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Increased Anion Permeability During Volume Regulation in Human Lymphocytes

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Increased anion permeability during volume regulation in human lymphocytes

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Peripheral blood lymphocytes (p.b.ls) readjust their volumes after swelling in hypotonic media. An essential component of the regulatory response is an increase in K^+ and Cl^- permeability. No evidence was found for a tightly coupled co-transport of K^+ and Cl^-. The flux of either ion proceeds normally in the virtual absence of the transported counterion. Furthermore, alterations in membrane potential recorded during the phase of volume readjustment can be qualitatively accounted for by an increase in Cl^- conductance. In tonsillar lymphocytes, a failure of the K^+-permeation path to respond to swelling leads to deficient volume recovery, but Cl^- permeability is nevertheless increased upon swelling. This further suggests that K^+ and Cl^- are transported during volume regulation through independent pathways. Cytoplasmic free Ca^{2+} appears to be involved in regulatory volume decrease. K^+ and Cl^- fluxes similar to those elicited by swelling can also be produced by A23187 plus Ca^{2+}. Moreover, swelling and shrinking can be induced in isotonic K^+-rich and K^+-free media, respectively, by the Ca^{2+} ionophore. The ion flux and volume changes produced by either swelling or internal Ca^{2+} can be inhibited by similar concentrations of quinine and phenothiazines. The inhibitory activity of the latter drugs, which are powerful antagonists of calmodulin, suggests the participation of this Ca^{2+}-regulator protein in volume regulation.

Introduction

Cellular volume is a remarkably constant parameter, which indicates that it is precisely regulated. The mechanisms underlying volume control can be conveniently studied by exposing cells to anisotonic conditions, which result in sudden, large volume changes. These volume alterations elicit substantial regulatory responses that are readily measurable. A number of cell types have been reported to regulate their volume in both hypotonic and hypertonic media (see MacKnight & Leaf (1977) and Kregenow (1981) for reviews). Hypotonic swelling is followed by a regulatory volume decrease (r.v.d.), whereas shrinking in hypertonic media is offset by a compensatory reswelling. In both cases volume displacements appear to be the result of net salt fluxes associated with osmotically obliged water. The molecular basis of volume regulation is poorly understood and is likely to be different in different cell types.

This article is concerned with the ionic mechanisms responsible for r.v.d. in peripheral blood lymphocytes (p.b.ls). These cells provide a convenient experimental system, because they display rapid and nearly complete regulatory responses and are amenable to electronic sizing, a fast and accurate technique.

Involvement of K^+ in r.v.d.

The participation of K^+ in r.v.d. in lymphoid cells from mouse and human was suggested by Roti-Roti & Rothstein (1973) and Doljanski *et al.* (1974). The latter authors found that the

shrinking normally observed in hypotonic Na^+-rich media failed to occur if K^+ was substituted for Na^+. Instead, a secondary swelling phase followed the initial (osmotic) swelling. More recent observations (Cheung *et al.* 1982*a*) indicate that the rate and direction of the secondary volume change are a function of the external K^+ concentration, $[K^+]_o$, and that at 50 mM no regulatory response is observed. It was concluded that the K^+ gradient appears to be a primary determinant of the volume changes and that, in physiological (Na^+-rich) media, a loss of K^+ down its electrochemical gradient brings about r.v.d. A loss in cellular K^+ content after shrinking has in fact been repeatedly documented (Doljanski *et al.* 1974; Bui & Wiley 1981; Cheung *et al.* 1982*a*). Na^+ content, in contrast, remains unaltered after r.v.d. The release of K^+ during shrinking can also be monitored isotopically by using $^{86}Rb^+$ as an analogue and the increased efflux can be shown to be due to an increased permeability, rather than to a change in driving force, because the ouabain-insensitive component of $^{86}Rb^+$ uptake is similarly increased. Interestingly, influx of $^{86}Rb^+$ through the Na^+–K^+ pump is also markedly increased, and this is not the result of increased Na^+ leakage (Cheung *et al.* 1982*a*). However, participation of the pump is not essential for r.v.d.; normal regulatory responses are recorded in the presence of fully inhibitory doses of ouabain. Partial inhibition is only noted after prolonged incubations with the glycoside, presumably due to a diminished K^+ gradient resulting from the depletion of cellular K^+.

Role of anions in r.v.d.

It has been generally assumed that in p.b.ls electroneutrality during r.v.d. is preserved by the comigration of anions (most likely Cl^-) with K^+, and that the relatively low K^+ permeability determines the rate of salt and water loss. Hence, an increase in K^+ permeability resulting from swelling would suffice to account for r.v.d. However, recent studies in other cell types have suggested that other, more complex, mechanisms may become operative during volume regulation. In Ehrlich ascites cells, Hoffman (1978, and this symposium) has found that, in addition to an increase in K^+ conductance, the conductance of Cl^- is also markedly enhanced, whereas Na^+ conductance decreases. In sheep red cells, swelling promotes the efflux of K^+ through a Cl^- dependent furosemide-sensitive pathway (Dunham & Ellory 1981), and in *Amphiuma* and other nucleated erythrocytes r.v.d. is accomplished by the simultaneous operation of a K^+/H^+ and a Cl^-/HCO_3^- (or Cl^-/OH^-) antiporter (Cala 1980; Kregenow 1981).

In view of these reports a more detailed analysis of the ionic basis of r.v.d. in p.b.ls was warranted. Two of the original premises were questioned: (*a*) is K^+ conductance rate-limiting for salt loss? and (*b*) given the adequate driving forces, can an increase in K^+ conductance alone produce a volume change? These questions were answered by the use of ionophores to increase the cation permeability, and by measurements of membrane potentials under a variety of conditions. The cell volumes of p.b.ls suspended in media of different K^+ concentration were measured before and after the addition of valinomycin. Unlike red blood cells, which shrink upon addition of the ionophore in K^+-free media due to the increased K^+ permeability, p.b.ls did not change their volumes perceptibly during equivalent or longer incubations (Grinstein *et al.* 1982*b*), and very little swelling was detected in K^+-rich media. The ineffectiveness of valinomycin in p.b.ls was *not* due to its failure to act as a K^+-specific ionophore. The ionophore was incorporated into the membrane, increasing K^+ ($^{86}Rb^+$) permeability severalfold. The effectiveness of valinomycin in increasing the K^+ conductance in p.b.ls was also established by measuring its effects on membrane potential. By using 3,3′-dipropylthiadicarbocyanine

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(diS-C_3-(5)) as a probe (Waggoner 1979), it was found that a definite hyperpolarization followed addition of valinomycin to cells in K^+-free medium, whereas a small depolarization was observed in K^+-rich solution.

Some experiments were also performed with the channel-former gramicidin. This antibiotic, which increases monovalent cation conductance much more effectively than valinomycin, similarly *failed* to induce cell swelling in K^+-rich medium. In parallel experiments gramicidin was shown to release over 90% of the intracellular $^{86}Rb^+$ within 10 min, and in cells suspended in normal phosphate-buffered saline (p.b.s.) it exchanged virtually all the intracellular K^+ for Na^+ in the same period. Potential measurements of cells in low K^+ media showed that gramicidin depolarized the cells when Na^+, but not when Tris, was the predominant extracellular cation.

Given that valinomycin and gramicidin increase the conductive permeability to monovalent cations, their failure to induce volume changes in p.b.ls suggests that the conductive permeability to anions (largely Cl^-) is relatively low. This conclusion is supported by other experiments with valinomycin. It has been noted above that valinomycin increases K^+ ($^{86}Rb^+$) efflux from p.b.ls. This increase was much smaller, however, if the cells were suspended in K^+-free medium, presumably because in the absence of exchangeable cations, ^{86}Rb efflux was limited by the low rate of anion outflow.

The assumption that anion (Cl^-) conductance is considerably lower than that of K^+ was directly tested by measurement of transmembrane voltage at different concentrations of external ions. The transference number (T_i) was calculated as

$$T_i = \frac{\Delta E_m}{(RT/F)\ln(C_1/C_2)},$$

where ΔE_m is the change in membrane potential recorded upon varying the concentration of the relevant ion from its initial (C_1) to its final value (C_2). When measured in the 9–140 mM interval the transference numbers of K^+, Na^+ and Cl^- were $T_{K+} = 0.57$, $T_{Na^+} < 0.1$ and $T_{Cl^-} < 0.1$. These data indicate that K^+ conductance is substantially lower than that of Cl^-, and explain why volume changes were not observed when ionophores were added: anion conductance is limiting in p.b.ls. It follows that if the fluxes that bring about r.v.d. are conductive (see below), an increase in anion (most likely Cl^-) conductance must also occur during shrinking.

Cl^- fluxes have been directly measured in p.b.ls (Grinstein *et al.* 1982*b*). The efflux recorded in isotonic conditions is relatively high compared with that of K^+ ($^{86}Rb^+$). In view of the low anion conductance discussed above, the relatively high $^{36}Cl^-$ fluxes must be largely due to electroneutral anion exchanges similar to those that have been described extensively for red blood cells and that are present in other cell types as well (Cabantchik *et al.* 1978). On exposure of p.b.ls to hypotonic conditions the Cl^- efflux shows a dramatic increase (Grinstein *et al.* 1982*b*). This increase is assumed to be an essential part of the r.v.d. mechanism.

Are the volume-induced fluxes of K^+ and Cl^- coupled?

In other cell types, the volume-induced fluxes of K^+ have been reported to be coupled to those of other ions. Coupling may be in the form of co-transport with anions (Dunham & Ellory 1981; Thornhill *et al.* 1982; Kregenow & Caryk 1979) or as an obligatory counter-transport with cations (Cala 1980; Kregenow 1981). These coupled systems are generally thought to be electroneutral. In contrast, as mentioned above, conductance changes have been

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found to occur in Ehrlich ascites cells during r.v.d. (Hoffman 1978). In p.b.ls, two approaches were used to establish the relation between anion and cation movements during r.v.d.: (*a*) we determined whether the volume-induced fluxes of K^+ and Cl^- required the presence of the permeating counterion; (*b*) we investigated the changes in membrane potential associated with the fluxes observed during r.v.d. The dependence of K^+ ($^{86}Rb^+$) fluxes on the presence of Cl^-

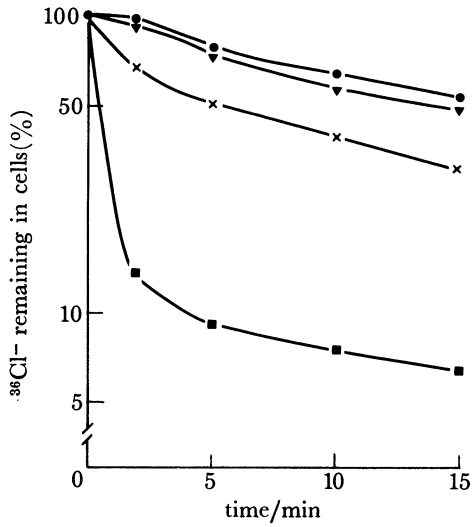

FIGURE 1. Effect of ionophores and hypotonic swelling on $^{36}Cl^-$ efflux from human p.b.ls. Cells were preloaded with the isotope, and the efflux measured by centrifugation as described (Grinstein *et al.* 1982*b*). The incubation media were as follows: ●, isotonic K^+-free PBS; ▼, isotonic K^+-free p.b.s. containing 0.5 μM gramicidin D; ×, isotonic p.b.s. containing 0.68 mM $CaCl_2$ and 5 μM A23187; ■, hypotonic (0.66 × isotonic) K^+-free p.b.s. with 0.5 μM gramicidin. Where present, gramicidin was added 10 min before the initiation of the efflux determinations.

was studied by replacement of Cl^- with SO_4^{2-}, a relatively impermeant anion that does not support secondary swelling in hypotonic high-K^+ media (Grinstein *et al.* 1982*a*). It was found that $^{86}Rb^+$ uptake was equally stimulated by hypotonicity in either Cl^- or SO_4^{2-} media, suggesting that volume-induced cation permeation occurs in the absence of Cl^- and of secondary volume changes.

In order to measure $^{36}Cl^-$ fluxes in the absence of K^+, cells were initially depleted of this cation by preincubation with gramicidin in Na^+-rich, K^+-free solution. Ion content analysis by flame photometry demonstrated that over 90% of the intracellular K^+ was lost after 10 min. Gramicidin-treated cells were loaded with $^{36}Cl^-$ and the efflux of the isotope was measured under isotonic and hypotonic conditions. As shown in figure 1, the antibiotic did not alter the rate of $^{36}Cl^-$ efflux in isotonic medium. A large flux increase was observed, however, when K^+-depleted cells were transferred to a hypotonic solution (figure 1). This stimulation was comparable in extent with that observed in normal, high-K^+ cells (Grinstein *et al.* 1982*b*). Once again, the data are consistent with non-interacting fluxes of Cl^- and K^+ during r.v.d.

When the volumes of gramicidin-pretreated cells were studied after hypotonic stress, a striking secondary *swelling* was found to follow the expected initial swelling. The cells reached over twice their normal size, and the phenomenon was present only when Cl^-, but not SO_4^{2-} or gluconate, were the extracellular anions. These findings can be explained as follows: after treatment with gramicidin in K^+-free isotonic solution, the membrane permeability to Na^+

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and K^+ is greatly increased, the potential has collapsed and the concentration of Na^+ across the membrane is near equilibrium. Because, as suggested by the transference numbers, anion conductance is low in isotonic conditions, an inwardly directed gradient of Cl^- persists long after Na^+ loading is completed. Upon hypotonic swelling, however, Cl^- conductance through the volume-dependent pathway is suddenly increased, allowing an inward Cl^- flux that drags

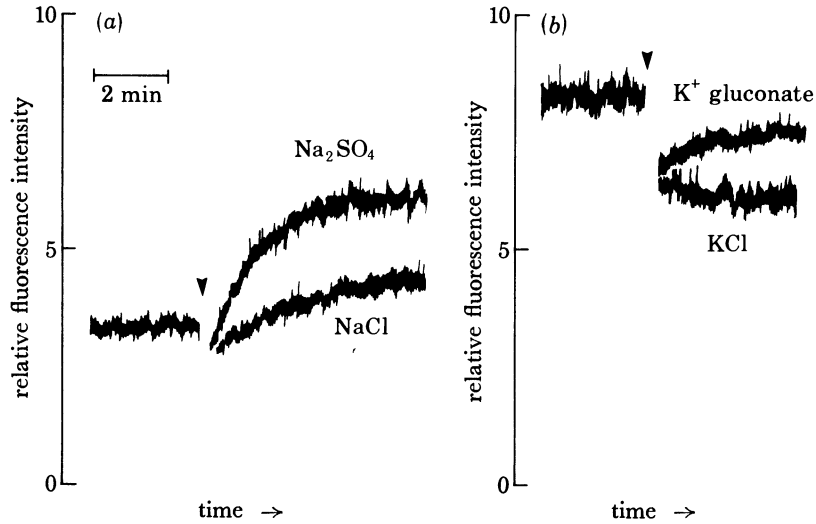

FIGURE 2. Effect of hypotonic dilution on the membrane potential of p.b.ls. Membrane potential was measured fluorimetrically by using 3,3′-dipropylthiadicarbocyanine (a gift from Dr A. Waggoner) as described elsewhere (Grinstein *et al.* 1982*b*).

(*a*) P.b.ls were suspended in phosphate-buffered solutions containing either NaCl or Na_2SO_4 as the principal salt, and 0.6 μM cyanine dye. After a period of equilibration (not illustrated in its entirety), the suspension was diluted to 0.66 × isotonic where indicated by the arrowhead and recording resumed. The fluorescence levels in isotonic Cl^- and SO_4^{2-}-solutions were indistinguishable. The rapid, step-like change in fluorescence immediately after hypotonic stress (arrowhead) is an artefact of dilution.

(*b*) P.b.ls were suspended in phosphate-buffered solutions containing 140 mM of either KCl or K^+ gluconate, and 0.6 μM cyanine dye. After equilibration, the suspension was diluted to 0.66 × isotonic where indicated (arrowhead), and recording was rapidly resumed. The fluorescence levels in isotonic Cl^- and gluconate solutions were similar. The same number of cells and recording magnification were used in (*a*) and (*b*), which are therefore comparable.

Na^+ and water into the cell, with the resulting secondary swelling. It must be stressed that Na^+ is not taken up by the volume-sensitive pathway, but it can enter the cell through the gramicidin channel, whereas Cl^- traverses the volume-regulatory pathway. This finding provides further evidence for the independent nature of the K^+ and Cl^- fluxes.

Membrane potential determinations during r.v.d. indicate that the fluxes are electrogenic. P.b.ls suspended in physiological (low-K^+) solutions consistently depolarized when the medium was made hypotonic (figure 2*a*). The depolarization was larger when the impermeant SO_4^{2-}, rather than Cl^-, was the predominant external anion. These results are consistent with an increase in Cl^- conductance. Measurements of intracellular Cl^- concentration indicated that this ion is not at electrochemical equilibrium ($E_{Cl^-} = -32$ mV, whereas in isotonic steady state $E_m = -53$ mV), so that a depolarization is predicted if Cl^- conductance increases. Furthermore, the depolarization ought to be more pronounced in SO_4^{2-} media, as observed. In high-KCl media the resting isotonic membrane potential was lower than in Na^+-rich media

(indicated by the more intense fluorescence of figure 2*b* than in figure 2*a*). The potential of these cells is lower than E_{Cl^-}, so that increasing Cl^- conductance should hyperpolarize the membrane. That this was so is shown in figure 2*b*. On the other hand, replacement of external Cl^- by a non-permeating anion reverses E_{Cl^-} to an internally positive value. Under these circumstance enhancing Cl^- conductance should further depolarize the cell. Figure 2*b* shows that in K^+ gluconate solution, hypotonic dilution leads to a potential change (depolarization) opposite to that seen in KCl. Thus, under a variety of circumstances, the potential change induced by hypotonic swelling seems to be dominated by an increase in Cl^- conductance.

Volume regulation in B and T lymphocyte populations

Further evidence for the independence of the volume-induced cation and anion permeation paths was obtained from studies of r.v.d. in lymphocytes of different lineage. Volume regulation in human tonsillar lymphocytes subjected to hypotonic stress was found to be extremely slow and incomplete when compared with p.b.ls (Cheung *et al.* 1982*b*). Tonsillar cell populations contain roughly 20–25% T cells and over 60% B cells, whereas p.b.ls are mostly (70% or more) T lymphocytes. Thus it was conceivable that the differential volume-regulatory behaviour was a reflection of the different composition of the cell populations. To analyse this possibility, populations of B and T cells from both tonsils and blood were separated by rosette depletion and gradient centrifugation. Further analysis showed that T cells from both sources were able to readjust their volume after hypotonic swelling at a rate comparable with that of unfractionated p.b.ls. In contrast, B cells from either blood or tonsil failed to demonstrate significant r.v.d. during an equivalent period. Studies of $^{86}Rb^+$ efflux and K^+ content closely paralleled cell volume determinations: T cells showed a volume-induced increase in $^{86}Rb^+$ efflux, and their K^+ content was reduced after r.v.d. B cells, in contrast, showed only a modest increase in flux and lost little K^+ (Cheung *et al.* 1982*b*). The differential behaviour of B and T cells provided another means of studying the relation between K^+ and anion fluxes in r.v.d. By using gramicidin as described above, it was possible to establish that B cells also respond to swelling with an increase in anion conductance, as described for p.b.ls, even though their K^+ response is impaired. Figure 3 illustrates the results of a typical experiment in which tonsillar B cells were used. No significant volume change was produced by the antibiotic in isotonic medium, but dilution brought about the two phases of volume change already described for p.b.ls: the initial rapid swelling, followed by a pronounced secondary swelling. The latter phase did not occur in solutions of large, impermeant anions such as gluconate, or in choline-based media. These findings indicate that swelling increases the anion conductance in B cells, even though K^+ permeation is not activated, and provide additional support for the notion that the cation and anion pathways are separate, non-interacting entities.

Role of Ca^{2+} in r.v.d.

In a number of cell types, increases in the level of cytoplasmic free Ca^{2+} concentration can lead to substantial and specific increases in K^+ permeability (see Lew & Ferreira (1978) for review). Considering that a K^+-selective permeability is activated upon swelling p.b.ls in hypotonic media, it was logical to assume that changes in cytoplasmic $[Ca^{2+}]$ might be involved in the genesis of r.v.d. We performed a series of experiments aimed at defining the role of this

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divalent cation in regulatory shrinking (Grinstein *et al.* 1982*a*). The rationale of these investigations, summarized below, was to establish a number of physiological and pharmacological parallels between the transport and volume changes induced by swelling or by artificially raising the intracellular Ca^{2+} concentration. The latter was accomplished by means of A23187, a Ca^{2+}-selective ionophore.

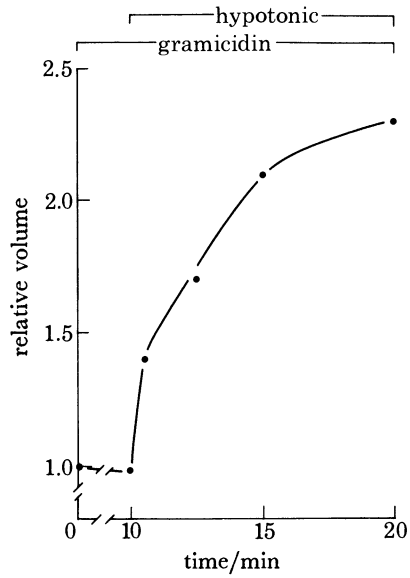

FIGURE 3. Hypotonically induced increase in anion conductance in human tonsillar B cells. The volume of B cells isolated by rosette depletion was measured electronically by using a Coulter-counter–channelyser combination. The cells were suspended in isotonic K^+-free p.b.s., and 0.5 μM gramicidin was added at the beginning of the experiment. Where indicated, the medium was diluted to 0.66 × isotonic by addition of two volumes of 50 % K^+-free p.b.s.

(*a*) *Can* Ca^{2+} *change* K^+ *permeability and cellular volume in p.b.ls?*

Rink *et al.* (1980) provided indirect evidence for the existence of a Ca^{2+}-activated K^+ conductance in lymphocytes. Direct measurements of $^{86}Rb^+$ efflux provided confirmatory evidence: addition of A23187 in the presence of external Ca^{2+} induced a rapid loss of the isotope (Szasz *et al.* 1981; Grinstein *et al.* 1982*a*), but the ionophore had little effect in Ca^{2+}-free solutions. In cells suspended in media with large outward or inward K^+ gradients, increasing K^+ permeability with A23187 plus Ca^{2+} led to measurable volume changes. The direction of these changes resembled the secondary responses elicited in hypotonic media, i.e. swelling in K^+-rich and shrinking K^+-free solutions. However, the magnitude of the volume alterations was different, suggesting that the effects are only partly comparable.

(*b*) *Pharmacological similarities between volume-induced and* Ca^{2+}*-induced fluxes*

Ca^{2+}-induced K^+ fluxes in various cell types, including erythrocytes, are blocked by extracellular addition of quinine or quinidine (Armando-Hardy *et al.* 1975). In p.b.ls, the efflux of $^{86}Rb^+$ promoted by A23187 plus Ca^{2+} was largely blocked by 75 μM quinine. To explore further the relation between Ca^{2+} and r.v.d., the effects of quinine on volume regulation were also assessed. Quinine was found to produce a dose-dependent inhibition of r.v.d., with complete

block at 75 μM. As expected, the loss of intracellular K^+ and the increased $^{86}Rb^+$ efflux were both abolished by the inhibitor.

Another group of drugs reported to inhibit Ca^{2+}-dependent K^+fluxes are the phenothiazines. Although conflicting results have been reported (compare Plishker *et al.* (1980) with Hoffman *et al.* (1980) and Lackington & Orrego (1981)), at least in some cases the phenothiazines have been shown to block Ca^{2+}-induced K^+ channels, and their inhibitory potency correlated with their affinity for calmodulin (Lackington & Orrego 1981). We compared the effects of trifluoperazine, the most widely studied calmodulin antagonist, on Ca^{2+}-induced and hypotonically induced volume changes. Micromolar concentrations of the phenothiazine blocked r.v.d., as well as the associated $^{86}Rb^+$ fluxes and K^+ loss. The drug also prevented ionophore plus Ca^{2+}-promoted swelling in K^+-rich media and shrinking K^+-free media. The phenothiazine concentrations needed for inhibition in both cases were alike, adding additional, albeit circumstantial, evidence that r.v.d. is a Ca^{2+}-dependent phenomenon.

(*c*) *Does* Ca^{2+} *modulate anion permeability?*

As pointed out above, increases in K^+ permeability are a necessary but not sufficient condition to produce volume changes. Indeed, in cells suspended in K^+-rich medium, addition of gramicidin enhances K^+ permeability substantially more than A23187 plus Ca^{2+}, but fails to produce a comparable volume gain. These findings imply that elevated intracellular Ca^{2+} levels must also enhance anion permeability, enabling the cell to change its volume. Two types of experiments were carried out to test this prediction. In the first, the efflux of $^{36}Cl^-$ from p.b.ls was measured in the presence and absence of A23187 and Ca^{2+}. As shown in figure 1, a significant increase in efflux rate was induced by Ca^{2+} plus ionophore. The ionophore had no effect in Ca^{2+}-free solutions (not illustrated). The second approach involved, once again, the use of gramicidin to provide a high cation permeability. The purpose of the experiments was to find out whether in K^+-free cells with equal Na^+ concentrations across the membrane, a volume increase could be induced by raising internal [Ca^{2+}]. Such a volume change ought to be driven by Cl^-, which is the only major ion that is not at electrochemical equilibrium. The result of one such experiment is illustrated in figure 4. As noted, addition of gramicidin to p.b.ls in K^+-free solution did not significantly modify their size. Subsequent inclusion of A23187 in the medium produced a small but reproducible swelling that was only observed in Ca^{2+}-containing media. The results described in this and the previous paragraph are compatible with an increase in anion permeation brought about by an increased intracellular [Ca^{2+}] resulting from exposure of the cells to Ca^{2+} plus A23187.

Concluding remarks

It has been shown that K^+ permeability is increased during r.v.d. This is undoubtedly an essential part of the volume-regulatory response as evidenced by the deficient regulatory behaviour of B-type lymphocytes. These cells display a normal enhancement of anion conductance, but nevertheless fail to regulate volume normally since K^+ permeability is not sufficiently increased. In agreement with Hoffman's findings (Hoffman 1978, and this symposium), p.b.ls were also found to increase their permeability to Cl^- when subjected to hypotonic stress. Because anion conductance limits salt fluxes in resting cells, this increase is critical for the achievement of regulatory shrinking.

The fluxes of K^+ and Cl^- during r.v.d. seem to be only electrically coupled. The permeability

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changes appear to be conductive and the membrane potential during r.v.d. becomes dominated by P_{Cl-} under a variety of circumstances. No evidence was found for a direct coupling between anion and cation fluxes: both occurred independently in the virtual absence of transported counterions. Moreover, cation efflux stimulation was deficient in B-cells, whereas the stimulation of anion transport was 'normal', i.e. indistinguishable in B and T cells. This finding indicates that volume-dependent K^+ and Cl^- fluxes are mediated by independent molecular entities.

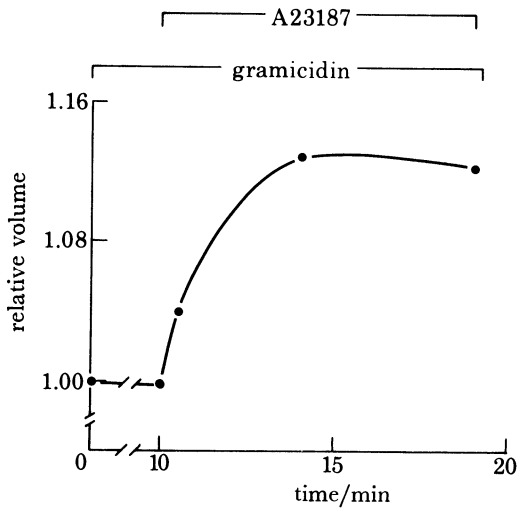

FIGURE 4. A23187 plus Ca^{2+}-induced swelling of K^+-depleted p.b.ls. The volume of p.b.ls suspended in K^+-free p.b.ls. was measured in the Coulter counter. The experiment was started by the addition of 0.5 μM gramicidin. After 10 min, A23187 (5 μM) was added and recording continued. The medium was isotonic throughout the experiment.

Physiological and pharmacological criteria suggest that Ca^{2+} plays a role in the modulation of ion permeability during hypotonic volume regulation. The source of the Ca^{2+} required for r.v.d. is probably intracellular, since regulation occurs normally in Ca^{2+}-free, EGTA-containing media, but is impaired after prolonged incubations in these conditions. Finally, calmodulin can be assigned a tentative role in r.v.d., based on the inhibitory effects of trifluoperazine and other calmodulin-blocking phenothiazines.

Tentatively, the regulatory response after exposure to hypotonic media involves the following events: osmotic swelling triggers a release of Ca^{2+} from an intracellular source; the increase in free cytoplasmic [Ca^{2+}] activates independent K^+ and Cl^- 'channels'; K^+, Cl^- and osmotically obliged water move in the direction of the combined K^+ plus Cl^- electrochemical gradient (normally outward); as the cell volume approaches normal values, cytoplasmic Ca^{2+} is sequestered and returns to normal levels, reverting the K^+ and Cl^- 'channels' to their inactive state.

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