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ATP stimulates GRK-3 phosphorylation and β -arrestin-2-dependent internalization of P2X₇ receptor

Ying-Hong Feng¹, Liqin Wang¹, Qifang Wang², Xin Li², Robin Zeng², and George I. Gorodeski^{2,3,4}

¹Department of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, Maryland

²Department of Reproductive Biology, Case Western Reserve University School of Medicine, Cleveland, Ohio

³Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, Ohio ⁴Department of Oncology, Case Western Reserve University School of Medicine, Cleveland, Ohio

Abstract

The objective of this study was to understand the mechanisms involved in P2X₇ receptor activation. Treatments with ATP or with the P2X₇ receptor-specific ligand 2',3'-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate (BzATP) induced pore formation, but the effect was slower in CaSki cells expressing endogenous P2X₇ receptor than in human embryonic kidney (HEK)-293 cells expressing exogenous P2X₇ receptor (HEK-293-hP2X₇-R). In both types of cells Western blots revealed expression of three forms of the receptor: the functional 85-kDa form present mainly in the membrane and 65- and 18-kDa forms expressed in both the plasma membrane and the cytosol. Treatments with ATP transiently decreased the 85-kDa form and increased the 18-kDa form in the membrane, suggesting internalization, degradation, and recycling of the receptor. In CaSki cells ATP stimulated phosphorylation of the 85-kDa form on tyrosine and serine residues. Phosphorylation on threonine residues increased with added ATP, and it increased ATP requirements for phosphorylation on tyrosine and serine residues, suggesting a dominant-negative effect. In both CaSki and in HEK-293-hP2X₇-R cells ATP also increased binding of the 85-kDa form to G protein-coupled receptor kinase (GRK)-3, β -arrestin-2, and dynamin, and it stimulated β -arrestin-2 redistribution into submembranous regions of the cell. These results suggest a novel mechanism for P2X₇ receptor action, whereby activation involves a GRK-3-, β -arrestin-2-, and dynamin-dependent internalization of the receptor into clathrin domains, followed in part by receptor degradation as well as receptor recycling into the plasma membrane.

Keywords

purinergic receptor; recycling; dynamin; clathrin; cervix; epithelium

The P2X₇ Receptor Belongs to the P2X receptor subfamily of P2 nucleotide receptors (6,45), which are membrane-bound, ligand-operated K⁺-, Na⁺-, and Ca²⁺-permeable channels that function as homo- or heteromultimeric complexes (38,48). Activation of the P2X₇ receptor can stimulate various cell-specific signaling cascades (11,40). Effects unique to the receptor are the induction of membrane fusion and blebbing associated with microvesicle generation (9,10), interleukin-1 β processing and secretion (11,24,33), and opening of membrane pores (11,37,40,46). Epithelial cells of the female lower reproductive tract express the P2X₇ receptor

(3), and in human cervical epithelial cells activation of the receptor induces apoptosis by a mechanism that involves calcium-dependent activation of the mitochondrial pathway (51). Apoptosis plays an important role in the regulation of cell cycle and control of neoplastic transformation (12). Because dysfunction of apoptosis could be associated with cervical dysplasia and cancer (13), our long-term objective is to better understand the mechanisms involved in P2X₇ receptor-mediated apoptosis of cervical cells.

The function of P2X₇ receptor depends on receptor expression and its cellular distribution (40), and the present study was undertaken to better elucidate some of these mechanisms. The experiments used human cervical epithelial cells, which express the P2X₇ receptor endogenously, and results were compared with those obtained in the heterologous system of human embryonic kidney (HEK)-293 cells transfected with the full-length human P2X₇ receptor (HEK-293-hP2X₇-R cells). In both types of cells stimulation of the receptor caused plasma membrane pore formation. In both types of cells treatment with ATP also transiently decreased the 85-kDa form of the P2X₇ receptor in the plasma membrane and increased its level in the cytosol. These effects were followed by an increase of the 18-kDa form both in the plasma membrane and in the cytosol and are therefore compatible with internalization, degradation, and recycling. Treatment with ATP also increased phosphorylation of the receptor and increased binding of the 85-kDa form to G protein-coupled receptor (GPCR) kinase (GRK)-3, β -arrestin-2, and dynamin. These novel data could be important for our understanding of P2X₇ receptor activation, compartmentalization, and signal transduction.

METHODS

Cell Culture Techniques and Transfections

CaSki and HEK-293 cells used in the experiment were obtained from the American Type Culture Collection. CaSki cells are a line of transformed cells that retain phenotypic characteristics of human endocervical epithelial cells and are a useful model with which to study cervical cell functions (19,20). HEK-293 cells lack endogenous expression of P2X receptors and are frequently used for heterologous expression of the receptors. The HEK-293 cells were maintained in a medium composed of MEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 5 mM sodium pyruvate, 100 U/ml penicillin, and 100 μ g/ml streptomycin (GIBCO, Los Angeles, CA). For experiments, cells were shifted to a modified Ringer solution composed of (in mM) 120 NaCl, 1.2 CaCl₂, 1.2 MgSO₄, 5 KCl, 10 NaHCO₃ (before equilibration with 95% O₂-5% CO₂), 10 HEPES, and 5 glucose, with 0.1% bovine serum albumin.

For transfections, the full-length human P2X₇ receptor [*Homo sapiens* purinergic receptor P2X ligand-gated ion channel 7 (P2RX₇) mRNA human (GenBank accession no. NM_002562); Ref. 48] was cloned into pcDNA-6 vector with subcloning sites *Eco*RI and *Not*I, along with a c-Myc epitope tag attached at the NH₂ terminus of the P2X₇ gene. The plasmid DNA was transfected into HEK-293 cells with GenePorter reagent (Gene Therapy, San Diego, CA) according to the manufacturer's instructions.

The plasmid containing the construct of the fusion protein β -arrestin-2 and green fluorescent protein (GFP) (β -arrestin-2-GFP; 1 μ g/ μ l) was kindly provided by Dr. Marc Caron of Duke University (39). Transfections in CaSki cells used 3 μ g of β -arrestin-2-GFP plasmid DNA per 100-mm dish and were carried out with the GenePorter II reagent, following the supplier's (GTS) instructions. Transfections were performed 48 h before the experiment in dishes with cells at confluence of ~70%. A similar method was used for cotransfection of HEK-293 cells with the P2X₇ receptor and β -arrestin-2-GFP plasmids, with a mixture of the two plasmids (3 μ g for each per 100-mm dish).

Fluorescence Experiments with Attached Cells

The method for fluorescence experiments with attached cells was described previously (21). Changes in cytosolic calcium (Ca_i) were determined in fura-2-loaded cells as described previously (21). For experiments with the nuclear stain ethidium bromide (molecular mass 394 Da), the agent was added to the perfusing solutions from a concentrated (100 $\times$) stock at a final concentration of 5 μ M. On influx into cells, the dye binds to nuclear chromatin and elicits specific fluorescence. Changes in fluorescence were measured online at 518- and 605-nm wavelengths (excitation and emission, respectively). Agents and solutions were added to both the luminal and subluminal solutions.

Immunostaining Technique

Light microscopy experiments were conducted on cells grown on glass coverslips. Cells were washed with PBS and fixed with fresh 2% paraformaldehyde in PBS for 10 min at room temperature. After washes with PBS, cells were permeabilized in PBS containing 0.05% Nonidet P-40 for 10 min at room temperature, incubated for 30 min at room temperature in blocking solution (PBS, 1% bovine serum albumin, 5% goat serum), and incubated overnight at 4°C in the same solution with rabbit anti-P2X₇ receptor polyclonal antibody (PAb). Cells were washed once with the blocking solution and once with PBS and incubated with horseradish peroxidase-labeled goat anti-rabbit IgG, heavy- and light-chain peroxidase (Calbiochem, San Diego, CA), at a dilution of 1:2,000 for 30 min at room temperature. The reaction was visualized using Fast Red (Dako, <http://www.dakocytomation.com>), and nuclear staining was done with hematoxylin according to standard methods. Confocal laser scanning microscopy experiments were done on cells cultured on Transwell filters (Costar, Cambridge, MA) or on Millicell-CM filters (Millipore, Bedford, MA). Cells were fixed in cold methanol for 15 min at room temperature, immersed in blocking buffer (3% bovine serum albumin, 0.1% Triton X-100 in PBS) for 30 min at room temperature, and incubated overnight at 4°C in the same solution with rabbit anti-P2X₇ receptor PAb. After three washes with PBS, cells attached to the filter were incubated with FITC-labeled anti-rabbit IgG secondary antibodies for 1 h at room temperature. The filters were mounted in Vestashield with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Vector H-1200). Immunolocalization was observed with a Zeiss MRC 1024 confocal laser scanning microscope. Representative fields were selected, and images were processed with the Adobe Photoshop software package.

Western Blot Analysis

The postnuclear supernatant of cells was prepared in lysis buffer [50 mM Tris-HCl, pH 6.8, 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM EDTA, pH 8.0] containing 50 μ g/ml PMSF, 10 μ g/ml benzamidine, 10 μ g/ml bacitracin, 10 μ g/ml leupeptin, and 2 μ g/ml aprotinin. Aliquots normalized to 15 μ g of protein were loaded on SDS-polyacrylamide gels, fractionated using gel electrophoresis (PAGE), and examined using Western blot analysis. Receptor polypeptides were visualized with 1.5 μ g/ml of rabbit anti-P2X₇ antibody. Anti-rabbit peroxidase-conjugated secondary antibody was used for visualization (ECL kit; Santa Cruz Biotechnology, Santa Cruz, CA).

Cell Fractionation by Freeze-Thaw Method

Cells were washed and released with cold HBSS containing 5 mM EDTA, 50 μ g/ml PMSF, 10 μ g/ml benzamidine, 10 μ g/ml bacitracin, 10 μ g/ml leupeptin, and 2 μ g/ml aprotinin. The cell suspension was centrifuged at 380 g, and the pellet was resuspended in cold, sterile 0.25 M sucrose solution containing the protease inhibitors and recentrifuged at 380 g for 5 min. Cells were resuspended in 10 ml of the same solution, dispersed into single cells by repeat pipetting, and centrifuged at 380 g for 5 min. The pellet was resuspended in 1/10 volume of HME buffer (50 mM HEPES, pH 7.4, 12.5 mM MgCl₂, 1.5 mM EGTA, and the protease

inhibitors) plus 5 mM EDTA and vortexed. After incubation at -75°C for 1 h, the pellet was thawed at room temperature and the freeze-thaw procedure was repeated two additional times. After being spun at 380 g for 5 min, the supernatant containing cytosol and plasma membranes was collected and centrifuged at 13,500 g for 20 min. After being spun, the supernatant (cytosol) was collected and the pellet (membrane-enriched fraction) was resuspended in 100 μl of HME buffer plus 10% glycerol. Protein concentration was estimated with a Bio-Rad protein assay kit.

Phosphorylation Assays

CaSki cells were shifted for 1 h to phosphate-free DMEM containing 10 mM HEPES, pH 7.4, at 37°C , and treated with 100 $\mu\text{Ci/ml}$ [^{32}P]orthophosphate (PerkinElmer Life Sciences, Boston, MA) plus 1 $\mu\text{g/ml}$ microcystin L-R (Calbiochem, San Diego, CA) to label the ATP pool. After treatment, ATP cells were washed with ice-cold PBS, lysed in lysis buffer as described below, and immunoprecipitated with rabbit anti-P2X₇ PAb. Samples containing equal amounts of protein were resolved on 10% polyacrylamide gels and dried under vacuum. Radioactive bands were visualized using PhosphorImager software [Molecular Dynamics (Amersham), Piscataway, NJ] and exposure to X-ray film.

Immunoprecipitation and Immunoblotting Assays

After treatment, cells collected from 100-mm culture dishes were lysed in lysis buffer (in mM: 2 Na-orthovanadate, 150 NaCl, 5 EDTA, 50 NaF, 40 sodium pyrophosphate, 50 KH_2PO_4 , 10 sodium molybdate, and 20 Tris-HCl, pH 7.4, with 1% Triton X-100, 0.5% Nonidet P-40, 10 mM DTT, 5 mg/ml aprotinin, 5 mg/ml leupeptin, 100 mg/ml bacitracin, and 100 mg/ml benzamidine), and samples were normalized by adjusting total protein level in each sample to 500 μg . For experiments using the anti-phosphotyrosine, -serine, and -threonine antibodies, the composition of the lysis buffer was 1% Triton X-100, 50 mM NaCl, 60 mM *n*-octyl- β -D-glucoside, 5 mM EDTA, and 50 mM HEPES, pH 7.5, plus the mixture of protease inhibitors. Lysis was carried out at 4°C for 20 min; the mixture was spun at 10,000 g for 15 min, and lysates were immunoprecipitated with the primary antibody first for 1–3 h and then precleared with protein A/G agarose overnight at 4°C . Immune complexes were washed three times with RIPA buffer (in mM: 20 Tris-HCl, 150 NaCl, 1 EDTA, and 10 DTT, with 1% Triton X-100, pH 8.0) and separated on 4–12% linear gradient SDS-acrylamide Laemmli gels. Immunoblotting and immunostaining were performed as described above.

Confocal Microscopy

Confocal microscopy was done as described previously (2,39) with some modifications. CaSki cells transfected with β -arrestin-2-GFP or HEK-293 cells cotransfected with the full-length human P2X₇ receptor and β -arrestin-2-GFP were plated on 35-mm glass-bottomed culture dishes (MatTek, Ashland, MA). Dishes contained a centered 1-cm well formed from a glass coverslip-sealed hole in the plastic. Two hours before experiments the medium was replaced with serum-free medium supplemented with 10 mM HEPES. The distribution of β -arrestin-2-GFP was visualized before and after treatment with the agonist using real-time confocal microscopy. Imaging of β -arrestin-2-GFP fluorescence in the same cells was performed on a Zeiss laser-scanning confocal microscope (LSM-510) with a heated (37°C) microscope stage. Images were collected sequentially before and after treatment with agonist for 0–30 min at 37°C using single-line excitation (488 nm).

Densitometry

Densitometry was done with an AGFA Arcus II scanner (AGFA, New York, NY) and Un-Scan-It gel automated digital software (Silk Scientific, Orem, UT).

Statistical Analysis

Data are presented as means ($\pm$ SD), and significance of differences among means was estimated using Student's *t*-test. Trends were calculated with GB-STAT version 5.3 (Dynamic Microsystems, Silver Spring, MD) and analyzed using ANOVA.

Chemicals and Supplies

Fura-2 AM and ethidium bromide were obtained from Molecular Probes (Eugene, OR). All other chemicals, unless otherwise specified, were obtained from Sigma (St. Louis, MO).

Antibodies

The anti-P2X₇ receptor PAb was from Alomone Laboratories (Jerusalem, Israel) and was raised against the purified peptide (C)K-IRK EFPKT QGQYS GFKYP Y (corresponding to residues 576–595 of rat P2X₇ with an additional NH₂-terminal cysteine; Ref. 48). The following were from Santa Cruz Biotechnology: anti-c-Myc MAb, anti-GRK-3 PAb, anti- β -arrestin-2 MAb, anti-dynamin MAb, and anti-clathrin MAb. Anti-phosphotyrosine PY20 and P99, anti-phosphoserine, and anti-phosphothreonine MAbs were from Transduction Laboratories (BD Biosciences, San Jose, CA).

RESULTS

Stimulation of P2X₇ Receptor Induces Plasma Membrane Pore Formation

Treatment of CaSki cells attached on filters with ATP-induced transient calcium mobilization and acute calcium influx (Fig. 1A and Refs. 17,18). In the continued presence of ATP, there was also a slow and sustained increase in Ca_i that began about 10 min after the ATP was added (Fig. 1A). The late, slow increase in Ca_i could be blocked by lowering extracellular calcium to <0.1 mM, indicating calcium influx, and attenuated by pretreatment with 1-[*N,O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), oxidized ATP (oATP), with polyethylene glycol 6000 (PEG-6000) (Fig. 1B). Among different ATP agonists, only the P2X₇ receptor agonist 2',3'-*O*-(4-benzoylbenzoyl)adenosine 5'-triphosphate (BzATP) could induce the late Ca_i increase (Fig. 1C). To determine whether the late sustained calcium influx is the result of P2X₇ receptor pore formation (11,40), CaSki cells were treated with ATP or BzATP in the presence of ethidium bromide. Under baseline conditions there were no changes in fluorescence at 518 and 605 nm (excitation and emission, respectively), but treatment with ATP or BzATP increased the fluorescence (Fig. 1, A, C, and D), indicating increased nuclear binding of ethidium bromide and suggesting influx of ethidium bromide. The ethidium bromide effect correlated in time with the Ca_i changes (Fig. 1, A and C), and it also could be attenuated by KN-62, oATP, and PEG-6000 (Fig. 1D). However, lowered extracellular calcium had no effect on ATP- or BzATP-induced influxes of ethidium bromide (Fig. 1D).

In wild-type HEK-293 cells attached to filters, treatment with ATP did not produce significant change in Ca_i or influx of ethidium bromide (not shown). Similarly, in HEK-293-hP2X₇-R cells, ATP did not induce Ca_i transients and the nucleotide UTP did not have any appreciable effect on Ca_i levels (not shown), effectively ruling out involvement of a putative P2Y₂ receptor in the Ca_i increase (42). In contrast, in HEK-293-hP2X₇-R cells, ATP and BzATP induced sustained Ca_i increase and influx of ethidium bromide (Fig. 2, A and C). Both effects could be inhibited by KN-62, oATP, and PEG-6000, and the former could be blocked by lowering extracellular calcium to <0.1 mM (Fig. 2, B and D). Both effects correlated in time (Fig. 2, A and C), but in HEK-293-hP2X₇-R they began earlier than in CaSki cells (Figs. 1 and 2). Collectively, these results suggest that in CaSki cells and HEK-293-hP2X₇-R cells, activation of the P2X₇ receptor induces Ca²⁺-independent pore formation.

The late ATP-induced calcium influx could be prevented if CaSki cells were washed and reincubated in fresh medium 1–7 min after treatment. Washes at later times resulted in partial or no effect on the responses (Fig. 3A). Increasing Mg^{2+} in the extracellular fluid from 1 mM to 5 mM did not significantly affect the magnitude of the ATP-induced late, prolonged increase in Ca_i (not shown), suggesting that, in contrast to other types of cells, the active ligand for the $P2X_7$ receptor is not the tetrabasic acid ATP^{4-} . In both types of cells the effects of ATP and BzATP were concentration dependent. Increases in Ca_i and in ethidium bromide fluorescence began with low micromolar concentrations of the ligands and increased with higher added amounts of the nucleotides (Fig. 3B). Although the dose-response curves did not reach saturation even at millimolar concentrations of the ligands (Fig. 2B), the data confirm findings (11,40) that BzATP produces more potent and efficacious effects than ATP.

Expression of $P2X_7$ Receptor Protein

Immunofluorescence staining of CaSki and HEK-293-h $P2X_7$ -R cells with the Alomone rabbit anti- $P2X_7$ receptor PAb revealed diffuse, uniform, and homogeneous speckled cellular decoration that could be blocked by preincubation with the $P2X_7$ receptor antigen (Fig. 4A). Confocal laser scanning microscopy provided a more detailed analysis of the $P2X_7$ receptor cellular distribution in CaSki cells (Fig. 4B): specific granular staining along the plasma membrane, patchy staining in the cytoplasm, and, in some cells, staining in clumps confined to the nucleus. Western immunoblot analysis of total homogenates from CaSki cells revealed specific reactivity to 85-, 65-, and 18-kDa forms that could be blocked by preincubation with the $P2X_7$ receptor antigen (Fig. 4C). Previous studies showed that the 85-kDa form is the mature and functional form of the $P2X_7$ receptor, residing mainly in the plasma membrane (50). The 65-kDa form is the native form of the $P2X_7$ receptor corresponding to the reported 70-kDa $P2X_7$ receptor isoform, whereas the 18-kDa form is a degradation product (41,50).

The $P2X_7$ receptor antibody did not stain untransfected HEK-293 cells or HEK-293 cells transfected with GFP- β -arrestin-2 fusion protein alone (not shown). In homogenates of HEK-293-h $P2X_7$ -R cells specific immunoreactivity to 85-, 65-, and 18-kDa forms of $P2X_7$ receptor was found, similar to CaSki cells (Fig. 4C). Cotransfection of HEK-293-h $P2X_7$ -R cells with GFP- β -arrestin-2 had no effect on the expression of the three $P2X_7$ receptor forms (Fig. 4C).

Redistribution of $P2X_7$ Receptor

One of the interpretations of the data in Fig. 4A is compartmentalization of the $P2X_7$ receptor. This speculation was confirmed in the experiments shown in Figs. 5–7. In both CaSki and HEK-293-h $P2X_7$ -R cells the 85-kDa form was expressed mainly in the plasma membrane-enriched fraction. Assays using the anti- $P2X_7$ receptor antibody did not detect expression of the 85-kDa form in the cytosol (Figs. 5 and 6). The 65- and 18-kDa forms were expressed in both the plasma membrane-enriched fraction and in the cytosol. Western blot analysis of homogenates of HEK-293 cells transfected with the full-length human $P2X_7$ receptor tagged with c-Myc epitope at the NH_2 terminus of the $P2X_7$ gene (HEK-293-c-Myc-h $P2X_7$ -R cells), using the anti c-Myc antibody, revealed immunoreactivity at 85 and 65 kDa in both the plasma membrane and cytosolic fractions but did not detect specific 18-kDa staining (Fig. 7).

To determine the degree to which ligand binding induces redistribution of the receptor, *day 6* CaSki cells were immunostained with the anti- $P2X_7$ receptor antibody at different time intervals after treatment with ATP. Thirty minutes after treatment with 10 μM ATP receptor, staining remained diffuse, uniform, and relatively homogeneous (Fig. 4A). In cells treated with 50 or 250 μM ATP, the staining became nonuniform, coarse, and condensed toward perinuclear regions of the cells (Fig. 4A). In control slides that were processed in the presence of the $P2X_7$ receptor antigen, no significant morphological changes could be discerned (Fig. 4A).

These results indicate that the ATP-induced changes in P2X₇ receptor staining are not the result of changes in cell structure, but rather represent specific changes in receptor distribution.

A more direct approach was used in the experiments shown in Figs. 5-7. In CaSki cells, treatment with ATP stimulated a transient decrease in the level of the 85-kDa form in the membrane-enriched fraction, with a parallel transient increase in the 65-kDa form. Maximal effects were observed 10 min after treatment with ATP, with gradual return to baseline levels after ~20 min. About 15 min after treatment with ATP, there was an increase in the densities of the 18-kDa isoform in the membrane-enriched fractions and of the 65- and 18-kDa forms in the cytosol. Those increases persisted throughout the 30 min of the experiment (Fig. 5).

In HEK-293-hP2X₇-R cells, treatment with ATP stimulated transient decrease in the 85- and 65-kDa forms in the membrane-enriched fraction (Fig. 6). Maximal effects were observed 3–5 min after the treatment, with gradual return to baseline levels after ~15 min. In the membrane-enriched fraction ATP also stimulated a gradual increase in the 18-kDa form that began 10 min after the treatment and persisted throughout the 30 min of the experiment. ATP also stimulated biphasic change in the 65- and 18-kDa forms in the cytosol: a transient increase that peaked at ~10 min after ATP was added, followed by a decrease to subbaseline levels that persisted throughout the 30 min of the experiment (Fig. 6). In HEK-293-c-Myc-hP2X₇-R cells, treatment with ATP stimulated transient decreases in the 85- and 65-kDa forms in the membrane-enriched fraction, with reciprocal transient increases in the 85- and 65-kDa forms in the cytosol (Fig. 7).

In summary, the data in Figs. 5-7 suggest that treatment with ATP increases the P2X₇ receptor 85-kDa form and decreases the 18-kDa form in the plasma membrane and increases the levels of the three receptor forms in the cytosol. The main differences between the effects in CaSki cells and in HEK-293-hP2X₇-R cells were faster effects in HEK-293-hP2X₇-R cells and an increase in the plasma membrane in CaSki cells of the 65-kDa form, in contrast to a decrease in HEK-293-hP2X₇-R cells.

Mechanism of Internalization of P2X₇ Receptor

Phosphorylation—To determine whether ligand binding induces phosphorylation of the P2X₇ receptor, the intracellular ATP pool of CaSki cells was labeled by incubation of cells with [³²P]orthophosphate. After treatment with ATP, cell lysates were immunoprecipitated with the anti-P2X₇ antibody. Autoradiography at baseline conditions before ATP revealed some phosphorylation of the 65-kDa form and negligible phosphorylation of the 85-kDa form (Fig. 8A). Treatment with ATP increased phosphorylation of the 85-kDa form by at least 10-fold, with no significant effect on the 65-kDa form (Fig. 8A). Western immunoblot analysis revealed no significant change in total receptor content after treatment with ATP (Fig. 8A), suggesting that the 85-kDa form of the P2X₇ receptor is the preferred target of phosphorylation.

To determine the mechanism of ATP-induced phosphorylation of the 85-kDa form, CaSki cells were treated with 250 μM ATP for different time intervals from 0 to 30 min. Cell lysates were immunoprecipitated in a mixture containing anti-phosphotyrosine PY20 and P99 antibodies and anti-phosphoserine and anti-phosphothreonine antibodies. Immunoblotting with the anti-P2X₇ antibody revealed negligible specific immunoreactivity at 85 kDa (Fig. 8B) at 0 min, which increased by 0.5 min and disappeared at 5–10 min after treatment with ATP (Fig. 8B).

To better understand which residues of the 85-kDa form are phosphorylated, CaSki cells were treated for 1 min with ATP at concentrations ranging from 0 to 500 μM; cell lysates were immunoprecipitated with anti-phosphorylation antibodies alone or in combination and immunoblotted with the anti-P2X₇ antibody. Immunoprecipitates with anti-phosphotyrosine PY20 and anti-phosphoserine antibodies revealed maximal immunoreactivity in cells treated

with 5 μ M ATP, with a gradual decrease in cells treated with higher concentrations of the nucleotide (Fig. 8C). Immunoprecipitates with anti-phosphothreonine antibodies, alone or in combination with anti-phosphotyrosine PY20 and anti-phosphoserine, revealed a lesser degree of immunoreactivity at 5 μ M ATP that increased with higher concentrations of ATP. These results indicate that low concentrations of ATP (5 μ M) sufficed to stimulate phosphorylation of the 85-kDa P2X₇ receptor form on tyrosine and serine residues, whereas ATP at higher concentrations tended to block the effect. In contrast, phosphorylation of the 85-kDa form on threonine residues increased with ATP, and it also increased ATP requirements for phosphorylation of the 85-kDa form on tyrosine and serine residues in combination (Fig. 8C).

Involvement of GRK-3—Phosphorylation of GPCRs by GRKs is often required for binding of β -arrestin and the consequent initiation of receptor endocytosis (31). The role of GRK phosphorylation is to increase the affinity of the receptor for arrestins (29,30). In preliminary experiments we found that CaSki and HEK-293 cells constitutively express GRK-2 and GRK-3 and that the latter also express GRK-5 and GRK-6 (not shown). To determine whether and which GRK phosphorylates the P2X₇ receptor, lysates of ATP-treated CaSki cells were immunoprecipitated with the anti-P2X₇ antibody and immunoblotted with commercially available anti-GRK antibodies. The immunoblots revealed specific immunoreactivity only to anti-GRK-3 antibody at ~80 kDa 1–3 min after ATP was added. This immunoreactivity increased in a time-related manner for at least 30 min after addition of ATP (Fig. 9Aa). A similar effect was seen in HEK-293-c-Myc-hP2X₇-R cells (Fig. 9Ac). However, in CaSki cells, the association between the P2X₇ receptor and GRK-3 persisted for at least 30 min after adding ATP, compared with 15 min in HEK-293-c-Myc-hP2X₇-R cells (Fig. 9A). In both types of cells immunoblots with anti-GRK-3 antibody revealed no significant change in total cellular GRK-3 protein (Fig. 9, Ab and Ad). Similarly, treatment with ATP did not significantly affect total cellular levels of the 85-, 65-, and 18-kDa P2X₇ receptor forms (Fig. 9D).

Recruitment of β -arrestin-2—GRK-mediated phosphorylation of GPCRs leads to recruitment of β -arrestins to the plasma membrane; β -arrestin binding to the phosphorylated GPCR both uncouples the receptors from heterotrimeric G proteins and targets them to clathrin-coated pits for endocytosis (31). β -Arrestin-1 (30) and β -arrestin-2 (1) are ubiquitously expressed proteins, and preliminary results showed expression of both in CaSki and HEK-293 cells. There are three potential β -arrestin-2 binding motifs in the P2X₇ receptor sequence, T357YSS, T508TS, and S540TNS (39), but until recently little was known about the involvement of β -arrestins in P2X₇ receptor activation. To determine whether and which β -arrestin associates with phosphorylated P2X₇ receptor, lysates of CaSki cells treated with ATP were immunoprecipitated with anti-P2X₇ antibody and immunoblotted with anti- β -arrestin-1 and β -arrestin-2 antibodies. The immunoblots revealed specific immunoreactivity only to β -arrestin-2 at ~50 kDa, which began 1 min after the nucleotide was added and increased in a time-related manner for at least 30 min after treatment (Fig. 9Be). A similar effect was seen in HEK-293-c-Myc-hP2X₇-R cells (Fig. 9Bg). In both types of cells, immunoblots with anti- β -arrestin-2 antibody revealed no significant change in total cellular β -arrestin-2 protein (Fig. 9, Bf and Bh).

This novel finding was confirmed with the secondary method of real-time confocal laser microscopy. The experiments used CaSki cells overexpressing β -arrestin-2 tagged with GFP and HEK-293-hP2X₇-R cells cotransfected with β -arrestin-2-GFP. We avoided using cells transfected with P2X₇ receptor tagged with GFP, because tagging a GFP onto the NH₂ terminus or the COOH terminus of the P2X₇ receptor could affect receptor functionality (44). Figure 10A demonstrates the feasibility of the method in CaSki cells. In control cells (not transfected with the β -arrestin-2-GFP), no GFP fluorescence was detected (not shown). In contrast, fluorescence was observed in cells transfected with β -arrestin-2-GFP. Under steady-state

conditions GFP fluorescence (i.e., β -arrestin-2) was distributed diffusely and homogeneously throughout the cytoplasm (Fig. 10*A**c*). Treatment with ATP resulted in redistribution of GFP fluorescence toward the cell periphery within 3 min of treatment (Fig. 10, *Ad* and *Ae*), suggesting recruitment of β -arrestin-2 into submembranous regions. A similar effect was observed with BzATP (not shown).

To determine more directly P2X₇ receptor recruitment of a β -arrestin, HEK-293-hP2X₇-R cells were cotransfected with β -arrestin-2-GFP, to determine P2X₇ receptor-mediated changes in β -arrestin-2 redistribution in real-time. Figure 10*B* shows the feasibility of the method, and it demonstrates three important points. First, in HEK-293 cells transfected with only β -arrestin-2-GFP under steady-state conditions, GFP fluorescence was diffuse and homogeneous (Fig. 10*Bc*). Second, in those cells, treatment with ATP had no significant effect on GFP fluorescence (Fig. 10*Bd*), ruling out activation of β -arrestin-2-associated ATP receptors. Third, GFP fluorescence was not significantly affected by cotransfection with the full-length human P2X₇ receptor (Fig. 10, *Be*, *Ca*, *Cd*, and *Cg*). Transfection with the P2X₇ receptor alone did not elicit GFP fluorescence (not shown).

The system described in Fig. 10*B* was used to determine the degree to which stimulation of the P2X₇ receptor induces recruitment of β -arrestin-2. Control experiments were treatments with angiotensin or UTP; the rationale for the former was that HEK-293 cells do not express angiotensin receptors endogenously. UTP was used to determine whether activation of the supposedly expressed P2Y₂ receptor in HEK-293 cells (42) can contribute to recruitment of β -arrestin-2. Neither angiotensin (Fig. 10, *Ca–Cc*) nor UTP (agonist of the P2Y₂ receptor, Ref. 8) (Fig. 10, *Cd–Cf*) had a significant effect on GFP fluorescence. In contrast, treatment with ATP resulted in redistribution of GFP fluorescence toward the cell periphery within 5 min of treatment, and the effect lasted for at least 10 min (Fig. 10, *Cg–Ci*). This result suggests that stimulation of the P2X₇ receptor induces recruitment of β -arrestin-2 into submembranous regions of the cell.

Role of clathrin and dynamin—In addition to the initiation of signal transduction events, ligand binding to GPCRs can induce dynamin-dependent receptor endocytosis via clathrin-coated pits (14,31,35,43,53). To determine whether the redistribution of the P2X₇ receptor after treatment with ATP is associated with dynamin and clathrin, lysates of ATP-treated CaSki cells were immunoprecipitated with anti-dynamin or anti-clathrin antibodies and immunoblotted with the anti-P2X₇ antibody. Immunoprecipitates obtained with anti-dynamin antibody revealed specific immunoreactivity to anti-P2X₇ antibody at ~85 kDa 3–5 min after treatment with ATP, which returned to baseline levels at 10–30 min (Fig. 9*Ci*). Immunoprecipitates obtained with anti-clathrin antibody revealed monotonic specific immunoreactivity to anti-P2X₇ antibody at ~85 kDa (Fig. 9*Ck*). Treatment with ATP had no effect on the cellular levels of dynamin (Fig. 9*Cj*) or clathrin (Fig. 9*Cl*).

DISCUSSION

The present results show that treatment with ATP induces redistribution of the P2X₇ receptor between the plasma membrane and the cytosol. Receptor redistribution was associated with changes in receptor phosphorylation status and with increased binding of the receptor to GRK-3, β -arrestin-2, and dynamin. These findings suggest a novel mechanism of P2X₇ receptor activation.

The receptor localization assays used three different systems that provided complementary data. Western blot analysis in CaSki cells and HEK-293-hP2X₇-R cells with the anti-P2X₇ receptor antibody provided cleaner results than assays in HEK-293-c-Myc-hP2X₇-R cells using the anti-c-Myc antibody. The former method failed to detect the 85-kDa P2X₇ receptor

form in the cytosol, probably because of the relatively low sensitivity of the antibody that could not detect low levels of the 85-kDa form in that compartment. The latter method failed to detect the 18-kDa form, possibly because of dissociation of the c-Myc tag from the 18-kDa fragment. Despite those differences, similar trends for receptor redistribution were observed, which allowed us to draw conclusions regarding the effects of treatment with ATP on the compartmentalization of the P2X₇ receptor.

Treatment with ATP transiently decreased the 85-kDa form in the plasma membrane and increased its level in the cytosol. These effects were followed by an increase of the 18-kDa form both in the plasma membrane and in the cytosol. Because the 85-kDa form is the functional form of the receptor (50) and the 18-kDa form is a degradation product (41,50), it is suggested that ligand-dependent activation of the P2X₇ receptor is associated with transient downregulation of the 85-kDa form in the membrane, followed by receptor degradation and replenishment in the membrane.

Redistribution of the 85-kDa form was preceded by transient upregulation of phosphorylation on tyrosine, serine, and threonine residues. The phosphorylation depended on the concentration of ATP, and phosphorylation of threonine residues had a dominant-negative effect on phosphorylation of tyrosine and serine residues. The results in human CaSki cells differ from those in the rat, where, under resting conditions, the P2X₇ receptor is phosphorylated on tyrosine residues and activation of the receptor increases receptor protein tyrosine phosphatase- β activity and induces dephosphorylation of the receptor (27). Of the seven threonine residues that the human P2X₇ receptor possesses, two reside in the NH₂ terminus. Because the receptor protein tyrosine phosphatase- β is also associated with the NH₂ terminus of the P2X₇ receptor (27), it is possible that the NH₂ terminus is involved in regulation of phosphorylation of the P2X₇ receptor. Furthermore, both in CaSki and in HEK-293 cells, the ligation-induced colocalization of P2X₇ receptor and GRK-3 was longer than the ligation-induced phosphorylation of the P2X₇ receptor. If, as proposed (see below), GRK-3 is the kinase that induces the phosphorylation of the receptor, a possible explanation would be dephosphorylation downstream of GRK-3 that is induced by GRK-3. A possible phosphatase is the receptor protein tyrosine phosphatase- β , which is expressed both in CaSki cells (not shown) and in HEK-293 cells (27).

Treatment with ATP also increased binding of GRK-3 and β -arrestin-2 to the 85-kDa form and recruitment of β -arrestin-2 to submembranous domains, and the effects were correlated in time with phosphorylation of the 85-kDa form. All of these effects could be epiphenomena, namely, unrelated events caused by different but simultaneous signaling cascades. On the other hand, it is possible that they are causatively related and result from a structured signaling pathway. Thus ligand binding could induce receptor activation via GRK-3 phosphorylation of the 85-kDa form in the membrane, followed by recruitment of β -arrestin-2. Ligand binding-induced phosphorylation is a well-described phenomenon of GPCRs (49), ion channels (23), γ -aminobutyric acid receptors (36), and *N*-methyl-D-aspartate receptors (52), but the present study shows for the first time ligand binding-induced phosphorylation of the P2X₇ receptor. A possible consequence could be internalization of the receptor as previously described for GPCRs (49). Receptor internalization is a complex series of cell-specific events that may involve endocytosis, receptor sequestration into various cellular domains, recycling, and degradation. The present results in two different human cell types show similar trends with regard to changes that are compatible with receptor internalization, degradation, and recycling.

β -Arrestin-2 involvement in the GRK-3-associated internalization of the 85-kDa form suggests that it may act as an adapter protein targeting the receptor for sequestration into clathrin-coated pits (31). Under baseline conditions in CaSki cells, the 85-kDa form colocalized tonically with clathrin, but treatment with ATP transiently increased the binding of the 85-kDa form to

dynamin. The mechanism of these findings is unclear; clathrin can interact with β -arrestins unrelated to endocytosis, and dynamin can regulate vesicle fission in non-clathrin cascades (14). However, in view of the receptor redistribution data discussed above and the general similarity to the mode of GPCR action (14,31,34,35,43,53), it is possible that activation of the P2X₇ receptor induces receptor endocytosis into clathrin-coated endosomes by a signaling cascade that begins with receptor phosphorylation. Endocytosis via clathrin-coated pits is an effective mechanism to attenuate ligand-activated responses and modulate receptor downregulation and/or resensitization (22,35). Signaling continues on the endocytotic pathway and may allow for transport of signaling complexes to specific subcellular locations (35). In this context, the present results could explain the translocation of P2X₇ receptors to the nucleus and provide better understanding of mechanisms for P2X₇ receptor signaling that were previously unknown, such as activation of MAP kinases (5), ERK1/2 (16), NF- κ B (15), and JNK3 (SAPK; Ref. 25).

Both in CaSki and in HEK-293-hP2X₇-R cells, treatments with ATP or BzATP induced pore formation. The results in HEK-293-hP2X₇-R cells are in agreement with works of others (see, e.g., Ref. 4), but the relatively slow course of the effect in CaSki cells cannot be readily explained according to our current understanding of P2X₇ channel/pore kinetics. Studies at the channel level showed relatively fast P2X₇ channel/pore opening transitions in the range of seconds (32). If one considers the summation of opening times of single channels/pores over an entire monolayer of cells, pore opening should have been observed within 1–5 min, which is the case in HEK-293-hP2X₇-R cells but not in CaSki cells.

The mechanism underlying P2X₇ pore formation is unknown. One possibility is a conformational change in the P2X₇ channel's filter selectivity (26). An alternative mechanism suggests that oligomerization of monomeric subunits creates a pore in the plasma membrane (7). The finding of oligomerization of P2X receptors (38,48), including the P2X₇ receptor (8, 28), supports the latter hypothesis. However, in HEK-293 cells transfected with the fused P2X₇ receptor and GFP receptor, cell density in the plasmalemma did not change significantly before pore formation (44). Our present results provide additional understanding of the regulation of pore formation. In both CaSki and HEK-293-hP2X₇-R cells, the ATP-induced phosphorylation of the 85-kDa form and the increased binding to GRK-3, β -arrestin-2, and dynamin occurred within seconds or minutes and significantly earlier than pore formation in CaSki cells. It is possible that activation of the 85-kDa form is the initial event triggering P2X₇-dependent cascades and that pore formation is a secondary signaling cascade distal to the activation of the membrane receptor. In this regard the 85-kDa form operates like a GPCR.

The corollary to this hypothesis is the 65-kDa P2X₇ receptor form, which could be involved in formation of the pores. The 65-kDa form is abundantly distributed both in the membrane and in the cytosol, but at present its function is unclear. In CaSki and HEK-293-hP2X₇-R cells, treatment with ATP induced transient increase in the 65-kDa form in the cytosol, which is compatible with internalization of that form. However, in the membrane fraction of CaSki cells, ATP induced a transient increase in the 65-kDa form, whereas in the membrane fractions of HEK-293-hP2X₇-R cells, ATP induced a transient decrease. The significance of these findings is unclear. One possibility, according to the theory of plasma membrane oligomerization of P2X receptors, is aggregation of the 65-kDa form in the plasma membrane. This effect occurred 10–15 min after ATP was added in CaSki cells, which could explain the delayed pore formation in those cells. In HEK-293-hP2X₇-R cells, on the other hand, we did not detect an increase in the 65-kDa form in the plasma membrane, despite the fact that ATP induced pore formation. Therefore, more studies are needed to clarify the specific role of the 65-kDa form.

In summary, we propose a new mechanism of action for the P2X₇ receptor on the basis of the present findings. Binding of the ligand stimulates GRK-3-dependent phosphorylation and activation of the functional 85-kDa form of the receptor, as well as β -arrestin-2-mediated internalization into dynamin and clathrin domains. Receptor internalization is associated in part with receptor degradation, as well as with receptor recycling into the plasma membrane. This novel hypothesis could contribute to better understanding of P2X₇ receptor-dependent actions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Effects of ATP (250 μ M; A and B) and 2',3'-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate (BzATP, 100 μ M; C and D) (arrows) on levels of cytosolic calcium (Ca_i , solid lines) and influx of ethidium bromide [Flu, dashed lines; in arbitrary units (AU)] in CaSki cells attached on filters for 6 days. B and D: means ($\pm$ SD, 3–5 experiments in each point) of net increases in Ca_i (B) and ethidium bromide fluorescence (D) in response to ATP or BzATP. Extracellular calcium (Ca_o) was lowered to <0.1 mM by the addition of 1.2 mM EGTA. 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), oxidized ATP (oATP), and polyethylene glycol 600 (PEG-6000) were added at 100 nM, 75 μ M, and 1 mM, respectively, 10–15 min before ATP was added. Change in Ca_i (ΔCa_i) above baseline for the late sustained increase in Ca_i was determined 30 min after adding ATP in cells pretreated with 75 μ M suramin or pyridoxal phosphate-6-azophenyl-2',4-disulfonic acid (PPADS) to block the transient acute increases in Ca_i (20,21).

Fig. 2.

Effects of ATP (A and B) and BzATP (C and D) (arrows) on levels of Ca_i (solid lines) and influx of ethidium bromide (dashed lines) in HEK-293 cells expressing exogenous human P2X₇ receptor (HEK-293-hP2X₇-R cells) attached to filters for 3 days. Experiments (repeated 3–5 times) were done as described in Fig. 1.

Fig. 3.

A: reversibility of ATP effect. Six-day fura-2-loaded CaSki cells attached on filters were treated with 250 μ M ATP, and at different time intervals the bathing solutions were replaced with fresh medium. Experiments were repeated 3–7 times, with similar trends. **B:** concentration-dependent effects (means of 2 experiments) of ATP and BzATP on Ca_i and ethidium bromide fluorescence in *day 6* CaSki cells attached on filters. ΔCa_i values for the late sustained increase in Ca_i were determined 30 min after ATP was added in cells pretreated for 15 min with 75 μ M suramin and PPADS. Dashed lines, changes in fluorescence 30 min after ethidium bromide was added.

Fig. 4.

A: immunostaining of *day 6* CaSki and HEK-293-hP2X₇-R cells for the P2X₇ receptor protein (×20). CaSki cells were treated 30 min before staining with 1 of the indicated concentrations of ATP. +Ag, coincubation with the P2X₇ antigen. **B:** cellular distribution of the P2X₇ receptor in *day 6* CaSki cells as determined using confocal laser scanning microscopy (×40). *a:* Coincubation of anti-P2X₇ receptor antibody with P2X₇ antigen. *b:* Nuclear stain. *c:* Immunostaining with anti-P2X₇ receptor antibody. *d:* Combined immunostaining of nuclei with anti-P2X₇ receptor antibody. **C:** Western immunoblot analysis of P2X₇ receptor protein using total homogenates from *day 6* cultured CaSki (*a*) and HEK-293 (*b*) cells. In experiments with HEK-293 cells, we used wild-type cells, cells transfected with β -arrestin-2-GFP (β -Arr-2-GFP), or cells transfected with the full-length human P2X₇ receptor (hP2X₇-R) alone or in combination with β -arrestin-2-GFP. The experiments were repeated 2–5 times, with similar trends observed.

Fig. 5.

Top: ATP effects on the distribution of P2X₇ receptor isoforms in the plasma membrane and cytosol. Day 6 CaSki cells were treated with 250 μ M ATP (arrows), and membrane-enriched and cytosolic fractions were prepared at time intervals of 0–30 min after treatment. Fifteen-microgram samples of protein were fractionated using gel electrophoresis and assayed using Western immunoblot analysis for the P2X₇ receptor. The experiments were repeated twice, with similar trends observed. *Middle and bottom:* densitometric analysis of the data at *top*; for each isoform the density levels were normalized to the level of expression at time 0 before ATP was added.

Fig. 6.

ATP effects (arrows) on the distribution of P2X₇ receptor isoforms in the plasma membrane and cytosol of HEK-293-hP2X₇-R cells. *Top*: immunoblot analysis. *Middle and bottom*: densitometric analysis of the data at *top*. The experiments were repeated twice, with similar trends observed, and data analysis was done as described in Fig. 5.

Fig. 7.

ATP effects (arrows) on the distribution of P2X₇ receptor isoforms in the plasma membrane and cytosol of HEK-293-c-Myc-hP2X₇-R cells. *Top*: immunoblot analysis. *Middle* and *bottom*: densitometric analysis of the data at *top*. The experiments were repeated twice, with similar trends observed, and data analysis was done as described in Fig. 5, except that Western blot analysis was performed with anti-c-Myc antibody.

Fig. 8.

ATP-induced phosphorylation of the P2X₇ receptor. *A, left:* day 6 CaSki cells were labeled with [³²P]orthophosphate and treated with 250 μ M ATP for 5 min. Cell lysates were fractionated using gel electrophoresis and immunoprecipitated with the anti-P2X₇ antibody. *Right:* Western immunoblots (IB) with anti-P2X₇ antibody of parallel protein samples. *B:* day 6 CaSki cells were treated with 250 μ M ATP; at time intervals of 0–30 min after treatment cells lysates were immunoprecipitated (IP) in a mixture of antibodies containing anti-phosphotyrosine PY20 and P99 antibodies and anti-phosphoserine and anti-phosphothreonine (PST) antibodies and immunoblotted with the anti-P2X₇ antibody. *C:* day 6 CaSki cells were treated for 1 min with ATP at concentrations ranging from 0 to 500 μ M. Cells lysates were

immunoprecipitated with the indicated anti-phosphorylation antibodies, alone or in combination, and immunoblotted with the anti-P2X₇ antibody. The experiments were repeated twice, with similar trends observed.

Fig. 9.

ATP-induced colocalization of the P2X₇ receptor with GRK-3 (A), β -arrestin-2 (B), and dynamin and clathrin (C). Day 6 CaSki cells (A–C) or HEK-293-c-Myc-hP2X₇-R cells (A and B) were treated with 250 μ M ATP, and cell lysates were immunoprecipitated or immunoblotted at time intervals of 0–30 min after treatment as indicated. D: effects of treatment with ATP on total cellular levels of the P2X₇ receptor forms in day 6 CaSki cells. Each experiment was repeated 2–3 times, with similar trends observed.

Fig. 10.

A: ATP-induced recruitment of β -arrestin-2 into submembranous regions: CaSki cells were transfected with β -arrestin-2-GFP and analyzed using real-time confocal microscopy. *a*: Phase images. *b*: Nuclei staining. *c–e*: Fluorescence images at excitation wavelength of 488 nm ($\times 20$) at baseline (*c*) and 3 (*d*) and 5 (*e*) min after 250 μ M ATP was added. The experiment was repeated 3 times, with similar trends observed. **B:** *a* and *b*: low magnification ($\times 20$) of HEK-293-hP2X₇-R cells cotransfected with β -arrestin-2-GFP (*a*, nuclei staining; *b*, fluorescence at excitation wavelength of 488 nm). *c–e*: Higher magnification ($\times 40$). *c*: HEK-293 cells transfected with β -arrestin-2-GFP only. *d*: HEK-293 cells transfected with β -arrestin-2-GFP after treatment with 250 μ M ATP. *e*: HEK-293-hP2X₇-R cells transfected with β -arrestin-2-GFP. **C:** ATP-induced recruitment of β -arrestin-2 into submembranous regions: HEK-293-hP2X₇-R cells cotransfected with β -arrestin-2-GFP and treated with 10 μ M angiotensin (*a–c*), 10 μ M UTP (*d–f*), or 250 μ M ATP (*g–i*). Real-time confocal laser microscopy was used to determine fluorescence at excitation wavelength of 488 nm at baseline (*a, d, g*) or 5 (*b, e, h*) or 10 (*c, f, i*) min after treatments ($\times 20$). The experiments were repeated 2–3 times, with similar trends observed.