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Phospholipase C and cAMP-dependent positive inotropic effects of ATP in mouse cardiomyocytes via P2Y₁₁-like receptors

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Abstract

ATP is released as a cotransmitter together with catecholamines from sympathetic nerves. In the heart ATP has been shown to cause a pronounced positive inotropic effect and may also act in synergy with β -adrenergic agonists to augment cardiomyocyte contractility. The aim of the present study was to investigate the inotropic effects mediated by purinergic P2 receptors using isolated mouse cardiomyocytes.

Stable adenine nucleotide analogs were used and the agonist rank order for adenine nucleotide stimulation of the mouse cardiomyocytes was AR-C67085 > ATP γ S > 2-MeSATP $\gg$ 2-MeSADP = 0, that fits the agonist profile of the P2Y₁₁ receptor. ATP γ S induced a positive inotropic response in single mouse cardiomyocytes. The response was similar to that for the β_1 receptor agonist isoproterenol. The most potent response was obtained using AR-C67085, a P2Y₁₁ receptor agonist. This agonist also potentiated contractions in isolated trabecular preparations. The adenylyl cyclase blocker (SQ22563) and phospholipase C (PLC) blocker (U73122) demonstrated that both pathways were required for the inotropic response of AR-C67085. A cAMP enzyme immunoassay confirmed that AR-C67085 increased cAMP in the cardiomyocytes. These findings are in agreement with the P2Y₁₁ receptor, coupled both to activation of IP₃ and cAMP, being a major receptor for ATP induced inotropy. Analyzing cardiomyocytes from desmin deficient mice, *Des*^{-/-}, with a congenital cardiomyopathy, we found a lower sensitivity to AR-C67085, suggesting a down-regulation of P2Y₁₁ receptor function in heart failure.

The prominent action of the P2Y₁₁ receptor in controlling cardiomyocyte contractility and possible alterations in its function during cardiomyopathy may suggest this receptor as a potential therapeutic target. It is possible that agonists for the P2Y₁₁ receptor could be used to improve cardiac output in patients with circulatory shock and that P2Y₁₁ receptor antagonist could be beneficial in patients with congestive heart failure (CHF).

Keywords

P2 receptors; Heart; Cardiomyocytes; ATP; cAMP; IP₃; Desmin; Contractility

1. Introduction

Extracellular adenosine 5'-triphosphate (ATP) exerts various potent actions in the cardiovascular system, e.g. regulation of vascular tone and platelet aggregation [1-5]. In the cardiomyocyte, ATP has been shown to cause a pronounced positive inotropic effect and may also act in synergy with β -adrenergic agonists to augment myocyte contractility [3,6-9].

ATP is released as a cotransmitter together with catecholamines from sympathetic nerves but it may also be released from other sources in the heart such as endothelium, platelets, red blood cells and ischemic myocardium [3,10,11]. Using microdialysis, ATP in the interstitial space has been estimated to be 40 nM but the levels may increase markedly during electrical stimulation, ischemia, challenge with cardiotonic agents, increase in blood flow, mechanical stretch and increased work load (for review see Vassort, 2001). At the place of release the concentration of ATP is high, but within seconds it is rapidly degraded into ADP, AMP and adenosine by ectonucleotidases.

Extracellular ATP mediates its effects via membrane bound P2 receptors [12]. P2 receptors can be divided into two classes: ligand-gated intrinsic ion channels, P2X receptors (P2X₁-P2X₇), and G-protein-coupled P2Y receptors [13,14]. P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₁ receptors are coupled to G_q, promoting phospholipase C (PLC) catalyzed generation of inositol phosphates (IP₃) and subsequent mobilization of intracellular calcium. P2Y₁₂, P2Y₁₃ and P2Y₁₄, are coupled to G_i inhibiting adenylyl cyclase.

In heart, ATP has been shown to stimulate an increase in cytosolic calcium and evidence for the involvement of IP₃ coupled P2Y₂ receptors and ion-channel coupled P2X receptors have been presented [7,8,15-17]. Increase of cAMP mediates the inotropic effects of catecholamines acting on β -receptors and antagonists of these receptors are important drugs for blood pressure lowering and reduce mortality in congestive heart failure (CHF). ATP stimulates increase in cAMP in cardiac myocytes and may act in synergy with the β_1 -adrenergic agonist isoproterenol by differential activation of adenylyl cyclase isoforms [3,18,19]. However, the ATP receptor mediating this increase in cAMP has not been related to any particular P2 receptor subtype in cardiac cells (Vassort, 2001). A candidate that has been suggested (Vassort, 2001), is the P2Y₁₁ receptor that is additionally coupled to G_s and activates adenylyl cyclase [20-22]. The P2Y₁₁ receptor has previously not been shown to be involved in heart physiology. It seems to play a role in the immune system in granulocytic differentiation [23] and dendritic cell maturation [24].

mRNA for the P2Y₁₁ receptor has been detected in both atria and ventricles of human hearts [25]. Furthermore, 2-propylthio- β , γ -dichloromethylene-d-ATP (AR-C67085) has been shown to be a specific agonist for the P2Y₁₁ receptor [21], giving us a tool to discriminate between the different ATP receptors. The purpose of the present study was therefore to examine a possible role for the P2Y₁₁ receptor in mediating the positive inotropic effects of ATP. We also examined the response to the P2Y₁₁ receptor agonist on cardiomyocytes from transgenic (desmin deficient) mice with cardiomyopathy [26-28] to further explore if alterations in P2Y₁₁ receptor responses occur in the heart in pathophysiological conditions.

2. Methods

2.1. Animals

The major part of this study was performed using adult female NMRI mice (B & K AB Sollentuna). We also examined genetically modified desmin deficient mice (*Des*^{-/-}) and their wild type controls (*Des*^{+/+}), obtained of the strain C57BL/6J in the laboratory of Dr. Li et al. [26] at University Paris VII. The animals were kept in the university animal facilities

with free access to food and water according to regulations of the local animal ethics committee. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). The local animal ethics committee approved the experiments.

2.2. Isolation of cardiomyocytes

The mice were killed by cervical dislocation and the hearts were dissected and transferred to a Krebs–Henseleit solution containing (in mM): 4.74 KCl, 25 NaHCO₃, 1.19 MgCl₂, 1.19 KH₂PO₄, 11 glucose, 118 NaCl, 2.5 CaCl₂. The hearts were gently massaged to avoid blood clotting in the ventricles. The aorta was cannulated and perfused with Krebs–Henseleit solution and its beating was stopped by perfusion with a cardioplegic solution (Krebs–Henseleit solution with addition of 30 mM KCl). The hearts were connected to a pump with a flow of 2.2 ml/min and perfused first with 37 °C Krebs–Henseleit solution oxygenated with O₂/CO₂ (95%/5% resulting in a pH of 7.4) containing 2.5 mM Ca²⁺ for 5 min and then perfused for 6 min with an oxygenated Hepes-buffered control solution containing 1 mg/ml bovine serum albumin (BSA) and in mM 133.5 NaCl, 4 KCl, 1.2 Na₂HPO₄, 1.2 MgSO₄, 10 Hepes, 11 glucose, 10 2,3-butanedione monoxime (BDM), pH 7.4 at 37 °C. The cells were isolated using perfusion with the same Hepes-buffered solution containing 0.3 mg/ml Collagenase D (0.39 U/mg, Boehringer Mannheim) and 25 μM Ca²⁺ for 13 min. The hearts were thereafter removed and cut into pieces and incubated at 37 °C in the control solution with 100 μM Ca²⁺ and 1% BSA during shaking for 2–3 min. Cells were then collected through a mesh and slowly equilibrated to higher Ca²⁺ concentration via two washing procedures which also minimized the number of fibroblasts and macrophages in the cell suspension. The first washing solution was the control solution containing 10% BSA and 0.36 mM Ca²⁺ and the second wash 6% BSA with 1.8 mM Ca²⁺ [29].

2.3. Cardiomyocyte contractility

The cells could be kept with unaltered properties for up to 4 h in room temperature. The cells were stimulated electrically in Krebs–Henseleit (1.8 mM Ca²⁺) solution adjusted to give pH 7.4 at 22 °C and oxygenated with O₂/CO₂. Stimulation voltage was set to 20% above threshold and impulse duration was 4 ms at 0.5 Hz. To observe cell shortening we used an inverted microscope (TMD, Nikon, Japan), an image processor (Hamamatsu, Japan) and a video recording system. The video signal was digitized at 25 Hz. The cell length during shortening was analyzed using an optical edge tracking method. The shortening amplitude, measured in pixel units on the digitized images was compared before and after addition of substance to the electrically stimulated cardiomyocyte in the cuvette. For each condition the maximal shortening amplitude was determined for an individual cell prior to and after addition of the drug (average amplitude of three contractions). The contractile amplitude following addition of the drug was expressed relative to control amplitude prior to the drug administration (about 5% shortening of the cell length). The maximal responses were obtained within 1 min and the average contractility at 1 min was determined. The cells were stimulated using isoproterenol, adenine nucleotide analogues (2-methylthio-adenosine diphosphate (2-MeSADP), 2-methylthio triphosphate (2-MeSATP), adenosine 5'-O-(1-thiotriphosphate) (ATPγS), 2-propylthio-β,γ-dichloromethylene-*D*-ATP (AR-C67085), N⁶-(2-Methylthioethyl)-2-(3,3,3-trifluoropropylthio)-*D*-ATP (AR-C69931)), apyrase and adenosine. To further investigate the signaling pathway of the response after stimulation with the contracting cardiomyocytes were incubated with SQ22563 (10 μM), an inhibitor of adenylyl cyclase, which decreases the production of cAMP. The PLC and IP₃ pathway after stimulation with AR-C67085 was studied, when inhibiting the production of IP₃ using the PLC inhibitor U73122 (10 μM).

Cardiomyocytes from desmin deficient mice were isolated essentially as described above, but with extended collagenase incubation (17 min). The cells were stimulated with AR-C67085.

2.4. Isolated trabeculae

The mouse heart was excised from the thorax and gently massaged in cardioplegic solution. The heart was then perfused with the cardioplegic solution via aorta. The left ventricle was opened and trabecular preparations were excised. The preparations were mounted with silk thread between a steel rod and a force transducer (AE 801, SensoNor, Horten, Norway) in a 37 °C Krebs–Henseleit solution oxygenated with O₂/CO₂ (95%/5%). The trabeculae were electrically stimulated just above threshold at a frequency of 0.5 Hz and 4 ms pulse duration.

2.5. cAMP assay

Cardiomyocytes were isolated as described above. After 1 h resting at 37 °C, the myocytes were stimulated with AR-C67085 (10 µM) for 10 min. The concentration of cAMP was determined in duplicate in unstimulated and stimulated cells using the cAMP Enzyme Biotrak (EIA) System (Amersham Biosciences).

2.6. RNA extraction and RT-PCR assay

Total RNA was prepared from cardiomyocytes using a RNeasy column (Qiagen, CA, USA), according to the manufacturer's protocol. The RNA was then reverse-transcribed using Multiscribe RT Kit (Qiagen), as described by the manufacturer.

2.7. Real time PCR

Real time PCR reactions on RNA were performed with primer pairs designed using the VectorNTI software (Invitrogen, Informax, UK). The transcribed cDNA was amplified in a LightCycler (Roche, Basel, Switzerland) in a 10 µl PCR containing 3 mM MgCl₂, 0.5 µM of each primer (see below) and 1 × LightCycler DNA Master SYBR Green I mix (Roche). The samples were incubated for an initial denaturation at 95 °C for 600 s, followed by 45 PCR cycles. Each cycle consisted of 95 °C for 1 s, 55 °C for 6 s, and 74 °C for 23 s. SYBR Green I fluorescence emission was monitored after each cycle and mRNA levels of the receptors were quantified by using the second-derivative maximum method of the Light-Cycler software. For each experiment, a baseline was set just above the background and the quantitative results were obtained by determination of crossing point values, which mark the cycle number at which sample fluorescence crosses a predetermined value. The amount of receptor was expressed relative to the housekeeping gene GAPDH. To confirm amplification of specific transcripts, melting curve profiles (cooling the sample to 65 °C and heating slowly to 95 °C with continuous measurement of fluorescence) were produced at the end of each PCR. The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis. To amplify P2Y₁ forward (fw) GACTGACTGGATCTTCGGGGA and reversed (rd) CCACCACAATGAGCCACACC were used. For P2Y₂ fw GCTTCAACGAGGACTTCAAG, rw GTAATAAACCAACAGCGGCA; P2Y₄ fw TCACTTGCCATGACACCTCGG, rw AATGGTGCGCACAGACTTGC; GAPDH fw GGTCATCCCAGAGCTGAACG, rw TTGCTGTTGAAGTCGCAGGA.

2.8. Drugs

Isoproterenol, 2-MeSADP, 2-MeSATP, ATP_γS, SQ22563, U73122, apyrase and adenosine were purchased from Sigma Co., USA. AR-C69931 and AR-C67085 was a gift from Astra-Zeneca, Mölndal. All the drugs were dissolved in 0.9% saline.

2.9. Calculation and statistics

Calculations and statistics were performed using the Graph Pad Prism 3.02 software. The negative logarithm of the drug concentration that elicited 50% relaxation (pEC_{50}) was determined by fitting the data to the sigmoidal dose-response curve equation. E_{max} refers to maximum contraction induced by agonist calculated as the percentage of the corresponding contraction without agonist. Statistical significance was accepted when $P < 0.05$. Raw data submitted to paired Student's t -test. In the analysis of the cAMP assay one-tailed, paired analysis was used. Values are presented as mean $\pm$ S.E.M. and n denotes the number of cells if otherwise not stated.

3. Results

3.1. Cardiomyocyte cell shortening

The amplitude of cardiomyocyte shortening was increased following addition of inotropic substances, such as the selective β_1 -agonist isoproterenol. The amplitude of cardiomyocyte shortening due to electrical stimulation in the absence of drug was $9.3 \pm 0.6 \mu m$. The mean relaxed cardiomyocyte length was $257 \pm 12 \mu m$. Isoproterenol ($1 \mu M$) caused $65 \pm 17\%$ ($n = 24$) increase in the cardiomyocyte shortening amplitude, i.e. 14.9 ± 2.1 . An increase in shortening was also observed after addition of the stable 2-MeSATP ($1 \mu M$) as well as ATP γ S ($1 \mu M$) causing an increase in the myocyte contractility ($34 \pm 16\%$, $n = 12$, resp. $65 \pm 17\%$, $n = 17$, corresponding to 10.2 ± 1.0 resp. 14.9 ± 2.2). Stimulation with the P2Y₁, P2Y₁₂ and P2Y₁₃ receptor agonist, 2-MeSADP, did not result in any significant change in myocyte contraction, neither did adenosine ($-2 \pm 12\%$, $n = 11$ resp. $-12 \pm 14\%$, $n = 6$). Stimulation with AR-C67085, a blocker for P2Y₁₂ but also an agonist for the human P2Y₁₁ receptor, induced a significant inotropic response ($91 \pm 19\%$, $n = 33$, i.e. 18.5 ± 2.1). Results are presented in Fig. 1. Fig. 2 shows traces of cardiomyocyte contractions, with increased cell shortening in response to isoproterenol, AR-C67085 and 2-MeSATP.

The inotropic response to AR-C67085 ($1 \mu M$) and to ATP γ S ($1 \mu M$) was inhibited by the adenylyl cyclase inhibitor, SQ22563 ($10 \mu M$, change in contraction amplitude: $3 \pm 7\%$ $n = 12$ resp. $-5 \pm 6\%$ $n = 23$). To exclude that the response to AR-C67085 was mediated by its P2Y₁₂ antagonistic action or by ectonucleotidase inhibition control experiments were performed using AR-C69931 (P2Y₁₂ antagonist), $1 \mu M$, and apyrase (nucleotidase), $1 U/ml$. No significant change in myocyte shortening was obtained (AR-C69931 $-3 \pm 8\%$ $n = 29$, apyrase $-2 \pm 14\%$ $n = 14$, three to six mice) suggesting that these two mechanisms are not involved in the action of AR-C67085.

Inhibiting the PLC pathway using U73122 ($10 \mu M$) also resulted in a total inhibition of the inotropic response to AR-C67085 (Fig. 3). Contractility was not altered by addition of the adenylyl cyclase inhibitor SQ22563 alone ($-6 \pm 9\%$ $n = 21$, n.s.) neither by addition of the PLC inhibitor U73122 ($-2 \pm 14\%$ $n = 15$, n.s.). The results are presented in Fig. 3. As a control for the efficiency of SQ22563 as an adenylyl cyclase inhibitory substance we incubated cells with SQ22563 and thereafter stimulated with isoproterenol. The inotropic response of isoproterenol was absent under these conditions ($5 \pm 15\%$ $n = 8$, n.s.).

3.2. cAMP assay

After 1 h at $37^\circ C$, the cardiomyocytes were stimulated with AR-C67085 ($10 \mu M$) or ATP γ S ($10 \mu M$) for 10 min. Stimulation using AR-C67085 was also performed in presence of U73122 ($10 \mu M$). The concentration of cAMP was then determined in duplicate using the cAMP Enzyme assay. The cAMP levels were significantly increased by both AR-C67085 and ATP γ S, $60 \pm 31\%$, $n = 11$ for AR-C67085, resp. $79 \pm 24\%$, $n = 7$ for ATP γ S. No

significant elevation of cAMP was obtained adding AR-C67085 to cardiomyocytes blocked with U73122. The results are presented in Fig. 4. Data expressed relative to control value.

3.3. Isolated trabecular preparations

To study the effect of AR-C67085 on the heart tissue level, we examined trabecular preparations. One original recording of a preparation stimulated electrically with addition of AR-C67085 is shown in Fig. 5a. We noticed an increase in contractility with about 23% ($E_{\max} = 25\% \pm 8$, $EC_{50} = 6.9 \pm 0.5 -\log M$, $n = 7$) (Fig. 5b). We used isoproterenol ($0.1 \mu M$) as a control for an inotropic substance, which gave a 90% ($90 \pm 24\%$, $n = 4$) increase in contractility of the isolated trabeculae.

3.4. Desmin deficient mice

Experiments were performed on cardiomyocytes obtained from desmin deficient mice, *Des*^{-/-}, and their wild type controls, *Des*^{+/+}. Due to limited animal availability the present study included cells isolated from three *Des*^{+/+} and two *Des*^{-/-} mice. The cardiomyocyte shortening due to electrical stimulation in the absence of drug was 13.3 ± 3.3 for *Des*^{+/+} and $10.6 \pm 2.0 \mu m$ for *Des*^{-/-}, *ns*. No difference in relaxed cell length was noted (*Des*^{+/+}: 262 ± 8 ; *Des*^{-/-}: $260 \pm 12 \mu m$). The mean data from eight cells in each group are presented in Fig. 6 showing a significantly lower increase in contractile response to AR-C67085 ($0.1 \mu M$) in the *Des*^{-/-} cardiomyocytes. No significant difference in response to maximal dose ($1 \mu M$) AR-C67085 was detected between *Des*^{+/+} and *Des*^{-/-} cardiomyocytes.

3.5. mRNA quantification

The expression of purine receptor mRNA in the myocytes was quantified. P2Y₁, P2Y₂, P2Y₄. P2Y₁ was the most highly expressed ($156\% \pm 24$, expressed as percent of P2Y₂ expression). P2Y₄ had an expression of $133\% \pm 19$ expressed as percent of P2Y₂ expression.

4. Discussion

In this study inotropic effects of adenine nucleotides were studied in cardiomyocytes from mouse. Different ATP analogs were examined and the most potent response was obtained using AR-C67085, a P2Y₁₁ receptor agonist. The response to AR-C67085 recruited a P2Y₁₁ receptor like signaling pathway associated with changes in both cAMP and IP₃. Analyzing the cardiomyocytes from the desmin deficient mice, *Des*^{-/-}, with cardiomyopathy, we found a lower sensitivity to AR-C67085.

To limit the contribution of ATP degradation products (ADP and adenosine) we used the more stable ATP analogue ATP γ S. In this study ATP γ S induced a potent positive inotropic effect in isolated cardiomyocytes to a similar degree as isoproterenol. ATP γ S is an agonist acting on P2Y₁₁ but also on P2Y₂, P2Y₄ and on P2X receptors. Significant, but somewhat weaker inotropic effects were also seen for 2-MeSATP, acting on P2Y₁ and P2Y₁₁ receptors. However, 2-MeSATP is also an agonist for P2Y₁ and P2X receptors. Involvement of agonistic effects via the ADP receptors P2Y₁, P2Y₁₂ and P2Y₁₃ receptors was excluded by the lack of effect by their agonist 2-MeSADP. Furthermore, no inotropic effects were obtained when stimulating the cardiomyocytes with adenosine, excluding contribution of adenosine receptors.

The ATP derivative AR-C67085 is a potent inhibitor of P2Y₁₂ receptors, but also a potent agonist of the human P2Y₁₁ receptor [21,30]. AR-C67085 induced a positive inotropic effect in the cardiomyocytes that was of equal or even of higher magnitude than for ATP γ S and isoproterenol. As far as we know, AR-C67085 is not an agonist of any other P2 receptor except P2Y₁₁, giving strong support for our hypothesis that ATP mediates inotropic effects

via P2Y₁₁ receptors. The inotropic effects of the different examined P2 receptor agonists are in agreement with the agonist rank order for stimulation of the human P2Y₁₁: ATP γ S = AR-C67085 > ATP > 2-MeSATP $\gg$ 2-MeSADP = 0 [21].

There is a continuous release of nucleotides from most cells creating a background level of naturally occurring nucleotides [10,12]. Theoretically it could be argued that AR-C67085 mediated its effect by inhibition of endogenous ADP acting on P2Y₁₂ receptors. This double negation with inhibition of P2Y₁₂ G_i-mediated cAMP inhibition could then result in increased cAMP and positive inotropic effects. We therefore tested the related compound AR-C69931 that is an inhibitor of the P2Y₁₂ receptor but with no P2Y₁₁ receptor selectivity. However, no inotropic effect was obtained when stimulating the cardiomyocytes with AR-C69931. Furthermore, the nucleotidase apyrase did not result in any inotropic effects.

AR-C67085 could mediate its positive inotropic effects after degradation or conversion to other purines or by contamination in the sample. In order to characterize this batch of substance AR-C67085 was tested on rat mesenteric artery using vascular tissue bath. For methods see Malmjö et al. [31]. No contractile or dilatatory effects were detected, excluding contaminating or degradation products acting on contractile P2X or P2Y receptors, or dilatatory P2Y receptors (data not shown). This also supports the selective agonistic effects of AR-C67085 on P2Y₁₁ receptors.

The P2Y₂, P2Y₄ and P2Y₆ receptors couples to G_q, activating PLC, increasing inositol triphosphate (IP₃), resulting in increased intracellular calcium level. The P2Y₁₁ receptor has a unique feature; it couples to both G_q and G_s, acting through a PLC coupled pathway and an adenylyl cyclase coupled signaling pathway [20-22]. Interestingly, we found that inhibition of either pathway abolished the total inotropic response of AR-C67085, suggesting that both pathways are required for the inotropic response. These results indicate a potent inotropic effect in mouse heart mediated by a P2Y₁₁-like receptor pathway. We could reproduce the cAMP stimulation in cardiomyocytes in response to ATP γ S previously demonstrated by Puceat et al. [19]. Furthermore, AR-C67085 had a similar stimulatory effect on cAMP as ATP γ S indicating that the P2Y₁₁ receptor could mediate a major part of the ATP effect.

Effects of 2-MeSATP, on mouse heart has been examined by Mei and Liang [9] concluding an effect mediated by P2X receptors due to minor effects on the response of the PLC inhibitor U73122. In that study a cAMP inhibitor was not used. Previous stimulation of cardiomyocytes using $\alpha\beta$ -MeATP have not showed any inotropic effect, excluding P2X₁ mediating the ATP effects but suggesting other P2 receptors [8]. A P2X₄ mediated response to 2-MeSATP has been proposed [32]. The full inhibition of the ATP γ S response seen for the adenylyl cyclase inhibitor SQ22563 in the present study indicates that most of the ATP effect was mediated via P2Y receptors and not ion channel coupled P2X receptors in our preparation.

The inotropic effect induced by AR-C67085 was also confirmed studying isolated mouse trabeculae. This experiment confirms that stimulation with AR-C67085 most likely via P2Y₁₁ receptors increases shortening amplitude of isolated cardiomyocytes as well as contractile strength in intact heart tissue.

P2Y₁₁ receptor mRNA has been detected in human heart [25], but no information is available about expression of P2Y₁₁ in mouse heart, since the gene has not been cloned in mouse or rat. So far P2Y₁₁ is only cloned in man and dog [20,22].

We quantified the mRNA expression of the P2 receptors and P2Y₁ was found to be the highest expressed P2 receptor. This is surprising because repeated stimulation with the P2Y₁ receptor agonist 2-MeSADP [33-36] did not result in any inotropic effects. The presence of

P2Y₁ receptor mRNA and the lack of inotropic effects of ADP analogues in cardiomyocytes need to be investigated further. P2Y₁ receptors could mediate other important effects in the heart.

As discussed above, our data indicate that ATP acting on P2Y₁₁ receptors may have positive inotropic effects mediated via cAMP. This is in homology with their sympathetic co-transmitters, the catecholamines acting on β_1 -receptors activating cAMP pathway. Sympathetic activation is of basal physiological importance to raise blood pressure and increase cardiac output. It is possible that ATP in the heart contributes to this effect, but this needs to be examined with specific P2Y₁₁ antagonists that are not available at the moment. On the other hand, chronic sympathetic activation can be deleterious, as exemplified in patients with CHF. β_1 -Receptor blockers have been highly successful in the treatment of CHF reducing both mortality and morbidity. It would be interesting to examine if P2Y₁₁ receptor blockers could have similar effects.

Another effect of the chronic sympathetic activation in CHF is a down-regulation of β_1 -receptors [37]. To examine if a similar attenuation of P2Y₁₁ receptor expression is seen in CHF, we used a mouse model for CHF, where the desmin gene is ablated [26]. Desmin is a 53 kDa protein forming intermediate filaments at the Z-disk of the sarcomere, anchoring the contractile filaments myosin and actin, aligning the sarcomere structures and connecting sarcomeres and the sarcolemma. The phenotype of the desmin deficient mouse, *Des*^{-/-}, is cardiomyopathy with hypertrophic left ventricle wall and regions of calcifications as well as general muscle weakness [27,28,38-43]. We found a decreased sensitivity to the P2Y₁₁ receptor agonist AR-C67085. One explanation could be a down-regulation of this receptor function in the cardiomyocytes in a similar manner as seen for β_1 -receptor in CHF.

5. Conclusion

We present data suggesting that P2Y₁₁ is expressed in the heart myocytes mediating ATP stimulated positive inotropic effects. The signaling pathway includes activations of adenylyl cyclase with an increase of cAMP and was dependent on IP₃ generation. Both pathways seem to be required for an inotropic response. Analyzing cardiomyocytes from desmin deficient mice which have a cardiomyopathy, we found a decreased response to AR-C67085. Several similarities with the sympathetic nervous system and adrenergic effects in the heart are striking. Both ATP and catecholamines are released from sympathetic nerves, acting through cAMP-stimulating receptors to mediate positive inotropic effects. It is possible that agonists for the P2Y₁₁ receptor could be used to improve cardiac output in patients with circulatory shock. However, an even more important drug candidate would be a P2Y₁₁ receptor antagonist that may be beneficial in patients with CHF.

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Fig. 1.

β_1 -Stimulation and nucleotide analogs increased shortening amplitudes on cardiomyocyte contractility. The shortening amplitude was compared before and after addition of substance to the electrically stimulated cardiomyocytes. Isoproterenol (1 μ M), 2-MeSATP (1 μ M) and ATP γ S (1 μ M) increased the contraction amplitude. AR-C67086 (1 μ M) also increased contraction amplitude. 2-MeSADP (1 μ M) and adenosine (1 μ M) did not have any significant effects. The contractions are expressed as the cardiomyocyte shortening in percent of the respective control. Data are shown as mean $\pm$ S.E.M., $n = 5$ –33 cells from three to six mice.

Fig. 2.

Isoproterenol and ATP-analogues increase the contractility of cardiomyocytes. Cell shortening traces of contracting cardiomyocytes in response to electrical stimulation, before (control) and after addition of drug (1 μM). A) Isoproterenol, B) AR-C67085 and C) 2-MeSATP.

Fig. 3.

Effects of PLC and adenylyl cyclase inhibition on AR-C67086 responses of cardiomyocytes. The shortening was compared before and after addition of substance to the electrically stimulated cardiomyocyte in the cuvette. The response to AR-C67085 (1 μ M, same data as in Fig. 1) can be blocked by both adenylyl cyclase inhibitor SQ22563 (10 μ M) and PLC inhibitor U73122 (10 μ M). Responses to ATP γ S (1 μ M), same data as in Fig. 1, was also blocked by SQ22563. The contractions are expressed as the cardiomyocyte shortening in per cent of the control. Data are shown as mean $\pm$ S.E.M., $n = 10$ –33 cells from three to six mice.

Fig. 4. cAMP levels in cardiomyocytes determined in enzyme immunoassay. AR-C67085 (10 μ M) and ATP γ S (10 μ M) induced increased levels of cAMP after stimulation of the cardiomyocytes for 10 min. AR-C67085 stimulation in presence of U73122 did not induce increased cAMP levels. The cAMP level is expressed as per cent of the control. Data are shown as mean $\pm$ S.E.M., $n = 8-11$ samples from three mice.

Fig. 5.

The upper panel shows original recording of a trabecular preparation from mouse heart. AR-C67085 induced an increase in contractile amplitude. The lower panel B shows dose-response curve of AR-C67085 on isolated trabeculae from mouse heart. The agonist was added cumulatively. The contractile force is expressed relative to the force in the absence of the agonist. A sigmoidal dose-response curve is fitted to the data. Data are shown as mean $\pm$ S.E.M., $n = 9$ preparations from three mice.

Fig. 6.

Effects of AR-C67085 on contractility of cardiomyocytes from wild type (*Des*^{+/+}, unfilled bars) and desmin deficient mice (*Des*^{-/-}, filled bars). The shortening was compared before and after addition of substance to the electrically stimulated cardiomyocyte in the cuvette. AR-C67085 (0.1 μ M) induced positive inotropic responses in both groups. The cell shortening was significantly smaller in the *Des*^{-/-} cardiomyocytes at 0.1 μ M compared to the *Des*^{+/+} group, $P < 0.05$. No significant difference in *Des*^{+/+} and *Des*^{-/-} cell shortening was obtained at 1 μ M AR-C67085. The cell shortening is expressed relative to maximal cardiomyocyte shortening obtained at 1 μ M AR-C67085. Data are shown as mean $\pm$ S.E.M., $n = 8$ cells from two to three mice.