Mutation of a Dibasic Amino Acid Motif Within the C Terminus of the P2X<sub>7</sub> Nucleotide Receptor Results in Trafficking Defects and Impaired Function<sup>1</sup>

Loren C. Denlinger,<sup>2,4</sup> Julie A. Sommer,<sup>2,3</sup> Karen Parker,<sup>3</sup> Lalitha Gudipaty,<sup>3</sup> Philip L. Fisette,* Jyoti W. Watters,* Richard A. Proctor,<sup>7</sup> George R. Dubyak,<sup>3</sup> and Paul J. Bertics<sup>4</sup>*

Activation of the P2X<sub>7</sub> receptor by extracellular nucleotides modulates multiple immune functions, including inflammatory mediator production, membrane fusion events, and apoptosis. Previous studies have revealed that the C terminus of this multimeric cation channel possesses a lipid-interaction motif that has been proposed to regulate receptor function. This domain is homologous to the LPS binding region of the LPS binding protein, and we demonstrated that two basic residues (Arg<sup>578</sup>, Lys<sup>579</sup>) within this motif are essential for LPS binding to P2X<sub>7</sub> in vitro. Because P2X<sub>7</sub> can influence LPS action, and because lipid interaction motifs modulate the trafficking of other ion channel-linked receptors, we hypothesized that this motif of P2X<sub>7</sub> is critical for receptor function and trafficking. In these studies we mutated Arg<sup>578</sup> and Lys<sup>579</sup> of P2X<sub>7</sub>, and the expression profile, channel activity, and pore formation of the mutant were characterized in transfected human embryonic kidney 293 cells. In contrast with the wild-type receptor, the P2X<sub>7</sub>-R578E/K579E mutant fails to demonstrate surface immunoreactivity despite normal levels of total protein expression. This effect on the mutant receptor is unlikely to result from widespread defects in protein folding, because surface localization, determined using conformation-specific Abs, can be restored by growing the cells at 25°C, conditions that slow receptor recycling. Despite surface expression at reduced temperatures, at 25°C the P2X<sub>7</sub>-R578E/K579E mutant still exhibits greatly reduced sodium, potassium, and calcium channel activity when compared with the wild-type receptor, and cannot induce pore formation. These data suggest that the lipid interaction motif of the P2X<sub>7</sub> C terminus controls receptor trafficking and modulates channel activity.

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The nucleotide receptor P2X<sub>i</sub>, modulates multiple immune functions, including the production of inflammatory cytokines, PG, superoxide, and nitric oxide (1–6). Additionally, several events requiring membrane fusion have been linked to P2X<sub>i</sub>, such as phagolysosomal maturation, giant cell formation, and the blebbing associated with microvesicle generation and IL-1β processing (7–9). Furthermore, P2X<sub>i</sub>-initiated events control selective activation of the proteolytic cascade leading to apoptosis (10).

The P2 receptor family has been divided into the P2X and P2Y subfamilies according to whether the individual member acts as a ligand-gated ion channel (P2X) or a G protein coupled receptor (P2Y). P2X<sub>7</sub> belongs to the P2X subfamily on the basis of structural similarity with the six other members, each possessing two predicted membrane-spanning domains (11, 12). Several features of P2X<sub>7</sub> make it unique within the family of purinergic receptors. For example, various P2X receptors are thought to form hetero- or homotrimer; however, P2X<sub>7</sub> has only been observed to form homotrimers (12). Additionally, the P2X<sub>7</sub> C terminus is ≥119 aa longer than those of other family members, which is a property that has been correlated with several of the unique functions of the receptor (11).

The reversible formation of pores is also a characteristic feature of P2X<sub>7</sub> (1). This receptor normally functions as a nonselective cation channel that allows agonist-dependent conductance of sodium, potassium, and calcium (11). After brief stimulations (≤1 s), removal of the agonist is associated with cessation of these nonselective currents with minimal desensitization, such that repetitive brief agonist applications do not attenuate the maximum achievable current amplitude (11). Longer applications of agonist allow passage of cations with progressively larger diameters. The pattern of time constants associated with the passage of increasingly large cations is not consistent with a model of simple diffusion, suggesting that the channel diameter dilates with chronic administration of agonist (13). Pore activity is arbitrarily defined as the passage of larger molecules, including fluorescent dyes with an upper size limit of 900 Da, probably as a result of modulation of the selectivity filter of the channel (14). The process associated with pore dilation requires at least a few seconds of agonist administration, is reversible upon agonist removal, and is modulated by temperature. The process is also modulated by the concentrations of sodium and divalent cations in the extracellular solution (11, 13).
There are several lines of evidence demonstrating that P2X7 expression is tightly regulated at the level of protein localization (2). Diverse cell types including PBMC, tissue macrophages, platelets, fibroblasts, neurons, and epithelial cells express P2X7 mRNA. However, P2X7 protein expression has a much more restrictive pattern under normal conditions (1). For example, monocytes express similar levels of P2X7 mRNA, but they have much lower levels of receptor surface expression than macrophages (15). Additionally, P2X7 function in monocytes is relatively attenuated when measured using physiological agonists and buffers (16). However, the receptor can still induce pore formation in response to cell treatment with potent pharmacological agonists (4) or by altering the ionic environment using buffers that do not contain NaCl (16). Multimerization also appears important for receptor function, although there are tissue-specific differences in this process. For example, microglia largely express the receptor as monomers, whereas peritoneal macrophages contain P2X7 multimers (17). This is likely to be specific to the cell type and/or differentiation state in that transfected fibroblasts do not appear to undergo P2X7-enhanced green fluorescence protein clustering in response to agonist administration (14). Furthermore, analogous to other P2X family members, P2X7 receptor trafficking is crucial to its functionality (18–20). Specifically, there appears to be an intracellular pool of P2X7 in monocytes that is recruited to the cell surface during macrophage differentiation (15, 20). Thus, there is differential expression, trafficking, and localization of P2X7 in various cell types, and elucidation of the details governing these processes will likely contribute to a better understanding of its physiological actions and functional diversity. The unique P2X7 C terminus (residues 353–595) is thought to mediate many receptor biological activities (2). Early data supporting this notion included the observation that truncation of the last 177 aa of the rat receptor abolished P2X7 pore-inducing activity while preserving channel function (11). From these data, channel and pore activities appear distinct, and there may be additional C-terminal features that direct pore formation. In addition to participation in channel and pore activity, the P2X7 C terminus may direct receptor trafficking, consistent with observations showing that P2X7 C-terminal mutations can lead to constitutive surface localization (21). However, it is unclear whether P2X7 also contains stabilization domains that interact with cell surface constituents or retention signals that prevent surface expression under certain conditions. Because of the differential control of P2X7 expression and localization in various cell types and differentiation states, it is likely that there are multiple domains controlling this process.

Several protein- and lipid-interaction motifs within the C terminus may contribute to channel-independent signaling and other receptor functions (22). Of note, P2X7 contains a conserved LPS/lipid-binding motif, and mutation of 2 aa (Arg578, Lys579) within this domain abolishes LPS binding in vitro (22), analogous to the effects of similar mutations in LPS binding protein (23). Interest-ingly, highly basic amino acid motifs have been shown in other ion channel systems to regulate receptor trafficking (24) and promote lipid-dependent endocytosis (25). Thus, we hypothesized that the lipid-interaction motif of P2X7 is critical to receptor trafficking and/or function. In this study, we demonstrate that point mutations within this domain promote a temperature-sensitive defect in surface localization, and that alterations within this domain are associated with attenuation of channel activities. Thus, the dibasic amino acid motif within the distal C-terminal portion of P2X7 contributes to receptor trafficking as well as ligand binding or channel gating.

Materials and Methods

Materials

Cell culture reagents were purchased from Mediatech (Hernand, VA) unless otherwise specified. The fluorescent dye YO-PRO-1 was obtained from Molecular Probes (Eugene, OR), Mouse monoclonal IgG2b and FITC-labeled goat anti-mouse (Life Technologies). Using control buffer (20 mM Tris-HCl (pH 8.0), 10 mM EDTA) or HEK 293 transfectant cDNA as templates, PCR was performed with the rat P2X7-specific primers that have been described previously (27). The predicted size of the PCR product was 939 bp. PCR products were resolved on a 1% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

Abbreviations used in this paper: Bz-ATP, 2′,3′-O-benzoylbenzoyl-ATP; HEK, human embryonic kidney; fura-2 AM, fura-2-acetoxymethyl ester; HBSS, HEPES-buffered saline.

Construction of the human P2X7-R578E/K579E mutant

Two point mutations in the P2X7 cDNA that are necessary for the desired 2 aa change were introduced by PCR using the following primers (Operon Technologies, Alameda, CA): BsGI sense: 5′-AATTGGACATTAATCGCTGGTGGTGTACA; BsGI antisense: 5′-AAACTCTTCTCTCCTGACTCCTCCAGCGCCA; NotI sense: 5′-GAGGAGTCCAGAGAAGGTTCCCGAGAG; NotI antisense: 5′-ATAGCGCCGCAAAAAGGTGGGATTACA.

The P2X7 cDNA from the pcDNA3/human P2X7 receptor expression vector was cleaved out with NotI and ligated the pIREShyg expression vector (Clontech Laboratories, Palo Alto, CA). Using the pIREShyg/P2X7 expression vector as a template, two separate primary PCR sets were run with the BsrGI primer pairs and the NotI primer pairs using Pyrococcus furiosus DNA polymerase. The PCR products were gel purified using the Quaex II gel purification kit (Qiagen, Valencia, CA) and were used as both template and megaprimer sets for the secondary PCR product in the presence of the antisense NotI and the sense BsrGI primers. The resulting PCR product was gel purified, digested with NotI and BsrGI, and ligated into the pIREShyg/P2X7 expression vector that was also digested with NotI and BsrGI. The correct insertion of the P2X7-R578E/K579E cDNA into the expression vector was verified by a restriction digest analysis. The PCR-generated portion of the P2X7-R578E/K579E cDNA was sequenced in both directions. Sequencing reactions were performed using Big Dye-labeled dideoxy nucleotides and resolved by electrophoresis at the University of Wisconsin Biotechnology Center (Madison, WI). The P2X7-S342F, S342Y, and S342A mutants were created by a similar strategy using different primers.

HEK 293 cell transfection

HEK 293 cells were transfected with the pIREShyg, pIREsh/2P2X7, and the pIRESh/p2X7-R578E/K579E expression vectors using lipofectin reagent (Life Technologies, Alameda, CA): BsrG I sense: 5′-AATTGGACATTAATCGCTGGTGGTGTACA; BsrGI antisense: 5′-AAACTCTTCTCTCCTGACTCCTCCAGCGCCA; NotI sense: 5′-GAGGAGTCCAGAGAAGGTTCCCGAGAG; NotI antisense: 5′-ATAGCGCCGCAAAAAGGTGGGATTACA.

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Qualitative analysis of mRNA expression

Total RNA was prepared from confluent 10-cm plates of HEK 293 cells using RNA-STAT-60 (Tel-Test, Friendswood, TX). The resulting RNA (5 µg/reverse transcriptase reaction) was annealed with random hexamer primers (Operon Technologies), and cDNA was synthesized using SuperScript First-Strand Transcriptase (Life Technologies). Using control buffer (20 mM Tris-HCl (pH 8.0), 10 mM EDTA) or HEK 293 transfectant cDNA as templates, PCR was performed with the rat P2X7-specific primers that have been described previously (27). The predicted size of the PCR product was 939 bp. PCR products were resolved on a 1% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

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Determination of total P2X7 protein expression levels

Total cell membranes were isolated by differential centrifugation from parallel cultures of the HEK 293 transfectants. Membrane protein was quantified using the Micro BCA method (Pierce, Rockford, IL). Equal amounts of protein (25 μg) were resolved on 10% SDS-PAGE gels and transferred to Immobilon polyvinyldene difluoride membranes (Millipore, Bedford, MA) as previously described (28). Membranes were blocked in TBST (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk. The polyclonal anti-P2X7 Ab was used at a 1/1000 dilution, and HRP-conjugated anti-rabbit IgG was used at a 1/5000 dilution. Proteins were visualized using LumiGlo-ECL (Kirkgaard & Perry Laboratories, Gaithersburg, MD).

Functional screening of P2X7 mutants with a low-divalent cation cell-killing assay

At times 18–24 h before each experiment, HEK 293 transfectants (2 × 10^6 cells per well) were plated in 200 μl of medium using 96-well tissue culture plates. Before treatment the medium was removed, and 50 μl of the indicated concentration of the potent P2X agonist BzATP, prepared in low divalent cation bathing solution (96 mM NaCl: 2 mM KCl: 0.1 mM CaCl2: 5 mM HEPES, pH 7.55), was added to each well. These conditions are similar to those previously shown to facilitate agonist-dependent cell lysis in the presence of functional P2X7 receptors (13, 29, 30). After a 6-h incubation at 37°C, 50 μl of 2 mg/ml MTT in distilled water was added to each well and the plates were incubated for 2 h at 37°C. A 100-μl volume of solubilization buffer (10% SDS in 50% dimethylfluoride) was added to each well, and the plates were incubated for 12–18 h at 37°C before OD was measured at 562 nm. The data were normalized to that obtained using untreated cells within the same experiment, which were considered to be 100% viable. Each treatment was performed in triplicate.

Confirmation of P2X7 surface localization

Approximately 2 × 10^5 transfected HEK 293 cells were plated in 6-well plates or 35-mm dishes 2 days before experimentation. After overnight attachment at 37°C, half of these cultures were shifted to room temperature (24–27°C) for another 18 h. Cells were harvested on the day of experimentation by brief exposure to 0.1% trypsin, and the cultures were divided (24–27°C) for another 18 h. Cells were harvested on the day of experimentation. Cells were treated at room temperature (24–27°C) in NaCl or sodium gluconate solutions (25 mM Na-HEPES, 130 mM sodium gluconate or NaCl, 5 mM KCl, 1.5 mM CaCl2, 0.5 mM MgCl2, 0.5% BSA, 10 mM NaCl) for 18 h before patch-clamp analysis. After transfer of the culture dish to the incubator, the cells were superfused at room temperature (22–25°C) with an extracellular saline solution: 140 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 0.5 mM MgCl2, 10 mM Na-glucose, 10 mM Na-HEPES (pH 7.4). The cell suspension was supplemented with 1 μM fura 2-acetoxymethyl ester (fura 2-AM) (Molecular Probes) and incubated at room temperature for 45 min. The cells were pelleted, washed once, and resuspended in a fresh balanced saline solution to a density of 10^6 cells/ml. Cytosolic Ca^2+ levels were fluorometrically assayed at 24°C using equipment and calibration protocols that have previously been described (31). HEK 293 cells were first challenged with 30 μM ADP and 30 μM UTP to activate and desensitize the endogenously expressed Ca^2+-mo bislizing P2Y and P2Z receptors before stimulation with 3 mM ATP to activate the heterologously expressed P2X7 receptors. The cells were preincubated with 50 μg/ml digitonin to facilitate calibration of fura 2-AM as a function of extracellular Ca^2+ levels.

Detection of nucleotide-induced pore activity

Transfected HEK 293 cells were plated, shifted to the indicated temperatures, and harvested as described previously. After washing in HEPES-buffered saline (HBS: 130 mM NaCl, 5 mM KCl, 20 mM HEPES (pH 7.4), 0.1% BSA, 10 mM glucose, and 0.5 mM CaCl2), the cells were resuspended in 450 μl of fresh HBS. The dye uptake reaction was started by adding 50 μl of 10X stock solutions to provide a final concentration of 2 mM Yo-Pro-1 with and without 300 μM BzATP. After incubating at room temperature for 5 min, the reactions were equilibrated to 10 mM MgCl2, followed by washing and resuspension in 250 μl of HBS containing 5 μg/ml propidium iodide. Flow cytometry and data analysis with the exclusion of dead cells was performed as described previously.

Results

P2X7 mutant design, generation, and screening

To test the hypothesis that the distal and potential LPS/lipid-binding portion of the P2X7 C terminus (residues 571–595) is critical for receptor function, we mutated this region within the cDNA corresponding to the human P2X7 gene. Specifically, 2 aa (Arg578, Lys579) were targeted for point mutation because of previous work demonstrating their importance in the binding of LPS in vitro (22) and because of recent evidence suggesting that basic residues within lipid interaction motifs are necessary for the trafficking of certain receptors and other proteins (24, 25, 32). Stable HEK 293 transfectants expressing a human P2X7-R578E/K579E double point mutant exhibited normal levels of P2X7 mRNA and protein as assessed by RT-PCR and immunoblotting, relative to HEK cells expressing the wild-type P2X7 control (Fig. 1, A and B). To screen for receptor function, we treated these transfected HEK cells with increasing concentrations of a P2X agonist, Bz-ATP, and evaluated the capacity of this treatment to initiate a rapid low-divalent analogs (dissolved in the following low divalent cation-containing solution: 140 mM NaCl, 10 mM HEPES, 10 mM glucose, 5 mM KCl, 0.5 mM CaCl2) were applied through a gravity-fed superfusion system with solution switching controlled by parallel pinch valves (Cell MicroControls, Norfolk, VA). The patch-clamped cell was directly placed in the stream of the superfusing solution.

Measurement of cell K+ content

HEK 293 cells that stably express the wild-type human P2X7, or the human P2X7-R578E/K579E mutant were plated at a density of 10^6 cells/well in 12-well dishes and grown at room temperature for 24 h before the experiment. Cells were treated at room temperature (24–27°C) in NaCl or sodium gluconate solutions (25 mM Na-HEPES, 130 mM sodium gluconate or NaCl, 5 mM KCl, 1.5 mM CaCl2, 0.5 mM MgCl2, 0.5% BSA, 10 mM Na-glucose (pH 7.4)). The cells were stimulated with 3 mM ATP or with 0.3 mM BzATP for 10 min. The potassium content of the cells was assayed by atomic absorbance.

Fluorometric analysis of cytosolic Ca^2+

HEK 293 cells stably expressing either the wild-type human P2X7, or the human P2X7-R578E/K579E mutant receptor were plated at low density (10^5 cells/ml) on 100-mm dishes. Cells were incubated at room temperature (24–27°C) for at least 18 h before fluorometric analysis of agonist-induced changes in cytosolic Ca^2+ concentration. HEK cells expressing either the wild-type or mutant receptor were removed from the culture dishes by trypsinization. The suspended cells were washed, resuspended to 10^6 cells/ml in a balanced saline containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM Na-glucose, 1 mg/ml BSA, and 10 mM Na-HEPES (pH 7.5). The cell suspension was supplemented with 1 μM fura 2-acetoxymethyl ester (fura 2-AM) (Molecular Probes) and incubated at room temperature for 45 min. The cells were pelleted, washed once, and resuspended in a fresh balanced saline solution to a density of 10^6 cells/ml. Cytosolic Ca^2+ levels were fluorometrically assayed at 24°C using equipment and calibration protocols that have previously been described (31). HEK 293 cells were first challenged with 30 μM ADP and 30 μM UTP to activate and desensitize the endogenously expressed Ca^2+-mobilizing P2Y and P2Z receptors before stimulation with 3 mM ATP to activate the heterologously expressed P2X7 receptors. The cells were preincubated with 50 μg/ml digitonin to facilitate calibration of fura 2-AM as a function of extracellular Ca^2+ levels.

Electrophysiological analysis of ATP-gated ion channels expressed in HEK 293 cells

HEK 293 cells stably expressing either the wild-type human P2X7, or the human P2X7-R578E/K579E mutant were plated at low density (10^5 cells/ml) on 35-mm dishes. Cells were incubated at room temperature for at least 18 h before patch-clamp analysis. After transfer of the culture dish to the recording chamber, the cells were detached by incubation in Ca^2+- and Mg^2+-free Hank’s saline. Individual detached cells were sealed to patch electrodes (Garner Glass TW-150) with resistances of 2–10 MΩ. The bathing medium was then switched to a Ca^2+ - and apyrase-containing balanced saline (140 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 0.5 mM MgCl2, 10 mM Na-glucose, 10 mM Na-HEPES (pH 7.5), 0.1 U/ml potato apyrase) to reduce the background level extracellular nucleotides and minimize the contribution to receptor activation and/or desensitization. The whole-cell configuration was then established by suction. Cells were dialyzed internally with an intracellular saline containing 120 mM CsCl, 20 mM tetraethylammonium chloride, 10 mM EGTA, 5 mM MgCl2, and 10 mM tetraethylammonium-HEPES (pH 7.3) for several minutes, during which time they rounded up and detached from the chamber floor. Cells were continuously superfused at room temperature (22–25°C) with an extracellular saline solution containing 140 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 0.5 mM MgCl2, 10 mM Na-glucose, and 10 mM Na-HEPES (pH 7.5). All cells were held at −40 mV and were dialyzed internally for at least 5 min before being challenged with ATP. Rapidly activated inward Na+ currents were recorded by computer using the pClamp series of programs and an Axo- lamp 200A patch clamp with a Digidata 1200 Series interface. ATP and its

CONTROL OF P2X7, TRAFFICKING BY A LIPID-INTERACTION MOTIF
dependent channel and pore activity in P2X7-transfected HEK cells. This Ab has been shown to block agonist-induced cell killing response, using conditions similar to those previously described to facilitate pore formation and cell lysis (13, 29, 30). Under these conditions, Bz-ATP treatment for 6 h caused a reduction in the viability of HEK cells expressing wild-type P2X7 with an EC50 between 30 and 100 μM Bz-ATP (Fig. 1C). By contrast, HEK cells transfected with the control vector or the P2X7-R578E/K579E mutant are resistant to Bz-ATP-induced death in this low-divalent cation killing assay, using agonist concentrations as high as 1 mM (Fig. 1C). Thus, HEK cells require P2X7 expression for Bz-ATP-mediated toxicity, and mutation of the distal C-terminal domain of this receptor does not allow the transfected cells to undergo agonist-induced cell death, despite the observation that the transfected cells have normal levels of total P2X7 protein expression.

Surface localization of P2X7 protein

Because mutation of the P2X7 C-terminal region does not allow for Bz-ATP-induced cell death, it was postulated that either the mutant receptor was not effectively expressed on the cell surface and/or that this mutation abrogates receptor function. To delineate whether the distal C-terminal domain contributes to receptor trafficking and membrane localization, agonist binding, or channel gating, we first analyzed P2X7 cell surface expression by flow cytometry using a neutralizing Ab reactive against a conformation-specific epitope (26). This Ab has been shown to block agonist-dependent channel and pore activity in P2X7-transfected HEK cells as well as IL-1β processing in THP-1 monocytes (24). Relative to the isotype control, HEK cells transfected with wild-type P2X7 exhibit a 4- to 10-fold increase in the geometric mean of the intensity of P2X7 cell surface immunostaining (Fig. 2A). By contrast, the P2X7-R578E/K579E mutant cells exhibit a staining profile identical to the isotype control (Fig. 2B), suggesting a lack of surface expression. Given that another P2X family member, P2X4, has been shown to undergo rapid agonist-dependent endocytosis and recycling in transfected HEK cells and neurons (19), we evaluated the surface expression of P2X7 when the HEK cells were grown at room temperature to slow down endocytic pathways. Relative to growth at 37°C, room temperature cultures of HEK cells expressing wild-type P2X7 showed a trend toward a modest increase in levels of surface expression (7.3 ± 2.0-fold increase in the staining relative to the isotype control at 37°C, and 11.4 ± 4.2-fold increase in staining at room temperature; Fig. 2, A and C). This increase in surface P2X7 immunostaining at room temperature is more substantial with HEK cells transfected with P2X7-R578E/K579E (1.2 ± 0.1-fold at 37°C vs 3.2 ± 0.7-fold at room temperature; Fig. 2, B and D), suggesting that slowing down endocytic pathways can partially restore surface expression of this mutant. Consistent with these data are the observations that permeabilized HEK cells expressing the P2X7-R578E/K579E mutant exhibit P2X7 immunostaining at 37°C that is not detected with intact cells (Fig. 3). Collectively, these data regarding immunoreactivity with a conformational-specific Ab and the temperature-dependent restoration of mutant protein surface localization support the concept that the R578E/K579E mutation does not induce widespread deformation of P2X7 structure, but rather that the mutation affects proper receptor trafficking, membrane localization, and/or endocytosis.

As an additional control for nonspecific effects of our mutagenesis and transfection methods, we evaluated the expression profiles of other P2X7 mutants. One such mutant is P2X7-S342F, which we have identified to facilitate pore formation and cell lysis (13, 29, 30). Under these conditions, Bz-ATP treatment for 6 h caused a reduction in the viability of HEK cells expressing wild-type P2X7 with an EC50 between 30 and 100 μM Bz-ATP (Fig. 1C). By contrast, HEK cells transfected with the control vector or the P2X7-R578E/K579E mutant are resistant to Bz-ATP-induced death in this low-divalent cation killing assay, using agonist concentrations as high as 1 mM (Fig. 1C). Thus, HEK cells require P2X7 expression for Bz-ATP-mediated toxicity, and mutation of the distal C-terminal domain of this receptor does not allow the transfected cells to undergo agonist-induced cell death, despite the observation that the transfected cells have normal levels of total P2X7 protein expression.

**FIGURE 1.** Expression profile and functional screening of HEK 293 cells stably transfected with the wild-type human P2X7, or the human P2X7-R578E/K579E double-point mutant. A, RT-PCR analysis of P2X7 mRNA expression in HEK cells transfected with either the vector control, the wild-type human P2X7, or the double-point mutant. B, Immunoblotting of membrane fractions prepared from transfected HEK cells to evaluate total P2X7 protein expression levels. In all lanes, 25 μg of membrane-associated protein was loaded, and the samples were processed as detailed under Materials and Methods. C, MTT dye reduction by transfected HEK cells treated for 6 h with varying doses of Bz-ATP as a measure of cell viability. Equal numbers of cells were plated. Data are normalized to the respective untreated controls (100% viability) after subtracting the background generated by transfected HEK cells treated with water (0% viability). All results are representative of at least three experiments.

**FIGURE 2.** Cell surface expression of human wild-type and mutant (R578E/K579E) P2X7 in transfected HEK cells cultured at reduced temperatures. HEK cells expressing either wild-type (A and C) or mutant (B and D) P2X7 receptors were grown at 37°C (A and B) or 27°C (C and D) for 18 h before immunostaining and flow cytometry. The black lines represent the use of a primary Ab that recognizes an extracellular P2X7 epitope, whereas the gray lines are the isotype control. Dead cells labeled with propidium iodide were excluded from the analysis after collecting 10,000 events. Similar results were observed in three separate experiments.
created as a result of a partial sequence analysis of the P2X7 gene in a spontaneous murine RAW 264.7 macrophage clone (F6-2) that was selected for resistance to lethal doses of Bz-ATP. Because mutations introducing bulky residues in transmembrane domains have been shown to influence protein folding of other receptor systems (33), we also created the tyrosine and alanine mutants, P2X7-S342Y and P2X7-S342A. In this regard, the bulky point mutants (S342F and S342Y) are not expressed on the surface and have reduced levels of total protein expression (Fig. 4). By contrast, the S342A mutant is robustly expressed and is effectively routed to the cell surface (Fig. 4, A and B, left panel). Preliminary data suggest that the S342A mutant is functional (29.7-fold stimulation of Bz-ATP-induced YO-PRO-1 uptake vs 1.0-fold for the S342F and S342Y mutants, respectively), and that growth at room temperature is not sufficient to substantially restore the pore activities of the S342F and S342Y mutants (2.3- and 0.9-fold, respectively). Taken together, the protein expression profiles in HEK cells of P2X7-S342F and P2X7-S342Y, which are mutations that are predicted to exhibit disturbances in receptor folding, are different from the pattern observed with the P2X7-R578E/K579E mutant. Specifically, receptors with a transmembrane mutation likely to cause misfolding or inappropriate membrane insertion are expressed at very low levels (probably because of elevated degradation), whereas the C-terminal mutation allows for normal levels of total receptor expression. These data together with receptor reactivity of conformationally directed Abs suggest that the temperature-dependent surface immunoreactivity of the P2X7-R578E/K579E mutant is caused by alterations in receptor trafficking events, and not simply a factor of receptor misfolding or defective membrane insertion.

Channel and pore activity of the P2X7-R578E/K579E mutant

To determine whether surface expression is sufficient for P2X7 agonist binding capacity and inducible ion channel activity, we performed patch clamping experiments evaluating inward sodium currents of ATP-treated HEK cells grown at room temperature (Fig. 5). Untransfected HEK cells do not have ATP-inducible sodium currents (Ref. 11 and data not shown); however, P2X7 expression confers ATP-stimulated responses that do not desensitize until agonist removal (Ref. 11 and Fig. 5A). The P2X7-R578E/K579E-transfected cells are also responsive to ATP with respect to inward sodium currents; however, these cells require higher concentrations of ATP to generate smaller sodium currents (Fig. 5B). Additionally, fewer of the P2X7-R578E/K579E-transfected cells are responsive at all (Fig. 5C), despite growth at the permissive temperature, which is consistent with a subpopulation within the culture that has very low levels of surface expression (Fig. 2D). Furthermore, the peak ATP-induced currents are reduced in the mutant cells ~60% relative to those found in the wild-type HEK/P2X7 cells, even when only measuring cells that are responsive to ATP. Thus, mutation of a dibasic amino acid motif of the distal C-terminal domain of P2X7 also attenuates inward sodium channel activity.

Potassium efflux by P2X7 has been linked to ATP-stimulated IL-1β processing in monocytes (34). Therefore, we measured this channel function by atomic absorption spectrophotometry after treatment of the transfected HEK cells with either buffer, 3 mM ATP, or 0.3 mM Bz-ATP (Fig. 6). Treatment of HEK cells expressing wild-type P2X7 with either ATP or Bz-ATP for 10 min results in depletion of ~50–60% of the cellular potassium stores relative to the buffer control, whereas this reduction was only ~10% after treatment of the mutant HEK cells. Because chloride ions have been shown to negatively regulate channel activity (35), we also measured potassium efflux in a sodium gluconate buffer system. The removal of chloride ions reduced the total potassium
content of both the mutant and wild-type P2X7-expressing HEK cells and facilitated a small increase in the agonist-stimulated potassium efflux regulated by the wild-type receptor (70–80%). However, depending upon the agonist, the P2X7-R578E/K579E mutant cells exhibited a variable effect of chloride removal on potassium efflux (0 ± 25% for ATP and Bz-ATP, respectively). In sum, there is reduced potassium conductance of the channels formed by P2X7-R578E/K579E subunits.

Calcium fluxes are also regulated by P2X7 channels and appear to be important for the nucleotide-stimulated expression of inducible nitric oxide synthase in LPS-treated RAW cells (28). Thus, we evaluated the contribution of the dibasic amino acid motif to this P2X7 channel function by monitoring changes in fura 2-AM fluorescence in transfected HEK cells grown at room temperature (Fig. 6). HEK cells expressing either wild-type or R578E/K579E mutant human P2X7 were grown at room temperature for at least 18 h before patch clamping of single cells treated with increasing doses of ATP. Whole-cell inward sodium currents triggered by 3 mM ATP were recorded from 12 cells expressing wild-type P2X7 and 19 cells expressing the P2X7-R578E/K579E mutant. Cells that exhibited ATP-gated inward currents that could be readily distinguished from background current noise and slow fluctuations in the holding were scored as ATP-responsive cells (11/12 P2X7 wild-type vs 10/19 P2X7 mutant cells). Differences in maximal ATP-gated inward current in single HEK 293 cells stably transfected with wild-type or mutant receptors. The peak inward current triggered by 3 mM ATP was normalized to cell surface area (membrane capacitance: 13.7 ± 1.84 pF in HEK/P2X7 cells vs 8.8 ± 1.24 pF in HEK/P2X7-R578E/K579E cells) in 10 ATP-responsive cells in each case.

FIGURE 5. ATP-induced sodium currents in transfected HEK cells grown at room temperature. HEK cells expressing either wild-type (A) or R578E/K579E mutant (B) human P2X7 were grown at room temperature for 18 h before patch clamping of single cells treated with increasing doses of ATP. C, Whole-cell inward sodium currents triggered by 3 mM ATP were recorded from 12 cells expressing wild-type P2X7 and 19 cells expressing the P2X7-R578E/K579E mutant. Cells that exhibited ATP-gated inward currents that could be readily distinguished from background current noise and slow fluctuations in the holding were scored as ATP-responsive cells (11/12 P2X7 wild-type vs 10/19 P2X7 mutant cells). D, Differences in maximal ATP-gated inward current in single HEK 293 cells stably transfected with wild-type or mutant receptors. The peak inward current triggered by 3 mM ATP was normalized to cell surface area (membrane capacitance: 13.7 ± 1.84 pF in HEK/P2X7 cells vs 8.8 ± 1.24 pF in HEK/P2X7-R578E/K579E cells) in 10 ATP-responsive cells in each case.

FIGURE 6. P2X7 agonist-stimulated potassium efflux in transfected HEK cells grown at room temperature. HEK cells expressing either wild-type or R578E/K579E mutant human P2X7 were grown at room temperature for at least 18 h before trypsinization and resuspension in NaCl saline or sodium gluconate solutions at 106 cells/ml. Cells were stimulated for 10 min with the indicated concentrations of ATP or BzATP, and cell-associated potassium content was assayed by atomic absorbance spectrophotometry.

FIGURE 7. Partial rescue of ATP-induced calcium current in HEK cells transfected with P2X7-R578E/K579E by removal of NaCl. HEK 293 cells that stably express the P2X7 wild-type (A and C) or the P2X7-R578E/K579E mutant (B and D) receptors were cultured at room temperature for at least 18 h before treatment. Cells were trypsinized and resuspended at 106 cells/ml in NaCl (A and B) or K-gluconate (C and D) saline solutions at room temperature before treatment. Cells were treated with 0.1 mM ADP, 0.1 mM UTP, 3 mM ATP, and 50 μg/ml digitonin, respectively, and calcium levels were monitored by fura 2-AM fluorescence.

FIGURE 8. P2X7-regulated pore activity of transfected HEK cells grown at room temperature. HEK 293 cells that stably express human wild-type or R578E/K579E mutant receptors were cultured at room temperature for at least 18 h before treatment with 0 or 250 μM Bz-ATP for 15 min at room temperature. Pore activity was assessed by the uptake of YO-PRO-1, using flow cytometry as described under Materials and Methods. Similar results were observed in three separate experiments.
wild-type P2X7-expressing cells appears to be maximal in that subsequent treatment with digitonin does not cause further increases in fura 2-AM fluorescence. Removal of NaCl in the buffer attenuates the fura 2-AM fluorescence induced by ADP treatment of either mutant or wild-type P2X7-expressing cells, but not that stimulated by the addition of UTP. Interestingly, the potassium gluconate buffer system also causes a partial restoration of ATP-induced calcium flux in the P2X,-R578E/K579E mutant. These data suggest that the dibasic amino acid motif of the distal P2X7 C-terminal domain also regulates calcium channel activity.

In addition to its capacity to mediate ion fluxes, the P2X7 pore activity has been linked to IL-1β processing, membrane fusion events, and apoptosis (2). Therefore, we evaluated the pore-forming capacity of the P2X,-R578E/K579E mutant cells to determine whether the channel and pore activities can be dissociated. Transfected HEK cells expressing wild-type P2X7 have a 6.4 ± 0.5-fold increase in YO-PRO-1 uptake in response to Bz-ATP (Fig. 8A). Similar to the trend in protein expression, cell growth at ambient temperatures slightly increases the total amount of YO-PRO-1 uptake after 5 min (14.5 ± 3.2-fold; Fig. 8C), a time sufficiently long to overcome the temperature-induced delay in initial formation of the pore (13). By contrast, the P2X,-R578E/K579E mutant is unable to form pores at ambient temperatures, despite surface expression and partial channel activity (Fig. 8, B and D). Collectively, point mutations within the lipid-interaction motif of the distal C terminus of P2X7 affect surface immunoreactivity as well as the formation of agonist-regulated channels and pores. These data suggest that this domain is critical to receptor function and trafficking events.

Discussion

The data presented in this study are supportive of a model wherein residues within the lipid interaction domain of P2X7, specifically Arg578 and Lys579, are required for normal trafficking of the receptor. Point mutation of these two residues in P2X7 prevents receptor expression on the cell surface as assessed by flow cytometry. This defect is partially restored at lower temperatures (24–27°C), which is a condition known to slow down receptor recycling events and has been used previously to demonstrate different P2X7 channel permeability states (37). Because the P2X7-R578E/K579E mutant is recognized by conformation-sensitive Abs, and because its cell surface expression is detectable at 24–27°C, it is likely that this mutation does not promote large-scale receptor deformation, misfolding, or improper membrane insertion. In addition, the total protein expression level of the double-point mutant is not diminished, which contrasts with the reductions in protein expression that occur after disruption of conserved cysteine residues in the human P2X7 receptor that contribute to conformation stabilization of the extracellular domain (38). Additionally, two P2X7 mutants with bulky amino acid substitutions within the second transmembrane domain (S342F and S342Y, but not S342A) have reduced protein expression (Fig. 4). Similar mutations in other systems have been shown to affect protein folding (33). Thus, the dibasic amino acid motif within the distal C-terminal domain of P2X7 appears to regulate receptor trafficking rather than specify long-range conformational effects.

Previous studies have shown that all leukocytes contain a large intracellular pool of P2X7, which is recruited by monocytes to the surface during macrophage differentiation (16, 20). It is unclear whether recombinant P2X7 trafficking in HEK cells uses similar pathways, compartments, and cofactors; however, the trafficking of P2X7, P2X2, and P2X4 has been studied in these cells (19, 39) and has been shown to exhibit similarities to the patterns observed in neurons and smooth muscle. Interestingly, a YXXGL motif in the distal C terminus of P2X7 has been shown to direct agonist-dependent endocytosis, and mutation of this domain results in constitutive surface localization (21). That this motif is not present in P2X5, combined with the observations that leukocytes have a large intracellular pool of the receptor, suggests that P2X5, has compartmental retention and/or surface stabilization domains. A P2X7 retention domain might exist between residues 418 and 578, given that a 177-aa C-terminal truncation of the rat receptor allows for receptor expression on the surface, whereas the P2X7-R578E/K579E mutant is not stabilized on the cell surface unless the growing temperature is reduced. This observation suggests that Arg578 and/or Lys579 of P2X7 are part of a surface stabilization domain, such that mutation of these residues traps or routes the receptor to intracellular compartments.

We also observed that P2X7 channel activity is attenuated and that pore activity is completely abolished following mutation for Arg578/Lys579, despite the partial restoration of cell surface expression of P2X7-R578E/K579E at ambient growing temperatures. One interpretation is that this may simply be caused by a threshold effect of receptor number, such that there is a requirement for higher receptor expression to achieve efficient channel and pore activity. In this regard, we have observed a correlation between receptor expression level and the relative capacity to form pores in sorted populations of HEK cells expressing P2X7-S342A receptors (data not shown). This notion of a threshold receptor number has also been observed when comparing monocytes and macrophages (16). However, select P2X7 truncation mutants (P2X7-1–540, P2X7-1–550) have recently been shown to have reasonably normal levels of channel activity, despite low levels of surface expression that are undetectable by flow cytometry (40), thus making the threshold model less likely. Other possibilities include the scenario that the distal C terminus may be involved in modulating ligand binding and/or channel gating interacting with accessory proteins and lipids, perhaps by folding back toward the membrane. The present data would also be consistent with a role of the distal C terminus in P2X7, multimerization, although this function has been suggested to be largely controlled by the second transmembrane domain in the rat P2X7 receptor system (12). In sum, HEK cells expressing P2X7-R578E/K579E may be a useful tool to elucidate signaling events or other receptor activities, such as association with effector molecules or the cytoskeleton, that are independent of channel and pore formation.

The composition of the lipid microdomain within the membrane may also be a factor regulating P2X7 trafficking events and/or activity (41). We previously reported that P2X7 residues 574–589 represent a lipid interaction motif, and that mutation of Arg578 and Lys579 abolished the ability of synthetic peptides to bind LPS in vitro (22). Because highly basic C-terminal amino acid motifs have been shown in other ion channel systems to regulate receptor trafficking (24) and promote lipid-dependent endocytosis (25), one model is that this P2X7 domain interacts with endogenous lipids that influence receptor localization. Another possibility is that during infection LPS released within the cell from phagosomes, and this may promote local P2X7 recruitment, which is an event that has been shown to facilitate phagolysosomal maturation needed for microbial killing (8).

In conclusion, our data support a model wherein the lipid interaction motif of P2X7 is involved in receptor trafficking. Because mutations within this motif allow for cell surface expression at lower temperatures, which is a condition that slows down receptor endocytosis, it appears likely that the C-terminal domain functions to control surface stabilization, consistent with recent observations by Smart et al. (40). Together, these data suggest that the binding of endogenous lipids or LPS to this region of P2X7 may serve to...
modulate receptor availability to the extracellular milieu and thereby regulate its contribution to the inflammatory response.

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