Osmotic cell swelling-induced ATP release mediates the activation of extracellular signal-regulated protein kinase (Erk)-1/2 but not the activation of osmo-sensitive anion channels

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Human intestine 407 cells respond to hypo-osmotic stress by the rapid release of ATP into the extracellular medium. A difference in the time course of activation as well as in the sensitivity to cytochalasin B treatment and BAPTA-AM [1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid acetoxymethyl ester] loading suggests that ATP leaves the cell through a pathway distinct from volume-regulated anion channels. To evaluate a putative role for nucleotides as autocrinic/paracrinic factors in osmotic signalling, the effects of extracellular ATP on the regulation of volume-sensitive anion channels as well as on the hypotonicity-induced activation of extracellular signal-regulated protein kinases (Erk-1/2) were investigated. Micromolar concentrations of ATP were unable to elicit an isotope efflux from $^{125}I^-$-loaded cells by itself, but strongly potentiated the hypotonicity-provoked anion efflux through a Ca^{2+}-dependent mechanism. The order of potency of nucleotides (ATP = UTP = ATP[S] > ADP = AMP ≫ adenosine = cAMP) indicated the involvement of $P2Y_2$ receptors. In contrast, millimolar concentrations of ATP markedly inhibited both the osmotically induced isotope efflux and whole-cell Cl^- currents. Inhibition of whole-cell Cl^- currents, not only by millimolar ATP but also by the purinoceptor antagonists suramin and reactive blue, was observed most prominently at depolarizing holding potentials, suggesting a direct interaction with volume-sensitive Cl^- channels rather than interaction with purinoceptors. Both ATP and UTP, at submicromolar levels, were found to act as potent activators of Erk-1/2 in intestine 407 cells. Addition of the ATP hydrolase apyrase to the bath greatly reduced the hypotonicity-induced Erk-1/2 activation, but did not affect the swelling-induced isotope efflux or whole-cell Cl^- currents. Furthermore, pre-treatment with suramin or reactive blue almost completely prevented the hypo-osmotic activation of Erk-1/2. The results indicate that extracellularly released ATP functions as an autocrinic/paracrinic factor that mediates hypotonicity-induced Erk-1/2 activation but does not serve as an activator of volume-sensitive compensatory Cl^- currents.

Key words: chloride channels, mitogen-activated protein kinases, regulatory volume decrease.

INTRODUCTION

Most cell types studied to date respond to alterations in cell volume by evoking specific compensatory mechanisms. In general, osmotic cell swelling leads to the activation of K^+- and Cl^--selective ionic channels and, depending on cell type and experimental conditions, to an accompanying efflux of organic osmolytes (amino acids, polyols and methylamines; reviewed in [1]). Although the mechanism of activation of the volume-sensitive Cl^- channels is as yet elusive, protein tyrosine phosphorylation was found to be critically involved in multiple cell types [2–6]. Furthermore, activation of the ras-related G-protein $p21^{Rho}$ appeared to be a prerequisite for activation of the volume-regulated Cl^- channels in bovine endothelial cells and human intestine 407 cells [6,7]. Hypo-osmotic cell swelling is accompanied by a rapid phosphorylation and/or activation of a number of other signalling enzymes, including members of the MAP kinase (mitogen-activated protein kinase) family [2,8–14]. Activation of MAP kinases, however, was not mandatory for the regulation of osmo-sensitive Cl^- channels [13,14]. Although the molecular identity of the Cl^- channels involved in cellular volume regulation is not yet clear, the bioelectrical properties of osmo-sensitive Cl^- currents have been studied in many cell types. These currents are characterized by a marked outward rectification, a strong inactivation at positive holding potentials and an inhibition by extracellularly applied nucleotides (reviewed in [15,16]).

Recent evidence indicates that ATP is released rapidly from cells in response to increases in cellular cAMP, mechanical stress or osmotic cell swelling [17–19]. Furthermore, extracellular ATP has been recognized as an autocrinic factor involved in increasing transmembrane Cl^- permeabilities and in the activation of volume-sensitive Cl^- channels [17,18,20]. Binding of ATP to a specific subclass of G-protein-coupled purinoceptors (the P2Y subtypes) not only leads to phospholipase C-mediated activation of protein kinase C and mobilization of Ca^{2+}, but additionally results in the activation of MAP kinases, including the extracellular signal-regulated protein kinases (Erk-1/2) [21–27] and the stress-activated protein kinase SAPK/JNK [28,29].

In this study, we used the human fetal jejunum-derived intestine 407 cell line as a model to investigate the role of extracellularly released ATP in the regulation of volume-sensitive Cl^- channels as well as its putative involvement in the activation of Erk-1/2 by hypotonicity. Hypo-osmotic cell swelling was found to result in a dose- and time-dependent release of ATP into the medium, through a mechanism independent of the activation of volume-sensitive Cl^- channels. Furthermore, in contrast with previous observations in other cell models

Abbreviations used: MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; BAPTA-AM, 1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid acetoxymethyl ester; Erk, extracellular signal-regulated protein kinase.

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[18,30,31], autocrinic/paracrinic ATP signalling did not serve as a trigger to activate volume-sensitive Cl^- channels. ATP signalling through P2Y receptors, however, was found to be fully responsible for the hypo-osmotic activation of Erk-1/2.

MATERIALS AND METHODS

Materials

Radioisotopes ($^{125}I^-$, $^{45}Ca^{2+}$ and [γ-^{32}P]ATP) and enhanced chemiluminescence (ECL) kit were purchased from Amersham Netherlands B. V. ('s Hertogenbosch, The Netherlands). Polyclonal anti-Erk antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The Ca^{2+}-ionophore A23187 and cytotoxicity detection kit (lactate dehydrogenase) were from Boehringer Mannheim (Mannheim, Germany), and MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide], Protein A–Sepharose and luciferin/luciferase reagent were obtained from Janssen Chimica (Geel, Belgium), Pharmacia Biotech (Uppsala, Sweden) and Promega (Madison, WI, U.S.A.) respectively. Other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Cell culture

Intestine 407 cells were grown routinely as monolayers in Dulbecco's modified Eagle's medium supplemented with 25 mM Hepes, 10% fetal calf serum, 1% non-essential amino acids, 40 mg/l penicillin and 90 mg/l streptomycin under a humidified atmosphere of 95% O_2/5% CO_2 at 37 °C. Prior to the experiments, cells were serum-starved overnight. Cell viability was assessed by determining lactate dehydrogenase leakage according to the instructions provided by the manufacturer (Boehringer Mannheim) and by quantifying MTT conversion, as described by Hansen et al. [32].

Efflux assays

Confluent monolayers of intestine 407 cells were loaded with 5 μCi/ml $^{125}I^-$ or 0.5 μCi $^{45}Ca^{2+}$ for 2 h in modified Meyler solution (108 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM $NaHCO_3$, 0.8 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 20 mM Hepes and 10 mM glucose, pH 7.4) under a 95% O_2/5% CO_2 humidified atmosphere. Subsequently, the cultures were washed three times with isotonic buffer (80 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 95 mM mannitol and 20 mM Hepes, pH 7.4). Hypotonic buffers were prepared by adjusting the concentration of mannitol. Radioisotope efflux was determined by replacing the medium at 1–2 min intervals, quantified by β- or γ-radiation counting and expressed as fractional efflux per min, as described previously [33].

Luciferin/luciferase assay

Cells were seeded at a concentration of 10^4/cm^2 and incubated for 4 h under a humidified atmosphere of 95% O_2/5% CO_2 at 37 °C. Thereafter, cells were washed four times with isotonic buffer (80 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 95 mM mannitol and 20 mM Hepes, pH 7.4). ATP release was measured in real time immediately after adding isotonic or hypotonic buffer containing the luciferin/luciferase reagent using a Topcount.NXT luminometer (Packard, Meriden, CT, U.S.A.). Values are expressed as accumulation of extracellular ATP in time and corrected for the consumption of ATP by the luciferase reaction. Hypotonic buffers were prepared by adjusting the concentration of mannitol, and osmolality was assessed using a cryoscopic osmometer (Osmomat 030; Salm & Kipp B. V., Breukelen, The Netherlands).

Measurement of whole cell Cl^- currents

Cells were bathed in a solution containing 110 mM CsCl, 5 mM $MgSO_4$, 3.5 mM sodium gluconate, 12 mM Hepes, 8 mM Tris and 100 mM mannitol, pH 7.4. The intracellular pipette solution contained 110 mM CsCl, 2 mM $MgSO_4$, 25 mM Hepes, 1 mM EGTA, 1 mM Na_2ATP and 50 mM mannitol, pH 7.4. Patch pipettes were pulled from borosilicate glass (Clark Electromedical Instruments, Pangbourne, Berks., U.K.) and had a resistance of 2–3 MΩ. To monitor the time course of current activation, alternating step pulses (100-ms duration) from 0 to $\pm$100 mV were applied every 30 s. Voltage dependence of whole-cell current was monitored by applying step pulses (2-s duration, 7-s interval) from $-$100 mV to $+$100 mV with 25-mV increments. For command pulse control, data acquisition and analysis, pCLAMP 6 software (Axon Instruments, Foster City, CA, U.S.A.) was used. All data were sampled at 5 kHz after being low-pass filtered at 500 Hz with a Bessel filter (Axon Instruments).

Erk-1/2 activity assay

After stimulation, cells were washed once with ice-cold PBS and 1 ml of lysis buffer was added (1% Triton X-100, 150 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 1% aprotinin and 50 μg/ml leupeptin, pH 7.4). After 10 min (0 °C), lysates were collected, cleared by centrifugation and the supernatants were incubated with polyclonal anti-Erk antibodies (recognizing both Erk-2 and, to a lesser extent, Erk-1) for 1 h. Thereafter, Protein A–Sepharose was added and the mixture was incubated for a further hour. The immunoprecipitates were washed three times with lysis buffer and three times with kinase buffer (10 mM Tris/HCl, 150 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM dithiothreitol, pH 7.4) and Erk-1/2 activity was determined by *in vitro* phosphorylation of myelin basic protein (MBP). Phosphorylation was carried out in 40 μl of kinase buffer supplemented with 0.5 mg/ml MBP, 25 μM ATP and 5 μCi of [γ-^{32}P]ATP (30 min, 37 °C). After termination of the reactions by rapid centrifugation, boiling SDS sample buffer was added to the supernatants. The samples were boiled for 5 min and subjected to SDS/PAGE. Radioactivity of MBP was quantified by a phospho-imaging system (Molecular Imaging System GS-363; Bio-Rad, Hercules, CA, U.S.A.).

RESULTS

Osmotic cell swelling stimulates ATP release

The release of ATP from hypotonicity-provoked intestine 407 cells was monitored continuously using a luciferin/luciferase bioluminescence assay. As shown in Figure 1, a shift from isotonic to hypotonic medium caused a rapid increase in extracellular ATP. The ATP release was stimulus-dependent and continued for at least 15 min; only after mild hypo-osmotic stimulation (a 20$-$30% reduction in tonicity) was a tendency to level off observed. Notably, the regulatory volume decrease response under these conditions is completed within approx. 2–3 min [2]. Under all conditions tested, no detectable increase in luminescence was observed in the presence of the nucleotide hydrolase apyrase (results not shown).

Pre-treatment of the cells with 1,2-bis-(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid acetoxymethyl ester (BAPTA-AM) or cytochalasin B significantly inhibited the hypotonicity-

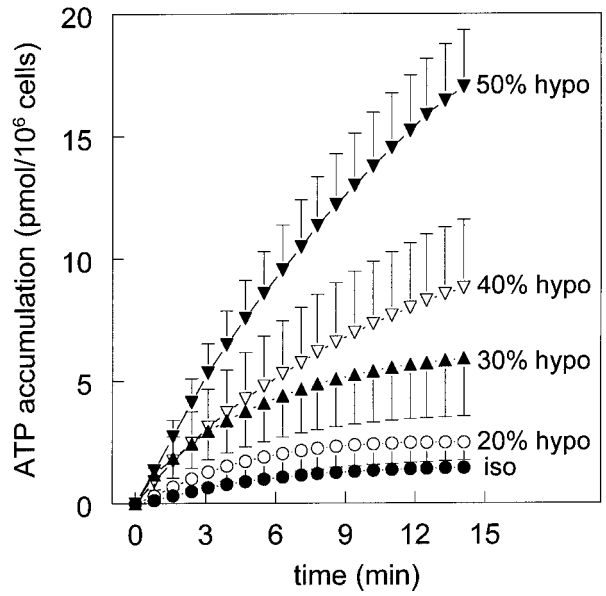

Figure 1 ATP release in response to hypo-osmotic cell swelling

ATP release from intestine 407 cells was measured in real time using the luciferin/luciferase assay immediately after addition of isotonic or hypotonic buffer. Data are expressed as means ± S.E.M. ($n = 5$). The amount of ATP present at time = 0 [immediately after adding isotonic (iso) or hypotonic (20 — 50% hypo) buffer containing luciferin/luciferase reagent] was taken as a baseline and was not significantly different between the experiments and the buffers used (9.5 ± 0.1 pmol/10^6 cells).

induced ATP release (Table 1). In contrast, the hypotonicity-provoked conductive $^{125}I^-$ efflux was unaffected by BAPTA-AM loading and was strongly potentiated by pre-treatment with cytochalasin B after submaximal stimulation (20% hypotonicity; Table 1). Cytochalasin B, however, did not affect the response to a saturating stimulus (30–50%; Table 1). These results, together with a clear difference in the time course of activation/inactivation of osmo-sensitive ATP release and anion efflux (peaks within 1–2 min), suggest that ATP leaves the cell through a pathway separate from the volume-sensitive anion channels. Notably, no significant differences were observed in either lactate dehydrogenase leakage or MTT conversion within 15 min of osmotic stimulation (50% tonicity; results not shown), indicating, together with our findings that the release of ATP is regulated by intracellular calcium and requires an intact cytoskeleton, that the increase in extracellular ATP is due to enhanced release from viable cells and not due to cell lysis.

Effects of extracellular nucleotides on osmo-sensitive anion efflux

The effects of extracellular ATP on anion transport were investigated by quantifying radioisotope efflux from $^{125}I^-$-loaded monolayers of intestine 407 cells. Whereas extracellular ATP (0.1 μM–10 mM) was unable to evoke a $^{125}I^-$ efflux by itself (Figure 2A), micromolar concentrations (10–100 μM) greatly potentiated the anion efflux in response to a submaximal (20% reduction in tonicity) osmotic stimulus (Figure 2A). Potentiation of the swelling-induced anion efflux by ATP could have been due to an increase in iodide permeability (genuine potentiation) or to an increase in driving force (stimulation of Ca^{2+}-activated K^+ channels). Two different experiments were performed to investigate whether the isotope efflux is mainly a function of the number of activated anion channels or is affected also by the membrane potential. (i) Potentiation of the efflux by ATP was not observed after maximal (30% reduction in tonicity) hypotonic stimulation: peak $^{125}I^-$ efflux, 38.7 ± 0.8%/min in the absence and 39.8 ± 1.5%/min in the presence of 100 μM ATP (mean ± S.E.M., $n = 3$). Under these conditions the $^{86}Rb^+$ efflux (indicative of K^+ permeability) was increased approx. 2-fold: peak $^{86}Rb^+$ efflux, 3.5 ± 0.2%/min in the absence and 5.9 ± 0.4%/min in the presence of 100 μM ATP ($n = 3$). (ii) Treating the cells with the K^+ ionophore valinomycin (10 μM) did not further increase the $^{125}I^-$ efflux in response to a 30% hypotonic medium: peak $^{125}I^-$ efflux was 32.2 ± 2.8%/min in the control versus 31.7 ± 1.2%/min in the presence of valinomycin ($n = 3$). Furthermore, as published previously [34], brief treatment of the cells with PMA completely inhibited the hypotonicity-induced $^{86}Rb^+$ efflux, but did not affect the osmo-sensitive $^{125}I^-$ efflux. These results show that the isotope efflux is virtually independent of the membrane potential. Taken together, micromolar concentrations of extracellular ATP potentiate the $^{125}I^-$ efflux through an increase in membrane permeability rather than by increasing the driving force for anion efflux by enhancing the K^+ conductance.

Increasing concentrations of ATP ($\geqslant$ 1 mM) not only reduced the potentiation of the hypotonicity-provoked $^{125}I^-$ efflux, but eventually abolished the isotope efflux almost completely (Figure 2A). Similar results (not shown) were obtained when Mg-ATP was used, indicating that the inhibition observed was not due to a reduction in extracellular [Mg^{2+}].

Pre-treatment of the cells with BAPTA-AM (25 μM, 1 h) did

Table 1 Effect of BAPTA-AM and cytochalasin B treatment on ATP release and hypotonicity-induced anion efflux

ATP release and fractional $^{125}I^-$ efflux were quantified from control, BAPTA-AM-loaded (25 μM, 1 h) and cytochalasin B-pre-treated (50 μM, 30 min) intestine 407 cells. ATP accumulation was determined after 15 min of incubation with isotonic or hypotonic medium. Activation of the anion conductance was determined by measuring the initial increase in fractional $^{125}I^-$ efflux at 90 s after changing the medium for an isotonic or hypotonic medium. Data are expressed as means ± S.E.M. ($n = 3$).

	ATP accumulation (pmol/10^6 cells)		Increase in fractional iodide efflux (%/min)		
Experimental condition	Isotonic	50% Hypotonic	Isotonic	20% Hypotonic	50% Hypotonic
Control	4.3 ± 1.0	23.1 ± 4.1	−0.7 ± 0.4	6.7 ± 1.7	32.4 ± 0.3
BAPTA-AM	1.9 ± 0.8	7.6 ± 2.3*	−0.2 ± 0.1	6.4 ± 0.5	31.1 ± 1.4
Cytochalasin B	0.3 ± 0.2*	8.0 ± 2.0*	1.4 ± 1.5	15.3 ± 1.1*	29.5 ± 1.9

* Significant difference from the control ($P < 0.05$, Student's t test).

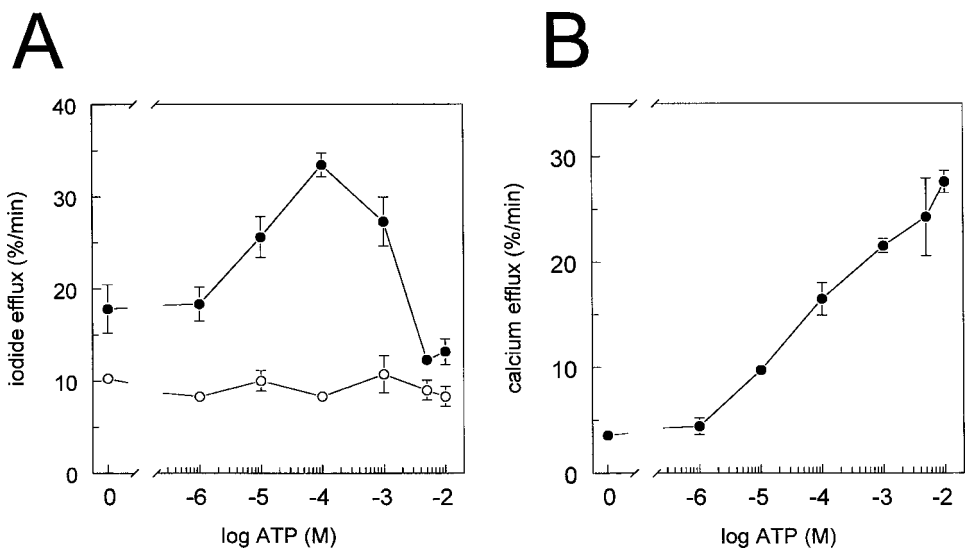

Figure 2 Effects of ATP on radioisotope release from $^{125}I^-$- or $^{45}Ca^{2+}$-loaded cells

(**A**) Dose-dependency of the effects of ATP on the fractional $^{125}I^-$ efflux from control (○) and osmotically stimulated (20% hypotonicity, ●) cultures. (**B**) Dose-dependency of the ATP-induced fractional $^{45}Ca^{2+}$ efflux from radioisotope-loaded cells under isotonic conditions. Data are expressed as means ± S.E.M. ($n = 3$).

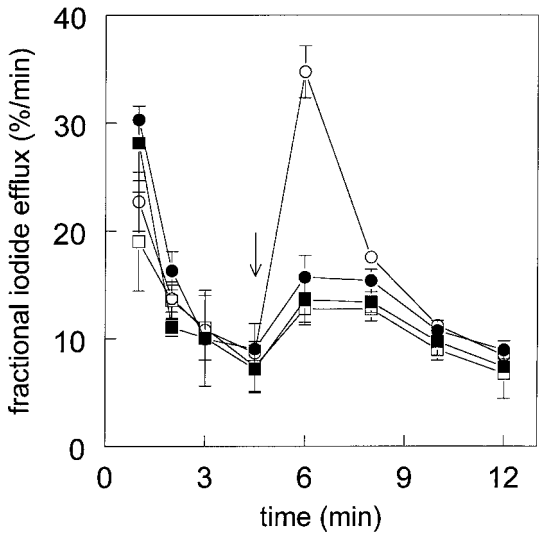

Figure 3 Calcium dependence of ATP-mediated potentiation of the osmo-sensitive anion efflux

Time course of the osmo-shock-induced (20% hypotonicity) fractional $^{125}I^-$ efflux from control (○, □) or BAPTA-AM-loaded (25 μM, 1 h; ●, ■) cultures in the presence (○, ●) or absence (□, ■) of ATP (50 μM). ATP or vehicle was added from 3 min onwards; the arrow indicates the shift to hypo-osmotic medium. Data are expressed as means ± S.E.M. ($n = 3$).

Table 2 Order of potency of nucleotides in potentiating the hypotonicity-induced $^{125}I^-$ efflux in intestine 407 cells

Increase in fractional $^{125}I^-$ efflux in response to a 20% hypotonic shock was determined in the absence or presence of equimolar concentrations (10 μM) of extracellular nucleotides. Data are expressed as percentages of the control and are given as means ± S.E.M. (with n values in parentheses). ATP[S], adenosine 5′-[γ-thio]-triphosphate; GTP[S], guanosine 5′-[γ-thio]-triphosphate.

Experimental condition	Increase in fractional $^{125}I^-$ efflux (%)
Control	100 ± 6 (8)
ATP	194 ± 8* (8)
ATP[S]	186 ± 7* (5)
UTP	185 ± 17* (3)
ADP	153 ± 8* (3)
GDP	140 ± 12 (3)
GTP[S]	138 ± 9* (3)
AMP	134 ± 14 (3)
GTP	122 ± 2* (3)
Adenosine	107 ± 3 (3)
cAMP	91 ± 15 (3)

* Significant difference from the control ($P < 0.05$, Student's t test).

not affect the anion-efflux response to hypo-osmotic stimulation but completely abolished the potentiation of the efflux by micromolar concentrations of ATP (Figure 3). These results suggest that the potentiation of the hypotonicity-induced anion efflux by extracellular ATP is due to an increase in intracellular Ca^{2+}. Indeed, addition of ATP to the medium was found to result in an increased cellular mobilization of Ca^{2+}, as quantified by $^{45}Ca^{2+}$ efflux from isotope-loaded cells (Figure 2B). The ATP-mediated Ca^{2+} release starts, like the potentiation of the hypo-tonicity-provoked anion efflux, at micromolar conditions and saturates at approx. 0.1–1 mM, but, in contrast with ATP effects on chloride-channel activation, no inhibition of the Ca^{2+} efflux was observed at millimolar concentrations (Figure 2). Taken together, these results suggest that different mechanisms underlie the potentiation and inactivation of the anion conductance.

Extracellular ATP is known to induce Ca^{2+} mobilization by activation of G-protein-coupled purinoceptors, which are divided into subtypes on the basis of differing agonist-affinity series (reviewed in [35]). To investigate the receptor involved in the ATP effect on the osmo-sensitive anion efflux, the relative potencies of adenine- and uridine-containing nucleotides were determined. As shown in Table 2, this affinity profile (UTP = ATP = ATP[S] > ADP = AMP; cAMP and adenosine ineffec-

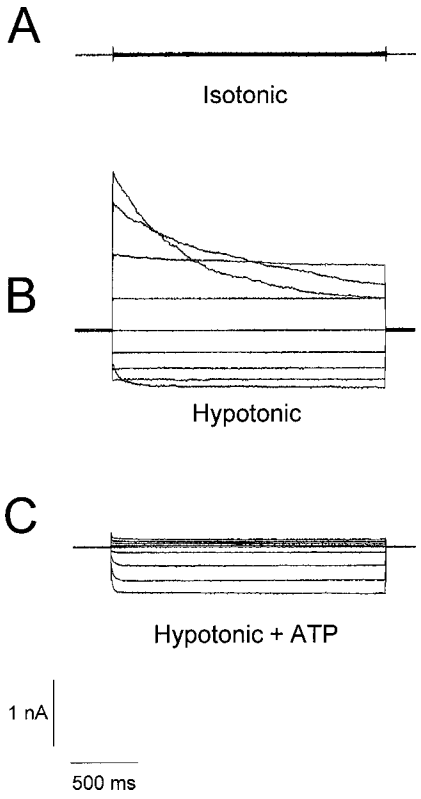

Figure 4 Volume-sensitive chloride currents in intestine 407 cells

Whole-cell Cl^- currents in control (isotonic; **A**) and hypo-osmotic stimulated (**B**) cells in response to step pulses from −100 to +100 mV (in 25-mV increments). (**C**) Inhibition of the cell-swelling-induced Cl^- current by extracellular ATP (5 mM). Traces are representative of 20 (**A**, **B**) and 6 (**C**) experiments.

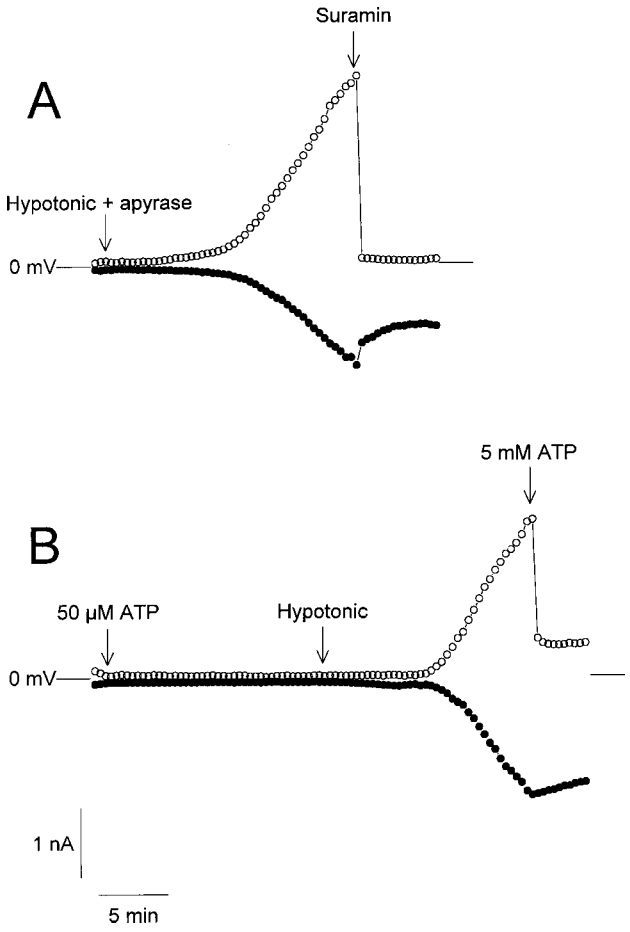

Figure 5 Time course of activation and inhibition of osmo-sensitive chloride currents

(**A**) Hypotonicity-provoked whole-cell Cl^- currents at −100 and +100 mV in the presence of apyrase (3 units/ml) and after subsequent addition of suramin (100 μM). (**B**) Magnitude of the whole-cell Cl^- currents after addition of ATP (50 μM), a shift to a hypotonic medium (30% hypotonicity) and subsequent addition of 5 mM ATP. Traces are representative of six experiments.

tive) closely resembles the agonist specificity of the $P2Y_2$ (= P2U) type of receptor as characterized by Lustig et al. [36].

Effect of purinoceptor antagonists and the ATP hydrolase apyrase on osmo-sensitive Cl^- currents

Although extracellular ATP alone was not sufficient to activate an anion current in Intestine 407 cells, hypo-osmotically released ATP may play an important role in the feed-forward regulation of compensatory Cl^- channels. To verify this possibility, both the hypotonicity-provoked whole-cell Cl^- currents as well as the $^{125}I^-$ efflux in intact cells were measured in the absence and presence of apyrase (3 units/ml).

When exposed to a hypo-osmotic medium, Intestine 407 cells respond by developing a large, Cl^--selective, outwardly rectifying current (Figures 4A and 4B). The anion current displayed a slow activation after strong hyperpolarizing pulses and a marked voltage-dependent inactivation at depolarizing holding potentials. Addition of millimolar concentrations of extracellular ATP blocks the hypotonicity-induced anion efflux in a voltage-dependent manner (Figure 4C). Similar currents have been reported by others for this cell line and for many other cell systems (for review, see [16,37]). Addition of apyrase to the bathing solution did not prevent the activation of the osmo-sensitive current (Figure 5A), nor did it affect the magnitude of the cell-swelling-induced $^{125}I^-$ efflux from intact cells (Table 3). Similar results were obtained when a hexokinase/glucose system was used to trap released ATP (results not shown). Furthermore, addition of micromolar concentrations of ATP to the bathing solution did not induce an increase in Cl^- current under isotonic conditions (Figure 5B). Taken together, these findings again indicate that, under our conditions, extracellularly released ATP triggered by osmotic cell swelling is not involved in the activation or potentiation of volume-sensitive Cl^- currents.

Paradoxically, after treatment of the cells with the purinoceptor antagonists suramin and reactive blue, a strong inhibition of the hypotonicity-induced anion efflux was observed (Table 3), suggesting a role for purinoceptors in the activation of the chloride efflux. Furthermore, addition of suramin (Figure 5A) or reactive blue (results not shown) to the bathing solution effectively decreased swelling-induced whole-cell Cl^- currents. Inhibition by both antagonists, like the inhibition by millimolar concentrations of extracellular ATP (Figure 5B), was voltage-dependent (outward currents were inhibited much more prominently than inward currents). Combined with the observation that addition of apyrase to the bathing solution had no effect on the development of hypotonicity-induced Cl^- currents (Figure 5A and Table 3), these results indicate that the inhibition of hypotonicity-

Table 3 Effect of ATP hydrolase apyrase and purinoceptor antagonists on hypotonicity-induced $^{125}I^-$ efflux

Fractional $^{125}I^-$ efflux was determined in response to 20 and 30% hypotonic shocks in the presence or absence (control) of 3 units/ml apyrase, 100 μM suramin or 100 μM reactive blue. Data are expressed as percentages of control (means ± S.E.M., $n = 3$).

	Hypotonicity	
Experimental condition	20%	30%
Control	100 ± 5	100 ± 5
Apyrase	93 ± 8	108 ± 5
Suramin	38 ± 4*	55 ± 6*
Reactive blue	34 ± 4*	75 ± 2*

*Significant difference from the control ($P < 0.05$, Student's t test).

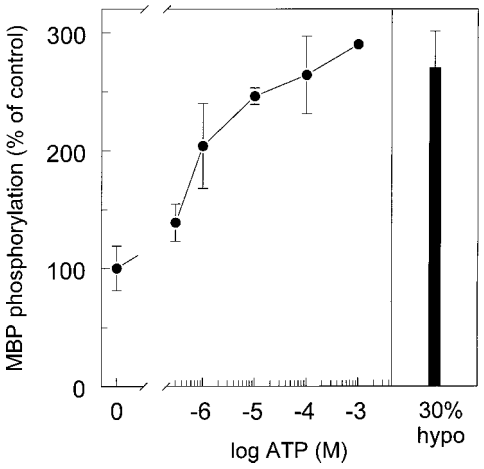

Figure 6 Dose-dependency of ATP-induced Erk-1/2 activation

Cells were exposed for 5 min to isotonic medium containing different concentrations of ATP or to a 30% hypotonic medium in the presence of 200 μM Na_3VO_4. After lysis of the cells, Erk-1/2 was immunoprecipitated and enzyme activity was determined using MBP as a substrate (as described in the Materials and methods section). Data are expressed as percentages of the control (means ± S.E.M., $n = 3$).

induced Cl^- channels by suramin and reactive blue is unrelated to their action as purinoceptor blockers.

Autocrinic release of ATP mediates swelling-induced Erk-1/2 activation

Recently, we demonstrated that hypo-osmotic stimulation of intestine 407 cells transiently activates Erk-1/2 through the Ras/Raf signalling pathway [14]. Because ATP has been reported to activate MAP kinases in some other cell types [21–27], we investigated the possible autocrinic/paracrinic involvement of osmotically released ATP. It was found that extracellular ATP under isotonic conditions acts as a potent activator of Erk-1/2 in intestine 407 cells (Figure 6). For comparison, the effect of a 30% hypotonic shock on Erk-1/2 activation is also shown in Figure 6. Stimulation of Erk-1/2 started at concentrations of 0.1–0.5 μM, indicating that the threshold for MAP kinase activation by ATP is two orders of magnitude lower than its threshold for the activation of Ca^{2+} efflux and for the potentiation of osmo-sensitive anion efflux (see Figure 2). As shown in Figure

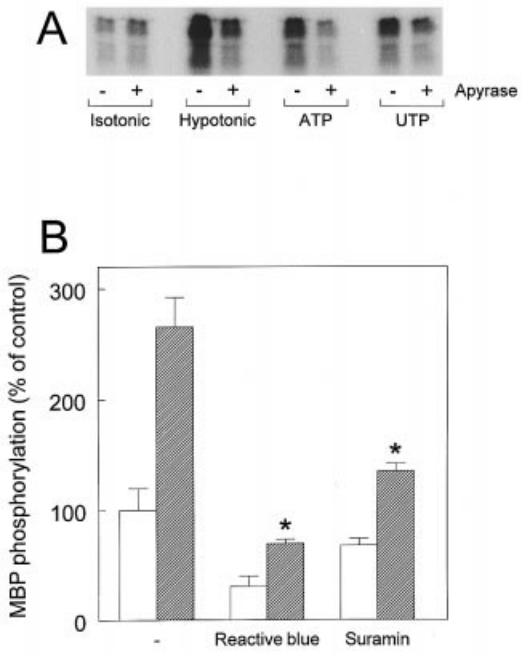

Figure 7 Extracellularly released ATP activates Erk-1/2

Erk-1/2 was immunoprecipitated and enzyme activity was determined using MBP as a substrate. (**A**) Following a 5-min incubation with vehicle (−) or with 3 units/ml apyrase (+), cells were exposed for another 5 min to isotonic medium, 30% hypotonic medium, 100 μM ATP or 100 μM UTP in the presence of Na_3VO_4 (200 μM). Shown is an autoradiograph of ^{32}P-labelled MBP separated by SDS/PAGE; results are representative of three independent experiments. (**B**) Following a 15-min incubation with vehicle (−), reactive blue (100 μM) or suramin (100 μM), cells were exposed for 5 min to isotonic (open bars) or 30% hypotonic (hatched bars) medium. Data are expressed as percentages of the untreated control (means ± S.E.M., $n = 3$). An asterisk indicates a significant difference from the control ($P < 0.05$, Student's t test).

7(A), treatment of the cells with apyrase (3 units/ml) not only abolished the ATP/UTP-induced Erk-1/2 activation, but also almost completely inhibited its activation by hypo-osmotic stress. Moreover, pre-treatment of the cells with suramin (100 μM, 15 min) or reactive blue (100 μM, 15 min) significantly inhibited the hypotonicity-induced Erk-1/2 activation (Figure 7B). These results indicate that extracellular ATP, acting as an autocrinic factor, is largely responsible for the hypotonicity-induced Erk-1/2 activation and, because UTP was equipotent, mediates its effects through $P2Y_2$-receptor signalling.

DISCUSSION

Hypo-osmotic cell swelling induces a rapid increase in the K^+- and Cl^--selective conductances and the concomitant activation of multiple signalling pathways. In intestine 407 cells, some components of these pathways, like $p21^{rho}$ and PtdIns 3-kinase, were found to be indispensable for a proper activation of the volume-sensitive Cl^- channels, whereas others, like the members of the MAP kinase superfamily, are apparently not involved [2,7,13,14]. Recent studies in HTC hepatoma cells suggest that both the hypotonicity-induced Cl^--current activation and the subsequent cell-volume recovery completely depend on a cell-swelling-induced conductive ATP efflux [18,30]. Similar to HTC hepatoma cells, intestine 407 cells respond to a hypo-osmotic shock with a release of ATP into the medium. The ATP release was stimulus-dependent and relatively sustained, lasting for at

least 10–15 min. Notably, the regulatory volume decrease response under these conditions is completed in approx. 2–3 min [2]. Both the ATP-binding-cassette proteins CFTR (cystic fibrosis transmembrane-conductance regulator) and P-glycoprotein have been proposed to function as ATP-release channels or to regulate an as-yet-unidentified associated ionic channel [17,30,38–40]. Other investigators however, have failed to establish a link between CFTR/P-glycoprotein and ATP release [19,41–43]. In intestine 407 cells, CFTR Cl^- channels are not expressed [2] and, in the subclone we use, P-glycoprotein expression is not detectable by RNAse protection assay (results not shown), arguing against a role for these ATP-binding-cassette transporters in ATP release. This notion is supported by the findings of Tominaga et al. [44], using a differ(inent, P-glycoprotein-expressing, subclone of intestine 407 cells. They found that inhibition of P-glycoprotein expression or function by treatment with anti-sense oligonucleotides or verapamil did not affect the osmo-sensitive Cl^- current [44]. Hypotonicity-induced ATP release was inhibited by BAPTA-AM and cytochalasin B whereas cell-swelling-induced anion efflux was strongly potentiated by cytochalasin B treatment and unaffected by Ca^{2+} chelation (see Table 1). These findings, together with the difference in kinetics between hypotonicity-induced ATP release and anion efflux, argue against conductive ATP efflux through volume-regulated Cl^- channels. In contrast, although rather speculative, the dependency of the ATP release on intracellular Ca^{2+} and an intact cytoskeleton suggests the involvement of an exocytotic process.

Based upon the inhibitory action of apyrase and of the purinoceptor antagonists suramin and reactive blue, Wang et al. [18] concluded that, in HTC rat hepatoma cells, extracellularly released ATP, through a P2-type receptor other that $P2Y_2$, was obligatory for the activation of volume-sensitive anion channels. The results of our study, however, demonstrate clearly that extracellularly released ATP is not involved in the activation of osmo-regulated Cl^- channels in intestine 407 cells, as evidenced by the inability of applied ATP to evoke an anion conductance under isotonic conditions and by the insensitivity of the hypotonicity-provoked anion efflux to apyrase. Instead of a direct activation of Cl^- conductances, micromolar concentrations of ATP were found to greatly potentiate the ionic response to a submaximal hypotonic stimulus, most likely through $P2Y_2$-receptor activation (as evidenced by the order of potency of different nucleotides) and subsequent Ca^{2+} mobilization (as evidenced by $^{45}Ca^{2+}$-efflux studies and potentiation studies in BAPTA-AM-loaded cultures).

Potentiation of the ionic responses to osmotic cell swelling is not unprecedented. Indeed, in intestine 407 cells, Ca^{2+}-mobilizing hormones as well as the phosphotyrosine phosphatase inhibitor (peroxo)vanadate and cytochalasin B were found to increase the anion efflux [2,7,34]. These data confirm the previous notion that intestine 407 cells do not express Ca^{2+}-activated anion channels [2,34], implying that ATP/Ca^{2+} potentiation of anion efflux is a regulatory property of the volume-sensitive Cl^- channels.

Importantly, the inhibition of volume-sensitive Cl^- channels by millimolar concentrations of ATP appeared to be independent of $P2Y_2$-receptor signalling because at those concentrations the ATP activation of $^{45}Ca^{2+}$ efflux was unaffected. Not only high concentrations of extracellular ATP (5 mM), but also the purinoceptor antagonists suramin and reactive blue, were found to inhibit both the volume-sensitive anion efflux and the hypotonicity-provoked whole-cell Cl^- current significantly. These results are in line with the recent findings of Galietta et al. [45], who reported an inhibition of swelling-induced taurine efflux and Cl^- currents by purinoceptor antagonists in $9HTE_0^-$ cells. The inhibition by ATP, suramin and reactive blue (i.e. by both purinoceptor agonist and antagonists) was voltage-dependent and therefore suggests a direct interaction with the extracellular domains of the channel protein. This property renders these antagonists less suitable for investigating the involvement of purinoceptors in Cl^--channel activation, but makes it tempting to speculate that volume-regulated anion channels share homologous nucleotide-binding domains with the $P2Y_2$ receptors. Notably, the ATP concentrations reached in the close proximity of the cells *in vivo* are difficult to assess. As recently shown by Beigi et al. [46], who used cell-surface-attached luciferase to measure ATP release from platelets, the ATP concentration measured by soluble luciferase in bulk extracellular fluids, as done in our studies, may greatly underestimate the concentration of ATP at the cell surface. Considering the micromolar rather than millimolar concentrations of released ATP found at the cell surface by Beigi et al. [46], the physiological role for cell-swelling-induced ATP release is most plausibly potentiation of channel activity (requiring micromolar levels of extracellular ATP) rather than channel inhibition.

A general feature of cell signalling in response to hypotonic stress is the activation of the extracellular signal-regulated protein kinases Erk-1 and Erk-2 [2,8–14]. In this study, we demonstrate that the activation of these MAP kinases is not a direct consequence of osmotic cell swelling, but instead involves an autocrinic/paracrinic loop with ATP as the first messenger. The ability of exogenously added ATP to potentiate the osmo-sensitive anion efflux is somewhat contradictory to the observations that released ATP is responsible for Erk-1/2 activation but is apparently not involved in the regulation of $^{125}I^-$ efflux or Cl^- current (e.g. no reduction in magnitude in the presence of apyrase; Table 3 and Figure 5A). We suggest that there are at least two plausible explanations for this apparent discrepancy. First, comparison of Figures 2(A) and 6 shows that the threshold for Erk-1/2 activation by ATP is about two orders of magnitude lower compared with ATP potentiation of the anion efflux or activation of Ca^{2+} channels. Second, in contrast with the conditions of the Erk-1/2 experiments, in the efflux assay, the extracellular medium is replaced at 1 min intervals to assess the release of radioisotope, thereby reducing unstirred layer dimensions and preventing ATP from accumulating.

The finding that UTP is as effective as ATP suggests a second role for the $P2Y_2$ receptor, a G-protein-coupled receptor, which was also found to be responsible for the potentiation of the swelling-induced anion efflux. In several cell systems, receptors coupled to the G-protein subfamilies $G_{q/11}$, $G_{i/0}$, G_s or $G_{12/13}$ were found to activate the Ras/Raf/Erk cascade (reviewed in [47,48]). However, since the hypotonicity-provoked activatiof Erk-1/2 was insensitive to pertussis toxin in intestine 407 cells [14], involvement of $G_{i/0}$ is not likely. Notably, Erk-1/2 activation was found to be more sensitive to extracellular ATP than the Ca^{2+}-mediated potentiation of hypotonicity-induced anion efflux (submicromolar versus micromolar levels). The reason for this difference is unknown, but may relate to the involvement of multiple $P2Y_2$-receptor subtypes, or to a different sensitivity of the signalling pathways to submaximal receptor activation.

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