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

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Introduction:

Adenosine 5'triphosphate (ATP) is often released as a cotransmitter with noradrenaline from sympathetic nerve endings including those that terminate in the heart (Forrester and Williams, 1987). This extracellular ATP (ATP_o) interacts with specific receptors on the surface membrane on a variety of cell types, including cardiomyocytes, resulting in an increase in contractility (Gordon, 1986). The mechanisms underlying the effects of extracellular ATP on the mammalian heart and isolated cardiomyocytes are not fully understood. Most investigations of the actions of ATP have concentrated on sacrolemmal ionic currents thought responsible for the changes in contractility. These studies have provided some useful insight into possible mechanisms. However variable results and conflicting conclusions suggest a different or more complex mechanism of action. Furthermore, it is not currently known whether extracellular ATP affects the calcium ion (Ca^{2+}) storage capacity of the sarcoplasmic reticulum (SR) or the calcium release process in cardiac cells. The aim of the present study is to observe the effects of extracellular ATP on internal calcium handling in isolated cardiomyocytes. This will be achieved by observing the effects that biological concentrations of extracellular ATP have on calcium waves in isolated rat cardiomyocytes.

Project Background:

Sources of extracellular ATP

As well as being a cotransmitter with noradrenaline released from sympathetic nerves that terminate in the heart, ATP is released during ischaemia and cardiac arrhythmias. Large quantities of ATP (ATP_o) are also liberated from damaged cardiac cells, activated platelets, and the endothelium during trauma and ischaemia caused by both physiological and pathophysiological conditions (Clemens and Forrester, 1980, Forrester and Williams, 1987). Under these conditions the amount of ATP present in the coronary circulation can approach several micromoles per litre (Gordon, 1986, Forrester and Williams, 1987). These observations suggest that ATP may be involved in the regulation or modulation of cardiac function during periods of cardiovascular stress.

Calcium Induced Calcium Release & Cardiac Contraction

In cardiac muscle, contraction is dependent upon a large transient increase in intracellular calcium ion concentration ([Ca^{2+}]i). The cardiac action potential opens voltage gated L-type Ca^{2+} channels on the t-tubule membrane, allowing a small influx of extracellular calcium (termed I_{Ca}^{2+}) into the cytoplasm of the cardiomyocyte. The release of intracellular calcium ions (Ca^{2+}) from the sarcoplasmic reticulum (SR) is then triggered by the opening of the Ryanodine receptor channels (RyR's) in response to this small increase in [Ca^{2+}]i. This results in a large flux of calcium from the SR into the cytoplasm, initiating contraction (Fabiato, 1983). This process of calcium release from the SR, induced by a small quantity of "trigger" calcium is termed Calcium-Induced Calcium release (CICR).

Physiological Effects of extracellular ATP on Cardiac Preparations

Recent studies based on whole mammalian heart models have shown that ATP_o can enhance the strength of contraction and thus increase cardiac output by altering the cardiac CICR mechanism (Song and Belardinelli, 1994, Mei and Liang, 2001). ATP_o is thought to exert this positive effect on cardiomyocytes by binding to either of 2 types of P_2-purinergic cell-surface receptors. P_2X-purinoceptors are a family of direct ligand-gated ion channels. Their activation by endogenous ATP_o is thought to enhance cardiac contractility by increasing intracellular [Ca^{2+}]i, without significantly increasing the heart rate (Mei and Liang, 2001, Hu *et al*. 2002). In contrast, P_2Y-purinoceptors are coupled to G-proteins, the majority of which activate phospholipase-C, leading to the production of inositol 1,4,5-trisphosphate (IP_3) and the subsequent release of calcium from intracellular stores (Hirano *et al*. 1991, Ralevic and Burnstock, 1991). There also exists a third P_1-purinoceptor class, which selectively bind adenosine and are linked to depressant effects on cardiac contractility.

Several observations support the concept that stimulation of P_2 receptors by ATP_o can enhance the performance of the intact heart. Firstly, Mei and Liang (2001) demonstrated in both the Langendorff and the work-performing rat heart models, that the P_2 receptor agonist ATP_o was able to stimulate a marked increase in the myocyte and whole heart contractile amplitude. Secondly, the stimulatory effect on cardiac function by ATP_o is present in more than one species. Similar to the data obtained in

work-performing rat heart, ATP_o stimulated significant increases in the force of contraction and rate of relaxation in the working mouse heart, as well as isolated rat, guinea pig and chick cardiomyocytes, (De Young and Scarpa, 1989, Hirano et al. 1991, Song and Belardinelli, 1994, Vassort, 2001, Hu et al. 2002).

In direct contrast to these observed results Kabbara, and Stephenson (1997) demonstrated in the intact rat heart, that exposure to1-8mM ATP_o immediately depressed systolic contractions and caused a marked, gradual elevation in the diastolic force level. They therefore demonstrated that large (millimolar) concentrations of extracellular ATP cause negative inotropy in rat ventricular muscle. Such negative inotropy induced by ATP was previously observed in the rat and ferret ventricle as well as in whole hearts (Qu *et al*. 1993, Vassort, 2001).

Thus, in both rat ventricular and isolated cardiomyocyte studies, multiple ATP_o related effects have been reported. It is possible that the differing observations are due to the rapid degradation of ATP to adenosine by enzymes present in intact cardiac tissue, or to its lability. In general, the negative inotropic effects of ATP_o on cardiac muscle have been attributed to its metabolism to adenosine and subsequent activation of adenosine (P_1) receptors (Gordon, 1986, Scamps et al. 1990). This theory is consistent with other studies, which have shown that in isolated cardiomyocytes, the stimulation of P_1-purinoceptors by adenosine is linked to depressant effects through mechanisms such as the activation of an outward hyperpolarising potassium current. Thus metabolism of ATP_o can complicate the results obtained by stimulating P_1 purinoceptors and other P_2 receptor sub-types present in the tissue. It is also possible that at higher concentrations, ATP may directly interact with P_1 receptors to induce the negative inotropic effects.

Effect of extracellular ATP on $[Ca^{2+}]i$

Consistent with the ATP-induced enhancement in heart contractility, ATP_o has also been shown to enhance the amplitude of electrically stimulated $[Ca^{2+}]i$ transients and contraction in field stimulated cardiomyocytes (Danziger et al. 1988, De Young and Scarpa, 1989). These studies have shown that ATP added to the extracellular solution bathing the cardiomyocytes activates an inward non-selective cation current

(Na^+, K^+ and to a lesser extent Ca^{2+}) termed I_{ATP}. The resulting partial depolarisation of the cell membrane increases the resting [Ca^{2+}]i concentration which may be attributed to the direct calcium influx through the non-selective cation channel (Puceat et al. 1991, Sheng et al. 1992). This increase in [Ca^{2+}]i allows the SR to become loaded with larger concentrations of calcium. Thus, following field stimulation, the L-type Ca^{++} channels are activated, promoting a larger transient increase in [Ca^{2+}]i caused by the increased SR calcium release and hence the stronger contraction. In contrast to these results, the ATP analogues AMP, ADP and adenosine (at concentrations of up to 2.5mM), had no significant effect on resting [Ca^{2+}]i (Christie et al. 1992, Vassort, 2001).

Effect of extracellular ATP on Transmembrane Currents

According to the accepted hypothesis, ATP increases [Ca^{++}]i by binding to P_2X-purinoceptors and partially depolarising the myocyte membrane leading to an increased [Ca^{2+}]i transient (Sheng et al. 1992). Recent studies have shown that rapid application of ATP (1μM to 100μM) to cardiomyocytes also elicits a rapid inward, non-selective cation current (I_{ATP}) (De Young and Scarpa, 1987, Hirano et al. 1991). This results in a transient depolarisation of the cell membrane (via I_{ATP}) that activates the L-type Ca^{2+} channels allowing an influx of Ca^{2+} from the extracellular medium. The resulting small increase in [Ca^{2+}]i may then initiate CICR and mobilise calcium from the SR, thereby transiently increasing [Ca^{2+}]i resulting in myocyte contraction. (Mei and Liang, 2001, Vassort, 2001). The order of agonist potency in eliciting this response in rat cardiomyocytes is as follows: ATP $\geq$ 2-MeSATP > α,β-met-ATP (2 slowly hydrolysable ATP analogues) > ADP, UTP, GTP were ineffective (Danziger *et al.* 1988, Hirano *et al.* 1991, Christie *et al*. 1992, Hu *et al*. 2002). However, the mechanism of action by which ATP can cause [Ca^{2+}]i to increase is still not clear. Some uncertainty appears to exist regarding the effect of ATP on the [Ca^{2+}]i transient responsible for cardiac contractility; both an increase (Christie *et al.* 1992, Hirano *et al.* 1993) and a decrease (Qu *et al*. 1993, Kabbara and Stephenson, 1997) have been demonstrated.

Removal of extracellular Calcium

Upon removal of extracellular calcium, the only source for a transient increase in $[Ca^{2+}]i$ is an intracellular calcium store, such as the SR. Consistent with the proposed mechanism, the majority of studies mentioned demonstrate that the ATP-induced $[Ca^{2+}]i$ transient is significantly suppressed without the influx of external Ca^{++} through the L-type Ca^{2+} channels (De Young and Scarpa, 1987, Danziger *et al.* 1988). This result indicates that extracellular calcium is essential for the ATP_o induced transient $[Ca^{2+}]i$ increase and increase in contractility. In contrast to this mechanism, Song and Belardinelli (1994) demonstrated that extracellularly applied ATP, alone, caused no significant membrane depolarisation of cardiomyocytes in the guinea pig model, and thus could not have activated the voltage gated L-type Ca^{2+} channels. Furthermore the $[Ca^{2+}]i$ response to ATP_o was not overly affected by the removal of extracellular calcium. This result suggests that the increase in $[Ca^{2+}]i$ is partially due to the mobilisation of calcium from intracellular stores and not by the influx of extracellular calcium as required by the CICR mechanism

Thus, several ionic mechanisms and their effect on intracellular Ca^{2+} regulation in cardiac cells have been proposed to mediate the positive (and negative) inotropic effects of extracellular ATP that have already been discussed. The majority of studies have however, have shown that agents which either diminish transmembrane calcium fluxes or reduce the magnitude of intracellular stores (such as caffeine and ryanodine application) decrease the transient increase in $[Ca^{2+}]i$ induced by ATP_o. This demonstrates that both calcium sources are involved in the observed increase in the $[Ca^{2+}]i$ transient (De Young and Scarpa, 1989). In contrast to the proposed mechanism of ATP_o-induced membrane depolarisation, in ferret ventricular myocytes an inhibition of L-type Ca^{2+} current by ATP_o in the same concentration range was observed (Qu *et al*. 1993). It is possible that these varying results reflect genuine species differences between the ferret, guinea pig and rat models. These variable effects of ATP_o may also be due to the presence of more than one subtype of purinergic receptor (e.g. P_{2x}, P_{2y} and P_1) in ventricular myocytes, where activation of both subtypes could produce opposing effects. Therefore, the overall response to extracellular ATP would depend upon which receptor subtype is more prominent.

Furthermore, a major downfall with these studies is that limited information on the regulation of intracellular calcium can be obtained using the calcium-fluorescent dye fura-2. This is because the calcium fluorophore may not be sufficiently sensitive to detect small changes in $[Ca^{2+}]i$ induced by ATP_o. In the current study, fluo-3 will be used to detect changes in $[Ca^{2+}]i$, as it exhibits a higher binding affinity for calcium ions and produces a larger fluorescence enhancement with calcium binding than does fura-2 (Molecular Probes, 2001). This will also allow detection of small changes in the gain of the CICR system caused by ATP_o which previous techniques have not detected. Differentiation of the calcium sources (extracellular and SR) is important for determination of the mechanism of ATP-receptor regulation of intracellular calcium handling in cardiomyocytes.

The Present Study

In the present study, use of a preparation of isolated adult rat cardiac ventricular myocytes will eliminate many of the interpretive difficulties of ATP metabolism in the whole heart system. Moreover, assessment of ATP's intracellular effects using other techniques would appear necessary in order to determine the true nature of the ATP response and how ATP receptor stimulation alters CICR and intracellular calcium handling (Hirano *et al.* 1991). Differentiation of these calcium sources and of calcium exchange between them is important for determination of the mechanism by which extracellular ATP alters the CICR mechanism. Calcium waves, in principle, may provide information regarding changes to the CICR mechanism induced by ATP_o. To date, experiments specifically involving the intracellular effects (i.e. calcium waves) of extracellular ATP or its slowly hydrolysable analogues have not been carried out. Such experiments would give specific information on the effects of P_2-receptor stimulation on calcium handling, that is, CICR and SR store load in isolated cardiomyocytes.

Calcium Waves

Propagating waves of raised calcium concentration are spontaneously generated when the SR becomes overloaded with calcium. This increases calcium leakage through stochastic opening of the calcium release channels (RyR's) to the point where CICR becomes locally regenerative. The propagation of a calcium wave is driven by the diffusion of released calcium ahead of a wavefront of CICR, produced when the free calcium reaches a threshold concentration. The propagation velocity of the wave in the cell thus depends primarily on the rate of diffusion of calcium in the cytoplasm and the threshold or "gain" of the CICR system (Ishide *et al.* 1990). Changes in calcium wave propagation can be monitored by incubating cells with a calcium-sensitive dye (such as fluo-3), whose fluorescence intensity depends upon the ambient calcium concentration ($[Ca^{2+}]i$). Thus a lower threshold (higher gain) will result in faster wave propagation, as a smaller concentration of trigger calcium is required to release calcium from the SR. Equally, a greater release of calcium from the SR will result in faster wave propagation since the calcium concentration at a given distance ahead of the wavefront is higher, and hence reaches threshold earlier during the approaching wave. By contrast, a decrease in the gain of the CICR system will result in a slower wave propagation due to a decrease in the calcium concentration at a given distance ahead of the wave. Furthermore, changes in resting $[Ca^{2+}]i$ between waves can also be monitored. Thus the effects of ATP_o on SR store load can be determined by monitoring the propagation velocity of spontaneous calcium waves (Ishide *et al.* 1990).

Aims and Hypotheses

The aims of this study are to observe the effects of biological concentrations of extracellular ATP on spontaneous and electrically induced calcium waves. The hypotheses are that P_2-purinoceptor activation by ATP_o will change calcium wave properties, primarily increasing wave velocity and resting $[Ca^{2+}]i$ as well as altering the frequency of spontaneous calcium waves.

Research Strategy:

Preparation of Heart

Experiments will be done on cardiomyocytes isolated from rat hearts. In brief, male Sprauge Dawley rats are fully anaesthetised with sodium pentabarbitone and the heart quickly excised and mounted on a plastic cannula attached to a Langendorff perfusion system. The heart is then perfused retrogradely through the aorta at the rate of 10ml/min with a Tyrode's based 1mM calcium solution to flush the heart of blood. The solution is then changed to a calcium free solution for 5minutes, followed by a crude bacterial collagenase and protease solution, (all are bubbled with 100% O_2 at 37^0C).

The basic Tyrode solution contains: 133.5mM NaCl, 10mM Hepes, 11mM D-glucose, 1.2mM NaH_2PO_4, 4mM KCl, 1.2 mM $MgCl_2$, pH adjusted to 7.4 with NaOH.

Isolation of heart Cells

Once the heart becomes flaccid and digested, the right ventricle is then removed and gently teased to dissociate the cells. The isolated cardiomyocytes are then stored in a basic Tyrode's solution supplemented with 2,3-Butanedione Monoxime and Bovine Serum Albumin. The calcium concentration of the storage solution is then progressively raised to 1mM by the addition 0.5M $CaCl_2$.

Fluorophore loading of isolated cardiomyocytes

The isolated cells suspended in the storage solution are plated onto laminin coated glass coverslips. The isolated cells are then loaded with fluo-3, a calcium-sensitive fluorescent dye, by incubating the cells in a solution containing the acetoxy-methyl ester form of the dye. Following fluo-3 loading, the dye is removed and replaced with 1mM Ca^{2+} Tyrode's solution, and incubated at room temperature to allow cellular enzymes to cleave the AM ester from the dye.

Individual coverslips are then placed in a perfusion chamber on the stage of a confocal microscope, and varying biological concentrations of ATP (from 100nM to 100μM) will be applied to bathing solution surrounding isolated cardiomyocytes. The effect of the ATP on spontaneous calcium waves will be observed using fluorescence confocal microscopy. Changes in fluorescence intensity (directly related to $[Ca^{++}]i$) will be recorded using Optiscan F900 computer software. The cells are used on the day of isolation and preparation.

Action potentials and hence $[Ca^{++}]i$ transients in isolated cells can be triggered using field stimulation (Trafford *et al.* 1993). Furthermore, it has been demonstrated that ATP application increases the $[Ca^{++}]i$ transient intensity in rat cardiomyocytes (Danziger et al. 1988, De Young and Scarpa, 1989). In the present study, the effects of ATP on both $[Ca^{++}]i$ transients and Ca^{++} waves will be observed in the same cardiomyocyte. Thus, the effects of ATP on the $[Ca^{++}]i$ transient will be used as a primary control for comparison with observed ATP-induced changes in calcium waves.

Significance and Outcomes

It is well accepted that β-adrenergic stimulation of the heart increases both heart rate and contractility and hence O_2 consumption . However P_2 receptor activation by ATP_o has been shown to increase heart contractility without the expense of a largely heart rate-related increase in O_2 consumption (Mei and Liang, 2001, Hu *et al*. 2002). This property may make such an agonist a beneficial agent in the treatment of left ventricular dysfunction and heart failure. The current study aims to further confirm the internal actions of ATP in cardiac myocytes by observing how ATP alters internal calcium handling by using calcium waves to monitor changes in the calcium-induced calcium release mechanism. It is anticipated that during this project we will develop a greater understanding as to how ATP alters calcium handling (CICR) within the cardiomyocyte. Moreover, the use of fluo-3 as a calcium indicator in these experiments will allow a more accurate assessment of the effects of ATP on CICR and any changes in the gain of this system.

References:

Christie. A, Sharma. V, & Sheu. S. 1992. Mechanism of extracellular ATP-induced increase of cytosolic Ca^{++} concentration in isolated rat ventricular myocytes. Journal of Physiology **445**: 369-388

Clemens. M, and Forrester. T. 1980. Appearance of adenosine triphosphate in the coronary sinus effluent from isolated working rat heart in response to hypoxia. Journal of Physiology **312**: 143-158

Danziger. R, Raffaeli. S, Moreno-Sanchez. R, Sakai. M, Capogrossi. M, Spurgeon. H, Hansford. R, and Lakatta. E. 1988. Extracellular ATP has a potent effect to enhance cytosolic Ca^{++} and contractility in single ventricular myocytes. Cell Calcium **9**(4): 193-199.

De Young. MB, and Scarpa. A. 1987. Extracellular ATP induces Ca^{++} transients in cardiac myocytes which are potentiated by norepinephrine. FEBS Letters **223**(1): 53-58

De Young. MB, and Scarpa. A. 1989. ATP receptor-induced Ca^{++} transients in cardiac myocytes: sources of mobilised Ca^{++} . The American Journal Of Physiology **257**(4 Pt 1): C750-C758

Fabiato. A. 1983. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. The American Journal of Physiology **245**(1): C1-C14

Forrester. T, and Williams. C. 1977. Release of Adenosine Triphosphate from isolated adult heart cells in response to hypoxia. The Journal of Physiology **268**(2): 371-390.

Gordon. J. 1986. Review Article: Extracellular ATP: Effects, Sources and fate Biochemistry Journal **233**: 309-319

Hirano. Y, Abe. S, Sawanobori. T, and Hiraoka. M. 1991. External ATP-induced changes in [Ca^{++}]i and membrane currents in mammalian atrial myocytes. The American Journal Of Physiology **260**(4 Pt 1): C673-680

Hirano. Y, Sawanobori. T, and Hiraoka. M. 1993. External ATP induced increase in intracellular free Ca^{++} in mammalian atrial myocytes. Japanese journal of physiology **43**, suppl.1: S101-S103, 1993.

Hu. B, Senkler. C, Yang. A, Soto. F, and Liang. B. 2002. P2X4 receptor is a glycosylated cardiac receptor mediating a positive inotropic response to ATP Journal of Biological Chemistry **277**(18): 15752-7

Ishide. N, Urayama. T, Inoue. K, Komaru. T, and Takishima. T. 1990. Propagation and collision characteristics of calcium waves in rat myocytes. The American Journal of Physiology **259**(3 Pt 2): H941-H950

Kabbara. A, and Stephenson. D. 1997. Ca^{++} handling by rat myocardium exposed to ATP solutions of different [Ca^{++}] and Ca^{++} buffering capacity. The American Journal Of Physiology **273**(3 Pt 2): H1347-1357

Mei. Q and Liang. B. 2001. P2 purinergic receptor activation enhances cardiac contractility in isolated rat and mouse hearts. American Journal of Physiology, Heart Circulation Physiology
281(1): H334–H341.

Molecular Probes Online Handbook. 2001. Indicators For Ca^{++} , Mg^{++},Zn^{++} and other metals, Chapter 20: Sections 20.2 and 20.3
www.probes.com/handbook/sections/2000.html

Puceat. M, Clement. O, Scamos. F, and Vassort. G. 1991. Extracellular ATP-induced acidification leads to cytosolic Calcium transient rise in single cardiac myocytes. Biochemistry journal **274**(1): 55-62

Qu. Y, Himmel. H, Campbell. D, and Strauss. H. 1993. Effects of extracellular ATP on Ica, [Ca^{++}]i, and contraction in isolated ferret ventricular myocytes. The American Journal of Physiology **264**(3 Pt 1): C702-708

Ralevic. V, and Burnstock. G. 1991. Roles of P_2-Purinoceptors in the Cardiovascular system
Circulation, **84**(1): 1-14

Scamps. F, Legssyer. A, Mayoux. E, and Vassort, G. 1990. The Mechanism of Positive Inotropy Induced by Adenosine Triphosphate in Rat Heart. Circulation Research **67**(4): 1007-1016

Sheng. J, Christie. A, De Young. MB, Levy. M, and Scarpa. A. 1992. Synergism between cAMP and ATP in signal transduction in cardiac myocytes. The American Journal Of Physiology, **262** (2 Pt 1): C128-135

Song. Y, and Belardinelli. L. 1994. ATP Promotes development of afterdepolarisations and triggered activity in cardiac myocytes. The American Journal of Physiology **267** (5 Pt 2): H2005- 2011

Trafford. A, O'neill. S, Eisner. D. 1993. Factors affecting the propagation of locally activated systolic Ca^{++} transients in rat ventricular myocytes European Journal Of Physiology **425**: 181-183

Vassort. G. 2001. Adenosine 5'-Triphosphate: a P2-Purinergic Agonist in the myocardium
Physiological Reviews **81**(2): 767-806