Release of ATP induced by hypertonic solutions in *Xenopus* oocytes

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ATP mediates intercellular communication. Mechanical stress and changes in cell volume induce ATP release from various cell types, both secretory and non-secretory. In the present study, we stressed *Xenopus* oocytes with a hypertonic solution enriched in mannitol (300 mM). We measured simultaneously ATP release and ionic currents from a single oocyte. A decrease in cell volume, the activation of an inward current and ATP release were coincident. We found two components of ATP release: the first was associated with granule or vesicle exocytosis, because it was inhibited by tetanus neurotoxin, and the second was related to the inward current. A single exponential described the correlation between ATP release and the hypertonic-activated current. Gadolinium ions, which block mechanically activated ionic channels, inhibited the ATP release and the inward current but did not affect the decrease in volume. Oocytes expressing CFTR (cystic fibrosis transmembrane regulator) released ATP under hypertonic shock, but ATP release was significantly inhibited in the first component: that related to granule exocytosis. Since the ATP measured is the balance between ATP release and ATP degradation by ecto-enzymes, we measured the nucleoside triphosphate diphosphohydrolase (NTPDase) activity of the oocyte surface during osmotic stress, as the calcium-dependent hydrolysis of ATP, which was inhibited by more than 50 % in hypertonic conditions. The best-characterized membrane protein showing NTPDase activity is CD39. Oocytes injected with an antisense oligonucleotide complementary to CD39 mRNA released less ATP and showed a lower amplitude in the inward current than those oocytes injected with water.

Purinergic signalling is almost ubiquitous. It is well known that ATP is kept inside cells because it is highly hydrophilic and does not permeate through lipidic cell membranes. However, ATP release has been reported in various cell types. Neuronal cells release ATP during synaptic transmission (Silinsky & Redman, 1996; Vizi *et al.* 2000), and it is generally supposed that all synaptic vesicles contain ATP, which is co-released with the main neurotransmitter, albeit in some cases some synaptic vesicles may only contain ATP (Bodin & Burnstock, 2001). Several laboratories have recorded ATP release from other non-neuronal cells such as astrocytes (Cotrina *et al.* 1998; Guthrie *et al.* 1999; Newman, 2001; Verderio & Matteoli, 2001; Stout *et al.* 2002) and from skeletal muscle fibres (Forrester, 1972). The purinergic signalling is especially relevant in the heart-vascular system, where P2 receptors have been detected in epithelial and smooth muscle fibres. This and the presence of P2 receptors in the same cells strongly suggest an autocrine purinergic loop. Endothelial cells also release ATP during shear stress or under hypotonic challenge (Yegutkin *et al.* 2000; Hashimoto *et*

al. 2001; Buxton *et al.* 2001; Koyama *et al.* 2001; Hisadome *et al.* 2002; Schwiebert *et al.* 2002). Mechanically induced release of ATP has been confirmed in every cell type studied (Grygorczyk & Guyot, 2001). The sensitivity to gadolinium suggests that ATP release is related to some type of mechanically activated ionic channel(s) (Hamill & Martinac, 2001).

Purinergic receptors have been described in the ovarian follicle of the amphibian *Xenopus laevis* (Lotan *et al.* 1982; 1986; Arellano *et al.* 1996; King *et al.* 1996*a,b*) and also in granulosa and luteal cells from pigs or humans (Kamada *et al.* 1994). Moreover, ionic currents activated or regulated via purinergic receptors have been described in *Xenopus* follicles (Arellano *et al.* 1998; Pérez-Sanmartín *et al.* 2000) and mouse cumulus cell-enclosed oocytes (Arellano *et al.* 2002). Although the stimulation of these receptors and ion channels has been implicated in the development of the oocyte (Eppig *et al.* 1985; Billig & Rosberg, 1986; Downs *et al.* 1986), the cellular mechanisms involved are poorly understood.

Xenopus oocytes release ATP in resting conditions, but they increase the efflux of ATP when expressing purinergic PY receptors (Nakamura & Strittmatter, 1996) and CFTR (cystic fibrosis transmembrane regulator; Jiang *et al.* 1998), and under local mechanical stress (Maroto & Hamill, 2001) and hyperpolarizing pulses (Bodas *et al.* 2000). Some endogenous currents of *Xenopus* oocytes are linked to mechanically gated channels (Zhang & Hamill, 2000). Currents activated by hyperpolarization or hypertonic solutions share some common features: they are inward currents in oocytes clamped at –60 mV, they are non-selective and, finally, they are permeable to large cations and anions (Zhang & Hamill, 2000). Since we have already demonstrated that hyperpolarization pulses induce the release of ATP (Bodas *et al.* 2000), we aimed to explore the release of ATP in oocytes shrunk by hypertonic solutions that activate an inward current.

METHODS

Solutions and chemicals
Isotonic Ringer solution (NR, also Normal Ringer solution, 238 mosmol l^{-1}) contained (mM): 115 NaCl, 2 KCl, 10 (Hepes/NaOH), 1.8 $CaCl_2$, 1.8 $MgCl_2$ (pH 7.4). Modified Barth's solution (MBS) contained (mM): 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 20 Hepes, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$ (pH 7.5), supplemented with 100 IU ml^{-1} of penicillin and 0.1 mg ml^{-1} of streptomycin. Hypertonic solution (HS) was prepared by adding 300 mM mannitol to NR, which resulted in an osmolarity of 543 mosmol l^{-1}, which was measured in a 5520 Wescor Osmometer.

Preparation of *Xenopus* oocytes
Mature female frogs (*Xenopus laevis*) were purchased from Centre d'Elevage de Xenopes (Montpellier, France). Frogs were maintained alive and handled in accordance with the EU laws and under the supervision of the Council for Animal Research Ethics of University of Barcelona. Oocytes were prepared as follows: frogs were anaesthetized in cold distilled water containing 1.7 g l^{-1} of tricaine (ethyl 3-aminobenzoate methanesulfonic acid, Sigma). Ovarian sacs were extracted by sterile surgical procedures and placed in NR. The frogs were immediately placed in a small water tank until they started to swim. Approximately 3 months after the first surgery, the frogs underwent contralateral surgery and were killed by decapitation. Oocytes of stages V and VI were collected using fine tip forceps and kept at 16–17 °C in MBS. The follicle envelope was removed by collagenase (Sigma, 1A; 0.5 mg ml^{-1}) treatment in NR for 45–50 min. Electrophysiological measurements were made 3–48 h after defolliculation.

Electrophysiological records
Whole-cell data were recorded using a two-microelectrode voltage-clamp configuration. The voltage and the current microelectrodes were filled with KCl (3 M) and had resistances ranging from 1 to 2 MΩ. The bath electrode was an Ag–AgCl pellet that made contact with the recording solutions through an agar bridge. Membrane potential and current were digitized by a PC computer through a Digidata-1200A (Axon Instruments) converter. The board was controlled by the Whole Cell Analysis Program (kindly provided by John Dempster, University of Strathclyde, UK). The signal was filtered at half the frequency of acquisition. Solutions were changed by gravity and controlled by electrovalves (ALA, Scientific Instruments, New York).

Preparation of luminescent reaction
A vial of luciferase extract lantern from *Photinus pyralis* (Sigma) was resuspended in 1 ml of HS and desalted in a 10 ml 10 DG-Biorad column equilibrated with HS. 200 μl of D-luciferin (2.5 mg ml^{-1}, Boehringer Mannheim) was added to the eluate and the resulting suspension was centrifuged for 30 s in a bench-top centrifuge. The eluate was frozen and maintained at –20 °C until use. An aliquot was thawed on ice and maintained at 4 °C until it was added to the recording chamber (125 μl).

Measurement and analysis of ATP release
We followed a procedure described elsewhere (Bodas *et al.* 2000). Briefly, oocytes were placed in the recording chamber and the two electrodes were inserted. After the chamber was equilibrated with HS (1–2 min), a small volume (5–20 μl) of luciferin–luciferase mixture was added to the recording chamber, which was completely light-proof. The light generated by the enzymatic reaction was transmitted through six optic fibres placed in front of the six planes of the recording cuvette. The end of the optic fibres was focused in front of a photomultiplier (Hamamatsu, R374) and controlled by a slit. The resulting electric signal was amplified in a P16 Grass amplifier (USA), filtered at 5 Hz in a Bessel filter (Frequency Devices, USA) and collected by the Digidata-1200A converter (Axon Instruments). To calculate the amount of ATP released, a known dose of ATP was added through a third pipette, with an electronic nanoinjector (Nanoject, Drummond). This dose was usually registered 2–6 min after starting the electrophysiological recording in HS, which was made at room temperature (22–23 °C).

To analyse ATP release, we integrated the light signal over a 1 min period and the amount of ATP enclosed in the area was calculated by comparison with the dose signal.

In some experiments, the light chain of tetanus toxin (L-TeNT) was injected 20–30 h before recording. The final concentration in the oocytes was around 10 nM. Tetanus toxin was prepared and kindly provided by Professor J. Blasi (University of Barcelona).

Estimation of *Xenopus* oocyte volume
Oocytes placed in a Petri dish containing NR were observed on an inverted microscope (Olympus CK 40) and the images digitized through an Olympus DP10 camera. Individual oocytes were transferred to a new Petri dish containing HS. Images were recorded for the period indicated. Assuming that oocytes are spherical in shape, cell volume was $4/3\,\pi r^3$, where r is the radius. At $t = 0$, the volume of the oocyte was taken as 100 %.

NTPDase activity (ecto-ATPase activity)
The NTPDase activity of defolliculated *Xenopus* oocytes was assessed as described elsewhere (Ziganshin *et al.* 1995). Groups of six oocytes were placed in 96 ELISA wells, in a volume of 100 μl of NR. Two groups of oocytes were assayed in the presence of NR or HS. In both cases, a subgroup of oocytes was incubated in the corresponding media, and calcium was replaced by 1 mM EGTA. Oocytes were first washed and subsequently incubated in the above medium for 30 min at room temperature. Media were then carefully replaced by fresh solutions supplemented or not by 100 μM ATP. After an additional 30 min incubation period, the media were removed and their phosphate contents were determined using the malachite green method (Lanzetta *et al.* 1979). Each condition was assayed in triplicate and results are shown as the means ± S.E.M. from four independent experiments. In some experiments, the mannitol in HS was replaced by sucrose or sorbitol. In other experiments, NTPDase activity was assayed in potato apyrase (grade VII, Sigma).
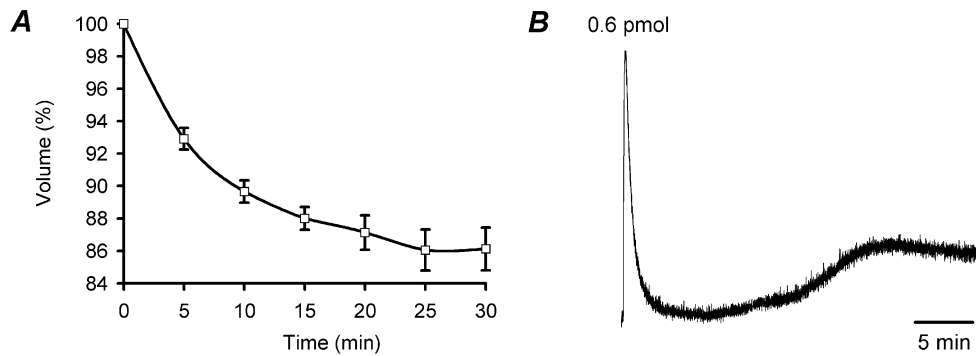
Figure 1. Macroscopic changes induced by hypertonic shock in *Xenopus* oocytes

A, reduction in cell volume (%) in 6 oocytes. *B,* example of ATP release measured in a single oocyte by means of the luciferin–luciferase reaction. This oocyte was not penetrated with fine-tipped electrodes. The trace corresponds to changes in light intensity, reflecting the presence of ATP in the medium. Just at the beginning of the recording, a known dose of ATP (0.6 pmol) was added to evaluate the amount of ATP released by the oocyte.

Expression of human CFTR in *Xenopus* oocytes

Plasmid containing full length cDNA for CFTR (pBS6.2-CFTR) was generously supplied by Professor Johanna Rommens (Department of Genetics, Hospital for Sick Children, Toronto, Canada). The plasmid (10 μg) was linearized with *Xho*I (Promega) and the resultant product was used for mRNA synthesis *in vitro* using the mCAP RNA Capping Kit (Stratagene). The mRNA obtained was injected (50 nl, 1–2 mg ml^{-1}) into oocytes 3 days before membrane current recordings. CFTR-activated ionic currents were elicited with NR supplemented with 100 μM 8-Br-cAMP (Sigma), 1 mM IBMX (3-isobutyl-1-methylxanthine, Sigma) and 1 μM forskolin (Sigma). Only oocytes exhibiting the activated current were further stimulated with the hypertonic protocol.

Injection of DNA antisense oligonucleotide complementary to CD39 mRNA in *Xenopus* oocytes

An antisense oligonucleotide complementary to CD39 mRNA (antiCD39): 5′-TGTTGGTCAAGTTCAGCATGTAGCCCAG-3′ was designed from consensus sequences of human, rat, mouse, cow, chicken and *Torpedo marmorata* and synthesized by Invitrogen-Life Technologies. The oligonucleotide was re-hydrated in ultra-pure water and injected (50 nl, 2 mg ml^{-1}) into oocytes 40–72 h before membrane current recordings.

Statistical analysis

Curves were fitted using Sigma Plot (SPSS). Statistical analysis was performed using Sigma Stat software (SPSS). Unless otherwise specified, values given in the text correspond to the mean ± S.E.M. When comparing two-group means of normally distributed data, the Student's *t* test was used. Otherwise, the Mann-Whitney rank sum test was applied. A significance level of $P < 0.05$ was adopted in all comparisons.

RESULTS

Oocytes in hypertonic (543 mosmol l^{-1}) conditions showed a reversible decrease in volume of 13 ± 3 % ($n = 6$), and reached a steady state within ~10 min, depending on the oocyte batch (Fig. 1*A*). During shrinkage, the oocytes released ATP (Fig. 1*B*). When they were recorded individually in voltage-clamp conditions with a membrane voltage (V_m) of –70 mV, the hypertonic medium induced an inward current and ATP release (Fig. 2). The time course of currents varied from one oocyte to another. At the beginning of recording, the amount of ATP released was apparently independent of

Figure 2. Simultaneous recording of ATP release and ionic currents from a single oocyte stressed by hypertonic solution

A, single oocyte superfused in hypertonic solution 2 min before the experiment. Upper trace: membrane voltage. Middle trace: membrane current. Bottom trace: ATP release. Note that in the light trace, we added a known dose of ATP (1.2 pmol) to evaluate the release of ATP. *B,* example illustrating a plot of ATP release *vs.* current and time (each point represents the ATP release *vs.* current amplitude during each minute of recording from minute 4 to minute 20). An exponential curve fitted the data obtained between minute 10 and minute 20 ($n = 20$).
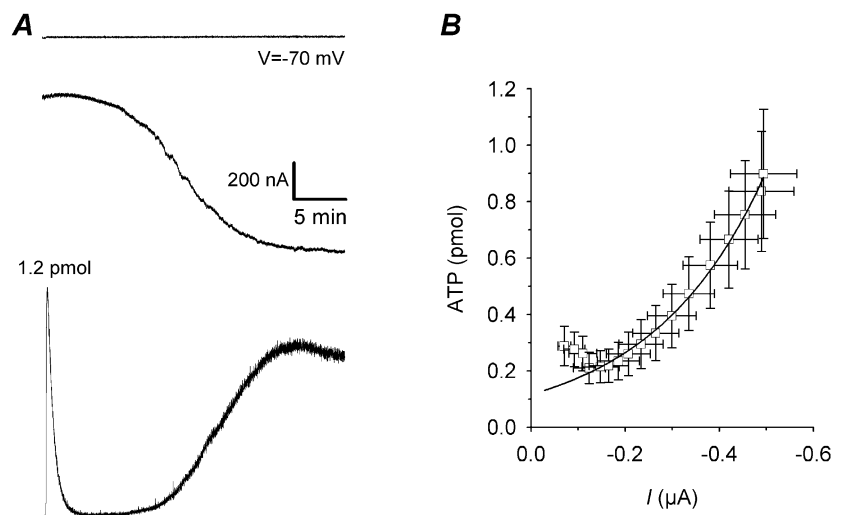
the amplitude of the current. However, after 8 min, this correlation fitted an exponential curve (ATP release = $a \times \exp(b \times \text{current})$, where b was 10^{-4} and a was 116; $R = 0.997$). The total amount of ATP released at the end of minute 20 of recording was 7 ± 2 pmol ($n = 24$).

The effect of the hypertonic solution applied for periods of 20 min was reversible (Fig. 3) and the initial volume was restored a short time after returning the oocytes to the isotonic solution. A second challenge with hypertonic solution reduced the cellular volume again, reaching the same values as recorded in the first perfusion. However, the hypertonic shock-activated inward current was blocked after a second hypertonic shock and ATP release was also inhibited. This inhibitory effect was not observed when oocy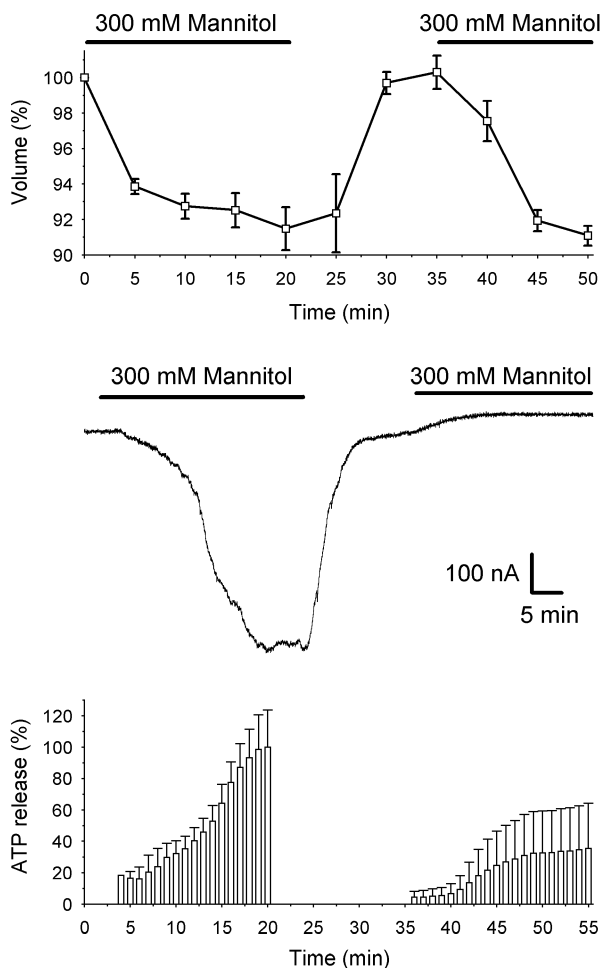tes were perfused with hypertonic solution for shorter periods such as 10 min. In these cases, hypertonic-activated current and ATP release after a second hypertonic stimulus were slightly inhibited (data not shown).

To test the possible exocytotic source of ATP, we treated some oocytes with tetanus neurotoxin (TeNT). Oocytes previously micro-injected with the light chain of tetanus neurotoxin (L-TeNT) did not show any significant change in the hypertonic shock-activated membrane currents (Fig. 4). However, the correlation between the current and the amount of ATP released could be fitted with an exponential function (ATP release = $a \times \exp(b \times \text{current})$, where b was 10^{-4} and a was 25; $R = 0.975$). This fit included all points measured. The amount of ATP released at the beginning of the recording was decreased (compared with Fig. 2).
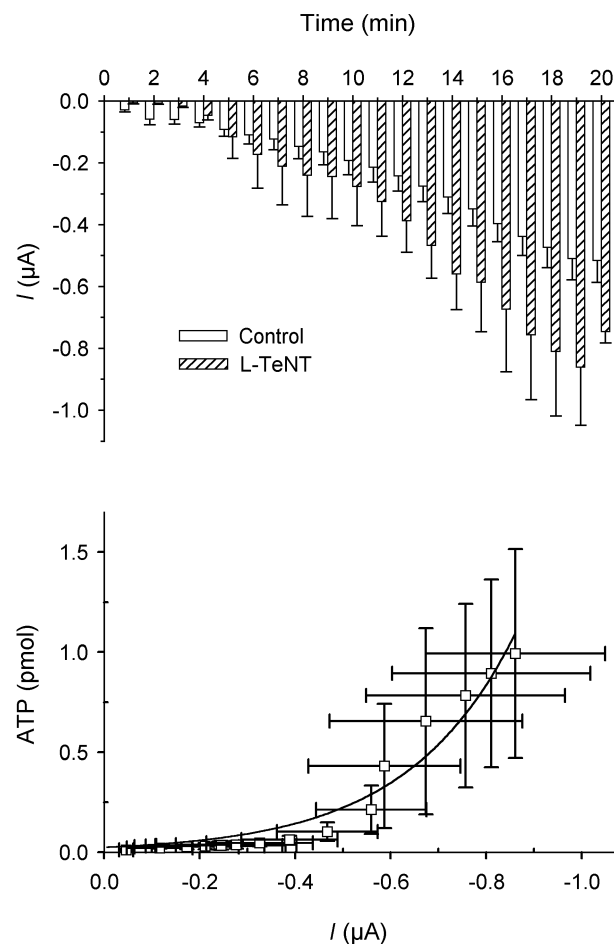

Figure 3. Effect of two consecutive hypertonic shocks on cell volume, inward current and ATP release

Upper graph: decrease in cell volume in 6 oocytes superfused twice (indicated with horizontal bars) in hypertonic solution. Middle trace: example of ionic currents recorded in one oocyte clamped at $V_m = -70$ mV in which two consecutive hypertonic superfusions (indicated with horizontal bars) were applied. Lower graph: percentage of ATP release with respect to the maximal release measured after the first period of 20 min of hypertonic perfusion ($n = 3$).

Figure 4. Effect of tetanus toxin on hypertonic shock-activated membrane current and ATP release

Upper graph: average of current amplitudes during 1 min of recording in non-injected oocytes ($\square$, $n = 33$) and in L-TeNT injected oocytes ($\boxtimes$, $n = 4$). Lower graph: plot of ATP release vs. current and time (each point represents the ATP release vs. current amplitude during each minute of recording from minute 4 to minute 20). An exponential function fitted the values of all the data represented. Compare the amount of ATP released in this condition and that in Fig. 2.
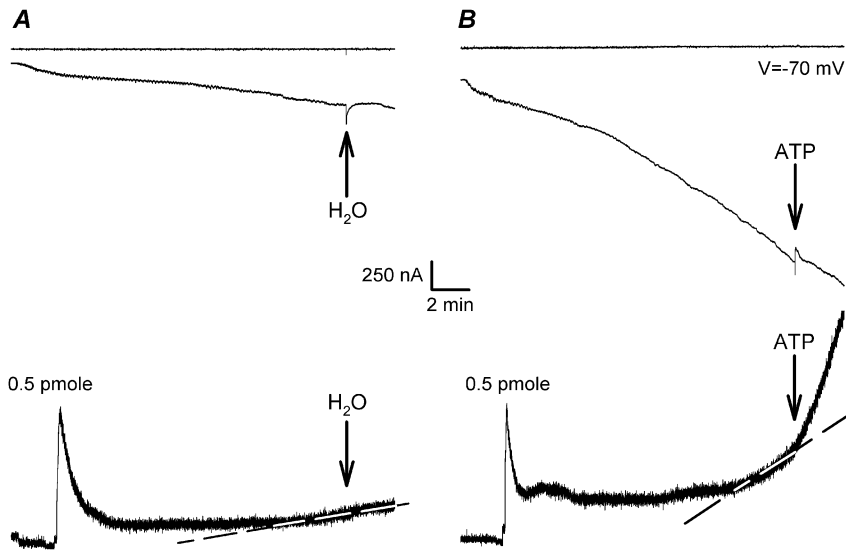
A

B

V=-70 mV

ATP

H$_2$O

250 nA

2 min

ATP

0.5 pmole

H$_2$O

0.5 pmole

Figure 5. Effect of intracellular ATP injection on ATP release and ionic current elicited by a hypertonic solution

The cytoplasmic ATP concentration was increased by a direct injection of a small volume of an ATP solution. Examples of recordings in two oocytes superfused in hypertonic solution into which we injected 9.2 nl of distilled water (*A*) and 9.2 nl of 250 mM ATP (*B*). Upper traces: membrane voltage. Middle traces: membrane current. Bottom traces: ATP release. Note that in both light traces, we added a known dose of ATP (0.5 pmol) to evaluate the release of ATP. Arrows indicate the injection of solutions. Dashed lines indicate the slope of ATP release before injections.

We increased the cytoplasmatic ATP concentration by injecting 9.2 nl of K$_2$ATP (250 mM, pH ≈ 7). In six oocytes we found an increase in the rate of ATP release (Fig. 5). The increase of ATP release was only recorded when oocytes were shrunken and the inward current was activated. When the same solution of ATP was injected into oocytes in isotonic conditions, no ATP release was detected (data not shown). In hypertonically stressed oocytes injected with ultra-pure water (9.2 nl), no increase in ATP was detected (Fig. 5).

To determine the influence of mechanically activated ionic channels, oocytes were treated with gadolinium. In all experiments, a known dose of ATP was applied in the presence of gadolinium, in order to check for any interference by gadolinium ions in the luciferin–luciferase–ATP reaction. When oocytes were preincubated for 10 min with an isotonic solution containing GdCl$_3$ (200 μM), and then with a hypertonic solution containing the same concentration of GdCl$_3$, we did not observe any change in the reduction of their

Figure 6. Effect of gadolinium on cell volume, membrane current and ATP release during a hypertonic shock

Upper graph: decrease in cell volume in oocytes superfused in hypertonic solution. The value at time 0 was set at 100 %. ●, hypertonic solution (*n* = 6); ○, hypertonic solution plus 200 μM GdCl$_3$ (*n* = 6). Middle graph: average of current amplitudes during 1 min of recording. □, non-treated oocytes (*n* = 33); ▨, gadolinium-treated oocytes (*n* = 11). *$P < 0.05$. Lower graph: plot of ATP release *vs.* current and time (each point represents the ATP release *vs.* current amplitude during each minute of recording from minute 4 to minute 20) showing the inhibition of ATP release and the hypertonic shock-activated currents.
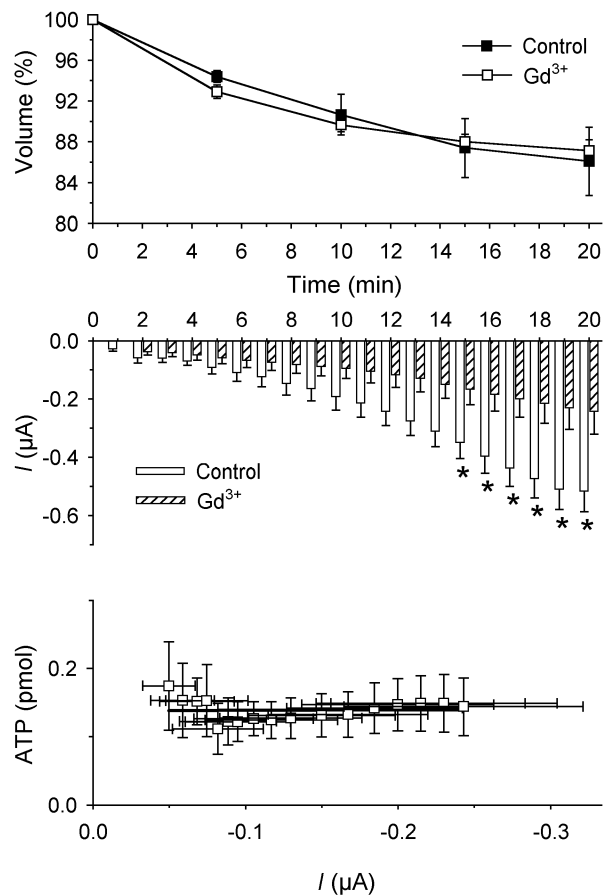
volume (Fig. 6). Hypertonic shock-activated currents were significantly inhibited (66 %) after 20 min, throughout all the phases of the currents. The plot of ATP release *vs.* inward current is a flat horizontal line, revealing that the second component of ATP release (the ionic mechanism) was blocked, but the first component (the exocytotic mechanism) was not affected by gadolinium ions. We also explored the relationship between voltage and current in shrunken oocytes: the effect of gadolinium on the *I–V* ratio of the hypertonic shock-activated currents is summarized in Fig. 7. While the currents in hypertonic conditions were linear, we found a slight rectification in the presence of gadolinium. The reversal potential was -30 ± 1 mV in isotonic solution, -28 ± 1 mV in isotonic solution plus gadolinium, -23 ± 1 mV in hypertonic solution and -29 ± 1 mV in hypertonic solution plus gadolinium ($n = 6$, in all conditions).

Some specific proteins like CFTR have been implicated in the extrusion of ATP from cells. Oocytes injected with 50–100 ng of mRNA encoding CFTR were tested with a solution containing 100 μM 8-Br-cAMP, 1 mM IBMX and 1 μM forskolin before being stimulated with hypertonic solutions. The current elicited by this solution was reversible and we did not detect any release of ATP. When
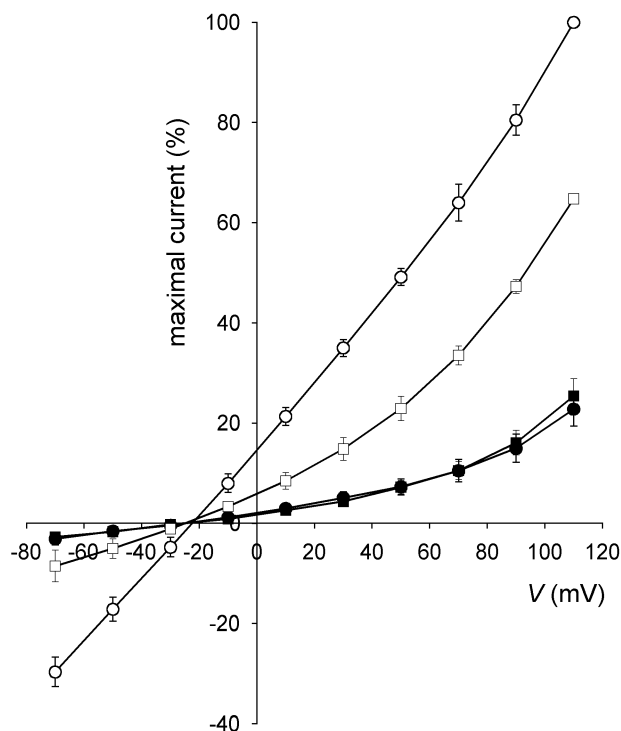
the oocytes expressing CFTR were stressed by hypertonic solution, we found a significant ($P < 0.05$) decrease in the activated inward current (up to minute 15) and in ATP release (up to minute 9) (Fig 8). The plot of ATP released *vs.* current reveals an exponential function from minute 4 to minute 20 (ATP release $= a \times \exp(b \times \text{current})$, where b was 10^{-4} and a was 48; $R = 0.998$).

The ATP measured above is the result of the equilibrium between ATP release and ATP degradation by ecto-enzymes such as NTPDases. The NTPDase activity of the intact oocyte surface was measured in isotonic and hypertonic conditions (Fig. 9) as the calcium-dependent ATPase activity. At low calcium concentrations (1 mM EGTA), the hydrolysis of ATP was much lower (0.03 ± 0.01 nmol inorganic phosphate (P_i) oocyte^{-1} (30 min)$^{-1}$) than in the presence of 1.8 mM $CaCl_2$ (0.28 ± 0.02 nmol P_i oocyte^{-1} (30 min)$^{-1}$). After 30 min in hypertonic solution, the activity in medium containing

Figure 7. *I–V* relationship of hypertonic shock-activated currents

Current amplitudes are represented as a percentage of the amplitude of the current measured in non-treated oocytes in hypertonic medium at +110 mV. Circles: non-treated oocytes ($n = 6$) in isotonic (●) and hypertonic (○) media. Squares: gadolinium-treated oocytes ($n = 6$) in isotonic (■) and hypertonic (□) media.
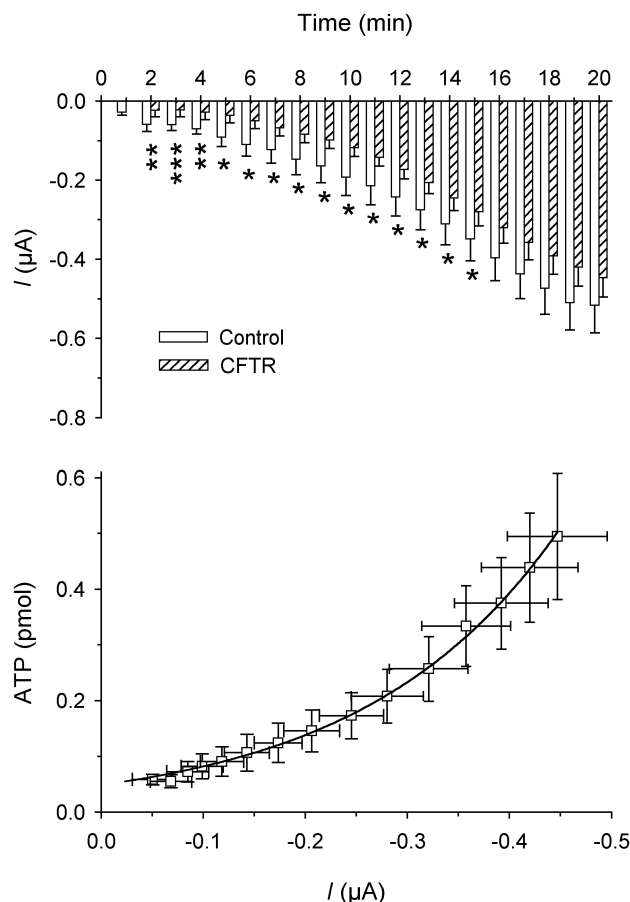

Figure 8. Hypertonic shock-activated membrane currents and ATP release in oocytes expressing CFTR

Upper graph: average of current amplitudes during 1 min of recording:. □, non-injected oocytes ($n = 33$); ▨, oocytes expressing CFTR ($n = 17$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Lower graph: plot of ATP release *vs.* current and time (each point represents the ATP release *vs.* current amplitude during each minute of recording from minute 4 to minute 20). An exponential curve fitted all the data represented.

1 mM EGTA did not differ (0.03 ± 0.02 nmol P_i oocyte^{-1} (30 min)$^{-1}$) from that measured in isotonic conditions. However, the calcium-dependent activity was reduced to 0.12 ± 0.02 nmol P_i. oocyte^{-1} (30 min)$^{-1}$, which represents about a 56 % inhibition of NTPDase activity. To rule out the direct inhibition of NTPDase activity by mannitol, we assayed other NTPDases. NTPDase activity from potato was $(112 \pm 20) \times 10^3$ nmol (mg protein)$^{-1}$ (30 min)$^{-1}$ and $(113 \pm 20) \times 10^3$ nmol (mg protein)$^{-1}$ (30 min)$^{-1}$ when 300 mM mannitol was added ($n = 3$).

Finally, we recorded ionic current and ATP release from CD39 antisense-injected oocytes ($n = 13$) and from water-injected oocytes ($n = 8$). In 13 of the 14 oocytes CD39 antisense oligonucleotide significantly reduced ATP release and the ionic current after 13 min of hypertonic exposure (Fig. 10).

DISCUSSION

Cell volume regulation is a complex cellular response, that involves at least three steps (Montrose-Rafizadeh & Guggino, 1990). First, the cell must be able to detect changes in cell volume; second, it must initiate regulatory processes that alter intracellular solute content, and third, the cell must remember its initial volume and shut down the volume regulatory process. Mammalian cells show regulatory cell volume decrease under hypotonic conditions, but they do not always exhibit regulatory cell volume increase under hypertonic conditions (Montrose-Rafizadeh & Guggino, 1990).

Cell shrinkage elicited by a hyperosmotic solution promotes subsequent influx of ions and water; regulatory volume increase is mediated either by Na^+–K^+–Cl^- cotransport or by the coupled activities of Na^+–H^+ exchange (NHE) and Cl^-–HCO_3^- anion exchange (Goss *et al.* 2001). Several isoforms from mammalian and non-mammalian cells have been cloned, including *Xenopus* oocytes. To achieve a regulatory volume increase *Xenopus* oocytes must co-express the endogenous ion transporter xoNHE and at least the anion exchanger AE2 (Jiang *et al.* 1997), cloned from mouse (Grinstein *et al.* 1992). This indicates that *Xenopus* oocytes share the same first steps of cell volume regulation as mammalian cells and therefore the release of ATP induced by cell shrinkage should be a considered a general phenomenon related to volume adaptation.

Changes in cell volume are associated with ATP release, which is usually detected during cell swelling in hypotonic
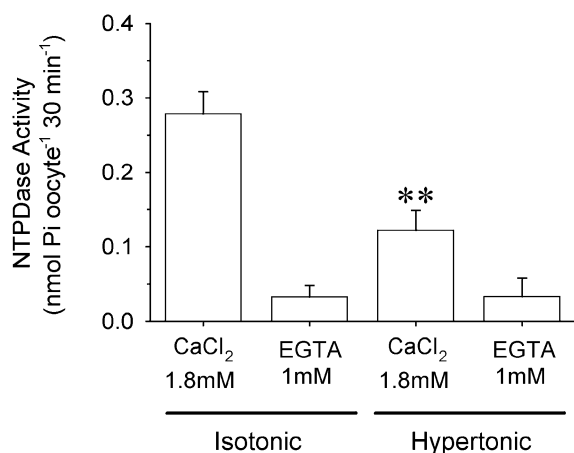
Figure 9. External NTPDase activity of oocytes during hypertonic shock

The bar histogram shows the NTPDase activity as ATP hydrolysis stimulated by calcium. We investigated the activity in isotonic and hypertonic conditions. Calcium-independent activity was very low and was insensitive to hypertonic shock. However, the calcium-stimulated activity was inhibited by 56 %. ** $P < 0.01$ ($n = 4$).
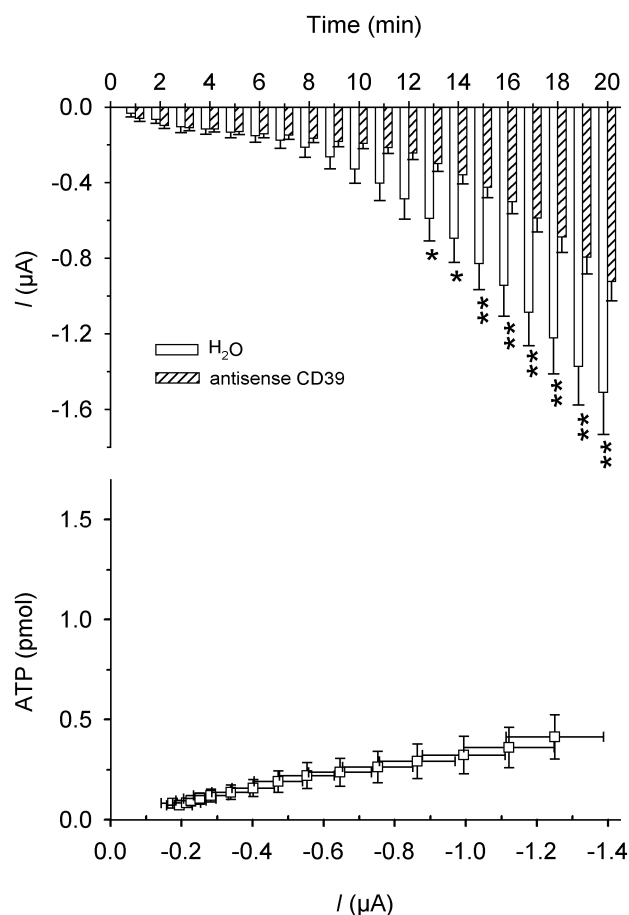
Figure 10. Hypertonic shock-activated membrane currents and ATP release in oocytes injected with an antisense oligonucleotide complementary to CD39 mRNA

Upper graph: average of current amplitudes during 1 min of recording. □, water-injected oocytes ($n = 8$); ▨, CD39 antisense-injected oocytes ($n = 13$). * $P < 0.05$, ** $P < 0.01$. Lower graph: plot of ATP release *vs.* current and time in CD39 antisense-injected oocytes (each point represents the ATP release *vs.* current amplitude during each minute of recording from minute 7 to minute 20).

conditions (Hazama *et al.* 2000; Grygorczyk & Guyot, 2001; Koyama *et al.* 2001; Okada *et al.* 2001; Sabirov *et al.* 2001; Boudreault & Grygorczyk, 2002). Several reports have shown that hypotonic shock-activated currents are observed only in follicle-enclosed *Xenopus* oocytes (Arellano & Miledi, 1993, 1995; Ackerman *et al.* 1994; Aleu *et al.* 1997). We tested defolliculated *Xenopus* oocytes in hypotonic conditions (95 mosmol l⁻¹) and we did not detect any release of ATP or activation of any ionic current. Moreover, we did not detect ATP release in oocytes in which even the vitelline envelope had been completely removed (data not shown). In contrast, oocytes challenged with hypertonic solution released ATP and showed a reduction in volume. We also recorded ATP release in oocytes that were not impaled by glass microelectrodes, which revealed that the release was not due to leakage around the electrodes during cell shrinkage. A hypertonic challenge in *Xenopus* oocytes also activated an inward current that has been described elsewhere (Zhang & Hamill, 2000) and is reminiscent of that activated by mechanical stress (Saitou *et al.* 2000).

The release of ATP was accompanied by activation of an inward current. While the effect of hypertonic solution on cell volume was reversible, even after a second application, activation of the inward current was totally blocked during a second hypertonic challenge and release of ATP, though still detectable, represented only 35 % of the amount of ATP released during the first hypertonic stimulus.

The hypertonic shock-activated inward current was completely reversible, but was blocked after a second hypertonic challenge, suggesting an association between the opening of the current and some disruption between the cytoskeleton and the plasma membrane. In fact, hypertonic solutions applied to *Xenopus* oocytes have been shown to induce the formation of individual cytoskeleton-free vesicles (Zhang *et al.* 2000). Such vesicles showed a reduced sensitivity to mechanically activated channels. The association between the cytoskeleton and mechanically activated channels has been reported in other experimental models such as neurons, in which the use of cytochalasin or colchicines disrupts the assembly of cytoskeleton and decreases the activity of mechanically activated channels (Cho *et al.* 2002).

The ATP release–current relationship described two major components of hypertonic shock-activated ATP release. The first component took place during the first 10 min of shrinkage, where the amount of ATP released and the amplitude of the inward current were not correlated. This indicates that a non-ionic process is involved, for example exocytosis. The second component began when oocyte volume reached a minimum. In this phase the amount of ATP released correlated with the amplitude of the inward current.

It is well known that exocytosis is inhibited by clostridial toxins (Rosseto *et al.* 2001; Aleu *et al.* 2002). To analyse the possible exocytotic origin of ATP release we used tetanus neurotoxin (TeNT). To verify the action of this toxin, we injected the light chain of TeNT (L-TeNT) instead of superfusing the oocytes with the holotoxin, because it is not known whether *Xenopus* oocytes express any receptors to clostridial toxins on their surface. We found that L-TeNT injected into oocytes inhibited the first component of ATP release induced by the hypertonic stimulus. In oocytes injected with L-TeNT, all points analysed fitted an exponential curve, and the fitted curves in L-TeNT-treated and non-L-TeNT-treated oocytes were very similar, suggesting that both describe the same cellular mechanism. Since only the release of ATP during shrinkage (the first component) is sensitive to L-TeNT, this component may correspond to an exocytosis-mediated release. In agreement with these results, a recent study provided evidence that ATP release from *Xenopus* oocytes stressed with local mechanical stimulation is sensitive to Brefeldin A, which interrupts the traffic of vesicles from the Golgi apparatus to the cell surface, suggesting an exocytotic pathway for ATP release (Maroto & Hamill, 2001). In other non-secretory cells also, such as those from the pig ureter epithelium (Knight *et al.* 2002), the release of ATP is associated with the exocytosis of small vesicles.

The second component of ATP release (once oocyte volume reached the minimum) was strongly inhibited by gadolinium, a classical blocker of mechanically activated ionic channels, but during the shrinking process (first component) there was no significant difference between control and gadolinium-treated oocytes: this implies that the first component of ATP release (the exocytotic process) is not affected by gadolinium. It has been reported that gadolinium also enhances ATP release during cell swelling owing to its membrane fusogenic activity (Boudreault & Grygorczyk, 2002), but we ruled out this possibility in *Xenopus* oocytes because, at the concentrations tested, ATP release did not increase.

During hypertonic challenge, gadolinium did not inhibit shrinkage of the oocytes, but the hypertonic shock-activated current was strongly inhibited. Similar results were obtained during the activation of osmoreceptors in supraoptic neurons (Oliet & Bourque, 1996) and the concentrations at which gadolinium was more active were in the range of 100–500 μM. *Xenopus* oocytes may thus be sensitive to hypertonic solutions in a manner similar to neurons. However, in oocytes the gadolinium-induced inhibition of the hypertonic-activated current was coincident with the inhibition of ATP release. The inhibitory effect of gadolinium on both the current and ATP release was observed throughout the period of current activation.

The reversal potential of the hypertonic-activated current was the same under all conditions tested and was very close to the reversal potential of Cl⁻ in *Xenopus* oocytes calculated according to their intracellular Cl⁻ concentration (Dascal, 1986). Gadolinium did not change the reversal potential in isotonic or hypertonic solutions. If ATP is released as a charged molecule, only a small fraction of the current (1 %) would be supported by the anionic ATP, and this net ATP current would be masked by the background noise of the amplifier. This explains why, when the intracellular concentration of ATP was increased, we recorded a clear increase in ATP release but not in current amplitude. An increase in ATP release was only detected when the inward current was activated and the oocyte had shrunk.

A link between ATP release and chloride flux has been demonstrated in *Xenopus* oocytes expressing functional CFTR (Jiang *et al.* 1998). We did not detect ATP release when activating CFTR with cAMP, as reported by other groups, supporting the view that CFTR is not permeable to ATP (Grygorczyk & Hanrahan, 1997; Watt *et al.* 1998; Braunstein *et al.* 2001). In oocytes injected with CFTR during the shrinking process, the current activated by the hypertonic stimulus was inhibited, but there was little effect on the ionic current recorded after the oocytes reached their minimum volumes. CFTR and L-TeNT inhibited ATP release during the first minutes of recording but not later, suggesting that they affect the same processses of ATP secretion. This would support one of the hypotheses formulated by Devidas & Guggino (1997), who propose that CFTR indirectly controls the exocytosis of ATP.

The membrane protein CD39 is also related to ATP transport through the cell plasma membrane, as suggested elsewhere (Wang *et al.* 1998; Bodas *et al.* 2000; Abraham *et al.* 2001). CD39 has two transmembrane domains and a small hydrophobic loop (Wang *et al.* 1998), which is analogous to other ionic channels. CD39 is a member of the family of type 1 NTPDases, since it hydrolyses ATP or ADP in a calcium-dependent manner (see Zimmermann, 2000 for review). Endogenous NTPDase activity has been described in *Xenopus* oocytes (Ziganshin *et al.* 1995; Bodas *et al.* 2000; Aguilar *et al.* 2001). In our study, NTPDase activity was inhibited by more than 50 % at the end of the period of hypertonic challenge. This inhibition is likely to contribute to the exponential increase in ATP detected because the probability of ATP being degraded decreases with time. We ruled out a direct effect of mannitol, since mannitol had no inhibitory effect on the NTPDase activity of potato apyrase. The amino acid sequence responsible for the activity of NTPDase has been conserved in all types of NTPDases, including potato apyrase, so any direct inhibitory effects of mannitol would be apparent in all of them. The inhibition of NTPDase activity may also be due to endocytosis of the endogenous CD39, which has been

associated with caveolae (Koziak *et al.* 2000). However, under the condition of hypertonic stimulus, we detected a release of ATP that was sensitive to clostridial toxins, indicating an increase in the rate of exocytosis. Since the increase in ATP release is coincident with inhibition of NTPDase activity, and we have suggested that CD39 is involved in plasma membrane ATP transport (Bodas *et al.* 2000), CD39 may have two conformations: one in which it behaves as a hydrolysing enzyme and another in which it behaves as a transporter. In hypertonic conditions, a significant part of the endogenous CD39 of oocytes would adopt the transporter conformation, thus reducing the number of molecules acting as NTPDase. The experiments performed in oocytes injected with CD39 antisense oligonucleotide showed a decrease in ATP and also in inward current (second component), reinforcing the view that CD39 is involved, directly or indirectly, in ATP release induced by hypertonic stress. Moreover these experiments also relate the transport of ATP to the inward current. In addition, our group has demonstrated that in *Xenopus* oocytes expressing human CD39 there is an increase in the hyperpolarizing-activated current associated with ATP release (Bodas *et al.* 2000).

Our studies have focused entirely on ATP release induced by hypertonic stress, but ATP release may also be induced in *Xenopus* oocytes by mechanical stress (Maroto & Hamill, 2001) and hyperpolarizing pulses (Bodas *et al.* 2000). Other mechanisms implicated in ATP release involve hemi-gap junctions, as recently described in astrocytes (Stout *et al.* 2002). *Xenopus* oocytes express endogenous hemi-gap channels Cx38 (Ebihara, 1996), and they may be involved in ATP release in this system.

Finally, we conclude that ATP release is induced by hypertonic stress in *Xenopus* oocytes via two different mechanisms: one related to exocytosis and the other related to an ionic current. CD39 is, directly or indirectly, implicated in this process.

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