

Calcium-independent Cell Volume Regulation in Human Lymphocytes

Inhibition by Charybdotoxin

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ABSTRACT The properties of the K^+ pathway underlying regulatory volume decrease (RVD) in human blood lymphocytes were investigated. Evidence is presented for the existence of three types of K^+ conductance in these cells. Ionomycin, a Ca^{2+} ionophore induced a K^+ -dependent hyperpolarization, indicating the presence of Ca^{2+} -activated K^+ channels, which were blocked by charybdotoxin (CTX). CTX also induced a depolarization of the resting membrane potential, even at subphysiological cytosolic $[Ca^{2+}]_i$, which suggests the existence of a second CTX-sensitive, but Ca^{2+} -independent conductance. A CTX-resistant K^+ conductance was also detected. RVD in blood lymphocytes was partially (~75%) blocked by CTX. However, volume regulation was not accompanied by detectable changes in $[Ca^{2+}]_i$, nor was it prevented by removal of extracellular Ca^{2+} and depletion or buffering of intracellular Ca^{2+} . These observations suggest that K^+ loss during RVD is mediated by Ca^{2+} -independent, CTX-sensitive channels or that Ca^{2+} -dependent channels can be activated by cell swelling at normal or subnormal $[Ca^{2+}]_i$. The former interpretation is supported by findings in rat thymic lymphocytes. These cells also displayed a CTX-sensitive Ca^{2+} -dependent hyperpolarization. However, CTX did not significantly alter the resting potential, suggesting the absence of functional Ca^{2+} -independent, toxin-sensitive channels. Volume regulation in thymic lymphocytes was less efficient than in human blood cells. In contrast to blood lymphocytes, RVD in thymocytes was not affected by CTX. These observations indicate that, though present in lymphocytes, Ca^{2+} -activated K^+ channels do not play an important role in volume regulation. Instead, RVD seems to be mediated by Ca^{2+} -independent K^+ channels. We propose that two types of channels, one CTX sensitive and the other CTX insensitive, mediate RVD in human blood lymphocytes, whereas only the latter type is involved in rat thymocytes.

INTRODUCTION

Most animal cell types maintain a relatively constant volume despite variations in the tonicity of the extracellular milieu (see Siebens, 1985 and Eveloff and Warnock, 1987 for reviews). Thus, when subjected to hypoosmolar solutions, the cells initially

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swell but subsequently regain near normal size through loss of solutes and osmotically obliged water. The mechanism underlying this process, known as regulatory volume decrease (RVD), varies in different cell types. Organic osmolytes constitute the majority of the solutes lost in some cells, whereas inorganic monovalent ions are mainly lost in others (Siebens, 1985). The latter, typified by K^+ and Cl^- , can be transported by electroneutral systems, such as K^+/H^+ exchange in parallel with Cl^-/HCO_3^- exchange (Cala, 1980), or by coupled K-Cl cotransport (Lauf, 1982). Alternatively, KCl loss can proceed through independent conductive pathways, as occurs in Ehrlich ascites cells (Hoffmann, 1985, 1986) and in lymphocytes (Grinstein et al., 1984b).

The mechanism(s) whereby specific ion transport pathways are activated during volume regulation are not well understood. Changes in the concentration of free cytosolic calcium ($[Ca^{2+}]_i$) have been suggested to mediate RVD in different tissues (Cala, 1983; Chase and Wong, 1985; Christensen, 1987). In lymphocytes, elevated $[Ca^{2+}]_i$ was proposed to be responsible for the increased K^+ conductance (Grinstein et al., 1982b), based on the following observations: (a) RVD and the associated K^+ fluxes are precluded by quinine, which can block Ca^{2+} -activated K^+ channels, and by trifluoperazine, a calmodulin antagonist; (b) RVD is also inhibited by prolonged preincubation in Ca^{2+} -free media containing EGTA; and (c) in isotonic media, shrinking can be induced by simply elevating $[Ca^{2+}]_i$, using divalent cation ionophores (Grinstein et al., 1982b). It was therefore suggested that cell swelling might increase $[Ca^{2+}]_i$, with consequent opening of K^+ (and perhaps also Cl^-) channels, leading to outward diffusion of these ions down their electrochemical gradients, which results in RVD. Consistent with this model, Christensen (1987) has demonstrated the existence of stretch-activated cation-selective, Ca^{2+} -permeable channels in cells of the amphibian choroid plexus, which could account for an elevated $[Ca^{2+}]_i$ in osmotically swollen cells. Indeed, direct measurements have demonstrated that hypotonic swelling produces an elevation of $[Ca^{2+}]_i$ in toad bladder cells (Chase and Wong, 1985).

Despite the accumulated evidence listed above, two important observations made in lymphoid cells are seemingly inconsistent with $[Ca^{2+}]_i$ mediation of RVD. Direct measurements using quin2, a fluorescent Ca^{2+} -sensitive probe, showed that unlike the report in bladder cells, no significant increase in $[Ca^{2+}]_i$ was detectable in peripheral blood lymphocytes treated hypotonically (Rink et al., 1983). Moreover, repeated attempts by several laboratories initially failed to demonstrate the presence of Ca^{2+} -activated K^+ channels in a variety of lymphoid cells by direct electrophysiological (patch-clamp) techniques (e.g., DeCoursey et al., 1985; Bregestovski et al., 1986; however, see Grissmer and Cahalan, 1989 and Schlichter and Mahaut-Smith, 1989 for recent abstracts reporting of Ca^{2+} -activated K^+ channels in lymphoid cells). Patch-clamping methods have, in addition, demonstrated that both quinine and trifluoperazine are powerful blockers of voltage-gated K^+ channels in lymphocytes, raising the possibility that this drug prevented RVD by inhibiting channels that were not Ca^{2+} activated. Therefore, the evidence supporting involvement of Ca^{2+} during volume regulation in lymphocytes is inconclusive.

The present study was prompted by two intervening developments that could shed light on the role of Ca^{2+} during RVD in lymphocytes: the discovery of potent,

more selective inhibitors of Ca^{2+} -activated K^+ channels and the introduction of more efficient Ca^{2+} -sensitive probes. In the earlier study (Rink et al., 1983), the occurrence of a $[\text{Ca}^{2+}]_i$ change upon cell swelling may have been obscured by the Ca^{2+} -buffering effect of quin2, since relatively large (millimolar) intracellular concentrations of this probe are required for reliable Ca^{2+} detection. This potential problem can be overcome using the more sensitive "second generation" Ca^{2+} indicators fura-2 or indo-1 (Grynkiewicz et al., 1985), which can be used at much lower cytosolic concentrations and therefore contribute less significantly to the intracellular Ca^{2+} buffering power. In this study, we used indo-1 to determine whether osmotic swelling can increase $[\text{Ca}^{2+}]_i$ in lymphocytes to the levels required to activate Ca^{2+} -gated K^+ channels. The presence of the latter and their role in RVD was assessed using charybdotoxin (CTX), a component of the venom of the scorpion *Leiurus quinquestriatus*. CTX was recently shown to be a potent, reversible blocker of Ca^{2+} -activated K^+ channels in several cell types (Miller et al., 1985; Hermann and Erxleben, 1987; Gimenez-Gallego et al., 1988). Unlike quinine and other blockers used earlier, which are effective only at concentrations in the micro- to millimolar range, CTX effectively blocks Ca^{2+} -activated K^+ channels in the nanomolar range (Miller et al., 1985; Hermann and Erxleben, 1987; Gimenez-Gallego et al., 1988). The toxin, however, is not perfectly selective and block of Ca^{2+} -independent K^+ channels has also been reported (Lewis and Cahalan, 1987; Cahalan and Lewis, 1988; Price et al., 1988). Our results indicate that $[\text{Ca}^{2+}]_i$ does not increase in swollen cells and that, though present, Ca^{2+} -gated K^+ channels are unlikely to mediate RVD in lymphocytes. Two other types of conductive K^+ pathways are proposed to underlie the loss of K^+ during volume regulation.

MATERIALS AND METHODS

Materials and Solutions

Bis(1,3-diethylthiobarbituric)trimethine oxonol (*bis*-oxonol), 3,3'-dipropylthiadicarbocyanine iodide [diS-C₃(5)], and the acetoxymethyl ester (AM) forms of indo-1 and 1,2-*bis*-(2-aminophenoxy)ethane-*N,N,N,N'*-tetraacetic acid (BAPTA) were obtained from Molecular Probes, Inc. (Eugene, OR). Ionomycin was from Calbiochem Behring Corp. (San Diego, CA). Three times recrystallized choline chloride, quinine, EGTA, gramicidin, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and medium RPMI 1640 (HCO_3^- free) were purchased from Sigma (St. Louis, MO). *N*-methyl-D-glucamine (NMG^+) was from Aldrich Chemical Co. (Milwaukee, WI). [^3H]2-deoxy-D-glucose, ^{86}Rb , and ^{36}Cl were from Amersham Corp. (Lachine, Quebec, Canada).

CTX was the very kind gift of Dr. C. Miller (Graduate Department of Biochemistry, Brandeis University, Waltham, MA). The toxin was prepared from the lyophilized venom of *Leiurus quinquestriatus* by the method described in Anderson et al. (1988). For most experiments, the eluate of the SP-Sephadex C-25 column was used without further purification. This preparation is ~43% pure CTX. For some studies we used a CTX preparation further purified by high pressure liquid chromatography (Anderson et al., 1988), which was $\geq 95\%$ pure. Similar results were obtained with both preparations when equimolar CTX concentrations were used.

Na^+ solution contained (in millimolar) 140 NaCl, 1 KCl, 1 MgCl_2 , 1 CaCl_2 , 10 glucose, and 20 HEPES-Na, pH 7.3. Where indicated, CaCl_2 was omitted and 0.1 mM EGTA was added.

NMG⁺ solution, choline⁺ solution, K⁺ solution, and Na-gluconate solution were prepared by isoosmotic replacement of NaCl by NMG-chloride, choline chloride, KCl, or Na-gluconate, respectively. In the latter case, the gluconate⁻ salts of K⁺, Ca²⁺, and Mg²⁺ were used and the total Ca²⁺ content was increased to obtain the same free Ca²⁺ concentration present in Na⁺ solution, as determined with a Ca²⁺-selective electrode (Orion Research Inc., Cambridge, MA). All media were adjusted to 295 ± 5 mosM with the major salt, using an Osmette freezing point osmometer. Stock solutions of ionomycin, gramicidin, and the acetoxymethyl esters of BAPTA and indo-1 were made up in dimethylsulfoxide and kept at -2°C.

Cell Isolation and Manipulation

Peripheral blood mononuclear cells (PBM) were isolated from fresh, heparinized blood from healthy human donors on a Ficoll-Isopaque gradient as described (Grinstein et al., 1982b). The cells were collected from the interface, washed twice in HEPES-buffered medium RPMI 1640 and resuspended at ~10⁷/ml in the same medium. The mononuclear cell population obtained by this method contains ~15% monocytes and a small number of platelets. These cells can be clearly discerned from the lymphocytes during electronic volume determinations and have been shown earlier not to contribute importantly to isotopic flux measurements. For some experiments, T and B cells were separated by E-rosetting, as described (Cheung et al., 1982). Thymic lymphocytes were isolated from male Wistar rats (130–200 g) as described earlier (Grinstein et al., 1984a). The cells were counted using the Coulter Counter and maintained at room temperature for up to 5 h in HEPES-buffered RPMI 1640 at a concentration of 3–5 × 10⁷ cells/ml. Where indicated, PBM were loaded with BAPTA and depleted of cytosolic Ca²⁺ by incubation in a Ca²⁺-free medium with 15 μM BAPTA-AM for 30 min at 37°C. The cells were then sedimented and resuspended in the indicated solution. In some experiments, intracellular Cl⁻ was depleted by incubating the cells for 60 min at 37°C in Cl⁻-free, Na⁺-gluconate solution.

Cell Volume Determinations

Cells were sized electronically using a Coulter Counter (model ZM; Coulter Electronics, Inc., Hialeah, FL) coupled to a Coulter Channelyzer and a flatbed plotter. Calibration was based on the use of fixed chicken red blood cells or latex beads of known diameter. Cell volumes were calculated from the median channel of the distribution generated by the Channelyzer. Incubation of the cells (2.5 × 10⁶) in media of different tonicity was carried out in small (0.5 ml) volumes, to minimize the amount of CTX required. Aliquots of this suspension were withdrawn at the indicated times and diluted in 7 ml of ice-cold media of identical tonicity, but without the inhibitors, immediately before sizing. Electronic sizing was completed within 30 s of dilution, and preliminary experiments indicated that the volume changes made after dilution were insignificant.

[Ca²⁺]_i Determinations

For [Ca²⁺]_i determinations, PBM suspensions (2.5 × 10⁷ cells/ml) were loaded with indo-1 by incubation with 1.5 μM of the AM precursor for 30 min at 37°C. The cells were then washed and suspended in fresh HEPES-buffered RPMI 1640. Aliquots of this suspension (2.5 × 10⁶ cells) were used for fluorimetric determinations within 2 h of loading. The cells were sedimented immediately before the measurement and resuspended in 1 ml of the medium indicated in the figure legends. [Ca²⁺]_i was measured using a fluorescence spectrometer (650-40; Perkin-Elmer Corp., Norwalk, CT) with excitation at 331 nm (3 nm slit) and emission at 410 nm (10 nm slit). Calibration of indo-1 fluorescence vs. [Ca²⁺]_i was performed with ionomycin and Mn²⁺, essentially as described (Nasmith and Grinstein, 1987), using a value of 250 nM for the dissociation constant of the indo-1/calcium complex.

Membrane Potential Measurements.

Membrane potential was estimated fluorimetrically using *bis*-oxonol or, in a few cases, diS-C₃(5). The fluorescence of the negatively charged oxonol dye was measured with excitation at 540 nm (2 nm slit) and emission at 580 nm (10 nm slit). Aliquots of the cell suspension (1.25×10^6 cells/ml, final) were added to the appropriate medium preequilibrated with 0.2 μ M *bis*-oxonol and the membrane potential was recorded as described by Rink et al. (1980). External calibration was made by adding gramicidin to cells suspended in isotonic media containing varying ratios of Na⁺ and NMG⁺. The membrane potential was then calculated assuming comparable rates of Na⁺ and K⁺ permeation through gramicidin, and/or that intracellular K⁺ exchanged rapidly with external Na⁺ through the ionophore (Grinstein et al., 1984a).

The positively charged cyanine dye diS-C₃(5) was used at 1 μ M in a suspension containing 1.25×10^6 cells/ml, as previously described (Grinstein et al., 1984a). Calibration of fluorescence vs. potential was made by setting the membrane potential equal to the K⁺ equilibrium potential using 1 μ M valinomycin. An intracellular K⁺ concentration of 145 mM, determined by flame photometry, was used to calculate the K⁺ equilibrium potential by the Nernst equation.

⁸⁶Rb and ³⁶Cl Efflux Determinations

For ⁸⁶Rb efflux determinations, lymphocytes were suspended in HEPES-buffered RPMI ($2.5\text{--}5 \times 10^7$ cells/ml) and loaded with the isotope by incubation with 5 μ Ci/ml for 60 min at 37°C. For experiments using Cl⁻-depleted cells, loading was carried out in Na-gluconate medium using otherwise comparable conditions. After loading, the cells were sedimented and resuspended in the indicated nonradioactive efflux solution, at a final concentration of $2.7\text{--}5.3 \times 10^6$ cells/ml. The initial isotope content was determined suspending ⁸⁶Rb-loaded cells into ice-cold efflux medium followed immediately by sedimentation through oil, as described below. The remaining samples were suspended at 21–23°C and after 5 min efflux was terminated by sedimenting the cells through a hydrophobic cushion composed of 3 parts corn oil and 10 parts dibutylphthalate. When the denser gluconate-containing solutions were used, the density of the cushion was modified accordingly by reducing the corn oil content to 2 volumes. After sampling and aspirating the overlying supernatant, the top part of the tube was rinsed with water, the oil phase was aspirated and the tip of the tube containing the pellet was cut off into a scintillation vial containing 0.75 ml distilled water. After vigorous vortexing the solubilized cells were counted using Ecolume (ICN, Biomedicals Inc., Irvine, CA) scintillation fluid. Cell integrity and recovery were monitored by double-labeling the cells with [³H]2-deoxy-D-glucose (5 μ Ci/ml), which is taken up by the cells and rapidly converted to the nondiffusible, nonmetabolizable [³H]2-deoxy-D-glucose-6-phosphate. Upwards of 95% of the tritium was recovered in all samples, indicating that cell loss during the manipulations was minimal.

For ³⁶Cl efflux determinations, $3\text{--}5 \times 10^7$ cells/ml were loaded in HEPES-buffered solution RPMI with 2 μ Ci/ml Na ³⁶Cl for 45 min at 37°C. After sedimentation, aliquots containing $2\text{--}3 \times 10^6$ cells/ml were suspended in 0.75 ml of the indicated efflux medium, with or without CTX, and incubated for 5 min at 21–23°C. The initial isotope content was determined by suspension of cells in ice-cold medium as described above. Cell separation, assessment of recovery, and scintillation counting were as described above for ⁸⁶Rb efflux.

Other Methods

Cell viability, measured by dye exclusion using trypan blue, was not significantly affected by any of the procedures or chemicals studied. Unless otherwise specified, all experiments were performed at room temperature (21–23°C). This enabled comparison with earlier data on

volume regulation in similar cells and with patch-clamping results, most of which were obtained at equivalent temperatures. Data are presented as representative traces of at least three similar experiments using cells from different donors, or as the mean \pm SE of the number of experiments indicated. The $[Ca^{2+}]_i$ and E_m traces illustrated proceed from left to right.

RESULTS

Ca^{2+} -induced Hyperpolarization: Effect of CTX

This section describes experiments designed to assess the presence of Ca^{2+} -gated K^+ channels in human PBM and the effect of CTX on their activation. For this purpose, membrane potential (E_m) was measured using *bis*-oxonol, while $[Ca^{2+}]_i$ was manipu-

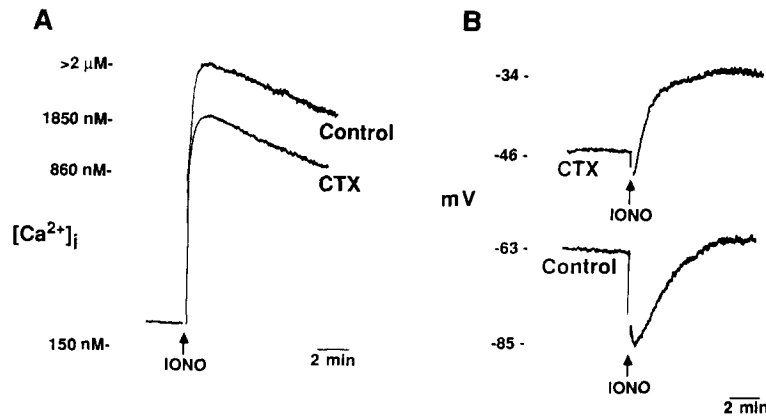


FIGURE 1. Ionomycin induces the elevation of $[Ca^{2+}]_i$ and a CTX-sensitive hyperpolarization in PBM. Human PBM were suspended in Ca^{2+} -containing Na^+ solution and $[Ca^{2+}]_i$ or E_m were measured using indo-1 and *bis*-oxonol, respectively, as described under Methods. Where indicated, 50 nM ionomycin was added. (A) $[Ca^{2+}]_i$ measurements. *Top trace*, control; *bottom trace*, cells preequilibrated with 25 nM CTX. The toxin did not affect the resting $[Ca^{2+}]_i$ significantly. (B) E_m determinations. The cells were preequilibrated with *bis*-oxonol in the presence (*top trace*) or absence (*bottom trace*) of 25 nM CTX. The calibration was obtained using gramicidin and varying the external $[Na^+]$, and is applicable to both traces. The traces are representative of eight experiments.

lated using the nonfluorescent ionophore, ionomycin. As shown in Fig. 1, addition of 50 nM ionomycin to cells suspended in Ca^{2+} -containing Na^+ solution induced a sizable, transient hyperpolarization, followed by a return towards the resting E_m . In eight determinations, the resting membrane potential averaged -65 ± 10.8 mV, while the peak hyperpolarization induced by ionomycin, attained generally within 1 min, reached -86 ± 2 mV.¹ The steady state E_m was reached within 6–8 min after addition of the ionophore, at -61 ± 3 mV. Addition of ionomycin did not produce a hyperpolarization in cells suspended in K^+ solution (not shown), suggesting that

¹ Because the relationship between E_m and the fluorescence of *bis*-oxonol becomes markedly non-linear below -80 mV, the determination of the peak hyperpolarization is imprecise. However, it is clear that the maximal E_m change exceeds 20 mV, approaching the K^+ equilibrium potential.

an outwardly directed K^+ gradient is required for the potential change, which is consistent with opening of K^+ channels by the elevated $[Ca^{2+}]_i$.

Fig. 1 illustrates the effects of 25 nM CTX on the ionomycin-induced E_m changes. This concentration of CTX was chosen because it is about two to fourfold higher than the K_i for inhibition of Ca^{2+} -activated K^+ channels in other cells, including lymphocytes (Grinstein and Smith, 1989). The presence of CTX almost completely eliminated the initial hyperpolarizing phase induced by ionomycin, despite the more positive resting E_m (-44 ± 1 mV vs. -65 mV in untreated cells). The residual hyperpolarization observed in the presence of CTX, though small, was observed consistently. The source and significance of the depolarization caused by CTX will be discussed in detail below. The toxin likely prevented the hyperpolarization by blocking the increase in K^+ conductance, as reported in other systems (Miller et al., 1985; Hermann and Erxleben 1987; Gimenez-Gallego et al., 1988) and not by preventing the ionomycin-induced increase in $[Ca^{2+}]_i$. Direct measurements using indo-1 demonstrated that, in both control and CTX-treated samples, addition of 50 nM ionomycin increased $[Ca^{2+}]_i$ to a level at least 10-fold greater than the resting concentration (Fig. 1). The levels attained with CTX were frequently somewhat lower than those in control cells, possibly due to stimulation of the Ca^{2+} pump by the depolarizing effect of CTX (Ishida and Chused, 1988). However, this small difference cannot account for the failure of the cells to hyperpolarize in the presence of the toxin, since marked hyperpolarizations were also found in control cells at lower $[Ca^{2+}]_i$, which were obtained by lowering the concentration of ionomycin (not illustrated). In addition to blocking the hyperpolarization induced by the Ca^{2+} ionophore, CTX unmasked a significant depolarization. In the presence of the toxin, E_m equilibrated at -32 ± 3 mV ($n = 6$) 4–6 min after addition of ionomycin. The biphasic nature of the response was confirmed using diS-C₃(5), a cationic fluorescent E_m probe, which yielded results that were very similar to those obtained with *bis*-oxonol (not illustrated). Taken together, these results suggest that the response of PBM to 50 nM ionomycin has two components: a CTX-sensitive hyperpolarization, likely reflecting Ca^{2+} -activated K^+ channels and a CTX-insensitive depolarizing component. The latter could result from inactivation of voltage-gated K^+ channels by Ca^{2+} , as described for both T and B lymphocytes (Bregestovski et al., 1986; Choquet et al., 1987; Cahalan and Lewis, 1988), or from an increase in conductance to other ions with an equilibrium potential more positive than E_m .

The causal relationship between $[Ca^{2+}]_i$ and the CTX-sensitive hyperpolarization was further investigated in cells loaded with BAPTA, an intracellular Ca^{2+} chelator. When such cells were suspended in Ca^{2+} -free media, the ionomycin-induced change in $[Ca^{2+}]_i$ was almost entirely abolished: the ionophore increased $[Ca^{2+}]_i$ from the baseline level of 156 ± 10 nM to only 178 ± 21 nM ($n = 4$). Under these conditions, the ionophore failed to induce a significant hyperpolarization. This indicates that a $[Ca^{2+}]_i$ increase is necessary for the appearance of the CTX-sensitive hyperpolarization, which is consistent with mediation by Ca^{2+} -gated K^+ channels.

Effect of CTX on Volume Regulation

Since the experiments described above demonstrated the susceptibility of Ca^{2+} -gated K^+ channels in PBM to inhibition by CTX, we proceeded to test whether the

toxin also impaired RVD. The results are summarized in Fig. 2 A. As reported earlier (Grinstein et al., 1982b), osmotically swollen PBM regain approximately normal volume within 10 min (*open squares*) and this response is precluded by quinine (75 μ M; *solid squares*), a K^+ channel blocker. At a concentration that prevents the ionomycin-induced hyperpolarization (i.e., 25 nM), CTX also inhibited RVD (*solid triangles*). The inhibition was not complete (75% when measured at 5 min) and could not be improved by further increasing the concentration of CTX. The incomplete nature of the inhibition was not due to cellular heterogeneity, as shown in Fig. 2 B. Analysis

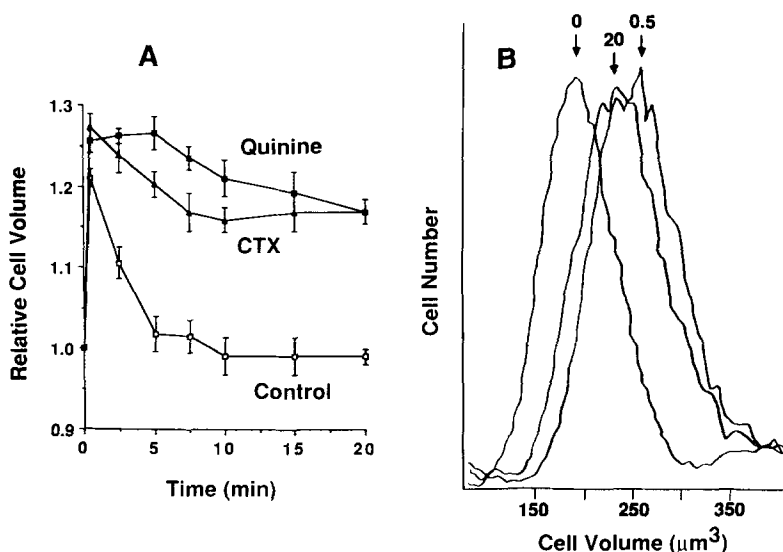


FIGURE 2. Inhibition of RVD by CTX. (A) Human PBM were initially suspended in isotonic Na^+ medium with (*solid symbols*) or without inhibitors (*open symbols*). The inhibitors used were quinine (75 μM ; *solid squares*) or CTX (25 nM; *solid triangles*). At zero time, the cells were subjected to hypotonic challenge ($0.60 \times$ isotonic). Cellular volume was estimated electronically from the median of the distribution obtained using the Coulter counter-Channelyzer combination. The points are means \pm SE of five experiments. The initial (isotonic) volume of PBM in Na^+ medium (295 ± 5 mosM) averaged $205 \mu\text{m}^3/\text{cell}$ and was not affected by the inhibitors. (B) Typical volume distributions of PBM at varying times after resuspension in hypotonic ($0.60 \times$ isotonic) Na^+ medium containing CTX. The distribution curves were obtained using a Coulter Channelyzer, as described under Methods. Representative distributions, obtained at the times (in minutes) indicated on the curves are illustrated.

of the distribution of cellular volumes using the Channelyzer revealed that the PBM population increased volume homogeneously upon hypoosmotic exposure and that partial volume restoration occurred in all cells, without appearance of distinct subpopulations with differential CTX sensitivity. These observations indicate that a CTX-sensitive pathway is largely responsible for RVD, but that other, CTX-resistant processes also contribute to the response.

Because RVD is driven by the outward K^+ gradient, PBM suspended in hypotonic, K^+ -rich solutions do not shrink back towards their original volume. Instead, as described earlier (Grinstein et al., 1982b) and shown in Fig. 3 A, the initial

osmotic swelling phase is followed by a secondary volume gain. The inhibitory effect of CTX on the volume-activated transport pathway was also discernible under these conditions. The secondary swelling component was greatly reduced by 25 nM CTX.

The experiments in Fig. 3 also indicate that the effect of CTX on volume regulation is attributable to inhibition of the K^+ pathway, without effect on the parallel increase in anion permeability. This was concluded from two observations. First, secondary swelling could be restored in CTX-treated cells by the addition of gramicidin, a cation-selective conductive ionophore (Fig. 3 A, *solid squares*). This suggests

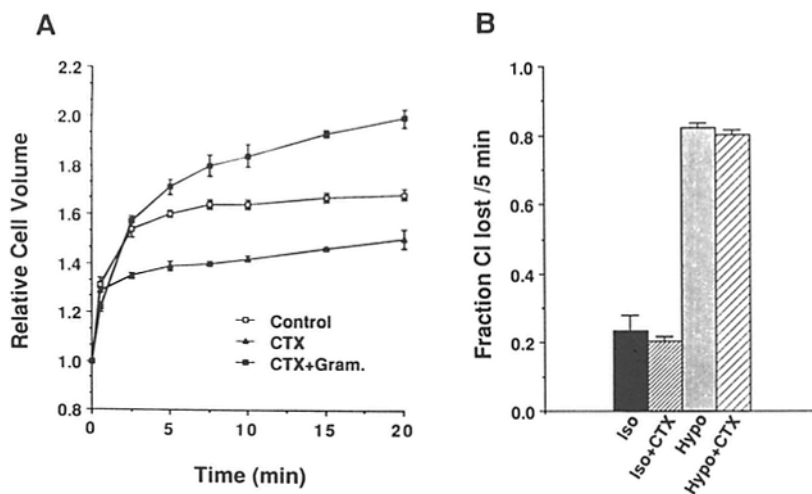


FIGURE 3. Specificity of the inhibitory effect of CTX. (A) Cell volume measurements in PBM suspended in K^+ -rich solution in the presence (*solid symbols*) or absence (*open symbols*) of 25 nM CTX. Where indicated (*solid squares*) the medium also contained 1 μ M gramicidin. At zero time, the medium was made hypotonic ($0.60 \times$ isotonic). Volume was measured electronically as described in Fig. 2. The data are means \pm SE of three experiments. Neither CTX nor gramicidin significantly altered the initial (isotonic) volume. (B) Measurement of ^{36}Cl efflux from PBM under isotonic or hypotonic conditions. Cells were preloaded with the isotope as described in Methods, sedimented and resuspended in isotonic or hypotonic medium in the presence or absence of 25 nM CTX, as indicated. After 5 min, the cells were sedimented and counted. The mean \pm SE of the fractional ^{36}Cl loss recorded in six experiments is reported.

that anion conductance remains elevated and that the block of K^+ transport exerted by CTX has been bypassed by the exogenous ionophore. Secondly, the large increase in the rate of unidirectional ^{36}Cl efflux elicited by hypotonicity (Grinstein et al., 1982a) was unaffected by CTX (Fig. 3 B). Taken together, these results indicate that CTX inhibits RVD by interfering with the loss of K^+ , without discernible effects on anion conductance.

Cytosolic Free Ca^{2+} and RVD

Inhibition of the ionomycin-induced hyperpolarization and of RVD by comparable concentrations of CTX is consistent with the involvement of Ca^{2+} -activated K^+ chan-

nels in both processes. As discussed in the Introduction, $[Ca^{2+}]_i$ could conceivably increase upon cell swelling, leading to the activation of K^+ channels. This hypothesis was tested using cells loaded with comparatively low concentrations of indo-1, to minimize the change in cytosolic Ca^{2+} buffering power (see Introduction). Typical results are shown in Fig. 4. No significant increase in $[Ca^{2+}]_i$ was detectable after hypotonic stress of PBM. In five experiments $[Ca^{2+}]_i$ after swelling averaged 184 ± 11.9 nM, which is not significantly different from the resting (isotonic) level of 169 ± 10.7 nM determined in parallel samples from the same donors. The contribu-

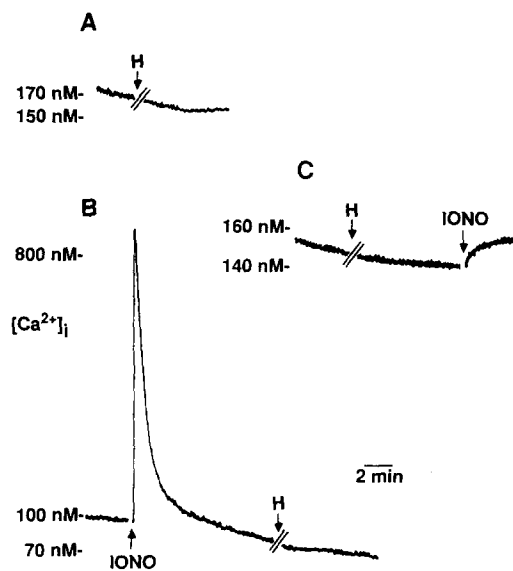


FIGURE 4. $[Ca^{2+}]_i$ determinations during RVD. Human PBM were loaded with indo-1 and used for determination of $[Ca^{2+}]_i$, as described in Methods. Calibration was made using ionomycin and Mn^{2+} . (A) Cells were suspended in isotonic Na^+ solution. Where indicated (H), the medium was made hypotonic ($0.60 \times$ isotonic) by the addition of water. The calibration was made *a posteriori* and therefore strictly applies only to the hypotonic period. Calibration of the isotonic segment of the trace was made in a parallel sample from the same donor, which was exposed to ionomycin and Mn^{2+} without prior hypotonic dilution. The constancy of $[Ca^{2+}]_i$ after hypotonic dilution was validated by diluting samples with an identical volume of isotonic medium.

The traces were virtually indistinguishable. (B) PBM were suspended in Ca^{2+} -free medium containing 0.1 mM EGTA. Where indicated, 0.5 μM ionomycin was added. The sample was next made hypotonic, as described for A. Calibration and alignment of the two segments of the trace were as in A. (C) Cells were double-loaded with indo-1 and BAPTA, to increase the cytosolic Ca^{2+} buffering capacity and then suspended in Ca^{2+} -free Na^+ medium containing 0.1 mM EGTA. Where indicated, the cells were challenged hypototically and subsequently treated with 0.5 μM ionomycin. Calibration and alignment of the two segments of the trace were as in A. Traces A–C are representative of at least four determinations, using blood from different donors.

tion of indo-1 to the intracellular buffering power probably cannot be held accountable for the failure to detect an increase in $[Ca^{2+}]_i$. This was concluded from experiments in which cells were suspended in Ca^{2+} -free solutions and then challenged with ionomycin (0.5 μM ; Fig. 4 B). Release of Ca^{2+} from intracellular stores produced a clearly discernible elevation in $[Ca^{2+}]_i$, from the baseline level of 120 ± 9 to 894 ± 10 nM ($n = 3$). This differs from the results obtained with quin2, in which case the response to ionomycin was largely obliterated (Rink et al., 1983) due to buffering by the fluorescent probe.

These results indicate that RVD is not accompanied by a measurable rise in $[Ca^{2+}]_i$. It is nevertheless possible that very small or localized $[Ca^{2+}]_i$ changes escaped detection, yet are necessary for RVD. The combined data in Figs. 4 and 5 argue against this possibility. As shown in Fig. 4 B, little change in $[Ca^{2+}]_i$ was recorded when hypotonic stress was imposed on cells pretreated with ionomycin while suspended in Ca^{2+} -free medium. In such cells Ca^{2+} entry from the medium is prevented and the membrane-bound intracellular Ca^{2+} stores have been depleted. Nevertheless, substantial volume regulation was observed under these conditions (Fig. 5). These observations suggest that flux of Ca^{2+} across the plasma membrane or internal membranes is not required for RVD. Mobilization of Ca^{2+} from ionomycin-insensitive stores (e.g., Ca^{2+} bound to intracellular proteins or lipids) could also conceivably be involved in RVD. Experiments using intracellular Ca^{2+} chelators, however, appear to rule out this possibility. As illustrated in Fig. 4 C, hypotonic stress did not modify $[Ca^{2+}]_i$ in cells double-loaded with indo-1 and the chelator

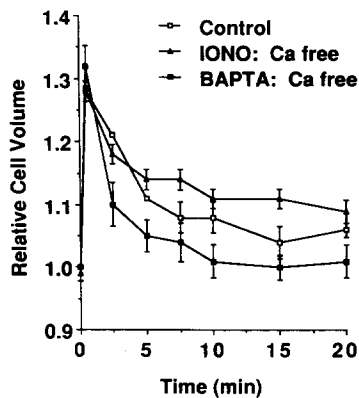


FIGURE 5. Effect of Ca^{2+} chelation and depletion on RVD. Cell volume changes were measured electronically in three groups of cells: untreated PBM (*open squares*), BAPTA-loaded cells (*solid squares*), and cells pretreated with $0.5 \mu M$ ionomycin for 5 min while suspended in Ca^{2+} -free medium with $0.1 mM$ EGTA (*triangles*). The cells were initially suspended in Na^+ solution containing Ca^{2+} (*open symbols*) or in Ca^{2+} -free medium with EGTA (*solid symbols*). At zero time, the medium was made hypotonic ($0.60 \times$ isotonic) and the volume was recorded at the indicated intervals using the Coulter-Channelyzer. Data are means \pm SE of three experiments.

BAPTA and suspended in Ca^{2+} -free solution. The effectiveness of the buffering procedure can be appreciated by comparing the effects of ionomycin in Figs. 4 B and C. Unlike the cells in Fig. 4 B, the large $[Ca^{2+}]_i$ transient elicited by the ionophore was greatly blunted in BAPTA-loaded cells (Fig. 4 C). Thus, if Ca^{2+} were released from intracellular sources during RVD, this comparatively small amount of the cation, incapable of altering the indo-1 signal, is expected to be readily buffered by BAPTA. Under these conditions, RVD was found to proceed at a rate and to an extent comparable to those of untreated cells (Fig. 5). Together, the experiments in Figs. 4 and 5 argue strongly against the requirement for a rise in $[Ca^{2+}]_i$ during RVD. This implies that the CTX-sensitive, Ca^{2+} -activated K^+ channels can be opened during RVD without changes in $[Ca^{2+}]_i$, or that other CTX-sensitive processes are responsible for volume regulation.

Effect of CTX on the Resting E_m

The experiments in Fig. 6 provide evidence for the existence of Ca^{2+} -independent, yet CTX-sensitive, K^+ channels in PBM. In other systems, including blood cells,

Ca^{2+} -dependent K^+ channels are largely inactive at physiological $[\text{Ca}^{2+}]_i$ (e.g., Simons, 1976; Gallin, 1986; see Schwarz and Passow, 1983 and Petersen and Maruyama, 1984 for reviews). Therefore, addition of CTX to otherwise untreated cells is expected to have little effect on the resting E_m . This was indeed found to be the case in rat thymocytes (Grinstein and Smith, 1989, and see below). However, in resting PBM the addition of 25 nM CTX induced a sizable depolarization (Fig. 6 A, bottom trace). In eight experiments E_m increased from the baseline level of -65 ± 3.5 to -44 ± 4 mV after CTX. The CTX-induced depolarization recorded with bis-oxonol was also observed using diS-C₃(5) (not shown). Because the resting E_m in PBM is dictated largely by K^+ (Grinstein et al., 1982a), the observed depolarization

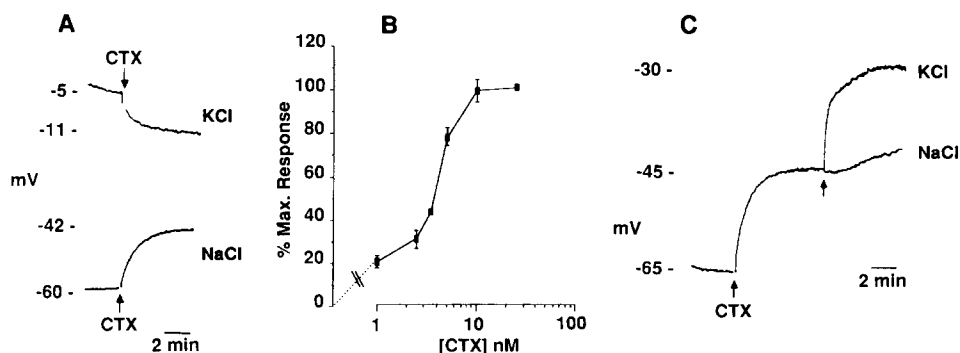


FIGURE 6. Effect of CTX on resting E_m . (A) Membrane potential measurements using bis-oxonol. PBM were suspended in Na^+ solution (bottom trace) or K^+ solution (top trace). Where indicated, 25 nM CTX was added. The calibration on the left was obtained as described for Fig. 1 and is applicable to both traces. The traces are representative of seven experiments. (B) Concentration dependence of the depolarizing effect of CTX. Cells were equilibrated with bis-oxonol in Na^+ solution and then the indicated concentration of CTX was added. The magnitude of the depolarization induced by the toxin was recorded and is expressed as a fraction of the maximal effect, obtained at 25 nM CTX ($\Delta E_m = 21$ mV). No further depolarization was induced by 50 nM CTX. The data are means \pm SE of three to six determinations. Where absent, the error bars were smaller than the symbol. (C) Cells were equilibrated with bis-oxonol in Na^+ medium. Where indicated, 25 nM CTX was added. Finally, 50 mM of either KCl (top trace) or NaCl (bottom trace) was added. The composite is representative of at least three determinations.

is likely due to decreased conductance to this ion. Accordingly, in cells suspended in K^+ -rich solution the resting potential is greatly reduced (to -4.5 ± 3 mV; $n = 4$) and addition of CTX results in a small hyperpolarization (to -8.3 ± 2.6 mV), bringing E_m closer to the equilibrium potential for Cl^- , the second most permeant ion under these conditions (Fig. 6 A, top trace). In addition, the CTX-induced E_m changes are probably not because of increased conductance to Na^+ , as they are observed in the absence of this ion (NMG⁺ solution, unpublished observations), or to Cl^- , inasmuch as the toxin had no effect on the flux of ^{36}Cl (Fig. 3). Thus, CTX depolarizes PBM by reducing K^+ conductance.

A detailed concentration dependence of the "spontaneous" depolarization elicited by CTX is presented in Fig. 6 B. Half-maximal effects were obtained at ~ 4 nM

CTX, which is similar to the concentrations of the toxin required to block K^+ channels in other cell types (Anderson et al., 1988; Gimenez-Gallego et al., 1988). Near-maximal effects were attained with 10 nM CTX and no further E_m change was observed with concentrations of up to 50 nM, despite the fact that the depolarization was not complete (i.e., E_m equilibrated at ~ -45 mV). The partial nature of the depolarization cannot be attributed to cellular heterogeneity. When either PBM or human tonsillar lymphocytes were fractionated, a spontaneous depolarization was induced by CTX in both purified T and B cells (not illustrated). Instead, the data suggest that multiple types of K^+ channels exist in most human PBM.

The spontaneous depolarization elicited by CTX in otherwise untreated PBM could be due to inhibition of Ca^{2+} -sensitive K^+ channels, which are open at resting $[Ca^{2+}]_i$. Alternatively, depolarization could result from blockade of a separate type of K^+ channels. In this regard, Lewis and Cahalan (1987; see also Cahalan and Lewis, 1988) and Price et al. (1988) have reported an inhibitory effect of CTX on two types of voltage-gated K^+ channels that are ostensibly Ca^{2+} -independent. To test whether Ca^{2+} -independent K^+ channels are responsible for the depolarization produced by CTX, the resting $[Ca^{2+}]_i$ levels of PBM were reduced using Ca^{2+} chelators and Ca^{2+} -free solutions. A reduction in $[Ca^{2+}]_i$ was anticipated to reduce the number of open Ca^{2+} -activated K^+ channels, without affecting Ca^{2+} -independent channels. In cells treated with 15 μ M BAPTA for 30 min while suspended in Ca^{2+} -free medium, $[Ca^{2+}]_i$ dropped 53% from the resting level of ~ 165 to 78 ± 7 nM. Under these conditions, the cells did not depolarize, suggesting that Ca^{2+} -activated K^+ channels do not contribute importantly to the establishment of the resting E_m .² More importantly, the CTX-induced depolarization persisted under these conditions. Although the existence of Ca^{2+} -activated K^+ channels with very high Ca^{2+} affinity cannot be ruled out, the results suggest the presence of Ca^{2+} -independent, CTX-sensitive K^+ channels in PBM.

As described above, the membrane potential is not totally depolarized even in the presence of maximally effective doses of CTX. The determinants of the residual E_m recorded under these conditions were investigated. As shown in Fig. 6 C, elevation of the external KCl concentration after the addition of 25 nM CTX produced a pronounced depolarization. In contrast, an equimolar addition of NaCl had comparatively little effect on E_m , ruling out nonspecific effects due to changes in Cl^- concentration, osmolarity, or ionic strength. Detailed studies of the cation concentration dependence of E_m in cells treated with saturating concentrations of CTX indicated that the residual membrane potential was still markedly dependent on the K^+ distribution. The K^+ transference number, calculated in the 17.5–70 mM range as

$$T_{K^+} = \Delta E_m / (RT/F) \ln([K^+]_i/[K^+]_f)$$

where ΔE_m is the potential change recorded upon varying the concentration of K^+ from its initial ($[K^+]_i$) to its final ($[K^+]_f$) value, decreased from 0.93 in untreated cells to 0.57 after 25 nM CTX. It therefore appears that three different types of K^+

² BAPTA-loaded cells appeared to undergo a significant hyperpolarization. It is difficult to ascertain whether this apparent E_m change is real or a consequence of an interaction between the chelator and the fluorescent potential probe.

conductances exist in PBM: Ca^{2+} -activated and CTX-sensitive channels, a second type of CTX-sensitive but seemingly Ca^{2+} -independent channels, and CTX-insensitive channels.

Effects of CTX on E_m and Volume Regulation in Thymocytes

In human PBM, volume recovery after hypotonic stress was found to be greatly inhibited by CTX (Fig. 2). However, because CTX-sensitive Ca^{2+} -independent as well as Ca^{2+} -dependent K^+ channels appear to exist in these cells, it is not clear whether the latter type of channel is responsible for RVD, as suggested originally.

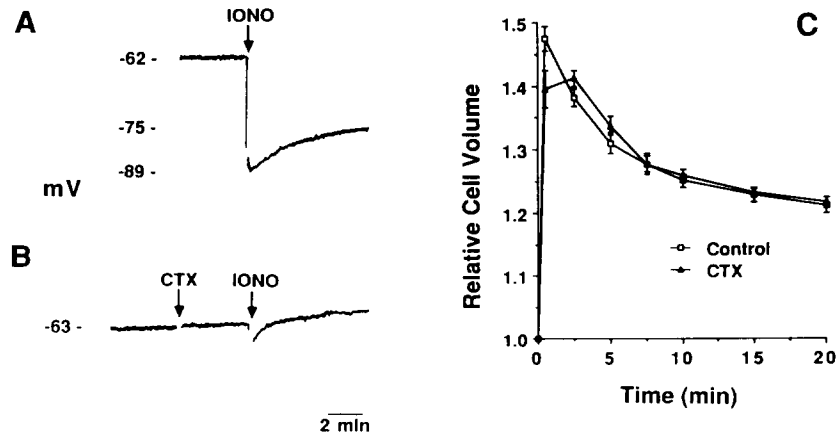


FIGURE 7. Effect of CTX on E_m and volume regulation in rat thymic lymphocytes. *A* and *B* are membrane potential measurements using *bis-oxonol*. The traces are representative of seven experiments. (*A*) Thymocytes were suspended in Ca^{2+} -containing Na^+ solution and, where indicated, $[\text{Ca}^{2+}]_i$ was increased by the addition of 50 nM ionomycin. (*B*) Cells suspended in Ca^{2+} -containing Na^+ solution. CTX (25 nM) was added where noted. Next, $[\text{Ca}^{2+}]_i$ was increased by the addition of 50 nM ionomycin. The change in $[\text{Ca}^{2+}]_i$, measured in parallel with indo-1, was comparable under the conditions used in *A* and *B*. (*C*) RVD in thymocytes. Cells were suspended in isotonic Na^+ solution with (solid triangles) or without (open squares) 25 nM CTX. At zero time the cells were subjected to hypotonic challenge ($0.6 \times$ isotonic) and their volume was monitored at the intervals indicated, as described for Fig. 2. Data are means \pm SE of four determinations. The initial (isotonic) volume of thymocytes averaged $114 \mu\text{m}^3$.

Indeed, our failure to measure increases in $[\text{Ca}^{2+}]_i$ during RVD suggests a Ca^{2+} -independent mechanism. Comparison of the CTX concentration dependence of the inhibition of RVD with those of the ionomycin-induced hyperpolarization and the spontaneous depolarization of the resting E_m failed to resolve these alternatives. All three processes were affected by CTX in the low nanomolar range (not shown).

We obtained evidence against the involvement of Ca^{2+} -activated K^+ channels in RVD from studies in lymphocytes from another source, namely, the rat thymus. As shown in Fig. 7, and as described elsewhere in more detail (Grinstein and Smith, 1989), elevation of $[\text{Ca}^{2+}]_i$ with ionomycin also results in a marked hyperpolarization in these cells.¹ The occurrence of a hyperpolarization was validated with diS-

C₃-(5) and indirectly by measurements of cytosolic pH (Grinstein and Smith, 1989). As in the case of PBM, the E_m change depends on the outward K⁺ gradient and its shape and duration closely resemble the changes in [Ca²⁺]_i (not illustrated). As shown in Fig. 7 B, the hyperpolarization was virtually abolished by CTX (apparent $K_i = \sim 8$ nM). The toxin did not affect the rise in [Ca²⁺]_i produced by the ionophore. Importantly, comparable and even higher (up to 50 nM) concentrations of CTX had no noticeable effect on the resting E_m (Fig. 7 B), indicating that in the thymocytes Ca²⁺-activated K⁺ channels do not contribute significantly to the resting potential.³ Similar results have been reported for a T cell hybridoma by Wilson et al. (1988). This finding also suggests that, if present, Ca²⁺-independent CTX-sensitive channels are much less abundant in thymocytes than in PBM. This difference in the density of CTX-sensitive Ca²⁺-dependent and -independent channels provided an indirect means for testing the role of these channels in RVD. As illustrated in Fig. 7 C, thymic lymphocytes can also regulate their volume after hypotonic challenge, but the rate and extent of the response are less than those observed in PBM. Unlike PBM, the RVD in thymocytes was insensitive to CTX, at concentrations that fully inhibit Ca²⁺-activated K⁺ channels. Moreover, the secondary swelling observed in K⁺-rich hypotonic solutions was also insensitive to CTX (not illustrated). These observations suggest that, though present in thymocytes, CTX-sensitive Ca²⁺-activated K⁺ channels do not play an important role in volume regulation in these cells. Moreover, it is possible to speculate that the more efficient and rapid RVD displayed by human PBM is attributable to the existence in these cells (but not in thymocytes) of the CTX-sensitive but Ca²⁺-independent K⁺ channels that mediate the depolarization of the resting E_m induced by the toxin. These channels seem to be scarce or absent in thymocytes, perhaps accounting for their incomplete RVD.

Does Depolarization Activate Voltage-gated K⁺ Channels during RVD?

Earlier work had demonstrated that independent, conductive K⁺ and Cl⁻ pathways mediate RVD in lymphoid cells (see Grinstein et al., 1984b for review). The relationship between these pathways and the temporal sequence of events during RVD is not well understood. However, Deutsch and her collaborators (Deutsch et al., 1986; Lee et al., 1988) and later Cahalan and Lewis (1988) suggested that opening of Cl⁻ channels by cell swelling may be the initial response. Indeed, stretch-activated Cl⁻ channels have been reported in lymphoid cells (Cahalan and Lewis, 1988). Opening of anion-selective channels would depolarize the cells, by bringing E_m closer to the Cl⁻ equilibrium potential. This E_m change has been hypothesized to lead to activation of voltage-gated K⁺ channels, providing a pathway for the efflux of a counterion to Cl⁻ (Lee et al., 1988; Cahalan and Lewis, 1988). Our data, pointing to involvement of Ca²⁺-independent K⁺ channels in RVD are consistent with this model. Moreover, Cahalan and collaborators (Lewis and Cahalan, 1987; Cahalan and Lewis, 1988) as well as Price et al. (1988) have recently reported the existence of CTX-sensitive voltage-gated K⁺ channels in murine and human lymphocytes. We

³ It is noteworthy that the resting [Ca²⁺]_i of human blood lymphocytes and of rat thymic lymphocytes was not significantly different. When measured with indo-1, [Ca²⁺]_i was ~ 160 nM in both cell types, whereas a lower value (~ 100 nM) was obtained using quin2.

therefore tested the hypothesis that the Cl^- -mediated depolarization accounts for the activation of K^+ efflux during volume regulation.

Fig. 8 A illustrates the depolarization that accompanies RVD in human PBM. Increased *bis*-oxonol fluorescence, indicative of depolarization, was obtained when the cell suspension was diluted with hypotonic, but not with isotonic medium. That

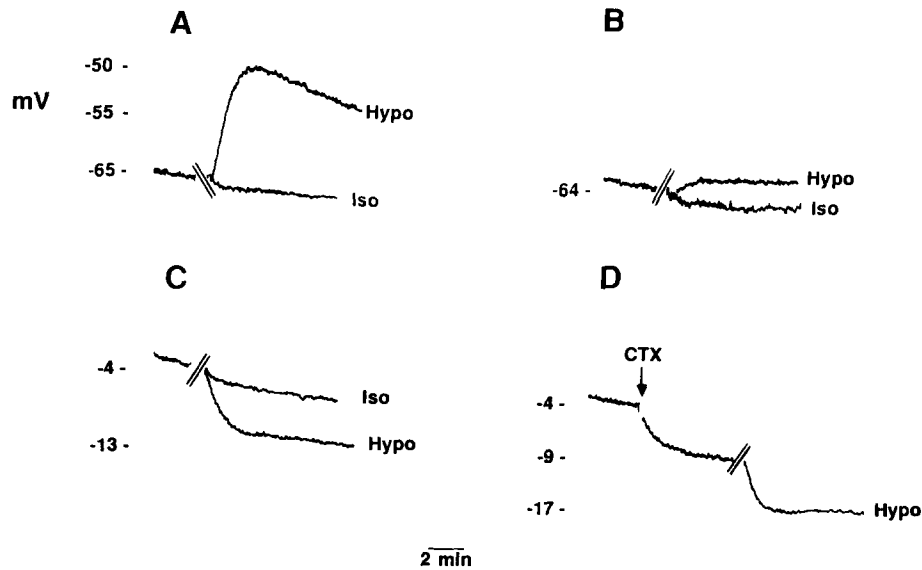


FIGURE 8. Cl^- -dependent E_m changes during RVD. (A) PBM were suspended in isotonic Na^+ solution. Where indicated by the break in the trace, one aliquot of cells was diluted with water to reduce the osmolarity of the medium to $0.6 \times$ isotonic (*top trace*). A second aliquot was diluted with a comparable volume of isotonic medium (*bottom trace*). The calibration strictly applies only to the segment of the trace after dilution. Alignment with the initial portion of the trace was made based on the assumption that E_m does not change upon dilution in homologous medium. This assumption is borne out by the fact that the decrease in fluorescence is essentially that expected from dilution of the fluorophore. The initial rapid fluorescence decrease upon dilution was identical for isotonic and hypotonically-treated cells, indicating that no instantaneous E_m (fluorescence) change occurred and that the alignment of the traces is valid. (B) PBM were Cl^- -depleted by incubation for 60 min at 37°C in Cl^- -free, gluconate $^-$ medium. The cells were then equilibrated with *bis*-oxonol in isotonic Cl^- -free solution. At the break, the cells underwent dilution with hypotonic (*top trace*) or isotonic (*bottom trace*) Cl^- -free solution, as described for A. (C) PBM were suspended in isotonic K^+ solution. At the break, the cells underwent dilution with hypotonic (*bottom trace*) or isotonic (*top trace*) K^+ solution, as described for A. (D) PBM suspended in K^+ solution. At the arrow, 25 nM CTX was added. Then, at the break, the medium was made hypotonic ($0.60 \times$ isotonic) by the addition of water. The traces in A–D are representative of at least four determinations. All E_m scales (ordinate) are in millivolts. The time scale is applicable to all traces.

this depolarization was due to increased anion conductance could be demonstrated using Cl^- -depleted cells suspended in Cl^- -free (gluconate $^-$) solution (Fig. 8 B). Under these conditions, hypotonic dilution induced only a marginal E_m change. Further evidence was obtained in cells suspended in K^+ -rich medium (Fig. 8 C). Such cells are initially depolarized, but undergo a significant repolarization upon hypo-

tonic dilution, as would be expected for an increase in Cl^- conductance, since E_{Cl^-} has been estimated to be in the range of -20 to -40 mV in these cells (Grinstein and Dixon, 1989). The selectivity of the inhibitory effect of CTX and the independence of the K^+ and Cl^- pathways was also verified in these experiments. As shown in Fig. 8 D, the hypotonically induced E_m change attributed to Cl^- channels was also detectable in cells pretreated with CTX. These results indicate that opening of Cl^- channels can occur despite the inhibition of the K^+ conductance.

Measurements of efflux of ^{86}Rb , a K^+ analogue transported by the volume-activated pathways, suggest that depolarization induced by increasing the Cl^- conductance is not the sole mechanism responsible for opening K^+ channels during RVD. This was concluded from experiments in which Cl^- induced depolarization was prevented by Cl^- depletion or by incubating the cells in high K^+ medium. As shown in Fig. 8, under these conditions the membrane potential undergoes little change or actually becomes more negative, respectively, upon cell swelling. We therefore

TABLE I
Effect of Hypotonic Stress on ^{86}Rb Efflux in PBM

Condition	Fraction ^{86}Rb lost/5 min			n
	Pretreatment	Mean	SE	
KCl isotonic	—	0.160	0.028	7
KCl hypotonic	—	0.321	0.018	7
Na-gluconate isotonic	Cl^- depleted	0.030	0.020	4
Na-gluconate hypotonic	Cl^- depleted	0.106	0.023	4

Cells were preloaded with ^{86}Rb in isotonic solution. Where indicated, the cells were depleted of Cl^- by incubation in Na-gluconate medium for 60 min at 37°C . In this case, ^{86}Rb loading was simultaneous with the Cl^- depletion. Cl^- -containing cells were then sedimented and resuspended in either isotonic or hypotonic ($0.60 \times$ isotonic) K^+ medium. Cl^- -depleted cells were resuspended in isotonic or hypotonic Na-gluconate medium. After 5 min, the cells were sedimented. The radioactivity present in the cells before and after the 5-min incubation was determined and the data are expressed as the fractional loss of ^{86}Rb over this period. n = number of experiments, each with duplicate determinations.

tested whether K^+ (^{86}Rb) permeability could still be increased by the volume changes under these conditions. The results are summarized in Table I. A significant increase in the rate of ^{86}Rb efflux was observed in cells treated with hypotonic K^+ medium, despite the concomitant hyperpolarization⁴ (Fig. 8 C). Similarly, ^{86}Rb permeability also increased upon hypotonic challenge in Cl^- -depleted cells suspended in Cl^- -free solution. The lower rate of efflux in the latter set of experiments is likely due to the unavailability of a counterion for ^{86}Rb (K^+) efflux. Under these conditions the isotope can only exit the cells accompanied by a minor anion or in exchange for extracellular K^+ , which is present at low concentrations. Together, these results indicate that depolarization due to opening of stretch-activated Cl^- channels is not the only mechanism whereby Rb^+ (K^+) fluxes are stimulated during RVD.

⁴ It is conceivable that, when the cells hyperpolarize upon swelling, the $^{86}\text{Rb}^+$ (K^+) flux increased due to relief of the voltage-dependent inactivation of the channels.

DISCUSSION

Three Types of K⁺ Channels in Lymphocytes

The data presented above suggest the existence of at least three distinct K⁺-conductive pathways in lymphocytes: a Ca²⁺-activated, CTX-sensitive channel, a Ca²⁺-independent but similarly CTX-sensitive channel, and a CTX-insensitive channel. The evidence documenting the existence and properties of these pathways is summarized below. Both PBM and thymic lymphocytes displayed a hyperpolarization when [Ca²⁺]_i was increased upon addition of ionomycin. The onset of the hyperpolarization, which was dependent on the outward K⁺ gradient, closely paralleled the increase in [Ca²⁺]_i, suggesting a causal relationship. Together, these observations point to the existence of Ca²⁺-activated K⁺ channels in the lymphocyte membrane. Consistent with this conclusion, the ionomycin-induced hyperpolarization was inhibited by CTX, a potent blocker of Ca²⁺-activated K⁺ channels in other cells (Miller et al., 1985; Hermann and Erxleben, 1987; Gimenez-Gallego et al., 1988). The present observations are also consistent with earlier measurements of hyperpolarization in splenocytes and other lymphoid cells under conditions where [Ca²⁺]_i was known or expected to increase (see Rink and Deutsch, 1983 for review), and with the report of Ca²⁺ ionophore-stimulated efflux of ⁸⁶Rb from PBM (Grinstein et al., 1982b). Considering the accumulated evidence favoring the existence of Ca²⁺-activated K⁺ channels in lymphoid cells, the failure of several laboratories to detect their presence by patch clamping (e.g., Bregestovski et al., 1986; DeCoursey et al., 1985; Choquet et al., 1987) is puzzling. It is possible that the single-channel conductance is very small and was obscured by noise during electrophysiological recordings. Alternatively, the channels or the events leading to their activation might have been impaired by disruption of the membrane while recording in the whole-cell or excised-patch modes, the preferred recording configurations used thus far in lymphocytes. Patch clamping in the cell-attached mode, which has been used sparingly with lymphoid cells, may clarify this apparent discrepancy in the future.

Addition of CTX to resting, unstimulated PBM in Na⁺ solution produced a spontaneous, sizable depolarization. This E_m change was also observed in Na⁺-free media and in Cl⁻-depleted cells, ruling out an effect of the toxin on the permeability to these ions and suggesting that the depolarization was due to inhibition of K⁺ conductance. Consistent with this view, CTX elicited a small hyperpolarization when added to cells suspended in K⁺-rich medium. The conductance blocked by CTX in otherwise untreated PBM could represent Ca²⁺-activated K⁺ channels that are open at physiological [Ca²⁺]_i. However, in other blood cells, such channels are usually inactive in resting cells, i.e., at normal [Ca²⁺]_i and E_m (Simons, 1976; Gallin, 1986). Moreover, the depolarizing effect of CTX persisted even in cells that were partially depleted of cytosolic Ca²⁺ by preincubation in Ca²⁺-free medium and intracellular accumulation of the Ca²⁺ buffer, BAPTA. Thus, while the possibility that K⁺ channels activated by very low [Ca²⁺]_i are present in PBM cannot be discounted, it is more likely that the depolarization is attributable to a distinct population of CTX-sensitive, Ca²⁺-independent channels. In fact, the existence of two types of CTX-sensitive, voltage-gated, and ostensibly Ca²⁺-insensitive K⁺ channels (labeled n and n') was reported in murine lymphocytes by Lewis and Cahalan (1987; see also

Cahalan and Lewis, 1988). If open under resting conditions, such channels could account for CTX-induced depolarization recorded in human PBM.

The depolarization induced by 25 nM CTX was not complete; E_m equilibrated at -44 mV and higher CTX concentrations (up to 50 nM) produced no further depolarization. Studies of the cation concentration dependence of E_m in cells treated with saturating concentrations of CTX indicated that the residual membrane potential was still markedly dependent on the K^+ distribution. Thus, the membrane retained a considerable CTX-insensitive K^+ conductance, distinct from the two types of channels described above, which are inhibited by the toxin. This residual K^+ conductance may represent the CTX-insensitive, voltage-gated I channel described by Lewis and Cahalan (1987; Cahalan and Lewis, 1988). In thymocytes, the CTX-resistant channel(s) are largely responsible for the resting E_m , which is practically unaffected by the addition of the toxin. Taken together, the data suggest the existence of three types of K^+ channels in human PBM. Because PBM populations are known to be heterogeneous in terms of lineage, it is presently not clear whether all three channel types are present in each cell, or if different subpopulations of cells selectively express a particular type of channel. However, in preliminary experiments, no gross differences were observed when comparing purified B and T cells from peripheral blood and from human tonsils.

K⁺ Channels Activated during RVD

Ca^{2+} has been suggested to mediate RVD in several biological systems (Cala, 1983; Chase and Wong, 1985; Christensen, 1987). The evidence supporting this notion is probably strongest in epithelial cells, where external Ca^{2+} appears to be essential for RVD. Cytosolic free Ca^{2+} has been reported to increase after osmotic swelling (Chase and Wong, 1985), possibly because of entry through stretch-activated Ca^{2+} -transporting channels (Christensen, 1987). The increased $[Ca^{2+}]_i$ is thought to activate K^+ efflux through Ca^{2+} -gated channels, which have been detected electrophysiologically (Christensen, 1987). A similar mechanism was originally proposed to operate in lymphoid cells (Grinstein et al., 1982b), based on pharmacological evidence and on the effects of Ca^{2+} ionophores (see Introduction). However, the evidence summarized below indicates that this hypothesis is no longer tenable.

Direct measurements of $[Ca^{2+}]_i$ using comparatively low concentrations of indo-1 failed to demonstrate any significant increase during RVD. Under comparable conditions, release of the intracellular stores by ionomycin was easily detectable, illustrating the sensitivity of the method. It is nevertheless conceivable that mobilization of small amounts of Ca^{2+} , sufficient to elicit RVD, remained undetected. This possibility is deemed unlikely for two reasons: RVD was unaffected when cells were first treated with ionomycin in Ca^{2+} -free media in order to deplete Ca^{2+} contained within membrane-bound compartments. Moreover, if the buffering power of indo-1 was responsible for our inability to detect increased $[Ca^{2+}]_i$, inhibition of RVD would be expected to accompany the introduction of comparable or higher concentrations of Ca^{2+} buffers. However, greatly increasing the cytoplasmic buffering power using BAPTA failed to prevent RVD, even when the cells were suspended in Ca^{2+} -free media. Under these conditions, the Ca^{2+} released from internal stores by ionomycin was readily buffered.

In the absence of a detectable change in $[Ca^{2+}]_i$, two possibilities can be envisaged to account for the susceptibility of RVD to inhibition by CTX in human lymphocytes. First, it is possible that the CTX-sensitive Ca^{2+} -activated K^+ channels can be activated at constant $[Ca^{2+}]_i$ during cell swelling. This could occur, for instance, if the Ca^{2+} affinity of the channels increased in response to membrane stretching. This hypothesis, however, cannot explain the failure of CTX to inhibit RVD in thymic lymphocytes, even though these cells clearly display Ca^{2+} -activated K^+ channels. An alternative model would implicate the Ca^{2+} -insensitive CTX-inhibited channels in RVD in human PBM. These channels, which are probably responsible for the spontaneous depolarization produced by CTX (see above), are present in PBM but not in thymic cells, which is consistent with the pattern of sensitivity of RVD to the toxin.

A fraction of the RVD response in human PBM and practically all the RVD in thymocytes were unaffected by the presence of CTX. These responses were nevertheless due to movement of K^+ down its electrochemical gradient, as evidenced by the secondary swelling recorded in high K^+ solution. Therefore, during RVD, K^+ appears to be exiting the cells by a third type of pathway, perhaps similar to the Ca^{2+} -independent and CTX-resistant channels that are partly or totally responsible for establishing the resting E_m in PBM and thymocytes, respectively. Interestingly, the contribution of the latter type of channels to the resting E_m is proportional to the fraction of CTX-insensitive RVD in the two cell types. This may simply reflect the relative abundance of these channels. The participation of voltage-gated and seemingly Ca^{2+} -insensitive K^+ channels during RVD was proposed earlier by Deutsch and her collaborators (Deutsch et al., 1986; Lee et al., 1988). These authors found that, in a mouse T cell line, the magnitude of the RVD response increased markedly when the cells were stimulated with interleukin 2. The improved volume regulation correlated with an increased expression of voltage-gated K^+ channels, prompting the suggestion that such channels are also involved in RVD (Lee et al., 1988).

As reviewed in the Introduction, evidence obtained earlier using quinine, EGTA, and Ca^{2+} ionophores was suggestive of a role for Ca^{2+} -activated K^+ channels during RVD, in contrast to the conclusion reached in the present manuscript. Further studies have led to a different interpretation and have provided alternative explanations. First, patch-clamping experiments have since shown that, in addition to the reported block of Ca^{2+} -activated K^+ channels, quinine is also a powerful inhibitor of Ca^{2+} -independent K^+ channels (DeCoursey et al., 1984; Matteson and Deutsch, 1984), such as those proposed here to mediate RVD. Secondly, the observation that cells can shrink after the addition of Ca^{2+} ionophores is consistent with the opening of Ca^{2+} -activated K^+ channels. The existence of such channels and their sensitivity to CTX was confirmed in this report. Volume loss is expected to ensue, provided that the cells have a substantial conductive permeability to Cl^- and/or that Cl^- conductance also increases due to Ca^{2+} , as has been demonstrated for other cells (Hoffmann, 1985, 1986). These observations, however, are not in disagreement with activation of alternative, Ca^{2+} -independent K^+ and Cl^- pathways during RVD. Finally, the inhibition of RVD observed after prolonged incubation in the presence of EGTA could be due to depletion of intracellular K^+ . Quastel et al. (1981) described

an increase in monovalent cation permeability in lymphocytes incubated with divalent cation chelators. Continued incubation under such conditions could be expected to result in exchange of intracellular K^+ for Na^+ , reducing the force driving RVD. Other nonspecific effects of EGTA could also have occurred.

Cahalan and Lewis (1988) recently described the existence of volume-sensitive Cl^- channels in lymphocytes. Such channels presumably account for the increased Cl^- conductance observed in osmotically swollen PBM (Grinstein et al., 1982a and Figs. 3 and 8). Under these conditions, the Cl^- permeability becomes greater than that of K^+ and, as a result, E_m depolarizes, approaching the Cl^- equilibrium potential. Deutsch and collaborators (Deutsch et al., 1986; Lee et al., 1988) and later Cahalan and Lewis (1988) suggested that this depolarization could in turn activate voltage-sensitive K^+ channels, providing a pathway for efflux of the counterion required for volume loss. However, this explanation cannot fully account for the increase in K^+ permeability, for the following reasons. First, swelling increased K^+ ($^{86}Rb^+$) efflux in cells that were already depolarized by resuspension in high K^+ solution.⁴ When osmotically swollen, such cells undergo a slight hyperpolarization, as E_m moves towards the more negative E_{Cl^-} (Fig. 8). In addition, a small yet significant increase in $^{86}Rb^+$ efflux was also observed when Cl^- -depleted cells were suspended in hypotonic Cl^- -free medium. Under these conditions, swelling is not accompanied by depolarization (Fig. 8). Hence, even though opening of K^+ channels by depolarization may, and in fact is expected to contribute to the overall increase in K^+ permeability during RVD, potential-independent activation processes must also exist. Like the Cl^- channel, K^+ channels may also be activated directly by stretching of the membrane. Recent patch-clamping studies by Sackin (1987) have shown that renal cells from *Necturus* possess a basolateral K^+ -selective channel that is reversibly activated by negative hydrostatic pressures of -9 to -15 mmHg at constant voltage. A similar channel may underlie at least part of the response in PBM. Membrane stretching may activate the channels directly or through more elaborate mechanisms, perhaps involving the cytoskeleton, as suggested for skeletal muscle channels (Guharay and Sachs, 1984), and/or fusion of intracellular vesicles with the plasma membrane (Lewis and DeMoura, 1982). The occurrence of vesicular fusion during RVD in the urinary bladder has been inferred from capacitance measurements (Lewis and DeMoura, 1982) and from the appearance of antigenic determinants in the surface membrane (Novak et al., 1988). However, there is no direct evidence that incorporation of vesicular components is directly related to volume regulation (Novak et al., 1988).

In summary, two types of K^+ channels, distinguishable by their sensitivity to CTX, can be activated during RVD in lymphoid cells. Though Ca^{2+} -activated K^+ channels are present in these cells, the increased K^+ permeability responsible for RVD does not appear to be accompanied by changes in $[Ca^{2+}]_i$. Depolarization-induced opening of voltage-gated K^+ channels is likely to contribute to the permeability change. However, additional modes of K^+ channel activation must also be invoked. These alternative mechanisms remain to be defined.

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