Research Paper

**PIP₂ Regulates the Ionic Current of P2X Receptors and P2X₇ Receptor-Mediated Cell Death**

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**ABSTRACT**

P2X₇ receptors are non-selective cation channels gated by extracellular ATP. Prolonged stimulation of the P2X₇ receptor leads to an increase in cell permeability, cytokine release and apoptosis/necrosis. Application of PIP₂ to inside-out patches strongly activated all homomeric members of the P2X receptor family, including P2X₂ channels. Blockade of PIP₂ re-synthesis or induction of PIP₂ hydrolysis diminished ATP-gated P2X₇ currents. Several positively charged residues in the proximal C-terminus of P2X₇ were found to be important for PIP₂ interactions, as mutation of these sites reduced the apparent affinity for PIP₂ and enhanced current inhibition by PIP₂ depletion. In addition, we demonstrated the dependence of ATP-mediated cell death on P2X₇ receptor interaction with PIP₂ in three different cell systems: HEK cells stably transfected with P2X₇, primary T cells and a macrophase cell line. These results identify PIP₂ as a critical regulator of the function of the extracellular ligand-gated P2X₇ receptor/channels and provide a novel way to control ATP-mediated cell death.

**INTRODUCTION**

P2X receptors are ion channels gated by extracellular ATP. In contrast to P2Y nucleotide receptors, which are coupled to G proteins, P2X receptors themselves form ion-conducting pathways that are permeable to small cations. Seven subunits (P2X₁₋₇) have been identified in vertebrates. Each P2X subunit consists of two transmembrane domains with a bulky extracellular domain (~280 a.a.) in between. Homomeric and heteromeric P2X receptors are widely distributed. Their functions range from controlling smooth muscle contraction and sensing inflammatory signals to stimulating cytokine release and inducing cell death.¹

Among the members of the P2X family, the P2X₇ receptor displays several distinct features. The P2X₇ subunit does not associate with other P2X receptors, thus it forms only homomeric channels.² P2X₇ channels are permeable to Na⁺, K⁺ and Ca²⁺, and their currents show little or no inward rectification. In T cells and other immune cells, prolonged (seconds of) agonist stimulation of the P2X₇ receptor initiates several downstream cellular events, including a permeability increase, cytokine release, cytolsis and/or apoptosis.³

In macrophages, the activation of P2X₇ receptors leads to apoptosis of the cells as well as the killing of the mycobacteria inside them.³ In contrast, the death of macrophages induced by cross-linking of cell surface receptors with antibodies to CD95/Fas/Apo-1 (apoptosis inducing protein 1), CD69 (a member of the natural killer cell gene family) or complement-mediated lysis is not accompanied by death of the intracellular bacteria.³ Therefore, ATP-induced apoptosis via P2X₇ receptors is critical for protective immunity to pathogenic mycobacteria, such as *Mycobacterium tuberculosis*.⁴

PIP₂ is a negatively charged phospholipid in the inner leaflet of plasma membranes. PIP₂ has been shown to be a key modulator of many ion channels and transporters,⁵ such as the inward rectifier potassium (Kir) channels,⁶ the rod cyclic nucleotide-gated (CNG) channels,⁷ the hyperpolarization-activated, cyclic nucleotide-regulated, cation-nonselective (HCN) channels,⁸ the transient receptor potential (TRP) channels,⁹ the sodium-calcium exchanger,⁷ etc. The interactions between PIP₂ and ion channels are mainly electrostatic, mediated through the positively charged amino acids on the channels and the negatively charged headgroup of PIP₂. PIP₂ interactions result in modulating channel activity, either to activate or inhibit, with the direction of regulation depending on the particular channel.

**KEY WORDS**

phosphoinositide, PIP₂, P2X, P2X₇, apoptosis, macrophase

**ACKNOWLEDGEMENTS**

We thank Dr. G. Dubyak for the human P2X₇ cDNA and HEK P2X₇ cells; Drs. S. Silberberg for the rat P2X₂ and P2X₄ cDNAs; E. Findeis, B. Liu, A. Pappas, and T. Borges for oocyte preparation; Drs. S. Lira, G. Furtado, T. Rohacs, Ms. P. Skountzos and members of the Logothetis lab for invaluable advice and discussions. This research was funded by an NIH grant HL 59949 to D.E.L. and in part by an Extended Fellowship from the Systemic Lupus Erythematosus Foundation to M.Y.
P(4,5)P₂ is produced by the phosphorylation of phosphatidylinositol. The sequential reactions are catalyzed by PI 4-kinase (from PI to PIP₁) and PIP₁ 5-kinase (from PIP₁ to PIP₂). Alterations of the enzymatic activity of PI4K or PIP5K can, therefore, cause changes in PIP₂ levels in the plasma membrane. For example, wortmannin at micromolar concentrations inhibits some PI4K isoforms, reducing PIP₂ re-synthesis and thereby PIP₂ levels. On the other hand, PIP₂ is hydrolyzed by phospholipase C (PLC), which can be activated through many plasma membrane receptors. These receptors include G protein-coupled receptors (i.e., M₁-muscarinic receptors) that activate PLCβ, as well as growth factor receptors (i.e., PDGF receptors) that activate PLCγ.\(^{19,20}\)

Although extensive studies have documented the functional importance of P2X₇ receptors in various types of immune cells, little is known about the regulation of these receptors. In this study, we investigated the effects of PIP₂ on P2X₇ receptors and the physiological consequences of such regulation. We show that the activation of all six homomeric P2X receptors depends on PIP₂, and proximal C-terminal basic residues of P2X₇ are involved in PIP₂ sensitivity. The regulation of P2X₇-mediated cell death by manipulating the levels of PIP₂ was also examined in a heterologous expression system, in native T cells and in a macrophage cell line.

**MATERIALS AND METHODS**

**Expression in Xenopus oocytes.** cRNAs coding for P2X receptors were transcribed in vitro using the mMessage mMachine kit (Ambion, Austin, TX). The concentration of the cRNAs was estimated by comparing three successive dilutions with a standard ran on formaldehyde gels. Oocyte isolation and injection were performed as previously described.\(^{21}\) The protocol for the maintenance of the frogs and oocyte isolation was approved by the Institutional Animal Care and Use Committee (IACUC) of Mount Sinai School of Medicine. For two-electrode voltage clamp experiments, 25 ng of P2X cRNA per oocyte was injected. For macropatch experiments, 50 ng was injected. All oocytes were maintained at 18°C. Electrophysiological recordings were performed 2–3 days after injection.

**Electrophysiology.** Two-electrode voltage clamp recordings were carried out with a GeneClamp 500 amplifier (Axon Instruments, Union City, CA). The external bath solution contained (in mM): 96 NaCl, 2 KCl, 0.1 CaCl₂, 5 HEPES, pH 7.5. Currents were recorded using a ramp protocol from -100 mV to +100 mV. The currents at -80 mV were analyzed.

Inside-out macropatch experiments were performed using an Axopatch 200B amplifier (Axon Instruments, Union City, CA). PClamp 9 was used for data acquisition and analysis. The sampling rate was usually 2 kHz. A ramp protocol from -100 mV to +100 mV was applied once per second. The vitelline membrane of Xenopus oocytes was removed using fine forces before trying to form seals. Electrodes with resistance of 0.5–1 MΩ were used. The internal (bath) solution contained (in mM): 96 KCl, 0.5 EGTA, 10 HEPES, pH 7.4. For P2X₇ experiments, the external solution contained (in mM): 96 NaCl, 2 KCl, 0.1 CaCl₂, 5 HEPES, pH 7.5. For P2X₅ experiments, the external solutions contained 1.8 mM CaCl₂. ATP at the following concentrations (in µM) was also included in the external solution for P2X₅: 150; P2X₇; 20; P2X₇ and P2X₅, 100; P2X₅ and P2X₇, 50. The solutions were applied through a gravity-driven perfusion system.

For each experiment, a minimum of two batches of oocytes was tested. The error bars in the figures indicate standard errors (s.e.m.). Statistical analysis was performed using two-tailed student t tests or one-way ANOVA (analysis of variance) and Bonferroni post tests.

**HEK cell culture and recording.** HEK cells stably transfected with P2X₇ (HEK-P2X₇) were maintained at 37°C with 5% CO₂, in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin and 0.5 mg/ml hygromycin. The cells were plated on polylysine-coated coverslips one day before patch-clamp experiments. Recordings were performed using the EPC9 amplifier (Heka, Germany) and the “Pulse” software (Heka, Germany). The electrode resistances were 2–3 MΩ. Solutions were also applied by a gravity-driven system. After the seal was formed, a gentle suction was applied to break the membrane and establish the whole-cell mode of patch-clamp. Whole-cell currents at -65 mV were measured. The internal solution contained (in mM): KCl 100, NaCl 10, MgCl₂ 5, KOH-EGTA 30/10, KOH-HEPES 10/10, pH 7.3. The external solution contained (in mM): D-glucose 10, KCl 5, NaCl 140, CaCl₂ 1.5, MgCl₂ 0.5, NaOH-HEPES 10/10, pH 7.5. The solution used to dissolve ATP was the same as the external solution except that it contained 0.5 mM CaCl₂.

**T cell isolation.** Mouse T cells were magnetically isolated from spleen using the Pan T cell isolation kit (Miltenyi Biotech, Auburn, CA) by depletion of CD45R (B220), DX5,CD11b(Mac1) and Ter-119* cells. The purity of the isolated T cells was approximately 97% CD3+ positive as analyzed by flow cytometry. Mouse spleens were obtained from 4–6 week old female BALB/c mice purchased from Charles River Laboratories (Wilmington, MA). The mice were maintained in pathogen-free facilities in accordance with IACUC guidelines.

**Expression of PIP5K in RAW 264.7 macrophages.** The murine macrophage cell line RAW 264.7 was maintained in Dulbecco’s modification of Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone Laboratories, Logan, Utah), 50 µM β-mercaptoethanol, 2 mM L-glutamine and 15 µg/ml gentamicin.\(^{22}\)

The mouse PIP5Kβ was cloned into a previously described Moloney murine leukemia virus-based retroviral vector upstream of an internal ribosome entry site (IRES)-GFP cassette.\(^{22}\)

Infection of RAW 264.7 macrophages with vesicular stomatitis virus G protein-pseudotyped retrovirus was conducted as previously described.\(^{22}\) Human 293 EbnaT cells were seeded at a density of 4 x 10⁶ cells in a 10-cm-diameter dish. The next day, cells were transfected by using calcium phosphate with 2.5 µg of plasmid pMD.G encoding vesicular stomatitis virus G protein and 7.5 µg of plasmid pMD.OGP encoding gag-pol, together with 10 µg of the retrotransfection expression construct encoding either the irrelevant control GST, the wild-type or either of two mutant PIP5Ks. At 48 h posttransfection, the viral supernatant was collected, centrifuged at 800 g, and used to infect RAW 264.7 cells. A quantity of 5 x 10⁶ RAW 264.7 cells was resuspended in 10 ml of viral supernatant in the presence of 4 µg of Polybrene/ml, aliquoted into a 24-well plate, and spun in a microtiter rotor at 800 g for 1 h at room temperature. 48 hours after infection, RAW 264.7 cells were treated with ATP and assayed for cell death, caspase activation and PARP cleavage.

**Cell death assay.** HEK-P2X₇ cells were treated with 5 mM ATP in serum-free media for 2 hours. When indicated, the cells were treated with wortmannin or LY294002 for 2 hours before and during ATP treatment. Mouse splenic T cells were stimulated with 200 µM ATP for 30 minutes following pre-incubation of the cells for 2 hours with wortmannin or LY294002. RAW 264.7 macrophages were incubated with 1 µM ATP for two hours. The cells were then harvested, washed once with PBS and stained with propidium iodide (PI) (BD Pharmingen, Rockville, MD). Cell death was measured by flow cytometry.

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**RESULTS**

**Western blotting.** Cells were gently lysed in ice-cold buffer containing 1% Nonidet P-40, 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 30 mM NaF supplemented with protease inhibitors. Protein concentrations in the extract were determined using the bicinchoninic acid protein assay kit (Pierce, Woburn, MA) and equivalent amounts of protein were resolved on SDS-PAGE gels. Western blotting was performed using standard methodologies. Cleaved PARP mouse mAb and cleaved caspase-3 mAb were purchased from Cell Signaling Technology (Danvers, MA).

**PIP₂ activated P2X receptors in excised patches.** To study the effect of PIP₂ on heterologously expressed P2X₇ receptor, we applied the naturally occurring long-chain, arachidonyl stearyl (AAS) PIP₂ to inside-out macropatch membranes from *Xenopus* oocytes. Upon patch excision, a decrease (run-down) in the current was observed (Fig. 1A). Current run-down is a common feature shared by PIP₂-sensitive channels, and has been attributed to the dephosphorylation of PIP₂ by lipid phosphatases. Application of 5 µM PIP₂ strongly...
activated P2X<sub>7</sub> currents. Poly-lysine (polyK), which binds and sequesters PIP<sub>2</sub>, reduced currents to basal levels. The summary histograms show that the P2X<sub>7</sub> current in the presence of PIP<sub>2</sub> is ~280% of that recorded in the inside-out (I/O) mode following current run-down (Fig 1A). Using the same experimental protocol, we showed that PIP<sub>2</sub> also activates homomeric P2X<sub>1</sub>, P2X<sub>2</sub> and P2X<sub>3</sub>, P2X<sub>4</sub> and P2X<sub>5</sub> channels (Fig. 1B–F). The effect on P2X<sub>2</sub> is in agreement with a recent report. The P2X<sub>6</sub> receptor that does not form homomeric receptors was not tested.

The ramp protocol we used did not evoke significant basal whole-cell currents (less than 0.5 µA at -80 mV) in non-injected oocytes or in oocytes injected with P2X<sub>7</sub> cRNAs (data not shown). The ATP-induced whole-cell currents at -80 mV evoked by the ramp from non-injected oocytes were about 10% of those recorded from P2X<sub>7</sub>-expressing oocytes (data not shown). PIP<sub>2</sub> and polylysine did not cause any significant changes in the endogenous currents in excised macropatches from non-injected oocytes (data not shown). Therefore, the majority of the currents resulted from the activation of P2X<sub>7</sub> receptors.

PIP<sub>2</sub> depletion inhibited P2X<sub>7</sub> currents in Xenopus oocytes. The platelet-derived-growth-factor receptor (PDGFR) is one of the receptor tyrosine kinases that activate both phospholipase C-γ (PLCγ) and PI-3K upon ligand binding. PLCγ hydrolyzes PIP<sub>2</sub> into inositol 1, 4, 5 triphosphate (IP3) and diacyl-glycerol (DAG). Therefore, stimulation of PDGFR receptor decreases PIP<sub>2</sub> levels in the plasma membrane. IP3 stimulates Ca<sup>2+</sup> release from internal stores. Elevation of intracellular Ca<sup>2+</sup> concentrations in Xenopus oocytes activates a Ca<sup>2+</sup>-sensitive, outwardly-rectifying native Cl<sup>-</sup> current. Thus, the activation of this Cl<sup>-</sup> current serves as an indicator of PIP<sub>2</sub> hydrolysis by PLC. Fig 2A and B show two-electrode voltage-clamp experiments in oocytes expressing P2X<sub>7</sub> and PDGFR β receptors. First, P2X<sub>7</sub> currents were activated by 1 mM ATP for 10 seconds. Then oocytes were perfused with PDGF (50 ng/ml) for 10 minutes. Stimulation of the PDGFR receptor hydrolyzed PIP<sub>2</sub> and activated an outwardly rectifying Cl<sup>-</sup> current. Following PDGF perfusion, P2X<sub>7</sub> currents were activated by 1 mM ATP for a second time. Stimulation of the wild-type PDGFR substantially decreased ATP-induced P2X<sub>7</sub> currents. A mutant PDGFR receptor (Y740F, Y751F), in which the PI-3K activation is eliminated, retained its ability to inhibit P2X<sub>7</sub> currents. In contrast, the PDGFR mutant (Y1009F, Y1021F), which
the phosphorylation of phosphatidylinositol at the 4’-position by PI-3K. Wortmannin inhibits PI-3K at nanomolar concentrations (10 nM or 100 nM) without significantly affecting its potency (Fig. 2D). Therefore, the effects of wortmannin resemble those of non-competitive inhibitors, suggesting that ATP and PIP_2 act onto different sites of the P2X_7 receptor. This is not surprising since ATP acts on the receptor extracellularly, while PIP_2 being localized in the inner leaflet of the membrane acts intracellularly.

**Figure 3.** PIP_2 interaction sites on P2X_7 receptors. (A) Topology of the P2X_7 receptor with cluster of basic residues proximal to the plasma membrane denoted. (B) Whole-cell currents of wild-type and mutant P2X_7 receptors. Each batch of oocytes, the currents were normalized to the mean current of the oocytes expressing the wild-type P2X_7 receptors. (C) DiC8 PIP_2 concentration-response relationships. (D and E) Current inhibition by wortmannin or PDGF in wild-type and mutant P2X_7 channels. ***, p < 0.05 and ***, p < 0.01 (n = 7–14).

Disrupts PLC_γ binding to the activated receptor, significantly reduced P2X_7 inhibition. These results suggested that the activation of P2X_7 channels depends on PIP_2 in the plasma membrane. Thus lowering of PIP_2 levels by PLC_γ following PDGF stimulation led to inhibition of P2X_7 currents (Fig. 2A and B).

Synthesis of PIP_2 requires PI 4-kinase (PI-4K), which catalyzes the phosphorylation of phosphatidylinositol at the 4’-position to form PI(4)P. PI(4)P is then phosphorylated into PIP_2 by PI 5-kinase. Wortmannin inhibits PI 3-kinase (PI-3K) at nanomolar concentrations. At higher (micromolar) concentrations, wortmannin also inhibits specific PI 4-kinase isoforms and attenuates PIP_2 synthesis. Pretreatment with wortmannin can provide a way to lower PIP_2 levels in cells. We examined the effects of PI-4K inhibition on P2X_7 currents in Xenopus oocytes. The P2X_7 currents activated by 1 mM ATP were greatly reduced by PI-4K inhibitors phenylarsine oxide (PAO, 30 µM) and wortmannin (35 µM). Inhibition of PI-3K only by LY294002 (50 µM) or wortmannin at low concentrations (10 nM or 100 nM) did not show any effects on P2X_7 activity (Fig. 2C). These results suggest that activation of P2X_7 receptors depends on PIP_2 levels and reduction of PI(4,5)P_2 levels inhibits P2X_7 currents.

Dependence of P2X_7 activity on PIP_2 in HEK cells. In addition to oocytes, we used HEK-293 mammalian cells stably transfected with the human P2X_7 receptors to study the effects of PIP_2 on channel activity. P2X_7 currents were activated by a series of ATP concentrations before and after perfusion of the cell with wortmannin (35 µM) for 10 minutes. Wortmannin reduced the efficacy of ATP without significantly affecting its potency (Fig. 2D). Therefore, the effects of wortmannin resemble those of non-competitive inhibitors, suggesting that ATP and PIP_2 act onto different sites of the P2X_7 receptor. This is not surprising since ATP acts on the receptor extracellularly, while PIP_2 being localized in the inner leaflet of the membrane acts intracellularly.

**PIP_2-interaction sites on P2X_7 receptors.** In several families of channels that are regulated by PIP_2, the C-terminal regions proximal to the plasma membrane contain basic residues that serve as important PIP_2-interacting sites. These clustered basic amino acids presumably interact electrostatically with the negatively charged PIP_2, which is water-soluble and can be washed into and out of the patch membrane several times. Fitting the concentration-response data with the Hill equation provided an EC_50 which indicates the apparent binding affinity of the channel for PIP_2. Mutants R385Q, K387Q and K395Q exhibited rightward shifts in the PIP_2 concentration-response curves, suggesting that these mutants have decreased apparent affinities for PIP_2 (Fig. 3C).

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Figure 4. Regulation of P2X<sub>7</sub>-mediated cell death by PIP<sub>2</sub> in HEK cells stably transfected with P2X<sub>7</sub> (HEK-P2X<sub>7</sub>). (A) Effects of PI-4K inhibition. The cells were pretreated with wortmannin (WMN) or LY294002 (LY) for 2 hours before and during stimulation with ATP for another 2 hours. *p < 0.05 (n = 5). (B) Transfection of PIP5K in HEK-P2X<sub>7</sub> cells increased P2X<sub>7</sub>-mediated cell death. *p < 0.05 (n = 4).

Figure 5. PIP<sub>2</sub> regulated P2X<sub>7</sub>-mediated cell death in mouse splenic T cells. The cells were pre-incubated with DMSO (Ctr), wortmannin (WMN) or LY294002 (LY) for 2 hours before they were stimulated with 200 µM ATP for 30 minutes. **p < 0.01 (n = 7).
We also examined, in each mutant channel, the inhibition of P2X7 currents caused by reducing PIP2 levels through wortmannin treatment or PDGFR stimulation. Depletion of PIP2 has been shown to display differential effects on the channel activity depending on the strength of the channel-PIP2 interactions. For channels that interact strongly with PIP2, depletion of PIP2 has little or no effect on the current; while for channels that interact weakly, depletion of PIP2 can substantially inhibit the current. The P2X7 receptors, R385Q, K387Q and K395Q showed enhanced current inhibition by wortmannin or PDGF (Fig. 3D and E), to an extent that corresponded consistently to the strength of channel-PIP2 interactions seen for each mutant in Figure 3C. These results suggested that R385, K387 and K395 are important channel sites for interactions with PIP2.

**PIP2 regulates P2X7-mediated cell death.** It has been well established that prolonged stimulation of P2X7 receptors leads to cell death in endothelial cells, T cells, macrophages, and a number of other immune cells. Several kinases (e.g., stress-activated protein kinases or SAPKs) and proteases (e.g., caspases 1 and 3) have been suggested to be involved in the P2X7-mediated apoptosis. 

The activation of these enzymes can be triggered by abrupt changes in the cytoplasmic ionic composition that result from P2X7 activation. For example, depletion of intracellular K+ has been implicated in the activation of SAPKs and caspases as well as in apoptotic cell death.

Since ionic perturbation activates the protein enzymes and leads to apoptosis, alteration of the ionic currents through P2X7 receptors could as well cause changes in P2X7-induced apoptosis. We have shown that PIP2 activates P2X7 currents, and that depletion of PIP2 resulted in reduced channel activation by ATP. To test the hypothesis that modulating-channel-PIP2 interactions regulates P2X7-mediated cell death, we treated HEK cells stably transfected with P2X7 with wortmannin (24 µM) for 2 hours before and during stimulation of P2X7 receptors with ATP. As mentioned earlier, wortmannin at nanomolar concentrations also inhibits PI 3-kinase (PI-3K), the enzyme known to promote cell survival. To differentiate the effects of wortmannin on cell death as a PI-4K inhibitor from those of a PI-3K inhibitor, we used the PI3K-specific inhibitor LY294002 as a control (also see Fig. 2C). As shown in Fig. 4A, cell death with ATP treatment was ~50% in the control cells, while the basal cell death without ATP treatment was ~11%. Both wortmannin and LY294002 in the absence of ATP caused about 30% cell death. ATP, on top of wortmannin and LY294002, caused cell death in 57.7% and 94.4% of the cells, respectively. ATP-induced cell death, as determined by the difference in the absence and presence of ATP, was 40.5%, 25.1% and 64.6% respectively in the control cells, the wortmannin-treated cells and the LY294002-treated cells. The summary data showed that wortmannin and LY294002 have distinct effects on ATP-induced cell death (Fig. 4A). Inhibition of PI-3K by LY294002 did not significantly affect P2X7-mediated cell death. Wortmannin significantly inhibited ATP-induced cell death, demonstrating its effects of PI-4K inhibition, which causes reduction in PIP2 levels and a decrease in P2X7−mediated cell death. To further investigate the effects of altering PIP2 levels on P2X7−mediated cell death, we transiently transfected the stable HEK-P2X7 cells with PIP 5-kinase (PIP5K) 1β, the enzyme that catalyzes the phosphorylation of PI(4)P to PI(4,5)P2. Expression of PIP5K significantly increased the ATP-induced cell death in HEK-P2X7 cells (Fig. 4B). These results suggested the dependence of P2X7−mediated cell death on membrane PIP2 levels.

To further test whether P2X7−mediated cell death depended on PIP2 in immune cell systems as well, we turned to T cells and macrophages. Prolonged stimulation of P2X7 receptors in T cells leads to apoptotic cell death. We studied the effects of altering PIP2 levels on P2X7−mediated cell death in mouse splenic T cells. The cells were pretreated with DMSO alone (Ct) (the solvent used for wortmannin and LY294002), wortmannin (WMN, 24 µM) or LY 294002 (LY, 50 µM) for 2 hours before they were stimulated with 200 µM ATP for 30 minutes. Micromolar levels of wortmannin caused a significant decrease in ATP-induced cell death. The effects of wortmannin as a PI-3K inhibitor are most likely not evident here, since inhibition of PI-3K by LY294002 did not affect P2X7−mediated death in these cells under our experimental conditions (Fig. 5). Treatment with wortmannin or LY294002 in the absence of ATP did not cause cell death that was significantly different from control. Again, these results suggested that PIP2 regulates P2X7−mediated cell death in native immune cells as it did in the heterologous expression system.

In macrophages, activation of P2X7 receptors causes concomitant killing of the cells and the intracellular mycobacteria. To study the regulation of ATP-induced death of macrophages by PIP2, we infected the RAW 264.7 macrophage cell line with the wild-type (wt) or kinase-inactive mutants (ΔI-238 or D307A) PIP5K. P2X7−mediated cell death was determined after treating the macrophages with 1 mM ATP for 2 hours. The cells expressing the wild-type PIP5K showed a significant increase in cell death while both the kinase-deficient mutants did not show a significant effect (Fig. 6A). Presumably, over-expression of PIP5K increased the membrane PIP2 levels, which upregulated the activity of P2X7 receptors and increased cell death.

Caspase-3 has been shown to be activated in P2X7−mediated apoptosis. One of the main cleavage targets of caspase-3 is PARP, a nuclear poly(ADP-ribose) polymerase, which is involved in DNA repair in response to environmental stress. Cleavage of PARP by caspase-3 serves as a marker of cells undergoing apoptosis. We examined ATP-induced caspase-3 and cleavage of PARP in RAW 264.7 macrophages. The cells were treated with 1 mM ATP for 2 hours. Western blots showed an increase in the activated form of caspase-3 and the cleaved form of PARP in cells transfected with the wild-type PIP5K compared with the control cells or cells transfected with either of the kinase-inactive form of PIP5K (Fig. 6B). These results showed that PIP2 regulates the activation of caspase-3 and the resulting cell death through P2X7 receptor stimulation. Thus, modulating the receptor-PIP2 interactions provides a novel way to regulate P2X7−mediated apoptosis in macrophages.

In oocytes, hydrolysis of PIP2 following PDGF receptor activation inhibited P2X7 currents. We examined the effects of PDGF receptor stimulation on ATP-mediated cell death in Raw 264.7 macrophages. Incubation of the cells with PDGF (50 ng/ml) for 15 minutes before and during the ATP treatment (1 mM, 2 hours) reduced ATP-induced cell death by 20% (Fig. 6C). The surface expression of endogenous PDGFβ receptors was confirmed by flow cytometry after staining the cells with phycoerythrin (PE) anti-mouse CD140β (PDGFRβ) or with PE-conjugated control IgG (isotope control) (Fig. 6C, bottom left panel).

**DISCUSSION**

P2X receptors have attracted great interest because of their significant roles in regulating diverse physiological processes in various types of immune cells, such as T, macrophage and dendritic cells.
The end point of prolonged activation of P2X<sub>7</sub> receptors is inevitable cell death. Despite an extensive documentation on P2X<sub>7</sub> functions, the regulation of these receptors remains largely unknown. In this study, we identified PIP<sub>2</sub> as a novel modulator of the P2X<sub>7</sub> receptor and demonstrated that the functions of the P2X<sub>7</sub> receptor in passing ionic currents and in promoting cell death depend on its interaction with the membrane phospholipid PIP<sub>2</sub>. We showed that PIP<sub>2</sub> activates in excised patches, all members (P2X<sub>1</sub> - P2X<sub>6</sub>) of the P2X family that form homomeric receptors, while hydrolysis or inhibition of synthesis of PIP<sub>2</sub> leads to decreased P2X<sub>7</sub> currents (Figs. 1 and 2). We also showed that modulating channel-PIP<sub>2</sub> interactions resulted in altered P2X<sub>7</sub>-mediated cell death in HEK cells, T cells and macrophages.

The juxtamembrane region of PIP<sub>2</sub>-interacting proteins. As with several other ion channels and membrane proteins that are regulated by PIP<sub>2</sub>, the C-terminal region of P2X<sub>7</sub> proximal to the plasma membrane contains a potential PIP<sub>2</sub>-interacting domain characterized by a cluster of positively charged residues interspersed with hydrophobic residues (Fig. 3A). Through mutagenesis studies, we identified P2X<sub>7</sub> residues R385, K387 and K395 in this region as putative interaction sites with PIP<sub>2</sub>. Mutation of these residues weakened the interaction between P2X<sub>7</sub> and PIP<sub>2</sub>, evidenced by the decreased currents, the decreased apparent affinity for PIP<sub>2</sub> and the increased current inhibition by PIP<sub>2</sub> depletion (Fig. 3). The C-terminal domains proximal to the plasma membrane of all other members of the P2X family (P2X<sub>1</sub>-P2X<sub>6</sub>) contain similar clusters of basic amino acids in a pattern similar to P2X<sub>7</sub>. Therefore, the region immediately following the second transmembrane helices may be of general importance for the regulation of P2X receptors by PIP<sub>2</sub>, as it seems to be for other PIP<sub>2</sub>-regulated channels (e.g. TRP channels<sup>15</sup>).

Macromolecular signalling complexes in the hydrolysis of PIP<sub>2</sub>. Stimulation of the PDGF receptor inhibited the activity of P2X<sub>7</sub> receptors (Fig. 2). This effect is exerted presumably through activation of PLC<sub>γ</sub> which hydrolyzes PIP<sub>2</sub>. Depletion of PIP<sub>2</sub> has differential effects on the channel activity depending on the strength of the channel-PIP<sub>2</sub> interactions.
For channels that strongly interact with PIP$_2$, depletion of PIP$_2$ may have little or no effect on the current (e.g., TRPM8 channels$^{15}$); for channels that show weak or moderate interactions, depletion of PIP$_2$ can substantially inhibit the current (e.g., P2X receptors). Even for the same channel, stimulation of different PLC-coupled receptors results in distinct effects on channel activity depending on the particular receptor. For example, receptor-specific inhibition of GIRK (Kir3) channels by Gq-coupled receptors that activate PLC$\beta$ has been demonstrated.$^{18}$ In order to explain this phenomenon, the suggestion has been made that GIRK channels and specific regulating Gq-coupled receptors might localize closely together in the membrane. Interestingly, P2X$_7$ receptors were shown to interact with several other proteins, including phosphatidylinositol 4-kinase, the enzyme that catalyzes in vivo the synthesis of PIP$_2$ and $\alpha$-actinin, a cytoskeletal protein that also binds to PIP$_2$. It is tempting to hypothesize that P2X receptors, PIP$_2$ and other proteins that modulate PIP$_2$ levels co-localize in microdomains to form signalling complexes. This architectural design would provide a mechanism by which the cross talk of P2X$_7$ with other receptors enables fine-tuning of ATP responses in controlling immune cell functions.

**P2X$_7$-mediated cell death and apoptosis.** Apoptotic cell death has been recognized in several vascular diseases, including atherosclerosis, hypertension and restenosis.$^{49,50}$ In atherosclerosis, vascular endothelial cells release a substantial amount of ATP.$^{51}$ Extracellular ATP activates P2X$_7$ receptors, leading to apoptosis in endothelial cells, T cells and macrophages.$^{1,5,29,40,51}$ We demonstrated a regulation of P2X$_7$-mediated cell death by membrane PIP$_2$. Increases in PIP$_5$ kinase and presumably PIP$_2$ levels increased ATP-stimulated cell death and markers of apoptosis. Furthermore, decreases in PIP$_2$ levels by treatment of cells with wortmannin or stimulation with PDGF decreased ATP-stimulated cell death. Our results not only have identified PIP$_2$ as a novel, crucial modulator of the P2X$_7$ receptor, but they also provide potential targets for drug discovery in these vascular diseases.

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