Biochimica et Biophysica Acta 1830 (2013) 4692–4707

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Kinetics of extracellular ATP in mastoparan 7-activated human erythrocytes

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ARTICLE INFO

Article history:
Received 18 February 2013
Received in revised form 24 April 2013
Accepted 20 May 2013
Available online 4 June 2013

Keywords:
Extracellular ATP
ATPases
Erythrocyte
Pannexin 1

ABSTRACT

Background: The peptide mastoparan 7 (MST7) stimulated ATP release in human erythrocytes. We explored intra- and extracellular processes governing the time-dependent accumulation of extracellular ATP (*i.e.*, ATPe kinetics).
Methods: Human erythrocytes were treated with MST7 in the presence or absence of two blockers of pannexin 1. ATPe concentration was monitored by luciferin–luciferase based real-time luminometry.
Results: Exposure of human erythrocytes to MST7 led to an acute increase in [ATPe], followed by a slower increase phase. ATPe kinetics reflected a strong activation of ATP efflux and a low rate of ATPe hydrolysis by ectoATPase activity. Enhancement of [ATPe] by MST7 required adhesion of erythrocytes to poly-D-lysin-coated coverslips, and correlated with a 31% increase of cAMP and 10% cell swelling. However, when MST7 was dissolved in a hyperosmotic medium to block cell swelling, ATPe accumulation was inhibited by 49%.
Erythrocytes pre-exposure to 10 μM of either carbenoxolone or probenecid, two blockers of pannexin 1, exhibited a partial reduction of ATP efflux.
Erythrocytes from pannexin 1 knockout mice exhibited similar ATPe kinetics as those of wild type mice erythrocytes exposed to pannexin 1 blockers.
Conclusions: MST7 induced release of ATP required either cell adhesion or strong activation of cAMP synthesis. Part of this release required cell swelling. Kinetic analysis and a data driven model suggested that ATP efflux is mediated by two ATP conduits displaying different kinetics, with one conduit being fully blocked by pannexin 1 blockers.
General significance: Kinetic analysis of extracellular ATP accumulation from human erythrocytes and potential effects on microcirculation.

1. Introduction

In healthy humans, about 40% of the blood volume is occupied by red blood cells (rbcs) containing a 1000-fold higher ATP concentration than plasma (mM *vs* μM; [1]). Thus even a limited release of ATP from the large rbc intracellular pool can result in nano to micromolar concentrations of ATP (ATPe) in the vascular lumen [1–3].

Human erythrocytes release ATP upon their exposure to β-adrenergic agonists, prostacyclin analogs, mechanical deformation, reduced oxygen tension acidosis, or swelling [4]. All of these conditions represent physiological stimuli to which rbcs are exposed in the vasculature, *e.g.* when passing through the small branches of the microcirculation [5–9].

A signaling route leading to ATP release from human rbcs involves the heterotrimeric G protein Gs, adenylyl cyclases and PKA [3,10]. In addition to Gs, Gi proteins of these cells have been proposed to transduce mechanical stress and hypoxia into ATP release [11,12].

Similarly, the tetradecapeptide MST extracted from wasp venom [13] binds to the plasma membrane and forms an amphiphilic helix that activates Gi without requiring the activation of a receptor [14]. MST7, synthesized by substituting alanine for the positively charged lysine in position 12, acts as a potent analog of MST in human rbcs and other cell systems [15,16]. Both MST and MST7 are known to activate ATP efflux of human rbcs [16].

The current postulated chain of events linking Gi activation to ATP efflux involves the dissociation of heterotrimeric Gi, allowing βγ

Abbreviations: ATPe, extracellular ATP; ATPi, intracellular ATP; CBX, carbenoxolone; CTZ, cilostazol; GEFs, guanine exchange factors; J_R, flux of ATP release; J_V, flux of ATPe hydrolysis; MST, mastoparan; PBC, probenecid; pnx, pannexin 1; $pnx^{+/+}$, pannexin 1 wild type mice; $pnx^{+/-}$, pannexin 1 heterozygous mice; $pnx^{-/-}$, pannexin 1 knockout mice, PTX, pertussis toxin; rbcs, red blood cells; Vr, relative cell volume; 3V, a cAMP activating cocktail containing isoproterenol, forskolin and papaverine

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http://dx.doi.org/10.1016/j.bbagen.2013.05.033

dimers to stimulate specific isoforms of adenylyl cyclases (including type II, an isoform that has been identified in rabbit and human erythrocytes [17]), and the subsequent increase in cAMP formation [11]. This is supported by the fact that all four of the Gβ subunits that have been reported to stimulate adenylyl cyclases are present in human rbc membranes [18] Moreover, blockage of Gi activation by pertussis toxin was shown to blunt deformation-induced ATP release of human rbcs [11].

These events are followed by a series of not-well defined intracellular signaling steps upstream of ATP release [11,12,19], where the cystic fibrosis transmembrane conductance regulator has been proposed to participate [20,21].

The idea that Gi activation is a critical step for ATP release from human rbcs was further strengthened by the demonstration that in rbcs from individuals with type 2 diabetes a reduction of Gi2 expression correlated with a reduction of both cAMP accumulation and MST7-induced release of ATP [16].

Besides the adenylyl cyclases, βγ subunits interact with various effector proteins such as ionic channels, phosphatidylinositol-3 kinase isoforms, proteins containing PDZ domains, and GEFs for small G proteins [22].

Released ATP can primarily act on specific P2X and P2Y purinergic receptors (receptors for di- and trinucleotides [2]) on adjacent endothelial cells, or be hydrolyzed by ectonucleotidases present on the plasma membrane of rbcs and other blood and vascular cells. Thus ATPe also serves as a reservoir to generate ADP and adenosine, which in turn activate P2Y and adenosine receptors functionally expressed in rbcs [23,24] and endothelial cells [25–27]. The physiological effects of such ligand-receptor interactions can be manifold, including the alteration of the vascular tone in the brain, coronary arteries and skeletal muscle [28,29].

Despite the accumulated knowledge on the mechanisms mediating ATP release, purinergic receptor signaling, and the cellular role of various ectonucleotidases [30], little is known regarding how the dynamic interaction of these processes controls the kinetics of ATPe accumulation in the immediate environment of rbcs. This is particularly important in the microvasculature, where rbcs and other blood cells interact over short distances (within the micrometer range), and local signals of high energy demand and/or stress can induce ATP release from erythrocytes. The subsequent extracellular diffusion would permit ATPe to trigger paracrine responses to these signals. Based on this, the following questions arise: (i) will ATP release, under a given metabolic condition, be counteracted by ATPe hydrolysis at the cell surface? This is not unexpected since in most cell types ectoATPase activity follows a function with [ATPe] [31], (ii) can the released ATP, as part of a postulated negative feedback system [32,33], inhibit one or more components that participate in ATP release? And (iii) what ATP transport mechanisms operate in rbcs?

The non-nucleated human rbcs offer a simple metabolic model to approach these questions because they possess glycolysis as the sole pathway for ATP generation and lack intracellular compartments, so that no exocytotic ATP release can occur in these cells. Moreover, these cells lack direct cell–cell communication that might increase the available signaling mechanisms inducing ATP release, which may complicate the analysis of ATPe regulation. Still, the kinetics of [ATPe] in the rbc model, as in any other cell, is complex enough due to the non-linear relationships among the different processes that alter ATPe concentration. For instance, various channels and transporters have been postulated in human rbcs as candidates for ATP release, although many of them have been rejected afterwards as main conduits [34–36].

In mammals, the prime candidate protein mediating conductive ATP release is the pannexon, a homohexamer of pannexin 1, which is expressed in rbcs [32]. The pannexon constitutes a large pore by itself or it can be part of a multiprotein complex capable of carrying anions and signaling molecules from the cytoplasm to the extracellular space [37].

Since MST7 is a robust activator of rbcs ATP release [38–40], in this study we intended to understand the kinetics of ATPe by exposing human erythrocytes to this peptide.

We present experimental evidence showing that several potential factors altering the kinetics of [ATPe] can be experimentally controlled in a way that allows a quantitative description of ATP transmembrane fluxes, and provides useful information on the underlying mechanisms enabling such transport.

By fitting a simple mathematical model to the experimental ATPe kinetics of erythrocytes, and from the analysis of ATP release of rbcs from human, canine and mice ($pnx^{+/+}$ and $pnx^{-/-}$) we demonstrate the existence of two kinetically different ATP pathways mediating ATP release.

2. Materials and methods

2.1. Chemicals

All reagents in this study were of analytical grade. Mastoparan 7, mastoparan 17, carbenoxolone, probenecid, firefly luciferase (EC 1.13.12.7), forskolin, isoproterenol, papaverine, H-89, laminin and collagen were purchased from Sigma-Aldrich (St. Louis, MO, USA). BCECF-AM, Fluo4-AM and D-luciferin were obtained from Invitrogen/Molecular Probes Inc. (Eugene, OR, USA). [γ-^{32}P]ATP (10 Ci/mmol) was purchased from Perkin Elmer Life Sciences (Santa Clara, California, USA).

2.2. Isolation of erythrocytes

Human rbcs were isolated as described before [3]. Before the experiments, rbcs were resuspended in RBC medium containing (in mM) 137 NaCl, 2.7 KCl, 4.72 Na_2HPO_4, 1.50 KH_2PO_4, 1.32 $CaCl_2$, 1.91 $MgSO_4$, 5 glucose, 0.5% bovine serum albumin, pH 7.4 at 25 °C, and 300 mOsm. All procedures conformed to the Declaration of Helsinki and written informed consent was given by the donors. Erythrocytes from dogs and mice were isolated similarly to human rbcs.

2.3. Pannexin 1 knockout mice

The pannexin 1 knockout mouse was generated and characterized, as previously described [41]. Heterozygous mice were bred to produce homozygous pannexin 1 deficient animals. All animal studies were approved by the Institutional Animal Care and Use Committee and were performed according to principles outlined by the Animal Welfare and the NIH guidelines for the care and use of animals in biomedical research. No morphological differences or cell volume variation were observed between wild type and pannexin 1 knockout mice rbcs through microscopy, hematocrit and cell counting techniques.

2.4. Treatments

ATP release was induced with 10 μM MST7 or a mixture called 3V, which contained 10 μM isoproterenol, 30 μM forskolin and 100 μM papaverine [3]. Carbenoxolone 10 μM or 100 μM and probenecid 10 μM were used as blockers of ATP release. H-89 10 μM was used as a PKA inhibitor.

2.5. Extracellular and intracellular ATP measurements

ATPe was measured using firefly luciferase, which catalyzes the oxidation of luciferin in the presence of ATP to produce light [42,43]. Two different types of luminometry determinations were performed, *real-time* and *off-line*.

Real-time luminometry measurements were carried out with cells laid on coverslips that were mounted in the assay chamber of a custom-built luminometer, as previously described [44]. Because luciferase activity at 37 °C is only 10% of that observed at 20 °C [45], to maintain