Extracellular ATP dissociates nonmuscle myosin from P2X7 complex: this dissociation regulates P2X7 pore formation

Ben J. Gu,1 Catherine Rathsam,2 Leanne Stokes,1 Andrew B. McGeachie,3 and James S. Wiley1

1Department of Medicine, Nepean Clinical School, Penrith; 2Institute of Dental Research, Westmead; 3Cell Signalling Unit, Children’s Medical Research Institute, Westmead, The University of Sydney, Australia

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Gu BJ, Rathsam C, Stokes L, McGeachie AB, Wiley JS. Extracellular ATP dissociates nonmuscle myosin from P2X7 complex: this dissociation regulates P2X7 pore formation. Am J Physiol Cell Physiol 297: C430–C439, 2009. First published June 3, 2009; doi:10.1152/ajpcell.00079.2009.—The P2X7 receptor is a ligand-gated cation channel that is highly expressed on monocyte-macrophages and that mediates the pro-inflammatory effects of extracellular ATP. Dilation of the P2X7 channel and massive K⁺ efflux follows initial channel opening, but the mechanism of secondary pore formation is unclear. The proteins associated with P2X7 were isolated by using anti-P2X7 monoclonal antibody-coated Dynabeads from both interferon-γ plus LPS-stimulated monocytes and P2X7-transfected HEK-293 cells. Two nonmuscle myosins, NMMHC-IIA and myosin Va, were found to associate with P2X7 in THP-1 cells and HEK-293 cells, respectively. Activation of the P2X7 receptor by ATP caused dissociation of P2X7 from nonmuscle myosin in both cell types. The interaction of P2X7 and NMMHC-IIA molecules was confirmed by fluorescent life time measurements and fluorescent resonance of energy transfer-based time-resolved flow cytometry assay. Reducing the expression of NMMHC-IIA or myosin Va by short interfering RNA or short hairpin RNA led to a significant increase of P2X7 pore function without any increase in surface expression or ion channel function of P2X7 receptors. S-1-blebbistatin, a specific inhibitor of NMMHC-IIA ATPase, inhibited both ATP-induced ethidium uptake and ATP-induced dissociation of P2X7-NMMHC-IIA complex. In both cell types nonmuscle myosin closely interacts with P2X7 and is dissociated from the complex by extracellular ATP. Dissociation of this anchoring protein may be required for the transition of P2X7 channel to a pore.

short hairpin RNA; blebbistatin; THP-1 cells; fluorescent resonance energy transfer; ethidium uptake; myosin IIA; myosin Va; phagocytosis

THE P2X7 RECEPTOR is a ligand-gated cation channel highly expressed on monocytes and macrophages that mediates the pro-inflammatory effects of extracellular ATP (23, 27, 41). The P2X7 receptor has two transmembrane domains with intracellular amino and carboxyl termini and an extracellular domain containing several β-pleated sheets within a tertiary structure maintained by five disulfide bonds (43). Activation of the receptor by brief exposure to extracellular ATP opens a cation-selective channel, whereas longer exposure to ATP leads to secondary pore formation and massive K⁺ efflux, which is a costimulus for secretion of pro-inflammatory cytokines IL-1β and IL-18 (13, 31). Other downstream effects of P2X7 activation include blebbing of the plasma membrane (49), shedding of surface L-selectin and CD23 (17), rapid secretion of matrix metalloproteinase-9 (18), and activation of the caspase cascade leading to a form of apoptotic cell death (8, 14). P2X7 has been shown to exist as a trimer within a large multimolecular complex in the membrane (29). Moreover, activation of P2X7 mediates actin reorganization and membrane blebbing in mouse macrophages (33, 50, 51), suggesting some functional connections between the P2X7 receptor and the actin cytoskeleton.

The myosin family, which consists of at least 20 structurally and functionally distinct classes, constitutes the major component of the cytoskeleton. Nonmuscle myosins are able to convert the energy of ATP hydrolysis into vectorial movement of organelles or cargo along actin filaments, although some nonmuscle myosins also have anchoring or tethering function for membrane molecules (9, 25). Nonmuscle myosin heavy chains usually consist of three distinct regions: one or two NH₂-terminal heads (or motors) that are responsible for actin binding and ATP hydrolysis; a neck region called light-chain binding domain that binds calmodulin-like regulatory light chain and essential light chain; and a long COOH-terminal tail that is responsible for cargo binding and/or dimerization of heavy chains (25). In nonmuscle cells, myosin II, as well as myosin V, are the two best characterized members of the myosin superfamily, and both are essential for viability in early life (7, 28). Myosin II has at least three isoforms: IIA, IIB, and IIC. Nonmuscle myosin IIA heavy chain (NMMHC-IIA) is present in almost all nonmuscle cells (39) and associated with membrane (9) involving preservation of cell morphology (53) and motility (21, 44), membrane repair (46), neuroblastoma cell adherence and retraction (55, 56), as well as vesicle transport (9) such as MHC class II trafficking and B cell receptor-driven antigen presentation (48). It is also essential for maintaining cell-cell adhesions in the early mammalian embryo (7). Mutations in MYH9, the gene encoding NMMHC-IIA, have been associated with May-Hegglin anomaly characterized by large platelets, thrombocytopenia, and neutrophil inclusions (38). NMMHC-IIA has also been shown to associate with many membrane proteins: It binds COOH-terminus of CXCR4 and CCR5 (36) and has been implicated in the endocytosis of CXCR4 via dynamic association with β-arrestin (35). NMMHC-IIA also binds a plasma-membrane-associated protein supervillin, which is involved in cell spreading (44). S-1-blebbistatin (S-bleb) is a specific inhibitor for the ATPase of NMMHC-IIA and IIB and rapidly and reversibly inhibits NMMHC-II-mediated cell blebbing during cytokinesis (42). This inhibitor has also been shown to prevent the dissociation of NMMHC-IIA from CD38, a membrane protein on activated killer cells thus inhibiting IL8-stimulated CD38 internalization (34). Like myosin II, myosin V also has three isomers, Va, Vb, and Vc. Myosin Va, also known as nonmuscle myosin heavy chain 12, is primarily expressed in neurons of the central...
nervous system and melanocytes and functions in rapid transport of various cargos in these cells (10).

There are few studies of the proteins associated with P2X7 in the cell membrane. In transfected HEK-293 cells, P2X7 has been reported in a membrane complex, which included laminin-α3 chain, β2-integrin, protein tyrosine phosphatase-β, β-actin, α-actinin 4, heat shock 71-kDa protein, heat shock 70-kDa protein 1, heat shock protein 90-β, phosphatidylinositol 4 kinase, membrane-associated guanylate kinase (MAGUK) P55, and supervillin (24). In transfected cervical endothelial cells, P2X7 has also been associated with a G protein-coupled receptor kinase (GRK-3), β-arrestin-2, dynamin, and clathrin (12). In mouse native lung epithelial cells, P2X7 has also been shown to bind with caveolin-1 present in lipid rafts (4). In this study, we isolated P2X7-associated proteins with anti-P2X7 monoclonal (L4)-coated Dynabeads using as a source of cells either interferon-stimulated human monocytic THP-1 cells or P2X7 transfected HEK-293 cells. In both cell types nonmuscle myosin was found in the P2X7 membrane complex as well as other proteins of the cytoskeleton, although the nonmuscle myosin was of different class in monocytes and HEK-293 cells. In both cell types, ATP dissociated nonmuscle myosin from the P2X7 complex, an event that was associated with P2X7 channel to pore transition.

EXPERIMENTAL PROCEDURES

**Materials.** ATP, BzATP, oxidized ATP (OxATP), phorbol 12-myristate 13-acetate (PMA), ML-7, ethidium bromide, sodium chloride, potassium chloride, barium chloride, d-glucose, bovine serum albumin (BSA), EDTA, tetramethylammonium hydroxide (TMA), lipopolysaccharides (LPS), ε-amino-n-capric acid, n-dodecyl β-maltoside, protease peptone, and Triton X-100 were purchased from Sigma (St. Louis, MO). Ficoll-Paque Plus and the GFX PCR DNA and Gel Band Purification Kit were from GE Healthcare (Uppsala, Sweden). 1-(N,O-Bis(5-sulfoquinoliny1 sulfonyl) N-methyl-L-tyrosyl)-4-phenylpiperazine (KN-62) was from R&D System (Minneapolis, MN). The recombinant human interferon-γ (IFNγ), minicomplete protease inhibitor cocktail, phenylmethylsulfonyl fluoride (PMSF) plus were from Roche Applied Science (Mannheim, Germany). ATP was dissolved in KCl buffer (in mM: 145 KCl, 5 KCl, and 10 HEPES, pH 7.5) at a stock concentration of 100 mM and neutralized with 18% (wt/vol) TMA to pH 7.4. HEPES, fetal calf serum (FCS), normal horse serum, Lipofectamine 2000 reagent, Opti-MEM I medium, Taq DNA polymerase, One-step RT-PCR kit for long template, vector pcdNA3, M-280 tosylactivated Dynabeads were from Invitrogen (Carlsbad, CA). Vector pEGFP-N1, pEGFP-C1, pAcGFP-N1 and pDsRed-monomer-N1 were from Clontech (Mountain View, CA). Wild-type P2RX7 construct was originally a gift from Dr. Gary Buell and was reconstructed into the above vectors in our lab. Rabbit anti-human NMMHC antibody was from Biomedical Technologies (Stoughton, MA). Rabbit anti-myosin Va antibody was from Santa Cruz (Santa Cruz, CA). Mouse anti-human NMMHC-IIA mAb, goat anti-human Ro-52 (SSA) polyclonal Ab, and anti-β-actin control Ab were from Abcam (Cambridge, UK). Mattek culture dishes with collagen-coated 1.0-mm slides were from Mattek (Ashland, MA). (S)-β-blebbistatin (S-bleb) and its inactive isomer (R)-β-blebbistatin (R-bleb) were from Toronto Research Chemicals (North York, Canada). Oligos [primers and small interfering RNA (siRNA)] were synthesized locally by Sigma-Proligo. Mouse monoclonal anti-human P2X7 receptor mAb (clone L4, kindly provided by Drs. Gary Buell and Ian Chessell) (5) and IgG2b isotype control mAb (clone WMD7, from the Millennium Institute, Westmead, NSW, Australia) were purified from clone hybridoma supernatants by chromatography on Protein A Sepharose Fast Flow as described previously (19). Sheep anti-human P2X7 polyclonal antibody was raised against a nonhomologous extracellular epitope of the human P2X7 receptor as described previously (54). Rabbit anti-rat P2X7 polyclonal antibody cross-reacting with human P2X7 has also been described previously (40). The SuperSignal West Pico Chemiluminescent Substrate kit was from Pierce Endogen (Rockford, IL). The HEKfectin, precast 4–20% gradient mini-gels, and prestained precise protein marker were from Bio-Rad (Hercules, CA).

**Sources of cells.** Human peripheral blood mononuclear cells were separated by density gradient centrifugation over Ficoll-Hypaque, washed once in RPMI-1640 medium, and resuspended in HEPES-buffered NaCl medium (in mM: 140 NaCl, 5 NaOH, 5 KCl, and 10 HEPES, pH 7.5, plus 5 mM glucose, 0.1% BSA, and 0.1 mM CaCl2). Human monocytic cell line THP-1 and human embryonic kidney cell line HEK-293 were cultured in RPMI-1640 medium containing 10% fetal calf serum and 5 μg/ml gentamycin. To differentiate THP-1 to macrophage-like cells, cells (0.5×10⁶/ml) were incubated with 1,000 U/ml IFN-γ plus 100 ng/ml LPS for 72 h (iTHP-1) according to previously described methods (20).

**Immunoprecipitation.** M-280 tosylactivated Dynabeads (2×10⁶/ml), which ensure correct orientation of antibody and have minimum nonspecific binding (supplemental Fig. S.1), were coated with anti-P2X7 mAb (clone L4) according to the manufacturer’s instruction and kept at 4°C for no more than 30 days. THP-1 cells (2×10⁶) or HEK-293 cells (5×10⁶) were washed twice following pretreatment with ATP or other compounds, and resuspended in 180 μl extraction buffer (750 mM ε-amino-n-capric acid and 50 mM Bistris, pH 7.2) with 40 μl Mini Complete Protease Inhibitor cocktail (stock: 2.0 ml extraction buffer/tablet) and 4 mM PMSF at 4°C, followed by addition of 20 μl 10% n-dodecyl β-maltoside, a mild nonionic detergent. The presence of high concentrations of ε-amino-n-capric acid enhances the solubility of membrane protein and helps to keep an intact complex (37). Cells were allowed to lyse for 30 min at 4°C. 200 μl of extraction buffer was added and centrifuged at 20,000 g for 15 min, and the supernatant was collected. Supernatants were preincubated with either WMD7-coated or sheep anti-mouse Ig polyclonal antibody-coated Dynabeads for 45 min at 4°C, and the beads were separated by magnetic force. L4-coated Dynabeads (50 μl for THP-1 or 200 μl for HEK-293) were washed once and mixed with the supernatant and incubated at 4°C in a rotator for 4 h. The Dynabeads were washed six times with PBS and stored at −30°C until the next day. The proteins on Dynabeads were eluted by boiling at 95°C for 5 min in Laemli sample loading buffer and separated on a 4–20% gradient SDS-PAGE under reducing conditions. Proteins were transferred to nitrocellulose membrane and blocked overnight in TTBS buffer (20 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 7.5) containing 5% skim milk powder. The membrane was washed and incubated with either a rabbit anti-NMHC antibody (1:2,000), a mouse anti-NMHC-IIA mAb (1:1,000), a rabbit anti-myosin Va antibody (1:500), a goat anti-Ro-52 (SSA1) antibody (1:1,000), or a sheep anti-human P2X7 antibody (1:1,000) in TTBS buffer for 2 h. The membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:2,500) for 1 h, and the bands were detected with the SuperSignal kit.

**Mass spectrum analysis.** Immunoprecipitation was performed as described above except that 10 times more cells were used and PMSF Plus reagent (Roche), which prevents PMSF-induced protein modification, was added when the cells were being lysed. Proteins eluted from Dynabeads were separated on a 4–20% SDS-PAGE (1 mm thick, 20×20 cm) under reducing conditions. The gel was stained with Brilliant Blue G and destained. The band was cut and destained
with 50 mM ammonium bicarbonate-50% acetonitrile and dried, followed by trypsin digestion. The samples were analyzed in a Voyage MALDI-TOF mass spectrometer. The peptide mass fingerprints were then analyzed using Peptide (http://kr.expasy.org/).

Cloning of MYH9. Total RNA was extracted from iTHP-1 cells. Two pairs of primers were used to clone the MYH9 gene, which contains the coding region for NMMHC-IIA: forward[1], 5'-CTG GCTAGC AGT CAC CAT GAC ACA GCA AGC-3' and reverse[1], 5'-CTC CGG GAA G GAATTC TTG TCC TC-3'; forward[2], 5'-GAG GAC GAC GA GAATTC CTG CCG GGA G-3' and reverse[2], 5'-CTA CGG GTG AAA AGG AGG TTT TGG CAG GTT TGGCCT CAG-3'. The RT-PCR was performed using the One-step RT-PCR Kit for Long Template (Invitrogen) according to the manufacturer's instructions. Amplified RT-PCR products were purified using the GFX PCR DNA and Gel Band Purification Kit. The two products were inserted into a pDsRed-monomer-N1 vector between the NheI/EcoRI sites, followed by a second insertion to the EcoRI/ AgeI sites. The pDsRed-monomer-N1-MYH9 construct containing the complete cDNA coding for NMMHC-IIA was fully sequenced. The pAcGFP-N1-MYH9 was constructed by replacing the NheUAgel fragment with the one cut from pDsRed-monomer-N1-MYH9.

Transfection of HEK-293 cells. The total 10 μg plasmid DNA or 1 μg plain vector was incubated in serum-free Opti-MEM I medium for 5 min followed by incubation with HEKfectin (15 μl, diluted with Opti-MEM I medium) for 20 min at room temperature. The solution was transfected into a near-confluent monolayer of HEK-293 cells (~1.5 × 10⁶ in 3 ml Opti-MEM I with 5% FCS). After 40–44 h, cells were either collected by mechanical scraping (transient transfection) or kept in complete RPMI-1640 medium containing 0.8 mg/ml G-418 (stable transfection). For live cell imaging, HEK-293 cells were cultured and transfected in collagen-coated 27-mm Mattek culture dish. For fluorescent resonance of energy transfer (FRET)-based assay, transient transfection was performed with mixed plasmid DNA with a copy number ratio of 1:4 (AcGFP: DsRed).

Fluorescence lifetime imaging. HEK cells cotransfected with AcGFP or DsRed tagged P2X7 and NMMHC-IIA were washed and kept in Na medium. Cells were visualized under a Nikon C1-LIMO culture dish incubator with perfusion control and temperature control technology, Rockingham, VT). A HQ525/50m; DsRed: HQ545/30x, Q570LP, HQ620/60m; FRET: HQ470/40x, Q495LP, HQ620/60m (exciter, dichroic, emitter) (Chroma Technology, Rockingham, VT). A ×40 objective lens and a DH-35i culture dish incubator with perfusion control and temperature control unit (Warner Instruments) were used. The images were captured and analyzed with the MetaMorph (version 6.0) software.

Time-resolved FRET flow cytometry. HEK cells cotransfected with pAcGFP-N1-MYH9 and pDsRed-monomer-N1-P2RX7 (1:4) were resuspended in Na medium with 0.1 mM CaCl₂ at a concentration of 2.0 × 10⁶/ml. Cell suspensions were stirred and temperature was maintained at 37°C using a time 0 module, and after 2 min, ATP (1.0 mM) or BzATP (100 μM) was added. Cells were analyzed at about 1,500 events/s on a FACSCalibur flow cytometer and were gated by forward and side scatter and by AcGFP and DsRed fluorescence intensity. The log mean channel of fluorescence intensity for each gated subpopulation over successive 5-s intervals was analyzed by WinMDI software and plotted against time.

siRNA blocking. To block P2X7 and NMMHC-IIA at transcription level, siRNA was designed using online program from Invitrogen, siDirector, and Wistar. The sequences were as the followings: P2RX7 siRNA, 5'-cga ugg uca cua cag auu u-3' and 5'-aca uce ugc gag uau uaa a-3' and 5'-cag agc ugg aag aca a-3'; MYH9 siRNA, 5'-cgu ggc ugc uca uaa a-3' and 5'-cca agc ugg aag aca a-3'; control siRNA, 5'-cag cau acu gua cgc uct t-3'. The duplex of each siRNA were synthesized by Sigma-ProLigo. The siRNA (50 μM each for P2RX7, for MYH9 or 100 μM for control) was mixed with 100 μl HiPerFectin and added into 8 ml THP-1 cells (0.5 × 10⁶/ml), which have been stimulated either with 100 nM PMA for 4 h or with 1,000 U/ml IFNγ plus 100 ng/ml LPS for 48 h in OPTI-MEM medium with 5% FCS. Cells were kept in culture for another 48 h before collection for ethidium bromide uptake and Western blotting.

shRNA blocking. Two shRNA vector sets each targeting five different sites of the human MYH9 and Myo5A genes were obtained from Sigma-Genosys. The plKO.1-puro control vector was used as a control. HEK-293 cells were transfected with 10 μg shRNA vector, and 2 days later the cells were transfected with 10 μg of AcGFP-P2X7 constructs with HEKfectin. Cells were cultured for an additional 44 h and collected for ethidium bromide uptake and Western blotting.

Electrophysiology. Patch-clamp recordings were performed at room temperature by using a HEKA EPC10 amplifier and Patchmast.

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Table 1. Identified P2X7-associated proteins in cells not exposed to ATP

Proteins were isolated from either interferon-γ plus LPS-stimulated THP-1 cells or P2X7-transfected HEK-293 cells using L4-coated Dynabeads and analyzed by mass spectrometry. Cells were not pretreated with ATP before lysis and incubation with L4-Dynabeads. Only proteins identified in at least two separate experiments are included. The Swiss-Prot accession number, the calculated molecular mass of protein band from SDS-PAGE, and the overall highest PeptIdent score are listed. IFN-γ, interferon-γ; TPR, tumor potentiating region; InsP6, inositol hexakisphosphate; PP-IP5 kinase 1, diphosphoinositol-pentakisphosphate kinase 1; HSP, heat shock protein.
Membrane potential was clamped at −60 mV, and agonist (1 mM ATP) was applied using a computer-controlled fast-flow system (Bio-Logic Instruments). Cells were plated onto 13-mm glass coverslips 24 h before recordings, which were performed 72 h posttransfection with human P2X7-AcGFP and 96 h posttransfection with shRNA constructs. Standard external/internal pipette solutions were (in mM) 145 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 1 D-glucose, 10 HEPES (pH 7.3), and 145 NaCl, 10 HEPES, 10 EGTA (pH 7.3), 300–310 mosmol/l, respectively. Pipettes were pulled from borosilicate glass using a Narashige PC-10 and resistance was 6–8 MΩ.

**Ethidium**

E uptake. Human mononuclear cells (2 × 10⁶) prelabeled with FITC-conjugated anti-CD14 mAb or HEK-293 cells (2 × 10⁶) transfected with AcGFP tagged P2X7 receptor constructs were washed once and resuspended in 1.0 ml HEPES-buffered KCl medium at 37°C. All samples were stirred and temperature maintained at 37°C using a time 0 module. Ethidium ** (25 μM) was added, followed 40 s later by addition of 1.0 mM ATP. Cells were analyzed at 1,000–1,500 events/s on a FACSCalibur flow cytometer and were gated by forward and side scatter and by cell type-specific antibodies or AcGFP intensity. The linear mean channel of fluorescence intensity (0–255 channel) for each gated subpopulation over successive 5-s intervals was analyzed by WinMDI software and plotted against time as described previously (19, 22). The areas under ATP-induced ethidium uptake curves were also calculated at 5 and 2.5 min as described previously (22) to yield arbitrary units of P2X7 function in monocyte and HEK-293 cells, respectively.

**RESULTS**

P2X7 membrane complex contains NMMHC-IIA in monocytic cells. Human monocytic THP-1 cells were stimulated with LPS together with interferon-γ (iTHP-1 cells), which is known to upregulate the expression and function of P2X7 (20). The iTHP-1 cells were treated with or without ATP for 5–15 min, washed to remove ATP, and lysed. P2X7 was separated using Dynabeads-coated with anti-P2X7 monoclonal antibody (clone L4). Several proteins associated with P2X7 were identified by mass spectrometry to be with a score greater than 0.3 (Table 1). In cells not exposed to ATP the most significant one
was a ~220-kDa protein of which its mass fingerprinting showed a high score of 0.95 of identity to NMMHC-IIA in iTHP-1 cells (Table 1, Fig. 1). The results were confirmed by Western blotting in which the P2X7-associated proteins were separated by gel electrophoresis and transferred to a nitrocellulose membrane. A 220-kDa band corresponding to the size of NMMHC-IIA (220 kDa molecular mass) was detected by a rabbit anti-nonmuscle myosin heavy chain polyclonal antibody in immunoprecipitates from iTHP-1 lysate (Fig. 1C). In contrast, no band was found in immunoprecipitated proteins from cell lysate of unstimulated THP-1 (Fig. 1B). Other proteins found in the immunoprecipitate were protein-tyrosine phosphatase, Ro52, β-actin, and myosin regulatory light chain (Table 1). Our finding of β-actin and protein tyrosine phosphatase in the P2X7 membrane complex is in line with previous data (24), although these authors did not report the presence of NMMHC-IIA, possibly because of differences in cell type.

Extracellular ATP dissociates the P2X7-NMMHC-IIA complex. To determine whether the complex was altered following P2X7 activation, the THP-1 cells were pretreated with ATP before washing. Both SDS-PAGE gel and Western blotting images showed that pretreatment of cells with 1 mM ATP for 5–10 min caused a marked reduction of NMMHC in L4-Dynabeads isolated proteins from cell lysates of iTHP-1 (Fig. 1C). In contrast, the protein amount of another immunoprecipitated protein, the Sjogren’s syndrome type A antigen Ro-52, was found unchanged following ATP activation (Fig. 1C). Other proteins that were unchanged in the immunoprecipitate include InsP6 and PP-IP5 kinase 1, protein-tyrosine phosphatase, and β-actin. KN-62, a potent P2X7 antagonist (16), abolished ATP-induced dissociation of the P2X7-NMMHC complex in iTHP-1 cells (Fig. 1C). KN-04, a weaker antagonist of P2X7, did not inhibit ATP-induced dissociation of NMMHC-IIA. These results suggest that activation of P2X7 disrupts its cytoskeletal attachments in iTHP-1 cells.

P2X7 membrane complex contains myosin Va in HEK-293 cells and is also dissociated by ATP. A similar approach was adopted to examine the proteins of P2X7 complex in HEK-293 cells transfected with P2X7 constructs. The cells were treated with or without ATP for 10 min, washed, and lysed, and P2X7 complex was pulled down using L4-Dynabeads. Of the seven proteins identified by mass spectrometry, only two with a score above 0.5 were the same as found in the complex from THP-1 cells (Table 1). Myosin Va was found to be the predominant nonmuscle myosin heavy chain associated with P2X7 in HEK-293 cells (Table 1), although other nonmuscle myosin heavy chains, such as NMMHC-IIA and IIB, were present with lower scores (data not shown). Western blot analysis confirmed a ~220-kDa band corresponding to the size of myosin Va (215.6 kDa molecular mass) was present and detected by rabbit anti-nonmuscle myosin heavy chain polyclonal antibody (Fig. 2B). In contrast, no band was found in immunoprecipitated proteins from mock transected HEK-293 cell lysates. Other proteins found in the immunoprecipitate were Nucleoprotein tumor promoting region (TPr), heat shock protein 90 and 70, Ro-52, tubulin, and nucleoside diphosphate kinase B (C-myc purine-binding transcription factor PUF) (Table 1). Our finding of heat shock protein 70 and 90 and tubulin in the P2X7 membrane complex is in line with previous data (24). Pretreatment of transfected HEK-293 cells with 1 mM ATP for 10 min dramatically reduced the amount of NMMHC associated with P2X7 complex, shown by both SDS-PAGE and Western blotting (Fig. 2). The P2X7 constructs in different vectors, pCI (Promega), EGFP-N1 or EGFP-C1 (Clontech), gave similar results. In contrast, Ro-52, which was associated with P2X7 in both THP-1 and HEK-293 immunoprecipitates, remained unchanged following exposure of cells to ATP (Fig. 2B).

Colocalization of P2X7 and NMMHC-IIA. The association between P2X7 and NMMHC-IIA was further studied by cloning the MYH9 gene (encoding NMMHC-IIA) from THP-1 cells and tagging NMMHC-IIA COOH-terminus with fluorescent AcGFP or DsRed monomer, which is known to maintain a functional molecule (3, 45). The use of monomeric form of AcGFP and DsRed reduces the possible false interaction caused by the fluorescent protein self-association. HEK-293 cells were cotransfected with NMMHC-IIA-DsRed and P2X7-AcGFP. The live cell confocal imaging showed that P2X7 and NMMHC-IIA were colocalized in plasma membrane and membranes of intracellular organelles (Supplemental Fig. S.2a). It is well established that a major fraction of P2X7 resides within intracellular membranes (19), and it can be readily visualized in membranes surrounding intracellular organelles (Supplemental Fig. S.2a). Blebbing of plasma membrane was induced by addition of BzATP to cotransfected HEK-293 cells. Fluorescent microscopy showed the presence of both P2X7 and NMMHC-IIA in the cell blebs induced by agonist (Supplemental Fig. S.2b and videos), suggesting that NMMHC-IIA was involved in the P2X7-mediated cell blebbing.

Fluorescence (FRET-FLIM) study proposes close P2X7-NMMHC interaction in live cells. Confocal microscopy is limited by optical resolution (200–300 nm), and simple colocalization of these two molecules does not prove their direct interaction. The direct interaction between P2X7 and NMMHC-IIA was
studied in HEK-293 cells, which were cotransfected with NMMHC-IIA-AcGFP and P2X7-DsRed monomer. The FRET-based FLIM was used to measure the lifetime of AcGFP in colocalized regions. After excitation by a 440-nm pulse laser, the lifetime of AcGFP was 1.7 ± 0.2 ns and increased to 4.3 ± 0.3 ns after incubation with 100 μM BzATP for 15 min (Fig. 3a). In the cells cotransfected with pAcGFP-N1 and pDsRed-monomer-N1 empty vector, the lifetime of AcGFP remained at 4.2–4.5 ns after BzATP treatment (data not shown). FRET-FLIM in conjunction with confocal microscopy operates over a scale of 1–10 nm and is a sensitive reporter of the close interaction between two molecules. Calculations from our data showed tight association of the two molecules with an interacting distance between the COOH-terminus of P2X7 and COOH-terminus of NMMHC-IIA of ~4.4 nm and a calculated FRET efficiency of ~0.6. After 15 min incubation with BzATP the lifetime of AcGFP increased, indicating there was no interaction between P2X7 and NMMHC-IIA.

To study the P2X7-NMMHC interaction in a large cohort of live cells, we developed a FRET-based time-resolved flow cytometry assay system. The protein interaction was assessed by monitoring the fluorescence intensity changes of AcGFP and DsRed in double-positive cells over several minutes. In HEK-293 cells cotransfected with NMMHC-IIA-AcGFP and P2X7-DsRed, ATP or BzATP led to a decrease of P2X7-DsRed fluorescent intensity in parallel with a slight increase of NMMHC-IIA-AcGFP intensity over a 15-min period in gated AcGFP+/DsRed+ cells (Fig. 3B). Pretreatment with KN-62 abolished the effect of ATP (Fig. 3B). These data confirm the immunoprecipitation results and show that P2X7 and NMMHC-IIA are tightly associated and that ATP is causing dissociation of P2X7 from NMMHC-IIA.

Both NMMHC-IIA and myosin Va are limiting factors for P2X7 pore formation. Channel to pore transition is also known as secondary pore formation, which occurs over 1 to 10 min following agonist activation and is a distinguishing feature of the P2X7 receptor. We investigated the role of NMMHC-IIA and Myosin Va in P2X7-transfected HEK-293 cells. HEK-293 cells were pretransfected with shRNA producing vectors targeting on either MYH9 or Myo5A (the gene coding for myosin Va) before the transfection of P2X7-AcGFP. Reducing the expression of either NMMHC-IIA or myosin Va in P2X7-transfected HEK-293 cells significantly increased ATP-induced ethidium uptake (Fig. 4A). The most significant results came from the HEK-293 cells in which both NMMHC-IIA and myosin Va were knocked down, and P2X7-mediated ethidium uptake was increased almost fourfold compared with the vector control (Fig. 4A). In contrast to this large increase in P2X7 pore permeability, the ATP-induced inward current was the same in control cells and cells transfected with shRNA against both nonmuscle myosin classes (Fig. 4B). To further confirm that P2X7 expression was unaltered by shRNA knockdown of nonmuscle myosins, we measured P2X7 surface expression by flow cytometry and found it did not change (Fig. 4C). Western analysis showed that the protein level of NMMHC in HEK-293 cells was reduced 20–40% with single shRNA blocking and 50–70% with double shRNA blocking (Fig. 4D). Similar results were seen when siRNA targeted against MYH9 was transfected into iTHP-1 cells. The siRNA blocked 30–50%
expression of NMMHC-IIA (Fig. 5B) and enhanced the ATP-induced ethidium+ uptake compared with cells transfected with scrambled siRNA (Fig. 5A). Control cells transfected with siRNA to P2X7 showed both reduced P2X7 protein and reduced ATP-induced ethidium+ influx (Fig. 5A).

Inhibition of NMMHC-IIA ATPase blocks P2X7 pore formation. Since NMMHC-IIA directly interacts with P2X7, the effect of the specific NMMHC-IIA ATPase inhibitor S-bleb (42) and its inactive isomer R-bleb were studied on pore formation. ATP-induced ethidium+ uptake in fresh isolated human monocytes was reduced significantly by pretreatment of 100 μM S-bleb for 1 h but not by R-bleb (Fig. 6B). In parallel experiments, S-bleb but not R-bleb inhibited the ATP-induced dissociation of NMMHC from the P2X7 complex in iTHP-1 cells (Fig. 6A). These results suggest dissociation of P2X7 from NMMHC-IIA may be required for P2X7 pore dilatation, and the dissociation requires ATP hydrolysis.

Inhibition of F-actin polymerization does not affect P2X7 pore formation. NMMHC-IIA is known to interact with F-actin, thus we examined the effect of depolymerizing F-actin on P2X7 pore formation. HEK-293 cells transfected with P2X7-AcGFP were pretreated with or without 20 μM cytochalasin D or 1 μM latrunculin A, two potent classical inhibitors for F-actin polymerization. Both cytochalasin D and latrunculin A had no effect on ATP-induced ethidium+ uptake (Supplemental Fig. S.3), suggesting that F-actin was not involved in P2X7 pore formation.

DISCUSSION

Our novel finding of a close molecular interaction between P2X7 and nonmuscle myosin and its dissociation by extracellular ATP provides insight into the possible mechanisms by which the P2X7 channel dilates to a larger pore. It is known...
that P2X₇ forms part of a multiprotein complex (24), and our initial results showed this complex included nonmuscle myosin, although the nonmuscle myosin was class IIA in monocytic cells and class Va in HEK-293 cells. Anchoring of P2X₇ on the membrane by its tight molecular attachment to NMMHC has not been previously described, although the effect of ATP to rearrange the actin cytoskeleton is well described (33). A number of previous studies have demonstrated membrane blebbing induced by activation of P2X₇ (33, 50, 51), and it is likely the dissociation of nonmuscle myosin from P2X₇ releases this receptor from cytoskeletal tethering and allows ATP-induced blebbing (see supplementary videos).

Previous studies have shown many different proteins associated with the P2X₇ receptor complex (4, 12, 24). Thus the differences in methodology, e.g., type of cells, lysing method and immunoprecipitation matrix, may result in differences in the coassociated proteins. We used both native monocyte-macrophage cell line THP-1 and P2X₇-transfected HEK-293 cells to extract the P2X₇ complex for analysis. Besides myosin IIA and myosin Va, two important components of the cytoskeleton, actin and tubulin, were also found in the complex, supporting the data above showing attachment of P2X₇ to the underlying cytoskeleton. Our finding of protein tyrosine phosphatase in the P2X₇ membrane complex is in line with the data of Kim et al. (24) as also was the finding of two chaperone proteins, heat shock protein 70 and 90. Nuclear membrane protein TPR was found only in P2X₇-transfected HEK-293 cells and is a component of the nuclear pore complex. It is involved in activation of oncogenic kinases (6), and its long coiled-coil COOH-terminus (over 1,600 amino acids) facilitates export of mRNA and other macromolecules from the nucleus (2). The association of TPR with P2X₇ in HEK-293 cells raises the possibility that P2X₇ may also be present in nuclear membrane. Interestingly, the Sjogren’s syndrome type A antigen Ro-52 (also known as SS-A or TRIM21) is found in the P2X₇ complex in both THP-1 and HEK-293 cells, and this associated protein level did not change following activation of P2X₇ by ATP (Figs. 1 and 2). Ro-52 is a RING-finger-type E3 ubiquitin ligase catalyzing ubiquitination of substrate (11, 52). The consistent association of P2X₇ and Ro-52 may be relevant to the finding of autoantibodies against Ro-52 in Sjogrens syndrome in which there is inflammatory destruction of salivary glands, a tissue with particularly high content of P2X₇ receptor (47).

The interaction between P2X₇ and NMMHC-IIA was studied by using FLIM-FRET. In this study, the AcGFP lifetime of 1.7 ns increased to 4.3 ns following the addition of BzATP to cotransfected HEK-293 cells. This latter value is the same as that of Kim et al. (24) as also was the finding of two chaperone proteins, heat shock protein 70 and 90. Nuclear membrane protein TPR was found only in P2X₇-transfected HEK-293 cells and is a component of the nuclear pore complex. It is involved in activation of oncogenic kinases (6), and its long coiled-coil COOH-terminus (over 1,600 amino acids) facilitates export of mRNA and other macromolecules from the nucleus (2). The association of TPR with P2X₇ in HEK-293 cells raises the possibility that P2X₇ may also be present in nuclear membrane. Interestingly, the Sjogren’s syndrome type A antigen Ro-52 (also known as SS-A or TRIM21) is found in the P2X₇ complex in both THP-1 and HEK-293 cells, and this associated protein level did not change following activation of P2X₇ by ATP (Figs. 1 and 2). Ro-52 is a RING-finger-type E3 ubiquitin ligase catalyzing ubiquitination of substrate (11, 52). The consistent association of P2X₇ and Ro-52 may be relevant to the finding of autoantibodies against Ro-52 in Sjogrens syndrome in which there is inflammatory destruction of salivary glands, a tissue with particularly high content of P2X₇ receptor (47).

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The interaction between P2X₇ and NMMHC-IIA was studied by using FLIM-FRET. In this study, the AcGFP lifetime of 1.7 ns increased to 4.3 ns following the addition of BzATP to cotransfected HEK-293 cells. This latter value is the same as
that found for AcGFP lifetime in cells transfected with empty plasmid vectors. These data provide strong evidence for a close molecular interaction over ~4.4 nm between AcGFP-tagged NMMHC-IIA and DsRed-tagged P2X7 with dissociation of these two molecules following activation of P2X7. To confirm the interaction of P2X7 and NMMHC-IIA in a large cohort of cells, a FRET-based time-resolved flow cytometry method was also developed. With careful compensation settings, the AcGFP and DsRed emissions can be readily studied in a real-time flow cytometry system using only a 488-nm laser. The decreased DsRed fluorescence intensity over a 15-min period following the addition of ATP also indicated the dissociation of P2X7 and NMMHC-IIA following P2X7 activation by ATP. Our live cell confocal imaging also showed that P2X7 and NMMHC-IIA were colocalized in apparent clusters not only in plasma membrane but also clustered at a limited number of locations in the cytoplasm (supplemental Fig. S.4). This finding supports the recent demonstration of P2X7 receptor in phagosomes membranes of J-774 macrophages, which have taken up latex beads (26).

The mechanism of channel dilation (or pore formation) following ATP activation of P2X7 is currently controversial. Our novel finding of a close molecular interaction between P2X7 and NMMHC-IIA and its dissociation by extracellular ATP provides insight into the possible mechanisms by which the P2X7 channel dilates to a larger pore. From this study, we propose that the P2X7 receptor is anchored in the membrane by nonmuscle myosin in the absence of ligand. Indeed some nonmuscle myosin are thought to function as both transporters and anchors (1). Once extracellular ATP reaches high concentrations, P2X7 changes its conformation to open the intrinsic ion channel and the downstream effects, e.g., ionic shifts, allow a slow detachment of nonmuscle myosin from P2X7. Dissociation of P2X7 from its cytoskeletal attachments may allow pore formation by P2X7, possibly by interaction with pore-forming proteins such as pannexin 1 (30). Regulation of P2X7 channel to pore transition is supported by the following observations. First, P2X7 is complexed with nonmuscle myosin in the absence of ATP. Second, ATP dissociates P2X7-nonmuscle myosin complex in 1–10 min, which is in line with the time frame for ATP to induce pore formation (0.5–5 min). Third, decrease of expression of NMMHC-IIA in iTHP-1 cells or transfected HEK-293 cells significantly increased ATP-induced ethidium + uptake without affecting the P2X7 channel function, suggesting that decreasing the amount of nonmuscle myosin in the cytoskeleton facilitates the transition of P2X7 channel to a pore. Fourth, as a specific inhibitor of NMMHC-IIA ATPase, S-Bleb or latrunculin A did not alter ATP-induced ethidium + uptake in human monocytes but also inhibited ATP-induced dissociation of P2X7 and nonmuscle myosin. In contrast, its inactive isomer R-bleb affected neither the function nor the dissociation in human monocyte cells, also suggesting that dissociation of P2X7–NMMHC-IIA complex is required for P2X7 pore formation. In contrast, F-actin, which is well known to associate with nonmuscle myosin, appears to play no role in the P2X7 pore formation since inhibition of actin polymerization by either cytochalasin D or latrunculin A did not alter ATP-induced ethidium + uptake. Whatever the molecular nature of the P2X7 large pore, it is likely that dissociation of P2X7 from nonmuscle myosin of the cytoskeleton is a critical early step in the P2X7 permeability transition.

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