

# A Volume Regulatory Response Can Be Triggered by Nucleosides in Human Erythrocytes, a Perfect Osmometer No Longer<sup>\*[5]</sup>

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Human erythrocytes have been regarded as perfect osmometers, which swell or shrink as dictated by their osmotic environment. In contrast, in most other cells, swelling elicits a regulatory volume decrease (RVD) modulated by the activation of purinic and pyrimidinic receptors (P receptors). For human erythrocytes this modulation has not been tested, and we thus investigated whether P receptor activation can induce RVD in these cells. Further, because ectonucleotidases may scavenge ATP or ADP or act as a source for extracellular adenosine and therefore modulate P receptor activation and RVD, we also determined their activity in intact erythrocytes. We found relatively low ectoATPase but significant ectoADPase and ectoAMPase activities. When erythrocytes were exposed to hypotonic medium alone, they swelled as expected for an osmometric response and showed no RVD. Activation of P2 receptors by exogenous ATP or ADP did not trigger RVD, whereas P1 agonists adenosine and adenosine-5'-N-ethylcarboxamide induced significant RVD. The effect of adenosine-5'-N-ethylcarboxamide was dose-dependent (maximal RVD of 27%; apparent  $K_{1/2}$  of  $1.6 \pm 1.7 \mu\text{M}$ ). The RVD induced by adenosine was blocked 80% with the non-selective P1 antagonist 8-(p-sulfophenyl) theophylline or the P1-A<sub>2B</sub> inhibitor MRS1754, but not by inhibitors of P1 subtypes A<sub>1</sub>, A<sub>2A</sub>, and A<sub>3</sub>. In addition, forskolin (an inducer of intracellular cAMP formation) could mimic the effect of adenosine, supporting the idea of P1-A<sub>2B</sub> receptor activation. In conclusion, we report a novel P1-A<sub>2B</sub> receptor-mediated RVD activation in mature human erythrocytes and thus indicate that these long held perfect osmometers are not so perfect after all.

Animal cells are much more permeable to water than to osmolytes. In consequence, a reduction of extracellular osmo-

larity or an increase in the intracellular osmolarity leads to a net influx of water and fast cell swelling. Because cells can only expand to a certain limit before bursting, it is essential to counteract this osmotic swelling. This is usually accomplished in a process termed regulatory volume decrease (RVD),<sup>4</sup> where intracellular osmolarity is reduced by the release of organic and inorganic osmolytes from the cell, together with osmotically obliged water and the concomitant reduction of cell volume (1).

Although RVD is observable in almost all animal cells, the mature anucleated mammalian erythrocyte appears to lack mechanisms of volume regulation. This is why as early as 1950 Eric Ponder postulated that, within certain limits normal human red blood cells exist in osmotic equilibrium with their solvent environments and behave as near-ideal volume osmometers (2). Early studies on red blood cells (RBCs) from normal adults, children, and newborn infants, varying considerably in size, shape, and hemoglobin content, showed a close agreement between the cell volume values for an expected osmometric response and the average cell volumes determined at each tonicity (3, 4). This lack of RVD is quite exceptional given that erythrocytes from almost all other vertebrate species studied so far display a measurable volume regulation (5–9).

The swelling activated response usually involves an increase of KCl co-transport, as in duck, sheep, rabbit, and mouse red cells (10, 11), but may also involve formation of a channel that mediates the efflux of osmolytes such as taurine, as in red cells from skate and trout (7, 12). In human RBCs, however, volume-sensitive efflux of KCl and RVD appear to be restricted to reticulocytes, which, under normal conditions, comprise only 0.5–1.5% of the whole erythrocyte population. Also, mouse anion exchanger band3/AE1 protein, the homolog of which is involved in RVD of trout erythrocytes (13), when expressed in *Xenopus* oocytes displays neither channel activity nor transport of organic osmolytes. This was consistent with the current idea that transport pathways for RVD remain inactivated in mature mammalian RBCs (14). In contrast, in isotonic conditions mammalian erythrocytes are capable of losing water via KCl

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<sup>4</sup> The abbreviations used are: RVD, regulatory volume decrease; RBC, red blood cell; ATPγS, adenosine 5'-O-(thiotriphosphate); 2Mes-ADP, 2-methylthioadenosine 5-diphosphate; NECA, adenosine-5'-N-ethylcarboxamide; 8-SPT, 8-sulfophenyl theophylline; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; HYPO, hypotonic medium; ADO, adenosine; ectoATPase, ectoadenosine triphosphate hydrolase; ectoADPase, ectoadenosine diphosphate hydrolase; ectoAMPase, ectoadenosine monophosphate hydrolase.

co-transport activity as well as  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels known as Gardos Channels (KCNN4 (15)).

Although these findings established an empirical and theoretical basis to explain the lack of volume regulation of mature mammalian erythrocytes, several lines of evidence demonstrated that, not only the absence or presence of effector mechanisms, but also signaling events have to be taken into account. Specifically, it was shown by Wang *et al.* (16) that swollen hepatoma cells required endogenous extracellular ATP to down-regulate cell volume (16).

Further examples of ATP-induced volume regulation were observed in nucleated erythrocytes from the salamander *Necturus* (9) and in hepatocytes, endothelial cells, astrocytes, and epithelial cells from human and several other mammalian species (1). Similarly, RVD was activated by UTP in salivary gland duct cells (17) and by ATP, UTP, UDP, or  $\text{ATP}\gamma\text{S}$  in trout and goldfish hepatocytes (18, 19).

Moreover, extracellular adenosine, derived from hydrolysis of extracellular ATP, was shown to inhibit RVD via P1 activation in fish hepatocytes (18, 19). Thus, it seems clear that understanding volume regulation requires an understanding of events involved in nucleoside signaling on the cell surface.

The main players in this regard appear to be extracellular nucleotides, cell surface P receptors (including purinic and pyrimidinic receptors; see Ref. 20), and ectonucleotidases. In mammals, 19 P receptors have been cloned, which belong to two main categories, *i.e.* P1 receptors with high affinity for adenosine and P2 receptors preferentially binding nucleoside di- and triphosphates (21). The P2 receptors have been further subdivided into P2X (ligand-gated ion channels) and P2Y (receptors coupled to a G-protein). Although P receptors are ubiquitous in almost every cell system, there is little information concerning their presence in vertebrate erythrocytes. In human mature RBCs the ADP receptor sub-type  $\text{P2Y}_{13}$  is the most abundantly expressed on the mRNA level, whereas other P2 receptors ( $\text{P2Y}_1$ ,  $\text{P2Y}_2$ ,  $\text{P2Y}_4$ ,  $\text{P2Y}_6$ ,  $\text{P2Y}_{11}$ ,  $\text{P2Y}_{12}$ ,  $\text{P2X}_1$ ,  $\text{P2X}_4$ , and  $\text{P2X}_7$ ) display very low levels (22, 23). Up to date, P1 receptors have not been studied in this cell system.

In human blood, several processes act to metabolize extracellular ATP; conversion of ATP to ADP and AMP is mainly promoted by the activities of ectonucleoside triphosphate dihydrolases (E-NTPDases) from leukocytes and endothelial cells, with little or no contribution of plasma, platelets, or erythrocytes. The dephosphorylation of AMP to adenosine is primarily mediated by a soluble 5'-nucleotidase of the plasma (24). Regardless of the bulk concentrations of nucleosides present in the plasma, P receptor signaling of erythrocytes will also depend on the capacity of ectonucleotidases present at the surface of these cells. Regarding this, isolated human erythrocytes display significant hydrolysis rates of extracellular AMP and ADP, and very low ectoATPase activity (25). However, no studies were made on ectonucleotidases of specific erythrocyte subpopulations that would allow discriminating between mature anucleated cells and reticulocytes.

Based on the main processes that govern RVD as well as the homeostasis of extracellular nucleotides, a model for autocrine/paracrine modulation of RVD by ATP was proposed (26). In this model, cell swelling causes a non-lytic, regulated release of

ATP, which would then stimulate specific P2 receptors and thereby activate the efflux of osmolytes and water, in this way promoting the recovery of cell volume (16). But although this is relatively well established, little is known regarding the potential action of P1 on volume regulation. This, however, would seem of importance, insofar as ATP can readily interconvert to its hydrolysis products in the extracellular space, and adenosine, derived from ATP, ADP, or AMP, may then accumulate in significant concentrations at the cell surface. For example, in pig red cells, adenosine and adenosine analogs were able to inhibit a volume-sensitive chloride-dependent  $\text{K}^+$  flux, although induction of the response required relatively long incubation times and millimolar concentrations that were several orders higher than the  $\text{EC}_{50}$  values of all known P1 receptors (27). In airway epithelial cells ATP released upon swelling was rapidly hydrolyzed to adenosine, followed by adenosine activation of volume-sensitive anion channels. Unfortunately, the corresponding kinetics of cell volume was not studied (28).

In the present study we sought to investigate whether extracellular adenine nucleotides and adenosine are able to trigger RVD of mature human erythrocytes. Our approach included an assessment of time-dependent volume changes of cells in the presence of exogenous nucleosides, as well as the use of selective antagonists to identify the specific P receptor mediating the regulatory response. Furthermore, because extracellular nucleosides can be metabolized in the extracellular space, generating different P receptor agonists, we determined ectonucleotidase activities using intact cells.

## EXPERIMENTAL PROCEDURES

### Reagents

Poly-D-lysine, ATP, ADP, 2-methylthioadenosine 5'-diphosphate (2MeS-ADP), AMP, adenosine, adenosine-5'-N-ethylcarboxamide (NECA), 8-sulfophenyl theophylline (8-SPT), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), nigericin, adenosine deaminase (type V), and Percoll were purchased from Sigma. 4-(2-([5-Amino-2-(2-furyl)[1,2,4]triazolo[1,5-a][1,3,5]triazin-7-yl]amino)ethyl)phenol (ZM241385), 2-phenoxy-6-(cyclohexylamino)purine hemioxalate (MRS3777), and *N*-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide (MRS1754) were from Tocris Bioscience (Bristol, UK). The 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) was obtained from Molecular Probes (Eugene, OR). [ $\gamma$ - $^{32}\text{P}$ ]ATP ( $5.4 \text{ Ci mg}^{-1}$ ,  $\sim 10 \text{ mCi ml}^{-1}$ ) was from PerkinElmer Life Sciences. All other reagents were of analytical grade.

### Cells

Ten milliliters of heparinized blood was obtained from healthy volunteers who had not received a transfusion within 3 months.

After blood centrifugation ( $900 \times g$  at  $25^\circ\text{C}$  for 3 min), plasma, platelets, and leukocytes were removed by pipetting, and erythrocytes were washed three times in RBC medium (in mM: 155 NaCl, 2.7 KCl, 1.5  $\text{KH}_2\text{PO}_4$ , 2.5  $\text{Na}_2\text{HPO}_4$ , 1 CaCl, 1  $\text{MgSO}_4$ , 5 glucose, pH = 7.4,  $20^\circ\text{C}$ , osmolarity = 300 mosm). Final hematocrit was  $\sim 45\%$ . All experiments shown in this

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study were performed with a subpopulation of RBCs containing only anucleated mature erythrocytes isolated by means of a discontinuous Percoll density gradient (29). Briefly, five solutions of modified RBC medium (in mM: 155 NaCl, 2.7 KCl, 1.5  $\text{KH}_2\text{PO}_4$ , 2.5  $\text{Na}_2\text{HPO}_4$ , 5 glucose, and 5% bovine serum albumin, pH = 7.4, 20 °C, osmolality = 300 mosM) were prepared with a final Percoll concentration of 60%, 66%, 70%, 74, and 80% (density 1.087–1.098 g ml<sup>-1</sup>). Discontinuous Percoll gradients were made by careful manual pipetting of 1 ml of 80% solution, 3 ml of 74%, 3 ml of 70%, 3 ml of 66%, and 1.5 of ml 60%. Finally 1.5 ml of erythrocyte suspension containing  $\sim 5.5\text{--}6.5 \times 10^9$  cells was seeded on top of the gradient. Centrifugation was carried out in a Sorvall RC-2B in a fixed angle rotor (SS-34) at 4000  $\times g$  for 15 min at 20 °C. Cell fractions were collected by aspiration, and the middle fractions (interphase 66–70% and 74–80%) were washed three times in RBC medium (900  $\times g$  at 25 °C for 3 min) to eliminate the remaining Percoll. In preliminary experiments no cells of these middle fractions stained with methylene blue, indicating that no reticulocytes were present (30).

In experiments of Fig. 1, where an assay medium lacking phosphate was required, separation of the erythrocytes was followed by three washes of the cell suspension in RBC-Hepes medium (in mM: 130 NaCl, 4.7 KCl, 1.5 CaCl, 1.1  $\text{MgSO}_4$ , 5  $\text{NaHCO}_3$ , 10 glucose, 20 HEPES, pH = 7.4, 20 °C, osmolality = 300 mosM). To remove intracellular phosphate, cells were then incubated in RBC-Hepes for 1 h at 25 °C and then washed twice with RBC-Hepes. All procedures conformed to the Declaration of Helsinki, and written informed consent was given by the subjects.

### Ectonucleotidase Activity

Red blood cells do not take up ATP, ADP, or AMP. Therefore, following addition of adenine nucleotides to a cells suspension, the rate of phosphate accumulation in the medium is a measure of ectoATPase (from ATP), ectoADPase (from ADP), and ectoAMPase (from AMP) activities.

EctoATPase activity was determined by following the release of  $[\gamma\text{-}^{32}\text{P}]\text{P}_i$  from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  as described before (31, 32) with modifications. Briefly, the reaction was started by adding 300  $\mu\text{M}$   $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  to cell suspensions (20% average hematocrit) at 20 °C under continuous stirring. At different times, a 200- $\mu\text{l}$  aliquot of the suspension was withdrawn and centrifuged at 20,000  $\times g$  during 30 s, and 100  $\mu\text{l}$  of the supernatant were poured into 750  $\mu\text{l}$  of a stop solution containing 4.05 mM  $\text{Mo}_7\text{O}_{24}(\text{NH}_4)_6$  and 0.83 mM  $\text{HClO}_4$ . The ammonium molybdate solution formed a complex with the released phosphate, which was then extracted by adding 0.6 ml of isobutyl alcohol under vigorous stirring. After the phases were separated by centrifugation for 5 min at 1,000  $\times g$ , aliquots of 200  $\mu\text{l}$  of the organic phase containing  $[\gamma\text{-}^{32}\text{P}]\text{P}_i$  were transferred to vials containing 2.5 ml of 0.5 M NaOH. Radioactivity was measured by using the Cerenkov effect.

EctoADPase and ectoAMPase activities were determined with a malachite green method (33). Briefly, cell suspensions (15% average hematocrit) were incubated with 300  $\mu\text{M}$  of ADP or AMP at 20 °C under continuous agitation. At the indicated times, 200- $\mu\text{l}$  aliquots were centrifuged at 20,000  $\times g$  for 30 s, and 150  $\mu\text{l}$  of the supernatant was mixed with 650  $\mu\text{l}$  of ice-cold

bidistilled water. 200  $\mu\text{l}$  of the malachite green reagent (containing 3.05 mM malachite green, 4.72 N  $\text{H}_2\text{SO}_4$ , 12 mM  $\text{Mo}_7\text{O}_{24}(\text{NH}_4)_6$ , and 0.16% Tween 20) were added, followed by 100  $\mu\text{l}$  of 1.37 M  $\text{Na}^+$  citrate. Solutions were incubated at 20 °C for 30 min, and absorbance was determined at 630 nm. Absorbance values were converted to phosphate concentration by means of a calibration curve (2–20 nmol of  $\text{P}_i$  ml<sup>-1</sup>).

In both methods, care was taken to obtain initial rate values. Values of phosphate concentration in assay medium lacking cells were subtracted from the phosphate accumulation in the presence of cells at each time point. Although phosphate content in the absence of cells was higher than zero, it did not increase with time, *i.e.* phosphate production was negligible.

### Cell Volume

Relative cell volumes before and after osmotic cell swelling were estimated at room temperature by two different methods as follows.

**Method 1: BCECF Fluorescence Quenching**—Under certain conditions fluorescence intensity decreases with the increase in fluorophore concentration. This quenching property can be used for measuring water volume changes in cells (34). In the following, a brief description of the method used to estimate cell volume by fluorescence quenching of BCECF-loaded erythrocytes is given.

Human erythrocytes ( $3.5 \times 10^6$ ) were attached to 0.001% poly-D-lysine-coated coverslips. At this very low concentration of poly-D-lysine cells remain discoidal in shape (35).

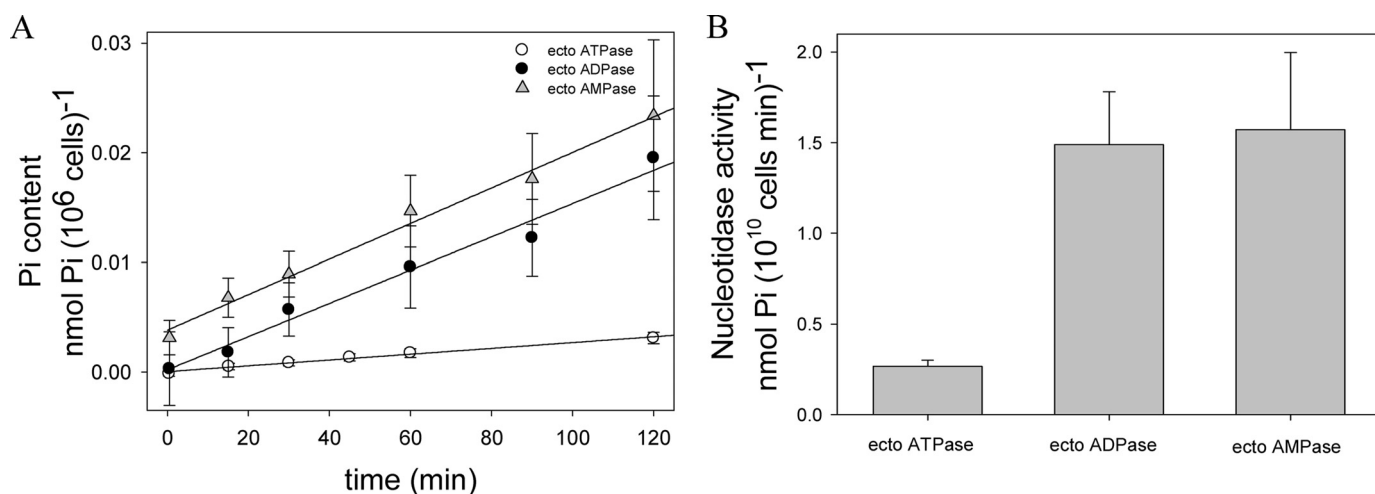
Erythrocytes were incubated with RBC medium containing 5  $\mu\text{M}$  BCECF-AM during 60 min at 20 °C. Subsequently, the solution was washed with RBC medium for 30 min to eliminate extracellular BCECF, and the coverslip was mounted on a recording chamber in a Nikon TE-200 epifluorescence inverted microscope. During experimental manipulations, all media were removed from or introduced in the chamber manually. Changes in cell water volume were inferred from readings of the fluorescence intensity recorded by exciting BCECF at 445 nm, where the fluorochrome is pH-insensitive (the isosbestic point; see below).

In preliminary experiments erythrocytes were exposed to anisotonic solutions so as to alter cell volume. It was seen that, as cell volume increases, fluorescence intensity increased, and similarly, as cell volume decreases, fluorescence intensity decreased with increasing fluorophore concentration. Thus, the directly proportional relationship between fluorescence intensity and cell volume was interpreted as a result of fluorescence quenching, and changes in fluorescence intensity recorded from a small region of dye-loaded cells (pinhole) reflect alterations of cell water volume. As an example, Fig. 2B shows results of relative cell volume ( $V_r$ ) versus relative osmolality of erythrocytes exposed to hypotonic media from the experiments performed in this report. Fluorescence images were acquired by use of a charge-coupled device camera (Hamamatsu C4742–95) and the Metafluor acquisition program (Universal Imaging).

Calibration was performed by sequentially exposing cells to assay media of osmolalities of  $\sim 300$ , 285, 270, and 255 mosM. The hypotonic media for cell volume calibrations and HYPO



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**FIGURE 1. Ectonucleotidase activity of intact erythrocytes.** A, time course of  $P_i$  accumulation (nanomoles of  $P_i$   $(10^{10} \text{ cells})^{-1}$ ) using 300  $\mu\text{M}$  of either ATP (open circles), ADP (closed circles), or AMP (gray triangles). The continuous lines are linear regressions to experimental data, with the corresponding slopes representing ectoATPase (from ATP), ectoADPase (from ADP), and ectoAMPase (from AMP) activities. For each condition, accumulation of  $P_i$  in the absence of cells was subtracted (see "Experimental Procedures"). B, ectoATPase, ectoADPase, and ectoAMPase activities calculated from experimental data of A. Results are mean  $\pm$  S.E.,  $n = 10$ .

medium had a similar composition as isotonic RBC medium, except that the NaCl concentration was lower.

Values of relative cell volume ( $V_r$ ) were obtained from the relative fluorescence ( $F_t/F_0$ ), with  $F_0$  representing the signal obtained from a pinhole region of the cell equilibrated with isotonic medium, and  $F_t$  denoting the fluorescence of the same region of the cell at time  $t$ . Thus  $V_r$  represents a fractional volume, where the initial isotonic cell volume value is 1, and volume changes are expressed as relative to the initial value. In some cases we found small but constant changes in fluorescence intensity in individual cells that can be due to a leak and/or photobleaching of the fluorophore. When this happened, the drift in fluorescent intensity was corrected using data from isotonic exposure. In other cases, individual cells had a non-constant change in fluorescence intensity or a fast loss of fluorescence intensity, a process compatible with a fast increase in plasma membrane permeability, and cell death. These cells were not used for analysis. Examples of these considerations can be found in supplemental Fig. S1.

Because BCECF is sensitive to pH, knowledge of the precise isosbestic point is critical to avoid pH interference on the volume signal. Therefore, pH-dependent changes of BCECF spectra were analyzed as follows.

Suspensions of human erythrocytes (1% hematocrit) were incubated with RBC containing 5  $\mu\text{M}$  BCECF during 60 min, and then washed three times by centrifugation at  $20,000 \times g$  for 30 s. Afterward the cells were incubated in 300  $\mu\text{l}$  of modified RBC containing 1  $\mu\text{M}$  of the  $K^+/H^+$  exchanger nigericin and a KCl concentration similar to the cytoplasmic  $K^+$  concentration. Under these conditions, this procedure assures rapid equilibration of  $K^+$  and  $H^+$  concentrations in the intra- and extracellular compartments, so that the values of intra- and extracellular pH are equal. For obtaining the spectra, erythrocyte suspensions (0.032% hematocrit) were incubated in an AMINCO-Bowman series 2 spectrofluorometer (Thermo Fisher, Waltham, MA) at 20  $^\circ\text{C}$  under con-

stant agitation. For each run the pH of the extracellular solution was modified by HCl addition, and time measurements of fluorescent intensity were recorded at  $530 \pm 4 \text{ nm}$  (with  $490 \pm 4 \text{ nm}$  excitation wavelength) until a stationary value was achieved. Then, excitation spectra were recorded at  $530 \pm 4 \text{ nm}$  with excitation wavelengths ranging from  $300 \pm 2 \text{ nm}$  to  $500 \pm 2 \text{ nm}$ .

**Method 2: Microhematocrit Method**—Cell suspensions ( $1.9$ – $2.8 \times 10^9 \text{ cells ml}^{-1}$ ) were loaded into 80- $\mu\text{l}$  capillary tubes (75 mm in length) and centrifuged at  $10,000 \times g$  for 2 min. Relative volume was calculated from the relative length occupied by cells, as compared with total length of the suspension.

### RVD Computation

The RVD associated with the cellular volumetric response was calculated by Equation 1,

$$\text{RVD} = \frac{V_{r(\max)} - V_{r(t)}}{V_{r(\max)} - 1} \times 100 \quad (\text{Eq. 1})$$

where  $V_{r(\max)}$  is the maximal value of  $V_r$  attained during hypotonic swelling, and  $V_{r(t)}$  represents the value of  $V_r$  observed at different times after reaching  $V_{r(\max)}$ . RVD thus denotes the magnitude of volume regulation, with 100% RVD indicating complete volume regulation. In all cases we calculated the extent of RVD observed 40 min after  $V_{r(\max)}$ .

### Mathematical Analysis

In experiments in Fig. 4, intact erythrocytes were used to assess the effect of exogenous NECA (0.5–4  $\mu\text{M}$ ) on RVD. Next, Equation 2 was fitted to experimental data,

$$\text{RVD} = \text{RVD}_{\text{HYPO}} + \frac{\text{RVD}_{\max}[\text{NECA}]}{K_{1/2} + [\text{NECA}]} \quad (\text{Eq. 2})$$

where  $\text{RVD}_{\max}$  denotes the apparent maximal RVD and  $K_{1/2}$  is the substrate concentration at which a half-maximal RVD is obtained under the specific conditions of the experiment.

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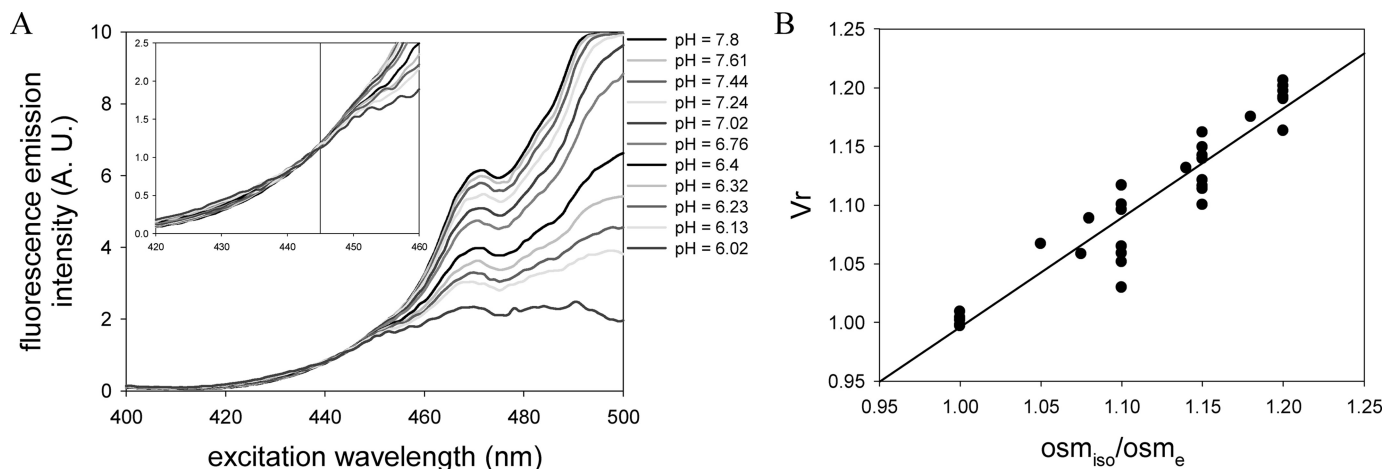


FIGURE 2. *A*, excitation spectra of BCECF-loaded erythrocytes. Cells (0.032% hematocrit) were incubated in isotonic high potassium media of different pH values (6.02–7.80) in the presence of 1  $\mu$ M nigericin. Emission wavelength was set to  $530 \pm 4$  nm, and excitation spectra were recorded by illuminating the sample with light of  $300\text{--}500 \pm 2$  nm. The inset shows a zoom of results for excitation range from 420 to 460 nm, with the vertical line denoting the isosbestic excitation wavelength. *B*, osmotic response of RBCs. Values of relative volume ( $V_r$ ) are plotted as a function of relative osmolarity in the intracellular ( $osm_{iso}$ ) and extracellular ( $osm_e$ ) compartments. The closed circles show  $V_r$  values for cells exposed to hypotonic calibration media. The continuous line represents a linear regression to  $V_r$  values.

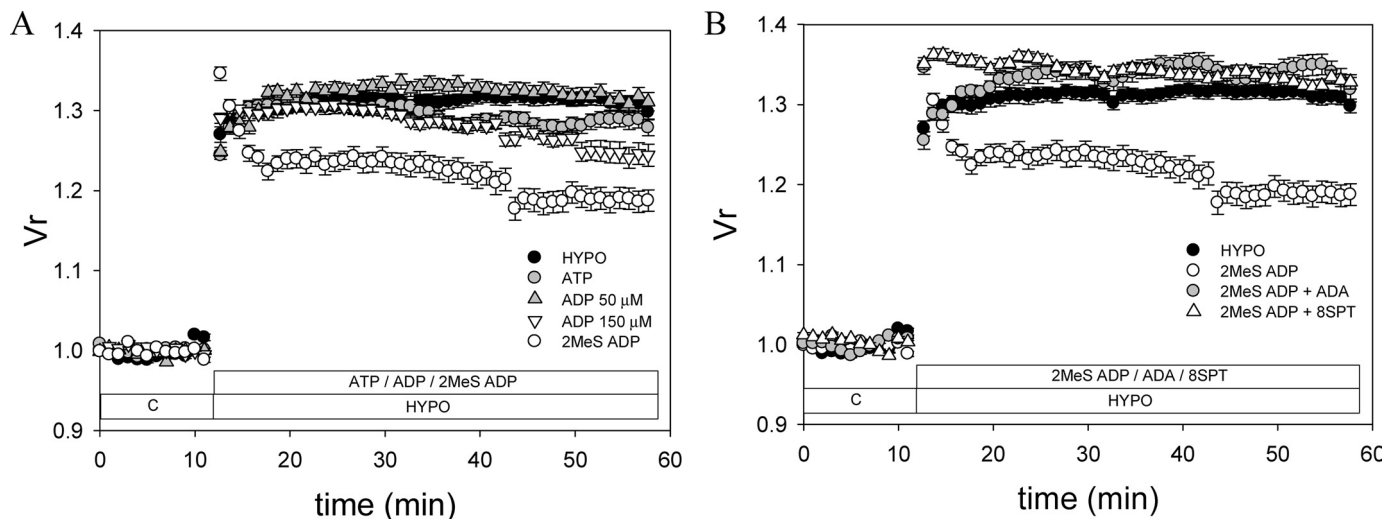


FIGURE 3. **Effect of P2 agonist on the relative volume ( $V_r$ ) of erythrocytes exposed to hypotonic medium.** *A*, kinetics of  $V_r$  estimated by fluorescence quenching of BCECF. Cells were exposed to isotonic medium (C) and hypotonic medium (HYP0) in the absence (closed circles) and presence of 300  $\mu$ M ATP (gray circles), 50  $\mu$ M ADP (gray triangles), 150  $\mu$ M ADP (open triangles), or 150  $\mu$ M 2MeS-ADP (open circles). Results are means  $\pm$  S.E.,  $n = 60$ ,  $n = 4$ . *B*, relative volume ( $V_r$ ) versus time of erythrocytes exposed to isotonic medium (C) and HYP0 in the absence (closed circles) and presence of 150  $\mu$ M 2MeS-ADP (open circles), 150  $\mu$ M 2MeS-ADP plus 1 unit ml<sup>-1</sup> adenosine deaminase (ADA; gray circles) or 150  $\mu$ M 2MeS-ADP plus 100  $\mu$ M 8-SPT (open triangles). Results are means  $\pm$  S.E.,  $n = 60$ ,  $n = 4$ . The same results for HYP0 (closed circles) and HYP0 plus 150  $\mu$ M 2MeS-ADP (open circles) were used in *A* and *B*.

$RVD_{HYP0}$  represents the RVD obtained in the absence of extrinsic modulators.

### Statistics

The absence or presence of RVD was evaluated by determining whether the slope of  $V_r$  versus time was significantly different from 0 ( $p < 0.05$ ), using a Pearson correlation test. The effect of the different treatments on RVD was evaluated by means of one-way analysis of variance followed by a Tukey-Kramer test of multiple comparisons. A  $p$  value of  $<0.05$  was considered significant. In all of the experiments, number of cells ( $n$ ) and number of independent preparations ( $N$ ) are indicated.

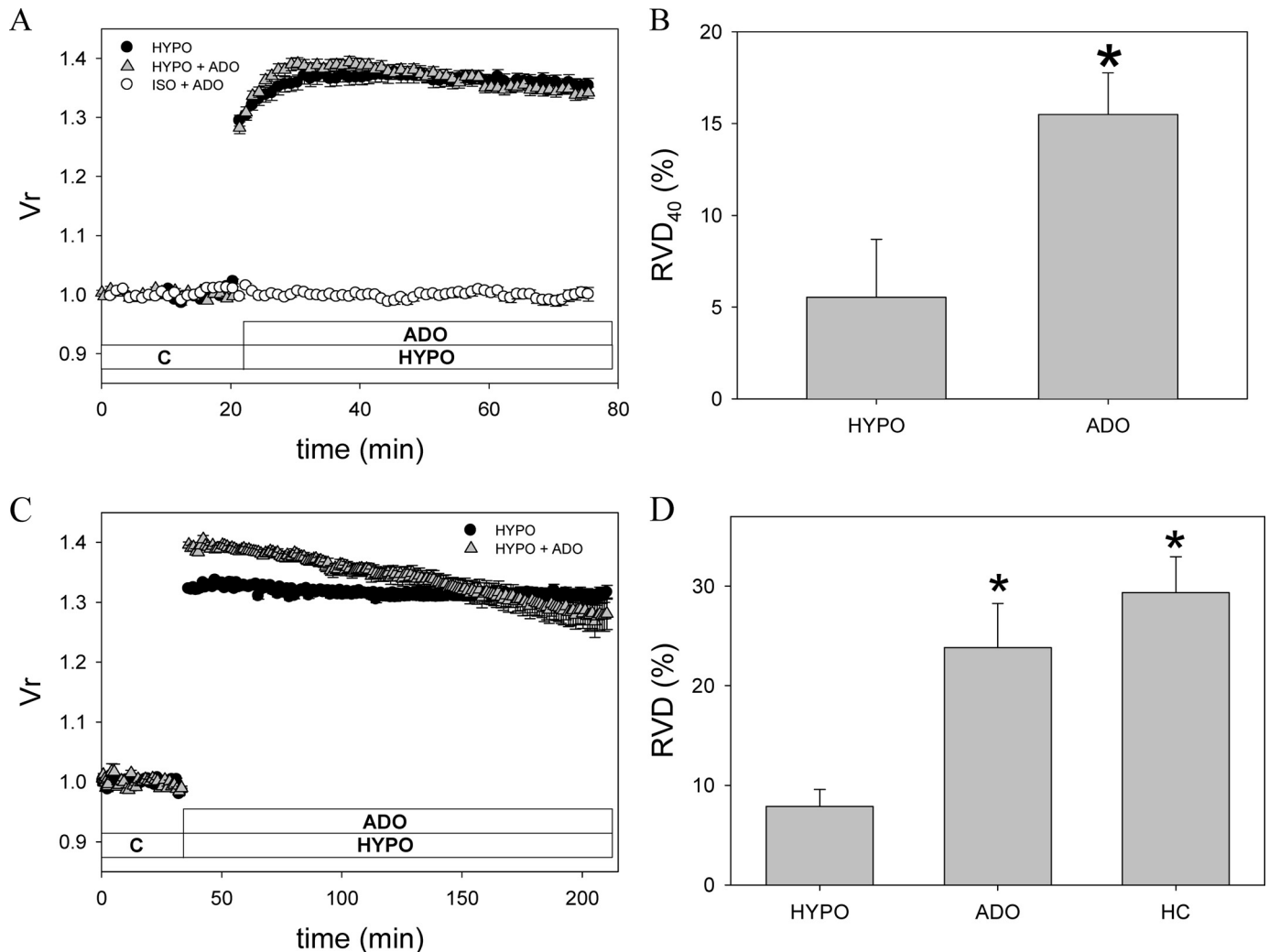
### RESULTS

**Ectonucleotidase Activity**—In light of the suggested role of ATP and its hydrolysis products in the volume regulatory

response of RBCs, the first series of experiments investigated the capacity of these cells to hydrolyze adenine nucleotides by membrane-bound enzymes called ectonucleotidases. Because nucleotides, unlike adenosine, are not taken up by intact cells, the rate of extracellular hydrolysis is a measure of ectoATPase (from ATP), ectoADPase (from ADP), and ectoAMPase (from AMP) activities.

In the presence of 300  $\mu$ M of any of the nucleotides,  $P_i$  accumulation increased linearly (Fig. 1A). EctoATPase activity was  $0.26 \pm 0.04$  nmol of  $P_i$  ( $10^{10}$  cells min)<sup>-1</sup>, whereas ectoADPase and ectoAMPase were  $1.49 \pm 0.29$  nmol of  $P_i$  ( $10^{10}$  cells min)<sup>-1</sup> and  $1.57 \pm 0.42$  nmol of  $P_i$  ( $10^{10}$  cells min)<sup>-1</sup>, respectively. Fig. 1B shows that ectoADPase and ectoAMPase activities were  $\sim 7$  times higher than ectoATPase activity. Similar experiments in the absence of cells showed no significant  $P_i$  accumulation ( $p = 0.37\text{--}0.55$ ).

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**FIGURE 4. Effect of P1 receptor modulation on the relative volume ( $V_r$ ) of erythrocytes under hypotonicity.** *A*, kinetics of  $V_r$  estimated by fluorescence quenching of BCECF at 20 °C. Cells were exposed to isotonic medium (C) and hypotonic medium (HYPO) in the absence (closed circles) and presence (gray triangles) of 2  $\mu$ M adenosine (ADO). The open circles show control experiments where cells were exposed to an isotonic medium containing 2  $\mu$ M adenosine. Results are means  $\pm$  S.E.,  $n = 60$ ,  $n = 4$ . *B*, values of RVD at 40 min estimated from experiments shown in *A*. \*,  $p < 0.05$  versus HYPO. *C*,  $V_r$  versus time for erythrocytes exposed to isotonic medium (C) and HYPO at 37 °C in the absence (closed circles) and presence (gray triangles) of 2  $\mu$ M adenosine (ADO). *D*, values of RVD at 120 min estimated from experiments shown in *C*; the RVD at 120 min obtained by microhematocrit method of cells exposed to HYPO at 37 °C plus 2  $\mu$ M adenosine (HC) is also plotted. \*,  $p < 0.05$  versus HYPO.

**Volumetric Measurements**—Relative cell volume ( $V_r$ ) was measured by BCECF fluorescence quenching and in selected experiments with the hematocrit method.

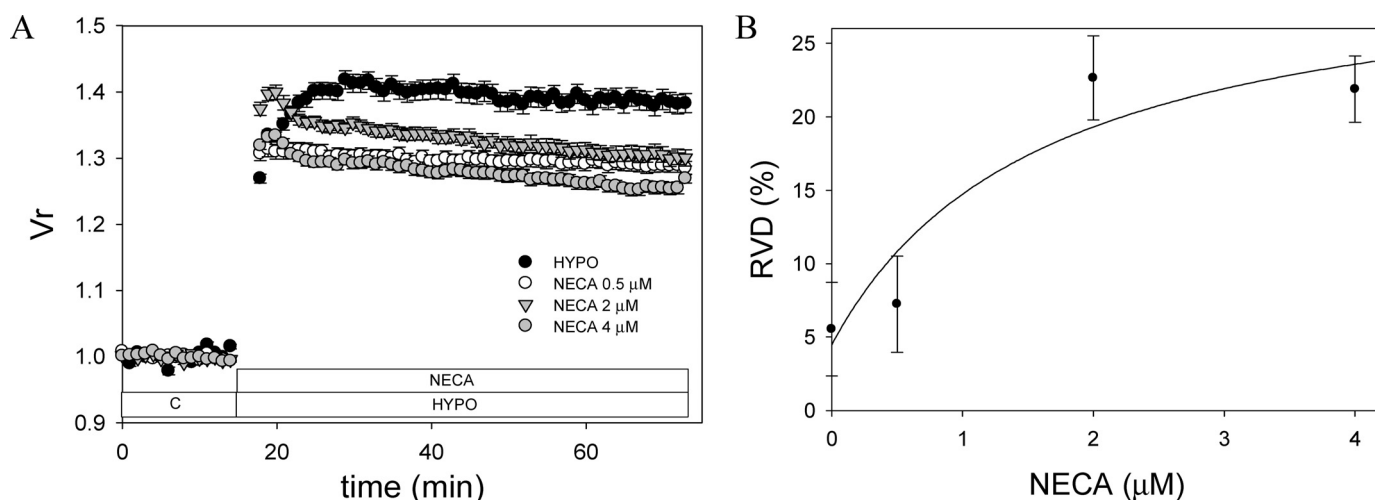
Because BCECF is sensitive to intracellular pH, volume measurements had to be performed by exciting cells at the excitation isosbestic wavelength. Accordingly, prior to the  $V_r$  measurements, excitation spectra of BCECF-loaded erythrocytes were analyzed using media with different pH values ranging from 6.02 to 7.8 (see “Experimental Procedures”). It can be seen that changes in fluorescence with pH become negligible at 445 nm (Fig. 2A), therefore all subsequent  $V_r$  measurements were made by evaluating fluorescence quenching at an excitation wavelength of  $445 \pm 5$  nm.

**Volumetric Response in Hypotonic Medium**—During calibration of the signal,  $V_r$  was measured in isotonic medium, followed by brief exposure to a series of media of increasing hypotonicity and later to isotonic medium again. Then, cells were exposed to a 210 mosM hypotonic medium (HYPO), and  $V_r$  was

monitored in the absence or presence of different compounds. As shown in Fig. 2B, there was a linear relationship between the osmotic ratio in the intracellular and extracellular compartments and the calculated  $V_r$  for the calibration media.

In a first series of experiments, we evaluated the effect of HYPO when applied alone or in the presence of either ATP, ADP, or 2MeS-ADP (a potent agonist of P2Y<sub>12</sub> and P2Y<sub>13</sub> receptors, known to be present in these cells). In HYPO, cells swelled to a maximum  $V_r$  value of  $1.30 \pm 0.01$ , which remained constant thereafter. In the presence of HYPO with 300  $\mu$ M ATP, a slight RVD could be calculated amounting to  $8.8 \pm 3.1\%$ , but this was not statistically significant ( $p = 0.31$ ). A similar result was obtained with 50  $\mu$ M ADP ( $9.6 \pm 3.8\%$ ;  $p = 0.10$ ). On the other hand, significant RVD values of  $21.8 \pm 4.6\%$  and  $45.5 \pm 3.9\%$  were obtained with 150  $\mu$ M ADP or 150  $\mu$ M 2MeS-ADP, respectively (Fig. 3A). However, these concentrations were 200–6000 times higher than the EC<sub>50</sub> values for all known P2Y receptors. This fact together with the finding that both 100  $\mu$ M

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**FIGURE 5. Dependence of RVD on the P1 agonist NECA.** *A*,  $V_r$  versus time for human erythrocytes exposed to isotonic medium (C), followed by hypotonic medium containing 0 (closed circles), 0.5 (open circles), 2 (gray triangles), or 5  $\mu$ M NECA (gray circles). Results are expressed as means  $\pm$  S.E.,  $n = 60$ ,  $n = 4$ . *B*, values of RVD (%) calculated for each NECA concentration shown in Fig. 4*A*. The continuous trace represents the fit by nonlinear regression of a single hyperbola to data. Results are expressed as means  $\pm$  S.E.,  $n = 60$ ,  $n = 4$ .

8-SPT (a P1 blocker) as well as 1 unit  $\text{ml}^{-1}$  of adenosine deaminase (that converts the P1 agonist adenosine to the non-P1 agonist inosine in the extracellular space) blocked 80–84% of the effect of 150  $\mu$ M 2MeS-ADP (Fig. 3*B*) make the observed responses unlikely to be mediated by P2 receptors and point toward a putative novel P1 modulation of RVD in erythrocytes.

**Effect of P1 Activation on RVD**—In isotonic medium containing 2  $\mu$ M adenosine no changes in cell volume were detected, whereas in HYPO plus 2  $\mu$ M adenosine, cells initially swelled to  $1.39 \pm 0.01$  and then displayed a significant RVD ( $15.9 \pm 2.2\%$ ; Fig. 4, *A* and *B*). Similar to experiments at room temperature, exposure of erythrocytes to HYPO at 37 °C (a more physiological temperature) caused an increase of  $V_r$  to  $1.34 \pm 0.01$ , and the cells subsequently remained swollen without significant loss of cell viability for 175 min (Fig. 4*C*). When the cells were exposed to HYPO at 37 °C plus 2  $\mu$ M adenosine, a significant RVD was detected ( $23.8 \pm 4.4\%$  at 120 min). Unexpectedly, the RVD found at 37 °C seemed to occur at a somewhat slower rate than the one found at 20 °C, because at 37 °C volume recovery after 55 min reached 16%, whereas a similar value was reached after only 40 min at room temperature. Although the reason for this remains unknown at present, these results nonetheless clearly show that the qualitative response remained unaltered at 37 °C.

The use of a microhematocrit method to measure cell volume under HYPO at fixed times provided similar results as those of the fluorescence-quenching method. In the absence of extrinsic modulators the hypotonic challenge led to no RVD, whereas the addition of adenosine (*i.e.* HYPO plus 2  $\mu$ M adenosine at 37 °C) led to RVD<sub>120</sub> of 24% (fluorescence quenching) and 29% (microhematocrit) (Fig. 4*D*).

The involvement of P1 in RVD was further evaluated by performing a dose-response curve for NECA (a potent analog of P1 receptors). Each data point in this dose-response curve is a value of the percentage of RVD calculated after exposing the cells to isotonic medium, followed by HYPO containing 0, 0.5, 2, and 4  $\mu$ M NECA and assessment of  $V_r$  kinetics in different experiments (Fig. 5*A*). There was a concentration-dependent

effect of NECA on RVD (Fig. 5*B*). A hyperbolic fit to experimental data yielded a maximal RVD (RVD<sub>max</sub>; asymptotic value of RVD at non-limiting agonist concentration) of  $26.8 \pm 9.5\%$  and an apparent  $K_{1/2} = 1.61 \pm 1.68 \mu\text{M}$ .

Then we analyzed the effect of P1 antagonists on the adenosine-induced RVD. As shown in Fig. 6 and supplemental Fig. S2, the RVD observed with 2  $\mu$ M adenosine could be inhibited  $81.3 \pm 15.5\%$  with 100  $\mu$ M 8-SPT, a known blocker for all P1 subtypes. In contrast, specific antagonists for  $A_1$  (DPCPX, 20 nM),  $A_{2A}$  (ZM241385, 10 nM), and  $A_3$  (MRS3777, 250 nM) only produced a non-significant 14–24% inhibition of RVD. Next, we used the P1 antagonist DPCPX at 100 nM, a concentration at which it can block both  $A_1$  and  $A_{2B}$  receptors (36). Accordingly, in cells exposed to 100 nM of DPCPX and 2  $\mu$ M ADO, RVD was reduced by  $65.9 \pm 8.4\%$ . Finally, we found that 10 nM of the  $A_{2B}$  antagonist MRS1754 was able to inhibit  $77.7 \pm 15.3\%$  of the RVD observed with adenosine. Regarding RVD in cells exposed to HYPO plus 100  $\mu$ M 8-SPT, after the initial swelling we observed a transient decrease of  $V_r$  followed by a slight increase (see supplemental Fig. S2). At present we have no explanation for this phenomenon, but because cell volume remained subsequently unaltered, this did not change the overall lack of volume regulation.

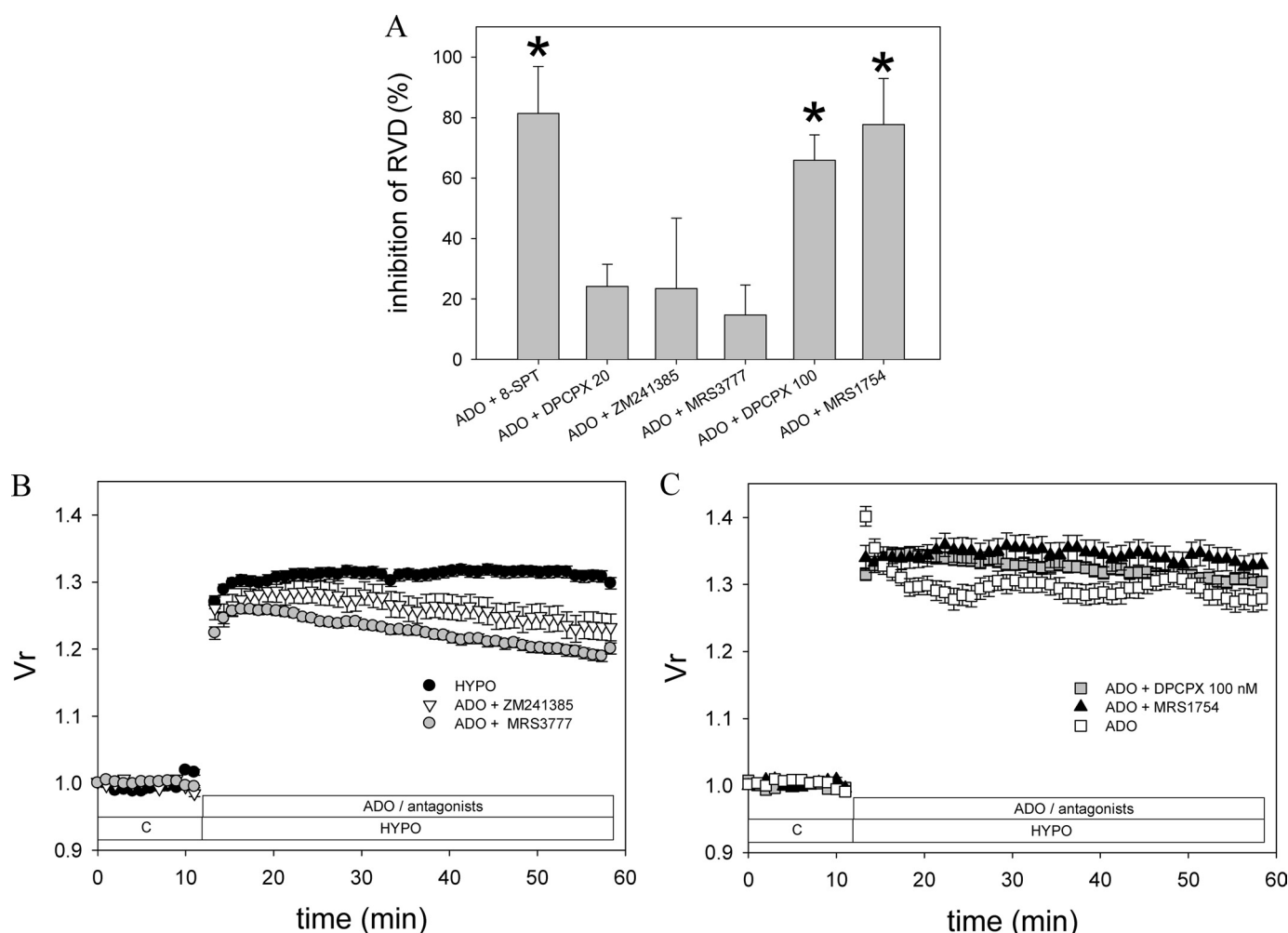
The experiments using selective antagonist of P1 receptors indicate that  $A_{2B}$  receptors are involved in RVD of human erythrocytes. Because these receptors signal toward an increase in cAMP concentration, in another set of experiments at 37 °C, we used 100  $\mu$ M forskolin to increase intracellular cAMP concentration. As shown in Fig. 7, forskolin was indeed able to induce RVD in these cells, supporting the idea of  $A_{2B}$  receptor activation during RVD.

## DISCUSSION

Within 1 day the human reticulocyte leaves the bone marrow and becomes a mature erythrocyte. During maturation reticulocytes undergo substantial morphological and biochemical changes where the loss of the nucleus and other organelles inactivates protein synthesis and oxidative phosphorylation.



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**FIGURE 6. Effects of antagonists of P1 receptors on RVD.** *A*, mature human erythrocytes exposed to hypotonic media in the absence (HYPO) or presence of 2  $\mu$ M adenosine alone (ADO) or 2  $\mu$ M adenosine plus: 100  $\mu$ M 8-SPT (ADO + 8-SPT); 20 nM DPCPX (ADO + DPCPX 20); 100 nM DPCPX (ADO + DPCPX 100); 10 nM ZM241385 (ADO + ZM241385); 10 nM MRS1754 (ADO + MRS1754); and 250 nM MRS3777 (ADO + MRS3777). Results are expressed as means  $\pm$  S.E. of the percentage of inhibition of the effect of HYPO plus 2  $\mu$ M adenosine,  $n = 60$ ,  $n = 4$ . \*,  $p < 0.05$  versus ADO. *B* and *C*, results of  $V_r$  versus time of mature human erythrocytes exposed to isotonic medium (C) followed by hypotonic media (HYPO) used to calculate results of *A*, only some of the results are shown to ease visualization (all plots are shown in supplemental Fig. S2). The cells were exposed to HYPO in the absence (closed circles in *B*) or presence of 2  $\mu$ M adenosine alone (ADO; open squares in *C*) or 100 nM DPCPX (ADO + DPCPX 100; gray squares in *C*); 10 nM ZM241385 (ADO + ZM241385; open triangles in *B*); 10 nM MRS1754 (ADO + MRS1754; closed triangles in *C*); and 250 nM MRS3777 (ADO + MRS3777; gray circles in *B*). Results are expressed as means  $\pm$  S.E.,  $n = 60$ ,  $n = 4$ .

In the course of this process, reticulocytes lose membrane material, including transporters, and this is accompanied by a reduction of water content and volume, and a progressive inactivation of volume regulatory mechanisms (37). Thus, in mature RBCs exposed to anisotonicity, changes in cell volume are assumed to be solely governed by transmembrane water fluxes that dissipate the difference in water chemical potential of the intra- and extracellular compartments. This is why, on average, cell volume follows a fairly good straight line when plotted against the inverse of the extracellular osmolality (2), *i.e.* mature RBCs behave as an osmometer.

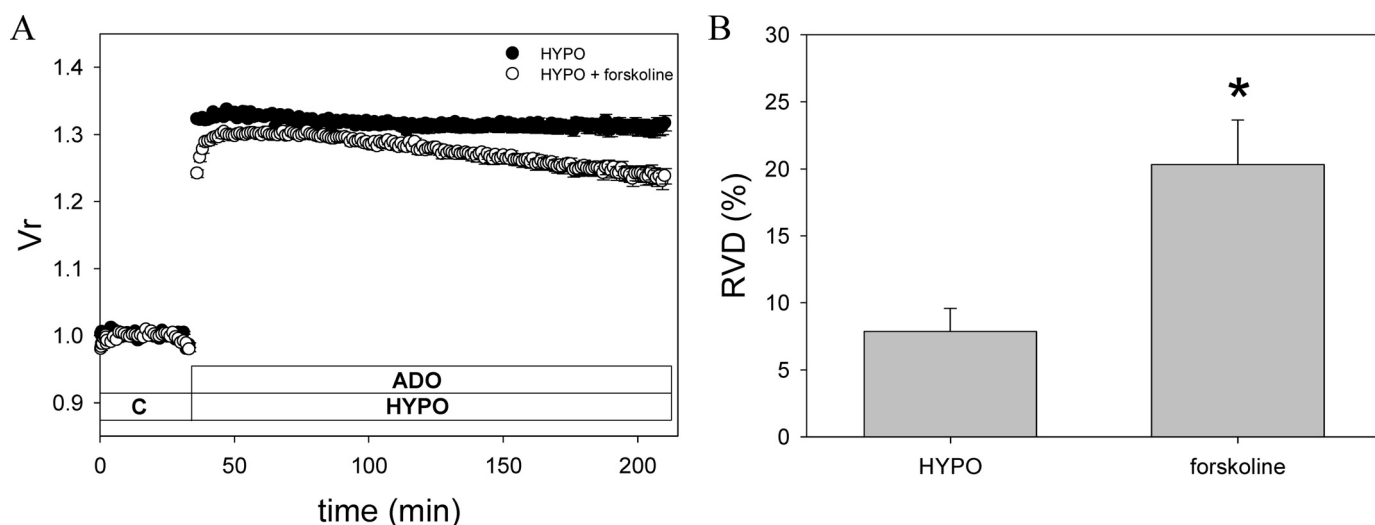
The main finding of the present study is that a small but significant volume regulation can nonetheless be activated when extracellular factors are considered, challenging the conventional view regarding the purely osmometric behavior of these cells. That is, following hypotonic swelling of mature human RBCs, extracellular nucleosides may interact with specific P receptors to trigger a significant compensatory shrinkage known as volume regulatory decrease.

As the stimulation of these receptors requires the presence of the suitable ligands as well as control of their effective concentration, we first investigated the presence and activity of corresponding membrane nucleotidases utilized for this function. In fact, our experiments examining the hydrolysis of exogenous nucleotides, a measure of ectonucleotidase activity of the cells, showed that mature RBCs are indeed capable of hydrolyzing ATP, ADP, and AMP, with ectoATPase activity being 7-fold lower than ADPase and AMPase activities. This agrees well with a previous study showing that, among RBCs examined in >40 vertebrate species, ectoATPase activity of human RBCs ranks as the lowest (38). Furthermore, as other studies have shown that ATP is released in human RBCs subjected to hypotonic medium (39), these enzymatic activities provide the physiological basis for the control over the action of these nucleotides on P receptors and in consequence one important level of control over volume regulation.

In mature human RBCs, the effect of extracellular nucleotides remained largely unexplored, mostly because of the abun-



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**FIGURE 7. Volumetric response of erythrocytes exposed to hypotonic medium in the presence of forskolin.** *A*,  $V_t$  versus time of cells exposed to isotonic medium (C) and hypotonic medium (HYPO) at 37 °C in the absence (closed circles) and presence (open circles) of 100  $\mu$ M forskolin. Results are means  $\pm$  S.E.,  $n = 19$ –22,  $n = 1$ . *B*, values of RVD at 120 min estimated from experiments shown in *A*. \*,  $p < 0.05$  versus HYPO.

dant literature describing the osmometric behavior of this cell model, a fact that agreed well with KCl co-transport (the main potential effector system of RVD) being inactivated in swollen cells (40). Only a single study, primarily devoted to volume regulation by extracellular ATP of *Necturus* erythrocytes, briefly reported that a similar treatment exerted on human erythrocytes produced no RVD (41), but this was not followed up.

In the present study, we first exposed mature human RBCs to hypotonic medium in the absence or presence of exogenous adenosine nucleotides. We observed that neither ATP (up to 300  $\mu$ M) nor ADP (up to 50  $\mu$ M) were able to induce RVD. It is important to mention that the concentrations of ATP and ADP used in this report are well beyond those needed to activate P2 receptors, particularly P2Y<sub>13</sub> receptors, which, according to Wang *et al.*, are highly expressed in human erythrocytes (23).

Accordingly, we turned our interest toward P1 receptors. In mammals four types of P1 receptors, A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>, have been cloned, displaying convergent biochemical and pharmacological profiles (36). Our first results indicated that relatively low micromolar concentrations of adenosine and NECA were required to indeed induce a significant RVD of the erythrocytes. This is, to our knowledge, the first report of volume regulatory decrease of anucleated mammalian erythrocytes exposed to hypotonic medium. Because previous information regarding expression of P1 receptor sub-types in mature RBCs was lacking, we decided to address this issue by evaluating the impact of various P1 blockers on ADO-induced RVD of RBCs. We observed that 8-SPT could inhibit 81% of the RVD, whereas specific agonists of A<sub>1</sub>, A<sub>2A</sub>, and A<sub>3</sub> receptors had no effect. In contrast, DPCPX, an agonist for A<sub>1</sub> and A<sub>2B</sub> receptors, and MRS1754, an agonist of A<sub>2B</sub> receptor, reduced 80 and 78% of the RVD observed with adenosine alone. Together, these findings suggest that, in mature human RBCs, RVD can be triggered by A<sub>2B</sub> receptor activation. In support for this conclusion we found that an activation of RVD may also be seen in the presence of forskolin, which increases intracellular cAMP concentrations (as does the activation of A<sub>2B</sub> receptor).

Altogether, our experiments thus suggested that in human RBCs both the receptors that trigger RVD as well as nucleotidases that may process these ligands are present. The fact that ATPase activity was rather minor, however, indicated that, at least in our *in vitro* model, the non-lytic release of ATP could not lead to accumulation of adenosine, and therefore, as directly observed in the volumetric experiments, an indirect action of ATP on RVD is discarded.

*In vivo* the situation is far more complex, and to understand the physiological relevance of the observed RVD response, the following points should be considered: 1) Under normal conditions low osmolarity does not appear to occur in the circulatory compartment. Also, the pH in the spleen and other organs is not sufficiently acidic to promote swelling of RBCs (42). Nonetheless, in certain diseases, altered cation permeability may result in volume perturbations, an example being overhydrated hereditary stomatocytosis, where RBCs may swell as solute and osmotically obliged water are gained (43). Thus, transient swelling may occur, at least under pathological conditions, and require compensatory net osmolyte fluxes leading to cell shrinkage. Furthermore, isosmotic swelling can take place in erythrocytes leaving the renal papilla as urea is taken up (44). In this respect, Joiner *et al.* have in fact previously reported the occurrence of RVD of mature RBCs when pretreated with nystatin (to increase cation content) and subsequently exposed to strong stimulation with urea (45). However, in our hands adenosine could not induce RVD upon induction of isosmotic swelling produced by replacing NaCl with 150 mM urea, neither in the absence nor in the presence of 32  $\mu$ M nystatin (data not shown). 2) Extracellular nucleotides can only act in an autocrine or paracrine fashion, because their mobility is restricted by nucleotidases present in the plasma, on cellular elements of the blood, and at the vascular wall. In contrast, RBCs are mobile and therefore encounter quite different environments across the circulatory system, not only in normal resting, but also in stimulated and pathological conditions. For example, transient high levels of ATP, ADP, and adenosine are involved in the regulation of platelet aggregation (46), and RBCs may them-

selves release adenylates in response to, *e.g.* mechanical stress (47) or reduced oxygen tensions (48). 3) Finally, the nucleotide metabolism may depend on the RBC-specific location, the action of ectonucleotidases from different cell types, and exonucleotidases of the blood plasma (49). For example, leukocytes and endothelial cells exhibit various degrees of ectoATPase and ectoADPase activities (50, 51), whereas AMP is mainly dephosphorylated by a plasma 5'-nucleotidase. The adenosine formed can interact with specific P1 receptors, be deaminated extracellularly, or taken up by erythrocytes through nucleoside transporters (52).

It is worth noting that the results obtained by fluorophore quenching, where cells are attached to a coverslip, could be reproduced by the use of a microhematocrit method using cells in suspension. This and the finding that RVD can also be observed at physiological temperature indicate that the RVD machinery can be activated *in vivo* during a hypotonic challenge.

For more than 50 years RBCs have been used as a simple cell model to characterize metabolic pathways as well as transport and signaling systems common to most animal cells. However, investigations were in part limited by the idea that, during maturation and aging, erythrocytes lose the machinery for many signaling systems, rendering many receptors obsolete (53–55). This is why most biophysical and physicochemistry textbooks used the anucleated erythrocyte as a model example to test the validity of the van't Hoff-Mariotte law, and, in general, volume regulation of erythrocytes focused on all but normal mature erythrocytes, *i.e.* on either nucleated non-mammalian cells, mammalian reticulocytes, or sickle RBCs (7, 56–58). As a logical consequence, volume regulatory mechanisms in mature RBCs remained largely unexplored.

The seminal work of Wang *et al.* (16) provided a crucial advancement of our understanding of volume regulatory mechanisms, because it postulated a link between activation of a surface "P" receptor and a mechanism to allow volume regulation of swollen cells. Although the proposed model and subsequent reports mostly described an action of ATP on specific P2Y receptors, the activities of ectonucleotidases made other metabolites available as alternative agonists to modulate cell volume. In line with this, Wurm *et al.* (59) recently described an activating effect of adenosine A<sub>1</sub> receptors on RVD of retinal glial cells using knockout mice. Results of our present study now add to the number of possible scenarios showing that in mature RBCs specific P1 receptors remain active, allowing these cells to partially compensate the hypotonic swelling. The complexity of volume regulatory signaling pathways apparently increases as we look at it, and it remains to be seen which further unexpected interactions between signaling pathways and effector mechanisms of volume regulation are to be discovered. In line, as this report was prepared, it was reported that in human RBCs and in other human cells a KCl co-transport involved in volume regulation and kept inactive by phosphorylation, KCC3, is dephosphorylated upon hypotonic exposure. However, although this led to activation and enhanced ion transport activity in other cells (60), this was apparently not the case in RBCs, confirming the presence of an additional level of control present in these cells.

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