Influence of external ATP on permeability and metabolism of dog red blood cells

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PARKER, JOHN C., AND ROGER L. SNOW. Influence of external ATP on permeability and metabolism of dog red blood cells. Am. J. Physiol. 223(4): 888 893. 1972. Dog red blood cells undergo a rapid increase in Na-K permeability and an alteration in physical properties when exposed to external ATP in concentrations greater than 0.1 mM. The effect is reversible on washing the cells, and it can be prevented by Ca and Mg ions. A variety of other nucleotides and chelators do not share this action on the dog red cell membrane. Simultaneous measurements of cation and anion flux show that only the former is affected by ATP. The implications of these results for models of membrane permeability are discussed.

Cell volume regulation; cell membrane; sodium transport; potassium transport; anion permeability

THE MECHANISMS governing ion movements across the dog red cell membrane are poorly understood. In particular, it is not clear how mature dog red cells control their volume, since they are almost in ionic equilibrium with plasma (1). Although they are quite permeable to sodium, it has not been possible to show that they are capable of pumping out this ion (7, 21). This report examines the effects of extracellular adenine nucleotides on dog red cell ion permeability. It is known that the volume of mitochondria is strikingly influenced by adenine nucleotides applied to their outer surface (13). Platelets respond to external ADP by changing shape and possibly volume (2, 24). Ascites tumor cells were found to undergo striking changes in permeability when bathed in a medium containing ATP (6, 26). The recent finding of ATP in the plasma of certain vascular beds (5) suggests that this nucleotide is available to cell surfaces in vivo. The present results, which are quite similar to the findings in ascites tumor cells (6, 26), provide some insights into the determinants of anion and cation movements in dog red cells, but they leave the question of volume control unresolved.

MATERIALS AND METHODS

Nucleotides (sodium salts) (Sigma Chemical Co., St. Louis, Mo., and Calbiochem, Los Angeles, Calif.), nucleotides (Calbiochem), 32NaCl and 42KCl (International Chemical and Nuclear Corp., Irvine, Calif.), Na235SO4 (Amersham-Searle Corp., Des. Plains, Ill.), and materials for enzymatic assays (Boehringer Mannheim Corp., N. Y.) were obtained from commercial sources.

Venous blood from healthy mongrel dogs was drawn on the day of an experiment into plastic syringes rinsed with heparin (1,000 U/ml).

Three standard solutions were used for most experiments, with additions and modifications noted in the presentation of results. Their composition in millimoles per liter was as follows: "standard NaCl buffer" contained 145 NaCl, 5 KCl, 15 glycylglycine, and 10 glucose. Standard KCl buffer was identical except for the replacement of NaCl by KCl. "Wash" consisted of 150 NaCl and 5 glycylglycine. All solutions were adjusted to pH 7.5 and readjusted to this pH after addition of other reagents.

All incubations were in stoppered, plastic flasks under air in a 37 C water-bath shaker oscillating at 100 cycles/min over a traverse of 1 inch. Unless otherwise noted, all cell suspensions had a hematocrit of 5–10 vol %.

The methods for cell water, Na, K, and chloride using 125I-labeled albumin as marker for trapped plasma have been published elsewhere (20). Cells were always washed 4 times by suspension and centrifugation in 10 volumes of wash solution prior to these determinations.

The efflux of 32Na+ and 42K+ was measured by preincubating whole blood for 1–2 hr with isotope (20–30 µc/ml) at 37 C. The cells were then washed 4 times in standard wash solution at 4 C to free them of external radioactivity. Aliquots of labeled, packed cells (0.3 ml) were then pipetted into 30-ml volumes of isotope-free solutions which had been prewarmed to 37 C. At intervals thereafter, portions of the cell suspension were centrifuged, and the radioactivity of the supernatant was determined (Auto-Gamma spectrometer, Packard Instruments Co., La Grange, Ill.)

A lysate of the suspension was then prepared by adding a drop of detergent (Acationox, Biological Research, Inc., Evanston, Ill.) to the supernatant. A correction for hemolysis was not necessary since in the isotope studies reported, less than 1% of hemoglobin was released during the incubation period. The results are expressed in terms of the percentage of total counts released into the supernatant in a given time interval:

\[
\text{counts/min per ml supernatant} \times (\text{time}_2 - \text{time}_1) \times 100 = \frac{\text{counts/min per ml hemolysate}}{\text{per cent counts released}}
\]

The reasons for this simplistic treatment of the data will be discussed.
TABLE 1. Constituents of dog red cells

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Value ± SE (mEq/kg dry wt)</th>
</tr>
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<tbody>
<tr>
<td>Na</td>
<td>323.6 ± 4.9</td>
</tr>
<tr>
<td>K</td>
<td>19.9 ± 0.6</td>
</tr>
<tr>
<td>Cl</td>
<td>198.1 ± 6.1</td>
</tr>
<tr>
<td>H₂O</td>
<td>1,828 ± 11</td>
</tr>
</tbody>
</table>

Values are means ± se for four studies.

RESULTS

Table 1 gives base-line data for the ion and water content of freshly drawn dog red cells. To facilitate presentation of net gains and losses, the values are expressed in units per kilogram dry cell solids.

Figure 1 shows the rate at which dog red cells suspended in standard NaCl buffer gain Na, Cl, and water when exposed to 0.5 mM ATP. The results are plotted so as to emphasize the point that the Na and Cl concentration of the fluid gained by the cells is substantially the same as that of the incubation medium. Potassium movements in this experiment amounted to less than 5 mEq/kg cell solids and are not shown.

Figure 2 presents data regarding the reversibility of the ATP effect. Washing the cells free of ATP at 10 and 60 min results in a decrease in the rapid rate of cell swelling. On no occasion did the washed cells tend to extrude the fluid they had gained in the presence of ATP.

Figure 3 further documents the reversibility of the ATP effect and shows that the nucleotide influences potassium and precise timing. The samples were counted for 35S by liquid scintillation (19) after allowing the 32PNa to decay at least 10 half-lives.

Enzymatic determinations of ATP, ADP, AMP, and lactate were accomplished by previously published techniques (18, 19).

An estimate of the viscosity of packed cells was obtained as follows: cell suspensions were centrifuged at 15,000 X g for 5 min, the supernatant was removed, and the cells were drawn into a 0.5 ml glass blood pipette (Microchemical Specialties Co., Berkeley, Calif.) with a minimum bore of 1 mm. The pipette was then connected to a Harvard infusion pump via a plastic tube which had a standard aneroid blood pressure cuff manometer attached via a sidearm. The infusion pump was run at a speed of 1.91 ml/min, and the maximum pressure required to expel the cells from the pipette was recorded.

Filterability of packed cells through Schleicher and Schuell no. 589 filter paper was determined exactly as described by Teitel (28). The results are expressed as the half-time for the passage of 2 ml of cell suspension (hematocrit 90 vol %) through the paper.

The simultaneous efflux of 35S0₄⁻ and 32PNa⁺ was measured in much the same way except for the following details: preincubation, wash, and efflux measurements were all done in standard NaCl buffer to which 1 mM nonradioactive Na₂SO₄ was added (30). The labeling preincubation was done at 37 C for 1 hr at a hematocrit of 50 vol % with 4–5 µc/ml Na₃5S0₄, added in addition to 32PNaCl as noted previously. Washing was carried out at 4 C. Samples of supernatant from the incubation flasks were obtained not by centrifugation but by Millipore filtration of the cell suspension into syringes as described by Mawe and Hempling (15). This technique permitted rapid sampling and precise timing. The samples were counted for 35S by liquid scintillation (19) after allowing the 32PNa to decay at least 10 half-lives.
J. C. PARKER AND R. L. SNOW

FIG. 4. Effect of ATP on release of Na\(^{24}\) and K\(^{42}\). Left-hand panels: isotope release at 2, 20, and 40 min in presence and absence of 0.5 mM ATP. Right-hand panel: release of radioactivity from 2 to 20 min in various concentrations of ATP. Standard NaCl buffer. Closed circles = Na\(^{24}\), open circles = K\(^{42}\).

FIG. 5. Effect of 1 mM ATP on release of Na\(^{24}\) and SO\(_{4}^{2-}\). Mean ± se for 4 studies. Standard NaCl buffer plus 1 mM Na\(_{2}\)SO\(_{4}\). Closed circles and solid lines = control. Open circles and dashed lines = ATP.

as well as sodium movements. When cells are suspended in potassium buffer containing ATP, they become loaded with potassium and sodium moves outward. Less than 1% hemolysis occurs (4). The K-loaded cells are then washed and reincubated in a sodium buffer in the presence and absence of ATP. With no ATP in the second incubation, the cation composition of the cells remains relatively stable, but in the presence of ATP the cells return to their high-Na, low-K state. This experiment also shows that ATP affects the movements of cations in both directions across the membrane.

Some of the effects of ATP were studied by measuring the movements of radioactive ions. Quantitative interpretation of isotopic flux data in dog red cells is difficult because of heterogeneity among cells in the compartmentalization and permeance of sodium and potassium (12). Furthermore, dog red cells respond to small changes in volume with large changes in cation permeability (7, 21). Since they always tend to swell slowly in vitro, the problems of compartmental analysis are compounded. Accordingly, the results of isotopic flux studies in this report will be simply expressed in terms of the percentage of total radioactivity which escapes preloaded cells in a given time period. The qualitative information thus gained will serve to illustrate some of the characteristics of the ATP effect.

Figure 4 (left-hand panel) shows the time courses of release of radioactivity from Na\(^{24}\) and K\(^{42}\)-loaded dog red cells in the presence and absence of 0.5 mM ATP. In subsequent tables the outflux of radioactivity will be given over a time interval beginning at 2 min and ending at 20 or 30 min following the suspension of labeled cells in flux medium at 37 C. The right-hand panel of Fig. 4 shows that the rate of release of both Na\(^{24}\) and K\(^{42}\) increases with increasing concentrations of ATP, reaching a maximum at about 0.5 mM. This relationship was shown to be independent of the proportion of red blood cells in the suspension over a range from 0.5 to 10 vol %.

Figure 5 shows the results of studies in which the effluxes of Na\(^{24}\) and SO\(_{4}^{2-}\) were measured simultaneously in the presence and absence of 1 mM ATP. It is clear that the large increase in Na efflux induced by the nucleotide is not accompanied by a change in the rate of transfer of sulfate.

Table 2 provides evidence that the agent responsible for the effect is in fact ATP and not some contaminant or metabolite thereof. Pretreatment of the incubation medium with hexokinase plus glucose prevents the ATP-induced increase in sodium and potassium flux.

Table 3 shows that both calcium and magnesium ions inhibit the effect of ATP. EDTA, which by itself has no effect on the ATP response, is capable of reversing the inhibition caused by the divalent cations.

TABLE 2. Abolition of ATP effect by hexokinase and glucose

<table>
<thead>
<tr>
<th>Pretreatment of Flux Medium</th>
<th>Percent Counts Released</th>
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<tbody>
<tr>
<td></td>
<td>(\text{Na}^{24})</td>
</tr>
<tr>
<td>Glucose Hexokinase</td>
<td>Control</td>
</tr>
<tr>
<td>----------------------</td>
<td>----------</td>
</tr>
<tr>
<td>0</td>
<td>9.2</td>
</tr>
<tr>
<td>0</td>
<td>8.5</td>
</tr>
<tr>
<td>0</td>
<td>11.2</td>
</tr>
<tr>
<td>0</td>
<td>9.2</td>
</tr>
</tbody>
</table>

Standard NaCl buffer containing 1 mM MgCl\(_2\), with and without 1 mM ATP, was preincubated for 1 hr at 37 C with 10 mM glucose and 0.02 mg/ml hexokinase in the combinations noted. EDTA was then added to all media in a final concentration of 2 mM, and labeled red cells were introduced. The counts released between 2 and 20 min of incubation are reported.

TABLE 3. Influence of divalent cations and EDTA

<table>
<thead>
<tr>
<th>Additions to Basic Medium, mM</th>
<th>Percent Counts Released</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\text{Na}^{24})</td>
</tr>
<tr>
<td>Cal++ Mg++ EDTA</td>
<td>Control</td>
</tr>
<tr>
<td>------------------</td>
<td>----------</td>
</tr>
<tr>
<td>0</td>
<td>10.5</td>
</tr>
<tr>
<td>0</td>
<td>11.2</td>
</tr>
<tr>
<td>2.5</td>
<td>12.0</td>
</tr>
<tr>
<td>2.5</td>
<td>12.1</td>
</tr>
<tr>
<td>2.5</td>
<td>10.5</td>
</tr>
<tr>
<td>2.5</td>
<td>10.6</td>
</tr>
</tbody>
</table>

Standard NaCl buffer plus the additions noted. Counts released between 2 and 20 min of incubation at 37 C.
The remarkable specificity of the ATP effect was demonstrated by the failure of the following compounds (all at a concentration of 1 mM) to influence Na and K movements in circumstances where ATP was maximally active: ADP, 5'-AMP, 3',5'-AMP adenosine, adenine, ITP, GTP, UTP, TTP, CTP, and deoxy-ATP.

Table 4 presents data which suggest that swelling of cells in the presence of ATP is not linked to breakdown of the nucleotide. When cells are suspended in standard NaCl buffer with ATP added, they swell rapidly, and some ATP is broken down to ADP. Addition of EDTA to the buffer greatly inhibits ATP breakdown, but rapid swelling is not prevented. Magnesium and calcium, which diminish the rate of cell swelling, accelerate the breakdown of ATP to ADP and AMP, although enough ATP remains at the end of 3 hr (>0.5 mM) to exert a maximum effect on permeability change (Fig. 4). The possibility that AMP made in the presence of divalent cation might inhibit the ATP effect is ruled out by the observation that with EDTA present, AMP added at the beginning of the experiment does not affect the rapid cell swelling.

In an attempt to assess whether ATP is bound to dog red cells, an aliquot of standard NaCl buffer containing 1 mM ATP and 2 mM EDTA was incubated 6 times for 10 min at 37°C with consecutive lots of fresh cells. No measurable decrease in ATP content of the medium was noted.

In addition to its effect on cation permeability, external ATP influences metabolism in dog red cells. Table 5 shows that the nucleotide causes increased lactate production and a fall in internal ATP. When the ATP content is expressed per liter of cells, the change is even more striking, because the swelling which occurs with external ATP dilutes the nondiffusible contents of the cell.

ATP added to the medium also induces an alteration in the physical properties of dog red cells. In early experiments it was noted that cells exposed to the nucleotide become viscous and difficult to pipette after packing in a centrifuge. This change is quantitated in Table 6. The ability of packed cells to pass through a filter is also reduced in the presence of ATP, although the experiments with hypotonic medium indicate that this is not due to an increase in cell volume.

ATP could not be shown to induce a change in cell shape as observed by light microscopy. Ouabain had no influence on the ATP effect.

### Discussion

These studies show that when ATP is applied to the surface of dog red cells a prompt increase in sodium-potassium permeability ensues. The effect does not appear to involve consumption or metabolism of the nucleotide extracellularly. As noted by others (9) the exogenous nucleotide influences glycolytic rate. The effect was reversible in the sense that the rapid swelling (Fig. 2) and the downhill movements of cations (Fig. 3) induced by ATP ceased when the cells were washed free of the nucleotide. The cells did not return to their normal volume, however, following exposure to ATP.

The abolition of the ATP response by calcium or magnesium would appear to relate to the chelating property of the nucleotide rather than to any effect of the divalent ions on the membrane. The data in Table 3 suggest that it is free ATP which interacts with the membrane to alter permeability. When divalent metal ions are present, the ATP chelates them and thus becomes unavailable to the membrane. EDTA, which is a stronger chelator than ATP
(22), restores the ATP effect, presumably by complexing with Ca or Mg, thereby freeing ATP to react with the cell surface. The possibility exists that ATP may bind to a divalent cation in the matrix of the membrane (14). If this is so, the amount bound must be small, or the binding constant must be low, inasmuch as it was impossible to demonstrate removal of ATP from an aliquot of solution by repeatedly exposing it to large numbers of cells in circumstances which favor the action of ATP on permeability. Also, the site of action of ATP must be highly stereospecific, since a variety of other chelators and nucleotides fail either to mimic or alter the effect of ATP on ion flux.

These observations and those on ascites tumor cells (6, 26) suggest that among the determinants of sodium and potassium permeability there are certain superficial membrane sites—possibly cationic—which are altered by exposure to free ATP. There are examples of contractile proteins which undergo a reversible change in viscosity when exposed to ATP in solutions containing no divalent cation (8, 29). One of these is platelet thromboplastin, which has been associated with the plasma membrane (17). The alteration in physical properties of packed dog red cells exposed to ATP (Table 6) is suggestive of an effect on surface structure or charge (23).

It was of interest to study the relative effects of ATP on the unidirectional movements of anions and cations. Many manipulations which cause red cell membranes to become leaky to cations result in a corresponding fall in anion permeability (22). Observations of this sort have suggested that ions permeate the membrane through aqueous channels (pores) which under normal conditions are lined with positive charges. Neutralization of these charges, it is held, causes the pores to become more permeable to cations and less so to anions. In preliminary studies it was determined that the flux of radioactive chloride was too rapid for accurate measurement by conventional techniques. Sulfate was therefore selected as a relatively slower moving, non-metabolized anion (22). The failure of ATP to exert opposing effects on anion and cation flux suggests that the action of the nucleotide is not due to neutralization of pore charges. A recently reported study employing a variety of amino- and sulfhydryl group inhibitors casts further doubt on the sufficiency of the charged pore model (10, 11).

There is evidence that Na+ and K+ move through the dog red cell membrane via separate routes. This conclusion has been derived from studies of experimentally induced volume changes showing large and disparate responses in the permeance of the two ions: shrunken cells are leaky to Na but tight to K, while swollen cells show just the reverse changes (7, 21). In no instance, however, have in vitro conditions been found in which mature dog red cells can be shown to use energy derived from metabolism to extrude ions and water. Such a mechanism must be operative in vivo, inasmuch as dog red cells survive in the circulation for 90–100 days (27). It seems likely that an understanding of the dog red cell membrane may lead to refinements in concepts of cell volume homeostasis.

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