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Characterization of myocardial extracellular ATP receptors by photoaffinity labelling and functional assays

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Extracellular ATP receptors in rat ventricular myocytes were investigated through intact cell photolabelling followed by protein isolation. 8-Azido-ATP (8Az-ATP) was used for labelling under specific conditions determined by parallel functional studies. In those studies ATP-induced cytosolic Ca^{2+} transients were irreversibly and specifically inhibited by UV-photolyzed 8Az-ATP, but not by 2-azido-ATP (2Az-ATP), even in the presence of high concentrations of phosphonucleotides not affecting myocardial ATP receptors. Under those conditions background labelling is minimized and radioactive 8Az-ATP specifically labels a band of 45–48 kDa on a SDS gel. Labelling under the above conditions in the presence of ATPγS or 2-methylthio-ATP (2-meSATP), which are distinct for two functionally different cardiac ATP receptors, shows two different proteins within the same band consistent with the possible labelling of these two receptors.

ATP receptor; Photolabelling; Myocyte; 8-Azido-ATP

1. INTRODUCTION

The exposure of many cell types to micromolar concentrations of extracellular ATP results in signals modulating several intracellular functions. Following the original finding that extracellular ATP serves as a neurotransmitter [1,2], the spectrum of activities elicited by ATP has significantly expanded and now includes regulation of most parenchymal and epithelial cells [3]. This resulted in the classification of P_2-purinergic receptors which are ATP specific as compared to adenosine-specific P_1-purinergic receptors (reviewed in [4]).

Recently, several P_2 receptor subtypes in various cells and within the same cell type have been classified on the basis of functional responses and agonist specificity. Accordingly, evidence has been provided for receptors linked to G-proteins, activating channels or forming pores (for review see [3–6]). Heretofore, no receptor has been isolated and no information exists on even partial primary structure, a condition which confounds classification of ATP receptors and limits our knowledge of ATP interaction with the receptor(s) and the resulting cellular signals.

Abbreviations: ATPγS, adenosine-5′-*O*-(3-thiotriphosphate); 2Az-ATP, 2-azido-adenosine-5′-triphosphate; 8Az-ATP, 8-azido-adenosine-5′-triphosphate; Bz-ATP, 3′-*O*-(4-benzoyl)benzoyl-[α-^{32}P]adenosine-5′-triphosphate; BSA, bovine serum albumin; 2-meSATP, 2-methylthio- adenosine-5′-triphosphate; fura2/AM, fura2-acetoxymethyl ester; GTP, guanosine-5′-triphosphate; SDS, sodium dodecyl sulfate; UTP, uridine-5′-triphosphate.

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Previous studies from our laboratory [7,8] and others [9] have shown that extracellular ATP regulates transmembrane potential and cytosolic Ca^{2+} in ventricular myocytes. At least two types of ATP receptors described in these cells have been shown to be functionally distinct from ATP receptors in other cells. The first type has stringent requirements for Mg-ATP, and responds to 2-meSATP and ATP, but not to ATPγS, UTP, GTP, ADP or adenosine. The major effect of Mg-ATP binding to this receptor is the activation of an inward non-selective cation depolarizing current. This event, which is not G-protein linked, is followed by the activation of voltage-sensitive Ca^{2+} channels, increase in Ca^{2+} influx and Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum [7]. The second type is activated by ATP or ATPγS, but not 2-meSATP, probably requires G-proteins, and initiates uptake of both P_i and Na^+ from the extracellular spaces [10].

Recently Boyers et al. [11] labelled a P_{2y} receptor by using [α-^{32}P](Bz-ATP) in turkey erythrocytes membranes. This analogue labelled a protein of M_r 53,000. In the present study we have attempted to characterize myocyte ATP receptors through the use of photoaffinity labelling. We took advantage of the high specificity of phosphonucleotides for these receptors and searched through functional assays for ATP analogues which could photolabel these receptors under conditions where unrelated ATP binding sites were saturated by large concentrations of nucleotides that did not affect ATP receptors.

Labelling with 8Az-ATP under these conditions identified a major protein band. This band may consist of two distinct proteins which are tentatively identified as

the ATP binding sites for the two receptors activating cation conductance or Na/P_i cotransport.

2. MATERIALS AND METHODS

2.1. *Cell preparation*

Ventricular myocytes were prepared from the hearts of Sprague-Dawley adult male rats as previously described [11]. The suspension of isolated myocytes was enriched for intact, rod-shaped myocytes by centrifugation on a Percoll step gradient: 3.7 ml of Joklik (Sigma Chemicals, St. Louis, MO) media with 1% BSA was layered on 10 ml of Joklik media made with 60% Percoll in a sterile conical tube. Cells from a single rat heart were suspended in 20 ml of Joklik (1% BSA) and aliquots of 10 ml were layered on parallel gradients. The gradients were centrifuged for 2–3 min in a table-top centrifuge (approximately 300 × *g*). The sediment was washed briefly in Joklik media, resedimented, and resuspended in low-phosphate Geigy buffer. The sedimented cells typically were 70% rod-shaped myocytes without other identifiable cell types. These cells are normally quiescent and the rate of spontaneous beating is less than one per minute. Yields of cells using this procedure was 2–3 × 10^6 cells/heart.

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

Loading with fura2/AM (Molecular Probes, Eugene, OR) was done as previously described [12]. After Percoll enrichment, the cells were suspended in 15–20 ml Geigy buffer. Aliquots of 1 ml were assayed at 37°C with magnetic stirring in a custom-built fluorimeter. A 75 W xenon lamp filtered at 340 nm served as the excitation source; fluorescence emission was collected after filtering through a 490 nm interference filter and measured at 90° from the source lamp by a photomultiplier tube. Changes in fluorescence intensity were plotted on a strip chart recorder.

As previously noted [8], precise calibration of the signal in terms of intracellular free calcium is difficult in myocytes. Hence, we used the ratio of the ligand generated signal to that obtained by cell depolarization with KCl (30 mM) as a method to standardize different cell samples [7,8].

2.3. *Labelling protocol*

8-Azido-ATP (8Az-ATP) solution was prepared from cold and radioactive stock; typically 10 μl of nonradioactive 8Az-ATP (5 mM) was added to 80 μl [α-^{32}P]8Az-ATP (ICN; 160 μM, 15.2 Ci/mmol) to give a working solution of 700 μM (2.1 μCi/μl, 3.2 Ci/mmol) and was stored in the dark.

Cell aliquots (1 ml) were stirred in plastic cuvettes; in the controls 2–5 mM ATP was added prior to 8Az-ATP addition, whereas 300–800 μM UTP was added to the experimental samples. Some samples contained UTP plus either 2-meSATP or ATPγS (200 μM). 8Az-ATP (10–15 μl of the working solution; final concentration 7–10 μM) was rapidly mixed with the samples and photolyzed under strong UV (A+B) light for 45–60 s. Cells were sedimented through oil (dibutylphthalate:dioctyl-phthalate 2:1 v/v) and resuspended in Geigy buffer supplemented with 5 mM ATP. Cells were then sonicated for 45–60 s with a Branson Sonifier model 185, using the microtip under maximum cavitation. The sample was briefly sedimented at 400 × *g*, and the supernatant frozen in liquid nitrogen and lyophilized overnight. The pellet was discarded. The samples were boiled in SDS loading buffer with dithiothreitol and run on 7.5% or 11% polyacrylamide gels.

Gel scanning was executed using a USB (United States Biochemicals) SciScan Tm 5000 optical scanner, and analysis was done using Bioanalysis software version 1.0.

3. RESULTS AND DISCUSSION

The addition of micromolar concentrations of extracellular ATP to isolated nonbeating ventricular myocytes activates Ca^{2+} transients [7]. Fig. 1A shows that the addition of ATP to fura2-loaded myocytes in physiological concentration of sodium and phosphate generates a Ca^{2+} transient measured as an increase in fluorescence. Consistent with results previously seen [7], a second addition of ATP results in smaller transients. Depolarization of the cells by KCl produces further increase in cytosolic Ca^{2+} as a result of calcium influx through voltage-dependent channels. We used KCl as an internal control in the evaluation of the ATP response that is expressed as Δligand/ΔKCl ratio. Fig. 1A also shows

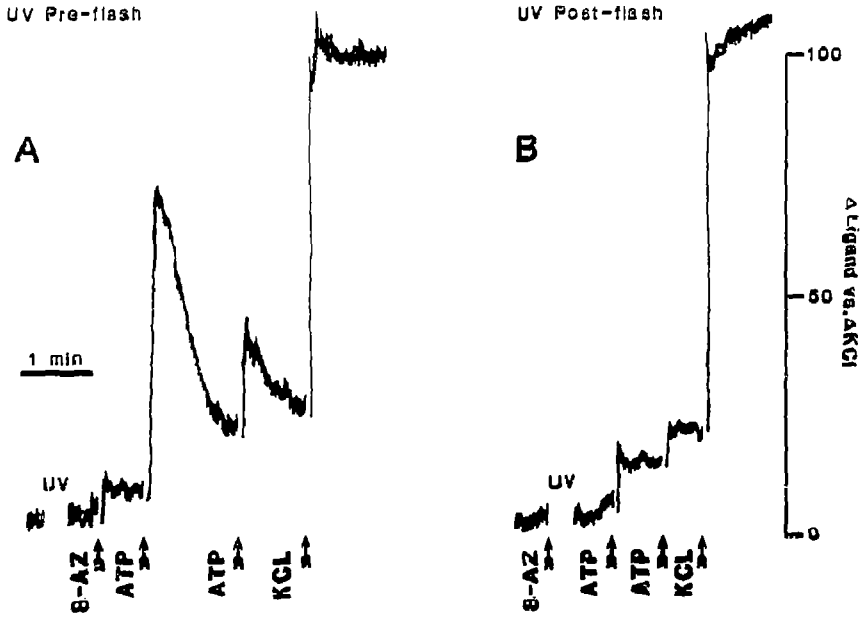

Fig. 1. Measurement of cytosolic free calcium in fura2/AM-loaded myocytes. The experimental conditions were as described in section 2. The concentrations of the reagents indicated were: ATP 20 μM, 8Az-ATP 10 μM, KCl 30 mM. UV light (A+B) was applied for 40–50 s before (Fig. 1A) or after (Fig. 1B) 8Az-ATP.

that, in the absence of photolysis, 8Az-ATP, in micromolar concentrations, acts neither as an agonist nor as an antagonist with respect to the ATP response. Additionally, Fig. 1 shows that pre-exposure with UV light (A+B) at the energy used for photolabelling has no effect on the transients of Ca^{2+} elicited by ATP or KCl. In fact, the response to added ATP was identical with or without previous UV light exposure (not shown). Light irradiation in the presence of 8Az-ATP results in a nearly complete inhibition of the increase in Ca^{2+} induced by ATP but not by KCl. Upon light activation, 8Az-ATP strongly inhibits the ATP response. The detailed mechanism for such an inhibition is presently unclear. It is possible that the inhibition is a consequence of receptor crosslinking upon photolysis of the arylazide and/or the inactivation or denaturation of receptors by the chemistry during photolabelling. In general the crosslinking efficiency of this reaction is low. As the structure of the receptors is unknown, the efficiency could be higher than that in other ATP binding proteins. From the gel shown later, it is apparent that a major protein band is labelled under those conditions and that this labelling is prevented by specific ligands of the receptors (ATPγS or 2-meSATP). Hence, possible explanations are: (i) that there is an unusually high labelling efficiency; or (ii) that an unknown percentage of the receptors is labelled and the remaining fraction is inactivated by chemical reactions during labelling. The alternative explanation that photolysis generates a product which, being a strong agonist, desensitizes the ATP response is not supported by the data of Fig. 1B, which shows that UV irradiation of 8Az-ATP does not itself produce an intracellular Ca^{2+} increase. Furthermore, the addition of the azide prephotolyzed in the absence of cells, results in no Ca^{2+} increase and the successive addition of ATP produces a normal or slightly lower response (not shown).

Fig. 2 shows that the 8Az-ATP inhibition is maintained in the presence of large amounts of other phosphonucleotides. Concentrations of 200 μM ADP (Fig. 2A) or UTP up to 800 μM (Fig. 2B) do not interfere with the 8Az-ATP inhibition. The slow drift in the baseline with ADP is due to slow formation of ATP by contaminant myokinase activity. The inhibition by 8Az-ATP is consistent with the agonist selectivity of the cardiac ATP receptors: UTP and ADP do not interfere with the ATP response [7] nor with the ability of photolyzed 8Az-ATP to inhibit the response. These experiments show that high concentrations of UTP could bind to and saturate other ATP binding sites and increase the labelling specificity of cardiac ATP receptors by 8Az-ATP (see below). The obvious experiment of competing 8Az-ATP with high concentrations of ATP in the fura2 studies was unfeasible because high ATP concentrations result in a long-lasting desensitization of the ATP dependent Ca^{2+} transient.

The specificities of photoaffinity analogues are shown in Table I: the action of various analogues as either agonists or antagonists, with or without UV light exposure is expressed relative to the ATP effect as a pattern

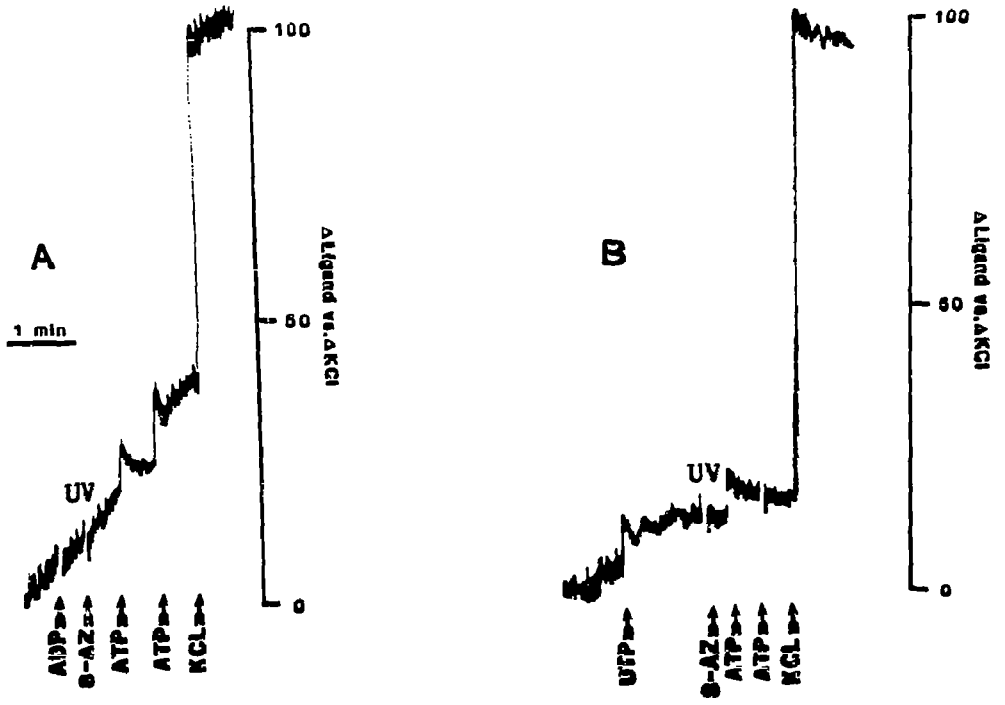

Fig. 2. Lack of effect of ADP or UTP on the 8Az-ATP inhibition. The conditions were similar to those of Fig. 1. Where indicated, ADP was 200 μM (Fig. 2A), or UTP 700 μM (Fig. 2B).

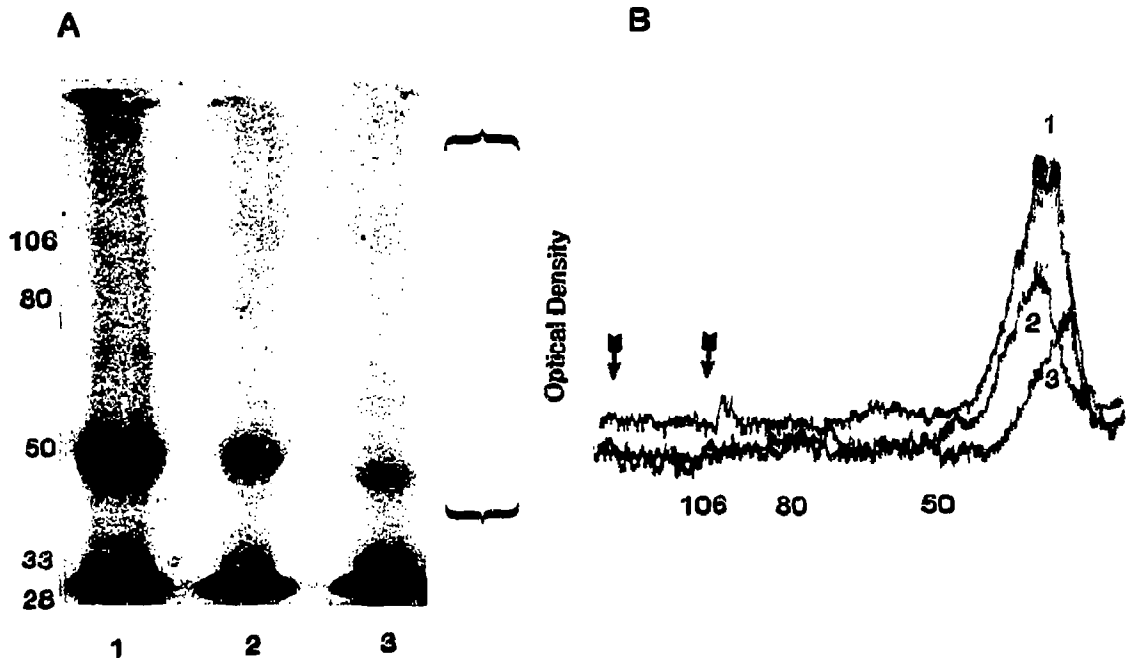

Fig. 3. (A) SDS-gel of myocyte proteins after 8Az-ATP labelling. Labelling in intact myocytes was carried out in: UTP 800 μM (lane 1), UTP plus 200 μM 2-meSATP (lane 2), UTP plus 200 μM ATPγS (lane 3). (B) A linear scanning of the same gel. The part of the gel scanned is within brackets. Intensity is in arbitrary units.

from several experiments. It is interesting to note that Bz-ATP which has previously been shown to label G-protein-coupled P_{2y} receptors [11], does not interfere with the ATP response. Even more striking is the finding that the 2Az-ATP, an analogue in N_9–$C_{1'}$ *anti* configuration which has been shown to label ATPase sites [12], has no activity. Non-photolyzed 8Az-ATP, an analogue in N_9–$C_{1'}$ *syn* configuration is a very weak agonist but becomes a strong antagonist after UV light exposure. This is an additional strong indication of the substrate specificity of these receptors which peculiarly prefer substrates in the N_9–$C_{1'}$ *syn* configuration rather than the usual N_9–$C_{1'}$ *anti* configuration.

Fig. 3 shows a representative experiment in which 7 μM radioactive 8Az-ATP in the presence of 700 μM UTP was added and photolyzed in freshly isolated intact myocytes. Upon cell filtration through oil and sonication and solubilization of their membrane a large band calculated from standards as near 48 kDa and a less pronounced band of 90 kDa (lane 1) are labelled. All the labelling is eliminated if 2–5 mM cold ATP was present before 8Az-ATP photolysis (not shown). Labelling of the 48 kDa band (lane 1) is partially inhibited by either 2-meSATP or ATPγS (200 μM) (lanes 2 and 3), and a shift of the labelled protein band is observed: 2-meSATP inhibits labelling of the lower portion of the band (lane 2), while ATPγS removes the upper portion. This is better seen from the scanning densitometry of the same gel (Fig. 3B), which indicates that the band of 48 kDa is the result of the comigration of two distinct proteins. The selectivity with which 2-meSATP and ATPγS compete with the labelling corresponds to the strict agonist specificity of two previously reported separate myocardial ATP receptors [10]. This conclusion is more quantitatively supported by the finding shown in Table II which shows that the sum of integrated optical densities (IOD) of the 2-meSATP and the ATPγS bands subtracted from the specific background is close to the total IOD of the 48 kDa total band. Since the labelling conditions in lanes 1 and 3 are identical, except for the specific competitor in lanes 2 and 3, the results suggest that the competitors selectively eliminate portions of the band in lane 1, as already hinted at by the slight difference in gel position observed in the linear scanning of Fig. 3.

In conclusion, ATP-induced calcium transients in myocytes are irreversibly and specifically inhibited by UV-photolyzed 8Az-ATP, even in the presence of high concentrations of several phosphonucleotides which do not affect myocardial ATP receptors. At these concentrations labelling of unrelated ATP binding sites is minimized and radioactive 8Az-ATP specifically labelled a large band of 45–48 kDa and one faint band of 90 kDa on autoradiographed SDS gels.

Labelling of the 45–48 kDa band can be inhibited by 2-meSATP and ATPγS. These two analogues are selective substrates for the activation of a fast calcium transient or sodium and phosphate uptake respectively. We have previously shown that extracellular ATP activates in myocytes both a fast calcium transient and a slower

sodium/phosphate cotransport [10]. 2-meSATP, but not ATPγS, activates the first rapid phase of Ca^{2+} transient and has no effect on Na^+/P_i cotransport. By contrast, the latter is activated ATPγS. Additional evidence for two distinct receptors are the preliminary data showing that two distinct mRNA fractions from rat hearts express either response in a *Xenopus* oocyte expression system [14,15]. Hence, these data are short of demonstrating but are consistent with the inference that the 45–48 kDa band is composed of two distinct ATP binding proteins that are involved in the different pathways of ATP signalling cascades in myocardial cells.

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