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Extracellular ATP induces Ca^{2+} transients in cardiac myocytes which are potentiated by norepinephrine

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Isolated rat ventricular cardiac myocytes loaded with the fluorescent calcium indicator fura2 showed significant changes in intracellular calcium concentrations upon exposure to >1 μM ATP ($EC_{50}=7.4\pm1.3$ μM, $n=4$, SE), suggesting that extracellular ATP may have an important influence on myocardial contractility. The response was found to be highly ATP specific and required extracellular calcium. Furthermore, 30 s pretreatment of the cells with 0.2–1 μM norepinephrine decreased the concentration of ATP required for the Ca^{2+} transient, shifting the EC_{50} for ATP to 1.7 ± 0.1 μM ($n=3$, SE). β-Propranolol (a β_1-receptor antagonist) prevented potentiation, whereas phentolamine (an α_1-receptor antagonist) did not, indicating that regulation is through the β_1-adrenergic receptor. ATP and norepinephrine released locally from sympathetic neurons may act in concert through the ATP and β_1-adrenergic receptors to regulate myocardial calcium homeostasis.

Myocyte; ATP; Norepinephrine; Ca^{2+} transient; Fura2

1. INTRODUCTION

It has been known for some time that a large amount of ATP is stored within many types of secretory granules, including chromaffin granules [1], adrenergic granules of the sympathetic nervous system [2], acetylcholine-containing granules of the parasympathetic nervous system [3], and platelet dense granules [4]. Recent work on ATP receptors [5–7] has renewed interest in the possibility that exocytosed ATP performs a signalling function in some cell types, and raised questions of whether co-release of ATP with other transmitters may induce an intracellular response greater than the sum of the individual responses. Although ATP is known to cause complex changes in heart function when infused through the circulation [8], the observations are difficult to interpret from a mechanistic or cellular perspective because of the variety of cell types present, the complexity of the cardiac function parameters measured, and rapid degradation of ATP to adenosine (which also has powerful functional effects). Use of a preparation of isolated adult rat cardiac ventricular myocytes obviates many of the interpretive difficulties of the whole heart system, and permits the use of the fluorescent Ca^{2+} indicator fura2 in intracellular [Ca^{2+}] measurements. Addition of extracellular ATP to fura2-loaded myocytes in suspension resulted in a transient increase in intracellular Ca^{2+} concentration which has not been

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Abbreviations: BSA, bovine serum albumin; ITP, inosine 5′-triphosphate; UTP, uridine 5′-triphosphate; AMP-PNP, 5′-adenylyl imidophosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate)

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reported in the literature except in abstract form [9]. The agonist specificity of the ATP receptor response was investigated. As norepinephrine and ATP are co-stored in sympathetic nerve terminals, the combined effect of these compounds on cardiac calcium metabolism was also examined. A marked potentiation of the ATP-calcium response by norepinephrine pretreatment was observed and characterized with respect to dose requirements and the adrenergic receptor subtype involved.

2. MATERIALS AND METHODS

2.1. *Cell preparation*

Ventricular myocytes were prepared from 250–350 g male Sprague-Dawley rats according to a modification of the procedure of Hohl et al. [10]. In brief, the heart was mounted on a modified Langendorf perfusion apparatus and perfused at 10 ml/min with oxygenated Joklik modified Eagles medium (Sigma) supplemented with 1.25 mM $CaCl_2$ and 10 mM Hepes, pH 7.20, at 37°C for 5 min followed by 5 min in the same medium without added calcium. Type I collagenase (Cooper Biomedical) was added to this medium to a concentration of 125 U/ml with 0.1% BSA (Miles Pentex fraction V). After 20 min in the Ca^{2+}-free medium, $CaCl_2$ was added to the perfusion medium at 5-min intervals to final concentrations of 0.25, 0.50, 1.00 and 1.25 mM. After 35–45 min of collagenase digestion by perfusion, ventricular tissue was dissociated by shaking in a collagenase solution for 5 min under oxygen at 37°C (pH 7.20). Following a brief trituration, the residue was filtered through 250 and 100 μm mesh screens (Tetko Precision Screening Media, Elmsford, NY), sedimented by centrifugation at 100 × g for 1 min and resuspended in fresh (Ca^{2+}-containing Joklik solution with 2% BSA (w/v). Cells were then purified over a 43% Percoll step-gradient at 500 × g in a swinging-bucket centrifuge for 2 min. Overall, cell preparations were 17 ± 8% trypan blue-permeant (with a final trypan blue concentration of 0.8 mg/ml), 73 ± 6% of rod-shaped morphology, and 9 ± 4% distorted ($n = 16$, SD). 3 ± 2% ($n = 6$, SE) were observed to contract spontaneously following the final Percoll separation. Yields were typically 1–2 × 10^6 rod-shaped cells per heart.

2.2. *Intracellular Ca^{2+} measurements*

Cells were loaded with 4 μM fura2-AM at 37°C for 40 min in Joklik medium supplemented with 1.25 mM $CaCl_2$, 10 mM Hepes and 2% BSA. After a wash (1 min, 100 × g), cells were reincubated at 37°C for 10 min in the supplemented Joklik medium to permit reaction of fura2-AM bound to cell membranes. Following a second Percoll separation and two washes to remove remaining Percoll, the cells were stored at room temperature in the Joklik medium. 1–2 × 10^4 cells were washed and resuspended per cuvet in 1.5 ml fresh Krebs-Henseleit solution (98 mM NaCl, 10 mM $NaHCO_3$, 10 mM $NaHPO_4$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 10 mM Hepes, pH 7.40) with 2% BSA and 10 mM glucose. Fluorescence measurements were carried out under constant stirring and temperature (37°C) in a custom-built fluorimeter designed by the University of Pennsylvania Biomedical Instrumentation Group [5]. A 75 W xenon lamp was used as an excitation source. The excitation wavelength was established using a 339 nm interference filter (Omega Optical, Brattleboro, VT) with an 8 nm half-bandwidth and emitted light was collected through a 510 nm filter (9 nm half-bandwidth). Intracellular calcium concentrations were calculated according to the equation $(F - F_{min})/(F_{max} - F) \cdot 224\ nM = [Ca^{2+}]_{in}$ as described previously for fura2 studies completed at a single emission wavelength [11]. F_{max} was determined as the fluorescence of fura2 after cell lysis with 33 μg/ml digitonin, and F_{min} was the fluorescence remaining after removal of Ca^{2+} from the medium with 5 mM EGTA and 50 mM Tris. F and F_{max} were corrected for fura2 released from the cells after stirring in the fluorimeter by subtracting either the fluorescence of fura2 remaining in the medium after sedimentation of the cells at 100 × g or the rapid changes in fluorescence induced by addition of 5 mM EGTA to loaded cells. Under identical conditions, comparable stimulation of unloaded cells did not produce detectable fluorescence changes. The concentration of fura2 in loaded cells was determined using fura2 acid standards to determine cellular fura2 content and a cellular volume measurement performed according to the isotopic ratio method of Rottenberg [12]. Cells were used for fluorescence measurements for up to 5 h after cell isolation with little variation in the measured parameters.

2.3. *Chemicals*

ATP, ADP, AMP, adenosine, AMP-PNP, α,β-methylene ATP, β,γ-methylene ATP, nucleotide triphosphates, norepinephrine and β-propranolol were obtained from Sigma. ATPγS was obtained from Boehringer Mannheim. Phentolamine was from Ciba-Geigy.

3. RESULTS AND DISCUSSION

Fig. 1 shows the time course of changes in the intracellular free Ca^{2+} concentration of fura2 loaded myocytes exposed to a half-maximal dose of ATP without (A) and with (B) pretreatment with 1 μM norepinephrine (NE). In this experiment, the magnitude of the Ca^{2+} transient was more than doubled after exposure to NE. Although the change in dye saturation due to addition of ATP was not large in the representative experiment shown (fig. 1), the ATP response was comparable to the maximal cellular response to K^+ depolarization. Calculated intracellular calcium concentrations were typically 67 ± 26 nM at rest and 167 ± 55 nM after K^+ stimulation ($n = 16$, SD). Smaller than predicted changes in cytosolic (Ca^{2+}) are not unusual for cardiac cell suspensions prepared using this digestion procedure: similar responses were observed by Cheung et al. [13] and Bjornsson et al. [14], although Powell et al. [15] have reported a peak Ca^{2+} value of 600 nM with exposure to 80 mM KCl. It is possible that the low cellular responsiveness is due to selection of a very stable and calcium tolerant population of cells, or that the intracellular stores of the cells are somewhat depleted as a result of lack of stimulation during the isolation, purification, and loading procedure. Although buffering of cytosolic Ca^{2+} by intracellular fura2 (400 ± 125 μM, $n = 3$, SE) is possible, the use of less fura2 in the loading procedure did not enhance the resting intracellular Ca^{2+} concentration or increase the relative magnitude of Ca^{2+} transients.

Two representative ATP dose responses of the potentiation by norepinephrine are given in fig. 2, demonstrating that NE pretreatment enhanced responsiveness at submaximal ATP doses. The data are expressed as the change in (Ca^{2+}) due to addition of ATP relative to the (Ca^{2+}) changes of the cells following K^+-depolarization. In a separate experiment (not shown) it was verified

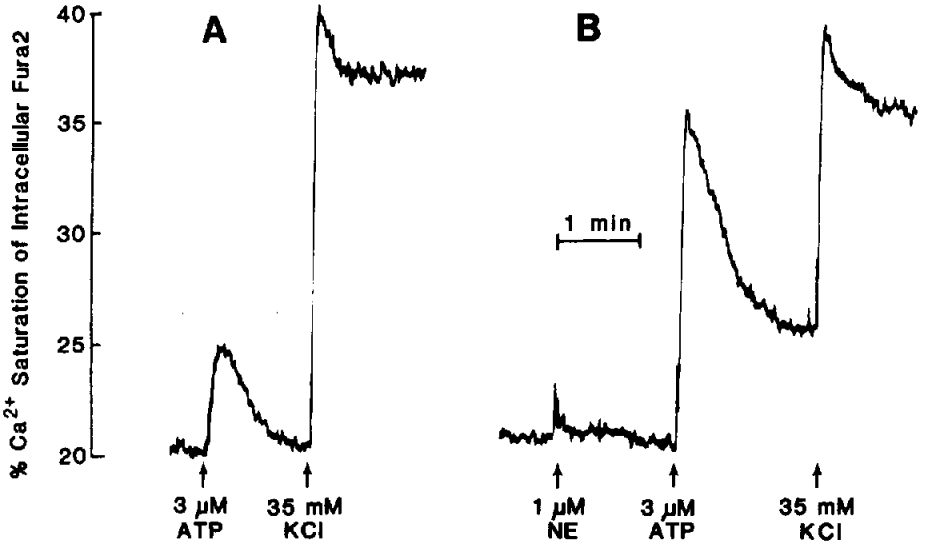

Fig. 1. NE potentiation of ATP-induced Ca^{2+}-transients. Fura2-loaded myocytes were exposed to 3 μM ATP without (A) and with (B) 1 min of pretreatment with 1 μM norepinephrine. K^+-depolarization increased calculated cytosolic calcium concentrations in this preparation from 81 to 168 nM. The cells were 75% rod-shaped, 15% trypan-blue permeant, and 10% distorted.

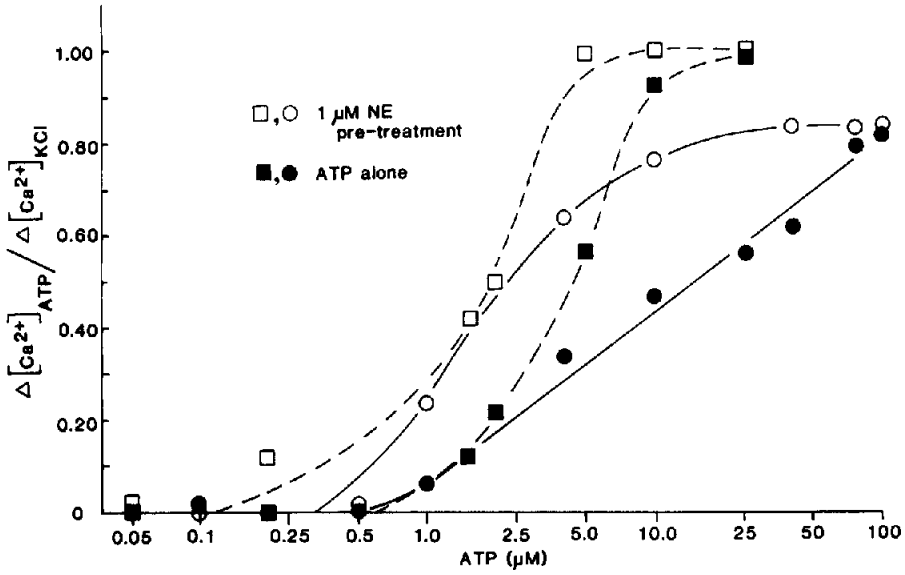

Fig. 2. Cytosolic calcium responses as a function of ATP concentration and NE pretreatment in two cell preparations. The ATP dose responses are given without (●, ■) and with (○, □) 1 min of 1 μM NE pretreatment. Data are expressed as the change in cytosolic calcium concentration due to ATP relative to the maximal cellular responsiveness to K^+-depolarization. The curves to the right (●, ○) are from a fura2-loaded cell preparation with 77% rod-shaped, 17% trypan-blue permeant, and 6% distorted cells; calculated cytosolic calcium levels were 50 nM at rest and 150 nM after K^+ stimulation. The cell suspension giving the curves to the left (□, ■) was composed of a 82% rod-shaped, 11% trypan-blue permeant and 8% distorted cell population; cytoslic calcium levels increased from 100 to 270 nM after K^+-depolarization.

that pretreatment with norepinephrine had no influence on the magnitude of the K^+-induced response. Two curves are shown to illustrate the widest range of inter-preparation variability in cellular responsiveness to ATP. Data points from 15 preparations were consistently between the extremes shown: an average EC_{50} for ATP was found to be 7.4 ± 1.3 μM ($n = 4$, SE). Pre-exposure to norepinephrine increased the apparent sensitivity of the receptor for ATP in all but one experiment, resulting in a new EC_{50} of 1.7 ± 0.1 μM ($n = 3$, SE). It is presumed that the sensitivity of the receptor for ATP in both cases is actually higher than this due to the action of surface ATPases. Interpreparation variability in the apparent ATP dose response may be due to modification of ecto-ATPase and/or receptor activity following the digestion procedure.

The Ca^{2+}-signalling characteristics of the ATP receptor were further examined. It was determined (not shown) that the receptor to ATP alone (not ADP, AMP, or adenosine in concentrations up to 100 μM) and that other nucleotide triphosphates (GTP, ITP, UTP, CTP and TTP) were without effect in concentrations up to 100 μM. These agonist specificities are similar to results obtained with ATP in embryonic rat myoblasts [7], but are more stringent than the responses of Ehrlich ascites tumor cells [5] and DDT smooth muscle cells (Dubyak, G.R., personal communication) which respond to GTP, ITP and UTP as well. Nonhydrolyzable analogues (AMP-PNP, α,β-methylene ATP and β,γ-methylene ATP) had no influence below 100 μM. ATPγS, which is partially hydrolyzable [16], was a partial agonist at concentrations > 10 μM and also prevented further Ca^{2+} responses to ATP in a dose-dependent fashion. ATPγS has been reported to be as potent as ATP in Ehrlich ascites tumor cells, DDT cells (Dubyak, G.R., personal communication), and embryonic chick muscle cells [7], suggesting a difference in the cardiac receptor subtype. The ATP response was inhibited by complex formation of extracellular calcium with 1.25 mM EGTA (15 s exposure) or in

the presence of 10 μM verapamil or 1 μM nifedipine, demonstrating that the increase in cytosolic Ca^{2+} is dependent on Ca^{2+} influx. The magnitude of the calcium response at maximal doses of ATP is equal (but not additive) to the effect of K^+ depolarization, suggesting that ATP could have a significant influence on heart metabolism and contractility.

The relationship between norepinephrine concentration and stimulation of ATP-induced intracellular Ca^{2+} responses is shown in fig. 3. The half-maximal dose is 70 ± 20 nM ($n=3$, SE). Epinephrine has the same effect at similar doses (not shown). Potentiation with 1 μM NE was maximal after 30 s of exposure, and maintained for at least 10 min. The effectiveness of several adrenergic receptor antagonists is shown in table 1. A 10-fold excess of β-propranolol (a β_1-receptor antagonist) over NE reduced the potentiation to near control levels whereas even a 100-fold excess of phentolamine (an α_1 antagonist) was slightly stimulatory. This suggests that potentiation is mediated by the β_1-receptor, perhaps via a cAMP-dependent mechanism. This is in contrast to

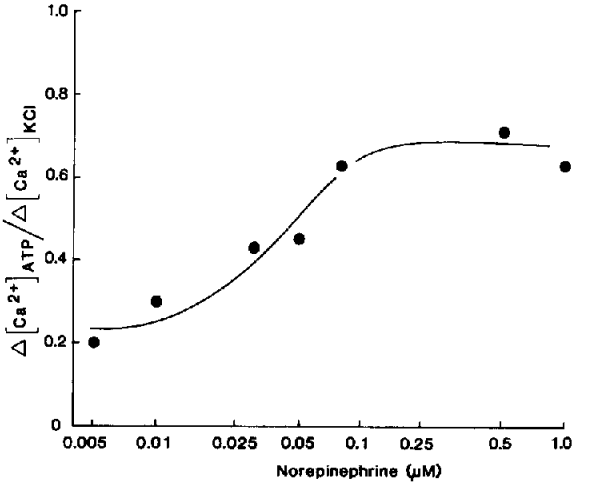

Fig. 3. Potentiation of ATP-induced calcium transients as a function of norepinephrine concentration. Fura2-loaded cells were exposed to the indicated dose of NE for 1 min before addition of 5 μM ATP. Intracellular Ca^{2+} concentrations were calculated as 30 and 110 nM without and with K^+ depolarization, respectively. Cells were 86% rod-shaped, 9% trypan-blue permeant, and 5% distorted. The EC_{50} of NE activation for this representative experiment is 0.04 μM.

Table 1

Characterization of the adrenergic receptor subtype involved in potentiation of ATP-induced Ca^{2+} transients

Addition	$d[Ca^{2+}]_{ATP}/d[Ca^{2+}]_{KCl}$
Control ATP	0.35 ± 0.02
0.1 μM NE, ATP	0.64 ± 0.01
1 μM β-propranolol, 0.1 μM NE, ATP	0.40 ± 0.02
10 μM phentolamine, 0.1 μM NE, ATP	0.76 ± 0.04

Each value is the average of results from 3 preparations, SE. ATP concentrations ranged from 7 to 10 μM. Antagonist was added 30 s before NE which was added 1 min before the ATP

similar findings of NE potentiation of ATP-induced contractile responses in vas deferens [17] which are mediated by the α_1-adrenergic receptor [18].

The activated target of the norepinephrine-mediated potentiation is unknown, and its determination will probably require knowledge of the mechanism by which ATP alters intracellular calcium levels. Other ATP receptors have been proposed to alter intracellular calcium in a variety of ways. Phosphoinositide hydrolysis, inositol 1,4,5-phosphate production and increased cytosolic calcium levels result from the addition of micromolar concentrations of ATP to Ehrlich ascites tumor cells [19], H-35 hepatoma cells [20], hepatocytes [21], DDT smooth muscle cells (Dubyak, G.R., personal communication), and rat aortic myocytes [22]. A calcium-permeable channel is activated by extracellular ATP in arterial smooth muscle cells [23]. Secondary voltage-activated calcium changes would be expected in rat dorsal horn neurons [6], which respond to added ATP with depolarization due to increased sodium influx. The dependence of the cardiac ventricular effect on extracellular calcium suggests that the ATP response is due to one of the latter mechanisms. The role of Na^+ influx may be minimal as the ATP response was present when all Na^+ in the medium had been replaced by *N*-methyl-D-glucamine. Norepinephrine may act at the level of the channel or carrier mediating the

ATP response, act upon the receptor itself, or reduce ATPase activity through an indirect action on ectoATPases.

The physiological importance of ATP-induced calcium transients and their modulation by norepinephrine has not been established, however, ATP is found extracellularly under a variety of circumstances which might be influenced by sympathetic enervation. Norepinephrine may enhance responses to ATP released from the same or nearby neurons, nearby hypoxia-damaged cells [23], platelet dense granules exocytosed during clotting [4], acetylcholine-containing granules [24] released during parasympathetic activity, or activation of putative 'purinergic nerves' [25]. ATP may be involved in short-range perturbations of calcium homeostasis which may significantly affect inotropic responses of the heart.

The demonstration that ATP has specific, highly reproducible effects on cytosolic Ca^{2+} levels in isolated cardiac ventricular myocytes coupled with the modulation of these effects by norepinephrine strongly suggests that ATP acts as a transmitter in this system and should be further investigated as a regulator of cell response.

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