Pseudoapoptosis Induced by Brief Activation of ATP-gated P2X<sub>7</sub> Receptors*<sup>5</sup>

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P2X<sub>7</sub> receptors are ATP-gated ion channels primarily expressed on antigen-presenting immune cells where they play a role in the acute inflammatory response. These ion channels couple not only to influx of cations, including calcium, but also to rapid alterations in cell morphology (membrane blebbing, phosphatidylserine exposure, microvesicle shedding). These features resemble the extraneuronal events associated with end stages of apoptosis but cell death does not occur if receptor activation is brief. Here we delineate two signaling pathways underlying these apoptotic-like processes. Loss of membrane asymmetry occurs within seconds, which directly triggers cytoskeletal disruption and zeotropic membrane blebbing; this is readily reversible and requires both calcium influx through P2X<sub>7</sub>-channels and mitochondrial calcium increase but is not associated with cytochrome c release. A slower, calcium-independent, ROCK-1-dependent cascade that does not involve rapid loss of membrane asymmetry but is associated with cytochrome c release is secondarily activated. The ROCK-1 pathway appears largely responsible for cell death, which occurs after prolonged stimulation of P2X<sub>7</sub> receptors. We suggest that the former mechanism underlies the reversible pseudoapoptotic events induced by brief activation of P2X<sub>7</sub> receptors.

In antigen-presenting cells of the immune system, prolonged application of high concentrations of extracellular ATP leads to apoptotic and/or necrotic cell death via activation of the ATP-gated ionotropic P2X<sub>7</sub> receptor (1–3). P2X<sub>7</sub> receptor coupling has also been associated with classical apoptotic signaling cascades such as activation of stress-activated kinases/c-Jun N-terminal kinases and caspase-3 (4–7). However, P2X<sub>7</sub> receptors in the immune system can also couple to responses distinct from cell death including cell fusion, cell proliferation, release of pro-inflammatory cytokines, and bone formation (8–11). Indeed, brief activation of P2X<sub>7</sub> receptors, on the order of seconds to minutes, causes rapid morphological changes, which appear to mimic the extraneuronal hallmarks of apoptosis, in particular membrane blebbing, phosphatidylserine exposure (PS flip),<sup>2</sup> and microvesicle shedding. Surprisingly, subsequent cell death does not occur (3, 10). Is this a type of pseudoapoptosis? That is, might this receptor couple to the same biochemical and cell biology signaling cascades that are now associated with apoptosis (12–15) and yet not lead to cell death (16)? Or, might a mechanism distinct from the "classical" apoptotic cytochrome c/caspase3/RhoA-associated kinase (ROCK-1) signaling cascade (17, 18) underlie the cellular events associated with brief receptor activation? Elucidation of signaling mechanisms underlying the rapid sequelae of cellular events induced by P2X<sub>7</sub>, receptor activation is not only an important aim in the area of ion channel function in immune cells but may also provide significant insight into mechanisms underlying what is regarded as "classical" apoptosis (19–21). We have addressed this issue by studying the kinetics of cell morphology and mitochondrial alterations in response to brief activation of P2X<sub>7</sub>-receptors heterologously expressed in HEK293 cells. We have focused on alterations in cytoskeletal protein elements, changes in lipid asymmetry and mitochondrial function, and the dynamics of membrane blebbing because these are some of the most intensively studied extraneuronal events associated with active cell death (i.e. apoptosis) (22–26).

We find that plasma membrane PS flip and gross mitochondrial swelling with collapse of mitochondrial membrane potential both occur in less than 5–10 s with actin filament/microtubule network disruption and synchronous membrane blebbing occurring some 30–60 s later. All of these events are fully reversible and not associated with cytochrome c release when receptor activation is less than 20–30 min. However, longer activation of this receptor leads to release of cytochrome c into the cytosol, which is associated with inevitable cell death. Thus, it appears that P2X<sub>7</sub>-receptors couple to at least two distinct mechanisms: a calcium-dependent, PS flip-initiated pathway resulting in reversible cytoskeletal disruptions without subsequent cell death and a calcium-independent, ROCK-1-dependent pathway that may be primarily responsible for cell death following prolonged P2X<sub>7</sub>-receptor activation.

**MATERIALS AND METHODS**

Reagents—All chemicals and reagents were purchased from Sigma, except the following: Mitotracker Green (stock 1 mM in Me<sub>2</sub>SO<sub>4</sub>), Fura-2-AM, Rhod-2, IC-1 (all 2 mM stock in Me<sub>2</sub>SO<sub>4</sub>), rhodamine phalloidin (stock solution of 200 units ml<sup>-1</sup> in methanol), and 2–6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) hexanol-1-hexadecanoyl-sn-glycero-3-phosphocholine (NBD) were obtained from Molecular Probes (Eugene, OR). The caspase inhibitor 1, zVAD-FMK (stock 100 mM in Me<sub>2</sub>SO<sub>4</sub> used at a final concentration of 100 μM), anti-human α-tubulin monoclonal antibody, Y-27632 (supplied as a 5 mM stock solution in H<sub>2</sub>O and used at 5 μM final concentration), LY294002 (10 mM stock in Me<sub>2</sub>SO<sub>4</sub> used at 10 μM final concentration), ML-9 (10 mM stock in Me<sub>2</sub>SO<sub>4</sub> used at 10 μM final concentration), BAPTA-AM (10 mM stock in Me<sub>2</sub>SO<sub>4</sub> used 1 μM), and cyclosporin A (1 mM in ethanol, used at 1 μM) were obtained from Calbiochem-Novabiochem Corp (La Jolla, CA). Unless otherwise stated, normal extracellular saline contained (in mM) NaCl 147, KCl 2,
saline contained NaCl 147; KCl 2; Hepes 10; glucose 12; MgCl2 1; EGTA 1 to pH 7.3 with NaOH.

**Microscopy**—For transmission electron microscopy, cells were fixed (30 min) in suspension in 4% glutaraldehyde in 0.1 M PIPES buffer at pH 7.2 with 200 ml of 33% hydrogen peroxide per 10 ml of fixative. The cell suspension was centrifuged, the pellet was fixed for a further 30 min, and then washed in 0.1 M PIPES buffer for 1 h. Cells were postfixed in 1% osmium tetroxide in PIPES buffer for 1 h then washed in 0.05 M maleate buffer over 1 h. The preparation was then bulk-stained in 1% uranyl acetate in maleate buffer for 12 h then dehydrated in graded alcohols, infiltrated in Spurr resin, and sectioned at 60 nm on a Riechert ultramicrotome. The sections were examined using a Philips CM 100 electron microscope. Standard video microscopy was performed using a Zeiss Axiosvert 100 with Fluor ×40 or ×100 objective, and a JVC TK-C1380 color video camera coupled with a VHS video recorder. Images were converted from analogue to digital images using the Formac Studio and ProTV software (Formac Electronics, Germany). Image J software (NIH) was used to time-compress videos, which were then saved as Quicktime Movies.

To assess mitochondrial morphology, cells were loaded with 2 μM Mitotracker Green for 2–5 min at room temperature. Fluorescent images were collected using either a cooled CCD camera (IMAGO) or a laser scanning confocal microscope (Molecular Dynamics CLSM2010). In fluorescent imaging experiments, images were obtained using an oil immersion ×100 Fluor objective by a cooled CCD camera at 0.5–2 Hz, NBD labeling of plasma membrane was as described previously (10). A scanning monochromator selected excitation wavelengths under computer control. Digital images were analyzed using TILLvision imaging software (Photronics, Planegg, Germany). Excitation/emission wavelengths were 480/488 nm for BCT-AM, 340/380 nm for Fura-2, 560/510 for JC-1, 500 for Rhod-2, and 560 nm for rhodamine phalloidin. Agonists were applied via a gravity-driven superfusion system. Confocal fluorescent imaging of mitochondria (Mitotracker Green) was carried out using the excitation wavelength 488 nm and emission collected via the 530-nm band pass filter (green channel). Images were acquired using an inverted microscope using a ×63 oil immersion objective. All experiments were conducted at ambient room temperature 22–24 °C. Data are mean ± S.E.

**Immunohistochemistry**—Cells were washed in PBS and fixed in Zamboni’s Fixative (20 min), blocked in 5% goat serum in PBS containing 2% Triton X-100 (30 min), and then incubated with the primary antibody (mouse anti-human vinculin or anti-human α-tubulin) for 1–2 h at room temperature or overnight at 4 °C. Cells were washed and incubated with the secondary antibody (fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (lgG) at 1:100 dilution) for 1 h at room temperature. For actin distribution, fixed cells were labeled with rhodamine phalloidin (filamentous actin/F-actin) or DNase1 (monomeric actin/G-actin) either separately, together, or following labeling for vinculin. Cells were washed in PBS, fixed with Zamboni’s (10 min), blocked with 1% bovine serum albumin in PBS containing 2% Triton X-100 (20 min), and incubated with rhodamine phalloidin (5 units ml⁻¹) or FITC-DNase1 for 1 h. For simultaneous labeling of vinculin and F-actin, cells were incubated with rhodamine phalloidin (30 min) following labeling with secondary antibody.

**Analysis of Bleb Kinetics and Dimensions**—Still images of blebbing cells were used for measurements. Because images were in two dimensions, we estimated the area of each bleb and of the cell itself by approximating each to a circle (drawing a circular region of interest around each) and calculating the area (πr²). For each image the sum of the areas of all blebs was expressed as a percent of the area of the cell. This protocol adjusted for differences in cell size and focal plane during video acquisition. Time to onset of membrane blebbing was taken as time when first fully protruding bleb was observed and is accurate to ± 4 s. All measurements were performed blind in terms of knowledge of the experimental condition applied. Curves following either onset of blebbing or extent of blebbing were fit by a least squares method using Kaleidagraph software (Synergy Software) in the form: 

\[ Y = Y_{\text{min}} + \frac{(Y_{\text{max}} - Y_{\text{min}})}{1 + (V_{50}/V_{\text{test}})^n} \]

where \( Y \) is response, \( t \) is time, \( V_{50} \) is half-maximal response, and \( n \) is slope of the line. The reversal of blebbing was fit only by a smoothing curve using Kaleidagraph.

**Cytochrome c Release**—To determine cytochrome c release from mitochondria, cytosolic fractions were isolated as described (27). Briefly, 4 × 10⁶ cells per sample, were exposed to 100 μM BzATP for various times or left untreated. The cells were then lysed under vigorous vortexing in PBS plus 500 μM sucrose containing protease inhibitors (Complete-TM, Roche Applied Science) and further incubated at room temperature for 60 s. Cytosolic fractions were quickly isolated and removed from organelles and cell debris by centrifugation at 14,000 × g for 5 min at 4 °C. As reference for total cytochrome c content of the cells, organelle-comprising fractions of untreated cells were lysed in 20 mM dodecylmaltoside in PBS plus protease inhibitors for 2 h under vigorous shaking. The presence of the detergent allowed solubilization of membrane proteins that were then separated from cell debris by centrifugation at 14,000 × g for 5 min at 4 °C. Proteins were visualized by Western blotting: SDS sample buffer was added, and proteins were denatured at 100 °C for 10 min. They were subsequently separated by SDS-PAGE (15%) and electrophoretically transferred to polyvinylidene difluoride (Immu-no-blot PVDF, Bio-Rad) membranes. PVDF membranes were rinsed in 10 mM Tris, 250 mM NaCl, pH 7.5, then blocked in 1% bovine serum albumin in TTBS: 10 mM Tris, 250 mM NaCl, 0.1% Tween 20, pH 7.5, for 1 h at room temperature. After a wash with TTBS, a monoclonal anti-cytochrome c Ab (2 μg/ml, BD Pharmingen clone 7H18.2C12) was added, and the membrane was incubated overnight at 4 °C. The membrane was washed twice in TTBS, and a 1:3000 dilution of goat anti-mouse IgG (H+L)-AP-conjugated Ab (Bio-Rad) was added in blocking solution. After 2 h of incubation at room temperature the membrane was washed three times and Immun-star substrate from Bio-Rad was applied for 5 min in the dark. Protein bands were revealed by standard photographic procedures. Densitometric semiquantitation of Western blot bands was obtained using the Gene Genius image system from Syngene. Images were acquired and analyzed from films using GeneSnap and Genetools software (Syngene). The reported data represent the ratio between the calculated density of each sample and a control untreated sample present on the same film (Fig. 7D) or relative to β-actin (Fig. 7 B and C) resolved on the same gel using rabbit β-actin polyclonal Ab (Abcam, Cambridge, UK).

**RESULTS**

P2X<sub>7</sub> Receptor-induced Membrane Blebbing and Cytoskeletal Rearrangements—HEK293 cells stably expressing the rat P2X<sub>7</sub> receptor were used because previous electrophysiological and immunohistochemical studies have shown that virtually all cells exhibit uniform and approximately equal receptor density at the plasma membrane (10, 28). The ATP analogue, BzATP, was used in most experiments, because it does not activate the endogenous metabolotropic purinergic (P2Y) receptors in these cells (29). The time-to-initial onset of blebbing decreased with increasing agonist concentrations, from 48 ± 3 s (n = 144) at 10 μM to 23 ± 1 s (n = 131) at 200 μM. As well, the proportion of cells undergoing blebbing was similarly concentration-dependent with 10 μM BzATP
inducing blebs in <2% of cells whereas >97% of cells blebbed in response to 100 μM BzATP (Fig. 1, A and B). When these parameters were plotted as a function of the agonist concentration, they were well fit by standard sigmoidal dose-response equations (Fig. 1, C and D); half-maximal EC₅₀ values for time-to-onset and percent total cells blebbed were 33 ± 2 μM and 72 ± 3 μM, respectively. These values are within the range of EC₅₀ values (3–40 μM) for membrane current, dye uptake or calcium entry in these cells under identical conditions (10, 28, 30) and thus indicate that the signaling pathway(s) underlying these rapid morphological changes are engaged over the same range as that for activation of the P2X₇ ion channel.

Membrane blebs readily reversed within 5–15 min in all cells when BzATP (30, 100 μM) was washed out following applications of 1–10 min (Fig. 1, A and E and supplemental Movie 1). Upon washout of agonist, blebs were observed to resorb at a rate corresponding to the total level of blebbing that occurred; that is, smaller blebs reversed more quickly than larger blebs (Fig. 1E). Blebbing was a dynamic process even during washout of agonist, as new blebs appeared while others were resorbed until complete retraction occurred (supplemental Movie 1). This type of dynamic blebbing is termed “zeiotic” and has been associated with apoptotic but not necrotic cell death (31–34).

Electron microscopy showed that P2X₇ receptor-induced blebs exhibited the same characteristics as classical apoptotic blebs (31, 33): they contained only amorphous cytosolic material and no organelles; the double nuclear membrane remained intact during activation for up to 10 min (longest time examined), although the chromatin appeared slightly more condensed when compared with unactivated cells (Fig. 2A).

Because the actin cytoskeleton is the major factor dictating cell shape and movement, we studied the distribution of polymerized (F-actin) and monomeric (G-actin) actin after 2–10 min exposure to BzATP (Fig. 2). In unactivated cells, a dense network of F-actin fibers traversed the periphery of the cell directly under/at the plasma membrane whereas monomeric G-actin was diffusely localized throughout the cytosol (Fig. 2B, upper panel). One minute after exposure to BzATP (30 μM), membrane blebs were rich in monomeric G-actin but relatively devoid of F-actin (n = 8; Fig. 2B, lower panel). F-actin stress fibers are complexed to integrin receptors via focal adhesion sites, a complex of actin-binding proteins, and signal transduction elements such as tyrosine kinases (35). Disassembly of focal adhesion complexes may account for formation of large blebs devoid of organelles caused by sudden loss of the membrane-cytoskeletal interaction (35, 36). Accordingly, we examined the localization of the focal adhesion protein vinculin; an actin-binding protein, which forms part of the focal adhesion complex (37). In control cells, vinculin was distributed at perinuclear regions and at punctate focal complexes at the termini of peripheral F-actin fibers (Fig. 2C, upper panel), but after P2X₇ receptor activation for 2 min, vinculin had accumulated at the cell periphery and in the blebs (Fig. 2C, lower panel). Finally, because the microtubule network regulates contractile and propulsive actions of the actin cytoskeleton, with extensive cross-talk between these two structural elements particularly at the level of focal adhesions (35, 37), we also...
examined α-tubulin distribution after P2X<sub>7</sub> receptor-induced blebbing. In unactivated cells, microtubule filaments extended throughout the cell and terminated within lamellipodia (Fig. 2D, left panel), after 2-min BzATP stimulation, α-tubulin was seen to cluster at the leading edge of the bleb (Fig. 2D, middle panel). Distribution of α-tubulin had returned to normal within 20 min of agonist removal (Fig. 2D, right panel), by which time all blebs had retracted.

A Calcium Influx Pathway and a Calcium-independent ROCK-1 Pathway Account for Two Types of Membrane Blebbing—Despite these dramatic cytoskeletal rearrangements, neither membrane blebbing nor its reversal were significantly altered by several agents that interfere with cytoskeletal proteins. Specifically, the following treatments were without effect on blebbing or reversibility of blebbing: cytochalasin D (n = 10), which disrupts the actin cytoskeleton by preventing F-actin polymerization, the myosin light chain kinase inhibitor ML-9 (n = 6), or the microtubule depolymerizer, nocodazole (n = 6; Fig. 3A). Because MAPK and Rho-dependent pathways are well known to be intimately involved with cytoskeletal function, we examined the following inhibitors of these pathways but did not observe any significant alterations in the kinetics or extent of P2X<sub>7</sub> receptor-induced membrane blebbing: dominant negative and positive mutations of RhoA (N19Rho and L61Rho), the C3 exoenzyme Rho inhibitor, the p38 MAPK inhibitor SB203580, the p42/44 MAPK inhibitor PD98059, and LY294002, which is a relatively specific inhibitor of phosphatidylinositol 3-kinase at the concentrations used in this study (n = 3–9 for all compounds, final concentrations of compounds used are listed under “Materials and Methods,” Fig. 3A). In contrast, the p160 ROCK-1 inhibitor, Y-27632, significantly increased the time to onset of membrane blebbing (Fig. 3A) but had no significant effect on the extent or type of membrane blebbing that occurred. More strikingly, removal of extracellular calcium (with 1 mM EGTA to ensure maximum calcium reduction), clearly altered the features of the blebs as well as increasing the time to onset of initial blebbing (Fig. 3B). The multiple blebbing around the cell circumference seen in control solutions (Fig. 1A and supplemental Movie 1) was replaced by many fewer (usually only 1 or 2) and much larger blebs, whose neck diameters were up to 10 μm (Fig. 3B and supplemental Movie 2). In contrast to the zeiotic nature of the blebbing observed in the presence of calcium, features of the calcium-independent blebs resembled those previously ascribed to necrotic blebs (33). Additional pretreatment with BAPTA-AM to prevent changes in intracellular calcium (with or without additional preincubation with BAPTA-AM). Removal of external calcium markedly inhibited the number of blebs/cell but increased the total area of individual blebs that formed (see supplemental Movie 2). Numbers above bars represent total number of cells examined.
P2X<sub>7</sub> Receptors and Pseudoapoptosis

BzATP occurred only in P2X7-expressing cells but not in untransfected control. This increased cell proliferation following brief (2 min) stimulation with BzATP; indeed, a significant increase in cell number occurred (Fig. 4A). Moreover, no loss of cell number or viability was observed 12 h following the 2-min stimulation with BzATP (Fig. 4B). The prolonged stimulation (40 min) resulted in almost complete loss of cells subsequent to P2X7 receptor activation (Fig. 4, lower and right graphs, respectively) in response to a 2-min application of BzATP in normal solution (filled circles), in calcium-free solution with cells pretreated with BAPTA-AM and with reintroduction of external calcium after BzATP (open circles). Open squares represent control experiments in which cells were pretreated with BAPTA-AM and calcium-free solution introduced for 2 min but without BzATP stimulation. Each point represents the mean ± S.E. from three separate experiments in which at least 20 cells were averaged. B, cell death does not occur following brief BzATP stimulation in the presence or absence of calcium. Histograms show number of viable cells 12-h post-BzATP stimulation (expressed as % matched controls). Brief stimulation in normal solution (but not in calcium-free solution) led to a significant increase in cell proliferation over the subsequent 12 h. The prolonged stimulation (40 min) resulted in almost complete loss of cells subsequently: this was partially prevented by incubation with zVAD-FMK. n = 3–6 for each case.

FIGURE 4. PS flip is associated with calcium-dependent but not calcium-independent blebbing. A, kinetics of annexin-V binding and cell blebbing (upper and lower graphs, respectively) in response to a 2-min application of BzATP in normal solution (filled circles), in calcium-free solution with cells pretreated with BAPTA-AM and with reintroduction of external calcium after BzATP (open circles). Open squares represent control experiments in which cells were pretreated with BAPTA-AM and calcium-free solution introduced for 2 min but without BzATP stimulation. Each point represents the mean ± S.E. from three separate experiments in which at least 20 cells were averaged. B, cell death does not occur following brief BzATP stimulation in the presence or absence of calcium. Histograms show number of viable cells 12-h post-BzATP stimulation (expressed as % matched controls). Brief stimulation in normal solution (but not in calcium-free solution) led to a significant increase in cell proliferation over the subsequent 12 h. The prolonged stimulation (40 min) resulted in almost complete loss of cells subsequently: this was partially prevented by incubation with zVAD-FMK. n = 3–6 for each case.

experiments performed in calcium-free solution on cells preloaded with BAPTA-AM, showed a delay to initial onset of blebbing comparable to that observed in experiments presented in Fig. 3, but no significant annexin V binding was observed at any time point after calcium was reintroduced (Fig. 4A). Interestingly, retraction of these blebs was much delayed; minimal bleb retraction had occurred 20 min after wash into calcium-containing solution although virtually all blebs had retracted within 3 h of wash (Fig. 4A). Subsequent cell death was also not observed under these circumstances although the increased rate of cell proliferation observed in normal solution was not seen (Fig. 4B). We consistently observed membrane blebbing without associated PS flip only when both removal of external calcium and chelation of intracellular calcium with BAPTA-AM loading were carried out (n = 9). When either of these manipulations were carried out separately, membrane blebbing was consistently observed (as described above) but annexin V binding was highly variable and inconsistent (n = 6). As expected (1, 3, 10), prolonged application of BzATP (40 min with calcium) led to death of all cells within 12 h (Fig. 4B). The cell death subsequent to prolonged BzATP application was significantly, but not completely, abrogated by incubation with zVAD-FMK (Fig. 4B). It is most likely that the zVAD-FMK-insensitive cell death following prolonged P2X<sub>7</sub> receptor activation results from necrotic, rather than apoptotic, cell death, and is in keeping with previous studies showing that prolonged activation of P2X<sub>7</sub> receptors can be associated with either or both apoptotic and necrotic cell death (1–3).

Rapid and Reversible Mitochondrial Changes: Depolarization and Swelling without Cytochrome c Release—Electron microscopic examination of unstimulated HEK293 cells showed typical mitochondrial morphology with clear double membranes and internal cisternae (Fig. 5A); dramatic global mitochondrial swelling with loss of cisternae were observed within 2 min of P2X<sub>7</sub> receptor activation (Fig. 5B). We also used confocal microscopy to follow the kinetics of these mitochondrial changes by labeling cells with Mitotracker Green, which accumulates in mitochondria independent of mitochondrial membrane potential, and used this to monitor changes in mitochondrial mass. In resting cells, Mitotracker Green labeled multiple mitochondria ~1-μm wide, extending as long filaments throughout the cytoplasm (Fig. 5C). Mitochondria began to round up and swell within 5–15 s of BzATP applica-
tion (Fig. 5C). Global mitochondrial swelling of this extent has been considered a trigger for cell death and a terminal event in several cell types (24, 25, 40, 41), but we found that 1–3 h after brief (2 min) activation of P2X7 receptors, the majority of mitochondria were no longer swollen and had recovered their original filamentous morphology (Fig. 5C). Changes in intramitochondrial free calcium and mitochondrial membrane potential \( (\Psi_{m}) \) during P2X7 receptor activation were measured using Rhod-2 and JC-1 dyes, respectively (Fig. 6A). A rapid increase in Rhod-2 fluorescence occurred within 5 s of receptor activation, whereas a decrease in JC-1 fluorescence began within 5–10 s (Fig. 6A). Thus, mitochondrial depolarization, increased intramitochondrial calcium, and mitochondrial swelling occur in <10 s of maximal receptor activation. None of these mitochondrial effects were altered by inhibition of the mitochondrial permeability transition pore using cyclosporin A (1 \( \mu \)M pretreatment at 37 °C for 1 h, \( n = 7 \)). The caspase inhibitor zVAD-FMK (100 \( \mu \)M for 1 h at 37 °C) delayed the onset of mitochondrial disruption (from <10 s in control to 24 ± 4 s after zVAD-FMK, \( n = 5 \)), but no other obvious inhibition of mitochondrial disruption was noted. Neither cyclosporin A, nor zVAD-FMK, altered the P2X7 receptor-mediated membrane blebbing (Fig. 3A). However, removal of extracellular calcium completely prevented mitochondrial swelling (Fig. 6C).

Finally, we asked whether cytochrome c release was associated with these rapid cytoskeletal and mitochondrial rearrangements by immunohistochemistry and immunoblotting (Fig. 7). Fig. 7A shows cytochrome c immunostaining (green) with a nuclear counterstain (DAPI, blue). In control cells and after 2 min in the presence of BzATP, all staining was confined to mitochondria even though gross mitochondrial swelling was obvious after 2 min of stimulation (Fig. 7A, middle panel inset). However, after 30 min in BzATP cytochrome c staining was now diffuse throughout the cytoplasm, and many of the swollen mitochondria could be seen to be ruptured (Fig. 7A, lower panel, arrowheads in inset). Ruptured mitochondria were not observed in cells stimulated for only 2 min. Semiquantitative analysis was carried out by Western blot (Fig. 7, B–D). No detectable release of cytochrome c occurred when P2X7 receptors were stimulated for 1, 2, or 5 min, whereas after 60 min of continuous stimulation, total cellular cytochrome c was now found in the cytosolic fraction.

**DISCUSSION**

Here we have identified a sequence of events that we refer to as pseudoapoptosis, which is induced by brief activation of P2X7 receptors by extracellular ATP. Pseudoapoptosis is an appropriate term because an almost complete repertoire of extranuclear events classically associated with apoptosis is observed: mitochondrial depolarization with gross mitochondrial swelling, rapid increase in both cytosolic and mitochondrial calcium, PS flip, actin filament disruption, and zonotic membrane blebbing. Despite these dramatic mitochondrial and cytoskeletal alterations, subsequent cell death does not occur, and all events are
P2X<sub>7</sub> Receptors and Pseudoapoptosis

**FIGURE 7.** Cytochrome c release occurs only with prolonged receptor stimulation. A, immunolocalization of cytochrome c (green) with nuclear counterstain using DAPI (blue) before, 2 min, and 30 min in the presence of BzATP as indicated. Cytochrome c remains localized to the swollen mitochondria at 2 min but at 30 min is diffuse throughout the cytoplasm as well as in the mitochondria; arrowheads in inset at 30 min of stimulation show ruptured mitochondria. B, Western blot detection of cytoplasmic cytochrome c and corresponding β-actin in the absence and presence of BzATP for times as indicated. C, summary of all experiments as illustrated in B; results are plotted as a ratio of β-actin level for each case; each time point represents 5–7 separate experiments. D, summary of separate set of experiments as in B but compared in each case to total cellular cytochrome c rather than β-actin levels; each value represents 5–11 separate experiments. Asterisks represent significant difference from control.

**FIGURE 8.** Possible mechanisms underlying P2X<sub>7</sub>-R-induced pseudoapoptosis and apoptosis. A, summary of sequence of events associated with the calcium-dependent and -independent pathways determined in the present study. Solid lines represent events we have shown directly in this study; we do not know whether cytochrome c release is calcium-independent but we have shown it occurs only after prolonged stimulation and therefore have placed this under the slower pathway. B, proposed mechanisms underlying reversible (pseudoapoptosis) and irreversible (apoptosis) actions of P2X<sub>7</sub> receptor activation. Here we propose that rapid loss of lipid asymmetry (PS flip) directly underlies cytoskeletal protein disruption and zoetic membrane blebbing. We hypothesize that PS flip is induced by extremely high calcium levels at the plasma membrane because of both calcium influx through the P2X<sub>7</sub> ion channel and mitochondrial calcium release. This pathway is associated with mitochondrial swelling but not with cytochrome c release or subsequent cell death. Prolonged stimulation does result in cytochrome c release, which triggers the well known calcium-independent caspase-3/7-ROCK1 signaling cascade and subsequent cell death. Both pathways are likely to interact with each other, and both pathways may be activated simultaneously under different physiological conditions. See text for further discussion.
onophore, carbonyl cyanide m-chlorophenylhydrazone (CCCP), has been shown to induce mitochondrial permeability transition and mitochondrial swelling without cytochrome c release or subsequent cell death (45, 46). There is little doubt that release of intramitochondrial cytochrome c into the cytoplasm leads to apoptotic cell death by binding to APAF and procaspase-9 to form the so-called apoptosome, which in turn leads to activation of the caspase cascade (25). On the other hand, there is much debate as to how cytochrome c is released from mitochondria, with at least five distinct models currently proposed (25). Two of these models involve mitochondrial swelling and loss of mitochondrial membrane potential leading to closure of the voltage-dependent anion channel (VDAC) or opening of the permeability transition pore (PTP) although it should be noted that the strongest evidence for these models of cytochrome c release comes from experiments on isolated mitochondria and other organelles (47). Our present results, together with those of Nagley and co-workers (46, 47), fairly conclusively rule out either of these two mechanisms as a primary means for mitochondrial release of cytochrome c in living cells. Remaining models involve formation of a Bax or Bax/VDAC channel or a type of lipid-protein pore (25, 48).

All P2X receptors are integral plasma membrane protein ion channels activated by extracellular ATP. They are all cation selective with high permeability to calcium ions, but only P2X2 receptors couple to the downstream events described in this and previous studies. The intracellular C terminus of the P2X2 receptor is some 100–200 amino acid residues longer than the other six P2X subunits, and an intact C terminus is required for downstream signaling (but not for ion flux through the channel). Proteomic analysis has identified protein-protein interactions between the P2X2 receptor and several cytoskeletal and signal transduction proteins (3, 49). Taken together with the present results, we propose the following model that may account for the P2X2,R-induced pseudoapoptosis elicited by brief agonist application and for the cell death that follows prolonged agonist application (Fig. 8). This model may also be relevant to at least some forms of apoptotic and/or necrotic cell death elicited by other stimuli.

We propose that mitochondria may be tethered to P2X2 receptors by direct or indirect protein-protein interactions with cytoskeletal proteins. Activation of P2X2 receptors leads to influx of extracellular calcium through the ion channel and rapid mitochondrial calcium increase, resulting in a localized calcium overload at the plasma membrane-mitochondria-cytoskeleton matrix. This leads to PS flip, either by activation of calcium-dependent flipase-like enzymes, or by calcium-induced lipid-protein interaction, perhaps between PS and VDAC or one of its interacting proteins. The consequent loss of lipid topology disrupts the normal interaction between cytoskeletal proteins and plasma membrane culminating in actin filament disruption and induction of zonotropic membrane blebbing. Because cytochrome c is still retained within the swollen, depolarized mitochondria, no caspase cascade to cell death occurs, and all events reverse as calcium levels fall and lipid-protein homeostasis is re-established. However, prolonged stimulation leads to development of mitochondrial channels (Bax or Bax/VDAC) or lipid-protein pores, which release cytochrome c and thus initiate apoptosis formation with classical apoptotic ROCK-1-dependent downstream signaling. What controls this shift from cytochrome c retention to release during P2X2 receptor activation is unknown, but our results suggest it is initiated by a distinct signaling pathway independent of the rapid, initial mitochondrial calcium rise and plasma membrane lipid-protein alterations, which we propose as the primary mechanism underlying P2X2,R-induced pseudoapoptosis. We base this suggestion on our findings that the ROCK-1-dependent blebbing was clearly observed only when both calcium influx and intracellular calcium rises were blocked, and because this calcium-independent blebbing was not associated with PS flip. Thus, in our model, PS flip is the initial upstream effector underlying P2X2,R-induced pseudoapoptosis whereas it is only a late downstream consequence of the cytochrome c-based apoptotic cascade resulting from prolonged receptor activation.

Apoptotic membrane blebbing has often been distinguished from other types of blebbing by its zonotropic nature; that is, the process of continuous and dynamic protrusions and retractions, in contrast to necrotic membrane blebbing, which is characterized by blebs that do not retract but gradually enlarge to diameters >10–20 μm (33). In the presence of calcium, activation of P2X2 receptors induces zonotropic blebbing, albeit on a time scale at least an order of magnitude faster than the currently reported apoptotic zonosis (e.g. supplemental Movie 1 and Ref. 10). We and others (28, 34, 43, 44) have previously described membrane blebbing in response to activation of P2X2 receptors heterologously expressed in HEK cells or endogenously expressed in macrophage and other immune cells, and had assumed it was, indeed, an indication of forthcoming cell death. Results from the present study show that this is not the case for brief receptor activation. Whereas the physiological role(s) for the rapid, reversible actin filament rearrangements and subsequent zonosis induced by PS flip in response to brief P2X2 receptor activation are not known, previous studies have linked activation of this receptor to cell proliferation in human lymphocytes and to cell-cell fusion in other immune cells (8, 9, 50, 51), both processes of which require concerted cytoskeletal reorganization and regulated plasma membrane disruption. The pseudoapoptosis identified in the present study may provide a mechanism underlying P2X2,R-generated giant cell formation at sites of inflammation or cell proliferation during tumorigenesis.

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P2X<sub>7</sub> Receptors and Pseudoapoptosis

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