The effect of extracellular ATP on spontaneous calcium waves in isolated rat cardiomyocytes

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Abstract:
The roles of different purinergic mechanisms in mediating an increase in cardiac contractility were investigated in rat ventricular myocytes using laser-scanning confocal microscopy to measure changes in field-stimulated calcium transients and spontaneous calcium waves. Prolonged exposure (>3 minutes) of cardiomyocytes to 1 and 100 μmol/L extracellular ATP (ATP_o) significantly increased fluorescence intensity of field-stimulated calcium transients from 101.2 $\pm$ 2.3 % (mean $\pm$ SEM) to 119.8 $\pm$ 6.2 and 136.5 $\pm$ 9.7 %, respectively ($P<0.05$). Following washout of ATP_o, the increase in transient intensity induced by 1 μmol/L ATP_o was reduced to 105.2 $\pm$ 1.5 % ($P<0.05$). Background fluorescence prior to each transient increased from 100.1 $\pm$ 3.4 % to 141.8 $\pm$ 10.8 % following exposure of cardiomyocytes to 100 μmol/L ATP_o ($P<0.05$). Exposure of cardiomyocytes to ATP_o (1-100 μmol/L) did not change mean calcium wave velocity or intensity. Spontaneous calcium wave frequency increased during sham experiments, and did not significantly change upon exposure of cardiomyocytes to 1 and 10 μmol/L ATP_o. Prolonged exposure (>3 minutes) of cardiomyocytes to 100 μmol/L ATP_o significantly augmented the increase in calcium wave frequency seen in sham experiments (from 205.3 $\pm$ 40.2 % without ATP_o to 355.18 $\pm$ 63.51 %, $P<0.05$). This increase in frequency was not reversible following washout. It is concluded that 100 μmol/L alters the calcium-induced calcium-release mechanism via P_{2Y}-purinoceptors and the involvement of a second messenger system other than IP_3. By contrast, 1 μmol/L ATP_o may act to increase the L-type calcium current directly during field-stimulation.

Key Words: extracellular ATP ■ calcium wave ■ calcium transient ■ purinergic receptor ■ cardiomyocyte

Introduction:

Sources and Effects of Extracellular ATP

Extracellular adenosine 5'triphosphate (ATP_o) is known to induce a significant increase in cardiac contractility.[1-3] ATP_o is released as a cotransmitter with noradrenaline from sympathetic nerves that terminate in the heart, and larger quantities of ATP_o are liberated from damaged cardiac cells, activated platelets and the endothelium during cardiovascular trauma and ischaemia.[4,5] Under these conditions the amount of ATP_o present in the coronary circulation can approach several μmol/L.[5,6]

Effects of Extracellular ATP on Cardiac Contraction

Studies on whole mammalian heart models have shown that ATP_o (1 μmol/L to 1 mmol/L) enhances cardiac contractility without significantly increasing heart rate.[1-3] Consistent with this observation, ATP_o has been shown to increase the resting intracellular calcium concentration, $[Ca^{2+}]i$, and to enhance the amplitude of calcium transients (and hence contraction) in field-stimulated cardiomyocytes.[7,8] ATP_o is thought to exert this positive effect on cardiomyocyte contractility by binding to either of two types of P_2-purinergic cell-surface receptors.[9]

P_{2Y}-purinoceptor Stimulation of Cardiac Contractility

P_{2Y}-purinoceptors are G protein-coupled receptors, the majority of which activate phospholipase-Cγ (PLCγ). Activation of PLCγ results in the production of inositol 1,4,5-trisphosphate (IP_3). IP_3 then facilitates the release of calcium ions (Ca^{2+}) from the sarcoplasmic reticulum (SR) and, hence, an increase in contractility and resting $[Ca^{2+}]i$.[9-11] Other studies suggest that P_{2Y}-purinoceptors enhance the L-type calcium current.[12-14] Thus, during cardiomyocyte depolarisation, there is a larger calcium influx through the L-type Ca^{2+} channels that promotes greater SR Ca^{2+} release and hence a greater contraction.[15]

P_{2X}-purinoceptor Stimulation of Cardiac Contractility

By contrast, P_{2X}-purinoceptors are a family of direct ligand-gated ion channels. Their activation by endogenous ATP_o results in an inward non-selective cation current (Na^+ and Ca^{2+}) termed I_{ATP}.[3,9,14] The resulting Ca^{2+} influx through the non-selective cation channel increases the resting $[Ca^{2+}]i$ concentration and this is thought to then allow the SR to become loaded with larger concentrations of Ca^{2+}.[2,7] Thus, during depolarisation, the L-type Ca^{2+} channels promote a larger transient increase in $[Ca^{2+}]i$ via the calcium-induced calcium-release (CICR) mechanism due to the increased SR Ca^{2+} store load.[15]

In contrast to the theory of P_2-purinoceptor stimulation of cardiomyocyte contractility, other studies suggest that ATP_o exerts its positive inotropic effect on contractility through either direct stimulation of L-type Ca^{2+} channels[16,17] or extracellular phosphorylation of other membrane proteins.[18-20] Thus, the purinergic mechanism by which ATP_o predominantly increases cardiac contractility and the resting $[Ca^{2+}]i$ remains unclear. Uncertainty exists regarding which of the above mechanisms is predominantly responsible for the increased calcium transient and hence cardiac contractility. That is: purinergic-P_{2X} or P_{2Y} mechanisms or changes in L-type Ca^{2+} channel properties. The aim of the present study was to observe the effects of biologically-relevant concentrations of extracellular ATP (1 –100 μmol/L) on spontaneous calcium waves and field-stimulated calcium transients. This was done in order to determine which of the purinergic mechanisms is predominantly responsible for the observed ATP_o-induced increase in cardiac contractility.

Calcium Waves

Calcium waves are propagating waves of raised $[Ca^{2+}]i$, that are spontaneously generated when the SR becomes overloaded with Ca^{2+}.[21] Overloading of the SR increases Ca^{2+} leakage during stochastic opening of the SR calcium-release channels to the point where CICR becomes locally regenerative.[21,22] The propagation of a calcium wave is driven by the diffusion of this released (leakage) calcium ahead of a wavefront of CICR, produced when the free calcium reaches a threshold concentration. Thus, the propagation velocity of the wave in the cell depends primarily on the rate of diffusion of Ca^{2+} in the cytoplasm, the quantity of free Ca^{2+} in the SR and the threshold or "gain" of the CICR system.[23,24] This leads to three hypotheses underlying the current study:

Firstly, we hypothesise that P_{2X}-purinoceptor stimulation will result in more frequent calcium wave propagation with little change in intensity and velocity. The increase in resting $[Ca^{2+}]i$ and resultant SR Ca^{2+} content will cause an increase in the frequency of stochastic openings during which SR Ca^{2+} is released through the calcium-release channels.[21,22] This would allow the SR Ca^{2+} content to stabilise (around the threshold concentration), as the excess calcium will trigger its more frequent release. This would result in an increase in calcium wave frequency, but little change in calcium wave intensity or propagation velocity, because the quantity of calcium released during each calcium wave will be similar. In addition, these effects on calcium waves would be rapidly reversible following the washout of ATP_o, because the effects in question are due to the activation of a ligand-gated ion channel (the P_{2X}-purinoceptor).

By contrast, IP_3 is suggested to facilitate Ca^{2+} release from the SR[10,11] and results in a reduction in SR Ca^{2+} content.[25] We hypothesise that this will result in more frequent, slower, and less intense calcium waves (due to the reduction in SR Ca^{2+} content). Furthermore, due to the presence of second messenger molecules in the cytosol (a result of P_{2Y}-purinoceptor activation), this effect would not be rapidly reversible following the washout of ATP_o.

Finally, we hypothesise that any effect of ATP_o on the L-type Ca^{2+} channels (direct or via P_{2Y}-purinoceptors) will not alter calcium wave properties since L-type Ca^{2+} channels are least active at resting membrane potential.[26] However, there would be a corresponding increase in the field-stimulated calcium transient. This increase would be rapidly reversible following the washout of ATP_o if it were due to a direct interaction between ATP_o and the L-type Ca^{2+} channels. By contrast, the increase in the transient would not be rapidly reversible if P_{2Y}-purinoceptor second messenger systems were involved.

Thus, by monitoring ATP_o-induced changes in calcium transients and calcium waves as well as the resting $[Ca^{2+}]i$, it is possible to determine the purinergic mechanism by which ATP_o predominantly increases cardiac contractility.

Methods:
Animals used throughout this study were cared for according to the Australian National Health and Medical Council *Guidelines for the Care and Use of Animals*. The University of Adelaide Animal Ethics Committee approved all experimental procedures.

Preparation of Isolated Cardiomyocytes:
Male Sprague Dawley rats (300-350 g) were fully anaesthetised by intraperitoneal injection of 0.6 -0.7 ml sodium pentobarbitone in 2000 units of heparin. The heart was then quickly excised and mounted on a plastic cannula attached to a modified Langendorff perfusion system, as described previously.[27] Cardiomyocytes were isolated from the right ventricle of the rat hearts using a collagenase and protease technique.[28]

Solutions:
The basic Tyrode's solution contained (in mmol/L) NaCl 133.5, Hepes 10, D-glucose 11, NaH_2PO_4 1.2, KCl 4, $MgCl_2$ 1.2, pH adjusted to 7.4 with NaOH. The collagenase solution contained 60 mL of the basic Tyrode's solution supplemented with 2.5 μL of 0.5mol/L $CaCl_2$, 150 mg BDM, 50 mg BSA (Sigma Chemicals), 6 mg collagenase (Yakult) and 2.5 mg of protease (Sigma).

Fluo-3 loading of isolated cardiomyocytes:
Isolated cells were allowed to attach onto small glass cover slips coated with laminin (10 μmol/L, Sigma). Attached cells were loaded with the Ca^{2+}-sensitive fluorescent dye fluo-3 (AM) as described previously.[29]

Experimental-Setup
Individual cover slips were placed in a perfusion chamber on the stage of a confocal microscope. Cover slips were perfused at the rate of -3 ml/minute with the basic Tyrode's solution, containing 1.5 or 5 mmol/L $CaCl_2$ for field-stimulated calcium transients and spontaneous calcium waves respectively. Cells were electrically stimulated at a frequency of 0.5 Hz, with 4 ms voltage pulses delivered through parallel platinum wires lining the edge of the perfusion chamber. Field stimulation was performed for 30 seconds at the beginning of calcium wave experiments and maintained throughout calcium transient experiments.

Solution changes:
During the experiments, Na_2ATP (Sigma, 1, 10 or 100 μmol/L) was applied to the perfusing solution around the isolated cardiomyocytes (pH of all solutions was adjusted to 7.4). A two-way valve was used to add either the control or the ATP solutions perfusing the experimental bath (approximate 1 -2 ml volume). With this arrangement, the maximum time of change of solution was in the order of 1.5 minutes.

Image Analysis:
Calcium transients and waves were observed using a 40x Nikon oil-immersion objective and the line scan mode of a fluorescence confocal microscope. Fluo-3 fluorescence was excited at 488 nm and measured above 515 nm. Changes in emitted fluorescence intensity (used as an index of $[Ca^{2+}]i$) were recorded using Optiscan F900 computer software. All experiments were carried out at room temperature (21 -23^{0}C). The laser scanning confocal microscope was used in the single line-scan acquisition mode to generate images 512 lines x 512 pixels in size. Data files of waves and transients were archived in sets of 50 bitmap image files. Image processing and analysis was performed using Scion Image data analysis software (Scion Corporation, 1998).

Data Analysis:
Changes in fluo-3 fluorescence during both calcium wave and calcium transient experiments were recorded in 4 sets of 50 images (512 lines x 512 pixels). One set of 50 images was obtained prior to ATP exposure (control), during gradual ATP exposure, following 3 minutes of ATP exposure (prolonged exposure) and following wash out of ATP. Propagation velocity (slope), frequency, intensity and background fluorescence of spontaneous calcium waves were analysed for the majority of waves in each image set. Fluorescence intensity alone was analysed for transients. All measurements were determined using Scion Image bit-map analysis tools.

Statistics:
All quantitative data are expressed as means $\pm$ SEM for n cells. Data were evaluated with a one-way analysis of variance and a Tukey's post hoc test (GraphPad Prism, (Graphpad Software, San Diego, USA). Where appropriate, a two-tailed, unpaired t-test was used, and significance was accepted at $P < 0.05$ for all experiments.

Results:
To investigate the effects of ATP_o on $[Ca^{2+}]i$, cells were gradually exposed to the various ATP_o concentrations (1, 10 and 100 μmol/L), to avoid the initiation of ATP_o-induced calcium transients.[30] All Figure values for both calcium waves and transients are expressed as a percentage of basal measurements made in control Tyrode's solution containing 5 and 1.5 mmol/L $CaCl_2$, respectively, at the beginning of each experiment. Sham experiments represent the same time period as ATP_o experiments, except that results were obtained in the absence of ATP_o (to test the consistency of the measurements over the same time period).

Effect of ATP_o on Field-Stimulated Calcium Transients
Transient intensity was examined in order to demonstrate that ATP_o augmented the peak $[Ca^{2+}]i$ during field-stimulated transients. Figure 1A demonstrates that transient intensity (proportional to peak $[Ca^{2+}]i$) did not significantly change throughout the duration of sham experiments, but significantly increased upon exposure of cardiomyocytes to 1 and 100 μmol/L ATP_o. Transient intensity increased from 99.5 $\pm$ 5.2 % in sham experiments to 119.8 $\pm$ 6.2 and 136.5 $\pm$ 9.7 % during prolonged exposure to 1 and 100 μmol/L ATP_o, respectively. Following washout of ATP_o, the increase in transient intensity induced by 1 μmol/L ATP_o was significantly reduced to 105.2 $\pm$ 1.5 %, an intensity that remained significantly above that in sham experiments (97.3 $\pm$ 2.2 %). In contrast, there was no significant decrease in transient intensity following the washout of 100 μmol/L ATP_o.

Effect of ATP_o on Calcium Transient Background Intensity
Figure 1B depicts changes in the background intensity (proportional to resting $[Ca^{2+}]i$) immediately prior to each calcium transient. There was no change in background intensity observed throughout the duration of sham experiments or in cardiomyocytes exposed to 1 μmol/L ATP_o. In contrast, 100 μmol/L ATP_o significantly increased background fluorescence throughout the duration of the experiments. This increase in background intensity peaked following the washout of 100 μmol/L ATP_o at 141.8 $\pm$ 10.8 %, compared to 100.1 $\pm$ 3.4 % obtained from the sham experiment during the same period.

Basal Calcium Wave Activity For Sham Experiments

In order to evaluate the effect of ATP_o on calcium waves, only cells that expressed at least four calcium waves during control initial conditions were selected. In general, for sham experiments, mean calcium wave velocity and intensity under control conditions (data not shown) were 100 $\pm$ 3.8 % and 100 $\pm$ 8.2 % respectively (n = 6, pooled data). These values did not significantly change over the experimental period (Figures 2A and B). In contrast, for the sham experiments, mean calcium wave frequency and background fluorescence immediately prior to each calcium wave (Figures 3A and B) gradually increased throughout the experimental period. This increase was from 100 $\pm$ 0.0 and 100 $\pm$ 6.5 % (under control conditions, data not shown) to 233.5 $\pm$ 59.4 and 116.1 $\pm$ 8.9 % (during the period equivalent to ATP_o washout) for calcium wave frequency and background fluorescence, respectively ($n = 6$, $P < 0.05$).

Effect of ATP_o on Calcium Wave Velocity and Intensity

Exposure of cardiomyocytes to ATP_o (1 -100 μmol/L) did not change mean calcium wave velocity or intensity between any of the ATP_o-exposed groups and the sham experiments throughout the experimental period (Figure 2A and B).

Effect of ATP_o on Background fluorescence

Gradual exposure of cardiomyocytes to ATP_o significantly increased background fluorescence immediately prior to each calcium wave from 98.2 $\pm$ 5.1 % in sham experiments to 115.8 $\pm$ 3.2, 127.1 $\pm$ 9.0 and 121.8 $\pm$ 3.0 % upon gradual exposure to 1, 10 and 100 μmol/L ATP_o, respectively (Figure 3A). Following this period, there was no significant difference in the background fluorescence of ATP_o exposed cardiomyocytes and sham experiments.

Effect of ATP_o on Calcium Wave Frequency

Figure 3B shows the number of spontaneously occurring calcium waves that were captured in the 50-bitmap images recorded during each experiment. Spontaneous calcium wave frequency did not significantly increase during exposure to 1 and 10 μmol/L ATP_o when compared to the increase observed in sham experiments. In contrast, prolonged exposure of cardiomyocytes to 100 μmol/L ATP_o significantly increased calcium wave frequency to 355.18 $\pm$ 63.51 % compared to the increase to 205.3 $\pm$ 40.2 % seen in sham experiments during the same time period. Following the washout of ATP_o, the increase in calcium wave frequency stimulated by 100 μmol/L ATP_o persisted, and further increased to 459.29 $\pm$ 99.84 % compared to 233.5 $\pm$ 59.4 % observed during sham experiments.

Discussion:

The present findings show that extracellular ATP (1 -100 μmol/L) produces a significant increase in the field-stimulated calcium transient in isolated rat right ventricular cardiomyocytes, consistent with previous studies on rat left ventricular myocytes.[7,8] This result is also in agreement with whole-heart studies in rats, which demonstrate that ATPo significantly increases whole-heart contractility.[2,3] In addition, the present study has demonstrated, for the first time, that exposure of cardiomyocytes to 100 μmol/L ATP_o results in a significant increase in the frequency of spontaneous calcium waves, a result not observed at lower ATP_o concentrations (1,10 μmol/L). This increase in frequency was accompanied by relatively constant calcium wave intensities and velocities, and persisted following the washout of 100 μmol/L ATP_o.

Effects of Extracellular ATP on Field-Stimulated Calcium transients:

The present results demonstrate that 1 μmol/L ATP_o significantly increased the peak transient intensity during ATP_o exposure, but did not significantly change background fluorescence immediately prior to each calcium transient (Figure 1). This result suggests that 1 μmol/L ATP_o was primarily increasing the L-type Ca^{2+} current during field stimulation and, to a lesser extent, interacting with $P_{2X/Y}$-purinoceptors. This can be concluded since L-type Ca^{2+} channels are predominantly inactive at resting membrane potentials,[26] and hence promote little change in resting $[Ca^{2+}]i$. In contrast, both P_{2X} and P_{2Y}-purinergic receptors act to increase the resting $[Ca^{2+}]i$.[7,9,16,18] However, the effect of 1 μmol/L ATP_o was not completely reversed following the washout of 1 μmol/L ATP_o. This residual effect on the calcium transient following the washout of ATP_o, and the increase in background fluorescence intensity observed prior to each calcium wave (Figure 3A), may suggest some P_{2Y}-purinoceptor activity as opposed to P_{2X}-purinoceptor activity.

In contrast to this result, 100 μmol/L ATP_o appears to stimulate an increase in the calcium transient via actions on P_{2Y}-purinoceptors. This conclusion seems reasonable since the observed increase in the calcium transient and background fluorescence intensity prior to each transient persisted following ATP_o washout. This result demonstrates the presence of a second messenger system that is able to maintain the increased calcium transient intensity and background fluorescence following termination of purinoceptor stimulation. However, the present findings are unable to distinguish between P_{2Y}-purinoceptor-facilitated SR Ca^{2+} release (by IP_3) and P_{2Y}-purinoceptor enhancement of the L-type Ca^{2+} current, both of which increase the field-stimulated transient.[9-11,15] The significantly increased resting background fluorescence intensity, however, would suggest facilitated SR Ca^{2+} release.

Effect of Extracellular ATP on Ca^{2+} waves:

Spontaneous calcium waves are thought to result from calcium sparks, via the stochastic opening of groups of SR Ca^{2+}-release channels under calcium overload conditions.[21] Thus, it would be expected that calcium wave frequency should be proportional to the frequency of the stochastic openings of the group(s) of SR Ca^{2+}-release channels (ie spark frequency). The present results from sham experiments show that calcium wave frequency gradually increased over time under calcium-overload conditions, consistent with studies on calcium sparks,[22,31] and that 100 μmol/L ATP_o augmented this process. However, this ATP_o-induced increase in wave frequency may have resulted from an increase in the number of calcium sparks that successfully initiated calcium waves. Therefore, exposure of cardiomyocytes to ATP_o may not have changed calcium spark frequency, but may have increased calcium spark size. The present results are unable to distinguish between these two processes due to the temporal resolution of the images obtained (512 lines x 512 pixels in ~ 1.58 seconds). In order to overcome this problem, a faster scan mode and analysis of calcium sparks would have been more appropriate.

Does P_{2X}-purinoceptor Activation by ATP_o Increase Calcium Wave Frequency?

In support of both theories, recent work indicates that both calcium spark frequency and size increase with increasing resting $[Ca^{2+}]i$ (due to calcium overload)[21,22] and thus both may account for the increase in calcium wave frequency induced by ATP_o. Nevertheless, whilst the modest increase in mean calcium wave frequency in sham experiments (Figure 3B) can be attributed to the increase in resting $[Ca^{2+}]i$ (Figure 3A), and hence increased SR Ca^{2+} store load, it is unlikely that this "overload" mechanism resulted in the significant increase in calcium wave frequency induced by 100 μmol/L ATP_o as first hypothesised. The results from Figure 3A demonstrate that there was no significant difference between

the increase in calcium wave background fluorescence induced by the different ATP_o concentrations throughout the experimental period. In addition, during and following the period of prolonged ATP_o exposure, there was no difference in background fluorescence intensity between ATP_o-exposed cardiomyocytes and the sham experiments. This result is at odds with the proposed relationship between increasing resting $[Ca^{2+}]i$ and increasing calcium wave frequency. This is because there was only a significant increase in wave frequency, above that of sham experiments, upon exposure to 100 μmol/L ATP_o. This suggests that ATP_o is altering the wave frequency by a mechanism other than an increase in SR Ca^{2+} content induced by an increase in resting $[Ca^{2+}]i$ (via P_2 X-purinoceptors), as first proposed.

Thus, whilst the effects of ATP_o on the calcium transient have been attributed to P_{2X}-purinoceptor stimulation in other studies,[1,2,7] the present effects are unlikely to be the result of P_{2X}-purinoceptor stimulation. Furthermore, since P_{2X}-purinoceptors are ligand-gated ion channels, any effects of P_{2X}-purinoceptors on the calcium transients and waves should have been rapidly reversible upon the washout of ATP_o, a result also not supported by the present findings.

Does the Presence of IP_3 Explain the ATP_o-Induced Increase in Calcium Wave Frequency or Transient Intensity?

The present results demonstrate that the increase in calcium wave frequency and transient intensity induced by 100 μmol/L ATP_o persisted for at least 1.5 min following washout. In contrast, IP_3 in the cytosol of other cell types has been shown to have a half-life of approximately 10 -20 seconds,[32,33] and thus should have had rapidly reversible effects on the calcium transient and calcium waves after the washout of ATP_o. Therefore, the present results are not consistent with the actions of the second messenger IP_3. Furthermore, the expected decrease in calcium wave intensity and frequency were not observed during purinergic stimulation. However, the significant reduction in calcium transient intensity observed following the washout of 1 μmol/ ATP_o (Figure 1A) may suggest some involvement of IP_3.

Support for P_{2Y}-purinoceptor-Induced Second Messenger System

There appears to be a mechanism, independent of an increase in resting $[Ca^{2+}]i$ (via P_{2X}-purinoceptors), and IP_3 generation by which 100 μmol/L ATP_o increases calcium wave frequency and transient intensity. The results of this study support the involvement of P_{2Y}-purinoceptors and the presence of a second messenger system since purinergic activity continued following the washout of 1 and 100 μmol/L ATP_o (Figures 1, 3). Thus, the increase in calcium wave frequency may be a result of P_{2Y}-purinoceptor-induced changes in L-type Ca^{2+} channels and/or other unknown changes.[12,14,17] These may explain both the poorly reversible increases in transient intensity and calcium wave frequency observed following cardiomyocyte exposure to 100 μmol/L ATP_o, while taking into account similarities in resting $[Ca^{2+}]i$ and the short half-life of IP_3.

Evidence of Enhanced L-type Ca^{2+} Channel Opening by P_{2Y}-purinoceptors

The present findings may be better explained by the less accepted theory of calcium sparks in which the stochastic opening of L-type Ca^{2+} channels triggers localised calcium release from the SR.[34] This theory is not universally accepted because when the L-type Ca^{2+} current is completely inhibited, a reduced number of sparks are clearly present.[35] However, ATP_o has been demonstrated to increase the probability of open L-type Ca^{2+} channels,[14,17]

and thus the frequency of stochastic openings. This would result in a proportional increase in calcium spark frequency, and hence may account for the observed increase in calcium wave frequency induced by 100 μmol/L ATP_o. Furthermore, enhancement of L-type Ca^{2+} channel opening during field stimulation by ATP_o[12,14] explains the significant increase in the field-stimulated transient shown in Figure 1A.

Thus, the mechanism by which 100 μmol/L ATP_o increases calcium wave frequency may involve second messenger-induced changes in the stochastic opening frequency of the L-type Ca^{2+} channels. This theory is able to explain the lack of reversibility in the purinergic response to 100 μmol/L ATP_o following washout. However it is unable to account for the associated increase in background fluorescence.

Other Mechanisms Responsible for the Increase in Calcium Wave Frequency

Others have demonstrated that increasing SR Ca^{2+} store load and resting $[Ca^{2+}]i$ are associated with more frequent and larger calcium sparks.[21,22,31] Hence, it remains possible that 100 μmol/L ATP_o is increasing SR Ca^{2+} store load by some mechanism, possibly by increasing the uptake of Ca^{2+} into the SR. This may explain the lack of any differences in resting $[Ca^{2+}]i$ between the different groups (Figure 3A), since increased Ca^{2+} uptake would act to reduce the ATP_o-induced increase in resting $[Ca^{2+}]i$. Moreover, an increase in the rate of Ca^{2+} uptake into the SR may explain why calcium wave velocity and intensity did not decrease in the presence of 100 μmol/L ATP_o. The present findings suggest that even though there was a significant increase in wave frequency, the quantity of Ca^{2+} released during each propagating calcium wave did not change, causing no change in calcium wave velocity or intensity (Figures 3, 4). An increase in the rate of SR Ca^{2+} uptake would enable the SR Ca^{2+} stores to equilibrate such that the ATP_o-induced increase in calcium wave frequency would not significantly lower SR Ca^{2+} content. Hence there would be little or no decrease in SR Ca^{2+} release during each calcium wave over the duration of the experiments. However, an increase in SR Ca^{2+} uptake would also be expected to significantly decrease the resting $[Ca^{2+}]i$ following the washout of ATP_o. An observation not supported by the present results.

Summary

The operative mechanism of ATP_o-induced increase in cardiac contractility appears to be governed, at least in part, by ATP_o concentration. Calcium transient experiments have demonstrated that lower concentrations of ATP_o (around 1 μmol/L) are more likely to stimulate an increase in cardiomyocyte contractility through direct interaction of ATP_o with the L-type Ca^{2+}channels and to a lesser extent, $P_{2X/Y}$-purinoceptors. In contrast, calcium transient and wave experiments have demonstrated that higher ATP_o concentrations (100 μmol/L) appear to stimulate an unknown second-messenger system via P_{2Y}-purinoceptors. In addition, the present study rules out P_{2X}-purinoceptor stimulation and IP_3 generation as a predominant source of the ATP_o-induced increase in cardiac contractility. Thus, there appears to be another mechanism (other than IP_3) by which P_{2Y}-purinoceptors stimulate cardiac contractility. This mechanism may involve changes in the rate of Ca^{2+} uptake into the SR, or long term changes in the open probability of L-type calcium channels.

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Figures:

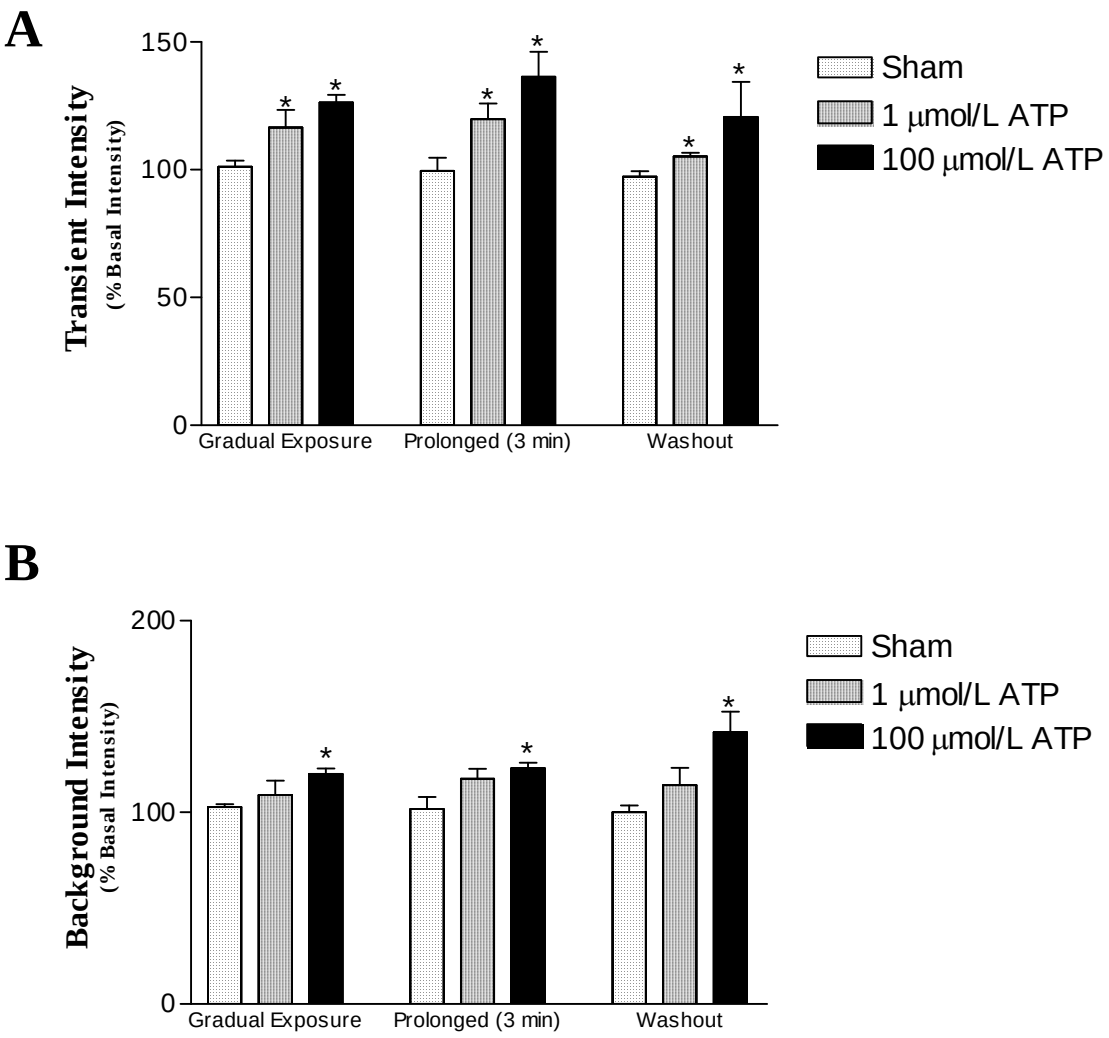

Figure 1. Effect of 1 and 100 μmol/L ATP_o exposure on **(A)** calcium transient intensity and **(B)** background fluorescence immediately prior to each calcium transient. Cells were superfused with Tyrode's solution containing 1.5 mmol/L $CaCl_2$, and stimulated at a rate of 0.5 Hz with pulse duration of 4ms at threshold. **(A)** Exposure of cardio-myocytes to 1 and 100 μmol/L ATP_o significantly increased transient intensity (n = 5 and 4 cells, respectively) throughout the experimental period. Washout significantly decreased the 1 μmol/L ATP_o-induced increase in calcium transient intensity, $P < 0{\cdot}05$. **(B)** 100 μmol/L ATP_o significantly increased background fluorescence intensity. Values presented are expressed as percentage of basal measurements made in control Tyrode's solution containing 1.5 mmol/L $CaCl_2$ at the beginning of each experiment (data not shown). Sham experiment results were obtained in the absence of ATP_o (n = 6). * Indicates statistical significance when compared to sham result obtained during same time period, $P < 0{\cdot}05$.

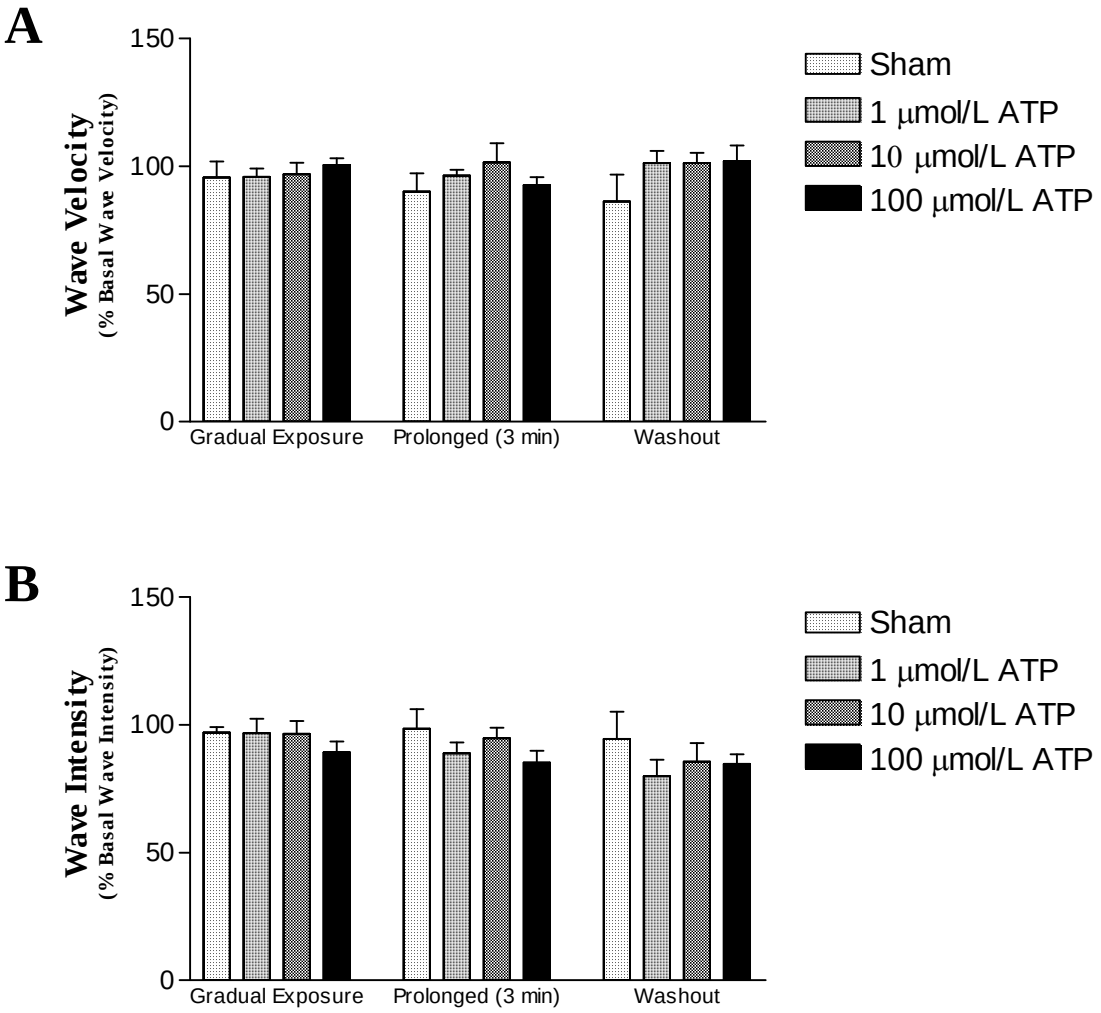

Figure 2. Effect of ATP_o exposure (1, 10 and 100 μmol/L) on **(A)** calcium wave velocity and **(B)** intensity for n = 7, 10 and 9 cells, respectively). Mean velocity and intensity values were calculated for calcium waves that appeared in the recorded 50 bitmap images. There was no significant change in calcium wave velocity or intensity when compared to sham experiments, upon exposure of cardiomyocytes to ATP_o. Values presented are expressed as a percentage of basal calcium wave velocity/intensity observed in control Tyrode's solution containing 5 mmol/L $CaCl_2$ at the beginning of each experiment (data not shown). Sham experiment results were obtained in the absence of ATP_o (n = 6).

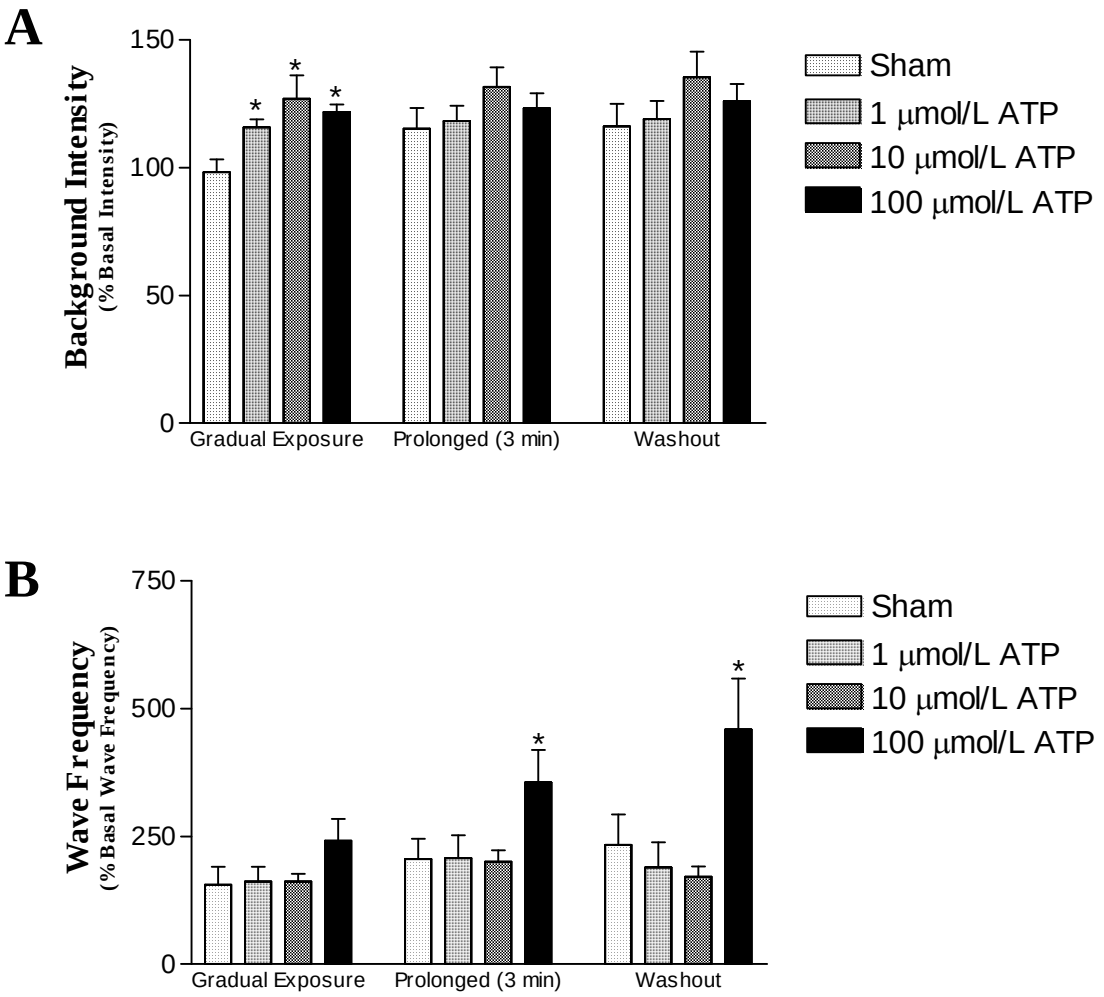

Figure 3. Effect of ATP_o exposure (1, 10 and 100 μmol/L) on **(A)** background fluorescence immediately prior to each calcium wave and **(B)** calcium wave frequency (for same cells in Figure 2). **(A)** Mean background fluorescence during sham experiments gradually increased from 98.2 $\pm$ 5.1 % to 116.1 $\pm$ 8.9 % over the experimental period ($P < 0.05$). Gradual exposure of cardiomyocytes to 1 -100 μmol/L ATP_o significantly increased background fluorescence above that of sham experiments. **(B)** Calcium wave frequency gradually increased from 100 $\pm$ 0.0 to 233.5 $\pm$ 59.4 % in sham experiments over the experimental period. Prolonged exposure of cardiomyocytes to 100 μmol/L ATP_o significantly increased calcium wave frequency above that of sham experiments. Values presented are expressed as a percentage of basal calcium wave background fluorescence/frequency observed in control Tyrode's solution containing 5 mmol/L $CaCl_2$ at the beginning of each experiment (data not shown). Sham experiment results were obtained in the absence of ATP_o ($n = 6$). * Indicates statistical significance when compared to sham result obtained during same time period, $P < 0{\cdot}05$.