

Potential of Regulatory Volume Decrease by a P2-Like Receptor and Arachidonic Acid in American Alligator Erythrocytes

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Abstract This study examined the role of a P2 receptor and arachidonic acid (AA) in regulatory volume decrease (RVD) by American alligator red blood cells (RBCs). Osmotic fragility was determined optically, mean cell volume was measured by electronic sizing, and changes in intracellular Ca^{2+} concentration were visualized using fluorescence microscopy. Gadolinium (50 μM), hexokinase (2.5 U/ml), and suramin (100 μM) increased osmotic fragility, blocked volume recovery after hypotonic shock, and prevented a rise in intracellular Ca^{2+} that normally occurs during cell swelling. The P2X antagonists PPADS (50 μM) and TNP-ATP (10 μM) also increased fragility and inhibited volume recovery. In contrast, $\text{ATP}\gamma\text{S}$ (10 μM), α,β -methylene-ATP (50 μM) and Bz-ATP (50 μM) had the opposite effect, whereas 2-methylthio-ATP (50 μM) and UTP (10 μM) had no effect. In addition, the phospholipase A_2 (PLA_2) inhibitors ONO-RS-082 (10 μM), chlorpromazine (10 μM), and isotetrandrine (10 μM) increased osmotic fragility and blocked volume recovery, whereas AA (10 μM) and its nonhydrolyzable analog eicosatetraenoic acid (ETYA, 10 μM) had the reverse effect. Further, AA (10 μM), but not $\text{ATP}\gamma\text{S}$ (10 μM), prevented the inhibitory effect of a low Ca^{2+} -EGTA Ringer on RVD, whereas both AA (10 μM) and $\text{ATP}\gamma\text{S}$ (10 μM) caused cell shrinkage under isosmotic conditions. In conclusion, our results are consistent with the presence of a P2-like receptor whose activation stimulated RVD. In addition, AA also was important for volume recovery.

Keywords ATP · Eicosanoid · Gramicidin · Hexokinase · Phospholipase A_2 · Suramin

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

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

The cellular mechanisms that activate and regulate permeability pathways during RVD are not completely understood and differ between cell types (Jakab et al. 2002; Hoffmann et al. 2009; Lang et al. 1998; Wehner et al.

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2003). However, Ca²⁺ has been shown to play a key role during RVD in a number of cases (Jakab et al. 2002; Pasantes-Morales and Morales 2000; Tinel et al. 2000). Although Ca²⁺ may directly activate ion channels during RVD (Lang 2007; Pasantes-Morales and Morales 2000), there also is evidence that several Ca²⁺-dependent intracellular messengers and enzymes are involved with cell volume regulation (Hoffmann et al. 2009; Tinel et al. 2000). One such messenger is arachidonic acid (AA), a 20-carbon polyunsaturated fatty acid that is formed primarily from membrane glycerophospholipids by the action of phospholipase A₂ (PLA₂), a Ca²⁺-activated enzyme (Jenkins et al. 2009; Lee 2009). AA and eicosanoid metabolites (Jenkins et al. 2009) regulate volume in a number of cell types, including Ehrlich ascites tumor cells (Hoffmann et al. 2009), kidney cells (Tinel et al. 2000), fish hepatocytes (Ollivier et al. 2006b), and mouse distal colon (Mignen et al. 1999).

It is well known that adenosine 5'-triphosphate (ATP) is a ubiquitous intracellular source of energy. However, ATP also may act as a transmitter substance at autonomic neuromuscular junctions, as well as play a significant role in a number of other biological processes (Burnstock 2009). In particular, a number of studies have demonstrated extracellular nucleotides are important for regulating ion channels and cell volume (Dezaki et al. 2000; Okada et al. 2001; Ollivier et al. 2006a; Shinozuka et al. 2001; Varela et al. 2010). Extracellular ATP exerts its influence by acting as an autocrine/paracrine signal, binding to specific cell surface receptors termed purinoceptors (Burnstock 2009). Purinoceptors have been subdivided into two main categories: P1 receptors that recognize nucleosides, such as adenosine, and P2 receptors that bind ATP and other nucleotides (Burnstock 2009). The P2 receptors have been further subdivided into two main groups: P2X (ATP-gated, Ca²⁺-permeable, nonselective channels) and P2Y (ATP-activated receptors coupled to a G-protein).

Despite recent reports concerning the physiology of P2 receptors and AA, there is still a lack of data on their role in RVD, especially for reptilian cells. Accordingly, the purpose of this study was to investigate the basis by which American alligator red blood cells (RBCs) regulate volume under hypotonic conditions. This study also builds on recent work that demonstrates RVD in this cell type depends on Ca²⁺ influx to stimulate K⁺ efflux (Elperin et al. 2011). We initially chose this cell type as a model system because alligators are known to “hibernate” from September until late spring (Hernandez and Coulson 1952). During this state of torpor, they do not consume food, which may cause variations in the concentration of plasma electrolytes and nonelectrolytes (Lance and Elsey 1999). In addition, kidney function is greatly reduced during hibernation, which also may lead to osmotic imbalance

(Zancanaro et al. 1999). Consequently, we speculated that alligator RBCs should have a well-developed RVD to cope with the homeostatic challenges of torpor.

Methods

Cells

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

Osmotic Fragility

Osmotic fragility was examined by determining the degree of cell lysis for a suspension of RBCs in hypotonic Ringer. The level of hemolysis was determined via a turbidity shift (cloudy to clear) that occurs when the integrity of the plasma membrane is compromised. This was detected with a spectrophotometer (Spectronic 21D, Milton Roy Co.) 5 min after blood (30 µl) was added to hypotonic Ringer solutions (3 ml) of different compositions. Spectrophotometric experiments were conducted at 625 nm because an absorption spectrum indicated that this wavelength provided the greatest difference in optical density (OD) between intact and lysed cells.

A hemolytic index (HI) was determined using the formula: HI (%) = (OD of test compound – OD of negative control)/(OD of positive control – OD of negative control) × 100, where OD of test compound refers to the OD of a cell suspension in diluted Ringer to which a test compound was added, OD of negative control refers to the OD of a cell suspension in diluted Ringer, and OD of positive control refers to the OD of a cell suspension in distilled water (used to set spectrophotometer OD to 0).

All reported hemolytic indices were calculated using Ringer solutions with a concentration of 85.0 ± 1.5 mosm/kg H₂O. We chose this concentration because it was sufficiently dilute to lyse approximately half the cells in suspension (determined empirically by challenging cells with solutions ranging from 0 to 300 mosm/kg H₂O). Consequently, we could assess whether a test compound increased osmotic fragility by a subsequent reduction in OD compared to the control solution. Conversely, a rise in OD indicated that a test compound reduced osmotic fragility. Because nonmammalian vertebrates have nucleated RBCs, which causes suspensions of lysed RBCs to remain slightly turbid, NaHCO₃ (1 mM) was added to all solutions. The slight alkalinity leads to better clearing of solutions without influencing experimental results. Cell lysis also was confirmed microscopically (Nikon Diaphot, DIC optics, 400×).

Fluorescence Microscopy

Intracellular free Ca²⁺ levels were monitored using the fluorescent Ca²⁺ indicator fluo-4-AM (10 μM, Molecular Probes, Eugene, OR). This indicator has a high binding affinity for Ca²⁺ (K_{id} = 345 nM) and a large fluorescence intensity increase in response to Ca²⁺ binding (>100-fold; Thomas et al. 2000). The acetoxymethyl (AM) ester derivative permeates cell membranes and, once inside a cell, the lipophilic blocking groups are cleaved by non-specific esterases. This results in a charged form that is relatively impermeable.

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

Cell swelling was accomplished by adding an equal volume of hypotonic (0.1×) Ringer to the isotonic Ringer (this reduced osmolality (0.5×) without changing the Ca²⁺, Mg²⁺, glucose, and buffer concentrations). Cells in isotonic Ringer served as the negative control, whereas cells in isotonic Ringer with A23187 (Ca²⁺-ionophore, 0.5 μM, Tiffert and Lew 1997) served as the positive control. Cells were photographed with a Zeiss Axiovert 100 microscope using Metamorph 6.1 software (Universal Imaging Corp.) or with a Nikon TE 2000-U microscope equipped with Hoffman DIC optics (400×) and epi-fluorescence (mercury lamp and FITC filter cube) using Metamorph 6.2 software. For any given experiment, only cells photographed using the same microscope and imaging parameters were used for qualitative comparisons.

Coulter Counter

Cell volume distribution curves were obtained by electronic sizing using a Coulter counter model Z2 with channelyzer (Beckman Coulter, Brea, CA). Mean cell volume was taken as the mean volume of the distribution curves. The diameter of the aperture tube orifice was 100 μm and the metered volume was 0.5 ml. Absolute cell volumes were obtained using polystyrene latex beads (9.565 μm diameter or 459.9 fl volume) as standards (Coulter). Experiments with the latex beads showed that measured volumes were unaffected by changes in

osmolality and ionic composition within the ranges used for this study. Cell suspensions were diluted to give a final cell density of approximately 10,000 cells per ml.

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

Solutions

Isosmotic reptilian Ringer consisted of (in mM) 140 NaCl, 6 KCl, 2.0 CaCl₂, 1.5 MgCl₂, 5.5 glucose, and 10 HEPES (titrated to pH 7.4 with NaOH or HCl, 300 ± 2 mosm/kg H₂O). A low Na⁺ Ringer, used for all experiments with gramicidin, was prepared by substituting choline chloride for NaCl (Mastrocola et al. 1991), and a hypotonic (~0.5×) Ringer was obtained by reducing the NaCl concentration to 70 mM. For some experiments, the free Ca²⁺ concentration in the extracellular solution was adjusted to 10 nM using ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) and an appropriate concentration of CaCl₂ (calculated using <http://www.stanford.edu/~cpatton/webmaxc/webmaxclite115.htm>). A stock solution of gramicidin was dissolved in methanol at 1000× the final concentration, and 1,4-di[3-(3-isothiocyanatophenyl)thioureido]butane (MRS-2578), 2-(p-aminocinnamoyl)-amino-p-chloro-benzoic acid (ONO-RS-082), 5,8,11,14-eicosatetraenoic acid (ETYA), isotetrandrine, chlorpromazine hydrochloride, palmityl trifluoromethylketone (PACOCF₃), arachidonic acid (cis-5,8,11,14-eicosatetraenoic acid), and nordihydroguaiaretic acid (NDGA) were dissolved in ethanol or dimethyl sulfoxide (DMSO), and then diluted to give a final working concentration. All stock aqueous solutions (gadolinium, suramin, ATP analogs, adenosine, hexokinase, apyrase) were diluted 100× to give an appropriate final concentration.

For osmotic fragility experiments, pharmacological agents or their vehicle were present before the addition of cells. For cell volume studies, pharmacological antagonists were added with hypotonic exposure, whereas agonists were added at the peak cell volume (1 min after hypotonic shock). The osmolality of solutions was measured with a vapor pressure osmometer (#5500, Wescor, Logan, UT) and the pH was determined using an Accumet Basic AB15 pH meter (Fisher Scientific). Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), Axxora, LLC (San Diego, CA), and MP Pharmaceuticals Inc. (Solon, OH). All

solutions were filtered with a 0.8 μM Advantec cellulose acetate membrane filter before use, and all experiments were conducted at room temperature (21–23°C).

Statistical Analysis

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

Results

Osmotic Fragility Studies

Although osmotic fragility depends on several factors, we first examined this property as one assessment of a cell's ability to regulate volume in a hypotonic medium. The OD, measured at a concentration of Ringer where approximately 50% of the cells in suspension were intact was 0.071 ± 0.002 ($n = 10$ experiments, Fig. 1a). In a previous study (Elperin et al. 2011), we showed that a low Ca^{2+} -EGTA Ringer increased osmotic fragility. To determine whether Ca^{2+} influx occurs via a stretch-activated (SA) channel, we repeated this assay with gadolinium (50 μM), a nonspecific blocker of many mechanosensitive channels (Hua et al. 2010). In this case, the OD measured at the same osmolality as the control decreased to 0.042 ± 0.003 , giving a hemolytic index of 41% ($n = 10$, $P < 0.001$, Fig. 1a). Gramicidin (1 μM) prevented the inhibitory effect of gadolinium (gramicidin is a cationophore that was used to maintain a high K^+ permeability in a choline Ringer, Mastrocola et al. 1991). In the presence of gadolinium, gramicidin increased the OD to 0.105 ± 0.003 , consistent with gadolinium inhibiting K^+ efflux ($n = 10$, $P < 0.001$, Fig. 1a). Vehicle for gramicidin (methanol, diluted 1:1000, $n = 6$) or replacing Na^+ with choline ($n = 6$) has no demonstrable effect (Elperin et al. 2011).

Because it has been reported that Gd^{3+} may interfere with the activation of P2 receptors by inhibiting ATP efflux (Brown et al. 1993; Boudreault and Grygorczyk 2002), we examined whether the effect we observed with this lanthanide was due to the presence of a purinoceptor (as opposed to an SA channel). This was accomplished by using the ATP scavenger hexokinase, an enzyme that traps ATP by transferring its γ -phosphoryl group to a variety of six-carbon sugars (Skals et al. 2009). Hexokinase (2.5 U/ml) decreased the OD from 0.069 ± 0.002 to

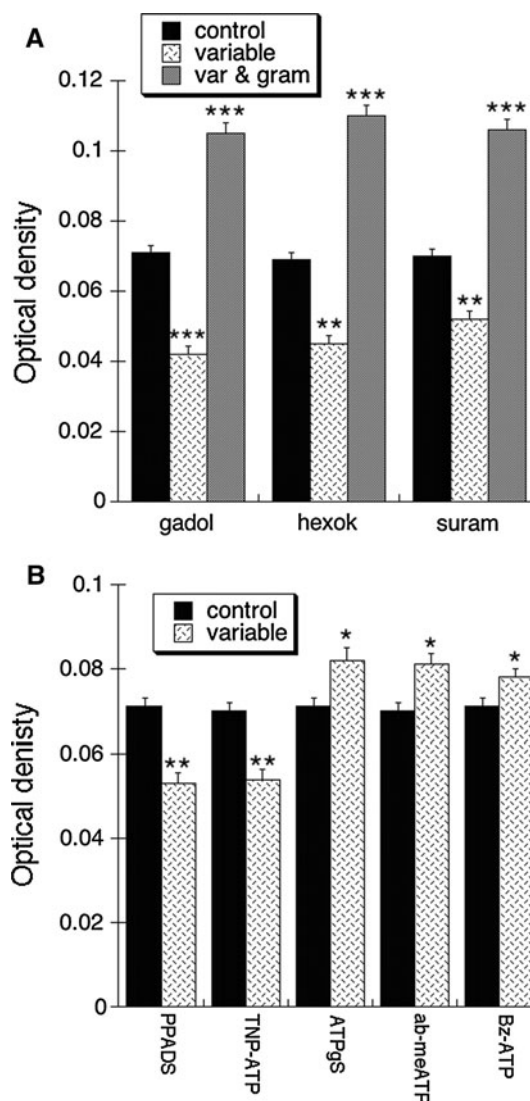


Fig. 1 Osmotic fragility depended on the activation of a P2 receptor. The control (solid bar) was diluted reptilian Ringer (85 mosm/kg H_2O , a concentration at which approximately 50% of the control cells lysed). **a** The stretch-activated channel antagonist gadolinium (gadol, 50 μM , $n = 10$), the ATP scavenger hexokinase (hexok, 2.5 U/ml, $n = 10$), and the P2 receptor inhibitor suramin (suram, 100 μM , $n = 8$) increased osmotic fragility. The cationophore gramicidin (gram, 1 μM) prevented the inhibitory effect of these agents (choline was substituted for Na^+ when conducting experiments with gramicidin). **b** The P2 receptor inhibitors PPADS (50 μM), and TNP-ATP (10 μM) increased osmotic fragility ($n = 6$ for each). In contrast, ATP γ S (ATP γ S, 10 μM , $n = 8$), α, β -meATP (ab-meATP, 50 μM , $n = 6$), and Bz-ATP (50 μM , $n = 6$) decreased osmotic fragility. Values are means \pm SEM of optical density. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

0.045 ± 0.003 ($n = 10$, $P < 0.01$, Fig. 1a), giving a HI of 35%. Gramicidin (1 μM), in the presence of hexokinase, increased OD to 0.110 ± 0.003 ($n = 10$, $P < 0.001$, Fig. 1a).

Similar to hexokinase, the P2 receptor antagonist suramin (100 μM , Ollivier et al. 2006a; Skals et al. 2009) decreased

OD from 0.070 ± 0.002 to 0.052 ± 0.003 ($n = 8$, $P < 0.01$, Fig. 1a), a hemolytic index of 26%. The inhibitory effect of suramin also was prevented with gramicidin (1 μM , Fig. 1a). In addition, an inhibitory response was observed with the P2X receptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS, 50 μM ; Neelands et al. 2003; Crumling et al. 2009), which decreased the OD from 0.071 ± 0.002 to 0.053 ± 0.002 ($n = 6$, $P < 0.01$, Fig. 1b), a hemolytic index of 25%. Similarly, the P2X inhibitor 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP, 10 μM , Neelands et al. 2003; Sugihara et al. 2011) increased osmotic fragility, giving a hemolytic index of 23% ($n = 6$, $P < 0.01$). However, the P2X antagonist 5-[[[(3-phenoxyphenyl)methyl][(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]-amino]-carbonyl]-1,2,4-benzenetricarboxylic acid sodium salt hydrate (A-317491, 1 μM ; Jarvis et al. 2002; Neelands et al. 2003) and the P2Y antagonist MRS-2578 (10 μM) had no effect ($n = 6$ for each, not shown).

The P2 receptor agonist adenosine 5'-O-(3-thiotriphosphate) (ATP γS , 10 μM , a poorly hydrolysable analog of ATP, Pafundo et al. 2004) increased the OD from 0.071 ± 0.002 to 0.082 ± 0.003 ($n = 8$, $P < 0.05$, Fig. 1b). In addition, the P2X agonists α,β -methylene-ATP (α,β -meATP, 50 μM , Neelands et al. 2003; Davies and Robson 2010) and 2'(3')-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate (Bz-ATP, 50 μM , Davies and Robson 2010) also decreased osmotic fragility ($n = 6$ and $P < 0.05$ for both, Fig. 1b). However, the P2Y agonists 2-methylthio-ATP (2-meS-ATP, 50 μM) and UTP (10 μM), as well as the P1 agonist adenosine (10 μM ; Burnstock 2009; Pafundo et al. 2004), had no significant effect ($n = 6$ for each, not shown).

As stated earlier, RVD in this cell type requires the influx of extracellular Ca^{2+} (Elperin et al. 2011). With this in mind, we next examined whether osmotic fragility depended on the formation of AA via the activation of the Ca^{2+} -activated enzyme PLA₂ (Jenkins et al. 2009; Lee 2009). To this end, we used the antagonist ON-RS-082 (10 μM ; Doody et al. 2009). In this case, the OD decreased from 0.071 ± 0.002 to 0.039 ± 0.003 , giving a hemolytic index of 44% ($n = 10$, $P < 0.001$, Fig. 2). A similar response was observed with the PLA₂ antagonist chlorpromazine (10 μM), which gave a hemolytic index of 38% ($n = 8$, $P < 0.001$, Fig. 2). In contrast, AA (10 μM) had the opposite effect on fragility, and increased OD from 0.070 ± 0.002 to 0.078 ± 0.003 ($n = 6$, $P < 0.05$, Fig. 2).

Having established the involvement of PLA₂, we next determined whether osmotic fragility depended on AA directly, or alternatively whether eicosanoid metabolites were responsible for modulating fragility. This was accomplished using ETYA, a nonhydrolyzable acetylenic analog of AA that inhibits cyclooxygenase, lipoxygenase, and cytochrome P-450 monooxygenase enzymes (Danthi

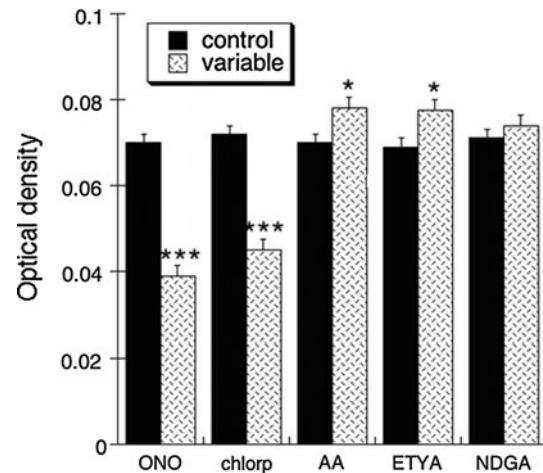


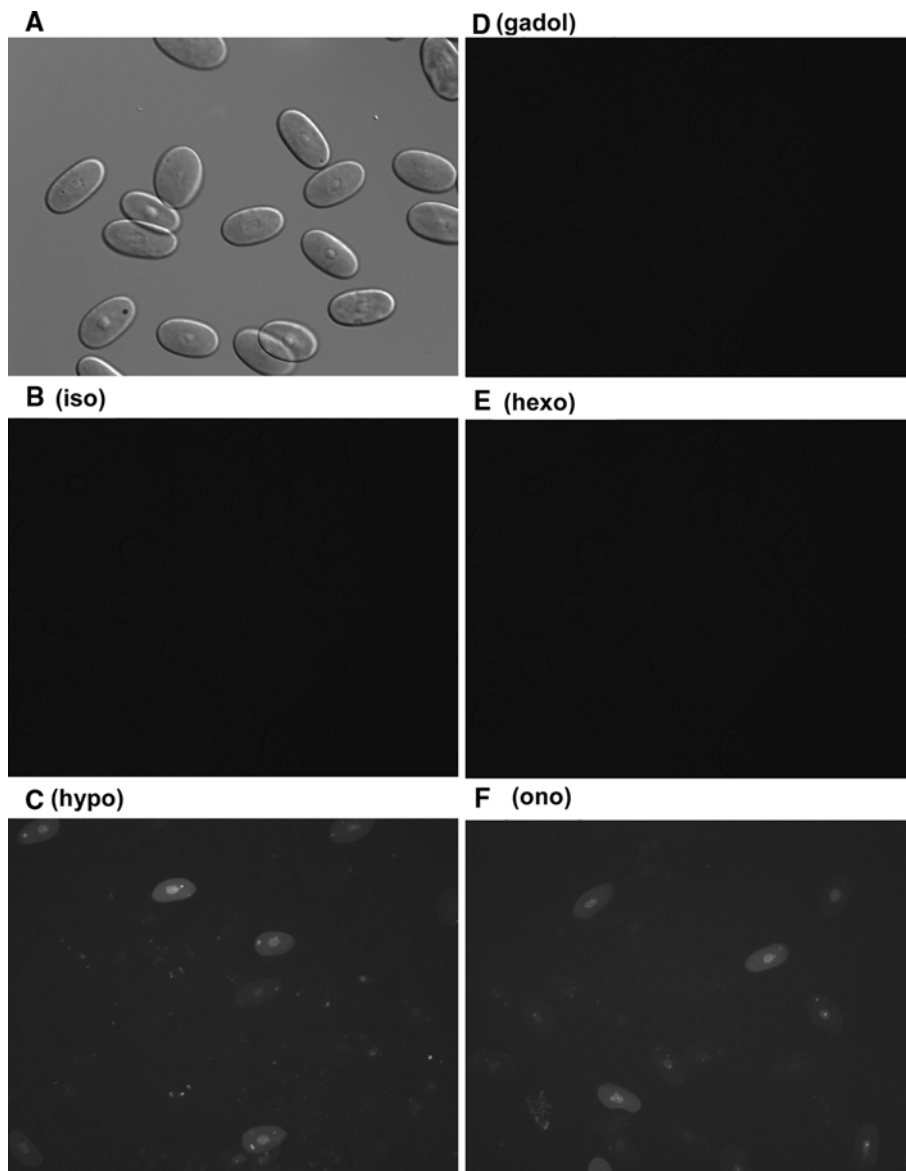
Fig. 2 Inhibition of PLA₂ increased osmotic fragility. The control (solid bar) was diluted Ringer (85 mosm/kg H₂O). The PLA₂ inhibitors ONO-RS-082 (ONO, 10 μM , $n = 10$) and chlorpromazine (chlorp, 10 μM , $n = 8$) increased osmotic fragility. In contrast arachidonic acid (AA, 10 μM) and its nonhydrolyzable analog ETYA (10 μM) decreased fragility ($n = 6$ for each). In addition, the lipoxygenase inhibitor NDGA (10 μM , $n = 6$) had no effect. Values are means \pm SEM of optical density. * $P < 0.05$, *** $P < 0.001$

et al. 2003; Lee 2009; Tinel et al. 2000). As illustrated in Fig. 2, ETYA (10 μM) also reduced osmotic fragility ($n = 6$). In contrast, nordihydroguaiaretic acid (NDGA, 10 μM), a selective inhibitor of lipoxygenase (LOX; Lee 2009; Ollivier et al. 2006b) had no significant effect ($n = 6$, Fig. 2). Similarly, the selective cyclooxygenase (COX) inhibitor indomethacin (10 μM ; Lee 2009) had no effect on osmotic fragility ($n = 6$, not shown).

Fluorescence Microscopy Studies

We previously demonstrated that there is an increase in cytosolic Ca^{2+} after hypotonic shock and this effect is not observed in a low Ca^{2+} -EGTA Ringer (Elperin et al. 2011). Accordingly, we next determined whether antagonists of P2 receptors and PLA₂ would influence the level of intracellular Ca^{2+} during cell swelling. A photograph of cells in isosmotic Ringer under DIC optics is shown in Fig. 3a. As illustrated in Fig. 3b, cells loaded with fluo-4-AM (10 μM) did not display fluorescence under isosmotic conditions ($n = 10$). In contrast, exposure of cells to a hypotonic (0.5 \times) Ringer increased the level of fluorescence, indicating a rise in intracellular Ca^{2+} occurred in swollen cells ($n = 6$, Fig. 3c). However, exposing cells to a hypotonic (0.5 \times) Ringer containing gadolinium (50 μM , Fig. 3d), hexokinase (2.5 U/ml, Fig. 3e), or suramin (100 μM , not shown) prevented the swelling-induced fluorescence ($n = 6$ for each). On the other hand, ONO-RS-082 (10 μM , $n = 6$, Fig. 3f) had no demonstrable effect on fluorescence after hypotonic challenge, indicating that inhibition of PLA₂ occurred downstream to Ca^{2+} influx.

Fig. 3 Inhibition of P2 receptors prevented a swelling-induced increase in intracellular Ca²⁺. Alligator RBCs were loaded with fluo-4 (10 μM) and exposed to UV light emitted from a mercury vapor bulb, filtered through a FITC cube (400×). **a** Cells in isosmotic Ringer visualized with DIC optics (cells were ~21 μm × 11 μm or ~390 fl). **b** Cells did not display fluorescence under isosmotic conditions (iso, *n* = 10). **c** Hypotonic shock (0.5× Ringer) increased fluorescence compared to basal conditions (hypo, *n* = 6). **d, e** Hypotonic shock with gadolinium (gadol, 50 μM) or hexokinase (hexo, 2.5 U/ml) in the extracellular medium prevented demonstrable fluorescence (*n* = 6 for each). **f** The PLA₂ inhibitor ONO-RS-082 (ono, 10 μM) had no effect on fluorescence after hypotonic challenge (*n* = 6)



Cell Volume Studies

When RBCs were placed in a hypotonic (0.5×) Ringer, they quickly swelled, and then slowly and spontaneously decreased in volume, giving a percentage volume recovery of 50% (Fig. 4a). In contrast, gadolinium (50 μM) completely inhibited volume recovery after hypotonic shock. In this case, the percentage recovery was -11%, indicating the cells had actually continued to swell further (*n* = 10, *P* < 0.001 at >5 min, Fig. 4a). However, the percentage recovery with gramicidin (1 μM), in the presence of Gd³⁺ (50 μM), at 90 min was 105% (*n* = 10, *P* < 0.001, Fig. 4a; gramicidin was added 1 min after hypotonic shock when maximum cell volume was observed).

Volume regulation also was inhibited with hexokinase (2.5 U/ml). In this case, although the recovery for control

cells at 70 min after hypotonic shock was 41%, and it was only 7% with hexokinase (*n* = 10, *P* < 0.01 > 30 min, Fig. 4b). Apyrase (5 U/ml), a diphosphohydrolase that hydrolyzes ATP into AMP and two orthophosphate anions (Dezaki et al. 2000; Ollivier et al. 2006a), had a similar effect (*n* = 6, not shown). As expected from the osmotic fragility studies, inhibition with hexokinase was prevented with gramicidin (1 μM); in this case, the percentage volume recovery at 70 min was 97% (*n* = 10, Fig. 4b). Interestingly, the effect of gadolinium (50 μM) and hexokinase (2.5 U/ml) were not additive (*n* = 6, not shown), and hexokinase (2.5 U/ml) was ineffective in the absence of glucose (*n* = 6, not shown).

As predicted from the osmotic fragility studies, P2 receptor antagonists inhibited volume recovery. Suramin (100 μM) reduced the percentage recovery from 41% for

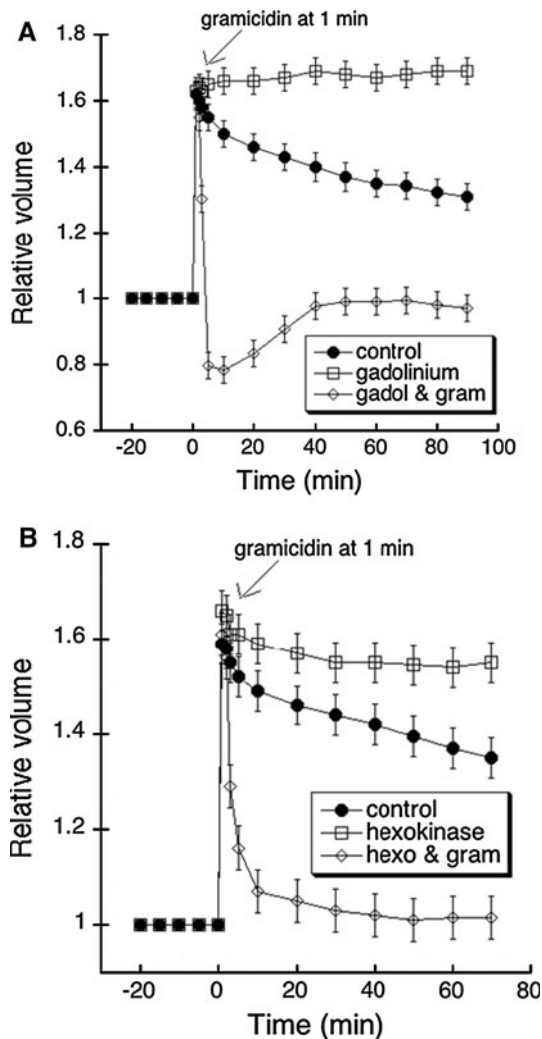


Fig. 4 Effect of a mechanosensitive channel antagonist and an ATP scavenger on volume recovery. At time 0, cells were abruptly exposed to a hypotonic ($\sim 0.5\times$) Ringer, which caused a rapid initial increase in volume followed by a gradual recovery toward basal values, despite the continued presence of a hypotonic medium. **a** Gadolinium ($50\ \mu\text{M}$) completely blocked volume recovery ($n = 10$) and this effect was prevented with gramicidin ($1\ \mu\text{M}$, added at 1 min after hypotonic shock, choline was substituted for Na^+ when conducting experiments with gramicidin). **b** The rate of volume recovery was reduced when cells were exposed to hexokinase ($2.5\ \text{U/ml}$, $n = 10$). Gramicidin ($1\ \mu\text{M}$, added at 1 min after hypotonic shock, potentiated volume recovery, even in the presence of hexokinase ($n = 10$). Values are means \pm SEM

the control to -21% with antagonist at 70 min ($n = 8$, $P < 0.001 > 5$ min, Fig. 5a). Similarly, at 70 min PPADS ($50\ \mu\text{M}$) changed the percentage recovery from 45% for the control to 11% with antagonist ($n = 6$, $P < 0.001 > 10$ min, Fig. 5b), and TNP-ATP ($10\ \mu\text{M}$) reduced the percentage recovery at 70 min from 45% to 7% ($n = 6$, $P < 0.001 > 10$ min, Fig. 5b). However, A-317491 ($1\ \mu\text{M}$) and MRS-2578 ($10\ \mu\text{M}$) had no effect ($n = 6$ for each, not shown). In contrast, ATP γ S ($10\ \mu\text{M}$) enhanced volume

recovery, which at 70 min was 48% for the control and 70% with nucleotide ($n = 6$, $P < 0.05 > 30$ min, Fig. 5a); and as expected for a poorly hydrolyzable analog, ATP γ S ($10\ \mu\text{M}$) prevented the inhibitory effect of hexokinase ($2.5\ \text{U/ml}$, $n = 6$, not shown). In addition, ATP γ S ($10\ \mu\text{M}$) with Gd^{3+} ($50\ \mu\text{M}$) was not significantly different from the control ($n = 6$, Fig. 6a). Further, α,β -meATP ($50\ \mu\text{M}$) potentiated volume recovery, which at 70 min was 42% for the control and 68% with agonist ($n = 6$, $P < 0.05 > 30$ min, Fig. 6b). Similarly, Bz-ATP ($50\ \mu\text{M}$) potentiated volume recovery to 59% at 70 min ($n = 6$, $P < 0.05 > 30$ min, Fig. 6b). In contrast, 2-meS-ATP ($50\ \mu\text{M}$, $n = 6$, Fig. 6b) and UTP ($10\ \mu\text{M}$, $n = 4$, not shown) had no significant effect.

We next examined the effect of several PLA₂ antagonists on volume recovery. At 90 min after hypotonic challenge, ONO-RS-082 ($10\ \mu\text{M}$) reduced the percentage recovery from 57% for the control to only 19% with drug ($n = 10$, $P < 0.001 > 30$ min, Fig. 7a). In addition, gramicidin ($1\ \mu\text{M}$) reversed the inhibitory effect of ONO-RS-082, giving a percentage recovery of 95% at 90 min after hypotonic shock ($n = 10$, Fig. 7a). Chlorpromazine ($10\ \mu\text{M}$) also had an inhibitory effect, and at 60 min changed the percentage recovery from 52% for the control to -18% with inhibitor ($n = 6$, $P < 0.001 > 1$ min, Fig. 7b). Further, the PLA₂ inhibitor isotretandrine ($10\ \mu\text{M}$) reduced volume recovery. At 60 min, percentage recovery for the control was 40%, but only 13% with antagonist ($n = 6$, $P < 0.01 > 20$ min, Fig. 7c). A similar response was seen with the PLA₂ inhibitor PACOCF₃ ($10\ \mu\text{M}$, $n = 6$, not shown).

In contrast to the effect of PLA₂ antagonists, addition of AA ($10\ \mu\text{M}$) caused a modest but significant potentiation of volume decrease. In this case, the percentage recovery for the control at 60 min was 45%, but was 62% with AA ($n = 6$, $P < 0.05 > 40$ min, Fig. 8a). We next determined whether antagonists of eicosanoid production would affect volume recovery. To accomplish this, we used ETYA ($10\ \mu\text{M}$), which stimulated volume recovery similar to AA: 50% for the control and 69% with agonist at 90 min ($n = 6$, $P < 0.05 > 50$ min, Fig. 8b). In addition, NDGA ($10\ \mu\text{M}$, $n = 6$) and indomethacin ($10\ \mu\text{M}$, $n = 6$) had no significant effect (not shown).

Having established that activation of a P2 receptor and PLA₂ stimulated volume recovery, we looked at a potential connection between ATP γ S, AA, and extracellular Ca^{2+} . As illustrated in Fig. 8c, ATP γ S ($10\ \mu\text{M}$) did not prevent the inhibitory action of bathing cells in a low Ca^{2+} -EGTA Ringer ($n = 6$). In contrast, AA ($10\ \mu\text{M}$) in a low Ca^{2+} Ringer was not significantly different from the control ($n = 6$, Fig. 8c). Finally, we examined the effect of extracellular agonists on cells bathed in an isosmotic Ringer. As illustrated in Fig. 9, there was a significant

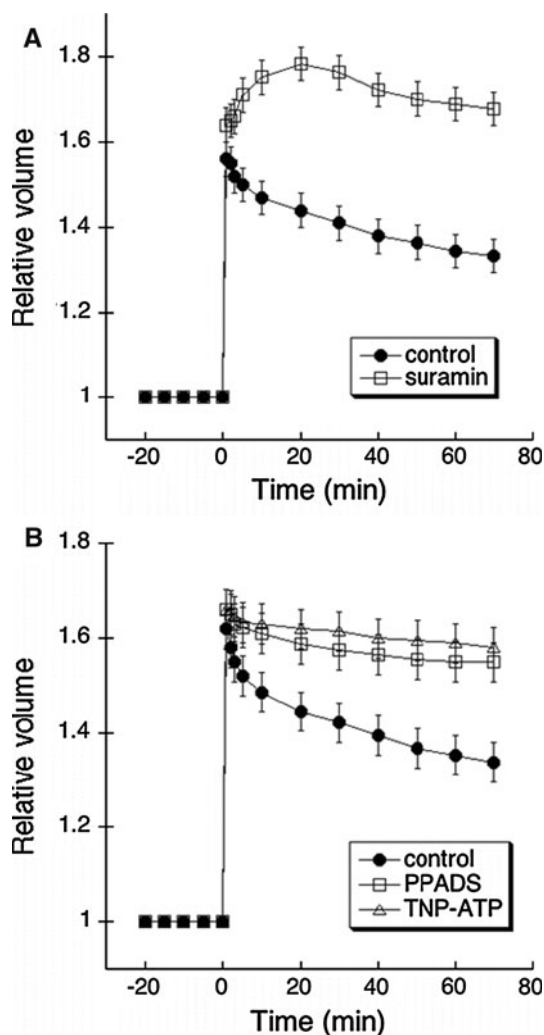


Fig. 5 Inhibition of P2 receptors blocked volume recovery. At time 0, cells were exposed to a hypotonic ($\sim 0.5\times$) Ringer. **a** The P2 receptor antagonist suramin (100 μM) inhibited volume recovery ($n = 8$). **b** Similarly, PPADS (50 μM) and TNP-ATP (10 μM) blocked volume recovery ($n = 6$ for each). Values are means \pm SEM

reduction in volume after the addition of ATP γ S (10 μM , $n = 6$). Similarly, AA (10 μM) caused cell volume to decrease ($n = 6$, Fig. 9).

Discussion

A major finding of this study was that a P2-like receptor regulates, at least in part, cell volume recovery in alligator RBCs when exposed to a hypotonic medium. Key evidence for this was obtained from a series of experiments using pharmacological agents that are known to alter levels of extracellular ATP or modulate the activity of P2 receptors. For example, endogenous extracellular ATP levels were presumably reduced with hexokinase, which increased osmotic fragility and inhibited volume recovery, as well as

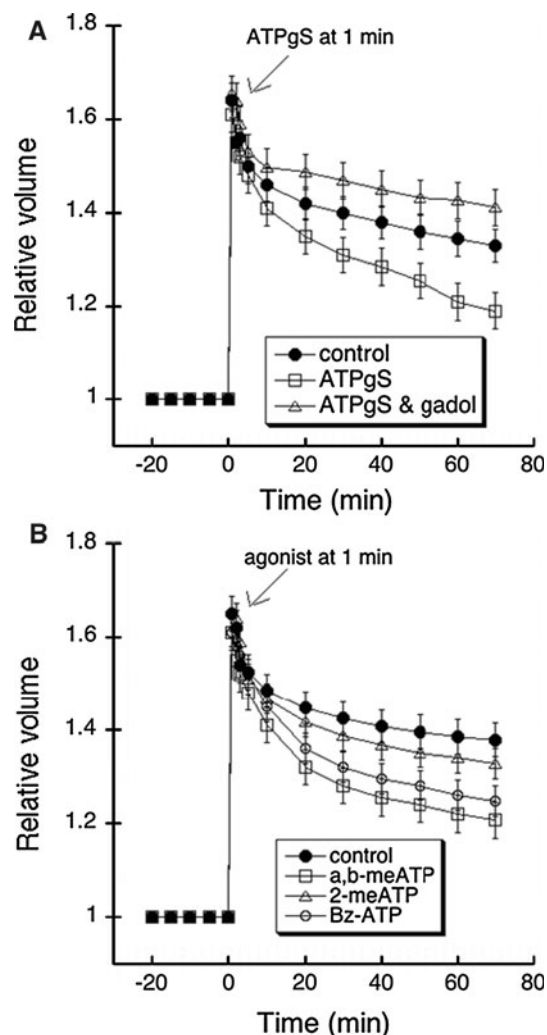
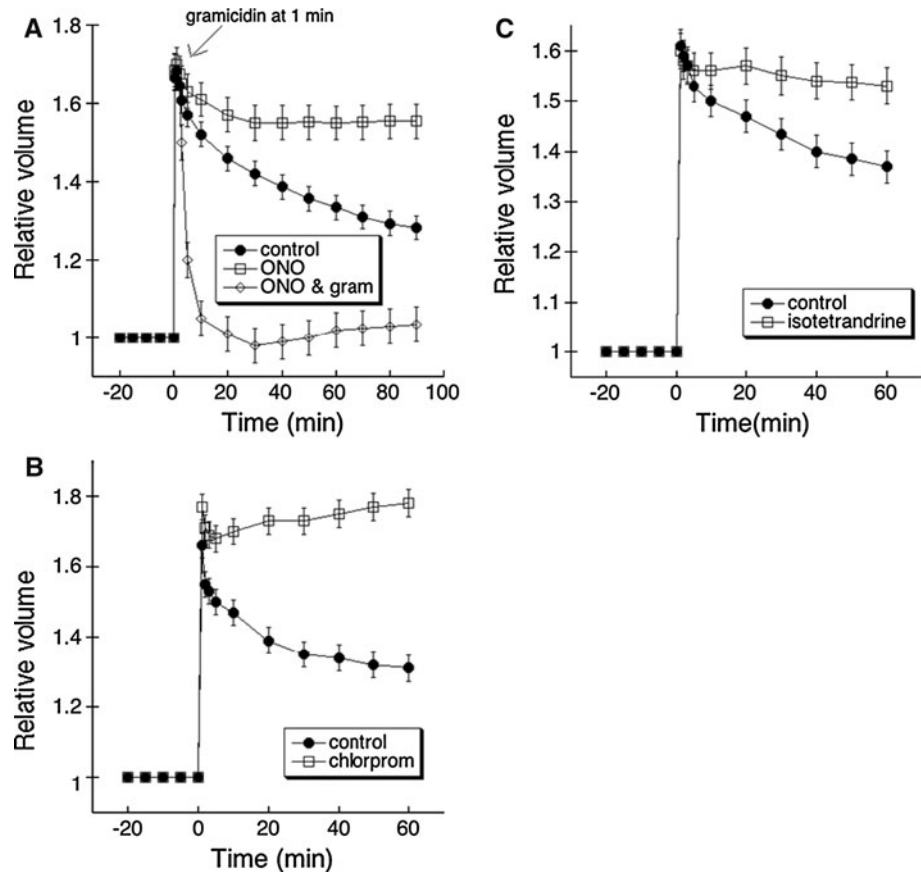


Fig. 6 Volume recovery was potentiated by the activation of a P2 receptor. At time 0, cells were exposed to a hypotonic ($\sim 0.5\times$) Ringer. **a** Application of ATP γ S (10 μM) potentiated volume recovery ($n = 6$), and ATP γ S (10 μM) with Gd $^{3+}$ (50 μM) was not significantly different from the control ($n = 6$). **b** The P2X agonists α,β -meth-ATP (a,b-meATP, 50 μM) and Bz-ATP (50 μM) potentiated volume recovery, whereas the P2Y agonist 2-meth-ATP (2-meATP, 50 μM) had no effect ($n = 6$ for each). Values are means \pm SEM

prevented a rise in cytosolic Ca $^{2+}$ that accompanies cell swelling. It is unlikely that hexokinase acted in a nonspecific manner because it was ineffective in the absence of glucose or in the presence of ATP γ S, and apyrase had a similar inhibitory effect on volume recovery.

In contrast, addition of extracellular ATP γ S, α,β -meATP, and Bz-ATP had the opposite effect of hexokinase. Further, ATP γ S caused cells to shrink under isosmotic conditions, presumably by eliciting a change that mimicked the response that normally occurs when cells are stimulated with hypotonic shock. Furthermore, the P2 receptor antagonists suramin, PPADS, and TNP-ATP increased osmotic

Fig. 7 Volume recovery depended on the activation of PLA₂. At time 0, cells were exposed to a hypotonic (0.5×) Ringer. **a** The rate of cell volume recovery was reduced when cells were treated with ONO-RS-082 (10 μM, *n* = 10), and this effect was prevented with the addition of gramicidin (1 μM, added 1 min after hypotonic shock, *n* = 10). **b**, **c** The PLA₂ antagonists chlorpromazine (10 μM) and isotretandrine (10 μM) also blocked volume recovery (*n* = 6 for each). Values are means ± SEM



fragility and inhibited volume recovery, whereas the P1 receptor agonist adenosine had no effect. Although we did not measure levels of endogenous extracellular ATP, the concentration of pharmacological agents we added was similar to those used by others (Dezaki et al. 2000; Jarvis et al. 2002; Neelands et al. 2003; Pafundo et al. 2004; Nakamura et al. 2011). Thus, taken together, our observations demonstrate a role for a P2-like receptor in the regulation of cell volume.

Interestingly, there was a demonstrable increase in relative volume elicited by suramin that was not observed with PPADS or TNP-ATP. This suggests that suramin had a nonselective effect on solute flux. Related to this, it has been shown that suramin can be a ryanodine receptor agonist, and this receptor, a Ca²⁺ channel in some tissues, can be responsible for Ca²⁺-induced calcium release (Wolner et al. 2005). However, given that we found suramin prevented a rise in cytosolic Ca²⁺ associated with cell swelling, it doesn't appear that it activated a Ca²⁺ conductance in alligator RBCs.

Our experimental protocols and their effect on volume regulation are consistent with reports by others. For example, Ollivier et al. (2006a) report that RVD is partially abolished in fish hepatocytes with apyrase or suramin. In addition, Dezaki et al. (2000) found apyrase and suramin

partially inhibit volume recovery in a human epithelial cell line, whereas application of extracellular ATP accelerates the rate of RVD in a concentration-dependent manner. On the basis of pharmacological evidence using various nucleotides and their analogs, they concluded that RVD is facilitated by release of ATP upon cell swelling and this nucleotide stimulates a P2Y₂ receptor. Using similar protocols with cultured endothelial cells, Shinozuka et al. (2001) concluded that RVD also is mediated by the activation of a P2Y receptor. Varela et al. (2010) found that hypotonic swelling induces ATP release in rat hepatoma cells, and they found that this nucleotide modulates the time course of a Cl⁻ current via activation of a P2X₄ receptor. Concerning our study with alligator RBCs, the specific purinoceptor subtype remains to be elucidated. However, a potentiation of RVD with ATPγS, α,β-meATP and Bz-ATP, but not 2-meS-ATP and UTP, as well as inhibition of RVD by PPADS and TNP-ATP, is suggestive of a receptor with P2X-like properties (Gever et al. 2006; Crumling et al. 2009; Lambrecht 2000; Sugihara et al. 2011; Nakamura et al. 2011). Similarly, Davies and Robson (2010) report a P2X-like Ca²⁺-permeable cation conductance in frog proximal tubule cells that is activated with ATP and α,β-meATP. They also found that suramin, PPADS, and TNP-ATP inhibited both this conductance and RVD.

Fig. 8 Potentiation of volume recovery with AA. At time 0, cells were exposed to a hypotonic (0.5×) Ringer. **a**, **b** Exposure of cells to AA (10 μM) or ETYA (10 μM) caused a small but significant increase in the rate of volume recovery ($n = 6$ for each). **c** ATP_γS (10 μM) did not prevent the inhibitory action of bathing cells in a low Ca²⁺-EGTA Ringer (10 nM free Ca²⁺, $n = 6$). In contrast, AA (10 μM) in a low Ca²⁺ Ringer was not significantly different from the control ($n = 6$). Values are means ± SEM

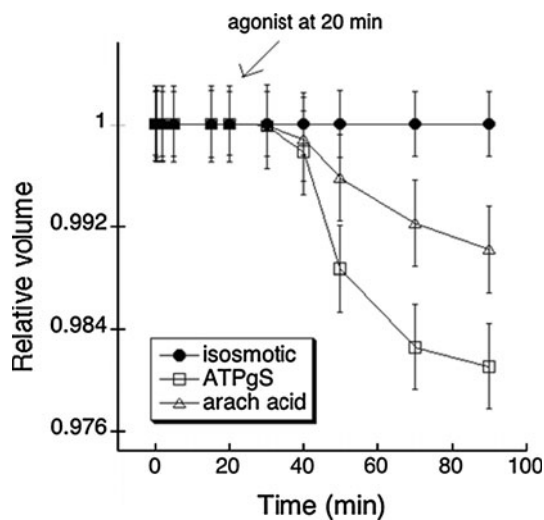
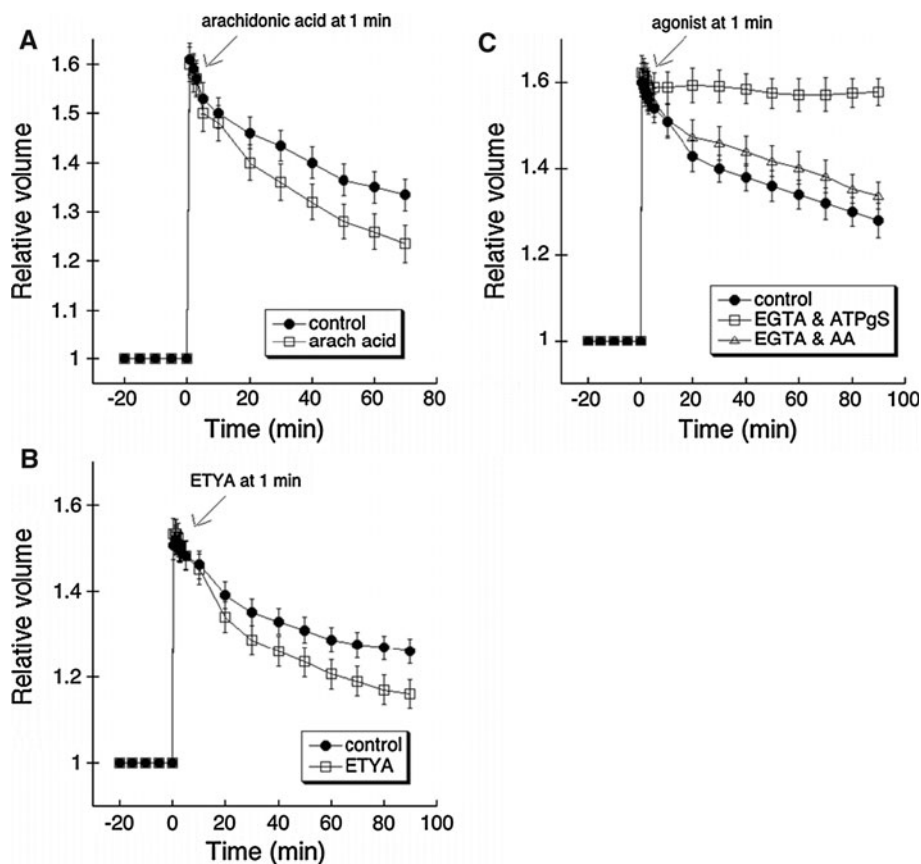


Fig. 9 Agonist-induced volume-decrease in an isosmotic Ringer. Addition of ATP_γS (10 μM) or AA (10 μM) to an isosmotic Ringer caused cells under basal conditions to shrink ($n = 6$ for each). Values are means ± SEM

Given our finding that a P2 receptor is important for modulating cell volume, it is reasonable to consider the source of endogenous external ATP that accompanies cell swelling. Although this nucleotide was not a component of our reptilian Ringer, there are a number of reports that ATP

can be released from RBCs during mechanical deformation (Ellsworth et al. 2008), and ATP is consistently measured in the circulation of humans and experimental animals (Gonzalez-Alonso et al. 2002). In fact, there is evidence supporting a controlled release of ATP from RBCs in response to mechanical deformation, which involves the heterotrimeric G protein Gi, adenylyl cyclase, cAMP, phosphodiesterase 3, protein kinase A, the cystic fibrosis transmembrane conductance regulator (CFTR), and pannexin 1 (Braunstein et al. 2001; Olearczyk et al. 2004; Lovcovei et al. 2006). Whether this mechanism exists in alligator RBCs remains to be elucidated.

We also found gadolinium prevented a swelling-induced rise in intracellular Ca²⁺ and blocked cell volume recovery. Others report a similar response in several different cell types (Boudreault and Grygorczyk 2002; Hua et al. 2010). One explanation for our results is that Ca²⁺ influx occurred via a stretch-activated channel. However, the inhibitory effect of Gd³⁺ was not additive with hexokinase, as could happen if they blocked mechanisms acting in parallel. In addition, the inhibitory action of Gd³⁺ was absent in the presence of ATP_γS, suggesting the two influenced processes working in series. Related to this, it has been shown that Gd³⁺ can bind to class C-type G-protein coupled receptors, including the Ca²⁺-sensing

receptor, thereby modifying intracellular signaling (Brown et al. 1993). This is relevant to our study because, as stated above, such a G protein is associated with deformation-induced ATP release (Olearczyk et al. 2004; Locovei et al. 2006). In addition, Boudreault and Grygorczyk (2002) report that Gd³⁺ has an indirect and nonspecific effect on lipid membrane and membrane-dependent processes, and concluded that this lanthanide inhibits a Ca²⁺-dependent exocytosis that plays a major role in mechanosensitive ATP release. Regardless of the mechanism for ATP release (conductive pathway or exocytosis), our results are consistent with Gd³⁺ inhibition of ATP efflux.

Related to the above discussion, we also demonstrated that a P2 receptor is important for the influx of Ca²⁺ during cell swelling. Evidence for this conclusion included a lack of fluorescence when hexokinase or suramin was present in the extracellular medium. In addition, ATP_γS did not prevent the inhibitory effect of a low Ca²⁺-EGTA Ringer. Taken together, our observations demonstrate that the Ca²⁺ influx step was “downstream” to P2 receptor activation. This conclusion has support from other studies. For example, Shinozuka et al. (2001) report that a swelling-induced increase in intracellular Ca²⁺ in cultured endothelial cells is prevented with the P2 receptor antagonist PPADS. They concluded that ATP participates in RVD via a P2 receptor-stimulated increase in cytosolic Ca²⁺. In addition, using a human epithelial cell line, Dezaki et al. (2000) found apyrase and suramin partially suppress a swelling-induced rise in intracellular Ca²⁺, whereas application of extracellular ATP causes an increase in cytosolic Ca²⁺. However, Ollivier et al. (2006a) found that chelation of extracellular Ca²⁺ with EGTA in fish hepatocytes abolishes ATP release under hypo-osmotic conditions, and the calcium ionophore ionomycin elicits a rapid efflux of ATP in an iso-osmotic medium.

We also demonstrated indirectly that ATP enhanced RVD by stimulating a K⁺ permeability pathway. This was shown pharmacologically using the cationophore gramicidin in a choline Ringer. With this solution, K⁺ and Cl⁻ were the only two permeable ions of significance, and addition of gramicidin ensured a continued high K⁺ permeability. We found gramicidin consistently reversed the inhibitory effect of hexokinase. These results, along with a previous finding that gramicidin causes alligator RBCs to shrink under isosmotic conditions and K⁺ efflux is a rate-limiting step for volume recovery (Elperin et al. 2011), are consistent with this cell type having a low K⁺ permeability under basal conditions and an elevated K⁺ permeability during hypotonic stress that is stimulated via the activation of a P2 receptor. Interestingly, in the presence of Gd³⁺, gramicidin caused cells to decrease in volume well below basal levels, which was then followed by RVI, and this phenomenon was not seen to the same extent with

hexokinase or ONO-RS-082. Because the initial level of cell shrinkage would depend on the concentration of gramicidin that intercalated into the plasma membrane and Gd³⁺ is known to have nonspecific effects (Boudreault and Grygorczyk 2002), it is conceivable the amount of gramicidin incorporated into RBCs was influenced by the presence of pharmacological agents.

Another major finding of this study was that RVD depended upon the formation of AA. This was demonstrated by exposing cells to several different inhibitors of PLA₂. In addition, application of AA potentiated volume recovery and also caused cell shrinkage in an isosmotic bath. Our results also indicate that metabolites of AA were not necessary for RVD. This was shown by using ETYA, a nonhydrolyzable analog of AA, which blocks the formation of eicosanoids (Danthi et al. 2003; Lee 2009; Tinel et al. 2000). Further, NDGA and indomethacin had no demonstrable effect. Taken together, our results are consistent with AA acting directly to modulate cell volume.

Others have reported membrane transport being affected by AA and its metabolites. For instance, Lee (2009) found exogenous application of AA or ETYA induces K⁺ efflux in a dose-dependent manner in human hepatoblastoma cells. In addition, this flux is suppressed when PLA₂ is inhibited, but not by treatment with NDGA or indomethacin. Further, using whole-cell patch-clamp recordings on bovine adrenal cells, Danthi et al. (2003) found AA increases the activity of bTREK-1 K⁺ channels. Because ETYA failed to inhibit activation of these channels, they concluded that AA acts directly.

Interestingly, and unlike ATP_γS, we found AA was effective at enhancing RVD in a low Ca²⁺-EGTA Ringer. In addition, inhibition of PLA₂ did not prevent a rise in cytosolic Ca²⁺ in response to hypotonic shock. These observations are consistent with Ca²⁺ being necessary to activate PLA₂ (Jenkins et al. 2009; Lee 2009) and that the action of AA was downstream to a rise in intracellular Ca²⁺. Others have reported similar findings using a variety of cell types, including isolated crypts of mouse distal colon (Mignen et al. 1999), turbot hepatocytes (Ollivier et al. 2006b), and Ehrlich ascites tumor cells (Hoffmann et al. 2009). However, Tinel et al. (2000) found that ETYA reduces the calcium response to hypotonic stress in rat inner medullary collecting duct cells, indicating the signaling process for a rise in intracellular Ca²⁺ involves AA metabolism.

In conclusion, our results demonstrate that alligator RBCs have a well-developed RVD, which is consistent with our original hypothesis. In particular, this process was mediated, at least in part, by activation of a P2-like receptor. This, in turn, stimulated Ca²⁺ influx, thereby activating PLA₂ and forming AA, which resulted in volume recovery by increasing the K⁺ permeability.

Additional investigations are needed to characterize the P2 receptor and also to further elucidate the Ca²⁺-dependent processes. Nonetheless, our results contribute to an increased understanding of volume regulation, especially for reptilian cells, and further demonstrate the myriad of circumstances whereby cells regulate membrane transport to cope with osmotic challenges.

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