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Effects of purinergic stimulation on the Ca current in single frog cardiac cells

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Abstract. Ca current (I_{Ca}) was measured by whole-cell voltage clamp in single cells isolated from frog ventricle, in which the Na current was inhibited by tetrodotoxin (0.3 μM) and K currents were blocked by substituting K with 120 mM intracellular and 20 mM extracellular Cs. The influence of stimulation by ATP (0.1–100 μM) was assessed in the presence of propranolol (1 μM) or pindolol (0.1 μM), prazozin (0.1 μM) and atropine (10 μM). ATP, in the micromolar range, had two types of effect. Like other P_1-purinoagonists, it antagonized the increase in I_{Ca} elicited by β-adrenostimulation. When added alone, 1 μM ATP could increase I_{Ca} up to twofold. An increase in I_{Ca} was also observed even after it had been maximally enhanced by intracellularly applied cAMP (50 μM). Voltage dependence and kinetics of I_{Ca} were not affected. These effects were considered to be related to P_2-purinoceptor activation. At higher ATP concentrations the increase in I_{Ca} was less; at 100 μM, ATP reduced I_{Ca}. The ATP-induced increase in I_{Ca} was prevented by internal perfusion of the cells with GDP [β-S] or neomycin, respectively, to block signal transduction to phospholipase C or its phosphodiesterase activity on the polyphosphoinositides. We conclude that P_2-purinoceptor stimulation increases the Ca current in frog ventricular cells by a pathway that might involve phosphoinositide turnover.

Key words: Heart – Ca current – Patch clamp – ATP – P_2-purinostimulation

Introduction

The force of myocardial contraction is modulated by catecholamines. It is generally agreed that the adrenoceptors involved are predominantly of the β-type. Their stimulation leads to an accumulation in the cell of adenosine cyclic 3′,5′-monophosphate (cAMP) through activation of the stimulatory guanine nucleotide regulatory protein. This results in the activation of the catalytic subunit of membrane-bound adenylate cyclase (Gilman 1987). The movements of Ca through the sarcolemma and the sarcoplasmic reticulum membrane are then increased and the Ca sensitivity of the contractile apparatus decreased. This leads to a large increase in force of contraction associated with its abbreviation (see Winegrad 1984).

ATP and other adenine compounds are often associated with sympathomimetic amines in nerve terminals and may be released in the coronary circulation. The existence of specific plasma membrane receptors for adenosine was first demonstrated in a crude membrane fraction prepared from dog ventricles (Dutta and Mustafa 1979). Adenine compounds are generally reported to have a negative inotropic effect particularly in the atrium of mammals (Burnstock and Meghji 1973). They mediated an increase in K current (Belardinelli and Isenberg 1983b), a slight decrease in Ca current (Cerbai et al. 1988) and antagonized the β-adrenergic stimulation (Isenberg and Belardinelli 1984; Linden et al. 1985). These effects could be related to P_1-purinergic stimulation and are similar to those of acetylcholine. On the other hand, ATP has been shown to have positive inotropic effects in a few studies on mammalian ventricular preparations (Danziger et al. 1988; Legssyer et al. 1988) and in the frog heart (Goto et al. 1976; Flitney and Singh 1980; Niedergerke and Page 1981). This could be related to the fact that ATP facilitates a slow action potential in guinea-pig hearts (Schneider and Sperelakis 1975), increases the Ca current slightly in frog trabeculae (Goto et al. 1976) and enhances the free intracellular calcium concentration synergistically with β-agonists in mammals (De Young and Scarpa 1987). These effects are generally attributed to P_2-purinergic stimulation.

The intermediate steps for P_2-purinergic stimulation are poorly understood. There is some recent evidence that cellular biochemical processes associated with positive

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inotropy in mammalian hearts might involve an increased breakdown of membrane phosphoinositides as a result of either α_1-adrenergic and muscarinic (Brown and Jones 1986; Poggioli et al. 1986; Schmitz et al. 1987b) or P_2-purinergic stimulations (Legssyer et al. 1988). Phosphoinositide breakdown products, inositol trisphosphate and diacylglycerol can both act as intracellular second messengers with diacylglycerol activating protein kinase C. As in other tissues, following receptor occupation phospholipase C is reported to be activated via a membrane-bound guanine nucleotide protein or G-protein (Bockaert et al. 1987; Gilman 1987). An alternative possibility is that the G-protein could be directly coupled to one of the membrane channels. This was recently proposed for the Ca channel both for its inhibition by α_2-adrenoagonists and 4-aminobutyric acid in dorsal root ganglion cells (Holz et al. 1986) or for its stimulation by β-adrenoagonists in cardiac cells (Yatani et al. 1987).

We have investigated the effects of ATP on the Ca current in isolated frog ventricular cells since positive inotropy has been described in frog tissues. Our results show a limited increase in the Ca current amplitude, which seems to require the activation of phospholipase C by a G-protein.

Materials and methods

The methods used for dissociation of frog ventricular cells (*Rana esculenta*), whole-cell patch-clamp recording, superfusion of the cells, and data analysis have been extensively described (Fischmeister and Hartzell 1986) and were used here with minor modifications.

For routine monitoring of calcium current (I_{Ca}), the cell was depolarized every 8 s from −80 mV holding potential to 0 mV for 200 ms. To measure I_{Ca} accurately with no contamination of other ionic currents, the cells were bathed in K-free Cs/Ringer solution containing (mM): NaCl 88.4, CsCl 20, $NaHCO_3$ 22.9, NaH_2PO_4 0.6, $CaCl_2$ 1.8, $MgCl_2$ 1.8, D-glucose 5, sodium pyruvate 5 and 0.3 μM tetrodotoxin (Sankyo Japan); it was gassed with 95% − 5% O_2/CO_2. Control and drug-containing solutions were applied to the exterior of the cell by placing the cell at the opening of one of six capillary tubes, 250 μm inner diameter, through which solutions were flowing at a rate of about 10 μl/min. In some experiments the patch pipette was internally perfused. Current-voltage relationships, inactivation curves and recovery from inactivation (reactivation) curves were obtained with voltage-clamp protocols as previously described (Fischmeister and Hartzell 1986). The standard 'intracellular' solution in the patch electrode (1 − 3 MΩ resistance) contained (mM): CsCl 120, K_2EGTA 5, $MgCl_2$ 4, disodium phosphocreatine 5, Na_2ATP 3, Na_2GTP 0.4, adjusted to pH 7.1 with KOH; $[Ca^{2+}]$ free below 0.01 μM. Under these conditions, I_{Ca} was measured on-line as the difference between peak inward current and the current at the end of the 200-ms pulse. This gives the same results as subtracting the current left after blockade of I_{Ca} with Cd^{2+} (Fischmeister and Hartzell 1986). Currents were digitized at 5 kHz (12 bits A/D converter) and analysed on-line by a Compaq 286 Deskpro computer using programs written in Pascal language.

Three different pulse protocols were used in this study. In every case the cells were held at −80 mV. A train of repetitive pulses, 200 ms to 0 mV every 8 s, allowed us to analyse the time course of the variations in I_{Ca}. A double pulse sequence consisting of a 200-ms prepulse of variable amplitude (−100 mV to +100 mV) and of a test pulse (200 ms to 0 mV) was used to establish both the *I-V* relationship and the availability curve. The I_{Ca}-*V* relationship was obtained by plotting I_{Ca} elicited by the prepulse against the prepulse potential. The availability curve is the plot of I_{Ca} during the test pulse, after a given prepulse relative to maximum I_{Ca} in the absence of prepulse, against the prepulse potential. The prepulse and the test pulse were separated by a 3-ms interval to avoid most of the capacitive current following the prepulse while estimating peak I_{Ca} during the test pulse. Two identical pulses (200 ms to 0 mV) separated by a variable interval of increasing duration allowed us to estimate the recovery from inactivation by plotting I_{Ca} during the second pulse relative to I_{Ca} elicited by the first pulse as a function of the time interval.

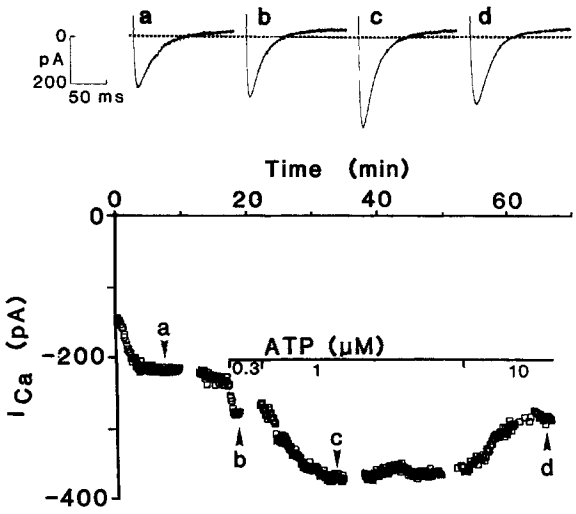


Fig. 1. Time course of the effects of ATP on peak I_{Ca}. I_{Ca} was significantly increased when ATP was added at 0.3 μM. ATP at 10 μM was less effective than at 1 μM. Individual current traces recorded at the indicated times (*a* − *d*) are shown above

Results

Effects of purinostimulation on I_{Ca}

The Ca current of single ventricular frog cells was markedly increased by applying external ATP at low concentrations (in the micromolar range). It was increased less or even reduced by ATP at larger concentrations. The results of one such an experiment are reported in Fig. 1. When the peak inward current had stabilized after the onset of whole-cell recording, addition of 0.3 μM ATP induced an increase of 30% in I_{Ca}. Switching to a solution containing 1 μM ATP further increased I_{Ca} by 70% of control. Prazozin (0.1 μM), pindolol (0.1 μM) and propranolol (1 μM) or atropine (10 μM) did not prevent this effect of ATP (not shown). A further increase in the ATP concentration to 10 μM reduced the current elevated by 1 μM ATP. Figure 2 shows the dose-response curves of extracellular application of ATP. Generally not more than two increasing ATP concentrations were used since a second application of the same solution to a given cell was less potent in increasing I_{Ca}. I_{Ca} was already significantly increased by 0.1 μM ATP and the increase was maximal at 1 μM with a half-activation value at 0.2 μM ATP. Larger concentrations were less effective; even a reduction in peak I_{Ca} was observed with 100 μM ATP. Both increase and decrease in I_{Ca} were fully reversible.

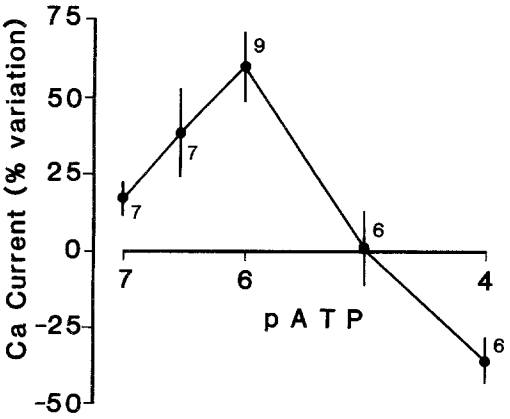


Fig. 2. Dose-response curve of the variation in I_{Ca} induced by ATP. At most two increasing ATP concentrations were applied to the same cell. Only cells that responded to ATP were taken into account

ATP below 10 μM did not alter the holding current at −80 mV in agreement with the results of Friel and Bean (1988), who reported a sustained inward current in bullfrog atrial cells only with ATP concentrations over 10 μM.

Besides the variations in amplitude of I_{Ca}, ATP induced little change in I_{Ca} kinetics. The inactivation of the Ca currents during the voltage pulse at 0 mV (inset of Fig. 1) could be fitted by the sum of two exponentials (see Argibay et al. 1988). The two time constants were not significantly changed in the presence of ATP as compared to control values. The two time constants of I_{Ca} inactivation were 11.6 ± 1.8 ms and 33.2 ± 4.0 ms in control, and 10.8 ± 2.3 ms and 31.4 ± 3.5 ms in the presence of ATP (1 μM; $n = 9$, traces from five cells).

Figure 3 shows the effects of 1 μM ATP on the *I-V* relationships and on the inactivation and reactivation of I_{Ca}. ATP did not alter the shape of the I_{Ca}-*V* relationships nor did it alter the steady-state current measured at 200 ms (Fig. 3A). ATP either did not change the half-inactivation value or shifted slightly it towards less negative values (Fig. 3B). In some experiments a slight reduction in the current available after prepulse depolarization over +20 mV was seen particularly with 100 μM ATP. Reactivation was unchanged in the presence of ATP (Fig. 3C) except that when an overshoot was observed, it was suppressed by ATP. It should be noticed that while all the effects of externally applied ATP were reversible, the recovery of the overshoot in the reactivation curve was never seen, in agreement with the fact that anyway it disappeared with time during the experiment.

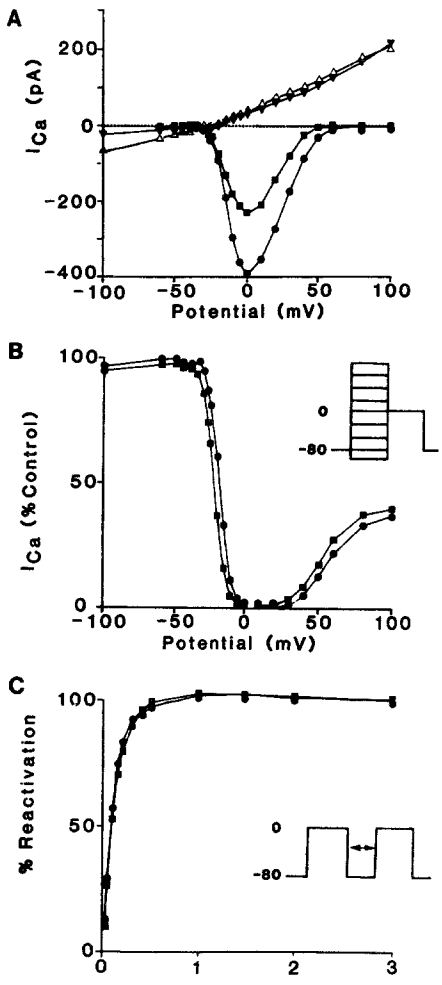


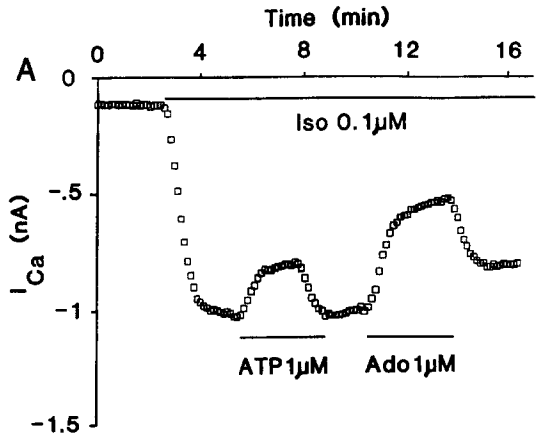
Fig. 3A–C. Kinetics of the Ca current after P_2-purinergic stimulation by 1 μM ATP. **A** The current/voltage relationships are shown for the peak Ca current (■, ●) and the current at the end of the 200-ms pulse (△, ▼). **B, C** Availability and reactivation curves were established by two types of voltage protocols as indicated in the insets (see also Materials and methods). The delay between the two pulses in **B** was 3 ms. These three relations were recorded after at least 5 min in the presence of ATP on the same cell. (■, △) control; (●, ▼) ATP 1 μM

Antagonistic effects of ATP on the β-adrenergic stimulation

On a frog ventricular cell after I_{Ca} had been increased tenfold by 0.1 μM isoprenaline, the addition of 1 μM ATP induced a 25% reduction in peak I_{Ca}. After recovery on switching back to the isoprenaline-containing solution, the subsequent addition of 1 μM adenosine reduced peak I_{Ca} by 50% (Fig. 4A). The lesser antagonistic effect of ATP on β-adrenergic-stimulated I_{Ca} compared to that of adenosine could be related to the fact that ATP is less effective than adenosine on the P_1-purinergic stimulation. It could also be related to the fact that ATP simultaneously activates the P_2-purinergic pathway and thus may increase I_{Ca} more than adenosine. This interpretation is supported by the following observation. On a cell that was stimulated by isoprenaline at a higher concentration (2 μM), ATP (1 μM) increased I_{Ca} slightly further while adenosine (1 μM) decreased it (Fig. 4B). When applied at a higher concentration, ATP (100 μM) reduced β-adrenergic-stimulated I_{Ca}. This could be due to both its P_1-purinergic effect and its as yet unexplained inhibitory effect at this high concentration.

Mechanisms of I_{Ca} increase by ATP

First to check whether or not the increase in I_{Ca} induced by ATP was related to the cAMP pathway, ATP (0.1 μM)



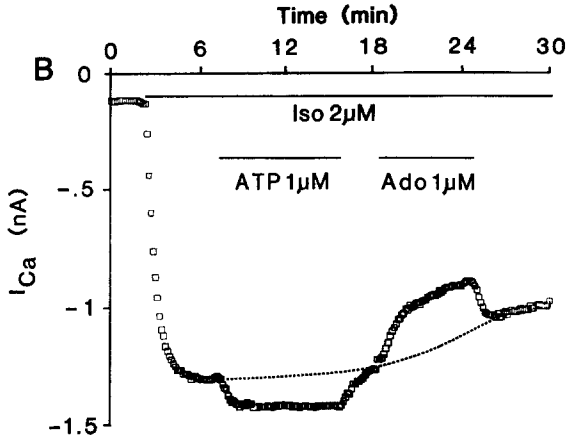


Fig. 4 A, B. Effects of ATP and adenosine (*Ado*) on the Ca current stimulated by isoprenaline (*Iso*). **A** After I_{Ca} was increased tenfold by isoprenaline (0.1 μM), ATP (1 μM) induced a 25% decrease in I_{Ca}. Adenosine (1 μM) added after recovery of I_{Ca} under isoprenaline alone, reduced I_{Ca} by 50%. **B** Effects of ATP and adenosine both at 1 μM on another cell stimulated by a higher concentration of isoprenaline (2 μM). In this case ATP was able to increase I_{Ca} by 10%, while adenosine showed its inhibitory action

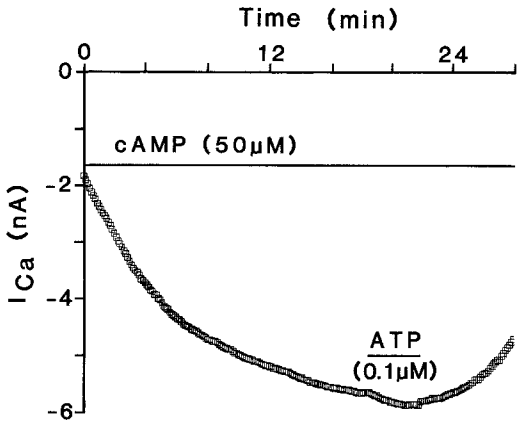


Fig. 5. Time course of the effects of ATP (0.1 μM) on the Ca current maximally stimulated by cAMP (50 μM) added to the pipette solution

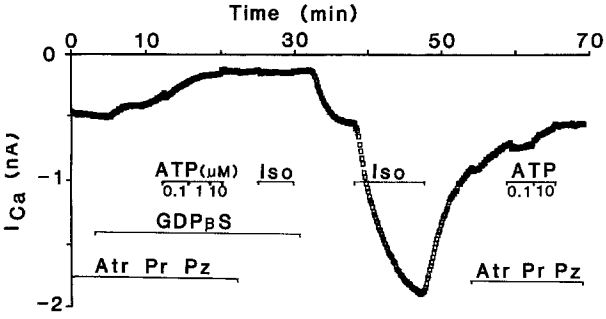


Fig. 6. Inhibition of P_2-purinergic responses by internally applied GDP[β-S]. After the whole-cell recording configuration had been established the pipette solution that contained GTP was exchanged for one that contained GDP[β-S] by internal perfusion of the pipette. This induced a reduction in I_{Ca}. ATP at 0.1, 1 and 10 μM had no effect upon I_{Ca} in the presence of atropine (*Atr*, 10 μM), propranolol (*Pr*, 1 μM) and prazozin (*Pz*, 0.1 μM). Isoprenaline (1 μM) was also ineffective. Switching back to an internal perfusing solution that contained GTP restored peak I_{Ca}. Isoprenaline (1 μM) could then increase I_{Ca} four-fold; ATP at 0.1 μM, and to a lesser extent at 10 μM, also slightly increased I_{Ca}

was applied on a cell previously stimulated by intracellular perfusion with a supramaximal concentration of cAMP (50 μM) added to the pipette solution. A few minutes after breaking the patch I_{Ca} had already reached 2 nA, it levelled off at 6 nA. Applying the low ATP concentration then further increased I_{Ca} (Fig. 5). Similar results were obtained in three other cells.

In view of previous observations on rat heart that P_2-purinergic agonists increased the turnover of phosphoinositides (Legssyer et al. 1988), we checked whether phospholipase C activation was required for ATP to increase I_{Ca} and whether this was mediated through a G-protein. One way to inhibit this coupling, in the absence of specific tools like cholera and pertussis toxins is to use GDP[β-S], a non-degradable form of GDP, which should prevent the dissociation of the trioligomeric G-protein.

In this series of experiments, we used a perfused pipette so that the usual internal solution could be exchanged for one containing GDP[β-S] (500 μM) but not GTP. This solution induced a slow decrease in I_{Ca}. Externally applied ATP had no effect on I_{Ca}; also 1 μM isoprenaline did not increase I_{Ca} (Fig. 6). After return to the GTP-containing internal pipette solution, I_{Ca} recovered its initial amplitude. Isoprenaline then induced a more than fourfold increase in I_{Ca} and, although weak, an increase in I_{Ca} was always observed upon applying ATP. Two other experiments using the same protocol gave similar results. Notice that in some initial experiments, when only GTP was omitted, I_{Ca} was sustained; it could still be increased by isoprenaline but only up to twofold and not at all by ATP.

In other experiments neomycin was added to the intracellular solution in the pipette to inhibit the degradation of polyphosphoinositides by the phospholipase C (Schacht 1976; Vergara et al. 1985). Neomycin (0.3 mM) induced a run down of I_{Ca} as recently reported in clonal pituitary cells in relation with the general properties of aminoglycosides to displace Ca ions (Suarez-Kurtz and

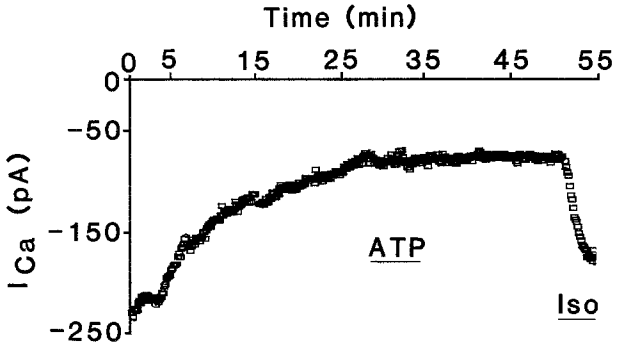


Fig. 7. Inhibition of the agonistic effects of P_2-purinergic stimulation on I_{Ca} by internally applied neomycin. The addition of neomycin at 0.3 mM to the internal solution in the pipette prevented the effects of ATP (0.1 μM) on a cell taken from a dissociation batch that generally showed clear responses to P_2-purinergic stimulations. Neomycin on its own significantly reduced I_{Ca} in a slow time-dependent fashion. It did not prevent isoprenaline (1 μM) from increasing I_{Ca} threefold from its new control value

Reuben 1987). On frog cells, after I_{Ca} had decreased to about half its initial amplitude in the presence of neomycin, ATP at 0.1 μM had no agonist effects (Fig. 7). At the same concentration, on cells from the same batches in the absence of neomycin, ATP increased I_{Ca} to $121.3\% \pm 11.1\%$ ($n = 5$) of control. Although after 50 min in the presence of neomycin I_{Ca} was reduced to one-third of its original amplitude, isoprenaline still proved to be efficient and increased I_{Ca} threefold. Similar observations were obtained in four cells. In three other cells internally perfused with neomycin, 100 μM ATP significantly reduced I_{Ca} to a stable value; these effects were poorly reversible on washing out the ATP.

Discussion

Positive inotropy can be induced by β-adrenergic stimulation, the many intermediate steps of which have been described. Other positive inotropic actions include α_1-adrenergic and P_2-purinergic stimulations. However, the complex, generally triphasic time course of the increase in mechanical activity (in frog: see Flitney and Singh 1980; Niedergerke and Page 1981; in mammal: see Danziger et al. 1988; Legssyer et al. 1988) suggests that several mechanisms are involved. We focused our work on the effects of ATP on the Ca current. The above results show that P_2-purinergic stimulation leads to an increase in I_{Ca} without significant alterations in its kinetics. Such alterations in I_{Ca} were prevented by internal application of neomycin and of GDP[β-S], which are expected to inhibit degradation of the polyphosphoinositides or dissociation of the trioligomeric G-protein respectively.

Adenine nucleotides depress atrial myocardial contractility, probably as a consequence of a shortening of the action potential mediated by an increase in a K current (Hartzell 1979; Belardinelli and Isenberg 1983b). ATP is also known to increase contractility in both amphibian and mammalian heart ventricles (Flitney and Singh 1980; Niedergerke and Page 1981; Legssyer et al. 1988) as well as inducing slow action potential in partially depolarized guinea-pig cardiac cells (Schneider and Sperelakis 1975). Using the double sucrose-gap voltage-clamp method on frog atrial bundles, Goto et al. (1976) demonstrated that ATP at concentrations up to 0.2 mM increased both I_{Ca} and the phasic tension but that at higher concentrations it reduced I_{Ca}. Our results on single cells isolated from the frog ventricle extend their observations. We further show that none of the Ca conductance kinetics are significantly modified by ATP. Besides, ATP markedly reduces the I_{Ca} when it has been previously increased by low doses of isoprenaline. This should be related to the antagonizing effects of P_1-agonists, which are similar to that of adenosine on the stimulatory action of isoprenaline, reported in mammalian ventricular myocytes (Belardinelli and Isenberg 1983a, Linden et al. 1985).

It should be noticed that some cells (up to one-third; generally from the same dissociation batch) showed only the negative effect or a transient positive followed by a negative effect even at ATP concentrations as low as 1 μM. Moreover cells from a few dissociations were not at all sensitive to ATP although they responded to β-adrenoagonists. Buffering free Ca to 0.17 μM or/and increasing GTP to 1 mM in the intracellular pipette solution did not allow ATP to increase I_{Ca} on these cells. The broad range of the quality of cell responses to ATP stimulation, as already noticed by Friel and Bean (1988), could result from technical aspects of either cell preparation or experimental conditions. But most probably these variations can be related to the physiological state of the isolated cells. One set of possibilities relates to protein kinase C. It has been demonstrated in polymorphonuclear leukocytes that activation of the protein kinase C by diacylglycerol leads to attenuation of receptor-stimulated phospholipase C by disruption of the coupling of the activated G-protein to phospholipase C (Smith et al. 1987). It has also been shown that there may be several phosphorylation sites on the Ca channel protein that could be affected by protein kinase C (Nastainczyk et al. 1987) and may have either stimulatory or inhibitory effects. Several other mechanisms of the dual action of protein kinase C activation have been recently reviewed by Nishizuka (1988). Another set of possibilities is that several P_2-purinoceptor subtypes may exist in cardiac tissues and they may have opposite effects. Stimulation of P_2-purinoceptors by various purine agonists mediates either constriction or dilatation in the rabbit mesentery artery (Burnstock and Warland 1987) and, as well, either increases inositol trisphosphate production or inhibits cAMP accumulation in hepatocytes (Okajima et al. 1987). In frog cells this view is supported by the biphasic effect of ATP at low concentration in some cells and its negative effect at high concentration (100 μM) including the response after neomycin treatment or in the presence of cAMP. This negative effect could be similar to the inhibitory modulation of the Ca channel by adenosine agonists reported in neurons (MacDonald et al. 1986) and in guinea pig atrium (Cerbai et al. 1988). Further studies are obviously needed that require the use of well-defined, highly selective antago-

nists to characterize the receptor subtypes and their intracellular second messengers in cardiac tissues. Our attempts with 8-phenyltheophylline and reactive blue 2 were rather unsuccessful.

Stimulation of the P_2-purinoceptors does not involve the cAMP pathway since ATP further increased I_{Ca} after supramaximal β-adrenergic stimulation or in the presence of high internal cAMP (Figs. 4B, 5). The phospholipase C and the guanine regulatory binding protein seem to be required intermediates in the agonist effect of ATP since GDP[β-S] and neomycin are able to prevent an increase in I_{Ca} during P_2-purinoceptor stimulation (Figs. 6, 7). The evidence for a role of a regulatory GTP-binding protein in phosphoinositide breakdown has been established in many tissues (Bockaert et al. 1987; Gilman 1987). Unfortunately in heart, the inhibition by pertussis toxin of the G-protein involved in α_1-adrenergic transduction is controversial (Schmitz et al. 1987a; Steinberg et al. 1987), and no results are available for P_2-purinergic transduction. This leaves us with few tools to investigate this pathway besides its inhibition by GDP[β-S] (Eckstein et al. 1979; Hescheler et al. 1987). Neomycin is known to inhibit phospholipase C degradation of polyphosphoinositides (Schacht 1976) and when applied internally in skeletal muscle fibres was shown to inhibit mechanical activity as well as inositol trisphosphate production (Vergara et al. 1985). In our experiments neomycin induces a rundown in I_{Ca} (see also Suarez-Kurtz and Reuben 1987); however, this current remains sensitive to β-adrenergic stimulation. In the presence of GDP[β-S] or neomycin, ATP was unable to increase I_{Ca}. Thus during P_2-purinergic stimulation, the activation of phospholipase C could be a necessary step following the activation of a G-protein. The activation of the phospholipase C leads to two products, inositol trisphosphate and diacylglycerol, the effects of which are still poorly understood in cardiac tissues but which may account for the complexity and variability of the present and previous results. These observations are in line with the increase in I_{Ca} induced by phorbol esters (Dösemeci et al. 1988; Lacerda et al. 1988) and suggest that following P_2-purinergic stimulation, activation of protein kinase C is an intermediate step leading to I_{Ca} increase.

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