e.g. Christie, Sharma & Sheu, 1992; Scamps, Rybin, Puceat, Tkachuk & Vassort, 1992). However, the extent to which the effects of extracellular ATP observed in rat ventricular myocytes can be applied to myocytes isolated from cardiac tissues of other mammalian species has not yet been determined.

In the preceding paper we demonstrated that adenosine inhibits basal L-type I_{Ca} in ferret right ventricular myocytes by binding to an A_1 receptor coupled to a pertussis toxin (PTX)-sensitive G protein (Qu, Campbell, Whorton & Strauss, 1993*a*). Adenosine reduced basal I_{Ca} without altering either its steady-state or kinetic gating characteristics. We have also previously demonstrated in these

myocytes that, in contrast to results reported in rat myocytes (see Discussion), extracellular ATP decreases basal L-type I_{Ca}, $[Ca^{2+}]_i$ transients, and cell length changes elicited upon depolarizing voltage clamp stimulation without changing free myoplasmic $[Ca^{2+}]_i$ under resting conditions (Qu, Himmel, Campbell & Strauss, 1993*b*). Our previous results imply an important functional role for both extracellular adenosine and ATP in ferret right ventricular myocardium. Therefore, it is critical to determine if ATP produces its inhibitory effect on basal I_{Ca} in ferret right ventricular myocytes independently of hydrolysis to adenosine or activation of adenosine A_1 receptors.

In the present study we characterize the effects of extracellular ATP on basal L-type I_{Ca} in ferret right ventricular myocytes. We demonstrate that micromolar levels of extracellular ATP inhibit basal L-type I_{Ca}, and that these inhibitory effects can be clearly distinguished electrophysiologically, pharmacologically and biochemically from those produced by adenosine. The involvement of ATP-specific P_{2Y} receptors and a PTX-insensitive G protein in the receptor-coupling process for inhibition of I_{Ca} is demonstrated, and the possible involvement of four different second messenger pathways ($[Ca^{2+}]_i$, protein kinase C, inositol phosphates, cAMP-dependent protein kinase A) is examined.

Preliminary accounts of this work have appeared in abstract form (Qu, Campbell, Whorton & Strauss, 1991; Qu, Campbell, Himmel & Strauss, 1992).

METHODS

Myocyte isolation and electrophysiological recording techniques

The myocyte isolation (right ventricles of 10 to 16-week-old male ferrets) and storage procedures, voltage clamp amplifier, stimulating, recording and data-acquisition equipment and software, perfusion system and chamber, patch pipette fabrication for whole-cell recordings of I_{Ca}, and whole-cell internal perfusion technique were all identical to those described in detail in the preceding paper (Qu *et al.* 1993*a*). Voltage clamp protocols for measuring current–voltage and steady-state inactivation relationships, kinetics of recovery, and drug application sequences, and the methods of quantitative analysis applied for each of these protocols, were also identical to those described in the preceding paper. For cell-attached recordings of single L-type Ca^{2+} channels borosilicate glass tubing with 1·5 mm o.d., 0·86 mm i.d. was used (Corning 7052, A-M Systems, Inc., Everett, WA, USA). The shanks of the electrodes were coated with 'Sylgard' (Dow Corning) to within approximately 50–100 μM of the tip.

Solutions and drugs

In all experiments myocytes were initially perfused and gigaseals formed in 'control extracellular Na^+-containing solution' containing (mM): 144 NaCl, 5·4 KCl, 1·8 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, pH = 7·40. For whole-cell recording of I_{Ca} myocytes were then perfused with a 'Na^+-free NMDG solution' containing (mM): 144 *N*-methyl-D-glucamine chloride (NMDG), 5·4 CsCl, 1·8 $CaCl_2$, 1 $MgCl_2$, 12 μM-tetrodotoxin (TTX), 10 Hepes, pH = 7·40. The patch pipettes were filled with 'K^+-free intracellular solution containing (mM): 120 CsCl, 20 TEA-Cl, 1 $MgCl_2$, 5 EGTA, 5 Mg-ATP, 5 disodium creatine phosphate, 0·2 Na-GTP, 10 Hepes, pH = 7·40. Pipettes had resistances of 2–4 MΩ. For measurements of whole-cell I_{Ba}, 1·8 mM $CaCl_2$ was replaced with 1 mM $BaCl_2$ and $MgCl_2$ was removed (e.g. Campbell, Giles & Shibata, 1988). Complete replacement of Na^+ by NMDG suppressed or greatly minimized I_{Na} and Na^+–Ca^{2+} exchanger current, 12 μM TTX minimized any residual flow of other cations through the Na^+ channel, and complete replacement of K^+ by Cs^+ and TEA^+ suppressed any voltage- and agonist-activated K^+ currents. In a limited series of experiments designed to test for the possible effects of Cl^-, both extracellular and intracellular

solutions were simultaneously modified so as to reduce Cl^-: extracellularly, NMDG solution was modified by replacing 144 mM NMDG-Cl + 5·4 mM CsCl with 144 mM NMDG-methanesulphonate (MSA) + 5·4 mM Cs-MSA, and the patch pipette solution was modified by replacing 120 mM CsCl with 120 mM caesium aspartate (all other compounds were the same as control solutions).

For recording of single Ca^{2+} channel activity in the cell-attached configuration, membrane potential was 'zeroed' by perfusing with 'isotonic K^+ solution' containing (mM): 140 KCl, 12·1 NaCl, 1 EGTA, 1 $MgCl_2$, 10 Hepes, pH = 7·40). Pipettes were filled with isotonic Ba^{2+} solution containing (mM): 110 $BaCl_2$, 10 Hepes, pH = 7·40. Pipette resistances were 8–15 MΩ. Assuming a transmembrane potential of 0 mV in isotonic K^+ solution, the patch holding potential was set at −70 to −90 mV (i.e. close to the normal resting potential), and 200 ms test pulses typically to +10 to +30 mV were applied. Two hundred to 300 consecutive traces of channel activity were recorded in control, during bath application of 10 μM ATP, and wash-out. These 200–300 traces were then used to construct ensemble-average currents. To control for 'run-down' or other experimental variability, each series of 200–300 pulses was bracketed by a similar control or ATP application series (i.e. either control–ATP–control or ATP–control–ATP).

Depending upon experiments, solutions were modified by appropriate addition/substitution of compounds and drugs. Stock solutions of 1 mM-ATP (sodium salt) were made fresh daily in NMDG I_{Ca} solution (without TTX) and refrigerated until used. Staurosporine was dissolved in dimethyl sulphoxide (DMSO) to give 1 mM stock and stored at 0–4 °C until used. 8-Cyclopentyl-1,3-dipropylxanthine (CPDPX) was dissolved in DMSO to give 10 mM stock and stored at −20 °C until used. Phorbol ester was dissolved in DMSO to give 1 mM stock and stored at −70 °C until used. Drugs and compounds were obtained from the following companies: CPDPX and 2-methyl-thio-ATP, Research Biochemicals Incorporated, Natick, MA, USA; PTX, List Biological Laboratories, Inc., Campbell, CA, USA; adenosine, ATP, α,β-methylene-ATP, GTPγS, GDPβS, ATPγS, atropine (sulphate salt), 3-isobutyl-1-methyl-xanthine (IBMX), phorbol ester (4β-phorbol-12-myristate-13-acetate; PMA), methanesulphonic acid, and aspartic acid, Sigma, St Louis, MO, USA; $BaCl_2$, Aldrich, Milwaukee, WI, USA; WIPTIDE (PKI), (Peninsula Laboratories, Inc., Belmont, CA, USA; D-*myo*-inositol 1,4,5-trisphosphate, D-*myo*-inositol 1,3,4,5-tetrakisphosphate, and 5,5′-difluoro-BAPTA (potassium salt), Calbiochem, La Jolla, CA, USA; cAMP (free acid), Boehringer Mannheim, Indianapolis, IN, USA; and TTX, Sigma and Calbiochem. Suramin was kindly provided as a gift from Dr T. K. Harden (Department of Pharmacology, University of North Carolina, Chapel Hill, USA).

All experiments were conducted at room temperature (21–23 °C) and within 8–12 h of myocyte isolation. Currents have not been 'leakage corrected'. Results are given as means ± S.D. Statistical analysis was conducted using Student's paired *t* test. Differences were considered significant at $P < 0·05$.

RESULTS

ATP-mediated inhibition of I_{Ca}: basic electrophysiological characterization

In all myocytes examined application of extracellular ATP in the micromolar range reversibly inhibited I_{Ca} in a concentration-dependent manner (see also Qu *et al.* 1993*b*). Under control conditions 10 μM extracellular ATP inhibited peak I_{Ca} at 0 mV by 45·9 ± 11·0 % ($n = 10$ myocytes). To determine if the inhibition of I_{Ca} mediated by ATP displayed different electrophysiological characteristics from the inhibition mediated by adenosine (Qu *et al.* 1993*a*), the effects of ATP on the I_{Ca} current–voltage (I–V) relationship, steady-state inactivation relationship, and kinetics of inactivation and recovery were determined.

Figure 1*A* illustrates the mean peak different I_{Ca} I–V relationship ($I_{Ca,peak} - I_{500ms}$) and the isochronal (500 ms) I–V relationship obtained before and after application of 10 μM ATP ($n = 5$ myocytes). Ten micromolar ATP reduced the amplitude of the peak I_{Ca} I–V relationship without appreciably altering its voltage dependence (ATP produced at most a 5 mV depolarizing shift, a value well within the range of error of our measurements). The effect of ATP on the isochronal I–V relationship was minimal. The mean apparent reversal potentials, E_{rev}, in the absence and presence

of 10 μM ATP were not statistically different (control: $E_{rev} = +64{\cdot}7 \pm 3{\cdot}1$ mV, $n = 5$; ATP: $E_{rev} = +63{\cdot}6 \pm 3{\cdot}4$ mV, $n = 6$; $P > 0{\cdot}2$). The effect of ATP on I_{Ca} was also measured in low-Cl^- solutions (see Methods). Under such low-Cl^- conditions, 10 mM ATP inhibited I_{Ca} (-10 or 0 mV) by $39{\cdot}0 \pm 5{\cdot}4\%$ ($n = 3$ myocytes), a value

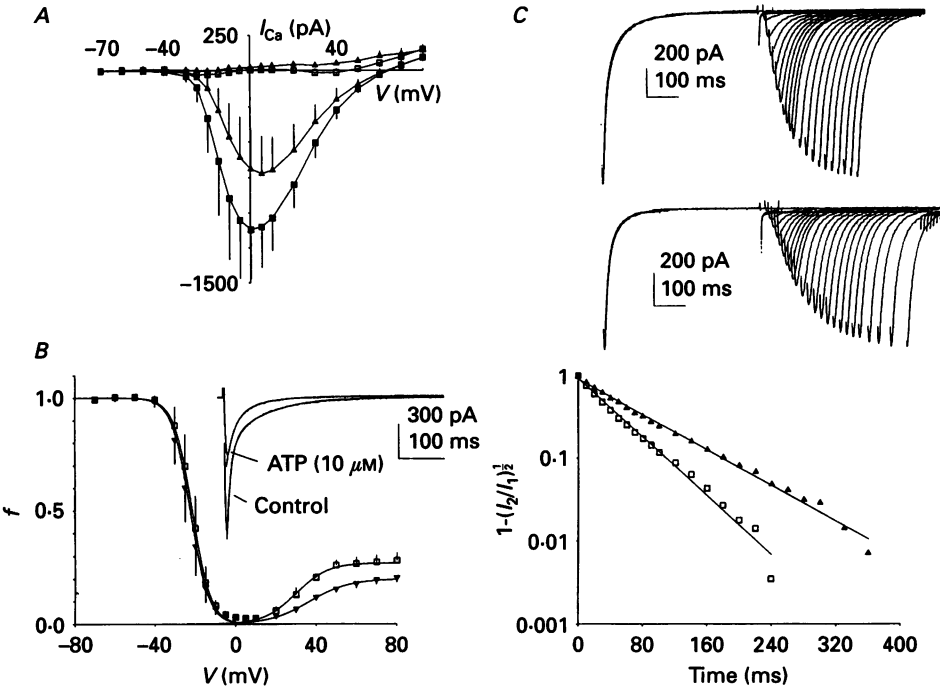

Fig. 1. Basic electrophysiological effects of 10 μM extracellular ATP on I_{Ca}. *A*, mean peak difference ($I_{Ca,peak} - I_{Ca,500ms}$) and isochronal (500 ms) *I–V* relationships in control (■) and 10 μM ATP (▲). *B*: main figure, steady-state I_{Ca} inactivation relationship in control and 10 μM ATP. Continuous lines are fits to the data sets using two Boltzmann relationships and the equations for '*f*' and '*r*' given in the text. Inset, effects of 10 μM ATP on I_{Ca} inactivation kinetics at 0 mV. Representative double-exponential fits to I_{Ca} inactivation in control (□) and 10 μM ATP (▲). Best-fit values of time constants were: control, $\tau_1 =$ 10 ms, $\tau_2 = 82$ ms; ATP, $\tau_1 = 19$ ms, $\tau_2 = 72$ ms. *C*, the effect of 10 μM ATP on the kinetics of I_{Ca} recovery from inactivation at a holding potential (HP) -70 mV. Upper panels, I_{Ca} recovery waveforms in control (upper panel) and 10 μM ATP (middle panel) elicited at 0 mV. Lower panel, semilogarithmic transformation of the I_{Ca} recovery time course for the currents illustrated in the upper panels □, control; ▲, 10 μM ATP. The data have been fitted with a general equation $I = I_{max}(1 - \exp(-t/\tau))^2$, where $I_1 = I_{max} =$ the peak amplitude of I_{Ca} elicited by the first pulse, and $I_2 = I =$ the peak amplitude of I_{Ca} elicited by the second pulse (Qu *et al.* 1993*a*). Best-fit time constants of recovery: control, $\tau_{rec} = 49$ ms; 10 μM ATP, $\tau_{rec} = 81$ ms.

similar to that observed under control conditions ($45{\cdot}9 \pm 11{\cdot}0\%$). In combination, these results indicate that extracellular ATP is indeed inhibiting I_{Ca}, i.e. that the reduction of macroscopic I_{Ca} is not due to activation of some other conductance (see also Fig. 8).

The effects of 10 μM ATP on the inactivation characteristics of I_{Ca} are illustrated in Fig. 1*B* and *C* and are summarized in Table 1. Figure 1*B* illustrates the effect of 10 μM ATP on the I_{Ca} steady-state inactivation relationship. As described in the previous paper (Qu *et al.* 1993*a*), the inactivation curves in both control and ATP were non-monotonic or 'U-shaped', and could be described as the sum of two

TABLE 1. Summarized effects of 10 μM ATP on I_{Ca} inactivation characteristics

	Control	10 μM ATP	n (significance)
A. Mean r value (+80 mV):			
Control	0·299 ± 0·062	0·208 ± 0·022	8 (0·001 < P < 0·002)
Ba*	0·467 ± 0·082	0·284 ± 0·020	3 (0·02 < P < 0·05)
BAPTA†	0·511 ± 0·032	0·178 ± 0·082	4 (0·002 < P < 0·005)
B. Kinetics of inactivation (0 mV):			
τ_1(ms)	11·2 ± 2·4	18·7 ± 4·3	8 (P < 0·001)
τ_2 (ms)	80·5 ± 7·8	71·7 ± 9·9	8 (0·01 < P < 0·025)
C. Kinetics of recovery (−70 mV):			
τ_{rec} (ms)	52 ± 13	100 ± 28	6 (0·001 < P < 0·002)

* 1·8 mM Ca^{2+} replaced with 1 mM Ba^{2+}.

† 5 mM EGTA in the patch pipette replaced with 10 mM BAPTA; myocytes perfused for at least 30 min prior to conducting measurements.

n = number of myocytes.

Results are given as means ± S.D. Statistical analysis was conducted using Student's paired t test. Differences considered significant at P < 0·05.

Boltzmann relationships, 'f' + 'r' (where the r relationship characterizes the degree of 'bend-up' or incomplete inactivation at depolarized potentials; e.g. Rasmusson *et al.* (1990)):

$$\text{Control:}\quad f = 1/(1+\exp((V+21{\cdot}5)/4{\cdot}5))+r,$$
$$r = 0{\cdot}27/(1+\exp((30-V)/8{\cdot}0)),$$
$$\text{ATP:}\quad f = 1/(1+\exp((V+22{\cdot}5)/4{\cdot}5))+r,$$
$$r = 0{\cdot}20/(1+\exp((37-V)/10)),$$

where V is in millivolts. Ten micromolar ATP had no significant effect on the inactivation relationship for potentials hyperpolarized to 0 mV. However, ATP altered the r relationship by reducing the degree of 'bend-up' at depolarized potentials. At +80 mV the mean r value was significantly reduced by ATP (Table 1). This reduction of the mean r value at +80 mV by 10 μM ATP was also observed in both (i) 1 mM Ba^{2+} and (ii) 1·8 mM Ca^{2+} when 5 mM intracellular EGTA was replaced with 10 mM BAPTA (Table 1). Ten micromolar ATP also altered both time constants of I_{Ca} inactivation at 0 mV (Fig. 1*B*, inset). Finally, 10 mM ATP produced a marked slowing of the kinetics of recovery from inactivation at −70 mV (Fig. 1*C*). As summarized in Table 1, all of these effects were significantly different from control.

These basic electrophysiological measurements indicate that both adenosine and ATP inhibit basal L-type I_{Ca} in ferret right ventricular myocytes, and that neither compound significantly affects I_{Ca} activation characteristics. However, adenosine did

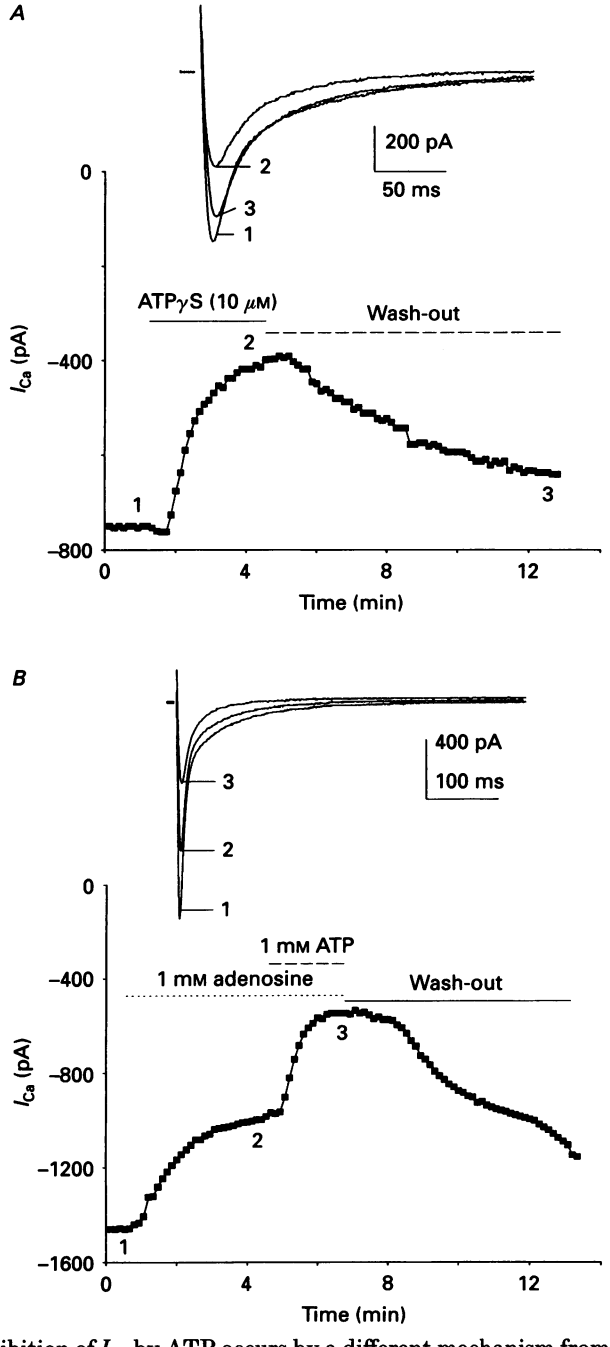

Fig. 2. Inhibition of I_{Ca} by ATP occurs by a different mechanism from inhibition produced by adenosine. *A*, extracellular ATP does not have to be hydrolysed to adenosine to inhibit I_{Ca}. Ten micromolar ATPγS, a non-hydrolysable ATP analogue, inhibited I_{Ca} to a similar degree as 10 μM ATP. *B*, the inhibitory effects of 1 mM adenosine and 1 mM ATP on basal I_{Ca} are additive. The myocyte was exposed to 1 mM adenosine initially to maximally

not produce any significant effects on either the r relationship or the kinetics of I_{Ca} inactivation or recovery (Qu *et al.* 1993*a*). These electrophysiological results therefore strongly suggest that the effects of ATP and adenosine on I_{Ca} are mediated via different cellular mechanisms.

ATP-mediated inhibition of I_{Ca}: demonstration of direct effects distinguishable from those produced by adenosine

Micromolar levels of extracellular adenosine reversibly inhibit basal I_{Ca} in these ventricular myocytes by binding to an A_1-specific receptor and activating a PTX-sensitive G protein (Qu *et al.* 1993*a*). It was therefore critically important to demonstrate that the inhibition of I_{Ca} was being produced by ATP directly, i.e. not through hydrolysis to adenosine and/or activation of adenosine A_1 receptors. To address this issue three different experimental protocols were conducted.

Does ATP have to be hydrolysed to adenosine to inhibit I_{Ca}?

To address this question a non-hydrolysable ATP analogue, ATPγS, was employed. Extracellular perfusion of 10 μM ATPγS produced a reversible inhibition of I_{Ca} (Fig. 2*A*). In a total of four myocytes 10 μM ATPγS inhibited peak I_{Ca} at 0 mV by $40{\cdot}3 \pm 12{\cdot}8\%$, a value similar to that produced by 10 μM ATP ($45{\cdot}9 \pm 11{\cdot}0\%$). These results indicate that ATP does not have to be hydrolysed to adenosine to inhibit I_{Ca}.

Are the effects of adenosine and ATP additive?

If adenosine and ATP inhibit I_{Ca} by the same pharmacological and biochemical mechanisms, then their maximal inhibitory effects should not be additive. Representative results from such an additive experiment using supramaximal concentrations (1 mM) of adenosine and ATP on I_{Ca} at 0 mV are illustrated in Fig. 2*B*. The myocyte was initially exposed to 1 mM adenosine to maximally inhibit I_{Ca} (Qu *et al.* 1993*a*). After reaching steady-state inhibition 1 mM ATP was added. In the presence of 1 mM adenosine ATP was still able to decrease I_{Ca}, i.e. the effects of adenosine and ATP were additive. In a total of four myocytes subsequent addition of 1 mM ATP in the presence of 1 mM adenosine caused an additional inhibition of I_{Ca} of $36{\cdot}6 \pm 6{\cdot}4\%$. These results strongly suggest that adenosine and ATP inhibit I_{Ca} by different pharmacological and biochemical mechanisms.

Are adenosine A_1 receptors involved in mediating the ATP effect?

To address this question, CPDPX, a specific A_1 receptor antagonist, was perfused before and during application of ATP. Fifty nanomolar CPDPX completely blocked the inhibitory effect of adenosine on basal I_{Ca} in these myocytes (Qu *et al.* 1993*a*). However, 50 nM CPDPX did *not* block the inhibitory effect of 10 μM-ATP (Fig. 3). In

---

inhibit I_{Ca}, and then 1 mM adenosine + 1 mM ATP was perfused after the inhibitory effect of 1 mM adenosine reached steady state. In this and all subsequent similar figures I_{Ca} was elicited at 0 mV (500 ms) from HP = −70 mV.

a total of four myocytes in the presence of 50 nM CPDPX, 10 μM ATP inhibited I_{Ca} by 41·5 ± 7·6%, a value similar to the inhibition observed in control (45·9 ± 11·0%). Increasing the concentration of CPDPX to 100 nM also did not block the effect of 10 μM ATP ($n = 2$ myocytes). These results indicate that ATP exerts its inhibitory

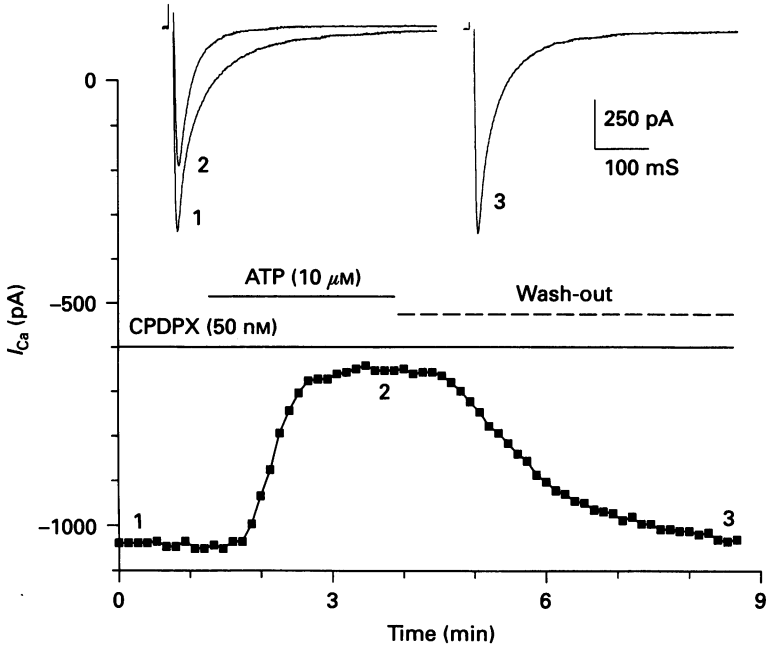

Fig. 3. Inhibitory effect of 10 μM ATP on I_{Ca} in the presence of 50 nM CPDPX, an adenosine A_1 receptor antagonist. Note that CPDPX did not attenuate the effect of ATP on I_{Ca}, indicating that ATP inhibits I_{Ca} independently of adenosine A_1 receptors.

effect through a receptor distinct from the adenosine A_1 type. In addition, ATP also inhibited I_{Ca} in the presence of 10 μM atropine, a muscarinic cholinergic antagonist ($n = 3$ myocytes).

Pharmacological characterization of ATP's effect on I_{Ca}

The results described above suggest the involvement of an ATP-selective P_2-type purinergic receptor in mediating inhibition of I_{Ca}. Two sets of experiments were therefore conducted to further demonstrate the involvement of P_2 receptors and to determine the specific receptor subtype.

Suramin

The trypanocidal compound suramin has recently been reported to be a selective antagonist of P_2 receptors (e.g. vas deferens: von Kügelgen, Bültmann & Starke, 1989; PC12 cells ($IC_{50} = 30$ μM): Nakazawa, Fujimori, Takanaka & Inoue, 1990; bladder and taenia coli: Hoyle, Knight & Burnstock, 1990; coeliac ganglion neurons ($IC_{50} = 1·5$ μM): Evans *et al.* 1992; medial habenula neurones: Edwards *et al.* 1992).

As illustrated in Fig. 4, 100 μM suramin was able to nearly completely antagonize the inhibitory effect of 10 μM ATP on I_{Ca} at 0 mV. In a total of five myocytes, during initial perfusion of 10 μM ATP I_{Ca} at 0 mV was inhibited by 34·8 $\pm$ 12·7 %. Subsequent perfusion of 10 μM ATP + 100 μM suramin caused I_{Ca} to increase to

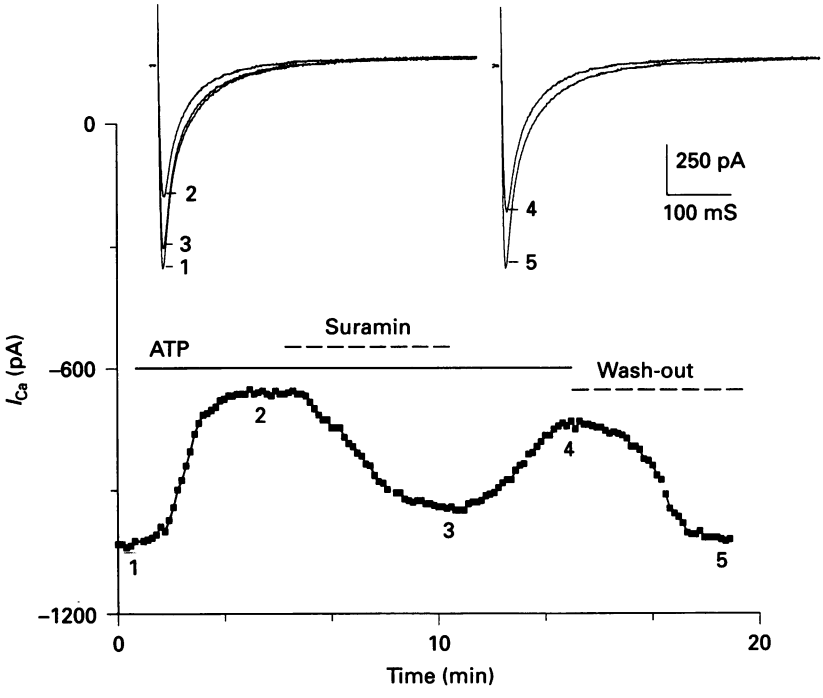

Fig. 4. Suramin reversibly antagonizes the inhibitory effect of ATP on I_{Ca}. After inhibition of I_{Ca} by 10 μM ATP had reached steady state, 10 μM ATP + 100 μM suramin was applied. In this particular myocyte, 100 μM suramin in the presence of 10 μM ATP brought I_{Ca} back to approximately 92 % of its mean initial control value. The effect of suramin was reversible upon wash-out.

92·4 $\pm$ 8·9 % of its mean initial control value. In additional control experiments application of 100 μM suramin alone consistently produced a small and reversible inhibition of I_{Ca} at 0 mV of 6 $\pm$ 1 % (n = 3 myocytes). If this 6 % inhibition is taken into account, then 100 μM suramin completely antagonized the inhibitory effect of 10 μM ATP.

P_2 receptor subtype

While the results obtained with suramin indicate the involvement of ATP-selective P_2-type receptors, the use of suramin alone does not allow distinction between subclasses of P_2-type receptors (e.g. Hoyle *et al.* 1990). P_2 receptors have been subclassified as P_{2X} and P_{2Y}. The reported potency order among ATP analogues for binding to P_{2Y} receptors is 2-methyl-thio-ATP > ATP > α,β-methylene-ATP, while the order is reversed for P_{2X} receptors (e.g. Williams, 1987; Burnstock, 1990;

Harden, Boyer, Brown, Cooper, Jeffs & Martin, 1990). However, ATP receptors on platelets, mast cells and lymphocytes apparently do not fit into this subclassification (e.g. Gordon, 1986; Burnstock, 1990).

The mean concentration–response curves for ATP, 2-methyl-thio-ATP, and α,β-methylene-ATP are illustrated in Fig. 5. The curves were constructed as the

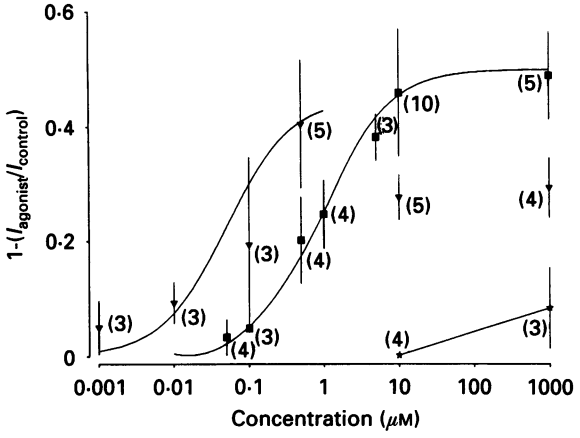

Fig. 5. ATP inhibits I_{Ca} by binding to a P_{2Y} receptor. Concentration–response relationships for inhibition of I_{Ca} by 2-methyl-thio-ATP (▼), ATP (■) and α,β-methylene-ATP (✱). Curves constructed as mean reduction in peak I_{Ca} (1-($I_{agonist}/I_{control}$)) at 0 mV (HP = −70 mV). The numbers in parentheses indicate the number of myocytes measured from at each concentration. The data were fitted by conventional Michaelis–Menten relationships ($E = E_{max}$[agonist]/(IC_{50} + [agonist]) (continuous curve), with E_{max} = 45%, IC_{50} = 0·05 μM for 2-methyl-thio-ATP; and E_{max} = 50%, IC_{50} = 1 μM for ATP. The effect of α,β-methylene-ATP on I_{Ca} was minimal even at 1 mM. Due to rapid desensitization at high concentrations (10 μM, 1 mM) the effect of 2-methyl-thio-ATP was smaller than that produced by lower concentrations, thereby precluding measurements of true steady-state effects. Therefore, at 10 μM and 1 mM the maximal effects of 2-methyl-thio-ATP were simply measured and the mean values plotted.

mean reduction in normalized peak I_{Ca} ($1-I_{agonist}/I_{control}$) at 0 mV. The data were fitted (continuous curves) by conventional Michaelis–Menten relationships ($E = E_{max}$ [agonist]/(IC_{50} + [agonist]) with the following values: 2-methyl-thio-ATP, E_{max} = 45%, IC_{50} = 0·05 μM; ATP, E_{max} = 50%, IC_{50} = 1 μM. The effect of α,β-methylene-ATP was minimal even at 1 mM.

The potency order for inhibition of peak I_{Ca} (2-methyl-thio-ATP > ATP ≫ α,β-methylene-ATP) indicates that ATP binds to a P_{2Y} receptor. In addition, the inhibitory effects of 1 mM ATP and 1 mM 2-methyl-thio-ATP on I_{Ca} were *not* additive, confirming that both ATP and 2-methyl-thio-ATP were acting at the same receptor type.

Involvement of G proteins in the P_{2Y} receptor-coupling mechanism

Is a PTX-sensitive G protein involved in the P_{2Y} receptor-coupling process?

To address this question a ribosylation assay was conducted as described in the preceding paper (Qu *et al.* 1993*a*). Figure 6*A* illustrates a representative result from a myocyte that had been incubated with PTX for 4 h at 37 °C (a time sufficient to

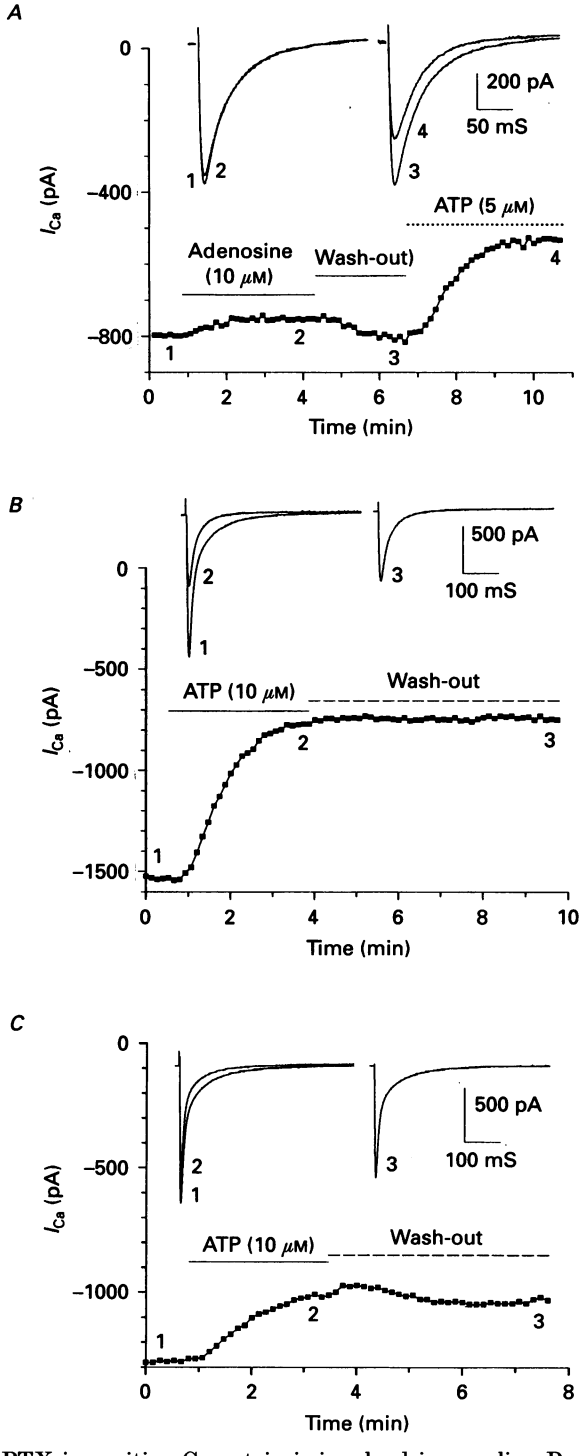

Fig. 6. A PTX-insensitive G protein is involved in coupling P_{2Y} receptor activation to inhibition of I_{Ca}. *A*, PTX pretreatment (4 h; Qu *et al.* 1993*a*) significantly attenuated the inhibitory effect of adenosine on I_{Ca}, but did not block the inhibitory effect of ATP. *B*,

produce greater than 90% ribosylation of PTX-sensitive G proteins). PTX pretreatment essentially abolished the inhibitory effect of 10 μM adenosine on basal I_{Ca} (Qu *et al.* 1993*a*). However, in the same myocyte PTX pretreatment did *not* attenuate the effect of 5 μM ATP. In a total of five myocytes pretreated with PTX for 4 h, 5 μM ATP consistently inhibited I_{Ca} to a similar extent as in control (non-PTX pretreated) myocytes obtained from the same isolation (PTX, $33{\cdot}4 \pm 2{\cdot}6$% inhibition; control, $35{\cdot}6 \pm 4{\cdot}3$% inhibition). These results indicate that, in contrast to the inhibition of basal I_{Ca} mediated by adenosine, ATP inhibits I_{Ca} through a mechanism independent of activation of a PTX-sensitive G protein.

Is a PTX-insensitive G protein involved in the P_{2Y} receptor-coupling process?

To address this question the non-hydrolysable GTP analogue, GTPγS, and the non-degradable GDP analogue, GDPβS, were employed. For these measurements 200 μM GTP in the control patch pipette solution was replaced with either 1 mM GTPγS or 1 mM GDPβS. These compounds were then perfused into myocytes for at least 30 min prior to conducting measurements.

When 200 μM GTP was included in the pipette (i.e. control conditions) 10 μM ATP produced a completely *reversible* inhibitory effect on I_{Ca}. When GTP was replaced with 1 mM GTPγS (Fig. 6*B*), 10 μM ATP still inhibited I_{Ca} to an extent similar to control ($41{\cdot}0 \pm 12{\cdot}1$%, $n = 4$ myocytes). However, the inhibitory effect of ATP was completely irreversible in the presence of GTPγS. This irreversibility was consistently observed in all myocytes perfused with 1 mM GTPγS ($n = 4$). When 1 mM GDPβS was included in the patch pipette (Fig. 6*C*) the inhibitory effect of 10 μM ATP on I_{Ca} was significantly attenuated compared to control ($21{\cdot}2 \pm 12{\cdot}2$%, $n = 4$ myocytes), and the effect was only partially reversible. Such partial reversibility of agonist effects in the presence of intracellular GDPβS has been observed in other preparations (e.g. Bosma, Bernheim, Leibowitz, Pfaffinger & Hille, 1990). The underlying reasons are presently unclear.

These results indicate that activation of a PTX-insensitive G protein is involved in the P_{2Y} receptor-coupling mechanism mediating the inhibitory effect of ATP on I_{Ca}.

Tests of possible intracellular mechanisms mediating the inhibitory effect of ATP on I_{Ca}

Is the inhibitory effect of ATP dependent on changes in $[Ca^{2+}]_i$?

To address this question, the effects of replacing (i) extracellular Ca^{2+} with Ba^{2+} and (ii) control intracellular EGTA with BAPTA were investigated.

Ba^{2+} is capable of carrying current through L-type Ca^{2+} channels while eliminating or greatly minimizing contributions of 'Ca^{2+}-dependent inactivation' (Eckert & Chad, 1984; Campbell & Giles, 1990). Figure 7*A* illustrates representative results on

---

ATP inhibited I_{Ca} irreversibly when 200 μM GTP in the patch pipette was replaced with 1 mM GTPγS. *C*, the effect of ATP on I_{Ca} was significantly attenuated when 200 μM GTP in the patch pipette was replaced with 1 mM GDPβS. All experiments were conducted at least 30 min after the whole-cell recording configuration was obtained.

the effect of 10 μM ATP on the peak I_{Ba} I–V relationship obtained from a single myocyte. Ten micromolar ATP decreased I_{Ba} at -25 mV (the mean peak I–V potential) by $49{\cdot}7 \pm 11{\cdot}8\%$ ($n = 5$ myocytes), a value not significantly different from its inhibitory effect on I_{Ca} recorded under control conditions ($45{\cdot}9 \pm 11{\cdot}0\%$, 0 mV). Therefore, ATP reversibly inhibited peak I_{Ba} to the same extent as peak I_{Ca}.

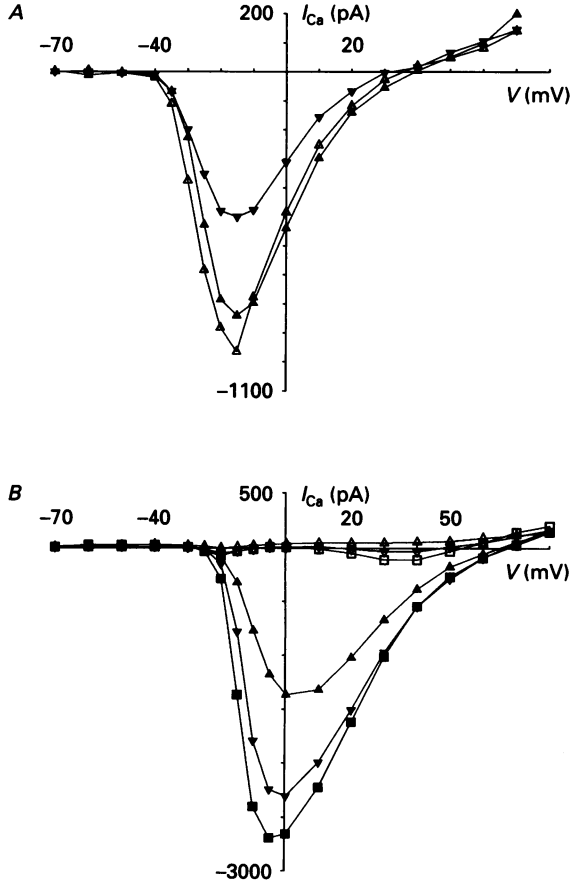

Fig. 7. *A*, effect of 10 μM ATP on Ba^{2+} flux through the Ca^{2+} channel. Peak current–voltage relationships in 1·0 mM Ba^{2+} (control) (▲), 1·0 mM Ba^{2+} + 10 μM ATP (▼) and 1·0 mM Ba^{2+} (wash-out) (△). *B*, effect of 10 μM ATP on I_{Ca} (1·8 mM Ca^{2+}) with 10 mM BAPTA in the pipette. Peak difference ($I_{Ca,peak} - I_{Ca,500ms}$) and isochronal (500 ms I–V relationships in control (■), 10 μM ATP (▲) and wash-out (▼).

BAPTA is a much stronger and faster Ca^{2+} chelator than EGTA. By replacing EGTA with BAPTA in the patch pipette, the $[Ca^{2+}]_i$ transient is expected to be more rapidly and efficiently buffered. Figure 7*B* shows representative results on the effect of 10 μM ATP on the I_{Ca} I–V relationship obtained from a single myocyte when 5 mM EGTA was replaced with 10 mM BAPTA in the patch pipette solution and the myocyte was perfused for longer than 30 min prior to conducting measurements. In 10 mM BAPTA, 10 μM ATP inhibited I_{Ca} at 0 mV by $43{\cdot}3 \pm 8{\cdot}8\%$ ($n = 4$ myocytes),

a value similar to that observed with normal control 5 mM EGTA (45·9 ± 11·0 %). These results indicate that the inhibitory effect of ATP is not attenuated by strong buffering of $[Ca^{2+}]_i$ with BAPTA.

In combination, the Ba^{2+} and BAPTA results suggest that the inhibition of L-type I_{Ca} by ATP is not obligatorily dependent upon Ca^{2+} ion influx or changes in $[Ca^{2+}]_i$ (see also Fig. 8).

Single-channel second messenger test

To determine if ATP inhibits I_{Ca} by activating intracellular diffusible second messenger(s), single Ca^{2+} channel activity was recorded using the cell-attached patch clamp configuration (see Methods). ATP was applied by externally perfusing myocytes (but *not* the patch surface under the pipette). If ATP exerts its effects by activating intracellular diffusible second messengers, effects on Ca^{2+} channels recorded under the patch should be demonstrable even if ATP does not have direct access to Ca^{2+} channels isolated in the patch (e.g. Hille, 1992).

The upper panels of Fig. 8 illustrate six consecutive traces of single L-type Ca^{2+} channel activity recorded in control and after bath application of 10 μM ATP elicited at +10 mV from a holding potential of −80 mV. In this particular patch, there were at least three Ca^{2+} channels. The lower panel of Fig. 8 shows ensemble averages of channel activity constructed from 200 traces obtained from the same myocyte in control, 10 μM ATP and wash-out. Bath application of ATP decreased the amplitude of the ensemble-average current. The effect was reversible upon wash-out. An inhibitory effect of bath-applied 10 μM ATP on ensemble-average Ba^{2+} currents was observed in a total of three patches (each containing at least three Ca^{2+} channels). The mean single-channel conductance measured from these three cell-attached patches in control and bath-applied 10 μM ATP were not significantly different (control, 19 ± 2 pS; ATP, 20 ± 2 pS; $P < 0·05$).

These single-channel results directly confirm the inhibitory effects of ATP on I_{Ca}, and suggest that the effect may involve cytosolic diffusible second messenger(s) generated following P_{2Y} receptor activation. However, these results do not exclude the additional possibility that P_{2Y} receptors may also modulate Ca^{2+} channels by a direct G protein–channel interaction, i.e. similar to the muscarinic receptor activation of I_{KACh} described in atrial myocytes (e.g. Breitwieser & Szabo, 1988).

Possible second messengers: are protein kinase A, protein kinase C, or inositol phosphates involved?

The second messenger systems associated with P_2 receptor subtypes have not been established unambiguously. In a number of cells ATP and other nucleotides produce an increase in inositol trisphosphates and elevation of free $[Ca^{2+}]_i$, suggesting the involvement of phospholipase C (PLC) (e.g. hepatocytes: Charest, Blackmore & Exton, 1985; HL60 cells: Dubyak, Cowen & Meuller, 1988; adrenal medullary cells: Boeynaems, Pirotton, van Coevorden, Raspe, Demolle & Erneux, 1988; rat ventricular myocytes: Danziger *et al.* 1988; Legssyer, Poggioli, Renard & Vassort, 1988; reviewed in Burnstock, 1990; El-Moatassim *et al.* 1992). It has also been demonstrated in turkey erythrocytes that the P_{2Y} receptor is coupled to PLC by GTP-dependent proteins (Harden *et al.* 1990). It is therefore possible that activation

of PLC, increases in $[Ca^{2+}]_i$, activation of protein kinase C (PKC), or production of inositol phosphates (IP_3, IP_4) might be important in mediating the effect of ATP on I_{Ca}. Finally, it is well documented that simulation of the cAMP-dependent protein kinase A pathway increases L-type I_{Ca} (e.g. Hartzell, 1988). However, it is not clear

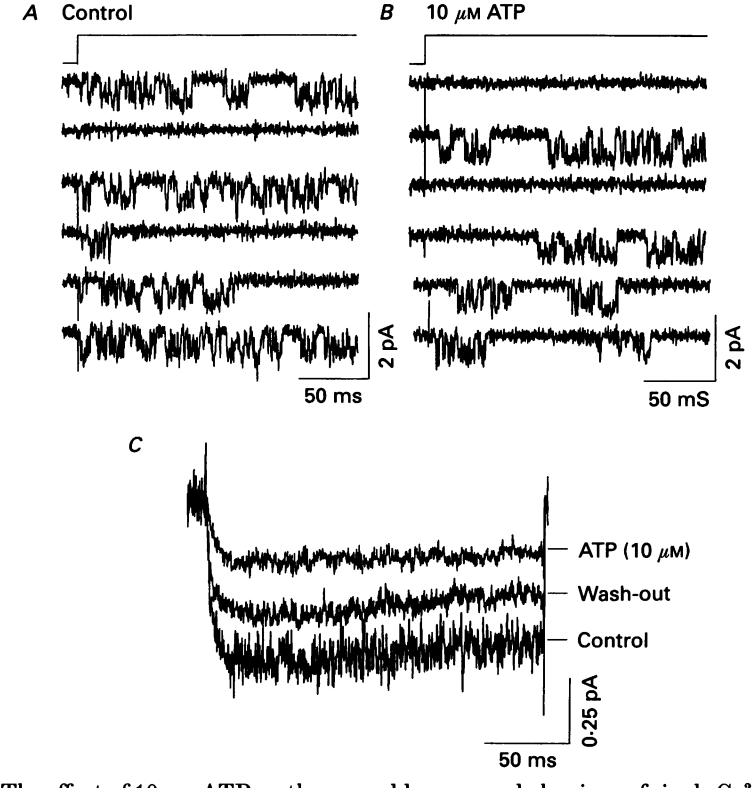

Fig. 8. The effect of 10 μM ATP on the ensemble-average behaviour of single Ca^{2+} channels recorded in the cell-attached configuration. In this particular patch there were at least three Ca^{2+} channels. The upper panel shows a selected series of six consecutive Ca^{2+} channel recordings in both control (*A*) and 10 μM ATP (*B*) at +10 mV (HP = −80 mV, pulses applied at 0·5 Hz, currents filtered at 2 kHz, digitized at 10 kHz). *C*, ensemble averages each constructed from 200 consecutive traces in control, 10 μM ATP and wash-out from the same myocyte in *A* and *B*. Bath-applied ATP decreased the amplitude of the ensemble-current and the effect was reversible.

if inhibition of this pathway under basal conditions decreases I_{Ca} (see Qu *et al.* 1993*a*).

To test for the possible involvement of PKC two approaches were used. First, the effect of 4β-phorbol-12-myristate-13-acetate (PMA), which is a direct activator of PKC, was examined to determine if it could imitate the effect of ATP. In three myocytes, application of 200 nM PMA did not inhibit I_{Ca}; rather, there was a slight stimulatory effect. Increasing PMA to 1 μM failed to produce any effect on I_{Ca} (n = 2 myocytes). Second, a non-specific PKC inhibitor, staurosporine (100 nM), was used to determine if it could block the effect of ATP on I_{Ca}. To determine if inositol

phosphates could imitate and attenuate the effect of ATP on I_{Ca}, 100 μM IP_3 + 7·7 μM IP_4 were internally perfused for 20 min prior to application of ATP. Finally, to determine if ATP reduces I_{Ca} by inhibiting basal activity of PKA two approaches were used: (i) 2 μM PKI, a specific peptide inhibitor of cAMP-dependent

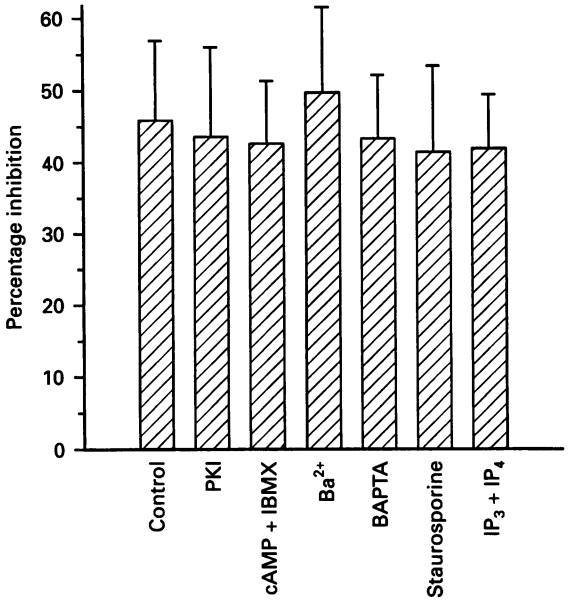

Fig. 9. Tests for possible involvement of different second messenger pathways ($[Ca^{2+}]_i$, PKA, PKC and inositol phosphates) in mediating the inhibitory effect of 10 μM ATP on I_{Ca} at 0 mV. Summarized results obtained using the different experimental protocols described in the text. Data give as means ± S.D. for $n \geqslant 4$ myocytes for each protocol. None of the experimental manoeuvres significantly affected the inhibition of I_{Ca} by ATP.

PKA, was internally perfused for 30 min prior to application of ATP, and (ii) a 'cAMP clamp' was imposed, i.e. 1 mM cAMP + 10 μM IBMX (a phosphodiesterase inhibitor) was internally perfused for an additional 10 min after reaching steady-state effects so as to maximally activate the cAMP–PKA system (Qu *et al.* 1993*a*).

The results of all of these different experimental manoeuvres are summarized in Fig. 9 (results of $n \geqslant 4$ myocytes for each case). None of the interventions affected the inhibition of I_{Ca} by 10 μM ATP. Therefore, none of the second messenger systems which we tested for appears to be critically involved in coupling P_{2Y} receptor activation to I_{Ca} inhibition.

DISCUSSION

Our results are the first to demonstrate that both adenosine and ATP inhibit basal L-type I_{Ca} in ferret isolated right ventricular myocytes. The present study is also the first in isolated cardiac myocytes to demonstrate that (i) adenosine and ATP exert their effects by different and distinct pharmacological and biochemical mechanisms,

(ii) that ATP is able to exert direct electrophysiological effects on ventricular L-type I_{Ca} through ATP-selective P_{2Y} receptors independently of adenosine A_1 receptors, and (iii) that a PTX-insensitive G protein is involved in coupling activation of the P_{2Y} receptor to inhibition of I_{Ca}. Finally, while we have not identified the second messenger systems involved, our results indicate that $[Ca^{2+}]_i$, protein kinase C, inositol phosphates and cAMP-dependent protein kinase A are not essential for mediating the inhibitory effect of ATP on I_{Ca} in these myocytes.

Effect of ATP on the electrophysiological properties of I_{Ca}

Similar to our previous results obtained with adenosine (Qu *et al.* 1993*a*), ATP reversibly inhibited the basal L-type I_{Ca} of ferret right ventricular myocytes in a concentration-dependent manner. However, while neither adenosine nor ATP significantly altered the voltage dependence of I_{Ca} activation, ATP did produce effects on I_{Ca} inactivation characteristics that were distinctly different from those produced by adenosine. Ten micromolar adenosine reduced peak I_{Ca} at 0 mV by $36{\cdot}0 \pm 12{\cdot}0\,\%$ ($E_{max} = 35\,\%$, $IC_{50} = 0{\cdot}3\ \mu M$) without altering either the time constants of inactivation of I_{Ca} at 0 mV or the time constant of recovery from inactivation at -70 mV (Figs 5 and 6 in Qu *et al* 1993*a*). Adenosine also did not affect either the '*f*' or '*r*' relationships of the I_{Ca} steady-state inactivation relationship (Figs 3 and 4 in Qu *et al.* 1993*a*). In contrast, 10 μM ATP reduced I_{Ca} by $45{\cdot}9 \pm 11{\cdot}0\,\%$ ($E_{max} = 50\,\%$, $IC_{50} = 1\ \mu M$) while significantly altering both time constants of inactivation of I_{Ca} at 0 mV and markedly slowing recovery from inactivation at -70 mV (Fig. 1*B* and *C*).

ATP also significantly altered the inactivation '*r*' relationship (Fig. 1*B*). This effect could be interpreted in terms of extracellular ATP acting like an open Ca^{2+} channel blocker. However, our data clearly indicate that this is not the case: (i) the inhibitory effect saturated at 50%; (ii) the effect was irreversible with GTPγs and significantly attenuated by GTPβS; (iii) ATP inhibited single Ca^{2+} channel activity without direct exposure to Ca^{2+} channels in the patch; and (iv) the inhibitory effect could be antagonized by 100 μM suramin. The last two points are clear evidence that ATP is not acting as a direct open Ca^{2+} channel blocker.

The mechanisms underlying the differences in the electrophysiological effects of adenosine and ATP on I_{Ca} are presently unclear; however, the effects of ATP on I_{Ca} inactivation and recovery were consistently observed and were significantly different from both control conditions and the effects produced by adenosine. These results combined with the fact that the same voltage clamp protocols and methods of analysis were used for both compounds, indicate that the electrophysiological differences observed between adenosine and ATP on I_{Ca} inactivation are genuine. Unfortunately, the mechanisms governing both inactivation and recovery of L-type I_{Ca} in cardiac muscle are still not understood (e.g. Campbell & Giles, 1990). While it is now generally accepted that inactivation of cardiac L-type I_{Ca} displays both voltage- and current-dependent components, the relative contributions of these two components have not been accurately quantified. Attributing the differences in the effects of adenosine and ATP to one specific component of I_{Ca} inactivation is therefore not possible at present.

Possible biochemical mechanisms by which ATP inhibits basal I_{Ca}

Our results demonstrate separate pharmacological and biochemical mechanisms by which adenosine and ATP inhibit basal I_{Ca}, both at the receptor level and the GTP-binding protein level. In contrast to adenosine, (i) ATP inhibits basal I_{Ca} by binding to an ATP-specific P_{2Y} receptor (Figs 3, 4 and 5), and (ii) the effect of ATP on basal I_{Ca} is insensitive to pretreatment with PTX (Fig. 6*A*). However, the effect of ATP is modulated by GTP analogues (GTPγS, GDPβS; Fig. 6*B* and *C*), indicating that P_{2Y} receptors are coupled through a PTX-insensitive G protein to mechanisms regulating I_{Ca}.

It has been demonstrated in rat ventricular myocytes that the rank potency order for ATP analogues in increasing resting $[Ca^{2+}]_i$ is consistent with the presence of ATP-selective P_{2Y} receptors (Bjornsson, Monck & Williamson, 1989). In the same preparation, Scamps *et al.* (1992) have proposed that ATP produces both a stimulatory and an inhibitory effect on L-type I_{Ca}, possibly mediated by subtypes of purinergic P_2 receptors. These investigators proposed that the stimulatory effect is mediated by an isoform of a G_s protein. However, in contrast to the findings of Scamps *et al.* (1992), we consistently observed a decrease in peak I_{Ca} upon application of ATP which remained stable over a period of up to 10 min.

The cell-attached single-channel experiments suggest that some type of intracellular diffusible second messenger(s) is produced upon exposure to extracellular ATP (although our measurements do not allow us to conclusively rule out direct G protein modulation of I_{Ca}). The intracellular mechanisms involved are presently not clear. Nonetheless, our experimental results (summarized in Fig. 9) strongly suggest that ATP does *not* inhibit I_{Ca} by (i) increasing $[Ca^{2+}]_i$ (I_{Ba}, BAPTA; and fura-2 measurements: Qu *et al.* 1993*b*), (ii) reduction of basal protein kinase A activity (PKI, 'cAMP clamp'), (iii) activation of protein kinase C (PMA, staurosporine), or (iv) production of inositol phosphates (IP_3, IP_4). One group of potential second messengers which we have not yet tested for are arachidonic acid and its metabolites, i.e. prostaglandins, thromboxanes, leukotrienes and epoxides. Arachidonic acid can be produced either by activation of phospholipase A_1 or from diacylglycerol (by the action of diacylglycerol lipase) following activation of phospholipase C (Rana & Hokin, 1990). It has been reported in a number of organs that ATP stimulates the production and release of prostaglandins (e.g. Needleman, Minkes & Douglas, 1974), and that in vascular endothelial cells this effect is mediated by P_{2Y} receptors (Needham, Cusack, Pearson & Gordon, 1987; Carter, Hallam, Cusack & Pearson, 1988). As another possibility, it has been observed in bovine pulmonary artery (Martin & Michaelis, 1989) and aortic (Pirotton, Robaye, Lagneau & Boeynaems, 1990) endothelial cells that ATP activates phospholipase D, which hydrolyses phosphatidylcholine to phosphatidic acid and choline. It will be interesting to see if any of these pathways can mimic or alter the effect of ATP in these myocytes.

Comparison to previous results and possible physiological implications

The responses to extracellular ATP are diverse and variable among different cell types (e.g. Gordon, 1986; Burnstock, 1990; El-Moatassim *et al.* 1992). Extracellular ATP increases free $[Ca^{2+}]_i$ in a variety of cell types (Burnstock, 1990; Bean, 1992),

and such increases enhance Ca^{2+}-dependent inactivation of I_{Ca} (Eckert & Chad, 1984). For example, in guinea-pig bladder smooth muscle cells ATP decreased I_{Ca} by increasing $[Ca^{2+}]_i$ (Schneider, Hopp & Isenberg, 1991). However, in mammalian cardiac myocytes the effects of ATP have only been studied in rat ventricular myocytes, with variability in results. In this preparation extracellular ATP has been reported to activate a non-specific cation current, which appears to be the main mechanism by which ATP increases $[Ca^{2+}]_i$ levels (Christie *et al.* 1992). However, while there does seem to be a consensus that extracellular ATP increases resting $[Ca^{2+}]_i$, both ATP-mediated increases and decreases in I_{Ca} have been reported (Scamps *et al.* 1992), as well as both increases (Daziger *et al.* 1988) and decreases (Bjornsson *et al.* 1989) in the $[Ca^{2+}]_i$ transient. The effects of extracellular ATP on Ca^{2+} homeostatic mechanisms in rat ventricular myocytes therefore appear to be complicated and not entirely agreed upon at present.

In contrast to results obtained in rat, we consistently failed to observe any increases in resting $[Ca^{2+}]_i$ in ferret ventricular myocytes upon application of micromolar levels of extracellular ATP (Qu *et al.* 1993*b*). Furthermore, we have yet to find any consistent evidence for the presence of ATP-activated non-selective cation channels, as evidenced by the lack of any consistent changes in holding current or the apparent E_{rev} of I_{Ca} (in either NMDG or normal Na^+, K^+ solutions: see Qu *et al.* 1993*b*). These results, combined with the Ba^{2+} and BAPTA results (Figs 7 and 8), suggest that in ferret right ventricular myocytes increases in $[Ca^{2+}]_i$ are not primarily involved in mediating the inhibitory effect of ATP on I_{Ca}.

In addition to being released as a co-transmitter from nerve terminals (Burnstock, 1990), ATP can also be released from endothelial cells, aggregating platelets and hypoxic myocardium (Paddle & Burnstock, 1974; Clemens & Forrester, 1980; Gordon, 1986). ATP in the coronary circulation can approach micromolar levels (Born & Kratzer, 1984), and higher levels can be reached under ischaemic conditions (Clemens & Forrester, 1980). Despite uncertainty on the mechanisms underlying cardiac L-type I_{Ca} inactivation, it is now clear that alteration of the inactivation characteristics of I_{Ca} has a significant effect on repolarization of the cardiac action potential (e.g. Rasmusson *et al.* 1990). The effects of ATP on I_{Ca} should therefore produce physiologically important effects in ferret ventricular myocytes. This is clearly indicated by the fact that extracellular ATP inhibits the basal action potential, $[Ca^{2+}]_i$ transients and cell length shortening in these myocytes (Qu *et al.* 1993*b*). Our results would suggest that in ferret ventricular myocardium extracellular ATP could serve a cardioprotective role by being potentially antiarrhythmogenic. In contrast, in rat ventricle extracellular ATP would appear to be arrhythmogenic. The effects of ATP therefore appear to be fundamentally different between the two species. It will be interesting to examine the effects of ATP on I_{Ca} in other mammalian myocytes, especially human, to assess the applicability of the effects which we have observed in ferret ventricular myocytes.

We would like to thank Ms Steffani Webb for skilled secretarial assistance, Ms Anne Crews and Ms Ying Zhang for technical assistance, Dr T. K. Harden for supplying us with suramin, Dr H. A. Brown for helpful comments on suramin and purinergic pharmacology, Drs D. Armstrong and R. White for technical instructions on the internal perfusion technique, and Drs G. Oxford. A. R. Whorton and R. L. Rasmusson for very valuable suggestions during the course of this project. This

work was supported by a predoctoral training grant (Graduate Award from Duke University) to Y. Qu, and grants from the American Heart Association (NC Affiliate; NC-91-G11) to D.L.C. and National Institutes of Health (HL-17670, HL-19216, HL-45132) to H.C.S. This work was submitted by Yusheng Qu to the Department of Pharmacology, Duke University, as part of a Dissertation for the degree Doctor of Philosophy.

REFERENCES

BEAN, B. P. (1992). Pharmacology and electrophysiology of ATP-activated ion channels. *Trends in Pharmacological Sciences* **13**, 87–90.

BJORNSSON, O. G., MONCK, J. R. & WILLIAMSON, J. R. (1989). Identification of P_{2Y} purinoceptors associated with voltage-activated cation channels in cardiac ventricular myocytes of the rat. *European Journal of Biochemistry* **186**, 395–404.

BOEYNAEMS, J. M., PIROTTON, S., VAN COEVORDEN, A., RASPE, E., DEMOLLE, D. & ERNEUX, C. (1988). P_2-purinogenic receptors in vascular endothelial cells: from concept to reality. *Journal of Receptor Research* **8**, 121–132.

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

BOSMA, M. M., BERNHEIM, L., LEIBOWITZ, M. D., PFAFFINGER, P. J. & HILLE, B. (1990). Modulation of M current in frog sympathetic ganglion cells. In *G Proteins and Signal Transduction*, ed. NATHANSON, N. M. & HARDEN, T. K., pp. 43–60. Society of General Physiologists Series, vol. 45. The Rockefeller University Press, NY, USA.

BREITWIESER, G. E. & SZABO, G. (1988). Mechanism of muscarinic receptor-induced K^+ channel activation as revealed by hydrolysis-resistant GTP analogues. *Journal of General Physiology* **91**, 469–494.

BURNSTOCK, G. (1990). Purinergic mechanisms. In *Biological Actions of Extracellular ATP*, ed. DUBYAK, G. R. & FEDAN, J. S., pp. 1–17. Annals of the New York Academy of Sciences, vol. 603. The New York Academy of Sciences, New York, NY, USA.

CAMPBELL, D. L. & GILES, W. (1990). Calcium Currents. In *Calcium and the Heart*, ed. LANGER, G. A., pp. 27–83, Raven Press Ltd, NY, USA.

CAMPBELL, D. L., GILES, W. R. & SHIBATA, E. F. (1988). Ion transfer characteristics of the calcium current in bull-frog atrial myocytes. *Journal of Physiology* **403**, 239–266.

CARTER, T. D., HALLAM, T. J., CUSACK, N. J. & PEARSON, J. D. (1988). Regulation of P_{2Y}-purinoceptor-mediated prostacyclin release from human endothelial cells by cytoplasmic calcium concentration. *British Journal of Pharmacology* **95**, 1181–1190.

CHAREST, R., BLACKMORE, P. F. & EXTON, J. H. (1985). Characterization of responses of isolated rat hepatocytes to ATP and ADP. *Journal of Biological Chemistry* **260** (1912), 15789–15794.

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

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.

DANZIGER, R. S., RAFFAELI, S., MORENO-SANCHEZ, R., SAKAI, M., CAPOGROSSI, M. C., SPURGEON, H. A., HANSFORD, R. G. & LAKATTA, E. G. (1988). Extracellular ATP has a potent effect to enhance cytosolic calcium and contractility in single ventricular myocytes. *Cell Calcium* **9**, 193–199.

DUBYAK, G. R., COWEN, D. S. & MEULLER, L. M. (1988). Activation of inositol phospholipid breakdown in HL60 cells by P_2-purinergic receptors for extracellular ATP. Evidence for mediation by both pertussis toxin-sensitive and pertussis toxin-insensitive mechanisms. *Journal of Biological Chemistry* **263**, 18108–18117.

ECKERT, R. & CHAD, J. E. (1984). Inactivation of Ca channels. *Progress in Biophysics and Molecular Biology* **44**, 215–267.

EDWARDS, F. A., GIBB, A. J. & COLQUHOUN, D. (1992). ATP receptor-mediated synaptic currents in the central nervous system. *Nature* **359**, 144–147.

EL-MOATASSIM, C., DORNARD, H. & MANI, J.-C. (1992). Extracellular ATP and cell signalling. *Biochimica et Biophysica Acta* **1134**, 31–45.

EVANS, R. J., DERKACH, V. & SURPRENANT, A. (1992). ATP mediates fast synaptic transmission in mammalian neurons. *Nature* **357**, 503–505.

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

HARDEN, T. K., BOYER, J. L., BROWN, H. A., COOPER, C. L., JEFFS, R. A. & MARTIN, M. W. (1990). Biochemical properties of a P_{2Y}-purinergic receptor. In *Biological Actions of Extracellular ATP*, ed. DUBYAK, G. R. & FEDAN, J. S., pp. 256–266. Annals of the New York Academy of Sciences, vol. 603.

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.

HILLE, B. (1992). *Ionic Channels of Excitable Membranes*, 2nd edn. Sinauer Associates, Sunderland, MA, USA.

HOYLE, C. V. & BURNSTOCK, G. (1986). Evidence that ATP is a neurotransmitter in the frog heart. *European Journal of Pharmacology* **124**, 285–289.

HOYLE, C. H. V., KNIGHT, G. E. & BURNSTOCK, G. (1990). Suramin antagonizes responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea pig urinary bladder and taenia coli. *British Journal of Pharmacology* **99**, 617–621.

LEGSSYER, A., POGGIOLI, J., RENARD, D. & VASSORT, G. (1988). ATP and other adenine compounds increase mechanical activity and inositol triphosphate production in rat heart. *Journal of Physiology* **401**, 185–199.

MARTIN, T. W. & MICHAELIS, K. (1989). P_2-purinergic agonists stimulate phosphodiesteratic cleavage of phosphatidylcholine in endothelial cells. Evidence for activation of phospholipase D. *Journal of Biological Chemistry* **264** (15), 8847–8856.

NAKAZAWA, K., FUJIMORI, K., TAKANAKA, A. & INOUE, K. (1990). Reversible and selective antagonism by suramin of ATP-activated inward current in PC12 phaeochromocytoma cells. *British Journal of Pharmacology* **101**, 224–226.

NEEDHAM, L., CUSAK, N. J., PEARSON, J. D. & GORDON, J. L. (1987). Characteristics of the P_2-purinoceptor that mediates prostacyclin production in pig aortic endothelial cells. *European Journal of Pharmacology* **134**, 199–209.

NEEDLEMAN, P., MINKES, M. S. & DOUGLAS, J. R. (1974). Stimulation of prostaglandin biosynthesis by adenine nucleotides. *Circulation Research* **34**, 455–460.

OLSSON, R. A. & PEARSON, J. D. (1990). Cardiovascular Purinoceptors. *Physiological Reviews* **7** (3), 761–845.

PADDLE, B. M. & BURNSTOCK, G. (1974). Release of ATP from perfused heart during coronary vasodilatation. *Blood Vessels* **11**, 110–119.

PIROTTON, S., ROBAYE, B., LAGNEAU, C. & BOEYNAEMS, J. M. (1990). Adenine nucleotides modulate phosphatidylcholine metabolism in aortic endothelial cells. *Journal of Cellular Physiology* **142**, 449–457.

QU, Y., CAMPBELL, D. L., HIMMEL, H. M. & STRAUSS, H. C. (1992). ATP reduces L-type I_{Ca} independently of Ca^{2+}-dependent inactivation in ferret ventricular myocytes. *Biophysical Journal* **61**, A400.

QU, Y., CAMPBELL, D. L., WHORTON, A. R. & STRAUSS, H. C. (1991). Modulation of I_{Ca} by ATP in isolated ferret right ventricular myocytes. *Circulation* **84**, II–172.

QU, Y., CAMPBELL, D. L., WHORTON, A. R. & STRAUSS, H. C. (1993*a*). Modulation of basal L-type I_{Ca} by adenosine in isolated ferret right ventricular myocytes. *Journal of Physiology* **471**, 269–293.

QU, Y., HIMMEL, H. M., CAMPBELL, D. L. & STRAUSS, H. C. (1993*b*). The effects of extracellular ATP on I_{Ca}, $[Ca^{2+}]_i$, and contraction in isolated ferret ventricular myocytes. *American Journal of Physiology* **264**, C702–708.

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.

RANA, R. S. & HOKIN, L. E. (1990). Role of phosphoinositides in transmembrane signaling. *Physiological Reviews* **70** (1), 115–164.

RASMUSSON, R. L., CLARK, J. W., GILES, W. R., ROBINSON, K., CLARK, R. B., SHIBATA, E. F. & CAMPBELL, D. L. (1990). A mathematical model of electrophysiological activity in a bullfrog atrial cell. *American Journal of Physiology* **259**, H370–389.

SCAMPS, F., RYBIN, V., PUCEAT, M., TKACHUK, V. & VASSORT, G. (1992). A G_s protein couples P_2-purinergic stimulation to cardiac Ca channels without cyclic AMP production. *Journal of General Physiology* **100**, 675–701.

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

VON KÜGELGEN, I., BÜLTMANN, R. & STARKE, K. (1989). Effects of suramin and α,β-methylene ATP indicate noradrenaline–ATP co-transmission in the response of the mouse vas deferens to single and low frequency pulses. *Naunyn-Schmiedeberg's Archives of Pharmacology* **340**, 760–763.

WILLIAMS, M. (1987). Purine receptors in mammalian tissues: pharmacology and functional significance. *Annual Review of Pharmacology and Toxicology* **27**, 315–345.