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ORIGINAL ARTICLES

Swelling-Induced Ca^{2+} Influx and K^+ Efflux in American Alligator Erythrocytes

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Abstract The American alligator can hibernate during winter, which may lead to osmotic imbalance because of reduced kidney function and lack of food consumption during this period. Accordingly, we hypothesized that their red blood cells would have a well-developed regulatory volume decrease (RVD) to cope with the homeostatic challenges associated with torpor. Osmotic fragility was determined optically, mean cell volume was measured by electronic sizing, and changes in intracellular Ca^{2+} concentration were visualized using fluorescence microscopy and fluo-4-AM. Osmotic fragility increased and the ability to regulate volume was inhibited when extracellular Na^+ was replaced with K^+, or when cells were exposed to the K^+ channel inhibitor quinine, indicating a requirement of K^+ efflux for RVD. Addition of the ionophore gramicidin to the extracellular medium decreased osmotic fragility and also potentiated volume recovery, even in the presence of quinine. In addition, hypotonic shock (0.5× Ringer) caused an increase in cytosolic Ca^{2+}, which resulted from Ca^{2+} influx because it was not observed when extracellular Ca^{2+} was chelated with EGTA (ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid). Furthermore, cells loaded with BAPTA-AM (1,2-bis(2-aminophenoxymethyl)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid *tetrakis*(acetoxymethyl) ester) or exposed to a low Ca^{2+}-EGTA hypotonic Ringer had a greater osmotic fragility and also failed to recover from cell swelling, indicating that extracellular Ca^{2+} was needed for RVD. Gramicidin reversed the inhibitory effect of low extracellular Ca^{2+}. Finally, and surprisingly, the Ca^{2+} ionophore A23187 increased osmotic fragility and inhibited volume recovery. Taken together, our results show that cell swelling activated a K^+ permeable pathway via a Ca^{2+}-dependent mechanism, and this process mediated K^+ loss during RVD.

Keywords Regulatory volume decrease · Fluo-4 · EGTA · BAPTA · A23187 · Quinine · Gramicidin

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The ability of animal cells to regulate their volume is a fundamental property common to a large number of cell types and has been reviewed extensively (Hoffmann et al. 2009; Koivusalo et al. 2009; Lang et al. 1998; Wehner et al. 2003). Volume regulation is necessary when cells are exposed to anisotonic extracellular conditions, and when the transport of solutes or pathophysiological conditions change intracellular osmolality (Lang 2007). Membrane transport pathways associated with volume regulation also have been implicated in processes as diverse as apoptosis, lymphocyte activation, and cell proliferation (Jakab et al. 2002; Lang et al. 2006; McManus et al. 1995; Okada et al. 2001; Wehner et al. 2003).

Exposure of vertebrate cells to a hypotonic solution results in an initial increase in cell volume as a result of the relatively rapid influx of water. During continuous hypotonic challenge, increases in cell volume are followed by a slower, spontaneous recovery toward the preshock level, a process known as regulatory volume decrease (RVD). This recovery is accomplished by selectively increasing the permeability of the plasma membrane during cell swelling to allow for efflux of specific intracellular osmolytes, thereby reversing the driving force for water influx (Hoffmann et al. 2009; Koivusalo et al. 2009; Lang 2007; Wehner et al. 2003). Vertebrate cells typically lose K^+ and

Cl^- during RVD (Hoffmann et al. 2009; Lang 2007; Wehner et al. 2003). This may occur by electroneutral ion transport pathways or by the separate activation of K^+ and Cl^- channels (Hoffmann et al. 2009; Lang 2007; Wehner et al. 2003). Loss of organic anions and osmolytes also may occur during RVD (Kirk and Strange 1998; Puffer et al. 2006; Wehner et al. 2003).

Calcium is a pivotal signaling agent for a wide variety of physiological processes. An elevation in the concentration of intracellular free Ca^{2+} from a resting level of approximately 50–100 nM to a stimulated level of 1–10 μM occurs as a rapid response to a number of cellular stimuli, including growth factors, neurotransmitters, hormones, peptides, toxins, and cell swelling (Foskett 1994). Although the cellular mechanisms that activate and regulate permeability pathways during RVD are not completely understood and differ between cell types (Hoffmann et al. 2009; Jakab et al. 2002; Land et al. 1998; Wehner et al. 2003), Ca^{2+} has been shown to play a key role during RVD in a number of cases (Foskett 1994; Jakab et al. 2002; Pasantes-Morales and Morales 2000; Tinel et al. 2000).

In some instances, RVD depends on Ca^{2+} influx across the plasma membrane, whereas in other cell types the Ca^{2+} response is mediated by Ca^{2+} release from intracellular stores (Foskett 1994; Tinel et al. 2000). In addition, although Ca^{2+} may directly activate ion channels during RVD (Foskett 1994; Lang 2007; Pasantes-Morales and Morales 2000), there is evidence that several Ca^{2+}-dependent intracellular messengers and enzymes (e.g., calmodulin, phospholipase A_2, eicosanoids, and protein kinases) are involved with cell volume regulation (Hoffmann et al. 2009; Tinel et al. 2000). Furthermore, the cytoskeleton is thought to play a role in volume regulation and actin polymerization is dependent on intracellular Ca^{2+} levels (Hoffmann et al. 2009; Pedersen et al. 2001).

Recent studies in our laboratory demonstrate that American alligator red blood cells (RBCs) display a robust RVD in response to hypotonic shock. We chose this species as a model system because alligators are known to hibernate from September until late spring (Hernandez and Coulson 1952; Rosenblatt 1936). During this state of torpor, they do not consume food, which may cause variations in the concentration of plasma electrolytes and nonelectrolytes (Rosenblatt 1936; Lance and Elsey 1999). In addition, kidney function is greatly reduced during hibernation, which also may lead to osmotic imbalance (Zancanaro et al. 1999). Accordingly, we speculated that alligator RBCs should have a well-developed RVD to cope with the homeostatic challenges of hibernation. The purpose of this study was to investigate the cellular basis by which alligator RBCs regulate volume under hypotonic conditions, with an emphasis on the potential role of Ca^{2+} influx and K^+ efflux. To this end, we used three different complementary experimental approaches: (1) hemolysis studies to examine osmotic fragility, (2) fluorescence microscopy to detect changes in intracellular Ca^{2+} levels, and (3) a Coulter counter to measure the volume of osmotically stressed cells.

Methods

Cells

American alligator (*Alligator mississippiensis*) blood, Alsever's anticoagulant, was obtained from Carolina Biological Supply (Burlington, NC). Blood was kept at 4°C for no more than 2 weeks before use and was mixed daily.

Osmotic Fragility

Osmotic fragility was examined by determining the degree of cell lysis for a suspension of RBCs in hypotonic Ringer. The level of hemolysis was determined via a turbidity shift (cloudy to clear) that occurs when the integrity of the plasma membrane is compromised. This was detected with a spectrophotometer (Spectronic 21D, Milton Roy Co.) 5 min after blood (15–30 μl) was added to hypotonic Ringer solutions (3 ml) of different compositions. (Slight differences in the values of mean OD between the controls in Figs. 1 and 2 resulted from using different volumes of blood. However, the same volume of blood was used

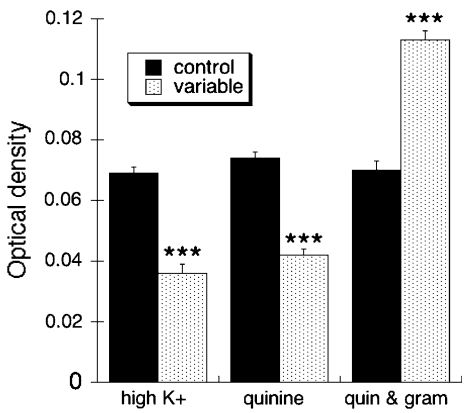

Fig. 1 Inhibition of K^+ efflux increased the osmotic fragility of alligator RBCs. The control (*solid bar*) was diluted reptilian Ringer (85 mosm/kg H_2O, a concentration at which approximately 50% of the control cells lysed). The high K^+ solution, diluted Ringer with the concentrations of NaCl and KCl switched with one another, increased osmotic fragility ($n = 12$). Quinine (1 mM, $n = 12$), a nonselective K^+ channel antagonist, also increased fragility, whereas the cationophore gramicidin (gram, 1 μM, $n = 12$) reversed this effect (both agents were added to the extracellular medium before the addition of RBCs, and choline was substituted for Na^+ when conducting experiments with gramicidin). Values are means ± SEM of OD. *** $P < 0.001$

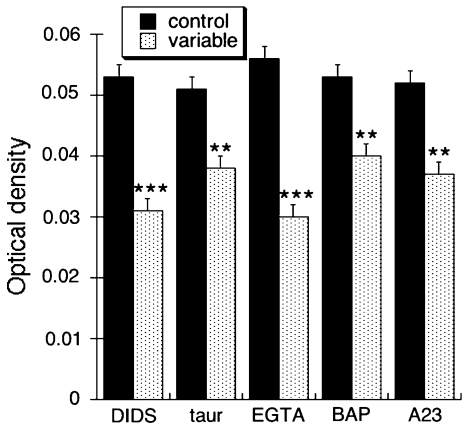

Fig. 2 Inhibition of Cl^- efflux or chelating Ca^{2+} increased osmotic fragility. The control (*solid bar*) was diluted Ringer (85 mosm/kg H_2O). The Cl^- transport inhibitor DIDS (10 μM), added to Ringer before the addition of RBCs, increased osmotic fragility ($n = 12$). The taurine Ringer (30 mM taurine with NaCl reduced accordingly to maintain osmolality) also increased fragility ($n = 12$). EGTA, used to reduce the level of free Ca^{2+} in the extracellular medium to 10 nM ($n = 12$), and BAPTA-AM (100 μM), used to chelate cytosolic Ca^{2+} ($n = 8$), increased fragility. The Ca^{2+} ionophore A23187 (0.5 μM) caused an increase in osmotic fragility ($n = 12$). Values are means ± SEM of OD. ** $P < 0.01$, *** $P < 0.001$

within each experimental set.) Spectrophotometric experiments were conducted at 625 nm because an absorption spectrum indicated that this wavelength provided the greatest difference in optical density (OD) between intact and lysed cells.

A hemolytic index (HI) was determined using the following formula: HI(%) = (OD of test compound − OD of negative control)/(OD of positive control − OD of negative control) × 100, where OD of test compound refers to the OD of a cell suspension in diluted Ringer to which a test compound was added, OD of negative control refers to the OD of a cell suspension in diluted Ringer, and OD of positive control refers to the OD of a cell suspension in distilled water (used to set spectrophotometer OD to zero).

All reported hemolytic indices were calculated using Ringer solutions with a concentration of (mean ± standard error of the mean [SEM]) 85.0 ± 1.5 mosm/kg H_2O. We chose this concentration because it was sufficiently dilute to lyse approximately half the cells in suspension (determined empirically by challenging cells with solutions ranging from 0 to 300 mosm/kg H_2O). Consequently, we could assess whether a test compound increased osmotic fragility by a subsequent reduction in OD compared to the control solution. Conversely, a rise in OD indicated that a test compound reduced osmotic fragility. Because nonmammalian vertebrates have nucleated RBCs, which causes suspensions of lysed RBCs to remain slightly turbid, $NaHCO_3$ (1 mM) was added to all solutions. The slight alkalinity leads to better clearing of solutions without influencing experimental results (Light et al. 2003). Cell lysis also was confirmed microscopically (Nikon Diaphot, Hoffman differential interference contrast optics, 400×).

Fluorescence Microscopy

Intracellular free Ca^{2+} levels were monitored using the fluorescent Ca^{2+} indicator fluo-4-AM (10 μM, Molecular Probes, Eugene, OR). This indicator has a high binding affinity for Ca^{2+} ($K_{id} = 345$ nM) and a large fluorescence intensity increase in response to Ca^{2+} binding (>100 fold, Thomas et al. 2000). The acetoxymethyl (AM) ester derivative permeates cell membranes, and once inside a cell, the lipophilic blocking groups are cleaved by nonspecific esterases. This results in a charged form that is relatively impermeable.

Aliquots of fluo-4-AM were mixed with dimethyl sulfoxide (DMSO) and diluted to give a final concentration of 10 μM. The nonionic detergent Pluronic F-127 was used to assist in dispersion of the nonpolar AM ester in aqueous media. This was accomplished by mixing an aliquot of AM ester stock solution in DMSO with an equal volume of 20% (w/v) Pluronic in DMSO before dilution into the loading medium. Cells were incubated with the AM ester for 60–90 min at room temperature. Cells were then washed in indicator-free medium to remove any dye that was not specifically associated with the cell surface and then incubated for another 30–60 min to allow for complete de-esterification of intracellular AM esters.

Cell swelling was accomplished by adding an equal volume of hypotonic (0.1×) Ringer to the isotonic Ringer (this reduced osmolality [0.5×] without changing the Ca^{2+}, Mg^{2+}, glucose, and buffer concentrations). Cells in isotonic Ringer served as the negative control, whereas cells in isotonic Ringer with A23187 (Ca^{2+}-ionophore, 0.5 μM; Tiffert and Lew 1997) served as the positive control. Cells were photographed with a Zeiss Axiovert 100 microscope using Metamorph 6.1 software (Universal Imaging Corp.) or with a Nikon TE 2000-U microscope equipped with Hoffman DIC optics (400×) and epifluorescence (mercury lamp and FITC filter cube) using Metamorph 6.2 software. For any given experiment, only cells photographed using the same microscope and imaging parameters were used for comparison purposes.

Coulter Counter

Cell volume distribution curves were obtained by electronic sizing using a Coulter counter model Z2 with channelizer (Coulter Electronics, Hialeah, FL). Mean cell volume was taken as the mean volume of the distribution curves. The diameter of the aperture tube orifice was 100 μm, and the metered volume was 0.5 ml. Absolute cell

volumes were obtained using polystyrene latex beads (9.565 μM diameter or 459.9 fl volume) as standards (Coulter). Experiments with the latex beads showed that measured volumes were unaffected by changes in osmolality and ionic composition within the ranges used for this study. Cell suspensions were diluted to give a final cell density of approximately 10,000 cells/ml.

Relative cell volume is defined as the average volume of cells compared to that in an isotonic medium. As described by others (Jorgensen et al. 1997), a percentage volume recovery at X minutes after hypotonic exposure was calculated as $[(V_{max} - V_{X\ min})/(V_{max} - V_0)] \times 100$, where V_{max} is the peak relative cell volume, V_0 is the initial relative volume (or one), and $V_{X\ min}$ is the relative cell volume measured X minutes after hypotonic exposure. A percentage volume decrease was calculated as [(percentage $\text{recovery}_{experimental}$)/(percentage $\text{recovery}_{control}$)] × 100, where maximal recovery in hypotonic Ringer is 100%.

Solutions

Isosmotic reptilian Ringer consisted of (in mM) 140 NaCl, 6 KCl, 2.0 $CaCl_2$, 1.5 $MgCl_2$, 5.5 glucose, and 10 HEPES (N-[2-hydroxyethyl] piperazine-*N*′[2-ethanesulfonic acid]; titrated to pH 7.4 with NaOH or HCl, 300 ± 2 mosm/kg H_2O). A low Na^+ Ringer, used for all experiments with gramicidin, was prepared by substituting choline chloride for NaCl (Mastrocola et al. 1991), and a hypotonic (∼0.5×) Ringer was obtained by reducing the NaCl concentration to 70 mM. A high K^+ Ringer was made by swopping the concentrations of NaCl and KCl, and a high taurine Ringer contained 30 mM taurine (with an appropriate reduction in the concentration of NaCl). For some experiments, NaCl was replaced with N-methyl-D-glucamine (NMDG) chloride, and in other experiments the free Ca^{2+} concentration in the extracellular solution was adjusted from 10 nM to 5000 nM using ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and appropriate concentrations of $CaCl_2$ (calculated by http://entropy.brneurosci.org/egta.html or http://www.stanford.edu/∼cpatton/webmaxc/webmaxclite115.htm). Cells were loaded with 1,2-bis(2-aminophenoxymethyl) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid *tetrakis*(acetoxymethyl) ester (BAPTA-AM) in a manner similar to that described above for fluo-4-AM.

A stock solution of gramicidin was dissolved in methanol, A23187 was prepared in DMSO, and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) was dissolved in ethanol, all at 1000× the final concentration and then diluted to give an appropriate working concentration. All stock aqueous solutions were diluted 100× to give an appropriate final concentration. For osmotic fragility experiments, pharmacological agents or their vehicle were present before the addition of cells. For cell volume studies, pharmacological antagonists were added with hypotonic exposure, whereas agonists were added at the peak cell volume (1 min after hypotonic shock). The osmolality of solutions was measured with a vapor pressure osmometer (#5500, Wescor, Logan, UT), and the pH was determined using an Accumet Basic AB15 pH meter (Fisher Scientific). Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), Axxora (San Diego, CA), and ICN Pharmaceuticals Inc. (Costa Mesa, CA). All solutions were filtered with a 0.8 μM Advantec cellulose acetate membrane filter before use, and all experiments were conducted at room temperature (21–23°C).

Statistics

Data are reported as means ± SEM. The statistical significance of an experimental procedure was determined by a paired Student's *t*-test or least significant difference test with paired design of analysis of variance (ANOVA)/multivariate ANOVA (MANOVA), as appropriate (Data Desk software, Ithaca, NY). $P < 0.05$ was considered significant. Cell volumes at specific times were tested against each other.

Results

Osmotic Fragility Studies

Although osmotic fragility depends on several factors, we first examined this property as one assessment of a cell's ability to regulate volume in a hypotonic medium. The OD, measured at a concentration of Ringer where approximately 50% of the cells in suspension were intact, was 0.069 ± 0.002 ($n = 12$ experiments, Fig. 1). To determine whether osmotic fragility depended on the efflux of K^+, we repeated this assay with a high K^+ (low Na^+) medium to reduce the electrochemical gradient for this cation. In this case, the OD measured at the same osmolality as the control decreased to 0.036 ± 0.003 ($n = 12$, $P < 0.001$, Fig. 1), giving a HI of 48%.

Consistent with these results, the nonselective K^+ channel antagonist quinine (1 mM; Yeung et al. 2005) also increased osmotic fragility. In this instance, the OD decreased from 0.074 ± 0.002 to 0.042 ± 0.002, giving a HI of 43% ($n = 12$, $P < 0.001$, Fig. 1). Quinidine (1 mM) had the identical response as quinine ($n = 8$); however, tetraethylammonium (TEA, 10 mM, $n = 5$) had no effect (not shown). In contrast, the K^+–Cl^- cotransport inhibitors bumetanide (10 μM) and furosemide (10 μM and 100 μM; Ellory et al. 1990) had no demonstrable effect (not shown). However, gramicidin (1 μM) prevented the inhibitory

effect of quinine (gramicidin is a cationophore that was used to maintain a high K^+ permeability in a choline Ringer, Mastrocola et al. 1991). In fact, even in the presence of quinine, gramicidin increased the OD from 0.070 ± 0.003 to 0.113 ± 0.003 ($n = 12$, $P < 0.001$, Fig. 1), indicating that quinine did block K^+ efflux. Vehicle for gramicidin (methanol, diluted 1:1000, $n = 6$) or replacing Na^+ with choline ($n = 6$) had no demonstrable effect (not shown).

We next examined whether the level of Cl^- efflux influenced osmotic fragility. Cells exposed to the Cl^- transport antagonist DIDS (10 μM; Yeung et al. 2005) had a decrease in OD, from 0.053 ± 0.002 to 0.031 ± 0.002 ($n = 12$, $P < 0.001$, Fig. 2), giving a HI of 42%. In addition, the efflux of taurine, an amino acid commonly eliminated by cells during RVD (Hoffmann et al. 2009; Puffer et al. 2006), also was assessed. Cells bathed in a high taurine Ringer displayed a decrease in OD, from 0.051 ± 0.002 to 0.038 ± 0.002, a HI of 25% ($n = 12$, $P < 0.01$, Fig. 2).

To determine whether osmotic fragility depended on extracellular Ca^{2+}, we repeated this assay with a low Ca^{2+}-EGTA Ringer (10 nM free Ca^{2+}). In this case, the OD decreased from 0.056 ± 0.002 to 0.030 ± 0.002, giving a HI of 46% ($n = 12$, $P < 0.001$, Fig. 2). Because a low concentration of extracellular Ca^{2+} may make cells more fragile by reducing membrane integrity (Orlov et al. 2005), we also directly lowered intracellular Ca^{2+} by loading cells with BAPTA-AM (100 μM). This procedure decreased the OD from 0.053 ± 0.002 to 0.040 ± 0.002, a HI of 25% ($n = 8$, $P < 0.01$, Fig. 2). Having established that a low concentration of Ca^{2+} increased osmotic fragility, we next used A23187, a Ca^{2+}-specific ionophore (Yeung et al. 2005), to artificially increase levels of intracellular Ca^{2+}. Surprisingly, A23187 (0.5 μM) decreased the OD from 0.052 ± 0.002 to 0.037 ± 0.002 ($n = 12$, $P < 0.01$, Fig. 2), indicating a greater number of cells had lysed (HI of 29%). Finally, the calcium channel blockers verapamil (10 μM, $n = 6$) and nifedipine (10 μM, $n = 6$; Mignen et al. 1999; Tinel et al. 2000) had no effect on osmotic fragility (not shown).

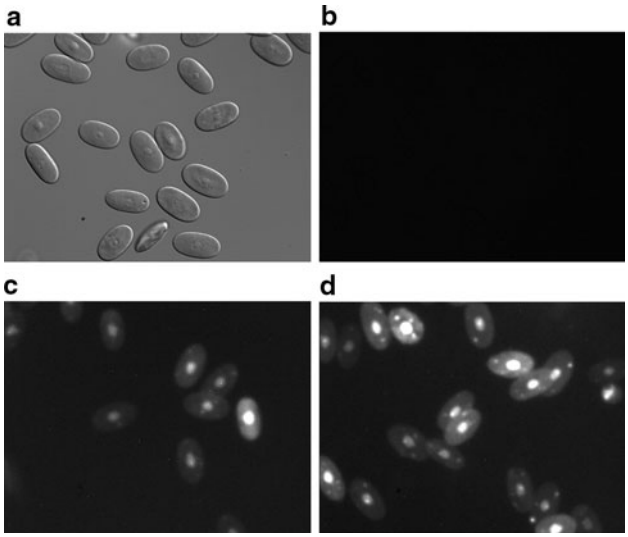

Fig. 3 Alligator erythrocytes loaded with fluo-4 (10 μM) and exposed to ultraviolet light emitted from a mercury vapor bulb, filtered through a FITC cube (400×). **a** Cells in isosmotic Ringer visualized with DIC optics (cells were ~21 μm × 11 μm or ~390 fl). **b** Cells did not display fluorescence under isosmotic conditions ($n = 8$). **c** Addition of A23187 (0.5 μM) to the extracellular medium, a positive control, increased fluorescence under isosmotic conditions ($n = 8$). **d** Exposure to a hypotonic (0.5×) Ringer increased fluorescence compared to basal conditions ($n = 8$)

Fluorescence Microscopy Studies

Having shown that osmotic fragility was dependent on the concentration of extracellular Ca^{2+}, we next determined whether levels of intracellular Ca^{2+} changed during cell swelling. A photograph of cells in isosmotic Ringer under DIC optics is shown in Fig. 3a. As shown in Fig. 3b, cells loaded with fluo-4-AM (10 μM) did not display fluorescence under isosmotic conditions ($n = 8$). In contrast, addition of A23187 (0.5 μM), used as a positive control, stimulated fluorescence under isosmotic conditions. This indicated that cells were properly loaded with dye and that we could detect qualitative changes in the level of cytosolic Ca^{2+} ($n = 8$, Fig. 3c). Exposure of cells to a hypotonic (0.5×) Ringer increased the level of fluorescence, indicating a rise in intracellular Ca^{2+} occurred in swollen cells ($n = 8$, Fig. 3d). However, exposing cells to a low Ca^{2+}-EGTA hypotonic (0.5×) Ringer did not cause demonstrable fluorescence ($n = 8$, not shown). Finally, verapamil (10 μM, $n = 5$) and nifedipine (10 μM, $n = 5$) had no effect on fluorescence after hypotonic challenge (not shown).

Cell Volume Studies

When RBCs were placed in a hypotonic (0.5×) Ringer, they quickly swelled, and then slowly and spontaneously decreased in volume, giving a percent volume recovery of 44% (Fig. 4a). In contrast, a high K^+ hypotonic Ringer caused a dramatic inhibition of volume recovery ($n = 12$, $P < 0.001$ at > 20 min, Fig. 4a). In this case, the percent volume recovery was −25%, indicating the cells had actually continued to swell further.

Volume recovery in RBCs also was inhibited with quinine (1 mM). In this case, although the recovery for control cells at 70 min after hypotonic shock was 79%, and it was only 38% with quinine ($n = 12$, $P < 0.001 > 20$ min, Fig. 4b). Quinidine (1 mM) had the same level of inhibition on volume recovery ($n = 6$); however, TEA (10 mM, $n = 5$) had no effect (not shown). In addition, bumetanide

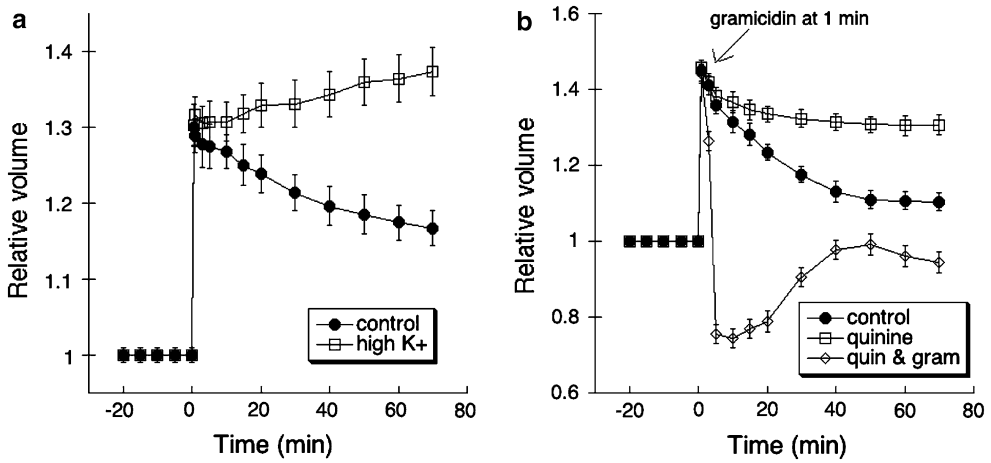

Fig. 4 Inhibition of K^+ efflux blocked volume recovery. At time 0, cells were abruptly exposed to a hypotonic (~0.5×) Ringer, which caused a rapid initial increase in volume followed by a gradual recovery toward basal values, despite the continued presence of a hypotonic medium. **a** High K^+ hypotonic Ringer blocked volume recovery ($n = 12$). **b** Quinine (1 mM), added to the bath before the addition of cells, also blocked volume recovery ($n = 12$). In contrast, gramicidin (1 μM), added at 1 min after hypotonic shock, potentiated volume recovery even in the presence of quinine ($n = 10$). Choline was substituted for Na^+ when conducting experiments with gramicidin. Values are means ± SEM

(10 μM) and furosemide (10 μM or 100 μM) had no demonstrable effect (not shown). However, gramicidin (1 μM), even in the presence of quinine (1 mM), enhanced volume recovery. The percent volume recovery with gramicidin at 70 min was 111% ($n = 12$, $P < 0.001$, Fig. 4b; gramicidin was added 1 min after hypotonic shock, when maximum cell volume was observed).

We next examined the effect of two ionophores using an isosmotic Ringer. As illustrated in Fig. 5, there was a significant reduction in volume after the addition of the cationophore gramicidin (1 μM, $n = 8$, $P < 0.001 > 5$ min). A lack of subsequent volume recovery to basal conditions with gramicidin was most likely due to the use of a choline Ringer, which could inhibit regulatory volume increase (RVI). Interestingly, the Ca^{2+}-specific ionophore A23187 (0.5 μM) caused a slight but significant increase in cell volume under isosmotic conditions ($n = 8$, $P < 0.05$, Fig. 5).

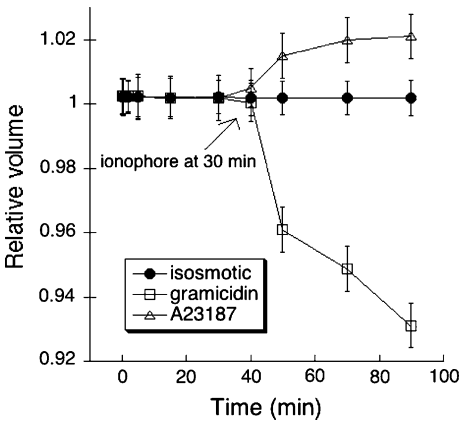

Fig. 5 Pharmacologically increasing the K^+ permeability caused volume decrease under isosmotic conditions. That is, addition of the ionophore gramicidin (1 μM) to isosmotic Ringer caused cells under basal conditions to shrink ($n = 10$, choline was substituted for Na^+ when conducting experiments with gramicidin). In contrast, the Ca^{2+} ionophore A23187 (0.5 μM) caused a slight but significant increase in volume under isosmotic conditions ($n = 8$). Values are means ± SEM

The influence of extracellular Ca^{2+} on volume recovery also was assessed. As predicted from the osmotic fragility studies, reducing extracellular Ca^{2+} to 10 nM inhibited recovery of cell volume after hypotonic shock ($n = 12$, $P < 0.001 > 5$ min, Fig. 6a). Under control conditions, recovery at 90 min was 44%, whereas it was −15% for the low Ca^{2+}-EGTA Ringer. Interestingly, as shown in Fig. 6a, gramicidin (1 μM) prevented the inhibitory affect of the low Ca^{2+} Ringer ($n = 12$). However, both nifedipine (10 μM, $n = 6$) and verapamil (10 μM, $n = 6$) had no demonstrable affect on volume recovery (not shown).

Having established that decreasing extracellular Ca^{2+} to 10 nM inhibited volume recovery, we next determined whether there was a concentration-dependence for this ion in the extracellular medium. To accomplish this, we used EGTA to chelate Ca^{2+} to different levels, ranging from 10 nM to 5000 nM. As illustrated in Fig. 6b, the percent volume recovery, measured 40 min after hypotonic challenge, was inhibited for all concentrations less than or equal to 500 μM ($n = 6$ for each concentration). In addition, the steep portion of this sigmoidal relationship was between 10 μM and 500 μM Ca^{2+} in the external medium.

We also examined the effect of reducing intracellular Ca^{2+} directly by loading cells with BAPTA-AM (100 μM). As illustrated in Fig. 7a, this procedure inhibited volume recovery. At 90 min, the percent recovery for control cells

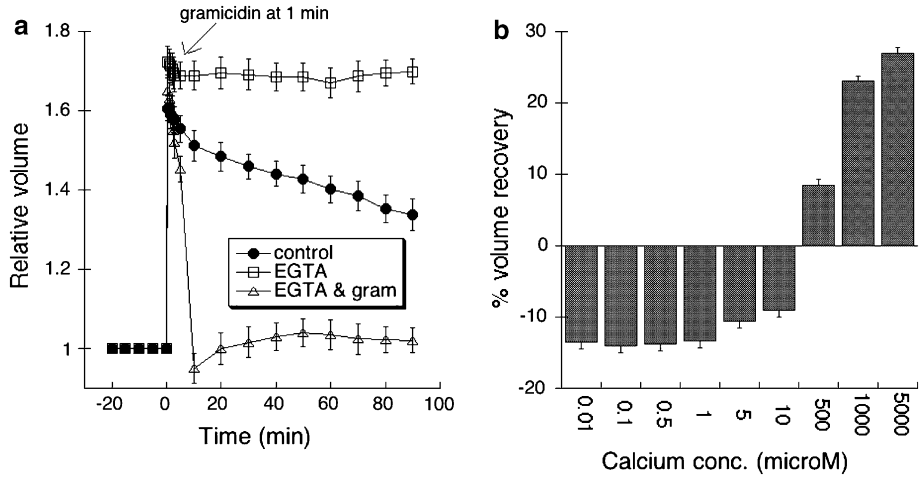

Fig. 6 Chelating Ca^{2+} in the extracellular medium inhibited recovery of cell volume. **a** The rate of cell volume recovery was reduced dramatically when cells were bathed in a low Ca^{2+}-EGTA hypotonic Ringer ($n = 12$). In contrast, the inhibitory affect of low extracellular Ca^{2+} was prevented with the addition of gramicidin (1 μM) to the extracellular medium (added 1 min after hypotonic shock, $n = 12$). **b** Dose–response relationship between the extracellular Ca^{2+} concentration and percent volume recovery measured 40 min after hypotonic challenge ($n = 6$ for each concentration of Ca^{2+}). Values are means ± SEM

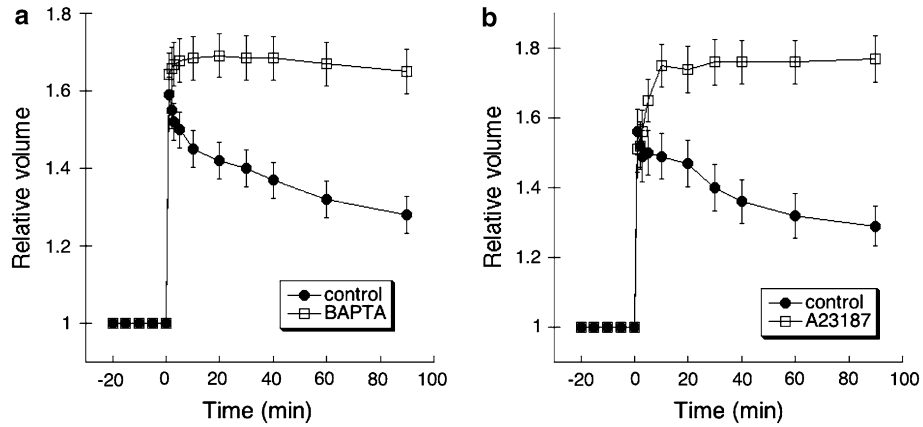

Fig. 7 Chelating intracellular Ca^{2+} and pharmacologically increasing Ca^{2+} influx had similar effects on the recovery of cell volume after hypotonic shock. At time 0, cells were abruptly exposed to a hypotonic (0.5×) Ringer. **a** Loading cells with BAPTA-AM (100 μM), to reduce the level of cytosolic Ca^{2+}, inhibited volume recovery ($n = 8$). **b** Similarly, volume recovery was blocked with A23187 (0.5 μM) in the extracellular medium ($n = 12$). Values are means ± SEM

was 53%, but was −10% with BAPTA-AM ($n = 8$, $P < 0.001 > 5$ min, Fig. 7a). As described above, we demonstrated with the osmotic fragility studies that addition of A23187 to the extracellular medium made cells more fragile. Consistent with these results, we found A23817 (0.5 μM) inhibited volume recovery. In this case, the recovery for control cells at 90 min was 48%, whereas it was −38% with the ionophore ($n = 12$, $P < 0.001 > 5$ min, Fig. 7b). Similar results with A23187 also were obtained when NaCl in the extracellular solution was replaced with choline chloride ($n = 6$) or NMDG chloride ($n = 8$, not shown).

We next examined whether the inhibition of volume regulation by increasing intracellular Ca^{2+} to pharmacologically high levels had a time-dependent nature. This was accomplished by adding A23187 (0.5 μM) at 5 min ($n = 10$, Fig. 8a) and at 30 min ($n = 10$, Fig. 8b) after hypotonic shock. In both cases, the ionophore quickly and dramatically inhibited volume recovery. Further, we determined whether artificially increasing K^+ permeability with gramicidin would reverse the inhibitory affect of A23187. For this part of the study, gramicidin was added 5 min or 30 min after hypotonic challenge to an extracellular medium that already contained A23187 (0.5 μM). As shown in Fig. 9, gramicidin (1 μM) quickly and profoundly reduced cell volume, reversing the inhibitory affect of A23187 in a time-independent manner ($n = 8$).

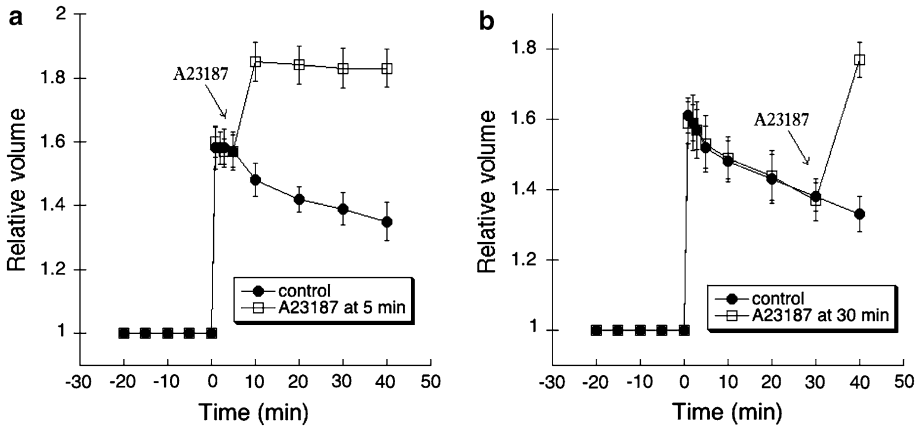

Fig. 8 Calcium ionophore inhibited volume recovery after hypotonic shock in a time-independent manner. At time 0, cells were abruptly exposed to a hypotonic (0.5×) Ringer. **a** Volume recovery was inhibited when A23187 (0.5 μM) was added to the extracellular medium 5 min after hypotonic challenge ($n = 10$). **b** Similar results were obtained when ionophore was added at 30 min ($n = 10$). Values are means ± SEM

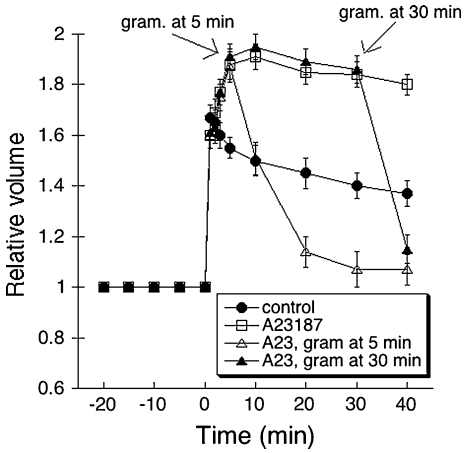

Fig. 9 Increasing K^+ permeability pharmacologically reversed the inhibitory effect of Ca^{2+} ionophore in a time-independent manner. At time 0, cells were abruptly exposed to a hypotonic (0.5×) Ringer. A23187 (0.5 μM) was present for all conditions, except the control, and was placed in the extracellular medium before the addition of cells. Gramicidin (1 μM) was then added to the extracellular solution at either 5 min ($n = 8$) or 30 min ($n = 8$) after hypotonic shock. Cell volume recovery was enhanced regardless of when it was added. Choline was substituted for Na^+ when conducting experiments with gramicidin. Values are means ± SEM

Discussion

A major finding of this study is that RVD by alligator RBCs depended on K^+ efflux during cell swelling. We demonstrated this by bathing alligator RBCs in a high K^+ medium, which increased osmotic fragility and also reduced the degree of volume recovery after hypotonic shock. In a similar manner, Kurbannazarova et al. (2003) reported that RVD was abolished in rat thymocytes when external Na^+ was replaced with K^+. We also found that RVD was accelerated in the presence of gramicidin. This indicates that the ionophore-induced K^+ permeability (P_K) was greater than the swelling-induced P_K, and thus P_K was a rate limiting process during volume regulation. Related to this, we found gramicidin caused cells to shrink under isosmotic conditions. This also is consistent with alligator RBCs having a low K^+ permeability under basal conditions and an elevated K^+ permeability during hypotonic stress. This conclusion is consistent with reports for bovine trabecular meshwork cells (Srinivas et al. 2004), murine spermatozoa (Yeung et al. 2005), and *Necturus* RBCs (Light et al. 2003), where RVD also was limited by the rate of K^+ efflux.

In addition, we found gramicidin reversed the inhibition of RVD by quinine and quinidine, indicating that these antagonists did indeed block K^+ efflux. In a similar manner, Yeung et al. (2005) used valinomycin to show the involvement of K^+ channels in RVD by murine spermatozoa. Our studies also demonstrated that in addition to swollen cells having a high K^+ permeability, there was presumably an accompanying high Cl^- permeability. This was further supported by a significant increase in osmotic fragility when the Cl^- transport blocker DIDS was used. A similar phenomenon is reported for trabecular meshwork cells (Srinivas et al. 2004), human fibroblasts (Mastrocola et al. 1991), Ehrlich ascites tumor cells (Niemeyer et al. 2001), *Necturus* RBCs (Light et al. 2003), and human bronchial epithelial cells (Caplanusi et al. 2006). In all of these cell types, hypotonic shock activates a separate P_K and P_{Cl}.

Our pharmacological studies also provide indirect evidence that K^+ efflux occurred through a K^+ channel. That is, the nonselective K^+ channel blockers quinine and quinidine increased the limit of osmotic fragility and also inhibited volume recovery, which is consistent with K^+

efflux occurring via a conductive pathway (Yeung et al. 2005). Similarly, others have shown that quinine inhibits RVD. For example, this antagonist blocks RVD in murine spermatozoa (Yeung et al. 2005), human bronchial epithelia cells (Caplanusi et al. 2006), and boar and bull spermatozoa (Petrunkina et al. 2001). Further, many cell types lose K^+ during RVD via the activation of a K^+ conductance. For instance, human bronchial epithelial cells (Caplanusi et al. 2006), bovine trabecular meshwork cells (Srinivas et al. 2004), Ehrlich cells (Niemeyer et al. 2001), murine spermatozoa (Yeung et al. 2005), human osteoblasts (Weskamp et al. 2000), human nasopharyngeal carcinoma cells (He et al. 2009), and others (Hoffmann et al. 2009; Koivusalo et al. 2009; Lang 2007) all are reported to have hypotonic challenge activate a K^+ conductance. However, the loss of K^+ by an ion channel during cell swelling is relatively unusual for RBCs. To our knowledge, the only other reports where a K^+ conductance is activated during RVD in a RBC is for the lamprey (Virkki and Nikinmaa 1995) and *Necturus* (Light et al. 2003). Thus, it appears that KCl efflux from RBCs of most species is mediated by an electroneutral transport mechanism. For example, a KCl cotransporter is used during RVD by RBCs from dog (Fujise et al. 2001), human (Joiner et al. 2004), and other species (Hoffmann et al. 2009).

It has been suggested by Niemeyer et al. (2001) that K^+ channels activated during cell swelling belong to the background K^+ channel group, voltage-independent channels which underlie the resting potential of many cells. However, unlike a number of cell types that have a high resting permeability to K^+, RBCs have a high basal permeability to Cl^-, and thus their resting potential is determined primarily by P_{Cl}. Accordingly, K^+ channels that are activated during RVD in alligator RBCs may be different from the background K^+ channels described by Niemeyer et al. (2001). In addition, we found inhibition of RVD by quinine was not as great as the inhibition resulting from a high K^+ Ringer. This suggests that quinine did not completely inhibit the K^+ efflux that occurred during volume recovery. However, we also found that traditional K–Cl cotransport inhibitors, such as bumetanide and furosemide, had no demonstrable effect, nor did TEA. Thus, the specific permeability pathways for K^+ remain to be elucidated.

Although K^+ efflux played a significant role during RVD by alligator RBCs, taurine efflux also was important for volume regulation. This was demonstrated by an increase in osmotic fragility with a high taurine Ringer. We point out that the loss of taurine during RVD is not unique to alligator RBCs. In fact, it has been reported for other cell types, including little skate RBCs (Puffer et al. 2006), catfish liver cells (Goswami and Saha 2006), and others (Hoffmann et al. 2009), all of which release taurine in response to volume expansion.

Another major finding of this study is that hypotonic swelling stimulated a rise in intracellular Ca^{2+}, which was necessary for RVD. This conclusion was demonstrated, in part, by using epifluorescence microscopy and the fluorescent Ca^{2+} indicator fluo-4. We found no fluorescence for cells exposed to isosmotic Ringer, indicating low levels of free cytosolic Ca^{2+}. In contrast, hypotonic shock caused demonstrable fluorescence, demonstrating a rise in intracellular Ca^{2+} associated with cell swelling. Although swollen cells displayed fluorescence as soon as we observed them, we could not assess the initial time course of events immediately after hypotonic challenge. Therefore, we cannot say for certain whether a rise in intracellular Ca^{2+} preceded volume recovery, and thus triggered RVD, or whether fluorescence lagged behind the volume response because it resulted from damage to membrane integrity. However, given this set of studies was conducted using a 0.5× Ringer with a normal extracellular level of Ca^{2+}, which did not cause cells to lyse, our results are consistent with Ca^{2+} influx preceding and stimulating volume recovery. In addition, our finding that hypotonic shock increased cytosolic Ca^{2+} is consistent with reports for several other cell types. For instance, reducing the osmolality of the bathing medium causes cytosolic Ca^{2+} to increase in rat inner medullary collecting duct cells (Tinel et al. 2000) and human osteoblasts (Weskamp et al. 2000).

Our findings also show that extracellular Ca^{2+} in particular was necessary for the swelling-induced rise in cytosolic Ca^{2+}. This was shown by a lack of fluorescence associated with hypotonic shock when cells were exposed to a low Ca^{2+} ($\sim$10 nM) medium. We should note that once internalized, fluo-4 is diluted as cells swell in response to hypotonic shock. This in turn decreases fluorescence intensity in a corresponding manner. Because we did not measure relative fluorescence intensity concomitant with cell volume changes, it is possible there was a small rise in intracellular Ca^{2+} in swollen cells exposed to the low Ca^{2+}-EGTA Ringer that we could not detect. However, we do not believe this was a significant problem in our study because cells in a normal Ca^{2+} hypotonic Ringer displayed bright fluorescence even though they initially swelled to the same extent as cells in a low Ca^{2+}-EGTA solution. Conversely, although cells in a low Ca^{2+} hypotonic Ringer had an inhibited RVD, they still initially swelled to approximately the same size as cells in normal Ca^{2+} hypotonic Ringer, yet did not fluoresce. Taken together, these observations support out conclusion that extracellular Ca^{2+} was necessary for an increase in fluorescence associated with cell swelling. We cannot, however, rule out the presence of a Ca^{2+}-induced calcium release from intracellular stores.

A dependence on extracellular Ca^{2+} for RVD was further supported by an increase in osmotic fragility and a lack

of volume recovery after hypotonic shock when we exposed cells to a low Ca^{2+}-EGTA Ringer. It should be noted that Orlov et al. (2005) have shown extracellular Ca^{2+} is required for the maintenance of plasma membrane integrity in nucleated cells. In particular, they found RBCs from fish, but not from mammals, underwent hemolysis when exposed to low extracellular Ca^{2+}, and this effect was absent when instead cells were loaded with BAPTA-AM. Although we cannot rule out definitively that there was loss in membrane integrity when we used a low Ca^{2+}-EGTA Ringer, we do not believe our observations can be adequately explained by this phenomenon. That is, we found alligator RBCs loaded with BAPTA-AM also had an increase in osmotic fragility and an inhibition of volume recovery, indicating the effect we observed with a low Ca^{2+}-EGTA Ringer was likely due to a low availability of intracellular Ca^{2+} and not the loss of membrane integrity. The importance of extracellular Ca^{2+} for RVD also has been shown for a number of other cell types (Hoffmann et al. 2009; Koivusalo et al. 2009; Lang 2007; Wehner et al. 2003). For instance, human osteoblasts (Weskamp et al. 2000), human nasopharyngeal carcinoma cells (He et al. 2009), and murine spermatozoa (Yeung et al. 2005) all display a reduced RVD when placed in a low Ca^{2+} medium.

We also conducted preliminary experiments to assess the specific nature of a Ca^{2+} entry pathway during cell swelling. We found conventional Ca^{2+} channel blockers, such as verapamil and nifedipine, had no effect on fluo-4 fluorescence or on cell volume recovery. This is in contrast to several other reports that show these antagonists inhibit a rise in intracellular Ca^{2+} after hypotonic challenge. For instance, Mignen et al. (1999) report that verapamil and nifedipine dramatically reduce volume recovery by intact mouse distal colon crypt cells and Tinel et al. (2000) found these antagonists partly reduced the calcium response during swelling of kidney cells. Park et al. (2007) found that whereas nifedipine had no affect on RVD, verapamil significantly inhibits volume recovery. Thus, the Ca^{2+} permeability pathway in alligator RBCs remains ill defined. An intriguing candidate, however, is a TRP channel. For instance, Foller et al. (2008) have shown that human and mouse erythrocytes express TRPC6 cation channels that can be activated by osmotic shock and subsequently contribute to Ca^{2+} leak, as well as Ca^{2+}-induced suicidal death.

Interestingly, we found that a pharmacologically high level of intracellular Ca^{2+}, obtained by using A23187, had a similar effect as a pharmacologically low level of extracellular or intracellular Ca^{2+} (obtained with EGTA or BAPTA-AM, respectively). In other words, both situations increased osmotic fragility and inhibited volume recovery after hypotonic shock. In addition, A23187 caused cells under isosmotic conditions to swell, indicating that this phenomenon was not unique to hypotonic challenge. The inhibitory results obtained with A23187 were quite surprising because they were the opposite of what we found when using *Necturus* RBCs (Light et al. 2003) and salmon RBCs (unpublished results). It is possible the level of intracellular Ca^{2+} that resulted with A23187 stimulated solute flux related to RVI. However, we obtained similar results with A23187 when the NaCl in the extracellular medium was replaced with choline chloride or NMDG chloride, both of which were presumably impermeable to the plasma membrane. This indicates it was unlikely that the Ca^{2+} ionophore was permeated with Na^{+}, thereby causing cell swelling. In addition, the concentration of Ca^{2+} in the extracellular medium was 2 mM, and even if the intracellular concentration of this ion increased to the same level in the presence of A23187, it would not explain the amount of swelling that was observed. Further, given the rapid time course of swelling with A23187, it seems unlikely the ionophore stimulated metabolic activity that increased intracellular osmolality (e.g., hydrolysis of proteins into peptides and amino acids). Thus, the inhibitory effect caused by A23187 is intriguing and remains unexplained.

It also should be noted that RVD is not always reported to be dependent on Ca^{2+}. For example, Pasantes-Morales and Morales (2000) found Ca^{2+}-dependent (predominantly epithelial cells) and Ca^{2+}-independent (nonepithelial cells) K^{+} fluxes activated by cell swelling. In the latter case, they suggest that a rise in intracellular Ca^{2+} elicited by hypotonic challenge may be an epiphenomenon (Pasantes-Morales and Morales 2000). Further, Park et al. (2007) report that mouse cholangiocytes show no increase in intracellular Ca^{2+} during RVD, and that chelation of extracellular Ca^{2+} with EGTA has no affect on volume recovery. Nonetheless, they found that BAPTA-AM decreases intracellular Ca^{2+} and also inhibits RVD, which is reversed with valinomycin, suggesting that the BAPTA-AM-induced inhibition is due to a K^{+} conductance or other cellular process stimulated by intracellular Ca^{2+}. They concluded that although an increase in intracellular Ca^{2+} or extracellular Ca^{2+} is not required for RVD, Ca^{2+} might have a permissive role (Park et al. 2007).

Nonetheless, our overall results are consistent with a rise in cytosolic Ca^{2+} being necessary for RVD. We make this claim because exposing cells to a low Ca^{2+}-EGTA Ringer or loading cells with BAPTA-AM increased osmotic fragility and also reduced the rate of volume recovery, demonstrating the importance of Ca^{2+}, and specifically extracellular Ca^{2+}, for volume regulation. Thus, if a rise in Ca^{2+} we observed during cell swelling was an epiphenomenon accompanying RVD, as described by others (Pasantes-Morales and Morales 2000), then chelating extracellular or intracellular Ca^{2+} should not have inhibited volume decrease.

Although we have not determined the specific Ca^{2+} requirements for activating K^+ efflux in alligator RBCs, we have shown that Ca^{2+} influx and K^+ efflux are necessary steps for RVD. Further, we demonstrated that in swollen cells, Cl^- permeability was high, and K^+ permeability was rate limiting to volume recovery. We therefore suggest that a rise in intracellular Ca^{2+} specifically stimulated K^+ efflux. This was shown, in part, pharmacologically using the cationophore gramicidin with a choline Ringer. With this solution, K^+ and Cl^- were the only two permeable ions of significance and addition of gramicidin ensured a continual high K^+ permeability. In this case, gramicidin prevented the inhibitory effect of a low Ca^{2+}-EGTA Ringer, as well as the inhibitory effect of quinine. These results indicate that K^+ efflux occurred "downstream" to the Ca^{2+}-dependent processes, and that our results are most consistent with a rise in cytosolic Ca^{2+} leading to the stimulation of K^+ efflux, either directly (Ca^{2+}-activated K^+ channel, Weskamp et al. 2000) or indirectly (a Ca^{2+}-stimulated biochemical pathway; Jakab et al. 2002). Taken together, our observations are most consistent with this cell type having a low K^+ permeability under isotonic conditions, and an elevated K^+ permeability in response to a rise in cytosolic Ca^{2+} during hypotonic stress.

The scenario we propose is similar with RVD described for a number of other cell types (Hoffmann et al. 2009; Koivusalo et al. 2009; Lang 2007; Wehner et al. 2003). For instance, swelling of human osteoblast-like cells causes an increase in intracellular Ca^{2+}, which in turn activates two types of Ca^{2+}-activated K^+ channels (Weskamp et al. 2000). In addition, hypotonic shock opens a Ca^{2+}-activated K^+ channel in human nasopharyngeal carcinoma cells (He et al. 2009) and Ca^{2+}-dependent K^+ fluxes are activated by cell swelling in a number of epithelial cells (Pasantes-Morales and Morales 2000).

Finally, our results support our original hypothesis that alligator RBCs would have a well-developed RVD as an adaption to cope with the homeostatic imbalances during torpor. Although we have not examined other cell types in this species, it would be interesting to know whether volume regulation by RBCs differs from other tissues, especially those that may not face the same osmotic challenges. It also would be interesting to examine whether the adaptations in alligator RBCs are universal among reptiles or limited to those that "hibernate." Regardless, our results further demonstrate the myriad of different circumstances whereby cells regulate membrane transport in order to deal with osmotic challenges. In conclusion, American alligator RBCs displayed a robust RVD in response to hypotonic shock. In addition, hypotonic challenge led to a rise in cytosolic Ca^{2+}, thereby stimulating volume decrease via the activation of a quinine-inhibitable K^+ efflux pathway. We propose that this rate-limiting step mediates RVD and is therefore important for maintaining cell volume under hypotonic conditions.

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References

Caplanusi A, Kim KJ, Lavriviere E, Van Driessche W, Jans D (2006) Swelling-activated K^+ efflux and regulatory volume decrease efficiency in human bronchial epithelial cells. J Membr Biol 214:33–41

Ellory JC, Hall AC, Ody SO, Englert HC, Mania D, Lang HJ (1990) Selective inhibitors of KCl cotransport in human red cells. FEBS Lett 262:215–218

Foller M, Kasinathan RS, Koka S, Lang C, Shumilina E, Birnbaumer L, Lang F, Huber SM (2008) TRPC6 contributes to the Ca^{2+} leak of human erythrocytes. Cell Physiol Biochem 21:183–192

Foskett JK (1994) The role of calcium in the control of volume regulatory transport pathways. In: Strange K (ed) Cellular and molecular physiology of cell volume regulation. CRC Press, Boca Raton, pp 259–278

Fujise H, Higa K, Kanemaru T, Fukuda M, Adragna NC, Lauf PK (2001) GSH depletion, K–Cl cotransport, and regulatory volume decrease in high-K/high GSH dog red blood cells. Am J Physiol Cell Physiol 281:C2003–C2009

Goswami C, Saha N (2006) Cell volume regulation in the perfused liver of a freshwater air-breathing cat fish *Clarias batrachus* under aniso-osmotic conditions: roles of inorganic ions and taurine. J Biosci 31:589–598

He ST, Zhu LY, Yang LJ, He SC, Mao JW, Wang LW, Chen LX (2009) Ca^{2+}-dependent potassium channels play important roles in regulatory volume decrease in human nasopharyngeal carcinoma cells. Sheng Li Xue Bao 61:485–492

Hernandez T, Coulson RA (1952) Hibernation in the alligator. Proc Soc Exp Biol Med 79:145–149

Hoffmann EK, Lambert IH, Pedersen SF (2009) Physiology of cell volume regulation in vertebrates. Physiol Rev 89:193–277

Jakab M, Furst J, Gschwentner M, Botta G, Garavaglia ML, Bazzini C, Rodughiero S, Meyer G, Eichmueller S, Woll E, Chwatal S, Ritter M, Paulmichl M (2002) Mechanisms sensing and modulating signals arising from cell swelling. Cell Physiol Biochem 12:235–258

Joiner CH, Rettig RK, Jiang M, Franco RS (2004) KCl cotransport mediates abnormal sulfhydryl-dependent volume regulation in sickle reticulocytes. Blood 104:2954–2960

Jorgensen NK, Christensen S, Harbak H, Brown AM, Lambert IH, Hoffman EK, Simonsen LO (1997) On the role of calcium in the regulatory volume decrease (RVD) response in Ehrlich mouse ascites tumor cells. J Membr Biol 157:281–299

Kirk K, Strange K (1998) Functional properties and physiological roles of organic solute channels. Annu Rev Physiol 60:719–739

Koivusalo M, Kapus A, Grinstein S (2009) Sensors, transducers, and effectors that regulate cell size and shape. J Biol Chem 284:6595–6599

Kurbannazarova RS, Tashmukhamedov BA, Sabirov RZ (2003) Osmotic water permeability and regulatory volume decrease of rat thymocytes. Gen Physiol Biophys 22:221–232

Lance VA, Elsey RM (1999) Hormonal and metabolic responses of juvenile alligators to cold shock. J Exp Zool 283:566–572

Land F, Busch GL, Volkl H (1998) The diversity of volume regulatory mechanisms. Cell Physiol Biochem 8:1–45

Lang F (2007) Mechanisms and significance of cell volume regulation. J Am Coll Nutr 26:613S–623S

Lang F, Bush GL, Ritter M, Volkl H, Waldegger S, Gulbins E, Haussinger D (1998) Functional significance of cell volume regulatory mechanisms. Physiol Rev 78:247–306

Lang F, Shumilina E, Ritter M, Gulbins E, Vereninov A, Huber SM (2006) Ion channels and cell volume in regulation of cell proliferation and apoptotic cell death. Contrib Nephrol 152:142–160

Light DB, Attwood AJ, Siegel C, Baumann NL (2003) Cell swelling increases intracellular calcium in *Necturus* erythrocytes. J Cell Sci 116:101–109

Mastrocola T, Flamigni A, Rugolo M (1991) Hypotonic shock activated Cl^- and K^+ pathways in human fibroblasts. Biochim Biophys Acta 1069:201–208

McManus ML, Churchwell KB, Strange K (1995) Regulation of cell volume in health and disease. N Engl J Med 333:1260–1266

Mignen O, Le Gall C, Harvey BJ, Thomas S (1999) Volume regulation following hypotonic shock in isolated crypts of mouse distal colon. J Physiol 515:501–510

Niemeyer MI, Cid LP, Sepulveda FV (2001) K^+ conductance activated during regulatory volume decrease. The channels in Ehrlich cells and their possible molecular counterpart. Comp Biochem Physiol A Mol Integr Physiol 130:565–575

Okada Y, Maeno E, Shimizu T, Dezaki K, Wang J, Morishima S (2001) Receptor-mediated control of regulatory volume decrease (RVD) and apoptotic volume decrease (AVD). J Physiol 532:3–16

Orlov SN, Aksentsev SL, Kotelevtsev SV (2005) Extracellular calcium is required for the maintenance of plasma membrane integrity in nucleated cells. Cell Calcium 38:53–57

Park JS, Choi YJ, Siegrist VJ, Ko YS, Cho WK (2007) Permissive role of calcium on regulatory volume decrease in freshly isolated mouse cholangiocytes. Pflugers Arch 455:261–271

Pasantes-Morales H, Morales SM (2000) Influence of calcium on regulatory volume decrease: role of potassium channels. Nephron 86:414–427

Pedersen SF, Hoffman EK, Mills JW (2001) The cytoskeleton and cell volume regulation. Comp Biochem Physiol A Mol Integr Physiol 130:385–399

Petrunkina AM, Harrison RA, Hebel M, Weitze KF, Topfer-Peterson E (2001) Role of quinine-sensitive ion channels in volume regulation in boar and bull spermatozoa. Reproduction 122:327–336

Puffer AB, Meschter EE, Musch MW, Goldstein L (2006) Membrane trafficking factors are involved in the hypotonic activation of the taurine channel in the little skate (*Raja erinacea*) red blood cell. J Exp Zool A Comp Exp Biol 305:594–601

Rosenblatt MB (1936) A chemical study of the blood of *Alligator mississippiensis*. J Biol Chem 11:81–86

Srinivas SP, Maertens C, Goon LH, Goon L, Satpathy M, Yue BY, Droogmans G, Nilius B (2004) Cell volume response to hyposmotic shock and elevated camp in bovine trabecular meshwork cells. Exp Eye Res 78:15–26

Thomas D, Tovey SC, Collins TJ, Bootman MD, Berridge MJ, Lipp P (2000) A comparison of fluorescent Ca^{2+} indicator properties and their use in measuring elementary and global Ca^{2+} signals. Cell Calcium 28:213–223

Tiffert T, Lew VL (1997) Cytoplasmic calcium buffers in intact human red cells. J Physiol 500:139–154

Tinel H, Kinne-Saffran E, Kinne RK (2000) Calcium signaling during RVD of kidney cells. Cell Physiol Biochem 10:297–302

Virkki LV, Nikinmaa M (1995) Regulatory volume decrease in lamprey erythrocytes: mechanisms of K^+ and Cl^- loss. Am J Physiol 268:R590–R597

Wehner F, Olsen H, Tinel H, Kinne-Saffran E, Kinne RK (2003) Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. Rev Physiol Biochem Pharmacol 148:1–80

Weskamp M, Seidl W, Grissmer S (2000) Characterization of the increase in $[Ca^{2+}]_i$ during hypotonic shock and the involvement of Ca^{2+}-activated K^+ channels in the regulatory volume decrease in human osteoblast-like cells. J Membr Biol 178:11–20

Yeung CH, Barfield JP, Cooper TG (2005) The role of anion channels and Ca^{2+} in addition to K^+ channels in the physiological volume regulation of murine spermatozoa. Mol Reprod Dev 71:368–379

Zancanaro C, Malatesta M, Mannello F, Vogel P, Fakan S (1999) The kidney during hibernation and arousal from hibernation. A natural model of organ preservation during cold ischaemia and reperfusion. Nephrol Dial Transplant 14:1982–1990