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Extracellular ATP Causes ROCK I-dependent Bleb Formation in $P2X_7$-transfected HEK293 Cells[V]

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The $P2X_7$ ATP receptor mediates the cytotoxic effect of extracellular ATP. $P2X_7$-dependent cell death is heralded by dramatic plasma membrane bleb formation. Membrane blebbing is a complex phenomenon involving as yet poorly characterized intracellular pathways. We have investigated the effect of extracellular ATP on HEK293 cells transfected with the cytotoxic/pore-forming $P2X_7$ receptor. Addition of ATP to $P2X_7$-transfected, but not to wt $P2X_7$-less, HEK293 cells caused massive membrane blebbing within 1–2 min. UTP, a nucleotide incapable of activating $P2X_7$, had no early effects on cell shape and bleb formation. Bleb formation triggered by ATP was reversible and required extracellular Ca^{2+} and an intact cytoskeleton. Furthermore, it was completely prevented by preincubation with the P2X blocker oxidized ATP. It was recently observed that the ROCK protein is a key determinant of bleb formation. Preincubation of HEK293-$P2X_7$ cells with the ROCK blocker Y-27632 completely prevented $P2X_7$-dependent blebbing. Although ATP triggered cleavage of the ROCK I isoform in $P2X_7$-transfected HEK293 cells, the wide range caspase inhibitor z-VAD-fluoromethylketone had no effect. These observations suggest that $P2X_7$-dependent plasma membrane blebbing depends on the activation of the serine/threonine kinase ROCK I.

INTRODUCTION

A distinctive feature of apoptosis is the occurrence of coordinated modifications that involve virtually every cell compartment: the plasma membrane, the cytoplasm, the endoplasmic reticulum, the mitochondria, and the nucleus (Ferri and Kroemer, 2001; Yu *et al.,* 2001). One of the earliest changes observed in apoptosis is the formation of plasma membrane blebs, a dramatic phenomenon still poorly understood in its mechanism (Torgerson and McNiven, 1998). Apoptotic blebs consist of membrane expansions that encircle cytoplasm devoid of organelles and filamentous actin. Their formation has been shown to be dependent on myosin light chain (MLC) phosphorylation, a process modulated by the Ca^{2+}/calmodulin-dependent MLC kinase and the serine/threonine kinase ROCK (Amano *et al.,* 1996; Kimura *et al.,* 1996; Fukata *et al*., 2001). It has previously been shown that during apoptosis caspase-3 cleaves the Rho-kinase I (ROCK I), thus leading to a deregulated activity of this kinase and consequently to bleb formation (Coleman *et al.,* 2001).

Accruing evidence suggests that substantial amounts of ATP can accumulate in the pericellular space under several physiological or pathological conditions (Ferrari *et al.,* 1997; Beigi *et al.,* 1999; Schwiebert *et al.,* 2002; Warny *et al.,* 2001). This nucleotide, by acting at plasma membrane P2 receptors, triggers different cell responses, such as secretion, chemotaxis, proliferation, transcription factor activation, or even cytotoxicity (Di Virgilio *et al.,* 2001). In addition, ATP can also be a powerful apoptotic agent via activation of the purinergic $P2X_7$ receptor, a plasma membrane nucleotide-gated ion channel endowed with the peculiar ability to generate a nonselective pore upon sustained stimulation (Zanovello *et al.,* 1990; Zheng *et al.,* 1991; Surprenant *et al.,* 1996). The intracellular pathways responsible for $P2X_7$-dependent apoptosis are only partially known, although they

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Abbreviations used: GFP, green fluorescent protein; MLC, myosin light chain; oATP, oxidized ATP; TNFRI, type I TNF receptor.

seem to largely coincide with those activated by other better-known apoptotic receptors such as Fas and the type I TNF receptor (TNFRI). $P2X_7$ induces a massive depletion of intracellular K^+, caspase-3 activation, degradation of nuclear lamin, DNA fragmentation, nuclear condensation, and apoptotic body formation (Steinberg and Silverstein, 1987; Ferrari *et al.*, 1999; Morelli *et al.*, 2001). In addition, a typical feature of ATP-stimulated cells is the occurrence of a dramatic membrane blebbing (MacKenzie *et al.*, 2001). The biochemical basis of membrane blebbing has not been studied in detail, nor has it been unequivocally shown to be solely dependent on the activation of the $P2X_7$ receptor/channel. In the present work, we have investigated the kinetics and biochemical mechanisms of ATP-induced bleb formation in a HEK293 cell clone stably expressing a GFP (green fluorescent protein)-tagged version of the $P2X_7$ receptor ($P2X_7$-HEK293). This cell line lacks endogenous P2X receptors and is therefore a good model system to study responses due to stimulation of the transduced $P2X_7$ receptor.

Our data demonstrate that $P2X_7$ expression is sufficient to cause membrane blebbing in response to ATP; furthermore, we show that bleb formation 1) is rapid, reversible and Ca^{2+} dependent, 2) is independent of caspase-3, 3) requires an intact cytoskeleton, and 4) is prevented by an inhibitor of ROCK.

Taken together, these data suggest that ROCK activation is a key step in the early membrane modifications triggered by the $P2X_7$ receptor.

MATERIALS AND METHODS

Cells, Solutions, and Reagents

HEK293 cells were cultured in DME/F-12 1:1 medium (Sigma, St. Louis, MO) containing 15% heat-inactivated FCS (Life Technologies, Paisley, Scotland), 100 U/ml penicillin, and 100 μg/ml streptomycin. Stable clones were cultured in the same medium containing G418 sulfate (Geneticin; Calbiochem, La Jolla, CA) at a concentration of 0.2 mg/ml. Visualization of transfected cells was performed in a saline solution (standard saline solution) containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$,1 mM Na_2HPO_4, 5.5 mM glucose, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, and 20 mM HEPES (pH 7.4).

ATP and UTP were used at a concentration of 3 and 1 mM, respectively, and were both from Roche Diagnostics (Mannheim, Germany). Oxidized ATP (oATP) was synthesized in our laboratory and used at the concentration of 300 μM. Cells were preincubated for 2 h with oATP, rinsed several times with saline solution, and exposed to the different stimuli. Hexokinase (Sigma) was used at the concentration of 100 μg/ml. Cells were preincubated for 45 min and kept in the continuous presence of hexokinase throughout the experiment. z-VAD fluoromethylketone (z-VAD-fmk; 100 μM; Bachem, Bubendorf, Switzerland) and cytochalasin B (10 μM; Sigma) were added to the cell monolayer 10 min before the addition of ATP. The ROCK inhibitor Y-27632 was purchased from Tocris Cookson Ltd. (Bristol, UK). The anti-Fas mAb was a kind gift of Professor Klaus Schulze-Osthoff (University of Munster, Germany) and was used at a concentration of 1 μg/ml.

Transfection of HEK293 Cells and Selection of Stable Clones

HEK293 wt cells were transfected with calcium phosphate. Briefly, the first day, 2.5×10^6 HEK293 cells were plated in Petri dishes. The second day, for each dish, 30 μg of plasmid DNA was resuspended in a total volume of 450 μl TE (10 mM Tris, 1 mM EDTA, pH 8) and then 50 μl of a 2.5 M $CaCl_2$ solution was added. This solution was added dropwise under vortexing to a tube containing 500 μl of 2× HBS (280 mM NaCl, 50 mM HEPES, 1 mM Na_2HPO_4, pH 7.12, at 25°C). After a 30-min incubation at room temperature, the DNA precipitate was added to the dish dropwise. The third day medium was changed. The fourth day, G418, 0.8 mg/ml, was added to fresh medium to select transfected clones. After clone selection, the selective medium contained G418, 0.2 mg/ml.

Transient transfection was performed using the same procedure directly on coverslips for 0.5×10^6 cells, 4–8 μg of plasmid DNA in a total volume of 90 μl TE, 10 μl of $CaCl_2$ solution, and 100 μl of 2× HBS. The plasmid containing rat $P2X_7$-GFP in pcDNA3 used for stable transfection was kindly provided by Dr. Annemarie Surprenant (University of Sheffield, UK).

Collection and Analysis of the GFP Images

Transfected cells seeded on coverslips were observed using a Nikon Eclipse TE-300 fluorescence microscope (Nikon Co., Tokyo, Japan) equipped with a thermostated chamber and the following filter set: excitation HQ480/40, dichroic Q480LP, and emission HQ510LP. The microscope was also equipped with the following devices to form a system for high-speed acquisition and processing of fluorescent images: a computer-controlled light shutter, a six-position filter wheel, a piezoelectric *z*-axis focus device, a back-illuminated 1000× 800 CCD camera (Princeton Instruments, Princeton, AZ), a computer equipped with Metamorph software (Universal Imaging Corporation, Downingtown, PA) for image acquisition, 2-D and 3-D visualization and analysis. All experiments were performed at 37°C in the standard saline solution described in Cells, Solutions, and Reagents. Microscopic observations were carried out with recently thawed $P2X_7$-HEK293, never exceeding the 10th in vitro passage. Recordings were performed with at least 10 different recently thawed cell batches, and the effect of inhibitors was consistently reproduced in three or more separate experiments. Criteria for induction of blebbing were 1) that at least one bleb per cell was produced within 1–2 min of ATP addition, 2) that within 3–5 min all cells in the field were actively blebbing, and 3) that, unless ATP was removed, this process continued as long as cells did not detach from the substrate. An inhibitor was considered effective if it was able to fully block blebbing (no blebs at any time points).

Rho Pull-down Assay

The pull-down assay was performed as described in Coleman *et al.*, (2001). Briefly, cells (5×10^{10}) were plated in 10-cm Petri dishes, grown for 48 h, and serum-starved for further 24 h. Lysis was performed in 50 mM Tris, pH 7.2, 500 mM NaCl (TBS), 1% (vol/vol) Triton X-100, 5 mM $MgCl_2$, 1 mM DTT, and protease inhibitors. An aliquot of cell lysates was used for determination of protein concentration, whereas the remainder was spun at $10{,}000 \times g$ for 5 min. The supernatant was then mixed with 20 μg of bacterially expressed GST-Rhotekin (murine amino acids 7–89) prebound to 75–100 μl glutathione-Sepharose beads and incubated at 4°C with tumbling for 30 min. Samples were spun, the supernatant was removed, and beads were washed three times in TBS containing 1% (vol/vol) Triton X-100, 5 mM $MgCl_2$, and 1 mM DTT before mixing with Laemmli buffer and analysis by Western blotting (see below).

Cell-permeable Tat-C3 Botulinum Toxin

The cell-permeable C3 toxin was prepared as described in Coleman *et al.* (2001). Briefly, the C3 recombinant protein, modified to include the nucleotide sequence 5′-GGA GGA TAC GGC CGA AAG AAG CGA CAG CGA CGC CGT GGA GGA of the thrombin cleavage site, was produced in *E. coli* BL21 and induced with 0.3 mM isopropyl-β-thiogalactopyranoside (IPTG) for 3 h at 32°C. Cells were lysed in TBS containing 5 mM $MgCl_2$, 1 mM DTT, and protease inhibitors by quick freezing followed by sonication. After centrifugation at $10{,}000 \times g$ for 10 min at 4°C, the supernatant was incubated with

glutathione-Sepharose for 2 h at 4°C. The beads were then washed with TBS plus 5 mM $MgCl_2$ and 1 mM DTT. To cleave Tat-C3, beads were incubated in TBS plus 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT, and 30 U of thrombin overnight at 4°C. The supernatant was then removed and incubated with *p*-aminobenzamidine beads for 1 h. Supernatants containing Tat-C3 were frozen and used at 0.5–1.0 μM in culture medium.

Immunoblotting

Cells were lysated in lysis buffer containing TBS Triton 0.1%, 1 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.2 μg DNase, and 0.2 μg Rnase (only for ROCK I analysis, lysis buffer also contained 1 mM diisopropylfluorophosphate [Fluka Chemie, Buchs, Switzerland], and cells were preincubated in the presence of this inhibitor for 1 h before lysis). Proteins were separated on 6% (ROCK I) or 14% (RhoA) SDS-polyacrylamide gel according to Laemmli, blotted on nitrocellulose paper (Amersham Life Sciences, Cologno Monzese, Italy), and hybridized with anti-ROCK I (clone 46; BD Transduction Laboratories, Los Angeles, CA) or anti-RhoA (Santa Cruz Biotechnology Inc., Santa Cruz, CA) mAbs.

RESULTS

Membrane bleb formation is a widely conserved event occurring during the early phases of apoptosis. It has long been known that extracellular ATP, mainly acting at the $P2X_7$ receptor (but see Chvatchko *et al.*, 1996), is a powerful proapoptotic agent in several cell types and induces most of the changes that are caused by typical proapoptotic stimuli such as FasL or TNF-α (Zanovello *et al.*, 1990; Zheng *et al.*, 1991; Ferrari *et al.*, 1999). Figure 1 shows the effect of ATP on $P2X_7$-HEK293 cell morphology. The plasma membrane was uniformly stained by the fluorescent $P2X_7$-GFP chimeric receptor. It is interesting to notice that a substantial amount of receptor also accumulated within cytoplasmic organelles, but not within the nucleus. Furthermore, $P2X_7$-HEK293 cells appeared rounded and slightly swollen when compared with wt $P2X_7$-less HEK293 (see Figure 5 below), even in the absence of added stimuli. Within 2 min of the addition of the nucleotide, $P2X_7$-HEK293 cells underwent a massive process of bleb formation that persisted at a sustained level for >20 min.

Blebs originated as projections of the plasma membrane that surrounded an optically empty cytoplasm apparently free of intracellular organelles. Cells appear to undergo a "boiling process" that caused a continuous formation and resorption of blebs of various sizes. The usual pattern included formation of small size blebs during the early phases, followed by protrusion of increasingly larger blebs often generated by fusion of smaller blebs. The observation was continued for >20 min, and at the end of this time cells started to detach from the substrate and disappear from the microscopic field. Removal of ATP stopped bleb formation and allowed full recovery of cell shape and volume (Figure 2, lower panels), thus showing that the process was reversible. We compared blebbing in HEK293 cells transfected with either the chimeric $P2X_7$-GFP or wt$P2X_7$ and observed a longer lag in the beginning of blebbing in the former cells, which, after this initial delay, proceeded on a similar time scale in both transfectants. Smart *et al.* (2002) have recently published a thorough analysis of the effect of GFP fusion at the N or C termini of $P2X_7$. They reported an approximately threefold decrease in sensitivity to ATP with GFP fused at

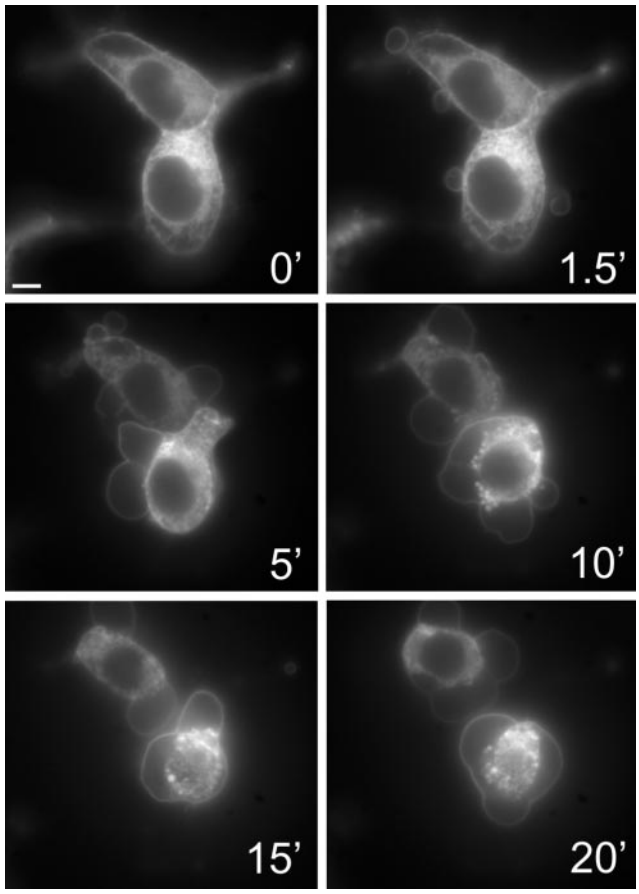

Figure 1. Extracellular ATP causes fast bleb formation. $P2X_7$-HEK293 cells were incubated in standard saline solution and treated with 3 mM ATP at time zero. Bar, 10 μm.

the $P2X_7$ C termini, although stimulation with a full dose of ATP (as that used in the present study) caused maximal channel activation and pore formation. Among the few known blockers of the $P2X_7$ receptor, one of the most useful is oATP, a di-aldehyde derivative that covalently binds and irreversibly blocks this receptor (Murgia *et al.*, 1993). Oxidized ATP could in principle also block other P2X receptors, but this is not a drawback in $P2X_7$-HEK293 because these cells do not express other P2X subtypes. Figure 3 shows that preincubation with this blocker completely prevented ATP-induced bleb formation throughout the observation time (45 min). After 20–25 min, some cell rounding became detectable but this did not lead to detachment or to major morphological alterations for up to 45 min.

All cells express powerful ecto-ATPases/nucleotidases that rapidly degrade added ATP to ADP and AMP, which in turn is hydrolized to adenosine by 5′-nucleotidase. Thus, it is possible that some of the effects of extracellular ATP might be mediated by ATP degradation products such as ADP or adenosine. To rule out participation of ATP degradation products, we added ATP in the presence of hexokinase, an enzyme that generates glucose-6 phosphate and ADP and accelerates the final degradation to AMP and adenosine (Figure 4). Hexokinase prevented blebbing throughout the

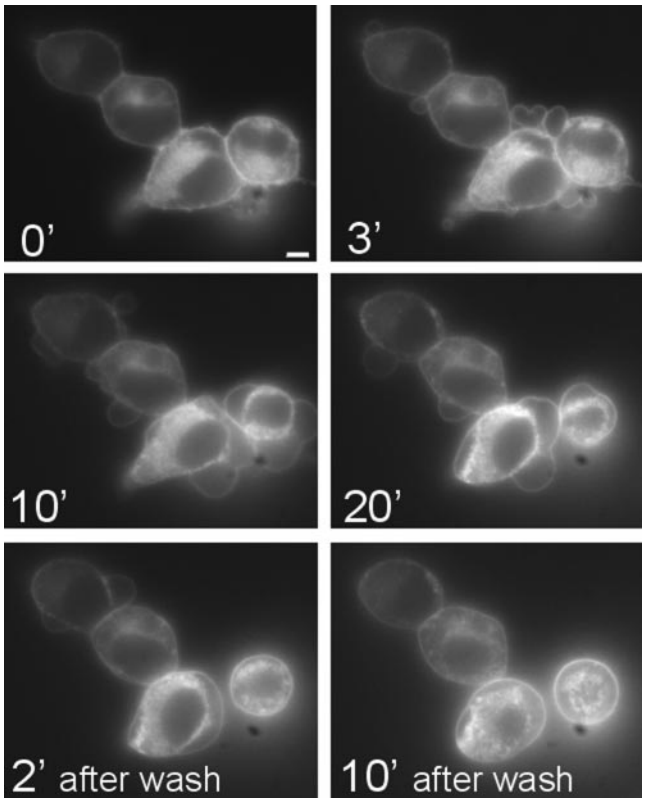

Figure 2. ATP-induced bleb formation is reversible. $P2X_7$-HEK293 cells were incubated in standard saline solution and treated with 3 mM ATP at time zero. After 20 min, the ATP-containing solution was replaced with fresh ATP-free solution and the observation was carried for an additional 10 min. Images shown in the bottom left and right panels were taken 2 and 10 min after ATP removal, respectively. Bar, 10 μm

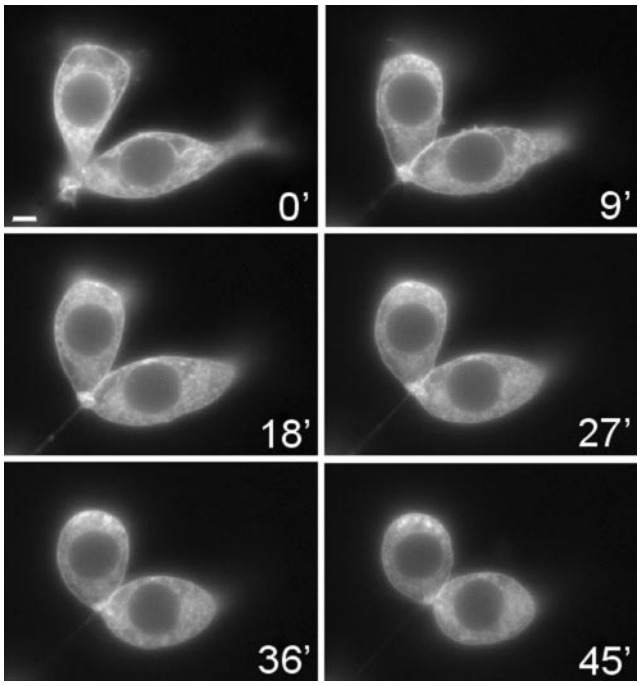

Figure 3. Bleb formation is blocked by oxidized ATP. $P2X_7$-HEK293 cells were pretreated for 120 min at 37°C in standard saline solution with 300 μM oATP, rinsed, placed in fresh solution, and stimulated with 3 mM ATP. The image in the top left panel (time zero) was taken soon after ATP addition. Bar, 10 μm.

incubation time, but for a brief outburst of bleb formation soon after addition. As in the case of the experiments performed in the presence of oATP, cells underwent some rounding. The HEK293 cell clone used in our laboratory endogenously express two P2Y subtypes: $P2Y_1$ and $P2Y_2$ (our unpublished results, see also Schachter *et al.*, 1997). ADP is a much better agonist than ATP for $P2Y_1$ (Ralevic and Burnstock, 1998; von Kugelgen and Wetter, 2000); thus, we can exclude a main role of this receptor in blebbing because in the presence of hexokinase large amounts of ADP are formed, and yet the cells did not bleb. UTP and ATP are equipotent as agonists of $P2Y_2$. To test the possible involvement of $P2Y_2$ receptors in blebbing, we incubated cells in the presence of 3 mM UTP. This treatment caused some blebbing but only after >30 min of incubation (our unpublished results). UTP-induced blebbing was fully abolished by hexokinase. We did not further investigate UTP-induced bleb formation; however, these latter observations show that it is a late event and suggest that it might be secondary to P2Y-triggered release of ATP.

As a further control for the specificity of the effect of extracellular ATP, we used the parental wt HEK293 cells. To closely compare plasma membrane dynamics of $P2X_7$less cells to that of $P2X_7$-GFP transfectants, wt HEK293 cells were transfected with a SNAP-25GFP–containing plasmid. SNAP-25 is a protein that localizes to the cytoplasmic side of

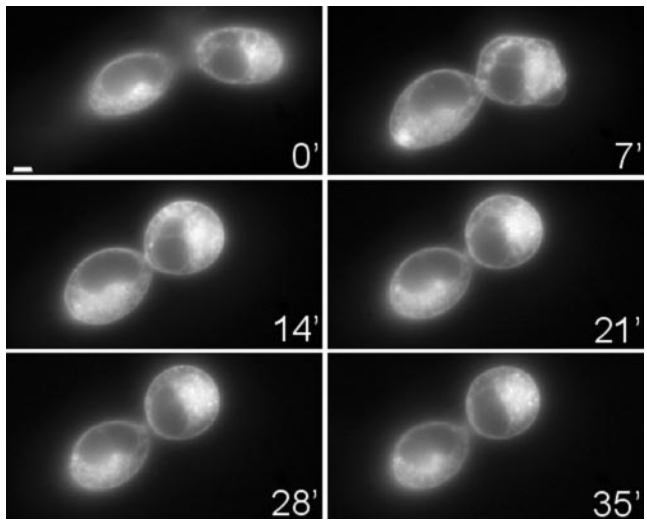

Figure 4. Bleb formation is inhibited by hexokinase. $P2X_7$-HEK293 cells were incubated at 37°C in standard saline solution. At time zero, 3 mM ATP and hexokinase (100 μg/ml) were added at the same time, and the observation was carried out for an additional 35 min. Bar, 10 μm.

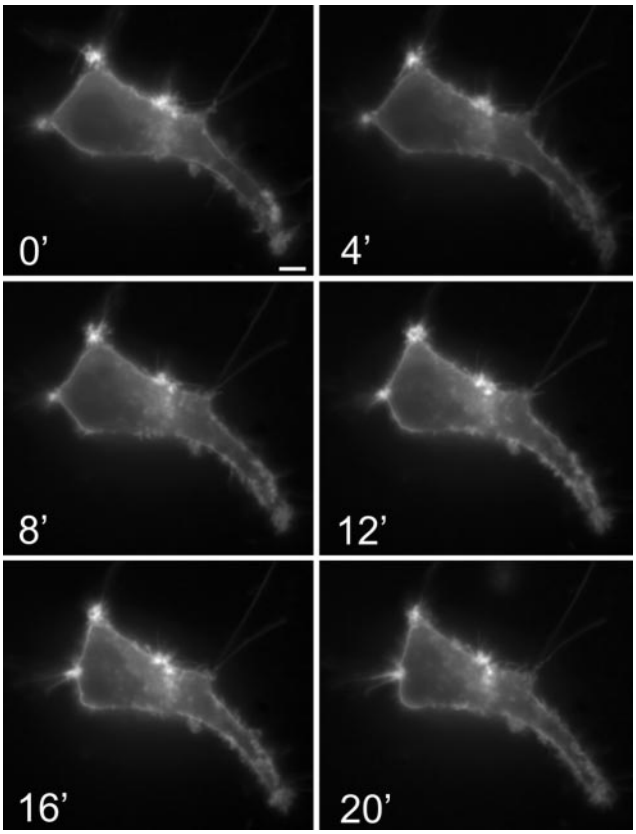

Figure 5. wt HEK293 cells transfected with SNAP25-GFP do not undergo blebbing. Cells were incubated in standard saline and challenged with 3 mM ATP at time zero. Bar, 10 μm.

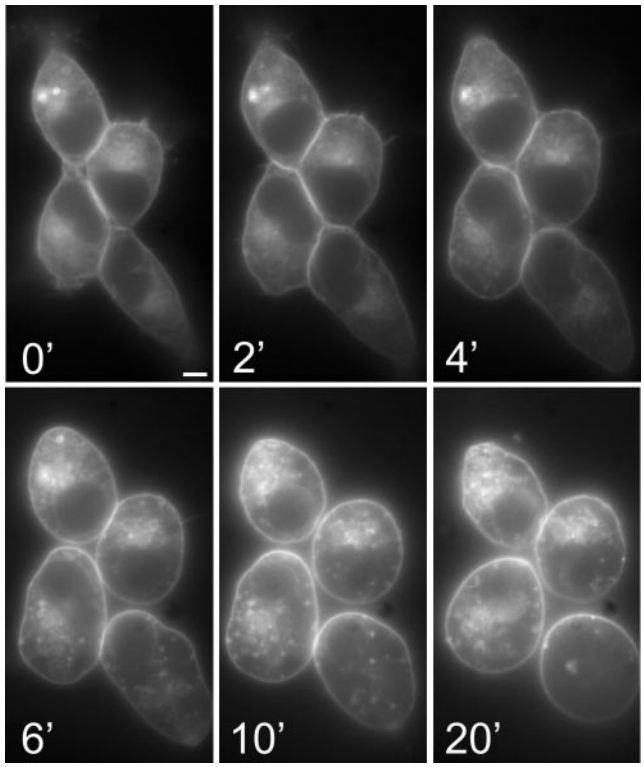

Figure 6. ATP-induced bleb formation is prevented by removal of extracellular Ca^{2+}. $P2X_7$-HEK293 cells were incubated in Ca^{2+}-free, 500 μM EGTA-containing saline solution and treated with 3 mM ATP at time zero. Bar, 10 μm.

the plasma membrane, thus allowing easy detection of cell shape changes and possible occurrence of bleb formation. Figure 5 shows that an incubation of cells lacking $P2X_7$ with ATP for up to 20 min had no effect on cell morphology, nor did it induce bleb formation.

Formation of membrane blebs is dependent on contraction of the cytoskeleton; therefore it is expected to be inhibited by chelation of Ca^{2+} or administration of the cytoskeletal poison cytochalasin B. Figure 6 shows that in the absence of added Ca^{2+} and in the presence of EGTA (nominal extracellular free Ca^{2+} concentration $<10^{-7}$ M) blebbing did not occur, although $P2X_7$ is known to be fully active under these conditions. Instead, cells swelled and rounded, a process likely due to the massive influx of Na^+, which is known to occur through $P2X_7$ in the absence of external Ca^{2+}. Thus, ATP-induced bleb formation requires a large and sustained increase in cytoplasmic Ca^{2+} due to influx across the plasma membrane. Bleb formation was also prevented by cell poisoning with cytochalasin, and as in the case of the experiments at low extracellular Ca^{2+}, cells underwent a large increase in volume (our unpublished results).

Bleb formation is a complex event dependent on the stimulation of specific intracellular pathways activated during apoptosis. It has been recently reported that TNFRI and Fas-induced blebbing is mediated by caspase-3 and ROCK I (Coleman *et al.*, 2001; Sebbagh *et al.*, 2001). Thus, we investigated whether these pathways were also stimulated during $P2X_7$-dependent bleb formation. First, we treated $P2X_7$-HEK293 cells with the wide range caspase blocker z-VAD-fmk before their exposure to ATP. This treatment did not prevent or delay blebbing (blebs were easily detected 1 min after the addition of ATP; our unpublished results). We then tested the ROCK inhibitor Y-27632, which completely blocked bleb formation (Figure 7). An incidental observation was that in the presence of Y-27632, $P2X_7$-HEK293 cells lost their rounded morphology and reverted to the usual spindle-like phenotype typical of wt HEK293 cells. An obvious implication of the inhibitory effect of Y-27632 is that extracellular ATP triggers ROCK activation via the $P2X_7$ receptor, and ROCK in turn mediates the cytoskeletal rearrangement responsible for membrane blebbing. To test this hypothesis, we investigated cleavage of the ROCK I isoform in $P2X_7$-HEK293 cells. As a control, we tested the effect of an anti-Fas mAb, a treatment shown previously to trigger ROCK I cleavage (Figure 8).

The effect of Fas stimulation was tested in Jurkat as well as in $P2X_7$-HEK293 cells. Fas activation lead to the formation of the cleaved 130-kDa active form of ROCK I, more extensively in Jurkat compared with $P2X_7$-HEK293 cells. ATP treatment of $P2X_7$-HEK293 cells caused accumulation of a 130-kDa band, and rather interestingly but in agreement with the experiments on ATP-induced bleb formation, this process was not inhibited by z-VAD-fmk. We assessed

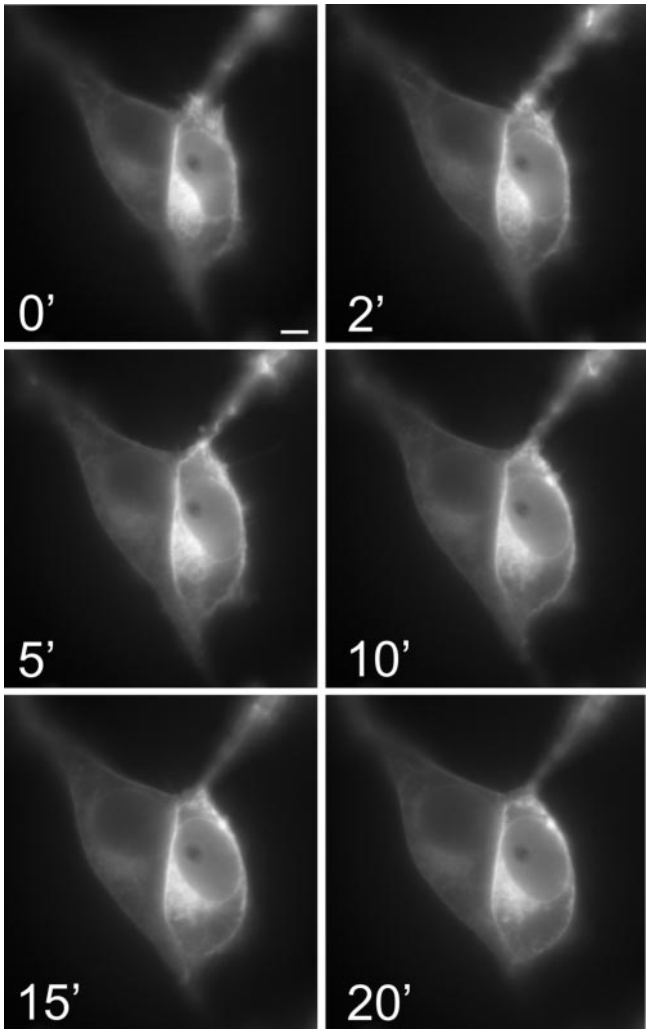

Figure 7. ATP-induced bleb formation is prevented by incubation in the presence of the ROCK inhibitor Y-27632. $P2X_7$-HEK293 cells were incubated in serum-free medium, pretreated with 10 μM Y-27632 for 2 h, rinsed, placed in fresh solution supplemented with 10 μM Y-27632, and treated with 3 mM ATP (time zero). Bar, 10 μm.

ROCK I proteolysis at various time points, starting 1 min after the addition of ATP. Even at this early time point the 130-kDa fragment was detectable (Figure 8B), suggesting that cleavage of ROCK I might be a very early event after ATP stimulation. ROCK I cleavage stimulated by ATP was z-VAD-fmk insensitive at all time points examined, whereas on the contrary it was z-VAD-sensitive when the stimulus was the anti-Fas antibody both in Jurkat (Figure 8A) and in HEK293 cells (Figure 8B).

Although clearly detectable, evidence of ROCK I cleavage does not rule out the possibility that ROCK I stimulation also occurs by other more conventional pathways, such as activation by Rho GTPases. To test this directly, we performed a pull-down experiment (Figure 9), to assay the RhoA activation status in ATP-stimulated cells. We found that in resting $P2X_7$-HEK293 cells RhoA was already in a partially activated state that was only very slightly increased by ATP stimulation after 20 min of incubation. We were unable to detect significant differences 1 min after ATP stimulation, when blebbing started. As a control, we show that histamine caused a large RhoA activation. Then, we tested the effect of the C3 botulinum toxin, a selective Rho ADP-ribosylating and -inactivating agent. The toxin was rendered cell permeable by fusion to a portion of the human immunodeficiency virus protein Tat. Figure 10A shows that 500 or 1000 nM Tat-C3 caused a nearly complete ADP-ribosylation of RhoA, as indicated by a MW shift. With 500 nM Tat-C3, ATP-induced blebbing was essentially unimpaired (Figure 10B); however, with 1 μM there was a substantial delay in blebbing that did not start until after ~20 min (Figure 10C), as if treatment with a supramaximal dose of toxin was necessary to fully inhibit all residual RhoA-dependent activity. This suggests that the Rho pathway may also play a role in $P2X_7$-dependent ROCK activation.

Finally, we checked whether $P2X_7$ might drive ROCK I cleavage by activating other intracellular proteases. Although calpains have been implicated in the control of cytoskeletal dynamics and apoptosis (Chan and Mattson, 1999), we found that specific calpain inhibitors (Calpain Inhibitor I, ALLN, and Calpain Inhibitor II, ALLM), were unable to prevent ATP-induced ROCK I cleavage and activation (our unpublished results).

DISCUSSION

The hypothesis that extracellular nucleotides play an important role in cell-to-cell communication has been well substantiated in recent years (Ralevic and Burnstock, 1998; Chizh and Illes, 2001; North, 2002). Several reports have demonstrated that ATP concentrations in the pericellular space attain levels as high as10–20 μM and are modulated by cell activity (Ferrari *et al.*, 1997; Beigi *et al.*, 1999; Schwiebert *et al.*, 2002). Furthermore, a large family of receptors for extracellular nucleotides, the P2 receptors, has been cloned and is currently a focus of intense investigation because of its involvement in many different responses such as pain sensation (Chizh and Illes, 2001), chemotaxis (Oshimi *et al.*, 1999), chloride secretion (Inoue *et al.*, 1997), cytokine and chemokine release (Di Virgilio *et al.*, 2001), mechanosensory transduction in the urinary bladder (Vlaskovska *et al.*, 2001), and cytotoxicity (Di Virgilio *et al.*, 1998). Finally, almost all cell types express very powerful ecto-ATPases that effectively modulate the concentration of extracellular nucleotides (Zimmermann, 2000). Thus, we must add to the known extracellular systems based on more conventional mediators (e.g., growth factors, neurotransmitters, cytokines), a complex and as yet poorly known system based on nucleotides (and nucleosides).

The responses elicited by extracellular ATP depend in the first place on the given P2 receptor subtype(s) expressed by the responding cell and the intensity of stimulation. Among the many different responses produced by ATP stimulation, cytotoxicity is one of the most intriguing and potentially most interesting. This phenomenon was initially described by Mirabelli and coworkers (1986) and later extensively investigated in several laboratories, including our own (Di Virgilio *et al.*, 1989; Filippini *et al.*, 1990; Zanovello *et al.*, 1990; Zheng *et al.*, 1991). Prolonged incubation in the presence of extracellular ATP can cause cell death by either necrosis or

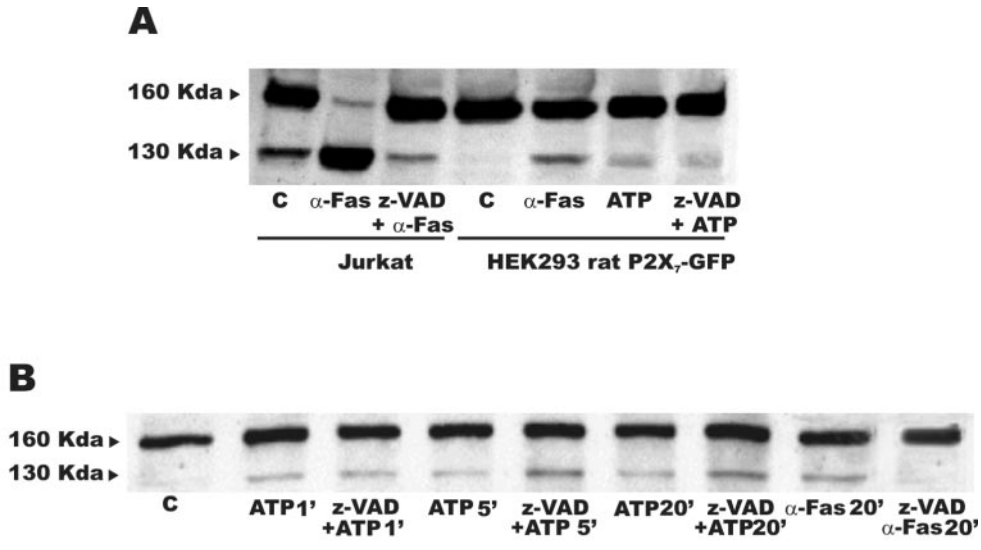

Figure 8. ATP causes ROCK I cleavage. Jurkat or $P2X_7$-HEK293 cells were treated with either anti-Fas mAb, in the presence or absence of z-VAD-fmk (100 μM) or with ATP (3 mM) in the presence or absence of z-VAD-fmk for 20 min (A) or for the times indicated below lanes (B) and then processed for protein extraction and immunoblot analysis (see MATERIALS AND METHODS). Controls received no treatment.

apoptosis. Whether one or the other pathway predominates depends on the cell type and the dose and length of exposure to the nucleotide. There are few doubts that the principal (and according to some investigators the only) P2 receptor capable of triggering cell death is $P2X_7$, a ligand-gated receptor channel, permeable to mono- and di-valent cations, whose only physiological ligand is ATP. This receptor is a homo-oligomer made of subunits (probably three or six) composed of 595 amino acids each (Surprenant *et al.*, 1996; Kim *et al.*, 2001b). In the presence of low ATP concentrations or in response to a single pulse of ATP, $P2X_7$ behaves as a conventional cation-selective channel. However, when exposed to high ATP concentrations or to repeated pulses of the agonist, it undergoes a channel to pore transition that allows hydrophilic solutes of molecular mass up to 900 Da through the plasma membrane (Di Virgilio, 1995; Surprenant *et al.*, 1996). Although $P2X_7$ is not the only P2X receptor for which such a peculiar behavior has been described (Khakh *et al.*, 1999; Virginio *et al.*, 1999), it is the receptor for which the channel/pore transition has been most extensively and reproducibly documented. The physiological meaning of this phenomenon is not understood, although some intriguing hypotheses have been forwarded (Di Virgilio, 1995, 2000; Di Virgilio *et al.*, 2001). Whatever the real physiological function of the $P2X_7$ pore might be, there is no doubt that such a lesion is lethal if it remains patent for any extended time (>10–15 min in most cell types).

$P2X_7$-mediated cell death occurs either via necrosis or apoptosis. We have observed that cells expressing this receptor to a high level are more prone to undergo fast necrotic death, probably because the rapid upset of intracellular ion homeostasis prevents the initiation of the complex chain of events necessary for apoptosis (Zanovello *et al.*, 1990). On the other hand, cells with a lower expression of $P2X_7$ or where this receptor forms a smaller sized pore, such as the lymphocytes, are more likely to die by apoptosis. Although seldom acknowledged as a proapoptotic receptor, $P2X_7$ triggers many of the changes typical of this process: membrane blebbing, phosphatidylserine exposure, cell shrinkage, activation of caspases 1, 3, and 8, cleavage of the

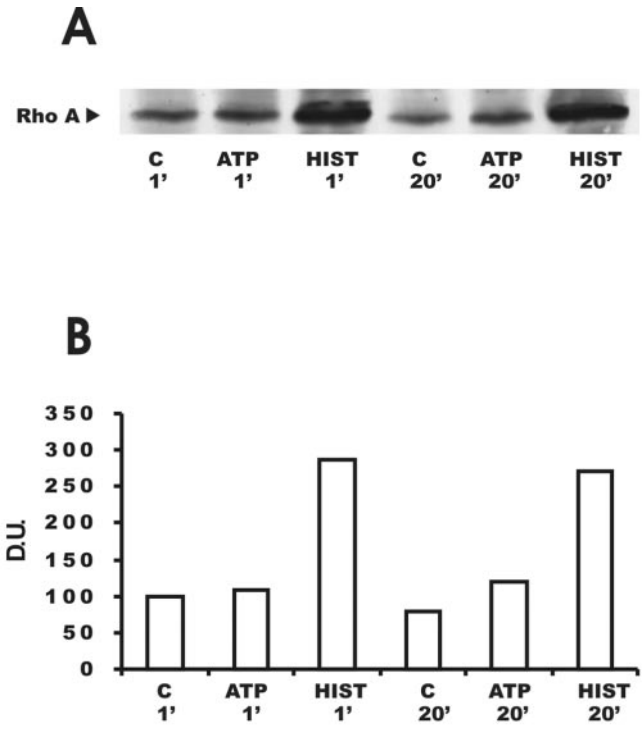

Figure 9. Effect of ATP stimulation on RhoA activation. Confluent $P2X_7$-HEK293 monolayers incubated in Ca^{2+}-containing saline solution were stimulated with 3 mM ATP or 100 μM histamine (HIST) for the indicated times, rinsed, and lysed. For each time point 1.5 mg of total cell protein was mixed with GST-Rhotekin–bound glutathione-Sepharose beads (100 μl). After this incubation, samples were spun, supernatants were discarded, and beads were washed three times and loaded onto the gel (100 μl/lane) for electrophoresis and immunoblot analysis (see MATERIALS AND METHODS for further details). (A) Immunoblot; (B) densitometric analysis of the bands. DU, arbitrary densitometric units. Density of the bands is expressed as percent variation of control band at the 1-min time point (C, 1').

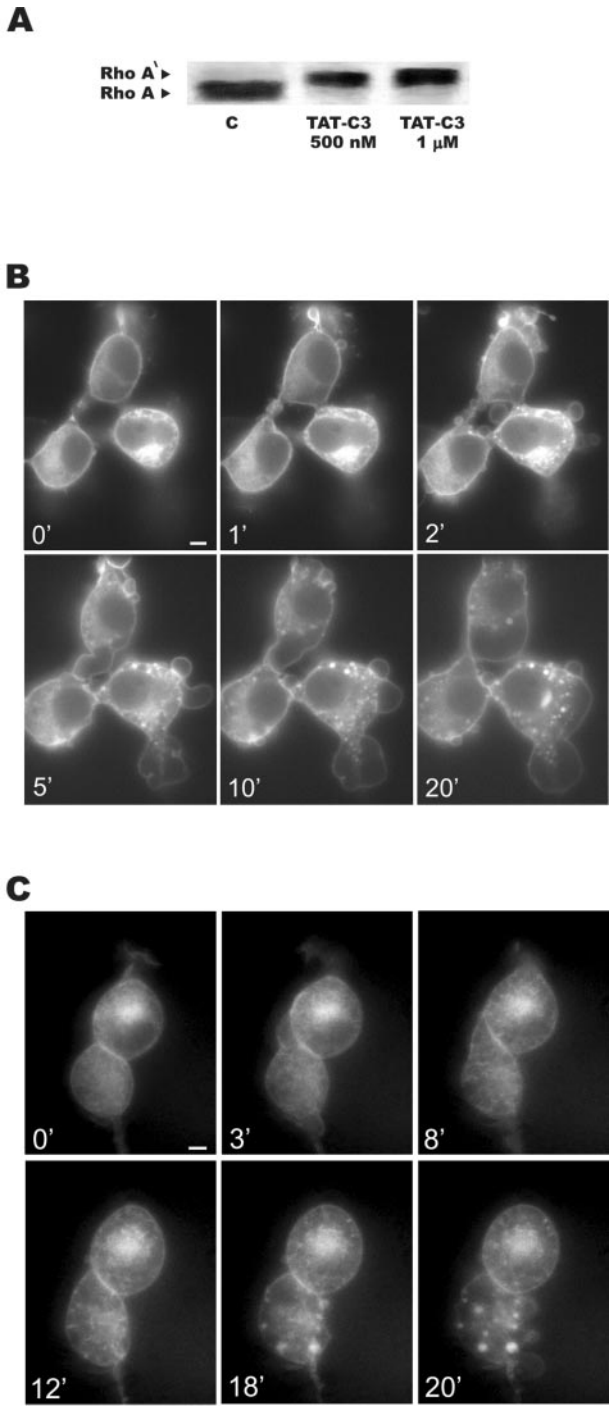

Figure 10. Effect of Tat-C3 treatment on Rho A and ATP-induced bleb formation. Confluent $P2X_7$-HEK293 monolayers were incubated in 15% FCS-containing DME/F-12 medium and treated with 500 nM or 1 μM Tat-C3 for 16 h. At the end of this incubation, cells were rinsed, lysed, and analyzed by SDS-PAGE (A). Treatment with Tat-C3 caused a shift in the mobility of RhoA to a slower mobility ADP-ribosylated form (RhoA'). Alternatively, monolayers were rinsed, incubated in Ca^{2+}-containing saline solution and subjected to microscopic analysis (see MATERIALS AND METHODS). (B and C) Cells treated with 500 nM or 1 μM Tat-C3, respectively. Bar, 10 μm.

caspase substrates PARP and lamin B, chromatin condensation, DNA fragmentation, and apoptotic body formation (Zanovello *et al.*, 1990; Zheng *et al.*, 1991; Ferrari *et al.*, 1999). Although originally bleb formation and phosphatidylserine exposure were regarded as unequivocal indications of apoptosis, it is now increasingly appreciated that these changes are not necessarily followed by cell death (see MacKenzie *et al.*, 2001). Our data also support this view because, provided ATP was removed within 15–20 min, blebbing was reversible. However, a more prolonged exposure to ATP almost invariably committed $P2X_7$-HEK293 cells to death, in agreement with MacKenzie *et al.* (2001).

The seemingly simple process of bleb formation is powered by the contractile forces generated by the acto-myosin cytoskeleton through the modulation of a complex array of intracellular signaling molecules that modulate MLC phosphorylation, control myosin ATPase activity, bridge the cytoskeleton with the plasma membrane, and stabilize filamentous actin. Cytoskeletal contractility is critically dependent on the activity of the Rho GTPases, which in their GTP-bound form stimulate the activity of the two ROCK isoforms I and II. Recent data show that ROCK I, but not Rho, activity is necessary for TNF-α–stimulated cells to undergo the full sequence of morphological alterations of apoptosis, from membrane blebbing to apoptotic body formation (Coleman *et al.*, 2001; Sebbagh *et al.*, 2001).

Extracellular ATP triggers via $P2X_7$ dramatic changes in cell shape and volume that are commonly thought to be the mere consequence of the ion unbalance caused by large pore formation. A large Ca^{2+} influx is indeed necessary because our study shows that in the absence of extracellular Ca^{2+} ATP-induced bleb formation is fully inhibited. A functioning cytoskeleton is also obviously required, because the powerful cytoskeletal poison cytochalasin B blocks blebbing. However, our data show that a large Ca^{2+} influx and a functioning cytoskeleton are not by themselves sufficient for bleb formation; rather, a complex chain of events downstream of $P2X_7$ is very rapidly triggered and is critically required for the full-blown pattern of morphological changes. Like the process triggered by the TNF-α receptor, by C2-ceramide, or by cycloheximide, ATP-induced membrane blebbing also is critically dependent on ROCK I activity. However, at variance with these stimuli and despite the fact that $P2X_7$ has been shown to activate caspases (Ferrari *et al.*, 1999), ATP-induced ROCK I cleavage and membrane blebbing are independent of caspase-3 activation. The identity of the pathway coupling $P2X_7$ to ROCK I is therefore an open question.

We think it likely that $P2X_7$ causes ROCK activation via a multiplicity of convergent pathways, some leading to direct ROCK I cleavage and activation, and therefore proapoptotic, and others to Rho-mediated ROCK I stimulation, and therefore nonapoptotic. Generation of a constitutive ROCK I kinase via cleavage of the C-terminal portion with ATP doses and kinetics compatible with blebbing is shown by Western blot analysis, but the identity of the protease responsible for the ATP-induced ROCK I cleavage is as yet unknown. We hypothesized that calpain might be involved, but inhibitors of this protease were unable to prevent the ATP effect. On the other hand, a role for Rho is supported by the delayed blebbing caused by treatment of $P2X_7$-HEK293 cells with a supramaximal dose of Tat-C3 toxin and by reversibility of

bleb formation when ATP is removed within 15–20 min of addition. However, two observations are puzzling: first, blebbing was unaffected by a low Tat-C3 dose (500 nM), which was nevertheless apparently able to modify all available RhoA; second, in the pull-down experiment we observed a minor increase in GTP-RhoA only at a late time point (20 min), when blebbing was well underway. We should also consider other possible pathways. For example, it might be that the $P2X_7$ is indirectly coupled to ROCK kinases via the Ca^{2+} increase (or the K^+ decrease; Sanz and Di Virgilio, 2000), or via one of the proteins known to coassemble with this receptor (Kim *et al.,* 2001a). Indirect coupling could also occur through the release of a diffusible soluble messenger generated at the plasma membrane, e.g., arachidonic acid, known to bind the C-terminal regulatory domain and to activate the ROCK I kinase (Feng *et al.,* 1999). Therefore, although our data strongly point to the involvement of ROCK I, the underlying mechanism of activation needs further investigation.

In our hands, sustained activation of $P2X_7$ beyond 20–30 min leads to $P2X_7$-HEK293 cell detachment from the substrate and death, not unlike other cell types expressing high $P2X_7$ levels. MacKenzie *et al.* (2001) reported that $P2X_7$-HEK293 stimulated with benzoyl ATP, a more potent and selective $P2X_7$ agonist, were still alive after 30 min, significant cell loss being detectable only after 6 h. We did observe that after several in vitro passages $P2X_7$-HEK293 cells became increasingly resistant to ATP (for example, they did not detach but rather became more adherent), but we did not perform a thorough characterization of phenotypic changes shown by $P2X_7$-HEK293 after prolonged culture.

In conclusion, we have described the dramatic plasma membrane dynamics caused by stimulation of the $P2X_7$ ATP receptor and have provided clues as to the intracellular mechanisms involved. Future experiments will hopefully disclose the protease(s) responsible for ROCK I cleavage and how it is coupled to $P2X_7$.

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REFERENCES

Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., and Kaibuchi, K. (1996). Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J. Biol. Chem. *271,* 20246–20249.

Beigi, R., Kobatake, E., Aizawa, M., and Dubyak, G.R. (1999). Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase. Am. J. Physiol. *276,* C267–C278.

Chan, S.L., and Mattson, M.P. (1999). Caspase and calpain substrates: roles in synaptic plasticity and cell death. J. Neurosci. Res. *58,* 167–190.

Chizh, B.A., and Illes, P. (2001). P2X receptors and nociception. Pharmacol. Rev. *53,* 553–568.

Chvatchko, Y., Valera, S., Aubry, J.P., Renno, T., Buell, G., and Bonnefoy, J.Y. (1996). The involvement of an ATP-gated ion channel, $P2X_1$ in thymocyte apoptosis. Immunity *5,* 275–283.

Coleman, M.L., Sahai, E.A., Yeo, M., Bosch, M., Dewar, A., and Olson, M.F. (2001). Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat. Cell Biol. *3,* 339–346.

Di Virgilio, F. (1995). The P2Z purinoceptor: an intriguing role in immunity, inflammation and cell death. Immunol. Today *16,* 524–528.

Di Virgilio, F. (2000). Dr. Jekyll/Mr. Hyde: the dual role of extracellular ATP. J. Autonom. Nerv. Syst. *81,* 59–63.

Di Virgilio, F., Bronte, V., Collavo, D., and Zanovello, P. (1989). Responses of mouse lymphocytes to extracellular adenosine 5′-triphosphate (ATP). Lymphocytes with cytotoxic activity are resistant to the permeabilizing effect of ATP. J. Immunol. *143,* 1955–1960.

Di Virgilio, F., Chiozzi, P., Falzoni, S., Ferrari, D., Sanz, J.M., Venketaraman, V., and Baricordi, O.R. (1998). Cytolytic P2X purinoceptors. Cell Death Differ. *5,* 191–199.

Di Virgilio, F., Chiozzi, P., Ferrari, D., Falzoni, S., Sanz, J.M., Morelli, A., Torboli, M., Bolognesi, G., and Baricordi, O.R. (2001). Nucleotide receptors: an emerging family of regulatory molecules. Blood *97,* 587–600.

Feng, J. *et al.* (1999). Rho-associated kinase of chicken gizzard smooth muscle. J. Biol. Chem. *274,* 3744–3752.

Ferrari, D., Chiozzi, P., Falzoni, S., Hanau, S., and Di Virgilio, F. (1997). Purinergic modulation of interleukin-1β release from microglial cells stimulated with bacterial endotoxin. J. Exp. Med. *185,* 579–582.

Ferrari, D., Los, M., Bauer, M.K.A., Vandenabeele, P., Wesselborg, S., and Schulze-Osthoff, K. (1999). P2Z purinoceptor ligation induces activation of caspases with distinct roles in apoptotic and necrotic alterations of cell death. FEBS Lett. *447,* 71–75.

Ferri, K.F., and Kroemer, G. (2001). Organelle-specific initiation of cell death pathways. Nat. Cell Biol. *3,* E255–E263.

Filippini, A., Taffs, R.E., Agui, T., and Sitkovsky, M.V. (1990). Ecto-ATPase activity in cytolytic T lymphocytes. Protection from the cytotoxic effects of extracellular ATP. J. Biol. Chem. *265,* 334–340.

Fukata, Y., Amano, M., and Kaibuchi, K. (2001). Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non muscle cells. Trends Pharmacol. Sci. *22,* 32–39.

Khakh, B., Bao, X., Labarca, C., and Lester, H. (1999). Neuronal P2X receptor-transmitter-gated cation channels change their ion selectivity in seconds. Nature Neurosci. *2,* 322–330.

Kim, M., Jiang, L.H., Wilson, H.L., North, R.A., and Surprenant, A. (2001a). Proteomic and functional evidence for a $P2X_7$ receptor signalling complex. EMBO J. *20,* 6347–6358.

Kim, M., Spelta, V., Sim, J., North, R.A., and Surprenant, A. (2001b). Differential assembly of rat purinergic $P2X_7$ receptor in immune cell of the brain and periphery. J. Biol. Chem. *276,* 23262–23267.

Kimura, K. *et al.* (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science *273,* 245–248.

Inoue, C.N., Woo, J.S., Schwiebert, E.M., Morita, T., Hanaoka, K., Guggino, S.E., and Guggino, W.B. (1997). Role of purinergic receptors in chloride secretion in Caco-2 cells. Am. J. Physiol. *272,* C1862–C1870.

MacKenzie, A., Wilson, H.L., Kiss-Toth, E., Dower, S.K., North, R.A., and Surprenant, A. (2001). Rapid secretion of interleukin-1β by microvesicle shedding. Immunity, *8,* 825–835.

Mirabelli, F., Bellomo, G., Nicotera, P., Moore, M., and Orrenius, S. (1986). Ca^{2+} homeostasis and cytotoxicity in isolated hepatocytes:

studies with extracellular adenosine 5′-triphosphate. J. Biochem. Toxicol. *1,* 29–39.

Morelli, A., Ferrari, D., Bolognesi, G., Rizzuto, R., and Di Virgilio, F. (2001). Proapoptotic plasma membrane pore: $P2X_7$ receptor. Drug Dev. Res. *52,* 571–578.

Murgia, M., Hanau, S., Pizzo, P., Rippa, M., and Di Virgilio, F. (1993). Oxidized ATP. An irreversible inhibitor of the macrophage purinergic P2Z receptor. J. Biol. Chem. *268,* 8199–8203.

North, R.A. (2002). The molecular physiology of P2X receptors. Physiol. Rev. *82,* 1013–1067.

Oshimi, Y., Miyazaki, S., and Oda, S. (1999). ATP-induced Ca^{2+} response mediated by P2U and P2Y purinoceptors in human macrophages: signalling from dying cells. Immunology *98,* 220–227.

Ralevic, V., and Burnstock, G. (1998). Receptors for purines and pyrimidines. Pharmacol. Rev. *50,* 413–492.

Sanz, J.M., and Di Virgilio, F. (2000). Kinetics and mechanism of ATP-dependent IL-1β release from microglial cells. J. Immunol. *164,* 4893–4898.

Schachter, J.B., Sromek, S.M., Nicholas, R.A., and Harden, T.K. (1997). HEK293 human embryonic kidney cells endogenously express the P2Y1 and P2Y2 receptors. Neuropharmacology *36,* 1181–1187.

Schwiebert, L.M., Rice, W.C., Kudlow, B.A., Taylor, A.L., and Schwiebert, E.M. (2002). Extracellular ATP signalling and P2X nucleotide receptors in monolayers of primary human vascular endothelial cells. Am. J. Physiol. *282,* C289–C301.

Sebbagh, M., Renvoizè, C., Hamelin, J., Richè, N., Bertoglio, J., and Breard, J. (2001). Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. Nat. Cell Biol. *3,* 346–352.

Smart, M.L., Panchal, R.G., Bowser, D.N., Williams, D.A., and Petrou, S. (2002). Pore formation is not associated with macroscopic redistribution of $P2X_7$ receptors. Am. J. Physiol. *283,* C77–C84.

Steinberg, T.H., and Silverstein, S.C. (1987). Extracellular ATP^{4-} promotes cation fluxes in the J774 mouse macrophage cell line. J. Biol. Chem. *262,* 3118–3122.

Surprenant, A., Rassendren, F., Kawashima, E., North, R.A., and Buell, G. (1996). The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor ($P2X_7$). Science *272,* 735–738.

Torgerson, R.R., and McNiven, M.A. (1998). The actin-myosin cytoskeleton mediates reversible agonist-induced membrane blebbing. J. Cell Sci. *111,* 2911–2922.

Virginio, C., MacKenzie, A., Rassendren, F.A., North, R.A., and Surprenant, A. (1999). Pore dilation of neuronal P2X receptor channels. Nat. Neurosci. *2,* 315–321.

Vlaskovska, M., Kasakov, L., Rong, W., Bodin, P., Bardini, M., Cockaine, D.A., Ford, A.P.D.W., and Burnstock, G. (2001). $P2X_3$ knock-out mice reveal a major sensory role for urothelially-released ATP. J. Neurosci. *21,* 5670–5677.

von Kugelgen, I., and Wetter, A. (2000). Molecular pharmacology of P2Y-receptors. Naunyn-Schmiedeberg's Arch. Pharmacol. *362,* 310–323.

Warny, M., Aboudola, S., Robson, S.C., Sevigny, J., Communi, D., Soltoff, S.P., and Kelly, C.P. (2001). $P2Y_6$ nucleotide receptor mediates monocyte IL-8 production in response to UDP or lipopolysaccharide. J. Biol. Chem. *276,* 26051–26056.

Yu, S.P., Canzoniero, L.M., and Choi, D.W. (2001). Ion homeostasis and apoptosis. Curr. Opin. Cell Biol. *13,* 405–411.

Zanovello, P., Bronte, V., Rosato, A., Pizzo, P., and Di Virgilio, F. (1990). Responses of mouse lymphocytes to extracellular ATP. II. Extracellular ATP causes cell type-dependent lysis and DNA fragmentation. J. Immunol. *145,* 1545–1550.

Zheng, L.M., Zychlinsky, A., Liu, C-C., Ojcius, D.M., and Young, J.D-E. (1991). Extracellular ATP as a trigger for apoptosis or programmed cell death. J. Cell Biol. *112,* 279–288.

Zimmermann, H. (2000). Extracellular metabolism of ATP and other nucleotides. Naunyn-Schmiedberg's Arch. Pharmacol. *362,* 299–309.