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MECHANISM OF EXTRACELLULAR ATP-INDUCED INCREASE OF CYTOSOLIC Ca^{2+} CONCENTRATION IN ISOLATED RAT VENTRICULAR MYOCYTES

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SUMMARY

1. Changes in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of isolated rat ventricular myocytes in suspension were measured in response to extracellular ATP using the fluorescent Ca^{2+} indicators Quin-2 and Fura-2.

2. ATP produced a concentration-, time- and Mg^{2+}-dependent, biphasic increase of $[Ca^{2+}]_i$ whereas slowly hydrolysable ATP analogues produced a slow, monophasic increase of $[Ca^{2+}]_i$ and the non-hydrolysable ATP analogues were without effect.

3. Extracellular Ca^{2+} was required for the ATP-induced increase of $[Ca^{2+}]_i$ and pre-treatment of the cells with caffeine, ryanodine, verapamil or nimodipine partially inhibited the $[Ca^{2+}]_i$ increase.

4. Whole-cell patch-clamp experiments revealed that ATP activated an ionic current that had a linear current–voltage relationship with a reversal potential near 0 mV. Quinidine, a putative P_2 purinergic receptor blocker, abolished the ATP-activated current. The ATP-activated current was Mg^{2+} dependent.

5. Associated with the ATP-activated current was cellular depolarization. In a physiological solution, ATP depolarized cells to the threshold for the firing of action potentials. In the presence of the voltage-activated ion channel blockers tetrodotoxin, 4-aminopyridine, caesium and nitrendipine, ATP depolarized cells to -44 ± 6 mV from a resting potential of -66 ± 4 mV ($n = 11$).

6. Sodium dodecyl sulphate (SDS) polyacrylamine gel electrophoresis and autoradiography demonstrated that extracellular ATP stimulated the phosphorylation of several extracellular membrane-bound proteins. The phosphorylation of these proteins was concentration, time and Mg^{2+} dependent. Pre-treatment of cells with the slowly hydrolysable ATP analogues inhibited the ATP-induced phosphorylation. Adenosine 5′-*O*-3-thiotriphosphate (ATPγS) thiophosphorylated proteins with the same apparent molecular weight as the proteins phosphorylated by ATP.

7. These results suggest that the ATP-induced increase of $[Ca^{2+}]_i$ is a result of the activation, possibly by protein phosphorylation, of a novel ion channel carrying

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inward current. The ATP-activated channel may be permeable to Na^+ and Ca^{2+} and causes $[Ca^{2+}]_i$ to rise. More importantly, this inward current depolarizes the cell to the threshold of inducing spontaneous firing of action potentials. The firing of action potentials results in the influx of Ca^{2+} through L-type Ca^{2+} channels which would trigger Ca^{2+} release from the sarcoplasmic reticulum and lead to the increase in $[Ca^{2+}]_i$.

INTRODUCTION

There is increasing evidence that ATP can serve as a potent extracellular signalling molecule (Burnstock, 1978, 1981). Several cell types, such as neurones, platelets and adrenomedullary cells, store ATP in secretory vesicles and release it through an exocytotic mechanism (Gordon, 1986). In addition, ATP is co-stored in synaptic vesicles with noradrenaline and acetylcholine (Silinsky, 1975; Johnson, 1988). The close association between neuronal activity and ATP secretion indicates that extracellular ATP may serve as a signal to target cells. In various cells, ATP stimulates ion fluxes which are related to physiological functions (Kimmich & Randles, 1982; Charest, Blackmore & Exton, 1986; Lückhoff & Busse, 1986; McMillian, Soltoff, Lechleiter, Cantley & Talamo, 1988). In smooth and cardiac muscle cells. ATP increases the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) (Sharma & Sheu, 1986; Phaneuf, Berta, Casanova & Cavadore, 1987; Tawada, Furukawa & Shigekawa, 1987; De Young & Scarpa, 1987, 1989; Danziger, Raffaeli, Moreno-Sanchez, Sakai, Capogrossi, Spurgeon & Lakatta, 1988; Björnsson, Monck & Williamson, 1989) and enhances contraction (Niedergerke & Page, 1981; Burnstock & Kennedy, 1985; Leggsyer, Poggioli, Renard & Vassort, 1988).

The sources of Ca^{2+} for the ATP-induced $[Ca^{2+}]_i$ increase have been attributed to trans-sarcolemmal influx of Ca^{2+} (Danziger *et al.* 1988) or a combination of trans-sarcolemmal influx of Ca^{2+} and release of Ca^{2+} from the sarcoplasmic reticulum (SR) (De Young & Scarpa, 1989; Björnsson *et al.* 1989). The mechanism of action of how ATP can cause $[Ca^{2+}]_i$ to increase is still not clear. It has been suggested that the $[Ca^{2+}]_i$ increase was due to an ATP-induced membrane depolarization that would lead to Ca^{2+} influx through the L-type Ca^{2+} channels (Danziger *et al.* 1988; De Young & Scarpa, 1989; Björnsson *et al.* 1989). However, there are no electrophysiological data in these studies to support this idea. Recently, in studies in which the voltage-clamp technique was used, an ATP-activated cation-permeable current that reversed near 0 mV has been found in sensory neurones (Krishtal, Marchenko & Pidoplichko, 1983; Bean, 1990; Bean, Williams & Ceelen, 1990), smooth muscle cells (Benham & Tsien, 1987; Nakazawa & Matsuki, 1987; Friel, 1988), atrial cells (Friel & Bean, 1988) and skeletal muscle (Hume & Honig, 1986). An intriguing point as to the mechanism of action of ATP is that the responses of $[Ca^{2+}]_i$ increase are induced only by ATP and its hydrolysable analogues such as ATPγS (Danziger *et al.* 1988; Björnsson *et al.* 1989). This brings up the possibility that protein phosphorylation is involved during the action of ATP.

The goal of this paper was to characterize the effect and determine the mechanism of action of extracellular ATP on $[Ca^{2+}]_i$ in isolated rat ventricular myocytes. Two specific questions were asked: (1) what is the electrophysiological basis for the ATP-induced $[Ca^{2+}]_i$ increase?; (2) is the structural specificity of the ligands related to

phosphorylation activity? To achieve these aims, the fluorescent Ca^{2+} indicators, Quin-2 and Fura-2, were used to monitor $[Ca^{2+}]_i$, whole-cell voltage- and current-clamp techniques were used to measure ionic currents and membrane potential and [γ-^{32}P]ATP and sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis were used to assay protein phosphorylation.

Preliminary reports of some of these results have been previously published in abstract form (Sharma & Sheu, 1986; Christie & Sheu, 1988, 1990).

METHODS

Myocyte isolation

The method for myocyte isolation was modified from previously described procedures (Powell & Twist, 1976; Sheu, Sharma & Banerjee, 1984). The heart and a 5 mm section of the aorta were excised from ether-anaesthetized male rats (225–250 g). The aortic section was immediately mounted on a plastic cannula attached to a perfusion system. Ca^{2+}-free Joklik tissue culture medium (GIBCO) was perfused through the heart at 37 °C for 5 min to cleanse the heart of blood. The perfusion solution was changed to Joklik medium containing 50 μM-$CaCl_2$, 0·5 mg/ml collagenase (Worthington, type II, 200 units/mg) and 0·1 % bovine serum albumin (BSA, Sigma, fraction IV). This enzyme solution was recirculated though the heart for approximately 30 min at which time the heart was removed from the perfusion system. The atria and aorta were separated from the ventricles and discarded. The ventricles were shaken vigorously and filtered through 250 μm nylon mesh so as to dissociate the muscle into single cells. The cells were washed with fresh enzyme-free Joklik medium, which contained 1 mM-$CaCl_2$ and 1 % BSA, and incubated for 1 h.

$[Ca^{2+}]_i$ *measurements*

Myocytes were loaded with the fluorescent Ca^{2+} indicators, Quin-2 or Fura-2, as described previously (Tsien, Pozzan & Rink, 1982; Sheu *et al.* 1984; Grynkiewicz, Poenie & Tsien, 1985). Briefly, cells were incubated with 50 μM-Quin-2 AM or 3 μM-Fura-2 AM (Molecular Probes, Eugene, OR, USA) in a modified Krebs–Henseleit solution containing (in mM): NaCl, 140; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 2; glucose, 10; *N*-2-hydroxyethylene piperazine-*N*′-2-ethanesulphonic acid (HEPES), 10; adjusted to pH 7·4 with NaOH and oxygenated with 100 % O_2. Dye-loaded cells were washed several times, centrifuged at 37 *g* for 30 s, and resuspended in fresh modified Krebs–Henseleit solution. Only cell suspensions consisting of > 80 % rod-shaped cells were used for experiments.

For fluorescent measurements, aliquots of dye-loaded cell suspensions in quartz cuvettes were placed in a Perkin–Elmer LS-5 spectrofluorimeter equipped with a magnetic stirrer and a thermostated cuvette holder. The cells were continuously stirred at 37 °C. Monochromator wavelength settings were 339 nm for excitation and 490 nm (Quin-2 experiments) or 510 nm (Fura-2 experiments) for emission. Testing agents were added directly into the cuvette at minimal volumes of 100 × concentration stock solutions.

To calculate the free intracellular Ca^{2+} concentration, the following equations were used (Cobbold & Rink, 1987):

$$[Ca^{2+}]_i = K_d[(F-F_{min})/(F_{max}-F)],$$

and

$$F_{min} = F_{Mn} + \tfrac{1}{6}(F_{max}-F_{min}),$$

where F_{max} represents the maximal fluorescence signal obtained by lysis of the cell suspensions by either 5 μM-ionomycin or 70 μM-digitonin in the presence of 20 mM-$CaCl_2$, F_{Mn} represents the fluorescence signal obtained after adding 20 mM-$MnCl_2$ and F_{min} represents the minimal fluorescence signal obtained after correcting for the decrease of autofluorescence produced by $MnCl_2$. The dissociation constants (K_d) were 115 nM for Quin-2 and 225 nM for Fura-2 (Tsien *et al.* 1982; Grynkiewicz *et al.* 1985).

Whole-cell current recordings

Whole-cell membrane currents were measured using standard patch-clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Patch electrodes with resistances of 2–5 MΩ were fabricated from Clay Adams Accu-fill 90 glass micropipettes. Series resistance was compensated

to provide the fastest capacity transient without oscillation. Currents were recorded using a List EPC-7 patch-clamp amplifier (Medical Systems Corp., Great Neck, N.Y, USA). Pulse generation, data acquisition, analysis and subtractions of linear leak currents were done using pCLAMP software (Axon Instruments, Burlingame, CA, USA). After digitization, current signals were stored on an IBM PC.

Myocytes were transferred to a tissue chamber with 2 ml of BSA-free solution containing (in mM): NaCl, 140; KCl, 4; $CaCl_2$, 1; $MgCl_2$,2; glucose, 10; HEPES, 10; with pH adjusted to 7·4 with NaOH. For recording L-type Ca^{2+} currents, Na^{2+} currents were eliminated by adding 5 μM-tetrodotoxin and by holding the cell at -45 or -40 mV. Patch electrodes were filled with a solution designed to eliminate K^+ currents. The composition was (in mM): CsCl, 136; $CaCl_2$, 2·5; EGTA, 7·5; $MgCl_2$, 1; phosphocreatine, 5; HEPES, 10; with pH adjusted to 7·2 with CsOH. The free Ca^{2+} concentration was calculated to be 100 nM according to the computer program of Fabiato (1988). This internal solution was also used to measure the ATP-activated current. For recording ATP-induced depolarization, KCl was used in lieu of CsCl. ATP was applied to the extracellular bathing solution from a 30 ml syringe through a broken pipette placed close to the cell. As indicated in Figs 3 and 5, the ATP-containing solution reached the cell in 1–3 s and would be expected to surround the cell within a few seconds. All electrophysiological measurements were carried out at room temperature.

Protein phosphorylation measurement in intact cells

Dissociated myocytes were centrifuged and resuspended at a concentration of 10^6 cells/ml. Approximately 10^5 cells were transferred to polyethylene microfuge tubes to which the appropriate amount of labelled ATP ([γ-^{32}P]ATP or [α-^{32}P]ATP, 3000 Ci/mmol, 1–250 μCi; [γ-^{35}S]ATP, 650 Ci/mmol, 50–500 μCi was added). Unlabelled ATP was added to initiate phosphorylation and increase ATP to the desired total concentration. Agents thought to modify the ATP-induced phosphorylation were added to cells either before or after the addition of labelled and unlabelled ATP. In some experiments, extracellular Mg^{2+} was omitted from the extracellular solution and 100–500 μM-EDTA was added to chelate residual Mg^{2+}.

After the desired length of time (1–600 s), the reaction was terminated by addition of SDS stop buffer so that the final volume of each microfuge tube was 200 μl. ATP was never washed out of the reaction mixture before addition of stop buffer. Dithiothreitol was added to each tube and the tubes were boiled for 10 min. The solubilized proteins (20–50 μg) were separated according to size by SDS polyacrylamide gel electrophoresis (SDS PAGE, 7·5, 10 or 15%) according to the basic design of Laemmli (1970) as modified by Studier (1973). The analysis of ^{32}P distribution on the gels was carried out by autoradiography of the dried gels on X-ray film (X-OMAT RP, Kodak, Rochester, NY, USA) followed by densitometric scanning. To improve the efficiency of detecting β-emissions from the weak β-emitter, ^{35}S, EN^{3}HANCE (New England Nuclear, Boston, MA, USA) was used. Protein quantification was carried out according to the method of Bradford (1976) using BSA as a standard.

The values given in the text are the means ± standard error of the mean.

RESULTS

ATP-induced $[Ca^{2+}]_i$ changes

Figure 1*A* shows a representative experiment where, in the presence of 1 mM-external Ca^{2+}, 50 μM-ATP increased $[Ca^{2+}]_i$ from a resting level of 169 nM to a maximum level of 430 nM within 30 s. The $[Ca^{2+}]_i$ slowly decreased over the next several minutes to a new steady level of 195 nM. This response showed the characteristics of desensitization because a second application of equimolar ATP on top of the existing 50 μM-ATP did not produce a larger change in $[Ca^{2+}]_i$. In some cases there was no change in $[Ca^{2+}]_i$ with the second addition of equimolar ATP (records not shown). Because of the diminished response to multiple applications of ATP, data presented were from cells exposed to ATP once. Cell viability was not decreased by the application of ATP and remained constant ($> 80\%$) for the duration of the experiments.

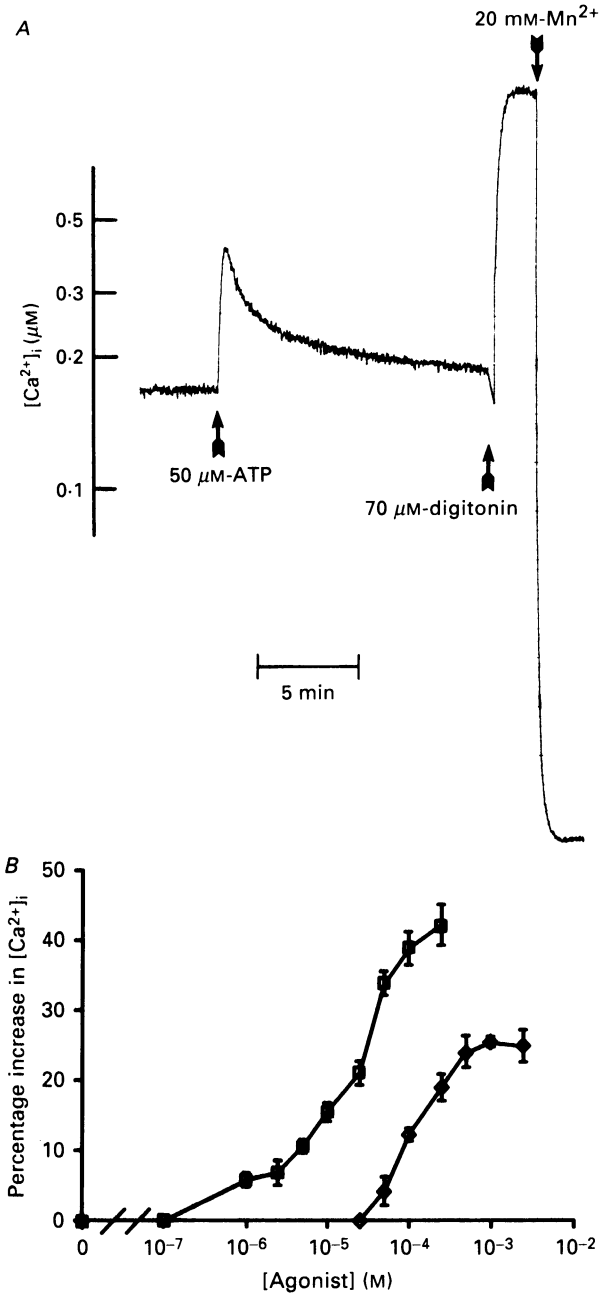

Fig. 1. *A*, change of $[Ca^{2+}]_i$ induced by 50 μM-ATP. Digitionin (70 μM) and Mn^{2+} (20 mM) were added subsequently to obtain maximum and minimum fluorescence, respectively. *B*, concentration–response curves for the increase of $[Ca^{2+}]_i$ produced by ATP (□) and AMP-CPP (◇) $n \geqslant 5$ for each point).

In contrast to the $[Ca^{2+}]_i$ response to ATP, the slowly hydrolysable ATP analogues adenosine 5′-(α,β-methylene)triphosphate (AMP-CPP) and adenosine 5′-(γ-thio)-triphosphate (ATPγS) increased $[Ca^{2+}]_i$ monophasically. In the presence of AMP-

CPP or ATPγS, the ATP-induced response was also desensitized since subsequent addition of 1–250 μM-ATP produced no change in $[Ca^{2+}]_i$. The non-hydrolysable ATP analogues adenosine 5′-(β, γ-methylene)triphosphate (AMP-PCP) and adenosine 5′-(β-γ-imido)triphosphate (AMP-PNP) had no effect on $[Ca^{2+}]_i$ at concentrations up to 1 mM and did not prevent ATP from increasing $[Ca^{2+}]_i$.

Figure 1*B* summarizes the $[Ca^{2+}]_i$ responses to ATP and AMP-CPP. The threshold for the ATP response was 1 μM (6±1% increase, $n = 5$). Higher concentrations of ATP produced larger increases of $[Ca^{2+}]_i$ until maximal responses were seen at 100 μM-ATP (42±3% increase, $n = 9$). The concentration–response curve for AMP-CPP was shifted to the right with a threshold concentration of 50 μM (5±2% increase, $n = 10$). The maximal response seen with 2·5 mM-AMP-CPP (24±3% increase, $n = 5$) was 60% of the maximal response to ATP.

In a further attempt to characterize the effect of ATP on $[Ca^{2+}]_i$, metabolites of ATP and other nucleotide triphosphates were tested for their effect on $[Ca^{2+}]_i$. At concentrations up to 2·5 mM, AMP and ADP had no significant effect on $[Ca^{2+}]_i$ whereas adenosine (250 μM) decreased $[Ca^{2+}]_i$ by 12±3% ($n = 4$). UTP, CTP and GTP had no effect on $[Ca^{2+}]_i$ at concentrations up to 1 mM.

The effect of ATP on $[Ca^{2+}]_i$ was dependent upon extracellular Mg^{2+} as summarized in Fig. 2. When Mg^{2+} was omitted from the extracellular solution (nominally Mg^{2+}-free solution) the concentration–response curve was shifted to the right and the maximal effect seen at 0·5–1 mM-ATP was less than the maximal response to ATP in the presence of 2 mM-$MgCl_2$ (34±4 *vs.* 42±3%, $n = 9$). When 100 μM-EDTA was included in the nominally Mg^{2+}-free solution, the maximal effect of ATP on $[Ca^{2+}]_i$ was reduced to 50% of the maximal effect of ATP in the presence of 2 mM-$MgCl_2$ (21±3%, $n = 6$). The resting $[Ca^{2+}]_i$ in the absence of Mg^{2+} was not significantly different from the resting $[Ca^{2+}]_i$ in the presence of 2 mM-$MgCl_2$. Resting $[Ca^{2+}]_i$ was 155±8 nM ($n = 126$) in control solution, 152±9 nM ($n = 21$) in a nominally Mg^{2+}-free solution and 168±14 nM ($n = 23$) in a nominally Mg^{2+}-free solution containing 100 μM-EDTA.

There were three possible mechanisms that could account for the ATP-induced $[Ca^{2+}]_i$ increase: (1) influx of Ca^{2+} from the extracellular solution; (2) release of Ca^{2+} from intracellular stores, such as the sarcoplasmic reticulum (SR); and (3) a combination of Ca^{2+} influx and Ca^{2+} release. When cells were bathed in a nominally Ca^{2+}-free extracellular solution, ATP did not produce any change in $[Ca^{2+}]_i$. This result indicated that extracellular Ca^{2+} was essential for the ATP-induced $[Ca^{2+}]_i$ increase. Consistent with this idea, when cells were pre-treated with L-type Ca^{2+} channel blockers, nimodipine (1 μM) or verapamil (10 μM), ATP (50 μM) increased $[Ca^{2+}]_i$ only by 6±2% ($n = 7$) and 14±3% ($n = 6$), respectively, above the resting level. Addition of 20 mM-caffeine to release Ca^{2+} from the SR caused a reduction of the increase in $[Ca^{2+}]_i$ produced by ATP (50 μM) to 21±5% ($n = 5$). Because it has been reported previously that caffeine inhibits Ca^{2+} current, ryanodine (10 μM) was also used to abolish Ca^{2+} release from the SR. Under this condition, ATP also produced a smaller increase in $[Ca^{2+}]_i$. These results are consistent with previously published results and substantiate the finding that the source of Ca^{2+} for the ATP-induced $[Ca^{2+}]_i$ increase is a combination of Ca^{2+} entry and Ca^{2+} release from the SR (Danzinger *et al.* 1988; De Young & Scarpa, 1989; Björnsson *et al.* 1989).

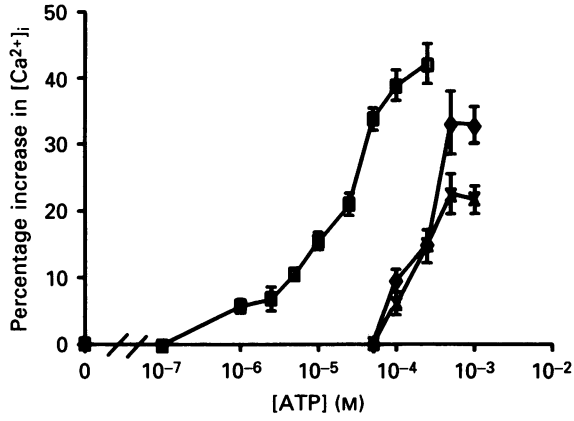

Fig. 2. Concentration–response curves for the effect of Mg^{2+} removal on the ability of ATP to increase $[Ca^{2+}]_i$ ($n \geqslant 5$ for each point): Mg^{2+}-containing solution (□); nominally Mg^{2+}-free solution (◇); nominally Mg^{2+}-free solution with 100 μM-EDTA (×)

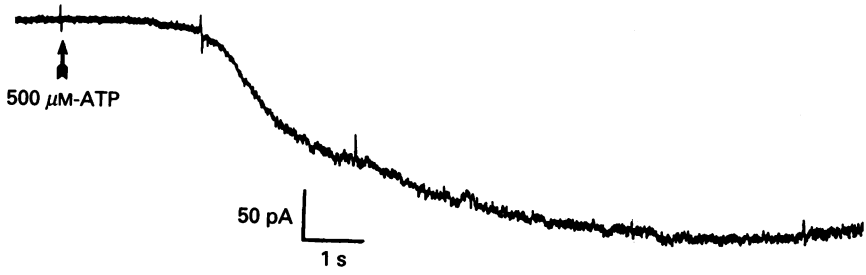

Fig. 3. Current activated by 500 μM-ATP. The cell was held at a holding potential of −70 mV and the current was continuously recorded. Bath solution contained 5 μM-tetrodotoxin, 2 mM-4-aminopyridine, 10 mM-caesium and 1 μM-nitrendipine. Pipette solution contained 136 mM-caesium. The delay of a few seconds in the response was due to the perfusing time for ATP to reach the cell.

Electrophysiological effects of extracellular ATP

The observation that extracellular Ca^{2+} was essential for the ATP-induced $[Ca^{2+}]_i$ increase suggested the presence of an ATP-activated current. To identify this current, cells were held at a constant holding potential ($V_H = -70$ mV) and the current was continuously recorded. Figure 3 is a representative experiment demonstrating the ability of 500 μM-ATP to produce an inward current. The cell was bathed in external solution containing 5 μM-tetrodotoxin, 2 mM-4-aminopyridine, 1 μM-nitrendipine and 10 mM-Cs^+ to block voltage-activated ion channels. The intracellular solution contained 136 mM-Cs^+ in lieu of K^+. Soon after the application of ATP, an inward current was elicited which had a peak current amplitude of 226 pA. The average peak amplitude of inward current was 221 ± 125 pA ($n = 24$) with a range of 80–554 pA. The current recovered 67 ± 20% within 3 min after the application of ATP. Pre-treatment of cells with quinidine (5–20 μM), a putative P_2-receptor blocker

(Burnstock, 1978), inhibited the activation of the ATP-induced current ($n = 5$). In addition, the current was dependent upon extracellular Mg^{2+}. In a nominally Mg^{2+}-free solution, only one of six cells responded to 100 μM-ATP with an inward current (peak current = 63 pA) and three cells responded to 1 mM-ATP with a peak inward

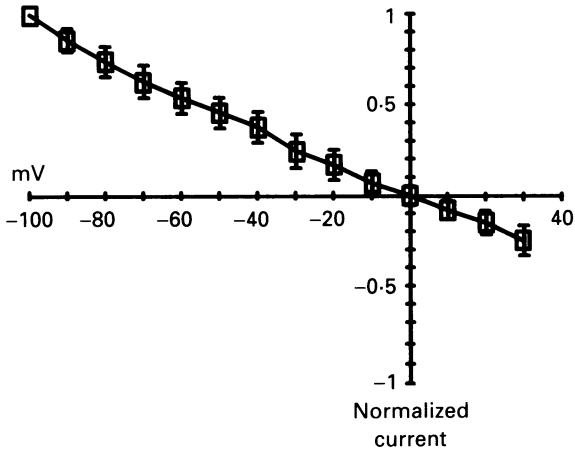

Fig. 4. Current–voltage relationship for the ATP-activated current. ATP-activated currents were measured by computer subtraction of pre- and post-ATP ramp currents (−100 to +30 mV). Each point is normalized to the maximum current measured at a holding potential of −100 mV ($n = 12$).

current of 143 ± 28 pA. These currents had a similar time course as currents in the presence of Mg^{2+} and recovered to a similar extent (51 ± 17 %).

The *I–V* relationship of the ATP-activated current is shown in Fig. 4. Cells were depolarized with a ramp protocol from −100 to +30 mV over 5 s. The *I–V* relationship was generated by computer subtraction of ramp currents before and during the application of ATP. The current of each cell, at 10 mV intervals, was normalized to the current at −100 mV which was the voltage where the inward current was the largest during the ramp protocol. The *I–V* relationship was linear and had a reversal potential of −1·9 ± 6·5 mV ($n = 12$).

The ATP-induced current was hypothesized to be accompanied by depolarization of the cell. To investigate the effect of extracellular ATP on membrane potential, the current-clamp mode of the patch-clamp technique was employed. When cells were bathed in an extracellular solution devoid of ion channel blockers, ATP depolarized cells to the threshold for the firing of action potentials (Fig. 5*A*). Upon application of ATP, the cell depolarized from a resting membrane potential of −68 mV to −49 mV, at which time the cell fired several action potentials. The first-appeared action potential usually had a fast spike, indicative of a Na^{2+}-dependent action potential. The firing of multiple slow action potentials followed ($dV_{max}/dt = 2{\cdot}85 \pm 0{\cdot}78$ V/s, $n = 62$). The firing of multiple slow action potentials was observed in sixteen of eighteen cells that depolarized from a resting membrane potential of −70 ± 5 mV in response to 10–500 μM-ATP. After firing of multiple slow action potentials, the cells became quiescent and stayed at a depolarized membrane

potential around −50 mV. Cells exposed to 1 mM-AMP-PNP or AMP-PCP did not depolarize but did depolarize and fire action potentials upon subsequent application of 100 μM-ATP ($n = 6$). When tetrodotoxin, 4-aminopyridine, Cs^+ and nitrendipine were added to the bathing solution, ATP caused depolarization without eliciting

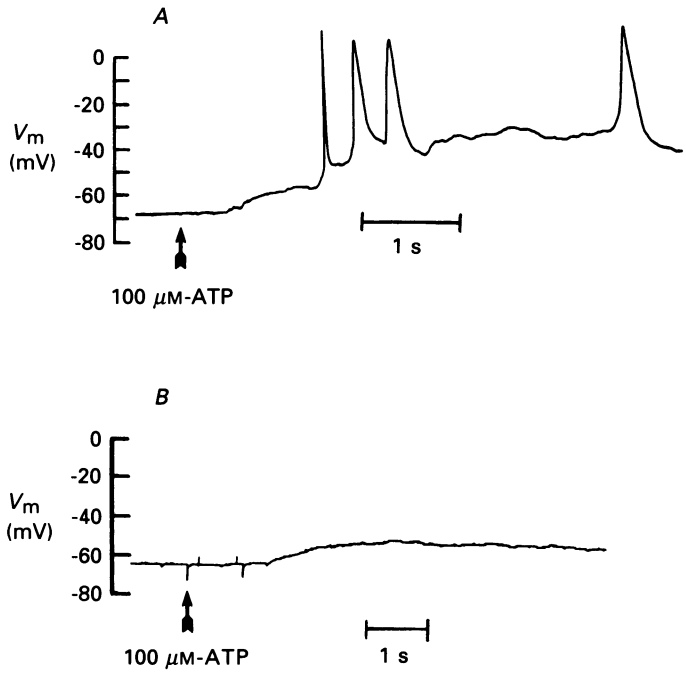

Fig. 5. Effect of 100 μM-ATP on membrane potential. *A*, bathing solution was devoid of voltage-activated ion channel blockers. Resting membrane potential was −68 mV before addition of ATP. *B*, bathing solution contained 5 μM-tetrodotoxin, 2 mM-4-amino-pyridine, 10 mM-caesium and 1 μM-nitrendipine. Resting membrane potential was −65 mV before addition of ATP.

action potentials. Figure 5*B* shows a representative cell which had a resting membrane potential of −65 mV and depolarized to −51 mV upon application of 100 μM-ATP. Under these conditions, cells depolarized 19±7 mV from a resting membrane potential of −66±4 mV but no action potentials were elicited ($n = 11$). In two cells tested, ATPγS (250 μM) also depolarized cells similar to the actions of ATP except with a slower time course.

ATP-induced protein phosphorylation

The data demonstrate that the ATP-induced increases in $[Ca^{2+}]_i$ and current are Mg^{2+} dependent. In addition, these responses require the presence of a hydrolysable terminal phosphate group on the polyphosphate chain of ATP. These observations suggest that ATP hydrolysis may be involved in the initiation of the cellular response to ATP. Therefore, experiments were designed to characterize the phosphorylation events induced by extracellular ATP.

Cells that were exposed to [γ-^{32}P]ATP and stimulated with unlabelled ATP displayed concentration-dependent phosphorylation of several proteins as seen on the autoradiogram in Fig. 6. Two proteins of apparent molecular masses 32 and 47 kDa were phosphorylated with a threshold of 1 μM and maximum intensity of

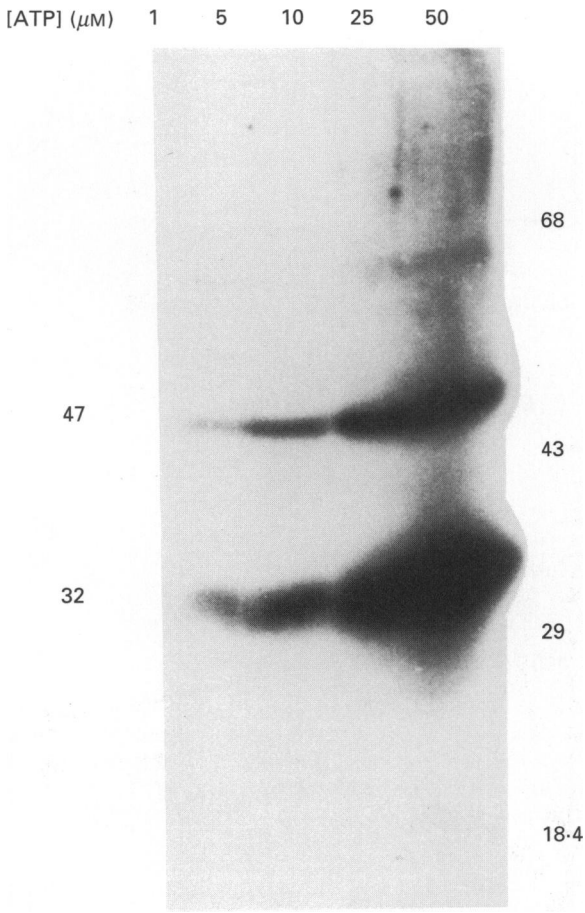

Fig. 6. Autoradiogram demonstrating the concentration–response effect of extracellular ATP-induced phosphorylation of intact myocytes. Numbers on the left indicate the apparent molecular weights of the phosphorylated proteins. Numbers on the right indicate the molecular weights of protein standards.

phosphorylation was seen at 100 μM-ATP. A third protein of apparent molecular mass 65 kDa was lightly phosphorylated at 25–100 μM of ATP. The phosphorylations were time dependent as seen in the autoradiogram in Fig. 7*A*. This experiment was one of five done in the absence of phosphatase inhibitors; seven additional experiments were done in the presence of 25 mM-NaF to inhibit phosphatases. Phosphorylation of the 32 kDa protein by 25 μM-ATP was evident at 5 s, increased to a maximum at 1 min and dephosphorylated. In contrast, the 47 kDa protein was phosphorylated less extensively, reached a maximum after the 32 kDa protein, and

did not dephosphorylate appreciably. In a separate experiment, the autoradiogram was quantified by densitometric scanning and plotted in Fig. 7*B*. The 32 kDa protein phosphorylated significantly faster and to a greater extent than the 47 kDa protein. The 47 kDa protein dephosphorylated only 14% from its maximum whereas the 32 kDa protein dephosphorylated 36% from its maximum at 300 s.

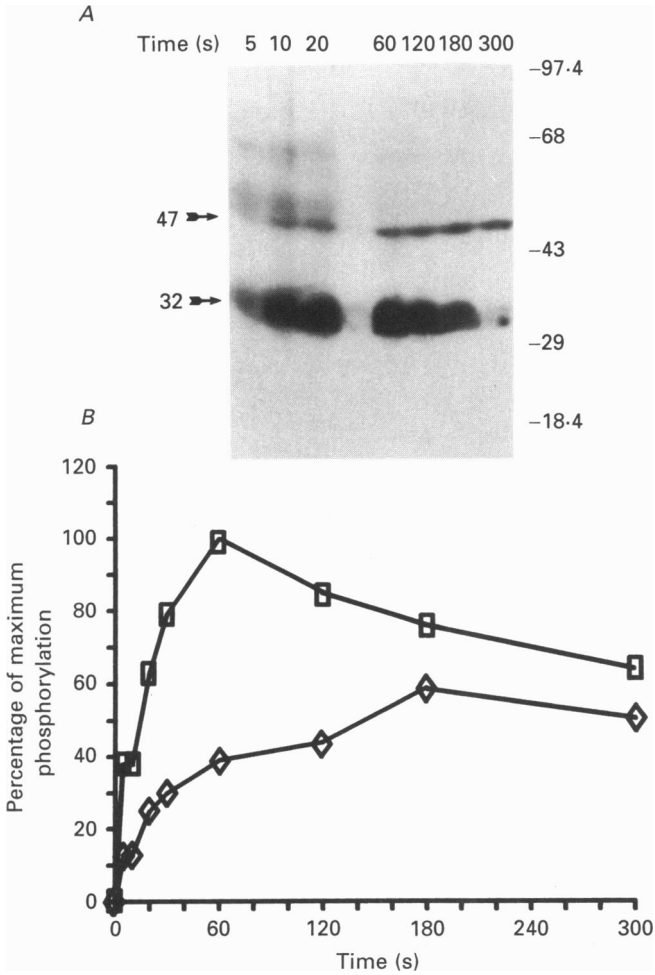

Fig. 7. *A*, autoradiogram demonstrating the time dependence of 25 μM-ATP-induced extracellular phosphorylation. *B*, graphic representation of time course of phosphorylation: 32 kDa protein (□); 47 kDa protein (◇). Phosphorylation was quantified by densitometric scanning and normalized to the maximum intensity (100%) of phosphorylation of the 32 kDa protein at 60 s.

ATP was needed to stimulate the reaction since addition of only [γ-^{32}P]ATP (10–50 μCi; 1–33 pM) did not stimulate phosphorylation. The kinase involved in the phosphorylation event was utilizing the γ-phosphate of ATP since only [γ-^{32}P]ATP

elicited the transfer of ^{32}P and not [α-^{32}P]ATP (25–50 μCi; $n = 3$). The phosphorylation was not inhibited by quinidine at concentrations up to 50 μM ($n = 3$). The kinase was not dependent upon extracellular Ca^{2+}, since removal of nominal Ca^{2+} with 500 μM-EGTA did not affect phosphorylation ($n = 3$). H7 (10 μM), a protein

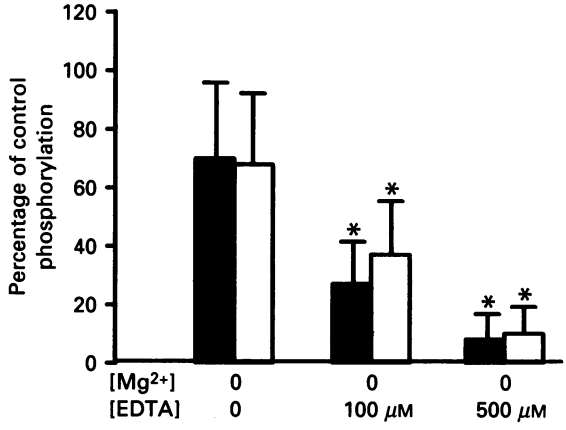

Fig. 8. Summary bar-graph of the effect of Mg^{2+} removal on the intensity of the protein phosphorylation induced by 100 μM-ATP: 32 kDa protein (■); 47 kDa protein (□). Phosphorylation was quantified by densitometric scanning and normalized to the intensity of phosphorylation of each protein in the presence of 1 mM-Mg^{2+} ($n = 5$). * Indicates statistical significance with P value < 0·05.

kinase C and cyclic nucleotide-dependent protein kinase inhibitor, did not inhibit phosphorylation ($n = 4$). In addition, phosphorylation was not affected by 10 μM-cyclic AMP ($n = 3$), 10 μM-verapamil ($n = 3$) or 500 μM-chlorpromazine ($n = 3$). Pre-treatment of cells, in the presence of 25 mM-NaF, with 0·1 % trypsin did not alter the ATP-induced phosphorylation. However, exposure of cells to trypsin after initiation of phosphorylation reduced phosphorylation of the 32 and 47 kDa proteins by 53 ± 6 and 52 ± 3 %, respectively ($n = 3$).

The kinase that was catalysing the transfer of ^{32}P to the 32 and 47 kDa proteins was Mg^{2+} dependent. Autoradiograms demonstrating the Mg^{2+} dependence of phosphorylation, induced by 100 μM-ATP, were quantified by densitometric scanning and plotted in Fig. 8. In a nominally Mg^{2+}-free solution, phosphorylation of the 32 and 47 kDa proteins was 70 ± 21 and 68 ± 15 % of control, respectively ($n = 4$). In the presence of 100 μM-EDTA, the 32 and 47 kDa proteins were reduced to 27 ± 6 and 37 ± 11 % of control, respectively, and in the presence of 500 μM-EDTA they were only 8 ± 2 and 10 ± 3 % of control ($n = 4$).

To correlate the ATP-induced phosphorylation with the ATP-induced current and $[Ca^{2+}]_i$ increase, the slowly hydrolysable and non-hydrolysable analogues were tested for their ability to alter the ATP-induced phosphorylation. Compared to the control phosphorylation induced by 50 μM-ATP, pre-treatment with 100 μM of the slowly hydrolysable ATP analogues reduced the intensity of phosphorylation of both the 32 and 47 kDa proteins dramatically. The non-hydrolysable ATP analogues at 1 mM or 100 μM were without significant effect on the intensity or pattern of phosphorylation.

Figure 9 is a summary bar-graph of the effects of 100 μM of the ATP analogues on the phosphorylation intensity induced by 25 μM-ATP ($n = 5$). ATPγS inhibited phosphorylation of the 32 and 47 kDa proteins by 90 ± 4 and 81 ± 8%, respectively, and AMP-CPP inhibited phosphorylation of both proteins by more than 50%. AMP-

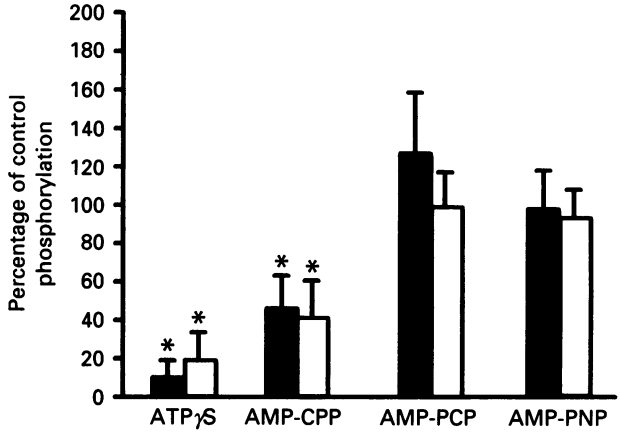

Fig. 9. Summary bar-graph demonstrating the alteration of 25 μM-ATP-induced phosphorylation produced by 2 min of pre-treatment with 100 μM of each of the ATP analogues: 32 kDa protein (■); 47 kDa protein (□). Phosphorylation was quantified by densitometric scanning and normalized to the intensity of phosphorylation in the absence of pre-treatment with the ATP analogues ($n = 4$). * Indicates statistical significance with P value $< 0{\cdot}01$.

PNP and AMP-PCP had no significant effect on either protein. ATPγS was further used to characterize the phosphorylation induced by ATP. If ATPγS and ATP were acting at the same site, then presumably ATPγS should thiophosphorylate the same proteins that were phosphorylated by ATP. Indeed, thiophosphorylation of the 32 and 47 kDa proteins was observed. Similar to the ATP-induced phosphorylation, the 32 kDa protein was thiophosphorylated more intensely than the 47 kDa protein.

DISCUSSION

Several major observations concerning the effects of extracellular ATP on single rat ventricular myocytes are made in this report: (1) ATP increases $[Ca^{2+}]_i$ in a time-, concentration- and Mg^{2+}-dependent manner; (2) ATP phosphorylates several membrane-bound cellular proteins, in a time-, concentration- and Mg^{2+}-dependent manner; (3) ATP activates an ionic conductance which can be manifested as cellular depolarization; (4) cellular depolarization initiates the firing of action potentials which causes influx of Ca^{2+} through L-type Ca^{2+} channels and leads to Ca^{2+}-induced Ca^{2+} release from SR; (5) the main sources of Ca^{2+} responsible for the $[Ca^{2+}]_i$ increase are the extracellular solution and the SR; (6) the presence of a hydrolysable terminal phosphate group on the polyphosphate chain of the adenine nucleotide is an absolute requirement for eliciting any of the aforementioned responses.

Ligand specificity and pharmacology of the ATP responses

Classification of purinergic receptors is based on the ligand specificity for eliciting a response (Burnstock, 1978). P_2-purinoceptors are characterized by their affinity for ATP over that for ADP, AMP and adenosine. Further subclassification of the P_2-purinoceptors is based on the relative responses elicited by structural analogues of ATP. From the responses elicited by ATP, ATP metabolites and ATP analogues, it is inferred that P_2-purinoceptors mediate the responses described in this report. This is also supported by the finding that quinidine inhibited the ATP-activated current. Björnsson *et al.* (1989) have further classified the purinoceptor as belonging to the P_{2Y} subclass which is substantiated by our data.

The effects of ATP metabolites and ATP analogues indicate that both the polyphosphate chain and the purine ring denote specificity. A complex picture emerges as to how the receptor recognizes the ATP molecule. One possibility is that ATP serves two functions: the purine ring serves as the recognition site for the receptor and the polyphosphate chain serves as a phosphate donor through the action of a protein kinase intimately associated with the receptor. Implicit in this scheme, an agonist would have to have the proper purine structure and hydrolysable terminal phosphate group to elicit a cellular response.

Role of ATP-induced protein phosphorylation

The involvement of a protein kinase and ATP hydrolysis in the ATP-induced responses is suggested by the rank order of potency for the ATP analogues (ATP > ATPγS $\geqslant$ AMP-CPP $\gg$ AMP-PNP = AMP-PCP), the Mg^{2+} dependence of the cellular responses, and the discovery of ATP-induced phosphorylated proteins. The action of the slowly hydrolysable ATP analogues is presumably elicited by the same mechanism as ATP since ATPγS thiophosphorylated proteins with the same apparent molecular weight as the proteins phosphorylated by ATP. In addition, pretreatment of cells with either of the slowly hydrolysable analogues inhibited the ability of ATP to phosphorylate proteins suggesting that a common mechanism of action exists between the related compounds.

The time course of the ATP-induced phosphorylation was rapid, reaching approximately 50% of the maximum within 5 s. This is similar to the time course of the ATP-induced $[Ca^{2+}]_i$ seen here and as reported by Björnsson *et al.* (1989). Resolution of phosphorylation within a few seconds was not technically possible due to inadequate exposure of cells to ATP and inability to stop the reaction fast enough.

As would be expected for any phosphorylation event, the ATP-induced phosphorylation was Mg^{2+} dependent. Even in a nominally Mg^{2+}-free solution, phosphorylation induced by a saturating concentration of ATP (50 μM) was reduced at both the 32 and 47 kDa proteins. The presence of any ATP-induced phosphorylation in the absence of Mg^{2+} is probably a result of residual Mg^{2+} in solution and/or the presence of Mg^{2+} tightly associated with the membrane.

The identities of the 32 and 47 kDa proteins are not known. It is possible that either or both of these proteins is involved in the formation of an aqueous pore that allows the influx of ions. As yet, no information exists on the size or identity of the ATP receptor or the ATP channel that has been described in the various tissues.

Electrophysiological effects of extracellular ATP

In the present study, an ATP-activated ionic conductance in rat ventricular myocytes was recorded with the whole-cell patch-clamp technique. A similar observation has been reported in neurones (Krishtal *et al.* 1983; Bean, 1990; Bean *et al.* 1990), embryonic skeletal muscle (Kolb & Wakelam, 1983; Hume & Honig, 1986), mammalian smooth muscle (Nakazawa & Matsuki, 1987; Benham, Bolton, Byrne & Large, 1987; Benham & Tsien, 1987) and frog atrial cells (Friel & Bean, 1988). The ATP-activated conductance described here is similar to those described in other tissues in that it is a novel conductance and not a modification of a known voltage-activated channel. The voltage-activated channels known to exist in rat myocardium were blocked with appropriate blockers when recording the ATP-activated current. The *I–V* relationship (Fig. 4) is linear with a reversal potential near 0 mV. This *I–V* relationship differs from that of neurones and of smooth muscle cells which show inward rectification (Benham & Tsien, 1987; Friel, 1988; Bean *et al.* 1990). The *I–V* relationship is characteristic of a non-selective cation conductance. This is supported by the ATP-activated conductance seen in frog heart (Friel & Bean, 1988), rabbit ear artery (Benham & Tsien, 1987), rat vas deferens smooth muscle (Nakazawa & Matsuki, 1987) and sensory neurones (Krishtal *et al.* 1983; Bean *et al.* 1990) which is carried by Na^+ and Ca^{2+}. The non-selectivity of the conductance can explain the residual $[Ca^{2+}]_i$ increase seen in the presence of Ca^{2+} channel blockers since Ca^{2+} can enter the cell through the ATP-activated channels (Benham, 1989) or through the Na^+–Ca^{2+} exchanger (as a result of increased $[Na^+]_i$).

The slow activation and inactivation of the ATP-activated current is quite different from the classical voltage-activated currents which activate and inactivate on a millisecond time scale. However, slow inactivation is similar to the neurotransmitter receptor-activated currents which activate on a seconds to minutes time scale. The ATP-activated conductance described in neurones has been shown to be directly activated by ATP with activation and inactivation kinetics in the tens to hundreds of milliseconds range. The slow activation kinetics seen here must be interpreted carefully due to slow solution exchange in our system.

This report also shows the direct measurement of an ATP-induced depolarization which can trigger the firing of action potentials. The first action potential fired always has a fast spike, typical of the normal Na^+-dependent action potentials seen in rat myocardium. Subsequent action potentials have a slower rate of rise, typical of Ca^{2+}-dependent action potentials. The lack of a Na^+ spike in the latter action potentials is most likely due to the fact that cells do not repolarize to a potential where the Na^+ channels can recover from inactivation. It is concluded that the Ca^{2+} influx during the plateau phase of the action potentials is the major initiator for the $[Ca^{2+}]_i$ increase which involves Ca^{2+} release from the SR. Finally, two papers have appeared recently which demonstrate an ATP-induced depolarization could trigger the firing of multiple action potentials in rat ventricular cells (Scamps & Vassort, 1990) and an ATP-induced depolarization could trigger the firing of one action potential in guinea-pig atrial cells (Hirano, Abe, Sawanobori & Hiraoka, 1991).

Effects of extracellular ATP on $[Ca^{2+}]_i$

The present report shows that micromolar concentrations of extracellular ATP produce a biphasic increase in $[Ca^{2+}]_i$. The primary source of the Ca^{2+} increase is the extracellular solution which enters the cell primarily through Ca^{2+} channels and

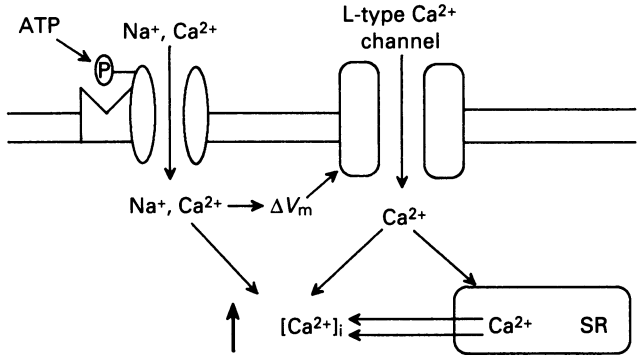

Fig. 10. Proposed scheme for the mechanism by which extracellular ATP increases $[Ca^{2+}]_i$ (see text for explanation). P indicates protein phosphorylation sites.

triggers Ca^{2+} release from the SR. This is consistent with observations made by us and others (Sheu & Sharma, 1986; De Young & Scarpa, 1987, 1989; Danzinger *et al.* 1989; Björnsson *et al.* 1989; Puceat, Scamps, Clement, Cornec & Vassort, 1990). In the presence of Ca^{2+} channel blockers, ATP still produced a small increase in $[Ca^{2+}]_i$. This could be due to (a) residual unblocked Ca^{2+} channels (Lee & Tsien, 1983); (b) depolarization-induced increase in $[Ca^{2+}]_i$ through Na^+–Ca^{2+} exchange (Sheu, Sharma & Uglesity, 1986); (c) Ca^{2+} influx through the ATP-activated conductance (Benham, 1989), and (d) Na^+ influx through the ATP-activated conductance that could lead to an increased $[Ca^{2+}]_i$ via Na^+–Ca^{2+} exchange.

The ATP-induced $[Ca^{2+}]_i$ increase was inhibited by pre-treatment with either ATPγS or AMP-CPP and by removing extracellular Mg^{2+}. These two facts support the possibility that phosphorylation precedes the $[Ca^{2+}]_i$ increase. The differences between the effects of ATP and the slowly hydrolysable analogues on $[Ca^{2+}]_i$ are explained by the affinity of the ligands for the receptor, the relative rates of hydrolysis of the terminal phosphate group on the ligands, and the presence of phosphatases which dephosphorylate the phosphorylated proteins. Since the rate of hydrolysis of ATP is greater than that for ATPγS and AMP-CPP (Welford, Cusack & Hourani, 1987), it was expected that ATP would induce changes faster than either of the slowly hydrolysable analogues. In addition, unlike phosphorylated proteins, thiophosphorylated proteins are impervious to the actions of phosphatases. Taken together, this suggests that ATP-induced changes would occur faster than those induced by either of the analogues and would be terminated faster than the actions of ATPγS. The $[Ca^{2+}]_i$ experiments confirm this prediction since the slowly hydrolysable analogues produced a slow monophasic increase of $[Ca^{2+}]_i$ whereas ATP produced faster changes in $[Ca^{2+}]_i$.

Correlation of responses to extracellular ATP

The three responses elicited by ATP (phosphorylation, electrophysiological, $[Ca^{2+}]_i$) are thought to occur in sequential fashion; phosphorylation creates a conductance pathway which depolarizes the cell and activates Ca^{2+} channels thereby allowing Ca^{2+} entry and the subsequent release of Ca^{2+} from the SR. In support of this hypothesis, manipulations which would decrease or inhibit phosphorylation (pre-treatment with ATPγS or AMP-CPP or Mg^{2+} removal) also decreased or inhibited the electrophysiological and $[Ca^{2+}]_i$ responses. However, the reverse does not hold true since Ca^{2+} removal inhibited the $[Ca^{2+}]_i$ response (by preventing Ca^{2+}-induced Ca^{2+} release) but did not affect phosphorylation or prevent the ATP-activated conductance (Christie & Scarpa, 1991).

The actions of the slowly hydrolysable analogues are thought to work through the same mechanism as ATP. This is supported by the observation that ATPγS thiophosphorylated both the 32 and 47 kDa proteins, activated an ionic conductance, and increased $[Ca^{2+}]_i$. Also, as previously discussed, pre-treatment with ATPγS prevented the actions of ATP.

Long-lasting desensitization of the electrophysiological and $[Ca^{2+}]_i$ responses was observed. Long-lasting desensitization is also evident with excitatory amino acid receptors (Mayer & Westbrook, 1985), vertebrate neuromuscular junction (Scubon-Mulieri & Parsons, 1977) and ATP receptors (Krishtal *et al.* 1983). In the $[Ca^{2+}]_i$ measurements, cells showed a variable degree of refractoriness to the actions of multiple applications of ATP. This can be explained, at least in part, by the prolonged membrane depolarization which would alter the availability of voltage-dependent Ca^{2+} channels and lead to a reduction in Ca^{2+} influx during subsequent application of ATP. In the case of the electrophysiological response, desensitization was seen in the continued presence of the agonist (ATP or ATPγS) and was maintained despite removal of the agonist. It does not seem likely that the mechanism of desensitization is related to the prolonged elevated $[Ca^{2+}]_i$ since desensitization of the ATP-induced electrophysiological response was observed in the absence of Ca^{2+}. It is possible that desensitization is maintained by phosphorylation and that the lack of a complete recovery is due to the partial absence of phosphatases. The time course of dephosphorylation is slow and incomplete, especially the 47 kDa protein.

In conclusion, the findings in this report suggest a mechanism by which ATP increases $[Ca^{2+}]_i$ as shown in Fig. 10. ATP application to cardiac myocytes stimulates phosphorylation (P) of several membrane-bound proteins which may plausibly induce the opening of a non-selective cation channel. The ATP-activated channel may be permeable to Na^+ and Ca^{2+} and causes $[Ca^{2+}]_i$ to rise. Furthermore, the activation of this channel causes depolarization (ΔV_m) and the generation of action potentials. During the firing of action potentials, Ca^{2+} enters the cell via L-type Ca^{2+} channels thereby triggering the release of Ca^{2+} from the sarcoplasmic reticulum (SR). Taken together, Ca^{2+} influx and Ca^{2+} release account for the ATP-induced $[Ca^{2+}]_i$ increase.

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