Calcium and Cell Volume Regulation in Alligator Mississippiensis Erythrocytes

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Summary

Maintenance of proper cell volume is critical for cell survival and proliferation; consequently, animal cells routinely control their volume to maintain homeostasis. For instance, biological processes such as the response to ischemia (insufficient blood flow), lymphocyte activation, and apoptosis (programmed cell death) are associated with changes in cell volume. It has been well established that osmotic swelling associated with a hypotonic medium is followed by a regulated volume decrease (RVD) resulting from the efflux of specific solutes. However, the signaling mechanisms of RVD are ill defined. The effect of calcium on RVD was studied because this ion is a common messenger that plays a key role in the maintenance of a stable intracellular environment. American alligator (Alligator mississippiensis) red blood cells' (RBCs) volume was measured electronically with a Coulter Counter® following hypotonic shock (0.5x Ringer). Studies using different extracellular concentrations of calcium, obtained with the chelator ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid (EGTA) (http://www.stanford.edu/~cpatton/maxc.html), showed that low extracellular calcium concentrations inhibited RVD. However, addition of the calcium ionophore A23187 in a normal Ringer caused a significant inhibition of RVD. Interestingly, substitution of the impermeant cation N-methyl-D-glucamine (NMDG) for sodium in the presence of A23187 also inhibited RVD, indicating that cell swelling was not dependent on sodium influx. Additional studies were also conducted to determine the mechanism(s) by which calcium ions cross the plasma membrane. A significant inhibition of RVD occurred in the presence of gadolinium, a blocker of stretch-activated channels. Other experiments with hexokinase (an ATP scavenger) suggested that calcium influx may occur via a purinoreceptor (P2 receptor). In conclusion, RVD by alligator RBCs depends on calcium influx via a stretch-activated channel and/or a P2 receptor.

Introduction

Cells are the basic building blocks of all living organisms. Although the cells of a eukaryotic organism are more complex than those of a prokaryote, all cells share a universal function of maintaining internal stability or homeostasis (Okada et al. 2001). Since living organisms are affected by their surroundings, fluctuations in the extracellular environment pose serious challenges to homeostasis. External changes in factors such as temperature, pH, pressure, and osmolality may present stress to cells and therefore, cells have developed important structures and mechanisms that help regulate their internal composition and physiology (Alberts et al. 2004). If a cell fails to maintain homeostasis it will become diseased and could eventually die.

One structure that is crucial for minimizing the internal fluctuations inside the cell is the plasma membrane. The plasma membrane is a phospholipid bilayer that separates the inside of the cell from the external environment (Alberts et al. 2004). The basic composition of the plasma membrane is a water insoluble hydrophobic region that is located within the membrane as well as a hydrophilic region that faces the aqueous solutions both on the inside and the outside of the cell (Yeagle 1989). The plasma membrane has several functions, such as giving the cell structure by anchoring to the cytoskeleton (Okada et al. 2001). However, the most important role of the plasma membrane is in controlling what goes in and out of the cell. That is, due to the amphipathic nature of the cell membrane, only lipid soluble molecules are able to pass freely into and out of the cell according to their concentration gradients. Ions and water soluble molecules are not able to easily cross a lipid membrane and thus there are other mechanisms for their transport (Alberts et al. 2004).

The plasma membrane is embedded with proteins, which account for almost 50% of its mass (Hooper et al. 1997). These proteins have many crucial functions. For example, some proteins work as anchors to help attach macromolecules on either side of the cell membrane. Others function as receptors and enzymes, recognizing chemicals and catalyzing reactions, respectively (Alberts et al. 2004). One of the most important functions of membrane proteins is transport of ions and water soluble molecules into and out of the cell (Hooper et al. 1997). Transport proteins form aqueous pores across the cell membrane shielding the charged and polar particles they are transporting through the lipophilic membrane. Based on function, some proteins are called ion channels because they only transport ions (Catterall 1992). Other proteins capable of facilitating membrane flux of polar molecules are termed "carriers" and they are the basis of facilitated diffusion (Singer & Nicolson 1972). Ion channels are extremely specific for a given solute and therefore require extensive regulation.

Since ion channels allow for passive travel across the cell membrane, they must be highly regulated to maintain homeostasis. There are 3 modes of ion channel regulation. Firstly, there are voltage-gated channels that open/close in response to a change in the membrane potential (Catterall 1992). Secondly, there are stretch-activated channels which are activated by mechanical stress or tension resulting in the distortion of the plasma membrane (Wellner et al. 1993). Finally, there are ligand-gated channels, which require the binding of a specific molecule with the channel protein to allow the pore to open (Alberts et al. 2004).

Ion channels facilitate the passive movement of ions down their concentration gradients (Carruthers,

*This author wrote the paper as a Senior thesis under the direction of Dr. Douglas Light.

1990). However, there are instances when it is necessary to transport ions and other molecules against their concentration gradients, a process termed active transport. Active transport requires the input of energy in the form of ATP, as well as protein pumps. Pumps are membrane proteins that change in conformation when a specific ligand and ATP are simultaneously bound. This binding allows for the transport of that ligand across the cell membrane against its concentration gradient (Singer & Nicolson 1972). Since these endergonic processes are coupled with the highly exergonic hydrolysis of ATP to ADP and inorganic phosphate, the overall reactions are favorable. An example of a pump is the proton pump in the inner mitochondrial membrane of all animal cells. This pump shuttles protons from the mitochondrial matrix into the intermembrane space against their concentration gradient. This is crucial since the energy derived from the passive return of the protons back into the mitochondrial matrix is used to generate ATP-the energy currency of all living organisms (Brand et al. 1999).

Thus, by being regulated and selective, transport proteins play an essential role in controlling the plasma membrane permeability to various molecules and ions, and thereby affect intracellular osmolality (the number of dissolved particles per kg H_2O). This is significant because the difference between the intracellular osmolality and the extracellular osmolality determines the osmotic gradient between the cell and its external environment. This gradient in turns dictates the osmotically obliged movement of water into and out of the cell. Therefore, solute transport across the cell membrane that is not compensated for will inadvertently influence the movement of water, which leads to the fluctuation of cellular volume. Even minor modifications in cellular volume may pose challenges to cell homeostasis.

Although the most common ways that cellular volume may change are due to modifications in the osmotic gradient established by solute transport, and by exposure to anisosmotic conditions, they are not the only way. Since there are many osmotically active molecules/ions within cells, cellular volume may change even under isosmotic conditions. This modification in cell volume is termed isosmotic volume change or IVC (O'Neill 1999). Isosmotic challenge to cellular volume results from changes in the number of solutes present inside the cell. This change can occur for various reasons such as metabolism, acidosis, and depolarization of the cell membrane potential (O'Neill 1999). For example, nerve cells can swell during the passage of action potentials, because neuronal activity leads to momentary changes in concentrations of K^+, Na^+, Cl^-, and Ca^{2+}, which alter neuronal cell volume (Strange 1992). The uptake of the amino acid glycine by Ehrlich ascites tumor cells, which is coupled to Na^+ influx, leads to a gradual increase of cellular volume (Hudson et al. 1988). Uptake of glucose and amino acids by intestinal cells after a meal causes swelling and cells of the nephron must compensate volume during urine formation as they selectively reabsorb and secrete solutes (Bankir et al. 1989). Cell volume also changes with protein degradation (increase in osmolality) or protein synthesis (decrease in osmolality) (Haussinger 1996). It is clear that cell volume modification may occur as a result of normal cellular processes.

No matter what the cause of cellular swelling or shrinking, osmotic stress modifies cellular functions. For instance, the swelling of rat liver cells increases protein synthesis as well as actin polymerization, but decreases the synthesis of urea, changing cellular metabolism (Haussinger 1996). Cell volume changes may also alter gene expression, which is often volume sensitive (Lang et al. 2000). For instance, cell shrinkage in Madine-Darby canine kidney cells induces transcription of the gene that codes for the betaine transporter increasing the activity of this transporter (Uchida et al. 1993). In addition, hypotonic swelling of rat hepatoma cells induces activation of a protein kinase Erk, which is followed by phosphorylation and auto-induction of the transcription factor c-Jun. This in turn leads to an increase in transcription of genes, such as MCP1, which codes for a protein responsible for recruiting monocytes to an area of injury (Schliess et al. 1995).

Although slight changes in the cell volume may be a way of regulating cellular activities, extreme changes in cell volume may inhibit proper functioning of the cell and can even result in its death. For example, the swelling of myocardial cells during ischemia lead to plasma membrane disruption, which may cause the cell to rupture (Steenbergen et al. 1985). Since volume modification clearly affects cellular operation, and may even prove detrimental, cells have developed ways of regulating it.

Cells have evolved several compensatory procedures to maintain a stable volume. These mechanisms; regulatory volume decrease (RVD) and regulatory volume increase (RVI), work to bring the cell volume back to its original size after the cell has been exposed to osmotic stress (Lang & Waldegger 1997). For instance, the extracellular environment may have a greater number of osmoles than the internal environment of the cell (hyperosmotic) or a smaller number of osmoles (hyposmotic). When the cell is in a hypertonic extracellular environment, water leaves the cell via osmosis down its concentration gradient, resulting in the shrinkage of the cell. Therefore, when the cell faces a hypertonic shock it must turn on an RVI type mechanism to increase the cell volume back to its original size. For example, intertidal organisms, such as starfish, often face variations in extracellular salinity with the coming and passing of the tide. If the extracellular environment is hypertonic to the intercellular environment of the starfish cell, the cell will rapidly shrink and an RVI mechanism will need to be activated to increase the cell volume (Vidolin et al. 2002). In contrast, in a hypotonic solution, water rapidly enters the cell requiring an RVD type mechanism to decrease the cell volume back to its size before the stress (Figure 1).

In order for RVD to take place, water must leave the cell even though there is a greater concentration of water on the outside of the cell. The cell accomplishes this by losing certain solutes, which forces water to follow out of the cell (Figure 1). For all cell types in which RVD has been studied, Cl^- and K^+ are the major ions that leave the cell and eventually lead to the efflux of water (Okada et al. 2001). Some organic molecules have also been implicated in facilitating the efflux of water, such as taurine, sorbitol, and betaine (Vitarella et al. 1994, Huxtable, et al. 1992, Hoffman et al. 2000, O'Neill 1999). Although the overall effect of RVD is the same in all animal cells, the specific signal transduction steps that lead to water efflux appears to vary among organisms and cell types. For instance, in response to hypotonic stress in human cervical cells a G-protein coupled receptor activates

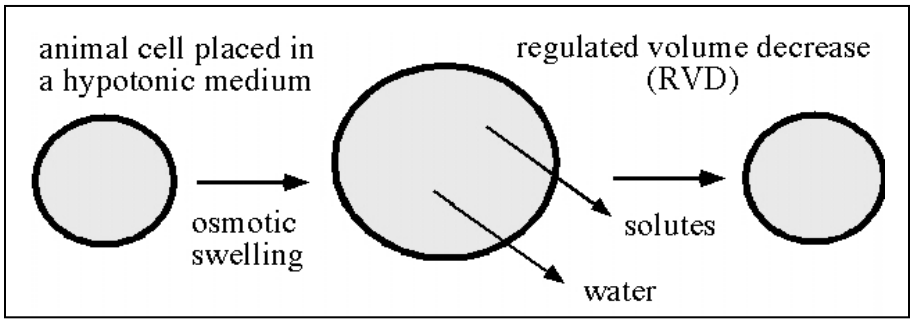

Figure 1. Regulatory volume decrease (RVD). When animal cells are exposed to a hypotonic medium, water rapidly enters the cell via osmosis. An RVD mechanism is a quick response initiated after cell swelling that results in efflux of selected ions such as K^+, Cl^-, organics, and others depending on the cell. Water passively follows the solutes out of the cell bringing the volume back to its original size (Lewis et al. 1990, Chan et al. 1992, Adragna et.al 2000, Garcia-Romeu et al. 1991).

phospholipase C (PLC) that in turn activates protein kinase C (PKC). This activation stimulates volume-sensitive Cl^- channels, which in conjunction with K^+, lead to efflux of water and the reduction of cell volume (Chou et al. 1998). However, volume reduction following swelling is brought about in a different manner in nonpigmented ciliary cells. Swelling in this case stimulates arachidonic acid turnover, triggering prostaglandin E (PGE)-mediated upregulation of K^+ channels. In addition, swelling reduces PKC activity and increases calcium/calmodulin (Ca^{2+}/CaM) activity, which both upregulate Cl^- channels. All together, these actions enhance KCl release from the cell reducing the cell volume (Civan et al. 1994). On the other hand, Necturus erythrocytes respond to hypotonic challenge by activating P_2 receptors. This leads to a rise in cytosolic Ca^{2+}, which stimulates volume decrease by activating a K^+ conductance (Light et al. 2002).

It is clear that the mechanism of RVD is complex and involves the coordination of several reactions to function properly. Although recent research has made progress in elucidating the processes that lead to RVD, the initial signal transduction steps still remain ill-defined. However, much attention has been focused on the role of calcium because it is a pivotal signaling agent in many physiological reactions. For example, upon fertilization of an oocyte, a rise in intracellular calcium brings about destruction of the cytostatic factors, leading to the breakdown of cyclin and progression into the second meiotic divison. This activates the egg and allows the zygote to begin cell division (Slack 2006). In addition, the release of Ca^{2+} by the sarcoplasmic reticulum causes tropomyosin to shift position allowing myosin heads to bind to the actin filaments, initiating a muscle contraction (Alberts et al. 2004). Further, Ca^{2+} evokes axonal exocytosis in neurons, which releases factors that effect other neurons, organs and/or muscles and possibly growth of the plasma membrane (Maletic-Savatic and Malinow 1998).

Since Ca^{2+} is a ubiquitous signaling agent, it was not a surprise when it was found that calcium also plays a role in RVD (Light et al. 2002). For example, the influx of Ca^{2+} increases the conductance of Cl^- via specific anion channels facilitating RVD in human epithelial cells (Shimizu et al. 2000). Frog kidney cells also require the presence of extracellular Ca^{2+} for the proper functioning of RVD (Mounfield and Robson 1998). In addition, Ca^{2+} allows for K^+ and Cl^- efflux in human astrocytes obliging water efflux that leads to RVD (O'Connor and Kimelberg 1993). Further, a certain amount of intracellular Ca^{2+} is required for the maintenance and regulation of human ciliary epithelial cell volume (Adorante and Cala 1995). Thus, much evidence indicates that Ca^{2+} plays an important role in RVD in many cells.

Nonetheless, some studies found that the presence of Ca^{2+} is not required for adequate RVD. For example, RVD in human lymphocytes is completely independent of the presence of extracellular Ca^{2+} (Grinstein and Smith 1990). In addition, although human nonpigmented epithelial cells require intercellular Ca^{2+} for activation of RVD, an increase in intercellular Ca^{2+} is not an absolute requirement for volume regulation (Adorante and Cala 1995). Further, osmotic swelling-induced changes in cytosolic Ca^{2+} in rat cultured suspended cerebellar astrocytes do not affect RVD in these cells (Morales-Mulia et al. 1998). Similarly, the efflux of solutes and subsequent RVD in cerebral granule neurons has been found to be Ca^{2+} independent (Moran et al. 1997). Thus, although it is clear that Ca^{2+} is a key player in RVD in many cell types, the precise involvement of this ion is not clearly known.

This study focused on elucidating the role Ca^{2+} plays in the RVD by alligator erythrocytes, as well as the transport mechanism(s) Ca^{2+} uses to enter these cells. The experiments that were conducted were designed to address the following questions: 1) Is Ca^{2+} influx necessary for proper RVD? I hypothesized that if Ca^{2+} influx during cell swelling is necessary for RVD, then blocking Ca^{2+} entry should inhibit RVD. However, if Ca^{2+} influx is not important for RVD or if Ca^{2+} is released from intracellular stores, then blocking Ca^{2+} influx would have no effect. 2) By what pathway(s) does Ca^{2+} enter the cells? Since RBCs are not highly excitable cells, it seemed unlikely that Ca^{2+} would enter the cell via voltage-gated channels. However, Ca^{2+} influx could occur via ligand-gated and/or stretch-activated channels. Further, if extracellular Ca^{2+} is required for proper RVD and enters via ligand-gated channels, then blocking those channels should inhibit RVD. An analogous scenario would occur in testing for the presence of stretch-activated Ca^{2+} channels.

There are several reasons why erythrocytes were chosen for this study. Firstly, it is convenient to study the osmotic fragility of red blood cells because it is easy to measure the amount of hemolysis of pigmented cells. Secondly, it is convenient to use RBCs to study RVD because these cells are not connected to

a substrate (as is the case for epithelial cells), facilitating the measurement of individual cell volumes. Previous studies in Dr. Light's lab used Atlantic salmon (Salmo salar) to examine RVD (see Abstract); however, the RBCs of this organism were no longer available. Therefore, using the Krogh Principle, I chose another model organism whose RBCs would be adapted to frequent volume changes (Alberts et al 2004).

I picked American alligator (Alligator mississippiensis) as my model organism because I assumed that RVD would be well developed in cells that are exposed to a variety of extracellular environments. That is, American alligators live in the southern region of North America and have been spotted in marshes, swamps, canals, as well as on land. Therefore, the osmolality of the extracellular fluid of alligators frequently changes simply due to their lifestyle. The kidneys and the salt glands of alligators are able to compensate for the osmolality changes under normal conditions; however, alligators hibernate annually which significantly decreases kidney functioning (Bankir et al.1989). Hibernation may result in a change in the osmolality in the extracellular fluid, which may lead to a modification in cellular volume (Zancanaro et al. 1999). Since these alligators are euryhaline (able to tolerate both freshwater and saltwater), as well as hibernate in the winter, I hypothesized that their RBCs have evolved a pronounced RVD to handle frequent volume changes.

I also hypothesized that alligator RBCs have a prominent RVD since these cells are nucleated (unlike mammalian RBCs). A nucleus allows for protein synthesis and degradation, which are activities that indirectly modify cellular osmolality (Lang & Waldegger 1997). This suggests that the RBCs of alligators may need to posses an RVD mechanism to maintain a stable volume.

Another reason to use alligator RBCs was dictated by the instrument that was used to measure individual cell volume which requires cells to be fairly large in size, compared to bacteria, for ease of measurement. Alligator RBCs are approximately four times the volume of human erythrocytes (82-98 fL) (Jensen et. al. 1998) (Figure 2). Therefore, the size of these cells made it easier to measure their volume. In addition, it was convenient to use a cold blooded animal because room temperature was physiological.

There are several clinical problems that may be better understood by examining RVD. For example, apoptosis, or programmed cell death, is cellular suicide that occurs as a result of severe cell damage or an external stimulus that signals the cell to die (Majno et al. 1995). For instance, in humans the death of interdigital cells during embryonic development occurs as a result of a brain stimulus to undergo apoptosis (Chautan et al. 1999). In fact, apoptosis is so important that if this mechanism is not functioning properly, uncontrolled cell growth arises, such as seen in cancer cells (Reed 1999). Apoptosis is initiated by a rise in intracellular Ca^{2+}, which results in the loss of solutes and water ultimately decreasing cell volume (an RVD-like mechanism) (Bortner & Cidlowski 2002). Therefore, a greater knowledge of RVD will allow us to better understand the mechanism of apoptosis. This in turn may potentially help in pharmaceutical research to create drugs that induce apoptosis and possibly help cancer patients.

Another clinical situation where RVD plays a role is ischemia, or insufficient blood delivery. Cells become ischemic by not receiving adequate amounts of blood and therefore become anoxic. Ischemia may occur as a result of either a blood clot that may block the flow of blood, or by a loss of blood vessel integrity, typically from an aneurism that hemorrages (Okada et al. 2001). As a result of anoxia, cellular metabolism is inhibited and the active process of sodium extrusion becomes inactivated (Leaf 1973). This leads to the accumulation of the sodium ion inside the cell, which osmotically draws water into the cell, resulting in cell swelling. Swollen cells may block the blood supply to an organ preventing the return of nutrient rich blood to a tissue. Therefore, swollen cells create a secondary vascular obstruction which prolongs the tissue ischemia and increases ultimate tissue injury (Leaf 1973). Consequently, if the process of RVD is better understood, cell swelling during ischemia may be prevented or the return to normal cell volume may be accelerated so as to decrease the damaging effects of ischemia.

Ischemia and apoptosis are only a few of the clinical applications where a better knowledge of RVD would prove beneficial. Fluid imbalance with other physiological conditions poses serious risks to all living organisms and therefore the regulatory mechanisms

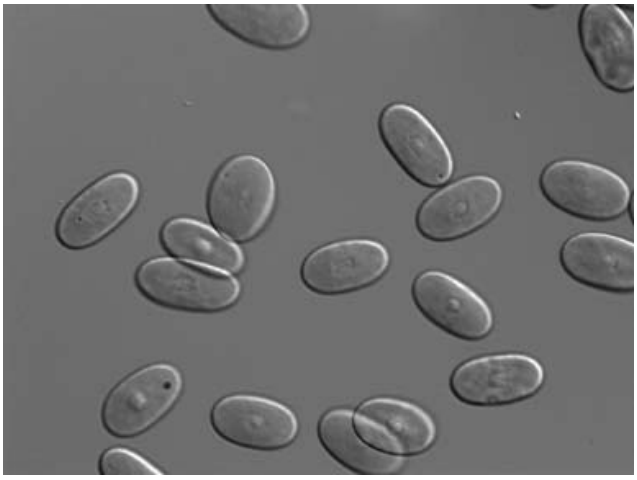

Figure 2. Alligator erythrocytes in isosmotic Ringer. 600x. Alligator erythrocytes are approximately 21 μm in length and 11 μm in width, oval in shape, and nucleated (Hartman & Lessler 1964).

that direct cell volume deserve attention and research. The purpose of this study was to examine the role of Ca^{2+} in RVD. I found that volume regulation in alligator RBCs did indeed depend on the extracellular Ca^{2+} concentration. Further, Ca^{2+} influx pathways were defined.

Materials and Methods

Experimental Design

My initial goal was to determine whether American alligator RBCs were indeed useful for the study of RVD. To accomplish this, I conducted osmotic fragility studies that allowed me to evaluate the ability of the RBCs to withstand osmotic stress when exposed to hypotonic solutions of various osmolalities. Using a spectrophotometer to determine the optical density of the cells in different hypotonic media, I was able to determine the percent hemolysis of a suspension of RBCs.

If the results of the above study showed that alligator RBCs are able to withstand significant osmotic challenge, then I would assume that these cells have a prominent RVD response. Accordingly, the main focus of my study would then be to determine whether Ca^{2+} plays a key role in RVD. To accomplish this, I would test whether the concentration of extracellular Ca^{2+} affects RVD and precisely what role Ca^{2+} plays in RVD. I hypothesized that if the source of Ca^{2+} was extracellular, then buffering the amount of Ca^{2+} present in the extracellular solution would modify the cells' RVD response. I studied the effect of decreasing the amount of extracellular Ca^{2+} on RVD by using a Ca^{2+} chelator ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetracetic acid (EGTA) (Zanoti and Charles 1987). If decreasing the amount of Ca^{2+} present in the extracellular solution inhibited RVD, then I would conclude that Ca^{2+} plays an important role in RVD and that the source of Ca^{2+} is extracellular. In contrast, if lowering extracellular Ca^{2+} had no effect on RVD, I would then conclude that extracellular Ca^{2+} does not play an important role in the RVD mechanism.

If the result of the above studies confirmed my hypothesis that Ca^{2+} plays a role in RVD and that low extracellular Ca^{2+} inhibited RVD, I would next examine whether a surplus of Ca^{2+} would potentiate RVD. I would test this by using a Ca^{2+} ionophore A23187. This ionophore makes specific Ca^{2+} channels in the plasma membrane of the RBCs to increase the permeability of the membrane to this ion (Palant et al. 1983). I hypothesized that if low Ca^{2+} inhibits RVD, then allowing for greater Ca^{2+} influx would improve volume regulation. However, if the Ca^{2+} ionophore A23187 inhibits RVD, then I would assume that Ca^{2+} affects RVD in a dose dependent manner.

If the result of the above study suggested that Ca^{2+} ionophore A23187 inhibits RVD, I would next want to examine how a surplus of Ca^{2+} is antagonistic to volume regulation. I hypothesized that if extra Ca^{2+} influx during hypotonic shock opens Na^+ channels, Na^+ would enter the cell and the osmotically obliged water would follow. I tested this hypothesis by replacing extracellular Na^+ with N-methyl-D-glucamine (NMDG), which is a monovalent cation that is too large to pass through Na^+ transport pathways (Kerschbaum & Cahalan, 1998). Consequently, if extra Ca^{2+} influx into the cell (induced by A23187) makes the cell swell by opening Na^+ channels, replacing Na^+ with NMDG would inhibit this process, and as a result, RBCs will not swell after A23187. In contrast, if in the absence of Na^+, the RBCs still swell, then extra Ca^{2+} influx inhibits RVD by a mechanism(s) other than allowing for Na^+ influx. This study would help elucidate the mechanism by which RVD was inhibited with increased Ca^{2+} influx.

If the results of above studies supported my hypothesis that Ca^{2+} plays a role in RVD, I next would study which pathway(s) are used by Ca^{2+} to enter the RBCs. One of the ways that Ca^{2+} has been known to enter cells after hypotonic shock is via stretch-activated channels (Hoffman, 2000). For example, in human bronchial epithelial cells, K^+ efflux results from Ca^{2+} influx via stretch-activated channels (Fernandez-Fernandez et al. 2002). Also, RVD in *Necturus* erythrocytes requires the entry of extracellular Ca^{2+} via stretch-activated channels (Light, et al. 2002). Similarly, Ca^{2+} enters via stretch-activated channels in rat lacrimal acinar cells during RVD (Speake, et al. 1998). Therefore to test my hypothesis that Ca^{2+} enters via a stretch-activated channel in alligator RBCs, I used an inhibitor of stretch-activated Ca^{2+} channels gadolinium (Boudreault & Grygoyczyk 2001). If Ca^{2+} does enter via these channels, this experimental result should mimic the result of bathing the cells in a low Ca^{2+} medium (in EGTA). However, if Ca^{2+} does not enter via stretch-activated channels, gadolinium should have no effect on RVD.

Although gadolinium is an effective stretch-activated Ca^{2+} channel blocker, some studies show that it might also block ATP-gated purinoreceptor channels (P_2 receptors) (Nakazawa et al. 1997). A P_2 receptor is a ligand-gated channel which requires the binding of extracellular ATP in order to function (Weisman et al. 1996). Several cell types exhibit Ca^{2+} influx during RVD through P_2 receptors. For example, *Necturus* erythrocytes initiate the RVD response by allowing Ca^{2+} to enter via P_2 receptors, which induces K^+ efflux (Light et al. 1999). The efflux of amino acids, which results in volume recovery in cultured astrocytes, is also dependent on Ca^{2+} entry via a P_2 receptor (Jeremic et al. 2001). In addition, Ca^{2+} is transported into retinal pigment epithelial cells through P_2 receptors allowing for volume regulation in the subretinal space (Peterson et al. 1997). Therefore, I wanted to examine whether Ca^{2+} enters via P_2 receptors in alligator RBCs during RVD.

I tested the hypothesis that Ca^{2+} enters the RBCs via P_2 channels by blocking these channels indirectly with hexokinase and observing that effect on RVD. Hexokinase is an enzyme that cleaves the terminal phosphate off of ATP and transfers it onto a glucose molecule (Dubyak 2002). Therefore, by using hexokinase, I would successfully deplete ATP present in the extracellular solution. Since P_2 receptors do not operate without extracellular ATP, if Ca^{2+} did enter the cell via a P_2 receptor, its transport into the cell would be inhibited with hexokinase. Consequently, if Ca^{2+} does enter via P_2 receptors then blocking this pathway should mimic the EGTA experiments. However, if Ca^{2+} does not enter via P_2 receptors, no effect on RVD would be observed with the addition of hexokinase.

If the experiments above indicate that Ca^{2+} enters RBCs both via stretch-activated channels and P_2 receptors, I would test whether the effect of combining these inhibitors is additive. I predicted that if Ca^{2+} enters the RBCs both via stretch-activated channels and P_2 receptors, and Ca^{2+} is necessary for RVD, then applying both of these inhibitors at once would severely limit or altogether inhibit RVD. However, if an additive effect is not observed, I would conclude that the RVD of

Table 1. Pharmacological agents used in this study.

Agent	Function	Predicted Effect
A23187	Ca2+ ionophore	Enhancement of Ca2+ influx stimulates RVD
EGTA	Extracellular Ca2+ chelator	Inhibition if of RVD if Ca2+ is necessary for this process
NMDG	A nonpermeant substitute for sodium ion	Enhancement of RVD if Na+ entry occurs as a consequence of surplus Ca2+ influx with A23187
Gadolinium	Stretch-activated Ca2+ channel inhibitor	Inhibition of RVD if Ca2+ entry occurs via a stretch-activated channel
Hexokinase	ATP scavenger	Inhibition of RVD if Ca2+ entry occurs via a P2 receptor

alligator erythrocytes depends on Ca^{2+} in a dose dependent manner and therefore blocking too much Ca^{2+} may have an effect that is different then predicted.

A list of the pharmacological agents, as well as their functions, used in my studies are shown in Table 1. In addition, the predicted effects of these pharmacological agents based on my hypotheses are also given.

Cells

Alligator mississippiensis (American alligator) whole blood in Alsevers anticoagulant was obtained from Carolina Biological Supply (Burlington, NC). Each sample of blood was obtained from a single adult alligator. The blood was stored at 4°C, inverted daily, and used within one month of delivery.

Osmotic Fragility

Osmotic fragility was assessed using a Spectrophotometer (Milton Roy Spectronic 21D). When a suspension of RBCs lyse, hemoglobin is released into the solution making it become clearer, thereby lowering its absorbance (optical density). Therefore, the osmotic fragility of RBCs in different solutions can be determined via light absorbance because it correlates with the number of cells that lysed. A wavelength of 620 nM was used because it provided the greatest optical density difference between intact and lysed cells. The red blood cells were incubated in Ringers of different osmolarities for 15 minutes prior to measuring the optical density. In order to assess osmotic fragility, the hemolytic index was calculated from: $[(OD_{test}-OD_{negative\ control})-(OD_{positive\ control}-OD_{negative\ control})]*100\%$, where the OD_{test} is the average optical density of the experimental cells, $OD_{negative\ control}$ is the average optical density of the control cells, and $OD_{positive\ control}$ is the optical density of cells in nanopure water ($OD_{positive\ control}=0$) (adapted from Light et al. 2002).

Cell Volume Measurements

Median cell volume was measured with a Coulter Counter® Z2™ with Channelyzer, fitted with a 100 µM diameter aperture. This apparatus measures cell volume electronically from changes in resistance (Ohm's Law) as cells pass through an aperture. A metering volume of 0.5 mL was used and whole blood was diluted 10,000x with alligator Ringer to obtain 15,000-40,000 RBCs per mL. Because the ratio of white blood cells to red blood cells was approximately 1:1000 (www.hsl.creighton.edu), we ignored the contribution of the white blood cells to cell volume. Time 0 was when the cells were added to a test solution. Cell volume measurements were taken every minute for the first 5 minutes following hypotonic shock and then volume was measured at 10 minute intervals.

Relative volume was determined by dividing the median cell volume for that day by the volume measurement of cells in isosmotic Ringer. This measure allowed me to assess the degree of swelling and recovery of the cells following hypotonic shock in comparison to basal conditions. Percent volume recovery was calculated by a formula adapted from Light et al. (1999) $[(V_{max}-V_x)/(V_{max}-V_0)]$ x100%, where V_{max} is the relative volume of control cells at maximum swelling (t=1 min for this study), V_x is the relative volume of control or experimental cells at time x, and V_0=basal volume (t=0 min). The percent recovery was calculated by the following formula (Light et al. 1999): [(% Recovery Experimental/ % Recovery Control) x100%]. In order to determine the percent swelling of cells in various extracellular calcium concentrations, the following formula was used: $[(V_{exp}-V_{control})/V_{control})]$ x100% where V_{exp} is the volume of experimental cells at maximum swelling (t=1min) and $V_{control}$ is the volume of control cells at maximum swelling (t=1min).

Osmolarity and pH Measurements

Osmolarity was measured using a Wescor 5500 Vapor Pressure Osmometer (SD +/-2 mmol/kg H_2O). The osmometer was standardized using Opti-Mole known standards of 100 +/- 2 mmol/kg H_2O, 290 +/- 2mmol/kg H_2O, and 1000 +/- 2mmol/kg H_2O. Accumet ® Basic AB15 pH Meter from Fisher Scientific was used to measure the pH of solutions. The pH meter was standardized using Orion pH Buffers of known pH: pH4, pH 7, and pH 10. All pH measurements were taken at room temperature (21-23°C). The acceptable pH for all Ringer solutions was pH 7.35-7.45, and 1N HCl and 1N NaOH were used to achieve this pH if the Ringer pH fell outside this range.

Solutions and Reagents

Ringer solutions were made to mimic alligator plasma. The osmolarity of alligator blood was measured to be 320 mOsm/kg H_2O. The isosmotic Ringer consisted of (in mM): 150 NaCl, 6.0 KCl, 6.0 $CaCl_2$, 1.5 $MgCl_2$, 4.55 Hepes base, 5.45 Hepes acid (310+/- 5 mOsm/kg H_2O). All solutions had 5.5 mM glucose in order to provide an energy source for the cells once they were taken out of plasma and 1 mM sodium bicarbonate to help clear up any lysed cell residue (Hoar & Hickman 1983).

The hypotonic (0.5x) Ringer contained 70 mM NaCl and all other ingredients were the same as in the isosmotic Ringer (165 +/-10 mOsm/kg H_2O).

Table 2. EGTA and Trizma base Concentrations for Ringers with Varying $[Ca^{2+}]_o$.*

Free Ca2+ (μM)	EGTA (M)	Trizma base (M)
Cell Volume Experiment		
0.01	4.36x10-3	1.74x10-2
0.1	8.94x10-4	3.58x10-3
0.5	5.78x10-4	2.31x10-3
1	5.35x10-4	2.14x10-3
5	5.03x10-4	2.01x10-3
10	4.00x10-4	1.6x10-3
500	0	0
Osmotic Fragility Experiment		
0.1	2.48x10-4	9.92x10-4
1	1.52x10-4	6.08x-4
150	0	0

*Based on Maxchelator 2004 calculations (http://www.stanford.edu/~cpatton/maxc.html)

Hypotonic EGTA Ringer for the cell volume experiment was made using the same ingredients as the regular hypotonic Ringer with the exception of $CaCl_2$. The EGTA Ringer contained 0.5 mM $CaCl_2$ in order to adequately chelate the extracellular Ca^{2+}. For the osmotic fragility studies, the same reagents were used as in the hypotonic Ringer, except instead of 6.0 mM $CaCl_2$, 150 μM $CaCl_2$ was added. The required concentration of EGTA was determined according to the desired value of free calcium using MaxChelator

downloads (http://maxchelator.stanford.edu/). Approximately four times as much Trizma base (M) as EGTA was added to the solutions in order to maintain proper pH (Table 2). The hypotonic sodium-free N-methyl-D-glucamine (NMDG) Ringer was prepared by adding (in mM): 66 NMDG, 66 HCl, 7.75 Hepes acid,

2.25 Hepes base, 2 Trizma base. The rest of the ingredients were the same as in the hypotonic Ringer.

Chemicals were obtained from Sigma Chemical Co. (St.Louis, MO), MP Biomedicals, Inc (Aurora, OH), or Alexis Corporation (San Diego, CA). Stock solutions were stored for a maximum of 3 days and all experiments were carried out at room temperature (21-23°C). Aqueous stock solutions were diluted 100x, whereas non-aqueous solutions (in ethanol, methanol, or DMSO) were diluted 1000x to achieve a final working concentration.

Data Analysis

Either a paired *t*-test or an ANOVA/ MANOVA were used, as appropriate (Data Desk Software Ithaca, NY). For cell volume experiments, variance was determined for the first (t=1 min) and the last (t=40 min) volume readings. The results were considered significant for $P \leq 0.05$.

Results

I first tested my hypothesis that alligator RBCs have a well-developed RVD response by conducting osmotic fragility studies. To accomplish this, I challenged the cells with hypotonic extracellular solutions of various concentrations, ranging from 0 Osm/kg H_2O (nanopure water) to 295 Osm/kg H_2O in order to determine the concentration that resulted in 50% hemolysis. This concentration was important to determine because, if used for my studies, I could determine whether an experimental manipulation either increased or decreased the level of hemolysis. The optical density of the samples was examined with a spectrophotometer to assess percent cell lysis. An exponential equation (x^5) was obtained from plotting osmolality (Osm/kg H_2O) vs. percent hemolysis using Microsoft Excel software. With this equation, I found that 50% lysis occurred at 84 Osm/kg H_2O, or 26.25% of the alligator blood plasma (Fig. 3).

After determining that alligator RBCs are able to withstand significant hypotonic shock and the concentration causing 50% hemolysis, I next examined whether RVD depends on extracellular Ca^{2+}. This was accomplished by observing the effect of low extracellular Ca^{2+} on the amount of osmotic swelling of RBCs upon exposure to hypotonic shock. I hypothesized that if swelling is increased with low extracellular Ca^{2+}, then Ca^{2+} influx is important for volume regulation. However, if low extracellular Ca^{2+} had no effect on cell swelling, then I would conclude that Ca^{2+} influx is not necessary for RVD. A Ca^{2+} chelator, EGTA, was used to achieve low extracellular concentrations of Ca^{2+} ranging from 0.01 μM to 500 μM (refer to methods). For all concentrations above 0.01 μM Ca^{2+}, cells swelled more than the control, which contained 6 mM Ca^{2+} (Fig. 4). Surprisingly, cells in a 0.01 μM Ca^{2+} solution swelled less than the control cells.

Given that hypotonic Ringer with low extracellular Ca^{2+} increased the amount of swelling for all concentrations above 0.01 μM, I next determined whether cells had an impaired RVD under these conditions. This was assessed by calculating the percent volume recovery after 40 minutes (the time of maximal recovery) for cells bathed in hypotonic solutions (0.5x Ringer) with various extracellular concentrations of Ca^{2+}. I hypothesized that because the RBCs swelled more in low extracellular Ca^{2+}, they should not be able to recover as extensively as the control cells under these conditions. In fact, a similar pattern was seen for the percent recovery of the RBCs

as was observed for the percent swelling. That is, for all concentrations of Ca^{2+} above 0.01 μM, RBCs recovered less than the control. In addition, the cells bathed in a hypotonic solution with 0.01 μM Ca^{2+} recovered better than control, exhibiting improved RVD (Fig. 5).

Observing that RVD was inhibited for all low extracellular Ca^{2+} concentrations above 0.01 μM, I examined whether Ca^{2+} affected RVD in a dose dependent manner. I predicted that as the extracellular Ca^{2+} in the Ringer was lowered (chelated by EGTA), concomitantly the percent volume recovery also would be decreased. To observe this, I plotted the percent volume recovery at each low extracellular Ca^{2+} concentration divided by the percent volume recovery of its control. Although the percent volume recovery of cells in 0.01 mM Ca^{2+} was statistically different from all other concentrations of Ca^{2+}, no significant trend was observed (Fig. 6).

Knowing that low levels of extracellular Ca^{2+} inhibited RVD, I hypothesized that high levels of Ca^{2+} should have the opposite affect. To examine this, I used the calcium ionophore A23187, which creates Ca^{2+} specific pores in the plasma membrane, allowing $Ca^{2+}$to enter the cell down its concentration gradient. Since, the hypotonic Ringer contained 6 mM Ca^{2+}, A23187 (1 μM) should allow for approximately 6 mM Ca^{2+} to enter the cell (presumably much more than would enter during normal RVD because generally Ca^{2+} influx pathways quickly inactivate). Surprisingly, the cells in hypotonic (0.5x) Ringer with A23187 exhibited a dramatic inhibition of RVD compared to the control cells (Fig. 7A, $P<0.05$ at 40 min). The percent volume recovery for cells exposed to A23187 Ringer is shown in Figure 7B ($P<0.05$ at 40 min and at 90 min).

The inhibition of RVD with the Ca^{2+} ionophore was not expected. To explain this observation, I hypothesized that Ca^{2+} influx stimulated the influx of another ion, forcing osmotically obliged water to follow, thereby leading to cell swelling. Given Na^+ is the most abundant extracellular cation, I predicted that A23187 induced Na^+ influx. To test this, I replaced extracellular Na^+ with *N*-methyl-D-glucamine (NMDG), a molecule that cannot enter via Na^+ channels (Kerschbaum and Cahalan, 1998). Under these conditions, I expected an adequate RVD to take place assuming supraphysiological Ca^{2+} influx stimulates the opening of Na^+ channels. Unexpectedly, the substitution of NMDG for Na^+ did not reverse the effect of A23187. In fact, cells exposed to NMDG and A23187 failed to display and RVD response (Figure 8A + 8B, $P<0.05$ at 40 min as compared to control).

Since my previous experiments showed that Ca^{2+} had a significant effect on RVD, I decided to examine how Ca^{2+} entered the cells. One of the ways that Ca^{2+} is known to enter cells following hypotonic shock is via stretch-activated channels (Fernandez-Fernandez et al. 2002, Speake et al. 1998, Light et al. 2002). In order to test for the presence of these channels, I used gadolinium (50 μM), a known antagonist of stretch-activated Ca^{2+} channels (Boudreault et al. 2001). Gadolinium significantly inhibited RVD over virtually the entire 40 minutes of observation (Fig. 9A, $P< 0.05$ at t=40 min as compared to control). The inhibition of percent volume recovery for cells exposed to gadolinium is shown in Figure 9B ($P< 0.05$ at 40 min). Although gadolinium is a known stretch-activated Ca^{2+} channel blocker, some studies suggest that it might also block purinoreceptor/channels (P_2 receptors) (Nakazawa et al. 1997). This is significant to my studies since Ca^{2+} has been shown to enter various cell types during RVD through P_2 receptors (Light et al. 1999, Jeremic et al. 2001, Peterson et al. 1997). For this aspect of my study, I used hexokinase (2.5 U/mL H_2O) to block P_2 receptors, an enzyme that cleaves the terminal phosphate off of ATP and transfers it to glucose (Dubyak, 2002). Therefore, by using hexokinase, it was assured that there would be insignificant free ATP in the extracellular solution thereby preventing the activation of P_2 receptors. I found that the cells in hypotonic Ringer with hexokinase exhibited an inhibition of RVD compared to control cells (Fig. 10A, $P<0.05$ at 40 min). Percent volume recovery was also reduced in cells exposed to hexokinase (Fig.10B, $P<0.05$).

Because both gadolinium and hexokinase inhibited RVD, I hypothesized that either both agents blocked RVD by inhibiting P2 receptors or, alternately, both P2 receptors and stretch-activated channels are present in this system. To help distinguish between these 2 possibilities, I determined the affect of using both inhibitors together. This would provide insight as to whether these inhibitors block the same pathway of Ca2+ entry or two different pathways depending on whether their effects are additive. That is, a non-additive affect would suggest that both agents blocked P2 receptors, whereas an additive response would imply that each agent blocks a distinct or separate pathway. Contrary to what I hypothesized, the cells exposed to gadolinium and hexokinase together actually had a better RVD response compared to gadolinium and hexokinase alone (Fig. 11A + 11B).

Discussion

Osmotic Fragility of Alligator RBCs

The findings of the osmotic fragility experiments confirmed my hypothesis that alligator RBCs are highly resistant to osmotic stress (Figure 3). That is, when the RBCs were bathed in hypotonic solutions of various osmolalities, 50% hemolysis was observed in cells exposed to 84 mOsm/kg H_2O Ringer, which is about 26% of alligator blood plasma. This indicates that alligator RBCs are able to resists a much stronger osmotic gradient before lysis compared to mammalian RBCs. For example, Sprague-Dawley rat RBCs lyse in about 50% blood plasma (Aldrich et al. 2006) as well as humans (Light, personal comm.). My results are also consistent with the findings of Aldrich et al. (2006) who found that a decrease of about 70% of blood plasma osmolality is required to cause 50% hemolysis in ornate box turtle, a reptile with nucleated RBCs. Light et al. (2002) also found that amphibian RBCs, which are nucleated, are very resistant to osmotic stress, 50% hemolysis in *Necturus* occurs in hypotonic Ringer, about 8 times more dilute than normal plasma. Since alligators live in diverse environments and are able to hibernate in the winter, it appears these ectothermic reptiles in particular, and other reptiles and amphibians in general, have evolved RBCs that are highly resistant to osmotic stress.

Alligator RBCs also may be able to withstand significant hypotonic shock because of anatomical and physiological reasons. For instance, alligator RBCs are nucleated, and a nucleus provides extra support for the cell membrane. In other words, the nucleus in RBCs is anchored in place by intermediate filaments that run between the nucleus and the cytoplasm and have linkages to the plasma membrane of RBCs (Centonze et al. 1986). This arrangement provides extra support allowing the cells to resist lysis under significant

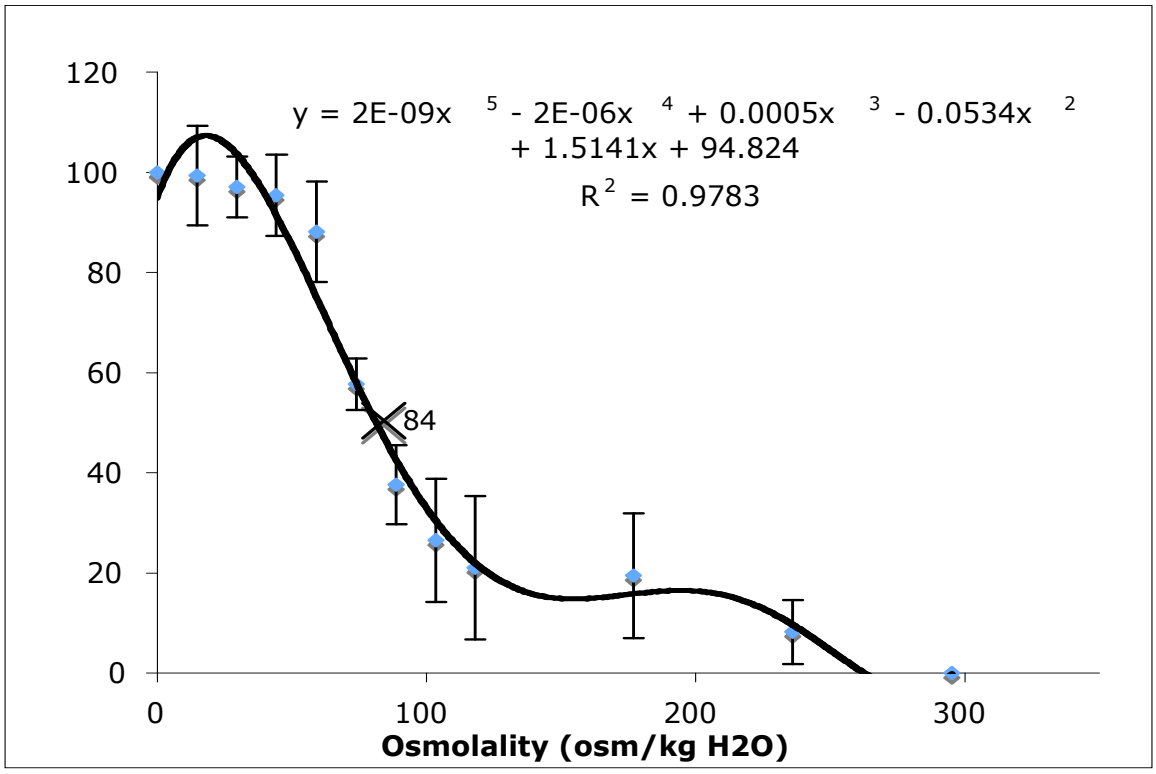

Figure 3. Percent hemolysis of alligator RBCs. Cells were exposed to hypotonic Ringer ranging from 0% to 100% of isosmotic conditions (n=7 for each concentration). Percent hemolysis was calculated from the spectrophotometer absorbance by using the Hemolytic Index equation (refer to methods). Fifty percent hemolysis occurred at 84 Osm/kg H_2O.

osmotic stress (Hagerstrand et al. 1999). It is also possible that alligator RBCs have a high osmotic resistance due to their large size and volume. Aldrich et al. (2006) suggest that larger RBCs may be more osmotically resistant based on LaPlace's law, which states that wall stress (Ws) is equal to pressure (P) times the cell radius (Rc) divided by wall thickness (Wt) (Ws=P x Rc/Wt). Therefore, under equal pressure a larger cell (bigger radius) would require stronger wall strength than a smaller cell (Aldrich et al. 2006). A stronger wall or plasma membrane would presumably provide a more osmotically resistant cell.

Role of Ca^{2+} in RVD

The most significant finding of this study is that extracellular Ca^{2+} plays a role in RVD in alligator RBCs. Cells that were bathed in low extracellular Ca^{2+} concentrations above 0.01 µM (obtained using EGTA) swelled significantly more than control cells (Figure 4). This indicates that extracellular Ca^{2+} is crucial for signaling in RVD, which ensures that RBCs only swell to a certain degree before they begin to decrease their volume back to its original size. This finding is in agreement with Goldberg and Choi (1993) who found that the removal of Ca^{2+} from the extracellular medium led to neuronal swelling following ischemic conditions of low oxygen and low glucose. Contrary to this finding, Liu et al. (2005) found that the removal of Ca^{2+} from the extracellular solution does not affect cell volume.

In addition, cells bathed in low extracellular Ca^{2+} concentrations (above 0.01 µM) recovered less than control cells during RVD (Figures 5 and 6). That is, an adequate amount of Ca^{2+} must be present in the extracellular medium for RBCs to have a proper RVD response. The finding that RBCs are not able to recover in low Ca^{2+} Ringers also indicates that the primary source of Ca^{2+} needed for RVD is extracellular. In other words, even if some Ca^{2+} is released from intracellular stores during RVD, it was not sufficient to stimulate a full volume recovery. Research in several cell types suggests that one of the effects of hypotonic shock is a rapid increase in intracellular Ca^{2+} due to the influx of Ca^{2+} from the external medium. O'Connor and Kimelberg (1993) found that the primary source of Ca^{2+} required for RVD in astrocytes is in the extracellular solution. Volume regulation in response to hypotonic shock in tilapia pituitary cells is also dependent on the influx of Ca^{2+} from the extracellular environment (Seale et al. 2002). In low Ca2+ hypotonic Ringer (chelated by EGTA) these cells failed to recover (Seale et al. 2002).

The conclusion that extracellular Ca^{2+} is critical for RVD is in agreement with a study by Davis and Finn (1987) who found a similar result in frog urinary bladder cells. Cells bathed in a Ca^{2+}-free hypotonic medium did not undergo RVD. McCarty and O'Neil (1992) also found a similar dependence on extracellular Ca^{2+} in the RVD by renal proximal tubule cells. In addition, the RVD in human osteopblast-like cells is dependent on the influx of extracellular Ca^{2+}, which stimulates RVD by opening K^+ channels (Weskamp et al. 2004). However, contrary to my findings, Tinel et al. (2000) found that extracellular Ca^{2+} does not play a role in rat inner medullary collecting duct cells' RVD, but Ca^{2+} released from intracellular

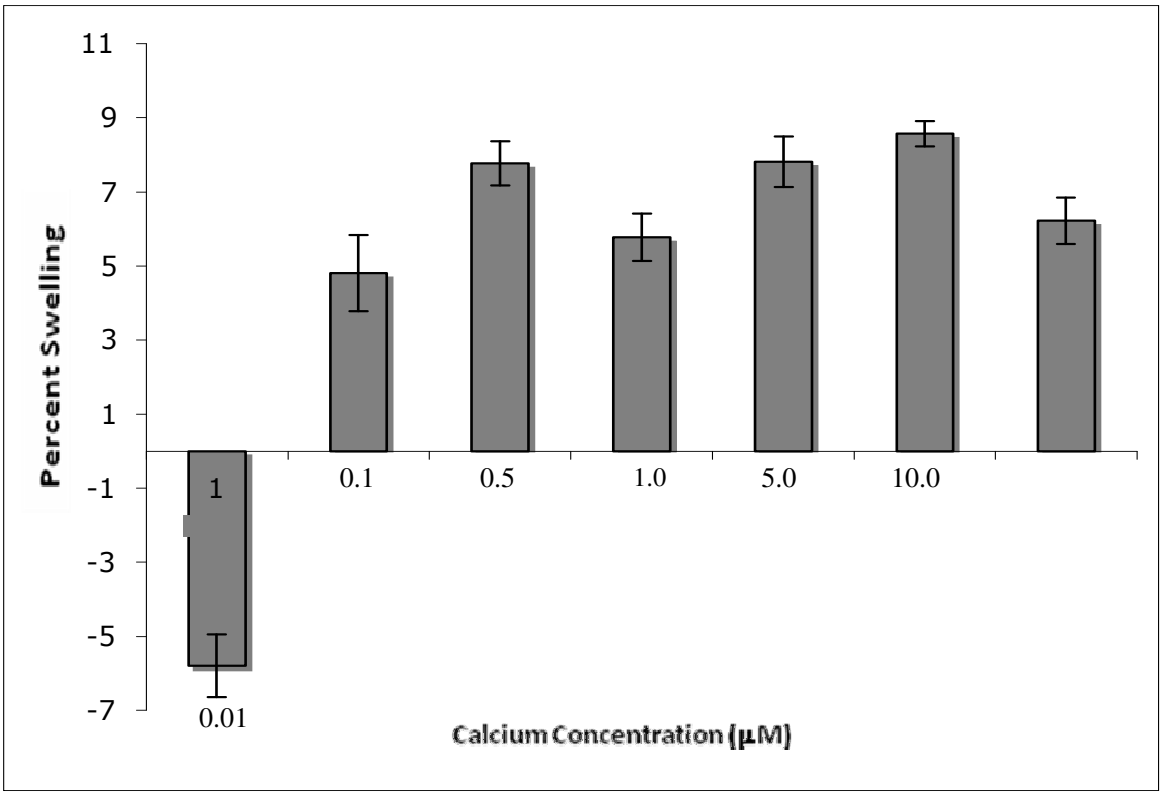

Figure 4. Red blood cells swell more in hypotonic Ringer when exposed to low Ca^{2+}. Percent swelling was obtained by dividing the relative volume of cells at t=1 min by the relative volume under basal conditions (see methods). For all concentrations of extracellular Ca^{2+} above 0.01 μM, RBCs swelled more that control (6 mM Ca^{2+}) at one minute following hypotonic shock (time when cell were maximally swollen). In contrast, cells in 0.01 μM Ca^{2+} solution swelled less than the control at one minute.

stores is necessary for proper RVD. The RVD of cerebral granule neurons is also independent on extracellular Ca^{2+} (Moran et al. 1997). Yellowley et al. (2002) found that both extracellular Ca^{2+} influx and Ca^{2+} released from intracellular stores play a role in RVD in bovine articular chondrocytes. Therefore, it is probable that the role Ca^{2+} plays in cell volume regulation depends on the species and/or cell type.

These findings are in agreement with other research on RVD that suggests that Ca^{2+} ion plays an important role in RVD in different animal cells (Mournfield et al. 1998, Shimizu et al. 2000, Light et al. 2002). Contrary to my findings, other studies show that Ca^{2+} is not necessary for adequate RVD (Grinstein et al. 1990, Adorante et al. 1995). It is clear that different cell types rely on various mechanisms for osmoregulation which may or may not include the participation of Ca^{2+}.

Interestingly, my data suggest that at very low extracellular Ca^{2+} concentrations, alligator RBCs both swell less than the control (Figure 4) and recover more after hypotonic shock (Figures 5 and 6). That is, RBCs bathed in 0.01 μM Ca^{2+} were able to withstand osmotic challenge better than control cells. This finding was in contrast to Adorante et al. (1995) who showed that a reduction of calcium to less than 0.01μM inhibited RVD in ciliary epithelium of human NPE cells.

This puzzling finding suggests a dose dependent effect of extracellular Ca^{2+} on RVD in alligator RBCs. Since extracellular Ca^{2+} is obviously important for RVD, I propose that the cell activates another mechanism to increase the internal Ca^{2+} concentrations in the situation when not enough Ca^{2+} is present. I suggest that for all concentrations of Ca^{2+} above 0.01 μM, there is enough extracellular Ca^{2+} for some RVD, although it is reduced compared to control. Yet, for extracellular concentrations of Ca^{2+} less than 0.01 μM, RVD was improved, compared to all other low Ca^{2+} concentrations. In this case, it is possible that RBCs released Ca^{2+} from intracellular stores for RVD, which has been shown by others for different species. Zanotti and Charles (1997) discovered that glial cells respond to a low Ca^{2+} external medium by activating the release of Ca^{2+} from intracellular stores. They suggest that low $[Ca^{2+}]_o$ -evoked Ca^{2+} signaling may occur in response to a decrease in $[Ca^{2+}]_i$. This is because a lower Ca^{2+} concentration in the outside medium may induce more Ca^{2+} to leave the cell, thereby decreasing the concentration of Ca^{2+} within the cell. This drop in $[Ca^{2+}]_i$ in turn may cause the subsequent formation of inositol phosphate 3 (IP_3), which would allow for the release of Ca^{2+} from intracellular stores (Zanoti and Charles, 1997). Therefore, pharmacologically low concentrations of Ca^{2+} in the extracellular medium may have induced Ca^{2+} to be released from intracellular stores in alligator cells, which in turn activated RVD (Fig. 12).

The idea that alligator RBCs may use one mechanism of RVD during physiological swelling and a different one during hypotonic shock in very low external Ca^{2+} is not novel. For example, rat somatotroph cells have two distinct RVD mechanisms,

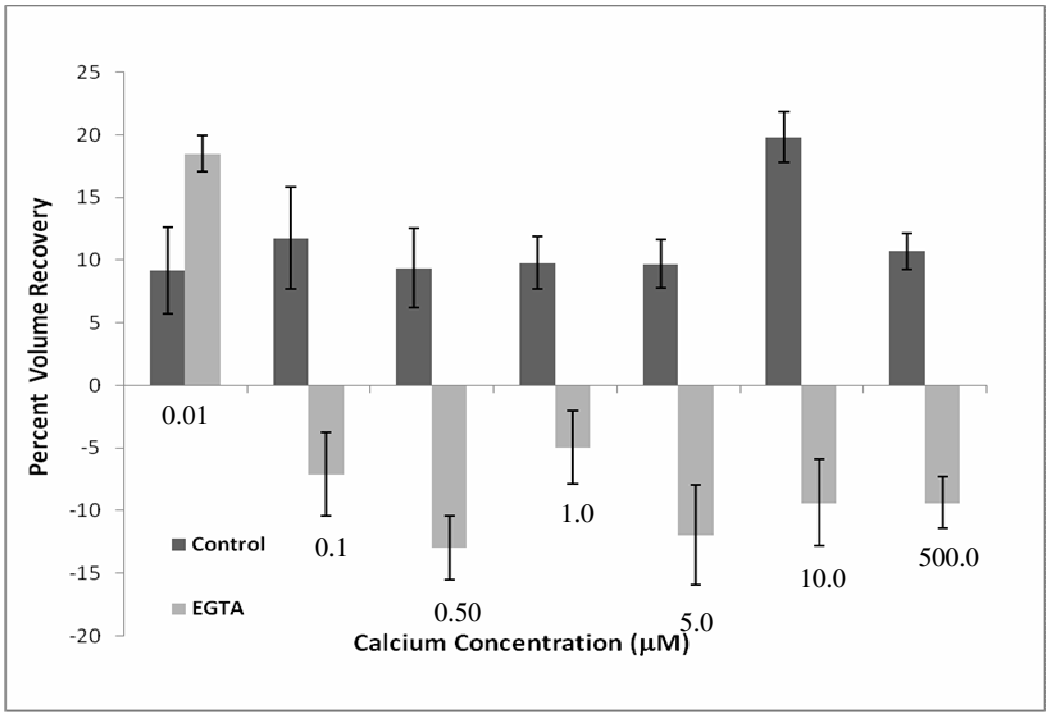

Figure 5. Relative volume is higher in low extracellular Ca^{2+}. For all concentrations of extracellular Ca^{2+} above 0.01 μM, RBCs have a larger relative volume compared to control (6 mM Ca^{2+}) at t=40 minutes following hypotonic shock (n=5 or 6 and P< 0.05 for all measurements). Cells in 0.01 μM Ca2+ solution had a smaller relative volume than control cells at t=40 minutes (n=4, P< 0.05).

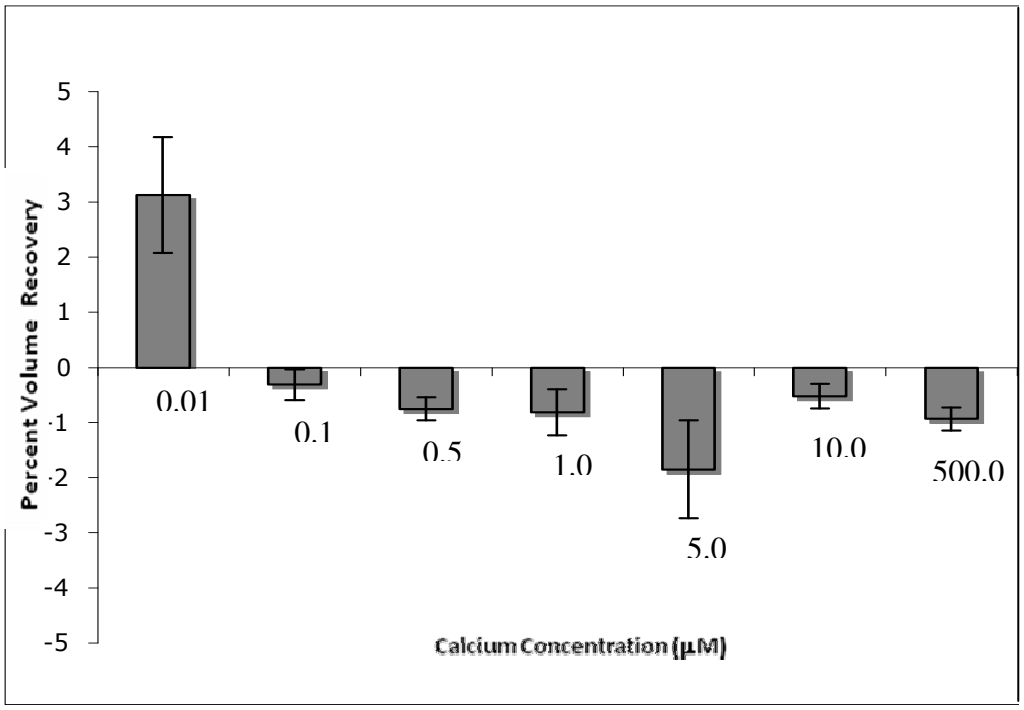

Figure 6. Recovery was inhibited in low extracellular Ca^{2+}. For all concentrations of extracellular Ca^{2+} above 0.01 μM, RBCs recovered less than control (6 mM Ca^{2+}) at t=40 minutes following hypotonic shock. Cells in 0.01 μM Ca^{2+} solution recovered more than the control at t=40 minutes. The percent volume recovery of cells in 0.01 μM Ca^{2+} solution divided by their control was statistically different from other Ca^{2+} solutions (0.01 μM n=4, P<0.05). In addition, the percent volume recovery of cells in 0.5 μM Ca^{2+} solution was statistically different from cells in 1.0 μM Ca^{2+} solution (0.05 μM n=6; 1.0 μM n=5. P<0.05).

one to deal with minor physiological volume changes, and a second that is activated during larger swelling (Engstrom and Savendahl, 1995). Bursell and Kirk (1996) found that eel erythrocytes also have two separate mechanisms for initiating RVD, one that is dependent on the presence of extracellular Cl^- and one that is independent of $[Cl^-]_o$. Therefore, it is possible that alligator RBCs also use more than mechanism for RVD and these mechanisms may utilize Ca^{2+} in different ways.

Regardless of the anomalous observation with Ca^{2+}<0.01 μM, it is clear that all other low concentrations of Ca^{2+} impeded RVD. With this in mind, I expected high levels of $[Ca^{2+}]_o$ to improve RVD. Interestingly though, increasing the amount of Ca^{2+} that entered RBCs with the Ca^{2+} ionophore A23187 did not improve RVD (Figure 7). In fact, bathing the cells in 1 μM A23187, which allowed for mM concentrations of Ca^{2+} to enter the RBCs, completely inhibited RVD (Fig. 13). This finding was in contrast to Light et al. (2002) who found that application of this ionophore caused a decrease in osmotic fragility and potentiated volume recovery in *Necturus* erythrocytes. Also, contrary to my findings, the application of this ionophore to mouse distal colon cells significantly improved their RVD response (Mignen et al. 1999).

The affect of A23187 seemed paradoxical to that of low extracellular Ca^{2+}. In other words, how could RVD be both inhibited and stimulated by Ca^{2+}? It is possible that a pharmacologically induced high level of Ca^{2+} influx with A23187 may be toxic to the RBCs and thus disrupt osmoregulatory mechanisms. A similar paradoxical relationship between Ca^{2+} and RVD was found in rat neuronal cells (Churchwell et al. 1996). They used veratridil (a Na^+ channel activator) to induce neuronal swelling. They then observed that chelating intracellular and/or extracellular Ca^{2+} inhibited RVD following veratridil-induced swelling. That is, both low extracellular and intracellular Ca^{2+} inhibit RVD. In addition, neuronal swelling leads to an accumulation of glutamate in excitatory synapses. Glutamate in turn activates NMDA receptors that open cation channels (Mayer and Westbrook, 1987). Churchwell et al. (1996) showed that the activation of NMDA receptors in rat neuronal cells leads to the influx of Ca^{2+} and an inhibition of RVD. Therefore, rat neuronal cells show a similar response to Ca^{2+} as alligator RBCs, where Ca^{2+} is both required for RVD and inhibits RVD. Churchwell et al. (1996) attributed this to a "threshold" level of Ca^{2+} necessary for RVD. These investigators proposed that the elevation of Ca^{2+} above this threshold, brought about by the stimulation of NMDA receptors, is toxic to neuronal cells and prevents osmoregulation. Berliocchi et al. (2005) also found that depending on the extent and the duration of the Ca^{2+} influx, neurons will either survive, die by apoptosis (i.e. sustained slow Ca^{2+} influx), or undergo necrotic lysis (i.e. rapid high Ca^{2+} influx).

Accordingly, I hypothesized that the inhibition of RVD caused by A23187 was due to the opening of Na^+ channels by the pharmacologically induced high Ca^{2+} influx. The opening of Na^+ channels would in turn lead to the influx of this ion, as well as osmotically obliged water. If the rate of volume-regulatory solute efflux does not exceed the rate of Na^+ entry, RVD would be inhibited. However, I found that A23187 did not lead to the opening of Na^+ channels (Figure 8). That is, when Na^+ was replaced with NMDG (a monovalent cation that is impermeable to Na^+ channels) in the extracellular medium, RVD remained inhibited. Therefore, contrary to my hypothesis, A23187-induced Ca^{2+} influx inhibited RVD by a mechanism other than enhancing Na^+ influx. One possibility to explain this observation is that high Ca^{2+} levels blocked the opening of K^+ channels, preventing the efflux of this ion and therefore inhibiting RVD. For example, Gomez-Logunas et al. (2002) found that elevated levels of Ca^{2+} block *Shaker* K^+ channels by changing the channel's ion occupancy state. Armstrong and Palti (1991) also observed that high levels of Ca^{2+} blocks K^+ channels in squid giant fiber lobe neurons. This hypothesis could be tested in alligator RBCs by monitoring K^+ efflux with and without A23187.

In summary, my studies indicate that extracellular Ca^{2+} plays a role in RVD in a dose dependent manner. Inadequate amounts of extracellular Ca^{2+} inhibit RVD and pharmacologically induced excessive Ca^{2+} influx also inhibit RVD. In contrast, extremely low extracellular Ca^{2+} allows for an enhanced RVD response. These results lead me to conclude that there may be a "threshold" level of Ca^{2+} needed to trigger RVD, and a certain amount of extracellular Ca^{2+} is needed to have a full RVD mechanism. A lack of this threshold amount of extracellular Ca^{2+} may initiate a second mechanism that allows for adequate RVD, possibly by the release of Ca^{2+} from intracellular stores. If so, it is possible that alligator RBCs use the release of intracellular Ca^{2+} as a last resort for initiating RVD in non-physiological instances, when almost no Ca^{2+} is present in the extracellular solution.

Future research should further investigate the paradoxical relationship between Ca^{2+} and RVD. To assess whether Ca^{2+} is released from intracellular stores in the RVD in alligator RBCs, pharmacological agents such as dantrolene (stimulates Ca^{2+} reabsorption by intracellular stores), ryanodine (releases Ca^{2+} from intracellular stores), and TMB-8 (inhibits intracellular Ca^{2+} mobilization) should be used (Moran et al. 1997). It would also be valuable to use an intercellular Ca^{2+} chelator, such as 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), to determine whether the effect of Ca^{2+} released from intracellular stores is concentration dependent (Altamirano et al. 1998). In addition, epi-fluorescence microscopy studies using the fluorescent Ca^{2+} indicator fluo-4-AM would be useful in analyzing whether intracellular Ca^{2+} levels change in hypotonic conditions (Light et al. 2002).

Mechanism of calcium entry into the RBCs

The findings of this study are consistent with the hypothesis that Ca^{2+} enters RBCs via stretch-activated channels following hypotonic shock. Evidence for this comes from cells in the hypotonic Ringer with gadolinium (Gd^{3+}), an inhibitor of stretch-activated Ca^{2+} permeable channels, which swelled more than the control and failed to undergo an RVD mechanism (Figure 9). A similar swelling-induced Ca^{2+} influx has been reported in renal proximal tubule cells (McCarty & O'Neill 1992), toad urinary bladder (Wong et. al, 1990), and *Necturus* gallbladder (Foskett & Spring 1985). This finding is also supported by Chen et al. (1996) who found that extracellular Ca^{2+} during RVD enters via Gd^{3+} sensitive stretch-activated channels in GH3 cells.

However, some studies suggest that Gd^{3+} not only blocks stretch-activated channels, but it might also inhibit purinoreceptors (Boudreault et al. 2001). Since P_2 channels require the presence of extracellular ATPDein order to function, I used hexokinase to

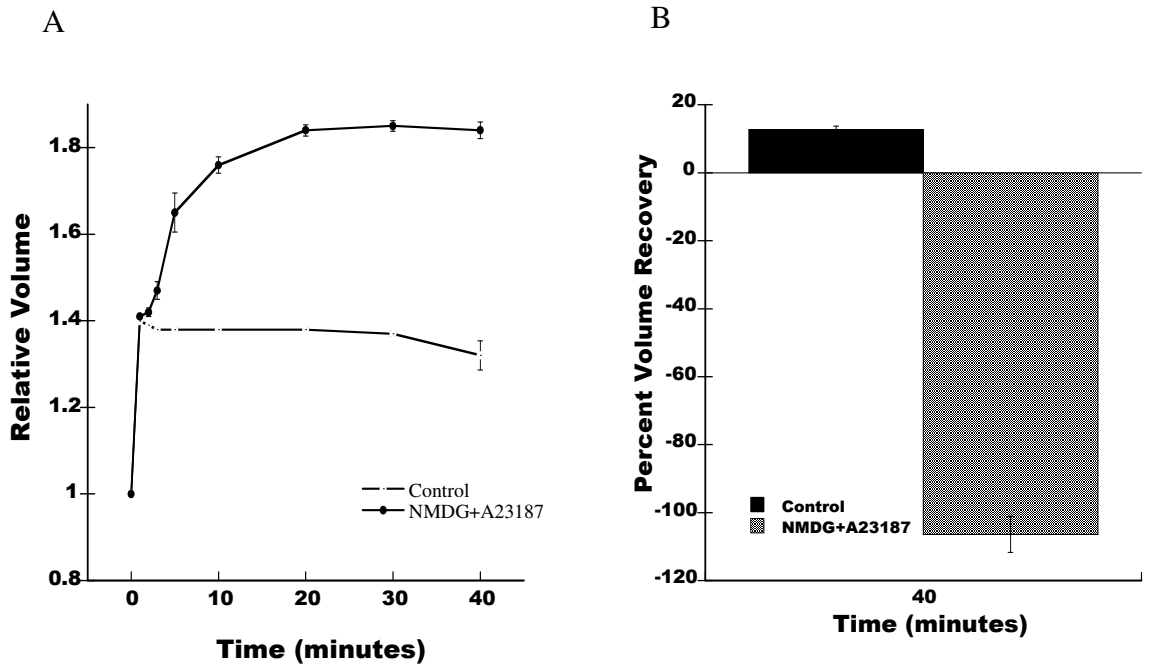

Figure 8. Calcium ionophore A23187 inhibited RVD in the absence of extracellular Na^+. Cells quickly swelled upon exposure to hypotonic Ringer at t=0, which was maintained for the duration of this experiment. (A) Unlike control cells which began to recover after 1 minute following hypotonic shock, volume recovery was not observed in the cells exposed to an NMDG + A23187 Ringer (n=6, P=0.0552 at 1 min and P<0.05 at 40 min as compared to control). (B) Red blood cells exposed to NMDG + A23187 Ringer showed a percent volume recovery of -106.39% at 40 minutes. This was in comparison to control cell percent volume recovery of 12.67% at 40 minutes (n=6, P< 0.05). Data shown as mean ± SE.

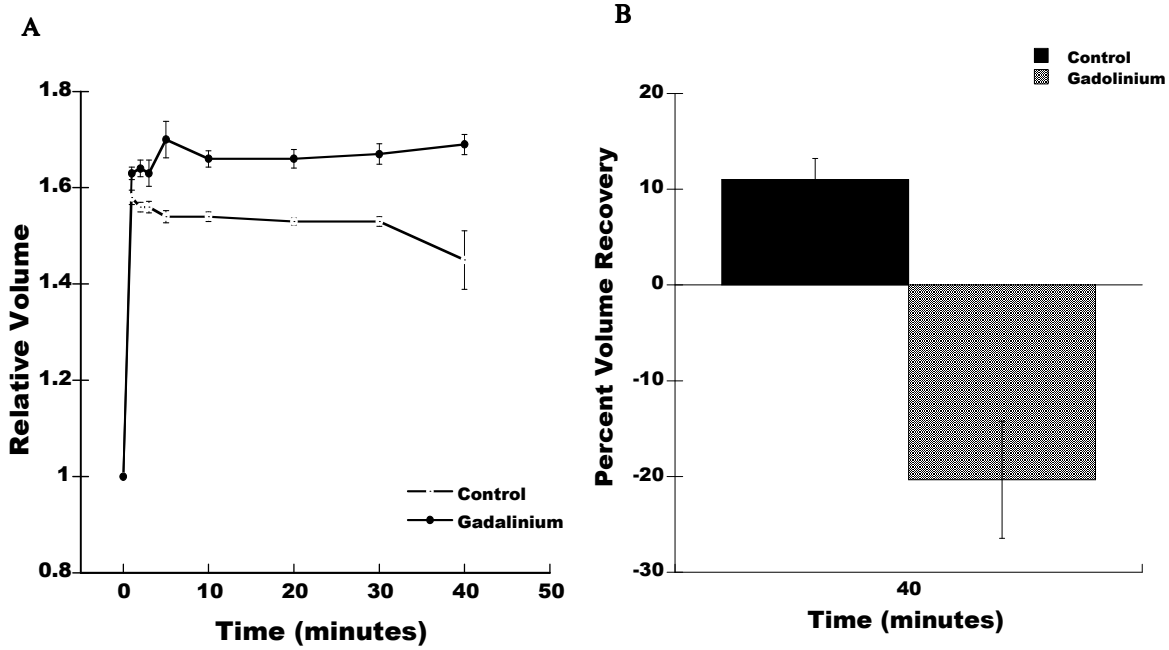

Figure 9. Stretch–activated channel blocker gadolinium inhibited RVD. (A) Unlike control cells which began to recover after 1 minute following hypotonic shock, volume recovery was not observed in the cells exposed to gadolinium (n=6, P=0.10 at 1 min and P<0.05 at 40 min as compared to control). (B) Red blood cells exposed to gadolinium showed a percent volume recovery of -20.34% at 40 minutes. This was in comparison to control cell percent volume recovery of 11.02% at 40 minutes (n=6, P<0.05). Data shown as mean ± SE.

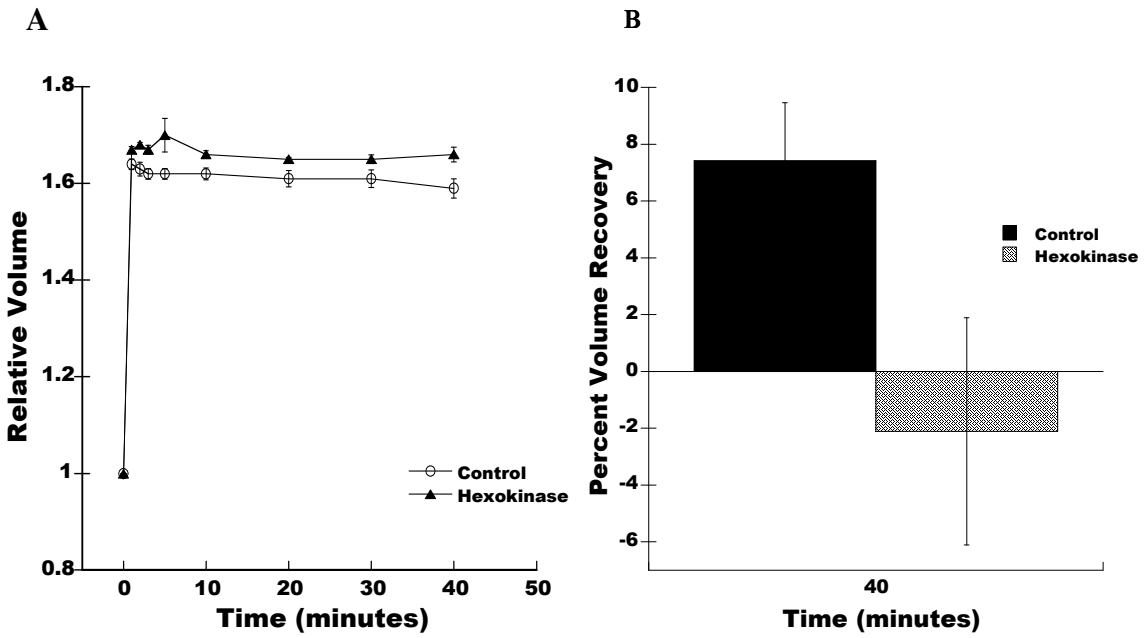

Figure 10. P_2 receptor antagonist hexokinase inhibited RVD. (A) Cells exposed to hexokinase, a blocker of P_2 receptors had a reduced RVD as compared to control cells (n=6, P<0.05 at 40 min). (B) Cells failed to recover in the presence of hexokinase (-2.11%) as compared to control cells (7.43%) (n=6, P<0.05). Data shown as mean ± SE.

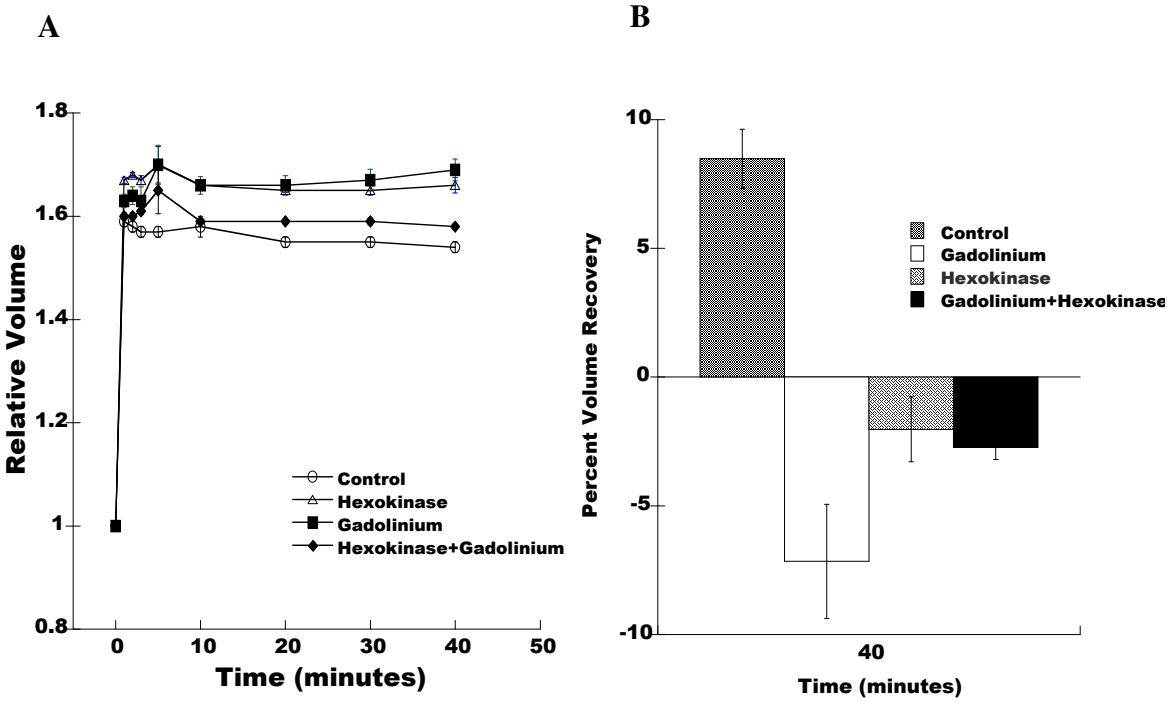

Figure 11. Gadolinium and hexokinase together did not have an additive effect. (A) Cells exposed to gadolinium and hexokinase at the same time had an inhibited RVD as compared to control. However, the RVD of the cells exposed to the two inhibitors together was better than in cells exposed to each inhibitor alone. (B) Cells exposed to the two inhibitors at once (-2.73% ± 0.47) failed to recover as compare to control (8.48% ± 1.15). However, the recovery of cells exposed to both inhibitors at the same time was similar to that of cells with hexokinase alone (-2.028 +/- 1.26), and better than those with gadolinium alone (-7.16 ± 2.22).

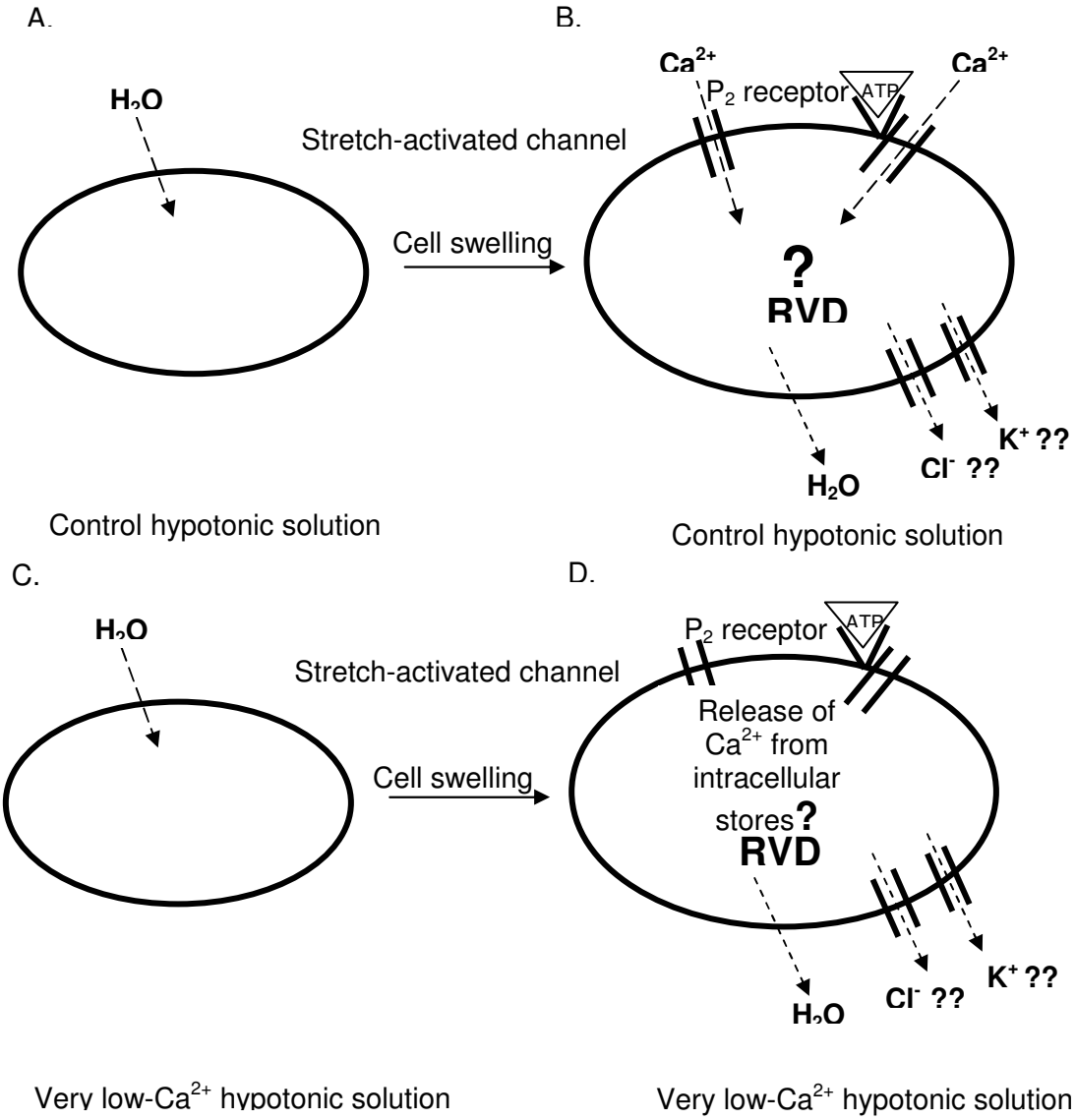

Figure 12. Proposed role of Ca^{2+} in RVD by alligator erythrocytes. (A), (C) When RBCs face a hypotonic challenge, water rapidly enters the cell causing swelling. (B). If the surrounding medium of the RBC contains an adequate concentration of Ca^{2+}, this ion enters via stretch-activated channels and/or P_2 receptors. The rise in cytosolic Ca^{2+} initiates a signal transduction mechanism that allows for efflux of osmoregulatory ions, as well as the osmotically obliged water, resulting in RVD. (D) If the RBC swells in very low-Ca^{2+} hypotonic solution, it is possible that Ca^{2+} is released from intracellular stores raising the cytosolic Ca^{2+} concentration. This rise in $[Ca^{2+}]_i$ may activate a signal transduction pathway that also leads to the efflux of key ions and water to shrink the cell back to its original size.

preventing activation of P_2 channels (Dubyak, 2002), (Taylor et al. 1998). I found that blocking P_2 channels with hexokinase inhibited RVD, which suggests that Ca^{2+}, at least in part, enters the RBCs via P_2 channels (Figure 10). This is consistent with studies by Garcia-Lecea et al. (1999) who showed that Ca^{2+} enters Perkinje neurons via P_2 channels during RVD. Similarly, Light et al. (1999) also concluded that RVD in *Necturus* RBCs requires the influx of Ca^{2+} via P_2 receptors. Contrary to my findings, Roe et al. (2001) found that even though hepatocell RVD requires extracellular Ca^{2+}, this Ca^{2+} does not enter via P_2 receptors. Thus, P_2 receptors do not appear to be universal for RVD.

Since I found that both gadolinium and hexokinase inhibit RVD, I investigated whether simultaneously using both agents would have an additive affect. Surprisingly, however, when RBCs were exposed to both gadolinium and hexokinase, RVD was improved compared to when the RBCs were exposed to either Gd^{3+} or hexokinase alone (Figure 11). It is possible that this finding is consistent with my previous observation that at extremely low concentrations of extracellular calcium (0.01 μM), RVD is potentiated. In other words, the combination of Gd^{3+} and hexokinase suggests that when both stretch-activated channels and P_2 channels are blocked, so little calcium (if any) was able to enter the cell, that it mimicked the situation

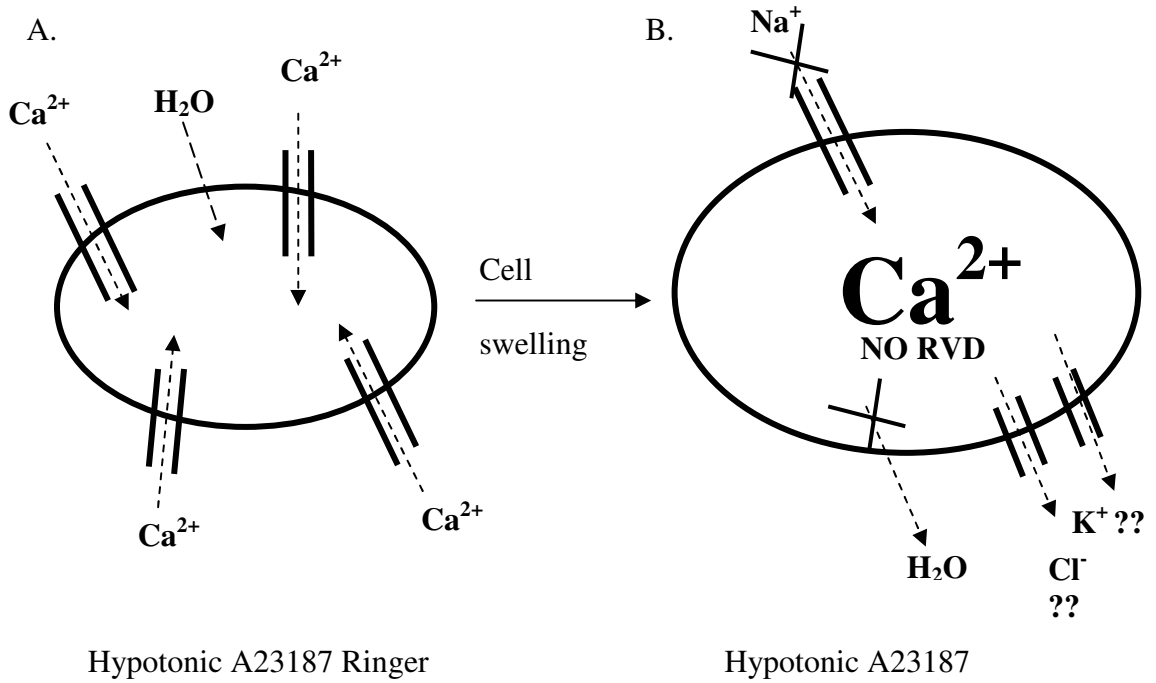

Figure 13. Inhibition of RVD after pharmacological induction of extra Ca^{2+} influx. (A) When RBCs are exposed to a hypotonic Ringer with Ca^{2+} ionophore A23187, H_2O and Ca^{2+} enter the cell causing swelling. (B) The pharmacologically high influx of Ca^{2+} into the RBC may be toxic to the RBC and inhibits RVD by a mechanism other than stimulating Na^+ influx.

when I exposed the cells to very little calcium in the extracellular environment. It is possible therefore, that when both stretch-activated channels and P_2 receptors are blocked, the cell is forced to release Ca^{2+} from intracellular stores to initiate an RVD mechanism. Although this finding is compelling, no definitive conclusion can be entertained without further confirming the involvement of P_2 channels in stimulating Ca^{2+} entry into the RBCs. Pharmacological agents such as ATP and ATPγS (non-hydrolysable form of ATP) should be used in future studies to determine whether added ATP or ATPγS potentiate RVD. If in fact these chemicals improve RVD, the role of P_2 receptors in stimulating Ca^{2+} influx would be further corroborated. Pharmacological agents such as suramin and Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS), known blockers of P_2 receptors, may also be used to assess whether inhibiting these channels affects RVD. In addition, the presence of P_2 receptors could be confirmed by immunocytochemistry and/or immunofluorescence if an antibody for the specific subtype of the P_2 receptor in attained.

Future studies also should focus on determining the precise role extracellular Ca^{2+} has in the activation of RVD. There are several signal transduction pathways that may involve Ca^{2+} and could lead to volume regulation. For example, a rise in intracellular Ca^{2+} could lead to the activation of protein kinase C (PKC), which upon autophosphorylation further propagates the signal that may lead to RVD (Bender et al. 1992). Another possible mode of Ca^{2+} action could be binding to calmodulin. This binding causes a conformational change that enables calmodulin to wrap around a wide range of target proteins in the cell, altering their activities. One target protein can be Ca^{2+}/calmodulin-dependent protein kinase (Cam-kinase) that phosphorylates other proteins relaying the signal that may initiate RVD (Bender & Norenberg, 1994). An experimental procedure using trifluoperazine, pimozide or chlorpromazine, calmodulin antagonists, should be carried out to discern whether Ca^{2+} affects RVD through this mechanism (Grinstein et al. 1982). Another target of Ca^{2+} could be phospholipase A2 (PLA2), which is an enzyme that produces arachidonic acid, a precursor of the eicosanoid pathway, a common signaling mechanism that has been shown to play a role in RVD in some cell types (Mitchell et al. 1997).

Another possibility is that Ca^{2+} may directly stimulate K^+ channels that allow for the efflux of this ion and the osmotically obliged water leading to the reduction in the cell volume (Fernandez-Fernandez et al. 2005). To test this hypothesis, a Ca^{2+} activated K^+ channel blocker, quinine, may be used to assess whether Ca^{2+} contributes to RVD by opening K^+ channels (Nilius et al. 1995). In addition, patch clamp studies may be carried out to determine properties of K^+ channels that are activated during cell swelling.

Conclusion

Calcium plays an important role in the RVD of alligator RBCs. RVD is attenuated in cells exposed to low extracellular Ca^{2+}; however, cells that are bathed in a nearly Ca^{2+}-free Ringer exhibit an improved RVD. Calcium enters the RBCs via stretch-activated channels and/or P_2 receptors. However, because Gd^{3+} with hexokinase added simultaneously had a different effect than either one alone, this suggests that both P_2

receptors and stretch-activated channels are present. Pharmacologically induced influx of Ca^{2+} into the RBCs inhibits RVD by a mechanism other than stimulating Na^{+} influx. This study confirmed that Ca^{2+} is an important signaling molecule in the RVD mechanism. More extensive research should be conducted to determine the precise role Ca^{2+} plays in cell volume regulation.

Acknowledgments

I would like to thank my advisor, Dr. Douglas Light for tremendous patience and support throughout the completion of my experiments and writing of the thesis. In addition, I thank Dr. William Martin and Dr. Pliny Smith for agreeing to evaluate my work by being on my thesis committee. Also, I would like to thank my colleagues in lab, particularly Shruti Pore, Lital Silverman, and Chloe Wormser for their helpful suggestions.

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