Dog Red Blood Cells: Na and K Diffusion Potentials with Extracellular ATP

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ABSTRACT   External ATP causes a prompt increase in the Na and K permeability of dog red blood cells. By manipulating intra- and extracellular ion composition it is possible to observe ATP-induced net fluxes which can be explained in terms of the contribution of Na or K diffusion potentials to the membrane potential. Measurements of membrane voltage by a fluorescent dye technique confirm the existence of such potentials. A rough calculation of chloride permeability gives a value of the order of 10^{-8} cm/s, which agrees with results in other species. The cells appear to be somewhat more permeable to bromide than to chloride.

INTRODUCTION

An interesting property of dog red blood cells is that their permeability to Na and K increases on exposure to external ATP in concentrations of 0.1–1.0 mM (Parker and Snow, 1972; Elford, 1975; Romualdez et al., 1976). The effect is ATP specific and reversible. It can be prevented by including physiologic concentrations of divalent cations in the medium and is therefore of uncertain significance for the cell's function in vivo. This action of exogenous ATP was first described in ascites tumor cells (Hempling et al., 1969; Stewart et al., 1969) and has also been reported in isolated renal tubules (Rorive and Kleinzeller, 1972) and other tissues (Trams, 1974). The mechanism of ATP's interaction with the membrane surface is obscure.

We suspected that in the presence of external ATP dog red cells might become as permeable to Na and K as they are to small anions. Our hypothesis was confirmed by measurements of net ion flux and membrane potentials. The data have furthermore allowed us to draw some conclusions about chloride and bromide movements in this species of cell. This approach has precedent in work with cation-specific ionophores such as gramicidin and valinomycin (Scarpa et al., 1968; Hunter, 1971; Tosteson et al., 1973).

It should be emphasized that the data in the present study concern net anion movements, presumably by electrodiffusion. Dog red blood cells have a tightly coupled, electrically silent anion exchange mechanism similar to that of all other species examined (Wieth et al., 1974). The possible relationship between net and exchange pathways will be briefly discussed in connection with our observations on the movements of different halide ions.

MATERIALS AND METHODS

Blood from mongrel dogs was drawn into heparinized syringes and used within 1 h of venipuncture. In preparation for experiments the cells were always washed four times by repeated mixing and centrifugation in a solution containing (mM): NaCl 150; HEPES 5 (pH 7.4). The "basic choline medium" in which net ion and water fluxes and membrane potentials were measured contained (mM): choline-Cl 120; KCl 4; HEPES 10; glucose 5; and ATP 1, brought to pH 7.40 at 37°C with Tris-hydroxide. Modifications of this medium are noted along with the results. The principal changes included equimolar substitution of NaCl for choline-Cl, variation of the ATP concentration, and substitution of Br and I salts for Cl (in which case $Na_2S_2O_5$ 0.5 mM was included to keep the anions in the reduced state). All incubations were done at a cell:medium ratio of 1:10–1:20 in a 37°C shaker bath.

Timed samples were obtained by pipetting a volume of cell suspension into an equal volume of incubation medium which had been precooled in salted ice. The cold suspension was thoroughly mixed and promptly centrifuged at 30,000 g (0°C). Cells were processed for Na, K, Cl, and water content, and ^{125}I-albumin trapped volume (always less than 2%) by methods previously published (Parker, 1971; Parker and Snow, 1972).

Determinations of the membrane potential were made by using the fluorescent probe Di-S-C₃(5), as described by Hoffman and Laris (1974). Fluorescence was measured in an Aminco Bowman spectrofluorophotometer (American Instrument Co., Silver Spring, Md.) with excitation and emission wavelengths set at 625 and 690 nm, respectively. These wavelengths are slightly different from those reported by Hoffman and Laris (1974) and reflect the addition of a narrow band pass filter (Baird-Atomic 646, Baird Atomic, Inc., Bedford, Mass.) and a cutoff filter (Schott RG-665, Schott Optical Glass, Inc., Duryea, Pa.) to eliminate interference in the signal due to the light scattering of the red blood cells.

Erythrocytes (0.33% hematocrit) were incubated at 37°C in a cuvette containing the basic choline medium described above, less ATP, and with the addition of 6.6 × 10⁻⁴ mg/ml Di-S-C₃(5) dissolved in ethanol. When indicated, Ca, Tris-ATP (pH 7.4), and valino-mycin in ethanol (10⁻⁶ M) were added by syringe and the resulting changes in fluorescence recorded. Hoffman and Laris (1974) have shown that a decrease in fluorescence is an indication of hyperpolarization, while an increase in the fluorescent signal is indicative of depolarization. All substances used were tested in the presence and absence of erythrocytes for reaction with the dye (i.e. quenching of fluorescence or alteration of the time course for equilibration of the dye with the cells).

The possibility that the fluorescent probe used to monitor potential alters P_{Na} or P_K was investigated. We found that the rate constant for both ^{24}Na and ^{42}K efflux from dog red blood cells is not affected by the presence of Di-S-C₃(5) in the flux medium. We also attempted to determine if dye or external ATP alters P_{Cl}. One can estimate relative values of P_{Cl} by determining the rate of shrinkage of cells treated with valinomycin and suspended in a K-free medium. Using a light-scattering technique to monitor relative cell volume, we found that the rate of shrinkage of high-K dog red cells prepared as noted in Fig. 11, in the presence of valinomycin, was not affected by the inclusion in the medium of the fluorescent dye or ATP in the concentrations mentioned above.

RESULTS

Fig. 1 A shows that as Na moves out from the cells into a choline medium containing 1 mM ATP and 4 mM K there occurs a transient increase in the ratio of cell to medium K concentration. The rise in cell K can be prevented by

making the medium high in Na (Fig. 1 B), low in K (Fig. 1 C), or by leaving out ATP (Fig. 1 D).

Several features of the experiment in Fig. 1 are brought out by replotting the data as in Fig. 2, with cell water and ion contents expressed in terms of cell solids. In the presence of external ATP there is a large net efflux of water, suggesting that the cells are less permeable to choline than to Na. Furthermore, the rise in cell K concentration in Fig. 1 A is not simply a reflection of water loss. Net movement of K from medium to cells occurs (Fig. 2 A) against a concentration gradient during the early time points.
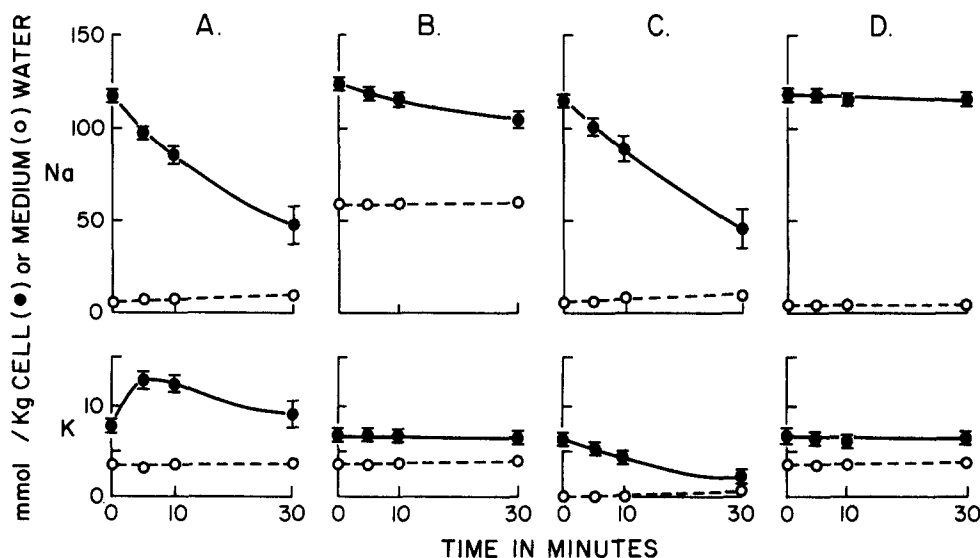

FIGURE 1. Na and K concentrations in cells (solid circles, solid lines) and medium (open circles, dashed lines) as a function of incubation time at 37°C. The basic 120 mM choline medium (see Materials and Methods) was used with the following modifications (mM): A, no modifications — Na 0, K 4, ATP 1; B, choline 60, Na 60, K 4, ATP 1; C, Na 0, K 0, ATP 1; D, Na 0, K 4, ATP 0. Mean ± SEM for four studies.

One way to explain the net movement of K up a concentration gradient would be to invoke a hyperpolarization of the cell membrane: net K uptake would occur in response to the cell interior's becoming more negative (Glynn and Warner, 1972). In order passively to concentrate K to the degree shown in Fig. 1 A, the Nernst equation predicts that at equilibrium the cell membrane would have to go from a normal resting potential of approximately −8 mV to a value of −31 mV. Since it is unlikely that K has reached equilibrium in 5 min (Fig. 1 A), this estimate of the membrane potential is a minimum. Such hyperpolarization could occur if the Na permeability were increased to such a degree that the Na gradient could make a substantial contribution to the membrane potential. Data from Fig. 1 B support the possibility of an Na-dependent hyperpolarization, since K is no longer concentrated when the external Na is high. To test the

possibility that ATP induces hyperpolarization of dog red cells the membrane potential was monitored by using a fluorescent probe (Hoffman and Laris, 1974). Fig. 3 shows that the addition of ATP to dog red cells suspended in a choline chloride medium leads to a rapid decrease in fluorescence, indicating hyperpolarization of the membrane. The effect can be rapidly dissipated by the addition of Ca in concentrations which inhibit the influence of ATP on cation movements (Parker and Snow, 1972). In other experiments (not shown) we found that ATP had no effect on the membrane potential of human or cat red cells. This agrees with the findings of Romualdez et al. (1976) in which ATP failed to increase Na movement in cat red cells.
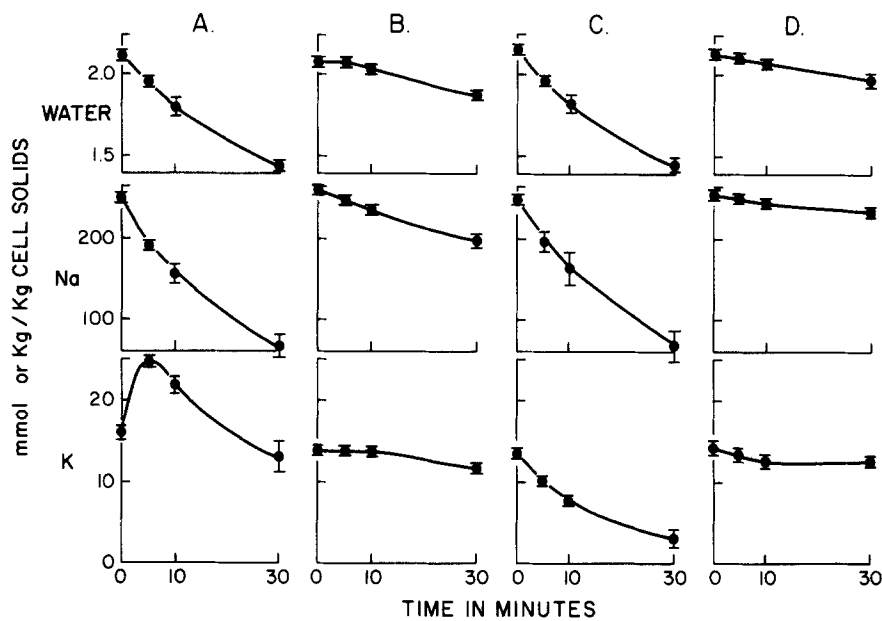

FIGURE 2. Same experiment as in Fig. 1 with cell constituents (water, Na, K) plotted per kilogram of cell solids.

Fig. 1 B indicates that the ATP-induced net K uptake is Na dependent. A detailed study of the relationship between external Na concentration and net Na and K movements is shown in Fig. 4. The general plan of the experiment is as in Figs. 1 and 2, except that cell ion and water contents were measured only at zero and 5 min incubation time. As medium Na concentration is progressively increased, the initial net K influx and net Na efflux both diminish. At 38 mM external Na, net K influx stops. Between 55 mM and 70 mM external Na a point is reached at which no net Na efflux occurs. At higher medium Na concentrations there is net K loss and Na entry during the 5 min after exposure to external ATP. At the point where net K flux is zero the mean concentration ratios of cell and medium ions are (mmol/liter cell or medium water): Na cells/Na medium = 107.5/37.5; K cells/K medium = 8.0/3.5; Cl medium/Cl cells = 125/94. These

numbers will be used to calculate membrane potentials and ion permeabilities in the Appendix.

The Na dependence of the ATP-induced net K uptake is supported by measurements of membrane potential: ATP-induced hyperpolarization is progressively diminished as the external Na concentration is raised from 0 to 70 mM
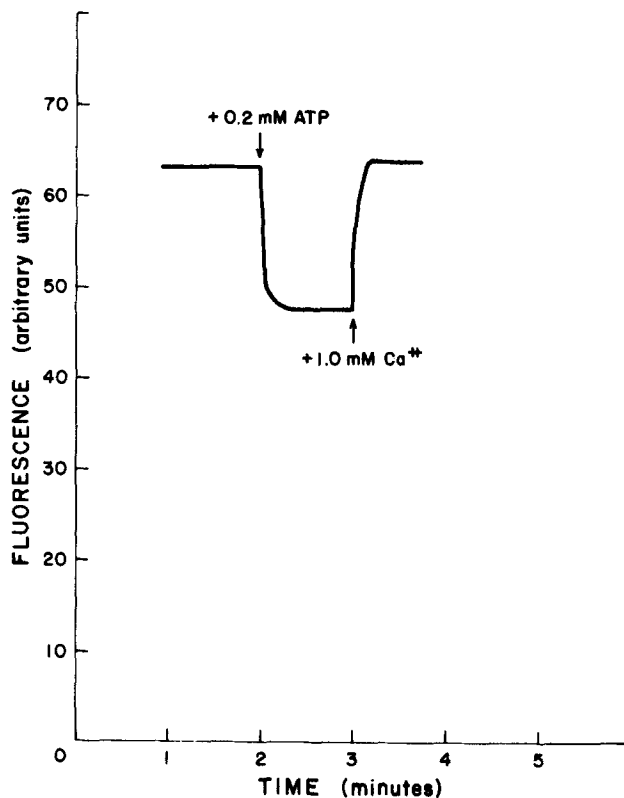

FIGURE 3. Fluorescence change in response to the addition of ATP (0.2 mM) and Ca (1.0 mM). Cells were incubated in a cuvette containing (mM): choline Cl 120, KCl 0.5, HEPES 10, glucose 5, as described in Materials and Methods, with Di-S-$C_3(5)$ 6.6 × 10^{-4} mg/ml. A downward deflection indicates hyperpolarization, while an upward deflection denotes depolarization.

(data not shown). This supports the conclusion that net K uptake is driven by a membrane potential to which the Na gradient makes a substantial contribution.

As noted above, ATP must hyperpolarize the membrane to at least −31 mV in order to explain the concentration of K shown in Fig. 1 A. The potential induced by ATP was measured by determining the null point for K both before and after the addition of ATP. At the null point, i.e. the external K concentration where no fluorescence change occurs after the addition of a K ionophore (valinomycin), the membrane potential can be defined as the Nernst potential for K. Fig. 5 shows null point determinations with and without ATP. From these data it is

estimated that the membrane potential in the absence of ATP is -9 mV. Upon the addition of ATP the membrane is hyperpolarized to -35.6 mV, i.e. a change of 26.6 mV from the resting potential. Note that the magnitude of this potential would account for the inward movement of K in Fig. 1 A.

Fig. 6 shows the concentration dependence on ATP of the net Na and K movements in circumstances similar to those of Figs. 1 A and 2 A. The influence of external ATP appears to be half-maximal at a concentration of about 50 μM.

Fig. 7 shows the concentration dependence on ATP of the membrane hyperpolarization. As with net Na and K movements, the ATP-induced potential change reaches a maximum at approximately 0.2 mM ATP.
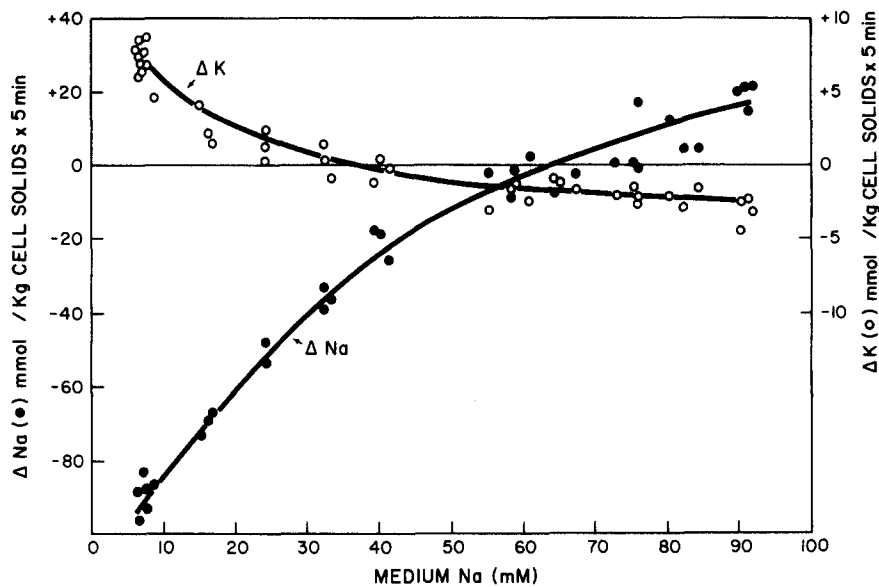

FIGURE 4.   Net change in cell Na (solid circles, left ordinate) and K (open circles, right ordinate) over the initial 5-min period after suspension of the cells in solutions containing (mM): sum of Na + choline chlorides = 120, KCl 3.6, HEPES 10, ATP 1. Measured medium Na concentrations are on the abscissa. Collected results of 10 studies plotted together.

Fig. 8 shows the results of substituting Br or I for Cl as the principal anion in experiments similar to those of Fig. 2 A. The degree of net K entry in the early time points decreases, while the rate of water loss increases, in the sequence Cl-Br-I. Net Na loss also increased in the same sequence, but the differences were small. Cell Na at the 10-min point (mmol/kg cell solids) was 123 ± SEM 2, 118 ± 2, and 113 ± 6 for Cl, Br, and I media, respectively. At 20 min the values for cell Na in the same order were 73 ± 1, 66 ± 2, and 65 ± 2. Note that in these experiments the cells were transferred from Cl medium in which they were washed to the three ATP incubation media containing the Cl, Br, or I salts of choline. At 37°C it would be expected that equilibrium of intra- and extracellular anions would take place via the exchange route within a few seconds (Dalmark

and Wieth, 1972). Similar results were obtained when cells were pre-equilibrated with Br and I solutions before ATP exposure.

The observation that ATP-induced cell shrinkage is faster in choline bromide and iodide than in choline chloride led to the performance of an experiment on cell swelling. It had been shown (Parker and Snow, 1972) that when suspended in an ATP-containing medium consisting of isotonic NaCl, dog red cells swell due to the net entry of Na, Cl, and water. Fig. 9 compares the rate of cell swelling in isosmotic solutions of NaCl, NaBr, and NaI (net K movements were negligibly small and are not presented). The base-line water content of dog red
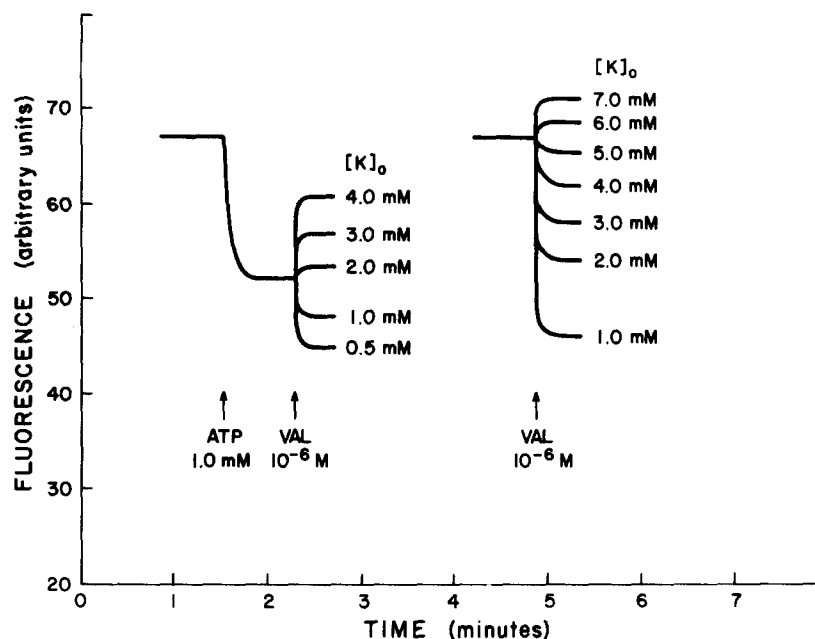

FIGURE 5.   Null point determination with valinomycin. Cells were incubated as in Fig. 3, except that the medium contained various concentrations of KCl as indicated. Shown are changes in fluorescence upon addition of ATP 1 mM and valinomycin 10^{-6} M as indicated by arrows. Results represent two experiments.

cells is not the same when they are suspended in equiosmolal solutions of the three salts. This was shown to be true for human red cells in NaI solutions by Dalmark and Wieth (1972) who attributed the discrepancy to intracellular iodide binding. But the rate of net entry of Na and water is faster in NaBr than in NaCl. The slowest rate is found with NaI.

Data from Fig. 8 indicate that net K entry decreases in the sequence Cl-Br-I. This would be expected if the degree of hyperpolarization induced by ATP were decreased as the anion is changed from Cl to Br or I. Fig. 10 shows that indeed the ATP-induced hyperpolarization is greater in Cl than Br. Null point determinations of the ATP-induced potential in these media indicate that the membrane potential is increased from a resting level of −9 mV to −24 mV in Br medium, as
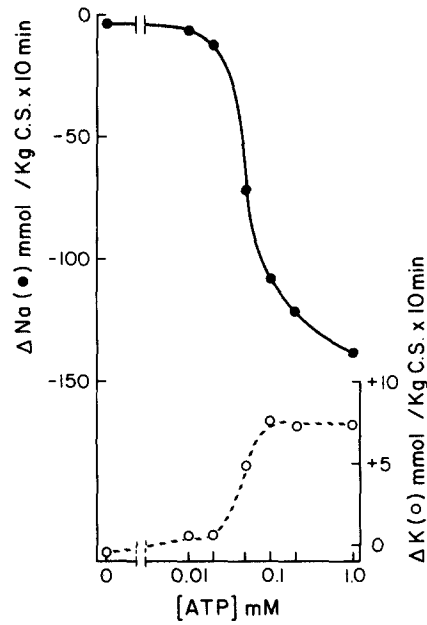
FIGURE 6. Net movements of Na (closed circles, solid lines, left ordinate) and K (open circles, dashed line, right ordinate) in fresh dog red cells during the first 10 min of exposure to the basic choline medium (see Materials and Methods) with varying concentrations of ATP. Note log scale on abscissa. Single study representative of three others.
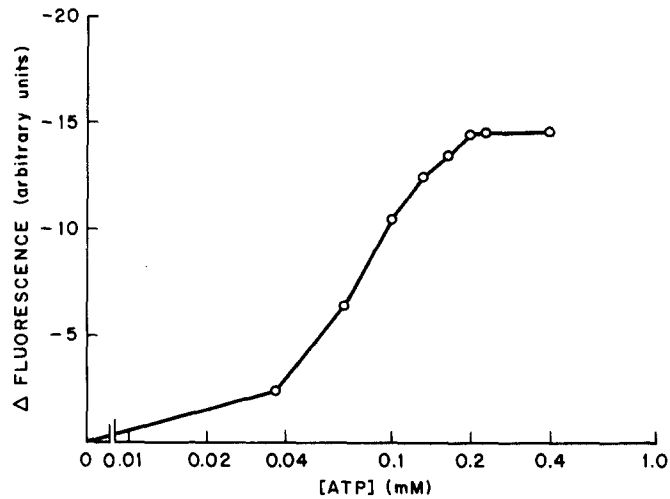

FIGURE 7. Fluorescence changes as a function of the ATP concentration. Cells were incubated in the choline medium described in Fig. 3. Shown are the changes in fluorescence observed after the addition of the indicated concentration of Tris-ATP, pH 7.4. Note log scale on abscissa.

compared with −35.6 mV in Cl medium (Fig. 5). Thus, hyperpolarization is about 11.6 mV less in the Br than the Cl medium. In a replicate experiment (not shown) with cells from another dog, the difference in hyperpolarization between Cl and Br media was 11.4 mV. The ATP-induced potential in I media could not be measured, because I quenches the fluorescence of the dye.

Net uptake of Na can be demonstrated when the gradients for the two ions are reversed, as shown in Fig. 11. Here the cells had been preincubated with external ATP in a high-K medium so as to exchange most of their Na for K.
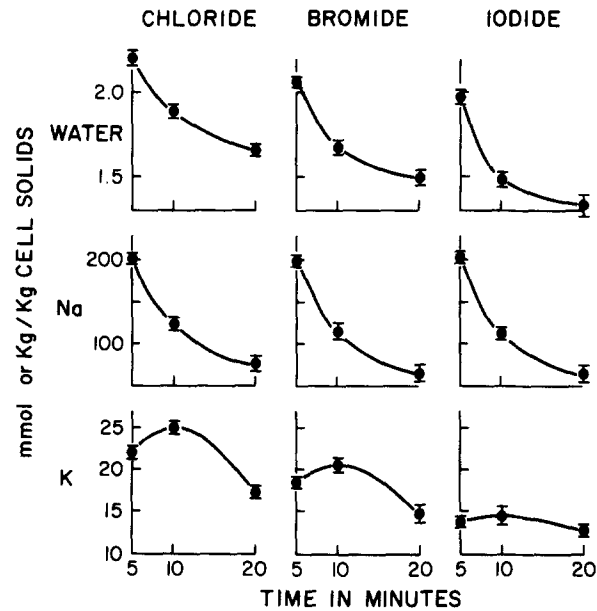

FIGURE 8. Net movements of water, Na, and K as functions of time at 37°C. Comparison of Cl, Br, and I media. Fresh cells were washed several times in (mM): NaCl 105; KCl 4; HEPES 5 (pH 7.4). After removal of all wash supernates cells were placed in (mM): choline X 105, K X 3.6, HEPES 10, glucose 5, $Na_2S_2O_5$ 0.5, ATP 1 (pH 7.4). X = Cl, Br, or I. Osmolalities of the three solutions were: Cl, 0.212; Br, 0.208; I, 0.205. Flame photometry of the 5-min supernates showed (mM): Na 4.5–5.0 and K 3.4–3.8. At 20 min the supernates contained (mM): Na 7.5–9.0 and K 3.6–3.8. There was less than 1% lysis. Mean ± SEM for four studies.

When the high-K cells are transferred to a choline medium containing Na 6 mM, there is a transient net Na entry as K and water flow outward. The net Na inflow can be prevented (Fig. 11 B) by raising external K to 60 mM. These data suggest that under the conditions of a high-K, low-Na cell, a K-dependent hyperpolarization may occur which drives Na up a concentration gradient.

DISCUSSION

We interpret these experiments to show that in the presence of external ATP the Na and K permeabilities of dog red cells increase to such a degree that gradients for these ions contribute importantly to the membrane potential. When cells are

exposed to ATP in a choline medium (where a steep outward gradient for Na exists) the Na diffusion potential is a sufficiently large component of the membrane potential that hyperpolarization occurs. It is this electrical driving force that accounts for the transient, inward K movement in Figs. 1 A and 2 A. By suitable manipulations Na can be made to move in response to the influence of a K diffusion potential, as shown in Fig. 11.

Attempts to derive quantitative relationships among P_{Na}, P_K, and P_{Cl} from the data presented here have not been completely satisfactory. A simple analysis of
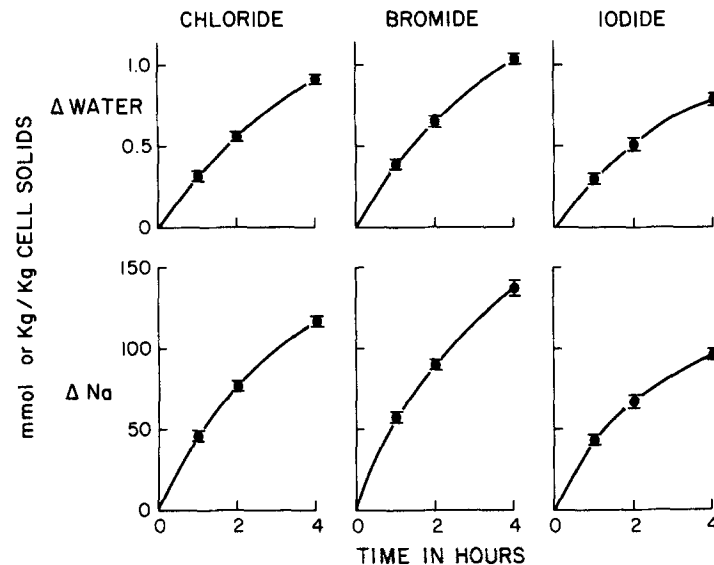
FIGURE 9. Swelling of ATP-treated cells in Na salts of Cl, Br, and I. Cells washed in ATP-free solutions containing (mM): HEPES 10, glucose 5, $Na_2S_2O_5$ 0.5, plus Na X 145, K X 5 (pH 7.4). X = Cl, Br, or I. At time zero cells were suspended in same solutions plus ATP 1 mM (pH 7.4). Samples taken at zero time (within 5 min), 1, 2, and 4 h of incubation at 37°C. All solutions were within 6 mosM of each other. Baseline (time zero) cell contents for the Cl, Br, and I media were, respectively, 1,836 ± 4, 1,767 ± 3, and 1,676 ± 21 g water per 1 kg cell solids and 266.1 ± 0.3, 262.9 ± 0.9, and 255.5 ± 1.9 mmol Na per 1 kg cell solids. Mean ± SEM for four studies.

Fig. 4 using the Goldman equation (Goldman, 1943; Hodgkin and Katz, 1949) is presented in the Appendix, where it becomes apparent that the experimental data are not sufficiently precise to test the applicability of the constant field formulation to this particular system. Accordingly, our discussion is presented in qualitative terms.

In the experiments where Br and I were substituted for Cl the degree of membrane hyperpolarization by ATP is diminished, as inferred from the net inward movement of K (Fig. 8). Estimation of the membrane potential by the dye technique confirms this observation in the case of Br (Fig. 10). Unfortunately, the fluorescence of the dye was quenched in the presence of I. If the action of ATP on dog red cells were changed in a Br or I medium in such a

direction that P_{Na} were less or $P_{choline}$ greater than when Cl is the major anion, then the lower degree of hyperpolarization could be accounted for. But if this were true, the cells ought to shrink less rapidly when Br or I are substituted for Cl. Fig. 8 shows that the opposite is the case.

An alternative explanation for the anion substitution results is that in dog red cells $P_{Cl} < P_{Br} < P_I$. If this were so, then the cells ought to swell more rapidly in
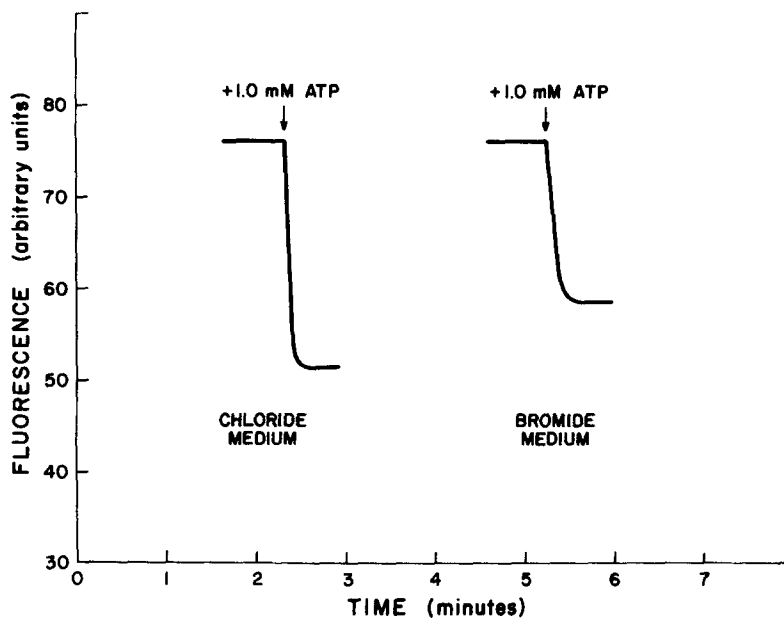

FIGURE 10. Anion dependence of fluorescence changes. Erythrocytes were preincubated for 10 min in a medium containing HEPES (pH 7.4) and isotonic NaCl or NaBr. The cells were then transferred to choline Cl or Br media and fluorescence was measured as in Fig. 3. The effect of adding ATP 1 mM is shown. Determinations of the null point for K in the presence of valinomycin were made with both Cl and Br media. At the null point the medium K was 2.0 mM for Cl and 2.4 mM for Br, while the cell K was 7.7 mM for Cl and 5.9 mM for Br. Thus at 37°C the membrane potential in the presence of ATP was −35.6 mV for Cl and −24.0 mV for Br.

ATP-containing solutions made up of NaBr or NaI than in NaCl media. Fig. 9 shows that this is true for Br but not for I, perhaps due to the binding properties of the latter (Dalmark and Wieth, 1972). This finding, plus the difficulty in measuring fluorescent dye potentials in I media, makes it hazardous to draw further conclusions about I permeability in dog red cells from the present data.

Our results suggest, then, that in dog red cells P_{Br} is greater than P_{Cl}. This contrasts with the data on tracer movements of these anions under steady-state conditions. In human red cells the rate of Cl exchange is eight times that for Br (Dalmark and Wieth, 1972). The difference in selectivity among anion species between net and exchange pathways has been noted by others (Hoffman and

Knauf, 1973; Knauf and Fuhrmann, 1974) and suggests that the two modes of anion permeation are, if not totally discrete, at least subject to different rate determinants.

APPENDIX

An approximate value for P_{Cl} can be computed from the data, granted the assumptions implicit in the Goldman equation and by regarding Na, K, and Cl as the only permeant
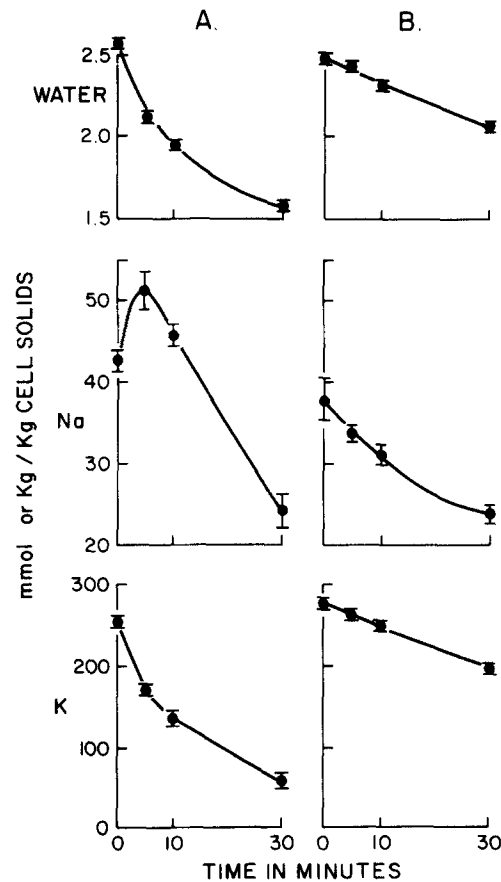

FIGURE 11. Movements of water, Na, and K in "high K" dog red cells, as a function of time at 37°C. Fresh cells were preincubated for 1 h at 37°C in (mM): KCl 150; HEPES 10; glucose 5; ATP 1, (pH 7.4). Cells washed in isotonic KCl, then placed in (mM): A, choline Cl 120; NaCl 6; HEPES 10; glucose 5; ATP 1; or B, choline Cl, 60; KCl, 60; NaCl, 6; HEPES, 10; glucose, 5; ATP, 1 (pH 7.4). Mean ± SEM for four studies.

species. In Fig. 4—at the point where K moves neither inward nor outward—the membrane potential, V_m, is given by the Nernst potential for K, and also by the Goldman equation:

$$V_m = -\frac{RT}{F} \ln \frac{(K_i)}{(K_o)}, \tag{1}$$

$$V_\mathrm{m} = -\frac{RT}{F} \ln \frac{P_\mathrm{K}(\mathrm{K}_i) + P_\mathrm{Na}(\mathrm{Na}_i) + P_\mathrm{Cl}(\mathrm{Cl}_o)}{P_\mathrm{K}(\mathrm{K}_o) + P_\mathrm{Na}(\mathrm{Na}_o) + P_\mathrm{Cl}(\mathrm{Cl}_i)}, \tag{2}$$

where the subscripts i and o refer to cells and medium, respectively. When one combines Eq. (1) and (2):

$$\frac{(\mathrm{K}_i)}{(\mathrm{K}_o)} = \frac{(\mathrm{Na}_i) + P_\mathrm{Cl}/P_\mathrm{Na}(\mathrm{Cl}_o)}{(\mathrm{Na}_o) + P_\mathrm{Cl}/P_\mathrm{Na}(\mathrm{Cl}_i)}. \tag{3}$$

When one substitutes the values for Na, K, and Cl given in Results for cells at the point where Eq. (1) holds, the ratio $P_\mathrm{Cl}/P_\mathrm{Na}$ comes out to be 0.24.

A value for P_Na (cm/s) in ATP-treated dog red cells can be calculated from the expression (Sachs et al., 1975):

$$P_\mathrm{Na} = K_\mathrm{Na}\frac{V}{A}\,\varnothing, \tag{4}$$

where K_Na (s^{-1}) is the rate constant for Na efflux, V (cm^3) and A (cm^2) are, respectively, the solvent volume and surface area of dog red cells, and $\varnothing$ is a dimensionless function of the membrane potential, V_m:

$$\varnothing = \frac{RT}{V_\mathrm{m}F}(e^{-V_\mathrm{m}F/RT} - 1). \tag{5}$$

Measured by net (Fig. 2 A) or isotopic (Parker and Snow, 1972) means, the half-time for Na efflux in the presence of 1 mM ATP is about 10 min, giving a value for K_Na of $-1.16 \times 10^{-3}\ s^{-1}$. The area of dog red cells (Wieth et al., 1974) is $117 \times 10^{-8}\ cm^2$. From Fig. 2 A a solvent volume of $70 \times 10^{-12}\ cm^3$ per cell can be calculated. Measurements of V_m by the dye technique in the circumstances of no net K movement give a value of -21.2 mV. P_Na by Eq. (4) then comes out to a value of 9.1×10^{-8} cm/s, and $P_\mathrm{Cl} = 0.24\ P_\mathrm{Na} = 2.2 \times 10^{-8}$ cm/s. The latter number is in agreement with estimates for human red cells of 2.8×10^{-8}, 1.8×10^{-8}, and 6×10^{-8} cm/s (Sachs et al., 1975, p. 662).

The estimate of $P_\mathrm{Cl}/P_\mathrm{Na}$ from Fig. 4 is imprecise because of the splay of the experimental data. If in Fig. 4 one selects the point at external Na 25 mM for the zero net flux calculation (Eq. 3), $P_\mathrm{Cl}/P_\mathrm{Na}$ comes out to be 0.41. Furthermore, if the same reasoning used in the derivation of Eq. (3) is applied to the point(s) at which no net Na movement occurs, the calculated values for $P_\mathrm{Cl}/P_\mathrm{K}$ show even greater variations.

Even if the precision were better, it is likely that appropriate use of the Goldman equation would require information about choline, hydroxyl, and bicarbonate movements. A further hazard to the use of a simple, two-compartment model might be the known heterogeneity of dog red blood cells (Lange et al., 1970; Castranova and Hoffman, 1977). For all these reasons the value of P_Cl calculated in this section should be regarded as a rough approximation.

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