P2X7 Receptor Cell Surface Expression and Cytolytic Pore Formation Are Regulated by a Distal C-terminal Region*

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The importance of the cytosolic C-terminal region of the P2X7 receptor (P2X7R) is unquestioned, yet little is known about the functional domains of this region and how they may contribute to the numerous properties ascribed to this receptor. A structure-function analysis of truncated and single-residue-mutated P2X7 receptors was performed in HEK-293 cells and Xenopus oocytes. Cells expressing receptors truncated at residue 581 (of 595) have negligible ethidium ion uptake, whereas those expressing the P2X7R truncated at position 582 give wild type ethidium ion uptake suggesting that pore formation requires over 95% of the C-terminal tail. Channel function was evident even in receptors that were truncated at position 380 indicating that only a small portion of the cytosolic region is required for channel activity. Surprisingly, truncations in the region between residues 551 and 581 resulted in non-functional receptors with no detectable cell surface expression in HEK-293 cells. A more detailed analysis revealed that mutations of single residues within this region could also abolish receptor function and cell surface expression, suggesting that this region may participate in regulating the surface expression of the pore-forming P2X7R.

The P2X7R1 is a ligand-gated ion channel and the seventh member of the P2X family (1). Exposure to ATP or the more potent agonist BzATP to the P2X7R renders the P2X7R permeant to Na⁺, K⁺, and Ca²⁺ (2). Repeated or prolonged application of either agonist induces the formation of a cytolytic pore that is permeable to larger cations such as positively charged ethidium or N-methyl-D-glucamine (3, 4). The ability of the P2X7R to form pores is dependent on experimental conditions. Factors such as the duration of the agonist application, the presence of divalent cations, extracellular pH, receptor density, and receptor species influence pore formation (1, 4–6).

The conversion from channel to pore state may reflect either a conformational change within the receptor’s selectivity filter (7) or the successive oligomerization of additional P2X7R subunits (8). We recently examined whether the P2X7R formed clusters (9) upon agonist activation, as a prelude to density-dependent pore formation. We found that, unlike the P2X2R, which clusters upon agonist activation (1–2 μm in size) (10), the P2X7R does not exist in clusters in the basal state or upon receptor activation (9). Although receptor density regulates pore formation in cells that endogenously express the P2X7R (6), a localized increase in receptor density or clusters is not associated with P2X7 pore formation.

The P2X7R shows structural homology with other P2X family members, consisting of two transmembrane domains connected by a large extracellular loop. The extracellular loop contains conserved cysteine, lysine, and glycine residues along with a number of potential N-linked glycosylation sites, all of which contribute to the structural constraints required for ATP binding (11–13). The C terminus of the P2X7R is 120 amino acids longer than any of the other P2X members. This long C-terminal domain (~240 amino acids) appears to modulate the function of the P2X7R, because the removal of this region tempers the receptor’s response to ATP. In particular, the removal of the last 177 amino acids (to yield P2X7-(1–418)) abolishes pore formation without affecting channel properties (1, 14). Investigation of a 1–439 truncated human P2X7 receptor in Xenopus oocytes revealed an altered kinetic response to ATP applications, thought to be related to a difference in the agonist binding site of the truncated receptor compared with the full-length P2X7R (14). Furthermore, when the human P2X7R is truncated at residue 415, it does not exhibit cell surface expression (15).

Sequence analysis of the cytosolic C-terminal of the P2X7R has supported the idea that the C-terminal region is important for receptor function. Denlinger and colleagues (16) identified a putative lipopolysaccharide binding domain and went on to corroborate this sequence analysis with biochemical evidence that this putative domain actually binds lipopolysaccharide. In a large scale proteomic analysis, Kim and co-workers (17) identified numerous putative protein partners, many of which are thought to interact with the C-terminal region. In particular, epithelial membrane proteins were found to interact with the C-terminal tail of the P2X7R and are involved in cell blebbing (18). Clinical investigations have shed further light on the role of the C-terminal region and have demonstrated that an E496A polymorphism seen in the human P2X7R is associated with a loss of function and the appearance of CLL in patients harboring this amino acid change (19).

Further evidence for a role of the C-terminal in regulating pore formation comes from studies of rat, human, and mouse
P2X7 Receptor Cell Surface Expression

The wild type clone of rat P2X7R (accession number X95882, a kind gift from Dr. Gary Buell) was used in these studies. Truncated P2X7 receptors were created using standard PCR protocols. A HindII and EcoRI restriction site was incorporated at the 5′ and 3′ ends of each PCR product. All PCR products were subcloned into the pcDNA3.1 (+) vector (Invitrogen, Mount Waverley, Victoria, Australia) and amplified using competent DH5α cells. The 1-Pro582Gly construct was created as above, but a point mutation was introduced at the appropriate position within the forward primer (reverse primer: 5′-GGC GAA TCT TCA GCC GTC CTT CCG GAT CTT CC). A total of seven separate sites were targeted for glycine substitution. The following residues were chosen for our studies: Cys54Gly, Arg575Gly, Lys576Glu, Glu580Gly, and Pro586Gly with each residue (in the full-length P2X7R) mutated to glycine. Site-directed mutagenesis was performed using Stratagene’s QuickChange site-directed mutagenesis kit (Integrated Sciences, Willoughby, New South Wales, Australia) as per the manufacturer’s instructions. The forward and reverse primers for each mutant were designed as recommended. The forward and reverse primers for each mutant are listed as follows: C54Gly forward: 5′-CAC AAG ATC CGG AAG GCC TAC AGG GCC TGC CGG TAA GAT CCG; C54Gly reverse: 5′-GGC ATC GCC CCT CTT CTA GGA ACC TCG CTT G; C572G forward: 5′-GCC ATT CCG GCC ACC GGC TCT CCG CTT G; C572G reverse: 5′-GGC GAA TCT TCA GCC GTC CTT CCG GAT CTT CC; R574G forward: 5′-CTG CCC AGC TGC TCG GCC TGG AAG ATC CCG GAG; R574G reverse: 5′-CTT CCG GAT CCT TCA GCA GCC GCA GCC GCT GGG CAG AAT GGC; R574G forward: 5′-CC AGC GCC GAA AGC CAG GCC GTC TGG AAT CAG; S581G forward: 5′-GGA AAG GAA AAG AGG CCC GGG CAG GCC ACC GAG; S581G reverse: 5′-CTG CCC GTG CTT CCG CTT CCT CCG GAT CTT CC; K576G forward: 5′-GG GAA TCT CTT CCG GAT CCC CCA GCA GCC GCA GCC GCA GCC GAT GG; E580G forward: 5′-GGG AAG ATC CGG AAG GCC TTC CCG ACC AAC CAG; E580G reverse: 5′-CTG GTT GCT GTG GAA GCC CTT CCT CCT CCT G; S581G forward: 5′-GGG AAG ATC CGG AAG GCC TTC CCG ACC AAC CAG; S581G reverse: 5′-CTG CCC GTG CTT CCG CTT CCT CCG GAT CTT CC. Base changes introducing the mutations are in boldface type and underlined.

Three mutants, Cys572Gly, Glu580Gly, and Phe586Gly, and the wild type rat P2X7R were subcloned into the pEGFP-N1 vector (Clontech, Palo Alto, CA) to create four EGFP fusion proteins. A single set of forward and reverse primers was used to subclone all mutants and the wild type rat P2X7R into the pEGFP-N1 vector, with an XhoI and a KpnI site introduced at the 5′ and 3′ ends, and the stop codon was deleted. All constructs were confirmed by sequencing (Australian Genome Research Facility, St. Lucia, Queensland, Australia). For electrophysiological studies, the truncated receptor constructs were linearized with XhoI in preparation for transcription run-off. In vitro transcription was performed using Ambion’s T7 mMessage mMachine mRNA kit (GeneWorks, Thebarton, South Australia, Australia) as per the manufacturer’s instructions. The full-length, capped cRNAs were purified using diethyl-pyrocarbonate H2O-equilibrated Clontech Chroma Spin-100 columns (Progen Industries, Brisbane, Australia). cRNA samples were electrophoresed on a denaturing agarose gel to determine the quantity, quality, and size of the transcript.

Cell Culture

HEK-293 and COS-7 cells were grown at 37 °C, 5% CO2 in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 units/ml). For ethidium ion uptake experiments HEK-293 cells were dislodged using Trypsin-EDTA and seeded into 96-well plates, then transfected 24 h later (or when confluency had reached 75%). 30 μM of Effectene buffer, 0.8 μl of enhancer, 2.5 μl of Effectene reagent (Qiagen, Clifton Hill, Victoria, Australia) were assembled with 0.8 μg of sample DNA. This mixture was used to transfect eight wells. Following a 12-h period the transfection reagent was replaced with fresh medium. For imaging studies, the HEK-293 and COS-7 cells were seeded onto poly-t-lysine-treated coverslips. Cells were transfected with the wild type rat P2X7-EGFP, Cys572Gly-EGFP, Glu580Gly-EGFP, or Phe586Gly-EGFP using the Effectene transfection reagents as described previously (9). HEK-293 cells were also transfected with the truncated P2X7 receptors and C-terminal mutants in preparation for calcium influx studies.

Ethidium Ion Uptake

Pore formation was examined in HEK-293 cells transiently transfected with the wild type rat P2X7R, truncated P2X7 receptors, and C-terminal mutants. A plate reader (Wallac 1420, PerkinElmer Life Sciences) was used to determine ethidium ion uptake in these cells. Three days post-transfection the cells were washed once with low divalent HEPES buffer (in millimolar; NaCl 147, KCl 10, CaCl2 0.1, pH 7.4). Basal ethidium ion fluorescence was assessed once the wash buffer was replaced with low divalent HEPES supplemented with ethidium bromide (10 μg/ml). Fluorescence signals from ethidium ion were excited with the 485 ± 15-nm band of a 75-watt tungsten-halogen lamp, and emission greater than 615 ± 8.5 nm was collected. Once background ethidium ion fluorescence was measured, this “ethidium only” solution was replaced with low divalent HEPES supplemented with BzATP (100 μM) and ethidium bromide (10 μg/ml). Ethidium ion fluorescence was measured at four separate locations within each well, and an entire plate was read ten times within a 30-min period. All ethidium ion uptake experiments were carried out at 37 °C.

Confocal Microscopy

Calcium Uptake—HEK-293 cells were loaded with fluo-4 AM for 20 min at 37 °C then washed twice before experiments. Cells were illuminated with the 488-nm line of a 100-milliwatt argon ion laser and viewed on an MRC-1024 (Bio-Rad, Regents Park, New South Wales, Australia) confocal microscope. Data was collected using the single-green fluorescence emission filter set. Fluorescence signals from fluo-4 were sampled every 3 s and collected using a software module (Laser Sharp Timecourse, version 3.1) for a period of 5 min. Recordings were made in high divalent HEPES buffer (in millimolar; NaCl 147, KCl 2, HEPES 10, glucose 10, CaCl2 0.1, pH 7.4). Basal calcium fluorescence was assessed once the wash buffer was replaced with high divalent HEPES supplemented with ethidium bromide (10 μg/ml). Ethidium ion fluorescence was measured at four separate locations within each well, and an entire plate was read ten times within a 30-min period. All ethidium ion uptake experiments were carried out at 37 °C.

Electrophysiology

Oocytes from adult female Xenopus laevis were surgically removed and prepared as outlined previously (21). Stage 5 or 6 oocytes were injected with cRNA-encoding wild type rat P2X7R, truncated P2X7 receptors, or the C-terminal mutants and were stored at 18 °C for 3 days prior to experimentation. For two electrode voltage clamp recordings, oocytes were impaled with two glass electrodes containing 3 M KCl and a membrane potential of ~70 mV was recorded. A 100-mV full scale recording amplifier (Axon Instruments, Union City, CA). Oocytes were perfused (at 2 mI/min) with a low divalent ND96 solution (in millimolar; NaCl 96, KCl 2, BaCl2 0.1, HEPES 5, pH 7.5) administered with a gravity-fed manifold perfusion system. 1 mM ATP was added for a period of 20 s and...
mutagenesis. A total of seven site-directed glycine mutants were made of the full-length P2X7R, truncated P2X7 receptors, or the C-terminal tail of the wild type rat P2X7R, truncated P2X7 receptors, or the C-terminal tail of the wild type rat P2X7R. Truncated receptors were then assayed for their ability to form ethidium ion permeant pores—i.e., to induce ethidium ion fluorescence that developed over a period of 30 min. The relative fluorescence intensity at this time point was chosen for all subsequent analyses of ethidium ion uptake. Plate reader analysis of ethidium ion uptake of truncated receptors revealed a strong relationship between the truncation position and ethidium ion uptake (Fig. 2B). Under no circumstances could we detect ethidium ion fluorescence for any receptor that was truncated upstream of residue 582. By contrast, all constructs that were 582 residues or longer in length displayed ethidium ion fluorescence levels identical to that of the full-length receptor. Thus, removal of one residue, proline 582, resulted in a switch from wild type ethidium ion uptake to complete absence of uptake. To examine this further we substituted this proline with glycine, and ethidium ion uptake was re-assessed (Fig. 2B). Interestingly, this 1-Pro582Gly truncation mutant displayed a level of ethidium ion uptake that fell almost halfway between the levels measured with the 1–582 truncation mutant and the full-length receptor.

The channel properties of truncated receptors were then investigated in transfected HEK-293 cells loaded with the Ca\(^{2+}\)-sensitive dye, fluo-4 AM, under ionic conditions that would not favor pore formation (2). Calcium influx was determined by the change in fluo-4 fluorescence upon the addition of 50 \(\mu\)M BzATP (Fig. 2C). The application of BzATP to HEK-293 cells transfected with the pcDNA3.1(+) vector caused an increase in the intracellular calcium concentration, presumably by the release of calcium from intracellular stores via activation of endogenously expressed P2Y receptors (Fig. 2C). This increase in calcium by P2Y receptors upon BzATP application has been previously reported for native HEK-293 cells (23). The addition of 50 \(\mu\)M BzATP to HEK-293 cells transfected with the full-length rat P2X7R caused a sustained increase in fluo-4 fluorescence, which was interpreted as an influx of calcium through the P2X7 channel (Fig. 2C). Reduction of this type of data by mathematical integration of the Ca\(^{2+}\) levels over time was a good discriminator of the difference between a sustained influx and the transient, P2Y-elicted store release of Ca\(^{2+}\). Furthermore, it correlated well with some whole cell current recordings made by patch clamp (data not shown) validating this approach as a rapid method for analysis of P2X7R channel properties. Analysis of truncated receptors showed that a sustained increase in Ca\(^{2+}\) was observed in transfected HEK-293 cells with the constructs, 1–380, 1–400, 1–418, 1–460, 1–500, 1–540, 1–550, 1–582, and 1-Pro582Gly (Fig. 2D), whereas the following constructs failed to produce a sustained increase in Ca\(^{2+}\) influx: 1–360, 1–560, 1–570, 1–580, and 1–581 (Fig. 2D). In contrast to pore function, channel function was clearly evident in many of the shorter truncation constructs.

We next investigated whether the loss of P2X7R channel function seen in certain truncated receptors may be associated with a decrease in plasmalemma expression. Cell surface expression was determined using flow cytometry with the L4 human P2X7 antibody (19). This FITC-conjugated antibody recognizes an extracellular epitope on the human P2X7R. HEK-293 cells were transfected with each of the truncated P2X7 receptors. Two days post transfection, cells were suspended in PBS and P2X7R surface expression was quantified using a FACScalibur flow cytometer. Clear differences in cell surface expression were observed, with expression levels varying from undetectable to twice that of wild type receptor. For the most part cell surface expression correlated well with channel function seen in Fig. 2D with two notable exceptions, the 1–540 and 1–550 truncations, which displayed significant channel function but had negligible surface expression. Clearly, function requires cell surface expression, and this may represent a limitation of the FACS technique in studying mutant receptors where differences in epitope presentation and
the consequent reduced antibody binding may be incorrectly interpreted as a lack of surface expression. The other noteworthy result is that the 1–582 truncation displayed almost twice the level of cell surface expression compared with that of control. Interestingly, activation of this truncation gave a robust Ca²⁺ response (Fig. 2D). In all cases, truncated receptors that displayed a loss of both channel and pore function could not be detected at the cell surface (Fig. 2D). As a further check for cell surface expression N- and C-terminal GFP-tagged 1–418, 1–570, and 1–580 truncation constructs were made and examined using confocal microscopy (data not shown). Although neither of the N- and C-terminal-tagged GFP truncations displayed surface expression, a fluorescence signal was detected in the cytoplasm, indicating that these receptors were being translated and expressed.

The channel properties of the truncated receptors were also examined in Xenopus oocytes. In most cases, the application of 1 mM ATP caused a rapid current (I₁) followed by a delayed current (I₂; Fig. 3A) and again correlated quite well with the Ca²⁺ influx data shown in Fig. 2D. As would be expected from the data, in HEK-293 cells negligible current responses were observed with the 1–360, 1–570, and 1–580 truncations (Fig. 3B). Two interesting exceptions are truncations 1–560 and 1–581, which showed no significant channel activity in HEK-293 cells (Fig. 2D) but gave robust current responses in Xenopus oocytes (Fig. 3B). The I₁ and I₂ components of current development were affected differently by the same truncation, suggesting the mechanisms responsible for these two distinct functional phases may also be structurally distinct.

Fig. 3B summarizes the findings above and presents in a clear fashion several key observations: 1) Truncation between residues 551 and 581 results in a complete abolition of
ethidium ion uptake, channel function, and absence of cell surface expression. 2) Truncation upstream results in restoration of cell surface expression and channel function to varying degrees but significantly above control. 3) The ability of the receptor to accumulate ethidium ions is not similarly restored. 4) Truncation at residue 380 results in a receptor that can still function as an ion channel. Thus, the region between 551 and 581 appears to regulate cell surface expression in conjunction with pore formation, defined by the ability to rapidly accumulate ethidium ions upon application of BzATP.

Functional Analysis of Point Mutations of the Distal Region of C-terminal of the Full-length P2X7R

To further elucidate the role of the 551–581 region, single residues within this domain were targeted for glycine mutagenesis. We also targeted a cysteine residue at position 548, close to the extremity of the region to examine its role in receptor function. A phenylalanine at position 581 was replaced with glycine in an attempt to elucidate why a normal channel response was observed in oocytes injected with the 1–581 truncation, yet no functional response was seen with this mutant when expressed in HEK-293 cells. The proline residue at position 582 was changed to glycine to investigate whether the reduced ethidium ion uptake observed with the 1-Pro582Gly truncation indicates that this position plays a role in the function of the receptor. Thus, two cysteines (Cys548 and Cys572), two positively charged residues (Arg574 and Lys576), and three residues adjacent to the site of truncation where pore formation was abruptly abolished (Glu580, Phe581, and Pro582) were chosen for more detailed structure function analysis (Fig. 1).

The ability of the C-terminal point mutants to accumulate ethidium ions in response to BzATP was tested in transfected HEK-293 cells using the plate reader assay described above. Three mutants, Cys572Gly, Arg574Gly, and Phe581Gly, did not accumulate ethidium ions in response to BzATP. In contrast, Cys548Gly, Lys576Gly, Glu580Gly, and Pro582Gly, all displayed a BzATP-elicited ethidium ion uptake.
uptake identical to that of wild type P2X7R (Fig. 4A).

Channel function was then determined using the fluo-4 assay as described above. Interestingly, the Cys<sup>572</sup> → Gly, Arg<sup>574</sup> → Gly, and Phe<sup>581</sup> → Gly mutants that failed to accumulate ethidium ions in response to 100 μM BzATP also failed to show BzATP-activated calcium influx (Fig. 4B). As we expected, mutants that displayed robust ethidium accumulation displayed wild type levels of calcium influx (Fig. 4B).

Cell surface expression of the non-functional C-terminal mutants was examined to determine whether loss of function was related to assembly or trafficking. Cell surface expression of the C-terminal mutants in HEK-293 cells was determined using flow cytometry with the L4 human P2X7 antibody (Fig. 4B). Analysis revealed that mutant receptors that displayed wild type pore formation and channel activity had similar levels of cell surface expression to the wild type rat P2X7R (Fig. 4B). Mutant receptors that displayed a loss of both channel and pore function did not have any cell surface expression (Fig. 4B).

To further examine the intracellular fate of these non-functional rat P2X7R mutants, EGFP was tagged to the C-terminal of Cys<sup>572</sup> → Gly, Glu<sup>580</sup> → Gly, and Phe<sup>581</sup> → Gly. Fusion proteins were expressed in both HEK-293 and COS-7 cells. Confocal microscopy revealed that the GFP fluorescence of both Cys<sup>572</sup> → Gly-EGFP and Phe<sup>581</sup> → Gly-EGFP constructs was localized to the endoplasmic reticulum and cytosol (Fig. 4C). However, the wild type rat P2X7R-EGFP and Glu<sup>580</sup> → Gly-EGFP proteins had a distinct plasmalemma distribution with identical expression patterns in both HEK-293 and COS-7 cells (Fig. 4C).

The channel properties of the C-terminal point mutants were examined in *Xenopus* oocytes. The foremost observation with the oocytes was that they did not reliably recapitulate the data obtained from HEK-293 cells. Of the three point mutants that failed to express at the surface and consequently failed to function as channel, only one of these, Cys<sup>572</sup> → Gly, had a marked functional deficit when expressed in *Xenopus* oocytes (Fig. 5, A and B). In this case the application of 1 mM ATP to oocytes injected with the Cys<sup>572</sup> → Gly mutant yielded a significantly reduced I<sub>1</sub> current and an absence of I<sub>2</sub> current (Fig. 5B). There was a less marked functional deficit seen in the, Phe<sup>581</sup> → Gly mutant, that displayed an attenuated I<sub>1</sub> current with a wild type I<sub>2</sub> current (Fig. 5B). The ability of expression in *Xenopus* oocytes to rescue function is likely to represent differences in protein trafficking, post-translational modifications, or presence of interacting proteins between mammalian and amphibian expression systems.

**DISCUSSION**

**Cell Surface Expression and Pore Formation Can Be Regulated by a Distal Region of the Cytosolic C-Terminal—Progressive deletion of the P2X7R reveals three functional classes of truncation mutant. The first class is truncated receptors retaining 10–80% of the C-terminal tail that display normal channel function but have complete loss of pore function. The second class is truncated receptors retaining 95% or more of the C-terminal tail that display normal channel and pore activity. The third class comprises truncated receptors retaining less than 10% (between 80 and 95%) of the C-terminal domain or those that have been truncated in the region between residues 551 and 581. Such truncations are completely non-functional and do not display surface expression.** These data are consistent with the idea that the 551–581 region regulates cell surface expression of the full-length pore-forming P2X7R. Furthermore, because we could not separate the cell surface expression region from the region required for pore formation, this indicates that the structural elements required for pore formation are closely associated with an additional trafficking domain.

Analysis of point mutations within this distal region of the cytosolic C-terminal provides additional evidence of its involvement in cell surface expression. Functional and receptor localization data of Cys<sup>572</sup> → Gly, Arg<sup>574</sup> → Gly, and Phe<sup>581</sup> → Gly P2X7R mutants that showed complete loss of function and a complete lack of cell surface expression indicate that these residues have key roles in determining receptor localization of pore-forming receptors. Although the C-terminal “surface-expression/pore-enabling” domain of the P2X7R is essential for pore formation, its presence is not necessary for channel function.

**Is the Putative Surface Expression/Pore-enabling Domain Identical to the Putative LPS Binding Domain?**—The functional role of the C-terminal pore-enabling domain may be to promote pore formation by ensuring an altered biogenesis pathway (as opposed to one for channel forming P2X7 truncations) or to anchor the C-terminal tail to the membrane by interacting with membrane phospholipids. The 551-RSYAT-
The number of oocytes used for each average is shown in parentheses within Cys251-Cys270 and Cys117-Cys165 (13).

taine residues (in the extracellular loop) are disrupted, specifically within Cys573-Cys612 and Cys117-Cys165 (13).

A Working Model of the Distal C-terminal Region—We have generated a simple working model (Fig. 6) that encapsulates much of the data presented in this study and permits further analysis of the role of the distal C-terminal region in biogenesis or in the regulation of the interaction between the P2X7R and protein partners. This model describes a pore-enabling region situated between the residues 551–582 that also contains two protein kinase A (Arg557-FVSQD-Met563) and protein kinase C (Ser552-IRKKEFPK) phosphorylation sites. This region may also promote heteromeric interactions between the P2X2R and other membrane proteins (17). Recently, the interaction of the P2X7R (through its C-terminal tail) with epithelial membrane proteins was found to mediate P2X7-induced cell blebbing (18).

Perhaps the presence of the pore-enabling domain within the C terminus tail augments the P2X7 receptor’s pore-forming ability as well as the cytolytic consequences, through the interaction with protein partners.

The Surface Expression/Pore-enabling Domain of the P2X7 Receptor Is Not Shared with Other P2X Receptors—Pore formation has been found to be a property shared by a number of P2X family members with both the P2X2R and P2X4R reported to form pores (24, 25). The fact that these receptors are able to form pores but do not contain the P2X7 pore-enabling domain (residues 551–581) indicates that a separate P2X pore-forming domain may exist. Khakh and Lester (7) proposed that the change between channel and pore states was achieved through a conformational change within the selectivity filter and have recently reported that the permeation of the Li+ state of the P2X2R is modulated by specific residues (432 and 444) within the C-terminal tail (26). The truncated P2X2R-(1–403) was reported to be permeable to large cations (positively charged N-methyl-D-glucamine) (25), which is in contrast to the minimum length requirement of 582 residues for the P2X7R to form pores seen in the present study.

Little is known about additional regions of the P2X7R that modulate surface expression. The region preceding the second transmembrane domain appears to determine surface expression of the P2X2R, because it regulates subunit interactions and receptor assembly (27). A splice variant of the P2X2R (residues 175–201 are absent) does not display cell surface expression, yet will co-assemble with wild type P2X1 subunits (28). Also, the trafficking of the human P2X2R to the cell membrane is reduced if the disulfide bridges between the cysteine residues (in the extracellular loop) are disrupted, specifically within Cys251-Cys270 and Cys117-Cys165 (13).

B

FIG. 5. Comparison of ATP-induced current recordings of wild type rat P2X7R and C-terminal point mutants expressed in Xenopus oocytes. A, application of 1 mM ATP for 20 s (indicated with a downward arrow above each trace) to oocytes expressing the C-terminal point mutations induced an inward current that recovered 8–10 min after application. All experiments were performed in a low divalent ND96 solution. B, current was measured isochronically (I1 (A), 20 s post 1 mM ATP addition; I2 (B), 4 min post 1 mM ATP addition) and averaged across experiments. The number of oocytes used for each average is shown in parentheses. #, p < 0.05 (one-way ANOVA) significantly different to the I1 of the wild type rat P2X7R.

WRFVSQDMADFAILPSCCRWKIRKEFPK-582 region partially overlaps a previously identified LPS binding domain found in the human P2X7R and seen in the rat, 573-CRWRIRKEFPK-SEGQYS-589 (residues that overlap are underlined). Denlinger and colleagues (16) found that a peptide made with the above residues of the human P2X7R could neutralize LPS-induced activation of intracellular kinases. This region may also promote heteromeric interactions between the P2X2R and other membrane proteins (17). Recently, the interaction of the P2X7R (through its C-terminal tail) with epithelial membrane proteins was found to mediate P2X7-induced cell blebbing (18).

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A Working Model of the Distal C-terminal Region—We have generated a simple working model (Fig. 6) that encapsulates much of the data presented in this study and permits further analysis of the role of the distal C-terminal region in biogenesis or in the regulation of the interaction between the P2X7R and protein partners. This model describes a pore-enabling region situated between the residues 551–582 that also contains two sections. The first section (retention region) inhibits receptor trafficking, whereas the second section overrides the first, enabling surface expression of the P2X7R. If the integrity of this region is disrupted by a truncation or point mutation, then disruption of the associated regions exposes the retention sequence thereby blocking cell surface expression. The retention of the P2X7R may occur because a protein partner can no longer interact with the P2X7R or the P2X7R precedes into an altered biogenic pathway. A preliminary Prosite (available at www.expasy.ch/prosite) analysis of the C-terminal tail of the rat P2X7R identified potential phosphorylation sites for protein kinase A (Arg357-FVSQD-Met563) and protein kinase C (Ser552-Mar1566)}
YATWR-Phe\textsuperscript{558}) within this region. It is tempting to suggest that a phosphorylation-dependent mechanism may govern the regulation of these two regions, thereby providing an additional pathway for controlling surface expression.

Physiological and Clinical Significance of P2X7 Receptor Cell Surface Expression—The P2X7R on hemopoietic cells is now known to play a role in host defenses against certain infective diseases. The function of monocyte P2X7R is increased by 5-fold on differentiation of these cells to macrophages (29) and activation of macrophage P2X7R by extracellular ATP leads to killing of intracellular Mycobacteria Tuberculosis by these cells (30). The polymorphic P2X7R E496A receptor traffics to the cell surface but is non-functional when expressed at low surface density (29). The pathological consequence of the Glu\textsuperscript{496} → Ala polymorphism is the lack of agonist-mediated P2X7R apoptosis in B-lymphocytes in patients with chronic B-lymphocytic leukemia (19), and patients’ susceptibility to tuberculosis is the subject of current investigation. Regulation of P2X7R cell surface expression may represent an important pathway for modulating receptor function in normal and pathological states.

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