Participation of cAMP in a signal-transduction pathway relating erythrocyte deformation to ATP release

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Sprague, Randy S., Mary L. Ellsworth, Alan H. Stephenson, and Andrew J. Lonigro. Participation of cAMP in a signal-transduction pathway relating erythrocyte deformation to ATP release. Am J Physiol Cell Physiol 281: C1158–C1164, 2001.—Previously, we reported that red blood cells (RBCs) of rabbits and humans release ATP in response to mechanical deformation and that this release of ATP requires the activity of the cystic fibrosis transmembrane conductance regulator (CFTR). It was reported that cAMP, acting through a cAMP-dependent protein kinase, PKA, is an activator of CFTR. Here we investigate the hypothesis that cAMP stimulates ATP release from RBCs. Incubation of human and rabbit RBCs with the direct activator of adenylyl cyclase, forskolin (10 or 100 μM), with IBMX (100 μM), resulted in ATP release and increases in intracellular cAMP. In addition, epinephrine (1 μM), a receptor-mediated activator of adenylyl cyclase, stimulated ATP release from rabbit RBCs. Moreover, incubation of human and rabbit RBCs with an active cAMP analog [adenosine 3’,5’-cyclic monophosphorothioate Sp-isomer (Sp-cAMP, 100 μM)] resulted in ATP release. In contrast, forskolin and Sp-cAMP were without effect on dog RBCs, cells known not to release ATP in response to deformation. When rabbit RBCs were incubated with the inactive cAMP analog and inhibitor of PKA activity, adenosine 3’,5’-cyclic monophosphorothioate Rp-isomer (100 μM), deformation-induced ATP release was attenuated. These results are consistent with the hypothesis that adenylyl cyclase and cAMP are components of a signal-transduction pathway relating RBC deformation to ATP release from human and rabbit RBCs.

pulmonary circulation; red blood cells; cystic fibrosis transmembrane conductance regulator; nitric oxide

RED BLOOD CELLS (RBCs) contain appreciable concentrations of ATP generated via a glycolytic pathway (4, 13, 19, 31, 42). In response to a number of stimuli, including reductions in oxygen tension and pH (4, 13, 18, 19) as well as mechanical deformation (40, 41, 42), RBCs of several species, but not all, release ATP in amounts adequate for activation of endothelial P2Y purinergic receptors (26, 32). Activation of these receptors results, in turn, in endothelial synthesis and release of nitric oxide (NO) and prostacyclin (7, 22), each a major contributor to the regulation of vascular tone.

ATP is a highly charged molecule and, as such, does not readily cross cell membranes by diffusion alone. Thus it would be anticipated that the efflux of ATP from RBCs would require a specific release mechanism(s). Several reports suggested that ATP egress from cells could be mediated via the activity of a family of ion channels, termed the ATP binding cassette (1, 36). One member of this family, the cystic fibrosis transmembrane conductance regulator (CFTR) was reported to facilitate the movement of ATP out of cells either by acting as an ATP conduit (34, 36) or by regulating another separate channel for ATP release (1, 25). Indeed, it was recently reported that CFTR, in response to changes in cell volume, can stimulate the activity of a separate ATP channel with which it is intimately associated in the cell membrane (5). Importantly, the finding that CFTR is not active in humans with cystic fibrosis (10, 12) provided a unique opportunity to investigate the role of CFTR in the release of ATP from RBCs. Indeed, we reported that RBCs of patients with cystic fibrosis do not release ATP in response to mechanical deformation (40). In addition, incubation of rabbit RBCs with either of two chemically dissimilar inhibitors of CFTR resulted in inhibition of deformation-induced ATP release (40). The finding that CFTR is required for deformation-induced release of ATP from the RBCs of humans and rabbits permitted studies to be designed that address characterization of the signal-transduction pathway(s) responsible for deformation-induced release of ATP from these cells.

Activation of CFTR requires phosphorylation by protein kinases (3, 17, 25, 45). It was reported that cAMP-dependent protein kinase [protein kinase A (PKA)] plays a major role in the activation of CFTR in several cell types (3, 17, 25). PKA is found in the membrane of RBCs (16, 38) and, when activated, was reported to phosphorylate erythrocyte proteins (20, 21). Importantly, RBCs were reported to contain adenylyl cyclase (37, 39), which, via the synthesis of cAMP, is the major activator of PKA activity (8, 30). Thus RBCs contain enzymes that are capable of increasing intracellular
cAMP, an activator of CFTR, the latter a requisite for deformation-induced ATP release (40).

In the present study, we investigated the hypothesis that cAMP is a major participant in the regulation of deformation-induced ATP release from rabbit and human RBCs. We examined the ATP release from human, rabbit, and dog RBCs in response to agents that increase endogenous cAMP concentration as well as in response to the exogenous administration of an active cAMP analog. In addition, we determined the effect of an inactive cAMP analog and inhibitor of PKA activity on deformation-induced ATP release from rabbit RBCs.

METHODS

Preparation of RBCs. For rabbit RBCs, animals (male New Zealand white rabbits, 2–3 kg) were anesthetized (ketamine 8 ml/kg and xylazine 1 mg/kg im followed by pentobarbital sodium 15 mg/kg iv). After tracheostomy, animals were mechanically ventilated (tidal volume 10 ml/kg body wt, rate 20–25 breaths/min, Harvard ventilator). A catheter was placed into a carotid artery, heparyn (500 units iv) was administered, and, after 10 min, animals were exsanguinated. For dog RBCs, animals (male mongrel dogs, 25–30 kg) were anesthetized (pentobarbital 15 mg/kg iv) and, following tracheostomy, were mechanically ventilated (tidal volume 15 ml/kg body wt, rate 20 breaths/min, Harvard ventilator). A catheter was placed into a femoral artery, heparyn (2,000 units iv) was administered, and, after 10 min, 100 ml of blood were removed. For human RBCs, venipuncture was performed in an antecubital vein, and, without the use of a tourniquet, 60 ml of blood were collected into a syringe containing heparyn (50 units). Immediately after collection of blood, RBCs were separated from other formed elements and plasma by centrifugation at 500 g at 4°C for 10 min. The supernatant anduffy coat were removed by aspiration. The packed RBCs were resuspended and washed three times in a physiological salt solution (PSS; in mM: 4.7 KCl, 2.0 CaCl₂, 1.2 MgSO₄, 140.5 NaCl, 21.0 Tris, and 11.1 dextrose with 5% BSA, pH 7.4). The oxygen tension of the suspension buffer was 85–90 mmHg. RBCs were prepared on the day of use. The protocols for removal of blood from rabbits, dogs, and humans were approved by the appropriate institutional review committees of Saint Louis University.

Deformation of RBCs. Rabbit RBCs were subjected to mechanical deformation using the St. George’s blood filtrimeter (Carri-Med, Dorking, UK) (15, 40, 42). This device develops a mechanical deformation using the St. George’s blood filtrimeter (15), it is an inherent property of the system that RBCs must be subjected to mechanical deformation to examine their deformability. If average filter pore size and hematocrit are controlled, then RCTT is an index of the degree of deformation to which the RBC is subjected. Thus, under these conditions, the longer the RCTT, the greater the degree of mechanical deformation to which the RBC is subjected. The concentration of ATP present in the effluent from the various filters as well as the number of RBCs/mm³ were determined. The basal release of ATP from RBCs was determined by measurement of ATP present in PSS to which RBCs not passed through a filter were added. Amounts of ATP released were normalized to a RBC count of 2 × 10⁶ cells/mm³. All solutions and RBC suspensions were warmed to 37°C for a minimum of 30 min before use.

Measurement of ATP and hemoglobin. ATP was measured by the luciferin-luciferase technique (4, 13, 19, 41, 42, 44), which utilizes the ATP concentration dependence of light generated by the reaction of ATP with firefly tail extract. Sensitivity was augmented by addition of synthetic d-luciferin to the crude firefly tail extract. A 200-μl sample of the RBC suspension was injected into a cuvette containing 100 μl crude firefly tail extract (5 mg/5 ml distilled water, ΦL 50; Sigma, St. Louis, MO) and 100 μl of a solution of synthetic d-luciferin (50 mg/100 ml distilled water; Sigma). The light emitted was detected using a luminometer (TD-20/20; Turner Designs, Oxnard, CA). A standard curve was obtained on the day of each experiment. To exclude the presence of significant hemolysis, after ATP determinations, samples were centrifuged at 1,000 g at 4°C for 10 min. The presence of hemoglobin in the supernatant was determined by light absorption at wavelengths of 385, 405, 560, 577, and 653 nm (47). In response to stimulation with pharmacological agonists or the application of deformation by filtration, the ATP signal increased in the absence of any consistent change in light absorption of hemoglobin. Moreover, in studies in which known numbers of RBCs were subjected to hypotonic lysis, increases in hemoglobin in the sample were detected concomitant with or before increases in ATP concentration were demonstrable with our assay. All data from experiments in which free hemoglobin increased were excluded. To ensure that the results of the assay were not altered by the agents with which RBCs were incubated, the effects of forskolin, IBMX, adenosine 3’-5’-cyclic monophosphorothioate Sp-isomer (Sp-cAMP), and adenosine 3’,5’-cyclic monophosphorothioate Rp-isomer (Rp-cAMP) on ATP measurement were determined. These agents, at the concentrations used in this study, did not alter the sensitivity of the assay for authentic ATP. Finally, total ATP content of RBCs was determined by measurement of ATP in solution following lysis of a known number of RBCs in distilled water.

Measurement of cAMP. A modified Gilman assay (6, 24) was performed to measure the concentration of cAMP in cell lysates. The assay measures the displacement of [³H]cAMP bound to a cAMP-dependent protein kinase (PKA) by unlabeled cAMP present in biological samples. A 500-μl aliquot of a solution containing RBCs that were incubated with forskolin and IBMX, or their vehicles, was added to 1,000 μl of acidic ethanol. This step stops further cAMP generation and/or degradation by disruption of the respective enzymes. The samples were placed in an ice bath at 0°C for 15 min and then were centrifuged at 1,000 g for 5 min. The supernatant was removed and evaporated to dryness in a vacuum centrifuge. The samples were resuspended in 100 μl of sodium
acetate (500 mM, pH adjusted to 4.5 with acetic acid), and 50 µl of this solution were added to scintillation vials. Histone (2 mg/ml in water) and [3H]cAMP (0.5 pmol) were added to each sample. PKA (20 µl of a solution of 5 mg in 1.7 ml of 10 mM phosphate buffer, 1% BSA) was then added to each sample, and the mixture was incubated for 60 min in an ice bath at 0°C. The reaction was terminated by the addition of 100 µl of hydroxyapatite. After 5 min, 2 ml of phosphate buffer were added and the samples centrifuged for 2 min at 1,000 g. The washing procedure was repeated a second time. The pellet present after the second wash was dissolved in 1 N HCl. Scintillation fluid was then added, and the tubes were counted in a scintillation counter.

**Incubation of RBCs with agents that alter cAMP concentration or activity.** RBCs of humans, rabbits, and dogs were resuspended in PSS (hematocrit 40%) and incubated for 2 min with the combination of forskolin (10 or 100 µM, dissolved in ethanol, final ethanol concentration 0.2%; Sigma) to stimulate adenylyl cyclase and IBMX (100 µM, dissolved in PBS, pH 7.40; Sigma) to prevent cAMP degradation or their respective vehicles (9). Amounts of ATP released in response to forskolin and IBMX as well as the effects of these agents on cAMP concentration were determined. In addition to an agent that directly stimulates adenylyl cyclase activity (forskolin), in separate studies, rabbit RBCs were incubated for 15 min with epinephrine (1 µM), an agonist that increases cAMP activity via activation of a heterotrimeric G protein-coupled receptor (35).

In separate studies, RBCs of humans, rabbits, and dogs (hematocrit 20%) were incubated for 30 min with the active, cell-permeable cAMP analog, Sp-cAMP (100 µM; Biomol Research Labs, Plymouth Meeting, PA), or its vehicle (PBS) (28). The amounts of cAMP and ATP measured were normalized to a RBC count of 5 × 10⁵ cells/mm³.

Finally, the effect of an inactive, cell-permeable cAMP analog and inhibitor of the activity of PKA, namely, Rp-cAMP (100 µM; Biomol Research Labs) (49), or its vehicle (PBS) on deformation-induced ATP release from rabbit RBCs was determined. In the latter studies, RBCs (hematocrit 10%) were incubated with the inhibitor for 30 min and then subjected to mechanical deformation in the St. George’s blood filtermeter. Amounts of ATP released were normalized to a RBC count of 2 × 10⁵ cells/mm³.

**Statistical methods.** Statistical significance between experimental periods was determined with an ANOVA. In the event that the F ratio indicated that changes had occurred, a least-significant difference test was used to identify individual differences. P values of 0.05 or less were considered statistically significant. Results are reported as means ± SE.

**RESULTS**

**Effect of forskolin and IBMX on cAMP concentration and ATP release from RBCs.** In response to incubation with forskolin (10 or 100 µM) and IBMX (100 µM), RBCs of humans (n = 5) released ATP in a dose-dependent fashion (Fig. 1). Similarly, incubation of RBCs of rabbits with forskolin (100 µM) and IBMX resulted in ATP release (n = 8; Fig. 2). The release of ATP in response to forskolin was associated with increases in cAMP concentration in human (n = 6) and rabbit (n = 6) RBCs (Fig. 3). Thus incubation of RBCs of two species that release ATP in response to mechanical deformation, reduced oxygen tension and acidosis, with a direct activator of adenylyl cyclase resulted in both ATP release and increases in cAMP.

**Effect of epinephrine on ATP release from RBCs.** To provide support for the hypothesis that the mechanism
responsible for forskolin-induced ATP release was via the stimulation of adenylyl cyclase, rabbit RBCs were incubated with a second agent, namely, epinephrine. Importantly, the mechanism by which epinephrine stimulates adenylyl cyclase, via activation of a heterotrimeric G protein of the Gs subtype, differs from the direct stimulation of the enzyme produced by forskolin (9, 35). Incubation of rabbit RBCs with epinephrine (1 μM) resulted in ATP release (n = 9; Fig. 4).

Effect of Sp-cAMP on ATP release from RBCs. Finally, to provide support for the hypothesis that ATP release from RBCs of humans and rabbits associated with the application of forskolin and IBMX or epinephrine was related to increases in cAMP, the effect of the active cAMP analog, Sp-cAMP (100 μM) (28) on ATP release was determined. RBCs of both humans (n = 5) and rabbits (n = 6) released ATP in response to the application of Sp-cAMP (Fig. 5).

Effect of forskolin and IBMX or Sp-cAMP on ATP release from dog RBCs. In contrast to RBCs of humans and rabbits, RBCs of dogs do not release ATP in response to deformation or reduced oxygen tension. Importantly, incubation of dog RBCs with forskolin and IBMX did not result in ATP release (n = 4; Fig. 2). In addition, dog RBCs did not release ATP in response to incubation with the active cAMP analog, Sp-cAMP (n = 4; Fig. 5). Total ATP content of RBCs of humans, rabbits, and dogs is depicted in Table 1. The ATP content of dog RBCs was not different from that of human RBCs, but was less than that of rabbit RBCs.

Effect of Rp-cAMP on deformation-induced ATP release from rabbit RBCs. To investigate the hypothesis that cAMP is required to demonstrate deformation-induced ATP release from RBCs, rabbit RBCs were subjected to mechanical deformation in the absence and presence of the inactive cAMP analog and inhibitor of PKA activity, Rp-cAMP (100 μM) (49). RBCs were deformed by passage through filters with average pore sizes of 12, 8, or 5 μm. In the absence of Rp-cAMP, as the pore size that the cells traversed was decreased, the RCTT, an index of RBC deformation, increased (Fig. 6A). Importantly, as RBCs were passed through filters with decreasing average pore size, i.e., as deformation of RBC increased, amounts of ATP released increased (Fig. 6B). Incubation of rabbit RBCs with Rp-cAMP resulted in attenuation of deformation-induced ATP release (Fig. 6B). Importantly, incubation with Rp-cAMP was without effect on RBC deformability, i.e., RCTT was not altered (Fig. 6A). Total ATP content of RBCs was not altered by incubation with Rp-cAMP (Table 2). Thus the effect of Rp-cAMP to limit deformation-induced ATP release could not be attributed to altered deformability or depletion of intracellular ATP.

**DISCUSSION**

RBCs are subjected to mechanical deformation when traversing microvascular beds such as in the pulmonary circulation. The finding that the presence of RBCs capable of releasing ATP in response to mechanical deformation (40, 42) was required to demonstrate flow-induced NO synthesis in the lung (42, 43) suggested a novel mechanism for the local control of pulmonary vascular caliber. In this construct, as the RBC is increasingly deformed by increments in the velocity of blood flow and/or by reductions in vascular caliber, it releases ATP, which, in turn, stimulates endothelial synthesis of NO. The released NO relaxes vascular smooth muscle, resulting, thereby, in a decrease in pulmonary vascular resistance as well as a decrease in the stimulus for RBC deformation and additional ATP release. Several lines of evidence provide support for the hypothesis that RBC-derived ATP is a participant

![Graph](https://example.com/graph.png)

**Table 1. Concentration of ATP in red blood cells of rabbits, humans, and dogs**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Total ATP, mM/RBC</th>
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<tbody>
<tr>
<td>Humans</td>
<td>9</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Rabbits</td>
<td>18</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Dogs</td>
<td>5</td>
<td>0.6 ± 0.1*</td>
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</tbody>
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Values are means ± SE; n, no. of experiments. *Different from value for rabbit red blood cells (RBCs), P < 0.05.

*Fig. 4. Effect of incubation of RBCs of rabbits (n = 9) with epinephrine (10 μM) or its vehicle (saline) on ATP release (per 5 × 10⁶ RBCs/mm³). *Different from vehicle value (P < 0.05).

*Fig. 5. Effect of incubation of RBCs of humans (open bars, n = 5), rabbits (solid bars, n = 6), and dogs (hatched bars, n = 4) with the active cAMP analog, adenosine 3'5'-cyclic monophosphorothioate Sp-isomer (Sp-cAMP; 100 μM) or its vehicle (PBS) on cAMP concentration (per 5 × 10⁶ RBCs/mm³). *Different from respective vehicle value (P < 0.05).
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in the regulation of vascular resistance in the pulmonary circulation and in other vascular beds as well.

Previously, we reported that, in the isolated perfused rabbit lung, RBCs that possess the capacity to release ATP in response to mechanical deformation (rabbit or human RBCs) were a required component of the perfusate for flow-induced endogenous NO synthesis to be demonstrable (42, 43). In support of these studies, it was reported that, in isolated rat cerebral arterioles, it was reported that rat RBCs were a required component of the perfusate to demonstrate ATP release and vasodilation in response to reduced oxygen tension (13, 14).

Taken together, the studies described above are consistent with the hypothesis that ATP, released from RBCs of several species, including humans, functions as a participant in local circulatory control mechanisms. In the case of the of the pulmonary circulation, such local control of the circulation would aid in the maintenance of low vascular resistance. In other tissues ATP-derived ATP would contribute to the regulation of the distribution of vascular resistance, resulting in the matching of oxygen supply with demand.

If ATP release from RBCs is a determinant of endogenous NO synthesis, then a signal-transduction mechanism that relates a stimulus, such as mechanical deformation, to ATP release should be demonstrable. The findings presented here support the hypothesis that ATP released from RBCs of rabbits and humans in response to mechanical deformation is regulated by intracellular cAMP. The contribution of increases in intracellular cAMP to ATP release is demonstrated in several ways. First, incubation of rabbit and human RBCs with agents that directly activate adenylyl cyclase (forskolin) and inhibit cAMP degradation (IBMX) resulted both in ATP release (Figs. 1 and 2) and increased cAMP concentration (Fig. 3). Second, incubation of rabbit RBCs with epinephrine, an agonist that stimulates cAMP synthesis via receptor-mediated activation of heterotrimeric G proteins of the Gs subclass, resulted in ATP release (Fig. 4). Finally, incubation of rabbit or human RBCs with an active, cell-permeable cAMP analog (Sp-cAMP) resulted in ATP release (Fig. 5).

In contrast to humans and rabbits, NO is not a determinant of vascular resistance in the pulmonary circulation of the dog (2, 27, 33). In addition, it was reported that dog RBCs do not release ATP in response to mechanical deformation (42) or reduced oxygen tension (18). In view of the finding that dog RBCs contain ATP in concentrations not different from those found in the RBCs of humans, we postulated that the dog RBC lacked one or more components of the signal-transduc-

### Table 2. Concentration of ATP in rabbit red blood cells in the absence and presence of Rp-cAMP

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total ATP, mM/RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>Rp-cAMP</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
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Values are means ± SE; n, no. of experiments. Rp-cAMP, adenosine 3’,5’-monophosphorothioate Rp-isomer.
tion mechanism(s) that related external stimuli to ATP release from these cells. The finding that dog RBCs do not release ATP in response to incubation with forskolin and IBMX (Fig. 2) or to treatment with Sp-cAMP (Fig. 5) demonstrates that ATP release from human and rabbit RBCs in response to these agonists is not the result of a nonspecific effect on the RBC membrane. In addition, these results suggest that the failure of dog RBCs to release ATP is related to the lack of a cAMP-responsive component or a component distal to one activated by cAMP in the signal-transduction mechanism for ATP release.

The activity of CFTR has been reported to be required for deformation-induced ATP release from RBCs of rabbits and humans (40). Although these studies did not address whether CFTR is itself a conduit for ATP or whether it influences another ATP channel, the findings suggest that CFTR is a component of a signal-transduction pathway for ATP release from RBCs of rabbits and humans (40). Increases in cAMP were reported to stimulate the activity of CFTR in several cell types (3, 17, 25, 45). Thus we anticipated that incubation of rabbit RBCs with an inactive, cell-permeable cAMP analog (Rp-cAMP) (49) would interfere with deformation-induced ATP release from these cells. The mechanism by which cAMP stimulates CFTR activity was reported to be via activation of PKA (3, 17, 45). Indeed, Rp-cAMP is a potent inhibitor of the activity of that protein kinase (49). In the absence of Rp-cAMP, mechanical deformation of rabbit RBCs by passage of the cells through filters with decreasing average pore size resulted in increments in ATP release (Fig. 6B). In contrast, incubation of rabbit RBCs with Rp-cAMP prevented deformation-induced ATP release (Fig. 6B). Importantly, Rp-cAMP was without effect on the deformability of the RBCs, i.e., the red cell transit time was unaltered (Fig. 6A), demonstrating that the effect of Rp-cAMP to limit ATP release could not be related to an effect on RBC deformability per se.

In summary, the results presented here provide support for the hypothesis that, in RBCs of rabbits and humans, increases in cAMP are required for deformation-induced ATP release. These findings, coupled with the reports that inclusion of RBCs of rabbits and humans in the perfusate of isolated rabbit lungs was required to demonstrate flow-induced NO synthesis, suggest that ATP released from RBCs via this signal-transduction pathway may be an important determinant of pulmonary vascular resistance. Moreover, this mechanism for the control of vascular caliber, i.e., deformation-induced ATP release from RBCs, would be anticipated to be operative in other vascular beds as well. A more comprehensive understanding of those mechanisms that regulate ATP release from RBCs may provide new insights into the pathophysiology of vascular disease.

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