Mitogen-Activated Protein Kinase and Caspase Signaling Pathways Are Required for P2X<sub>7</sub> Receptor (P2X<sub>7</sub>R)-Induced Pore Formation in Human THP-1 Cells

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Received September 4, 2003; accepted November 10, 2003

ABSTRACT

Brief activation of the ATP-sensitive P2X<sub>7</sub> receptor (P2X<sub>7</sub>R) stimulates the maturation and release of interleukin 1β (IL-1β) in macrophages, whereas prolonged agonist activation induces the formation of cytolytic pores in cell membranes. The present study investigated potential downstream mechanisms associated with native human P2X<sub>7</sub>R activation in lipopolysaccharide and interferon-γ differentiated THP-1 cells. 2,3-O-(4-Benzoylbenzoyl)-ATP (BzATP)-induced pore formation (EC<sub>50</sub> = 35 μM) was blocked by a selective P2X<sub>7</sub>R antagonist, [N,O-bis(5-isooquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) (IC<sub>50</sub> = 44 nM) and by pyridoxal phosphate-6-azophenyl-2,4-disulfonic acid (PPADS) (IC<sub>50</sub> = 344 nM). KN-62 and PPADS also blocked BzATP-induced IL-1β release (EC<sub>50</sub> = 617 μM) with IC<sub>50</sub> values of 75 and 3500 nM, respectively. The selective p38 mitogen-activated protein kinase (MAPK) inhibitor, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB 202190), potently inhibited BzATP-induced pore formation (IC<sub>50</sub> = 75 nM) but did not alter P2X<sub>7</sub>-mediated calcium influx or IL-1β release. SB 202190 and KN-62 also attenuated BzATP-mediated activation of phosphorylated p38 MAPK (pp38 MAPK). Two caspase inhibitors, YVAD (caspase 1) and DEVD (caspase 3), attenuated both BzATP-induced pore formation and IL-1β release in a concentration-dependent fashion. Neither DEVD nor p38-MAPK inhibitors blocked cell membrane pore formation evoked by maitotoxin or by activation of human P2X<sub>2a</sub> receptors. These results indicate that P2X<sub>7</sub>R-mediated pore formation results from a coordinated cascade involving both the p38 MAPK and caspase pathways that is distinct from other cytolytic pore-forming mechanisms. In contrast, P2X<sub>7</sub>R-mediated IL-1β release is dependent on caspase activity but not p38 MAPK. Taken together, these results support the hypothesis that downstream cellular signaling mechanisms, rather than channel dilation, mediate cytolytic pore formation after prolonged agonist activation, which underlies P2X<sub>7</sub> receptors.

The P2X<sub>7</sub>R is an ATP-sensitive, ligand-gated ion channel that functions as a nonselective cation channel and, upon prolonged agonist exposure, leads to the formation of progressively enlarged cytolytic pores (~900 Da) on the cell surface (North, 2002). Currently, seven different P2X receptor subtypes have been molecularly defined, and the P2X<sub>7</sub>R appears to be the most divergent member of this family in terms of molecular structure, pharmacology, and function (Jacobson et al., 2002; North, 2002). Homomeric P2X<sub>7</sub>Rs contain a longer intracellular C terminus compared with other P2X receptors. This C terminus is composed of a hydrophobic domain, as well as putative interaction sites for LPS (Denlinger et al., 2001), SH2 domains (Kim et al., 2001), and α-actin (Kim et al., 2001). P2X<sub>7</sub>R-mediated pore formation is dependent on an intact C terminus and is inhibited in the presence of extracellular divalent cations (Rassendren et al., 1997). Unlike many other P2XRs, the P2X<sub>7</sub>R does not stimulate the maturation and release of interleukin 1β (IL-1β) in macrophages, whereas prolonged agonist activation induces the formation of cytolytic pores in cell membranes. The present study investigated potential downstream mechanisms associated with native human P2X<sub>7</sub>R activation in lipopolysaccharide and interferon-γ differentiated THP-1 cells. 2,3-O-(4-Benzoylbenzoyl)-ATP (BzATP)- induced pore formation (EC<sub>50</sub> = 35 μM) was blocked by a selective P2X<sub>7</sub>R antagonist, [N,O-bis(5-isooquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) (IC<sub>50</sub> = 44 nM) and by pyridoxal phosphate-6-azophenyl-2,4-disulfonic acid (PPADS) (IC<sub>50</sub> = 344 nM). KN-62 and PPADS also blocked BzATP-induced IL-1β release (EC<sub>50</sub> = 617 μM) with IC<sub>50</sub> values of 75 and 3500 nM, respectively. The selective p38 mitogen-activated protein kinase (MAPK) inhibitor, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB 202190), potently inhibited BzATP-induced pore formation (IC<sub>50</sub> = 75 nM) but did not alter P2X<sub>7</sub>-mediated calcium influx or IL-1β release. SB 202190 and KN-62 also attenuated BzATP-mediated activation of phosphorylated p38 MAPK (pp38 MAPK). Two caspase inhibitors, YVAD (caspase 1) and DEVD (caspase 3), attenuated both BzATP-induced pore formation and IL-1β release in a concentration-dependent fashion. Neither DEVD nor p38-MAPK inhibitors blocked cell membrane pore formation evoked by maitotoxin or by activation of human P2X<sub>2a</sub> receptors. These results indicate that P2X<sub>7</sub>R-mediated pore formation results from a coordinated cascade involving both the p38 MAPK and caspase pathways that is distinct from other cytolytic pore-forming mechanisms. In contrast, P2X<sub>7</sub>R-mediated IL-1β release is dependent on caspase activity but not p38 MAPK. Taken together, these results support the hypothesis that downstream cellular signaling mechanisms, rather than channel dilation, mediate cytolytic pore formation after prolonged agonist activation, which underlies P2X<sub>7</sub> receptors.

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form functional heteromeric combinations with other P2X subunits (Torres et al., 1998).

The P2X7 receptor is also distinguished pharmacologically from other P2X receptors by the fact that ATP has weak affinity (EC_{50} > 100 \mu M) for this receptor, whereas 2,3-O-(4-benzoylbenzoyl)-ATP (BzATP) is approximately 30-fold more potent than ATP (Surprenant et al., 1996; Bianchi et al., 1999). P2X7-Rs are uniquely expressed on macrophages, epidermal Langerhans cells, and other antigen-presenting cells (Surprenant et al., 1996). Within the central nervous system, P2X7-R expression has been reported on microglia (Ferrari et al., 1996), astrocytes, Schwann cells (Irnhic et al., 2001), and presynaptic neuronal terminals in hippocampus and spinal cord (Duechars et al., 2001).

Another unique function of the P2X7-R is its contribution to a variety of proinflammatory events, including fusion of macrophages to form multinucleated giant cells (Falzoni et al., 1995). Activation of P2X7 receptors stimulates the release of the proinflammatory cytokine interleukin-1\( \beta \) (IL-1\( \beta \)) in immune cells (Surprenant et al., 1996). IL-1\( \beta \) is processed from a precursor to a mature form by the enzyme, caspase 1. ATP has been shown to both time- and dose-dependently induce the release of IL-1\( \beta \) from LPS-primed THP-1 cells (Grahames et al., 1999). This ATP-induced cytokine release appears to be specifically modulated by P2X7-Rs since IL-1\( \beta \) release can be blocked not only by the nonselective P2X antagonists, PPADS and oxidized ATP, but also by KN-62, which is a selective P2X7-R antagonist (Grahames et al., 1999). It has also been shown that the release of IL-1\( \beta \) is independent of cytolysis and appears not to require P2X7-mediated pore formation (Chessell et al., 2001). P2X7-R activation also results in cell membrane blebbing (Ferrari et al., 1997a) and changes in cellular morphology, which eventually lead to cell death by both necrotic and apoptotic mechanisms (Ferrari et al., 1999).

The present study evaluates the signaling pathways that contribute to two of the hallmark functional consequences of P2X7-R activation, pore formation and IL-1\( \beta \) release, in differentiated human THP-1 macrophage cells. By using pharmacologically selective inhibitors of intracellular signaling pathways, we show that P2X7-R-induced pore formation and IL-1\( \beta \) release are mediated via some common but also some distinct mechanistic pathways. The mechanism of P2X7-R-mediated pore formation was also compared with pore formation induced by non-P2X7-Rs such as the P2X5-R (Khakh et al., 1999) or via a toxin from marine dinoflagellates, maitotoxin (MTX) (Schilling et al., 1999a,b). The exact mechanism by which prolonged activation of the P2X7-R leads to pore formation remains unknown. Either dilation of the channel or recruitment of ancillary cellular mechanisms has been proposed (North, 2002). The present data provide evidence that P2X7-R-mediated pore formation is likely mediated by intracellular events rather than simply from an agonist-induced conformational change intrinsic to the channel.

**Materials and Methods**

**Materials.** Cell culture medium and phosphate-buffered saline, pH 7.4, were obtained from Invitrogen (Carlsbad, CA). BzATP, ATP, UTP, PPADS, protease inhibitor cocktail, and LPS were obtained from Sigma-Aldrich (St. Louis, MO). Maitotoxin was purchased from Wako Bioproducts (Richmond, VA). Fluoro-4 AM was purchased from TefLabs (Austin, TX), and Yo-Pro-1 (4-((3-methyl-2-3H)-benzoazolylidene) methyl]-1-[3-(trimethylammonio)propyl]-diiodide was purchased from Molecular Probes (Eugene, OR). PD 98059, U0126, U0124, SB 202190-HCl, SB 203580, SB 202474, PD 169316, JNK I and JNK II inhibitors, caspase 1 inhibitor, caspase 2 inhibitor, caspase 3 inhibitor (DEVD), and cathepsin B, a caspase-negative control inhibitor, were obtained from Calbiochem (San Diego, CA). The synthetic peptide dynorphin A was obtained from Bachem Biosciences (King of Prussia, PA). Human interferon-\( \gamma \) (IFN-\( \gamma \)) and the IL-1\( \beta \) ELISA kit were obtained from R&D Systems (Minneapolis, MN) or Pierce Endogen (Rockford, IL). KN-62 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). ELISA kits for the measurement of p38 MAPK [pTpY180/182] and total p38 were purchased from BioSource International (Camarillo, CA).

**Tissue Culture.** Cells of the THP-1 monocytic cell line (American Type Culture Collection, Manassas, VA) were maintained in the log phase of growth in RPMI 1640 medium containing high glucose and 10% fetal calf serum (Invitrogen) according to established procedures (Humphreys and Dubyk, 1996). Fresh vials of frozen THP-1 cells were initiated for growth every 8 weeks. To differentiate THP-1 cells into a macrophage phenotype, a final concentration of 25 ng/ml of LPS and 10 ng/ml of IFN-\( \gamma \) were added to the cells (Humphreys and Dubyk, 1996) either for 3 h for IL-1\( \beta \) release assays or overnight (16 h) for pore formation studies. 1321N1 cells stably expressing the recombinant human P2X7-R receptor were grown and used according to previously published protocols (Bianchi et al., 1999; Lynch et al., 1999). For both the pore formation and IL-1\( \beta \) release assays, cell density and viability were routinely assessed before each experiment by trypsin dye exclusion, and cells were found to be >90% viable after differentiation.

**Calcium Influx Assay.** The Fluorometric Imaging Plate Reader (FLIPR; Molecular Devices, Sunnyvale, CA) was used to measure agonist-induced Ca\(^{2+}\) influx using the calcium-chelating dye Fluo-4 as previously described by Bianchi et al. (1999) with the following modifications. One to two hours before the calcium flux assay, the differentiated THP-1 cells were washed in DPBS using a conical tube and loaded with Fluo-4 AM (2.28 \mu M in DPBS before centrifugation onto poly-lysine-coated black-walled 96-well plates. Cells were then maintained in a dark environment at room temperature. Immediately before the assay, each plate was washed three times with 250 \mu l of DPBS to well to remove extracellular Fluo-4 AM. Two 50-\mu l additions of test compounds (prepared in DPBS) were made to the cells during each experiment. The first compound addition was made after a 30-s baseline determination. Test compounds were incubated for 1 min before the addition of BzATP (final concentration of 30 \mu M), followed by incubation for an additional 1 min. Fluorescence data were collected at 1 s and then at 5-s intervals throughout the course of each experiment. Data shown are based on the maximal fluorescence response expressed in arbitrary relative fluorescence units (Bianchi et al., 1999). Concentration-response data were analyzed using GraphPad Prism (GraphPad Software Inc., San Diego, CA); the EC_{50} or IC_{50} values were derived from a single curve fit to the mean data of \( n = 6 \) in duplicates.

**Yo-Pro Uptake Assay.** Agonist-induced pore formation was also determined using the FLIPR by measuring cellular uptake of Yo-Pro (mol. wt. = 629 Da) in differentiated THP-1 cells with the modifications below. Differentiated THP-1 cells were rinsed once in PBS without Mg\(^{2+}\) or Ca\(^{2+}\) ions, which have been shown to inhibit pore formation (Michel et al., 1999). Yo-Pro iodide dye (1 \mu M in dimethyl sulfoxide) was diluted to a final concentration of 2 \mu M in PBS (without Mg\(^{2+}\) or Ca\(^{2+}\)) and then placed on the cells immediately before agonist addition. After the addition of various agonist concentrations (0.001 – 100 \mu M), Yo-Pro dye uptake was observed in the FLIPR equipped with an argon laser (wavelength = 488 nm) and a charge-coupled device camera. The exposure setting of the camera was 0.25 s with an f-stop setting of 2. Unless otherwise indicated, data points from each experiment were collected over a 60-min period after exposure to appropriate concentrations of BzATP. The intensity of the fluorescence was captured by the charge-coupled...
device camera every 15 s for the first 10 min of agonist exposure, followed by every 20 s for an additional 50 min. For antagonist activity measurements, the maximal intensity was expressed as a percentage of that induced by the EC70 value for agonist activation (50 μM BzATP for THP-1 cells) and plotted against the concentration of compound to determine IC50 values. For each experiment, differentiated control cells were also measured over the 60-min time course to assess background levels of fluorescence. This nonspecific background Yo-Pro uptake, averaging 6 to 10% of the maximum BzATP response (see Fig. 1), was subtracted from the maximum BzATP-induced fluorescence.

For experiments measuring P2X7R-mediated pore formation, a dose-response curve was generated for the agonist ATP under the same conditions used for the P2X-R Yo-Pro uptake assay. EC70 value was determined and used to examine antagonist effects. For MTX-mediated pore formation studies, various concentrations of MTX were examined under the conditions described above, except that the buffer, DPBS (Invitrogen) contained 0.9 mM CaCl2 and 0.5 mM MgCl2, as the presence of extracellular divalent cations are required for MTX activity (Schilling et al., 1999a,b) and inhibition studies were against the EC70 value.

**pp38 MAPK ELISA Measurements.** Undifferentiated THP-1 cells were plated into a T75 flask (Costar, Cambridge, MA) at a density of 5 x 10^6 cells/ml/well. The day before the experiment, the cells were differentiated with 25 ng/ml of LPS and 10 ng/ml of IFNγ for 16 h at 37°C. In the presence of the differentiation media, the cells were incubated with inhibitors for 30 min at 37°C followed by stimulation with 100 μM BzATP for an additional 60 min at 37°C. Pelleted cell lysates were rinsed in PBS, repelleted, and flash-frozen.

On the day of the ELISA, cell lysates were lysed and protein extracted in a 10 mM Tris buffer, pH 7.4, containing: 100 mM NaCl, 1 mM EDTA, 1 mM NaF, 20 μM Na3P2O7, 2 mM Na2VO4, 1% Triton X-100, 10% glycerol, 0.1% sodium deoxycyl sulfate, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and a 5% final concentration of a protease inhibitor cocktail. Lysates were clarified by centrifugation at 13,000 rpm for 10 min at 4°C before the detection of pp38 MAPK or total p38 MAPK activity using an ELISA, following the manufacturer’s instructions.

**IL-1β ELISA Measurements.** Undifferentiated THP-1 cells were plated in 24-well plates at a density of 1 x 10^6 cells/ml/well. On the day of the experiment, cells were differentiated with 25 ng/ml of LPS and 10 ng/ml of IFNγ for 3 h at 37°C. In the presence of the differentiation media, the cells were incubated with inhibitors for 30 min at 37°C followed by a challenge with 1 mM BzATP for an additional 30 min at 37°C. Supernatants were collected after a 5-min centrifugation in microfuge tubes and assayed for the presence of mature IL-1β by ELISA, following the manufacturer’s instructions. For each experiment, differentiated control cells were also measured over the 60-min time course of the assay to assess background IL-1β accumulation. This nonspecific background IL-1β release, typically averaged 3 to 8% of the maximum BzATP response, was subtracted from the maximum BzATP-induced release.

**Results**

**BzATP-Mediated Yo-Pro Uptake in THP-1 Cells.** Yo-Pro incorporation into differentiated THP-1 cells was detectable 15 min after the addition of BzATP (300 μM) and continued at a steady rate for 55 min (Fig. 1A). These kinetics were similar to those observed in human THP-1 cells following LPS/IFNγ stimulation (Fig. 1B).

**Fig. 1.** A, kinetics of BzATP-stimulated Yo-Pro uptake in LPS/IFNγ-treated and untreated human THP-1 cells. Traces represent responses from individual wells of confluent THP-1 cells. B, BzATP (300 μM) stimulated Yo-Pro uptake in LPS/IFNγ-treated (gray bar) and untreated (black bar) human THP-1 cells. Data represent means ± S.E.M. of three to five experiments.

**Fig. 2.** A, concentration-effect curves for P2 receptor agonists to stimulate Yo-Pro uptake in LPS/IFNγ-differentiated THP-1 cells. Data were normalized to the peak BzATP response (percentage of maximum response). B, concentration-effect curves for P2X7R receptor antagonists to block 50 μM BzATP-stimulated Yo-Pro uptake. Data represent mean ± S.E.M. of four to six experiments.
TABLE 1
Effects of MAPK and caspase inhibitors on 50 μM BzATP-induced Yo-Pro uptake in differentiated THP-1 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (nM)</th>
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<tbody>
<tr>
<td>MAPK inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SB 202190</td>
<td>75 ± 6</td>
<td>6</td>
</tr>
<tr>
<td>PD 188316</td>
<td>1800 ± 412</td>
<td>4</td>
</tr>
<tr>
<td>SB 203580</td>
<td>9734 ± 876</td>
<td>5</td>
</tr>
<tr>
<td>SB 202474</td>
<td>&gt;100,000</td>
<td>3</td>
</tr>
<tr>
<td>PD 98059</td>
<td>&gt;30,000</td>
<td>3</td>
</tr>
<tr>
<td>U0126</td>
<td>&gt;100,000</td>
<td>3</td>
</tr>
<tr>
<td>5-Iodotubercidin</td>
<td>&gt;100,000</td>
<td>3</td>
</tr>
<tr>
<td>Caspase Inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YVAD, caspase 1</td>
<td>8000 ± 800</td>
<td>4</td>
</tr>
<tr>
<td>DEVD, caspase 3</td>
<td>3200 ± 300</td>
<td>4</td>
</tr>
<tr>
<td>Cathepsin B, (–) control</td>
<td>&gt;30,000</td>
<td>3</td>
</tr>
<tr>
<td>Dynorphin A, control peptide</td>
<td>&gt;30,000</td>
<td>3</td>
</tr>
<tr>
<td>Oxytocin, control peptide</td>
<td>&gt;30,000</td>
<td>3</td>
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continued to increase over a 60-min period. These results are consistent with the effects previously described using cells expressing the stably transfected recombinant, human P2X7R (Rassendren et al., 1997). Exposure of differentiated THP-1 cells to BzATP for 60 min resulted in a 15-fold increase in fluorescence (Fig. 1, A and B). In contrast, ATP (at THP-1 cells to BzATP for 60 min resulted in a 15-fold increase in fluorescence (Fig. 1, A and B). In contrast, ATP (at

BzATP (IC50 value > 100 μM) using a 1321 astrocytoma cell line transfected with human P2X7R (Bianchi, et al., 1999). Using this same cell line for pore formation measurements, it was determined that SB 202190 had an IC50 value of 1500 ± 180 nM.

PD 98059, a MEK kinase inhibitor (Pang et al., 1995), exhibited only weak inhibition of P2X7R-mediated pore formation (IC50 ~ 100 μM; Fig. 3). In addition, in the 1321 astrocytoma cell line transfected with human P2X7R, PD 98059 (IC50 value > 100 μM) did not significantly block pore formation. Another MEK kinase inhibitor U0126 (Pang et al., 1995) did not significantly inhibit BzATP-stimulated Yo-Pro uptake (Table 1). In addition, 5-iodotubercidin, a potent inhibitor of the ERK2 and adenosine kinases (Fox et al., 1998), also had no effect on P2X7R-mediated pore formation (Table 1).

Inhibition of BzATP-Mediated pp38 MAPK Activation. Differentiation of THP-1 cells produced a significant increase in the level of pp38 MAPK relative to that observed for undifferentiated cells (Fig. 4). The level of pp38 MAPK in differentiated THP-1 cells was further enhanced by exposure to 100 μM BzATP. BzATP-induced pp38 MAPK activation was significantly attenuated (P < 0.05) by the p38 MAPK-specific inhibitor SB 202190 (10 μM) and not by the inert analog, SB 247474 (10 μM). This BzATP-induced pp38 MAPK activation was also significantly attenuated (P < 0.05) by the P2X7-selective antagonist, KN-62 (10 μM), to a level commensurate to that observed for differentiated con-
control THP-1 cells. BzATP stimulation had no effect on the total p38 MAPK detected (data not shown).

**Blockade of Pore Formation with Caspase Inhibitors.** YVAD, a caspase 1 inhibitor, inhibited BzATP-evoked Yo-Pro uptake in a concentration-dependent fashion (IC$_{50}$ value of 8 ± 0.8 μM) (Fig. 5). DEVD, a caspase 3 inhibitor, was slightly more potent in inhibiting BzATP-evoked Yo-Pro uptake with an IC$_{50}$ value of 3.2 ± 0.3 μM (Fig. 5). Cathepsin B, which serves as a negative control for caspase inhibitors as well as two synthetic peptides, oxytocin and dynorphin A, which have comparable molecular weights to the caspase inhibitors and are linked to an inert, longer peptide chain to increase cell permeability (Garcia-Calvo et al., 1998), were also examined for pore-forming inhibitory activity. None of these controls exhibited any significant effect on BzATP-evoked Yo-Pro uptake (Table 1).

**Effects of Other Signaling Pathway Inhibitors on P2X$_7$-Mediated Pore Formation.** Inhibitors of other signaling pathways were also assessed for effects on BzATP-injected Yo-Pro uptake. As shown in Table 2, pretreatment with thapsigargin (Won and Orth, 1995) or xestospongin C (Gafni et al., 1997) had no effect on BzATP-mediated Yo-Pro uptake, suggesting that neither mobilization of Ca$^{2+}$ from internal stores nor inositol 1,4,5-triphosphate-mediated Ca$^{2+}$ release is involved in pore formation (Table 2). In contrast, three PKC selective inhibitors, Go-6976, Ro-31-7549, and Ro-31-8220, exhibited concentration-dependent inhibition of BzATP-evoked Yo-Pro uptake with IC$_{50}$ values of 4.8, 2.5, and 2.4 μM, respectively (Table 2). Two of these PKC inhibitors, Ro-31-7549 and Ro-31-8220, were equally effective in blocking P2X$_7$-mediated calcium influx with IC$_{50}$ values of 5.2 ± 0.5 and 3.5 ± 0.4 μM, respectively.

**Inhibition of BzATP-Mediated IL-1β Release.** BzATP evoked a concentration-dependent increase in IL-1β release from differentiated THP-1 cells with an EC$_{50}$ value of 617 ± 57 μM (Fig. 6A). As shown in Fig. 5B, the selective P2X$_7$ antagonist, KN-62, potently blocked the BzATP-mediated release of IL-1β with an IC$_{50}$ value of 66 ± 3 nM. The nonselective P2XR antagonist PPADS also inhibited P2X$_7$-mediated IL-1β release with an IC$_{50}$ value of 5.8 ± 0.35 μM (Fig. 6B). BzATP-stimulated IL-1β release was not significantly inhibited by various MAPK inhibitors at concentrations up to 10 μM (Fig. 7). In contrast, both caspase inhibitors, YVAD and DEVD, inhibited the BzATP-induced IL-1β release (Fig. 7). Concentration-response determinations for the caspase 1 inhibitor, YVAD, and the caspase 3 inhibitor, DEVD yielded IC$_{50}$ values of 9 ± 1 and 23 ± 3 μM, respectively (Fig. 8). Two of the PKC inhibitors, Ro-31-7549 and Ro-31-8220, also blocked P2X$_7$-mediated IL-1β release with IC$_{50}$ values of 2.8 ± 0.6 and 5.0 ± 0.5 μM, respectively.

**Non-P2X$_7$-Mediated Pore Formation.** MTX, which produces cytolytic membrane pores via a non-P2X$_7$-related mechanism (Schilling et al., 1999a), induced pore formation in differentiated THP-1 cells with an EC$_{50}$ value of 6.8 ± 0.4 μM (Fig. 9A). This effect was blocked by calmidazolium (Fig. 9B, Table 3) consistent with previous reports (Schilling et al., 1999a).

Prolonged agonist-evoked activation of the P2X$_7$ receptors can also lead to the formation of cell membrane pores (Khakh

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**TABLE 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (μM)</th>
<th>n</th>
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<tbody>
<tr>
<td>Thapsigargin</td>
<td>&gt;100</td>
<td>3</td>
</tr>
<tr>
<td>Xestospongin C</td>
<td>&gt;100</td>
<td>3</td>
</tr>
<tr>
<td>Go-6976</td>
<td>4.9 ± 0.6</td>
<td>4</td>
</tr>
<tr>
<td>Ro-31-7549</td>
<td>2.5 ± 0.1</td>
<td>4</td>
</tr>
<tr>
<td>Ro-31-8220</td>
<td>2.4 ± 0.1</td>
<td>6</td>
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Fig. 5. Concentration-effect curves for a caspase 1 inhibitor, YVAD (black square), and a caspase 3 inhibitor, DEVD (white square), to attenuate 50 μM BzATP-induced Yo-Pro uptake. Data represent mean ± S.E.M. of three to five experiments.

Fig. 6. A, concentration-effect curve for BzATP stimulated release of mature IL-1β in LPS/IFN-γ differentiated THP-1 cells. B, concentration-dependent inhibition of 1 mM BzATP-stimulated IL-1β release by KN-62 and PPADS. Inhibition data were normalized to the peak BzATP response (percentage of maximum response), which ranged from 200 to 600 pg/ml of IL-1β released. Data represent means ± S.E.M. of three to five experiments.
Inhibitors on pore formation mediated by P2X7 receptors as assessed by Yo-Pro uptake. Calmidazolium blocked MTX-induced Yo-Pro uptake with an EC50 value of 3 nM (Fig. 9A). This ATP-induced P2X2a pore formation was blocked by PPADS with an IC50 value of 477 ± 12 nM (Fig. 9G, Table 3).

Table 3 summarizes the effects of several antagonists and inhibitors on pore formation mediated by P2X receptors as well as by other mechanisms. The nonselective P2X receptor antagonist PPADS potently blocked agonist-evoked Yo-Pro uptake mediated by activated P2X7, but was ineffective in blocking the effects of MTX. The P2X7 receptor-selective antagonist, KN-62, only blocked P2X7-mediated Yo-Pro uptake. Calmidazolium blocked MTX-induced P2X7 pore formation (IC50 = 8 μM) consistent with previous reports (Schilling et al., 1999b) but had no effect on P2X7- and P2X2a-R-mediated Yo-Pro uptake. U73122 [1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino[hexyl]-1H-pyrole-2,5-dione, a phospholipase C inhibitor (Smith et al., 1986) that has been shown to block MTX-mediated cell death (Estacion and Schilling, 2002), only blocked MTX-induced Yo-Pro uptake with an IC50 value of 1800 ± 400 nM.

The caspase 3 inhibitor, DEVD, significantly inhibited both P2X7- and P2X2a-mediated Yo-Pro uptake in a concentration-dependent fashion but did not block the MTX-induced pore formation (Table 3). Interestingly, SB 202190, the specific and potent p38 MAPK inhibitor, blocked P2X7-mediated pore formation but had no effect on Yo-Pro uptake mediated through either P2X2a receptors or MTX treatment.

Discussion

In the present study, we examined putative signaling mechanisms for two functional endpoints that are associated with P2X7R activation, agonist-evoked pore formation (Yo-Pro uptake) and IL-1β release. BzATP-stimulated Yo-Pro uptake provided a pharmacologically specific assay for native human P2X7R function on differentiated THP-1 cells based on the order of agonist (BzATP >> ATP and UTP) and antagonist (KN-62 >> PPADS) potency (Jacobson et al., 2002; North, 2002). Inhibition of BzATP-evoked IL-1β release showed a similar order of antagonist block. Although the precise signaling mechanisms governing each of these events is not completely understood, activation of P2X7Rs has been associated with a number of downstream pathways, including phospholipase D (Humphreys and Dubyak 1996), phospholipase A2, nuclear factor kappa B (NF-κB) (Ferrari et al., 1997b; Aga et al., 2002), pro-caspase 1 (Verhoef et al., 2003), and MAPK (Agan et al., 2002; Armstrong et al., 2002; Bradford and Solttof, 2002).

Agonist-evoked P2X7-R pore formation is a property shared by other non-desensitizing P2XRs (e.g., P2X2, P2X2a, and P2X3) but the time course and pore size formed by these receptors differ from that observed for the P2X7-R (Khakh et al., 1999; Virginio et al., 1999; North, 2002). MTX also induces pore formation via a non-P2X7-R-mediated mechanism (Schilling et al., 1999b). Both intrinsic P2X7-R channel dilatation and P2X7-R-dependent recruitment of accessory proteins have been proposed as candidate pore-forming mechanisms (North, 2002). Data demonstrating that P2X7-R pore formation is progressive, occurs in multiple cell types, and has an identical pharmacology for the opening of the cation channel support the intrinsic receptor hypothesis.

The present data demonstrate that nativey expressed human P2X7-R-mediated pore formation in differentiated THP-1 cells can be blocked by selective inhibitors of p38 MAPK and caspase activity. The present data also show that BzATP stimulated the activation of ps38 MAPK above the levels of control in a concentration-dependent fashion but did not block the MTX-induced pore formation (Table 3). Interestingly, SB 202190, the specific and potent p38 MAPK inhibitor, blocked P2X7-mediated pore formation but had no effect on Yo-Pro uptake mediated through either P2X2a receptors or MTX treatment.

![Fig. 7. Effect of MAPK and caspase inhibitors on BzATP-induced IL-1β release. The differentiated THP-1 cells were pretreated for 30 min with 10 μM listed MAPK inhibitors, caspase inhibitors, and control compounds before stimulation of IL-1β release with 1 μM BzATP. Data are shown as a percentage of BzATP-induced IL-1β release in the absence of inhibitors (which usually ranged from 200–600 pg/ml of IL-1β). Values shown represent the means ± S.E.M. of three to five experiments in duplicates. Statistical significance (*) represents a difference between control and experimental values with the use of analysis of variance. **, P < 0.01.](image-url)
induced by THP-1 cell differentiation with LPS and IFNγ (Ono and Han, 2000). The p38 MAPK inhibitor, SB 202190, as well as the P2X7-selective antagonist, KN-62, both significantly attenuated the BzATP-mediated production of pp38 MAPK, providing evidence for a direct interaction between P2X7R activation and the p38 MAPK pathway. The p38 MAPK-dependent pore formation was also demonstrated in 1321N1 cells expressing recombinant human P2X7 Rs. The differential potency of SB 202190 to block agonist-evoked pore formation in differentiated THP-1 cells and in the recombinant receptor cell line likely reflects differences in receptor expression as assessed by differences in agonist-evoked Yo-Pro fluorescence (D. L. Donnelly-Roberts, unpublished observations). This finding provides the first evidence that these downstream intracellular events play a role in P2X7R-mediated pore formation. The involvement of the p38 MAPK pathway may be mechanistically selective for P2X7R-mediated pore formation since inhibitors of this pathway were ineffective in blocking P2X7R-mediated IL-1β release and did not alter pore formation mediated by MTX or by activation of P2X7R receptors. The present demonstration that BzATP-evoked Yo-Pro uptake is modulated by both MAPK and caspase inhibition, whereas BzATP-stimulated IL-1β release is sensitive to only caspase inhibition, is consistent with previous work, suggesting that these P2X7 receptor-mediated events are at least in part mechanistically distinct (Schilling et al., 1999b; MacKenzie et al., 2001).

MAPKs are a family of serine and threonine kinases that are activated by various external stimuli to regulate a variety of intracellular events (English and Cobb, 2002). To date, there are three major signaling protein families involved in the MAPK cascades: ERK1/2, which is mainly involved in cellular growth and proliferation; JNK/stress-activated protein kinase, which is involved in cellular stress from ultraviolet irradiation, heat shock, or exposure to inflammatory...
cytokines; and the newest member, p38 kinase, which is activated upon exposure to inflammatory cytokines, endotoxins, or osmotic shock and often leads to apoptotic cell death (Su and Karin, 1996).

In contrast to the inhibitory effects of p38 MAPK inhibitors on BzATP-stimulated Yo-Pro uptake, no significant attenuation of BzATP-evoked pore formation was observed by inhibitors of MEK, ERK1/2, or by inhibitors of intracellular Ca\(^{2+}\) mobilization and inositol 1,4,5-triphosphate-mediated-Ca\(^{2+}\) release. Cell-permeable JNK I and JNK II inhibitors, as well as negative control (JNK-inactive) analogs were found to produce a nonspecific inhibition of BzATP-induced pore formation (D. L. Donnelly-Roberts, unpublished observations), thus limiting a mechanistic interpretation of these findings. Inhibitors of PKC were moderately effective in reducing BzATP-stimulated pore formation, consistent with a recent report demonstrating that PKC activation may be upstream from activation of p38 MAPK in some pathways (Bradford and Soltoff, 2002). However, representative PKC inhibitors are equally effective in blocking both Ca\(^{2+}\) influx and IL-1\(\beta\) release, indicating that this pathway may not be unique to pore formation but more generally involved in events mediated by P2X\(_R\) activation. Alternatively, it cannot be ruled out that the effects of PKC and JNK inhibitors may be mediated simply by blocking P2X\(_R\) activation (i.e., receptor antagonism). Taken together, the present data suggest that a P2X\(_R\)-linked PKC-p38 kinase pathway is involved in the cytopathic effects of prolonged P2X\(_R\) activation in differentiated THP-1 cells. Supportive evidence for the involvement of p38 MAPK activity in P2X\(_R\)-mediated depression of mossy fiber synaptic activity has been previously reported (Armstrong et al., 2002).

Another pathway triggered by P2X\(_R\) activation is the caspase pathway. The caspases are a family of cysteine proteases involved in both apoptosis and cytokine release (Thornberry and Lazebnik, 1995). Caspase 1 is involved in the release of mature IL-1\(\beta\) from leaderless pro-IL-1\(\beta\), whereas caspase 3 activity has been shown to be involved in P2X\(_R\)-mediated apoptotic cell death (Perregaux et al., 2001). In the present study, the caspase 1 and 3 inhibitors, YVAD and DEVD, exhibited concentration-dependent inhibition of P2X\(_R\)-mediated IL-1\(\beta\) release and Yo-Pro uptake. Interestingly, these compounds showed inverted potency orders in blocking these two P2X\(_R\)-mediated cleavage events. The caspase 1 inhibitor, YVAD, was more potent than the caspase 3 inhibitor, DEVD, in attenuating BzATP-stimulated IL-1\(\beta\) release, whereas the reverse was true for pore formation. These differences suggest some degree of mechanistic divergence in the generation of these two P2X\(_R\)-mediated events. This idea is further supported by the inability of p38 MAPK inhibitors to attenuate P2X\(_R\)-mediated IL-1\(\beta\) release, whereas they effectively block P2X\(_R\)-mediated pore formation. It should be noted that this apparent difference in mechanism cannot be explained by potential differences in cell death due to differentiation conditions, since similar levels of lactate dehydrogenase released were observed under both assay conditions (D. L. Donnelly-Roberts, unpublished observations). Although it has previously been shown that P2X\(_R\)-mediated stress-activated protein kinase-MAPK activity is independent of caspase 1 or caspase 3 activity (Humphreys et al., 2000), this does not rule out the possibility that the caspase isozymes may participate in P2X\(_R\)-stimulated pore formation along a parallel pathway. These two events are also temporally differentiated, since it has been shown that IL-1\(\beta\) containing microvesicle shedding occurs within seconds of P2X\(_R\)-mediated pore formation (MacKenzie et al., 2001), whereas P2X\(_R\)-mediated pore formation requires minutes of agonist exposure.

It was of interest to further investigate whether the involvement of the MAPK and caspase pathways were unique to P2X\(_R\)-mediated pore formation or common to other pore formation mechanisms such as those caused by MTX (Schilling et al., 1999a) or P2X\(_2\),P2X\(_3\)R activation (Khakh et al., 1999). In contrast to the effects on P2X\(_R\)-mediated pore formation, the p38 MAPK inhibitor, SB 202190, did not block pore formation induced by MTX or P2X\(_R\)-R activation. Both P2X\(_R\)-R- and P2X\(_2\),P2X\(_3\)R-induced pore formation were attenuated by the caspase 3 inhibitor, DEVD, suggesting an apoptotic cell death mechanism induced by prolonged activation of both P2X receptors (Thornberry and Lazebnik, 1995). Although not sensitive to MAPK or caspase inhibition, MTX-induced pore formation was significantly attenuated by calmidazolium and U73122, a putative inhibitor of phospholipase C (Estacion and Schilling, 2002). Taken together, these data demonstrate that pore formation mediated by activation of P2X\(_R\) is mechanistically distinct from that produced by MTX or agonist activation of P2X\(_R\)-Rs.

In conclusion, the present data demonstrate that both p38 MAPK and caspase pathways are involved in P2X\(_R\)-mediated pore formation, whereas the caspase pathway but not p38 MAPK is mechanistically linked to agonist-evoked IL-1\(\beta\) release. These data are consistent with other recent reports (MacKenzie et al., 2001; Verhoef et al., 2003), indicating that many of the functional sequelae engendered by P2X\(_R\) activation including IL-1\(\beta\) release, membrane blebbing, and pore formation may be mediated by parallel, rather than convergent, intracellular signal transduction pathways. These data also demonstrate that the involvement of p38 MAPK in pore formation is specific to P2X\(_R\) and does not contribute to the pore-forming mechanisms associated with activation of P2X\(_2\),P2X\(_3\)R or by exposure to MTX. Collectively, the data support the hypothesis that pore formation is a consequence of P2X\(_R\) activation but dependent on the involvement of downstream signaling interactions due to the mechanistic difference between pore formation and channel opening.

References


Protein Sci (Wash DC) 7:2249–2255.


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