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Extracellular ATP Activates a P2 Receptor in *Necturus* Erythrocytes During Hypotonic Swelling

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Abstract. We recently reported that ATP is released from *Necturus* erythrocytes via a conductive pathway during hypotonic swelling and that extracellular ATP potentiates regulatory volume decrease (RVD). This study was designed to determine whether extracellular ATP exerts its effect via a purinoceptor. This was accomplished using three different experimental approaches: 1) hemolysis studies to examine osmotic fragility, 2) a Coulter counter to assess RVD, and 3) the whole-cell patch-clamp technique to measure membrane currents. We found extracellular ATP and ATPγS, two P2 agonists, decreased osmotic fragility, enhanced cell volume recovery in response to hypotonic shock, and increased whole-cell currents. In addition, 2-methylthio-ATP potentiated RVD. In contrast, UTP, α,β-methylene-ATP, and 2′-& 3′-O-(4-benzoyl-benzoyl) adenosine 5′-triphosphate and the P1 agonist adenosine had no effect regardless of experimental approach. Furthermore, the P2 antagonist suramin increased osmotic fragility, inhibited RVD, and reduced whole-cell conductance in swollen cells. Consistent with a previous study that indicated cell swelling activates a K^+ conductance, suramin had no effect in the presence of gramicidin (a cationophore used to maintain a high K^+ permeability). We also found the P2 antagonist pyridoxal-5-phosphate-6-azophenyl-2′4-disulfonic acid (PPADS) increased osmotic fragility; however, reactive blue 2 and the P1 antagonists caffeine and theophylline had no effect. Our results show that extracellular ATP activated a P2 receptor in *Necturus* erythrocytes during hypotonic swelling, which in turn potentiated RVD by stimulating K^+ efflux. Pharmacological evidence suggested the presence of a P2X receptor subtype.

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Key words: Nucleotides — Purinoceptor — Suramin — Potassium channel — Cell volume regulation

Introduction

The ability of animal cells to regulate their volume is a fundamental property common to a large number of cell types and has been extensively reviewed [16, 18, 24, 27, 32]. 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 followed by a slower, spontaneous recovery towards the pre-shock 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 generating a driving force for water efflux [16, 18, 24, 27, 32]. Most vertebrate cells lose K^+ and Cl^- during RVD [16, 18, 24, 27, 32]. This may occur by electroneutral ion transport pathways [24] or by the separate activation of K^+ and Cl^- channels [2, 16, 18, 24, 28]. Loss of organic anions and osmolytes also may occur during RVD [23, 32, 33].

It is well known that intracellular ATP is a ubiquitous intracellular source of energy and that this nucleotide may be used to regulate ion channels [19]. However, over 25 years ago it was proposed that extracellular ATP acts as a transmitter substance at autonomic neuromuscular junctions [3]. Since then, there has been a growing body of evidence indicating extracellular ATP plays a significant role in a number of other biological processes [6, 9, 11, 17, 21, 26, 37, 38]. For example,

extracellular ATP has been implicated with the control of fluid secretion by salivary gland cells [31], ion and water balance of cochlear fluids [29], secretion of histamine by mast cells [17], vasodilation of coronary blood vessels [17], production of prostacyclin [17], and stimulation of Cl^- and fluid secretion in airway epithelia [30, 34]. In addition, ATP release is necessary for platelet self-aggregation and pain perception via neurotranmission in dorsal root ganglia [17]. Extracellular ATP also has been shown to stimulate cell volume decrease [35, 37], and a number of studies have demonstrated extracellular nucleotides are important for regulating ion channels [11, 29, 31, 34].

Extracellular ATP exerts its influence by acting as an autocrine/paracrine signal, binding to specific cell surface receptors [4, 7, 9, 12, 13, 22, 38]. Receptors for nucleosides and nucleotides are subdivided into two main categories: P1 receptors recognize nucleosides, such as adenosine, and P2 receptors bind ATP and other purine and pyrimidine nucleotides [7, 12, 13, 38]. The P2 receptors are further divided into two families: one family of ligand-gated, Ca^{2+}-permeable, cation-nonselective channels (P2X subtype) and another family of G protein-coupled receptors (P2Y subtype, [4, 12, 22]). In addition, each family of P2X and P2Y receptors have been further separated into a number of subtypes distinguished by their pharmacological properties [7, 12, 38]. Based on studies that have cloned P2 receptors, there appear to be at least seven isoforms for both the P2X and P2Y families [4, 13, 22, 38].

Nucleotide receptors modulate a number of diverse processes. P2X receptors, for example, mediate fast excitatory postsynaptic currents in cultured myenteric neurons [40], elicit an increase in intracellular Ca^{2+} in cultured Perkinje neurons [15] and Deiters' cells [6], and induce inward currents in rat pelvic ganglion neurons [39]. In addition, P2X receptors mediate the effects of ATP in smooth muscle leading to contraction in bladder [36] and vas deferens [7]. In contrast, P2Y receptor activation leads to relaxation of smooth muscle in guinea pig taenia coli [7]. Activation of P2Y receptors with UTP stimulates cation, K^+, and Cl^- currents in a liver cell line, presumably contributing to regulation of hepatic glycogenolysis [11]. In addition, purinoceptors have been linked to control of cell volume. For instance, ATP couples increases in cell volume to opening of Cl^- channels through stimulation of P2 receptors in HTC rat hepatoma cells [37]. Human hepatocytes exhibit volume-dependent ATP release, which stimulates Cl^- permeability and cell volume regulation via a P2 receptor [10]. Further, cell volume regulation in human epithelial cells is facilitated by ATP binding to P2Y receptors [8], whereas RVD in salivary gland duct cells is subject to extracellular control through UTP binding to P2Y receptors [21].

Despite recent reports concerning the physiology of extracellular ATP, there is still a paucity of data on the role of P2 receptors, especially concerning RVD. Thus, the potential connections between these receptors and cell volume regulation remain to be elucidated. Recently, we reported that ATP efflux occurs through a conductive pathway that is activated by hypotonic challenge in *Necturus* erythrocytes, and that extracellular ATP stimulates K^+ efflux during cell swelling [26]. The purpose of the present study was to evaluate the contribution of P2 receptor activation to RVD. To this 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.

Materials and Methods

Animals

Mudpuppies (*Necturus maculosus*) were obtained from a local vendor (Lemberger Co., Oshkosh, WI) and kept in well-aerated, aged tap water at 5–10°C for no more than 6 days prior to use. They were anesthetized with 3-aminobenzoic acid ethyl ester (MS-222, 1%) and sacrificed by decapitation. Blood was obtained from a mid-ventral incision and collected into heparin (10,000 units/ml)-coated tubes. Immediately following esanguination, the blood was spun in a centrifuge (Hermel-Z230, National Labnet Co., Woodbridge, NJ) at 100 × g for 1 min. The supernatant was aspirated and replaced with an equal volume of amphibian Ringer. This process of centrifuging and washing the cells was repeated twice.

Osmotic Fragility

A turbidity shift occurs in a suspension of RBCs when the integrity of the plasma membrane is compromised during hemolysis. Accordingly, we used optical density (OD), which is related to the degree of cell lysis, to assess osmotic fragility. This was accomplished with a spectrophotometer (Spectronic 20D, Milton Roy Co) 10, 15, or 20 min after blood (30–50 μl) was added to saline solutions (3 ml) of different osmolalities and compositions. Spectrophotometric experiments were conducted at 625 nm because this wavelength provided the greatest difference in optical density (OD) between intact and lysed cells (2).

A percent 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. All reported hemolytic indices were calculated using a concentration of Ringer (30 to 35 mosm/kg H_2O) that gave an OD reading between 0.025 and 0.040. We chose this concentration because it was suffi-

ciently dilute to lyse approximately half the cells in suspension (estimated by microscopic observation of samples at different concentrations). Consequently, we could assess whether a test compound increased osmotic fragility by a subsequent reduction in OD compared to the negative control solution. Conversely, a rise in OD indicated that a test compound decreased osmotic fragility.

Coulter Counter

Cell volume distribution curves were obtained by electronic sizing using a Coulter counter model Z2 with channelyzer (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 4.271×10^3 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 [25]. Cell suspensions were diluted to give a final cell count of 5,000–7,000 cells per ml.

Relative cell volume is defined as the average volume of cells compared to that in an isotonic medium. As described by others [20, 37], a percent volume recovery at X min after hypotonic exposure was calculated as $[(V_{max} - V_{Xmin})/(V_{max} - V_0)] \times 100$, where V_{max} is the peak relative cell volume, V_0 is the initial relative volume (or one), and V_{Xmin} is the relative cell volume measured X min following hypotonic exposure. A percent volume decrease was calculated as [(percent recovery$_{experimental}$)/(percent recovery$_{control}$)] × 100, where maximal recovery in hypotonic Ringer is 100%.

Patch Clamp

Patch pipettes were fabricated from Kovar sealing glass (Corning model 7052, 1.50 mm OD, 1.10 mm ID; Garner Glass, Claremont, CA) using a two-pull method (Narishige PP-7). Pipette tips were fire polished (Narishige MF-9) to give a direct current resistance of approximately 5–8 MΩ in symmetrical 100 mM KCl solutions. All pipette solutions were filtered immediately before use with a 0.22 μm membrane filter (Millex-GS, Bedford, MA), and the pipettes were held in a polycarbonate holder (E.W. Wright, Guilford, CT). Membrane currents were measured with a 10^{10} Ω feedback resistor in a headstage (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 4-pole low-pass Bessel filter and digitized at 5 kHz with an IBM-486 computer.

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

RBCs, attached to glass coverslips (5 mm diam., Bellco Biotech., Vineland, NJ) with poly-D-lysine (mol. wt. 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 Inc., Cotati, CA). The whole-cell configuration was achieved following formation of a gigaohm 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 $CaCl_2$, 0.5 $MgCl_2$, 5 glucose, and 10 HEPES (titrated to pH 7.4 with NaOH, 235 mosm/kg H_2O). A low Na^+ Ringer was prepared by substituting choline chloride for NaCl (used for all experiments with gramicidin), and a 0.5× or 0.67× Ringer were obtained by reducing NaCl accordingly. A stock solution of gramicidin was dissolved in methanol. All other non-aqueous stock solutions were mixed at 1000× the final concentration in ethanol or DMSO and then diluted 1000× to give an appropriate working concentration, thereby diluting the vehicle an equivalent amount. All stock aqueous solutions 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 $MgCl_2$, 1.0 $CaCl_2$, 2.0 EGTA, 5 glucose, 1.0 Mg-ATP, 0.5 GTP, and 5.0 HEPES (pH 7.4 with KOH, 235 mosm/kg/H_2O). During seal formation, the extracellular solution contained (in mM) 110 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5 glucose, and 10.0 HEPES (pH 7.4). An isosmotic high-K^+ bath contained (in mM) 110 KCl, 2.5 NaCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 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 $CaCl_2$, 0.5 $MgCl_2$, 5 glucose, and 10.0 HEPES (pH 7.4, 120 mosm/kg/H_2O).

For hemolysis experiments, pharmacological agents or their vehicle were present prior to the addition of cells. 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 (#5500, Wescor, Logan, UT). Chemicals were purchased from Sigma (St. Louis, MO), Alexis Biochemicals (San Diego, CA), and ICN (Costa Mesa, CA). All experiments were conducted at room temperature (21–23°C).

Statistics

Data are reported as means ± SEM. The statistical significance of an experimental procedure was determined by a paired Student's t-test or least significant difference test with paired design of analysis of variance (ANOVA)/multivariate ANOVA (MANOVA), as appropriate (Data Desk® software, Ithaca, NY). A $P < 0.05$ was considered significant. Each animal served as its own control, and cell volumes at specific times were tested against each other. For patch-clamp studies, each cell served as its own control.

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, where approximately 50% of the cells in suspension were intact (30.7 ± 1.3 mosmol/Kg H_2O), was 0.036 ± 0.003 ($n = 16$ experiments, Fig. 1). To determine

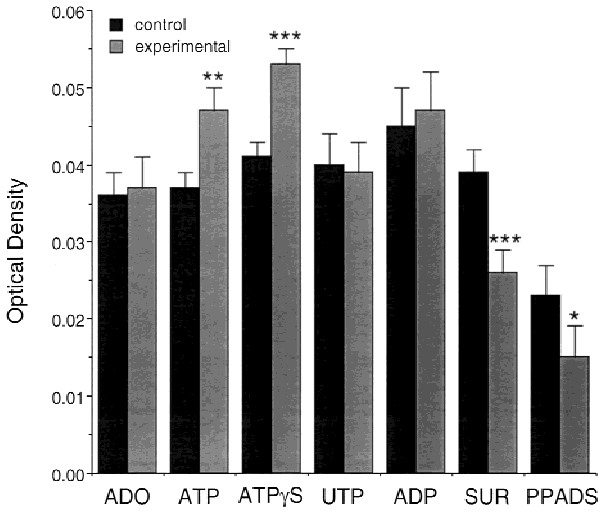

Fig. 1. Effects of P1 and P2 receptor agonists and antagonists on osmotic fragility. The *control* solution was diluted amphibian (high-Na^+) Ringer and the *experimental* solution was diluted Ringer with pharmacological agent. Adenosine (ADO), ATP, ATPγS, UTP, and ADP, all at 50 μM, and $n = 16$; suramin (SUR, 100 μM, $n = 16$), and pyridoxal-5-phosphate-6-azophenyl-2′4-disulfonic acid (PPADS, 100 μM, $n = 8$). Values are means ± SEM of optical density, obtained at concentrations of Ringer with approximately 50% lysed cells for the control. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

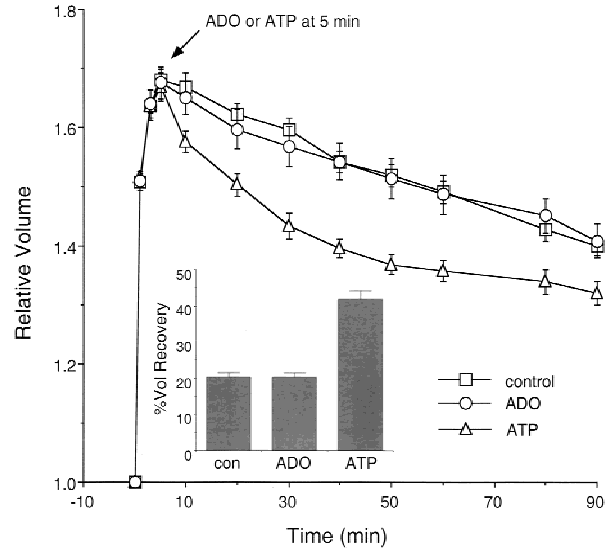

Fig. 2. Effect of extracellular adenosine (ADO) and ATP on cell volume following hypotonic shock. At time 0, cells were abruptly exposed to a hypotonic (0.5×) high Na^+ Ringer, which caused a rapid initial increase in volume followed by a gradual recovery toward basal values, despite the continued presence of hypotonic buffer (□). Cell volume recovery was enhanced by addition of extracellular ATP (50 μM, $n = 12$, △) to the extracellular medium at 5 min (arrow) but not by addition of ADO (50 μM, $n = 12$, ○). *Inset:* Percent volume recovery at 40 min; con, control. Values are means ± SEM.

whether osmotic fragility depended on activation of a P1 receptor, we repeated this assay with adenosine (ADO, 50 μM) in the extracellular medium [8, 12, 38]. In this case, the OD measured at the same concentration as the control was 0.037 ± 0.004 ($n = 16$, Fig. 1), a value that was not different from the control. In contrast, the P2 receptor agonist ATP (50 μM [7, 12, 37]) increased OD from 0.037 ± 0.002 to 0.047 ± 0.003 ($n = 16$, $P < 0.01$, Fig. 1). Adenosine 5′-O-(3-thiotriphosphate) (ATPγS, 50 μM, a poorly hydrolyzable analog of ATP [37]) also increased OD, from 0.041 ± 0.002 to 0.053 ± 0.002 ($n = 16$, $P < 0.001$, Fig. 1). Furthermore, UTP (50 μM, $n = 16$) and ADP (50 μM, $n = 16$), two agents known to activate a number of P2Y receptor subtypes [4, 12, 22], had no significant effect on osmotic fragility (Fig. 1). Additionally, 2′-& 3′-O-(4-benzoyl-benzoyl)adenosine 5′-triphosphate (BzBz-ATP, 50 μM, $n = 6$), a $P2X_R$-selective agonist that stimulates several P2X subtypes [4, 22], did not alter osmotic fragility.

We next examined the effect of inhibiting P1 and P2 receptors on osmotic fragility. The general P2 receptor antagonist suramin (100 μM [7, 12, 38]) decreased OD from 0.039 ± 0.003 to 0.026 ± 0.003 ($n = 16$, $P < 0.001$, Fig. 1), giving a hemolytic index of 33%. Similarly, the P2X inhibitor pyridoxal-5-phosphate-6-azophenyl-2′4-disulfonic acid (PPADS, 100 μM, [13, 21]) decreased OD from 0.023 ± 0.004 to 0.015 ± 0.004 ($n = 8$, $P < 0.05$, Fig. 1), a hemolytic index of 35%. In contrast, the P2Y antagonist reactive blue 2 (RB2, 10 μM, $n = 8$ [21]) had no significant effect. Additionally, caffeine (20 μM, $n = 6$) and theophylline (20 μM, $n = 6$), two antagonists of P1 receptors [7, 12], did not alter osmotic fragility. (*N.B.* variation in the average OD between control groups resulted from an unusually wide range of normal hematocrits found in this species, which extends from 12 to 35, with an average of 20 (ref. [2]). However, each animal served as its own control when assessing the effect of a pharmacological agent.)

Cell Volume Studies

When RBCs were placed in a hypotonic Na^+ Ringer, they quickly swelled, and then slowly and spontaneously decreased in volume (Fig. 2). As illustrated in Fig. 2, the relative volume with ADO (50 μM) was not significantly different from the control ($n = 12$). In contrast, cell volume recovery was enhanced with ATP (50 μM) when added 5 min after exposure to a hypotonic medium ($n = 12$, $P < 0.05 \geqslant 10$ min compared to control, Fig. 2). For example, by 40 min the percent volume decrease of the control was only 49% that of ATP (Fig. 2).

We next determined whether additional nucleotides would alter cell volume recovery. Both UTP (50 μM, $n = 12$) and ADP (50 μM, $n = 12$) had no significant effect (Fig. 3). In contrast, addition of ATPγS (50 μM) 5

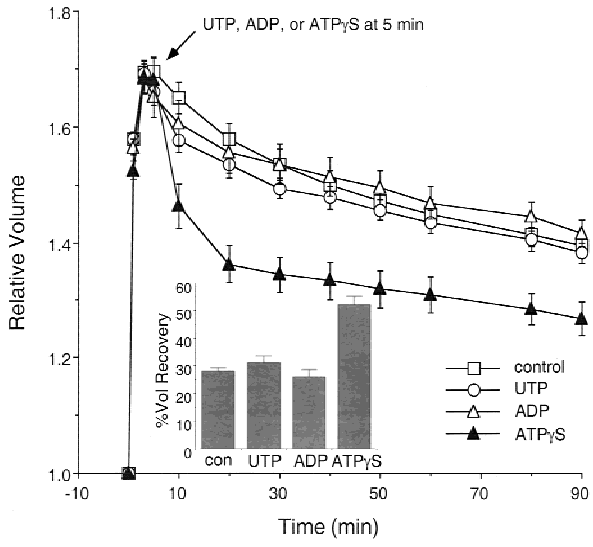

Fig. 3. Effect of extracellular UTP, ADP, and ATPγS on cell volume following hypotonic shock. At time 0, cells were abruptly exposed to a hypotonic (0.5×) high Na^+ Ringer, which caused a rapid initial increase in volume followed by a gradual recovery toward basal values (□). Cell volume recovery was enhanced by addition of extracellular ATPγS (50 μM, $n = 12$, ▲) to the extracellular medium at 5 min (arrow), but not by addition of UTP (50 μM, $n = 12$, ○) and ADP (50 μM, $n = 12$, △). *Inset:* Percent volume recovery at 40 min; con, control. Values are means ± SEM.

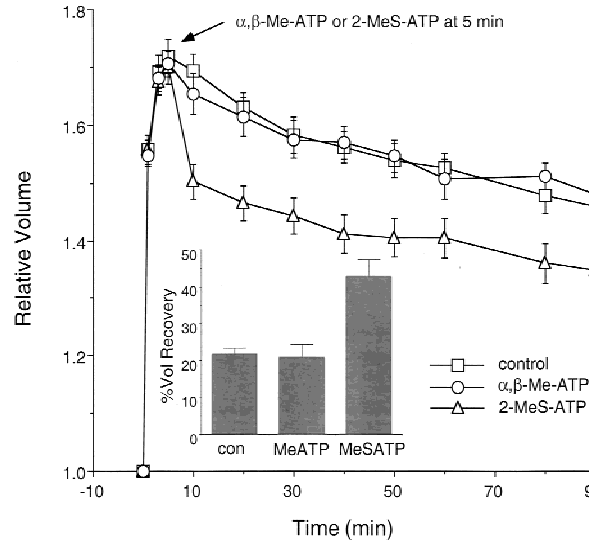

Fig. 4. Effect of extracellular 2-methylthio-ATP (2-MeS-ATP) and α,β-methylene-ATP (α,β-Me-ATP) on cell volume following hypotonic shock. At time 0, cells were abruptly exposed to a hypotonic (0.5×) high-Na^+ Ringer, which caused a rapid initial increase in volume followed by a gradual recovery toward basal values (□). Cell volume recovery was enhanced by addition of extracellular 2-MeS-ATP (50 μM, $n = 10$, △) to the extracellular medium at 5 min (arrow) but not by addition of α,β-Me-ATP (50 μM, $n = 10$, ○). *Inset:* Percent volume recovery at 40 min; con, control. Values are means ± SEM.

min after hypotonic shock led to a rapid decrease in cell volume ($n = 12$, $P < 0.05$ at > 5 min compared to control, Fig. 3). In this case, the percent volume decrease of the control was only 54% that of ATPγS by 40 min (Fig. 3). We also found addition of 2-methylthio-ATP (2-MeS-ATP, 50 μM) 5 min after hypotonic shock resulted in an enhanced cell volume recovery that was significantly greater than the control ($n = 10$, $P < 0.05$ at > 5 min, Fig. 4). In this instance, the percent volume decrease of the control was only 12% that of 2-MeS-ATP at 10 min and only 50% by 40 min (Fig. 4). In contrast, α,β-methylene-ATP (α,β-Me-ATP, 50 μM) had no effect on cell volume ($n = 10$, Fig. 4).

To further examine the nature of endogenous P2 receptors, we used three different pharmacological antagonists. As shown in Fig. 5, suramin (100 μM) inhibited cell volume recovery following hypotonic shock ($n = 16$, $P < 0.05 > 10$ min compared to control), reducing the percent volume decrease to 43% of control values at 40 min and 54% by 90 min. In addition, the cationophore gramicidin (5 μM, choline Ringer) prevented suramin from having an inhibitory effect on RVD, whether added at the time of hypotonic exposure (*not shown*) or 5 min after hypotonic shock (Fig. 5). In either case, mean values for relative cell volume with both suramin and gramicidin were significantly below control values for all measurements following addition of cationophore ($n = 8$, $P < 0.05$ compared to control). Furthermore, PPADS (100 μM) caused a slight reduction in the percent volume decrease, however this effect was shy of statistical significance ($n = 8$). In contrast, RB2 (10 μM, $n = 8$) was inactive. Additionally, caffeine (20 μM, $n = 6$) and theophylline (20 μM, $n = 6$) had no effect on cell volume.

Patch Clamp Studies

We previously reported that extracellular ATP activates whole-cell currents under isotonic conditions [25]. In this study, we examined whether extracellular ATP also would activate whole-cell currents under hypotonic conditions. For these experiments we used a high KCl Ringer in the pipette and a 0.5× KCl Ringer in the bath. The only major ions of significance with these solutions were K^+ and Cl^-, and the equilibrium potentials for perfectly cation- and anion-selective conductances were −16.2 mV and +14.7 mV, respectively. (*N.B.* these values assumed equilibration of the pipette's contents with the cell's cytoplasm, thereby imposing an artificial ionic gradient across the membrane.) After addition of ATP (50 μM) to the bath solution, the conductance gradually increased until a maximum stimulation occurred by approximately 2–5 min (Fig. 6). No increase in current was observed in control cells over a similar time period. With ATP, the whole-cell conductance increased by 41%, from 19.6 ± 2.7 nS to 27.6 ± 2.8 nS ($n = 6$, $P <$

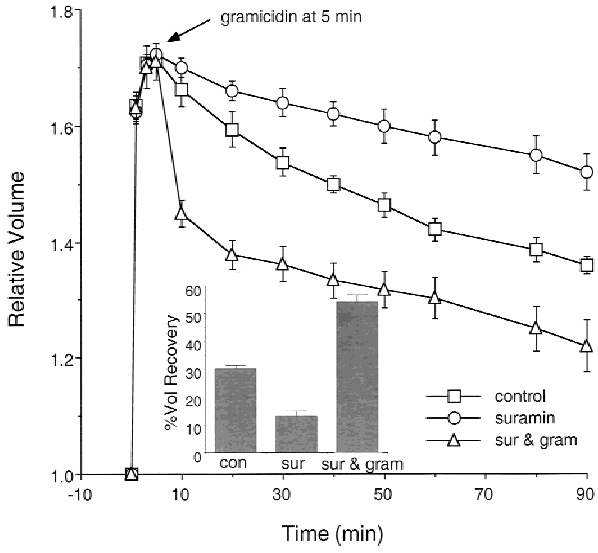

Fig. 5. Effect of suramin (sur) and gramicidin (gram) on cell volume following hypotonic shock. At time 0, cells were abruptly exposed to a hypotonic (0.5×) high-Na^+ Ringer, which caused a rapid initial increase in volume followed by a gradual recovery toward basal values (□). Cell volume recovery was inhibited by the addition of suramin (100 μM, $n = 16$, ○). In contrast, addition of gramicidin (5 μM) at 5 min (arrow) reversed the inhibitory effect of suramin ($n = 8$, △). *Inset:* Percent volume recovery at 40 min; con, control. Values are means ± SEM.

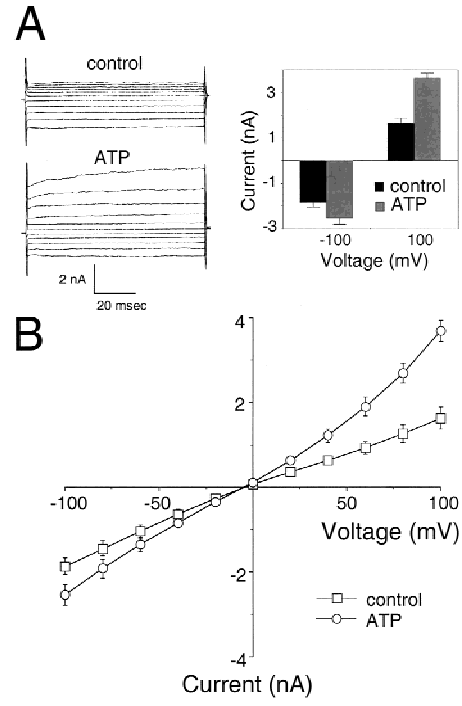

Fig. 6. Effect of ATP on whole-cell currents. Cells were maintained at a holding potential of −15 mV and stepped to potentials between −100 to +100 mV in 20 mV intervals. (*A*) whole-cell currents for a RBC exposed to a hypotonic KCl Ringer and enhancement of these currents by adding ATP (50 μM) to the bath. (*B*) Corresponding current-voltage (*I-V*) relationship for control (hypotonic, □) and ATP (○) solutions ($n = 6$). *Inset:* Current levels at −100 and +100 mV. Values are means ± SEM.

0.05, Fig. 6). There also was a slight shift in the reversal potential (E_{rev}) towards the K^+ equilibrium potential (E_K), from −4.4 ± 1.2 mV to −6.9 ± 2.6 mV ($n = 6$, $P < 0.05$). In contrast, ADO (50 μM, $n = 6$), UTP (50 μM, $n = 6$), and ADP (50 μM, $n = 6$) had no effect.

We next determined whether suramin would inhibit whole-cell currents under hypotonic conditions. Addition of suramin (100 μM) to the bath reduced whole-cell conductance by 45%, from 24.1 ± 3.2 nS to 13.2 ± 3.1 nS ($n = 9$, $P < 0.01$, Fig. 7). This antagonist also shifted the E_{rev} towards the Cl^- equilibrium potential (E_{Cl}), from −6.2 ± 1.4 mV to −7.8 ± 1.5 mV ($n = 9$, $P < 0.05$, Fig. 7). In addition, PPADS (10–100 μM, $n = 6$) caused a slight decrease in whole-cell conductance, however this effect was shy of statistical significance. Finally, RB2 (10 μM, $n = 6$), caffeine (20 μM, $n = 6$), and theophylline (20 μM, $n = 6$) did not alter whole-cell conductance nor E_{rev}.

Discussion

We recently reported that *Necturus* erythrocytes release ATP during cell swelling via a conductive pathway that is inactive under isosmotic conditions but stimulated during hypotonic stress [26]. Further, extracellular ATP enhances a K^+ conductance that also is inactive under basal conditions but active during cell swelling [26]. Thus, a connection between extracellular ATP and cell volume has been established for this cell type. This study provides evidence that extracellular ATP exerts its influence via a P2 receptor. Key evidence for this conclusion was obtained from a series of experiments that examined the pharmacological activity of P1 and P2 receptor agonists and antagonists on osmotic fragility, cell volume, and whole-cell currents.

Although we previously examined a role for extracellular ATP in the regulation of cell volume [26], we initially conducted experiments in this study to determine whether a P1 receptor also was involved. We consistently found ADO, a P1 receptor agonist [7, 12, 38], had no effect on osmotic fragility and cell volume recovery and on whole-cell currents. In addition, caffeine and theophylline, agents known to inhibit P1 receptors [7, 12, 38], were inactive for all experimental procedures. Thus, based on these observations, it is unlikely that P1 receptors are important components of RVD in this cell type. This finding is not surprising considering we are not aware of any published study that shows a connection between P1 receptors and RVD. Nonetheless, it has been shown that in response to ischemia,

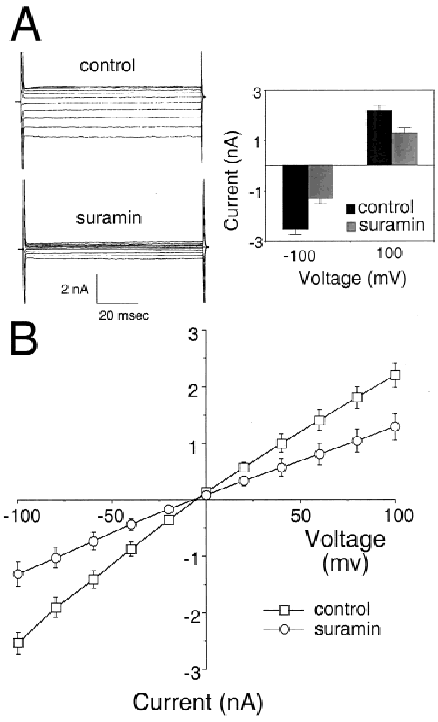

Fig. 7. Effect of suramin on whole-cell currents. Cells were maintained at a holding potential of −15 mV and stepped to potentials between −100 to +100 mV in 20 mV intervals. (*A*) whole-cell currents for a RBC exposed to a hypotonic KCl Ringer and inhibition of these currents by adding suramin (100 μM) to the bath. (*B*) Corresponding current-voltage (*I-V*) relationship for control (hypotonic, □) and suramin (○) solutions ($n = 9$). *Inset:* Current levels at −100 and +100 mV. Values are means ± SEM.

administration of an adenosine receptor antagonist blocks cell shrinkage in rat cardiac cells [1].

In contrast to ADO, addition of extracellular ATP and ATPγS consistently had a positive effect, indicating the presence of a P2 receptor [4, 7, 12, 38]. That is, addition of these nucleotides to the extracellular medium decreased osmotic fragility, enhanced cell volume recovery, and increased whole-cell conductance. Further, compared to ATP, ATPγS had a greater percent volume decrease during the first 30 min. This observation could have resulted from of a higher receptor affinity for ATPγS. Alternatively, it may have resulted from a reduced ability of an endogenous ATPase in the plasma membrane to hydrolyze this nucleotide analog [17]. Additional evidence for the presence of a P2 receptor was established using the general P2 receptor antagonist suramin [7, 12, 38], an agent that increased osmotic fragility and reduced percent volume decrease and whole-cell conductance.

Our finding that cell volume decrease was stimulated with extracellular nucleotides is consistent with reports for several other cell types. For example, Wang et al. [37] found an increase in cell volume in rat hepatoma cells leads to efflux of ATP through a conductive pathway. This nucleotide, in turn, acts as an autocrine by stimulating P2 receptors, which couples increases in cell volume to opening of Cl^- channels. Similarly, Kim et al. [21] demonstrated a potentiation of RVD in human submandibular salivary gland duct cells in response to extracellular UTP, which promotes Ca^{2+} mobilization and net K^+ efflux. Dezaki et al. [8] reported that ATP is released during cell swelling in human epithelial cells and facilitates RVD by augmenting a rise in intracellular Ca^{2+}. Taylor et al. [34] found that hypotonic shock triggers ATP release from human airway epithelial cells and suggest that extracellular ATP plays a role in RVD. Feranchak et al. [10] reported a volume-dependant ATP release in hepatocytes, which is a critical determinant of membrane Cl^- permeability and cell volume regulation. Interestingly, our previous studies with human RBCs indicate that these cells do not display a well-developed RVD response, nor does extracellular ATP effect their volume [26]. Apparently, there is a fundamental difference in the way nucleated and anucleated RBCs regulate their volume, at least in response to hypotonic shock.

Having established the presence of a P2 receptor, we next attempted to narrow down the subtype by using various nucleotides. For example, UTP and ADP are agonists in some systems for P2Y [4, 22], yet these agents were without effect on *Necturus* RBCs, suggesting the presence of a P2X receptor. In addition, the nucleotide analog 2-MeS-ATP had a stimulatory effect, whereas there was a lack of activity for α,β-Me-ATP, which also is consistent with the pharmacology of some P2X subtypes [4, 22]. To further characterize the receptor, we took into account that α,β-Me-ATP stimulates $P2X_1$ and $P2X_3$ in some systems, but not $P2X_2$ and $P2X_4$ [2, 22]. Because α,β-Me-ATP had no observable activity, our results suggested the presence of $P2X_{2, 4, 5, 6, 7}$. However, as stated above, ADP had no effect on *Necturus* erythrocytes, which also can act as a weak agonist for $P2X_6$ in some systems. Finally, we found a lack of an effect with BzBz-ATP, a nucleotide analog that stimulates $P2X_7$, and also probably $P2X_4$ and $P2X_5$ [4, 22]. Taken together, our results are most consistent with the presence of a $P2X_2$ receptor, which has been shown by others to be stimulated by ATP, ATPγS, and 2-MeS-ATP and to be insensitive to ADP, UTP, BzBz-ATP, and α,β-Me-ATP [4, 6, 15, 22, 39, 40]. Although *Necturus* erythrocytes could contain homomultimeric structures (e.g., all composed of $P2X_2$), they also could have a mixture of heteromultimeric complexes of receptor subtypes (e.g., $P2X_R$).

We also attempted to distinguish between P2X and P2Y receptors using pharmacological antagonists. The general P2X inhibitor PPADS [22] had mixed effects. Although it significantly increased osmotic fragility, suggesting the presence of a P2X subtype, its slight inhibi-

tion of cell volume recovery and whole-cell conductance was shy of statistical significance. In addition, the general P2Y receptor antagonist RB2 [22] had no apparent activity, regardless of experimental approach, suggesting the absence of a P2Y subtype. It should be noted, however, that PPADS and RB2, as well as suramin, are not highly selective. For example, suramin blocks a swelling-activated anion-selective channel directly, thereby exerting its effect on cell volume independently of its action on purinoceptors [14]. Thus, although it is not possible to draw a definitive conclusion regarding P2 receptor subtype from the use of antagonists, our results are consistent with a P2 receptor, and are not inconsistent with a P2X subtype.

Our studies suggesting the presence of a $P2X_2$ receptor based on pharmacology are similar to several other reports demonstrating the presence of this subtype. For example, by measuring inward currents in rat pelvic ganglion neurons, Zhong et al. [39] found an ATP response that was antagonized by suramin and PPADS. They also reported an agonist profile of ATP $\geqslant$ 2-MeS-ATP = ATPγS, whereas UTP, ADP, and α,β-Me-ATP were inactive. However, in contrast to our study, they found that RB2 antagonized the response to ATP. In addition, Garcia-Lecea [15] demonstrated the presence of $P2X_2$ receptors in cultured rat Purkinje neurons. They studied ATP-elicited Ca^{2+} signals that were mimicked with 2-MeS-ATP, but not with α,β-Me-ATP and other purinoceptor agonists. In addition, the Ca^{2+} signals were blocked with suramin and PPADS. The presence of $P2X_2$ receptors in Deiter's cells was demonstrated by Chen and Bobbin [6] who found the order of agonist potency for ATP-induced currents was 2-MeS-ATP > ATP. Zhou et al. [39] reported $P2X_2$ receptors in cultured myenteric neurons that are inhibited by PPADS. They also found a rank order potency of ATP > 2-MeS-ATP >> α,β-Me-ATP and ADP. Nonetheless, although there are a number of reports of $P2X_2$ receptors in several cell types that are involved with various physiological processes, we are not aware of a published study that describes the expression of P2X receptors in the plasma membrane of red blood cells from any species nor a connection between P2X receptors and cell volume decrease.

Our study also provides evidence that activation of a P2X receptor leads to an increase in K^+ efflux via a conductive pathway that is distinct from the P2X receptor channel. This was shown, in part, 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 continual high K^+ permeability. Gramicidin consistently negated the inhibitory effect of suramin. For the cell volume experiments, it did not matter whether gramicidin was added at 0 min or at 5 min. The reason for examining the effect of gramicidin at 5 min is because that point in time corresponded with maximum cell swelling, indicating it took several minutes for endogenous K^+ channels to activate following hypotonic stress (this also was shown in a previous study where patch clamp experiments demonstrated a similar time course for activation of a K^+ conductance following hypotonic challenge, ref. [2]). 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 the P2 receptor antagonist suramin. In addition, the response of swollen cells to a solution containing both gramicidin and suramin was virtually identical to the response we reported for a solution with gramicidin alone [2], indicating suramin did not influence the action of gramicidin. We also previously reported that addition of gramicidin causes cells to shrink under isosmotic conditions [25]. Taken together, these observations are consistent with this cell type having a relatively low K^+ permeability under isotonic conditions and an elevated K^+ permeability during hypotonic stress, and that K^+ efflux is a rate-limiting step during RVD (see ref. [2] for further details).

Moreover, our electrophysiological studies demonstrated that P2X activation led to stimulation of a K^+ conductance distinct from the nucleotide receptor. For example, addition of ATP to an isosmotic bath increases whole-cell conductance [26] and addition of ATP to a hypotonic bath not only increased whole-cell conductance, but also changed E_{rev} away from E_{Cl} and towards E_K, indicating stimulation of a K^+ channel. Nonetheless, we cannot rule out the possibility that ATP also stimulated a Cl^- conductance concomitantly with its activation of a K^+ channel. However, the putative presence of a voltage-sensitive, volume-sensitive, and/or ATP-sensitive Cl^- channel does not alter our conclusion that ATP stimulated a K^+ conductance during cell swelling. Furthermore, suramin had the opposite affect of ATP. That is, it decreased whole-cell conductance and shifted E_{rev} away from E_K and towards E_{Cl}, indicating inhibition of a K^+ channel. We also cannot rule out the possibility of a mixed current phenotype where K^+ was carried by both a P2X receptor channel and a K^+ channel regulated by the P2X receptor. However, rapid inactivation is a common property of P2X receptor channels [11] and the K^+ conductance we observed did not inactivate over the time course of our experiments [2].

The presence of a P2 receptor in this cell type also is consistent with a previous report from our laboratory that demonstrated release of ATP by swollen cells [26]. For instance, we found the ATP scavengers hexokinase and apyrase had no effect in an isotonic medium, but increased osmotic fragility and blocked cell volume recovery in response to hypotonic shock. These observations indicate efflux of ATP by swollen cells, but not under

basal conditions. Similarly, suramin had no effect under isotonic conditions, but inhibited ATP's ability to decrease osmotic fragility and enhance percent volume decrease. In addition, we previously reported that *Necturus* erythrocytes have an ATP conductance that is inactive under isotonic conditions, but active during hypotonic swelling, providing a pathway for ATP efflux [26]. Thus, although ATP is not released under basal conditions, there is a constitutive release of ATP by swollen cells, making the presence of P2 receptors logical. Furthermore, we recently demonstrated that extracellular ATP stimulates a K^+ conductance in swollen cells [26]. This observation is consistent with our present finding that activation of P2X receptors is linked to an increased K^+ efflux during cell swelling.

The presence of P2X receptors, as opposed to P2Y, also is consistent with recent reports from our laboratory demonstrating cell volume decrease is stimulated by Ca^{2+} [2] and that the Ca^{2+}-sensitive step occurs "down stream" to P2 receptor activation [25, 26]. Similarly, Dezaki et al. [8] reported that ATP is released during cell swelling in human epithelial cells and this nucleotide enhances RVD by augmenting a rise in intracellular Ca^{2+}. In addition, Kim et al. [21]) demonstrated UTP facilitates RVD in human submandibular salivary gland duct cells by promoting Ca^{2+} mobilization and net K^+ efflux. Because P2X receptors are Ca^{2+}-permeable nonselective cation channels, it is tempting to speculate that the Ca^{2+}-sensitive step we described earlier involves the influx of this ion through a P2X receptor.

Based on the evidence we present in this report, it is compelling to conclude that extracellular ATP regulates RVD in *Necturus* erythrocytes via a P2X receptor. We cannot, however, rule out the possibility that activation of this receptor may have caused superimposed cell shrinkage that was unrelated to RVD, thereby enhancing cell volume decrease. For example, relatively high concentrations of ATP can induce activation of K^+ and/or Cl^- channels. Although this would lead to a net loss of ions and subsequent cell shrinkage, it may not be involved with the normal volume-regulatory response to osmotic swelling. We also did not measure the extracellular concentrations of ATP achieved as a result of swelling-induced ATP release, which may be much lower than the 50 μM we used in this study. Additionally, ATP caused activation of currents greater than those seen in hypotonic Ringer, which may indicate that the ATP-activated channels are different from those channels normally involved with the RVD response. Furthermore, suramin did not reduce whole-cell currents to the level seen in an isosmotic medium, again raising the possibility that the ATP-stimulated channel may not be part of an RVD response.

Another factor to consider is that whole-cell currents induced by extracellular ATP under isosmotic conditions were significantly less than currents induced with hypotonic shock [26]. However, this observation may indicate that an additional mechanism is involved when cells are swollen, possibly analogous to a report concerning the calcium sensitivity of *Amphiuma* red blood cells [5]. With *Amphiuma*, Ca^{2+} stimulates K^+ loss in both isosmotic and hypotonic media; however, the Ca^{2+}-sensitivity of swollen cells is greater than that for cells at normal volume. The author concluded that cell swelling increases the calcium sensitivity of the Ca^{2+}-activated K^+ transport pathway [5]. By analogy, it is possible that swelling of *Necturus* erythrocytes increases their sensitivity to ATP.

In conclusion, this study provides evidence for the presence of a P2 receptor in *Necturus* erythrocytes. Activation of this receptor with extracellular ATP enhanced cell volume recovery during hypotonic swelling by stimulating K^+ efflux. Pharmacological evidence using various nucleotides and antagonists was consistent with a P2X subtype. The coupling of a P2X receptor to RVD represents a novel mechanism for osmotic regulation of cell function.

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