

Volume Regulation by Human Lymphocytes

Role of Calcium

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ABSTRACT Human peripheral blood lymphocytes regulate their volumes in hypotonic solutions. In hypotonic media in which Na^+ is the predominant cation, an initial swelling phase is followed by a regulatory volume decrease (RVD) associated with a net loss of cellular K^+ . In media in which K^+ is the predominant cation, the rapid initial swelling is followed by a slower second swelling phase. $^{86}\text{Rb}^+$ fluxes increased during RVD and returned to normal when the original volume was approximately regained. Effects similar to those induced by hypotonic stress could also be produced by raising the intracellular Ca^{++} level. In isotonic, Ca^{++} -containing media cells were found to shrink upon addition of the Ca^{++} ionophore A23187 in K^+ -free media, but to swell in K^+ -rich media. Exposure to Ca^{++} plus A23187 also increased $^{86}\text{Rb}^+$ fluxes. Quinine (75 μM), an inhibitor of the Ca^{++} -activated K^+ pathway in other systems, blocked RVD, the associated K^+ loss, and the increase in $^{86}\text{Rb}^+$ efflux. Quinine also inhibited the volume changes and the increased ^{86}Rb fluxes induced by Ca^{++} plus ionophore. The calmodulin inhibitors trifluoperazine, pimozide, and chlorpromazine blocked RVD as well as Ca^{++} plus A23187-induced volume changes. Trifluoperazine also prevented the increase in $^{86}\text{Rb}^+$ fluxes and K^+ loss induced by hypotonicity. Chlorpromazine sulfoxide, a relatively ineffective calmodulin antagonist, was considerably less potent as an inhibitor of RVD than chlorpromazine. It is suggested that an elevation in cytoplasmic $[\text{Ca}^{++}]$, triggered by cell swelling, increases the plasma membrane permeability to K^+ , the ensuing increased efflux of K^+ , associated anions, and osmotically obliged water, leading to cell shrinking (RVD).

INTRODUCTION

When suspended in hypertonic solutions, most mammalian cells behave as osmometers (Roti-Roti and Rothstein, 1973; Hempling et al., 1978; Pollock and Arieff, 1980). The Boyle-Van't Hoff relation holds within a considerable range of osmolarities, with deviations being observed only after long periods

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of time in some cell types (Poznansky and Solomon, 1972; Pollock and Arieff, 1980). In contrast, cells exposed to hypotonic solutions generally regulate their volume, showing marked departures from the ideal osmometric behavior (see MacKnight and Leaf, 1977, for review). As expected, cells initially swell in response to the hypotonic environment, but the swelling is followed by a shrinking phase, sometimes called regulatory volume decrease (RVD), which returns cell volume to near-isotonic values. The duration of the RVD varies considerably among tissues, from several hours in the case of brain (Pollock and Arieff, 1980) and red cells (Poznansky and Solomon, 1972), to a few minutes in mouse lymphoblasts (Roti-Roti and Rothstein, 1973; Buckhold-Shank and Smith, 1976) and Ehrlich ascites tumor cells (Hendil and Hoffman, 1974). Among the fastest responding cells are human peripheral blood lymphocytes. Shrinking in these cells is complete in ~5 min (Ben-Sasson et al., 1975) and the final volume attained is essentially identical to the isotonic one. This makes human lymphocytes a convenient system for the study of the phenomena that underlie this form of volume regulation.

The ionic basis of hypotonic volume regulation has been studied in several systems and is best understood in erythrocytes (Kregenow, 1971; Cala, 1980 and references therein) and lymphoid cells (Roti-Roti and Rothstein, 1973). RVD has been found to be associated with a decrease in the K^+ content of the cells that is dependent on the existence of an outwardly directed K^+ electrochemical gradient and which is brought about by a specific, volume-induced increase in the membrane permeability to this cation.

In recent years, it has become apparent that in a number of cell types, increases in the level of cytoplasmic Ca^{++} can lead to substantial and specific increases in K^+ permeability (see Lew and Ferreira, 1978, for review). It is tempting to assume that changes in cytoplasmic Ca^{++} levels might also be involved in the regulation of the volume-induced K^+ pathway. Ca^{++} could enter the cytoplasmic compartment from the medium or be released from intracellular stores during swelling, thus producing the observed changes in K^+ permeability and cellular volume. In the present communication, a role for Ca^{++} in volume regulation was established by using physiological as well as pharmacological criteria. The cytoplasmic concentration of Ca^{++} was modified with the ionophore A23187, and the resulting effects on K^+ (or Rb^+) permeability and cellular volume were compared with those induced by hypotonicity. The effects of drugs known to alter Ca^{++} -induced K^+ fluxes on the RVD and on ionophore-induced changes were also compared. Moreover, the possible involvement of calmodulin as a mediator of Ca^{++} action was analyzed using several phenothiazines, a family of antipsychotic agents known to be powerful calmodulin antagonists.

MATERIALS AND METHODS

A23187 was obtained from Calbiochem-Behring Corp., San Diego, CA; trifluoperazine hydrochloride and chlorpromazine sulfoxide were a gift of the Medical Department of Smith, Kline and French, Canada Ltd., Montreal; pimozide was a gift from McNeil Laboratories, Montreal; chlorpromazine hydrochloride and quinine were from Sigma Chemical Co., St. Louis, MO; trypan blue was from Grand Island

Biological Co., Grand Island, NY; $^{86}\text{Rb}^+$ and $^{45}\text{Ca}^{++}$ were from Amersham Corp., Arlington Heights, IL; $^3\text{H}_2\text{O}$ and [^{14}C]-polyethylene glycol (4,000 mol wt) and Aquasol II were from New England Nuclear, Boston, MA; RPMI 1640 medium and fetal bovine serum were from Grand Island Biological Co.

Cell Preparation

Peripheral blood mononuclear cells (PBM) were isolated on a Ficoll-Isopaque gradient (Dosch et al., 1979). Fresh heparinized human blood was diluted with an equal volume of RPMI 1640 medium and 10 ml of this mixture was carefully layered onto 3 ml of Ficoll-Isopaque in sterile plastic tubes. After centrifugation at 350 *g* for 30 min, the PBM were collected from the interface with a Pasteur pipette, washed twice with RPMI 1640, and finally resuspended in the same medium containing 10% fetal bovine serum at a concentration of $\sim 10^7$ cells/ml. The mononuclear cell population obtained by this method contained 15–20% monocytes and a small number of platelets. Purified peripheral blood lymphocytes from which monocytes and platelets had been essentially completely removed (as described below) were used for some experiments and identical results were obtained.

Where indicated, the cells were spun down and resuspended in one of the following media: phosphate-buffered saline (PBS: 137 mM NaCl; 2.7 mM KCl; 1.2 mM KH_2PO_4 ; 8.1 mM Na_2HPO_4 ; 10 mM glucose; 0.68 mM CaCl_2 ; 0.49 mM MgCl_2); K^+ -free PBS (similar to PBS but with NaCl substituted for KCl and NaH_2PO_4 for KH_2PO_4); and K^+ -rich PBS (KCl substituted for NaCl). The pH in all cases was adjusted to 7.2. All experiments were performed at room temperature.

Cell Volume and Viability Determinations

Cells were sized using a Coulter counter (model ZBI; Coulter Electronics, Hialeah, FL) adapted with a Coulter Channelyzer. Calibration was based on the use of latex beads of known diameter in solutions of appropriate salt concentrations. Cell volumes were calculated from the median channel of the distributions generated by the Channelyzer. Cell viability was measured by dye exclusion using trypan blue. Unless otherwise indicated, the viability was $>90\%$ and was not significantly affected by the hypotonic challenge or by the chemicals studied.

Determination of Cell Water Content

Cellular water content was determined by isotope dilution using [^{14}C]-polyethylene glycol as an extracellular marker. PBM (10^7 cells/ml) were suspended in a medium containing 10 $\mu\text{Ci/ml}$ of $^3\text{H}_2\text{O}$ and 1 $\mu\text{Ci/ml}$ of [^{14}C]-polyethylene glycol. Aliquots of this suspension were diluted at zero time with either 2 vol of PBS or 2 vol of 50% diluted PBS. At appropriate time intervals, duplicate aliquots of the suspension were removed and layered over 0.4 ml of an oil mixture (3 parts corn oil and 10 parts dibutyl phthalate), and centrifuged in an Eppendorf microfuge (Brinkmann Instruments, Westbury, NY), to which a variable transformer was attached. Centrifugation was for 30 s at 50 V followed by 1 min at 100 V. Aliquots of the supernatant were saved for counting. The rest of the supernatant and most of the oil were discarded and the tip of the tube containing the cell pellet covered by a thin layer of oil was cut off and transferred to a scintillation vial. The pellet was dissolved in 1 ml of 1% sodium dodecyl sulfate before addition of 10 ml of Aquasol II and counting. Cell water space was determined by subtracting the calculated trapped extracellular space (based on [^{14}C]-polyethylene glycol content) from the total pellet water content.

Validity of Volume Measurements by Electronic Sizing

Electronic sizing using a Coulter counter provides a rapid and convenient method for cell volume determinations. However, changes in cell shape or rigidity can be misinterpreted as volume changes, and the system is also sensitive to alterations in ionic strength of the medium in which the cells are suspended. Therefore, it is necessary to ascertain that, under the conditions used in our experiments, actual volume changes were being measured. For this purpose, cellular volume, as determined by the Coulter apparatus, and cellular water, as determined by isotopic dilution (as above), have been compared.¹ Both volume and water content determinations displayed qualitatively similar time patterns after hypotonic challenge (0.67 × isotonic). As expected, the relative change in water content exceeded the relative increase in total cell volume. This is because a fraction of the cellular space does not respond to changes in medium osmolarity. The observed difference (~30%) is consistent with the known fraction (32%) of osmotically inactive volume in human lymphocytes (Hempling et al., 1978).

During hypotonic challenge, the ionic strength of the buffer was decreased. However, a 33% reduction in ionic strength such as the one imposed during hypotonic shock did not by itself alter volume determinations made with the Coulter counter. This was determined by diluting the normal incubation medium with isotonic sucrose, in which case no volume change was perceptible.

Rubidium Efflux Measurements

PBM were loaded overnight with 10 $\mu\text{Ci/ml}$ of $^{86}\text{Rb}^+$ in RPMI 1640 with 10% serum at a concentration of 10^7 cells/ml. The cells were then diluted 10-fold with nonradioactive RPMI 1640 and sedimented at 350 g for 5 min. The PBM were resuspended in PBS, at a concentration of $\sim 10^7$ cells/ml. At time zero, aliquots of this suspension were diluted with either 2 vol of medium or 2 vol of 50% diluted PBS. For some experiments, quinine or trifluoperazine was added to the diluting medium. At appropriate time intervals, duplicate aliquots of the cell suspension were removed and processed as described above for water determination.

Calcium Efflux Studies

PBM were loaded with 5 $\mu\text{Ci/ml}$ of ^{45}Ca for 3 h in RPMI 1640 supplemented with 10% serum at a concentration of 2×10^7 cells/ml. The cells were washed, resuspended, and assayed for ^{45}Ca content as described above for $^{86}\text{Rb}^+$.

Intracellular K^+ Content

Cellular K^+ content was determined by flame photometry (model 443 Photometer; Instrumentation Laboratory, Inc., Lexington, MA), using Li^+ as an internal standard. Aliquots of a cell suspension in PBS were diluted at zero time with either PBS or with 2 vol of 50% diluted PBS. In some experiments, quinine or trifluoperazine was added to the medium to a final concentration of 75 or 10 μM , respectively. At appropriate intervals, aliquots containing $4\text{--}5 \times 10^6$ cells were removed in duplicate and layered onto 0.4 ml of the oil mixture. After centrifugation, the supernatant and oil were removed and the pellet was lysed in 1 ml of Li^+ standard diluent (15 meq/liter of LiNO_3). Potassium concentration of the lysate was determined by comparison with a series of standard solutions (Flame Photometer Standards; Instrumentation Labora-

¹ These observations, made by A. DuPre and P. Cheung in our laboratory, will be published elsewhere in detail in connection with related studies on volume regulation in lymphocytes.

tory, Inc.). No correction was made for extracellularly trapped K^+ because, as determined using $[^{14}C]$ -polyethylene glycol and 3H_2O , the extracellular space was <16% of the volume of the pellet in control cells and even smaller in swollen cells.

RESULTS

Role of K^+ in RVD

The bottom trace of Fig. 1 shows the time course of the volume change (measured by the Coulter counter) undergone by cells suspended in hypotonic media in which Na^+ is the predominant cation. Briefly, the cells swell rapidly,

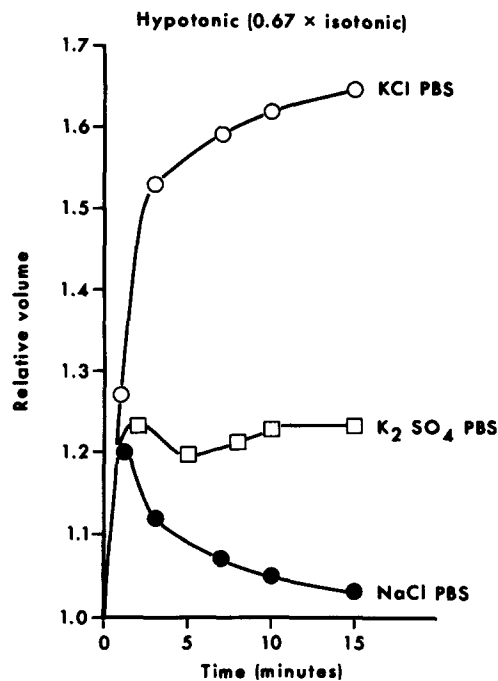


FIGURE 1. Effect of medium composition on RVD. PBM were suspended in isotonic phosphate-buffered media containing 137 mM NaCl (●), 137 mM KCl (○), or 92 mM K_2SO_4 (□). At zero time, cells were subjected to a hypotonic challenge ($0.67 \times$ isotonic), and their volume was measured by use of the Coulter counter. Data are representative of six such experiments.

reaching a peak volume before 1 min, and shrink thereafter. The extent and velocity of the RVD may vary among donors, but shrinking is usually 50% complete before 5 min and the cells regain near-normal volume within 10–15 min. As has been observed by Ben-Sasson et al. (1975), PBM shocked hypototically in high- K^+ media do not show an RVD but rather continue to swell rapidly (Fig. 1). This additional swelling is prevented if the more impermeant SO_4^- is substituted for Cl^- (middle trace in Fig. 1). In this case, only the initial, rapid swelling phase is noted, and the volume attained is preserved for at least 20 min.

These results suggest that, as has been proposed for other cell types (Kregenow, 1971; Roti-Roti and Rothstein, 1973), a loss of cellular K^+ down its electrochemical gradient is the mechanism underlying RVD. Indeed, when the K^+ content was determined in cells before and after hypotonic challenge in normal media (Table I), a loss was found to occur in parallel with shrinking. After 1 min the loss was 10% and after 10 min, 22%. In the controls, in contrast, the loss after 10 min was only 2%. Moreover, as shown in Fig. 2A, the permeability of the membrane to $^{86}Rb^+$, a K^+ analogue in many membrane systems (that is also transported by the volume-induced system),¹ was found to increase transiently as a result of swelling. A rapid loss of $^{86}Rb^+$ follows dilution of the cell suspension with >25% of the isotope released within 15 min, a value comparable with the loss of K^+ reported in Table I. Less than 6% of $^{86}Rb^+$ was lost from cells in isotonic media during the same period. The time course of the Rb^+ loss was essentially parallel to the loss of volume during RVD (Fig. 1).

TABLE I
EFFECT OF HYPOTONIC CHALLENGE ON THE RELATIVE K^+
CONTENT OF PBM

	1 min	10 min
Isotonic	1.00	0.98±0.01
Hypotonic	0.90±0.02	0.78±0.02
Hypotonic, 75 μ M quinine	0.96±0.01	0.91±0.02
Hypotonic, 10 μ M trifluoperazine	0.96±0.01	0.93±0.02

The data are the mean \pm SEM of four to seven determinations, each performed in duplicate. The K^+ content of control cells in isotonic medium is taken as unity. The column headings indicate the time elapsed after dilution.

Although the studies described above were performed upon heterogeneous cell suspensions that were 80–85% lymphocytes, similar studies upon purified lymphocyte preparations yielded identical results.

Effect of A23187 and Ca^{++} on ^{86}Rb Efflux and Cell Volume

Addition of A23187 (2–5 μ M) to PBM preloaded with $^{86}Rb^+$ induces a rapid efflux of the isotope if Ca^{++} is present in the medium. As shown in Fig. 2B, ~50% of the $^{86}Rb^+$ is lost in 10 min, compared with <5% in control cells (Fig. 2A, top line). The effect of the ionophore is substantially less in Ca^{++} -free media containing 1 mM EGTA, but the efflux is still higher than in control PBM.

On the basis of the finding that Ca^{++} plus ionophore leads to an enhanced Rb^+ (or K^+) permeability, it can be predicted that the cells should swell or shrink upon addition of Ca^{++} plus A23187, depending on the direction of the K^+ gradient. That such predictions are fulfilled is illustrated in Fig. 3. Cells were suspended in isotonic K^+ -free or K^+ -rich PBS, with or without Ca^{++} . No

volume changes were detected in the absence of ionophore or of extracellular Ca^{++} under any of the above conditions within the time frame illustrated. Addition of A23187 in the presence of Ca^{++} , however, caused the cells to shrink $\sim 15\%$ when suspended in K^+ -free PBS, and to swell $\sim 10\%$ in high- K^+ medium.

The swelling in K^+ -rich media was heterogeneous. The dispersion of individual cell volumes around the mean was expanded in both directions, and in some experiments a sharp decrease in viability was observed after some time. This was never the case for cells in K^+ -free media, in which the dispersion on either side of the mean volume was reduced upon shrinking.

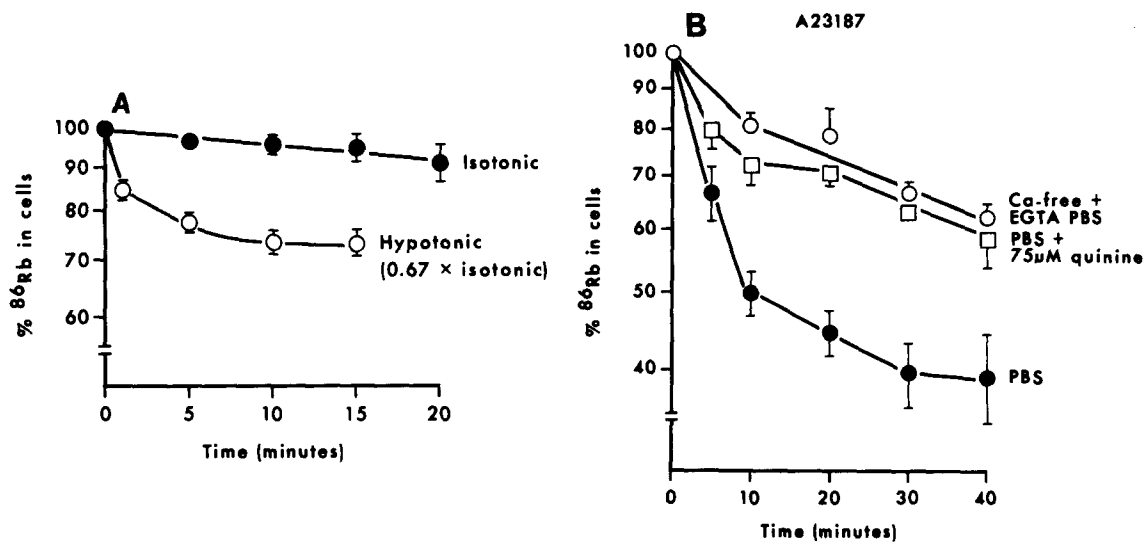


FIGURE 2. A. Effect of hypotonic challenge on $^{86}\text{Rb}^+$ efflux. PBM were loaded overnight with $^{86}\text{Rb}^+$ in RPMI 1640 supplemented with 10% fetal calf serum. The extracellular isotope was removed and the cells were suspended in nonradioactive medium. This suspension was then diluted with either two volumes of PBS (●) or of 50% diluted PBS (○). The loss of $^{86}\text{Rb}^+$ was monitored as a function of time. Ordinate: percent $^{86}\text{Rb}^+$ remaining in cells, log scale. Data are the mean \pm SEM of eight determinations. B. Effects of A23187, Ca^{++} , and quinine on $^{86}\text{Rb}^+$ efflux from PBM. Cells loaded overnight with $^{86}\text{Rb}^+$ were washed and suspended in either isotonic PBS containing 0.67 mM CaCl_2 (○), Ca^{++} -free PBS containing 2 mM EGTA (●), or PBS containing 0.67 mM CaCl_2 plus 75 μM quinine (□). A23187 (5 μM) was added to all samples at zero time. Data are the mean \pm SEM of 6–16 determinations.

Effect of Quinine on Volume Regulation and Cation Fluxes

If the increased Rb^+ (K^+) permeability and volume changes induced by Ca^{++} plus ionophore are relevant to those induced by hypotonic stress, then the RVD can be presumed to be a Ca^{++} -mediated process. Ca^{++} -induced K^+ fluxes in erythrocytes and other cells are inhibited by extracellular addition of quinine or quinidine (Armando-Hardy et al., 1975). Therefore, to further

explore the possible relationship between Ca^{++} and RVD, the effects of quinine on volume regulation were assessed. PBM were hypotonically challenged in media containing increasing concentrations of quinine (Fig. 4A). The swelling phase was not affected, but quinine produced a dose-dependent inhibition of RVD, with complete block at $75 \mu\text{M}$, a concentration that had no effect on cell viability as determined by trypan blue dye exclusion. As expected, quinine also blocked most of the loss of K^+ normally observed during RVD (Table I). The small loss of K^+ observed in the presence of quinine is probably due to marginal cell breakage that occurs while sedimenting the more fragile swollen cells.

The effect of quinine on $^{86}\text{Rb}^+$ efflux was also determined; the results are shown in Fig. 5A. Quinine did not significantly alter the control rate of $^{86}\text{Rb}^+$

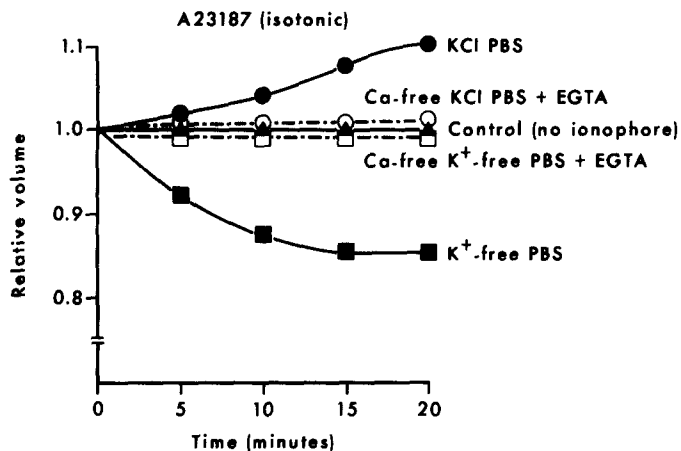


FIGURE 3. Effects of A23187, Ca^{++} , and K^+ on cell volume. PBM were suspended in Ca^{++} -containing (0.67 mM CaCl_2) or Ca^{++} -free (with 2 mM EGTA) isotonic media, which were either K^+ -rich (137 mM KCl) or K^+ -free. A23187 ($5 \mu\text{M}$) was added at zero time and volume was measured using the Coulter counter. Data are representative of three such experiments.

efflux, which supports the notion that no gross impairment of cellular function occurred. However, quinine largely prevented the increase in $^{86}\text{Rb}^+$ efflux induced by the hypotonic medium (compare with lower curve of Fig. 2A). After 15 min in hypotonic buffer, the loss of $^{86}\text{Rb}^+$ was reduced from 25% to <10%.

To extend the comparison between the volume- and Ca^{++} -induced K^+ permeability pathways, the effect of quinine on volume and isotope flux changes promoted by A23187 plus Ca^{++} were also determined. Experiments like those described above were carried out in the presence of concentrations of quinine that produce full inhibition of RVD (i.e., $75 \mu\text{M}$). Fig. 5B shows the effect of quinine on A23187 plus Ca^{++} -induced efflux of $^{86}\text{Rb}^+$. The drug was a potent inhibitor, eliminating much of the increase in flux induced by

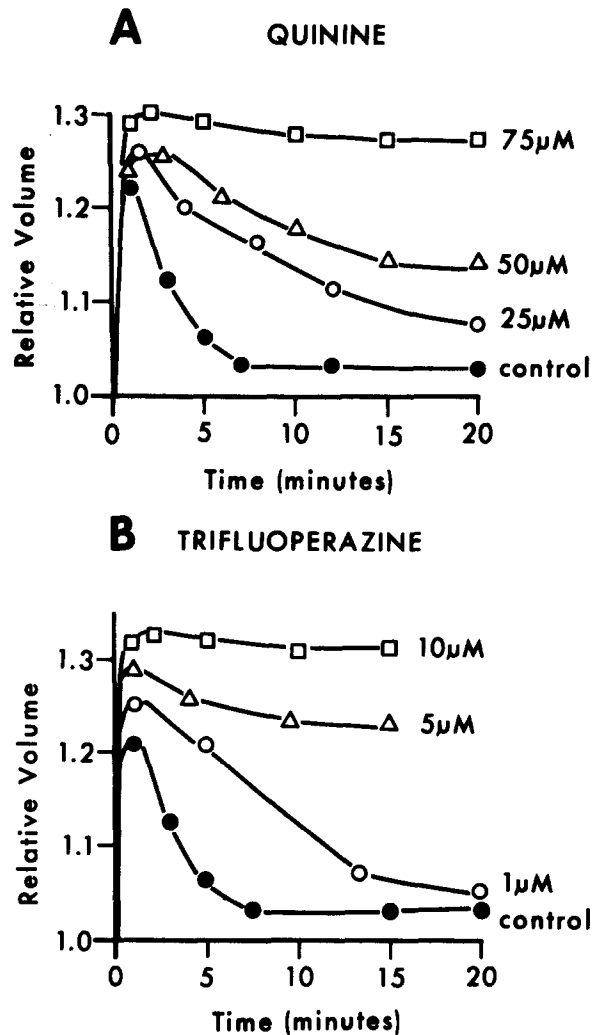


FIGURE 4. A. Effect of quinine on RVD. Cells were hypotonically challenged ($0.67 \times$ isotonic) in media containing quinine and their volume was recorded with the Coulter counter. Quinine hydrochloride, at the concentrations indicated, was added simultaneously with the diluting medium at zero time. Data are representative of four such experiments. B. Effect of trifluoperazine on RVD. Trifluoperazine was added at zero time, when the hypotonic challenge was initiated. Data are representative of three such experiments. Other details as in A.

the ionophore. Although it might be entirely coincidental, it is worth noting that the flux remaining in the presence of quinine is almost identical to that observed when the ionophore is added in the absence of extracellular Ca^{++} .

Volume changes induced by A23187 plus Ca^{++} were also affected by quinine. Cells in K^+ -free PBS, which normally shrink upon addition of the

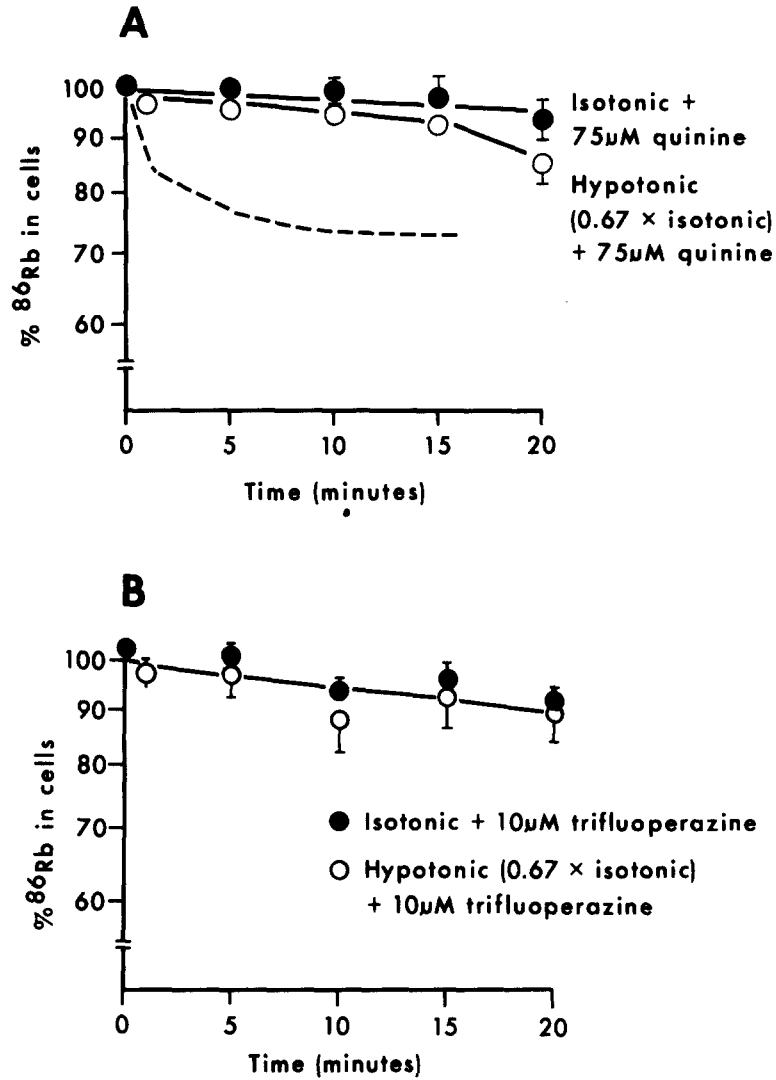


FIGURE 5. A. Effect of quinine on $^{86}\text{Rb}^+$ efflux during RVD. PBM loaded overnight with $^{86}\text{Rb}^+$ were washed and resuspended in nonradioactive medium. They were then diluted at zero time in either two volumes of PBS (●) or of 50% diluted PBS (○) containing quinine at a final concentration of 75 μM . Data are the mean \pm SE of six determinations. The dotted line represents the pattern normally observed during hypotonic challenge of PBM in the absence of drugs. It is redrawn from Fig. 2A and was included for comparison. B. Effect of trifluoperazine on $^{86}\text{Rb}^+$ efflux during RVD. PBM loaded overnight with $^{86}\text{Rb}^+$ were washed and resuspended in nonradioactive medium. They were then diluted at zero time in either two volumes of PBS (●) or of 50% diluted PBS (○) containing trifluoperazine at a final concentration of 10 μM . Data are the mean \pm SE of six determinations. Other details as in Fig. 2A.

ionophore (Fig. 3), were tested in the presence of 75 μM quinine. The ionophore-induced shrinking was reduced by $\sim 50\%$ (not illustrated). Surprisingly, however, swelling in K^+ -rich PBS was not significantly prevented by quinine.

Effect of Phenothiazines and Possible Role of Calmodulin

It has recently become clear that a number of enzyme-modulating activities previously thought to be performed by Ca^{++} itself are actually mediated by the Ca^{++} -binding protein calmodulin (Means and Dedman, 1980). An example in the field of transport is "goblin," a membrane protein of avian erythrocytes believed to control Na^+ - K^+ co-transport when phosphorylated and which can also be phosphorylated in vitro by addition of Ca^{++} and calmodulin (Alper et al., 1980). Calmodulin is known to be inactivated by a variety of antipsychotic drugs, particularly the phenothiazines (Weiss and Levin, 1978), which bind to a hydrophobic site on the Ca^{++} -containing form of the protein. The phenothiazines are currently being used to establish the participation of calmodulin in a variety of biological phenomena, including microtubule depolymerization (Schliwa et al., 1981), secretion (Krausz et al., 1980), cell aggregation (White and Raynor, 1980), and capping (Bourgignon and Balazovich, 1980). The possible role of calmodulin (or a similar activator) in RVD was explored using several phenothiazines of known affinities for calmodulin. The most widely studied phenothiazine, trifluoperazine, was studied in the greatest detail. Fig. 4B shows the effects of trifluoperazine on RVD. The initial swelling phase was not affected. However, micromolar concentrations suffice for complete inhibition of the shrinking phase and also for a substantial inhibition of the secondary swelling that occurs in high- K^+ media (Fig. 6A). The concentrations required for full inhibition of RVD did not affect cell viability. In other cell types, much higher concentrations are required before viability is altered (Osborn and Weber, 1980).

Trifluoperazine appears to inhibit RVD by blocking the increase in membrane K^+ permeability associated with hypotonic stress. Thus trifluoperazine largely prevented net K^+ loss (Table I) and the increased $^{86}\text{Rb}^+$ efflux normally seen in hypotonically treated cells, but did not significantly change the $^{86}\text{Rb}^+$ efflux from PBM suspended in isotonic solution (Fig. 5B).

The parallels between effects mediated by hypotonicity and by ionophore plus Ca^{++} also extend to the action of the phenothiazines. Fig. 6B shows that when trifluoperazine is present, no swelling was observed when ionophore plus Ca^{++} were added to cells in K^+ -rich PBS. Similarly, shrinking in K^+ -free PBS was also prevented.

Phenothiazines are amphiphilic molecules that can potentially interact with the hydrophobic matrix of membranes. This could result in a variety of "nonspecific" (calmodulin-unrelated) effects and, in fact, rather gross changes such as cellular lysis can be obtained if sufficiently high concentrations are used (see Seeman, 1972, for review). It is thus important to establish whether the observed effects of trifluoperazine are indeed related to inactivation of

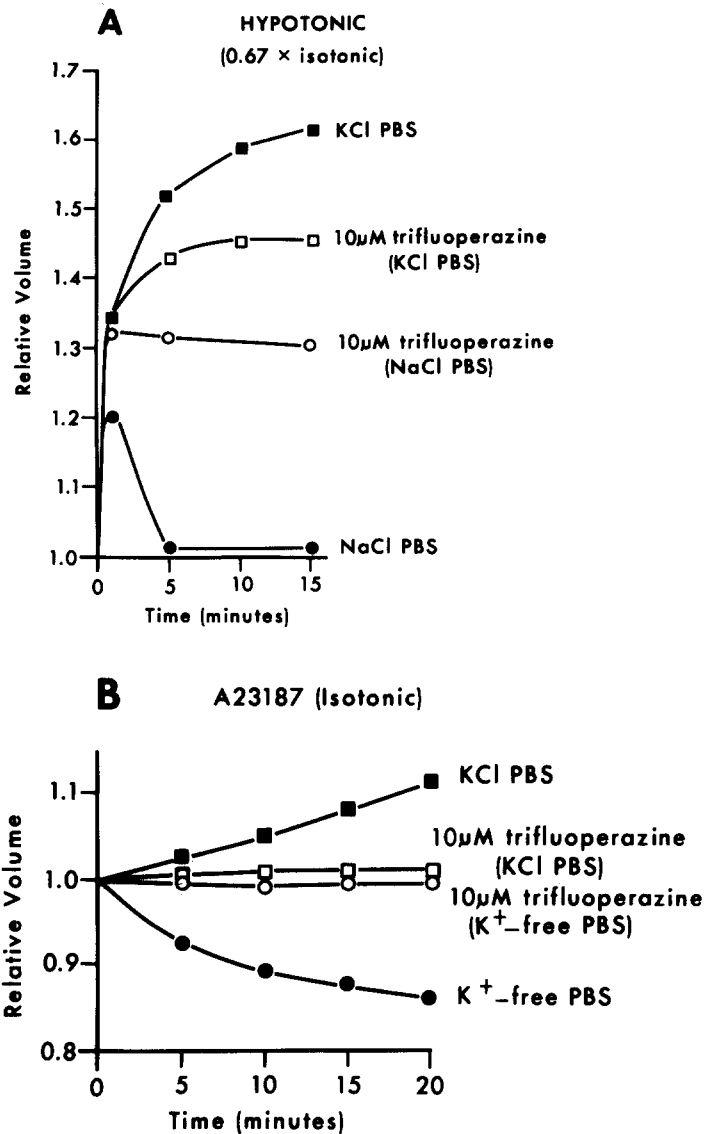


FIGURE 6. A. Effect of trifluoperazine and medium composition on volume changes induced by hypotonic challenge. PBM were suspended in isotonic phosphate buffered media containing 137 mM NaCl (circles) or 137 mM KCl (squares). At zero time, the suspension was diluted with 1 vol of the appropriate isotonic buffer and 1 vol of water. Where indicated, trifluoperazine (10 μ M) was added with the dilution medium (empty symbols). Data are representative of three experiments. B. Effect of trifluoperazine on changes in volume induced by A23187 plus Ca^{++} . At zero time, A23187 (5 μ M) was added to isotonic cell suspensions with (137 mM KCl) or without KCl. Trifluoperazine (10 μ M) was added at zero time. Data are representative of three experiments.

calmodulin. One approach involves the comparison of the inhibitory potency of the drug with its reported affinity for calmodulin. The case can be further strengthened by using not one but several analogues with widely different affinities. We compared the inhibitory effects of trifluoperazine, pimozide, chlorpromazine, and chlorpromazine sulfoxide on RVD with their reported binding constants to calmodulin. Volume vs. time curves like those of Fig. 4B were obtained for the different inhibitors, and dose-inhibition plots were constructed. The concentrations required for half-maximal inhibition calculated from these plots are compiled in Table II. A correlation exists between the calmodulin-inactivating capacity of the drugs and their efficiency as RVD inhibitors, particularly in the case of chlorpromazine and its structural analogue chlorpromazine sulfoxide. The latter is essentially inactive as a calmodulin antagonist and was also a very poor inhibitor of RVD. However, the correlation is not perfect, particularly in the cases of pimozide and chlorpromazine, which are far more effective as inhibitors of RVD than as calmodulin antagonists.

TABLE II
COMPARISON OF THE INHIBITORY EFFECT OF
PHENOTHIAZINES ON VOLUME REGULATION AND ON
CALMODULIN ACTIVATION OF PHOSPHODIESTERASE

	Inhibition of volume regulation*	Inhibition of calmodulin-activated phosphodiesterase‡
Trifluoperazine	3.0-5.0	10
Pimozide	0.125-0.15	7
Chlorpromazine	5.0-10.0	42
Chlorpromazine sulfoxide	>100.0	2,500

* The values indicate the concentration of drug (in micromolar) required for 50% inhibition of volume regulation. Volume recovery was determined 5 min after the hypotonic shock. The values given were determined graphically as described in the text and represent the range of four experiments.

‡ Concentrations (in micromolar) required for 50% inhibition of calmodulin-activated phosphodiesterase. From Weiss and Levin (1978).

Role of Extracellular and Intracellular Ca⁺⁺ in RVD

If Ca⁺⁺ is involved in the induction of the increased K⁺ permeability associated with RVD, it could originate from the extracellular milieu or from intracellular stores, such as mitochondria, endoplasmic reticulum, or the plasma membrane itself. To analyze the possible role of intra- and extracellular Ca⁺⁺, PBM were osmotically challenged in Ca⁺⁺-free solutions containing 1 mM EGTA. In normal (Na-rich) media, the cells displayed essentially normal volume regulatory responses (bottom curve in Fig. 7), and the usual prolonged swelling was observed in K⁺-rich solutions (top curve, Fig. 7). If, on the other hand, the intracellular Ca⁺⁺ stores were depleted before osmotic challenge, by

leaching the cells in Ca^{++} -free medium with chelating agents (2 mM EGTA), the capacity for RVD was diminished. A 2-h preincubation at room temperature in the absence of Ca^{++} resulted in complete inhibition of RVD in three cases, partial inhibition in one case, and had little effect in the remaining experiment. The source of this heterogeneity has not been identified.

The possibility that RVD was associated with a redistribution of Ca^{++} inside the cell was also assessed. Assuming a constant membrane permeability to the cation, an increase in cytoplasmic Ca^{++} concentration should be reflected in an enhanced rate of its efflux from the cell. Cells were loaded with

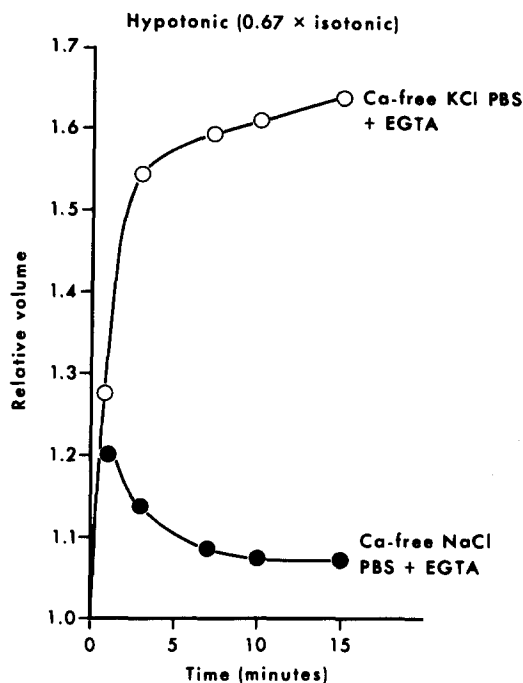


FIGURE 7. Volume changes in Ca^{++} -free media. PBM suspended in Ca^{++} -free isotonic medium containing either 137 mM NaCl (●) or 137 mM KCl (○) were hypotonicly ($0.67 \times$ isotonic) challenged at zero time. EGTA (1 mM) was included in both cases. Data are representative of three such experiments.

$^{45}\text{Ca}^{++}$ and the efflux was monitored under several conditions, as illustrated in Fig. 8. In agreement with previous reports, $^{45}\text{Ca}^{++}$ exchange was relatively fast under resting conditions: 30% of the isotope exchanged within 15 min. To assess the sensitivity of the experimental protocol, PBM were treated with A23187, which induced a massive loss of isotope within a very short time (bottom curve in Fig. 8). Efflux was also measured in cells resuspended in hypotonic PBS (middle curve of Fig. 8). The rate of $^{45}\text{Ca}^{++}$ release from the cells was increased, especially during the first few minutes. The efflux thereafter decreased somewhat but was still faster than that of control cells in

isotonic suspension. The results are consistent with a swelling-induced redistribution of Ca^{++} inside the cell, leading to a transient increase in cytoplasmic Ca^{++} concentration and an associated increase in its efflux. This interpretation assumes that the permeability of the membrane to Ca^{++} remains constant and that the membrane potential does not change (see Discussion).

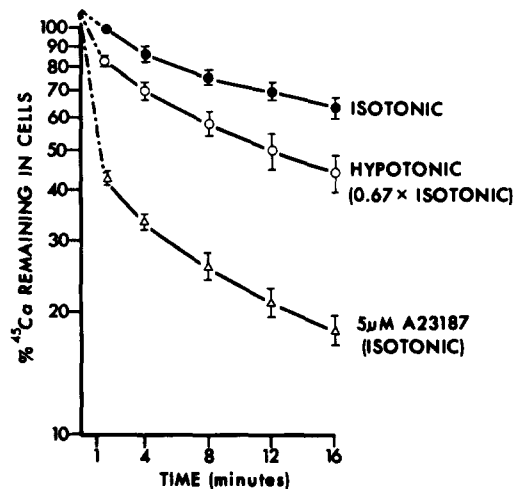


FIGURE 8. Effect of hypotonic challenge and the ionophore A23187 on the rate of Ca^{++} efflux from PBM. Cells were loaded with $^{45}\text{Ca}^{++}$ for 3 h and then washed and resuspended in nonradioactive PBS. Efflux of $^{45}\text{Ca}^{++}$ was monitored after diluting the cells in hypotonic (○) or in isotonic medium with (△) or without (●) 5 μM A23187. Ordinate: fraction of $^{45}\text{Ca}^{++}$ remaining in the cells. Data are the mean \pm SE of four experiments performed in duplicate.

DISCUSSION

The main purpose of this work was to analyze the possible participation of intracellular Ca^{++} in volume control. Four questions were asked in this regard: (a) is RVD accomplished by changing K^+ permeability upon swelling?; (b) is there a Ca^{++} -mediated K^+ transport pathway in human PBM?; (c) given the right conditions, can K^+ fluxes through this pathway modify cellular volume?; and (d) can parallels be established between the Ca^{++} - and volume-induced K^+ permeabilities?

Three lines of evidence indicate that an increase in K^+ permeability is involved in the mechanism whereby PBM regulate their volume during hypotonic stress. First, the direction and magnitude of the regulatory volume change depend on the direction of the K^+ concentration gradient; an outward gradient results in shrinking, but an inward gradient causes swelling. Second, ^{86}Rb efflux increased during RVD, but returned to normal values when the initial volume was regained. That this increased efflux reflects a change in permeability rather than an increased driving force is indicated by the fact that (a) an ouabain-insensitive component of ^{86}Rb uptake is also

enhanced by hypotonic stress; (b) the resting membrane potential, determined to be around -50 mV (i.e., significantly lower than E_k) is only slightly depolarized upon hypotonic treatment, the observed change being insufficient to account for the severalfold increase in ^{86}Rb efflux (this laboratory, unpublished observations); and (c) a loss in cellular K^+ content accompanies shrinking, and the magnitude of the loss is sufficient to account for most of the reduction in water content, if it is assumed that an anion co-migrates with K^+ , and that the cells remain in osmotic equilibrium. Indeed, 22% of the internal K^+ (Table I) and $\sim 26\%$ of the cell water are lost during RVD. A more precise correlation cannot be established because the total osmotic content of the cells and the activity coefficient of K^+ are not known, but it is reasonable to assume that K ions represent the bulk of the osmotically active cations. A role for K^+ in volume regulation by PBM had been proposed by Ben-Sasson et al. (1975) and similar mechanisms are known to operate in a variety of lymphoid and other cells (see Introduction).

It must be borne in mind, however, that preservation of electroneutrality requires a counter-ion to accompany the exit of K^+ (since the counterflow of a monovalent cation would be osmotically silent). This implies that a high anion permeability exists before the shock or else is being induced by the hypotonic challenge. Experiments in which anion permeability and conductivity were measured in resting and stressed cells indicate that the latter mechanism applies (Grinstein et al., manuscript in preparation).

Indirect evidence for the existence of Ca^{++} -dependent K^+ permeability in lymphocytes was presented by Rink et al. (1980), who could depolarize cells by addition of either quinine or 3,3'-dipropyl-thiadicarbocyanine, two compounds that are effective blockers of the Ca -induced K^+ permeability pathway in red cells (Armando-Hardy et al., 1975; Simons, 1976). This led the authors to suggest that part of the resting K^+ permeability of lymphocytes—which brings membrane potential close to the K^+ equilibrium potential—is contributed by Ca^{++} -dependent channels.

In the case of PBM, direct evidence of effects of Ca^{++} on Rb^+ fluxes are demonstrated by use of the Ca^{++} ionophore A23187 (Fig. 2B). Similar findings have been reported by Szasz et al. (1981). When a large outward gradient of K^+ was imposed, the ionophore produced cellular shrinking, but only if Ca^{++} was present in the external medium. Conversely, swelling occurred in media with high external K^+ concentrations.

Two sets of data suggest that more than one cation permeation pathway is activated by A23187: (a) a considerable efflux of ^{86}Rb is observed when the ionophore is added to cells in Ca^{++} -free media (Fig. 2B), whereas no significant volume is lost under similar circumstances (Fig. 3); (b) in Ca^{++} -containing solutions, A23187 induced a large efflux of ^{86}Rb (Fig. 2B), which does not appear to be commensurate with the modest volume changes recorded in identical media. It is conceivable that a rapid cation-exchange system is activated by the Ca^{++} ionophore, in addition to that catalyzing net K^+ flow, and that the former requires lower cytoplasmic Ca^{++} concentrations for activation.

Quinine, an inhibitor of the Ca^{++} -dependent increase in K^+ permeability

of red cells (Armando-Hardy et al., 1975) blocked A23187 plus Ca^{++} -induced $^{86}\text{Rb}^+$ efflux from PBM. Szasz et al. (1981) have made the same observation. RVD was also prevented by quinine, which indicates that some similarity exists between the volume-induced and Ca^{++} plus ionophore-induced systems. Importantly, the concentrations of quinine that are effective in red cells and PBM in media containing physiological K^+ concentrations are similar. The failure of quinine to inhibit the volume changes in high- K^+ media is presently not understood, but could be related to the competitive nature of the interaction between the drug and a K^+ site in the membrane (Reichstein and Rothstein, 1980). As a result, the potency of quinine is markedly reduced in high- K^+ media. The use of higher quinine concentrations to test this point is restricted by the appearance of signs of cellular damage.

The analogy between volume and A23187 plus Ca^{++} -induced K^+ fluxes also applies to their common sensitivity to a group of phenothiazines. Trifluoperazine completely blocked $^{86}\text{Rb}^+$ efflux, net K^+ fluxes, and the attending volume changes, whether induced by hypotonic stress or by the ionophore plus Ca^{++} . Although the concentration dependence of inhibition of A23187 plus Ca^{++} -mediated fluxes was not explored, the concentration that produced full inhibition of RVD also had maximal effects in the case of the ionophore. Whether calmodulin inactivation is the mechanism of this inhibition is not clear. Other phenothiazines, including chlorpromazine, that bind to and impair calmodulin action were also effective inhibitors of RVD. In contrast, chlorpromazine sulfoxide, a close structural analogue of chlorpromazine that does not interact with calmodulin, had little, if any, effect on RVD. Although it would be tempting to suggest that RVD is a Ca^{++} - and calmodulin-mediated process, the concentrations of the phenothiazines required for half-inhibition are not identical to their reported affinities for the Ca^{++} activator. In fact, in the cases of pimozide and chlorpromazine, RVD is more sensitive than calmodulin by one order of magnitude. Because of the hydrophobic nature of some moieties of these drugs and their known ability to interact with biological membranes (Seeman, 1972), a calmodulin-unrelated inhibition would not be surprising. However, an unequivocal answer is not available at this time, and a form of calmodulin or a calmodulin-like protein that is more susceptible to the phenothiazines cannot be ruled out.

In summary, both quinine and the phenothiazines inhibit the volume regulatory and ionophore-induced responses at similar concentrations, which suggests a common underlying mechanism. However, in establishing parallels between the Ca^{++} - and volume-induced responses, it must be stressed that the inhibitors used can interact with the membrane in a variety of ways, and that mechanisms other than the ones outlined could be responsible for the inhibition.

The source of the Ca^{++} involved in RVD was investigated. Removal of extracellular Ca^{++} did not prevent regulation, but prolonged preincubation in Ca^{++} -free, EGTA-containing medium slowed or eliminated shrinking altogether. These data, together with the increased $^{45}\text{Ca}^{++}$ efflux observed upon swelling, point to a role of intracellular Ca^{++} stores in volume regulation.

Unfortunately, methods for the direct measurement of transient Ca^{++}

redistribution inside small cells, such as PBM, are not available, so that the involvement of this ion in volume regulation must be inferred from analogies between the Ca^{++} ionophore and the hypotonically induced changes and from their common sensitivity to inhibitors. On this basis, a model can be proposed whereby swelling might release Ca^{++} from mitochondria, endoplasmic reticulum, or other sources into the cytoplasm. The elevation in internal $[\text{Ca}^{++}]$ would in turn activate K^+ channels in the membrane, with a concomitant efflux of K^+ , an accompanying anion, and osmotically obliged water.

In spite of the similarities between RVD- and A23187-induced fluxes and volume changes summarized above, differences also exist. For instance, quinine is a more effective blocker of volume-induced fluxes (Fig. 4A) than of ionophore-induced volume and flux alterations (Figs. 2B and 3). Also, the effect of A23187 on the volume of PBM requires the presence of extracellular Ca^{++} (Fig. 3), whereas RVD occurs in the absence of nominal Ca^{++} , and even in the presence of EGTA (Fig. 7).

The flux of K^+ induced in PBM by either swelling or A23187 plus Ca^{++} shares several common features, such as quinine sensitivity, with the analogous system reported in erythrocytes, but some differential properties exist. In PBM, micromolar concentrations of trifluoperazine have an inhibitory effect, whereas this is not the case in human red cells (Plishker et al., 1980). On the other hand, a variety of other calmodulin inhibitors have been recently reported to block the Gardos effect in erythrocytes (Lackington and Orrego, 1981). Also, the efflux of K^+ in the red cell system is stringently dependent on the presence of external K^+ , whereas RVD and the simultaneous $^{86}\text{Rb}^+$ efflux are not impaired by external K^+ removal (not illustrated).² Therefore the molecular mechanisms operating in both systems might differ.

In summary, it is suggested the PBM regulate their volume after osmotic swelling by a loss of K^+ through a Ca^{++} -sensitive pathway that is analogous but not identical to that of red cells. RVD is sensitive to phenothiazines and might be mediated by calmodulin or a similar protein.

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² The K^+ dependence of ^{86}Rb efflux in human PBM and red cells was studied under different experimental conditions, so the results, though suggestive of differences, are not strictly comparable. In addition, it is possible that the rapid leakage of K^+ out of the cells leads to a local concentration at the cell surface sufficient to stimulate further K^+ efflux. Moreover, no precautions were taken to remove the contaminating K^+ (probably in the micromolar range) that was present in our nominally " K^+ -free" medium.

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