

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of the collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE
1994

3. REPORT TYPE AND DATES COVERED
Reprint

4. TITLE AND SUBTITLE

(see title on reprint)

AD-A283 009

5. FUNDING NUMBERS

PE: NWED QAXM

WU: 00020

6. AUTHOR(S)

Gallin et al.



7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Armed Forces Radiobiology Research Institute
8901 Wisconsin Ave.
Bethesda, MD 20889-5603

8. PERFORMING ORGANIZATION
REPORT NUMBER

SR94-10

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Uniformed Services University of the Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20814-4799

10. SPONSORING/MONITORING
AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

11P

94-24129



12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

13. ABSTRACT (Maximum 200 words)

DTIC
ELECTE
AUG 01 1994
S B D

14. SUBJECT TERMS

15. NUMBER OF PAGES

9

16. PRICE CODE

17. SECURITY CLASSIFICATION
OF REPORT

UNCLASSIFIED

18. SECURITY CLASSIFICATION
OF THIS PAGE

UNCLASSIFIED

19. SECURITY CLASSIFICATION
OF ABSTRACT

20. LIMITATION OF
ABSTRACT

94 7 29 061

SECURITY CLASSIFICATION OF THIS PAGE

CLASSIFIED BY:

DECLASSIFY ON:

SECURITY CLASSIFICATION OF THIS PAGE

Characterization of Regulatory Volume Decrease in the THP-1 and HL-60 Human Myelocytic Cell Lines

ELAINE K. GALLIN,* TONI M. MASON, AND ARIE MORAN

Department of Physiology, Armed Forces Radiobiology Research Institute, Bethesda, Maryland 20889-5603 (E.K.G., T.M.); Department of Physiology, Faculty of Health Sciences, University of Negev, Beer Sheva, Israel 84105 (A.M.)

Exposure to hypotonic stress produces a transient increase in cell volume followed by a regulatory volume decrease (RVD) in both THP-1 and HL-60 cells. In contrast, cells exposed to hypotonic stress in a high K/low Na Hanks' solution not only failed to volume regulate, but displayed a secondary swelling. Thus, while an outward K gradient was required for RVD, the secondary swelling indicated that hypotonic stress increased permeability in the absence of a negative membrane potential. The K channel blocker quinine (1–4 mM) blocked RVD in both cell types. Gramicidin's ability to overcome the quinine block of RVD indicated that RVD is mediated by a quinine-sensitive cation transport mechanism that is independent of the swelling-induced anion transport mechanism. Barium (1–4 mM), another K channel blocker, slowed the rate of RVD, while 4-aminopyridine, charybdotoxin, tetraethylammonium chloride, tetrabutylammonium chloride, and gadolinium had no effect on RVD. Furthermore, RVD was not mediated by calcium-activated conductances, since it occurred normally in Ca-free medium, in medium containing cadmium, and in BAPTA-loaded cells. Gramicidin produced little or no volume change in isotonic medium, suggesting that basal C1 permeability of both THP-1 and HL-60 cells is low. However, swelling induced an anion efflux pathway that is permeable to both chloride and bromide, but is impermeable to methanesulfonate and glutamate. The anion channel blocker 3,5-diiodosalicylic acid (DISA) antagonized RVD in both cell types. In conclusion, RVD in THP-1 and HL-60 cells is mediated by independent anion and cation transport mechanisms that involve both a DISA-sensitive anion pathway and a quinine-inhibitable K efflux pathway, neither of which requires increases in intracellular calcium to be activated. ©1994 Wiley-Liss, Inc.**

The presence of impermeable intracellular charged particles results in a tendency for all cells to swell. Animal cells counterbalance this tendency by extruding ions through either conductive pathways or coupled transporters (for review, Sarkadi and Parker, 1991). Volume regulation also occurs when cells, such as kidney epithelial cells, are exposed to anisotonic environments (Lewis and Donaldson, 1990). The pathways involved in homeostatic volume regulation have been extensively studied *in vitro* by placing cells in anisotonic medium. Exposure to hypotonic medium produces a rapid swelling followed by a volume decrease referred to as a regulatory volume decrease (RVD) in many cell types. In some cells, including erythrocytes, RVD is associated with an increase in the activity of electro-neutral transporter(s) (Sarkadi and Parker, 1991), while in other cells, such as lymphocytes, K and C1 conductive pathways underlie RVD (Grinstein and Foskett, 1990).

While volume regulatory responses in lymphocytes and erythrocytes have been well characterized, only two studies have examined RVD in cells of the macrophage lineage (monocytes, macrophages, or promono-

cytic cells). Novak et al. (1988) demonstrated that, in rabbit alveolar macrophages, RVD depends on the K gradient and is due to a ouabain-insensitive loss of K and C1, which precedes the swelling-induced changes in surface receptor numbers associated with inhibition of receptor-mediated endocytosis. In the promyelocytic cell line HL-60, RVD is associated with a change in actin polymerization, but the shift in actin polymerization is not required for the transduction of the volume regulatory signal (Hallows et al., 1992). While these two studies demonstrated that myeloid cells, like lymphocytes, exhibit RVD, the ionic mechanisms underlying RVD were not investigated.

It is possible that one or more of the K and C1 conductances, which have been characterized in primary cells of the monocytic-macrophage lineage or in macrophage-like cell lines using electrophysiological tech-

DTIC QUALITY INSPECTED 8

Received August 2, 1993; accepted January 4, 1994.

*To whom reprint requests/correspondence should be addressed.

niques (for review see Gallin, 1991), mediate RVD in these cells. As a first step towards exploring this possibility, this study investigated RVD in two human leukemic cell lines; HL-60 cells, which can be induced to differentiate into either macrophage-like cells or neutrophil-like cells, and THP-1 cells, monocytic-like cells that can be differentiated into mature macrophages (Auwerx, 1991). We demonstrate that while RVD in both these cell lines involves the activation of independent cation and anion permeability pathways, the calcium-activated conductances described in macrophages are unlikely to mediate RVD. Furthermore, the cation permeability pathway activated in these two cell lines has a different sensitivity to pharmacological agents than the n-type K channel that mediates RVD in lymphocytes (Grinstein and Foskett, 1990; Deutsch and Lee, 1988).

MATERIALS AND METHODS

Cells

HL-60 cells and THP-1 cells were obtained from American Type Culture Collection (Rockville, MD). HL-60 cells and THP-1 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT), penicillin (20 units/ml), streptomycin (20 ng/ml), and glutamine (0.3%). Cells were grown in a 5% CO₂-air incubator at 37°C and split 3:1 twice a week. Cells were maintained for approximately 3 months, at which time new frozen stocks were thawed.

Volume measurements

Volume measurements were carried out electronically using a model ZM Coulter Counter equipped with a 100 μ m diameter orifice. The counter was coupled to an IBM compatible personal computer equipped with a Series II Personal Computer Analyzer card (PCAI; Nucleus Inc., Oak Ridge, TN). The PCAI card sorted incoming digitized signals into channels binned to provide histograms in which the channel number was proportional to cell volume. Median cell volume was computed from histograms of approximately 10,000 cells. Polystyrene beads 9–15 μ m in diameter (Coulter) in solutions with the same tonicity were used to calibrate the instrument.

Cells were resuspended at approximately 10⁶ cells/ml in NaCl Hanks' solution containing 10 mM glucose and 0.5% bovine serum albumin (BSA; normal Hanks') and used for volume analysis within 30 minutes. Volume measurements were performed at room temperature (21–23°C) with control cell volume determined in normal Hanks'. RVD was assessed following dilution with 41% water (0.59 $\times$ Hanks'). This degree of osmotic stress was chosen because it produced a significant swelling and regulatory response without affecting cell viability. Volume changes were measured over a 30-minute time period and are expressed relative to control cell volume. For later time points where volume changed more slowly, an averaged volume was determined from two to three separate measurements taken over 30 seconds.

Cell viability

Cell viability was assessed with a fluorescent microscope using ethidium bromide and acridine orange. Ex-

posure to 41% hypotonic medium for 30 minutes produced no significant change in cell viability. Addition of quinine (4 mM) in either isotonic or hypotonic medium had no effect on the number of viable cells.

Solutions

Normal Hanks' referred to as Hanks' contained in mM: 150 NaCl, 1.2 MgCl₂, 1.6 CaCl₂, 4.6 KCl, 10 HEPES, 10 glucose, and 0.5% BSA, titrated with NaOH to pH 7.3. KCl Hanks' contained in mM: 150 KCl, 1.6 MgCl₂, 1.2 CaCl₂, 5 NaCl, 10 HEPES, 10 glucose, 0.5% BSA, titrated with KOH to pH 7.3. Low sodium Hanks' was made either with N-methylglucamine chloride (NMDGCl) or tetramethylammonium chloride (TMACl) to replace all but 4 mM NaCl. For studies in which anion selectivity was examined, 150 mM chloride was replaced by other anions. For example, Kmethanesulfonate Hanks' contained in mM 150 Kmethanesulfonate, 4.6 KCl, 1.6 CaCl₂, 1.2 MgCl₂, 10 HEPES, 10 glucose, and 0.5% BSA.

Calcium measurements

Cells were loaded with fura in normal Hanks' containing 2.5 μ M fura-2-AM and 0.2 mg/ml of pluronic acid with or without 15 μ M BAPTA-AM for 1 hour at room temperature. Cells were centrifuged, and loading medium was replaced with normal Hanks'. Following a 15-minute incubation at room temperature fluorescence ratios (excitation 340 and 380; emission 510) were measured at room temperature using an SLM spectrofluorimeter. After obtaining baseline fluorescence measurements, 41% water was added (3 ml for final cuvette volume) and fluorescence changes were monitored for 5–10 minutes. Calibration was accomplished for each cuvette by adding 15 μ l of 10% Triton-X 100 to the cuvette to obtain F_{max}, followed by the addition of 50 μ l of 0.75 M EGTA/TRIS, pH 8, to obtain F_{min}. Complete hydrolysis of the fura-AM was confirmed by comparing the fluorescence ratio at 340/380 of lysed fura-loaded cells in high calcium to the fluorescence ratio of free fura recorded under the same conditions. Intracellular calcium ([Ca]_i) was estimated as described by Grynkiewicz et al. (1985) using a K_d of 135 nM for fura measured in 100 mM KCl, pH 7.2, at 20°C.

Chemicals

Gramicidin, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS), 3,5-diiodosalicylic acid (DISA), ouabain, quinine, and tetrabutylammonium chloride (TBACl) were obtained from Sigma (St. Louis, MO), tetraethylammonium chloride from Aldrich (Milwaukee, WI), and charybdotoxin (CTX) from Latoxan (Rosans, France). Gramicidin and DISA were made up as concentrated stocks (100X) in dimethylsulfoxide (DMSO). CTX was made up in Hanks' containing 0.5% BSA and stored in glass vials. CTX's activity was confirmed by demonstrating that it blocked an ionomycin-stimulated K efflux from guinea pig epithelium (Dr. Pamela Gunter-Smith, personal communication). Fura-2-AM, pluronic acid, and BAPTA-AM were obtained from Molecular Probes (Eugene, OR) and made up as 1 mM stock solutions in DMSO.

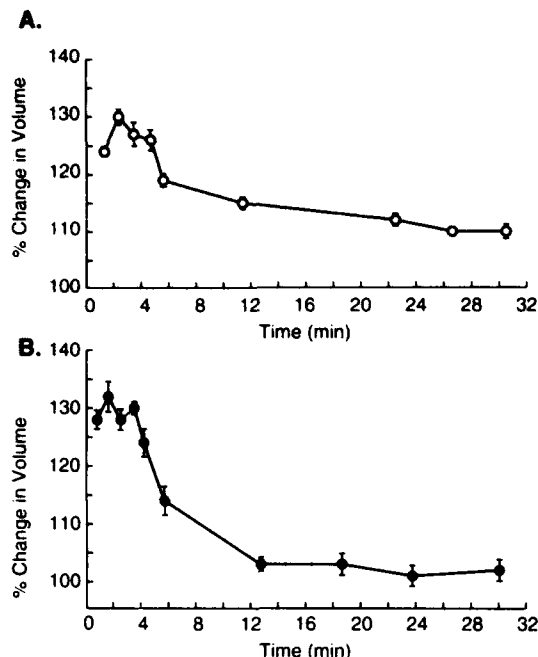


Fig. 1. Time course of regulatory volume changes in (A) HL-60 cells and (B) THP-1 cells following addition of 41% water at time 0. For this figure and other figures, data are expressed as the percentage volume change compared to control cell volume determined at the beginning of the experiment. Averaged data from three separate runs are shown for each cell type.

Statistics

All data are expressed as mean $\pm$ standard error of mean.

RESULTS

RVD occurs in both THP-1 and HL-60 cells

HL-60 and THP-1 cell populations exhibit unimodal volume distributions under isosmotic and hypotonic conditions. Their mean peak volume under isosmotic conditions was 970 ± 4.1 fL and 580 ± 2.4 fL for THP-1 cells and HL-60 cells, respectively. As reported previously by Hallows et al. (1991), HL-60 cells exhibit a transient swelling followed by a decrease in volume when exposed to hypotonic solution (Fig. 1A). A similar RVD response is also present in the THP-1 cell line following exposure to $0.59\times$ Hanks' (Fig. 1B). Peak swelling occurred between 2 and 4 minutes in both cell types, reaching 125–145% of control cell volume in $0.59\times$ solutions. Cell volume returned to within 10% of control levels within 30 minutes at $21\text{--}23^\circ\text{C}$. RVD was characterized by an initial rapid decrease followed by a slower change in volume. The initial rate of RVD measured during the first 5–6 minutes was $-3.31\%/ \text{min} \pm 0.12$ ($n = 17$) and $-5.48\%/ \text{min} \pm 0.7$ ($n = 11$) for THP-1 and HL-60 cells, respectively.

Role of cation permeability

Potassium channel blockers. Since K conductances have been implicated in RVD responses of other cells (Grinstein and Foskett, 1990; Okada and Hazama, 1989), the effects of several different K channel block-

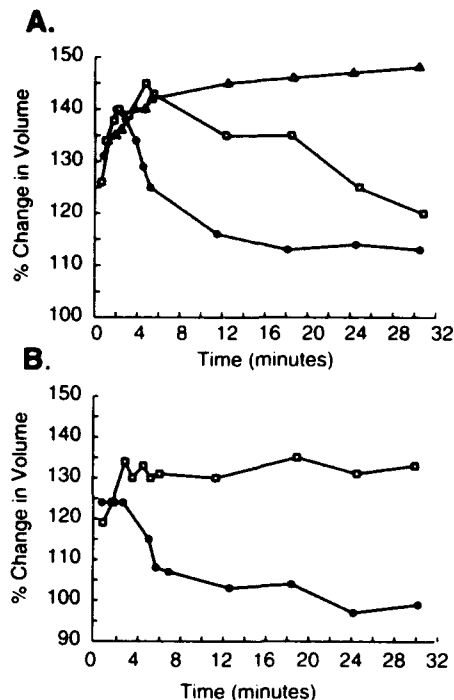


Fig. 2. Effect of quinine on RVD in (A) HL-60 cells and (B) THP-1 cells. Time course of volume responses of cells in $0.59\times$ NaCl Hanks' (●) and cells in $0.59\times$ NaCl Hanks' containing either 1 mM (□) or 4 mM (△) quinine. For this figure and subsequent figures, each time course is a representative run from a single cuvette of cells (three to four individual time courses were obtained for each experimental condition).

ers were tested on HL-60 and THP-1 cells exposed to $0.59\times$ Hanks. In HL-60 cells 1 mM quinine slowed but did not completely block RVD; cell volume at 30 minutes was $120\% \pm 4.9$ ($n = 7$) of initial control volume compared to a 30-minute volume of $107\% \pm 3.6$ for control measurements in the absence of 1 mM quinine (Fig. 2A). Increasing the quinine concentration to 4 mM blocked RVD completely in HL-60 cells (peak volume vs. 30-minute volume was $142\% \pm 3.9$ vs 139 ± 5.6 , $n = 3$). In contrast, in THP-1 cells, 1 mM quinine completely blocked RVD (volume at 30 minutes was $131\% \pm 2$ of initial control volume, $n = 4$; Fig. 2B).

Since in some cell types quinine has been reported to affect C1 conductances as well as K conductances (Gogelein and Capek, 1990), experiments were performed using both the cation pore former gramicidin and the K transporter valinomycin to determine whether quinine's block of RVD was due to a block of a cation permeability. Cell volume was monitored in hypotonically stressed cells bathed in medium that contained quinine to which gramicidin was added. Gramicidin, which forms channels permeable to K and Na, enables cells to volume regulate in the presence of a K channel blocker, as long as their anion permeability is unaffected by the K channel blocker. In $0.59\times$ Hanks' containing quinine, addition of gramicidin to THP-1 cells produced a secondary swelling presumably due to the Na influx through gramicidin channels. In contrast, HL-60 cells exposed to gramicidin in $0.59\times$ Hanks' plus

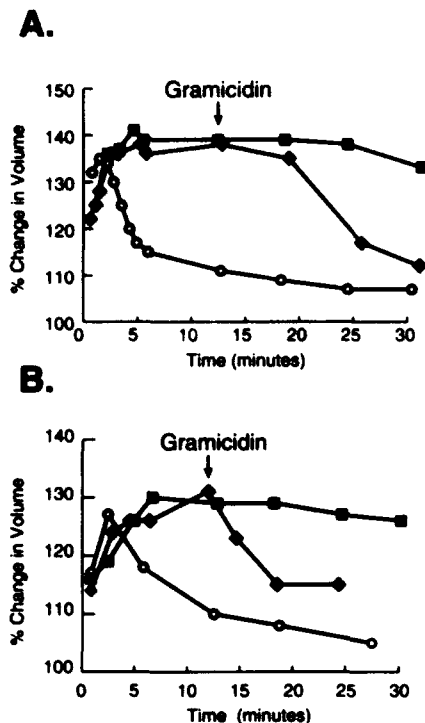


Fig. 3. Gramicidin induces RVD in the presence of 4 mM quinine. Time course of representative volume responses from (A) HL-60 cells in 0.59x NMDG Hanks' and (B) THP-1 cells in 0.59x TMA-Hanks'. Cells in (○) 0.59x Hanks'; (●) 0.59x Hanks' + 4 mM quinine; and (◆) 0.59x Hanks' + 4 mM quinine + 1 μM gramicidin added at time indicated by the arrows.

quinine exhibited an RVD response, suggesting that under these conditions the relative electrochemical gradients for K and Na in these two cell types is different. Therefore, to minimize gramicidin-induced influx of Na, experiments on both HL-60 and THP-1 cells were performed in either low Na-NMDGCl Hanks' or low Na-TMA Hanks'. Under these conditions, both HL-60 cells (Fig. 3A) and THP-1 cells (Fig. 3B) exhibit RVD in 0.59x low Na-Hanks' which was blocked by quinine and restored by gramicidin (1 μM) despite the presence of quinine. Similar experiments also were done using the mobile K carrier valinomycin (1 μM) to restore RVD responses in quinine-containing Na Hanks'. While the results of these experiments were qualitatively similar to those done using gramicidin in Na-free Hanks', valinomycin was less effective at restoring the quinine-inhibited RVD than gramicidin. The difference in effectiveness between gramicidin and valinomycin probably reflects the fact that the rate of cation flux induced by gramicidin (a channel-forming ionophore) will be considerably greater than that produced by valinomycin (a mobile carrier).

The divalent cation, barium blocks partially or completely several K conductances, including the voltage-gated inwardly rectifying K conductance and the calcium-activated inwardly rectifying K conductance present in macrophages (Gallin, 1991). In HL-60 cells, RVD was slowed, but not blocked by barium (1 mM).

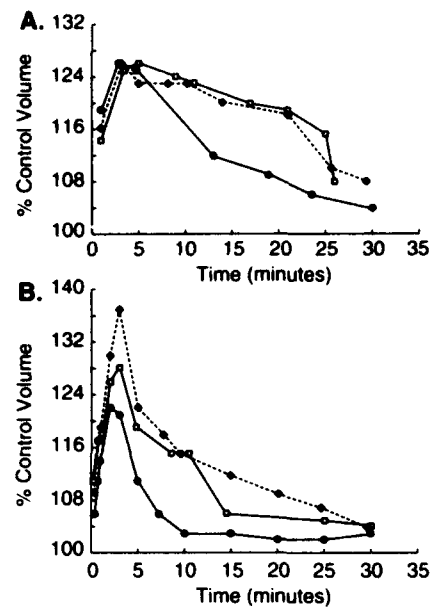


Fig. 4. Effect of barium on RVD response of (A) HL-60 cells and (B) THP-1 cells. Time course of representative volume responses of cells in 0.59x NaCl Hanks' (●), 0.59x NaCl Hanks' containing either 1 (□) or 4 mM BaCl₂ (◆).

TABLE 1. Ability of agents to block RVD

Agent ¹	Concentration	Block ²	
		HL-60	THP-1
Quinine	0.5–1 mM	++	++
Barium	2–4 mM	+	+
4-AP	5 mM	—	—
TEA	10 mM	—	—
TBA	10 mM	—	—
CTX	25 nM	—	—
Cadmium	1 mM	—	—
DISA	0.2 mM	++	++
SITS ³	0.5–1 mM	+	+
Ouabain	1 mM	—	—
Gadolinium	10–100 μM	—	—

¹Agents were added immediately before cells were exposed to 0.59x Hanks' and volume was monitored for the next 30 minutes.

²+, complete block; +, partial block.

³Cells were incubated in SITS for 30 minutes prior to hypotonic stress.

Increasing the barium concentration from 1 to 4 mM did not result in further inhibition of RVD (Fig. 4A), while decreasing the barium concentration to 0.5 mM produced a smaller inhibition of the RVD response (data not shown). In THP-1 cells, 4 mM barium was required to significantly slow RVD (Fig. 4B). The K channel blockers, CTX (25 nM), TEACl (10 mM), TBACl (10 mM), and 4-AP (5 mM) had little or no effect on RVD in either cell type (Table 1).

Is the K gradient required for RVD? To determine if the K gradient is required for RVD, both THP-1 and HL-60 cells were placed in a 0.59x KCl Hanks' and cell volume was monitored. Neither cell type exhibited RVD in 0.59x KCl Hanks'. Furthermore, 0.59x KCl Hanks' produced a secondary swelling in both cell types. This is evident in Figure 5 in which representative responses of THP-1 and HL-60 cells to 0.59x KCl

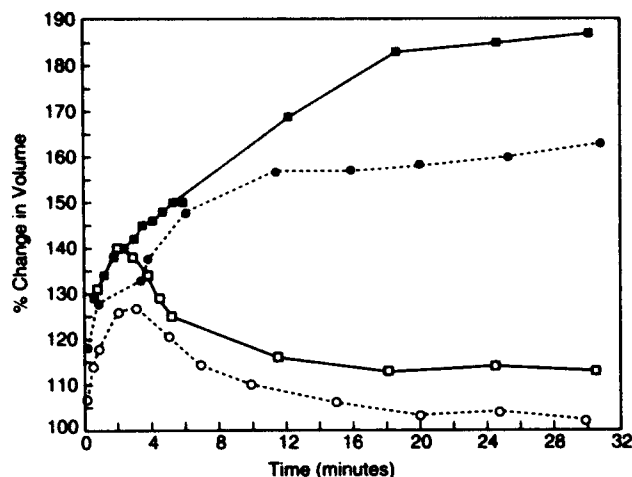


Fig. 5. Comparison of time course of representative volume responses of HL-60 cells (□) and THP-1 cells (○) in 0.59× KCl Hanks' (filled symbols) and 0.59× NaCl Hanks' (open symbols).

Hanks' are depicted along with RVD responses in 0.59× NaCl Hanks'. The secondary swelling response for HL-60 cells was considerably greater ($188\% \pm 2.1$ at 30 minutes [$n = 5$]) than that for THP-1 cells ($159\% \pm 1.7$ at 30 minutes [$n = 7$]). To determine if the diminished secondary swelling in THP-1 cells compared to HL-60 cells reflects lower cation permeability in THP-1 cells compared to HL-60 cells, gramicidin was added to the bath 10–12 minutes after the hypotonic stress and volume were monitored. Gramicidin had little or no effect on the final volume of THP-1 cells after 30 minutes in 0.59× KCl Hanks' (gramicidin-treated volume was 159%, $n = 4$), suggesting that the anion permeability or gradient of THP-1 cells accounted for the difference in the magnitude of the secondary swelling.

Anion permeability

Gramicidin produced little or no change in the mean volume of THP-1 or HL-60 cells bathed in isotonic Hanks', indicating that the basal Cl permeability of THP-1 and HL-60 cells is low. In contrast, Cl permeability increased in response to hypotonic stress since, as noted above (Fig. 3) the addition of gramicidin induced RVD when HL-60 and THP-1 cells were bathed in 0.59× Hanks' plus quinine. Furthermore, gramicidin's ability to overcome the quinine-induced block of RVD indicates that the swelling-induced anion and cation permeabilities are independent.

Anion channel blockers. RVD was blocked in both cell types by the anion channel blocker DISA, which has been shown to block RVD in neutrophils (Stoddard et al., 1993). In 0.59× Hanks' the 30-minute volume in DISA-treated cells (0.2 mM) was 136 ± 10 ($N = 3$) and 136 ± 8 ($n = 3$) in HL-60 cells and THP-1 cells, respectively. The DISA-induced block of RVD could not be overcome by addition of gramicidin (1 μ M). In contrast, SITS (0.5–1 mM) produced only a partial block in THP-1 cells ($112\% \pm 3$ of control volume at 30 minutes) and in HL-60 cells ($114\% \pm 4$ of control volume at 30

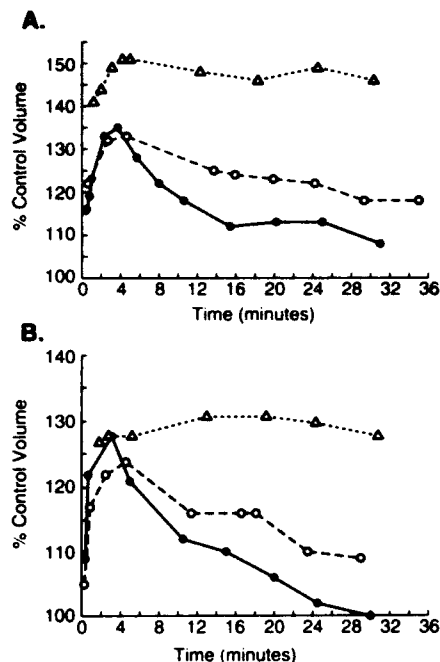


Fig. 6. Time course of representative volume responses of (A) HL-60 cells and (B) THP-1 cells in 0.59× NaCl Hanks' (●), 0.59× NaCl Hanks' containing either 1 mM SITS (○) or 0.2 mM DISA (△).

minutes), even when cells were preincubated with 1 mM SITS for 30 minutes before exposure to hypotonic medium (Fig. 6).

Anion selectivity. As noted earlier (Fig. 5), HL-60 cells in 0.59× KCl Hanks' failed to volume regulate and showed a secondary volume increase which was presumably due to an influx of both K and Cl. In order to investigate the selectivity of the anion efflux pathway, the magnitude of the secondary volume increase was monitored in HL-60 cells bathed in 0.59× high K Hanks' containing different anions. As shown in Figure 7, bathing HL-60 cells in Kmethanesulfonate Hanks' or Kglutamate Hanks' abolished the secondary volume increase evident in 0.59× KCl Hanks'. In contrast, cells in Kbromide Hanks' displayed a secondary volume increase similar to that of the cells in KCl Hanks'. Thus, the volume-induced anion efflux mechanism is permeable to both Cl and Br and is impermeable to methanesulfonate and glutamate.

Role of calcium

To determine if a calcium influx was required for the RVD response, volume was monitored in THP-1 and HL-60 cells exposed to 0.59× Hanks' which contained 1.0 mM EGTA and no calcium. RVD occurred normally in both cell types in this solution. Furthermore, cadmium (1 mM), a calcium channel antagonist in other cells, had no effect on RVD in either cell type (Fig. 8).

Despite the absence of a requirement for calcium influx during RVD, it is possible that swelling causes release of [Ca]_i stores that in turn can activate calcium-gated channels. To explore this possibility, THP-1 and HL-60 cells were loaded with either fura, or with

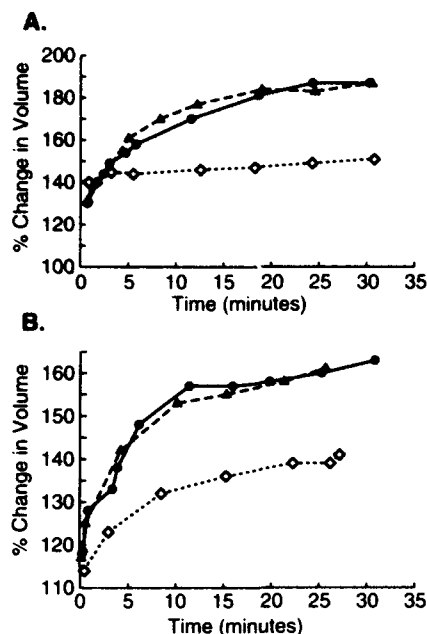


Fig. 7. Time course of representative volume responses of (A) HL-60 cells and (B) THP-1 cells in 0.59x KCl Hanks' (●), 0.59x KBr Hanks' (▲), and in 0.59x Kmethanesulfonate Hanks' (◇).

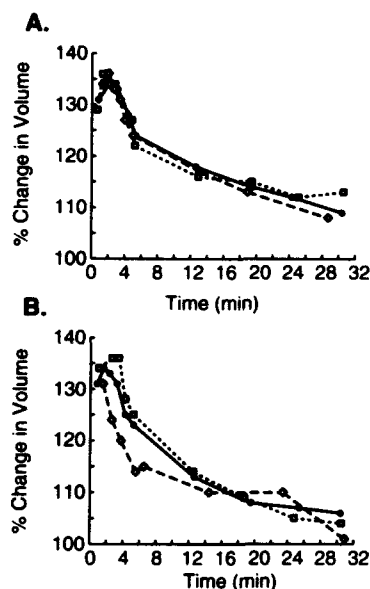


Fig. 8. Time course of representative volume responses of (A) HL-60 cells and (B) THP-1 cells in 0.59x NaCl Hanks' (●), 0.59x NaCl Hanks' containing either 0 calcium/1 mM EGTA (□) or 1 mM CdCl (◇).

BAPTA and fura, and both $[Ca]_i$ changes and cell volume were monitored during hypotonic stress. Since volume measurements were performed at room temperature, $[Ca]_i$ measurements also were carried out at room temperature. As shown in Figure 9, swelling induced increases in $[Ca]_i$ in both THP-1 and HL-60 cells, although the response of THP-1 cells was quite small; the

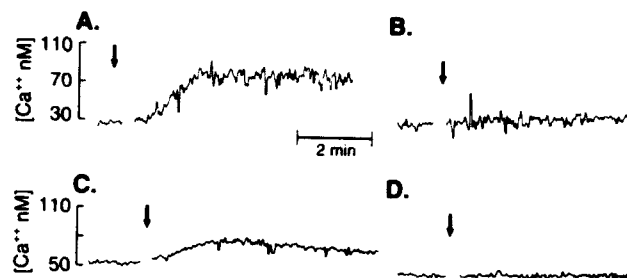


Fig. 9. Effect of swelling on $[Ca]_i$. A,B: HL-60 cells and (C,D) THP-1 cells were loaded with either fura-2 am (A,C) or fura-2-AM plus BAPTA-AM, (B,D). At time indicated by arrows, 41% water was added.

percentage increase in $[Ca]_i$ compared to baseline levels obtained before addition of 41% water was $273\% \pm 26$ ($n = 6$) for HL-60 cells and $154\% \pm 16$ ($n = 4$) for THP-1 cells. BAPTA loading both cell types abolished the induced $[Ca]_i$ rise (Fig. 9). However, BAPTA loading did not block RVD in either cell type in response to either 0.59x NaCl Hanks' or 0.59x Ca-free, EGTA-containing NaCl Hanks' (shown in Fig. 10).

Experiments were also performed to determine if increases in $[Ca]_i$ can induce volume changes in the absence of an osmotic stress. In these studies, the volume of cells exposed to either 1 μ M ionomycin or 32 nM thapsigargin was compared to the volume of control HL-60 and THP-1 cells in normal Hanks'. No changes in cell volume were apparent during a 30-minute exposure to either thapsigargin or ionomycin in either cell type.

Effect of gadolinium

Stretch-activated channels, which are inhibited by gadolinium (Yang and Sachs, 1989), have been implicated in RVD in astrocytes and other cells (Medrano and Gruenstein, 1993). However, in the presence of 1-100 μ M gadolinium chloride, THP-1 and HL-60 cells exhibited normal RVD (data not shown).

DISCUSSION

This study demonstrates that RVD in THP-1 cells, a monocytic cell line, and in HL-60 cells, a promonocytic cell line, involves efflux through independent anion and cationic transport mechanisms. While little data are available about the ionic conductances in HL-60 and THP-1 cells (Wieland et al., 1987, 1990), electrophysiological studies on other myeloid cells have characterized several different K and Cl conductances that may be activated during RVD (Gallin, 1991). In many cases, pharmacological blockers of these ionic conductances have been described. Thus we reasoned that examining the ability of these agents to block RVD in THP-1 and HL-60 cells would provide information about the involvement of these ionic conductances in RVD, and therefore, their presence in these cell lines.

Cation permeability pathway

Surprisingly, quinine was the only K channel antagonist that completely blocked RVD in both cell types; 1 mM quinine blocked RVD in THP-1 cells while 4 mM

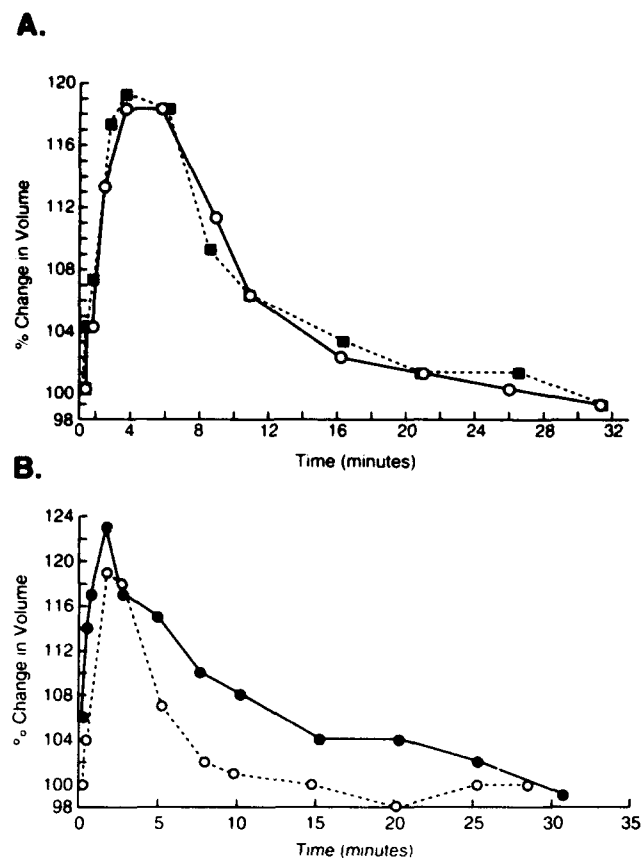


Fig. 10. A: Time course of representative volume responses of control (○) and BAPTA-loaded (■) HL-60 cells in 0.59×0 -calcium/1 mM EGTA NaCl Hanks'. B: Time course of representative volume responses of control (○) and BAPTA-loaded (●) THP-1 cells in 0.59×0 -calcium/1 mM EGTA NaCl Hanks'.

was required to block RVD in HL-60 cells. Although these concentrations of quinine have been reported to effect anionic conductances in some cells (Gogelein and Capek, 1990), the ability of gramicidin (a cationic channel former) to overcome quinine block indicates that in our experiments quinine is blocking RVD through its action on a cationic efflux. In macrophages, quinine blocks Ca-activated K conductances that have been implicated in RVD in other cell types (Okado and Hazama, 1989). Furthermore, we have demonstrated in this study that swelling induces a small increase in $[Ca]_i$ in both THP-1 and HL-60 cells which may be due to either a swelling-induced influx of extracellular calcium or release of intracellular calcium stores. Despite these observations, the inability of either BAPTA loading or CTX, an antagonist of Ca-activated K channels to block RVD makes it unlikely that calcium-activated K channels are involved in RVD in these cell lines. Furthermore, the observations that two agents which increase $[Ca]_i$, thapsigargin and ionomycin, fail to change control cell volume, indicates that a $[Ca]_i$ increase, which activates Ca-activated K conductances in macrophages (Gallin, 1989), does not itself induce volume changes in these two cell lines.

Barium, the other K channel blocker tested that affected RVD in THP-1 and HL-60 cells, only partially blocked RVD. Perhaps the best characterized K conductance in macrophages is a barium-inhibitable voltage-dependent inwardly rectifying K conductance (K_i). The K_i conductance has been cloned recently (Kubo et al., 1993) and, when present, sets the macrophage resting membrane potential near to the K equilibrium potential (Gallin, 1981). This conductance also enables cells to flip between two stable states of resting membrane potential (Gallin, 1981). Barium block of K_i occurs in 300–500 μ M extracellular barium (McKinney and Gallin, 1988). In contrast, 1–4 mM barium was required to slow RVD, but failed to completely inhibit it. Thus, the K_i conductance is unlikely to mediate RVD in HL-60 cells or in THP-1 cells. Partial block of RVD by barium could be due either to a partial block by barium of a single cation conductance or to the involvement of more than one cation conductance in RVD, a barium-sensitive conductance and a barium-insensitive conductance in RVD. In the human colon adenocarcinoma HT-29 cell line, where noise analysis has implicated only one type of K channel in volume regulation, the volume-sensitive K channel is completely blocked by quinine but only partially blocked by barium (Ilek et al., 1992). Volume regulation in macrophages may involve a similar K channel.

None of the other K channel blockers tested in THP-1 and HL-60 cells inhibited RVD. Consequently, unlike lymphocytes where a CTX-sensitive "delayed-rectifier type" K conductance has been implicated in RVD (Grinstein and Smith, 1990; Grinstein and Foskett, 1990) or cultured proximal tubule cells where a Ca-activated K conductance (Dube et al., 1990) has been implicated in RVD, volume regulatory responses in myeloid cells do not appear to involve either the Ca-activated K conductances or delayed-rectifier type K conductances that have been described in myeloid cells.

The secondary swelling evident in both THP-1 and HL-60 cells bathed in $0.59 \times$ KCl Hanks' indicates that opening of the swelling-induced cation permeability does not depend on the K gradient, nor is it inactivated by depolarization. Our experiments with gadolinium, an antagonist of stretch-activated cation channels, also suggest that these channels are not involved in RVD of HL-60 and THP-1 cells. Further studies using both patch clamp techniques and volume measurements are needed to determine the cation permeability pathway(s) that are involved in RVD in macrophages.

Anion permeability pathway

Addition of gramicidin to either cell type produced little change in cell volume unless cells were exposed to hypotonic medium, indicating that the basal anion permeability of both THP-1 cells and HL-60 cells is low and that it increases following swelling. As in lymphocytes, our studies with high K hypotonic medium containing different anions indicated that the swelling-induced anionic transport pathway is permeable to both Cl and bromide ions but relatively impermeable to methanesulfonate and glutamate (Grinstein et al., 1982). While SITS completely blocks RVD in neutrophils (Stoddard et al., 1993) and other cells, it only produced a partial block of the RVD response in THP-1

and HL-60 cells. On the other hand, RVD was blocked in both cell types by the anion transport blocker DISA and this block could not be overcome by gramicidin. DISA, unlike SITS, is lipophilic and probably crosses the membrane through nonionic diffusion (Simchowtiz et al., 1993). In human neutrophils DISA blocked both RVD and the swelling-induced increases in ^{36}Cl efflux and Cl currents (Stoddard et al., 1993; Simchowtiz et al., 1993). In addition to blocking RVD in several cell types, DISA is a noncompetitive inhibitor of Cl - Cl exchange and Na independent Cl/HCO_3 exchange (Restrepo et al., 1992). Thus, while we can conclude that under our experimental conditions (in HEPES-buffered media without bicarbonate) swelling increases a DISA-inhibitable anionic transport mechanism(s), DISA may block RVD by acting on several different anionic transport mechanisms. In this context, a preliminary report by Hallows et al. (1992) showed that for HL-60 cells suspended in bicarbonate-containing hypotonic medium, the Cl efflux was significantly less than the K efflux, suggesting that additional osmolytes are involved in the RVD response in HL-60 cells. In astrocytes, cell swelling induces a rapid release of taurine, which is markedly reduced by the Cl channel inhibitor DIDS (Pasantos-Morales et al., 1990). If the loss of additional osmolytes also is involved in the RVD response of HL-60 cells maintained in bicarbonate-free medium, then DISA must block both the Cl permeability change and the efflux of the additional osmolytes. Alternatively, it is possible that under the conditions of our experiment, the magnitude of the Cl efflux is similar to that of K efflux and DISA is acting to block only a Cl efflux. Chloride efflux studies will be needed to differentiate between these possibilities.

Delineating the ionic basis of RVD provides information about the ionic transport mechanisms present in myeloid cells, and may even be useful in assessing changes in transport that occur during activation and/or differentiation. Blood monocytes will be exposed to large changes in tonicity during passage through the kidney. On the other hand, tissue macrophages are probably exposed to only small changes in tonicity at sites of inflammation or following changes in metabolism. Volume regulation following swelling induced by enhanced amino acid uptake has been demonstrated in a number of cells including intestinal enterocytes (MacLeod et al., 1992). It is possible that stimulation of nitrous oxide formation and the subsequent increase in arginine uptake may lead to cell swelling in macrophages.

In conclusion, this study demonstrates that RVD in two myeloid cell lines is mediated by independent quinine-inhibitable cation and DISA-inhibitable anion transport pathways that are not gated by increases in $[\text{Ca}]_i$ and that the cation permeability pathway mediating RVD in these cells is different from the pathway mediating RVD in lymphocytes.

ACKNOWLEDGMENTS

The authors are grateful to Keith Brockgreitins and Brain Hively for their excellent technical assistance.

LITERATURE CITED

- Auwerx, J. (1991) The human leukemia cell line, THP-1: A multifaceted model for the study of monocyte-macrophage differentiation. *Experientia*, 47:22-31.
- Deutsch, C., and Lee, S.C. (1988) Cell volume regulation in lymphocytes. *Renal Physiol. Biochem.*, 3-5:260-276.
- Dube, L., Parent, L., and Sauve, R. (1990) Hypotonic shock activates a maxi K^+ channel in primary cultured proximal tubule cells. *Am. J. Physiol.*, 25:F348-F356.
- Gallin, E.K. (1981) Voltage clamp studies in macrophages from mouse spleen cultures. *Science*, 214:458-460.
- Gallin, E.K. (1989) Evidence for a Ca -activated inwardly rectifying K channel in human macrophages. *Am. J. Physiol.*, 257:C77-C85.
- Gallin, E.K. (1991) Ion channels in leukocytes. *Physiol. Rev.*, 71:775-811.
- Gogelein, H., and Capek, K. (1990) Quinine inhibits chloride and nonselective cation channels in isolated rat distal colon cells. *Biochem. Biophys. Acta*, 1027:191-198.
- Grinstein, S., and Foskett, J.K. (1990) Ionic mechanisms of cell volume regulation in leukocytes. *Annu. Rev. Physiol.*, 52:399-414.
- Grinstein, S., and Smith, J.D. (1990) Calcium-independent cell volume regulation in human lymphocytes. *J. Gen. Physiol.*, 95:97-120.
- Grinstein, S., Clarke, C.A., DuPre, A., and Rothstein, A. (1982) Volume-induced increase of anion permeability in human lymphocytes. *J. Gen. Physiol.*, 80:801-823.
- Gryniewicz, G., Poenie, M., and Tsien, R. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, 260:3440-3450.
- Hallows, S.K.R., Packman, C.H., and Knauf, P.A. (1991) Acute cell volume changes in anisotonic media affect F-actin content in HL-60 cells. *Am. J. Physiol.*, 261:C1154-C1161.
- Hallows, K.R., Restrepo, D., and Knauf, P. (1992) Control of volume and intracellular pH under hypotonic conditions in human promyelocytic HL-60 cells. *J. Gen. Physiol.*, 100:60a.
- Illek, B., Fisher, H., Kreuzel, K., Hegel, U., and Claus, W. (1992) Volume-sensitive basolateral K channels in HT-29/B6 cells: Block by lidocaine, quinidine, NPPB and Ba. *Am. J. Physiol.*, 263:C674-683.
- Kubo, Y., Baldwin, T.J., Jan, Y.N., and Jan, L.Y. (1993) Primary structure and functional expression of mouse inward rectifier potassium channel. *Nature*, 352:127-133.
- Lewis, S.A., and Donaldson, P. (1990) Ion channels and cell volume regulation: Chaos in an organized system. *News in Physiological Sciences* 5:112-119.
- MacLeod, R.J., Lembessis, P., and Hamilton, J.R. (1992) Effect of protein kinase C inhibitors on Cl conductance required for volume regulation after L-alanine cotransport. *Am. J. Physiol.*, 262 (Cell. Physiol.), 31:C950-955.
- McKinney, L.C., and Gallin, E.K. (1988) Inwardly rectifying whole-cell and single-channel K currents in the murine macrophage cell line J774.1. *J. Membr. Biol.*, 103:42-53.
- Medrano, S., and Gruenstein, E. (1993) Mechanisms of regulatory volume decrease in UC-11MG human astrocytoma cells. *Am. J. Physiol.*, 264:C1201-1209.
- Novak, J.M., Cala, P., Ward, D., Buys, S., and Kaplan, J. (1988) Regulatory volume decrease in alveolar macrophages: Cation loss is not correlated with changes in membrane recycling. *J. Cell. Physiol.*, 137:243-250.
- Okada, Y., and Hazama, A. (1989) Volume regulatory ion channels in epithelial cells. *NIPS*, 4:238-242.
- Pasantos-Morales, H., Moran, J., and Schousboe, A. (1990) Volume sensitive release of taurine from cultured astrocytes: Properties and mechanism. *Glia*, 3:427-432.
- Restrepo, D., Cronise, B.L., Snyder, R.B., and Knauf, P.A. (1992) A novel method to differentiate between ping-pong and simultaneous exchange kinetics and its application to the anion exchanger of the HL-60 cell. *J. Gen. Physiol.*, 100:825-846.
- Sarkadi, B., and Parker, J.C. (1991) Activation of ion transport pathways by changes in cell volume. *Biochim. Biophys. Acta*, 107:407-427.
- Simchowtiz, L., Textor, J.A., and Cragoe, E.J. (1993) Cell volume regulation in human neutrophils: 2-(aminomethyl)phenols as Cl^- channel inhibitors. *Am. J. Physiol.*, 265:C143-C155.
- Stoddard, J.S., Steinbach, J.H., and Simchowtiz, L. (1993) Whole-cell chloride currents in human neutrophils induced by swelling. *Am. J. Physiol.*, 265:C156-C165.

Wieland, S.J., Chou, R.H., and Chen, T.A. (1987) Elevation of a potassium current in differentiating human leukemic (HL-60) cells. *J. Cell. Physiol.*, 132:321-375.

Wieland, S.J., Chou, R.H., and Gong, Q. (1990) Macrophage-colony stimulating factor (CSF-1) modulates a differentiation-specific in-

ward-rectifying potassium current in human leukemic (HL-60) cells. *J. Cell. Physiol.*, 142:643-651.

Yang, X.C., and Sachs, F. (1989) Block of stretch activated ion channels in *Xenopus* oocytes by gadolinium and calcium ions. *Science*, 243:1068-1071.

Accession For	
NTIS GRA&I	☒
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A-1	20