Kinetics of ATP release and cell volume regulation of hyposmotically challenged goldfish hepatocytes

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Pafundo DE, Chara O, Faillace MP, Krumschnabel G, Schwarzbaum PJ. Kinetics of ATP release and cell volume regulation of hyposmotically challenged goldfish hepatocytes. Am J Physiol Regul Integr Comp Physiol 294: R220–R233, 2008. First published October 10, 2007; doi:10.1152/ajpregu.00522.2007.—In most animal cells, hypotonic swelling is followed by a regulatory volume decrease (RVD) thought to prevent cell death. In contrast, goldfish hepatocytes challenged with hypotonic medium (180 mosM, HYPO) increase their volume 1.7 times but remain swollen and viable for at least 5 h. Incubation with ATP·S (an ATP analog) in HYPO triggers a 42% volume decrease. This effect is concentration dependent ($K_{1/2}$ = 760 nM) and partially abolished by P2 receptor antagonists (64% inhibition). A similar induction of RVD is observed with ATP, UTP, and UDP, whereas adenosine inhibits RVD. Goldfish hepatocytes release more than 500 nM ATP during the first minutes of HYPO with no induction of RVD. The fact that similar concentrations of ATP·S did trigger RVD could be explained by showing that ATP·S induced ATP release. Finally, we observed that in a very small extracellular volume, hepatocytes do show a 56% RVD. This response was diminished by P2 receptor antagonists (73%) and increased (73%) when the extracellular ATP hydrolysis was inhibited 72%. Using a mathematical model, we predict that during the first 2 min of HYPO exposure the extracellular [ATP] is mainly governed by ATP diffusion and by both ecto-ATPase activity. We show that goldfish hepatocytes under standard HYPO (large volume) do not display RVD unless this is opposed by the compensatory efflux of osmolytes and water. Most vertebrate cells, when suddenly exposed to hypotonic conditions, rapidly swell due to the influx of water but subsequently, despite continuous osmotic perturbation, this swelling is opposed by the compensatory efflux of osmolytes and water. The resulting cell volume decrease is termed regulatory volume decrease, or RVD (20). RVD is mediated to a large extent by KCl loss through both K+ and Cl− channels, parallel activity of K+-H+ and Cl−-HCO3− exchangers or KCl cotransporters (5, 21). However, little is known on the extracellular factors inducing RVD.

In the past few years, a growing body of evidence has shown that extracellular nucleotides, mainly ATP, play a significant role in the control of cell volume regulation by binding to specific cell surface molecules termed “P” receptors (purinic and pyrimidinic receptors) (29). P1 receptors bind adenosine and other nucleosides, whereas P2 receptors bind mainly dinucleotides and trinucleotides. The P2 receptor family (8, 32, 36, 38) consists of two main subtypes, P2X and P2Y, representing ligand-gated cation channels and G protein-coupled receptors (linked to phospholipase C), respectively. At the cell membrane, the availability of agonists of P receptors is tightly regulated by specific membrane-bound enzymes located at the surface of the cell. These include E-NTPDases, a family of enzymes that hydrolyze nucleoside diphosphates and triphosphates, and also ecto-5′-nucleotidases that promote the dephosphorylation of monophosphate nucleotides. The coordinated action of E-NTPDases and 5′-nucleotidases reduces extracellular nucleotide levels and therefore restrains their action on P2 receptors.

All cell types appear to possess mechanisms that enable a controlled, nonlytic release of ATP, that is, a release not involving cell membrane rupture, which occurs in response to osmotic, mechanical, and neurohormonal stimuli (17, 22). As a consequence of this ATP efflux, cell surface ATP concentrations of up to 15 μM can be achieved, levels sufficient to activate P2 receptors (29).

Extracellular ATP (ATP)e is also important for liver function, where cholangiocytes and hepatocytes from different species (13, 14, 35, 37) show regulated release of ATP, particularly in response to a reduction of medium osmolarity. Under such conditions, hepatocytes can release ATP to signal to both nearby hepatocytes and bile duct cells (10). The significance of an autocrine/paracrine induction of RVD by extracellular ATP was first evidenced by Wang et al. (44). These authors showed that rat hepatoma cells exposed to hypotonicity first swelled and then released ATP in response to this cell volume increase. The resulting endogenous extracellular ATP was shown to bind to P2 receptors, which enhanced Cl− permeability and induced RVD. Conversely, either removal of extracellular ATP or P2 receptor blockade prevented both chloride channel activation and volume recovery. The ubiquity of such findings has been confirmed using primary cultured hepatocytes from humans (13) and trout (35).

A recent study using hepatocytes from trout and goldfish (two freshwater fish species) provided new insights into this physiological mechanism (35). First, the volume regulatory
response in trout hepatocytes was the result of an acute P2 receptor activation (by ATP, promoting RVD) followed by a delayed P1 receptor activation (by extracellularly derived adenosine), inhibiting RVD. Second, goldfish hepatocytes exposed to hypotonicity, unlike all other hepatic cells studied so far, swell and maintain viability without showing either RVD or net K⁺ efflux (12). However, the addition of exogenous ATP to goldfish hepatocytes in vitro triggered RVD (35), suggesting that, in principle, a similar mechanism should be present in these cells. This makes goldfish hepatocytes an excellent model to study the properties of nucleotide-dependent RVD activation. Accordingly, this study aimed at evaluating the role of extracellular nucleotides on the RVD of goldfish hepatocytes.

Our approach includes an assessment of time-dependent volume changes of cells in the presence of exogenous nucleotides, as well as determinations of ATP present on the surface of these cells. Furthermore, because goldfish hepatocytes were shown to sequentially dephosphorylate ATP (41), we investigated whether RVD can be regulated by the combined effects of ATP and adenosine. Finally, we present a mathematical model that accounts for the time course of ATP accumulation in the extracellular space when goldfish hepatocytes are challenged by hypotonicity.

MATERIALS AND METHODS

Chemicals

Collagenase (type IV), cibacron blue 3GA, hexokinase, adenosine, suramin, poly-D-lysine, luciferase, apyrase (grade III), ATP, ATPyS, ADP, UTP, UDP, AMP-PCP, CoA-SH, ionomycin, gentamicin, kanamycin, and 8-sulfophenyl theophylline (8-SPT) were purchased from Sigma (St. Louis, MO). Luciferin and the AM esters of calcein and FURA-2 were obtained from Molecular Probes (Eugene, OR). [γ³²P]ATP (5.4 Ci/mg, ~10 mCi/ml) was from NEN Life Science Products (Boston, MA). Leibovitz L-15 medium and DMEM were purchased from Invitrogen (Buenos Aires, Argentina). All other reagents were of analytical grade.

Animals

Goldfish Carassius auratus L (10–30 g) were obtained commercially from a local aquarium in Buenos Aires, Argentina. Fish were maintained in 200-liter tanks at 15°C for at least 2 wk before being used. Fish were kept under a 12:12-h light-dark cycle and fed ad libitum. The use of the animals and cell lines conformed to institutional animal care and use committee.

Primary Culture of Hepatocytes

Goldfish hepatocytes were isolated by incubating fragments of liver tissue with a medium containing collagenase (300 U/ml) and mechanical disruption as described before (25, 39). Isolation procedures were performed under sterile conditions.

After isolation, goldfish hepatocytes (3 × 10⁶ cells/ml) were suspended in modified Leibovitz L-15 medium containing 10 mM HEPES, 5 mM NaHCO₃, 50 μg/ml gentamicin, and 100 μg/ml kanamycin (pH 7.6 at 15°C, 305 mosM). Either 10⁴ or 10⁵ cells were seeded onto 0.02% poly-D-lysine-pretreated glass coverslips and cultured in atmospheric air at 15°C for 2 or 3 days. Medium was changed every 24 h.

The viability of isolated hepatocytes was routinely assessed by Trypan blue exclusion (before the onset of each experiment) and by retention of calcein or FURA-2 fluorescence (during the whole experimental period).

Cell Culture

The human hepatoma cell line HepG2-C3A was obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained at 37°C in an atmosphere of 5% CO₂ in DMEM, pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin.

Incubation Media

Except where otherwise stated, goldfish hepatocytes were incubated in media (pH 7.6) at 20°C, having the following composition (in mM): C (isotonic medium): 10 HEPES, 135.2 NaCl, 3.8 KCl, 1.3 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 10 NaHCO₃, osmolality: 286 mosM; D, medium C without NaCl, osmolality 52.5 mosM; E, medium C plus 400 mM sucrose, osmolality 660 mosM; A (hypotonic calibration medium), a 4:1 volume mixture of media C and D, osmolality 250 mosM; and B (hypertonic calibration medium), a 4:1 volume mixture of media C and E, osmolality 320 mosM.

Hypotonic media (denoted as HYPO) were prepared by mixing 1:1 volume mixture of isotonic and D medium, yielding an osmolality of 160–170 mosM, corresponding to an osmolality 54–58% of that of isotonic media.

In some experiments (Fig. 1A), Leibovitz media (L-15) with some modifications were used: L-15, with an osmolality of 305 mosM, and HYPO L-15, similar to L-15, but NaCl was reduced to 69 mM, with an osmolality 180 mosM.

For calibration of the fluorescent signal using these media, slightly hypertonic and hypotonic L-15 media were prepared by adding sucrose (hyper L-15) or reducing NaCl (hypo L-15), respectively. HepG2 cells were incubated in media (pH 7.4) at 20°C having the following composition (in mM): HepG2-C, 20 HEPES, 130 NaCl, 4.7 KCl, 1.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 5 NaHCO₃, 10 glucose, osmolality 290 mosM. HepG2-HYPO was similar to HepG2-C, but NaCl was reduced to 65 mM, with an osmolality 170 mosM.

Cell Volume Determinations

Fluorescence microscopy. The technique was described in detail in a previous report (35). Briefly, changes in cell water volume were inferred from readings of the fluorescence intensity recorded by exciting calcein at 470 nm. Temporal changes in fluorescence intensity recorded from a small region of dye-loaded cells reflect changes in intracellular fluorophore concentration, and therefore alterations of cell water volume (3, 4).

A calibration is needed to convert values of relative fluorescence for each cell to relative volume (Vr). During this fluorescence calibration, cells had to be sequentially exposed to isotonic media, followed by slightly hypotonic, isotonic, slightly hypertonic, and isotonic media. During all of these sequential media exchanges, the X, Y, Z position of the microscope field must remain unchanged. In experiments of Fig. 6, cells were incubated in 40 μl of medium, the technique described could thus not be used, since using this small incubation volume media exchange could not be performed. This is why in these experiments, we estimated cell volume by planimetry, where calibration of the fluorescence signal is not required.

Planimetry. 10⁴ cells attached to the coverslip placed on the chamber’s bottom were loaded with 2 μM of calcein-AM until fluorescence of the cells reached about 5–10 times the autofluorescence level. The solution was then washed out with medium C for 1 h before starting the experiment. At the beginning of the experiment, the chamber was placed in the previously described microscope set up, and medium C was replaced with 40 μl of hypertonic media containing 1% BSA.

Images of calcein emission from fluorescent cells were recorded by fluorescence microscopy. Cell volumes were estimated from the areas...
of one plane of the cells, assuming that cells have a spherical shape, and their volume was changed by the same magnitude in all radial directions as follows:

\[ V = \frac{4}{3} \pi \left( \frac{a}{2} \right)^{3/2} \]  

(1)

where the area of one plane of the cells (denoted as \( a \)) was measured with Metamorph software (ver. 6.0, Universal Imaging, Downingtown, PA) from the images recorded. Volume data were presented as relative volume (\( V_r \)), where

\[ V_r = \frac{V_t}{V_{iso}} \]  

(2)

with \( V_t \) being the value of \( V \) at time \( t \) and \( V_{iso} \) the volume in isotonic medium. The estimation of cell volume of goldfish hepatocytes using planimetry has been validated before (12).

**RVD computation.** The RVD associated with the volumetric response of cells exposed to hypotonic medium was calculated by the following equation (2):

\[ RVD = \frac{V_{r_{max}} - V_r}{V_{r_{max}} - 1} \times 100 \]  

(3)

where \( V_{r_{max}} \) is the maximal value of \( V_r \) attained during hypotonic swelling, and \( V_r \) 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% of RVD indicating complete volume regulation. We calculated the extent of RVD observed 20 min (RVD20) and 40 min (RVD40) after \( V_{r_{max}} \), so as to cover the whole time frame of the regulatory volume response studied.

**Cytosolic-Free Calcium**

The concentration of cytosolic-free calcium (\( \text{Ca}^{2+} \)) of individual hepatocytes was determined in cells loaded with 6 \( \mu \text{M} \) of the \( \text{Ca}^{2+} \)-sensitive fluorescent dye FURA-2-AM. The procedure for fluorophore cell loading and washout, addition and removal of media, and acquisition of fluorescence intensity using a dual-wavelength excitation system was described before (2). Cells loaded with FURA-2 were exposed to alternate illumination at 358 and 380 nm and fluorescent images recorded at 510 nm. At the end of the experiment, cells were exposed to a medium containing ionomycin dissolved in medium C (maximum \( \text{Ca}^{2+} \) concentration), and ionomycin plus 10 mM EGTA in a \( \text{Ca}^{2+} \)-free medium C (minimum \( \text{Ca}^{2+} \) concentration). Absolute \( \text{Ca}^{2+} \) values were calculated from the measured fluorescence ratios (358/380) using the formula given by Grynkiewicz et al. (18) with a dissociation constant (\( K_D \)) of 225 nM.

**Extracellular Concentration of ATP**

Firefly luciferase produces light by the ATP-dependent oxidation of luciferin and can thus be used to determine ATP concentrations in aqueous media (7). In the present study, cells were incubated in the presence of soluble firefly luciferase to determine the ATP present in the bulk extracellular space. Two different types of determinations were made. On the one hand, we used "exogenous" ATP to determine ecto-ATPase activity from the rate of disappearance of ATP (see "ecto-ATPase activity" for details). On the other hand, measurements of "endogenous" extracellular ATP were performed with 40 \( \mu \)l of incubation medium, a condition in which the medium has a height of about 105 \( \mu \)m (height at the coverslip bottom of the chamber equals 0). As shown by Okada et al. (33), continuous measurements of ATP using this small volume provide similar results as the measurement of ATP at the cell surface using a surface-attached chimeric luciferase.

We designed a luminometer apparatus, which allows the continuous quantification of extracellular ATP via light output detection of the luciferase-luciferin reaction. The setup consists of a photomultiplier tube (P3032–02; Electron Tubes, Coventry, UK) surrounded by a light-tight aluminum housing and a sample holder that can accommodate the coverslips used for primary culture of hepatocytes. An electronic shutter (Uniblitz, Vincent Associates, Rochester, NY) between the sample holder and the surface of the photomultiplier protects the tube from stray light during reagent addition. Output from the luminometer was recorded digitally using a PC with custom-built software (Sobral ver. 1.01, Buenos Aires, Argentina).

**Calibration process.** At the end of each experiment a calibration curve was performed by sequentially adding increasing concentrations of ATP from 0.25 to 2,500 nM. Also, a calibration curve was performed for each experiment in similar conditions but in the absence of cells. Both calibration curves displayed a linear relationship in the range tested. The slopes of these curves were directly related to luciferase activity. In 90% of these calibrations, luciferase activity with or without cells was similar, thereby yielding similar parameter values of the calibration function. When these calibrations were different, the experiments were discarded.

In experiments of Fig. 4B, ATP\( \gamma \)S was used during ATP measurements. Since the ATP\( \gamma \)S reagent used had 0.01 nM ATP for each nM, the contaminant ATP had to be eliminated. To this end, we dissolved 5 mM ATP\( \gamma \)S in a medium containing 5 mM \( \Delta \)-mannose, 5 mM MgCl\(_2\), 10 mM HEPES, 138 mM NaCl, and 5 mM KCl. Then, the ATP\( \gamma \)S solution was incubated with 0.1 U/\( \mu \)l hexokinase for 4 h at room temperature. This ATP\( \gamma \)S stock solution can be used in experiments without further treatment, since although hexokinase remains active in the stock solution, experimental media had not enough substrate (i.e., 0 mM \( \Delta \)-glucose, 1–4 mM \( \Delta \)-mannose) for that enzyme.

**Intracellular ATP Content**

The total mass of intracellular ATP was assessed using a luciferin-luciferase-based method. In brief, 10\(^6\) cells cultured on coverslips were incubated in 2% perchloric acid for 1 h to induce cell lysis and inactivation of enzymes. Then the acid mixture was neutralized with (final concentration in mM): 14 HEPES, 7 KCl, and 45.5 KOH. To measure ATP, the following reagents were added (final concentration): 1% BSA, 200 \( \mu \)M luciferin, 100 \( \mu \)g/\( \mu \)l CoA and 625 ng luciferase, with a final volume of 500 \( \mu \)l. Detection and calibration methods were performed as above.

**Ecto-ATPase Activity**

Because ATP release is a consequence of cell suspension, ATP release does not permeate the cell membrane (41), any hydrolysis of ATP into ADP and Pi in a cell suspension can be defined as an ecto-ATPase activity, the time course of which yields a measure of the rate at which ectoenzymes hydrolyze extracellular ATP. Ecto-ATPase activity was determined at 20°C by following the release of \( [\gamma^32\text{P}] \)Pi from \( [\gamma^32\text{P}] \)-ATP as described before (40, 41). Care was taken to obtain initial rate values.

In experiments of Fig. 3A (substrate curve), the initial rate of disappearance of ATP (\( V_{i_{\text{H}}\text{H}} \)) was estimated from the rate of disappearance of ATP by luminometry methods (see Extracellular concentration of ATP). In brief, coverslips with attached cells were
mounted in the measuring chamber of the luminometer and, as described before, after background luminescence recording, cells were exposed to HYPO containing 1% BSA, 50 ng luciferase, 200 μM luciferin, and 100 μg/ml CoA. Light emission was measured for 15 min, and then ATP was added to the chamber at final concentrations of 50, 100, 250, 500, 1,000, 2,500, and 5,000 nM (in different experiments), and light emission was recorded for 20 min.

Recorded light output was transformed into ATP concentration vs. time by means of a calibration curve.

Oxygen Consumption

Rates of oxygen consumption (VO2) were determined with a dual-chamber Cyclobios Oxysgraph (19), as described previously (25). Before each measurement, two groups of cells from one preparation, at a density of 30 × 10⁶ cells/ml, were incubated under either isotonic or hypotonic conditions for 30 min. Hepatocytes (5 × 10⁶ cells/ml) were then injected into one of two measuring chambers containing the equivalent incubation medium, and oxygen consumption was followed over ~10 min. At the end of the experiment, cells were recounted with a Neubauer chamber.

Mathematical Analysis

In experiments of Fig. 1D, we estimated the effect of ATPγS (0–50 μM) on RVD20 and RVD40 of primary cultured hepatocytes. Next, Eq. 4 was fitted to experimental data:

\[ Y = Y_0 + \frac{Y_{\text{max}} [\text{ATPγS}]}{K_{1/2} + [\text{ATPγS}]} \]  

where \( Y \) and \( Y_0 \) are the values of RVD at any ATPγS concentration and that at [ATPγS] = 0, respectively. The value of \( Y_{\text{max}} \) represents the apparent maximal value of RVD, and \( K_{1/2} \) is the concentration of ATPγS at which a half-maximal RVD is obtained under the specific conditions of the experiment.

Ecto-ATPase activity. As explained above, ecto-ATPase activity was estimated by two different methods: 1) by following the course of [γ32P]Pi release from [γ32P]ATP and 2) from the rate of disappearance of ATP, where the nucleotide was determined continuously by a luciferase-based luminescent method.

For method 1, Eq. 5 was fitted to experimental data.

\[ Y = Y_0 + A_1(1 - e^{-kt}) \]  

where \( Y \) and \( Y_0 \) are the values of Pi at any time (t) and that at t = 0, respectively; \( A_1 \) represents the maximal value for the increase of Pi at any ATP concentration (\( k.A \) i.e., the first derivative of Eq. 5 for t tending to 0).

For method 2, a double exponential decay (Eq. 6) was fitted to experimental data of ATP vs. time to obtain the parameters values of best fit. We chose this empirical function because it provided the smallest value of the Akaike criterion (1).

\[ Y = A_1 e^{-kt_1} + A_2 e^{-kt_2} \]  

In Eq. 6, \( Y \) stands for the values of ATP at any given time (t); \( A_1, A_2, k_1, \) and \( k_2 \) are the parameters that provided the best fit to the experimental data. Initial rate of ATP extinction was calculated as \( v_{\text{lum}} = A_1 k_1 t + A_2 k_2 t \) (the first derivative of Eq. 6 for t tending to 0). In Fig. 3A, values of \( v_{\text{lum}} \) vs. [ATP] were plotted, and a hyperbola (Eq. 7) was fitted to the experimental data.

\[ Y = \frac{Y_{\text{max}} [\text{ATP}]}{K_{1/2} + [\text{ATP}]} \]  

where \( Y \) is the value for \( v_{\text{lum}} \) at any ATP concentration, \( Y_{\text{max}} \) represents the apparent maximal value of ecto-ATPase activity, and \( K_{1/2} \) the concentration of ATP at which a half-maximal activity is obtained under the specific conditions of the experiment.

Effect of extracellular pH on ATP kinetics. Data of ATP vs. time measured in media at two different extracellular pH values were compared by fitting Eq. 8 to data.

\[ Y = Y_0 + a_1(1 - e^{-t/k_1}) - a_2(1 - e^{-t/k_2}) \]  

where \( Y \) is the value of ATP at any time t, \( Y_0 \) is the value of ATP at time \( t = 0 \), \( k_1 \) and \( k_2 \) are the rise and decay constants and \( a_1 \) and \( a_2 \) are constant parameters of the fit.

The maximum ATP achieved in each experiment was calculated from the first derivative of Eq. 8, as follows:

\[ Y_{\text{max}} = Y_0 + a_1(1 - e^{-t/k_1})^2 - a_2(1 - e^{-t/k_2})^2 \]  

Modeling of the kinetics of extracellular ATP. A mathematical model consisting of a system of differential equations was used to predict the time-dependent changes in the concentration of extracellular ATP when hepatocytes are challenged by hypotonicity.

In the model, the concentration of extracellular ATP is governed by lytic and nonlytic release of ATP, hydrolysis of ATP by ecto-ATPase activity at the cell surface, and ATP diffusion within the extracellular compartment. Details on this model can be seen in the appendix.

Statistics. The absence or presence of RVD was evaluated by determining whether the slope of \( V_r \) vs. 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 ANOVA followed by a Tukey-Kramer test of multiple comparisons. A \( P \leq 0.05 \) was considered significant. The F values of the ANOVAs were included in the figure legends. The mean value of the parameters obtained by fitting Eqs. 8 and 9 to data of Fig. 8B, were compared by a two-sided Student’s t-test.

For all of the experiments on the time course of \( V_r \) and Ca2+ in 55–60 cells from 4 or 5 independent preparations were used. In all of the other experiments, number of cells (N) and number of independent preparations (n) are indicated.

RESULTS

Volumetric Response of Goldfish Hepatocytes Under Hypotonic Medium

\( V_r \) was measured in isotonic, followed by hypotonic media. Whenever volume regulation took place, RVD was assessed by the extent of RVD after 20 min (RVD20) and after 40 min (RVD40; see MATERIALS AND METHODS).

Standard conditions. In a first series of experiments, goldfish hepatocytes were exposed to hypotonic medium (HYPO; 160–170 mosM) at low density (10⁶ cells in 500 μl) in the absence of any extrinsic modulator. Experiments were run with both freshly isolated hepatocytes (fresh hepatocytes) and hepatocytes maintained in culture for 48–72 h (cultured hepatocytes).

Cells swelled to a maximum \( V_r \) value of 1.88 ± 0.03 (fresh hepatocytes) and 1.74 ± 0.02 (cultured hepatocytes), but no significant RVD was observed (\( P = 0.15–0.16 \)). Cell viability diminished 2.0% during the first minute of HYPO exposure and remained constant thereafter. The apparent lack of RVD in goldfish hepatocytes could be due to a very slow volume decrease, which cannot be detected in the 45 min of the experimental period. To monitor volume changes for longer periods, hepatocytes were exposed to isotonic (L15) and hypotonic (hypo L15) Leibovitz media (see MATERIALS AND METHODS).

Under these conditions, cultured cells could be exposed to hypotonicity for up to 5 h with almost no loss of viability. In hypo L15, cells rapidly swelled to a \( V_r \) of 1.78 ± 0.04, and
thereafter, cell volume remained constant for the whole experimental 5-h period (Fig. 1A), so that no RVD could be detected ($P = 0.2$). Another series of experiments examined the metabolic integrity of the cells facing the hypotonic challenge. We found that in isotonic medium, oxygen consumption amounted to $0.35 \pm 0.02 \text{ nmol O}_2 (10^6 \text{ cells min}^{-1})$, whereas in hypotonic medium, it was $17\%$ higher, that is, $0.41 \pm 0.01 \text{ nmol O}_2 (10^6 \text{ cells min}^{-1})$.

Thus, both fresh and cultured hepatocytes exposed to hypotonic media showed the same volumetric response and no RVD. Therefore, in the following experiments, we used cultured hepatocytes in standard conditions.

**Effect of P2 receptor agonists and antagonists on RVD.**

Cultured goldfish hepatocytes were incubated in HYPO containing either 5 $\mu M$ ATP, ATPγS (a slowly hydrolyzable analog of ATP), UTP, ADP, or UDP. It was found that, except for ADP, all P2 receptor agonists induced RVD, with no significant differences among treatments (Fig. 1, B and C). The value of RVD$_{20}$ amounted to 26–29%, that of RVD$_{40}$ was 39–51% for ATP, ATPγS, UTP, and UDP. Values of RVD$_{20}$ and RVD$_{40}$ for ADP were not significantly different from control (HYPO) values.

Thereafter, a series of experiments was conducted to further characterize ATPγS-dependent activation of RVD. In isotonic conditions, cell volume ($V_r = 1.022 \pm 0.003$) was not affected by 5 $\mu M$ ATPγS during the 45 min of incubation with the nucleotide. In hypotonic medium, however, there was a concentration-dependent effect of ATPγS on RVD (Fig. 1D). A hyperbolic relationship provided the best fit to the experimental data with $Y_{\text{max}} = 35.6 \pm 1.55\% \text{ min}^{-1}$, $K_{1/2} = 0.86 \pm 0.25 \mu M$ for RVD$_{20}$ and $Y_{\text{max}} = 63.5 \pm 4.1\%$, $K_{1/2} = 0.76 \pm 0.37 \mu M$ for RVD$_{40}$. The effect of 5 $\mu M$ ATPγS was significantly reduced by 78.9% (RVD$_{20}$) and 63.4% (RVD$_{40}$) in the presence of 100 $\mu M$ of both cibacron blue and suramin, two well-known P2 receptor blockers (Fig. 1, B and C). Cibacron blue and suramin in the absence of ATPγS did not significantly affect the cell volume changes induced by HYPO alone. In the above-mentioned experiments, ATPγS was present during the 45 min of hypotonic exposure. When ATPγS was removed after 5 min of hypotonic exposure, cell volume changes were not restored, so that no RVD could be detected.

![Fig. 1. Effects of agonists and antagonists of P2 receptors on regulatory volume decrease (RVD). A: Time course of relative cell volume ($V_r$) of goldfish hepatocytes exposed to hypotonicity. Primary cultured hepatocytes were incubated in 500 $\mu M$ isotonic L-15 (L-15), and then 500 $\mu M$ hypo L-15. The first 25 min represent the volume response to calibration pulses using media with osmolarities of 270 mosM, 305 mosM and 330 mosM. Results are means $\pm$ SE of 55–60 cells from 4 independent preparations. B: $V_r$ changes of goldfish hepatocytes exposed to 500 $\mu M$ isotonic 286 mosM (C) and then 160–170 mosM (HYPO) media in the absence (○) or presence of 5 $\mu M$ ATP (●); 5 $\mu M$ ATPγS (gray circles); 5 $\mu M$ UTP (●); 5 $\mu M$ UDP (●); 5 $\mu M$ ATPγS + 100 $\mu M$ of both cibacron blue and suramin (SC-ATPγS; gray triangles) and 5 $\mu M$ ADP (●). In ATPγS 5′, cells were exposed to HYPO in the presence of 5 $\mu M$ ATPγS only for the initial 5 min; afterward, extracellular medium was replaced by HYPO alone. Calibration of relative cell volume (first 25 min) was performed in media 250 mosM (A) and 320 mosM (B). Results are expressed as means $\pm$ SE of 55–60 cells from 4 or 5 independent preparations. C: RVD$_{20}$ and RVD$_{40}$ from the experiments in 1B. *P $< 0.05$ vs. HYPO. $F = 6.83$ for RVD$_{20}$ and 21.60 for RVD$_{40}$. D: Dependence of RVD on ATPγS concentration. Goldfish hepatocytes were exposed to 500 $\mu M$ isotonic medium, followed by 500 $\mu M$ hypotonic medium containing 0, 1, 5, 10, or 50 $\mu M$ ATPγS, and the time course of $V_r$ was assessed similarly to experiments in Fig. 1B. Values of $V_r$ vs. time were then used to calculate RVD$_{20}$ (%; ○) and RVD$_{40}$ (%; ●) for any given concentration of ATPγS. The continuous lines represent the fit by nonlinear regression of a single hyperbola to data. Results are expressed as means $\pm$ SE of 55–60 cells from 4 or 5 independent preparations. **Fig. 1.**
addition of ATP to goldfish hepatocytes results in the production of extracellular adenosine (41), we analyzed the effect of P1 receptor activation on RVD of goldfish hepatocytes.

As shown in Fig. 2, when hepatocytes were exposed to 5 μM ATPγS plus 5 μM adenosine, RVD20 and RVD40 were inhibited by 47.9% and 24.6%, respectively, compared with 5 μM of ATPγS alone. The inhibition induced by adenosine was abolished by 100 μM 8-SPT, a P1 antagonist, indicating an inhibitory effect of P1 receptor activation on RVD of goldfish hepatocytes. The addition of 5 μM adenosine (not shown) or 100 μM 8-SPT alone in hypotonic medium did not affect RVD. In the presence of 5 μM ATPγS + 5 μM adenosine + 100 μM 8-SPT, RVD40 was ~1.7 times higher than with 5 μM ATPγS + 5 μM adenosine (P < 0.05).

Ecto-ATPase activity. In experiments shown in Fig. 3A, ecto-ATPase activity (denoted as vium) was measured using the luciferase-luciferin reaction. Ecto-ATPase activity followed a hyperbolic function of ATP concentration (50–5,000 nM), with Ymax = 0.72 ± 0.32 nmol hydrolyzed ATP (106 cells/min)−1 and K1/2 = 5.7 ± 4.1 μM.

Ecto-ATPase activity and RVD. As stated above, goldfish hepatocytes are able to hydrolyze extracellular ATP, that is, they display ecto-ATPase activity. Therefore, we reasoned, the absence of RVD in these cells could be the result of hypotonically induced ATP release accompanied by simultaneous removal of extracellular ATP by a relatively high degree of ecto-ATPase activity. Thus, a series of experiments was conducted to test whether inhibition of ecto-ATPase could lead to ATP accumulation and subsequent activation of RVD.

We first determined whether AMP-PCP could inhibit ecto-ATPase activity without altering Ca2+1 (an indicator of P2 receptor activation). For this purpose, cells were incubated for

![Fig. 2. Effects of agonists and antagonists of P1 receptors on regulatory volume decrease. RVD20 and RVD40 of goldfish hepatocytes exposed to 500 μl isotonic 286 mosM (C) and 160–170 mosM (HYPO) media in the absence (HYPO) or presence of 5 μM ATPγS (ATPγS); 5 μM ATPγS + 5 μM adenosine (ADO ATPγS); 100 μM 8-(p-sulphophenyl)theophylline (8-SPT); 5 μM ATPγS + 5 μM adenosine + 100 μM 8-SPT (ADO ATPγS 8-SPT). Results are expressed as means ± SE of 55–60 cells from 4 or 5 independent preparations. *P < 0.05 vs. HYPO; #P < 0.05 vs. ADO ATPγS.

Fig. 3. Ecto-ATPase activity. Substrate curve, inhibition by β,γ-methylenadenosine 5’-triphosphate (AMP-PCP) and effect on RVD. A: E-ATPase activity (vium) vs. ATP concentration (50–5,000 nM). Each point represents the initial rate of ATP extinction (vium) obtained by fitting a double exponential decay function (see MATERIALS AND METHODS) to data of ATP vs. time. The line represents the best fit by nonlinear regression of a single hyperbola to data. Results are expressed as means ± SE of four independent preparations. B: E-ATPase activity vs. AMP-PCP concentration. Cells were incubated with 5 μM [γ-32P]ATP in the presence of increasing concentrations of AMP-PCP (from 0 to 1,000 μM). Each point represents the slope ± SE obtained by linear regression of a time course of [γ-32P]Pi release from [γ-32P]ATP with at least five experimental points from four independent preparations. The line represents a hyperbolic function fitted by nonlinear regression to data (see MATERIALS AND METHODS). C: RVD20 and RVD40 of cells exposed to 500 μl isotonic medium C and to 500 μl hypotonic medium in the absence (HYPO) or presence of 5 μM ATP (ATP), 500 μM AMP-PCP (AMP-PCP), and 500 μM AMP-PCP plus 5 μM ATP (AMP-PCP ATP). Results are expressed as means ± SE of 55–60 cells from 4 or 5 independent preparations. *P < 0.05 vs. HYPO. F = 9.33 for RVD20 and 27.10 for RVD40.
10 min in the presence of various concentrations of AMP-PCP (0–1 mM), before addition of 5 μM [γ-32P]ATP. As shown in Fig. 3B, in the absence of AMP-PCP, ecto-ATPase activity was 0.524 ± 0.131 nmol Pi (10^6 cells min)^{-1}, and it was found to decrease with increasing concentrations of AMP-PCP. Maximal inhibition was 77.69%, with a Ki = 223.13 ± 98.51 μM.

With regard to changes of Ca\(^{2+}\)i, we observed that the addition of 5 μM of ATPyS and ATP transiently increased Ca\(^{2+}\)i from 19.2 ± 6.6 nM (resting Ca\(^{2+}\)i concentration) to 150 ± 21 nM and 310 ± 36 nM, respectively, whereas 500 μM AMP-PCP did not alter this parameter.

Finally, cell volume response was evaluated with hepatocytes exposed to hypotonic medium in the presence of 500 μM AMP-PCP, a condition where 72% of ecto-ATPase activity is inhibited. In Fig. 3C, it can be seen that under these conditions, no RVD was induced. Furthermore, the extent of volume regulation with 5 μM ATP was similar in the presence and in the absence of AMP-PCP.

**Extracellular ATP**

With 10^4 hepatocytes in 40 μl of hypotonic medium (Measurements of ATP were performed with 10^4 cells since higher number of cells in 40 μl of hypotonic medium produced an inhibition of luciferase activity.), ATPe increased to a maximum value of 7.25 ± 1.65 nM at 2 min, and this was followed by a single exponential decay of ATPe with t_{1/2} = 16.8 ± 5.7 min.

In the presence of 40 μl hypotonic medium and 500 μM AMP-PCP, significant differences were found—compared to HYPO alone—with ATPe reaching 17.15 ± 4.40 nM at 6.1 min (maximum) with t_{1/2} = 462 ± 127 min (Fig. 4A). For comparison, ATPe concentrations were measured in cultures of HepG2 cells under similar experimental conditions. When 10^4 HepG2 cells were exposed to 40 μl of hypotonic medium, ATPe amounted to 3.12 ± 1.03 nM at the maximum, followed by an exponential decay with t_{1/2} = 55 ± 12 min.

Interestingly, in goldfish hepatocytes under HYPO, ATPyS induced a dose-dependent increase of peak ATPe concentration. This increase was not due to an inhibition of ecto-ATPase activity, since enzyme activities with 250 nM ATPe in the absence of AMP-PCP.

**Intracellular content of ATP.** The total mass of intracellular ATP of cultured goldfish hepatocytes under isotonic conditions was 0.74 ± 0.03 nmol ATP (10^6 cells)^{-1} (means ± SE, n = 4). When these cells were exposed to HYPO for 5 min (prior to cell lysis), the total mass of intracellular ATP was 0.62 ± 0.05 nmol ATP (10^6 cells)^{-1} (means ± SE, n = 4), a value not significantly different from that found in the presence of HYPO alone.

Thus, ATPyS appears to activate P2 receptors and therefore RVD, both directly and by induction of ATP release, which, in turn, activates P2 receptors.

To distinguish both possibilities, we measured the extent of RVD of cells exposed to hypotonic medium with 5 μM ATPyS and 5 U/ml apyrase, a condition in which all available extracellular ATP and ADP is scavenged without affecting ATPyS. As shown in Fig. 5, when cells were exposed to HYPO plus 5 μM ATPyS and 5 U/ml apyrase, RVD_{50} was 15.6 ± 5.9% (not significantly different from HYPO alone) and RVD_{40} was 30 ± 6.6%, that is, 41–50% lower than in the absence of apyrase (i.e., under HYPO plus 5 μM ATPyS).

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Interestingly, in goldfish hepatocytes under HYPO, ATPyS induced a dose-dependent increase of peak ATPe concentration. This increase was not due to an inhibition of ecto-ATPase activity, since enzyme activities with 250 nM ATPe in the presence and in the absence of 5 μM ATPyS (the highest concentration tested) were not significantly different (P = 0.36, n = 5). Results are depicted in Fig. 4B, where each point (means ± SE) represents the maximal ATPe concentration determined when cells were exposed to HYPO plus 0–5 μM of ATPyS.

This activating effect can be blocked by P2 receptor antagonists, since the release of ATP in the presence of 1 μM ATPyS was 13.3 ± 3.4 nM, whereas in the presence of 1 μM ATPyS plus 100 μM of both suramin and cibacron blue, it amounted to 7.5 ± 1.5 nM. The latter result was not significantly different from that found in the presence of HYPO alone.

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RVD Under Low-Volume Hypotonic Exposure

Experiments shown in Figs. 1 and 4A indicate that goldfish hepatocytes do not perform RVD under HYPO conditions, despite of the fact that they are capable of releasing ATP. Upon addition of ATP, however, RVD can be triggered. This apparent lack of effect of endogenously derived ATPe could be due to dilution of the nucleotide in the extracellular space. Assuming that ATP[S] has equal potency with ATP to trigger RVD, we estimated that a 12.5-fold reduction of the extracellular ATP concentration is sufficient for RVD by 111% for RVD[S] and by 73% for RVD[40], compared with HYPO alone. Conversely, addition of 100 μM of suramin and cibacron blue to HYPO medium caused a decrease to 0.78% in RVD[20] and a 72% decrease in RVD[40], when compared with HYPO alone.

Modeling of the Kinetics of ATPe

Although swollen goldfish hepatocytes are able to release ATP (Fig. 4), the kinetics of ATPe depends on many factors affecting the concentration of the nucleotide simultaneously. In principle, the concentration of ATP can be enhanced by lytic (JNL), as well as nonlytic (JNL), release of intracellular ATP. Once in the extracellular medium, ATP can be hydrolyzed by ectoATPase activity—whose substrate curve was determined using results of Fig. 3A—or be diluted in the extracellular medium by diffusion.

Regarding JNL, in Fig. 4B, it can be seen that ATP[S] activates the release of ATP. Thus, if ATP behaves similarly as ATP[S], a positive feedback mechanism can be proposed, so that extracellular ATP activates JNL. Therefore, the JNL was described by a specific time function multiplied by a function accounting for the positive feedback mechanism (see Eqs. A6, A7 and A8).

Empirical equations derived from all experimental data of Figs. 3A, 4B and from experimental data on the time course of cell viability (not shown) were fed into a mathematical model. As shown in Fig. 7A, the model predicts the kinetics of ATPe with reasonable accuracy. The predicted JNL shows an acute increase to 1.93 10⁻¹⁸ mol/s after 2 s, followed by a nonlinear decrease towards 0 (Fig. 7B).

To see the relative contribution of the different processes affecting ATPe (ecto-ATPase activity, ATP diffusion, lytic, and nonlytic release of ATP), we simulated the kinetics of ATPe under a putative condition, in which each of these processes—one at a time—is blocked (Fig. 7C). It can be seen that the first part of the response (0–2 min) is mainly governed by ATP diffusion and ATP release (lytic and nonlytic), with little contribution from ecto-ATPase activity. On the contrary,
the last part of the response (>2 min) is essentially ruled by ecto-ATPase activity.

RVD Induction at Alkaline pH

In a previous work on volume regulation of goldfish hepatocytes, it was shown that increasing medium pH from 7.6 to 7.8 (within the physiological range of goldfish plasma), resulted in activation of a slight RVD (12). Accordingly, we found that at pH = 7.8 RVD was indeed present (RVD40 = 33.2 ± 5.3; Fig. 8A). Interestingly RVD40 could be abolished by 5 U/ml apyrase, indicating a role for ATPe in this effect. Thus, we conducted experiments displayed in Fig. 8B, in which the ATP released into hypotonic media with either pH = 7.6 or pH = 7.8 is plotted. Equation 8 was fitted to experimental data to obtain the parameters of best fit. This function includes an exponential rise to a maximum, followed by an exponential decay. It was found that the exponential rise constant was similar for both treatments (1.14 ± 0.32 min⁻¹ at pH = 7.8; 1.43 ± 0.42 min⁻¹ at pH = 7.6), whereas the estimated maximal ATPe was significantly higher at pH = 7.8 (10.8 ± 2.5 nmol) than at pH = 7.6 (5.5 ± 1 nmol). Furthermore, the decay constant for both curves was significantly different (0.09 ± 0.02 min⁻¹ at pH = 7.8; 0.03 ± 0.01 min⁻¹ at pH = 7.6).

DISCUSSION

In all previous studies on volume regulation of vertebrate cells, the assay volume was used at least 300 times that of the total volume of all the cells examined. Under these conditions, almost all of the cells studied so far display RVD in hypotonic medium, except for goldfish hepatocytes, where RVD is absent. Importantly, goldfish hepatocytes not only swell but remain swollen and viable for at least 5 h. Moreover, these swollen cells are capable of maintaining Ca²⁺ (Fig. 9) and K⁺ homeostasis (12) and, contrary to a postulated hypotonically driven slowdown of metabolism (28), they even increase their oxidative metabolism. Thus, swollen goldfish hepatocytes remain structurally intact and metabolically active, but nevertheless, they show no volume regulatory response in a standard hypotonic exposure experiment.

Interestingly, swollen cells could be induced to downregulate their volume in the presence of extrinsic nucleotides such as ATP, ATPγS, UTP, and UDP, whereas ADP exert no such effect. In line with other reports (30, 34, 44), this nucleotide-dependent activation of RVD was decreased by antagonists of P2 receptors, indicating that this effect is largely receptor-mediated. Using ATPγS, we showed that RVD activation by this nucleotide was dose dependent (K1/2 = 760–860 nM) and triggered within the first 5 min of hypotonic exposure, while the presence of this nucleotide beyond that time is not required to promote RVD.

As previously shown in goldfish hepatocytes, extrinsic extracellular ATP can be sequentially dephosphorylated to adenosine (41), which may then act on P1 receptors and, similar to its effect in trout hepatocytes (35), inhibit RVD. However, although 5 μM of extrinsic adenosine reduced RVD of goldfish hepatocytes, the P1 antagonist 8PT alone did not change RVD. Thus, ATP (via P2 receptors) and adenosine (via P1 receptors) cause opposing effects on RVD, but in the experimental...
conditions, applied adenosine does not accumulate to levels high enough to inhibit RVD. In agreement with this, the stimulatory effect on RVD by ATP, which produces adenosine, and ATPγS, which is not degraded to adenosine, were indistinguishable. There may be several reasons underlying the lack of RVD of goldfish hepatocytes under standard conditions. These may include 1) the lack of a release or a subthreshold release of ATP, 2) hypotonically released ATP is rapidly degraded due to high ecto-ATPase activity, so that the effective concentration of the nucleotide at the cell surface is insufficient to activate P2 receptors, and 3) extracellular ATP is substantially elevated, but the affinity of P2 receptors is too low to respond to the nucleotide. Our results showed that goldfish hepatocytes were capable of releasing ATP, but as they also exhibited substantial ecto-ATPase activity, we investigated if excessive ATP hydrolysis would counteract the induction of RVD. However, although AMP-PCP inhibited 72% of ecto-ATPase activity and significantly increased [ATP], this treatment failed to induce RVD, suggesting that ecto-ATPase activity was not preventing RVD induction.

Another possible reason for the lacking stimulation of RVD might be that the total mass of ATP released by the cells was insufficient to stimulate P2 receptors. However, experiments using the human hepatoma cell line HepG2, which undergo nucleotide-dependent RVD (RVD40 is ~50% and 2% in the absence and presence of apyrase, respectively; unpublished observation), did not support this notion. Both cell types released comparable ATP masses in similar hypotonic conditions. Thus not the total mass of ATP released but rather some other property, possibly different P2 receptor affinities, distinguishes the RVD response of these cell types. It is noteworthy that when the ATP released from 10,000 cells into 40 µl (Fig. 4) is extrapolated to the expected amount liberated from 10⁶ cells into 500 µl, a peak concentration of up to 630 nM for at least 5 min is obtained, with no RVD triggered. In contrast, ATPγS does trigger RVD at a comparable concentration. Nevertheless, ATPγS is capable of inducing the release of ATP in a dose-dependent manner, implying that ATPγS affects RVD both directly, by interaction with P2 receptors, and through stimulation of the release of ATP from the cells. Accordingly, when ATPγS is added to hypotonic medium with apyrase, RVD was significantly diminished. Assuming that ATP acts similarly to ATPγS, our findings suggest that a positive feedback mechanism accelerates ATP release. Relatively small amounts of ATPe could thus induce a self-amplifying cascade, so that a threshold concentration of ATPe could be rapidly achieved, above which RVD can be triggered. A similar effect of extracellular nucleotides on ATP secretion has been previously observed in other cell types (31; 42; 43), although this has not yet been described in the context of volume regulation. Unraveling the mystery. Considering the ATPe dependency of RVD induction described above, we reduced the standard
assay volume to such an extent that endogenous ATPe would accumulate to a level comparable to that of ATPγS required to induce RVD in the standard larger experimental volume.

Under this condition, RVD was induced in the absence of extrinsic ATP, providing clear evidence that it was triggered by endogenously released ATP. In line with the notion of a specific ATP-dependent effect, RVD was further stimulated by AMP-PCP, as this treatment caused a further increase of ATPe in this and other reports (23, 24), and RVD was completely blocked by P2 receptor antagonists (Fig. 6), showing that it was receptor mediated. Altogether, these results indicate that ATP released from goldfish hepatocytes upon hypotonic exposure is an important trigger of the RVD response when examined under appropriate conditions and that the lack of RVD in a larger experimental volume is due to excessive dilution of the nucleotides derived from the cells.

**Effect of extracellular pH.** Results of Fig. 8 showed that RVD was activated at slightly alkaline pH, and apyrase abolished this effect. In addition the maximal ATP concentration and the efflux of ATP were higher at pH 7.8 than at 7.6. Taken together, these findings suggest a role for ATP in alkaline pH-induced RVD. An effect of extracellular pH on RVD via intracellular pH must be ruled out since in goldfish alkaline pH-induced RVD. An effect of extracellular pH on RVD was activated at slightly alkaline pH, and apyrase abolishes this effect. In addition, the maximal ATP concentration and the efflux of ATP were higher at pH 7.8 than at 7.6. Taken together, these findings suggest a role for ATP in alkaline pH-induced RVD.

**Free cytosolic calcium.** Hypotonicity causes an acute Ca\(^{2+}\) spike that is not affected by apyrase. Thus, although this and a previous study on goldfish hepatocytes (41) show that extrinsic ATP can modulate Ca\(^{2+}\)i under isotonic conditions, ATP resulting from the hypotonic challenge does not affect Ca\(^{2+}\)i levels. Because ATPe is required to achieve RVD and hypotonicity causes an almost simultaneous—though with very different kinetics—increase of both Ca\(^{2+}\)i and ATPe, it is tempting to speculate that the observed calcium spike could function as a signal to induce ATP efflux. This hypothesis remains to be tested.

**Lessons from the mathematical model.** A mathematical model was used to understand the mechanisms that affect the concentration of endogenous ATPe (see Appendix). The model includes equations describing the kinetics of cell death (J\(_L\)), ecto-ATPase activity (J\(_P\)), nonlytic efflux of ATP (J\(_{NL}\)), and ATP diffusion in the extracellular compartment (J\(_D\)). The model also includes a positive feedback mechanism, which explicitly takes into account the linear function applied to data of ATPe vs. ATPγS of Fig. 4B.

The model provides a good fit to experimental data (Fig. 7A). The predicted ATP efflux (J\(_{NL}\)) acts mainly within the first minute of the response, with a maximum attained in 2 s (Fig. 7B). This maximum value lies in the order of the maximal ATP efflux estimated in A6 renal cells under hypotonicity (15). During the first 2 min of hypotonic exposure, the concentration of extracellular ATP is mainly governed by ATP nonlytic efflux, ATP diffusion (J\(_D\)) and cell death (J\(_L\)), with almost no contribution from ecto-ATPase activity (J\(_P\), see Fig. 7C). The latter becomes important in defining the last phase of the curve. The positive feedback loop is essential for activating ATP efflux, since in a simulation where the model uses the parameters of best fit in the absence of this mechanism, the resultant ATPe maximum is reduced by 50%. In the absence of cell death (i.e., J\(_L\) = 0) ATPe decreases about 6 times. Thus, a relatively low percentage of cell death (~3%) occurring during the first minute of the hypotonic challenge provides sufficient ATP to enhance ATPe both directly and via feedback activation of J\(_{NL}\).

This means that in an in vivo physiological scenario in which a minor proportion of cells die, the resultant steep increase of ATPe could signal to the cells that remained alive.

Furthermore, ATP from nonlytic flux could be envisaged as a source of ATP originating in another cell type, like e.g., red blood cells and/or endothelial cells (29). Our model predicts that, because of the ATP-induced ATP release, this extra

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**Fig. 10.** Scheme depicting the main mechanisms involved in RVD regulation in goldfish hepatocytes. The parameters considered in the mathematical model of ATP release are also depicted. e\(_1\), e\(_2\), and D are parameters of the mathematical model: e\(_1\) is the extracellular compartment near the cell surface, whereas e\(_2\) represent the extracellular compartment surrounding e\(_1\); a = interconversion of ATP into UTP (and vice versa) produced by a putative E-NTPKase; 1, β,γ-methylene-ATP; 2, suramin and cibacron blue, 3, ATPγS; 4, NTPs and NDPs (nucleoside diphosphates and triphosphates); 5, formycin B and dipyridamole; and 6, 8-sulpho phenyl theophylline (8-SPT).
supply of ATPe will enhance the release of ATP from hepatocytes to induce RVD.

Finally, in Fig. 10, we present a scheme for nucleotide-dependent RVD of goldfish hepatocytes compatible with the experimental results of this and previous studies.

Goldfish hepatocytes rapidly swell in hypotonic medium accompanied by both a sudden and reversible increase in intracellular Ca2+ and the release of ATP to the extracellular space. According to the mathematical model, a single, nonlinear burst of ATP is sufficient to increase ATP concentrations to a maximum within 2 min. In standard hypotonic conditions, the relatively low ATP concentration resulting from this ATP release, together with a hypothetical low affinity of P2 receptors, fails to induce RVD. However, micromolar concentrations of extrinsic ATP, UTP, UDP, and ATPγS interact with P2 receptors to trigger the volume regulatory response. Importantly, at very low assay volumes, sufficient extracellular ATP is accumulated to induce RVD. In this situation, the extracellular ATP appears to exert at least two different functions. It enhances the release of ATP in a positive feedback loop, possibly by interaction with P2 receptors (30, 42), and it activates effector mechanisms mediating the loss of osmolytes along with osmotically obligated water and thereby ultimately stimulates the recovery of cell volume.

Extracellular ATP concentrations will change rapidly and reflect a balance among the rates of ATP release, hydrolysis, and dilution into the bathing media. Although a full RVD response takes more than 45 min to be accomplished, the ATP-dependent trigger requires less than 5 min. According to the mathematical model, during this short time, ATPe concentration is highly governed by lytic- and nonlytic ATP efflux, irrespective of ecto-ATPase activity.

In the long term, ATPe is completely dephosphorylated to adenosine, and this may interact with P1 receptors to inhibit RVD. However, in the experimental conditions of this study, adenosine accumulation would be too low to significantly affect RVD.

**Perspectives and Significance**

The present study confirms previous evidence indicating that extracellular nucleotides are fundamental cues to regulate cell volume. Likewise, our data indicate for the first time in goldfish hepatocytes that ATP can activate a regulatory volume response tendency to recover steady-state cell volume, whereas its hydrolysis end product adenosine may inhibit this response. Hepatocytes from goldfish (present study) and trout (35), two fish cell models, are the only known examples so far where dephosphorylation of ATPe to adenosine tunes the RVD response by exerting opposing effects on volume changes. Whether this straightforward example of a self-controlling mechanism can be generalized to other cell types will require further research.

In vivo, vertebrate hepatocytes are indeed exposed to transient hypotonicity as a consequence of intestinal water absorption (28) to isosmotic swelling during the uptake of osmolytes (e.g., amino acids) and during metabolic inhibition. Furthermore, liver cells of many vertebrates are challenged by hypoxia in vivo, a condition in which liver cells might swell (16), and both red blood and endothelial cells are induced to release ATP (6). In this hypothetical scenario, as well as under physiological or pathological conditions, the present study predicts that a paracrine supply of ATP from a relatively small population of cells would enhance ATP release from hepatocytes and contribute significantly to the recovery of their steady-state volume.

**APPENDIX**

**Modelling of the Kinetics of Extracellular ATP of Hypotonically Challenged Hepatocytes**

A mathematical model consisting of a system of differential equations was used to predict the time-dependent changes in the concentration of extracellular ATP (denoted as [ATP]e(t)) when hepatocytes are challenged by hypotonicity (at time t = 0). The model (Fig. 11) has three compartments, so that ATP can be located either in an intracellular compartment (denoted as i) or in two extracellular compartments (e1 and e2).

In the model, the [ATP]e is controlled by 1) rate of nonlytic release of ATP (JNL), which accounts for any release of ATP by a yet unknown mechanism. JNL was defined by a function of time (F0) modulated by a positive feedback mechanism (F1): 2) Rate of lytic release of ATP (JL), i.e., the release of intracellular ATP from dead cells. This was estimated by using experimental data on cell volume, cell death and intracellular ATP concentration of hepatocytes under hypotonic conditions. That is, the intracellular compartment contains the total mass of intracellular ATP (mATP) for any given number of cells, a mass which, according to the model, is released and diluted instantly into e1 following cell death; 3) rate of ATP consumption by ecto-ATPase activity (JV). According to the model, JV occurs only at the surface of the cell, i.e., in e1; and 4) ATP diffusion between e1 and e2 (J0), where the latter compartment receives ATP only from e1, and is not accessible to ecto-ATPase activity.

The model is described by the following equations:

\[
\frac{d[ATP]_{e1}}{dt} = J_{NL} + J_L - J_V - J_D \quad (A1)
\]

\[
\frac{d[ATP]_{e2}}{dt} = J_D \quad (A2)
\]

where \(d[ATP]_{e1}/dt\) and \(d[ATP]_{e2}/dt\) represent the rate of extracellular ATP concentration in compartments e1 and e2, respectively. \([ATP]_{e1}\) and \([ATP]_{e2}\) are the concentrations of ATP in each compartment. \(J_{NL}, J_L, J_V\), and \(J_D\) are the fluxes mentioned above. Formally, the meaning of the

**Fig. 11.** Schematic summary of the mathematical model. The parameters used in the model are shown. The cells are represented by a circle; the discontinuous line circle stands for cell death. The two extracellular compartments e1 and e2 are shown in light and dark gray, respectively. \(J_L, J_{NL}, J_V,\) and \(J_D\) stands for lytic release of ATP, nonlytic release of ATP, diffusion between e1 and e2 and E-ATPase activity, respectively.
different fluxes modulating ATP_{e} is described as follows:  
1) Lytic release of ATP (J_{L})

The lytic flux is accounted by

\[ J_{L} = \left( \frac{m_{ATP\text{-}i}}{S_{x_{1}}} \right) \left( \frac{100 - \text{viability}(t)}{100} \right) \]  

(A3)

As stated above, \( m_{ATP\text{-}i} \) is the total mass of intracellular ATP. The total number of cells in the measuring chamber is denoted as \( f \), whereas \( \text{viability}(t) \) is the experimental time course of viability (see Volumetric response of goldfish hepatocytes under hypotonic medium. Standard conditions in Results section) and \( S \) is the area of the measuring chamber. \( \Delta x_{1} \) is the depth of compartment \( e_{1} \), arbitrarily taken as the diameter of one hepatocyte (15.44 \( \mu \text{m} \), taken from the mean diameter of 60 cells from four independent experiments).

2) Rate of ATP consumption (J_{V})

A hyperbolic function (Eq. 7 in MATERIALS AND METHODS) was fitted to experimental data of Fig. 4A. However, since the model was used to simulate ATP_{e} in a concentration range which is at least 300 times lower than the \( K_{1/2} \), under this condition \( J_{V} \) can be described by a linear function as

\[ J_{V} = \frac{V_{m}}{K_{1/2}} [\text{ATP}]_{1} \]  

(A4)

where \( V_{m} \) represents the apparent maximal value of ecto-ATPase activity, and \( K_{1/2} \) the concentration of ATPs at which a half-maximal activity is obtained under the specific conditions of the experiment.

During model-dependent fit, the value of the ratio \( V_{m}/K_{1/2} \) was allowed to vary within 1 SE of the mean value.

3) ATP diffusion (J_{D})

\[ J_{D} = \frac{D \cdot S \cdot 2}{\Delta x_{1} + \Delta x_{2}} ([\text{ATP}]_{1} - [\text{ATP}]_{2}) \]  

(A5)

where \( D \) is the diffusion coefficient of ATP in water (3 \( \times \) 10\(^{6} \) cm\(^{2}\)/s; Ref. 9), while \( S \) and \( \Delta x_{1} \) have the same meaning pointed out above. \( \Delta x_{2} \) is the depth of the outer compartment defined as \( \Delta x_{2} = V/S - \Delta x_{1} \) with \( V \) being the volume of the measuring chamber.

4) Nonlytic release of ATP (J_{NL})

\[ J_{NL} = J_{R} \cdot F \]  

(A6)

As stated above, the mechanism responsible for the nonlytic release of ATP remains unknown. This is why it proved necessary to test different expressions accounting for \( J_{R} \), so as to check which one allows the model to display the best fit to experimental data. The best \( J_{R} \) function was chosen according to an overall goodness-of-fit index called the Akaika criterion (1). Accordingly, the model was fitted to experimental data of Fig. 4A by using: 1) a constant \( J_{R} \) (\( J_{R} \) does not vary with time), 2) a step function in which \( J_{R} \) is zero until it becomes activated and remains constant thereafter, 3) an impulse function, where \( J_{R} \) has the form of a rectangular pulse that can be triggered and shut off at variable times, and 4) a log-normal function that includes a nonlinear fast increase to a maximum, followed by a relatively slowly nonlinear decrease.

Preliminary simulations of the model showed that functions 1-3 in the model did not provide a good fit to experimental data, i.e., the release of ATP can neither be described by a constant flux or a step time profile. Thus, according to the Akaika criterion the lognormal function was selected, where

\[ J_{R}(t) = \frac{A}{2\pi dt} e^{-\frac{(t - T)^{2}}{2dt}} \]  

(A7)

\( A, d, \) and \( c \) are parameters subjected to fitting. This function is similar to a Gaussian expression where the rise and decay phases might not be symmetrical.

The factor “F” accounts for a positive feedback process whereby ATP_{e} can amplify \( J_{NL} \), as follows:

\[ F([\text{ATP}]_{1}) = a[\text{ATP}]_{1} + b \]  

(A8)

where the values of \( a \) and \( b \) are obtained by fitting a linear function to experimental data of Fig. 4B. Two assumptions were made: 1) The increase of [ATP]_{e} as a function of [ATP]_{PS} implies that ATP_{PS} and similarly ATP_{e} amplifies \( J_{NL} \), and 2) only ATP_{e} present on the cell surface or \( e_{1} \) (i.e., \([\text{ATP}]_{1} \) ATP ) can act on \( J_{NL} \).

The model simulates the concentration of ATP_{e} at any given time (Fig. 7, A and B) during the hypotonic challenge as follows:

\[ [\text{ATP}]_{e}(t) = \int_{0}^{t} \frac{d[\text{ATP}]_{e}}{dt} dt \cdot V_{e} + \int_{0}^{t} \frac{d[\text{ATP}]_{e}}{dt} dt \cdot V_{e} \]  

\[ \frac{V_{e} + V_{e}}{V_{e} + V_{e}} \]  

(A9)

where

\[ \int_{0}^{t} \frac{d[\text{ATP}]_{e}}{dt} dt \cdot V_{e} \]  

and

\[ \int_{0}^{t} \frac{d[\text{ATP}]_{e}}{dt} dt \cdot V_{e} \]  

are the masses of the ATPs in the compartments \( e_{1} \) and \( e_{2} \) at time \( t \), respectively; \( V_{e} \) and \( V_{e} \) are the volumes of the corresponding compartments, defined as \( V_{e} = \Delta x_{1} S = \Delta x_{2} S \). Simulations were performed using the Euler method with a custom-made software developed in Visual Basic code (software and source code available) using an integration step of 1 s. This step guarantees the order of accuracy and the stability of the Euler method. The procedure yields a good description of the time course of extracellular ATP following the hypotonic challenge.

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