Calcium-Stimulated Regulatory Volume Decrease in *Salmo salar* and *Alligator mississippiensis* Erythrocytes

Chloe Wormser*
Department of Biology
Lake Forest College
Lake Forest, Illinois 60045

Summary

The mechanisms by which cells compensate for volume fluctuations are not clearly understood and vary among species. Research efforts in our lab have focused on elucidating the pathways involved in regulatory volume decrease (RVD), the process activated in response to cell swelling that allows for volume recovery. Previously, fluorescence microscopy studies performed by Light et al. (2005) revealed that in salmon red blood cells, cell swelling elicits a rise in intracellular Ca^{2+} concentration (visualized using fluorescence microscopy and the Ca^{2+} indicator fluo-4-AM). This was most likely due Ca^{2+} influx from the extracellular environment, because it was not observed in cells bathed in a hypotonic, low Ca^{2+} medium. The goal of this study, therefore, was to confirm a role for extracellular Ca^{2+} in the RVD response, using both *Salmo salar* (Atlantic salmon) and *Alligator mississippiensis* (American alligator) red blood cells. This was done by exposing cells to different extracellular environments and pharmacological agents that block Ca^{2+} influx pathways or Ca^{2+}-mediated intracellular signaling cascades. To asses the effects of these manipulations on RVD, median cell volume changes over a 90 minute time course were determined by electronic sizing using a Coulter counter. Salmon cells exposed to a low Ca^{2+} environment failed to recover from cell swelling, indicating that extracellular Ca^{2+} was needed for a successful RVD response. Similarly, volume regulation of alligator red blood cells occurred by a Ca^{2+}-dependent mechanism. Additionally, RVD in alligator cells appeared to occur through an intracellular signaling cascade involving Ca^{2+} activation of phospholipase A_2 and the subsequent formation of arachidonic acid. Arachidonic acid itself, as opposed to one of its potential breakdown products, aided in volume recovery by stimulating K^+ efflux. In conclusion, the results from this study indicate that Ca^{2+} plays a pivotal role in the RVD response of both salmon and alligator red blood cells.

Introduction

Cells are the basic building blocks of all life forms, no matter how simple or complex. In fact, despite the enormous diversity that exists among organisms, all have the same basic level of organization in that they are composed of one or more cells. Although cell morphology and physiology may vary, both within an organism and among species, all cells conform to a common function: maintaining a homeostatic balance, both within the intracellular environment and between the intracellular and extracellular environments (Lang & Waldegger 1997). Securing this steady-state equilibrium is not an easy task, as there are unavoidable instances over the course of a cell's life in which homeostasis may be challenged (O'Neill 1999). Fortunately, cells have acquired distinctive features that aid in preventing cellular imbalance and recovering from inevitable challenges.

One such feature is the plasma membrane, which is responsible for compartmentalizing the cell and allowing metabolic processes crucial for cell survival to take place in isolation from the external environment (Schultz 1989). The basic structure of the plasma membrane is a phospholipid bilayer embedded with proteins (Goodman 2002). The phospholipids that compose the membrane form a stable bilayer as a result of their amphipathic nature; they possess both hydrophobic phosphate moieties and hydrophilic hydrocarbon tails. As a result of this composition, the plasma membrane permits the selective movement of fat-soluble solutes, both in and out of the cell, in accordance with the concentration gradient established between the extracellular and intracellular environments (Goodman 2002). For ions or molecules that cannot readily traverse the membrane, either due to their size or charge, carriers and channels allow for their transport (Goodman 2002). This transport may be active, if the molecule or ion is moving *against* its concentration gradient, or passive, if the molecule or ion is moving *with* its concentration gradient (McCarty & O'Neil 1992). Both types of transport, active and passive, play a role in daily cellular function and are necessary for proper communication between the cell and its surroundings.

Active transport of molecules across the plasma membrane requires both energy and the assistance of a pump (O'Neill 1999). Protein pumps function to recognize and bind a specific substrate molecule and transfer that molecule across the membrane in the direction that *opposes* its concentration gradient (Singer & Nicolson 1972). This process requires energy input, often in the form of ATP (Singer & Nicolson 1972). In contrast, other carriers transport molecules *with* their concentration gradient; this passive process is known as "facilitated diffusion" (Singer & Nicolson 1972).

Protein channels function as pores that shield water-soluble ions from direct contact with the lipid bilayer, thus allowing the ions to travel passively into or out of the cell (Lewis & Donaldson 1990). These channels are often highly regulated, with this regulation being dependent on both the type of channel and the specific membrane in which the channel is embedded. For example, some channels are ligand gated, in which the binding of a signal molecule to the channel leads to its opening or closing (Singer & Nicolson 1972). In contrast, other channels are voltage gated, where a change in membrane potential either activates or deactivates the channel (Lewis & Donaldson 1990). Ion channels may also be mechanically gated such that mechanical stress or distortion to the plasma membrane controls the channel's activity (O'Neill 1999).

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Having numerous membrane channels and multiple gating mechanisms at their disposal allow cells to detect different types of perturbations and respond accordingly.

Carriers, pumps, and ion channels are distinct from one another in their affinity and specificity for a given solute. Despite these differences, they all function similarly to assist the cell in responding to physiological conditions by altering plasma membrane permeability to certain molecules and ions (O'Neill 1999). This change in permeability, in turn, adjusts intracellular solute concentration and affects the osmotic gradient between a cell and its surrounding medium (O'Neill 1999). As a result, the movement of osmotically obligated water occurs in coordination with solute transport (Lang & Waldegger 1997). This water and solute flux across the plasma membrane is linked to a major cellular threat: a change in cell volume (McCarty & O'Neil 1992).

The difference in osmolality (i.e., the number of dissolved particles per kg H_2O) existing between two media separated by a semi-permeable membrane determines the direction of water flow between them. Specifically, water will travel passively across the membrane from a medium of lower osmolality (more dilute) to higher osmolality (more concentrated); this process is termed *osmosis* (Lang & Waldegger 1997). Because cells are bathed in an extracellular medium, an osmotic gradient might exist between the intracellular and extracellular environments. Consequently, if the osmolality inside the cell is greater than the osmolality outside the cell, water will travel passively into the cell, resulting in cell swelling. In contrast, if the solute concentration within the cell is lower than the solute concentration of the extracellular environment, water will diffuse out of the cell, and the cell will shrink. Such cell volume changes are characteristic of organisms composed of cells that are faced with variable extracellular solute concentrations (O'Neill 1999). For example, many intertidal animals are exposed to an external medium that dramatically and continually changes salinity due to environmental factors, such as evaporation, rainfall, and tidal movements of water (Vidolin et al. 2002). Similarly, the gill epithelia of many teleosts, particularly euryhaline species, are bathed in external environments of fluctuating salinity (Kultz 2002). Such fluctuations may lead to volume changes at the cellular level.

Even cells bathed in a fairly invariable extracellular medium experience shifts in cell volume as a result of changes in intracellular osmolality, which can occur from a variety of cellular processes (O'Neill 1999). For example, the cortical proximal tubule cells of the kidney are challenged with significant fluctuations in intracellular osmolality as a consequence of their role in water and nutrient resorption (Linshaw 1991). Similarly, the crucial role that intestinal cells play in solute absorption leads to changes in the number of osmotically active particles within them and, in turn, changes in their volume (O'Neill 1999). Further, the protein synthesis and degradation that occur in nucleated cells lead to osmotic perturbations, as the synthesis of proteins from amino acids reduces intracellular osmolality, whereas the degradation of proteins into amino acid monomers increases intracellular osmolality (Lang & Waldegger 1997). Clearly, some cell volume fluctuations are unavoidable events that occur as a result of common cellular activities and metabolic functions.

Extreme volume changes, changes that a cell would not typically face under normal physiological conditions, can be induced *in vitro*. The direct effects of such perturbations on cell homeostasis have been determined experimentally by placing cells in either dilute or concentrated extracellular media. These experiments have shown that drastic shifts in extracellular osmolality result in rapid cell swelling or shrinking and are accompanied by altered cellular functioning (Schultz 1989). For example, the expression of certain genes is osmosensitive, and the activating or deactivating effects that changes in cell volume have on these genes alter the expression of the proteins for which they encode (Lang & Waldegger 1997). Additionally, some genes cannot tolerate abrupt changes in cell volume, so such perturbations result in deleterious effects on the metabolic and enzymatic activities they control (Kultz 2002). In the most basic sense, the mechanical strain imposed on a cell from volume fluctuations affects cell morphology and may even threaten cellular existence altogether; a cell can only expand to a given point before bursting or shrink to a given point before shriveling up in atrophy (McCarty & O'Neil 1992). Thus, to prevent the potential loss of cell integrity resulting from cell swelling or shrinkage, it is crucial that a cell be able to employ specific recovery mechanisms.

These mechanisms do exist in a wide number of cell types. Specifically, certain cells are able to respond to and recover from hypotonic or hypertonic shock by activating the compensatory processes known as regulatory volume decrease (RVD) or regulatory volume increase (RVI) (Chamberlin & Strange 1989). Regulatory volume increase is the process by which a cell counteracts cell shrinkage by activating intracellular processes that increase cell volume through solute and water uptake. In contrast, RVD is the compensatory response by which cells decrease their volume through the loss of solutes and water to recover from exposure to a dilute extracellular medium and subsequent swelling (McCarty & O'Neil 1992). The fact that RVI and RVD allow for cell volume restoration following instances of severe anisosmotic stress make them critical parts of the cellular machinery. This explains why they are such highly conserved mechanisms; indeed, rudimentary cell volume regulatory processes occur in most prokaryotic cells (Schultz 1989).

The processes involved in RVD have been studied extensively in many cell types. In those studied to date, such as human ciliary epithelial cells (Adorante & Cala 1995), *Necturus* red blood cells (Bergeron et al. 1996), Erlich ascites tumor cells (Hoffman et al. 1986), and Madin-Darby canine kidney cells (Rothstein & Mack 1992), the RVD mechanism activated by cell swelling ultimately leads to net K^+ and Cl^- efflux. This efflux is essential for volume recovery, as it reverses the driving force for water flux across the membrane. The intracellular signaling cascades that result in K^+ efflux have also been examined, and some of these cascades involve protein kinase C (PKC) (McCarty & O'Neil 1992). For instance, in *Necturus* red blood cells, RVD occurs through a PKC-dependent cascade that eventually results in the loss of K^+ and Cl^- from cells (Light et al. 1998). Similarly, changes in cell volume and cell volume recovery mechanisms in astrocytes are PKC-sensitive (Bender et al. 1992).

Additional intracellular messengers have been shown to play a role in activation of K^+-Cl^- loss, such as calmodulin and arachidonic acid. Calmodulin is a Ca^{2+} binding protein involved in many biological

pathways, including cell volume regulation in certain cell types (Huang et al. 2001). Specifically, activation of this cellular messenger is crucial for RVD in astrocytes (Quesada et al. 1999, Bender et al., 1992), and *Necturus* red blood cells depend on calmodulin for a successful RVD response (Bergeron et al. 1996). Arachidonic acid metabolites, called eicosanoids, are also involved in volume recovery mechanisms. For example, eicosanoids mediate RVD in trout proximal renal tubules (Kanli & Norderhus 1998). Similarly, a 5-lipoxygenase metabolite of arachidonic acid is involved in regulation of *Necturus* cell volume (Light et al. 1997). Clearly, the intracellular processes involved in volume decrease are often rather complex, with numerous cell signaling agents working in coordination to activate and carry out the RVD response.

Despite the necessity of K^+-Cl^- efflux for cell volume recovery, large shifts in the intracellular concentrations of such ions can have destabilizing effects on cells (Lang & Waldegger 1997). Therefore, the loss of organic solutes, such as sugars and amino acids, is also commonly involved in cell volume regulation (Chamberlin & Strange 1989). For example, loss of the amino acid taurine has been implicated in the RVD processes that take place in trout erythrocytes (Garcia-Romeu et al. 1991, Huang et al. 2001), and in Ehrlich ascites tumor cells, both taurine and glycine efflux occur following cell swelling (Hoffman & Lambert 1983). A cell's ability to use organic osmolytes to adjust intracellular solute concentration is advantageous, as the loss of many of these molecules does not cause deleterious effects on cell functioning (Chamberlin & Strange 1989). This is of key importance because preventing cell damage from volume fluctuations must not occur at the cost of ionic imbalance, as this could also be detrimental to cell integrity.

Despite what has been learned regarding cell volume regulatory mechanisms, much is still unknown concerning the initial steps that lead to activation of RVD. In particular, the role that Ca^{2+} plays in RVD initiation and signal transduction is widely debated. Calcium is known for the essential role it plays in many cellular processes, such as muscle contraction (Martonosi 2000) and neurotransmission (DeLorenzo 1981), but its utility extends much further. For example, Ca^{2+} is involved in activities that range from inducing apoptosis (programmed cell death) (Orrenius et al. 2003) to stimulating sperm motility in carp (Krasznai et al. 2000).

A role for this signal molecule has also been identified in the volume regulatory processes of numerous cell types, including human ciliary epithelial cells (Adorante & Cala 1995), choroids plexus epithelial cells (Christensen 1987), *Necturus* red cells (Light et al. 1999), Madin-Darby canine kidney cells (Rothstein & Mack 1992), proximal tubules (McCarty & O'Neil 1990), and rat lacrimal acinar cells (Speake et al. 1998). In these cell types, Ca^{2+} plays an important role in activating intracellular events that culminate in solute efflux. However, in other biological systems, Ca^{2+} is not essential for volume recovery, although it does assist in achieving an optimal RVD response. This is the case for rat liver cells (Bear 1990) and cultured astrocytes (Quesada et al. 1999). In contrast, findings from other research indicate either an RVD scheme completely independent of Ca^{2+} or a mechanism by which a rise in Ca^{2+} during cell swelling is a non-crucial epiphenomenon. For example, rat cerebellar astrocytes employ a volume recovery response that

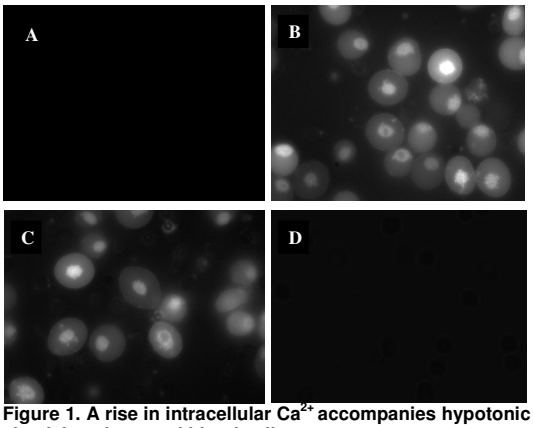

Figure 1. A rise in intracellular Ca^{2+} accompanies hypotonic shock in salmon red blood cells.

The amount of fluorescence visible in red blood cells loaded with fluorescent dye (fluo-4, 10 μ M) and exposed to UV light was dependent on the extracellular medium the cells were bathed in. (A) Cells bathed in isosmotic solution failed to fluoresce. (B) Cells treated with the Ca^{2+} ionophore A23187 (positive control) in an isosmotic solution displayed fluorescence. (C) Exposure to hypotonic shock increased the degree of fluorescence in the red blood cells. (D) When Ca^{2+} was buffered to 10 nM with EGTA in a hypotonic medium, no fluorescence was observed. The above fluorescent microscopy studies were conducted by Light et al. (2005).

lacks Ca^{2+} involvement (Morales-Mulia et al. 1998), as do trout proximal renal tubules (Kanli & Norderhus 1998) and trout erythrocytes (Garcia-Romeu et al. 1991). Clearly, whether a dependency on Ca^{2+} for RVD exists is controversial and appears to vary among cell types. Furthermore, for those cell types in which Ca^{2+} *does* appear to play a role in volume restoration, the exact pathway by which it operates has yet to be determined.

The goal of my research was to help clarify the inconclusive relationship between Ca^{2+} and regulatory volume decrease. To do so, I studied the effects of Ca^{2+} manipulation on RVD in both Atlantic salmon (*Salmo salar*) and American alligator (*Alligator mississippiensis*) erythrocytes, in an effort to determine whether the signal transduction pathways stimulated by cell swelling are Ca^{2+}-dependent or Ca^{2+}-independent. Previous research in our laboratory provides evidence for potential involvement of Ca^{2+} in the RVD response of salmon red blood cells (Light et al. 2005). Using fluorescent microscopy, Light et al. (2005) tracked changes in the intracellular Ca^{2+} of the cells (Figure 1). They observed that cells bathed in an isosmotic medium displayed virtually no fluorescence, which was indicative of low Ca^{2+} levels under basal conditions. However, exposure to a dilute extracellular environment elicited a dramatic increase in fluorescence, which suggests that salmon cells experience a rise in intracellular Ca^{2+} in response to hypotonic shock.

Based on the findings of Light et al. (2005), my aim was to characterize the role of Ca^{2+} in RVD further. Specifically, my study rested on the following three hypotheses: 1) Cell swelling is accompanied by a rise in intracellular Ca^{2+} necessary to stimulate RVD, 2) the source of this Ca^{2+} is extracellular, and 3) Ca^{2+} activates an intracellular event essential for volume recovery. If RVD does indeed depend on extracellular Ca^{2+}, then I expected removing this Ca^{2+} source would inhibit the volume recovery response. In contrast, if RVD is Ca^{2+}-independent, then Ca^{2+} removal should

have no effect on the ability of cells to regulate their volume following hypotonic challenge. Additionally, blocking Ca^{2+} entry into cells using Ca^{2+} transport antagonists should inhibit RVD if Ca^{2+} influx is required to facilitate the volume recovery process. However, if Ca^{2+} influx is not an essential step, then no effect should be observed following inhibition of Ca^{2+} permeability pathways. Lastly, if the role of Ca^{2+} is to stimulate an intracellular signaling cascade crucial for volume recovery, then blocking the signaling events occurring downstream of Ca^{2+} activation should also result in the failure of cells to respond appropriately to anisosmotic challenges. But if these Ca^{2+}-activated signaling events are not involved in RVD, antagonizing them should not inhibit volume regulation.

I chose to focus on salmon and alligator cells because the lifestyles of these organisms suggest that they would be good models for studies on cell volume recovery. In particular, salmon are euryhaline marine teleosts, meaning they can tolerate both freshwater and saltwater environments (Handeland et al. 1996). They are also anadromous, making multiple migratory trips between freshwater and saltwater throughout the course of their lives (Handeland et al. 1996). The fact that they are able to adapt to such varying degrees of salinity suggests that their cells, which may come in contact with the extracellular environment during transport through the gills, have a finely-tuned mechanism for coping with cell volume deviations.

Likewise, although alligators are primarily found in freshwater, they can tolerate abbreviated episodes in brackish environments (Richards et al. 2004). Additionally, they hibernate during the winter and can also endure freezing conditions for short periods of time (Lance & Elsey 1999). Hibernation results in reduced blood flow to the kidneys and consequent reduced renal activity (Zancanaro et al. 1999). Kidney cells play a crucial role in adjusting extracellular fluid osmolarity. Therefore, abrupt changes in kidney cell activity as a result of hibernation could result in cell volume changes. In addition, exposure of alligators to freezing conditions has a fairly rapid stimulatory effect on stress hormone production, which initiates a change in plasma ion concentration (Lance & Elsey 1999). A more long-term effect of such exposure includes retardation of protein synthesis (Lance & Elsey 1999). As stated earlier, such changes in osmolyte concentrations correspond with a shift in the osmotic gradient between intracellular and extracellular environments and, consequently, lead to either cell swelling or shrinkage (McCarty & O'Neil 1992). Therefore, the ability of alligators to cope with such challenges is presumably allowed for, at least in part, by a well-developed cell volume regulatory mechanism.

In addition, unlike mammalian erythrocytes, red blood cells from salmon and alligators are nucleated. This allows for protein synthesis and degradation to take place within them (Lang & Waldegger 1997), activities that alter intracellular osmolality. This suggests that the red blood cells of both species may be adapted to deal with shifts in solute concentration (Lang & Waldegger 1997). Furthermore, these cells are free floating, so they do not require a substrate on which to grow. Additionally, the characteristic pigmentation of these cells makes osmotic fragility studies possible.

The continuation of research in this field promotes a better understanding of cell physiology, and uncovering the underlying processes involved in RVD has practical applications, as well, including the study of certain pathophysiological conditions. For example, apoptosis is the process by which cells marked for destruction lose water and solutes in a feed-forward mechanism until they are reduced to a size that can be easily engulfed by phagocytic cells (Okada & Maeno 2001). Overactive or dysfunctional apoptosis are characteristic of diseases including neurodegeneration and cancer (Okada & Maeno 2001). The obvious parallels between apoptosis and RVD, mainly that both proceed in a controlled manner that allows for volume reduction (Okada & Maeno 2001), suggest that clarification of the RVD pathway may be useful when developing methods for either inducing apoptosis in apoptotically deficient cells or inhibiting it in cells displaying overactive apoptosis.

Another disease state related to RVD in its mechanics is ischemia, which is characterized by a lack of oxygenated blood flow to cells and often accompanies a blood clot or stroke (Pasantes-Morales et al. 2000). One of the hallmark events that occurs during ischemia is significant cell swelling. Subsequently, ischemic cells are able to recover from this swelling by the loss of K^+ and organic osmolytes (Pasantes-Morales et al. 2000). However, the recovery rarely occurs quickly enough to prevent some amount of cell damage (Diaz et al. 2003). It has been proposed that enhancing the rate of cell volume recovery may be the key to preventing irreversible damage to these cells (Diaz et al. 2003). To develop a method for quickening this rate, further research on cell volume recovery processes is needed.

Finally, while similarities can be recognized between RVD, apoptosis, and ischemia, other disorders are largely the direct consequence of insufficient or faulty cell volume regulation. These include renal disease, diabetes mellitus, dehydration, and cerebral edema (McManus et al. 1995). The potential for developing better techniques for managing these disease states lies in the ability to elucidate the pathways involved in the underlying cell volume regulatory mechanisms.

Materials and Methods

Experimental Design

The first hypothesis I set out to test was whether extracellular Ca^{2+} is necessary for RVD. Presumably, if extracellular Ca^{2+} is required for a successful RVD response, then in its absence cells should fail to regulate their volume effectively. However, this manipulation should have no effect if RVD is Ca^{2+}-independent. To distinguish between these two possibilities, I carried out calcium influx studies in which Ca^{2+} was removed from the extracellular environment using the extracellular Ca^{2+} chelator ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA). Additionally, I performed experiments where plasma membrane permeability to Ca^{2+} was increased using A23198, an ionophore that facilitates Ca^{2+} entry into cells (Light et al. 2003). I expected this to have the opposite effect of removing Ca^{2+}. That is, increasing Ca^{2+} influx should enhance RVD if Ca^{2+} plays a role in stimulating the volume regulatory response.

If the results of the above studies supported my first hypothesis (i.e., Ca^{2+} is necessary for RVD and is extracellular in origin), I next wanted to characterize the Ca^{2+} influx pathways activated subsequent to cell swelling to identify how Ca^{2+} was entering cells. There

are three main entry sites that could facilitate Ca^{2+} influx: stretch-activated channels, ligand-gated channels, and voltage-sensitive channels (McCarty & O'Neil 1992). Stretch-activated channels have been shown to be a major site for Ca^{2+} entry in numerous cell types (McCarty & O'Neil 1992). For example, Christensen (1987) concluded that hypotonic shock stimulates the opening of stretch-activated channels that are permeable to Ca^{2+} in the plasma membrane of choroids plexus epithelia. Also, Hoyer et al. (1994) named stretch-activated ion channels as the site of Ca^{2+} entry following hypotonic exposure of porcine endocardial endothelia. Ligand-gated cation channels, such as P_2 receptors, also allow significant amounts of Ca^{2+} to cross the plasma membrane of many cells, *including* red blood cells (Ralevic & Burnstock 1998). Light et al. (2003), for example, showed P_2 receptors as a likely site for Ca^{2+} entry into *Necturus* red blood cells following hypotonic shock. In contrast to stretch-activated and ligand-gated channels, voltage-sensitive channels are typically restricted to neurons and muscle cells (Jones 1998). Accordingly, I decided to first examine those entry pathways that have been strongly implicated in solute influx during RVD.

To address the role of stretch-activated channels in RVD, I used the pharmacological agents lanthanum and gadolinium. Lanthanum is a broad-spectrum Ca^{2+} channel inhibitor, but it shows some selectivity toward stretch-activated channels, which tend to be permeable to Ca^{2+} (Yang & Sachs 1989). Gadolinium primarily blocks stretch-activated channels, but there is some evidence suggesting that this agent also antagonizes P_2 receptors (Nakazawa et al. 1997). These agents have proven to be potent inhibitors in many cell volume studies. For example, Adorante & Cala (1995) and Rothstein & Mack (1992) found that treating cells with lanthanum has an inhibitory effect on cell volume recovery following hypotonic shock of nonpigmented human ciliary epithelial cells and Madin-Darby canine kidney cells, respectively. Light et al. (2003) discovered that Ca^{2+} entry into *Necturus* red blood cells after hypotonic shock occurs through a gadolinium-sensitive channel, as use of this agent inhibits volume recovery. Similar RVD inhibition in response to gadolinium treatment was observed in rat lacrimal acinar cells by Speake et al. (1998). Accordingly, I expected volume recovery to be weakened in the presence of these Ca^{2+} transport antagonists if their sites of action correlate with the calcium entry pathways active during RVD of salmon and alligator cells, whereas they should have no effect on volume recovery if they act on influx pathways not involved in the volume regulatory responses of these cell types.

To further determine the entry site for Ca^{2+}, I examined the effects of targeting P_2 receptors, specifically the P_{2X} class, which are ATP-gated, Ca^{2+} permeable channels (Nakazawa et al. 1997). To address whether they are involved in the RVD process, I utilized the ATP scavenger hexokinase, as extracellular ATP has been shown to stimulate RVD in several cell types by activating P_2 receptors (Dezaki et al. 2000, Light et al. 2001, Schwiebert et al. 1995). Hexokinase can be used to remove this ATP source in the presence of glucose, thus preventing P_2 receptor activation (Schwiebert et al. 1995). In addition, I directly inhibited P_2 receptors using suramin, an agent that binds to and inactivates this receptor type (Dezaki et al. 2000). If P_2 receptors play a role in Ca^{2+} entry following hypotonic shock, then these agents should have an attenuating effect on RVD. I also attempted to potentiate volume recovery by adding ATP to the extracellular medium. I predicted that if P_2 receptors are indeed involved in volume regulation, then this addition should have the opposite effect of hexokinase and suramin, and thus stimulate a decrease in cell volume.

After studying the processes by which Ca^{2+} entered cells, I wanted to address my hypothesis that Ca^{2+} functions as an intracellular messenger to activate an event necessary for volume recovery. I accomplished this by first determining whether K^+ efflux is required for a decrease in cell volume, and whether this efflux is Ca^{2+}-dependent, as K^+ loss from swollen cells is a necessary step in RVD of virtually all cell types that have been studied (Adorante & Cala 1995, Bergeron et al. 1996, Hoffman et al. 1986, Rothstein & Mack 1992). To do so, I observed the effects of enhancing K^+ efflux with gramicidin. This agent increases plasma membrane permeability to K^+ by forming pores in the cell membrane specific for monovalent cations, thereby allowing for the movement of cations either in or out of the cell as dictated by their electrochemical gradients (Light et al. 1998). In my studies, I was able to make these pores essentially K^+ specific by removing Na^+ from the extracellular environment and replacing it with choline (a large, impermeant cation that does not readily traverse the plasma membrane, nor alter RVD). In doing so, I could assess whether RVD is dependent on K^+ efflux, and whether K^+ efflux, in turn, is dependent on Ca^{2+}. If this is indeed the case, then bypassing the Ca^{2+}-dependent step (with gramicidin) should reverse the inhibitory effects of placing cells in a hypotonic, Ca^{2+}-free solution.

Then, I sought to establish whether Ca^{2+} acts as a direct modulator of K^+ efflux or, alternatively, if it plays an indirect role by acting as a second messenger. To this end, I inhibited Ca^{2+}-activated K^+ channels using quinine, a selective blocker of this channel type (Hoffman et al. 1986), expecting to observe a reduction in RVD upon doing so if K^+ loss from cells occurred by this route. I also examined the potential role Ca^{2+} might play as an intracellular messenger by targeting different Ca^{2+}-activated signal transduction pathways that could ultimately lead to K^+ efflux. One pathway I thought could be involved was calmodulin, which plays a role in the RVD signaling cascade of the astrocytes studied by both Quesada et al. (1999) and Bender et al. (1992), *Necturus* erythrocytes studied by Bergeron et al. (1996), and the human erythroleukemia cells studied by Huang et al. (2001). In these cells, inhibition of calmodulin resulted in a reduced ability of cells to regulate their volume while under hypotonic challenge. I assessed a potential role for calmodulin in volume recovery using pimozide, an antagonist of calmodulin activity (Bergeron et al. 1996, Hoffman et al. 1986, Huang et al. 2001). Hypothetically, if calmodulin-dependent signaling occurs downstream of Ca^{2+} and is required to stimulate volume decrease, then inhibiting calmodulin should correlate with a reduced RVD response.

In addition, I targeted the enzyme phospholipase A_2, whose activation results in the formation of arachidonic acid. Metabolites of arachidonic acid, in particular, leukotrienes, have been implicated in the RVD response in a diverse number of cell types (Kanli & Norderhus 1998, Light et al. 1998, Pasantes-Morales et al. 2000). The first step in determining whether arachidonic acid or its metabolites

Agent	Function	Predicted Effect on RVD
EGTA	Extracellular calcium chelator	Inhibition if Ca^{2+} is necessary for volume recovery
A23187	Calcium ionophore	Enhancement if Ca^{2+} influx stimulates RVD
Gadolinium	Stretch-activated channel blocker	Inhibition if Ca^{2+} influx occurs through a SA channel
Lanthanum	Stretch-activated channel blocker	Inhibition if Ca^{2+} entry occurs through a SA channel
Hexokinase	ATP scavenger	Inhibition if Ca^{2+} entry occurs through a P_2 receptor
Suramin	P_2 receptor antagonist	Inhibition if Ca^{2+} entry occurs through a P_2 receptor
ATP	Natural ligand for P_2 receptors	Enhancement if P_2 receptor activity facilitates RVD
Gramicidin	Ionophore for monovalent cations	Enhancement of K^+ efflux is required for volume recovery
Quinine	Ca^{2+}-activated K^+ channel blocker	Inhibition if K^+ efflux occurs via a Ca^{2+}-activated K^+ channel
Pimozide	Calmodulin antagonist	Inhibition if RVD requires calmodulin activation
ONO-RS-082	Phospholipase A_2 inhibitor	Inhibition if volume recovery is PLA_2-dependent
2,3 DBAP	Phospholipase A_2 inhibitor	Inhibition if volume recovery is PLA_2-dependent
ETYA	Antagonist of eicosanoid synthesis	Inhibition if an eicosanoid stimulates RVD
NDGA	Antagonist of leukotriene production	Inhibition if a leukotriene is needed for volume decrease

Table 1. Pharmacological agents used in cell volume studies, their functions, and my predictions as to their effects on regulatory volume decrease.

are involved in RVD of the cells I studied was to identify whether RVD is PLA_2 dependent, because PLA_2 (a Ca^{2+} activated enzyme) is responsible for arachidonic acid formation (Pasantes-Morales et al. 2000). I used the PLA_2 antagonists ONO-RS-082 and 2,4-dibromoacetophenone to do this, as these agents have been shown to be useful PLA_2 inhibitors in other RVD studies (Balsinde et al. 1999, Nakashima et al. 1989). If arachidonic acid or its metabolites are essential for volume recovery following hypotonic shock, then I expected to observe an inhibited volume recovery in response to preventing arachidonic acid formation.

If my PLA_2 inhibition studies implicated arachidonic acid formation as a necessary event in the RVD response, my next step would be to determine whether arachidonic acid itself modulates volume recovery, or, alternatively, if one of its metabolites (known as eicosanoids) does. To distinguish between these two possibilities, I treated cells with the general eicosanoid antagonist 5,8,11,14-eicosatetraynoic acid (ETYA). Additionally, I targeted the lipoxygenase pathway of arachidonic acid metabolism using nordihydroguaiaretic acid (NDGA), as this pathway has been shown to be activated during RVD of several cell types (Lambert et al. 1987, Light et al. 1997). I predicted that if arachidonic acid, but not an eicosanoid, is required for RVD, then inhibiting eicosanoid formation should not affect the volume recovery response. However, if an eicosanoid is involved in volume regulation, then antagonizing the pathways responsible for forming the metabolite should inhibit the ability of cells to recover from cell swelling.

Table 1 summarizes the experimental approach I used to test my hypotheses. Listed are the various pharmacological agents I used and their modes of action. Additionally, my predictions as to the effects of these agents are also given.

Animals

A common pool (3-4 fish) of salmon blood was purchased from Bioreclamation (Hicksville, NY). Alligator whole blood was purchased from Carolina Biological Supply (Burlington, NC). The blood was stored at 4°C for no longer than one month and was inverted daily. Visualization of salmon erythrocytes (Figure 2) was achieved using a Nikon phase contrast microscope (Fryer Co. Scientific Instruments, Carpentersville, IL) along with a mounted camera (Spot Insight Color and Spot software). For visualizing alligator erythrocytes (Figure 2), a Nikon Eclipse TE2000-U DIC microscope (Fryer Co. Scientific Instruments, Carpentersville, IL) was used.

Cell Volume

A Z2™ COULTER COUNTER® with Channelyzer was used to electronically size and count red blood cells (Figure 3, Coulter Electronics, Fullerton, CA). The aperture orifice was 100 μm in diameter, and the metered volume was 0.5 ml. The Coulter counter was calibrated using latex beads of known size. Median cell volumes (fl) were obtained from distribution curves between 50 fl and 1200 fl. The lower limit (50 fl) was chosen to prevent the counting of thrombocytes, which are smaller than red blood cells and comprise a significant portion of total blood volume (Histology of Blood, Creighton University Health Sciences Laboratory, www.hsl.creighton.edu). Although white blood cells were counted, as they are approximately the

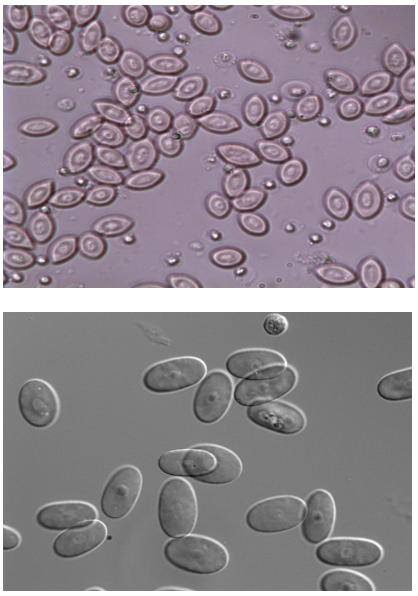

Figure 2. Salmon and alligator erythrocytes.
(A) Salmon erythrocytes photographed at 400X. The cells are approximately 15 μm long and 11 μm wide (Nash & Egginton 1993); as a comparison, this is about twice the size of human red blood cells. They are oval in shape, free-floating, and, unlike mammalian red cells, they are nucleated. (B) Alligator erythrocytes photographed at 1000X (taken by Spivak, Pore, & Silverman, Light Lab). These oval-shaped cells measure approximately 21 μm in length and 11 μm in width (Hartman & Lessler 1964). They are free-floating and nucleated.

same size as red blood cells, the proportion of white cell to red cell is typically 1/1000 (www.hsl.creighton.edu); therefore, their contribution was assumed to be insignificant. Readings were taken from cell suspensions containing between 20,000-40,000 cells over a time course of 90 min. Specifically, median cell volumes were recorded at 30 sec intervals from 0 to 3 min, at 5 min, and at 10 min intervals from 10 to 90 min.

Using the volume measurements obtained from electronic sizing, two different assessments were made. First, the final relative volume of experimental cells was compared to control cells, with relative volume being the absolute cell volume divided by the volume of untreated cells equilibrated in isosmotic solution. This was done to determine differences in the overall degree by which cells reduced in size following hypotonic challenge. Additionally, the rate of volume recovery was used to compare the RVD responses of experimental and control cells. This was done by comparing the relative volume of experimental and control cells at both 40 and 90 min to the maximum relative volume of control cells. To do so, the following formulas were used (Light et al. 1999): percent volume recovery at time *x* is equal to $\left[(V_{max} - V_x)/(V_{max} - V_0) \right]$ x 100%. In this equation, V_{max} represents maximum relative control cell volume, V_x represents the relative cell volume measured at time *x*, and V_0 represents relative cell volume at time 0 min. Overall percent volume decrease was determined as $\left[(\text{percent recovery}_{experimental})/\text{percent recovery}_{control}) \right]$ x 100%.

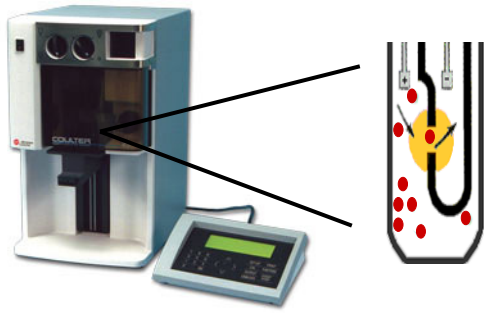

Figure 3. Z2™ COULTER COUNTER® and aperture.
The Coulter counter electronically sizes and counts cells based on a change in resistance that occurs as the cells pass through a narrow aperture opening. Cell volume is proportional to this change in resistance (figure is courtesy of Beckman Coulter, Inc., www.beckmancoulter.com).

Solutions and Reagents

Isosmotic and hypotonic Ringer solutions were used to mimic blood plasma environments. The compositions of the Ringers used in this study were based on those provided by the Marine Biology Laboratory in Woods Hole, MA (www.mbl.edu). The osmolality and pH of all solutions were determined using a Vapor Pressure Osmometer (Wescor 5500) and an Accumet Basic AB15 pH meter (Fisher Scientific), respectively.

For salmon, isosmotic high Na^+ Ringer contained (in mM) 178 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 1.8 $MgCl_2$, 8.75 Hepes hemisodium salt, 1.25 Hepes, 5.5 Glucose, and 1.0 $NaHCO_3$. Hypotonic high Na^+ Ringer had the same composition, except the NaCl content was reduced to 67 mM. Hypotonic low Ca^{2+} (10 nM free Ca^{2+}) contained (in mM) 67 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 4 EGTA, 1.5 $MgCl_2$, 10 Hepes, 8 Trizma base, 5.5 Glucose, and 1.0 $NaHCO_3$. The MAXC downloads website (http://www.stanford.edu/%7ECpatton/downloads.htm) was used to calculate the appropriate free Ca^{2+} concentration.

For alligator, isosmotic high Na^+ Ringer contained (in mM) 140 NaCl, 6 KCl, 6 $CaCl_2$, 1.5 $MgCl_2$, 5.45 Hepes hemisodium salt, 4.55 Hepes, 5.5 Glucose, and 1.0 $NaHCO_3$. For the hypotonic high Na^+ Ringer, NaCl content was reduced to 70 mM, and for the NaCl-free hypotonic Ringer, NaCl was replaced with 70 mM choline chloride. Hypotonic low Ca^{2+} (10 nM) Ringer contained (in mM) 62.5 NaCl, 6 KCl, 0.5 $CaCl_2$, 1.5 $MgCl_2$, 3.8 EGTA, 5.45 Hepes hemisodium salt, 4.55 Hepes, 9.16 Trizma base, 5.5 Glucose, and 1.0 $NaHCO_3$.

All chemicals and pharmacological agents were acquired from Sigma Chemical Co. (St. Louis, MO), Axxora LLC (San Diego, CA), and ICN Biomedicals (Aurora, OH). Aqueous stock solutions were made at 100X their final concentration and were then diluted 100X when used to achieve the final working concentration desired. Nonaqueous stock solutions (using methanol, ethanol, or DMSO as vehicles) were made at 1000X their final concentration and diluted 1000X to their final working concentration (Light et al. 2001). All experiments were carried out at room temperature (21-23°C).

Statistics

Cell volume changes were graphically represented using KaleidaGraph 4.0 (Synergy Software; Reading,

PA) and Microsoft Excel (Microsoft Corporation; Redmond, WA). The statistical significance of the experimental results (i.e., differences in final cell volume and percent volume decrease of control cells compared to experimental cells) was assessed by performing either paired *t*-tests or analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA). Statistics were computed using Data Desk Software (Ithaca, NY).

Each experiment was replicated at least five times. Each maneuver was compared to its own control, allowing for paired comparisons. A separate blood sample was used for each replicate experiment. All samples were taken from several common pools.

Results

Salmon

Challenging salmon cells with a hypotonic (~0.5x) extracellular environment caused them to swell rapidly. Following this initial increase in volume, the cells gradually and spontaneously recovered toward their normal size over a 90 minute time course (Figure 4A). I then bathed cells in a low Ca^{2+} medium to test whether RVD requires extracellular Ca^{2+}. There was a significant difference in final cell volume of cells bathed in the low Ca^{2+} hypotonic Ringer (buffered to 10 nM with EGTA) compared to that of control cells (Figure 4A, n=5, P<0.001 at 90 min). Additionally, in the low Ca^{2+} environment, percent volume recovery was inhibited both at 40 min and at 90 min (Figure 4B, n=5, P<0.001).

The next series of experiments were designed to inhibit Ca^{2+} influx. Surprisingly, cells treated with lanthanum (10 μ M, n=5), an inorganic inhibitor of Ca^{2+} channels (Rothstein & Mack 1992), displayed an enhanced volume recovery (Figure 5A, P<0.001 at 90 min compared to control). The percent volume recovery was dramatically potentiated at both 40 min and 90 min (Figure 5B, P<0.001 for both 40 and 90 min), and the total percent volume decrease of lanthanum-treated cells compared to control cells was 188%.

Alligator

Alligator erythrocytes swelled rapidly in response to hypotonic challenge (Figure 7A). The initial swelling was followed by a subtle volume recovery. However, in a hypotonic, low-Ca^{2+} extracellular medium, volume decrease was completely inhibited (Figure 7A, n=7, P<0.001 at 90 min compared to control cells). Specifically, in the absence of extracellular Ca^{2+}, percent volume recovery was abolished (Figure 7B, n=7, P<0.01 at 40 and 90 min). Upon examining whether the cationophore gramicidin would influence RVD by enhancing K^+ efflux, I found that addition of this agent (0.5 μ M added at peak cell volume, n=5) to a low Ca^{2+} choline Ringer reversed EGTA-mediated RVD inhibition. In fact, despite the lack of an extracellular Ca^{2+} source, in the presence of gramicidin, final cell volume recovery exceeded that of control cells (Figure 7A, n=5, P<0.05 at 90 min), as did percent volume recovery at 90 min (Figure 7B, n=5, P<0.05). This I attempted to address my hypothesis that facilitating Ca^{2+} influx should potentiate volume recovery by increasing intracellular Ca^{2+} concentration using the Ca^{2+} ionophore A23187 (0.5 μ M, n=6). Unexpectedly, the ionophore had a pronounced inhibitory effect on the ability of alligator red blood cells to regulate their volume following hypotonic shock (Figure 8A, P<0.001 at 90 min compared to control cells). Not only did A23187-treated cells fail to recover their volume, but they continued to swell throughout the 90 min time course (Figure 8B, P<0.001 at 40 min and P<0.001 at 90 min).

To examine the mechanism for Ca^{2+} influx into alligator cells, I used gadolinium (50 μ M, n=7). Unlike in salmon (Figure 6), this agent had a distinct inhibitory effect on cell volume recovery (Figure 9A, P<0.01 at 90 min compared to control cells). That is, unlike control cells, percent volume recovery of gadolinium-treated cells was completely inhibited (Figure 9B, P<0.001 at 40 min and P<0.01 at 90 min).

After observing an inhibitory effect following gadolinium treatment, I wanted to pinpoint the gadolinium-sensitive transport pathway involved in Ca^{2+} influx in response to cell swelling. To this end, I examined whether P_{2X} receptors were involved in the alligator RVD process. To accomplish this, I utilized the ATP scavenger hexokinase (2.5 units/mL H_2O, n=6), as extracellular ATP is known to enhance RVD by stimulating P_2 receptors (Dezaki et al. 2000, Light et al. 2001, Schwiebert et al. 1995). Consistent with my prediction, this agent had a significant inhibitory effect on cell volume recovery, as hexokinase-treated cells were more swollen than control cells at 90 min (Figure 10A, P<0.01). Furthermore, upon hexokinase addition, percent volume recovery was reduced (Figure 10B, P<0.01 at 40 min and P<0.001 at 90 min).

To further verify a role for P_2 receptors, I treated cells with suramin (100 μ M, n=5), which directly inhibits this receptor type (Dezaki et al. 2000, Light et al. 2001). Cells bathed in a hypotonic solution containing this agent showed a decreased ability to regulate their volume compared to control cells (Figure 11A, p<0.05 at 90 min). Furthermore, percent volume recovery of experimental cells was also limited in comparison to control cell percent volume recovery (Figure 11B, P<0.01 at both 40 and 90 min).

After successfully inhibiting the RVD response of alligator cells using antagonists of P_2 receptor activation, I attempted to potentiate volume recovery using an agonist of this receptor type. I provided cells with an exogenous source of ATP (100 μ M, n=6), as this has been shown to stimulate P_2 receptor-dependent RVD responses (Dezaki et al. 2000, Schwiebert et al. 1995). Although I predicted this would enhance RVD, the degree by which cells were able to regulate their volume was unaffected by increasing their exogenous ATP source. Specifically, volume recovery of ATP-treated cells matched that of control cells (Figure 12A), as did the percent volume recovery at both 40 and 90 min (Figure 12B).

One potential method by which Ca^{2+} could stimulate volume recovery would be to directly activate K^+ efflux. To assess this possibility, I used quinine (1 mM, n=5), a Ca^{2+}-activated K^+ channel antagonist (Bergeron et al. 1996, Hoffman et al. 1986). Quinine had no significant effect on the RVD response of alligator red blood cells (Figure 13A). Specifically, there was no difference in the overall volume recovery of

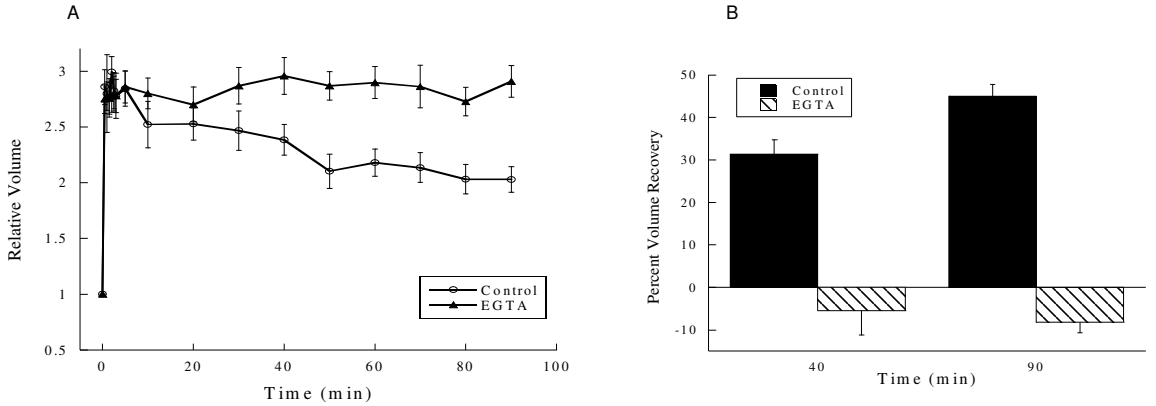

Figure 4. Cells failed to regulate their volume in the absence of extracellular Ca^{2+}.
(A) At time 0, cells were exposed to hypotonic shock, which resulted in a rapid rise in cell volume. Following this initial swelling, control cells recovered toward their normal size over a 90 min time course. In contrast, cells in a low Ca^{2+} medium remained swollen (n=5, P<0.001 at 90 min compared to control). (B) Percent volume decrease was significantly inhibited in the absence of extracellular Ca^{2+}, both at 40 min (n=5, P<0.001 compared to control at 40 min) and at 90 min (n=5, P<0.001 compared to control at 90 min). Cells swelled in both instances, as indicated by the negative percent volume recoveries of -5% (40 min) and -8% (90 min) compared to the control cells' percent volume recoveries of 31% (40 min) and 50% (90 min). Values represent mean ± standard error.

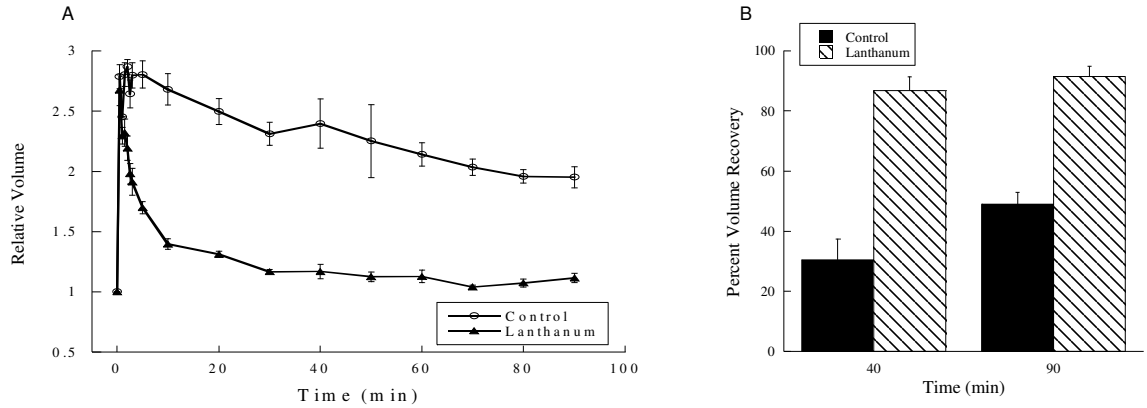

Figure 5. Lanthanum treatment potentiated cell volume recovery.
(A) At time 0, cells were exposed to hypotonic shock. Lanthanum-treated cells expressed a substantially enhanced cell volume recovery compared to control cells, both in terms of rate and final cell volume (n=5, P<0.001 at 90 min). (B) Control cells decreased in volume by 30% and 49% at 40 min and 90 min, respectively. However, cells treated with lanthanum had remarkably recovered by 87% and 92% at 40 min and 90 min, respectively (n=5, P<0.001 for both 40 and 90 minutes). Values represent mean ± standard error.

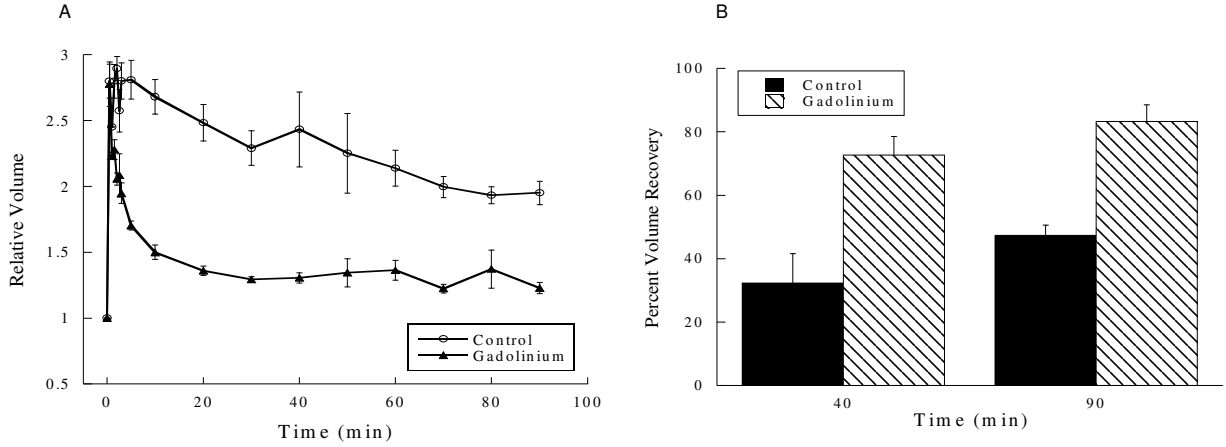

Figure 6. Treating cells with gadolinium enhanced cell volume recovery after exposure to a hypotonic medium.
(A) Gadolinium-treated cells had more successfully regulated their volume after 90 minutes when compared to control cells (n=5, P<0.05 at 90 min). (B) The rate of volume recovery of gadolinium-treated cells was elevated to 73% at 40 min and 83% at 90 min compared to control values of 32% at 40 min and 47% at 90 min (n=5, P<0.001 at both 40 and 90 min). Values represent mean ± standard error.

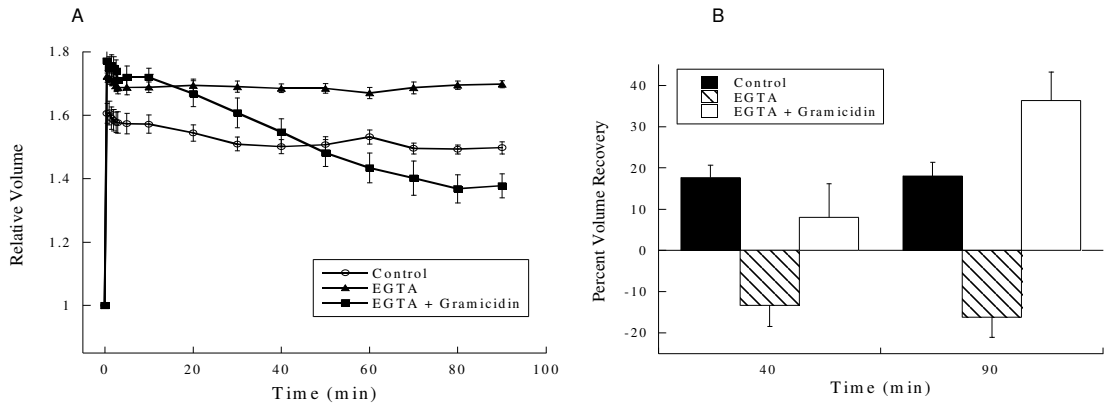

Figure 7. Removing extracellular Ca^{2+} inhibited volume recovery, and gramicidin reversed this inhibition.
(A) At time 0, cells were challenged with a hypotonic medium. RVD was inhibited by the removal of Ca^{2+} from the extracellular medium (*n*=7, P<0.001 at 90 min compared to control). However, with the addition of gramicidin (added at 30 sec, *n*=5), volume recovery exceeded that of control cells (P<0.01 at 90 min compared to control), even in the absence of extracellular Ca^{2+} (B) Percent volume recovery was -13% at 40 min and -16% at 90 min in EGTA-treated cells compared to control cell percent volume recovery of 18% at 40 and 90 min (*n*=5, P<0.01 at 40 and 90 min). Percent volume recovery of gramicidin-treated cells (*n*=5) was enhanced to 36% compared to control cell percent volume recovery of 18% at 90 min (P<0.05 at 90 min). Values are mean ± standard error.

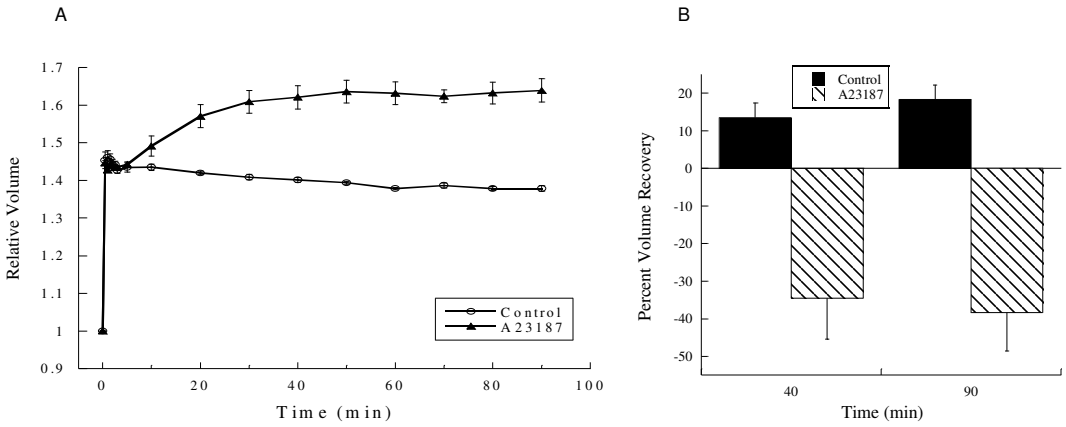

Figure 8. A23187 inhibited recovery following hypotonic shock in alligator cells.
(A) Control cells gradually recovered from cell swelling, whereas A23187-treated cells continued to increase in volume over the 90 min time course studied (n=6, P<0.001 at 90 min compared to control). (B) Following hypotonic shock, control cells recovered their volume by 13% and 18% at 40 and 90 min, respectively. In contrast, A23187-treated cells displayed a negative percent volume recovery of -42% at 40 min (n=6, P<0.001 compared to control) and -48% at 90 min (n=6, P<0.001 compared to control). Values are mean + standard error.

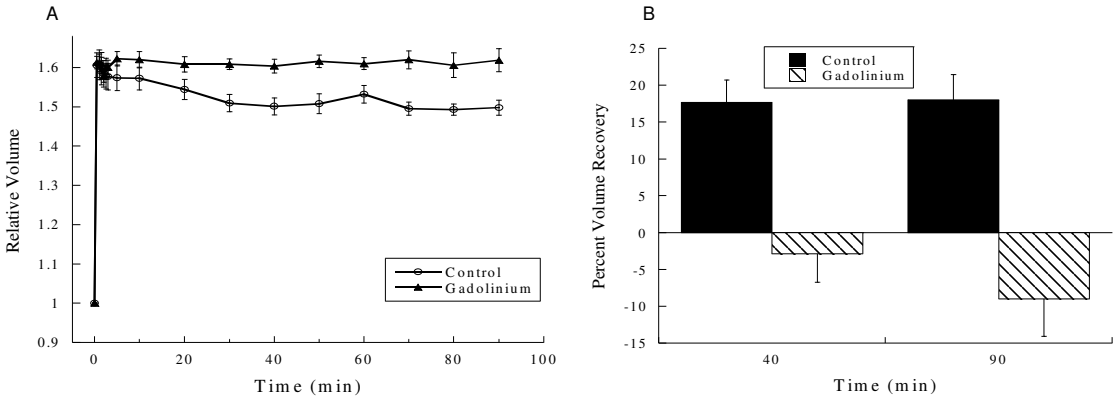

Figure 9. Gadolinium inhibited regulatory volume decrease of alligator red blood cells.
(A) Unlike control cells, which slowly recovered toward steady state cell volume following hypotonic shock, recovery was abolished in gadolinium-treated cells (*n*=7, P<0.01 at 90 min compared to control cells). (B) Alligator red cells exposed to gadolinium showed a percent volume recovery of -3% and -9% at 40 and 90 min respectively. This was in comparison to control cell percent volume recovery of 18% at the corresponding times (*n*=7, P<0.001 at 40 min and P<0.01 at 90 min). Values are means ± standard error.

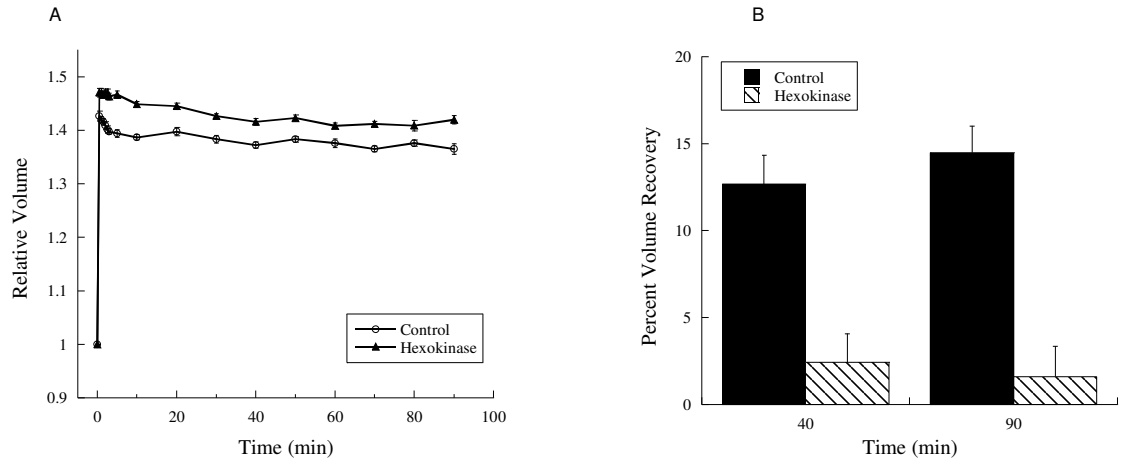

Figure 10. Cell volume recovery was attenuated in the presence of hexokinase.
(A) Hexokinase-treated cells remained more swollen throughout the 90 min time course than control cells (n=6, P<0.01 at 90 min compared to control). (B) Percent volume recovery of hexokinase-treated cells was limited to 2% at both 40 and 90 min. This was in contrast to control cells, which showed a percent volume recovery of 13% and 15% at 40 and 90 min, respectively (n=6, P<0.01 at 40 min and P<0.001 at 90 min compared to control). Values are mean ± standard error.

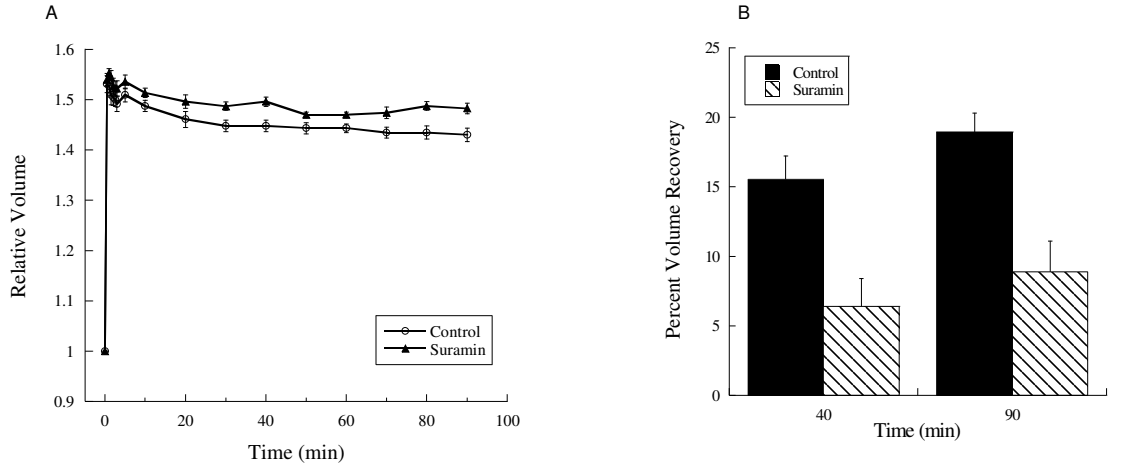

Figure 11. Suramin reduced alligator cell volume recovery.
(A) Addition of suramin to the extracellular medium limited the ability of cells to reduce in volume over the 90 min time course (n=5, P<0.05 at 90 min compared to control). (B) In the presence of suramin, percent volume recovery was reduced to 6% at 40 min and 9% at 90 min, in comparison to control cell percent volume recovery of 16% and 19% at the corresponding times (n=6, P<0.01 at 40 min and 90 min). Values are mean ± standard error.

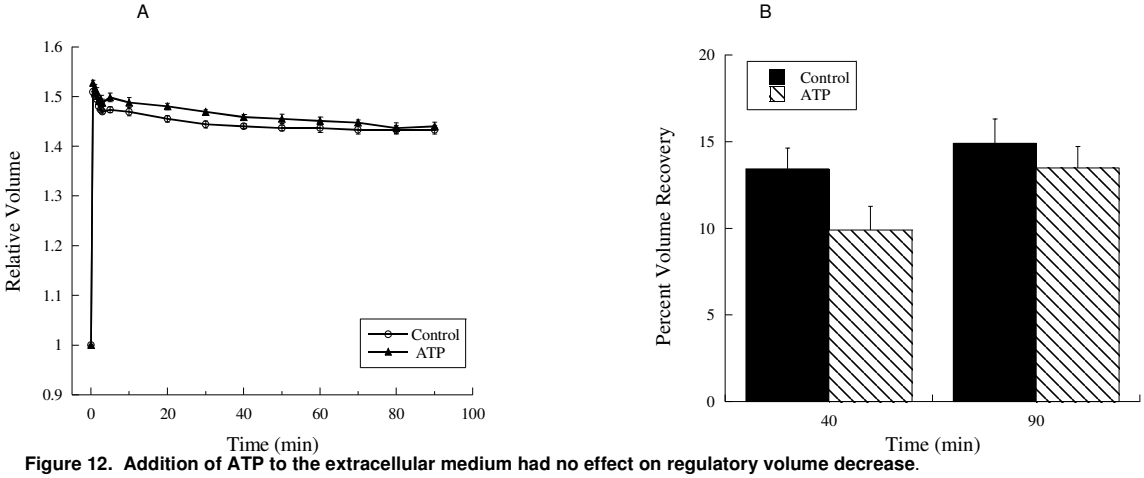

Figure 12. Addition of ATP to the extracellular medium had no effect on regulatory volume decrease.
(A) Cells bathed in a hypotonic medium containing ATP showed a volume recovery response that paralleled the response of control cells (n=6). (B) Percent volume recovery of ATP-treated cells was equivalent to control cell percent volume recovery at both 40 and 90 min (n=6). Values are mean ± standard error.

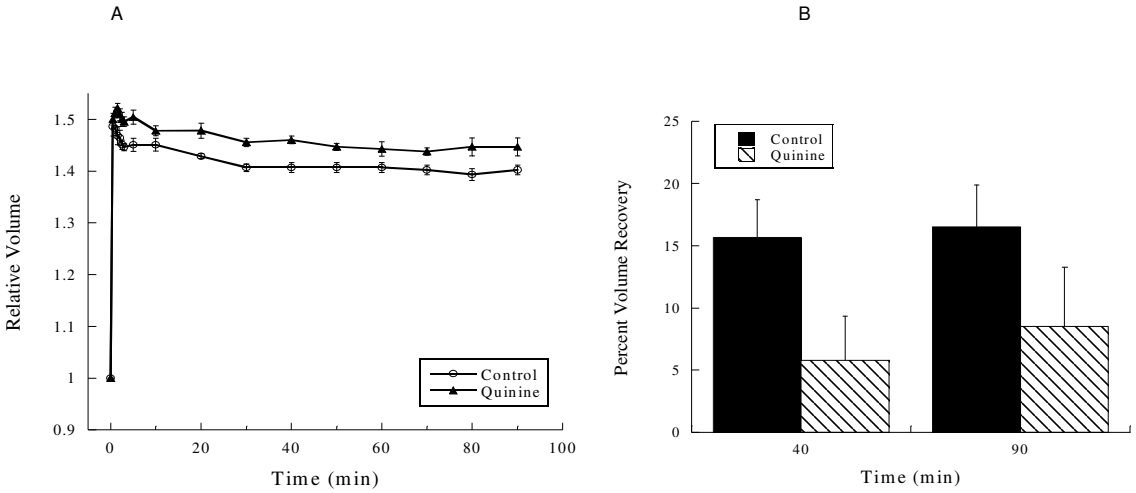

Figure 13. Quinine failed to inhibit volume recovery of alligator cells.
(A) After exposure to hypotonic shock, there was no significant difference in the overall volume recovery of quinine-treated cells when compared to control cells (*n*=5). (B) Quinine-treated cells and control cells displayed similar percent volume recoveries, both at 40 and 90 min (*n*=5). Values are means ± standard error.

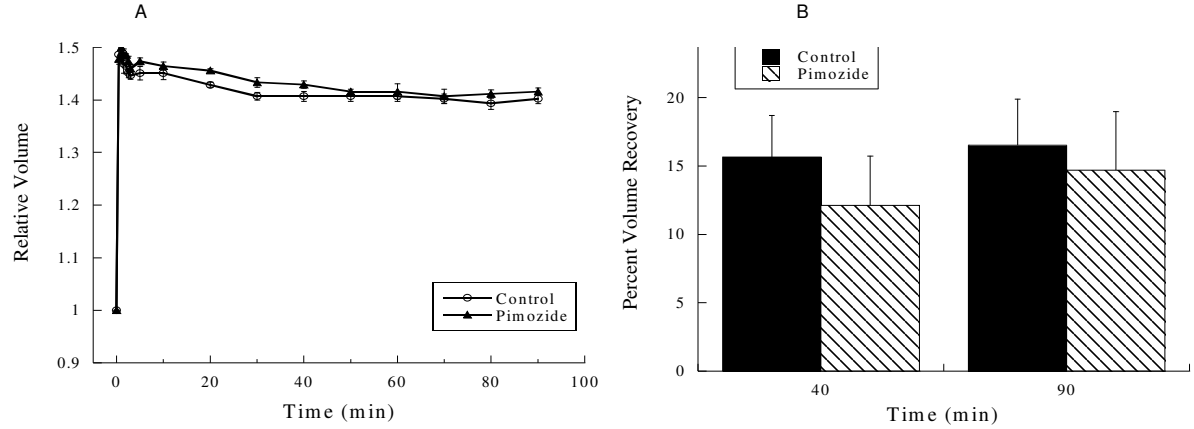

Figure 14. Pimozide-treated cells and control cells responded similarly to hypotonic challenge. (A) There was no significant difference between control cell volume recovery and cell volume recovery of pimozide-treated cells (*n*=5). (B) There was no difference between the percent volume recovery of control and pimozide-treated cells at either 40 min or 90 min (*n*=5). Values are means ± standard error.

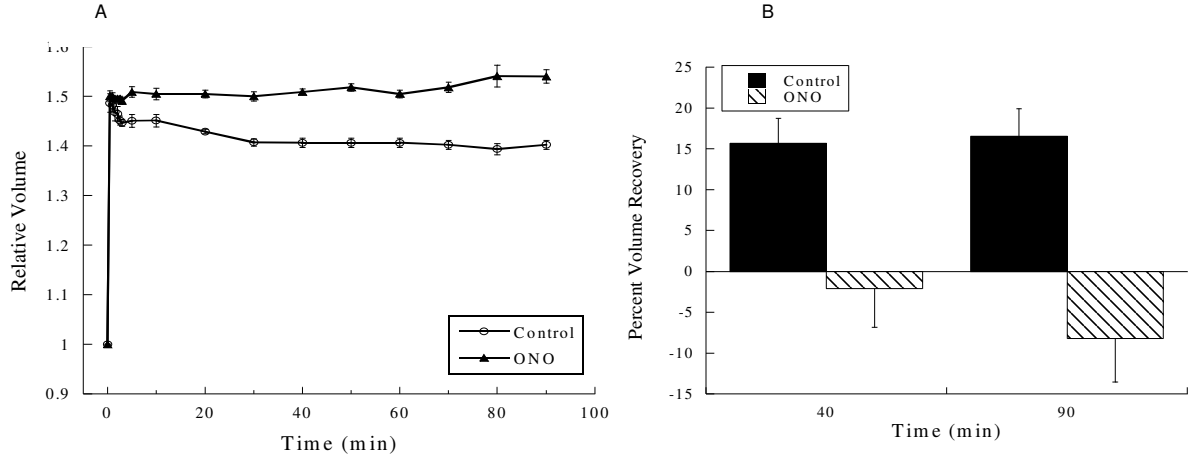

Figure 15. ONO-RS-082 abolished the RVD response of alligator cells.
(A) Control cells gradually recovered toward resting cell volume following hypotonic shock, but cells treated with ONO showed no volume recovery response and instead continued to swell over the 90 min time course (*n*=5, P<0.001 at 90 min compared to control cells). (B) Upon ONO treatment, a reduction in alligator cell percent volume recovery to -2% and -8% was observed at 40 and 90 min, respectively. This was in contrast to control cell percent volume recovery of 16% and 17% at the corresponding times (*n*=5, P<0.01 at 40 min and P<0.001 at 90 min). Values are mean ± standard error.

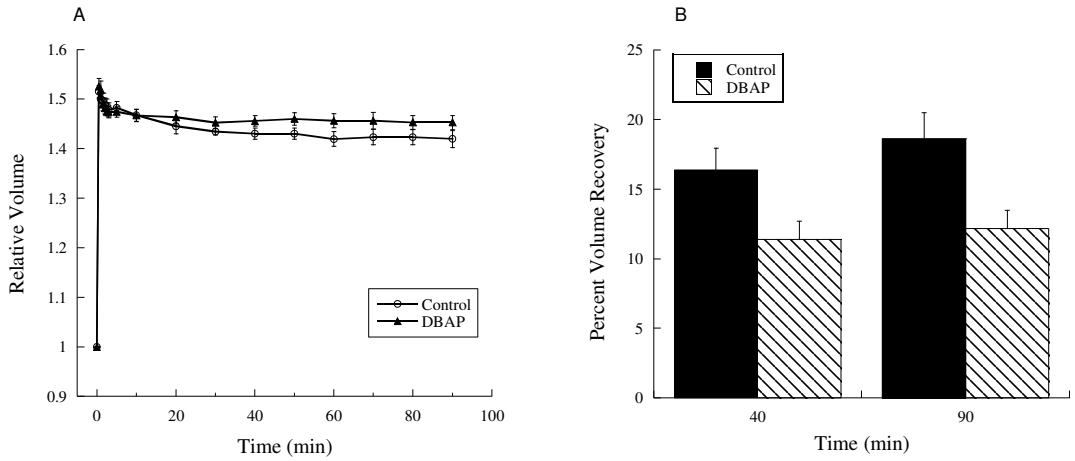

Figure 16. DHAP attenuated percent volume recovery of alligator cells. (A) There was no significant difference in the final volume of control cells and DHAP-treated cells. (B) Addition of DHAP reduced percent volume recovery to 11% and 12% at 40 and 90 min, respectively. This was in contrast to control cells, which recovered by 16% and 19% at the corresponding times (n=6, P<0.05 at 40 min and 90 min). Values are mean ± standard error.

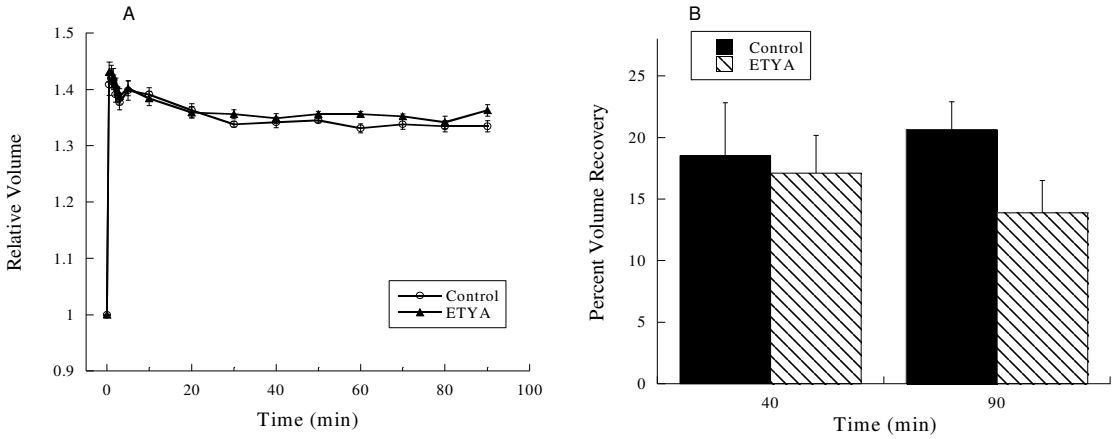

Figure 17. ETYA had no effect on cell volume recovery. (A) There was no difference in the volume of ETYA-treated cells and control cells at 90 min (n=6). (B) The percent volume recovery of ETYA-treated cell matched that of control cells (n=6). Values are mean ± standard error.

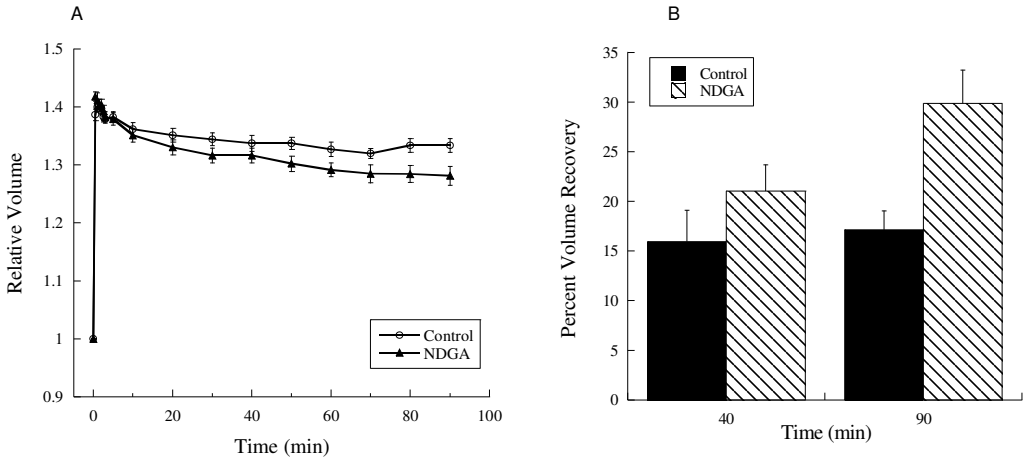

Figure 18. NDGA-treated cells displayed a potentiated volume recovery response following hypotonic challenge.
(A) Cells exposed to NDGA were slightly more successful in regulating their volume than control cells, as reflected in their smaller final volume (n=6, P<0.05 at 90 min compared to control cells). (B) The percent volume recovery of control cells and NDGA-treated cells was similar at 40 min (approximately 16%). However, the percent volume recovery of 30% experienced by NDGA-treated cells at 90 min surpassed the 17% volume recovery of control cells at this time (n=6, P<0.01). Values represent mean + standard error.

Agent	Prediction	Observed Effect on RVD
EGTA	Inhibition if Ca^{2+} is necessary for volume recovery	Inhibition in salmon Inhibition in alligator
A23187	Enhancement if Ca^{2+} influx stimulates RVD	Inhibition in alligator
Gadolinium	Inhibition if Ca^{2+} influx occurs through a SA channel	Enhancement in salmon Inhibition in alligator
Lanthanum	Inhibition if Ca^{2+} entry occurs through a SA channel	Enhancement in salmon
Hexokinase	Inhibition if Ca^{2+} entry occurs through a P_2 receptor	Inhibition in alligator
Suramin	Inhibition if Ca^{2+} entry occurs through a P_2 receptor	Inhibition in alligator
ATP	Enhancement if P_2 receptor activity facilitates RVD	No effect in alligator
Gramicidin	Enhancement of K^+ efflux is required for volume recovery	Reversed inhibition in alligator
Quinine	Inhibition if K^+ efflux occurs via a Ca^{2+}-activated K^+ channel	No effect in alligator
Pimozide	Inhibition if RVD requires calmodulin activation	No effect in alligator
ONO-RS-082	Inhibition if volume recovery is PLA_2-dependent	Inhibition in alligator
2,3 DBAP	Inhibition if volume recovery is PLA_2-dependent	Inhibition in alligator
ETYA	Inhibition if an eicosanoid stimulates RVD	No effect in alligator
NDGA	Inhibition if a leukotriene is needed for volume decrease	Enhancement in alligator

Table 2. Pharmacological agents used in cell volume studies, my predictions as to their effects, and the experimental outcomes of their usage.

quinine-treated cells in comparison to control cells (Figure 13A). Likewise, the percent volume recovery of cells was unchanged by the addition of quinine (Figure 13B).

I then turned my attention to the various Ca^{2+}-mediated intracellular signaling cascades that might be initiated during RVD. I used pimozide (10 μ M, n=5), a blocker of calmodulin activity (Bergeron et al. 1996, Hoffman et al. 1986, Huang et al. 2001), to address the possibility of the involvement in this Ca^{2+} binding protein in alligator RVD. Pimozide had no significant effect on alligator cell volume regulation, as the RVD response of pimozide-treated cells paralleled that of control cells (Figure 14A). There was no difference in percent volume recovery of pimozide-treated cells compared to unmanipulated control cells, either (Figure 14B).

Having ruled out calmodulin, I next targeted arachidonic acid metabolism because this also is a Ca^{2+}-activated system. I blocked arachidonic acid production using ONO-RS-082 (10 μ M, n=5). This agent is a potent inhibitor of phospholipase A_2 (Light et al. 1998, Nakashima et al. 1989), a Ca^{2+}-activated enzyme that functions to release arachidonic acid from the phospholipid bilayer (Balsinde et al. 1999). Treating cells with ONO abolished their ability to regulate their volume (Figure 15A, $P<0.001$ at 90 min compared to control cells). In fact, ONO-treated cells continued to swell over the time course studied (Figure 15B, $P<0.01$ at 40 min and 90 min).

The results of my ONO studies implicated PLA_2 involvement in alligator RVD, as inhibiting this enzyme resulted in the failed ability of cells to reduce in volume following cell swelling. To further confirm a role for PLA_2, I used 2,4-dibromoacetophenone (5 μ M, n=6), a PLA_2 inhibitor (Balsinde et al. 1999). Although this agent's effect on the relative volume of alligator cells at 90 min fell shy of statistical significance (Figure 16A), it *did* significantly reduce the percent volume recovery at both 40 and 90 min (Figure 16B, $P<0.05$ compared to control cells).

Arachidonic acid could act directly on ion channels or could be metabolized further into eicosanoids, which in turn can activate K^+ channels. To determine whether it was arachidonic acid or an eicosanoid stimulating K^+ efflux in alligator cells, I used 5,8,11,14-eicosatetraynoic acid (10 μ M, n=6), an agent that blocks the production of all eicosanoids (Lehr & Griessbach 2000). This antagonist had no effect on RVD. That is, the final cell volume of ETYA-treated cells was not significantly different to that of control cells (Figure 17A). Likewise, no difference in percent volume recovery was apparent at either 40 or 90 min (Figure 17B), suggesting eicosanoid formation is not necessary for volume recovery.

Nonetheless, I still examined the role of the lipoxygenase pathway of arachidonic acid metabolism to ensure that the lack of effect following ETYA treatment was not due to this agent's relatively low potency. I treated cells with nordihydroguaiaretic acid (10 μ M, n=6), an effective inhibitor of the lipoxygenase pathway (Lambert et al. 1987). This agent had a modest potentiating effect on alligator RVD. Specifically, cells treated with NDGA showed a more extensive volume recovery, indicated by their reduced final cell volume compared to control cells (Figure 18A, $P<0.05$ at 90 min compared to control). Additionally, the rate of volume recovery was potentiated in NDGA-treated cells, which showed a final percent volume

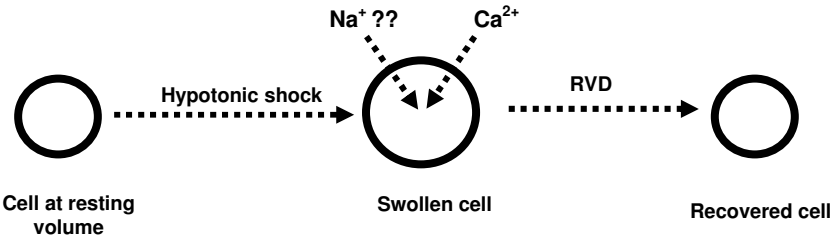

Figure 19. Proposed pathway for volume recovery of salmon red blood cells.
Hypotonic challenge leads to a rise in intracellular Ca^{2+} as a result of influx from the extracellular medium by way of a gadolinium- and lanthanum-insensitive transport pathway. The rise in Ca^{2+} activates the RVD response, allowing for volume recovery. Additionally, Na^{+} influx at the onset of RVD might attenuate volume decrease.

recovery exceeding that of control cells (Figure 18B, $P<0.01$ at 90 min).

The results of my cell volume studies are summarized in Table 2. Listed are the various agents I used in my experiments, the predictions I made as to the outcomes of my studies, and my actual experimental findings.

Discussion

A role for Ca^{2+} in salmon RVD

Cell volume recovery was dependent on extracellular Ca^{2+}

The findings of my study are consistent with a role for Ca^{2+} in regulatory volume decrease. That is, in a low Ca^{2+} environment, salmon red blood cells failed to recover from the rapid swelling that accompanied hypotonic shock (Figure 4). Furthermore, the Ca^{2+} source needed for the RVD response appears to be extracellular in origin, because if *intracellular* Ca^{2+} was the main source involved, then removal of *extracellular* Ca^{2+} should not have inhibited volume recovery. And if both intracellular and extracellular Ca^{2+} played a role in RVD, then even in the absence of an extracellular Ca^{2+} source, cells might have experienced a partial volume recovery. Therefore, my results indicate that extracellular Ca^{2+} plays the critical role in activating the RVD transduction pathway, thus supporting my original hypothesis.

This finding is in agreement with the microscopy studies of Light et al. (2005). Specifically, if the conclusion that extracellular Ca^{2+} is needed for volume regulation is correct, then one would expect a rise in intracellular Ca^{2+} levels to follow hypotonic exposure in order to activate the RVD response. Light et al. (2005) showed that an elevation in Ca^{2+} concentration does indeed occur (Figure 1). Additionally, they assessed the source of this Ca^{2+} by bathing salmon cells in a hypotonic, low Ca^{2+}-EGTA Ringer. In this environment, fluorescence was virtually abolished. This reinforces my suggestion that hypotonic shock is followed by Ca^{2+} influx from the extracellular medium as opposed to Ca^{2+} release from intracellular storages.

The conclusion that Ca^{2+} influx plays the predominant role in elevating intracellular Ca^{2+} levels is shared by Rothstein & Mack (1992), who determined that the increasing cytosolic Ca^{2+} concentration accompanying hypotonic shock of Madin-Darby canine kidney cells is due to influx from the extracellular environment as opposed to release from intracellular stores. They found that use of EGTA to form a low Ca^{2+} hypotonic medium inhibits this increase. The results of McCarty & O'Neil (1990) further corroborate this finding; they failed to observe a rise in cytosolic Ca^{2+} upon removal of Ca^{2+} from the hypotonic medium in proximal tubules. However, in other cell types, the rise in Ca^{2+} concentration appears to be the result of *both* influx from the extracellular medium as well as release from the intracellular medium. This is the case, for example, in rat cultured suspended cerebellar astrocytes (Morales-Mulia et al. 1998). Additionally, it should be noted that hypotonic shock does not invariably cause a rise in intracellular Ca^{2+}. For example, Kanli & Norderhus (1998), who tracked intracellular Ca^{2+} levels using epifluorescence, observed no change in the degree of fluorescence upon hypotonic stimulation of trout proximal renal tubules. Clearly, the specific events that occur as a result of changing extracellular osmolality vary as a function of cell type.

Although my results suggest that extracellular Ca^{2+} is the primary activator of the RVD response, more complex mechanisms than the one I proposed above could be responsible for the volume recovery mechanism employed by salmon cells. For example, extracellular Ca^{2+} might function to stimulate release of Ca^{2+} from intracellular storages, and the release of this Ca^{2+} source, in turn, might then be responsible for activation of downstream RVD processes (McCarty & O'Neil 1992). If such a sequence of events was responsible for volume recovery, then removal of extracellular Ca^{2+} would indeed inhibit this process, as the extracellular Ca^{2+}-dependent event occurs upstream from that of intracellular Ca^{2+} release. Such a cascade has been demonstrated by Tinel et al. (2002) using rabbit TALH cells. In this cell type, Ca^{2+} release from intracellular stores is dependent on Ca^{2+} influx from the extracellular medium. When Ca^{2+} influx and, in turn, Ca^{2+} release, was blocked using a low Ca^{2+} Ringer, cells were unable to recover from hypotonic challenge. Future studies could address this possibility in salmon red blood cells by exposing the cells to pharmacological agents that deplete intracellular Ca^{2+} stores, such as thapsigargin, ryanodine, or caffeine (Morales-Mulia et al. 1998, Quesada et al. 1999, Tinel et al. 2002). If the primary role of extracellular Ca^{2+} is to stimulate Ca^{2+} release, then use of these agents prior to hypotonic exposure should inhibit RVD, even in the presence of extracellular Ca^{2+}.

Extracellular Ca^{2+} influx did not occur through lanthanum- or gadolinium-sensitive transport pathways

Both lanthanum and gadolinium had similar, and unexpected, effects on the RVD response of salmon erythrocytes. Specifically, these agents dramatically *enhanced* the rate of cell volume recovery (Figures 5 & 6), which was contrary to my original prediction and the findings of other researchers (Adorante & Cala 1995, Rothstein & Mack 1992, Light et al. 2003). Furthermore, there is no clear explanation as to why these agents would potentiate volume decrease. One possibility, however, is that gadolinium and lanthanum had non-specific effects that caused the blockage of Na^+ influx. To explain, both the fluorescent studies (Light et al. 2005) and EGTA studies show unequivocally that RVD in salmon cells is Ca^{2+}-dependent. Therefore, it is unlikely that lanthanum and gadolinium blocked Ca^{2+} entry pathways, for if they did, cells would not have been able to respond appropriately to hypotonic challenge. However, if these agents instead prevented Na^+ entry into the cells, the result *could* be an enhanced volume recovery. For instance, Garcia-Romeu et al. (1991) showed that Na^+ influx at the onset of RVD attenuates volume recovery in rainbow trout erythrocytes. Although few studies identify a weakening effect of Na^+ on the RVD response as proposed here, the fact that rainbow trout is a close relative to Atlantic salmon suggests that RVD in salmon might share similar characteristics to the RVD process observed by Garcia-Romeu at al. (1991). Furthermore, there is evidence that lanthanides can be somewhat non-selective and block the influx of other cations besides Ca^{2+}, including Na^+ (Caldwell et al. 1998). Taking the limitations of these inhibitors' specificities into account and my observation, the suggestion that they might block Na^+ influx as opposed to Ca^{2+} influx in this cell type is not unreasonable.

Furthermore, Na^+ influx might play an important role in buffering the cell volume recovery response of salmon erythrocytes, because if lanthanum and gadolinium did inhibit Na^+ influx, the result was a strikingly rapid RVD response compared to control cells. In this sense, Na^+ entry might serve to ensure that cells do not decrease in volume too quickly or too much; just as cell swelling can have devastating effects, a reduction in cell volume beyond a critical point is equally threatening to cell integrity. Another possibility is that Na^+ influx is a concomitant occurrence. That is, it might serve no purpose in the overall RVD scheme, but at the same time, it might not be preventable. For example, if cell swelling initiates the opening of non-selective stretch activated cation channels, then these permeability pathways would inevitably allow for Na^+ entry.

Due to the unavailability of blood, I was unable to further study the salmon RVD response. Accordingly, future studies are needed to better pinpoint the events activated subsequent to cell swelling. To assess more directly whether Na^+ entry at the onset of RVD was indeed responsible for the observed effects of lanthanum and gadolinium, studies could be performed to examine the outcome of removing Na^+ from the extracellular environment and replacing it with an impermeant cation such as N-methyl-D-glucamine (NMDG). If the proposed scenario outlined above is indeed what is occurring in this cell type, then inhibiting Na^+ entry directly should also have an enhancing effect on RVD that mimics the effect observed in the gadolinium and lanthanum experiments.

Finally, future studies are needed to determine what pathways *are* responsible for the Ca^{2+} influx essential for cell volume regulation by salmon red blood cells. The findings of this study do not support Ca^{2+} entry through stretch-activated channels, as gadolinium and lanthanum failed to inhibit volume recovery. Because gadolinium has also been shown to block P_2 receptors (Nakazawa et al. 1997), this entry site does not seem likely, either. This is consistent with the findings of Light et al. (2005), who examined the potential role for P_2 receptors in salmon RVD. They performed experiments using the ATP-scavenger, hexokinase, which inhibits P_2 receptor activation. They found this agent to have no significant effect on volume recovery, which indicates a P_2 receptor-independent RVD response. Considering that the specific Ca^{2+} entry site has yet to be determined for this cell type, future experiments using additional Ca^{2+} transport antagonists are required.

Summary

Taken collectively, my findings suggest that swelling of salmon erythrocytes triggers an influx of Ca^{2+} and the subsequent rise in intracellular Ca^{2+} levels. This, in turn, plays a role in activating the volume regulatory response (Figure 19).

RVD in alligator erythrocytes is Ca^{2+}-dependent

Extracellular Ca^{2+} was necessary for volume recovery of alligator red blood cells

Similar to my results from salmon (Figure 4), alligator cells placed in a hypotonic medium lacking Ca^{2+} failed to recover toward steady state cell volume (Figure 7). This finding is consistent with Ca^{2+} playing a primary role in alligator volume recovery, which is not surprising; as mentioned previously, such a Ca^{2+}-dependent RVD response is common among numerous cell types (Christensen 1987, Hoffman et al. 1986, Light et al. 1998, Rothstein & Mack 1992). This most likely reflects the ubiquitous nature of this signal molecule.

Taking into account the Ca^{2+}-dependent nature of alligator RVD, I hypothesized that increasing plasma membrane permeability to Ca^{2+}, which allows for increased Ca^{2+} influx, should enhance the RVD process. However, the results of my studies using the calcium ionophore A23187 contradicted this prediction. Unexpectedly, this agent dramatically *inhibited* volume recovery following hypotonic shock (Figure 8). Therefore, it appears that in this cell type, a subtle rise in intracellular Ca^{2+} might be required to activate RVD, whereas a dramatic rise might have the opposite effect. The lack of RVD rate enhancement upon large increases in intracellular Ca^{2+} is not restricted to alligator erythrocytes (McCarty and O'Neil 1992). For instance, Montrose-Rafizadeh & Guggino (1991) found that in rabbit medullary thick ascending limb cells, the rate of RVD is proportional to intracellular Ca^{2+} concentration *only* when this concentration is lower than basal Ca^{2+} levels. They concluded that the resting Ca^{2+} concentration in this cell type is sufficient to fully maximize the RVD response, which explains why an increase in Ca^{2+} cannot further enhance the rate of volume recovery. Beck et al. (1991) also found that no rise in intracellular Ca^{2+} is necessary for RVD in rabbit proximal convoluted tubules. Although Ca^{2+} appears to

be involved in the RVD process in this cell type (as removing Ca^{2+} attenuates volume recovery), the observed increase in intracellular Ca^{2+} following addition of A23187 did not result in RVD rate enhancement.

Similarly, it might be that alligator cells have a low Ca^{2+} threshold such that only a slight increase in Ca^{2+} is needed to fully saturate the Ca^{2+}-stimulated volume recovery response. However, many cell types, including *Necturus* erythrocytes (Light et al. 2003), dissociated epithelial cells (Rothstein & Mack 1990), and proximal tubules (McCarty & O'Neil 1990) do not display such a low Ca^{2+} threshold. In these cell types, fairly large increases in cytosolic Ca^{2+} (achieved by A23187 addition) do, indeed, potentiate volume recovery. The dissimilarity in Ca^{2+} sensitivity among cell types might be due to differences in the cellular machinery involved in their RVD processes or differences in the specific role played by Ca^{2+} in their volume recovery mechanisms.

The fact that alligator cells responded in an inhibitory fashion to A23187, as opposed to showing no response, was rather puzzling. This observation is best explained as a concentration effect—*small* increases in Ca^{2+} levels stimulate RVD, whereas *pharmacological* increases inhibit the volume recovery process. Additionally, A23187 might have a permissive effect on both Ca^{2+} *and* Na^{+} entry into cells (Escobales & Canessa 1985). And sodium influx, in turn, could result in additional cell swelling (Garcia-Romeu at al. 1991). Because some researchers suggest that cell swelling *itself* plays a role in inactivating certain aspects of the RVD process (McCarty & O'Neil 1990), excessive Na^{+} entry might prematurely arrest the volume recovery process in alligator cells, accounting for the dramatic inhibition of RVD following A23187 treatment. However, experimental evidence must be obtained to draw more definitive conclusions. Specifically, studies in which the concentration of A23187 used or the concentration of Ca^{2+} present in the extracellular medium is adjusted would be useful. By observing the effects of such manipulations on volume recovery, insight into the effects of A23187 on alligator RVD might be gained.

Extracellular Ca^{2+} functioned to activate K^{+} efflux

It appears that Ca^{2+} is involved in stimulating K^{+} efflux from alligator cells. Support for this conclusion comes from the finding that the cationophore gramicidin reversed the inhibitory effect of extracellular Ca^{2+} removal (Figure 7), which was the expected result if Ca^{2+} has a permissive effect on K^{+} efflux. The finding that K^{+} efflux is a crucial component of alligator RVD is consistent with virtually all cell types studied to date (Chamberlin & Strange 1989, McCarty & O'Neil 1992, Pasantes-Morales et al. 2000). Furthermore, other researchers have similarly concluded that this efflux is Ca^{2+} dependent. For example, Adorante & Cala (1995) found that Ca^{2+} stimulates K^{+} efflux in nonpigmented human ciliary epithelial cells, as the inhibition of RVD by Ca^{2+} chelation was removed with the addition of gramicidin. Likewise, Light et al. (2003) found that gramicidin reverses the RVD inhibition that accompanies EGTA treatment of *Necturus* erythrocytes. These parallel findings lend support for the results of this study.

Ca^{2+} influx occurred through gadolinium-sensitive ion channels

Consistent with my original hypothesis, a gadolinium-sensitive channel appears to account for the Ca^{2+} influx needed to activate the RVD response in alligator cells, because gadolinium-treated cells showed a complete inability to regulate their volume (figure 9). The response of alligator cells to this antagonist is not unique, for similar results have been obtained from studies on rat lacrimal acinar cells (Speake et al. 1998) as well as on *Necturus* erythrocytes (Light et al. 2003). These similarities suggest that some continuity exists in terms of calcium entry pathways. However, interestingly, the membrane transport pathway active in alligator RVD appears to be fundamentally different to that stimulated in response to salmon cell swelling (figure 6). That is, salmon cells displayed the complete opposite response to gadolinium treatment.

In addition, it seems that the gadolinium-sensitive transport pathway involved in Ca^{2+} entry into alligator cells is, at least in part, a P_2 receptor in contrast to a stretch-activated channel. Evidence for this can be seen in figures 10 and 11, which reveal that inhibition of alligator RVD occurred following addition of the ATP-scavenger hexokinase and the P_2 receptor antagonist suramin. Thus, a role for P_2 receptors in the volume recovery response of alligator cells is supported by this study. Similarly, in *Necturus* red blood cells, RVD is dependent on P_2 receptor activation, which is followed by the increase in cytosolic Ca^{2+} required to stimulate RVD (Light et al. 1999). Dezaki et al. (2000) also showed that in a human epithelial cell line, hypotonic shock triggers the release of ATP into the extracellular medium, the subsequent activation of P_2 receptors, and an increase in intracellular Ca^{2+}. The fact that the findings of Light et al. (1999) and Dezaki et al. (2000) are consistent with this study strengthens the notion of a P_2 receptor-mediated RVD mechanism.

However, unlike *Necturus* erythrocytes (Light et al.1999) and human epithelial cells (Dezaki et al. 2000), it appears that alligator cells release sufficient ATP at the onset of the RVD response to fully saturate their P_2 receptors. Whereas ATP addition to the extracellular medium enhanced volume recovery in *Necturus* and human epithelial cells, no effect was observed upon ATP addition to alligator cells (Figure 12). Therefore, the most logical conclusion, based on the fact that P_2 receptors *do* appear to be necessary in alligator RVD, is that they are maximally active under normal conditions. In future studies, ATP γS, a non-hydrolyzable form of ATP (Light et al. 2001) could be used. This might discern whether the lack of effect upon ATP addition observed in this study was indeed the result of receptor saturation, or, alternatively, if these cells have very active exo-ATPases (Gordon 1986) that prevent the rise in extracellular ATP concentration necessary to observe an enhanced volume recovery.

It should be noted that the inhibition of RVD evoked by hexokinase and suramin was not as robust as that of gadolinium. This suggests that P_2 receptors might not be entirely responsible for Ca^{2+} entry during RVD. In addition to a P_2 receptor, another gadolinium-sensitive transport pathway (such as a stretch-activated channel) could also be active during the volume recovery process. In future studies, using additional P_2 receptor antagonists, in particular those specific for P_{2X} receptors, would be beneficial for further elucidation of

the specific Ca^{2+} transport pathways functioning in this cell type.

K^+ efflux does not occur through Ca^{2+}-activated K^+ channels

The effect of gramicidin on RVD in alligator erythrocytes suggested that the rate limiting step in volume recovery was K^+ efflux, as facilitating K^+ loss from cells with exogenous pores resulted in a more efficient RVD response (Figure 7). Furthermore, it appears that Ca^{2+} had a permissive effect on K^+ efflux, as I was able to reverse the inhibitory effect of a low-Ca^{2+} medium by artificially permitting K^+ loss from alligator cells. As mentioned earlier, this was the expected result if K^+ efflux occurs downstream of Ca^{2+} entry into cells. However, my results suggest that Ca^{2+} does not modulate K^+ permeability *directly*, because quinine, a Ca^{2+}-gated K^+ channel blocker (Hoffman et al. 1986), had no significant effect on volume recovery (Figure 13). That is, it is unlikely that Ca^{2+} stimulates K^+ efflux by binding to and activating a K^+ efflux site, as such a channel does not appear to be operating in alligator erythrocytes during the RVD response. Similar results were found by Arrazola et al. (1993) in rat thymocytes. In this cell type, RVD was unaffected by the addition of quinine. Similarly, K^+ efflux during cultured lens epithelial cell RVD occurs via a quinine-insensitive transport pathway (Diecke & Beyer-Mears 1997). However, in the case of alligator erythrocyte RVD, it is not possible to rule out the involvement of a Ca^{2+}-activated K^+ channel entirely, as a quinine *insensitive* Ca^{2+}-dependent K^+ channel might be present and operating during volume recovery. In order to account for this possibility, experiments using other Ca^{2+}-activated K^+ channel antagonists would have to be performed.

The lack of an effect following quinine addition to alligator red blood cells is in contrast to the studies performed by Hoffman et al. (1986) on Erlich ascites tumor cells, Adorante & Cala (1995) on nonpigmented human ciliary epithelial cells, and Bergeron et al. (1996) on *Necturus* red cells. In these cell types, quinine did have an inhibitory effect on RVD, which lends support to Ca^{2+} activated K^+ channels being present in these cell types. Therefore, although K^+ efflux is a universal step in the volume recovery process, the pathway for this efflux seems to vary among cell types.

RVD does not involve a Ca^{2+}/calmodulin signaling pathway

Since Ca^{2+} did not appear to *directly* activate K^+ efflux during alligator RVD, I assessed whether a signal transduction pathway involving calmodulin is at play in alligator erythrocytes. Although calmodulin has been implicated in the RVD process in many cell types (McCarty & O'Neil 1992), it does not seem to play a role in alligator volume recovery. That is, I failed to observe an inhibitory effect in response to preventing calmodulin activation with pimozide (Figure 14). Hence, it seems unlikely that RVD in alligator cells is modulated by a calmodulin system.

Calmodulin-independent transduction pathways similar to the one found in alligator cells have been identified in other cell types. For example, Fincham et al. (1987) showed that the response of erythrocytes from euryhaline fish species to hypotonic shock is independent of calmodulin activation. Additionally, Schliess et al. (1996) determined that calmodulin is not involved in the cell signaling events stimulated by cell swelling in rat cerebral astrocytes. The fact that both calmodulin dependent *and* independent signaling processes have been observed implies that there are variations in the steps ultimately leading to volume restoration.

RVD depends on Phospholipase A_2 activation and arachidonic acid production

The results of my PLA_2 inhibition studies were in agreement with my early predictions. Specifically, as was expected if PLA_2 activation is a crucial component to the RVD response, the PLA_2 antagonist ONO had a robust inhibitory effect on the ability of alligator cells to regulate their volume (Figure 15). Additionally, the PLA_2 inhibitor 2,4-dibromoacetophenone attenuated percent volume recovery in alligator cells (Figure 16). In combination, these findings imply that arachidonic acid and/or its metabolites are efficacious in alligator volume recovery. As stated previously, a role for arachidonic acid in volume recovery following hypotonic challenge is not unique to this cell type; RVD in neuroblastoma (Pasantes-Morales et al. 2000), *Necturus* erythrocytes (Light et al. 1998), and trout proximal renal tubules (Kanli & Norderhus 1998) all involve arachidonic acid metabolism.

It appears that in alligator erythrocytes, arachidonic acid *itself*, as opposed to one of its metabolites, potentiates the RVD signaling cascade. Support for this conclusion comes from the observation that suppressing the formation of all eicosanoids (the further breakdown products of arachidonic acid) using ETYA failed to inhibit volume regulation (Figure 17). Interestingly, the more specific antagonist NDGA (an inhibitor of the lipoxygenase pathway thereby blocking production of leukotrienes) slightly *potentiated* volume recovery (Figure 18). If anything, this result further supports the direct role I propose for arachidonic acid in alligator cell volume regulation. That is, by blocking arachidonic acid breakdown, NDGA might enhance RVD by increasing the concentration of arachidonic acid available to participate in volume restoration.

A primary role for arachidonic acid itself in volume regulation is consistent with the findings of other researchers. Basavappa et al. (1998) found that inhibiting arachidonic acid formation in human neuroblastoma cells results in a reduced RVD. However, similar to this study, RVD inhibition was not observed upon selective blockade of arachidonic acid breakdown. Sanchez-Olea et al. (1995) also showed that arachidonic acid stimulates RVD in astrocytes directly; RVD was significantly affected in the absence of the fatty acid itself, whereas antagonizing its further metabolism did not alter RVD. In contrast, if an arachidonic acid metabolite was required to stimulate RVD, then blocking its formation would attenuate volume recovery. This has been shown to occur in kidney cells (Tinel et al. 2000), where addition of ETYA results in a weakened RVD mechanism. Additional support for arachidonic acid metabolites as volume recovery agents comes from studies performed on *Necturus* erythrocytes (Light et al. 1997). In this cell type, both ETYA and NDGA had inhibitory effects on RVD, indicating a volume regulatory response dependent on a lipoxygenase metabolite.

Obviously, there is a clear continuity with respect to the RVD mechanisms among cell types, as many of them involve a signal transduction pathway

dependent on arachidonic acid. At the same time, however, obvious differences are apparent, particularly when it comes to the degree of arachidonic acid metabolism required to stimulate the RVD response. This may reflect differences in the specific role played by arachidonic acid in the overall RVD scheme, which has yet to be elucidated in many cell types.

Future studies should be performed to confirm a role for arachidonic acid in alligator volume

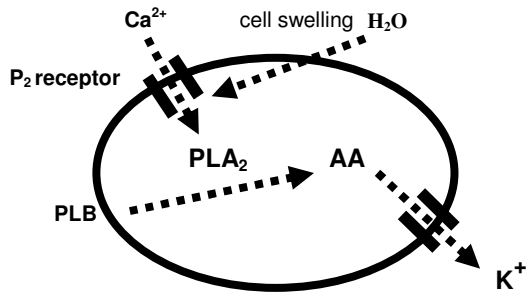

Figure 20. Proposed RVD signal transduction pathway in alligator red blood cells.
Cell swelling leads to Ca^{2+} influx, possibly by activating a P_2 receptor. The rise in intracellular Ca^{2+} stimulates PLA_2, which catalyzes the breakdown of membrane phospholipids to form arachidonic acid. Finally, the actions of arachidonic acid result in K^+ efflux, thereby allowing for cell volume recovery.

arachidonic acid is added to the extracellular medium. I would expect this to enhance volume recovery, if RVD is indeed stimulated by arachidonic acid.

Summary

The results from my studies on alligator RVD are consistent with cell swelling being followed by the influx of Ca^{2+} through a gadolinium-sensitive entry site, possibly a P_2 receptor. In turn, calcium stimulates the volume regulatory response through the activation of PLA_2 and the subsequent formation of arachidonic acid. Arachidonic acid leads to volume restoration by activating K^+ efflux (Figure 20).

Conclusion

Ca^{2+} as a signaling agent in regulatory volume decrease

Cell swelling appears to correlate with a rise in intracellular Ca^{2+} in both salmon and alligator red blood cells following an influx from the extracellular medium, as opposed to release from intracellular stores. The increase in Ca^{2+} levels is not an epiphenomenon, as Ca^{2+} is needed to activate the RVD response. Therefore, it seems that Ca^{2+} is a pivotal signaling agent in the intracellular processes that allow for volume decrease following hypotonic challenge.

Similarities and differences between the volume regulatory responses of salmon and alligator

I observed both similarities and differences in the RVD mechanisms of the two cell types I used as models in my studies. The most apparent similarity was the dependence on extracellular Ca^{2+} for volume recovery following cell swelling; neither cell type was able to respond appropriately to hypotonic challenge in the absence of this Ca^{2+} source. This might reflect similarities in their RVD pathways, if Ca^{2+} is operating in a similar fashion in salmon and alligator cells. However, Ca^{2+} serves as an activator of a wide variety of cellular processes. Thus, although RVD in both cell types clearly relies on Ca^{2+}, it might be serving a different function in each species. In alligator cells, Ca^{2+} appears to stimulate arachidonic acid formation, which then leads to K^+ efflux. I was unable to study the signaling cascade downstream of Ca^{2+} influx in salmon cells, but future research could examine this topic. It would be interesting to determine whether RVD in salmon similarly occurs through a pathway mediated by arachidonic acid.

The most obvious difference between salmon and alligator RVD is the rate and extent by which the cell types are able to recover from cell swelling. Salmon cells displayed a robust RVD response, whereas volume decrease by alligator cells was much less pronounced. This difference might indicate that salmon erythrocytes possess a more highly tuned and efficient RVD mechanism. This might be physiologically relevant, as severe hypotonic shock is probably a much greater threat to the cells of marine teleosts, especially those cells that travel through the gills. In contrast, alligator cells are not directly exposed to the external environment. Instead, it is likely that their RVD mechanism is adapted to counteract milder and more gradual osmotic fluctuations, such as those that accompany altered metabolic activity and kidney function during hibernation. In other words, the differing lifestyles of these two organisms should be taken into account when comparing their RVD responses.

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