Deformation-induced ATP release from red blood cells requires CFTR activity

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Deformation-induced ATP release from red blood cells requires CFTR activity. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1726–H1732, 1998.—Recently, it was reported that rabbit and human red blood cells (RBCs) release ATP in response to mechanical deformation. Here we investigate the hypothesis that the activity of the cystic fibrosis transmembrane conductance regulator (CFTR), a member of the ATP binding cassette, is required for deformation-induced ATP release from RBCs. Incubation of rabbit RBCs with either of two inhibitors of CFTR activity, glibenclamide (10 µM) or niflumic acid (20 µM), resulted in inhibition of deformation-induced ATP release. To demonstrate the contribution of CFTR to deformation-induced ATP release from human RBCs, cells from healthy humans, patients with cystic fibrosis (CF), or patients with chronic obstructive lung disease (COPD) unrelated to CF were studied. RBCs of healthy humans and COPD patients released ATP in response to mechanical deformation. In contrast, deformation of RBCs from patients with CF did not result in ATP release. We conclude that deformation-induced ATP release from rabbit and human RBCs requires CFTR activity, suggesting a previously unrecognized role for CFTR in the regulation of vascular resistance.

cystic fibrosis; chronic obstructive lung disease; nitric oxide; pulmonary circulation; vascular resistance; adenosine 5’-triphosphate; cystic fibrosis transmembrane conductance regulator

ENDOTHELIUM-DERIVED nitric oxide (NO) is considered to be an important participant in the regulation of the circulation via its ability to produce relaxation of vascular smooth muscle (11, 15). However, those mechanisms that stimulate the synthesis and release of NO under physiological conditions, for example, in response to changes in vascular resistance, are incompletely understood. A number of endogenous substances, when applied to endothelial cells in culture or to isolated blood vessels, stimulate the synthesis and release of NO (3, 5, 9). One of those substances, ATP, is contained in large amounts in red blood cells (RBCs; see Refs. 17, 24). Recently, it was reported that ATP is released in response to mechanical deformation of RBCs from humans and rabbits (24). Thus, in the intact circulation, ATP released from RBCs in response to this physiological stimulus could serve as an important determinant of NO synthesis and, thereby, vascular resistance. However, ATP is a highly charged molecule and, as such, does not readily cross cell membranes. It has been suggested that ATP may exit cells via the activity of one or more ion channels in a family referred to as the “ATP binding cassette” (27). One member of this family, the cystic fibrosis transmembrane conductance regulator (CFTR), was suggested to participate in the movement of ATP out of cells (1); however, the mechanism by which CFTR influences ATP release remains controversial. Several studies report that CFTR is, itself, an ion channel mediating ATP efflux (18, 21), whereas others suggest that ATP does not exit cells via this channel (13, 16, 20). In an attempt to reconcile these divergent points of view, it was proposed that CFTR is not the channel mediating ATP efflux but rather is a regulator of another ion channel(s) that permits the movement of ATP out of cells (14), i.e., CFTR is, as the name implies, truly a transmembrane conductance regulator for ATP (1, 14).

In the present work, we investigated the hypothesis that the release of ATP from RBCs of rabbits and humans, in response to mechanical deformation, requires the activity of CFTR. We used pharmacological inhibitors of CFTR activity as well as RBCs from patients with cystic fibrosis, who are genetically deficient in CFTR activity, to determine if CFTR is necessary for deformation-induced ATP release from RBCs.

METHODS

Preparation of RBCs. For obtaining rabbit RBCs, male New Zealand White rabbits (2.0–2.5 kg) were anesthetized with droperidol (0.1 ml/kg im) followed by pentobarbital sodium (15 mg/kg iv). After tracheostomy, the rabbits were mechanically ventilated (tidal volume 10 ml/kg, rate 20–25 breaths/min; Harvard ventilator). A catheter was placed into a carotid artery, heparin (2,000 units iv) was administered, and, after 10 min, animals were exsanguinated. For human RBCs, 60 ml of blood were collected into a syringe containing heparin (50 units) via venipuncture without the use of a tourniquet. Immediately after collection of blood from rabbits or humans, RBCs were separated from other formed elements and plasma by centrifugation at 1,000 g at 4°C for 10 min. The supernatant and Buffy coat were removed by aspiration. 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, and 21.0 tris(hydroxymethyl)aminomethane and 11.1 dextrose with 5% bovine serum albumin, pH adjusted to 7.4). RBCs were prepared on the day of use. The protocols for removal of blood from rabbits and humans were approved by the appropriate institutional review committees of Saint Louis University.

Deformation of RBCs. RBCs were subjected to mechanical deformation using the St. George’s Blood Filtrometer (Carri-
ATP content of RBCs was determined by measurement of ATP concentrations used in this study, altered the sensitivity was determined. Neither glibenclamide nor niflumic acid, at acid, the effect of these agents on the measurement of ATP which free hemoglobin was detected were excluded. To ensure the luciferin-luciferase assay. All data from experiments in which synthetic D-luciferin was added to the filtrate. The amount of ATP released in absorption at wavelengths of 385, 405, 560, 577, and 630 nm hemoglobin in the supernatant was determined by light centrifuged at 1,000 g for 5 min. The suspension fluid was injected into a cuvette containing 250 µl crude firefly tail extract. A 250-µl sample of the RBC suspension fluid was injected into a cuvette containing 250 µl crude firefly tail extract (5 mg/5 ml distilled water, FLE 50; Sigma, St. Louis, MO) and 250 µl of a solution of synthetic D-luciferin (50 mg/100 ml distilled water, Sigma). The cuvette was mounted opposite a photomultiplier tube (RCA 931A) in a light-excluding box. A standard curve to ATP was obtained on mounting this filter. The time taken for the fluid to pass each detector was recorded, and a transit time was calculated with a computer. This process was repeated until coefficients of variance between runs were 1% or less.

Deformation of RBCs was achieved by passing cells suspended in PSS at a hematocrit of 10% through the filters. The filtration rate of the RBC suspension relative to PSS alone, the RBC transit time (RCTT), was calculated as described previously (10, 24). The RCTT is dependent on the deformability of the RBCs, the hematocrit, the pressure gradient, and the size of the filter pores relative to the size of the RBC studied. Although the St. George's Blood Filtrometer was originally designed to measure the deformability of RBCs, if hematocrit, pressure gradient, and average filter pore size 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 each filter as well as the number of RBCs per cubic millimeter 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 an RBC count of 2 × 10^5 cells/mm^3. 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 (2, 26) in which the amount of light generated by the reaction of ATP with firefly tail extract is dependent on the ATP concentration. Sensitivity was augmented by addition of synthetic D-luciferin to the crude firefly tail extract. A 250-µl sample of the suspension fluid was injected into a cuvette containing 250 µl crude firefly tail extract (5 mg/5 ml distilled water, FLE 50; Sigma, St. Louis, MO) and 250 µl of a solution of synthetic D-luciferin (50 mg/100 ml distilled water, Sigma). The cuvette was mounted opposite a photomultiplier tube (RCA 931A) in a light-excluding box. A standard curve to ATP was obtained on the day of each experiment. To exclude the possibility that the ATP measured represented that released from the lysis of RBCs, after ATP determinations, the filter effluent was centrifuged at 1,000 g at 4°C for 10 min, and the presence of hemoglobin in the supernatant was determined by light absorption at wavelengths of 385, 405, 560, 577, and 630 nm (4, 28). This technique for hemoglobin determination is sufficiently sensitive to detect hemolysis of 0.1% of RBCs added to the photomultiplier. The amount of ATP released in association with this level of hemolysis was not detectable in the luciferin-luciferase assay. All data from experiments in which free hemoglobin was detected were excluded. To ensure that the results of the assay were not altered by the agents with which RBCs were incubated, glibenclamide or niflumic acid, the effect of these agents on the measurement of ATP was determined. Neither glibenclamide nor niflumic acid, at the concentrations used in this study, altered the sensitivity of the assay for authentic ATP. Finally, in all experiments, ATP content of RBCs was determined by measurement of ATP in solution after lysis of a known number of RBCs in distilled water.

Preparation of reagents. Glibenclamide was prepared as a 0.01 M stock solution by adding 49 mg of glibenclamide to a solution containing 2 ml of 0.1 N NaOH and 7.94 ml of dextrose in distilled water (50 mg/ml) and heating it slowly to 52°C. Niflumic acid was dissolved in distilled water. All compounds were purchased from Sigma.

Deformation of rabbit RBCs in the absence and presence of inhibitors of CFTR activity. Before the passage of rabbit RBCs through the filtermeter, the cells were incubated for 30 min with one of two purported inhibitors of CFTR activity, namely, glibenclamide (10 µM, n = 5; see Refs. 22, 23) and niflumic acid (20 µM, n = 5; see Ref. 7), or with PSS. The RBCs were passed through filters with average pore diameters of 12, 8, or 5 µm. The sequence of filters was randomly assigned.

To determine whether the effect of glibenclamide on deformation-induced ATP release from RBCs was irreversible, rabbit RBCs were incubated with glibenclamide (10 µM, n = 4) for 30 min and then washed three times in PSS. Four groups of RBCs were subjected to deformation by passage through filters with an average pore diameter of 5 µm in the filtermeter. The groups were as follows: 1) control RBCs not exposed to glibenclamide, 2) RBCs exposed to glibenclamide for 30 min but not washed, 3) RBCs exposed to glibenclamide for 30 min and then washed three times, and 4) three experiments in which fresh rabbit RBCs were incubated with the supernatant obtained after the final washing of RBCs exposed to glibenclamide.

Deformation of human RBCs. Human RBCs were obtained from healthy volunteers (n = 5), patients with cystic fibrosis (n = 5), and patients with chronic obstructive pulmonary disease (n = 4) and were deformed in the filtermeter. Patients with cystic fibrosis were being treated with medications, including inhaled β-agonists (5), inhaled ipratropium bromide (4), oral pancreatic enzyme replacement (4), inhaled corticosteroids (3), oral antibiotics (3), and oral theophylline (1). All patients with chronic lung disease were being treated with inhaled β-agonists, and two were using inhaled ipratropium bromide. Healthy subjects were on no medications.

Statistical methods. Statistical significance between experimental periods was determined with an analysis of variance. 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 glibenclamide on deformation-induced ATP release from rabbit RBCs. Mechanical deformation of rabbit RBCs by passage through filters with average pore sizes of 12, 8, or 5 µm resulted in increments in both RCTT and ATP release (Fig. 1, A and B). Thus, in the control group, ATP from the RBCs was greater than baseline values after passage through all filters. Moreover, amounts of ATP released after passage through filters with pores of 5 µm were greater than amounts released after passage through filters with pores 8 or 12 µm in diameter (Fig. 1B). Incubation of rabbit RBCs with glibenclamide (10 µM, n = 5) resulted in inhibition of deformation-induced ATP release. In the presence of glibenclamide, ATP concentration in the effluent of
filters with pores of 12, 8, and 5 µm decreased from control values by 37 ± 6, 55 ± 7, and 63 ± 6%, respectively (Fig. 1B).

Incubation of rabbit RBCs with glibenclamide did not result in a change in RCTT compared with control RBCs (Fig. 1A). In addition, concentrations of ATP present within rabbit RBCs after incubation with glibenclamide were not different from those of untreated RBCs (Table 1).

Table 1. Concentration of ATP in rabbit RBCs in the absence and presence of glibenclamide and niflumic acid

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total ATP, mM/RBC</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>2.3 ± 0.7</td>
</tr>
<tr>
<td>Control</td>
<td>2.4 ± 0.8</td>
</tr>
<tr>
<td>Niflumic acid</td>
<td>2.3 ± 0.8</td>
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</tbody>
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Values are means ± SE, n = 5 experiments/group. RBCs, red blood cells.

Fig. 1. Red blood cell (RBC) transit time (RCTT; A) and ATP release (ATP concentration in filter effluent per 2 × 10⁶ RBCs/mm²; B) at baseline and in response to passage of rabbit RBCs through filters with pores of decreasing size. Open bars, untreated RBCs (n = 5); filled bars, RBCs incubated with glibenclamide (n = 5). *Significantly different from 12 µm value (A) or baseline (B); †significantly different from untreated RBCs.

Effect of washing on deformation-induced ATP release from rabbit RBCs incubated with glibenclamide. Neither exposure of RBCs to glibenclamide nor repeated washing resulted in any change in RCTT from control values in response to passage of cells through filters with 5-µm pores (Fig. 2A). In contrast, glibenclamide treatment resulted in inhibition of ATP release when RBCs were subjected to mechanical deformation (Fig. 2B). The inhibition of ATP release in response to mechanical deformation was not diminished after repeated washing of the RBCs (Fig. 2C). Finally, the supernatant obtained after the final wash of RBCs treated with glibenclamide, when incubated with fresh RBCs, did not alter RCTT or inhibit ATP release (Fig. 2, A and B).

Effect of niflumic acid on deformation-induced ATP release from rabbit RBCs. In the presence of niflumic acid (20 µM, n = 5), the RCTT of RBCs was unaltered (Fig. 3A). In contrast, ATP release was reduced significantly in response to passage of RBCs through filters with average pore sizes of both 8 and 5 µm after incubation with niflumic acid (Fig. 3B). The inhibition of ATP release with niflumic acid was not unlike that seen after incubation of rabbit RBCs with glibenclamide (Fig. 1B), i.e., in the presence of niflumic acid, release of ATP was reduced by 37 ± 20, 54 ± 5, and 61 ±
6% in response to passage through filters with pore sizes of 12, 8, and 5 µm, respectively. Niflumic acid was without effect on the concentration of ATP in the RBCs (Table 1).

Effect of mechanical deformation on ATP release from RBCs of healthy humans and humans with cystic fibrosis. The finding that the genetic defects of cystic fibrosis result in abnormal expression or processing of CFTR (8) provides a unique opportunity to examine the contribution of this channel to mechanical deformation-induced ATP release from human RBCs. RBCs obtained from healthy humans (n = 5) released ATP in response to mechanical deformation in a stimulus-dependent fashion, i.e., ATP release increased with each decrement in average filter pore size (Fig. 4). In contrast, RBCs from patients with cystic fibrosis (n = 5) did not release ATP in response to mechanical deformation (Fig. 4B). Moreover, ATP values after passage through the filters did not differ from values for ATP determined for cells not subjected to deformation, i.e., the values did not differ from baseline. The RCTT for RBCs from healthy humans and patients with cystic fibrosis was not different for any pore size studied (Fig. 4A). Concentrations of ATP in the RBCs of healthy humans and patients with cystic fibrosis did not differ (Table 2).

Effect of mechanical deformation on ATP release from RBCs of humans with chronic obstructive lung disease. The RCTTs for RBCs of patients with chronic obstructive lung disease (n = 4) did not differ from those observed for RBCs from normal humans and patients with cystic fibrosis (Fig. 5A). RBCs of chronic lung disease patients released ATP in response to mechanical deformation in a manner not unlike that observed

Table 2. Concentration of ATP in RBCs of control subjects (healthy humans), patients with CF, and patients with COPD

<table>
<thead>
<tr>
<th>Subject</th>
<th>n</th>
<th>Total ATP, mM/RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy humans</td>
<td>5</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>CF</td>
<td>5</td>
<td>2.2 ± 0.7</td>
</tr>
<tr>
<td>COPD</td>
<td>4</td>
<td>5.9 ± 1.0*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of experiments. CF, cystic fibrosis; COPD, chronic obstructive lung disease. *P < 0.05 compared with control and CF groups.
with RBCs of healthy humans (Fig. 5B). Amounts of ATP in the RBCs of patients with chronic lung disease were greater than those in healthy humans and humans with cystic fibrosis (Table 2).

**DISCUSSION**

These studies reaffirm our previous finding that ATP is released from the RBCs of rabbits and healthy humans in response to mechanical deformation (24) and extend that observation to demonstrate that this release of ATP requires the activity of CFTR. The contribution of CFTR to deformation-induced ATP release was investigated in two ways, first, by incubation of rabbit RBCs with agents reported to inhibit the activity of CFTR and, second, by comparing the ability of RBCs from healthy humans and patients with cystic fibrosis to release ATP in response to mechanical deformation.

The sulfonylurea glibenclamide, in addition to effects on ATP-sensitive potassium channels (12, 23), has been reported to be a potent inhibitor of the activity of CFTR (22, 23). Incubation of rabbit RBCs with glibenclamide resulted in inhibition of ATP release in response to mechanical deformation (Fig. 1). Importantly, this agent had no effect on the RCTT, an index of the degree of deformation applied to the RBC. Thus the ability of glibenclamide to block ATP release cannot be explained by an effect on RBC deformability. In addition, glibenclamide was without effect on total amounts of ATP present in the RBCs; thus, the lack of ATP release in response to deformation could not be attributed to a failure of ATP synthesis or to depletion of total ATP (Table 1). As stated above, in addition to effects on CFTR, glibenclamide has been reported to inhibit the activity of ATP-sensitive potassium channels in some cells (12, 23). In separate studies in our laboratory, rabbit RBCs were incubated with cromakalim (10 µM, n = 5) and exposed to mechanical deformation. Incubation with cromakalim was without effect on either RCTT or deformation-induced ATP release (data not shown). These experiments provide additional support for the hypothesis that the observed inhibition of deformation-induced ATP release from rabbit RBCs in the presence of glibenclamide was not related to an effect of the latter compound on ATP-sensitive potassium channels.

It was reported that the ability of glibenclamide to inhibit the activity of CFTR is irreversible, i.e., the blockade remains in spite of removal of the inhibitor (23). In the present work, removal of glibenclamide from RBCs after a 30-min incubation did not restore the ability of the RBCs to release ATP in response to mechanical deformation (Fig. 2). The completeness of glibenclamide removal from the suspending medium is demonstrated by the finding that the supernatant of the final RBC wash, when added to RBCs that had not been exposed to glibenclamide, had no effect on ATP release from the latter cells (Fig. 2). Thus the effect of glibenclamide on deformation-induced ATP release from rabbit RBCs was not reversible over the time course of these experiments and is consistent with reports of the effect of this inhibitor on the activity of CFTR.

Finally, to establish that the ability of glibenclamide to inhibit deformation-induced ATP release was related to its effects on CFTR and was not a nonspecific effect of the drug, in separate experiments, rabbit RBCs were incubated with a second purported inhibitor of CFTR activity, namely, niflumic acid (7). The release of ATP in response to deformation was inhibited by incubation with niflumic acid in a manner identical to that seen after incubation with glibenclamide (Figs. 1 and 3). The finding that, in rabbit RBCs, deformation-dependent ATP release was significantly decreased by two chemically dissimilar inhibitors of CFTR activity provides strong pharmacological evidence in support of the hypothesis that the deformation-induced release of ATP from these cells requires CFTR activity.

The finding that human RBCs, like those of rabbits, release ATP in response to mechanical deformation presents a unique opportunity for the study of the contribution of CFTR to ATP release from RBCs. Cystic fibrosis is a genetic disorder characterized clinically by increased concentrations of chloride in sweat and a constellation of symptoms related to chronic pulmonary disease or pancreatic insufficiency or both (8). Despite the fact that patients may have different
patterns of organ involvement, severity of disease, and genetic mutation, they share a common pathophysiological problem: the expression of CFTR activity is deficient (6, 8). Depending on the nature of the specific genetic defect, CFTR may be incompletely synthesized, may be degraded in the cytoplasm before it can be incorporated into the cell membrane, may be incorporated into the membrane but fail to respond to activation signals, or may reach the membrane but have altered functional properties (6, 8, 19). The nature of the defect notwithstanding, in all cases of cystic fibrosis, the activity of CFTR is markedly diminished or lost.

In the present work, RBCs of healthy humans were compared with those of patients with cystic fibrosis with respect to deformation-induced ATP release. RBCs of healthy humans released ATP in a stimulus-dependent fashion, i.e., as the deformation applied to the RBC increased, ATP release increased (Fig. 4). In contrast, RBCs of patients with cystic fibrosis did not release ATP in response to mechanical deformation. Indeed, ATP in the effluent of the filters was not significantly increased over levels found under baseline conditions. The failure of RBCs of patients with cystic fibrosis to release ATP could not be attributed to decreased ATP present within the RBC (Table 2). To demonstrate that the lack of ATP release in response to deformation could not be related simply to the presence of chronic lung disease, RBCs from four additional patients with the clinical diagnosis of chronic obstructive lung disease were studied. RBCs from the latter group released ATP in response to mechanical deformation, and the response did not differ from that of healthy humans (Fig. 5). Although, as a group, the patients with cystic fibrosis were on more medications than were healthy humans (no medications) or patients with chronic lung disease, it is unlikely that the failure of RBCs from cystic fibrosis patients to release ATP in response to mechanical deformation can be explained on this basis. Importantly, in one patient with cystic fibrosis the sole medication was oral pancreatic enzyme replacement, and a second patient was treated only with the latter medication and an inhaled agonist. In neither case was there any measurable ATP release from RBCs in response to mechanical deformation.

The design of these experiments does not permit a determination of the exact role of CFTR in the egress of ATP from the RBC. Thus the possibility that CFTR functions as a conduit for ATP efflux cannot be excluded. However, the data are also consistent with the interpretation that CFTR activity is required for another protein present in the cell membrane to function as an ATP channel. These considerations notwithstanding, the results of the work presented here provide strong support for the hypothesis that ATP is released from the RBCs of rabbits and humans in response to mechanical deformation and that this release of ATP requires the activity of CFTR.

Previously, it was reported that, in isolated perfused rabbit lungs, RBCs were required for the synthesis of NO in response to increases in perfusate flow rate (25). Importantly, only those RBCs that possessed the capability to release ATP in response to mechanical deformation, namely, RBCs of rabbits and healthy humans, but not those of dogs, stimulated endogenous NO synthesis (24). The finding that ATP stimulates endothelial cells to synthesize and release NO (3, 5, 9) coupled with the observations that ATP is released from RBCs of humans and rabbits in response to mechanical deformation (24) and that these RBCs stimulate NO synthesis in the intact pulmonary circulation (24, 25) suggests that the RBC itself may participate in local circulatory control. In this construct, as the RBC is deformed by increments in blood flow through a vessel and/or by reductions in the vessel’s caliber, ATP is released. This ATP, in turn, stimulates local synthesis and release of NO by endothelial cells, leading, ultimately, to an increase in vascular caliber and, thereby, a reduced stimulus for ATP release.

The pulmonary hypertension associated with cystic fibrosis has generally been attributed to extensive alveolar hypoventilation leading to hypoxic pulmonary vasoconstriction (8). The results of the present work suggest that, in addition to hypoxic pulmonary vasoconstriction, the failure of deformation-induced ATP release from RBCs in cystic fibrosis may lead to a decrease in endogenous NO synthesis and thereby contribute to the development of pulmonary hypertension. Identification of the contribution of CFTR to deformation-induced ATP release from RBCs suggests that this channel may play a previously unrecognized role in the regulation of vascular resistance.

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