Homeostasis of Extracellular ATP in Human Erythrocytes*§

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We explored the intra- and extracellular processes governing the kinetics of extracellular ATP (ATPe) in human erythrocytes stimulated with agents that increase cAMP. Using the luciferin-luciferase reaction in off-line luminometry we found both direct adenyl cyclase activation by forskolin and indirect activation through β-adrenergic stimulation with isoproterenol-enhanced [ATP]e in a concentration-dependent manner. A mixture (3V) containing a combination of these agents and the phosphodiesterase inhibitor papaverine activated ATP release, leading to a 3-fold increase in [ATP]e, and caused increases in cAMP concentration (3-fold for forskolin + papaverine, and 10-fold for 3V). The pannexin 1 inhibitor carbenoxolone and a pannexin 1 blocking peptide (10Panx1) decreased [ATP]e by 75–84%. The residual efflux of ATP resulted from unavoidable mechanical perturbations stimulating a novel, carbenoxolone-insensitive pathway. In real-time luminometry experiments using soluble luciferase, addition of 3V led to an acute increase in [ATP]e to a constant value of ~1 pmol × (10^6 cells)^−1. A similar treatment using a surface attached luciferase (proA-luc) triggered a rapid accumulation of surface ATP levels to a peak concentration of 2.4 pmol × (10^6 cells)^−1, followed by a slower exponential decay (t_1/2 = 3.7 min) to a constant value of 1.3 pmol × (10^6 cells)^−1. Both for soluble luciferase and proA-luc, ATP efflux was fully blocked by carbenoxolone, pointing to a 3V-induced mechanism of ATP release mediated by pannexin 1. Ecto-ATPase activity was extremely low (~28 fmol × (10^6 cells min)^−1), but nevertheless physiologically relevant considering the high density of erythrocytes in human blood.

All cell types appear to possess mechanisms that enable a controlled, nonlytic release of ATP, that is, a release not involving cell membrane rupture, and which occurs in response to osmotic, mechanical, and/or neurohormonal stimuli (1). Specifically human erythrocytes release ATP following exposure to β-adrenergic stimulation, mechanical deformation, reduced oxygen tension, or acidosis (2). All of these represent physiological conditions to which erythrocytes are exposed in the vasculature, e.g. when passing through constricted vessels or in the contracting striated muscle (3). Once in the extracellular medium, extracellular ATP (ATPe) can trigger different cellular responses by interacting with P receptors on the cell surface while at the same time its concentration is controlled by the activities of one or more ectonucleotidases (4, 5). Several reports published over recent years have shown that an increase in intracellular cyclic AMP (cAMP) concentration triggered ATP release from human erythrocytes (6, 7). Receptor-mediated ATP release in human erythrocytes involves activation of heterotrimeric G proteins G_s or G_i, (3, 8, 9). Regarding the G_s pathway, activation of β-adrenergic receptors by various agonists was reported to stimulate adenyl cyclase, with concomitant increases in cAMP levels and protein kinase A activity (6, 10). Moreover, direct activation of adenyl cyclase by forskolin resulted in both ATP release and cAMP increases in human and rabbit erythrocytes (6).

Despite the accumulated knowledge regarding the intracellular signaling events mediating ATP release, comparatively little is known regarding the processes governing the kinetics of ATPe accumulation at the surface in animal cells (11, 12), with rates of intracellular ATP release and extracellular ATP hydrolysis being the main actors. The human non-nucleated erythrocyte is an excellent model in this respect, because it lacks intracellular compartments and direct cell-cell communication that would enhance the available signaling mechanisms inducing ATP release, and therefore complicate the analysis of ATPe homeostasis. Among potential candidate membrane-bound proteins enabling a regulated nonlytic ATP efflux, pannexin 1 was identified as a molecule that might associate with the ionotropic P receptor P2X7, and probably other P receptors (13). Pannexin 1 appears to either constitute a large pore capable of

5 The abbreviations used are: ATPe, extracellular ATP; BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5(and −6)-carboxyfluorescein acetoxymethyl ester; TME-cAMP, 2'-O-monosuccinyladenosine-3',5'-cyclic monophosphate tyrosyl methyl ester; CBX, carbenoxolone.
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carrying ions and signaling molecules between the cytoplasm and extracellular space (14). As such, pannexin 1 is a candidate ATP release channel in erythrocytes, because it is expressed at high levels in these cells (15).

In the present study, we investigated the homeostasis of ATPe from erythrocytes stimulated with agents that increase intracellular cAMP. We focused our attention on the activation of the Gs pathway by isoproterenol, a well known β-adrenergic agonist. Because of the relatively modest increases in cAMP level and ATP release observed with this agonist, addition of forskolin together with the phosphodiesterase inhibitor papaverine was used to enhance the dynamic range of the response.

We examined the role of pannexin 1 as a necessary mediator of ATP exit in human erythrocytes. Moreover, by studying the kinetics of ATP accumulation in the extracellular space and the capacity of cells to hydrolyze ATPe by ecto-ATPase activity, we were able to estimate, for the first time in human erythrocytes, the precise kinetics of pannexin 1-mediated ATP release. This allowed us to analyze the interplay between time-dependent extracellular ATP consumption and the release of cytosolic ATP. For a comparative purpose, critical experiments were conducted with canine and Xenopus erythrocytes.

EXPERIMENTAL PROCEDURES

Reagents—All reagents used in this study were of analytical grade. Isoproterenol, forskolin, papaverine, carbenoxolone, cAMP, dibutyryl cyclic AMP, 2′-O-monosuccinyladenosine-3′,5′-cyclic monophosphate tyrosyl methyl ester (TPM-cAMP), ATP, chlorpromazine, and chloroacetalddehyde were purchased from Sigma. Pannexin 1 blocking peptide WRQ-AAVFDSY (10Panx1), and its scrambled version (ScrPanx1) purchased from Abcam (Cambridge, MA). Pannexin 1 blocking peptide WRQ-AAVFDSY (10Panx1) and ScrPanx1 were synthesized at the University of North Carolina Microprotein Sequencing and Peptide Synthesis Facility. The Script were synthesized at the University of North Carolina Microprotein Sequencing and Peptide Synthesis Facility.

Isolation of Erythrocytes—Human blood was obtained by venipuncture from healthy volunteers on the day each study was done. Immediately after collection of blood, plasma, platelets, and leukocytes were removed by centrifugation (900 × g at 20 °C for 3 min). The supernatant and Buffy coat were removed by aspiration and discarded. Isolated erythrocytes were resuspended and washed three times in RBC medium containing (in mm) 155 NaCl, 2.7 KCl, 1.5 KH₂PO₄, 2.5 Na₂HPO₄, 1 CaCl₂, 1 MgSO₄, 5 glucose (pH adjusted to 7.4 at 20 °C and osmolarity to 300 mosmol). Packed erythrocytes were resuspended in RBC medium supplemented with 0.5% bovine serum albumin to the corresponding final hematocrit. All procedures conformed to the Declaration of Helsinki, and written informed consent was given by the donors. In preliminary experiments aliquots of the resulting erythrocyte suspensions were stained with the blue fluorescent Hoechst dye to check for the presence of nucleated cells. Results of these experiments showed only 0.05% contamination of our preparation with nucleated cells. Xenopus laevis erythrocytes were isolated as in humans. Canine erythrocytes were obtained from blood samples as above using a modified RBC medium without CaCl₂.

In supplemental Fig. S4, a subpopulation of RBCs containing only nucleated mature erythrocytes was used. Cells were isolated by means of a discontinuous Percoll density gradient as previously described (31).

Measurement of ATPe—Extracellular ATP was measured using firefly luciferase (EC 1.13.12.7), which catalyzes the oxidation of luciferin in the presence of ATP to produce light (17, 18).

Two different types of determinations were made, real-time and off-line luminometry. In most experiments soluble luciferase was used, whereas in a few real-time experiments determination of ATPe on the cell surface was estimated by a fusion protein denoted as proA-luc. In all cases (real-time or off-line luminometry, using luciferase or proA-luc) the same luciferase reaction was used to estimate ATPe via quantification of light emission.

Real-time luminometry measurements were performed with cells on coverslips that were mounted in the assay chamber of a custom-built luminometer as described earlier (19). Because luciferase activity at 37 °C is only 10% of that observed at 20 °C (20), to maintain full luciferase activity real-time ATP measurements were performed in a cool chamber acclimated at 20 °C. Under the experimental conditions, assay volume did not change during the course of the experiment. Measurements were performed with 3 × 10⁶ cells incubated in 40 μl of RBC medium. Under these conditions the medium has a height of about 104 μm (height at the coverslip bottom of the chamber equals 0). The time course of light emission was transformed into ATPe concentration versus time by means of a calibration curve. Increasing concentrations of ATP from 0.25 to 2,500 nM were sequentially added to the assay medium from a stock solution of ATP dissolved in RBC medium. Also, a calibration curve was performed for each experiment in similar conditions but in the absence of cells. Both calibration curves displayed a linear relationship in the range tested. The slopes of these curves were directly related to luciferase activity. To avoid errors in the calculation of absolute ATPe values only the experiments with similar parameter values of the calibration function with or without cells were used. In preliminary experiments we found no effect of 100 μM carbenoxolone on luciferase activity.

In experiments of Fig. 5, detection of cell surface ATP was performed using proA-luc, a fusion protein between staphylococcal protein A and firefly luciferase (21). Protein A specifically binds to the Fc domain of immunoglobulin G (IgG) and, as mentioned before, firefly luciferase can be used to detect ATP by luminescence emission. ProA-luc can then be stably adsorbed onto the surface of intact cells via interactions with primary IgG antibodies directed against native surface antigens (22). The spatial resolution of the method enables the detection of local changes on the surface of cells. For human erythrocytes we used an antibody against the N-terminal extracellular segment of glycophorin A, the most abundant sialoglycoprotein on human red cells (23).

In the cell-attached proA-luc assays, erythrocytes (3 × 10⁶) were attached on coverslips coated with 0.001% poly-d-lysine.
At such a very low concentration of poly-D-lysine, negatively charged human erythrocytes will adhere firmly and remain discoidal in shape (24). Adherent cells on 25-mm diameter circular coverslips were washed twice with 150 μl of RBC medium and then allowed to incubate for 1 h in 40 μl of RBC medium containing the anti-glycoporin A antibody (1/250 dilution from stock). Adherent cells were then washed three times and allowed to incubate for another 1 h in 30 μl of RBC medium containing proA-luc (500 μg/ml). Finally cells were washed three times and incubated in 100 μl of RBC medium until they were used for real-time luminescence assays as described above for soluble luciferase. In preliminary experiments using proA-luc, cells were challenged with exogenous ATP in the presence of 150 μM luciferin. Only those cells preincubated with primary antibodies were able to emit detectable light, demonstrating the specificity of proA-luc attachment to the cell surface. Similarly, when monitoring endogenous ATPe, no light was detected in experiments lacking the primary antibody.

For the off-line luminometry human red blood cells were incubated with different pharmacological agents. After the incubation the erythrocyte suspension was centrifuged 2 min at 1,000 × g. ATPe levels were determined in 45 μl of the resulting supernatant. A standard ATP curve was obtained for each experiment. Although most experiments were conducted at room temperature, to account for ATP release at a physiological temperature the main off-line experiments were conducted at both 20 and 37 °C.

Measurement of Intracellular ATP—For intracellular ATP content estimations, erythrocytes (3 × 10⁶) held on coverslips were permeabilized with digitonin (50 μg/ml) at the end of real-time lumimetry experiments. The released cytosolic ATP was measured with luciferin-luciferase as described for ATPe. After considering the total volume occupied by all erythrocytes present in the chamber, and the relative solvent cell volume, i.e. 70% (25), ATP values were expressed as the intracellular ATP concentration of erythrocytes.

Purification of ProA-luc—A gene expression plasmid pMALU7 was used that encodes the fused gene between protein A and the complete sequence of a mutated firefly luciferase (26).

Expression and purification methods were described before (21, 22, 26). Briefly, the JM109 strain of Escherichia coli cells was transformed with pMALU7. Transformed bacteria were grown to mid-log phase at 37 °C at which time 1 mM isopropyl β-D-thiogalactopyranoside was added to induce protein expression. Bacteria were harvested by centrifugation (4000 × g for 4 min) and lysed by three cycles of sonication at 4 °C in PBS medium containing 1 mM phenylmethysulfonyl fluoride. The bacterial lysate was centrifuged 7000 × g (4 °C for 20 min) and the supernatant was applied to a IgG-Sepharose affinity column to collect the fusion protein. Fractions were eluted in acetate acid buffer 0.5 M (pH 3.4) and allowed to drop directly into a neutralization buffer (Tris chloride, 1 M, pH 8.0). Fractions were tested for luciferase activity, and those containing the peaks of the activity were pooled and concentrated 6-fold using centrifugal filters (Centriprep, YM50, Millipore Corp., MA). Protein content was assessed by the method of Lowry et al. (27).

Measurement of Intracellular Cyclic AMP—Human erythrocytes (50% hematocrit) were incubated 10 min at room temperature either in the absence (control) or presence of stimulating agents. The reaction was stopped by the addition of 4 ml of ice-cold ethanol containing HC1 (10%) to the erythrocyte suspension. The resulting erythrocyte/ethanol mixture was centrifuged at 14,000 × g for 10 min at 4 °C. The supernatant was removed and stored overnight at −20 °C to precipitate the remaining proteins. Samples were then centrifuged a second time at 3,700 × g for 10 min at 4 °C. The supernatant was removed and dried under vacuum centrifugation and the residue was resuspended in 50 mM sodium acetate buffer (pH 6.0). Unknown samples and standards were acetylated and measured by standard radioimmunoassay system using the method described by Steiner et al. (28) with modifications (29, 30). The TME-cAMP was radiolabeled with Na125I by the method of chloramine T (specific activity, 600 Ci/mmoll) previously described by Del Punta et al. (31). The specific antibody for cAMP was provided by NIH (Dr. A. F. Parlow, National Hormone and Peptide Program, NIDDK, National Institutes of Health). The inter-assay and intra-assay variations of coefficients were lower than 10%.

Extracellular Hydrolysis of ATP, Adenosine Production, and Ecto-ATPase Activity—The hydrolysis rate of ATPe was determined by following the accumulation of [γ-32P]Pi, released from exogenous [γ-32P]ATP added to a erythrocyte suspension of known hematocrit, as described before (32–34) with modifications. Briefly, the reaction was started by adding 1 μM [γ-32P]ATP (0.27 Ci/mmoll) to cell suspensions at room temperature. At different times, a 200-μl aliquot of the suspension was withdrawn and centrifuged at 900 × g during 30 s, and 100 μl of the supernatant were poured into 750 μl of a stop solution containing 4.05 mM Mo7O24(NH4)6 and 0.83 mM HClO4. The ammonium molybdate solution formed a complex with the released phosphate, which was then extracted by adding 0.6 ml of isobutyl alcohol under vigorous stirring. After the phases were separated by centrifugation for 5 min at 1,000 × g, aliquots of 200 μl of the organic phase containing [γ-32P]Pi, were transferred to vials containing 2.5 ml of 0.5 M NaOH. Radioactivity was measured by the Cerenkov effect. Production of adenosine from ATP was estimated as described previously (33). The release of α-32P from [α-32P]ATP was followed using a method similar to that described for measuring ecto-ATPase activity. Under these conditions, one adenosine is formed for every α-32P, produced. Values of phosphate concentration in assay medium lacking cells were used to estimate the phosphate concentration at time 0 of experiments run with cell suspensions. Phosphate content in the absence of cells amounted to 1.5 ± 0.2% (n = 3) of total ATP concentration in the assay medium, and did not increase with time, i.e. the nonenzymatic phosphate production was negligible.

Because erythrocyte uptake of ATP is extremely low, any hydrolysis of [γ-32P]ATP into ADP + [γ-32P]Pi, in a cell suspension can be defined as ecto-ATPase activity, the time course of which yields a measure of the rate at which ecto-nucleotidases hydrolyze extracellular ATP. That is, whereas it is possible that
more than one ecto-nucleotidase can contribute to the total measured activity, the term ecto-ATPase has been used because all ATPase activity measured in intact cell suspensions resulted solely from the extracellular hydrolysis of the γ-P_i from ATP. There may be more than one ENTPDase accounting for ecto-ATPase activity, whereas no ecto-phosphatase activity could be detected in human erythrocytes (35).

To calculate ecto-ATPase activity, time dependent levels of P_i were fitted to Equation 1,

\[ Y = Y_0 + A \times (1 - e^{-kt}) \]

(Eq. 1)

where \( Y \) and \( Y_0 \) are the values of \( {}^{32}\text{P} \) at any time (\( t \)) and at \( t = 0 \), respectively; \( A \) represents the maximal value for the increase in \( Y \) with time, and \( k \) is a rate coefficient. The parameters of best fit resulting from the regression were used to calculate the initial rate of ecto-ATPase activity (\( v \)) as \( kA \) (i.e. the first derivative of Equation 1 for \( t \) tending to 0).

The \( {}^{32}\text{P} \) mass produced from \([\gamma-{}^{32}\text{P}]\text{ATP} \) and \([\alpha-{}^{32}\text{P}]\text{ATP} \) was calculated using the ATP specific activity and expressed in picomoles per 10^6 red blood cells. Ecto-ATPase activity is expressed as picomoles of \( {}^{32}\text{P} \times (10^6 \text{ cells min}^{-1})^{-1} \).

**Cell Volume Determinations by Fluorescence Microscopy**—In erythrocytes from several vertebrates species, adrenergic stimulation can lead to cell swelling leading to changes in solute intracellular content and activation of signaling mechanisms (36). Therefore, we checked whether the potential volume changes can affect cAMP concentration and/or the rate of ATP release assessed in this study. To this end we monitored cell volume of erythrocytes exposed to 3V, both in the presence and absence of carbeneoxolone. Relative cell volumes before and after addition of pharmacological agents were estimated at room temperature using the BCECF fluorescence quenching method, as described (34, 37, 38). Under quenching conditions, fluorescence intensity of human erythrocytes decreased with the increase in fluorophore concentration. This property can be used to continuously monitor changes in water cell volume (34).

In brief, human erythrocytes (\( 3 \times 10^6 \) cells) were attached to 0.001% poly-D-lysine-coated coverslips. Erythrocytes were incubated with RBC medium containing 5 \( \mu \text{M} \) BCECF-AM during 60 min at room temperature. Subsequently, the solution was washed with RBC medium for 30 min to eliminate extracellular BCECF, and the coverslip was mounted on a recording chamber in a Nikon TE-200 epifluorescence inverted microscope. During experimental manipulations, all media were removed from or introduced in the chamber manually. Changes in cell water volume were inferred from readings of fluorescence intensity recorded by exciting BCECF at 445 nm, fluorescence images were acquired by use of a charge-coupled device camera (Hamamatsu C4742–95) and the Metalfluor acquisition program (Universal Imaging). Values of cell volume were obtained from the fluorescence ratio \( (F_{x}/F_{0}) \). The value of \( F_{x} \) represents the signal obtained from a small circular digital region placed at the image plane of each fluorophore-loaded cell equilibrated with isotonic medium, whereas \( F_{0} \) denotes the fluorescence of the same region of the cell at time \( t \). Thus, this measure represents a fractional volume where the initial isotonic cell volume value is 1 and volume changes are expressed as relative to the initial value. A calibration is needed to convert values of relative fluorescence for each cell to relative volume. Calibration was performed by sequentially exposing cells to assay media of osmolarities (in milliosmolar) = 298, 285, 260, and 245. The hypotonic media for cell volume calibrations was similar in composition to isotonic RBC medium, except that the NaCl concentration was adjusted appropriately. During all of these sequential media exchanges, the \( X, Y, \) and \( Z \) positions of the microscope field remained unchanged.

**Hemolysis Measurements**—Erythrocyte suspensions used to measure extracellular ATP in off-line luminometry were centrifuged at 1000 \( \times g \) for 2 min at room temperature. The amount of hemoglobin present in the supernatant was determined by measurement of absorbance at 405 nm (oxyhemoglobin). A calibration curve was obtained by measuring the absorbance at 405 nm of a sequentially increasing known number of lysed erythrocytes. Experiments in which free hemoglobin was detected were discarded to avoid the contribution of ATPe content by hemolysis. For online measurements two methods were used to assess time-dependent hemolysis. First, fluorescence microscopy was used to measure hemolysis as described earlier (8). Briefly, \( 3 \times 10^6 \) erythrocytes were loaded with BCECF and the retention of the intracellular fluorophore was assessed before and after addition of pharmacological agents. A steep, acute loss of fluorophore was interpreted as cell death. Hemolysis was assessed each second during 60 min and expressed as percentage.

Second, an enzymatic method was used to detect microquantities of free hemoglobin as described by Vazquez et al. (39). Time-dependent hemolysis was measured before and after addition of 3V, and expressed as percentage.

**Data Analysis**—Statistical significance was determined using one-way analysis of variance followed by a Tukey-Kramer test of multiple comparisons. A \( p \) value \( \leq 0.05 \) was considered significant. In all of the experiments, numbers of determinations (\( n \)) from independent preparations (\( N \)) are indicated.

**RESULTS**

**Effect of Forskolin, Papaverin, and Isoproterenol on Extracellular ATP Concentration from Human Erythrocytes**

We first examined whether isoproterenol alone or in combination with agents that directly enhance cAMP concentration were able to increase the concentration of [ATP]e.

Exposure of human erythrocytes to forskolin for 10 min resulted in a dose-dependent enhancement of [ATP]e, with nucleotide concentration being further increased when forskolin was given together with 100 \( \mu \text{M} \) isoproterenol (Fig. 1A). Similarly, the \( \beta \)-adrenergic receptor agonist isoproterenol (EC_{50} = 2.9 \pm 0.7 \text{ nm}) promoted an [ATP]e increase in a dose-dependent manner (Fig. 1B) that was comparable in magnitude with that induced by forskolin. The [ATP]e was enhanced \( \sim 2 \)-fold by 30 \( \mu \text{M} \) forskolin and 100 \( \mu \text{M} \) papaverine (from 1.4 \pm 0.3 to 2.7 \pm 0.4 \text{ pmol} \times (10^6 \text{ cells})^{-1}; \( N = 5, n = 4; p < 0.01 \)) and 6-fold with an activating mixture (called “3V”) containing 10 \( \mu \text{M} \) isoproterenol, 30 \( \mu \text{M} \) forskolin,
and 100 μM papaverine (from 1.4 ± 0.3 to 8.5 ± 0.4 pmol × (10^8 cells)^{-1}; N = 5, n = 4; p < 0.01; Fig. 1C).

Next, we checked whether these changes in [ATP]e correlated with changes in intracellular cAMP concentration. As shown in Fig. 1D, incubation of human RBC with 30 μM forskolin and 100 μM papaverine resulted in a significant increase in cAMP concentration at 10 min (from 0.027 ± 0.006 to 0.14 ± 0.05 pmol × (10^9 cells)^{-1}; N = 15, n = 4, N = 10, and n = 4, respectively; p < 0.03). This effect was markedly increased for 3V-exposed cells, i.e. when forskolin and papaverine were added together with 10 μM isoproterenol (from 0.14 ± 0.05 to 0.32 ± 0.04 pmol × (10^9 cells)^{-1}; N = 10, n = 4 and N = 15, n = 4, respectively; p < 0.01). To further test the correlation between increases in cAMP and ATPe, cells were incubated with 1 mM of the cell-permeable cAMP analog dibutyryl cyclic AMP, which induced an increase of [ATP]e from 1.6 ± 0.3 pmol × (10^8 cells)^{-1} (N = 3, n = 2) to 3.7 ± 0.4 pmol × (10^8 cells)^{-1} (N = 3, n = 2; p < 0.01 (supplementary Fig. S1).

**Inhibition of Pannexin 1-mediated ATP Release from Human Red Blood Cells**

Pannexin 1 was suggested as one potential conduit for ATP release of human erythrocytes (40, 41). Thus, we investigated whether pharmacological inhibition of pannexin 1 by carbenoxolone or by 10^15Panx1 can inhibit ATP release in our experimental setup (Fig. 2).

The 3V stimulating mixture induced a significant increase in [ATP]e compared with unstimulated cells (Fig. 2A, from 2.0 ± 0.1 to 8.6 ± 0.4 pmol × (10^8 cells)^{-1}; N = 4, n = 4; p < 0.01). This effect was reduced 78–84% by preincubating the cells with either carbenoxolone at 10 μM (Fig. 2A, 3.4 ± 0.4 pmol × (10^8 cells)^{-1}; N = 4, n = 4; p < 0.01) or 100 μM (Fig. 2A, 3.1 ± 0.8 pmol × (10^8 cells)^{-1}; N = 4, n = 4; p < 0.01).

The 3V induced increase in [ATP]e was inhibited 75–80% by 30 μM 10^15Panx1 (from 8.6 ± 0.3 to 2.7 ± 0.2 pmol × (10^8 cells)^{-1}; N = 4, n = 4; p < 0.02) although a significant 30% inhibition was also observed with its scrambled control peptide (**15Panx1,
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**FIGURE 2. Inhibition of isoproterenol-induced ATP release from human red blood cells.** A, extracellular ATP levels (ATP<sub>e</sub>) were determined at 20°C in unstimulated cells (ctrl, empty bar), in cells stimulated 10 min with 3V alone (ctrl, right fine hatched bar), or with 3V preceded by exposure to the following inhibitors: carbenoxolone, 10 μM (CBX 10, left fine hatched bar) or 100 μM (CBX 100, coarse hatched bar), or 30 μM pannexin 1-selective blocking peptide (10Panx1, right coarse hatched bar) and 30 μM scrambled control peptide (scPanx1, left coarse hatched bar). Data represent mean ± S.E. of values of quadruplicate determinations from four independent experiments (**, p < 0.01 versus control; ##, p < 0.05 versus 3V control). B, extracellular ATP levels (ATP<sub>e</sub>) were determined at 37°C in unstimulated cells (ctrl, empty bar), in cells stimulated 10 min with 3V alone (ctrl, right fine hatched bar), or with 3V preceded by exposure to the following inhibitors: 10 μM carbenoxolone (CBX 10, left fine hatched bar), 30 μM pannexin 1-selective blocking peptide (10Panx1, right coarse hatched bar), or the scrambled control peptide (scPanx1, left coarse hatched bar). Data represent mean ± S.E. of quadruplicate determinations from three independent experiments (**, p < 0.01 versus control; #, p < 0.05, and ##, p < 0.02 versus 3V control).

From 8.6 ± 0.3 to 6.0 ± 0.4 pmol × (10<sup>9</sup> cells)<sup>−1</sup>; N = 4, n = 4; p < 0.05). Moreover, although the degree of [ATP]<sub>e</sub> inhibition followed a function with 10Panx1 concentration, with [ATP]<sub>e</sub> being 80% inhibited at 100 μM 10Panx1, the scrambled peptide also led to a significant concentration-dependent blockade of ATP<sub>e</sub> release (see supplemental Fig. S5).

The above mentioned results were obtained by incubating cells at room temperature; running experiments at 37°C produced a similar trend. That is, under 3V exposure the ATP<sub>e</sub> was reduced 70% by 10 μM carbenoxolone or 30 μM 10Panx1, whereas scPanx1 produced a slight (10–15%) but not significant inhibition (Fig. 2B).

**Effect of Forskolin, Papaverine, and Isoproterenol on the Concentrations of ATP<sub>e</sub> and cAMP from Xenopus and Canine Erythrocytes**

To test whether the above described dependence of ATP release on cAMP is a common feature of other vertebrates, we exposed nucleated erythrocytes from X. laevis to 3V stimula-

**FIGURE 3. Effect of isoproterenol on ATP release from Xenopus and dog erythrocytes.** A, [ATP]<sub>e</sub> of X. laevis erythrocytes incubated 10 min at room temperature under unstimulated conditions (Ctrl, empty bar), with the ATP stimulating mixture 3V (Ctrl, right hatched bar), or with 3V for cells pre-exposed 5 min to 100 μM carbenoxolone (CBX 100, left hatched bar). Data represent mean ± S.E. of quadruplicate assays from three independent experiments (**, p < 0.01 versus control; ##, p < 0.01 versus 3V control). B, Xenopus erythrocytes were incubated 10 min at room temperature under control conditions (Ctrl, empty bar) or in the presence of 3V (hatched bar), and cAMP concentrations were determined using a radioimmunoassay. Data represent mean ± S.E. of values of quadruplicate determinations from four independent experiments (**, p < 0.01 versus control). C, levels of ATP<sub>e</sub> in dog erythrocytes determined under control conditions (empty bar) or in cells stimulated for 10 min with 3V at room temperature (hatched bar). Data represent mean ± S.E. of quadruplicate determinations from three independent experiments. D, CAMP levels for dog erythrocytes exposed 10 min at room temperature under control conditions (empty bar) or in the presence of 3V (hatched bar). Data represent mean ± S.E. of quadruplicate determinations from three independent experiments.

Such a treatment produced a 15-fold increase in [ATP]<sub>e</sub> over basal values (Fig. 3A), together with a 10-fold elevation of intracellular concentrations of cAMP (Fig. 3B). Previously, it was shown that dog RBC do not exhibit a pathway for cAMP-dependent ATP release (6), so these cells can be used to test for nonspecific effects of the agents used to enhance cAMP levels. Following 3V activation of dog RBCs, there is a trend toward a [cAMP] increase that is not statistically significant (from 0.09 ± 0.06 pmol × (10<sup>10</sup> cells)<sup>−1</sup>; N = 4, n = 4; p > 0.05, and Fig. 3D), whereas no changes in [ATP]<sub>e</sub> are observed (Fig. 3C).

**Kinetics of cAMP-stimulated ATP Release from Human and Xenopus Erythrocytes**

Up to here, determinations of accumulated ATP<sub>e</sub> by off-line luminometry were performed at a fixed time point after stimulation. However, to better understand the mechanism(s) allowing a regulated ATP exit, we monitored the kinetics of [ATP]<sub>e</sub> by real-time luminometry using either soluble luciferase or proA-luc.

**Real-time Measurement of [ATP]<sub>e</sub> Using Luciferase—**In human erythrocytes, a fast 2-fold increase of [ATP]<sub>e</sub> was
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FIGURE 4. Kinetics of ATPe from cAMP-stimulated human and Xenopus erythrocytes. A, time course of ATPe concentration ([ATP]e) from human erythrocytes quantified by real-time luminometry. In the times indicated by the arrow, cells were exposed to the 3V mixture (3V). Levels of ATPe are expressed both as concentration (left axis) or as percentage of the intracellular ATP concentration (right axis). Data represent the mean from seven independent preparations. B, kinetics of [ATP]e from X. laevis erythrocytes exposed to 3V (3V arrow). Results represent the mean from six independent preparations. C, [ATP]e kinetics from human and Xenopus erythrocytes preincubated with 100 μM carbenoxolone (CBX arrow), followed by addition of 3V (3V arrow). Results represent the mean from six to seven independent preparations, respectively.

observed after 3V stimulation, with [ATP]e reaching a maximal value of 0.98 ± 0.02 pmol × (10^6 cells)^{-1}, which remained constant thereafter (Fig. 4A). Addition of 100 μM carbenoxolone completely abolished ATP release (Fig. 4C). A similar kinetics of [ATP]e was observed using a subpopulation of RBCs containing only mature erythrocytes (supplemental Fig. S4).

In Xenopus erythrocytes the kinetics of [ATP]e was different, because following stimulation of ATP release a fast increase in [ATP]e to a maximum value of 15.2 ± 0.06 pmol × (10^6 cells)^{-1} was followed by a slow nonlinear decay with t_{1/2} = 7.5 ± 0.6 min (Fig. 4B). As in human erythrocytes, 100 μM carbenoxolone completely abolished any [ATP]e increment (Fig. 4C).

To further validate the ATPe kinetics profile obtained, we checked for artifacts created by potential alterations in hemolysis and cell volume. Hemolysis could increase [ATP]e and thus alter its accumulation profile, whereas changes in cell volume could modify the concentration of cAMP, and other signaling molecules, and therefore indirectly affect ATP efflux. This is why cell volume and hemolysis were determined continuously by fluorescence microscopy of BCECF-loaded human erythrocytes. Results showed that 3V stimulation did not change cell volume (supplemental Fig. S2). On the other hand, hemolysis was assessed before and after exposure to 3V in the absence or presence of carbenoxolone (CBX). After evaluating 3125 cells (n = 5), we found only 6 hemolyzed erythrocytes before application of the treatment (see supplementary Table S1). If each hemolyzed erythrocyte would dilute instantly its entire intracellular ATP content in the extracellular medium, the measured [ATP]e (as shown in Fig. 4) would increase only 0.025%, i.e. a negligible contribution of cell death to [ATP]e. In agreement with these calculations, hemolysis between 8 and 17 min of the basal trace (prior to stimulation) of Fig. 4A do not cause any significant change in [ATP]e.

Parallel online experiments were run to assess hemolysis by quantitating the amount of released hemoglobin (see “Experimental Procedures”). In the absence of 3V (unstimulated condition) a basal hemolysis of ~0.16% was obtained (n = 5). Importantly, basal hemoglobin was content kept constant during the treatment (from 338 ± 80 to 320 ± 117 ng/ml of hemoglobin for untreated and 3V-treated cells, respectively; p > 0.5), so that 3V does not induce hemolysis.

Real-time Measurement of Surface ATP Using ProA-luc—When cell-attached luciferase was used as the ATP sensor, 3V activation triggered rapid accumulation of surface ATP levels to a peak concentration of 2.4 ± 1.13 pmol × (10^6 cells)^{-1}, followed by a slower exponential decay (t_{1/2} = 3.7 ± 1.0 min) to a constant value of 1.3 ± 0.05 pmol × (10^6 cells)^{-1} (Fig. 5). Similarly to real-time experiments with soluble luciferase, 100 μM CBX fully blocks ATP release, so that no significant differences in surface ATP levels were detected using cells in control medium, or in media with either CBX or 3V plus CBX (p > 0.9). The initial ATP release rate amounted to 2.6 ± 1.4 pmol × (10^{-6} cells min)^{-1}.

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The observed kinetics of [ATP]e (Fig. 4) does not only depend on the rate of ATP release, but also on the capacity of ecto-nucleotidases to promote the extracellular hydrolysis of ATP, i.e. ecto-ATPase activity. Thus, using suspensions of human, Xenopus, and canine erythrocytes, at low and high hematocrits, we determined the rate of ATP hydrolysis by following the time course of [γ-32P]P, accumulation released from 1 μM [γ-32P]ATP. Results were expressed as percentage of the total ATP hydrolyzed at each time point (Fig. 6). Furthermore, by calculating the initial rate values in each case we were able to
calculate ecto-ATPase activities (Fig. 7). As expected for mature mammalian erythrocytes, in human and dog erythrocytes ecto-ATPase activities were very low (human, 0.028 ± 0.004 pmol of Pi (10^6 cells min)^{-1}; dog, 0.024 ± 0.003 pmol of Pi (10^6 cells min)^{-1}), whereas in *Xenopus* erythrocytes ecto-ATPase activity assayed at the same ATP concentration was about 45-fold higher than that in its mammalian counterparts (1.2 ± 0.2 pmol (10^6 cells min)^{-1}; Fig. 7).

On the other hand, [α-^32^P]ATP was used to monitor total dephosphorylation of ATP to adenosine. Accordingly, addition of [α-^32^P]ATP to human erythrocyte suspensions resulted in the release of [α-^32^P]Pi at 0.014 ± 0.003 pmol (10^6 cells min)^{-1} (Fig. 6A). In the absence of cells, both the rates of [γ-^32^P]Pi, and [α-^32^P]Pi, release were negligible.

**DISCUSSION**

In humans, about 40% of blood is occupied by erythrocytes containing millimolar concentrations of ATP. Thus even a limited release of ATP from the large intracellular pool could result in micromolar concentrations of ATP under various physiological conditions (42). Because specific P receptors for ATP are lacking on the erythrocytes plasma membrane (43), the resulting ATPe can thus primarily act on P2Y receptors of the adjacent endothelial cells, or, in conjunction with ecto- and exonucleotidase activities of the vasculature, ATPe can also serve as a reservoir for the generation of the ADP or adenosine agonists that subsequently target various functional P receptor subtypes of erythrocytes (ADP-P2Y13 (43)/adenosine-A2B; (34)) and endothelial cells (44–46). The physiological effects of such ligand-receptor interactions can be manifold, including, e.g., the alteration of the vascular tone (see discussion below).

Thus, in this study we analyzed the time dependent balance of ATP efflux and ATPe hydrolysis to determine the factors governing the effective extracellular concentration of the nucleotide, focusing on a condition when ATPe levels are elevated. Specifically, we made use of the fact that over the past years a growing body of evidence indicated that in human erythrocytes an increase in cAMP elicited by various physiological stimuli can induce a regulated release of ATP (8, 43, 47, 49, 50). The kinetic properties of this ATP efflux has so far received little attention and therefore, before studying the main features of...
ATP efflux in human erythrocytes, we established the optimal conditions leading to a cAMP-dependent release of ATP. It has been found that exposure to forskolin, to directly activate adenylyl cyclase, to forskolin with papaverine, to simultaneously inhibiting cAMP degradation, or cyclase, to forskolin with papaverine, to directly activate adenylyl cAMP of 3- (for forskolin and carboxolone, resulted in a dose-dependent increase in [ATP]e. This increase in [ATP]e was potentiated when cells were exposed to a combination of isoproteonol, forskolin, and papaverine (6-fold). Because similar treatments produced significant increases in cAMP of 3- (for forskolin + papaverine) and 10-fold (for 3V mixture), a causal relationship linking increases in the concentrations of cAMP and [ATP]e is clearly indicated.

An important aspect of this study addressed the nature of the mechanism(s) leading to ATP release. Because mature mammalian erythrocytes lack vesicles and thus the capability to exocytose ATP, and because the negatively charged ATP is impermeant for the phospholipidic plasma membrane, it has been previously hypothesized that cAMP stimulated a conductive mechanism of ATP exit (6). Among various other potential transport proteins mediating such a conductive release, pannexin 1 has been suggested to function as a nonselective channel to molecules of $M_r$ less than 900 (40), so that it could also transport ATP ($M_r$ of ATP ~507). Assuming an ATP electrochemical gradient of the order of 3–4 across the plasma membrane, even brief activation of a nucleotide-permeable transport would rapidly increase the rate of ATP delivery to the cell surface (51) where it can be continuously detected by real-time luminometry. Recently, Sridharan et al. (41) demonstrated that treating human erythrocytes with three pannexin 1 inhibitors prevented hypoxia-induced exit of the nucleotide. Moreover, pannexin 1 is expressed at high levels in human erythrocytes, where large conductance consistent with the properties of pannexin 1 channel activity were observed (52). Accordingly, when in the present study ATP release was induced by the activating mixture 3V, carbenoxolone (a nonselective inhibitor of pannexins and connexins) decreased [ATP]e by 75–84% both at 20 and 37 °C. The specific blocking of pannexin 1 by the peptide 10Panx1 also led to significant blocking of 3V-induced increases in [ATP]e at 20 and 37 °C, but the fact that a scrambled peptide used as a control also inhibited ATPe release precluded any further use of 10Panx1. Noteworthy, this unspecific effect of pannexin 1 mimetic peptides has been observed before, and is in agreement with a steric effect on the channel rather than a sequence specific action (53). In addition to the efflux pathway as such, we wanted to characterize the main factors affecting [ATP]e kinetics, so as to fully appreciate the homeostatic control of ATPe of human red blood cells.

In real-time measurements, soluble luciferase was used in a small volume (40 μl) of a relatively wide chamber, resulting in a medium height of about 100 μm, so that under this condition luciferase acts as an indicator of bulk ATP levels within an extracellular microenvironment of cells, whereas proA-luc adsorbed to the extracellular domain of a membrane-bound protein (in our case, glycoporphin A) was used to detect ATP levels at the cell surface.

Using soluble luciferase as well as proA-luc, we observed that in unstimulated conditions, the steady level of [ATP]e (0 to 10 min) is compatible with a low ecto-ATPase activity being at balance with a basal rate of ATP release of the same magnitude (Figs. 4 and 5). Following 3V exposure, accumulation of ATP within the extracellular compartment will in principle reflect both an increase in the rate of ATP release (by lytic and/or nonlytic mechanisms) and the rate of ATP hydrolysis by membrane-bound ecto-nucleotidases. However, in human erythrocytes the observed kinetics of [ATP]e will primarily reflect the kinetics of ATP release, with little contribution from other processes. This conclusion is suggested by the following observations.

By continuously monitoring human erythrocytes we detected virtually no hemolysis during the experiments (see supplemental Table S1) that would enhance [ATP]e. Ecto-ATPase activity, an ATPe remover at the cell surface, is extremely low. The latter is a common feature of most anucleated mammalian erythrocytes as can also be seen for dog erythrocytes (Fig. 7), whereas nucleated erythrocytes from non-mammalian vertebrates, including Xenopus erythrocytes (Fig. 7), display considerably higher rates of ATP hydrolysis at the cell surface (54). The fact that, at least under the conditions prevailing in our real-time experiments, the resulting [ATP]e kinetics of human erythrocytes thus directly relates to the characteristics of ATP efflux.

Accordingly using soluble luciferase, addition of 3V led to a steep, fast increase in [ATP]e to a steady value at about ~1 pmol × (10^6 cells)−1, which corresponded to an initial ATP release rate of ~880 fmol × (10^6 cells min)−1, followed by rapid flux inactivation. Interestingly, the acute increase of such a potential extracellular signaling factor does not impose any energetic burden on cells, because it requires less than 0.5% of the available cytosolic ATP (Fig. 4A).

On the other hand, when the surface-attached proA-luc ATP sensor was used, exposure to 3V led to a steep increase of surface [ATP]e to a maximum value, followed by a slower nonlinear decay to a constant value. The calculated initial rate of ATP release amounted to 2.6 pmol × (10^6 cells min)−1, and compares well with the ~5 pmol × (10^6 cells min)−1 obtained in human astrocytoma cells using a similar proA-luc probe (51).

A comparison of [ATP]e kinetics from human erythrocytes using luciferase and proA-luc is interesting because, as mentioned before, soluble luciferase dissolved in a very small assay
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volume would sense the immediate microenvironment of cells, whereas proA-luc would sense the cell surface nanoenvironment. As seen in Fig. 5, following 3V activation of human erythrocytes, surface ATP showed a peak [ATP]e, which slowly decreases to a constant value. A fit of an exponential function to this decaying phase shows that ATPe would tend to stabilize at a value that is slightly higher than but nevertheless close to the constant maximal value of ATPe obtained with soluble luciferase. Unlike in most other cell systems, this slow nonlinear [ATP]e decrease cannot be due to ecto-ATPase activity, which we showed is negligible under the assay conditions. Thus, we hypothesized that the observed ATPe decrease is due to extracellular diffusion from cell surface into the bulk medium.

Regarding the differences in ATPe kinetics using both luciferase probes, it is worth noting that human erythrocytes have a net negative surface charge mainly due to ionized sialic acid (55), and that at physiological extracellular pH most ATP molecules exist in anionic form (56). Thus, it is possible that the kinetic profile of surface ATP can be modulated by this negatively charged surface of human erythrocytes. In the absence of experimental evidence, this statement remains speculative.

The fact that in human and Xenopus erythrocytes, the 3V-induced increase in [ATP]e was fully blocked by carbenoxolone (Fig. 3A), implies that, at least under the experimental conditions used here, pannexin 1 might act as the single conduit for cAMP-induced ATP release of these cells. However, in off-line experiments using human erythrocytes carbenoxolone only inhibited 75–84% of the [ATP]e increase, pointing to an additional, pannexin 1-independent mechanism of ATP release. This discrepancy could be explained by assuming that during the off-line experiments, cells held in suspension are subjected to mechanical perturbations due to shaking upon drug addition, whereas in real-time experiments cells are held relatively unperturbed on the bottom of a chamber and modulators are added by diffusion (i.e. the medium is not replaced). Accordingly, when in a new set of real-time experiments a gentle mechanical perturbation was introduced (to mimic the off-line condition) prior to 3V stimulation, a carbenoxolone-insensitive component was detected (see supplemental Fig. S3). This component proved to be induced by mechanical perturbation not directly associated with the cAMP-signaling cascade under study.

In Xenopus erythrocytes, the higher ATPe hydrolysis rate agrees well with 3V activation curves where [ATP]e decreased exponentially after a maximum (Fig. 4B). As in human erythrocytes, this [ATP]e kinetics agrees well with transient opening of the ATP efflux through a pannexin 1-mediated pathway during activation, suggesting that the observed release mechanism is a conserved pathway among different vertebrates.

Regarding cellular and systemic roles of ATPe, the quantitative ATPe profile described in this study for human erythrocytes is compatible with an in vivo scenario where, under unstimulated conditions, [ATP]e is maintained constant at a relatively low value (57), and acute increases occur only transiently and in response to certain physiological and/or pathological conditions. Assuming a 30–40% hematocrit in vivo and a linear relationship between [ATP]e and hematocrit, activation of the cAMP pathway in circulating erythrocytes would generate up to 1 μM ATPe, a concentration suitable for activating most P2 receptors in erythrocytes as well as in endothelial cells (44, 45, 58). This is important inasmuch as activation of P2Y1 and P2Y2 receptors in endothelial cells induces the generation and release of nitric oxide (59, 60), followed by relaxation of smooth muscles surrounding the capillaries of the microcirculation (3) and a corresponding relaxation of vascular tone. In the context of Fig. 5, the proA-luc ATP kinetics would show the time-dependent changes of surface ATP that can be used autocrinally for signaling. Soluble luciferase, on the other hand, would show the ATPe kinetics of the microenvironment (up to 100 μM) that can be used for paracrine signaling.

To the present, the role of red blood cells in promoting the hydrolysis of ATPe in blood has been neglected. However, the mentioned high density of erythrocytes in blood implied that the ecto-ATPase activity, even if extremely low compared with erythrocytes of non-mammalian vertebrates (49), can contribute significantly to the hydrolysis of micro- and submicromolar concentrations of ATPe. Moreover, as seen in Fig. 6A, human erythrocytes are also capable of completely dephosphorylating ATP to adenosine. This is compatible with the existence of an enzymatic cascade involving ecto-nucleosome trisphosphate diphosphohydrolases (E-NTPDases) and ecto-5′-nucleotidase (5, 33), so as to sequentially hydrolyze ATP, ADP, and AMP. Similarly to ATPe, the resulting extracellular adenosine may interact with A2 receptors of the endothelium to also mediate vasorelaxant effects (44). Thus, even if leukocytes and endothelial cells, as well as plasma exonucleotidases exhibit various degrees of ATP diphosphohydrolase activity (48, 61) the contribution of erythrocytes to ATPe hydrolysis in blood cannot be neglected, and should be taken into account for physiological interpretations of ATPe actions within the vascular system.

In summary, we have shown that agents that induce intracellular CAMP formation lead to a regulated release of ATP from human erythrocytes, with Xenopus erythrocytes displaying a qualitative similar pattern. Furthermore, our results are consistent with pannexin 1 acting as a pore permeable to ATP or being an essential mediator of cytoplasmic ATP exit. We show here, for the first time, the transient nature of pannexin 1-mediated ATP efflux of human erythrocytes, where CAMP increases lead to a steep activation that is rapidly inactivated. As a corollary from this study it became apparent that caution should be given to experiments where the effect of specific stimuli is analyzed in cells suspensions, because besides pannexin 1-mediated efflux, ATP can also be released via a carboxolone-insensitive, mechanosensitive component.

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