Extracellular ATP stimulates volume decrease in Necturus red blood cells

Douglas B. Light, Tracy L. Capes, Rachel T. Gronau, and Matthew R. Adler

Department of Biology, Ripon College, Ripon, Wisconsin 54971

Extracellular ATP stimulates volume decrease in Necturus red blood cells. Am. J. Physiol. 277 (Cell Physiol. 46): C480–C491, 1999.—This study examined whether extracellular ATP stimulates regulatory volume decrease (RVD) in Necturus maculosus (mudpuppy) red blood cells (RBCs). The hemolytic index (a measure of osmotic fragility) decreased with extracellular ATP (50 µM). In contrast, the ATP scavenger hexokinase (2.5 U/ml, 1 mM glucose) increased osmotic fragility. In addition, the ATP-dependent potassium channel antagonist glibenclamide (100 µM) increased the hemolytic index, and this inhibition was reversed with ATP (50 µM). We also measured cell volume recovery in response to hypotonic shock electronically with a Coulter counter. Extracellular ATP (50 µM) enhanced cell volume decrease in a hypotonic (0.5×) Ringer solution. In contrast, hexokinase (2.5 U/ml) and apyrase (an ATP diphosphohydrolase, 2.5 U/ml) inhibited cell volume recovery. The inhibitory effect of hexokinase was reversed with the Ca²⁺ ionophore A-23187 (1 µM); it also was reversed with the cationophore gramicidin (5 µM in a choline-Ringer solution), indicating that ATP was linked to K⁺ efflux. In addition, glibenclamide (100 µM) and gadinolium (10 µM) inhibited cell volume decrease, and the effect of these agents was reversed with ATP (50 µM) and A-23187 (1 µM). Using the whole cell patch-clamp technique, we found that ATP (50 µM) stimulated a whole cell current under isosmotic conditions. In addition, apyrase (2.5 U/ml), glibenclamide (100 µM), and gadinolium (10 µM) inhibited whole cell currents that were activated during hypotonic swelling. The inhibitory effect of apyrase was reversed with the nonhydrolyzable analog adenosine 5′-O-(3-thiotriphosphate) (50 µM), and the effect of glibenclamide or gadinolium was reversed with ATP (50 µM). Finally, anionic whole cell currents were activated with hypotonic swelling when ATP was the only significant charge carrier, suggesting that increases in cell volume led to ATP efflux through a conductive pathway. Taken together, these results indicate that extracellular ATP stimulated cell volume decrease via a Ca²⁺-dependent step that led to K⁺ efflux.

volume regulation; patch clamp; potassium channel; hexokinase; calcium

The ability of animal cells to regulate their volume is a fundamental property common to a large number of cell types (11, 20, 22, 24–26) and has been extensively reviewed (8, 12, 21, 23, 29). Volume regulation is of importance in cells exposed to anisotonic extracellular media and in cells where transport of solutes could change intracellular osmolality. Exposure of vertebrate cells to a hypotonic solution results in an initial increase in cell volume due to the relatively rapid influx of water. During continuous hypotonic stress, increases in cell volume are then 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 decreasing the driving force for water influx (8, 12, 21, 23, 29). Most vertebrate cells lose K⁺ and Cl⁻ during RVD (8, 12, 21, 23, 29). This may occur by electrophysiological ion transport pathways (21) or by the separate activation of K⁺ and anion channels (8, 11, 21, 26, 35). Loss of organic anions and osmolytes also may occur during RVD (18, 29).

The cellular mechanisms that activate and regulate permeability pathways during RVD are not completely understood and appear to differ between cell types. For example, in some instances, the activation mechanism for an RVD response is Ca²⁺ independent (16, 20, 24). In contrast, Ca²⁺ appears to play a role during cell volume regulation in several cell types (4, 22, 23, 25, 38). In addition, although it has been suggested that Ca²⁺ directly activates ion channels during RVD (11, 23, 35), there also is evidence that several Ca²⁺-dependent intracellular messengers and enzymes (e.g., calcmodulin, phospholipase A₂, 5-lipoxygenase, and protein kinase C) are involved with cell volume regulation (12, 21–23).

It is well known that ATP is an ubiquitous intracellular source of energy. However, over 25 years ago it was proposed that ATP acts as a transmitter substance at autonomic neuromuscular junctions (3). Since then, there has been a growing body of evidence indicating that extracellular ATP plays a significant role in a number of other biological processes (6, 9, 17, 36, 37). For example, extracellular ATP has been implicated in the control of fluid secretion by salivary gland cells (28), ion and water balance of cochlear fluids (32), secretion of histamine by mast cells (9), vasodilation of coronary blood vessels (9), and production of prostacyclin (9). Extracellular ATP also has been shown to stimulate cell volume regulation (33, 36), and a number of studies have demonstrated that extracellular nucleotides are important for regulating ion channels (1, 7, 28, 31, 32). Extracellular ATP exerts its influence by acting as an autocrine and paracrine signal, binding to specific cell surface receptors termed purinoceptors (7, 27, 32, 36, 37). Purinoceptors have been subdivided into two main categories: P₁ receptors, which recognize nucleosides, such as adenosine, and P₂ receptors, which bind ATP and other nucleotides (28, 32, 33, 37). The P₂ receptors have been further subdivided into two main groups: ATP-gated, Ca²⁺-permeable, nonselective channels (32,
and ATP-activated receptors coupled to a G protein (32, 37).

Despite recent reports concerning the physiology of extracellular ATP, there is a paucity of data on the role of ATP in RVD. Thus the potential connections between this nucleotide and cell volume regulation remain to be elucidated. In view of these uncertainties, the purpose of this study was to investigate whether extracellular ATP regulates K+ efflux during RVD in Necturus red blood cells (RBCs). The basis of this study also stemmed from our recent work in which we demonstrated that RVD in this cell type depends on a K+ conductance that is regulated during cell swelling by a Ca2+-dependent mechanism (22) and that extracellular ATP may elevate the intracellular free Ca2+ mechanism (22) and that extracellular ATP may el

end, we used three different approaches: 1) hemolysis studies to examine osmotic fragility, 2) a Coulter counter to measure the volume of osmotically stressed cells, and 3) the whole cell patch-clamp technique to study membrane currents.

METHODS

Animals. Mudpuppies (Necturus maculosus) were obtained from a local vendor (Lemberger, Oshkosh, WI) and kept in well-aerated, aged tap water at 5–10°C for ≤6 days before use. They were anesthetized with 3-aminobenzoic acid ethyl ester (MS-222, 1%) and killed by decapitation. Blood was obtained from a midventral incision and collected into tubes coated with heparin (10,000 U/ml). Immediately after exsanguination, the blood was spun in a centrifuge (Hermel-Z230, National Labnet, Woodbridge, NJ) at 1,000 rpm for 1 min. The supernatant was aspirated and replaced with an equal volume of amphibian Ringer solution. This process of spinning and washing the cells was repeated twice.

Osmotic fragility. Osmotic fragility was examined by determining the degree of cell lysis for a suspension of RBCs in hypotonic Ringer solution. The level of hemolysis was determined via a turbidity (cloudy-to-clear) shift that occurs when the integrity of the plasma membrane is compromised. This was detected with a spectrophotometer (Spectronic 20D, Milton Roy) 10, 15, or 20 min after blood (30–50 ml) was diluted Ringer solution, and the test compound was blood in distilled water. A stock solution of red blood cells in suspension were intact at this dilution.

A hemolytic index (HI percentile) was determined using the following formula: HI(%) = (OD of test compound – OD of negative control)/(OD of positive control – OD of negative control) × 100, where the positive control was blood in distilled water, the negative control referred to blood in diluted Ringer solution, and the test compound was blood in diluted Ringer solution containing a specific pharmacological agent. All reported HI values were calculated using a concentration for the negative control that gave an OD reading between 0.025 and 0.030. We chose this region of OD as a standard for comparison, because approximately one-half the cells in suspension were intact at this dilution.

Coulter counter. Cell volume distribution curves were obtained by electronic sizing with use of a Coulter counter (model Z2) with Channelizer (Coulter Electronics, Hialeah, FL). Mean cell volume was taken as the mean volume of the distribution curves. The diameter of the aperture tube orifice was 200 µm, and the metered volume was 0.5 ml. Absolute cell volumes were obtained using polystyrene latex beads (20.13 µm diameter or 4.271 × 103 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 4,000-fold with amphibian Ringer solution followed by a 1-fold dilution with distilled water to give a final cell density of ~5,000 cells/ml.

As described by others (16, 36), a percent volume recovery at x minutes after hypotonic exposure was calculated as follows: ([Vmax — Vmin]/[Vmax — V0]) × 100, where Vmax is the peak relative cell volume, V0 is the initial relative volume or 1, and Vmin is the relative cell volume measured x min after hypotonic exposure. We also used the peak relative volume for the control when assessing the effect of a pharmacological agent added at 0 min. A percent volume decrease was calculated as follows: (% recoveryexp%/recovery con) × 100, where recoveryexp and recovery con are experimental and control recovery, respectively, and maximal recovery in hypotonic Ringer solution is 100%.

Patch clamp. Patch pipettes were fabricated from Kovar sealing glass (Corning model 7052, 1.50 mm outside diameter, 1.10 mm inside diameter, Garner Glass, Claremont, CA) by means of a two-pull method (model PP-7, Narishige). Pipette tips were fire polished (model MF-9, Narishige) to give a direct-current resistance of ~5–8 MΩ in symmetrical 100 mM KCl solutions. All pipette solutions were filtered immediately before use with a 0.22-µm membrane filter (Miltonex-GS, Bedford, MA), and the pipettes were held in a polycarbonate holder (E. W. Wright, Guilford, CT). Membrane currents were measured with a 1012-Ω feedback resistor in a head stage (CV-201A, Axon Instruments, Foster City, CA) with a variable-gain amplifier set at 1 mV/pA (Axopatch 200A, Axon Instruments). The current signals were filtered at 1 kHz through a four-pole low-pass Bessel filter and digitized at 5 kHz with an IBM-486 computer.

Data were acquired and analyzed with P-Clamp (version 6, Axon Instruments). Data were acquired during 100-ms voltage pulses, and the command potential was set to −15 mV (close to the resting potential for RBCs) for 100 ms between each pulse. All voltage measurements refer to the cell interior.

RBCs, attached to glass coverslips (5 mm diameter, Bellco Biotechnology, Vineland, NJ) with poly-d-lysine (150,000–300,000; 1 mg/ml), were placed in a specially designed open-style chamber (250 µl volume, Warner Instruments, Hamden, CT). The bath solution could be changed by a six-way rotary valve (Rheodyne, Cotati, CA). The whole cell configuration was achieved after formation of a gigahm seal (cell-attached configuration) by applying suction to disrupt the patch of membrane beneath the pipette or by applying a large voltage (>200 mV) to the patch. A sudden increase in the capacitance current transient accompanied disruption of the membrane.

Solutions. Amphibian Ringer solution consisted of (in mM) 110 NaCl, 2.5 KCl, 1.8 CaCl2, 0.5 MgCl2, 5 glucose, and 10 HEPES (titrated to pH 7.4 with NaOH). A low Na+-Ringer solution was prepared by substituting choline chloride for NaCl (used for all experiments with gramicidin), and a 0.5× Ringer solution was obtained by mixing equal volumes of Ringer solution and distilled water. A stock solution of gramicidin was dissolved in methanol; stock solutions of A-23187 (Ca2+ ionophore calcium cyanide) and glibenclamide were prepared with DMSO. All nonaqueous stock solutions were mixed at 1,000× the final concentration and then diluted 1,000× to give an appropriate working concentration, thereby
diluting the vehicle an equivalent amount. All stock aqueous solutions (e.g., ATP, hexokinase, apyrase) were diluted 100× to give an appropriate final concentration.

Patch pipettes were filled with an intracellular Ringer solution containing (in mM) 100 KCl, 3.5 NaCl, 1.0 MgCl₂, 1.0 CaCl₂, 2.0 EGTA, 5 glucose, 1.0 Mg-ATP, 0.5 GTP, and 5.0 HEPES (titrated to pH 7.4 with KOH). During seal formation, the extracellular solution contained (in mM) 105 NaCl, 2.5 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 5 glucose, and 10.0 HEPES (pH 7.4). An isosmotic high-K⁺ bath contained (in mM) 105 KCl, 2.5 NaCl, 1.8 CaCl₂, 0.5 MgCl₂, 5 glucose, and 10.0 HEPES (pH 7.4). A hypotonic (0.5×) high-K⁺ bath contained (in mM) 2.5 NaCl, 50 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 5 glucose, and 10.0 HEPES (pH 7.4). Currents carried by ATP at physiological concentrations are below the limits of detection (36). Accordingly, we used abnormally high ATP concentrations in the bath and pipette solutions to examine the presence of a putative ATP conductance. An isosmotic ATP solution contained (in mM) 100 Tris-ATP, 1.0 CaCl₂, 1.0 MgCl₂, 5 glucose, and 10.0 HEPES (pH 7.4). An isosmotic high-K⁺-ATP solution contained (in mM) 100 Tris-ATP, 1.0 CaCl₂, 1.0 MgCl₂, 5 glucose, 10.0 HEPES (pH 7.4). A hypotonic ATP solution contained (in mM) 100 Tris-ATP, 1.0 CaCl₂, 1.0 MgCl₂, 5 glucose, and 10.0 HEPES (pH 7.4).

For hemolysis experiments, cells were incubated with a pharmacological agent or its vehicle for 1–10 min before experimentation. For cell volume studies, pharmacological agents were added with hypotonic exposure (0 min) or at peak cell volume (5 min after hypotonic stress). Osmolality of solutions was measured with a vapor pressure osmometer (model 5500, Wescor, Logan, UT). Chemicals were purchased from Sigma Chemical (St. Louis, MO), Alexis Biochemicals (San Diego, CA), and ICM (Costa Mesa, CA). All experiments were conducted at room temperature (21–23°C).

Statistics. Values are means ± SE. The statistical significance of an experimental procedure was determined by a paired Student’s t-test or least significant difference test with appropriate multiple comparisons. GraphPad Prism (San Diego, CA) was used for data analysis. Statistical significance was set at P < 0.05.

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 amphibian Ringer solution where ~50% of the cells in suspension were intact (20.7 ± 1.3 mosmol/kgH₂O), was 0.029 ± 0.002 (n = 7; n = 7 experiments; Fig. 1). To determine whether osmotic fragility depended on ATP, we repeated the hemolysis assay with this nucleotide at 50 µM in the extracellular medium. In this case, the OD measured at the same concentration for the control was 0.042 ± 0.004 (n = 7, P < 0.01; Fig. 1), indicating a 45% decrease in cell lysis compared with the control.

We next looked at the effect of the ATP scavenger hexokinase on osmotic fragility. Hexokinase is an enzyme that traps ATP by transferring its γ-phosphoryl group to a variety of C₆ sugars, such as to the hydroxyl group on C-6 of glucose (27). Hexokinase (2.5 U/ml, 1 mM glucose) decreased the OD from 0.025 ± 0.003 to 0.017 ± 0.003 (n = 6, P < 0.01; Fig. 1), giving an HI of 32%. In addition, we examined the effect of glibenclamide, an antagonist of ATP-dependent K⁺ channels, on osmotic fragility (10). This inhibitor (100 µM) decreased the OD from 0.027 ± 0.002 to 0.021 ± 0.002 (n = 6, P < 0.05; Fig. 1), which resulted in an HI of 22%. As illustrated in Fig. 1, ATP (50 µM) reversed the inhibitory effect of glibenclamide, increasing the OD to 0.025 ± 0.004, which was not significantly different from the control (n = 6).

As illustrated in Fig. 2A, the relative volume with ATP (50 µM) was significantly lower than the control for all measurements beyond 5 min (n = 7, P < 0.05 at ≥5 min). The percent volume decrease of the control was only 39% that of ATP at 30 min, whereas it was 73% that of ATP by 90 min. We also added ATP to the extracellular medium 5 min after hypotonic shock, when the cells were maximally swollen and when it appeared that endogenous K⁺ channels were activated. Even when added at this time, ATP (50 µM) still enhanced cell volume recovery (n = 6, P < 0.05 at ≥10 min). Interestingly, addition of ATP (50 µM) to human RBCs had no effect on the percent volume decrease for cells exposed to a hypotonic (0.67×) Ringer solution (n = 6, data not shown; human RBCs did not express a well-developed RVD response; nonetheless, on the basis of our studies with Necturus RBCs, we were interested in determining whether ATP also could influence volume in this cell type).

We previously demonstrated that volume decrease in Necturus RBCs is enhanced by a Ca²⁺-dependent process and that RVD depends on activation of a K⁺ conductance (2, 22). To further examine the importance of Ca²⁺ in cell volume recovery, we added the Ca²⁺ ionophore A-23187 to the extracellular medium 5 min after hypotonic shock. As illustrated in Fig. 2B, applica-
tion of A-23187 (1 µM) at this point in time enhanced cell volume recovery (\(n = 5\), \(P = 0.05\) at 90 min). For example, at 20 min, the percent volume decrease of the control was only 38% that of cells exposed to A-23187.

We next examined the effect of extracellular ATP, A-23187, and gramicidin (a cationophore that was used with a choline-Ringer solution to maintain a high K\(^+\) permeability) on cells bathed in an isosmotic Ringer solution. As illustrated in Fig. 3, there was a significant reduction in cell volume 2 min after the addition of ATP (50 µM) that was followed by a slower, spontaneous recovery to the original volume (\(n = 6\), \(P < 0.05\) at 11–70 min). The Ca\(^{2+}\) ionophore A-23187 produced a similar response, initially reducing cell volume, which was followed by a slower volume recovery (\(n = 9\), \(P < 0.05\) at 11–70 min; Fig. 3). By 80 min there was no significant difference in mean volumes between cells treated with ATP or A-23187 and the control cells (Fig. 3). Finally, gramicidin (5 µM, choline-Ringer solution) also caused a decrease in cell volume (\(n = 9\), \(P < 0.001\) at >4 min after gramicidin; Fig. 3). A lack of volume recovery in this instance was most likely due to the use of a choline-Ringer solution, which could inhibit a regulatory volume increase.

To further examine whether extracellular ATP enhanced volume decrease, we used enzymes that dephosphorylate ATP. As shown in Fig. 4A, hexokinase (2.5 U/ml) inhibited cell volume recovery after hypotonic shock (\(n = 6\), \(P < 0.05\) at >10 min), reducing the percent volume decrease to 44% of control values at 90 min. Hexokinase was ineffective when glucose was omitted from the bath solution (\(n = 4\), not shown). In addition, the inhibitory effect of hexokinase was reversed with the Ca\(^{2+}\) ionophore A-23187 (1 µM), such that mean values for relative cell volume with hexokinase and ionophore were significantly below control values for all measurements after hypotonic exposure (\(P < 0.001\); Fig. 4A). For example, at 90 min cells exposed to both hexokinase and A-23187 had a 232% greater volume recovery than cells bathed in hexokiniase alone and a 46% greater recovery than the control cells. Furthermore, gramicidin (5 µM) reversed the inhibitory effect of hexokinase when added 5 min after hypotonic shock (Fig. 4B). In this case, mean values for relative cell volume with both hexokinase and gramicidin were significantly below control values for all measurements after hypotonic shock (\(n = 6\), \(P < 0.001\) compared with control; Fig. 4B). At 90 min, cells with hexokinase and gramicidin had a 129% greater volume recovery than the control cells.
We next examined the effect of apyrase on RVD. Apyrase is a diphosphohydrolase that hydrolyzes ATP into AMP and two orthophosphate anions (19). Consistent with the hexokinase results, apyrase (2.5 U/ml, n = 6) reduced cell volume recovery (n = 6, P < 0.05 at >10 min; Fig. 4C). In this case, the percent volume decrease changed to 76% of the control values at 90 min. In addition, the inhibitory effect of apyrase was reversed with the nonhydrolyzable ATP analog adenosine 5'-O-(3-thiotriphosphate) (ATPγS, 50 µM, n = 6). Insets: percent volume recovery at 90 min; gram, gramicidin; apy, apyrase; hex, hexokinase. Values are means ± SE.

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Next we examined the effect of the ATP-dependent K^+ channel antagonist glibenclamide on RVD. As illustrated in Fig. 5A, glibenclamide (100 µM) inhibited cell volume recovery (n = 7, P < 0.05 at >10 min), reducing the percent volume decrease to 38% of control values at 30 min. In addition, the inhibitory effect of glibenclamide was reversed with ATP (50 µM), such that mean values for glibenclamide with ATP were significantly lower than for glibenclamide alone (n = 7, P < 0.05 at ≥10 min; Fig. 5A). At 30 min, cells treated with glibenclamide and ATP had a 239% greater volume recovery than cells treated with the antagonist alone. However, cell volume recovery with a combination of glibenclamide and ATP was not significantly different from the control (Fig. 5A). Furthermore, the Ca^{2+} ionophore A-23187 (1 µM) reversed the inhibitory effect of glibenclamide, such that mean values for glibenclamide with A-23187 were significantly lower than for glibenclamide alone and for the control (n = 7, P < 0.05 at >10 min; Fig. 5A). At 30 min, volume recovery was 517% greater in cells treated with glibenclamide and A-23187 than in cells treated with glibenclamide alone and 136% greater than in control cells.

The effect of the stretch-activated channel blocker gadolinium also was examined (39). As illustrated in Fig. 5B, gadolinium (10 µM) inhibited cell volume recovery (n = 6, P < 0.05 at >10 min), such that the percent volume decrease changed to 41% of control values at 90 min. Furthermore, the inhibitory effect of gadolinium was reversed with ATP (50 µM, n = 6; Fig. 5B). In this case, the percent volume decrease with both gadolinium and ATP was not significantly different from the control at all time points after hypotonic shock (Fig. 5B).

Patch-clamp studies. We first examined whether extracellular ATP would activate whole cell currents under isosmotic conditions. After addition of ATP (50 µM) to the extracellular bath (isosmotic high-K^+-
The equilibrium potentials for perfectly cation- and anion-selective conductances were −16.2 and +14.7 mV, respectively. Addition of apyrase (2.5 U/ml) to the bath reduced the whole cell conductance by 74%; from 20.7 ± 2.9 to 5.4 ± 1.3 nS (n = 9, P < 0.001; Fig. 6, C and D). Interestingly, this antagonist did not alter the \( E_{\text{rev}} \), which was −8.0 ± 1.0 mV for the control and −8.4 ± 0.6 mV with apyrase (n = 9; Fig. 6D). As might be anticipated, the inhibitory effect of apyrase was not affected by adding ATP (50 µM, n = 6, not shown). In contrast, it was reversed with the nonhydrolyzable analog ATPγS (50 µM), which increased the whole cell conductance by 170%; to 14.6 ± 3.6 nS (n = 9, P < 0.05 compared with apyrase), a value that was not significantly different from the control (Fig. 6, C and D). In addition, application of ATPγS to a bath containing apyrase changed \( E_{\text{rev}} \) to −10.3 ± 1.7 mV, a value closer to the equilibrium potential for K⁺ (50 µM; n = 9).

We next looked at the effect of glibenclamide on whole cell currents after hypotonic shock. This antagonist (100 µM) decreased the whole cell conductance by 77%; from 13.7 ± 2.4 to 3.2 ± 0.6 nS, which was close to that observed for cells under isosmotic conditions (n = 4, P < 0.01; Fig. 7; A and B). Glibenclamide also caused a slight shift in \( E_{\text{rev}} \) toward the equilibrium potential for Cl⁻ (\( E_{\text{Cl}} \)); from −8.0 ± 1.0 to −6.8 ± 2.4 mV. Interestingly, addition of ATP (50 µM) partially reversed the inhibitory effect of glibenclamide (Fig. 7A). In this case, the conductance with ATP and glibenclamide together increased to 7.7 ± 1.1 nS, and \( E_{\text{rev}} \) changed to −10.4 ± 1.3 mV, a displacement toward the equilibrium potential for K⁺ (n = 3; Fig. 7B).

Gadolinium (10 µM) also inhibited whole cell currents in cells exposed to a 0.5× KCl-Ringer solution (Fig. 7C). This agent decreased the conductance by 95%; from 11.6 ± 5.1 to 0.6 ± 0.1 nS (n = 3, P < 0.01; Fig. 7D). It also shifted \( E_{\text{rev}} \) toward \( E_{\text{Cl}} \); from −5.7 ± 0.6 to −0.8 ± 0.7 mV. Addition of ATP caused a reversal of the inhibitory effect caused by gadolinium, increasing the conductance to 9.1 ± 4.1 nS (n = 3; Fig. 7C and D), which was not significantly different from the control. In addition, \( E_{\text{rev}} \) changed to −8.2 ± 1.4 mV, a value nearer \( E_{K} \).

To further examine the properties of ATP efflux during cell swelling, we used solutions in which this nucleotide was the only significant charge carrier. For this purpose, abnormally high ATP concentrations were used in the bath and pipette solutions to enhance measurements of ATP transport (see METHODS). With symmetrical 100 mM ATP solutions, whole cell currents were very small but measurable (Fig. 8A). In this case, the conductance was 1.0 ± 0.3 nS (n = 3), and \( E_{\text{rev}} \) was −0.7 ± 0.5 mV (n = 3), which was not significantly different from zero (Fig. 8B). In contrast, cell swelling induced with a hypotonic ATP bath (50 mM) caused an increase in whole cell currents (Fig. 8A). Under these conditions, the conductance gradually increased until a maximum stimulation occurred by 3–5 min. No increase in current was observed in control cells over a similar time period. With the hypotonic ATP bath, the whole cell conductance increased 550%; to 6.5 ± 2.2 nS (n = 6, P < 0.05; Fig. 8B). In addition, there was a shift in \( E_{\text{rev}} \) from −0.7 ± 0.5 to +4.4 ± 2.4 mV, a value close
to that predicted for currents carried by ATP (at pH 7.4, the 4th OH on ATP would be 74% ionized, giving the molecule a net charge of $-2.37$ or a theoretical equilibrium potential for ATP of $14.7$ mV; at pH 7.4 Tris would be 83% ionized, giving an equilibrium potential for Tris of around $70$ mV). Consistent with these observations, a further reduction in extracellular ATP to 10 mM resulted in a larger shift in $E_{rev}$. With this 10-fold difference in the ATP concentration, $E_{rev}$ was $+12.2 \pm 2.5$ mV ($n = 9$, the theoretical equilibrium potential for ATP for these solutions was $+15.5$ mV; Fig. 7, A and B). This solution also caused an increase in conductance to $9.9 \pm 1.5$ nS, which probably resulted from a further decrease in osmolality.

Having established the presence of an ATP conductance, we next tried to inhibit it with pharmacological agents. Glibenclamide (100 µM) blocked ATP currents, reducing whole cell conductance by 77%; from $9.9 \pm 1.5$ to $2.3 \pm 0.6$ nS ($n = 7$, $P < 0.01$, measured with 100 mM ATP in the pipette and 10 mM ATP in the bath; Fig. 8, C and D). In addition, this agent shifted $E_{rev}$ from $+12.2 \pm 2.5$ to $+4.1 \pm 4.2$ mV, consistent with inhibition of an anion conductance. Gadolinium (10 µM) also inhibited an ATP conductance, reducing it by 46%; to $5.3 \pm 1.7$ nS ($n = 4$, $P < 0.05$; Fig. 8, C and D). However, gadolinium did not significantly alter $E_{rev}$. In addition, whereas glibenclamide essentially reduced inward and outward currents equally, gadolinium had a greater inhibitory effect on outward negative current, suggesting inhibition of ATP efflux.

**DISCUSSION**

The major finding of this study was that extracellular ATP enhanced cell volume recovery in Necturus RBCs when exposed to a hypotonic medium. Our results are most consistent with swelling-induced stimulation of ATP release through a glibenclamide- and gadolinium-sensitive conductance. This, in turn, led to a rise in intracellular Ca$^{2+}$, thereby increasing K$^+$ efflux, which contributed to solute loss and recovery of cell volume.

Key evidence for the dependence of cell volume decrease on ATP was obtained from a series of experiments in which the extracellular ATP concentration was altered. For example, endogenous extracellular ATP levels were reduced with hexokinase and apyrase, which had the effect of increasing osmotic fragility, decreasing cell volume recovery in response to hypotonic shock, and blocking whole cell currents that were activated with a hypotonic bath. It seems unlikely that these ATP scavengers acted in a nonspecific manner, because hexokinase was ineffective in the absence of glucose, and the inhibitory effect of apyrase was reversed with ATP$_S$ (27). In contrast, addition of micromolar amounts of exogenous extracellular ATP had the opposite effect, reducing osmotic fragility, enhancing...
the percent volume decrease, and activating whole cell currents in isosmotic and hypotonic media. Furthermore, ATP caused cells to shrink under isosmotic conditions, presumably by eliciting a change that mimicked the response that occurs when cells are stimulated with hypotonic exposure. Although we did not measure the level of endogenous extracellular ATP, the concentration of exogenous ATP we added was similar to that used by others (1, 31, 32, 36). Taken together, these observations demonstrate a positive role for extracellular ATP modulation of cell volume in response to hypotonic shock.

Our experimental protocols for reducing endogenous extracellular ATP and the effect of these changes on ion efflux are consistent with a report by Schwiebert et al. (27). They also used hexokinase and apyrase to reduce the extracellular ATP concentration and found that these agents prevented cAMP and protein kinase A activation of outwardly rectifying whole cell Cl− currents in a human airway epithelial cell line. In addition, our finding that cell volume decrease was stimulated by extracellular nucleotides is consistent with reports for several other cell types. Wang et al. (36) reported that increases in cell volume lead to efflux of ATP through a conductive pathway in rat hepatoma cells. This nucleotide, in turn, acts as an autocrine that couples increases in cell volume to opening of Cl− channels through stimulation of P2 receptors. Similarly, Kim et al. (17) demonstrated a potentiation of RVD in response to extracellular UTP, which promotes Ca2+ mobilization and net K+ efflux in human submandibular salivary gland duct cells. Furthermore, Taylor et al. (33) found that hypotonic shock triggers ATP release from human airway epithelial cells and suggest that extracellular ATP plays a role in RVD. Interestingly, our initial studies with human RBCs indicated that these cells do not display a well-developed RVD response. In addition, extracellular ATP had no effect on their size. Apparently, there is a fundamental difference in the way nucleated and anucleated RBCs regulate their volume, at least in response to hypotonic shock.

A logical question stemming from our observations is, What was the source of endogenous external ATP? This nucleotide was not a component of amphibian Ringer solution, nor was it normally added to the extracellular bath solution used for patch-clamp experiments. Furthermore, it has been shown by others that ATP cannot act as a blood-borne ligand, because it is subject to quick degradation in the general circulation (9). Thus, except for a few experiments where ATP was added as an agonist to the extracellular medium, the only source...
of this nucleotide was the RBCs themselves. In addition, our patch-clamp studies indicated the presence of an ATP-permeable conductance that was activated during cell swelling, thereby providing a pathway for ATP efflux in swollen cells. The presence of an ATP conductance is consistent with reports by Wang et al. (36) and Schwiebert et al. (27), who also showed that ATP can be released from cells via a conductive pathway.

Interestingly, the ATP conductance in mudpuppy RBCs was inhibited by glibenclamide. We originally chose this antagonist, because it has been shown to block ATP-dependent K⁺ channels (10) and because RVD by mudpuppy RBCs depends on a K⁺ conductance that is activated during cell swelling (2, 22). Although glibenclamide increased osmotic fragility, reduced cell volume recovery, and blocked whole cell currents in swollen cells, its inhibitory effects were reversed by the addition of extracellular ATP. This indicated that the site of action for glibenclamide was ”upstream” to the site affected by ATP, suggesting that glibenclamide blocked ATP release from the cell. This hypothesis was supported by our patch-clamp studies, in which glibenclamide was shown to be a potent inhibitor of the ATP conductance. Glibenclamide inhibition of an ATP conductance is not unique to this cell type; it also has been reported for a human airway epithelial cell line (27).

In this study we also demonstrated that ATP enhanced RVD by stimulating a K⁺ permeability. This was shown pharmacologically using the cationophore gramicidin with a choline-Ringer solution. With this solution, K⁺ and Cl⁻ were the only two permeable ions of significance, and addition of gramicidin ensured a continual high K⁺ permeability. Gramicidin consistently reversed the inhibitory effect of hexokinase. In addition, for the cell volume experiments, it did not matter whether gramicidin was added at 0 or 5 min. The effect of gramicidin was examined at 5 min, because 5 min corresponded with maximum cell swelling, indicating that several minutes were required for endogenous K⁺ channels to activate after hypotonic stress. Thus percent volume recovery was enhanced regardless of whether the K⁺ permeability was artificially enhanced with gramicidin at the time of hypotonic stress or at 5 min, even in the presence of an ATP scavenger. In addition, gramicidin caused cells to shrink under isosmotic conditions. This is consistent with these cells having a low K⁺ permeability under normal conditions and an elevated K⁺ permeability during hypotonic stress. In fact, in a previous report we showed that mudpuppy RBCs have a high basal Cl⁻ permeability and that K⁺ efflux is a rate-limiting step for cell volume recovery in response to hypotonic shock (2).
Moreover, our electrophysiological studies demonstrated that the ATP-stimulated K\(^{+}\) permeability was a conductive pathway. For example, addition of ATP or ATPγS to a hypotonic KCl bath consistently changed \(E_{\text{rev}}\) away from \(E_{\text{K}}\) and toward \(E_{\text{K}}\), indicating stimulation of a K\(^{+}\) conductance. Nonetheless, we cannot rule out the possibility that ATP also stimulated a Cl\(^{-}\) permeability concomitantly with its activation of a K\(^{+}\) channel. However, the putative presence of voltage-sensitive, volume-sensitive, or ATP-sensitive Cl\(^{-}\) channels does not alter our conclusion that ATP stimulated a K\(^{+}\) conductance during cell swelling.

Similar to gramicidin, the Ca\(^{2+}\) ionophore A-23187 also increased percent volume recovery whether it was added at 0 or 5 min, indicating that the rate of cell volume recovery was sensitive to the level of free Ca\(^{2+}\). Furthermore, A-23187 caused cells to shrink under osmotic conditions, presumably by eliciting a change in swelling-induced response. The Ca\(^{2+}\) ionophore also reversed the inhibitory effects of apyrase and hexokinase, indicating that the Ca\(^{2+}\)-dependent step is “downstream” to the site of action of ATP. Furthermore, the inhibitory effects of glibenclamide and gadolinium, two agents that blocked the ATP conductance, also were reversed with A-23187. Taken together, these observations are consistent with a presumed rise in intracellular Ca\(^{2+}\) occurring after cell release of ATP. It is worth noting that RBCs exposed to gramicidin or A-23187 stabilized their volume at a smaller size than the control cells. In fact, under control conditions, the percent volume recovery was only 40–50% by 90 min. Although this level of RVD was less than that expressed by several other cell types (8, 12, 13, 16), it is consistent with our previous studies on Necturus RBCs (2, 22). It is possible that, under the conditions of our study, control cells lacked a sufficient rise in intracellular Ca\(^{2+}\) or an adequate increase in K\(^{+}\) permeability to display a full RVD response. Alternatively, these cells may naturally never reach a level of RVD that is equivalent to cell volume recovery with A-23187 and gramicidin or that expressed by other cell types.

We previously reported that gadolinium increases osmotic fragility, inhibits cell volume recovery in response to hypotonic shock, and blocks whole cell currents in swollen cells (2). Furthermore, the inhibitory effects of this agent were reversed with A-23187. On the basis of the information we had at that time, we concluded that the Ca\(^{2+}\) influx step during cell volume decrease occurred through a Ca\(^{2+}\)-permeable, stretch-activated channel. However, in this study we show that the inhibitory effect of gadolinium on cell volume recovery and on whole cell currents was reversed by adding micromolar amounts of extracellular ATP, suggesting that this agent blocked ATP efflux. This was further supported by our patch-clamp studies in which it was shown that gadolinium blocked the ATP conduc-

![Fig. 9. Proposed model for regulatory volume decrease signaling by extracellular ATP during hypotonic swelling in Necturus RBCs. Cell swelling leads to ATP release via a glibenclamide- and gadolinium-sensitive conductance. Extracellular ATP then stimulates an increase in intracellular Ca\(^{2+}\), presumably by binding to a P\(_{2}\) receptor (P\(_{2}\)R, nature of which remains to be determined). This, in turn, activates a K\(^{+}\) conductance, thereby leading to solute efflux and cell volume recovery. Dashed lines, site of inhibition by specific antagonists. Narrow arrows, effect of adding exogenous ionophores.](http://ajpcell.physiology.org/)
hypotonic shock may only remain active for a short period of time. For instance, Ehrlich ascites tumor cells display a Cl− transport pathway that is activated with cell swelling but inactivates within the next 10 min (13). Thus it is conceivable that the initial phase of cell volume recovery in Necturus RBCs depends on a K+ permeability pathway that no longer contributes to K+ flux during the slower phase.

Finally, on the basis of the evidence we present in this report, it is compelling to conclude that extracellular ATP regulates RVD in Necturus RBCs. We cannot, however, rule out the possibility that this nucleotide may have caused superimposed cell shrinkage that was unrelated to RVD, thereby enhancing cell volume decrease. For example, under hypotonic conditions the apyrase-sensitive current was greater than the ATPγS-induced current for voltages less than −25 mV. However, these two currents were not significantly different for positive voltages. Furthermore, we have not established a pharmacological potency profile for ATP and its analogs. Another factor to consider concerning the role of ATP in RVD is that whole cell currents induced by extracellular ATP under isosmotic conditions were significantly less than currents induced with hypotonic shock. This observation suggests that an additional mechanism may be involved when cells are swollen, possibly analogous to a report concerning the Ca2+-dependent step, thereby leading to K+ loss in isosmotic and hypotonic media; however, the Ca2+-sensitivity of swollen cells is greater than that for cells at normal volume. The author concluded that cell swelling increases the Ca2+-sensitivity of the Ca2+-activated K+ transport pathway (5). By analogy, it is possible that swelling of Necturus RBCs increased their sensitivity to ATP and/or Ca2+.

In conclusion, cell volume decrease in mudpuppy RBCs was stimulated by extracellular ATP. Cell swelling activated an ATP conductance, which, in turn, stimulated a Ca2+-dependent step, thereby leading to K+ efflux and subsequent cell volume recovery. The coupling of swelling-activated ATP release and subsequent cell volume decrease represents a novel mechanism for osmotic regulation of cell function.

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Address for reprint requests and other correspondence: D. B. Light, Dept. of Biology, Ripon College, 300 Seward St., Ripon, WI 54971-0248 (e-mail: LightD@Ripon.edu).

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