0026-895X/03/6303-706–713
MOLECULAR PHARMACOLOGY
U.S. Government work not protected by U.S. copyright
*Mol Pharmacol* 63:706–713, 2003

Vol. 63, No. 3
2033/1047485
*Printed in U.S.A.*



# Poly(ADP-ribose) Polymerase Activation and Changes in Bax Protein Expression Associated with Extracellular ATP-Mediated Apoptosis in Human Embryonic Kidney 293-P2X$_7$ Cells

LONG T. WEN, CHARLES C. CALDWELL, and AILEEN F. KNOWLES

*Microchemical Core Facility, Department of Biology (L.T.W.) and Department of Chemistry (A.F.K.), San Diego State University, San Diego, California; and Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland (C.C.C.)*

Received July 29, 2002; accepted December 10, 2002

This article is available online at http://molpharm.aspetjournals.org



## ABSTRACT

Extracellular ATP is a potent signaling factor that modulates a variety of cellular functions through the activation of P2 purinergic receptors. Extracellular ATP at higher concentrations exerts cytostatic as well as cytotoxic effects in a variety of cell systems, the mechanism of which is not fully understood. In this study, we used cultured human embryonic kidney (HEK) cells stably transfected with human P2X$_7$ receptors (HEK-P2X$_7$) to investigate the mechanism of ATP-induced cell death. The cytotoxic effects of ATP in HEK-P2X$_7$ cells were dose- and time-dependent, whereas ADP, AMP, and UTP had no effect. ATP treatment induced a significant increase in apoptotic HEK-P2X$_7$ cells as ascertained by the terminal deoxynucleotidyl transferase dUTP nick-end labeling technique and flow cytometry. An ATP-induced decrease in the pro-apoptotic *bax* gene expression was detected by apoptosis-related cDNA microarray analysis, which correlated with a decrease of Bax protein expression. Western blot analysis revealed that ATP treatment resulted in the processing of pro-caspase 3 to its active form and cleavage of the nuclear enzyme, poly(ADP-ribose) polymerase (PARP). Both ATP-induced molecular alterations in HEK-P2X$_7$ cells (i.e., decrease of Bax expression and increase of PARP cleavage) were blocked by the purinergic P2X$_7$ receptor antagonist oxidized ATP. In conclusion, we demonstrated the importance of the P2X$_7$ receptor in ATP induced cell death of HEK-P2X$_7$ cells, which seems to be independent of *bax* expression; however, the activation of caspases is required.



The ability of extracellular ATP to kill cells is well established, especially toward cells of the immune system and hepatocytes (Nicotera et al., 1986; for review see Apasov et al., 1995; Zoeteweij et al., 1996). Extracellular ATP activates two cell surface receptors, P2X and P2Y. P2X receptors are ATP-regulated (ligand-gated) ion channels and P2Y receptors are G protein-coupled heptahelical receptors (North and Barnard, 1997). P2X receptors are emerging as one of the most interesting new families of plasma membrane receptors. The P2X$_7$ receptor (previously called the P$_{2Z}$ receptor), one of the P2X receptors, is especially interesting in the context of cell death. It has been implicated in induction of apoptosis and necrosis in several cell lines such as mesangial, microglial, and dendritic cells (Schulze-Lohoff et al., 1998; Coutinho-Silva et al., 1999; Ferrari et al., 1999; Nihei et al., 2000). Activation of the P2X$_7$ receptor results in pore opening and cell membrane permeabilization when ATP is continuously present (Surprenant et al., 1996; Rassendren et al., 1997). Although the mechanism of pore formation by the P2X$_7$ receptor is not completely understood, they allow the exchange of ions and small molecules of up to 900 Da.

Recent progress in apoptosis research has delineated the general pathways; however, the mechanisms of ATP-mediated cytotoxicity vary with cell types. ATP-dependent cytotoxicity in inflammatory cells was absolutely dependent on P2X$_7$ expression and involved dramatic alterations in plasma membrane permeability as well as DNA degradation into nucleosome-sized fragments that are typical in necrotic or apoptotic cell death (for review, see Chow et al., 1997). In macrophages and macrophage-like cell lines, the activation of P2X$_7$ receptor resulted in the induction of interleukin-1$\beta$ (IL-1$\beta$) release (Humphreys and Dubyak, 1996; Solle et al.,

This work was supported by a grant from the California Metabolic Research Foundation (to A.F.K).

**ABBREVIATIONS:** IL, interleukin; HEK, human embryonic kidney; HEK-P2X$_7$, human embryonic kidney 293 cells stably transfected with human P2X$_7$ cDNA; oxATP, periodate oxidized ATP; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; DMEM, Dulbecco's modified Eagle's medium; KN-62, 1-(*N*,*O*-bis[5-isoquinolinesulfonyl]-*N*-methyl-L-tyrosyl)-4-phenylpiperazine; bzATP, 3′-*O*-(4-benzoyl)benzoyl-ATP; PARP, poly(ADP-ribose) polymerase; Z-DEVD.fmk, *N*-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone; Z-FA.fmk, *N*-benzyloxycarbonyl-Phe-Ala-fluoromethyl ketone; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; FACS, fluorescence-activated cell-sorting; HPLC, high-performance liquid chromatography.

2001), which could be the result of caspase 1 activation (Schulze-Lohoff et al., 1998; Laliberte et al., 1999). However, the relationship between P2X$_7$ receptor stimulation, pro–IL-1$\beta$ cleavage by caspase 1, and cell death has not been clarified. Reduced cell death in monocytes and microglia from animals lacking caspase 1 has been reported (Kuida et al., 1995; Ferrari et al., 1999). On the other hand, it has also been shown that apoptosis in macrophages is not affected by caspase 1 inhibition (Nett-Fiordalisi et al., 1995; Perregaux and Gabel, 1998). Furthermore, prolonged stimulation of P2X$_7$ receptors by ATP activates the stress-activated protein kinase in human macrophages via a pathway independent of caspase 1- or caspase 3-like proteases (Humphreys et al., 2000). Therefore, the molecular mechanisms underlying the cytotoxic effects of extracellular ATP via the P2X$_7$ receptor in these cells are not fully understood.

Nevertheless, the induction of apoptotic cell death in many cell types is controlled by caspases (Thornberry and Lazebnik, 1998). These death proteases are part of a proteolytic caspase cascade that is activated by diverse apoptotic stimuli from outside and inside of the cell. An important family of the up-stream regulators of caspases is represented by Bcl-2 and its homologs, such as Bax (reviewed in Chao and Korsmeyer, 1998; Zamzami et al., 1998). Whereas Bcl-2 family clearly governs a cell death commitment step in many cells, Bcl-2–independent pathways for caspase activation and apoptosis induction also exist (reviewed in Vaux and Strasser, 1996). At present, the factors that determine Bax dependence and the molecular mechanism(s) by which Bax and its homologs exert their death-promoting function are also largely unknown.

Human embryonic kidney (HEK) 293 cells transfected with P2X$_7$ cDNA (HEK-P2X$_7$) have been used as a model system in the investigation of ATP-induced cytotoxicity. They were shown to undergo apoptosis when exposed to ATP by morphological and biochemical analysis (Ferrari et al., 1997). ATP-induced dye uptake and cation flux in these cells and the antagonistic effects of the isoquinolines have been reported (Humphreys et al., 1998). However, the molecular events leading to apoptotic cell death in HEK-P2X$_7$ after ATP treatment were not investigated. In this article, we report that extracellular ATP inhibits the expression of the pro-apoptotic-related gene Bax, as well as inhibiting the growth of HEK-P2X$_7$ cells, which is accompanied by the proteolytic processing of caspase 3 and its substrate PARP. Decreased expression of *bax*, activation of caspase 3, and cleavage of PARP are abolished by the P2X$_7$ receptor antagonist oxidized ATP (oxATP). These results suggest that P2X$_7$ receptor ligation causes Bax-independent activation of caspase 3 cascade, the latter being required for apoptotic alterations during ATP-induced cell death.

## Materials and Methods

**Materials.** Cell culture medium, Dulbecco's modified Eagle's medium (DMEM) and newborn and fetal calf sera were purchased from Invitrogen (Carlsbad, CA). All nucleotides, adenosine, uridine, dipyridamole, $N^6$-cyclohexyladenosine, 5-(*N*-cyclopropyl) carboxamidoadenosine, 1-(*N*,*O*-bis[5-isoquinolinesulfonyl]-*N*-methyl-L-tyrosyl)-4-phenylpiperazine (KN-62), 3′-*O*-(4-benzoyl)benzoyl-ATP (bzATP) and periodate oxidized ATP were obtained from Sigma (St. Louis, MO). 2-Chloro-$N^6$-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide was from Tocris (Ellisville, MO). Z-DEVD.fmk, Z-FA.fmk, and *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) were from Calbiochem (La Jolla, CA).

**Cell Culture.** Wild-type HEK 293 cells and 293 cells stably transfected with the human P2X$_7$ receptor (HEK-P2X$_7$ cells) (kindly provided by Dr. G.R. Dubyak, Case Western Reserve University) were maintained in DMEM supplemented with 5% newborn calf serum and 5% fetal calf serum and antibiotics (50 U/ml penicillin and 50 $\mu$g/ml streptomycin). Hygromycin (25 $\mu$g/ml) was also added to the media of HEK-P2X$_7$ cells. Cells were grown in 24-well or 10-cm diameter culture plates in a humidified atmosphere of 5% $CO_2$ at 37°C. Confluent HEK-P2X$_7$ cells were washed twice with DMEM without serum, and cultured in the absence or presence of ATP and other nucleotides. The presence of the P2X$_7$ receptor was confirmed using reverse transcriptase-polymerase chain reaction and Northern blot analysis.

**cDNA Microarray Analysis.** Total cellular RNA was prepared from HEK-P2X$_7$ cells cultured in the presence or absence of 6 mM ATP for 15 h. Apoptosis-related cDNA microarrays were obtained from Super Array Inc. (Bethesda, MD). Ten micrograms of total RNA were used as template for $^{32}$P-labeled cDNA probe synthesis. The RNA was first annealed with specific primer mix of apoptosis-related genes and the cDNA probes were synthesized according to the protocols provided by the manufacturer. The cDNA probes were denatured and hybridized with the cDNA array. Hybridization was carried out at 68°C overnight. The membrane was washed twice with wash solution (2× standard saline citrate, 1% SDS) for 20 min at 68°C. The membrane was wrapped in a plastic wrap and exposed to X-ray film with an intensifying screen at −70°C, developed or scanned by a PhosphorImager 344 (Amersham Biosciences, Piscataway, NJ). Each array is composed of 23 marker genes in duplicates, which include two positive controls, $\beta$-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and a negative control, bacterial plasmid pUC18. Signal intensities were quantified using a computing densitometer scanner and ImageQuant ver. 3.3 (Amersham Biosciences). The relative amount of a given gene transcript was estimated by comparing its signal intensity with those derived from GAPDH and $\beta$-actin.

**Cytotoxicity Assays.** The effect of nucleotides on the proliferation of HEK-P2X$_7$ cells was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) bioassay (Cory et al., 1991). Cells were cultured without or with various concentrations of nucleotides at different time intervals. The cells were then exposed for 2 to 3 h to MTT tetrazolium salt with phenazine methosulfate. Cell viability was determined by colorimetric assay for the activities of mitochondrial dehydrogenases, which convert MTT into a color-dense formazan. Absorbance was determined by dissolving the insoluble dye with dimethyl sulfoxide, and the absorbance was measured at 550 nm with a reference wavelength of 630 nm. At least three independent experiments were performed.

**Immunoblotting Analysis.** Cells were lysed and protein extraction was performed. Protein concentrations of the cell lysates were determined by the detergent-compatible protein assay (Bio-Rad, Hercules, CA). Cell proteins (25 $\mu$g) were separated in 10 to 13% SDS-polyacrylamide gel and electrophoretically transferred to polyvinylidene difluoride membrane (PerkinElmer Life Science, Boston, MA). The membranes were blotted with 5% nonfat milk, washed, and subsequently incubated with the corresponding primary antibodies, as indicated: mouse monoclonal antibodies against Bax (1:1000 dilution; Santa Cruz Biotech, Santa Cruz, CA), Bcl-2 (1:500 dilution; DAKO, Carpenteria, CA), PARP (1:1000 dilution; Santa Cruz Biotech), and a rabbit polyclonal antibody against caspase 3 (1:1000 dilution; Santa Cruz Biotech). After washing, the membrane was incubated with horseradish peroxidase-conjugated sheep anti-mouse antibody or donkey anti-rabbit antibody (Amersham Biosciences) and then visualized by enhanced chemiluminescence according to the manufacturer's recommendations (Amersham Biosciences).

**TUNEL Assay.** Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was performed using an in situ

cell detection kit, BrdUTP-FragEL, according to the manufacturer's instructions (Calbiochem, San Diego, CA). The cells were plated on chamber slides (Lab-Tek, Naperville, IL), grown overnight, and then treated with ATP or not. Cells were washed, fixed with 4% paraformaldehyde in phosphate-buffered saline solution, and incubated with hydrogen peroxide to block endogenous peroxidase. After rinsing, the cells were incubated with the TUNEL reaction mixture. Thereafter, the cells were washed and incubated with biotinylated monoclonal anti-bromodeoxyuridine and a streptavidin-horseradish peroxidase conjugate, followed by incubation with diaminobenzidine substrate solution and then counterstained with methyl green stain.

**Flow Cytometry.** HEK-P2X$_7$ cells were incubated with 0, 2, 4, or 6 mM ATP for 16 h, after which all the cells in each well were collected in tubes. Just before FACS collection, 10 $\mu$l of 50 $\mu$g/ml propidium iodide was added to the cell suspension. Flow cytometry data acquisition was done on a FACScan (BD Biosciences, San Jose, CA), whereas the FACS analysis was accomplished using the CellQuest FACS research software (BD Biosciences).

**HPLC Separation of Adenosine and Adenine Nucleotides.** Nucleotides were separated and quantified by HPLC ("Millennium" system; Waters, Milford, MA) via a DNApac PA-100 anion exchange column (4 × 250 mm; Dionex) using a 45-min linear gradient developed from 20 mM sodium phosphate, pH 7.0, to 20 mM sodium phosphate with 1 M sodium chloride, pH 7.0. The method allowed clear separation and identification of ATP, ADP, AMP, and adenosine. Peaks in the eluates were identified by comparison with known standards for their characteristic retention times.

**Statistical Analysis.** Statistical comparison was done by the Student's *t* test. Data are expressed as means ± S.D. *P* values less than 0.05 were considered significant.

## Results

**ATP-Induced Cytotoxicity in HEK-P2X$_7$ Cells.** Because it has previously been reported that millimolar concentrations of extracellular ATP are required to activate the P2X$_7$ receptor (Dubyak and El-Moatassim, 1993; Fredholm et al., 1994; Di Virgilio, 1995), we characterized the dose dependence of ATP-induced cytotoxicity in HEK-P2X$_7$ cells. HEK-P2X$_7$ cells were treated with different concentrations of ATP at various time intervals. Cell viability was quantified by the MTT colorimetric assay. Figure 1 shows that ATP, up to 10 mM, had no effect on cell viability if the incubation was carried out for only 2 h. With longer incubation, ATP caused a time- and dose-dependent decrease of the viability of HEK-P2X$_7$ cells. The effective concentration for 50% reduction (EC$_{50}$) of HEK-P2X$_7$ cell viability is 5 mM after 15 h of ATP treatment. Thus, the concentration dependence is similar to that observed for the P2X$_7$-associated permeabilization in macrophages and other cells. In the following experiments, 6 mM (higher than EC$_{50}$) ATP was used as the standard concentration.

**ATP-Induced Cell Death Is Caused by Apoptosis.** We conducted experiments to verify that ATP-induced cell death in HEK-P2X$_7$ was the result of apoptosis. Because apoptosis is characterized by internucleosomal degradation of genomic DNA, we used in situ TUNEL assay (Gavriell et al., 1992) and demonstrated that DNA cleavage had occurred and free 3′-OH groups were generated by cellular endonucleases in the ATP-treated cells (Fig. 2). A dark brown signal resulting from diaminobenzidine staining indicated nuclear DNA degradation, whereas shades of blue-green to greenish tan signify a nonreactive cell. HEK-P2X$_7$ cells, which express a high level of P2X$_7$ receptors, underwent some spontaneous cell death in cell culture (Fig. 2A). The number of TUNEL-positive cells increased with increasing ATP concentrations (Fig. 2, B to D). The results indicated that apoptosis was induced by ATP in HEK-P2X$_7$ cells.

The extent of cell death was also determined by flow cytometry. Cells were treated with 0, 2, 4, or 6 mM ATP and incubated for 16 h. The cells were then collected in FACS tubes without washing to collect both adherent cells and those cells that have become nonadherent. The collected cells were treated with propidium iodide to determine whether the cells were either apoptotic or necrotic. With the addition of 2, 4, and 6 mM ATP, the percentage of dead cells increased to 22.5, 40, and 58.7%, respectively (Fig. 2, bottom). In agreement with the TUNEL assay, the percentage of cells that were propidium iodide-positive increased with increasing amounts of exogenously added ATP. The results presented in Fig. 2, obtained by two independent assay methods, unambiguously indicated that apoptosis was induced by ATP in HEK-P2X$_7$ cells.

**Extracellular ATP Inhibits the Expression of *bax* Gene.** To define the molecular basis of the cytotoxic action of ATP, we examined the effect of ATP on the expression of apoptosis-related genes using apoptosis-specific cDNA microarray analysis. This approach profiles multiple genes simultaneously. Through side-by-side hybridization with cDNA probes prepared from RNAs of untreated and ATP-treated HEK-P2X$_7$ cells, the expression profiles of these genes under each condition were determined (Fig. 3). The bacterial plasmid pUC18, which should not be expressed in either untreated or ATP-treated HEK-P2X$_7$ cells, served as negative control (Fig. 3, spots 1G and 2G). ATP treatment of HEK-P2X$_7$ caused no changes in the expression of the housekeeping genes *β-actin* (spots 3G and 4G) and *GAPDH* (spots 5–8G, 8E, and 8F). Extracellular ATP caused a slight up-regulation of several genes in the HEK-P2X$_7$ cells [e.g., *mdm2* (spots 5E and 5F) and *pig8* (spots 7C and 7D)]. In contrast, Bax was significantly down-regulated (spots 1C and 1D). The expression of other cDNAs showed little or no change in ATP-treated cells.

To determine whether the down-regulation of *bax* expres-

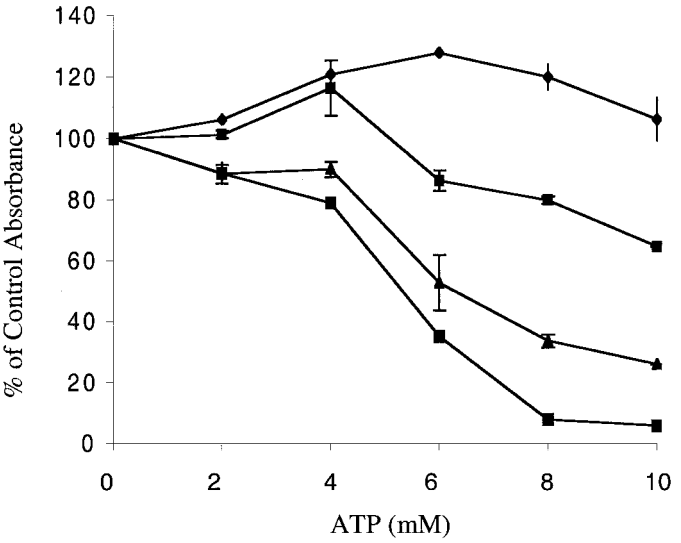


**Fig. 1.** Cytotoxicity of extracellular ATP in HEK-P2X$_7$ cells determined by MTT assay. Cells were treated with 0 to 10 mM ATP for 2 h (♦), 4 h (■), 7 h (▲), and 15 h (●). Results are expressed as percentages using values obtained from untreated cells as 100%.

sion correlates with an increase of ATP concentration, we have examined changes in protein levels of Bax in ATP-treated cells. Immunoblot analysis with Bax-specific antibody demonstrated that there was a significant decrease in Bax protein level 8 h after the addition of 6 mM ATP (Fig. 4A). Bax protein expression was reproducibly inhibited at 4 mM ATP post-treatment for 24 h (Fig. 4B). These results agreed with the decrease of *bax* gene expression in the microarray assays (Fig. 3). It is well known that proteins encoded by the *bcl-2* gene family play a major role in the regulation of apoptosis. It has been suggested that the balance between the levels of pro- and anti-apoptotic members of the Bcl-2 family plays an important role in the regulation of cell death or survival (Gajewski and Thompson, 1996; Michaelidis et al., 1996). Bcl-2 inhibits apoptosis induced by a variety of stimuli, whereas Bax promotes apoptosis. To determine whether the death signals induced by ATP can alter the inherent Bcl-2-to-Bax ratio, Western blot analysis of Bcl-2 expression in ATP-treated and untreated cells was performed. Figure 4C shows a decrease in the Bcl-2 level in ATP-treated HEK-P2X$_7$ cells (bottom). In contrast, the Bcl-2 protein level did not change significantly in the control cells over 24 h (top).

**Involvement of the P2X$_7$ Receptor in Cell Death and Reduction of Bax Expression.** The P2X$_7$ receptor has been shown to mediate ATP-induced apoptosis (Surprenant et al., 1996; Rassendren et al., 1997; Coutinho-Silva et al., 1999; Ferrari et al., 1999). We set out to determine whether the P2X$_7$ receptor is involved in the ATP-induced apoptosis of HEK-P2X$_7$ cells. First we confirmed the presence of the P2X$_7$ receptor in HEK-P2X$_7$ cells at the mRNA level with reverse transcriptase-polymerase chain reaction and Northern blot analysis. Using cDNA of HEK-P2X$_7$ cells as the template and the human P2X$_7$ receptor specific primers, a single PCR product was obtained. The amplified cDNA was subcloned and sequenced and found to encode the full-length P2X$_7$ receptor (data not shown). Northern blot analysis also verified the presence of the P2X$_7$ receptor transcripts (data not shown).

Because the ligand potencies for P2X$_7$ receptor are known (Surprenant et al., 1996; Rassendren et al., 1997; Coutinho-Silva et al., 1999; Ferrari et al., 1999), we tested the ability of different nucleotides other than ATP in inducing HEK-P2X$_7$ cell death. ADP, AMP, adenosine, and UTP were tested at 6 mM in all assays and their effects were compared with that of 6 mM ATP. Figure 5A shows that ATP selectively inhibited HEK-P2X$_7$ cell growth, whereas the other nucleotides had no effect. BzATP, which is a potent P2X$_7$ receptor agonist, induced cell death comparable to that of 6 mM ATP when used at 2 mM (Fig. 5B). These results, together with the fact that ATP had no effect on the viability of wild-type HEK 293 cells

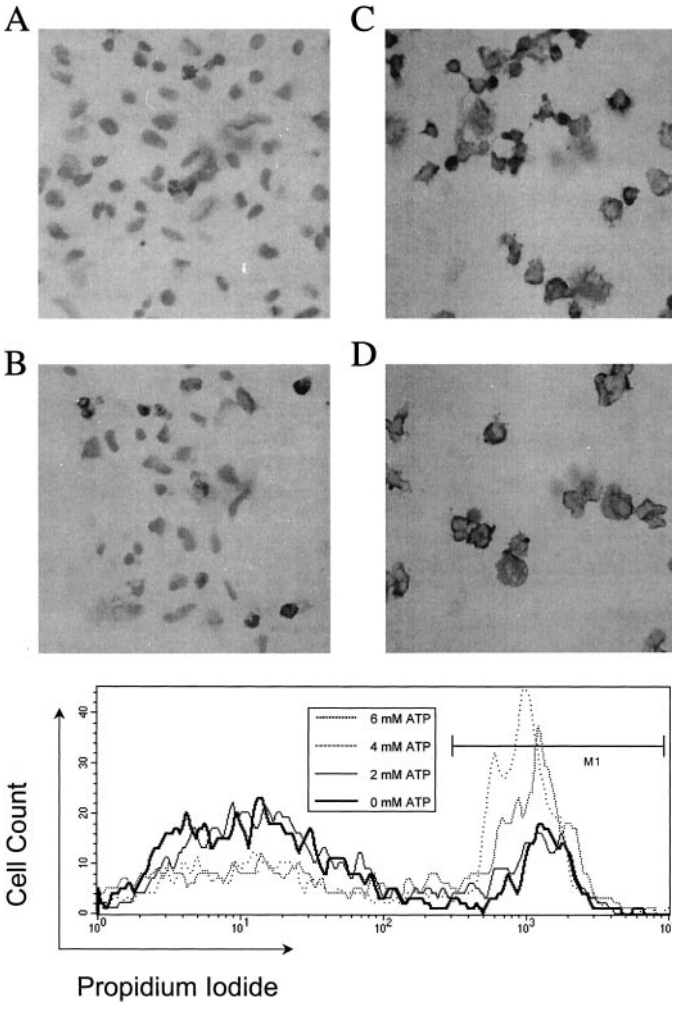


**Fig. 2.** Extracellular ATP induces apoptosis in HEK-P2X$_7$ cells. Top, in situ TUNEL assay for apoptosis. Cells were grown on Lab-Tek chamber slides and treated with the indicated concentrations of ATP for 16 h. In the TUNEL assay, diaminobenzidine reacts with the labeled cells to generate a brown product at the site of DNA fragmentation. Brown staining, therefore, indicates apoptotic cells. Cells were counterstained with methyl green to aid in the morphological evaluation of normal and apoptotic cells. Cells were examined by light microscopy. Magnification, 40×. A, no treatment; B, 2 mM ATP treatment; C, 4 mM ATP treatment; D, 6 mM ATP treatment. Bottom, flow cytometric analysis of HEK-P2X$_7$ cells treated with 0, 2, 4, and 6 mM ATP for 16 h. Cells were collected and stained with propidium iodide (PI) and analyzed as described under *Materials and Methods*. These data are representative of four independent experiments.

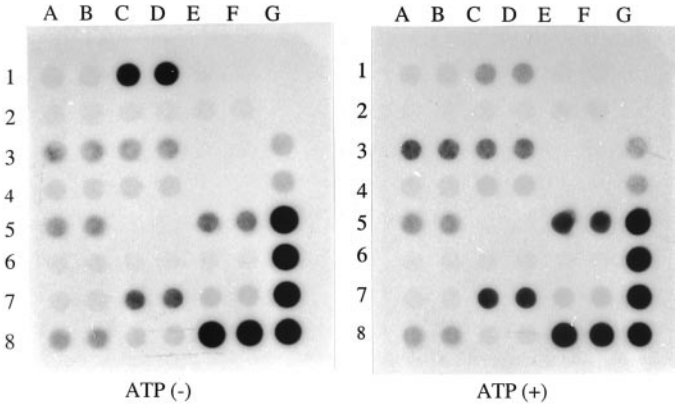


**Fig. 3.** ATP modulates the expression profile of cellular genes involved in apoptosis. HEK-P2X$_7$ cells were treated with or without 6 mM ATP for 15 h. RNA samples used for cDNA synthesis were prepared as described under *Materials and Methods*. Each gene is present on the arrays in duplicate. The expression of positive controls $\beta$-actin (3G, 4G), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 5G, 6G, 7G, 8G, 8E, and 8F) remained constant in the presence and absence of ATP. The negative control bacterial plasmid pUC18 (1G, 2G) was not expressed in either array. Bax mRNA (1C, 1D) was expressed at high level in the untreated cells, and ATP down-regulated this gene. A difference of >2-fold was detected between the untreated and ATP-treated cells. Mdm2 (5E, 5F) and pig8 (7C, 7D) were expressed at low levels, and ATP slightly up-regulated the expression of these genes.

(data not shown), indicated that ATP-induced cell death was specifically mediated by $P2X_7$ receptor of HEK-$P2X_7$ cells.

We then examined the effect of oxATP, described previously as a selective $P2X_7$ receptor antagonist that binds covalently and irreversibly to the receptor and inhibits its activation (Murgia et al., 1993). HEK-$P2X_7$ cells were first incubated with 0.25 and 1 mM oxATP for 2 h at 37°C. The cells were then incubated with or without ATP for 24 h. As shown in Fig. 5B, oxATP inhibited cell growth by itself when applied at 1 mM but not at 0.25 mM. Pretreatment of HEK-$P2X_7$ cells with 0.25 mM oxATP blocked ATP-induced cell death. Furthermore, we tested the effect of KN-62, which was shown recently to be a more potent inhibitor of $P2X_7$ receptor signaling in HEK-$P2X_7$ cells (Humphreys et al., 1998). Figure 5B shows that 3.5 $\mu$M KN-62, which had no deleterious effect on HEK-$P2X_7$ cells by itself, completely blocked the ATP-induced cell death of HEK-$P2X_7$ cells (Fig. 5B). These results support the conclusion that $P2X_7$ receptor is responsible for ATP-induced cell death.

To determine whether the effects of the nucleotides described above are accompanied by changes in Bax expression, immunoblot analysis was used to analyze the levels of Bax in these cells. Figure 6A shows that the expression of Bax in HEK-$P2X_7$ cells after AMP, ADP, and UTP treatment was similar to that in the untreated cells, whereas Bax could not be detected in ATP treated cells. More importantly, Fig. 6B shows that 0.25 mM oxATP, which significantly blocked the ATP effect on cell viability (Fig. 5B), also counteracted the inhibitory effect of ATP on Bax expression. These results indicate that the $P2X_7$ receptor present on HEK-$P2X_7$ cells is involved in the initiation of a series of events that result in reduction of cell growth as well as decrease of Bax expression.

**Activation of Caspase Cascade in ATP-Induced Cell Death.** Increased proteolysis of PARP, the substrate of

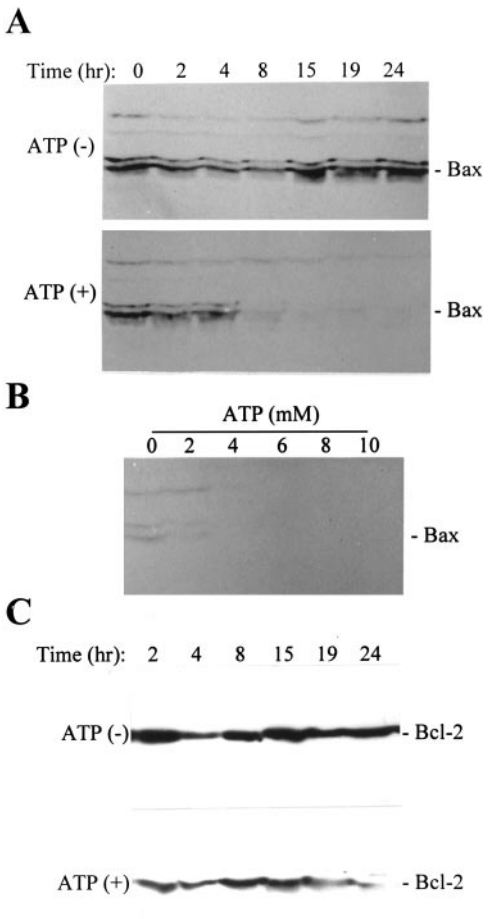


**Fig. 4.** Expression of Bax and Bcl-2 in untreated and ATP treated HEK-$P2X_7$ cells. A, cells were cultured with or without 6 mM ATP for 2, 4, 8, 15, 19, or 24 h, after which cells were harvested and lysed. Western blot analysis of cell lysates (25 $\mu$g of protein) was conducted with the Bax monoclonal antibody. B, cells were treated with various concentrations of ATP (0–10 mM). Expression of Bax protein was determined after 24 h. C, Western blots of the same cell lysates as described in A except immunoblotted with anti–Bcl-2 antibody.

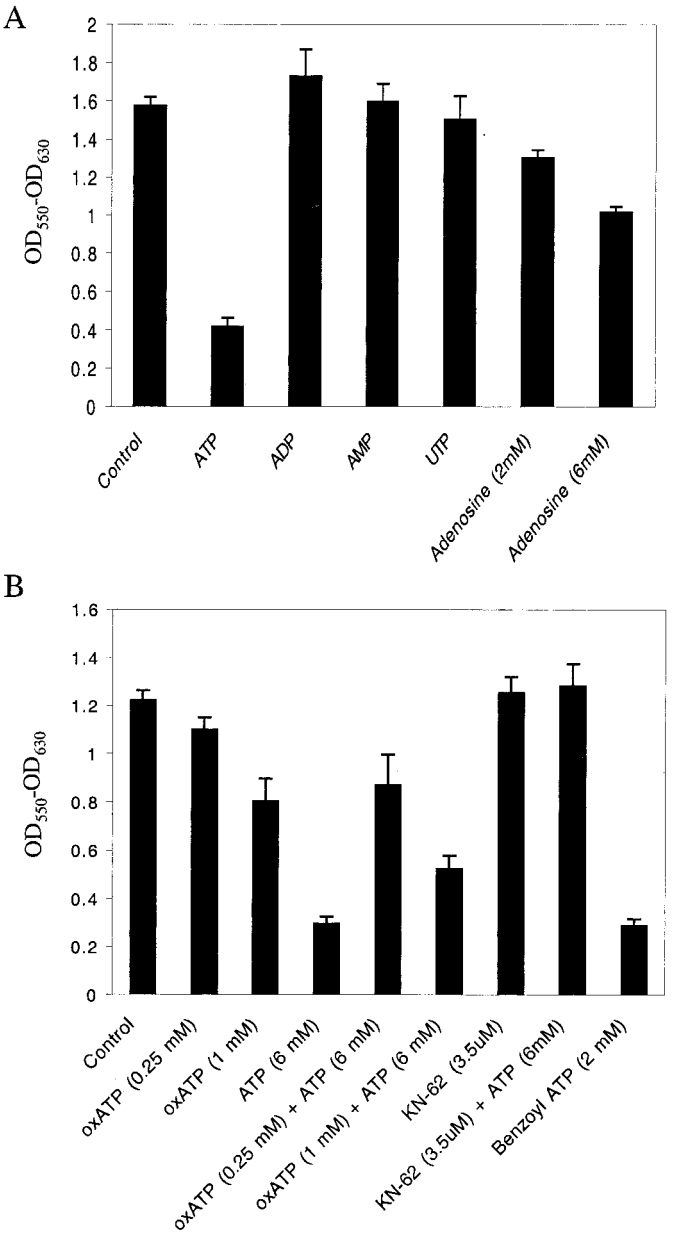


**Fig. 5.** Effect of extracellular nucleotides, adenosine, bzATP, KN-62, and oxATP on cell viability in HEK-$P2X_7$ cells. A, cells were either left untreated (control) or treated with 6 mM ATP, ADP, AMP, UTP, adenosine, or 2 mM adenosine for 24 h. Cell growth was determined by the MTT assay. B, HEK-$P2X_7$ cells were incubated at 37°C in the absence or presence of 0.25 mM, 1 mM oxATP or 3.5 $\mu$M KN-62. After 2 h, 6 mM ATP or 2 mM bzATP was added and incubation was carried out for an additional 22 h. Cell viability was determined by the MTT assay.

caspase 3/CPP32, has been shown to be an integral part of the apoptotic pathway (He et al., 1998). We used Western blot analysis to determine whether cleavage of PARP occurs during ATP-induced cell death of HEK-P2X$_7$ cells. Figure 7A demonstrates that PARP, a 117-kDa protein, was cleaved into the characteristic 89-kDa fragment after 15 h of treatment with 6 mM ATP. Cleavage of PARP into its 89-kDa fragment was also observed after 24-h treatment of HEK-P2X$_7$ cells by 2 mM ATP (Fig. 7B). PARP cleavage is usually attributed to caspase 3 activity. Caspase 3, like the other caspases, is synthesized as inactive zymogen, which can be converted to an active tetrameric complex composed of two heterodimeric subunits. As shown in Fig. 7C, there was a decrease in the proenzyme CPP32 levels in HEK-P2X$_7$ cells 24 h after ATP treatment, concomitant with the appearance of the proteolytically cleaved 17-kDa subunit. Importantly, Fig. 7C shows that 0.25 mM oxATP inhibited the action of ATP on the generation of the apoptosis-specific PARP cleavage fragment (89 kDa) and the caspase 3 17-kDa subunit.

To investigate whether ATP-induced cell death in HEK-P2X$_7$ cells is caspase 3-dependent, the effect of the caspase 3 inhibitor, Z-DEVD.fmk, was tested. Figure 8 shows that 350 $\mu$M Z-DEVD.fmk efficiently inhibited ATP-induced cell death by 56%. On the other hand, 350 $\mu$M Z-FA.fmk, a structural analog of Z-DEVD.fmk in which the amino acid sequence DEVD has been replaced, and ALLN, a proteasome inhibitor, did not affect ATP-induced cell death in HEK-P2X$_7$ cells. Taken together, these data are consistent with the conclusion that caspase 3 activation is required for ATP-mediated apoptosis of HEK-P2X$_7$ cells.

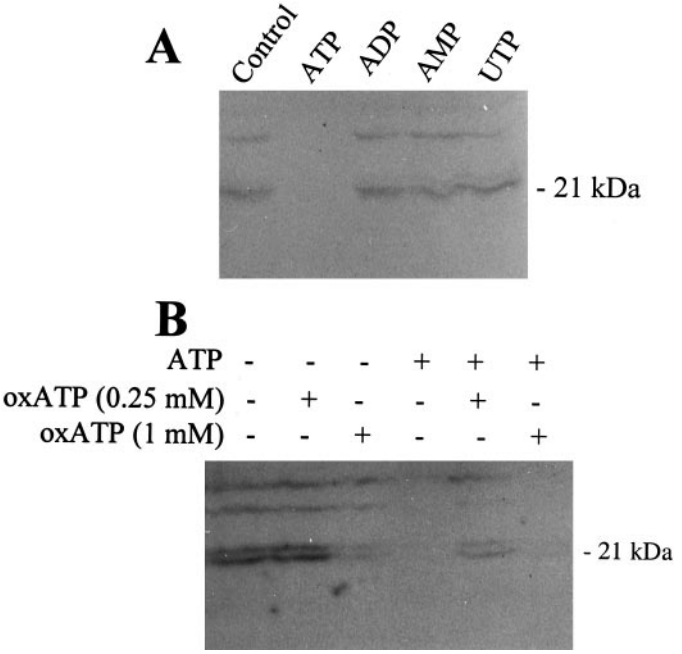


**Fig. 6.** Effect of extracellular nucleotides, and oxATP on *bax* expression in HEK–P2X$_7$ cells. A, cells were treated with 6 mM ATP, ADP, AMP, or UTP as described in the legend to Fig. 5A. Cellular lysates were prepared and subjected to immunoblotting using antibody against Bax. B, cells were pretreated with or without oxATP (0.25 and 1.0 mM), followed by addition of ATP (6 mM) as described in the legend to Fig. 5B. Subsequently, cell lysates were prepared and aliquots of the lysates were analyzed by Western blotting with antibody against Bax.

## Discussion

This study demonstrates that extracellular ATP induces apoptosis in HEK 293 cells stably transfected with P2X$_7$ cDNA. ATP treatment resulted in a significant increase in the number of cells bearing TUNEL-positive nuclei. This was accompanied by a marked increase of the 89-kDa product of PARP cleavage that resulted from caspase 3 activation. At the same time, expression of Bcl-2 and Bax was significantly reduced. The critical role of P2X$_7$ receptor in mediating these effects was substantiated by several findings. First, the effect of ATP on HEK-P2X$_7$ cell death was specific and other naturally occurring nucleotides were ineffective. Second, the inhibitors of P2X$_7$ receptor activation, oxATP and KN-62, completely inhibited ATP-induced cell death. It is known that extracellular ATP can also induce target cell lysis via P2Y receptors (Surprenant et al., 1996; Koshiba et al., 1997).

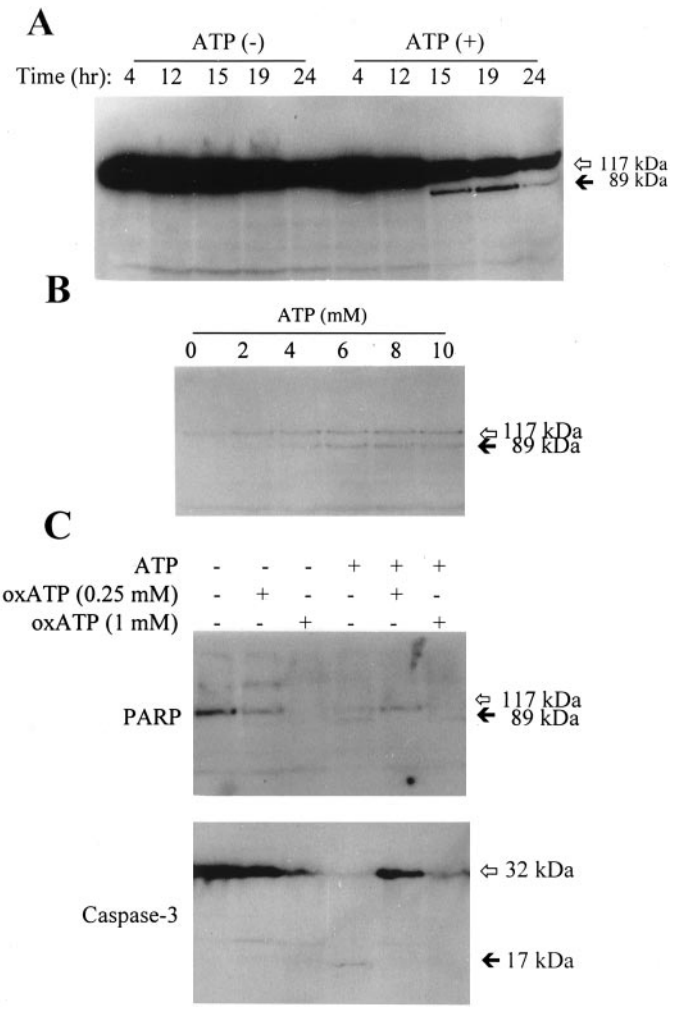


**Fig. 7.** ATP treatment causes cleavage of PARP and caspase-3. A, HEK-P2X$_7$ cells were treated with 6 mM ATP for the indicated times. Cellular lysates were prepared and subjected to immunoblotting using antibodies against the caspase substrate, PARP. B, cells were incubated for 24 h in the absence and presence of the indicated concentrations of the ATP. After SDS-PAGE of cellular lysates, full-length and cleaved PARP protein levels were determined by Western blot analysis with antibodies against PARP. C, cells were treated with 6 mM ATP in the absence or presence of oxATP as described in the legend to Fig. 5B. After SDS-PAGE of cellular lysates, processing of PARP (top) and caspase-3 (bottom) was analyzed. Open arrowheads, full-length forms of the proteins; closed arrowheads, cleaved forms of the proteins.

However, we found that UTP, an agonist of several P2Y receptor subtypes, was unable to induce apoptosis in HEK-P2X$_7$ cells.

Whereas ADP and AMP had no effect on the viability of HEK-P2X$_7$ cells, treatment of these cells with 6 mM adenosine resulted in ~20% decrease of viability (Fig. 5A). This raised the question of whether adenosine, which could be generated from ATP by ecto-nucleotidase activities, contributed partly to the apoptotic effect of ATP. This possibility was ruled out by the following experiments. 1) Selective agonists for the $A_1$, $A_2$, and $A_3$ receptors [i.e., $N^6$-cyclohexyladenosine, 5-(*N*-cyclopropyl)carboxamidoadenosine, and 2-chloro-$N^6$-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide] had no effect on cell viability when used at 200 $\mu$M (data not shown), indicating that adenosine receptors are not involved in ATP-induced cell death in HEK-P2X$_7$ cells. 2) The apoptotic effect of ATP was affected by neither dipyridamole, an inhibitor of nucleoside transporter, nor uridine, ruling out the possibility that adenosine acts intracellularly by causing a nucleotide imbalance (pyrimidine starvation). 3) More importantly, HPLC analysis of adenine metabolites in the culture media of cells treated for 24 h by ATP and ADP showed that similar amounts of adenosine were produced (~20%). However, ADP had no effect on cell viability (Fig. 5A). Taken together, the data suggest that the limited amounts of adenosine, which is generated from ATP by ecto-nucleotidase activities, is not involved in ATP-induced apoptosis in HEK-P2X$_7$ cells. However, it is possible that higher concentrations of adenosine (i.e., 6 mM in Fig. 5A) are cytotoxic to HEK-P2X$_7$ cells after being transported into the cells. This seems to be the case because dipyridamole completely abolished the cytotoxic effect of 6 mM adenosine (data not shown).

We conclude that the P2X$_7$ receptor mediates the apoptotic responses of HEK-P2X$_7$ cells to extracellular ATP. Similar to the findings in dendritic cells and microglial cells (Ferrari et al., 1997; Coutinho-Silva et al., 1999), ATP-induced apoptosis in HEK-P2X$_7$ cells clearly involved the activation of caspases, as evidenced by proteolysis of caspase 3 and its substrate PARP. Further supportive evidence that caspase 3 activation and PARP cleavage play a crucial role in ATP-induced cell death was provided through the use of a caspase 3 inhibitor (Fig. 8) that effectively abolished ATP-induced HEK-P2X$_7$ cell death.

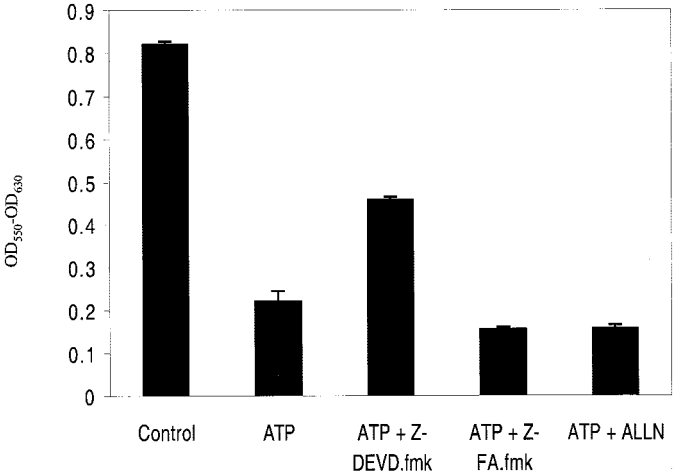


**Fig. 8.** Effect of caspase-3 inhibitor on cell viability in HEK-P2X$_7$ cells. HEK-P2X$_7$ cells were incubated at 37°C in the absence or presence of 350 $\mu$M caspase-3 inhibitor Z-DEVD.fmk, caspase inhibitor negative control Z-FA.fmk, and a proteasome inhibitor, ALLN. After 2 h, 6 mM ATP was added and incubation was carried out for an additional 22 h. Cell viability was determined by the MTT assay.

On the other hand, the effect of extracellular ATP on the expression of *bax* in HEK-P2X$_7$ cells was unexpected. Both Bcl-2 and Bax proteins were detectable in the HEK-P2X$_7$ cells under our culture conditions (Figs. 3 and 4). A sustained expression of *bax* may have contributed to the significant spontaneous cell death of HEK-P2X$_7$ cells and was expected to increase, whereas that of *bcl-2* might decrease in ATP-treated cells. However, 4 h of 6 mM ATP treatment markedly reduced the expression of both genes. Bcl-2 and Bax seem to have intrinsic independent functions as effectors of survival and death, respectively. Although Bcl-2 and Bax may be capable of functioning independently, it is also clear that the Bcl-2/Bax ratio dictates the relative sensitivity or resistance of cells to a wide variety of apoptotic stimuli (Oltvai and Korsmeyer, 1994). The fact that *bcl-2* expression was also decreased in ATP-treated cells (Fig. 4C) can be interpreted to mean that the down-regulation of both *bax* and *bcl-2* expression may play a role in fine-tuning the induction of HEK-P2X$_7$ cell death during ATP treatment. Similar decrease of Bcl-2 and Bax during apoptosis has been reported previously (Basnakian and James, 1994). These results and our own lend support to recent findings that Bax is not always required for promoting apoptosis. Using $bax^{-/-}$ mice, it was shown that *bax* expression was neither required nor sufficient for $\gamma$-irradiation- or dexamethasone-induced apoptosis (Kundson et al., 1995). Furthermore, it has been shown that Bcl-2-independent pathways for caspase activation and apoptosis induction also exist (Vaux and Strasser, 1996). In many types of cells, the tumor necrosis factor family of 'death receptors' activates apoptosis via a Bcl-2-independent pathway (Vaux and Strasser, 1996). Similar tumor necrosis factor family receptors that contain 'death domains' within their cytosolic tails directly induce caspase activation through ligand-induced recruitment of cytosolic procaspases via interactions with adaptor proteins that bind the death domains of these receptors (Wallach et al., 1997; Yuan, 1997). In this scenario, ATP might simply interact with P2X$_7$ purinoceptor and trigger a cascade of proteolysis, involving processing and activation of the zymogen forms of downstream caspases (e.g., caspase 3), and bypass Bax. The identification of loss of expression of *bcl-2* and *bax* indicates that neither Bcl-2 nor Bax is involved in the activation of caspases in ATP-treated HEK-P2X$_7$ cells. Nevertheless, our results clearly indicate that their expression is regulated by extracellular ATP via the P2X$_7$ receptor. The physiological significance remains to be elucidated.

In summary, we have presented evidence to show that the P2X$_7$ receptor is essential for ATP-induced cell death of HEK-P2X$_7$ cells. More importantly, our studies showed that Bax-independent caspase 3 activation and PARP cleavage play an important role in P2X$_7$ receptor-mediated cell death.

**Acknowledgments**

L.T.W. acknowledges the Biology Department, San Diego State University for supporting the Microchemical Core Facility. We gratefully acknowledge Dr. George R. Dubyak of Case Western Reserve University for providing the HEK-P2X$_7$ cells.

## References


Apasov S, Koshiba M, Redegeld F, and Sitkovsky MV (1995) Role of extracellular ATP and P1 and P2 classes of purinoceptors in T-cell development and cytotoxic T-lymphocyte effector functions. *Immunol Rev* **146:**5–19.

Basnakian AG and James SJ (1994) A rapid and sensitive assay for the detection of DNA fragmentation during early phases of apoptosis. *Nucleic Acids Res* **22:**2714–2715.

Chao DT and Korsmeyer SJ (1998) BCL-2 family: regulators of cell death. *Annu Rev Immunol* **16:**395–419.

Chow SC, Kass GEN, and Orrenius S (1997) Purines and their roles in apoptosis. *Neuropharmacology* **36:**1149–1156.

Cory AH, Owen TC, Barltrop JA, and Cory JG (1991) Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun* **3:**207–212.

Coutinho-Silva R, Persechini PM, Bisaggio RD, Perfettini JL, Neto AC, Kanellopoulos JM, Motta LI, Dautry VA, and Ojcius DM (1999) $P_{2Z}$/P2X7 receptor-dependent apoptosis of dendritic cells. *Am J Physiol* **276:**C1139–C1147.

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

Dubyak GR and El-Moatassim C (1993) Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. *Am J Physiol* **265:**C577–C606.

Ferrari D, Chiozzi P, Falzoni S, Dal Susino M, Collo G, Buell G, and Di Virgilio F (1997) ATP mediated cytotoxicity in microglia cells. *Neuropharmacology* **36:**1295–1301.

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

Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P, and Williams M (1994) Nomenclature and classification of purinoceptors. *Pharmacol Rev* **46:**143–156.

Gajewski TF and Thompson CB (1996) Apoptosis meets signal transduction: elimination of a BAD influence. *Cell* **87:**589–592.

Gavriell Y, Sherman Y, and Ben-Sasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* **3:**493–501.

He J, Whitacre CM, Xue LY, Berger NA, and Oleinick NL (1998) Protease activation and cleavage of poly (ADP-ribose) polymerase: an integral part of apoptosis in response to photodynamic treatment. *Cancer Res* **58:**940–946.

Humphreys BD and Dubyak GR (1996) Induction of the $P_{2Z}$/P2X$_7$ nucleotide receptor and associated phospholipase D activity by lipopolysaccharide and IFN- in the human THP-1 monocytic cell line. *J Immunol* **157:**5627–5637.

Humphreys BD, Rice J, Kertesy S, and Dubyak GR (2000) Stress-activated protein kinase/Jnk activation and apoptotic induction by the macrophage P2X7 nucleotide receptor. *J Biol Chem* **275:**26792–26798.

Humphreys BD, Virginio C, Surprenant A, Rice J, and Dubyak GR (1998) Isoquinolines as antagonists of the P2X7 nucleotide receptor: high selectivity for the human versus rat receptor homologues. *Mol Pharmacol* **54:**22–32.

Koshiba M, Apasov S, Sverdlov V, Chen P, Erb L, Turner JT, Weisman GA, and Sitkovsky MV (1997) Transient up-regulation of P2Y2 nucleotide receptor mRNA expression is an immediate early gene response in activated thymocytes. *Proc Natl Acad Sci USA* **94:**831–836.

Kuida K, Lippke JA, Ku G, Harding MW, Livingston DJ, Su MS-S, and Flavell RA (1995) Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science (Wash DC)* **267:**2000–2003.

Kundson CM, Tung KS, Tourtellotte WG, Brown GA, and Korsmeyer SJ (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science (Wash DC)* **270:**96–99.

Laliberte RE, Eggler J, and Gabel CA (1999) ATP treatment of human monocytes promotes caspase-1 maturation and externalization. *J Biol Chem* **274:**36944–36951.

Michaelidis TM, Sendtner M, Cooper JD, Airaksinen MS, Holtmann B, Meyer M, and Thoenen H (1996) Inactivation of bcl-2 results in progressive degeneration of motor neurons, sympathetic and sensory neurons during early postnatal development. *Neuron* **17:**75–89.

Murgia M, Hanau S, Pizzo P, Rippa M, and Di Virgilio F (1993) Oxidized ATP. An irreversible inhibitor of macrophage purinergic $P_{2Z}$ receptor. *J Biol Chem* **268:**8199–8203.

Nett-Fiordalisi M, Tomaselli K, Russell JH, and Chaplin DD (1995) Macrophage apoptosis in the absence of active interleukin-1 beta-converting enzyme. *J Leukoc Biol* **58:**717–724.

Nicotera P, Hartzell P, Davis G and Orrenius S (1986) The formation of plasma membrane blebs in hepatocytes exposed to agents that increase cytosolic $Ca^{2+}$ is mediated by the activation of a non-lysomal proteolytic system. *FEBS Lett* **209:**139–144.

Nihei OK, de Carvalho AC, Savino W, and Alves IA (2000) Pharmacological properties of $P_{2Z}$/P2X$_7$ receptor characterized in murine dendritic cells: role on the induction of apoptosis. *Blood* **96:**996–1005.

North RA and Barnard EA (1997) Nucleotide receptors. *Curr Opin Neurobiol* **7:**346–357.

Oltvai ZN and Korsmeyer SJ (1994) Checkpoints of dueling dimers foil death wishes. *Cell* **79:**189–192.

Perregaux DG and Gabel CA (1998) Post-translational processing of murine IL-1: evidence that ATP-induced release of IL-1$\alpha$ and IL-1$\beta$ occurs via a similar mechanism. *J Immunol* **160:**2469–2477.

Rassendren F, Buell GN, Virginio C, Collo G, North RA, and Suprenant A (1997) The permeabilizing ATP receptor, P2X7. Cloning and expression of a human cDNA. *J Biol Chem* **272:**5482–5486.

Schulze-Lohoff E, Hugo C, Rost S, Arnold S, Gruber A, Brune B, and Sterzel RB (1998) Extracellular ATP causes apoptosis and necrosis of cultured mesangial cells via $P_{2Z}$/P2X$_7$ receptors. *Am J Physiol* **275:**F962–F971.

Solle M, Labasi J, Perregaux DG, Stam E, Petrushova N, Koller BH, Griffiths RJ, and Gabel CA (2001) Altered cytokine production in mice lacking P2X$_7$ receptors. *J Biol Chem* **276:**125–132.

Surprenant A, Rassendren F, Kawashima E, North RA, and Buell G (1996) The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). *Science (Wash DC)* **272:**735–738.

Thornberry NA and Lazebnik Y (1998) Caspases: enemies within. *Science (Wash DC)* **281:**312–316.

Vaux DL and Strasser A (1996) The molecular biology of apoptosis. *Proc Natl Acad Sci USA* **93:**2239–2244.

Wallach D, Boldin M, Varfolomeev E, Beyaert R, Vandenabeele P, and Fiers W (1997) Cell death induction by receptors of the TNF family: towards a molecular understanding. *FEBS Lett* **410:**96–106.

Yuan J (1997) Transducing signals of life and death. *Curr Opin Cell Biol* **9:**247–251.

Zamzami N, Brenner C, Marzo I, Susin S, and Kroemer G (1998) Subcellular and submitochondrial mode of action of Bcl-2-like oncoproteins. *Oncogene* **16:**2265–2282.

Zoeteweij JP, van de Water B, de Bont HJGM, and Nagelkerke JF (1996) The role of a purinergic P2Z receptor in calcium-dependent cell killing of isolated rat hepatocytes by extracellular adenosine triphosphate. *Hepatology* **23:**858–865.


**Address correspondence to:** Long T. Wen, Microchemical Core Facility, Department of Biology, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182-1030. E-mail: lwen@sciences.sdsu.edu