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## Tumor necrosis factor $\alpha$ -induced apoptosis in astrocytes is prevented by the activation of P2Y<sub>6</sub>, but not P2Y<sub>4</sub> nucleotide receptors\*

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### Abstract

The physiological role of the uracil nucleotide-preferring P2Y<sub>6</sub> and P2Y<sub>4</sub> receptors is still unclear, although they are widely distributed in various tissues. In an effort to identify their biological functions, we found that activation by UDP of the rat P2Y<sub>6</sub> receptor expressed in 1321N1 human astrocytes significantly reduced cell death induced by tumor necrosis factor  $\alpha$  (TNF $\alpha$ ). This effect of UDP was not observed in non-transfected 1321N1 cells. Activation of the human P2Y<sub>4</sub> receptor expressed in 1321N1 cells by UTP did not elicit this protective effect, although both receptors were coupled to phospholipase C. The activation of P2Y<sub>6</sub> receptors prevented the activation of both caspase-3 and caspase-8 resulting from TNF $\alpha$  exposure. Even a brief (10-min) incubation with UDP protected the cells against TNF $\alpha$ -induced apoptosis. Interestingly, UDP did not protect the P2Y<sub>6</sub>-1321N1 cells from death induced by other methods, i.e. oxidative stress induced by hydrogen peroxide and chemical ischemia. Therefore, it is suggested that P2Y<sub>6</sub> receptors interact rapidly with the TNF $\alpha$ -related intracellular signals to prevent apoptotic cell death. This is the first study to describe the cellular protective role of P2Y<sub>6</sub> nucleotide receptor activation.

### Keywords

Apoptosis; Pyrimidines; G protein-coupled receptor; Phospholipase C; Cell death; Cytokines; TNF $\alpha$ ; Caspase; Nucleotide receptor

## 1. Introduction

Extracellular nucleotides have a ubiquitous role as signaling molecules in addition to their intracellular functions. The receptors for extracellular nucleotides, P2 receptors, are divided into two subfamilies: G protein-coupled (P2Y) and ligand-gated ion channels (P2X) [1–3]. The P2Y receptors have seven transmembrane domains and activate PLC, via coupling to

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$G_{q/11}$  proteins. This, in turn, increases inositol-1,4,5-trisphosphate ( $IP_3$ ) production and intracellular calcium, and/or stimulation/inhibition of adenylate cyclase. Several members of the P2 nucleotide receptor family are activated by uracil nucleotides. These pyrimidine nucleotide-responsive receptors include P2Y<sub>4</sub> and P2Y<sub>6</sub> receptors, which are activated by UTP and UDP, respectively. P2Y<sub>2</sub> receptors respond to activation by either ATP or UTP.

The P2Y<sub>6</sub> receptors are widely distributed in the placenta, spleen, thymus, bones, lungs, intestine, smooth muscle, heart, and epithelia. This distribution is not indicative of specific physiological roles in organs or tissues [4–8], and particular functions are largely unexplained. Recently there have been several reports on cellular functions of P2Y<sub>6</sub> receptors. In human THP-1 monocytic cells and 1321N1 astrocytoma cells, P2Y<sub>6</sub> receptors stimulated interleukin-8 production [9]. UTP potentiated LPS-induced PGE<sub>2</sub> release in J744 macrophages expressing P2Y<sub>6</sub> receptors [10]. It was suggested that UTP-evoked noradrenaline release in cultures of rat superior cervical ganglia is mediated through P2Y<sub>6</sub> receptor activation [11]. In rat pial arterioles, a combination of P2Y<sub>2</sub>- and P2Y<sub>6</sub>-like receptors was suggested to be responsible for the pyrimidine nucleotide-evoked sustained vasoconstriction [12].

Another subtype of nucleotide receptors, P2Y<sub>4</sub>, is activated by UTP, is insensitive to ADP and UDP, and in humans is antagonized by ATP. Its expression is found in the placenta, pancreas, lungs, and vascular smooth muscle [13–16]. In human colorectal carcinoma cells, P2Y<sub>2</sub>/P2Y<sub>4</sub> receptors have been identified and indicated to be associated with the ATP-mediated control of cell proliferation [17]. In vestibular dark cell epithelium, P2Y<sub>4</sub> receptors controlled K<sup>+</sup> secretion [18]. The possible involvement of P2Y<sub>2</sub>/P2Y<sub>4</sub> receptors in apoptosis in rat glomerular mesangial cells has also been suggested [19].

In an effort to identify their biological functions, we have now investigated the relationship of P2Y<sub>6</sub> and P2Y<sub>4</sub> receptors, expressed in the 1321N1 astrocytoma cell line, to cell death. Previously, we had studied the relationship between adenosine receptors and cell death phenomena [20]. In the present study, protective effects against apoptosis by UDP were found, and the mechanism of this modulation was probed.

## 2. Materials and methods

### 2.1. Materials

1321N1 astrocytoma cells stably transfected with either rat P2Y<sub>6</sub> (rP2Y<sub>6</sub>) or human P2Y<sub>4</sub> (hP2Y<sub>4</sub>) receptors were provided by Dr. Robert Nicholas (University of North Carolina). *myo*-[<sup>3</sup>H]Inositol (20 Ci/mmol) was obtained from American Radiolabeled Chemicals. Dowex AG 1-X8 resin was purchased from Bio-Rad. DMEM and FBS were from Life Technologies, Inc. Anti-caspase-3 (Asp175), anti-caspase-8 (1C12), HRP-linked anti-mouse IgG, and HRP-linked anti-rabbit IgG antibodies were purchased from Cell Signaling Technology. TNF $\alpha$  was purchased from Biosource International. All other reagents were purchased from the Sigma Chemical Co.

### 2.2. Cell culture and preparation

1321N1 cells stably transfected with the rP2Y<sub>6</sub> or hP2Y<sub>4</sub> receptors were grown at 37° in a humidified incubator with 5% CO<sub>2</sub>/95% air in DMEM supplemented with 10% FBS, 100 units/mL of penicillin, 100 mg/mL of streptomycin, and 2 mM L-glutamine. The cells were grown to ~60% confluence for the experiments.

### 2.3. Induction of apoptosis

One method used to induce apoptosis was subjecting the cells to chemical ischemia. Cellular ischemia was induced using chemicals that block respiration and the utilization of glucose [21]. rP2Y<sub>6</sub>-1321N1 or hP2Y<sub>4</sub>-1321N1 cells were washed with PBS and then incubated at 37° in chemical ischemia-inducing buffer (10 mM sodium azide, 10 mM 2-deoxyglucose, 120 mM NaCl, 5 mM KCl, 0.62 mM MgSO<sub>4</sub>, 1.8 mM CaCl<sub>2</sub>, 10 mM HEPES, pH 7.4) for 2 or 3 hr. Medium was replaced with fresh 10% serum-containing DMEM with or without UDP or UTP. Cell death was assessed 16 hr later.

Apoptotic cell death was also induced by oxidative stress. The cells were washed once with PBS and incubated at 37° in DMEM containing 3% BSA and 1 μM insulin without antibiotics for 1 hr. Hydrogen peroxide was added in appropriate concentrations. After an hour, the medium was replaced with fresh DMEM containing 10% FBS with or without UDP or UTP. Cell death was assessed 16 hr later.

Finally, TNFα was used to induce apoptosis. Medium containing 5 μg/mL of cycloheximide was added to the cells grown to ~60% confluence. In all the experiments concerning TNFα-induced cell apoptosis, cycloheximide was included. The cells were treated with appropriate concentrations of TNFα for 4 or 16 hr. UDP or UTP was added as indicated in each figure. Cell death was assessed 16 hr later.

### 2.4. Degree of cell death

After the treatment, the cells in the supernatant and the cells detached by trypsin-EDTA were combined and centrifuged. The cells were washed with PBS once and resuspended at 2–5 × 10<sup>5</sup> cells/mL in PBS. The cell suspension was stained with a propidium iodide solution (final concentration; 2 μg/mL), and the numbers of unstained (live) and stained (dead) cells were analyzed on a FACSCalibur instrument (Becton Dickinson).

### 2.5. Determination of sub-G<sub>1</sub> population

The apoptotic fraction after the various treatments was quantified by analyzing the sub-G<sub>1</sub> (sub-diploid) population following the method of Kim *et al.* [22]. After fixing with 2% formaldehyde, treating with trypsin followed by RNase and trypsin inhibitor, and staining with propidium iodide, the cells were applied to a FACSCalibur instrument to measure the fluorescence activity of propidium iodide-stained DNA.

### 2.6. Immunoblot analysis

The proteins from the cells were extracted using a lysis buffer (0.5% Nonidet P-40, 120 mM NaCl, 40 mM Tris, pH 8.0). After SDS-PAGE, the protein bands were transferred to nitrocellulose membranes that were subsequently blocked with 5% powdered non-fat milk. These membranes were incubated overnight with the primary antibodies and for 1 hr with the HRP-linked secondary antibody. Immunoblots were developed with an enhanced chemiluminescence (ECL) reagent (Pierce).

### 2.7. Determination of inositol phosphates

The amount of inositol phosphates was measured by a modification of the method of Kim *et al.* [22]. The P2Y<sub>6</sub>-1321N1 or P2Y<sub>4</sub>-1321N1 cells were grown to confluence in 6-well plates in the presence of *myo*-[<sup>3</sup>H]inositol (2 μCi/mL) for 24 hr. After an additional incubation of 20 min with 20 mM LiCl at room temperature, cells were treated with UDP or UTP for 30 min. The reaction was terminated upon aspiration of the medium and addition of cold formic acid (20 mM). After 30 min, supernatants were neutralized with NH<sub>4</sub>OH, and applied to Bio-Rad Dowex AG 1-X8 anion exchange columns. All of the columns were then washed

with water followed by a 60 mM sodium formate solution containing 5 mM sodium tetraborate. Total inositol phosphates were eluted with 1 M ammonium formate containing 0.1 M formic acid, and the  $^3\text{H}$  counts were measured using a liquid scintillation counter. Pharmacological parameters were analyzed using the Prism program (version 3.0, GraphPAD).

### 3. Results

It has been reported that both P2Y<sub>6</sub> and P2Y<sub>4</sub> nucleotide receptors are coupled to PLC [23]. As shown in Fig. 1, the production of inositol phosphates in 1321N1 human astrocytes expressing the rP2Y<sub>6</sub> receptor was increased by UDP in a concentration-dependent manner. The maximal effect was reached at 1  $\mu\text{M}$  with an EC<sub>50</sub> of 53.2 nM, similar to previous reports [23,24]. UTP also induced the production of inositol phosphates in hP2Y<sub>4</sub>-transfected 1321N1 cells in a concentration-dependent manner with an EC<sub>50</sub> of 740 nM. This indicated that functional UDP-or UTP-responsive receptors linked to PLC were expressed in these cells.

Death was induced in rP2Y<sub>6</sub>-1321N1 or hP2Y<sub>4</sub>-1321N1 cells by three different methods (chemical ischemia, oxidative stress, and death receptor activation by TNF $\alpha$ ) for investigation of the effects of P2Y<sub>6</sub> and P2Y<sub>4</sub> receptor activation. Figure 2 shows the cellular morphology under these conditions. Chemical ischemia caused significant cell detachment and the formation of attached cell aggregates. After the induction of oxidative stress with 1 mM hydrogen peroxide, the cells appeared shrunken and displayed membrane blebbing. TNF $\alpha$  treatment (5 ng/mL) in the presence of 5  $\mu\text{g/mL}$  of cycloheximide caused less disintegration of the cells than hydrogen peroxide treatment, but more fragmented cellular particles (apoptotic bodies) were observed. The morphological changes induced by each set of conditions were distinct, implying that the cell death mechanisms involved in each case were different. We observed that UDP antagonized TNF $\alpha$ -induced cell death in P2Y<sub>6</sub>-1321N1 cells and reduced the cell fragmentation (Fig. 2B). Microscopic examination of cells exposed to TNF $\alpha$  after detachment and propidium iodide staining indicated that UDP treatment decreased the fraction of cells stained. The activation of P2Y<sub>4</sub> receptors in a similar line of stably transfected 1321N1 cells by UDP neither elicited morphological recovery nor protected against death in any of the three conditions.

The cell death ratio was determined using FACS analysis following propidium iodide staining (Fig. 3). A 3-hr pulse-stress of 1321N1 cells with ischemia-causing chemicals increased the ratio of cellular detachment and cell death in rP2Y<sub>6</sub>-transfected 1321N1 human astrocytes. The activation of P2Y<sub>6</sub> receptors by UDP in this cell line did not affect the detachment ratio or cell viability. The addition of UDP before or during the ischemic pulse-stress did not affect cell viability (data not shown). The cell death induced by hydrogen peroxide was 7:0  $\pm$  0:8 and 28:0  $\pm$  4:4% at 0.25 and 1.0 mM, respectively, after overnight incubation. In these cases, the treatment of the cells with UDP did not affect the cell death ratio. Activation of the hP2Y<sub>4</sub> receptor in 1321N1 cells by the agonist UTP did not have preventive effects, either on chemical ischemia-or on hydrogen peroxide-induced cell death (Fig. 3).

Interestingly, however, we found that activation of the receptor by UDP (both at 1 and 10  $\mu\text{M}$  at which the inositol phosphate production was maximal) in rP2Y<sub>6</sub>-transfected 1321N1 human astrocytes significantly reduced the apoptotic process induced by TNF $\alpha$ . With the sensitization by cycloheximide (5  $\mu\text{g/mL}$ ), exposure of P2Y<sub>6</sub>-1321N1 cells to 5 and 20 ng/mL of TNF $\alpha$  killed 10:5  $\pm$  2:5 and 21:3  $\pm$  3:3% of the total cell population, respectively. Activation of the P2Y<sub>6</sub> receptor by UDP (10  $\mu\text{M}$ ) in the presence of TNF $\alpha$  (5 and 20 ng/mL) reduced cell death to 2:8  $\pm$  1:5 and 3:2  $\pm$  1:8%, respectively; i.e. ~70–80% inhibition in

each case. In the concentration–response experiments, the EC<sub>50</sub> for the protective effect of UDP against 20 ng/mL of TNF $\alpha$  was 13.9 nM. With a higher concentration of TNF $\alpha$  (80 ng/mL), cell death exceeded 50%, and the addition of UDP was not protective (data not shown). The protection by UDP was not observed in non-transfected 1321N1 astrocytes (data not shown). Furthermore, in human P2Y<sub>4</sub>-transfected 1321N1 astrocytes, UTP was not able to protect the cells from TNF $\alpha$ -induced death (Fig. 3).

The apoptotic cell death induced by TNF $\alpha$  occurred through recruitment of caspase-8. Activated caspase-8 is known to cleave and activate additional downstream caspases including caspase-3, one of the key lethal mediators of apoptosis [25,26]. Six hours after the TNF $\alpha$  treatment of P2Y<sub>6</sub> receptor-expressing 1321N1 cells, the cleavage of both caspase-3 and caspase-8 was observed. In the presence of 10  $\mu$ M UDP, the activation of these caspases was inhibited significantly (Fig. 4). Measurement by flow cytometry of the sub-diploid fraction of the population indicated that  $17 \pm 4\%$  of P2Y<sub>6</sub>-1321N1 cells were apoptotic following treatment with 20 ng/mL of TNF $\alpha$ . The addition of 10  $\mu$ M UDP reduced the apoptosis ratio to the control level,  $2.2 \pm 1.2\%$ . This suggested that a signal from the P2Y<sub>6</sub> receptor interfered at some step(s) in the apoptotic pathway following TNF $\alpha$  receptor activation.

TNF $\alpha$  was able to induce cell death in 1321N1 cells even after a short period of treatment. The cells were incubated for only 4 hr with TNF $\alpha$  and further incubated in fresh medium for an additional 12 hr. After a 4-hr incubation, cell death was not observed (data not shown), but 12 hr later death occurred in  $23 \pm 3\%$  of the cells, which was similar to the level of a 16-hr treatment with TNF $\alpha$  (Fig. 5). Upon a 4-hr incubation with TNF $\alpha$ , co-administered UDP was protective, reducing cell death to  $2.7 \pm 1.3\%$ . The cells were also protected when preincubated with UDP for 10 min, washed with PBS, and then treated with TNF $\alpha$  for 4 hr. Interestingly, post-treatment with UDP, in which UDP was added 4 hr after the TNF $\alpha$  treatment, did not reverse the effect of TNF $\alpha$ . Therefore, it was suggested that UDP rapidly induced intracellular events that played a crucial role in preventing the cells from undergoing TNF $\alpha$ -induced apoptosis.

#### 4. Discussion

This is the first study to describe a role for P2Y<sub>6</sub> nucleotide receptors in cellular protection. There have been several reports on the function of other P2Y receptors in cell death. The activation of P2Y<sub>1</sub> receptors in 1321N1 cells decreased cell number and increased caspase-3-like activities [27]. It was proposed that P2Y<sub>2</sub> and/or P2Y<sub>4</sub> receptors in glomerular mesangial cells could induce proliferation and apoptosis, where the balance between proliferation and apoptosis would depend on the relative stimulation and expression of the P2 receptor subtypes [19]. The increase in PC12 cell death by extracellular ATP appeared to be through P2Y receptor activation [28]. In this study we investigated the action of recombinant rP2Y<sub>6</sub> and hP2Y<sub>4</sub> receptors in three different models: chemical ischemia, oxidative stress, and cell death receptor activation.

In ischemic conditions, the loss of oxygen and nutrient supply causes stress on cells by various pathways and eventually induces cell death. In the case of adenosine receptors, a significant physiological role in modulating ischemic damage to various tissues has been reported [29–37]. Recently, neuroprotection by the P2 receptor antagonist Basilen blue under hypoglycemic or chemically induced hypoxic conditions was reported [38]. We investigated the potential effects of P2Y<sub>6</sub> and P2Y<sub>4</sub> nucleotide receptor activation under ischemic conditions. However, activation of these receptors in our ischemic model was not protective (Fig. 3), in contrast to other nucleoside/nucleotide receptors in other models [29–38].



There have been reports on the role of nucleoside/nucleotide receptors in oxidative stress. The P2 receptor antagonists suramin and reactive blue 2 prevented dequalinium-induced neuronal death [39]. In chick retinal cells, an A<sub>1</sub> adenosine agonist (CPA) and an A<sub>2A</sub> antagonist (DMPX) inhibited oxidative damage, while an A<sub>1</sub> antagonist (DPCPX) and an A<sub>2A</sub> agonist (CGS 21680) potentiated damage [40]. It was reported that A<sub>1</sub> receptor expression was increased by oxidative stress [41]. In the present study, activation of P2Y<sub>6</sub> and P2Y<sub>4</sub> receptors did not affect cell death in a model of oxidative stress (Fig. 3).

Among the three mechanistically different methods of inducing cell death, the administration of UDP to the P2Y<sub>6</sub> receptor-expressing cells interfered with cell death only when induced by TNF $\alpha$ . TNF $\alpha$  has been known to induce apoptosis through the coupling of caspase-8 with TRADD, which, in turn, activates caspase-3 [25,26]. It was also suggested that activation by TNF $\alpha$  of JNK, p38, and nuclear factor- $\kappa$ B would result in crosstalk to this pathway, although the precise mechanism remains unclear [42]. Figure 4 demonstrated that the activation of P2Y<sub>6</sub> receptors in 1321N1 cells by UDP drastically inhibited the cleavage of caspase-3 induced by TNF $\alpha$ , implying that the cell death induced by TNF $\alpha$  was apoptotic, and the signals from the receptor interfered with the apoptotic process to protect the cells. Since P2Y<sub>6</sub> receptor activation also blocked cleavage of caspase-8, it appeared that the signal from P2Y<sub>6</sub> receptor activation interfered with the death domain signaling upstream to, or at the level of caspase-8. The inhibition of caspase activation appeared to be responsible for the protection from cell death, as shown in Fig. 3.

Interestingly, activation of P2Y<sub>4</sub> receptors by UTP did not have protective effects in TNF $\alpha$ -induced cell death. Both P2Y<sub>6</sub> and P2Y<sub>4</sub> have been known to activate PLC through coupling to the G $\alpha_q$  subunit of G proteins. Therefore, these two receptors would be expected to have had common intracellular pathways in order to elicit their biological functions, especially when expressed in the identical cell line. However, only P2Y<sub>6</sub> receptors showed a protective effect in this astrocytoma cell line. Compared to P2Y<sub>6</sub> receptors, P2Y<sub>4</sub> receptors expressed in 1321N1 cells have been reported to desensitize very rapidly upon agonist exposure and to lose about 50% in surface expression within 10 min [43]. The P2Y<sub>4</sub> receptor also showed a relatively rapid recovery rate following removal of UTP. The steadier and much slower turnover rate of P2Y<sub>6</sub> receptors might be important for its observed protective effect.

To understand whether the signals from the receptor act at an early or late stage of the apoptotic pathway, we treated the cells with UDP prior to or following the addition of TNF $\alpha$ . In this case cell death was induced upon a short-term (4-hr) treatment with TNF $\alpha$ . Even though the residual UDP was washed away after a 10-min pretreatment, its protective signals persisted and prevented the cells from undergoing subsequent TNF $\alpha$ -induced death (Fig. 5). However, once the apoptotic signals were processed to some extent, UDP was not able to reverse the already initiated apoptosis. This implied that the P2Y<sub>6</sub> receptor signal interacted in the early stages of TNF $\alpha$  signaling.

The involvement of mitogen-activated protein (MAP) kinases in TNF $\alpha$ -induced apoptosis has been postulated. JNK has been reported to be responsible for death receptor-mediated cell death [25,42,44]. The activation of Erk appeared to regulate the death signal induced by the TNF receptor [45–47]. Overexpression of Erk prevented TNF $\alpha$ -induced apoptosis [48]. We have observed the increase of phospho-Erk by P2Y<sub>6</sub> receptor activation (unpublished data). The involvement of PKC in death receptor-mediated TNF $\alpha$ -induced apoptosis is also known [49,50]. Since the P2Y<sub>6</sub> receptor is a G protein-coupled receptor (GPCR) coupled to PLC, the activation of PKC by the receptor could be related to its biological effects [51].

In conclusion, the addition of UDP prevented cell death in rP2Y<sub>6</sub> receptor-expressing 1321N1 astrocytoma cells only when it was induced by TNF $\alpha$ . Therefore, it is suggested

that the P2Y<sub>6</sub> receptor may interact with the TNF $\alpha$ -related intracellular signals to prevent apoptotic cell death. The precise mechanisms are under continuing investigation.

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## Abbreviations

<b>FACS</b>	fluorescence-activated cell sorting
<b>PLC</b>	phospholipase C
<b>Ab</b>	antibody
<b>DMEM</b>	Dulbecco's modified Eagle's medium
<b>FBS</b>	fetal bovine serum
<b>TNF</b>	tumor necrosis factor
<b>HRP</b>	horseradish peroxidase
<b>LPS</b>	lipopolysaccharide
<b>PGE<sub>2</sub></b>	prostaglandin E <sub>2</sub>
<b>Erk</b>	extracellular signal-regulated protein kinase
<b>and JNK</b>	c-Jun N-terminal kinase

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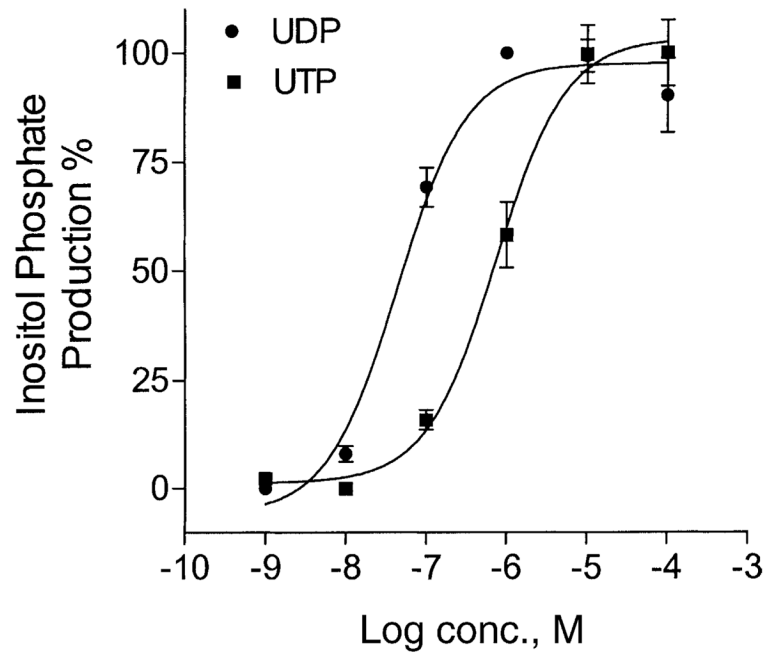
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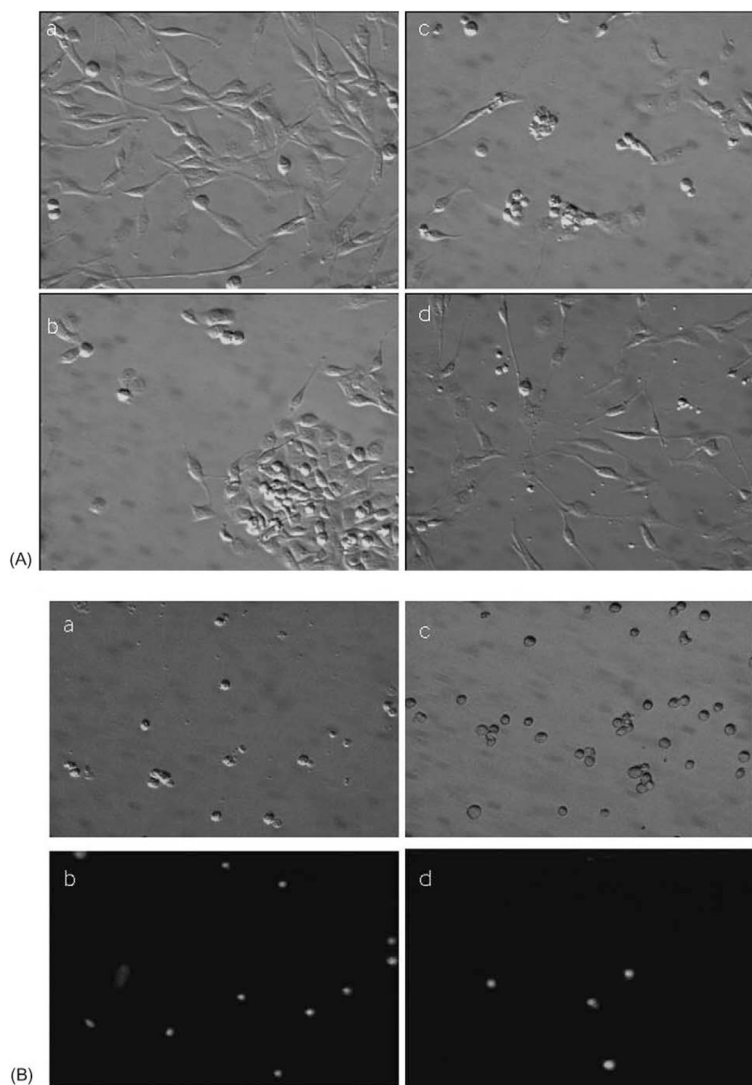


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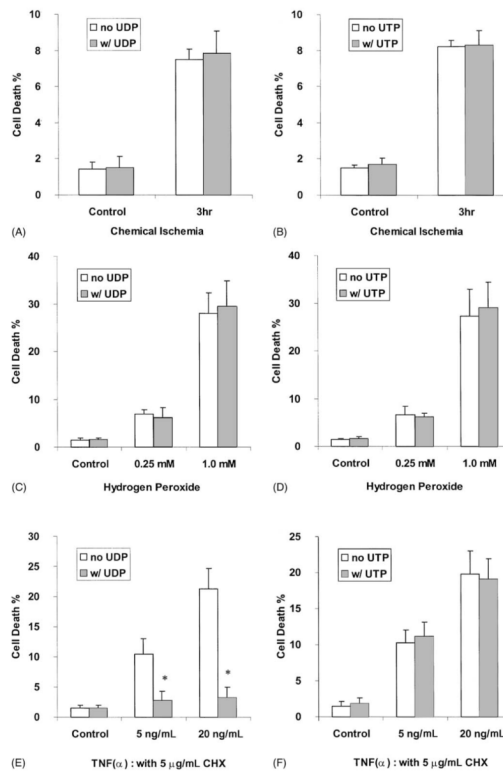
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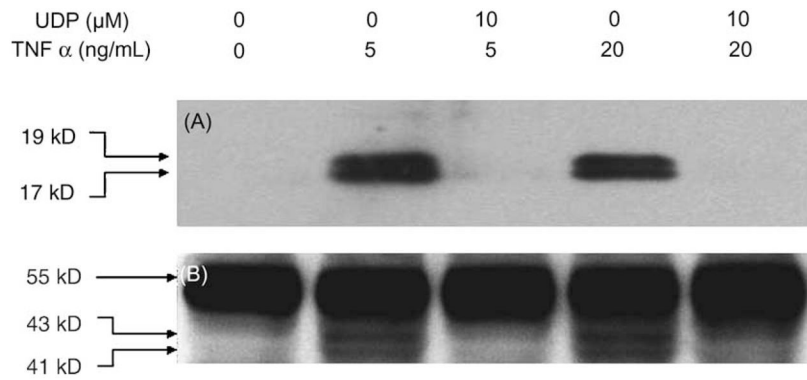
**Fig. 1.** Inositol phosphate production in P2Y<sub>6</sub>- or P2Y<sub>4</sub>-transfected 1321N1 human astrocytes. After being labeled with *myo*-[<sup>3</sup>H]inositol (1  $\mu$ Ci/10<sup>6</sup> cells) for 24 hr, the cells were treated for 30 min at 37° with agonists: UDP for P2Y<sub>6</sub> or UTP for P2Y<sub>4</sub>. The amount of inositol phosphates was analyzed after extraction through Dowex AG 1-X8 columns (see “Section 2”). Data are means  $\pm$  SD (N = 3). Where error bars are not visible, they are smaller than the symbol.



**Fig. 2.** Morphological changes of rP2Y<sub>6</sub> receptor-transfected 1321N1 astrocytoma cells 16 hr after the treatments indicated (see “Section 2”): (A) a, non-treated adherent control cells; b, 3 hr of chemical ischemia; c, hydrogen peroxide (1 mM) for 1 hr; d, 5 ng/mL of TNF $\alpha$  with 5  $\mu$ g/mL of cycloheximide. (B) Effect of UDP (10  $\mu$ M) on TNF $\alpha$  (5 ng/mL)-induced cell death. Cells were detached and stained with propidium iodide. a, b: TNF $\alpha$ -treated cells; c, d: TNF $\alpha$  and UDP-treated cells; b, d; fluorescence micrographs of the fields of a and c, respectively.

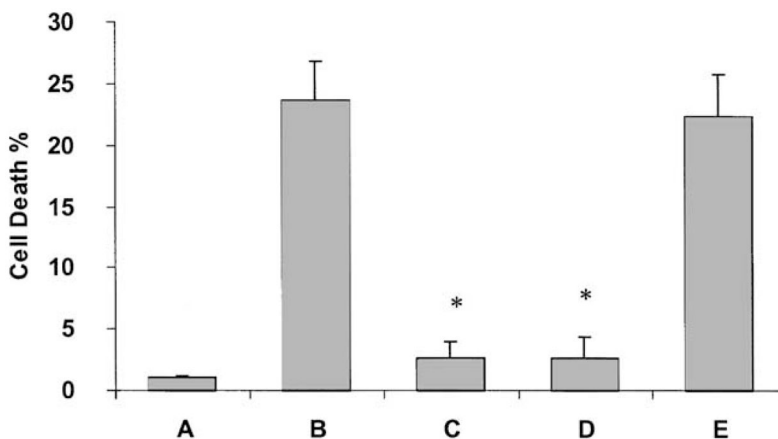


**Fig. 3.** Effects of UDP or UTP on 1321N1 astrocytoma cells expressing rP2Y<sub>6</sub> receptors (A, C, E) or hP2Y<sub>4</sub> receptors (B, D, F). (A, B) Cells were incubated in an ischemia-inducing buffer (see “Section 2”) for 3 hr and restored to normal medium with or without 10 μM UDP or UTP. (C, D) Cells were treated with 0.25 or 1.0 mM hydrogen peroxide for 1 hr in DMEM containing 3% BSA, 1 μM insulin, and no antibiotics, and then returned to normal medium with or without 10 μM UDP or UTP. (E, F) Cells were treated with 5 or 20 ng/mL of TNFα in DMEM containing 3 μg/mL of cycloheximide (CHX) with or without 10 μM UDP or UTP. Sixteen hours after the treatments, the degree of cell death was analyzed using a FACSCalibur instrument. Data are means ± SD from the combined data of two independent experiments performed in triplicate. Key: (\*) statistically significant ( $P < 0.01$ ) as determined by Student’s *t*-test.



**Fig. 4.** Inhibition of TNF $\alpha$ -induced cleavage of caspase-3 (A) and caspase-8 (B) by P2Y<sub>6</sub> receptor activation. The rP2Y<sub>6</sub> receptor-expressing 1321N1 astrocytoma cells were treated with 5 or 20 ng/mL of TNF $\alpha$  in DMEM containing 3  $\mu$ g/mL of cycloheximide in the absence or presence of 10  $\mu$ M UDP. Six hours after the treatment, the proteins were extracted and immunoblotted as described in “Section 2.” (A) Large fragments of activated caspase-3 (17/19 kDa) are indicated. (B) Full-length caspase-8 (57 kDa) and the cleaved intermediate fragments (p43/41) are indicated. Results are representative of three experiments.





**Fig. 5.**

Effects of UDP on short-term treatment of TNF $\alpha$  in rP2Y<sub>6</sub> receptor-expressing 1321N1 cells. (A) Control cells. (B) Cells treated for 4 hr with TNF $\alpha$ . (C) Cells co-treated with both UDP and TNF $\alpha$  for 4 hr. (D) Cells incubated for 10 min with UDP, washed once with PBS, and then treated with TNF $\alpha$  for 4 hr (pretreatment). (E) Cells treated with TNF $\alpha$  for 4 hr and then the medium was replaced with fresh medium containing UDP (post-treatment). In each case, cell death was observed after an additional 12-hr incubation. The medium always contained 5  $\mu$ g/mL of cycloheximide. The concentrations of UDP and TNF $\alpha$  were 1  $\mu$ M and 20 ng/mL, respectively. The degree of cell death was analyzed using a FACSCalibur instrument. Data are means  $\pm$  SD from the combined data of two independent experiments in triplicate. Key: (\*) statistically significant ( $P < 0.01$ ), as determined by Student's  $t$ -test, compared with TNF $\alpha$  treatment (B).