Volume-induced Increase of Anion Permeability in Human Lymphocytes

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ABSTRACT Peripheral blood mononuclear cells (PBM) readjust their volumes after swelling in hypotonic media. This regulatory volume decrease (RVD) is associated with a loss of cellular K⁺ and is thought to be promoted by an increased permeability to this ion. In contrast, no change in volume was observed when K⁺ permeability of PBM in isotonic media was increased to comparable or higher levels using valinomycin. Moreover, valinomycin-induced ⁸⁶Rb⁺ loss in K⁺-free medium was considerably slower than in K⁺-rich medium. These results suggest that anion conductance limits net salt loss in isotonic media. Direct measurements of relative conductance confirmed that in volume-static cells, anion conductance is lower than that of K⁺. In volume-regulating cells depolarization occurred presumably as a result of increased anion conductance. Accordingly, the efflux of ³⁶Cl⁻ from PBM was markedly increased by hypotonic stress. Since both membrane potential and intracellular ³⁶Cl⁻ concentration are reduced in hypotonically swollen cells, the increased efflux is probably due to a change in Cl⁻ permeability. Anions and cations seem to move independently through the volume-induced pathways: the initial rate of ⁶⁸Rb uptake in swollen cells was not affected by replacement of external Cl⁻ by SO₄⁻; conversely, ³⁶Cl⁻ fluxes were unaffected by substitution of K⁺ by Na⁺. The data indicate that anion conductance is rate-determining in salt and water loss from PBM. An increase in anion conductance is suggested to be the critical step of RVD of human PBM.

INTRODUCTION

When exposed to hypotonic media most nucleated mammalian cells, including lymphocytes, regulate their volumes, showing marked departures from the ideal osmometric behavior predicted by the Boyle-Van’t Hoff relation (MacKnight and Leaf, 1977; Hoffman, 1978). After the initial passive swelling phase, cells return to near-normal volume by poorly understood mechanisms involving, in most cases, the loss of K⁺ (Roti-Roti and Rothstein, 1973; Hendil and Hoffman, 1974). The involvement of K ions in regulatory volume decrease (RVD) is further indicated by the observation that cells suspended in K⁺-rich media fail to shrink after osmotic swelling and, in fact, display a...
secondary swelling phase (Doljanski et al., 1974). It is generally assumed that macroscopic electroneutrality is preserved during RVD by the co-migration of anions with K\(^+\), since the alternative method for charge neutralization—the exchange of K\(^+\) for extracellular monovalent cations other than H\(^+\)—would be osmotically ineffective (for a dissenting view see Buckhold et al., 1973). Three types of mechanisms have been proposed to explain the ionic fluxes observed during volume regulation: (a) uncoupled, conductive fluxes of K\(^+\) and Cl\(^-\) (Hoffman, 1978); (b) directly coupled co-transport of K\(^+\) and Cl\(^-\); this mechanism is suggested by the Cl\(^-\) dependence of K\(^+\) fluxes (Kregenow and Caryk, 1979; Dunham and Ellory, 1981) and by the electroneutrality and furosemide sensitivity of the volume changes (Thornhill et al., 1982); and (c) simultaneous but independently operating cation and anion antiporters (Cala, 1980; Kregenow, 1981).

In the case of lymphocytes, it has been implicitly assumed that the volume-induced fluxes are independent and presumably conductive, and that cation permeability is rate limiting for salt and water loss during RVD (Doljanski et al., 1974; Bui and Wiley, 1981; Grinstein et al., 1982). Thus, cation and particularly K\(^+\) permeability has been the central subject of most studies of RVD in lymphoid cells (Roti-Roti and Rothstein, 1973; Rosenberg et al., 1972; Ben Sasson et al., 1975; Bui and Wiley, 1981). In contrast, the role of anions has not been analyzed. Particularly, the assumption that anion conductance is initially high and unaffected by the hypotonic stress has not been tested.

The present study was prompted by the above considerations and by the observation that valinomycin, a K\(^+\)-specific ionophore, failed to induce volume changes in peripheral blood mononuclear cells (PBM) suspended in K\(^+\)-free or K\(^+\)-rich solutions. This finding suggested that under normal conditions PBM have a low anion conductance and that during RVD a substantial change in anion permeability must therefore occur. Evidence is presented in this report which indicates that osmotic swelling does indeed lead to a substantial increase in anion conductance. The specificity of the volume-induced anion pathway and its relationship to cation transport were also examined.

MATERIALS AND METHODS

Reagents

Valinomycin was obtained from Calbiochem-Behring Corp., San Diego, CA; gramicidin D was purchased from Sigma Chemical Co., St. Louis, MO; RPMI 1640 medium and fetal bovine serum were from Grand Island Biological Co., Grand Island, NY; trypan blue was from Grand Island Biochemicals, Grand Island, NY; 4,4'-diisothiocyanato 2,2'-stilbene disulfonate (DIDS) was synthesized by Dr. M. Ramjessingh, Hospital for Sick Children, Toronto; 3,3'dipropylthiadicarbocyanine [diS-C\(_3\)-(5)] was the kind gift of Dr. A. Waggoner, Amherst College, MA.

Isotopes

\(^{36}\text{Cl}^-\) (as the sodium salt) and \(^{86}\text{Rb}\) were from Amersham Corp., San Diego, CA; \(^{1}\text{H}\)polyethylene glycol (mol wt 4,000) and Aquasol II were from New England Nuclear, Boston, MA.
Solutions

Standard phosphate-buffered saline (PBS) contained 137 mM NaCl, 2.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 8.1 mM Na$_2$HPO$_4$, 0.68 mM CaCl$_2$, 0.49 mM MgCl$_2$, and 10 mM glucose. K$^+$-free PBS was similar to standard PBS with NaCl substituted for KCl and Na$_2$HPO$_4$ for KH$_2$PO$_4$. KCl-PBS, K-gluconate-PBS, Na gluconate-PBS, K acetate-PBS, K$_2$SO$_4$-PBS, KBr-PBS, KSCN-PBS, and KNO$_3$-PBS were prepared by isoosmotic replacement of NaCl by the indicated salts while maintaining the pH at 7.3. All other components were identical to those of standard PBS. Unless otherwise indicated, media were made hypotonic by the addition of 2 vol of 50%-diluted PBS. Valinomycin (1 mM) and gramicidin D (0.5 mM) stock solutions were prepared in ethanol and dimethylsulfoxide, respectively.

Methods

PBM were isolated from heparinized blood of healthy donors as described (Grinstein et al., 1982). The cells were kept at room temperature in RPMI 1640 medium with 10% fetal bovine serum and, where indicated, they were sedimented and resuspended in the specified PBS. The cells were always used on the day of collection, and their viability was >90% under all conditions.

Intracellular Na$^+$ and K$^+$ content were determined by flame photometry using $[^{3}H]$polyethylene glycol as an extracellular space marker. Cell viability was determined by dye exclusion using trypan blue; cell volume was measured electronically using a Coulter Counter ZBI (Coulter Electronics, Hialeah, FL) adapted with a Coulter Channelyzer. These methods have been described in detail elsewhere (Grinstein et al., 1982).

$^{86}$Rubidium Uptake and Efflux Measurements

For efflux determinations, PBM were loaded for 3 h with 7 $\mu$Ci/ml of $^{86}$Rb in RPMI 1640 with 10% serum at a concentration of 1-2 $\times$ 10$^7$ cells/ml. The cells were then diluted 10-fold with RPMI 1640 and sedimented at 350 g for 10 min. The PBM were resuspended in the indicated PBS at a concentration of 1-2 $\times$ 10$^6$ cells/ml. At appropriate intervals, duplicate aliquots of this suspension were removed, layered over a mixture of 3 parts corn oil (Mazola) and 10 parts dibutyl phthalate, and centrifuged in an Eppendorf microfuge (Brinkmann Instruments, Westbury, NY). The tip of the tube containing the pellet was cut off and transferred to a scintillation vial containing 1 ml of 1% sodium dodecyl sulfate. The dissolved pellet was counted in 10 ml Aquasol II.

Uptake of $^{86}$Rb was measured by resuspending 10-15 $\times$ 10$^6$ PBM/ml of the appropriate buffer containing 10 $\mu$Ci/ml of $^{86}$Rb and 30 $\mu$Ci/ml of $[^{3}H]$polyethylene glycol. The mixture was incubated for the indicated times, at which point 50-$\mu$l aliquots were diluted into 1 ml of nonradioactive buffer and rapidly sedimented through the oil:phthalate mixture as described above. The aqueous phase was aspirated and the sides of the tube were rinsed twice before aspirating the oil, cutting the tip, and counting.

$^{36}$Cl Efflux

PBM (10$^6$ cells/ml) were loaded for 3 h in a medium containing standard PBS supplemented with 10% fetal bovine serum and 20-25 $\mu$Ci/ml of $^{36}$Cl. The cells were then sedimented at 350 g for 10 min and resuspended in the indicated media at a final concentration of 1-2 $\times$ 10$^6$ cells/ml. Duplicate aliquots of this suspension were taken at different intervals and sedimented through oil as described above.
Fluorescence Determinations

All experiments were performed in an Aminco-Bowman Ratio Spectrofluorometer (Silver Spring, MD) whose output was connected to a 7044 A y vs. t pen recorder (Hewlett-Packard Co., Inc., Palo Alto, CA).

The fluorescent dye 3,3'-dipropylthiadicarbocyanine or diS-C3(5) (referred to hereafter as the cyanine or simply the dye) was added to a cuvette containing 1.5 ml of the indicated medium to a final concentration of 0.6 µM. Next, 2-3 x 10^6 cells were added to the solution, which was stirred briefly. The fluorescence signal was then recorded with excitation and emission wavelengths of 620 and 670 nm, respectively, with 5.5-nm slit widths. In a series of experiments with any one preparation, the same number of cells was used for each measurement. Additions were made to the cuvette after a steady level of fluorescence was reached; the sample was briefly stirred again and the recording continued.

Calibration of the membrane potential in fluorescence units was performed by isoosmotically varying the extracellular K⁺ concentration in the presence of 1 µM valinomycin, as described by Waggoner (1979). An intracellular K⁺ concentration of 145 mM (Segel et al., 1981) was used for the calculation of membrane potential with the Nernst equation, assuming that the valinomycin-induced conductance dominated all other conductances. This assumption is probably valid, since (a) the membrane potential, which is largely determined by K⁺ in resting cells (see below) is shifted further towards E_K upon addition of the ionophore (e.g., Fig. 4), and (b) higher concentrations of the ionophore (up to 5 µM) produced identical results.

Other Methods

Media osmolarity was determined with an Advanced Osmometer, model 3L (Advanced Instruments, Needham Heights, MA). All the experiments were carried out at room temperature (21-23°C). Unless otherwise indicated, the data are presented as the mean ± SE of at least six determinations. Within the time of observation, none of the conditions or agents used in this report significantly affected viability, as determined by dye exclusion (trypan blue).

RESULTS

(a) Comparison of Ionophore-induced and Osmotically Induced Volume Changes

As reported previously (Ben-Sasson et al., 1975; Bui and Wiley, 1981), exposure of PBM to hypotonic media results in a substantial increase in K⁺ permeability. The associated volume changes depend on the direction of the K⁺ gradient. Thus, suspension of cells in hypotonic K⁺-free media (Fig. 1a) results in two distinct phases of volume change: an initial passive swelling, followed by a regulatory volume decrease (RVD) that is associated with K⁺ outflow. Suspension in hypotonic KCl-PBS (Fig. 1b) also results in two phases: the rapid passive swelling, and a slower second swelling phase that is associated with K⁺ inflow (Grinstein et al., 1982). On the other hand, if the K⁺ permeability of PBM is increased by the K⁺-specific ionophore valinomycin (see Figs. 2 and 4 and Discussion), no substantial volume changes occur in isotonic K⁺-free (Fig. 1a) or KCl-PBS (Fig. 1b), even though the K⁺ gradients are comparable to those under hypotonic conditions. The cells swelled at a slow rate in valinomycin-containing KSCN-PBS (Fig. 1b).
For comparison, similar experiments with valinomycin were performed using human erythrocytes. Under identical conditions (i.e., the same cytocrit and valinomycin concentration), the ionophore caused a marked reduction of cellular volume in K⁺-free PBS (Fig. 1a), as had been reported by Knauf et al. (1977). Also in agreement with these authors is the observation that the stilbene derivative DIDS reduces the rate of shrinking of red cells by ~60%.

This effect is due to inhibition by DIDS of net anion transport, the rate-limiting step in the loss of KCl and osmotically obliged water in valinomycin-treated cells. Based on these observations, it seemed possible that the absence of a volume response of PBM to addition of valinomycin might also be due to a low anion conductance. Consistent with this interpretation is the observation that some swelling is observed in SCN⁻-containing solutions (Fig. 1b), presum-
ably because SCN\(^-\) permeates faster than Cl\(^-\). Moreover, addition of gramicidin to cells in KCl-PBS similarly produced little swelling (not illustrated). Because gramicidin increases cation conductance considerably more than valinomycin (see Fig. 3), the similarity of their effects on volume points to an anion-limited process.

(b) Comparison of Ionophore-induced and Osmotically Induced \(^{86}\)Rb Fluxes

The pattern of \(^{86}\)Rb (a K\(^+\) analogue) efflux from hypotonically stressed cells is shown in Fig. 2. In K\(^+\)-free solution the results were virtually identical to those previously observed in normal PBS (3.9 mM K\(^+\)), i.e., a rapid loss of nearly 25% of the isotope, with a time course similar to that of RVD. Hypotonicity also enhanced \(^{86}\)Rb efflux from cells in K\(^+\)-rich medium (KCl-PBS), though to a somewhat lesser extent, probably reflecting the increase in volume and K\(^+\) content that occurs under these conditions.

![Figure 2](image_url)

**Figure 2.** \(^{86}\)Rb efflux from PBM in iso- and hypotonic media. PBM were loaded and resuspended in K\(^+\)-free (circles) or KCl-PBS (squares) as above. At zero time these suspensions were diluted with 2 vol of the corresponding isotonic PBS (filled symbols) or with 50%-diluted PBS (open symbols). The loss of \(^{86}\)Rb was monitored as a function of time. Ordinate: \(^{86}\)Rb remaining in cells (log scale). Data are the mean ± SE of six to eight determinations.

In contrast to the hypotonically stressed cells, the results obtained with valinomycin in isotonic media were strikingly different for cells suspended in K\(^+\)-free as compared with K\(^+\)-rich solutions (Fig. 3a). As expected, the ionophore greatly increased \(^{86}\)Rb efflux from cells in K\(^+\)-rich medium, but in K\(^+\)-free solutions valinomycin produced only a small effect. These observations can be explained if it is assumed that a rapid \(^{86}\)Rb-K\(^+\) exchange occurred in K\(^+\)-containing media, whereas in K\(^+\)-free solution, cation exchanges were minimal and net outflow of \(^{86}\)Rb\(^+\) was limited by a low anion conductance.

Similar experiments were performed with the channel-former gramicidin. This antibiotic is very poorly selective and transports Na\(^+\) and K\(^+\) at comparable rates in model membrane systems (Ovchinnikov, 1979). As in the case
of valinomycin, gramicidin was added to $^{86}$Rb-loaded cells suspended in K+-free and K+-rich solutions (Fig. 3b). The channel-former rapidly depleted the cells of isotope, both in the presence and absence of extracellular K+. Although efflux was slightly faster in K+-rich solutions, >90% of the isotope was lost, in both cases within 5 min. These results indicate that most of the cellular $^{86}$Rb is located in a compartment that can readily exchange across the cell membrane after addition of ionophores, and that in K+-free media gramicidin induces a $^{86}$Rb+-Na+ exchange, rather than co-transport with an anion, since in the latter case a loss of volume would result. No significant volume loss was observed under these conditions (Fig. 10). Neither antibiotic reduced cell viability during the experimental period at the concentrations tested.

(c) Anion Conductance and Membrane Potential in Isoosmotic Media

The experiments in sections a and b are consistent with a low anion conductance of the plasma membrane of PBM under isoosmotic conditions. Direct evidence of the low relative conductance to anions was obtained by determining the transference numbers of the principal ions in the bathing and intracellular media: Na+, K+, and Cl-. For this purpose, the membrane potential of PBM was measured fluorimetrically (Waggoner, 1979) under various conditions, using 3,3′dipropylthiodiearbocyanine [diS-C$_3$(5)]. Because cyanine dyes have been reported to interact with mitochondria and other organelles (Johnson et al., 1981), it was essential to ascertain that the measurements reflected the potentials across the plasma membrane. A membrane-originated fluorescent signal should respond rapidly to changes in external ionic composition and to the addition of ionophores, whereas signals derived from mitochondria should be affected only marginally and more slowly. We analyzed the effect of ionophores on the fluorescence of PBM suspended in media of different ionic composition. Traces from a typical experiment are presented in Fig. 4.

$K^+$-free PBS

Addition of cells to K+-free medium containing 0.6 μM cyanine produced a rapid decline of fluorescence intensity, reaching a steady value after 6–8 min. Addition of 1 μM valinomycin (expected to hyperpolarize the cell) evoked a further decrease with no observable lag (Fig. 4), whereas addition of aliquots of concentrated (2 M) KCl rapidly enhanced fluorescence (not illustrated). When added to cells in K+-free medium (with or without valinomycin), gramicidin greatly increased the fluorescent signal, consistent with a drastic depolarization resulting from the increased Na+ permeability.

Tris-PBS

Cells suspended in Tris medium (prepared by isoosmotic replacement of NaCl by Tris-HCl) showed an initial fluorescence decline comparable to that in K+-free medium, which suggests that Na+ conductance does not contribute importantly to the membrane potential. As expected, addition of valinomycin induced a clear-cut hyperpolarization. However, the drastic depolarization produced by gramicidin in Na+-containing media was not observed in Tris-
Figure 3. (a) Loss of $^{86}\text{Rb}$ from cells treated with valinomycin in K$^+\text{-free}$ and K$^+$-rich media. PBM were loaded with $^{86}\text{Rb}$ in RPMI 1640 supplemented with 10% fetal calf serum, the extracellular isotope was removed, and the cells were suspended in nonradioactive K$^+$-free PBS (circles) or KCl-PBS (squares). The cells were then incubated in the presence (open symbols) or absence (filled symbols) of 1 μM valinomycin (final), and aliquots were taken at fixed intervals for the determination of the fraction of $^{86}\text{Rb}$ remaining in the cells (ordinate, log scale) as a function of time (abscissa). Data are the mean ± SE of five determinations. Where not indicated, error bars were smaller than the symbols. (b) Loss of $^{86}\text{Rb}$ from cells treated with gramicidin in K$^+$-free and K$^+$-rich media. PBM were loaded and resuspended as above in K$^+$-free PBS (circles) or KCl-PBS (squares). Aliquots of these suspensions were incubated in the presence...
PBS. This is expected, since this large organic cation is presumably not transported by the antibiotic.

**KCl-PBS**

The fluorescence of cells equilibrated with the dye in K⁺-rich solution was considerably more intense than in the previous two cases (Fig. 4), which indicates that external K⁺ depolarized the PBM. As expected, addition of valinomycin to cells in KCl-PBS medium produced a small increase of the signal (i.e., depolarization), and gramicidin slightly decreased fluorescence.

In all of the above experiments, the onset of the fluorescence change was virtually instantaneous, consistent with phenomena occurring at the plasma membrane level. Equilibration of the signal after a change in the experimental conditions required 5–8 min (the time required for complete redistribution of the dye).

![Figure 4. Validation of fluorescence methods for the determination of membrane potential of PBM. 2–3 × 10⁶ cells were added to 1.5 ml of either K⁺-free PBS, KCl-PBS, or Tris-PBS containing 0.6 μM diS-C₃(5), and fluorescence changes were recorded as a function of time. After fluorescence reached a steady level, 1 μM valinomycin was added and the suspension was stirred briefly. After reaching a second equilibrium, 0.5 μM gramicidin was added, the suspension was stirred, and recording continued. All the media were isotonic. Ordinate: fluorescence intensity in arbitrary units. The traces are representative of two to six such experiments.](image)

The procedure for determining absolute values of membrane potential requires calibration with varying external K⁺ concentrations in valinomycin-treated cells (see Methods). The linearity of the data (a typical calibration curve is shown as an inset in Fig. 5) is consistent with the K⁺ gradient being the determinant of the potential in the presence of valinomycin and with the dye distribution (measured as fluorescence intensity) being responsive to membrane potential.

(open symbols) or absence (filled symbols) of 0.5 μM gramicidin, and ⁸⁶Rb efflux was determined as described. Ordinate: ⁸⁶Rb remaining in cells (log scale). Abscissa: time in minutes. Data are the mean ± SE of six determinations.
The relative conductance or transference number of the different ions was estimated from the ionic concentration dependence of the membrane potential. Fig. 5 illustrates the results of these experiments. The dashed line indicates the Nernst potential for $K^+$, calculated by assuming an internal $K^+$ concentration of 145 mM. Reducing the concentration of either $Na^+$ (by replacement with choline) or $Cl^-$ (replaced by isethionate) produced little change in membrane potential. In contrast, increasing the concentration of extracellular $K^+$ above 9.5 mM substantially depolarized the cells. The transference numbers of the major ions were calculated as $T_i = \frac{\Delta E_m}{RT} \ln \frac{C_1}{C_2}$, where $\Delta E_m$ is the potential recorded upon varying the concentration of the relevant ion from its initial ($C_i$) to its final ($C_f$) value. In the 9.5–140-mM interval, the transference numbers of $K^+$, $Na^+$, and $Cl^-$ were $T_{K^+} = 0.57$, $T_{Na^+} < 0.1$, and $T_{Cl^-} < 0.1$, respectively. Below 9.5 mM the dependence of the potential on the external $K^+$ concentration is less clear. This observation may be a reflection of the reduced sensitivity of the fluorescence method in this range or, if real, may suggest that at low $K^+_o$, factors other than the passive $K^+$ permeability contribute substantially to the transmembrane voltage. Experiments using

![Figure 5. The membrane potential of PBM as a function of external ion concentration. Membrane potential was determined from calibration curves constructed using valinomycin as described in Methods. $K^+$, $Na^+$, and $Cl^-$ were isoosmotically replaced by $Na^+$, choline $^+$, and isethionate $^-$, respectively. Steady state fluorescence was measured as in Fig. 4. The continuous line was drawn by eye. The Nernst potential for $K^+$ ($E_{K^+}$) is indicated by the dashed line. Note log scale in abscissa. The points are the mean of three similar experiments. The inset shows a typical calibration curve, obtained by treating the cells with 1 μM valinomycin and measuring the fluorescence intensity at equilibrium (8 min) at the indicated $K^+$ concentrations.](image-url)
ouabain (100 μM) failed to demonstrate any substantial contribution of the Na⁺-K⁺ pump to the membrane potential in cells suspended in PBS (not illustrated). Perhaps other ions, present in low concentration, could account for the observed reduction of slope.

The resting membrane potential of PBM in standard PBS (3.9 mM K⁺) was found to be -53 mV (n = 9), which is similar to the upper limit of -52 mV reported by Deutsch et al. (1979) for the same cells, using radiolabeled lipophilic ions. These authors also found the PBM membrane to be K⁺ selective, particularly at high extracellular [K⁺], and could only slightly alter membrane potential by addition of up to 10 μM valinomycin.

Membrane Potential and Conductance in Hypotonic Media

The experiments in the three preceding sections indicate that valinomycin increases the K⁺ conductance of PBM, but fails to induce significant net K⁺ movements or volume changes. Anion conductance appears to be the limiting factor. This does not appear to be the case in hypotonically stressed cells, in which net K⁺ loss brings about RVD, which implies that anion conductance has increased. Experiments designed to test this prediction are reported in this and the following sections. The dye diS-C₅(5) was used to determine the potential difference across the membrane of cells undergoing volume regulation. Fluorescence intensity is expected to decrease upon dilution, a necessary step in the imposition of a hypotonic stress. Moreover, the dye would be expected to redistribute between cells and medium when the concentration in the latter drops. Thus, fluorescence intensity changes will occur upon dilution that are unrelated to alterations in membrane potential. Experiments were performed to estimate the magnitude of these changes and to assess whether actual membrane potential alterations, superimposed on the effects of dilution, could be detected (Fig. 6a). For this purpose, PBM were equilibrated with diS-C₅(5) in isotonic K⁺-free medium and then diluted with the same medium (without dye). As expected, a steplike fall of fluorescence intensity occurred, but only marginal changes were recorded thereafter, within the observation period. Similar results were obtained using isotonic sucrose as the diluent. In contrast, if valinomycin was included in the diluting K⁺-free medium, a comparatively slow hyperpolarization (decrease of fluorescence) followed the initial rapid drop. On the other hand, if isotonic K⁺-rich medium was used for dilution, bringing the extracellular K⁺ concentration to 46.9 mM, a clear-cut depolarization was recorded, in spite of the dilution of the dye. These data indicate that at least qualitative indications of changes in membrane potential can be recorded after a sudden dilution.

Fig. 6b illustrates the effect of hypotonic (0.67× isotonic) stress on the membrane potential of PBM suspended in different media. In K⁺-free buffer, the cells were reproducibly depolarized when the osmolarity was reduced. The magnitude of the potential change was increased if all the NaCl in the K⁺-free solution was isoosmotically replaced by Na₂SO₄ or Na-gluconate (not illustrated). PBM suspended in KCl-PBS medium were also subjected to osmotic challenge. As mentioned above, the equilibrium fluorescence intensity of these cells is high, i.e., they have a low resting potential. Upon hypotonic
dilution, the fluorescence of this cell suspension decreased (Fig. 6b). If KCl was replaced by K-gluconate, fluorescence intensity in isotonic medium was not significantly altered, but hypotonic dilution slightly depolarized the cells (not shown). These results are consistent with a volume-induced increase in Cl⁻ conductance. Assuming that in K⁺-free PBS $E_{Cl}$ is more positive than $E_m$ (see Discussion), a depolarization is expected if Cl⁻ conductance is increased; the depolarization ought to be larger in SO₄²⁻-PBS, where $E_{Cl}$ actually becomes positive. In high-K⁺ media, $E_m$ is near zero in isotonic conditions and will...
hyperpolarize towards $E_{C_{1}}$, which is negative in $C_{1}^{-}$ medium, or depolarize further in gluconate medium, where $E_{C_{1}}$ is positive.

**Efflux of $^{36}Cl$ in Hypotonic Solutions**

There are at least two reasons to suspect $Cl^{-}$ of being one (and possibly the major) of the anions transported with $K^{+}$ during RVD: (i) $Cl^{-}$ comprises a large fraction of the diffusible intracellular anion pool (38 mM, as determined by isotope equilibration), and (ii) $Cl^{-}$ is taken up by the cells when exposed to hypotonic KCl-PBS, as evidenced by the secondary swelling observed (Fig. 1b). Substitution of $Cl^{-}$ by impermeant anions eliminated this second phase of swelling (see below).

To establish the role of $Cl^{-}$ in RVD, we measured the efflux of $^{36}Cl^{-}$ from PBM in isotonic and hypotonic media (Fig. 7). In isotonic PBS, cells pre-equilibrated with $^{36}Cl$ lost 50% of the isotope in 13 min. This rapid efflux is

![Figure 7](image_url)  
**Figure 7.** Chloride efflux from PBM in iso- and hypotonic media. PBM were loaded with $^{36}Cl$ in standard PBS supplemented with 10% fetal calf serum for 2-3 h at room temperature. The cells were then sedimented and resuspended in nonradioactive PBS. At zero time, identical aliquots of the suspension were diluted with 2 vol of PBS (○) or with 2 vol of 50%-diluted PBS (□). Efflux was measured at regular intervals by sedimentation through an oil layer. The results are the mean ± SE of six determinations. Ordinate: $^{36}Cl$ remaining in cells (log scale).
much higher than predicted from the conductance determinations described above and probably represents an electroneutral anion exchange system like those described for other cell types (Cabantchik et al., 1978). In agreement with this notion, the $^{36}\text{Cl}^-$ efflux was found to be similar in isotonic low- and high-$\text{K}^+$ media, which indicates that it is potential insensitive. The rate of $^{36}\text{Cl}^-$ efflux from cells subjected to hypotonicity was, however, markedly increased, with 80% loss in 5 min. Because both the intracellular concentration of the isotope and the transmembrane potential are reduced upon cell swelling (i.e., the outward electrochemical gradient for $\text{Cl}^-$ is reduced), the increased efflux appears to reflect a volume-induced change in anion permeability. However, these isotopic determinations are not sufficient by themselves to establish the conductive nature of the $\text{Cl}^-$ fluxes or their direct involvement in the volume change.

**Selectivity of the Anion Transport Pathway**

The specificity of the anion pathway induced by hypotonic swelling was studied in media of high extracellular [K$^+$]. Under these conditions the electrochemical gradient for K$^+$ is virtually abolished and the driving force for net salt movement is largely provided by the anion gradient. The direction and magnitude of the secondary volume change (i.e., that following the initial passive swelling) will therefore be determined by the nature and concentration of extracellular anions. Fig. 8 shows a series of typical determinations of cellular volume after hypotonic stress in various high-K$^+$ media. Permeating ions, such as SCN$^-$, induce secondary swelling, whereas large, poorly permeant anions, like gluconate, allow a small volume decrease. The latter is probably due to loss of $\text{Cl}^-$ or other intracellular permeating anions down their gradients. The selectivity sequence of the volume-induced anion permeability, measured by the rate of secondary volume change, is as follows: SCN$^-$ > I$^-$ > NO$^-$ > Br$^-$ > $\text{Cl}^-$ > acetate$^-$ > SO$^{2-}_4$ = gluconate$^-$. 

**Studies on the Mode of Coupling of Volume-induced Anion and Cation Fluxes**

Is K$^+$ transport during RVD stringently dependent on the movement of anions? To define the mode of coupling of counterions during volume-induced permeability changes, we analyzed the fluxes of $^{86}\text{Rb}$ in K$^+$-rich media, with anion substitution. As described above, under these conditions net influx of K$^+$ will occur in KCl-PBS but not in K$_2$SO$_4$-PBS, which indicates that SO$_4^{2-}$ is poorly transported, if at all, by the volume-dependent pathway. Thus, it was of interest to find out whether $^{86}\text{Rb}$ influx, which is known to be increased in hypotonic media (Bui and Wiley, 1981), would be affected by substitution of the anion. Fig. 9 shows the results of these experiments. In either KCl-PBS or K$_2$SO$_4$-PBS the influx of $^{86}\text{Rb}$ was slow under isotonic conditions. As expected, hypotonic stress in KCl-PBS resulted in a substantial increase in $^{86}\text{Rb}$ uptake. In K$_2$SO$_4$-PBS, the initial rate of uptake was almost as high. The more persistent increase in rate observed in KCl-PBS is probably due to the prolonged swelling observed in this medium that results from the continued uptake of KCl (see Grinstein et al., 1982). These results suggest that cations can move independently of anions during RVD.
An alternative way of determining the interdependence of anion and cation fluxes during volume regulation was devised using gramicidin. The aim of these experiments was to determine whether a volume-induced anion flux could be observed in the absence of K⁺. In these investigations gramicidin served a dual purpose: (a) to rapidly deplete intracellular K⁺; as suggested by Fig. 3b nearly complete replacement of intracellular K⁺ (or ⁸⁶Rb) by external Na⁺ can be attained within minutes of addition of the antibiotic;¹ and (b) to provide a parallel conductive route for cation permeation, so that a counterion

Figure 8. Selectivity of the volume-induced anion permeation pathway. PBM were suspended in isotonic KSCN-PBS (○), KNO₃-PBS (▲), K₂SO₄-PBS (●), or K-gluconate-PBS (△). The experiment was initiated by dilution of the medium to 0.67X isotonic. Cell volume was measured by electronic sizing with a Coulter Counter-Channelyzer combination. The results are representative of at least three such experiments.

¹ Indeed, direct measurements of cation content by flame photometry indicated that >95% of the intracellular K⁺ was lost after a 10-min incubation of PBM in K⁺-free PBS with 0.5 μM gramicidin. That exchange of K⁺ for Na⁺ is occurring, as opposed to co-transport of an anion with K⁺, is indicated by the constancy of cellular volume and the depolarization observed under these circumstances.
via gramicidin channels, resulting in a measurable cell swelling. The results of one such experiment are shown in Fig. 10. PBM were suspended in K⁺-free PBS and electronically sized before and after addition of gramicidin. The antibiotic alone had either no effect or very slightly reduced cell volume. At
this point the cells were hypotonically stressed. In K+-free media with Cl− as the main anion, the initial swelling phase was followed by a dramatic secondary volume increase. These results indicate that a volume-induced uptake of Cl− can occur in the virtual absence of K+. In contrast, cells treated with gramicidin in a Na+ gluconate, K+-free medium failed to show secondary swelling and in fact shrank somewhat after the passive swelling phase. In cells suspended in the impermeant gluconate, the outward gradient of Cl− leads to an outflow of Cl− and volume loss that is not K+ dependent.

Further evidence that Cl− transport by the volume-induced pathway is not
impaired by removal of K⁺ was obtained by measuring isotopic fluxes. In these experiments the efflux of ³⁶Cl⁻ was measured in cells depleted of K⁺ by gramicidin addition in K⁺-free media, as described above. Efflux was measured in isotonic as well as in hypotonic conditions and compared with the fluxes observed in cells with normal K⁺ content (Fig. 7). The presence of the antibiotic in isotonic medium did not significantly affect the basal ³⁶Cl⁻ flux (not shown). On the other hand, a substantial volume-induced component of the efflux was observed in K⁺-depleted cells when the osmolarity was reduced. In fact, the results obtained in K⁺-containing (Fig. 7) or K⁺-depleted PBM were not significantly different. These results support the notion that anions and cations move independently during RVD.

DISCUSSION

Limitations of the Fluorimetric Measurements of Membrane Potential

Direct measurement of transmembrane voltage by impalement with microelectrodes is extremely difficult in small cells such as PBM. Thus, more indirect methods, based on the partition of lipid soluble dyes or radioactive ions between the cells and the medium, must be used. The indirect procedures, however, have possible shortcomings that must be taken into account. First, respiring mitochondria have substantial potentials with respect to the cytoplasm and therefore tend to accumulate dye (Johnson et al., 1981), which complicates the interpretation of results. In the case of PBM, however, mitochondria are unlikely to contribute significantly to the fluorescent signal since (a) the mitochondrial volume in lymphocytes is only 4% (Deutsch et al., 1979), and (b) the fluorescent signal recorded varied substantially and immediately upon changing the ionic composition of the medium or after the addition of ionophores. In agreement with these results, Mikkelsen and Koch (1981) estimated the contribution of mitochondria to the potential of splenic lymphocytes measured with triphenylmethylphosphonium (TPMP⁺) at a maximum of 5-10 mV.

In practice, the fluorescent method appears to work effectively for PBM in isotonic media. Various manipulations lead to expected changes in fluorescence and the calibration curve, involving alterations of extracellular K⁺ in the presence of valinomycin, gives a reasonably linear relationship. On the other hand, under conditions in which the potential is changing, the method is limited by the time required for equilibration of the dye between the cells and the medium. This process is rather slow (lasting 5-8 min), so that quantitative (i.e., equilibrium) measurements of comparatively rapid, transitory processes, such as RVD, cannot be obtained. Radioactive lipid-soluble ions such as TPMP⁺ and SCN⁻ equilibrate even more slowly (Deutsch et al., 1979; Kiefer et al., 1980). Nevertheless, as illustrated by Figs. 6a and b, qualitative indications of the direction of potential changes can be readily obtained with diS-C₃-(5). Another difficulty is posed by the dilution of the dye, which occurs during experiments involving hypotonic stress. As indicated
by the middle trace in Fig. 6a, isotonic dilution results in an immediate drop in the signal; this is followed by redistribution of the dye between cells and medium producing relatively small fluorescence changes. These are clearly discernible from the slower, presumably potential-induced alterations in fluorescence measured in hypotonically treated cells. The fact that hypotonically stressed cells depolarize in K⁺-free PBS and K₂SO₄-PBS but hyperpolarize in KCl-PBS strongly suggests that potential, rather than dilution-induced, alterations are being measured.

**Role of Cations and Anions in RVD**

Volume regulation by lymphoid cells in hypotonic media has generally been thought to be accomplished by a selective increase in K⁺ permeability (Roti-Roti and Rothstein, 1973; Doljanski et al., 1975; Bui and Wiley, 1981). Evidence documenting the increase in K⁺ permeability has been presented by Cheung et al. (1982), who demonstrated that both ⁸⁶Rb efflux and ouabain-insensitive uptake are increased during RVD. However, the premise that K⁺ permeability limits the rate of KCl and water fluxes in volume-static PBM is not supported by the experiments reported here using valinomycin or gramicidin. The ionophores failed to induce volume changes in cells suspended in isotonic solutions, despite producing increases in cation permeability greater than those resulting from hypotonic stress (compare Figs. 2 and 3). The ionophore-induced increase in permeability and conductivity could be detected by both changes in isotopically measured fluxes and in membrane potential. The results summarized above suggest that an increase in K⁺ permeability is not sufficient to account for the secondary volume alterations that follow hypotonic swelling of PBM. Because water permeability in PBM is comparatively high (Hempling et al., 1978), as also evidenced by the rapid initial swelling observed in anisotonic media (Fig. 1), it is most likely that anion conductance is the rate-determining factor in salt and water transport. It follows that the secondary volume changes following hypotonic stress could only occur if the anion conductance were substantially increased. The remainder of this Discussion is devoted to the analysis of this hypothesis and of the possible coupling mode of anion and K⁺ transport.

**Anion Permeability and Conductance in Volume-static PBM**

Three independent sets of observations indicate that anion conductance is low in resting PBM. First, cells incubated with valinomycin in KSCN-PBS increased significantly in volume compared with cells in KCl-PBS, presumably because of the greater permeability (higher lipid solubility) of SCN⁻ compared with Cl⁻. Second, measurements of the relative conductance of the main ions indicate that, at least at concentrations of K⁺ ≥ 9.5 mM, this cation contributes predominantly to the total conductance, which implies that anion conductance is substantially lower than that of K⁺ (Fig. 5). Furthermore, substitution of Cl⁻ by the purportedly less permeant isethionate did not affect membrane potential (Fig. 5), which stresses the nonconductive nature of most
of the Cl⁻ flux. Third, the increased K⁺ flux induced by valinomycin was dependent on the presence of external K⁺ (Fig. 3). K⁺ efflux by K⁺-K⁺ exchange could take place, but net efflux of K⁺ with an anion could not, which suggests low conductive fluxes of anions.

Evidence for an Increase in Anion Permeability during Hypotonic Stress

It has already been noted in the presentation of the results that the isotopically measured Cl⁻ flux in PBM suspended in isotonic media is substantially higher than the Rb⁺ (K⁺) flux under the same conditions. However, a number of experiments presented in this paper suggest that the Cl⁻ conductance is lower than the K⁺ conductance. It was suggested that the fluxes represent largely an electroneutral anion exchange system like that present in other cell types. Nevertheless, the efflux of ³⁶Cl⁻ from PBM was increased substantially by hypotonic challenge. Since both the intracellular concentration of the isotope and the transmembrane potential are decreased upon swelling, the enhanced efflux must be attributed to an increase in anion permeability. That part of the volume-induced anion permeation path is conductive is suggested by the depolarization that accompanies RVD. In fact, simultaneous addition of valinomycin during the hypotonic stress did not prevent the depolarization, which indicates that the volume-induced anion conductance dominated the K⁺ conductance produced by the ionophore. A depolarization is to be expected if anion conductance increases, since the Nernst potential for Cl⁻ (Eₐ₁ = -33 mV) is lower than the resting membrane potential (-53 mV). The former was calculated using an intracellular Cl⁻ concentration of 38 mM, as determined by isotope equilibration and assuming no compartmentation and an activity coefficient of 1.

Probably the most compelling proof for an increased anion conductance during RVD is presented in Fig. 10. Cells depleted of K⁺ and permeabilized to cations with gramicidin fail to swell in the presence of an inward anion gradient in isotonic media. However, hypotonic swelling, which renders the membrane more conductive to Cl⁻, results in a striking secondary swelling which is not observed in Cl⁻-free (gluconate) solutions.

Using direct measurements with microelectrodes, Hoffman and collaborators (Simonsen et al., 1976; Hoffman, 1978; Hoffman et al., 1979) found that

2 An approximate estimate of the permeability can be obtained from either ³⁶Cl exchange measurements or from valinomycin-induced volume changes, if the following assumptions are made: that [K⁺] = 145 mM, [Cl⁻] = 38 mM, and cell volume = 205 μm³; that Eₚ = -53 mV in normal cells and Eₚ = Eₐ₁ in valinomycin-treated cells, and that volume changes can be accounted for solely by net KCl fluxes. When measured isotopically during the initial 5-min interval, P₃Cl ranged between 0.7 × 10⁻⁹ and 1.6 × 10⁻⁹ 1·min⁻¹·10⁶ cells⁻¹ (the results are expressed per 10⁶ cells, since the surface area is unknown). In contrast, when calculated on the basis of the isotonic volume changes, P₃Cl was over 25-fold lower.

3 Although the membrane potential could not be determined accurately during the volume-induced transient, it is likely to vary between the resting potential (-53 mV) and Eₐ₁ (-33 mV). Using an intermediate value of -43 mV, P₃Cl during RVD can be estimated from the isotope fluxes as well as from the volume change. In the former case, P₃Cl was calculated to be 7.6 × 10⁻⁹ 1·min⁻¹·10⁶ cell⁻¹, whereas volume measurements produced a value of 3.6 × 10⁻⁹ 1·min⁻¹·10⁶ cells⁻¹. Thus, a large fraction of the volume-induced permeability is conductive.
the Cl\textsuperscript{−} conductance of Ehrlich ascites cells is (a) much lower than the isotopically measured permeability; (b) rate limiting for net salt loss; and (c) increased by hypoosmotic media. Our observations in human PBM are in agreement with these results.

**Anion Selectivity and Coupling to Cation Fluxes**

The volume-induced anion pathway of PBM was rather unselective. Of those tested, only very large monovalent anions (e.g., gluconate\textsuperscript{−}) or divalent anions (SO\textsubscript{4}\textsuperscript{2−}) were excluded (Fig. 8). This is in sharp contrast with other volume-induced systems, like that of LK sheep red cells, in which NO\textsubscript{3}\textsuperscript{−}, SCN\textsuperscript{−}, and I\textsuperscript{−} support K\textsuperscript{+} transport much less efficiently than Cl\textsuperscript{−} (Dunham and Ellory, 1981), or of duck (Kregenow and Caryk, 1979) and *Amphiuma* (Cala, 1980) red cells, which are also specific for Cl\textsuperscript{−}, or HCO\textsubscript{3}\textsuperscript{−} and Cl\textsuperscript{−}, respectively.

Several mechanisms have been proposed to explain the coupling of anions and cations during RVD. Because of the strict Cl\textsuperscript{−} (or Br\textsuperscript{−}) requirement of volume-induced K\textsuperscript{+} fluxes in duck and sheep red cells, a symport mechanism has been suggested (Kregenow and Caryk, 1979; Dunham and Ellory, 1981). A different model was suggested by Cala (1980), in which a K\textsuperscript{+}/H\textsuperscript{+} antiporter operates simultaneously, but independently, with an anion antiporter which exchanges HCO\textsubscript{3}\textsuperscript{−}/Cl\textsuperscript{−} or, as proposed by Kregenow (1981), Cl\textsuperscript{−}/OH\textsuperscript{−}. The anion antiporter is probably the normal stilbene-sensitive exchange system that operates in volume-static cells, since addition of DIDS blocks RVD in these cells.

Neither of the above models is consistent with the observations in PBM. (a) Both \textsuperscript{86}Rb and \textsuperscript{36}Cl fluxes are stimulated by hypotonicity in the absence of permeating counterions. If it is assumed that these isotopic movements reflect the net fluxes responsible for the volume change, then a tightly coupled KCl co-transport system must be ruled out as the mechanism underlying RVD. Moreover, furosemide, which is generally an inhibitor of coupled KCl co-transport systems, failed to inhibit RVD in PBM (unpublished observations). (b) Membrane potential and conductance are affected during RVD. (c) RVD was not inhibited by pretreatment of the cells (5 × 10\textsuperscript{5}/ml) with up to 10\textsuperscript{−4} M DIDS (unpublished observations). These findings are inconsistent with coupled cation-anion co-transport or with the electrically silent double antiporter model.

The results reported in this communication can best be explained by assuming that the K\textsuperscript{+} conductance is low and the Cl\textsuperscript{−} conductance even lower in volume-static PBM, and that the resting membrane potential lies between $E_K$ and $E_{Cl}$. Upon swelling, the conductance of K\textsuperscript{+}, and especially that of Cl\textsuperscript{−}, increase independently (i.e., the ions are not obligatorily co-transported), leading to a loss of salt and water from the cells and bringing the potential closer to $E_{Cl}$. This hypothesis is similar to the one offered by Hoffman (1978) to explain RVD in Ehrlich ascites tumor cells.

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