Cell signaling via the P2X₇ nucleotide receptor: linkage to ROS production, gene transcription, and receptor trafficking

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Abstract Extracellular nucleotides can act as important intercellular signals in diverse biological processes, including the enhanced production of factors that are key to immune response regulation. One receptor that binds extracellular adenosine triphosphate released at sites of infection and injury is P2X₇, which is an ionotropic receptor that can also lead to the formation of a non-specific pore, activate multiple mitogen-activated protein kinases (MAPKs), and stimulate the production of immune mediators including interleukin family members and reactive oxygen species (ROS). In the present report, we have investigated the signaling mechanisms by which P2X₇ promotes monocytic cell mediator production and induces transcription factor expression/phosphorylation, as well as how receptor-associated pore activity is regulated by intracellular trafficking. We report that P2X₇ stimulates ROS production in macrophages through the MAPKs ERK1/2 and the nicotinamide adenine dinucleotide phosphate oxidase complex, activates several transcription factors including cyclic-AMP response element-binding protein and components of the activating protein-1 complex, and contains specific sequences within its intracellular C-terminus that appear critical for its activity. Altogether, these data further implicate P2X₇ activation and signaling as a fundamental modulator of macrophage immune responses.

Keywords P2X₇ · ROS · CREB · AP-1 · Arg-based ER retention signals · Mediator production

Introduction

Extracellular nucleotides such as adenosine triphosphate (ATP), uridine triphosphate (UTP), and their metabolites (e.g., uridine diphosphate (UDP), adenosine diphosphate (ADP)) are now recognized as highly important signaling molecules that can mediate an array of physiological processes, including neurotransmission, muscle contraction, pain sensation, and diverse immune responses [1–4]. Adenine nucleotides are stored in high concentrations within vesicles of platelets and immune cells, such as basophils and mast cells, and can be released into the extracellular space upon degranulation or following cell lysis resulting from infection, inflammation, or tissue damage [5, 6]. These extracellular nucleotides initiate multiple downstream events through their binding to a family of cell surface receptors, including the P2 nucleotide receptors, which have been divided into two major subfamilies: G-protein-coupled P2Y receptors that can bind ATP, ADP, UTP, and UDP (depending of the receptor subtype) and the ionotropic P2X receptors that largely bind ATP [7–9].

The stimulation of P2X receptors, in particular P2X₇, has been implicated as a key process in a variety of inflammatory conditions, including sepsis, arthritis, extrapulmonary tuberculosis, granuloma formation, and Alzheimer's disease [10–16]. P2X₇ is unique among the other P2X receptors in that it requires high concentrations (millimolar) of ATP, such as those observed at sites of inflammation, to be activated. In addition, P2X₇ is sensitive to the synthetic

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ATP analog 3'-O-(4-benzoyl) benzoyl ATP (BzATP), and thus BzATP is often used to study P2X₇ action. Upon ligand binding, P2X₇ is linked to the formation of a cation channel that mediates the influx of Ca²⁺ and Na⁺ and the efflux of K⁺. Upon prolonged stimulation, P2X₇ can also allow for the formation of a non-specific pore that permits the passage of molecules of up to 900 Da.

Among the many critical functions of P2X₇ is its capacity to control various immune responses, e.g., P2X₇ activation leads to the enhanced release of multiple immune modulators, including reactive oxygen species (ROS), it mediates host defense against intracellular pathogens, it initiates membrane blebbing, and it promotes apoptosis [8]. Interestingly, P2X₇ ligation by extracellular ATP can also modulate lipopolysaccharide (LPS)-mediated macrophage activation, and P2X₇ stimulation is known to potentiate the release of several LPS-induced pro-inflammatory mediators, including interleukin-1 β (IL-1 β), tumor necrosis factor- α , and nitric oxide [17–22]. However, although there is a growing literature regarding several cell signaling and cytokine processing events that are initiated upon P2X₇ activation, relatively little is known about the regulation of receptor trafficking and the mechanisms by which P2X₇ activation can impact on long-term cellular processes such as the regulation of gene transcription. In this regard, we have reported that P2X₇ action can influence LPS-stimulated expression of inducible nitric oxide synthase (iNOS), and others have shown that P2X₇ activation is not always linked to apoptosis [23–26], which is an event often associated with cell exposure to P2X₇ agonists. These observations led to the hypothesis that, under certain circumstances, P2X₇ stimulation may not only elicit immediate cellular responses, such as the potentiation of cytokine processing, but that it can also have an important effect on longer term cellular behavior via effects on gene expression.

Given that animal and human genetic studies support the concept that P2X₇ is a major immune modulator and may be an effective therapeutic target [11–14, 27–29], in this report, we will discuss several key events associated with P2X₇ signaling and trafficking, with an emphasis on their role in mediating/regulating the immune responses of monocytic cells. Specifically, we will provide evidence that P2X₇ stimulation in macrophages promotes ROS production via the extracellular-regulated kinases-1 and 2 (ERK1/2) and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, and that ligand binding to P2X₇ results in activation of the transcription factor cyclic-AMP response element-binding protein (CREB) and the expression of key components of the transcription factor activating protein-1 (AP-1) complex. Because receptor trafficking appears critical for controlling cell responsiveness to extracellular ATP [7–9, 18, 30], we will also discuss the mechanisms by which P2X₇

activity/trafficking may be regulated by specific residues in its C-terminus. As an overview, we will first discuss the general properties of P2X₇ as well as human genetic and animal studies that have revealed several of the biological roles of this receptor.

General properties of P2X₇

As noted above, extracellular nucleotides can bind to P2 receptors to elicit diverse cellular responses. There are two P2 receptor subfamilies, the seven-transmembrane spanning P2Y G-protein-coupled receptors and the double membrane spanning P2X cation channels. There are at least eight P2Y family members (P2Y_{1–2}, P2Y₄, P2Y₆, P2Y_{11–14}) and seven P2X receptors (P2X_{1–7}) [8, 9]. All of the P2X receptors contain a short (<35 amino acids) intracellular N-terminal region, a large extracellular nucleotide-binding domain, and an intracellular C-terminus. The human nucleotide receptor P2X₇ is composed of 595 amino acids and is unique in that it contains a C-terminus that is ~120 amino acids longer than the next largest P2X receptor [8]. Deletions within the extended P2X₇ C-terminal region that are N-terminal to residue 582 have been shown to reduce its pore-forming ability and to inhibit its expression on the cell surface [30]. Furthermore, the P2X₇ C-terminus contains several potential protein–protein and protein–lipid interaction motifs, including an LPS/lipid-binding domain, PxxP sequences (which are known to bind to SH3 domains) [31], and an apparent trafficking domain (Fig. 1; also see “P2X₇ contains a trafficking domain in its C-terminus” section below).

P2X₇ in animal models of disease

Many in vivo animal studies have revealed the importance of P2X₇ in various diseases. Studies in rodents have directly implicated P2X₇ in immune response regulation especially in the context of exacerbating arthritic symptoms and inhibiting *Chlamydia* infection. For example, mice deficient in P2X₇ exhibit decreased monoclonal anti-collagen-induced arthritis in comparison to wild-type counterparts, and rats treated with oxidized ATP experience less adjuvant-induced pain than control animals [11, 12]. In support of the hypothesis that P2X₇ plays a role in host defense against pathogens, P2X₇-deficient mice were reported to exhibit increased vaginal *Chlamydia* infection in comparison to wild-type mice [27]. These in vivo results illustrate the involvement of P2X₇ in exacerbating arthritic pain and attenuating pathogen infection, supporting the idea that targeting P2X₇ may be useful in treating certain inflammatory diseases.

Fig. 1 General diagram of P2X₇ highlighting its two trans-membrane domains (TM), the extracellular ATP-binding domain, the potential SH3 domain-interacting region (PxxP), the trafficking domain (TD), and the LPS-binding domain (LPS BD). Several of the identified polymorphic amino acids along with a sequence within the putative trafficking domain of the receptor are also indicated

P2X₇ polymorphisms and altered expression levels in human disease

In addition to the aforementioned animal studies, there is growing evidence that P2X₇ plays an important role in human disease. In this regard, several single nucleotide polymorphisms (SNPs) have been identified in the promoter, introns, and coding sequences of P2X₇ [29, 32–41]. Investigations evaluating the significance of P2X₇ polymorphisms and overall protein expression levels support the idea that cells need to correctly respond to extracellular ATP via P2X₇ for the mediation of several functions, including neurotransmission, mycobacterial killing, and appropriate cell death. Genetic analyses have linked P2X₇ polymorphisms to *Mycobacterium tuberculosis* (TB) infection and bipolar affective disorder [13, 14, 28, 29]. The P2X₇ A1513C polymorphism, which results in mutation of Glu496 to Ala, has been associated with TB in two different population groups [13, 14], and one report has shown that a SNP located in the promoter at position –762 is protective against the bacterium [28]. These results indicate that analysis of P2X₇ may eventually be used to assess an individual's susceptibility to TB infection.

Because P2X₇ activation is often associated with apoptosis, it is noteworthy that several groups have proposed that P2X₇ may be used as a cancer biomarker [42–45]. In support of this concept, a few studies have shown that P2X₇ messenger RNA (mRNA) and protein levels are altered in cancer cells in comparison to normal tissue [43–47], and P2X₇ SNPs have been found in colorectal cancers [42]. Gorodeski and colleagues have observed that P2X₇ is expressed at lower levels in uterine cancer and cervix squamous carcinoma in comparison to normal tissue and that uterine cervical cells express a 258 amino acid-truncated P2X₇ variant that antagonizes the ability of the full-length receptor to promote apoptosis [45,

47, 48]. In contrast, thyroid papillary carcinoma cells express higher levels of P2X₇ in comparison to normal thyroid tissue, and it has been suggested that this increased level of P2X₇ and subsequent increase in intracellular Ca²⁺ results in the enhanced release of the pro-inflammatory cytokine interleukin-6 (IL-6) [44]. Altogether, these findings associating P2X₇ expression and SNPs to human disease reveals that P2X₇ is a highly complex protein that plays an essential role in responding to extracellular ATP.

P2X₇ modulation of innate immunity

Stimulation of P2X₇ is known to result in the activation of several cellular events including K⁺/Ca²⁺-mediated signaling and the activation of phospholipase D and mitogen-activated protein kinase (MAPK) cascades. In addition, many investigations have focused on the role of P2X₇ in the modulation of cellular responses to classical activators such as LPS, e.g., P2X₇ signaling in the classically activated macrophage has been shown to potentiate such diverse processes as the release of IL-1β, IL-6, and IL-18, the expression of iNOS, and the activation of metalloproteases and caspases (see [49–51] for reviews of P2X₇ signaling in immune cells).

Recent studies have revealed additional roles for P2X₇ signaling in mediator production and transcriptional regulation of unprimed immune cells. For example, reports by our lab and others have shown that P2X₇ activation results in ROS production, which may act as a second messenger in several P2X₇ agonist-induced events [16, 17, 21, 52, 53]. However, the mechanisms by which P2X₇ promotes ROS production are less well understood, and ROS generation in response to P2X₇ agonists has not been demonstrated in primary cells. However, in this report, we show that P2X₇ mediates ROS production in primary human monocytes and

that generation of ROS in RAW 264.7 likely involves ERK1/2 and the NADPH oxidase complex. In addition, although prolonged P2X₇ activation can result in apoptotic or necrotic cell death [8, 54], it has been found that transient stimulation of P2X₇ does not lead to apoptosis [24–26] but can induce cell proliferation [25, 26]. These reports, together with the findings that P2X₇ regulates the expression of iNOS and early growth response factor-1 (EGR-1) expression support the idea that P2X₇ can function in the control of gene transcription [17, 55]. To this end, we also tested the hypothesis that P2X₇ stimulation results in the activation of several transcription factors in macrophages including CREB and AP-1 (see “P2X₇-mediated transcription factor activation” section below).

P2X₇ activation mediates NADPH oxidase-dependent ROS production

The production of ROS, such as superoxide (O₂^{•−}), hydrogen peroxide (H₂O₂), and hydroxyl radicals (OH), is vital to a variety of biological processes. When properly regulated, ROS maintain an integral role in host defense, regulation of apoptosis, and modulation of cell signaling. The generation of ROS has also been implicated in several human disorders, including hypertension, atherosclerosis, ischemia/reperfusion injury, neurodegenerative disease, chronic granulomatous disease, and the deleterious effects of aging [18, 56]. The use of ROS by leukocytes in the defense against pathogens is well recognized, but the role of ROS as second messengers is still emerging, e.g., ROS generation has been linked to the activation of transcription factors, including apurinic/apyrimidinic endonuclease 1 (APE1)/Ref1, nuclear factor κB (NFκB), and AP-1, as well as the signaling kinases p90 ribosomal S6 kinase (p90RSK) and various MAPKs [21, 57–65]. Although the mechanisms of ROS-induced signaling have not been fully resolved, multiple processes including inactivation of phosphatases through cysteine modification and the activation of redox sensitive systems, have been proposed [57, 61, 62, 66].

With respect to P2X₇ and ROS generation, Ferrari et al. have noted that activation of NFκB in microglial cells by P2X₇ agonists is sensitive to antioxidants, indicating that ROS may play a role in this endpoint [67]. Similarly, we have observed that stimulation of the MAP kinases ERK1/2, p38, and c-Jun N-terminal kinase (JNK) in the murine macrophage cell line RAW 264.7 by P2X₇ agonists is sensitive to antioxidants, suggesting that ROS may play a more global role in P2X₇ signaling [21]. Although P2 receptor potentiation of ROS production has been seen with several immune cell types, the role of P2X₇ in nucleotide-dependent ROS production in macrophages is less well

known. To this end, we have shown that P2X₇ agonists stimulate ROS production in RAW macrophages but not in a RAW cell line containing a non-functional P2X₇ receptor [21]. However, because the behavior of cell lines may differ from primary cells, we have now tested whether P2X₇ agonists can promote ROS production in human primary cells. As shown in Fig. 2a, we have observed that the P2X₇ agonist BzATP can stimulate ROS production in primary human monocytic cells to levels even greater than that observed with murine RAW 264.7 macrophages. These results reveal that this effect of P2X₇ agonists is not restricted to cell lines and is characteristic of primary cells as well.

Past studies have shown that P2X₇ agonists are able to modulate macrophage activation in response to LPS, resulting in increased pro-inflammatory mediator production [17, 18, 23, 56, 67–76]. Because LPS is known to activate ROS production in leukocytes [77, 78], and because P2X₇ activation is able to augment several LPS-induced signaling events, we tested the hypothesis that P2X₇-stimulated ROS production is enhanced in LPS-primed macrophages. We observed that LPS-primed macrophages stimulated with P2X₇ agonists display synergistic ROS production greater than either stimulus alone [21]. However, it is unclear if P2X₇ activation can synergize with other Toll-like receptor (TLR) systems besides TLR4 (which is an LPS receptor). Thus, we have now tested the capacity of P2X₇ ligands to cooperate with other TLR ligands in the generation of ROS, and as shown in Fig. 2b, we detect synergistic ROS production after P2X₇ agonist treatment of macrophages primed with other TLR ligands, namely the lipopeptide Pam3CSK4 (a TLR2/1 ligand) and zymosan (a TLR2/6 ligand). These data further implicate P2X₇ activation and signaling as a fundamental modulator of macrophage immune responses in response to multiple TLR ligands.

Although ROS can be generated by various enzyme systems, including NADPH oxidase, nitric oxide synthases, xanthine oxidase, and the mitochondrial respiratory chain, we first tested the hypothesis that the major system controlling O₂^{•−} production by P2X₇ agonists in macrophages is NADPH oxidase. The NADPH oxidase complex is comprised of several differentially regulated subunits that accept an electron from NADPH and donate it to molecular oxygen (see [78, 79] for comprehensive reviews). A link between NADPH oxidase activation and P2X₇ signaling in monocytic cells appears plausible in that Parvathenani et al. demonstrated that P2X₇ agonists can modulate the localization of the NADPH oxidase subunit p67^{phox} in microglia [16], and Noguchi et al. found that P2X₇ agonist-induced ROS production is sensitive to the NADPH oxidase inhibitor apocynin and can be attenuated in RAW 264.7 macrophages by reducing the expression of the NADPH subunit gp91phox [52]. Similarly, we have

Fig. 2 The P2X₇ agonist BzATP stimulates ROS production in primary human monocytic cells. **a** RAW 264.7 cells ($n=12$) were cultured as described [21] and primary human monocytes ($n=5$) were purified and cultured as outlined earlier [105]. The cells were first treated with HEPES buffer (control) or 1 μ g/ml LPS (*E. coli*, serotype 0111:B4) for 10 h, and then transferred to flow cytometry tubes (5×10^5 cells/tube) and loaded with 10 μ M 2',7'-dichloro-dihydrofluorescein diacetate (DCFDA) for 30 min at 37°C. The cells were then treated with either HEPES buffer (control) or 250 μ M BzATP for 30 min. DCFDA fluorescence was assessed using flow cytometry as detailed previously [21]. The data are plotted as geometric mean ($\pm$ SEM) of DCFDA fluorescence. **b** RAW 264.7 cells were first treated with HEPES buffer (control), 1 μ g/ml LPS, 1 μ g/ml Pam3CSK4, or 250 μ g/ml zymosan for 10 h, and then transferred to flow cytometry tubes (5×10^5 cells/tube) and loaded with 10 μ M DCFDA for 30 min at 37°C. The cells were then treated with either HEPES (control) or 250 μ M BzATP for 30 min. DCFDA fluorescence was assessed using flow cytometry. The data are plotted as the mean ($\pm$ SEM) fold increase over control ($n=3$). * $p<0.05$, ** $p<0.005$, *** $p<0.001$, and $\psi p<0.0001$ as compared to control pretreated, control-induced DCFDA fluorescence

found that P2X₇ agonist-induced JNK phosphorylation in RAW macrophages is sensitive to the NADPH oxidase inhibitor diphenyliodonium and that ROS production stimulated by P2X₇ agonists in LPS-primed RAW 264.7 cells is sensitive to the NADPH oxidase inhibitor apocynin (data not shown).

Many stimuli, including LPS, phorbol 12-myristate 13-acetate (PMA), and zymosan, increase the expression of NADPH oxidase subunits in leukocytes and can induce their spatiotemporal assembly and activation [77, 78, 80].

To further characterize P2X₇ modulation of the NADPH oxidase complex in macrophages, we assessed the effect of P2X₇ agonists on the phosphorylation of a cytosolic subunit of NADPH oxidase, p40^{phox}. As shown in Fig. 3, treatment of RAW macrophages with BzATP or high doses of ATP results in p40^{phox} phosphorylation, and this process is enhanced after LPS priming. These stimuli had no detectable effect on total p40^{phox} levels (data not shown). To test the receptor specificity, we evaluated p40^{phox} phosphorylation in response to the administration of P2X₇ agonists to a RAW 264.7 macrophage cell line expressing a non-functional P2X₇ mutant. We found that although treatment with PMA was able to induce p40^{phox} phosphorylation in the P2X₇ mutant cell line, P2X₇ activity was critical for BzATP/ATP-induced phosphorylation of p40^{phox} (Fig. 3). Further support for P2X₇ involvement in p40^{phox} phosphorylation comes from the observation that agonists (UTP, UDP, ADP, and low ATP doses) selective for other nucleotide receptors did not stimulate p40^{phox} phosphorylation (data not shown), illustrating that the pharmacological profile of p40^{phox} phosphorylation is consistent with P2X₇ stimulation. Together, these data reveal that P2X₇ stimulates ROS production in a manner that is concomitant with the phosphorylation of NADPH oxidase subunits.

Many protein kinases have been implicated in the stimulus-induced assembly and activation of the NADPH oxidase complex, including protein kinase C (PKC) isoforms, MAPKs, and p21-activated kinase [78, 79, 81–83]. Given that P2X₇ activation results in the phosphorylation of NADPH oxidase subunits and subsequent ROS production, we examined the involvement of the MEK–ERK1/2 MAPK network in P2X₇-stimulated ROS production. To this end, RAW macrophages were pretreated with increasing concentrations of U0126, a MEK1/2 inhibitor, followed by stimulation with BzATP, and then ROS production was assessed by flow cytometry. We found that U0126 pretreatment is able to decrease P2X₇ agonist-stimulated ROS production and greatly attenuate the enhanced ROS production found in P2X₇ agonist-stimulated LPS-primed macrophages (Fig. 4). Conversely, general antagonists of several PKC isoforms (such as Go6976 and rottlerin), as well as p38 MAPK (i.e., SB203580), had little effect (0–3%) on P2X₇ agonist-stimulated ROS levels in LPS-primed macrophages. These data support the concept that the MEK/ERK pathway is critical for P2X₇-induced ROS production (Fig. 4).

P2X₇-mediated transcription factor activation

Activation of P2X₇ has been linked not only to apoptosis, but also to lymphocyte cell proliferation [25, 84] and transcriptional activation in immune cells [85]. As noted

Fig. 3 P2X₇ agonists promote p40^{phox} phosphorylation in murine macrophages. RAW 264.7 cells expressing either wild-type or defective P2X₇ were pretreated with either HEPES buffer (control) or 1 μ g/ml LPS for 10 h. The cells were then treated with either HEPES (control), 1 μ g/ml LPS, 250 μ M BzATP, or 3 mM ATP for 30 min or 1 μ g/ml PMA for 10 min. **Left panel: a** phosphorylation of p40^{phox} in murine macrophages expressing wild-type P2X₇ and treated with either 1 μ g/ml PMA (as a positive control) or 250 μ M BzATP. The results of nine independent experiments were combined and are presented as the mean ($\pm$ SEM). **b** Phosphorylation of p40^{phox} in

murine macrophages expressing wild-type P2X₇ and treated with 3 mM ATP. The results were obtained from six independent experiments and plotted as the mean ($\pm$ SEM). * p <0.05 as compared to control pretreated, control-induced p40^{phox} phosphorylation. **Right panel:** a representative immunoblot displaying p40^{phox} phosphorylation in response to the indicated stimuli is shown for RAW 264.7 cells expressing either wild-type (*upper blot*) or defective P2X₇ (*lower blot*). Immunoblotting was performed with anti-Grb2 antibody as a loading control

above, several studies have implicated P2X₇ activation in regulating the processing and release of interleukin family members and the promotion of iNOS expression. A recent mRNA microarray analysis comparing P2X₇ agonist-treated peripheral blood mononuclear cells (PBMCs) from tuberculosis patients with P2X₇ agonist-treated cells from control patients revealed differential gene expression, suggesting a potentially complex role of P2X₇ in gene regulation [86]. Thus, although P2X₇ stimulation appears to affect gene expression, the mechanisms by which these events occur are not well-defined. In this regard, a few studies evaluating the mechanisms of P2X₇-induced modulation of gene expression have suggested a role for several transcription factors including NF κ B, nuclear factor of activated T cells, signal transducers and activators of transcription [85], and, as discussed below, CREB and AP-1.

The transcription factor CREB is a ubiquitously expressed protein that contributes to many cellular processes such as glucose homeostasis, growth factor-dependent cell survival, and inflammatory mediator production [87]. In general, CREB mediates the activation of cAMP-responsive genes by binding as a dimer to conserved CREB response elements (CREs) (5'-TGACGTCA-3') in

the promoter regions of target genes. The activation of CREB requires the phosphorylation of the protein at Ser-133 (S133), which promotes the recruitment of the transcriptional co-activator CREB-binding protein (CBP) and p300, thereby enabling gene transcription [87, 88].

CREB regulation is recognized to play a role in leukocyte function [88], yet little is known concerning the effect of P2X₇ stimulation on CREB activation, although Potucek et al. observed BzATP-induced CREB phosphorylation in murine microglial cells [89]. In the present report, we show that stimulation of primary human PBMCs and RAW 264.7 with P2X₇ agonists (BzATP and high doses of ATP) induces the rapid and transient phosphorylation of CREB (at Ser-133) and the related factor activating transcription factor-1 (ATF-1; Fig. 5a, b). As shown in Fig. 5b, CREB phosphorylation in murine macrophages is detectable within 5 min of ligand addition, it occurs more rapidly than in response to LPS, and it is complete within 1 h. In addition, we have observed: (a) a lack of nucleotide-induced CREB phosphorylation in RAW 264.7 cells expressing a non-functional P2X₇, (b) a gain of nucleotide-induced CREB phosphorylation in HEK293 cells that heterologously express human P2X₇, and (c) the induction of CREB/CBP complex formation (which is necessary for

Fig. 4 The MEK/ERK cascade appears critical for P2X₇-induced ROS production. RAW 264.7 cells were treated with either HEPES buffer (control) or 1 μ g/ml LPS for 10 h, and then transferred to flow cytometry tubes (5×10^5 cells/tube) and loaded with 10 μ M DCFDA for 30 min at 37°C. The cells were then pretreated with DMSO or increasing concentrations of UO126 (Calbiochem, San Diego, CA, USA; 130 μ M) for 30 min at 37°C. Cells were then treated with either HEPES (control) or 250 μ M BzATP for 30 min. Flow cytometry was performed by measuring 10,000 cells and excluding propidium iodide-stained (dead) cells from the analysis. The data are plotted as the mean ($\pm$ SEM) fold increase over control ($n=3$). * $p<0.01$, ** $p<0.005$ as compared to LPS-pretreated, BzATP-induced DCFDA fluorescence

CREB transcriptional activation) in macrophages treated with P2X₇ agonists (data not shown, Gavala et al., in preparation). Altogether these data suggest that P2X₇ stimulation modulates monocytic gene expression through the activation of CREB.

Activating protein-1 is a collective term for dimeric transcription factors composed of Jun, Fos, or ATF subunits that bind to a common 12-*O*-tetradecanoylphorbol-13-acetate response elements (5'-TGAGTCA-3') found in the promoter regions of many inflammatory cytokines and chemokines [90, 91]. Previous evidence for AP-1 activation by extracellular ATP comes from studies examining B lymphocytes. In these experiments, administration of extracellular ATP to human tonsillar B lymphocytes was found to increase cFos mRNA levels within 30 min [92]. Furthermore, co-treatment of human fetal astrocytes with IL-1 β and ATP was seen to induce an activation of AP-1 that is more robust than that observed with IL-1 β alone [93], and treatment with P2 receptor antagonists inhibits cytokine-induced AP-1 activation [93, 94]. It has been suggested that stimulation of Jurkat T cells induces AP-1 DNA-binding activity that results from increased c-Jun and cFos expression [84]. Conversely, in the present report, we show that stimulation of human PBMCs with P2X₇ agonists does not appear to enhance cFos expression (Fig. 6a) but does substantially induce the expression of the FosB and JunB proteins (Fig. 6b, c). These results are the first report linking the activation of a nucleotide receptor to FosB and JunB induction. Interestingly, the

effect of P2X₇ activation on FosB levels is detectable within 1 h of treatment with BzATP alone and suggests that FosB may be a unique transcriptional regulator of P2X₇-initiated signaling. Electrophoretic mobility shift assays (EMSA) in BzATP-treated RAW 264.7 cells reveal an increase in protein binding to an AP-1 consensus oligonucleotide compared to vehicle treatment, and this AP-1 complex is supershifted with antibodies directed toward FosB, JunB, and c-Jun (not shown, Gavala et al., in preparation). However, stimulation of P2X₇ does not induce AP-1 activation in all phenotypic backgrounds. Stimulation of N9 and N13 microglial cells with 3 mM ATP inhibits the basal DNA-binding activity of AP-1, suggesting that P2X₇-

Fig. 5 P2X₇ agonists induce CREB Ser-133 phosphorylation in monocytic cells. **a** Isolated peripheral human blood monocytes (1×10^6 cells/well) were treated with either vehicle (HEPES buffer), 1 mM ATP, 250 μ M BzATP, or 1 μ g/ml LPS for 15 min. A representative immunoblot displaying CREB Ser-133 phosphorylation (*pCREB*) is shown ($n=4$). The *pCREB* antibody recognizes both *pCREB* and phosphorylated ATF-1 (*pATF1*), thus it is also shown. Immunoblotting was performed with anti-Grb2 antibody as a loading control. **b** Murine RAW 264.7 macrophages (3×10^5 cells/well) were stimulated with either vehicle (HEPES), 250 μ M BzATP or 1 μ g/ml LPS for the indicated time. A representative immunoblot displaying *pCREB*/*pATF1* is shown. Immunoblotting was performed with anti- β -tubulin antibody as a loading control. Results of the three independent experiments were combined and plotted as the mean ($\pm$ SEM)

Fig. 6 P2X₇ agonists induce AP-1 protein expression in primary human monocytes. Isolated peripheral human blood monocytes were stimulated with either vehicle (HEPES), 250 μM BzATP, 1 μg/ml PMA, or 10 μM ionomycin (c) for 5 min. The media was replaced and the cells were incubated at 37°C for the indicated times (PMA treatment was reintroduced to the new media) and immunoblotted for **a** cFos, **b** FosB, or **c** JunB expression. Immunoblotting was performed with anti-Grb2 antibody as a loading control. The results are representative of three independent experiments

mediated AP-1 activation is cell type-specific (data not shown).

The activation of certain protein kinases by P2X₇ agonists has been implicated in subsequent AP-1 activation in several immune cells. Stimulation of Jurkat T cells with P2X₇ agonists induces the activation of the lymphoid-specific cytoplasmic protein tyrosine kinase p56^{lck} [84]. Although the activation of cFos and c-Jun by P2X₇ ligands occurs in Jurkat T cells, it is absent in a p56^{lck}-deficient JCaM1T cell line [84], suggesting that this kinase may play a role in P2X₇-induced AP-1 activation in T cells. A role for MAPKs in P2X₇-dependent AP-1 activation in monocytic cells has also been supported. The transactivation of c-Jun is augmented by the phosphorylation of Ser-63 and -73 by JNK1/2. Studies from our lab and others have noted JNK1/2 activation following stimulation of monocytic cells with P2X₇ agonists [21, 68, 95]. Furthermore, co-stimulation of RAW 264.7 cells with LPS and BzATP results in enhanced JNK activation compared to either stimulus alone [68]. Of note, P2X₇ agonist-induced JNK activation in RAW 264.7 cells is attenuated by *N*-acetylcysteine and ascorbic acid, implicating a role for ROS in P2X₇-dependent AP-1 activation [21]. In addition, we have observed that P2X₇ agonist-induced FosB and JunB expression in RAW 264.7 macrophages is abrogated in a dose-dependent fashion by the MEK1/2 antagonist UO126, suggesting that the MEK/

ERK cascade is upstream of the expression of these AP-1 proteins in macrophages (Gavala et al., in preparation).

Although reporter assays and EMSAs have often been used to demonstrate activation of transcription factors by P2X₇ agonists, little is known about the target genes of these transcription factors. To this end, preliminary studies by our lab have suggested a novel role for CREB in P2X₇-

Fig. 7 Mutation of two arginine residues in the P2X₇ C-terminal region reduces receptor-mediated pore-forming activity. **a** The distal C-terminal sequences of mouse, rat, and human P2X₇ were aligned. The proposed Arg-based ER retention signal is underlined, and Arg578 and Lys579 are *highlighted in red*. **b** COS7 cells were transfected with pcDNA3, P2X₇/pcDNA3, or P2X₇ R576A/R578A/pcDNA3. Sixteen to 24 h later, the cells were treated with 250 μM BzATP in the presence of 1 μM YO-PRO-1 (Molecular Probes, Eugene, OR, USA) for 20 min at room temperature. The cells were fixed with 4% paraformaldehyde, washed, and imaged using a Zeiss Axioplan 2 microscope. These data represent one of five independent experiments. P2X₇ R576A/R578A/pcDNA3 was generated from P2X₇/pcDNA3 (accession number BC011913) by site-directed mutagenesis. **c** COS7 cells were transfected with eYFP-P2X₇/pCMV, fixed the next day with 4% paraformaldehyde, imaged using a Zeiss 200M Axiovert inverted microscope, and deconvolved. P2X₇ was subcloned into the plasmid eYFP/pCMV, which was a generous gift from Dr. Melanie Cobb (University of Texas Southwestern Medical Center)

mediated FosB expression, i.e., transfection of dominant-negative CREB vectors into HEK293 cells heterologously expressing P2X₇ abrogates P2X₇ agonist-induced FosB expression (Gavala et al., in preparation).

P2X₇ contains a trafficking domain in its C-terminus

The intracellular trafficking of plasma membrane receptors is a critical component of their regulation. P2X₇ trafficking has not been widely explored, and it is unclear how newly synthesized P2X₇ subunits are transported from the endoplasmic reticulum (ER) to the surface and whether P2X₇ internalization or trafficking to the plasma membrane is controlled by extracellular signals. It is also not clear whether P2X₇ recycles following internalization or whether the receptor contains signals within its sequence that regulate its transport through the cell. Addressing these issues will increase our understanding of how P2X₇ is globally regulated and how cells mediate their responsiveness to extracellular ATP.

Our lab and those of Wiley, Petrou, and colleagues have provided evidence supporting the concept that P2X₇ contains a trafficking domain in its distal C-terminal region that is critical for cell surface localization. Point mutations and deletions within this region reduce P2X₇ surface expression and pore activity [30, 34, 96]. When P2X₇ is truncated at residues 551–581, it exhibits significantly reduced activity and plasma membrane localization [30], and three polymorphisms, I568N, R574H, and R574L, have been identified in this area of the receptor [34, 42, 97]. T lymphocytes and natural killer cells from individuals who

are heterozygous for the I568N polymorphism have reduced ATP-induced pore-forming ability, and exogenously expressed P2X₇ with the I568N mutation is poorly expressed on the surface of HEK293 cells [34]. We have previously found that mutation of Arg578 and Lys579, which are localized in the LPS-binding motif (Fig. 1), drastically reduces the ability of P2X₇ to localize on the plasma membrane and to promote BzATP-induced cell death [96]. Although the P2X₇ R578E/K579E mutant does not appear to be stably expressed on the cell surface at 37°C, it does localize to the plasma membrane at 27°C, suggesting that the double mutant is internalized more readily than wild-type receptor. These findings lead us to speculate that P2X₇ contains specific signals within its C-terminus that direct its intracellular trafficking.

One aspect of P2X₇ trafficking that is less well characterized entails the mechanisms by which it is transported from the ER to the cell surface. Several studies have shown that newly synthesized subunits of integral membrane proteins, including *N*-methyl-D-aspartate (NMDA), glutamate receptor, and γ -aminobutyric acid type B (GABA_B) receptor, are retained in the ER before the properly assembled receptors and are further transported through the secretory pathway [98]. The NMDA and GABA_B subunits possess arginine (Arg)-based ER retention/retrieval signals that prevent improperly assembled subunits from trafficking to the Golgi apparatus until assembly is complete and the signal is masked [99, 100]. These ER retention/retrieval sequences were first discovered in major histocompatibility complex class II proteins and have also been characterized in inward rectifier

Fig. 8 Proposed model of several of the key events by which P2X₇ promotes the production of immune mediators and activates gene expression. Stimulation of P2X₇ leads to enhanced ROS formation via the MAPKs ERK1/2 and the NADPH oxidase complex and stimulates gene transcription by activating CREB and increasing the expression of the AP-1 proteins FosB and JunB. In addition, specific residues in the putative trafficking domain of P2X₇ are required for proper localization and full activation of the receptor

potassium channel and sulfonyleurea receptor subunits [101, 102]. The Arg-based ER retention/retrieval consensus sequence is ϕ/ψ /R-R-X-R, where ϕ/ψ is an aromatic or bulky hydrophobic residue and X is any amino acid but is generally not a negatively charged or small, non-polar residue [98].

In view of the above discussion, we predict that P2X₇ contains a RXR retention/retrieval signal at amino acid 576 that lies within the sequence WRIR, which conforms to the consensus sequence ϕ/ψ /R-R-X-R (Fig. 7a). To test the hypothesis that P2X₇ contains an RXR ER retention/retrieval sequence at residue 576, a pore activity assay was conducted as an indirect readout for cell surface expression. In this experiment, COS7 cells were transfected with wild-type or mutant P2X₇, stimulated with BzATP, and imaged for relative dye uptake as a measure of ligand-dependent pore formation. As shown in Fig. 7b, mutation of human R576 and R578 to Ala substantially reduces the activity of the receptor. Cells take up the dye YO-PRO-1 when wild-type P2X₇ is expressed but exhibit drastically reduced dye uptake in the presence of the RXR mutant or the vector control. These findings, together with the information available about Arg-based signals in other receptors, provide initial support for the idea that P2X₇ contains an RXR ER retention/retrieval sequence in its C-terminus in the area surrounding residue 576.

Although our previous findings revealing that the P2X₇ mutant R578E/K579E does not express on the cell surface seems counterintuitive to the idea that P2X₇ possesses an ER retention/retrieval sequence, we speculate that cells prematurely traffic the R578E/K579E P2X₇ mutant from the ER before it is properly assembled, and once it reaches the plasma membrane, it becomes quickly down-regulated because it is non-functional or lacks appropriate interaction with specific lipids. Alternatively, the P2X₇ R578E/K579E mutation and not the mutation of an RXR signal prevents P2X₇ from localizing to the cell surface. In this regard, Marshall and colleagues have shown that a RK/AA mutation in the C-terminus of the GluR5-2b kainate receptor subunit disrupts ER retention [103].

Another unresolved issue is whether the trafficking of P2X₇ from intracellular compartments to the plasma membrane is stimulated by extracellular signals, as it is for the major insulin-responsive glucose transporter GLUT4, and has been reported for the NADPH oxidase complex [79, 80, 104]. We analyzed the localization of P2X₇ fused to enhanced yellow fluorescent protein (eYFP) and found that it is predominantly localized in intracellular pools (Fig. 7c). We are now in a position to explore the critical hypothesis that the trafficking of P2X₇ to and from these intracellular pools to the plasma membrane is stimulated by extracellular signals.

Summary

The ionotropic receptor P2X₇ is thought to be a major immune modulator that responds to extracellular ATP at sites of inflammation and tissue damage. Although several human genetic and animal studies have demonstrated the importance of P2X₇ in immune function, many questions remain concerning P2X₇ signaling and the regulation of its activity via intracellular trafficking. We have investigated the mechanisms by which P2X₇ promotes inflammatory mediator production and the poorly understood link between activation of P2X₇ and gene transcription (Fig. 8). Specifically, we have reported here that P2X₇ promotes ROS production in human monocytes and that production of this immune regulator is dependent upon the MEK/ERK cascade and likely involves the phosphorylation of the NADPH oxidase subunit p40^{phox}. We have further demonstrated that P2X₇ activates the transcription factors CREB and specific members of the AP-1 complex. Activation of P2X₇ results in the phosphorylation of CREB at a serine residue that is critical for its activity, and it appears that activation of CREB in response to P2X₇-initiated signaling is linked to FosB induction by P2X₇ agonists. In addition, we provide support for the idea that P2X₇ contains an Arg-based ER retention/retrieval sequence that is located within the P2X₇ LPS-binding domain and is important for regulating its activity (Fig. 8). These findings undergird the concepts that P2X₇ modulates mediator production and transcriptional activity as an immune response mechanism and that P2X₇ activity is tightly controlled through trafficking regulation.

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