Ionic Events During the Volume Response of Human Peripheral Blood Lymphocytes to Hypotonic Media

I. Distinctions Between Volume-activated Cl⁻ and K⁺ Conductance Pathways

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ABSTRACT   Human peripheral blood lymphocytes (PBL), when placed into hypotonic media, first swell and then shrink back to their original volumes because of a rapid KCl leakage via volume-activated K^+ and anion permeation pathways. By using gramicidin, a cation channel-forming ionophore, cation transport through the cell membrane can be shunted so that the salt fluxes and thus the volume changes are limited by the rate of the net anion movements. The "gramicidin method," supplemented with direct measurements of volume-induced ion fluxes, can be used to assess the effects of drugs and of various treatments on cation and anion permeabilities. It is demonstrated that quinine and cetiedil are much more effective blockers of volume-induced K^+ transport than of Cl^- transport, while dipyridamole, DIDS, and NIP-taurine inhibit only volume-induced Cl^- movement. Oligomycins block both cation and anion transport pathways, oligomycin A being more effective in inhibiting K^+ transport and oligomycin C preferentially blocking Cl^- movement. Ca depletion of PBL abolishes volume-induced K^+ transport but has no effect on Cl^- transport. Repletion of cell calcium by ionophore A23187 immediately restores rapid K^+ transport without significantly affecting volume-induced Cl^- transport. These observations, taken together with other reported information, can be best explained by a model in which cell swelling activates independent Cl^- and K^+ conductance pathways, the latter being similar in properties to the Ca^{2+}-activated K^+ transport observed in various cell membranes.

INTRODUCTION

Volume regulation in hypotonically swollen cells has been shown to occur in a variety of mammalian cells (for reviews see MacKnight and Leaf, 1977; Krege-

now, 1981; Spring and Ericson, 1982). The general prevalence of this phenomenon suggests that it has a physiological importance, but the underlying molecular mechanisms may be different in different cell types.

Mouse lymphoblasts, as well as chicken and human peripheral blood lymphocytes (Roti-Roti and Rothstein, 1973; Buckhold Shank et al., 1973; Doljanski et al., 1974; Ben-Sasson et al., 1975), have been shown to first swell in hypotonic media and then to shrink back to their original volumes. As documented in the literature, the swelling results in an increased KCl transport and shrinkage is produced by this salt efflux down its electrochemical potential gradient combined with the exit of osmotically obliged water (Bui and Wiley, 1981; Grinstein et al., 1982c; Cheung et al., 1982a; Deutsch et al., 1982). If the gradient is reversed by suspending the cells in high-K^+ media, they undergo secondary swelling rather than shrinking (Grinstein et al., 1982a, b). The involvement of calcium ions in the induction of K^+ permeability increase has been implicated (Grinstein et al., 1982c), and an increase in the anion permeability has also been shown to play a basic role in regulatory volume decrease in hypotonically shocked human lymphocytes (Grinstein et al., 1982a, b).

In several types of animal cells the volume-induced K^+ and/or Cl^- fluxes are proposed to represent coupled ion movements. These are either described as K^+ anion co-transports or coupled obligatory counter-transport mechanisms for both cations and anions (see Kregenow, 1981; Cala, 1980; Hoffman, 1982). These coupled systems are characterized as electrically neutral transport processes and the counter-transport mechanisms may result in significant changes in the transmembrane pH gradient. In contrast, the data suggest that in lymphocytes volume-induced K^+ and Cl^- transports are mediated by two distinct conductive pathways (Grinstein et al., 1982b). In order to further characterize these pathways, we investigated the effects of various drugs and of Ca^{2+} depletion on hypotonicity-induced ion movements and applied the ionophore gramicidin to make the cell membrane leaky to cations, thus allowing an independent estimation of the effects on volume-induced anion transport. The results indicate that the K^+ and Cl^- pathways are independently inhibitable and therefore apparently distinct from each other.

MATERIALS AND METHODS

All the reagents used were of analytical grade. Gramicidin D, oligomycin and its separated components A, B, and C, dipyridamole (Persantin), quinine, ouabain, and ionophore A23187 were purchased from Sigma Chemical Co., St. Louis, MO. NIP-taurine [N-(2-nitro-4-isothiocyano-phenyl)-taurine], and H_2DIDS (4,4′-diisothiocyano-1,2-diphenyl-ethane-2,2′-disulfonate) were kind gifts of Dr. M. Ramjeesingh, The Hospital for Sick Children. Cetiedil citrate was a gift from Dr. L. R. Berkowitz, University of North Carolina at Chapel Hill. $Na^{36}Cl$ and $^{86}RbCl$ isotopes were obtained from Amersham Corp., Arlington Heights, IL.

The standard incubation solutions used in the experiments contained 103 mM NaCl, 5.4 mM KCl, 23.8 mM $NaHCO_3$, 5.6 mM Na_2HPO_4, 0.4 mM $MgSO_4$, 0.04 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.2). In several experiments all Na^+ was replaced by choline$^+$ or by K^+.

Human peripheral blood lymphocytes (PBL) were isolated by Ficoll gradient separation

(see Grinstein et al., 1982c), either from freshly drawn heparinized blood of healthy donors, or from buffy coat-rich fractions of bank blood stored for <24 h in citrate-phosphate-dextrose (CPD) preservative. The buffy coat-rich blood was kindly provided by Joan Green, Canadian Red Cross, Toronto. The cells were used on the day of separation and their viability, checked by trypan blue exclusion, was always >90%.

Cell volume changes were followed by using a Coulter Counter adapted with a Coulter Channelyzer (Coulter Electronics, Inc., Hialeah, FL). For a detailed description of this method, see Segel et al. (1981) and Grinstein et al. (1982c). For tracer efflux determinations, lymphocytes were loaded with the respective isotopes during a 2-h incubation in RPMI 1640 media. The radioactivity remaining in the cells during flux experiments was measured after separating the cells by centrifugation through an oil cushion, as described in detail by Grinstein et al. (1982b).

All the experiments reported here were carried out at room temperature (22–23°C). The volume measurement data are representative of single experiments, repeated at least four times with different cell populations. The data from flux experiments are the means of at least three separate experiments, each done in duplicate. Within the assay periods the viability of PBL was not significantly affected by the treatments or the drugs used in the experiments.

RESULTS

When human PBL are diluted in hypotonic media containing Na^+ or $choline^+$ as the major cation, a rapid initial swelling of the cells is followed by a shrinking to the isotonic cell volume (Fig. 1, control), a phenomenon known as regulatory volume decrease (RVD). The initial swelling is an osmotic response, whereas the shrinking phase is associated with a loss of KCl caused by volume-induced increases in K^+ and Cl^- permeabilities. The response is essentially complete within 10 min at room temperature (control), but it is completely inhibited by the addition of 50–100 μM quinine (Grinstein et al., 1982c). In the hypotonic (0.7× isotonic) choline-Cl media, the addition of gramicidin increases the rate of the RVD and produces a slight "overshoot," that is, a decrease of the cell volume below the isotonic value. In contrast to the normal RVD, the response in the presence of gramicidin is insensitive to quinine in concentrations up to 200 μM.

Gramicidin forms channels in membranes that allow rapid K^+ and Na^+ fluxes but do not allow the passage of the larger $choline^+$ cation (see Pressman, 1976; Finkelstein and Anderson, 1981). Nevertheless, addition of gramicidin to isotonic PBL has almost no effect on the cell volume. This finding, first reported by Grinstein et al. (1982a, b), indicates that the anion conductance in isotonic cells is very low, so that net changes in salt content do not occur, despite the gramicidin-induced cation permeability. On the other hand, these authors noted that in hypotonically shocked, gramicidin-treated cells, the rapid osmotic swelling was followed by a substantial continued increase in volume. These results demonstrated that the anion permeability of the shocked cells had been substantially increased, allowing net uptake of NaCl to occur. In these experiments the gramicidin treatment resulted in displacement of cellular K^+ by Na^+, and the swelling is driven by the inward Cl^- gradient.

In the experiments of Fig. 1, Na^+ in the medium is replaced by $choline^+$, a cation whose permeation is unaffected by gramicidin. The ionophore, under

these conditions, induces a secondary shrinkage resembling RVD, driven largely by the outward K^+ gradient (and also by the outward Na^+ gradient). The addition of gramicidin results in a more rapid shrinkage than the normal RVD, presumably because the gramicidin-induced K^+ permeability is larger than the volume-induced one (a conclusion confirmed by direct flux measurements; see Fig. 5). The Cl^- permeability is not the limiting factor to salt outflow in hypotonically shocked cells, because it substantially exceeds the K^+ permeability (Grinstein et al., 1982a, b).

Inhibitors of RVD may act by blocking either the volume-induced K^+ or Cl^- permeabilities (or both). On the basis of the above observations, gramicidin can be used in hypotonically shocked cells to specify the effects of each agent. In the
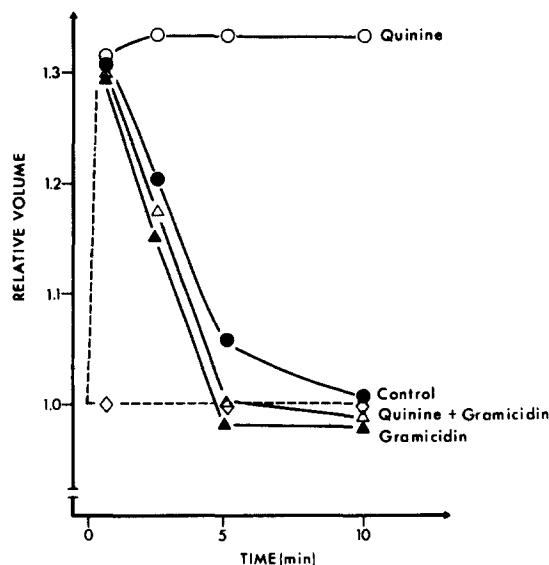

FIGURE 1.   Effects of quinine and gramicidin on the volume changes in hypotonically shocked human PBL. The media contained choline-Cl as the predominant salt. Isotonic media with or without 0.5 μM gramicidin or 100 μM quinine ($\Diamond$); 0.7× isotonic medium, control ($\bullet$); 0.7× isotonic plus 100 μM quinine (O); 0.7× isotonic plus 0.5 μM gramicidin ($\blacktriangle$); 0.7× isotonic plus 100 μM quinine plus 0.5 μM gramicidin ($\triangle$).

presence of gramicidin, the cation permeability will be substantial (assuming the agents do not directly block the gramicidin channel). Those that inhibit the volume response in the presence of gramicidin must therefore be inhibitors of the chloride pathway. They may also block the volume-induced K^+ pathway. In contrast, those RVD inhibitors that do not block the volume response in the presence of gramicidin must act specifically on the volume-induced K^+ pathway. For example, the sensitivity of normal RVD to quinine and the insensitivity of the response in the presence of gramicidin can be explained by the following simple assumptions: in red cells, quinine is a specific inhibitor of Ca^{2+}-induced K^+ transport (Armando-Hardy et al., 1975; Reichstein and Rothstein, 1980). A

similar ion specificity applies to the effects of quinine in RVD of lymphocytes. In the presence of gramicidin the quinine-blocked K^+ channels are bypassed by the ionophore so that the RVD becomes quinine insensitive (Fig. 1). These results indicate that both the gramicidin channels and the volume-induced anion permeability are insensitive to quinine at the concentration used (100 μM). Quinine concentrations higher than 300 μM, on the other hand, do produce inhibitions of the volume changes in the presence of gramicidin (data not shown), which indicates an effect of high concentrations on anion transport as well.

The effects of cetiedil, also reported to be a specific inhibitor of Ca^{2+}-induced K^+ transport in erythrocytes (Berkowitz and Orringer, 1983), were quantitatively assessed by comparing the percent inhibition of lymphocyte volume changes in
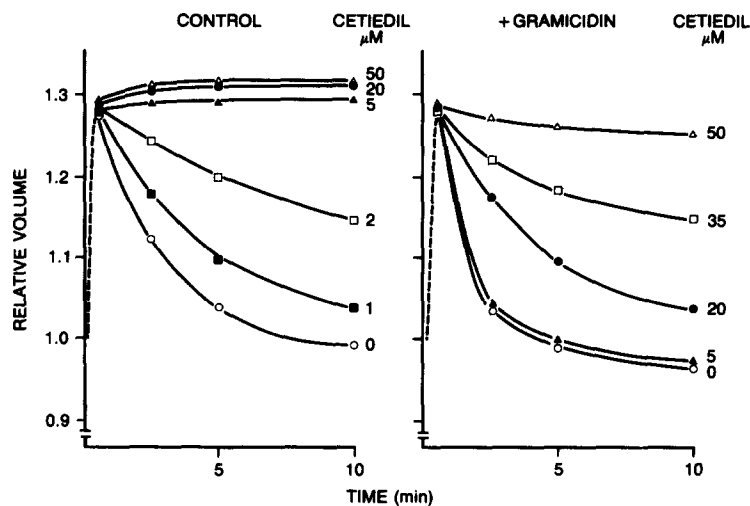

FIGURE 2.   Inhibition of volume changes in hypotonically shocked (0.7× isotonic) human PBL by cetiedil. All the media contained choline-Cl as the predominant salt, with or without 0.5 μM gramicidin. Cetiedil was added in the concentrations indicated in the figure.

control cells and in the presence of gramicidin with increasing concentrations of cetiedil. As shown in Fig. 2, this agent inhibits RVD in the control cells with an apparent K_i of ~2 μM, while in the presence of gramicidin this concentration is ineffective and the value of K_i is ~30 μM. Thus, the inhibitory behavior of cetiedil is similar to that of quinine, with a relatively high specificity for the volume-induced K^+ transport.

Agents that inhibit volume changes both in the absence and presence of gramicidin and are, therefore, inhibitors of volume-induced anion transport, include oligomycin (0.2–10 μg/ml), dipyridamole (50–150 μM), and H_2DIDS and NIP-taurine (100–500 μM; see Fig. 3). Isotonic cell volume is not affected by any of these drugs, either in the presence or absence of gramicidin. In the case of H_2DIDS and NIP-taurine, inhibition of volume decrease requires a 20-min preincubation of the isotonic cells with relatively high concentrations of

these drugs and the inhibition is never complete. Oligomycin or dipyridamole, on the other hand, produces 90–100% inhibition without any preincubation.

Another way of assessing the changes in permeability of PBL after hypotonic shock involves their suspension in media containing KCl as the predominant salt. In this situation the initial osmotic swelling is followed by a secondary swelling caused by KCl influx driven largely by the inward Cl^- gradient (Grinstein et al., 1982a, b). As illustrated in Fig. 4, the response is accelerated by gramicidin, which indicates that volume-induced Cl^- conductance is considerably higher than volume-induced K^+ conductance. Fig. 4 demonstrates that this secondary swelling
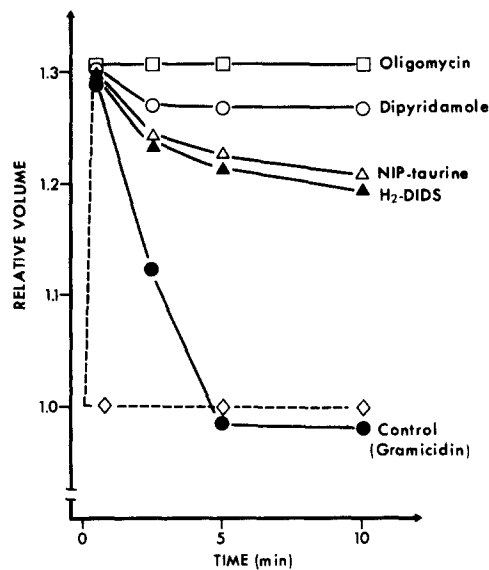

FIGURE 3. Effects of drugs on volume changes in hypotonically shocked human PBL. All the media contained choline-Cl as the predominant salt and 0.5 μM gramicidin. Isotonic medium ($\Diamond$); 0.7× isotonic medium, control ($\bullet$); 0.7× isotonic plus 5 μg/ml oligomycin ($\square$); 0.7× isotonic plus 100 μM dipyridamole ($\bigcirc$); 0.7× isotonic plus 200 μM NIP-taurine ($\triangle$); 0.7× isotonic plus 200 μM H_2DIDS ($\blacktriangle$). (In the case of NIP-taurine and H_2DIDS, the cells were preincubated with the drugs for 20 min in isotonic media.)

is completely blocked by an oligomycin mixture or dipyridamole, even in the presence of gramicidin. The drug concentrations required for inhibition are similar to those that block cell shrinkage in choline-Cl media. Preincubation of the cells with H_2DIDS or NIP-taurine produces the same qualitative, although less pronounced, inhibition. Because the results by the swelling procedure (Fig. 4) are similar to those described previously for the shrinking procedure (Figs. 2 and 3), it can be concluded that the inhibitory effects of the agents tested are independent of the direction of the ion flows.

Oligomycin is a mixture of several related antibiotics. When the effects of separated oligomycins A, B, and C were examined, it was found that oligomycin A and B were ~10 times more effective inhibitors of RVD in the control cells
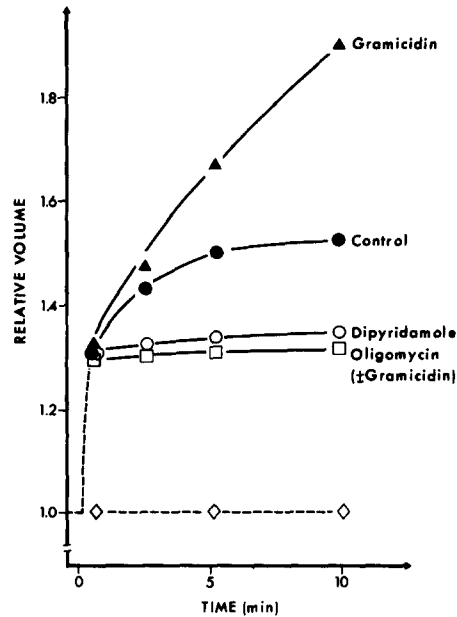
FIGURE 4.　Effects of oligomycin (unseparated compounds) and dipyridamole on volume changes in hypotonically shocked PBL. The media contained KCl as the predominant salt. Isotonic media with or without 0.5 μM gramicidin ($\diamond$); 0.7$\times$ isotonic control ($\bullet$); 0.7$\times$ isotonic plus 5 μg/ml oligomycin with or without 0.5 μM gramicidin ($\square$); 0.7$\times$ isotonic plus 100 μM dipyridamole with or without 0.5 μM gramicidin ($\bigcirc$); 0.5 μM gramicidin ($\blacktriangle$).

than in the gramicidin-treated ones. In contrast, oligomycin C, as well as dipyridamole, had the same potency for inhibiting volume response in the control and in the gramicidin-treated cells (see Table I, columns 1 and 2).

TABLE I

Effects of Drugs on Volume Changes and Ion Fluxes in Hypotonically Shocked PBL

	Approximate K_i values for the inhibition of:			
	Volume decrease in choline media		Volume-induced ion fluxes	
Drugs	Control	+Gramicidin	⁸⁶Rb⁺	³⁶Cl⁻
	μM	μM	μM	μM
Cetiedil	2	30	2–5*	30–50
Oligomycin A	0.5	5	0.5	5
Oligomycin C	0.3	0.5	5	0.5
Dipyridamole	50	50	No inhibition	50

Volume changes and ion fluxes were measured in 0.7$\times$ isotonic media as described in Materials and Methods and the legends to Figs. 1, 5, and 6. K_i values (drug concentrations required for 50% inhibition) were calculated on the basis of 5-min measurements of volume decrease or ion efflux. Mean values are from at least three different experiments for each drug.

* Values measured in media with low K⁺ (5.0 mM) and NaCl as a predominant salt, as high K⁺ concentrations antagonize the cetiedil effect.

The observations noted above concerning the effects of inhibitors on cell shrinking and swelling indicate that quinine, cetiedil, and oligomycin A are relatively specific blockers of volume-induced K^+ transport, whereas oligomycin C, dipyridamole, H_2DIDS, and NIP-taurine must block Cl^- transport but may also block K^+ transport.

In order to further characterize the nature of the volume-induced ion fluxes and their sensitivities to the above inhibitors, tracer Rb^+ and Cl^- movements in isotonic and in hypotonically shocked PBL were studied (Rb ions have been shown to behave identically to K ions in RVD; Cheung et al., 1982a). $^{86}Rb^+$ efflux experiments were carried out by incubating tracer-preloaded PBL in media containing low K^+ concentrations or K^+ as the predominant cation. Under the latter "equilibrium exchange" conditions the effect of net salt transport or
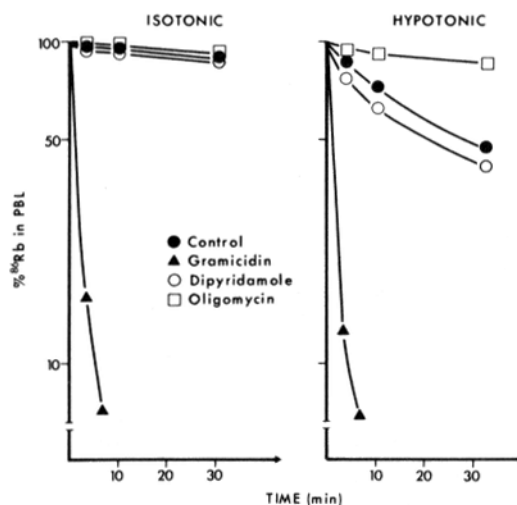

FIGURE 5. $^{86}Rb^+$ efflux from isotonic or hypotonically shocked (0.7× isotonic) human PBL. The incubation media contained KCl as the predominant salt and 0.5 mM ouabain. Control (●); 0.25 μM gramicidin (▲); 5 μg/ml oligomycin (unseparated compounds) (□); 100 μM dipyridamole (○).

the membrane potential on Rb^+ movement is negligible. The similar increase in ^{86}Rb efflux observed in virtually K^+-free and high-K^+ media after hypotonic shock (see Grinstein et al., 1982b) indicates that K^+ exchange is negligible and tracer Rb^+ movement is a good indicator of K^+ conductance in PBL. In order to prevent K^+ (Rb^+) movement related to the Na^+-K^+ pump, the fluxes were measured in the presence of 0.5 mM ouabain. In these short-term experiments ouabain had no effect on RVD or on volume-induced K^+ and Cl^- fluxes, in agreement with observations reported in the literature (Bui and Wiley, 1981; Grinstein et al., 1982c; Deutsch et al., 1982).

In PBL suspended in isotonic media, the half-time of Rb^+ efflux is between 80 and 100 min (Fig. 5). The addition of gramicidin accelerates this Rb^+ efflux by at least 50-fold. The half-time is ~2 min at 0.25 μM gramicidin and is less at higher concentrations (half-times of <2 min cannot be precisely determined by

the technique used). As reported previously (Grinstein et al., 1982*b*, *c*), in hypotonically shocked lymphocytes the rate of Rb^+ efflux is much higher than in the isotonic cells. The efflux is not linear with time; rather, it has a faster initial and a slower later phase. The approximate half-time for the initial efflux, which probably represents the volume-induced component, is ~10 min under the experimental conditions shown in Fig. 5, which indicates an ~10-fold increase in efflux rate. Dipyridamole has no inhibitory effect on Rb^+ efflux in isosmotic or in hypotonic PBL (a slight increase of Rb^+ efflux by dipyridamole in hypotonically shocked cells is often observed). NIP-taurine or H_2DIDS also have no effect
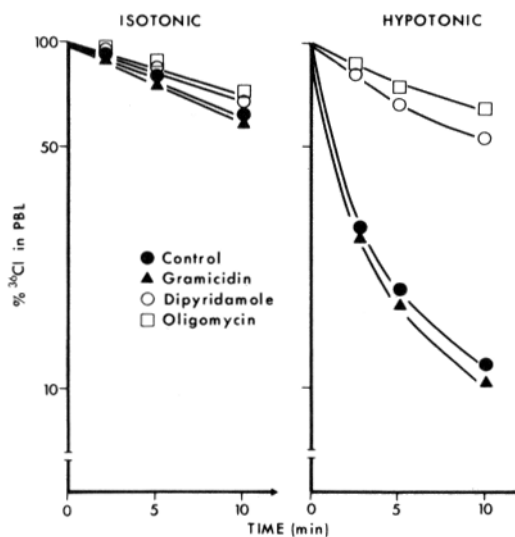

FIGURE 6. ^{36}Cl efflux from isotonic or from hypotonically shocked (0.7× isotonic) human PBL. The media contained NaCl as the predominant salt. Control (●); 0.5 μM gramicidin (▲); 5 $\mu g/ml$ oligomycin (□); 100 μM dipyridamole (○).

on Rb^+ fluxes in PBL (data not shown). On the other hand, quinine abolishes volume-induced acceleration of Rb^+ efflux (see Grinstein et al., 1982*c*) and the oligomycin mixture does likewise (Fig. 5). None of the drugs examined have any effect on the Rb^+ fluxes in isotonic cells or on the rapid Rb^+ efflux produced by gramicidin.

The experiments for measuring $^{36}Cl^-$ efflux from PBL, such as shown in Fig. 6, were carried out by placing the tracer-preloaded cells into isotonic or hypotonic media containing Na^+ as the major cation. Similar experiments, carried out in $choline^+$- or K^+-rich media, demonstrated no qualitative differences in the drug effects. As reported previously, in isotonic media the half-time for tracer Cl^- efflux from PBL is 15–20 min, while in hypotonically (0.7× isotonic) shocked cells this half-time decreases to <2 min (Grinstein et al., 1982*a*, *b*). As shown in Fig. 6, gramicidin, a cation-specific ionophore, has no effect on Cl^- movement, and the same is true for quinine and cetiedil in concentrations strongly inhibitory to RVD. In contrast, oligomycin and dipyridamole inhibit volume-induced Cl^-

effluxes by 90–100%, while H_2DIDS and NIP-taurine produce a partial inhibition (data not shown). Oligomycin and dipyridamole also produce a 15–25% inhibition of the Cl^- efflux from isotonic PBL, but these effects are absent in Na-gluconate media, where Cl^- efflux is somewhat slower. Substitution of gluconate medium had no effect, however, on the Cl^- efflux from hypotonic cells with or without drugs.

Table I compares the approximate K_i values for the drug inhibition of ion fluxes with those determined from volume changes in the presence or absence of gramicidin, all measured under hypotonic (0.7× isotonic) conditions. As documented, cetiedil and oligomycin A effectively inhibit volume-induced Rb^+
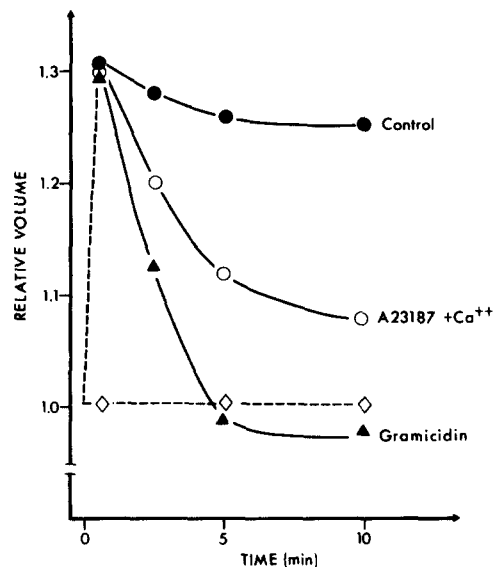

FIGURE 7. Effect of Ca^{2+} depletion on the volume decrease in hypotonically shocked human PBL. The media contained choline-Cl as the predominant salt. Isotonic media with or without 0.5 μM gramicidin (◇); 0.7× isotonic control (●); 0.7× isotonic plus 1 μM A23187 and 1 mM Ca^{2+} (O); 0.7× isotonic plus 0.5 μM gramicidin (▲).

transport and RVD in the control cells in at least 10 times lower concentrations than those affecting Cl^- transport or volume decrease in the presence of gramicidin. In contrast, oligomycin C and dipyridamole have about the same inhibitory potency on volume decrease both in the absence and presence of gramicidin; thus, these experiments cannot exclude a simultaneous inhibition of both K^+ and Cl^- transport pathways. However, the flux experiments indicate that they predominantly inhibit volume-induced Cl^- transport.

As reported by Grinstein et al. (1982c) and Bauer and Lauf (1983), the reduction of extracellular calcium by EGTA does not immediately influence RVD in lymphocytes, but prolonged preincubation with the Ca^{2+} chelator results in a loss of the response. An almost complete inhibition of the phenomenon was

observed in lymphocytes stored overnight in CPD preservative (which reduces free calcium ion concentration in the blood plasma). Under various experimental conditions, the loss of volume response is closely correlated with the time of calcium depletion and the effect does not depend on the nature of the calcium-chelating agent used to reduce extracellular free calcium.

As shown in Fig. 7, relatively unresponsive, calcium-depleted lymphocytes that were swollen in hypotonic choline-Cl media shrink rapidly upon addition of gramicidin. The ionophore gramicidin does not, however, produce volume changes in calcium-depleted isotonic cells (similar to the behavior of normal
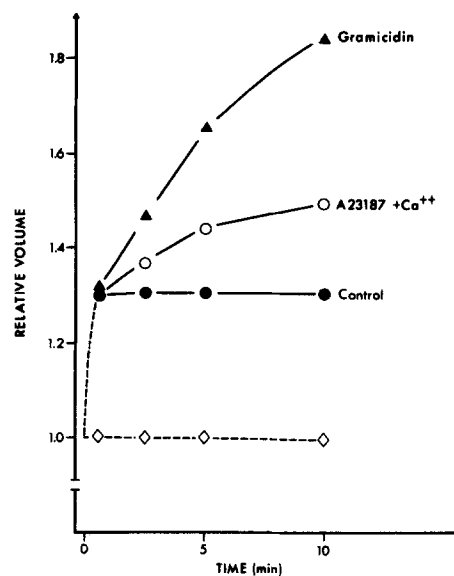

FIGURE 8. Effect of Ca^{2+} depletion on the secondary swelling of hypotonically shocked human PBL. The media contained KCl as the predominant salt. Isotonic media with or without 0.5 μM gramicidin ($\Diamond$); 0.7× isotonic control ($\bullet$); 0.7× isotonic plus 1 μM A23187 and 1 mM Ca^{2+} (O); 0.7× isotonic plus 0.5 μM gramicidin ($\blacktriangle$).

isotonic PBL). The hypotonic response in depleted cells can be immediately restored by the addition of ionophore A23187 plus Ca^{2+}. Restoration is slower (complete in ~1 h) in media containing 1–2 mM Ca^{2+} (without A23187). Fig. 8 demonstrates that volume-induced secondary swelling of PBL in KCl media is also abolished by calcium depletion. The addition of ionophore A23187 plus Ca^{2+} restores the response to the level seen in nondepleted cells and the addition of gramicidin produces a rapid swelling similar to that found in normal PBL treated with gramicidin. Thus, the effects of calcium depletion are independent of the direction of ion flow.

These findings indicate that depletion of intracellular calcium specifically reduces volume-induced K^+ transport in PBL but is without effect on volume-induced Cl^- transport. This conclusion was directly confirmed by measurements in which no alteration in $^{36}Cl^-$ efflux from cells preincubated in CPD or EGTA

was observed, while volume-induced [86]Rb^{+} efflux was substantially reduced in calcium-depleted PBL (data not shown).

DISCUSSION

Independence of K and Cl Transport Pathways

In human PBL, hypotonic shock induces the activation of K^{+} and Cl^{−} transport pathways, allowing ion movements down their respective electrochemical potential gradients (Ben-Sasson et al., 1975; Bui and Wiley, 1981; Grinstein et al., 1982a–c; Cheung et al., 1982a; Deutsch et al., 1982). Net salt flux and cell volume changes can only occur with simultaneous opening of both cation and anion transport routes, but the data presented in this paper support the conclusion that these do not represent inherently coupled molecular mechanisms. It has already been noted that both transports are electrogenic (Grinstein et al., 1982b) and that the fluxes of anions and cations are not interdependent. That is, the volume-induced Cl^{−} transport is not diminished in the absence of K^{+} (by Na^{+} replacement) and the volume-induced K^{+} transport is not diminished in the absence of external Cl^{−} (gluconate replacement). Furthermore, human B lymphocytes respond only slightly to a hypotonic shock by volume regulation, because they lack an increased K^{+} conductance (Cheung et al., 1982b), but hypotonic shock activates a Cl^{−} conductance (gramicidin-treated B lymphocytes shrink in hypotonic choline-Cl and swell in hypotonic KCl media to the same extent as T cells; Grinstein et al., 1983a).

In the present paper the concept of independent K^{+} and Cl^{−} transport pathways is supported by inhibition studies. By comparing volume changes with and without the ionophore gramicidin, combined with direct measurements of Rb^{+} (K^{+}) and Cl^{−} fluxes, it is demonstrated that Cl^{−} and K^{+} transports can be selectively inhibited.

(a) Quinine, cetiedil, and oligomycin A specifically inhibit volume-induced K^{+} movement at concentrations that are without effect on Cl^{−} movement. At concentrations ∼10 times as high, however, they also inhibit the volume-induced anion transport.

(b) Dipyridamole, H2DIDS, and NIP-taurine inhibit volume-induced Cl^{−} transport in PBL without affecting K^{+} transport. Oligomycin C is also a relatively specific inhibitor of anion transport, but at higher concentrations it also inhibits the K^{+} transport pathway.

(c) In hypotonically shocked lymphocytes, calcium depletion inhibits volume-induced K^{+} transport but has no effect on Cl^{−} movement.

Nature of the Cation Transport Pathway Involved in the Volume Response

As previously shown (Cheung et al., 1982a), the volume-induced increase in cation transport is highly selective for K^{+} and Rb^{+}, as compared with Li, Na^{+}, or choline[+]. A number of K^{+}-specific (Rb^{+}-specific) pathways have been reported (see reviews of Lew and Ferreira, 1978; Sarkadi and Gárdos, 1983) that are Ca^{2+}-activated, as typified by the "Gárdos phenomenon" (Gárdos, 1958). Several correlations suggest that the volume-activated K^{+} transport is similar to the

Gárdos phenomenon of red cells (Grinstein et al., 1982a, c, and the present paper). Both involve conductive K^+ transport pathways, inhibitable by quinine, cetiedil, trifluoperazine, and oligomycin. The Ca^{2+} dependence of the red cell system has been directly demonstrated, whereas that of the volume-activated is based on indirect evidence. Depletion of cellular Ca^{2+} by prolonged incubation in media containing Ca^{2+} chelators leads to inhibition of the volume-activated K^+ transport and RVD; the inhibition is reversed by the addition of Ca^{2+} to the medium, as illustrated in Figs. 7 and 8 (immediately in the presence of the divalent cation ionophore A23187, and more slowly in its absence). The depletion process is necessary in lymphocytes, presumably because of their large stores of Ca^{2+} in intracellular organelles (Lichtman et al., 1980; Deutsch and Price, 1982).

Addition of Ca^{2+} plus A23187 to normal isotonic PBL stimulates a specific, ouabain-insensitive K^+ transport (Szász et al., 1981; Grinstein et al., 1982c, 1983b). The transport is inhibited by the same concentrations of quinine and trifluoperazine as is the volume-induced K^+ transport, which suggests identity of the two pathways (Grinstein et al., 1982a).

Despite the evidence noted above, no changes in cytoplasmic Ca^{2+} were directly demonstrable during the regulatory volume response using the fluorescent Ca^{2+} indicator Quin 2 (Rink et al., 1983). The reason for the apparently contradictory results is not known. One possible explanation is that small, local changes of Ca^{2+} concentrations near the plasma membrane are involved that would not be detected by the Quin 2 method (which reports the average cytoplasmic Ca^{2+} concentration).

Nature of the Anion Transport Pathway Involved in Volume Response

The most extensively studied anion transport system is that of the red blood cell (Gunn et al., 1973; Knauf, 1979). It involves a rapid, nonconductive anion exchange with only a relatively small fraction of conductive flux. Dipyridamole, H_2DIDS, and NIP-taurine, specific inhibitors of the erythrocyte anion transport system, also inhibit volume-induced Cl^- transport in PBL without affecting K^+ transport. However, H_2DIDS and NIP-taurine concentrations required to inhibit red cell anion transport are at least one order of magnitude smaller than those reducing Cl^- movements in hypotonically shocked lymphocytes (0.1–0.5 mM). Furosemide and bumetanide, inhibitors of red cell anion transport, have no pronounced effect on volume changes in PBL (unpublished data), while low concentrations of oligomycin (especially oligomycin C) inhibit RVD and volume-induced anion transport in lymphocytes but not anion transport in red cells. On the basis of these observations, a close relationship between red cell anion transport and isotonic or volume-induced Cl^- transport in PBL is not apparent.

The anion fluxes of isotonic lymphocytes largely involve an exchange system (Grinstein et al., 1982b) that is relatively insensitive to the inhibitors of either the volume-induced anion fluxes or of the red cell exchange system. The volume-activated anion transport, on the other hand, is a conductive rather than an exchange system, and is influenced by the specific inhibitors as noted above. The anion specificity of the lymphocyte anion exchange system is not known, but the volume-induced anion transport carries Cl^-, NO_3^-, and probably bicarbonate, but not $SO_4^=$ or gluconate$^-$ ions (Grinstein et al., 1982a, b).

Comparison of the Volume-induced Transport Pathways in Lymphocytes with Those of Other Cells

It has been suggested that in several mammalian cell types an electroneutral exchange of K^+ and H^+ is involved in regulatory volume response to hypotonic media (see Hoffman, 1982). The simultaneous activation of a K^+-H^+ and a Cl^--OH^- (or HCO_3^-) electroneutral exchange pathway could produce net salt fluxes and volume changes, but several observations make such a mechanism unlikely in PBL.

(a) Changes in the membrane potential in PBL during volume response are consistent with the presence of conductive ion pathways. As shown by Grinstein et al. (1982b), in resting PBL, K^+ permeability is dominant and the membrane potential is mostly determined by the K^+ gradient, while during RVD, Cl^- conductance becomes dominant and this induces a membrane depolarization (see also the following paper). The potential in the isotonic cell is close to the Nernst potential for the K^+ gradient (about −60 mV) and it shifts toward the Nernst potential for the Cl^- gradient (about −33 mV). As indicated by these potential measurements and by the lack of gramicidin effect on the volume of resting cells, the relatively fast tracer Cl^- efflux from isotonic lymphocytes is carried by an obligatory anion exchange pathway and it is incapable of supporting net salt flux and volume changes.

(b) HCO_3^- depletion or replacement of extracellular Cl^- by gluconate$^-$ does not inhibit RVD or volume-induced tracer Cl^- efflux in PBL. Replacement of extra- and intracellular K^+ by Na^+ has no effect on volume-induced Cl^- fluxes (Grinstein et al., 1982b). Development of volume-regulatory shrinkage does not produce any change in intracellular pH (Rink et al., 1983), as might be expected if Cl^--HCO_3^- exchange flux were an essential component.

It can be concluded that in contrast to the reported volume-regulatory mechanisms in several other cell types, in lymphocytes hypotonicity-induced volume response involves activation of independent conductance pathways for K and Cl ions. The kinetic behavior of these ion pathways is the subject of the following paper.

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