Conserved ectodomain cysteines are essential for rat P2X7 receptor trafficking

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Abstract The P2X7 receptor (P2X7R) is a member of the ATP-gated ion channel family that exhibits distinct electrophysiological and pharmacological properties. This includes low sensitivity to ATP, lack of desensitization, a sustained current growth during prolonged receptor stimulation accompanied with development of permeability to large organic cations, and the coupling of receptor activation to cell blebbing and death. The uniquely long C-terminus of P2X7R accounts for many of these receptor-specific functions. The aim of this study was to understand the role of conserved ectodomain cysteine residues in P2X7R function. Single- and double-point threonine mutants of C119–C168, C129–C152, C135–C162, C216–C226, and C260–C269 cysteine pairs were expressed in HEK293 cells and studied using whole-cell current recording. All mutants other than C119T-P2X7R responded to initial and subsequent application of 300- μ M BzATP and ATP with small amplitude monophasic currents or were practically nonfunctional. The mutagenesis-induced loss of function was due to decreased cell-surface receptor expression, as revealed by assessing levels of biotinylated mutants. Coexpression of all double mutants with the wild-type receptor had a transient or, in the case of C119T/C168T double mutant,

sustained inhibitory effect on receptor trafficking. The C119T-P2X7R mutant was expressed on the plasma membrane and was fully functional with a slight decrease in the sensitivity for BzATP, indicating that interaction of liberated Cys168 with another residue rescues the trafficking of receptor. Thus, in contrast to other P2XRs, all disulfide bonds of P2X7R are individually essential for the proper receptor trafficking.

Keywords Purinergic P2X receptors · ATP-gated channels · Disulfide bonds · Trafficking · BzATP

Introduction

Seven mammalian P2X receptor (P2XR) subunits, termed P2X1–7, form homo- or heterotrimeric receptors [30, 35], which are ATP-gated nonselective cationic channels playing important roles in a variety of physiological functions, including calcium signaling, neurotransmitter and hormone release, inflammation, and pain sensation [5, 13, 27, 44]. Each subunit is composed of a large extracellular domain (hereafter ectodomain), two transmembrane domains, and cytoplasmic N- and C-termini [14]. The ten conserved cysteine residues of the ectodomain form five intrasubunit disulfide bonds (SS1–SS5) [9]. The crystal structure of zebrafish P2X4.1R shows that SS1–SS3 pairs are located in the receptor “head,” SS4 in the “dorsal fin,” and SS5 near the transmembrane domains [26].

To date, the importance of extracellular SS bonds for receptor function has been examined in human P2X1R [15], rat P2X2R [6], and rat P2X4R [39, 52]. The SS bonds in P2X1R and P2X2R are inaccessible to the reducing agents β -mercaptoethanol and dithiothreitol (DTT), indicating that they are not responsible for the formation of

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homotrimers [6, 15]. The P2XR expressed in *Dictyostelium discoideum* responds to ATP application [44], although it contains only one disulfide bond (SS1), suggesting that other bonds are not critical for cell surface expression and plasma membrane receptor function. However, this receptor shows a low sensitivity to ATP, which could also be interpreted that disulfide bonds may contribute to agonist binding and/or channel gating. Extracellular conserved cysteines are unlikely to be directly involved in ATP binding [6] but could contribute to the tertiary structure of the ATP binding pocket because several cysteine-to-alanine single or double point mutants of P2X1R, P2X2R, and P2X4R exhibit decreased sensitivity to ATP [6, 15, 39]. The cysteine residues 126, 132, 149, and 159 forming the P2X2R SS2 and SS3 bond may also contribute to the formation of a metal binding site [6, 8, 17].

Among P2XRs, the P2X7R is unique in many respects. Functional P2X7Rs are homotrimers [28] abundantly expressed in cells of immunological origin or function, in particular macrophages [43], mast cells [7], and microglia [49], as well as in some types of cancer [31, 42]. The receptor exhibits low sensitivity to ATP, does not desensitize, and gradually develops permeability to organic cations, causing a sustained current growth accompanied by cell blebbing and death [1, 12, 32, 45, 50]. The C-terminus of P2X7R is longest among P2XRs and was suggested to account for many of these receptor-specific characteristics [2, 41, 45]. In contrast, the relevance of the ectodomain structure in P2X7R function has not been studied extensively. This includes the lack of information about the role of SS bonds in the receptor function, which is an issue addressed in this manuscript using single- and double-point threonine mutants of five cysteine pairs on rat P2X7R and studying their expression and current responses.

Material and methods

DNA constructs

cDNAs encoding the sequences of rat P2X7R and its mutants were subcloned into the bicistronic enhanced fluorescent protein expression vector pIRES2-EGFP (Clontech, Mountain View, CA, USA). Mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and P2X/pIRES2-EGFP as the template with oligonucleotides introducing specific point mutations (synthesized by Integrated DNA Technologies, Coralville, Iowa, USA, and VBC-Genomics, Vienna, Austria). Plasmid DNAs for transfection were purified using a Jetquick plasmid spin kit (Genomed, Löhne, Germany). The presence of the mutations and the identity of the constructs were verified by dye terminator cycle sequencing (PerkinElmer Life and

Analytical Sciences, Boston MA—performed by the Laboratory of Molecular Technology, NCI, Frederick, MD and Veritas, Inc. Rockville, MD; ABI PRISM 3100, Applied Biosystems, Foster City, CA—performed by the Laboratory of DNA Sequencing, Institute of Microbiology, ASCR, Prague).

Patch clamp recording

Experiments were performed on human embryonic kidney 293 (HEK293) cells obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50-U/ml penicillin, and 50-μg/ml streptomycin in a humidified 5% CO₂ atmosphere at 37°C. Cells were transfected with the wild-type (WT) or mutant receptor using 2 μg of DNA and 4 μl of jetPRIME™ reagent in 2 ml of Dulbecco's modified Eagle's medium according to the manufacturer's instructions (PolyPlus-transfection, Illkirch, France) and examined 24–72 h after transfection. Coexpression of WT and mutant cDNA was performed using 1 μg of each DNA. Currents were recorded in a whole-cell configuration from cells voltage clamped at −60 mV using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA). Records were captured and stored using Digidata 1322A and pClamp9 software (Molecular Devices, USA). During recording, cells were continuously perfused with an extracellular solution containing (in mM): 142 NaCl, 3 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 D-glucose, adjusted to pH 7.3 with 1-M NaOH. Patch electrodes used for whole-cell recording were filled with an intracellular solution containing (in mM): 145 NaCl, 10 EGTA 10, and 10 HEPES; pH was adjusted with 10-M NaOH to 7.2. Control and 2'-3'-O-(4-benzoylbenzoyl)-adenosine 5'-triphosphate (BzATP) or ATP (Sigma-Aldrich, St. Louis, MO, USA)-containing solutions were applied using the RSC-200 Rapid Solution Changer system (BIO-LOGIC, Claix, France). If not otherwise stated, brief (2–10 s) applications of different concentrations of agonists were used to evoke inward currents, and one or two responses were recorded from one cell to prevent the sensitization of the receptor after repeated agonist applications [51]. To estimate the concentration producing 50% of the maximal response (EC₅₀) values, the responses from different cells were pooled, and dose response curves were constructed with mean values.

Surface biotinylation and isolation of biotinylated proteins

The WT and double cysteine-to-threonine mutants (C119T/C168T, C129T/C152T, C135T/C162T, C216T/C226T, and C260T/C269T) were transiently expressed in HEK293 cells and biotinylated 24 h following transfection. The efficiency of transfection was verified by microscope using UV light

and expressed as the number of fluorescent cells to the total number of cells. The procedure was deemed successful when at least 80% of cells were transfected. Following isolation, cells were incubated with Sulfo-NHS-SS-Biotin for 1 h and lysed by sonication. The resulting lysate was then incubated with NeutrAvidin agarose, which binds biotinylated membrane-associated proteins. Unbound intracellular proteins were eluted using a mild detergent-containing washing buffer and stored for subcellular localization experiments. The membranous fraction was eluted with 50-mM DTT solution. Protein concentration was determined using a bicinchoninic acid reaction-based kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA) that was read on a BioTek Synergy HT Multi-detection Microplate Reader (BioTek Instruments, Highland Park, Vermont, USA). Equal amounts of membrane-associated and eluted intracellular proteins were run on SDS-PAGE gels. Proteins were then transferred to polyvinylidene difluoride membranes and incubated with rabbit anti-P2X antibodies (1:2,000; Alomone Laboratories, Jerusalem, Israel) and secondary anti-rabbit antibodies (1:10,000; Sigma-Aldrich, St. Louis, MO, USA) and visualized with HRP Substrate Peroxide Solution/HRP Luminol Reagent (Millipore, Billerica, MA, USA). Images were collected immediately after HRP-incubation with an AIDA Image Analyzer LAS 1000 (GmbH, Straubenhardt, Germany), using the same exposure time for membranous and intracellular proteins. The amount of proteins was then determined by multiplication of band size and band intensity as assessed by ImageJ software (NIH). Differences in expressions of protein in membrane and cytoplasm were presented as a ratio of their amounts.

Data analysis

Dose–response data points were fitted by a three-parameter logistic equation using a nonlinear curve-fitting program that derives the EC_{50} and Hill coefficient values of the produced curves (SigmaPlot 2000 v9.01; SPSS Inc., Chicago, IL). The current amplitude was measured using the program CLAMP-FIT 9 (Axon Instruments). All numerical values in the text are reported as mean $\pm$ SEM. Significant differences between means were determined by one-way analysis of variance using SigmaStat 2000 v9.01 and Tukey's post-hoc test, with $p < 0.01$ (*) and $p < 0.05$ (**).

Results

The effect of cysteine pair mutants on P2X7R function

In this study, we examined the relevance of the following cysteine ectodomain bonds to P2X7R function: C119–C168 (SS1), C129–C152 (SS2), C135–C162 (SS3), C216–C226

(SS4), and C260–C269 (SS5). In initial experiments, we tested whether the functional properties of WT receptor could be modified by reducing agent DTT, which is able to disrupt accessible disulfide bonds in other proteins [33]. When applied for 3 min in 10-mM concentration, DTT had no effect on the amplitude of BzATP-evoked current in cells expressing WT receptors. The I_1 current response to 100- μ M BzATP (2 s application) in the presence of DTT was $91 \pm 7\%$ ($n=7$) of that observed before DTT application. These results are consistent with observations at P2X1R and P2X3R [6, 15], confirming that SS bonds of the WT-P2X7R were not accessible for testing with sulphhydryl active substances.

In further experiments, we used mutants in which both cysteines were substituted with threonines. The WT or double mutant receptors were expressed in HEK293 cells, and the current responses to BzATP (100–300 μ M) or ATP (300 μ M) application were examined 24–48 h after transfection using the whole-cell patch clamp recording. During the initial 60-s application of BzATP, the WT receptor responded with a rapid (I_1) current growth followed by a slow sustained (I_2) current growth (Fig. 1a, top). The amplitude of I_1 current generated by 300- μ M BzATP application was 1.9 ± 0.4 nA, whereas the mean value of $I_1 + I_2$ currents at the end of 60-s application was 2.9 ± 0.5 nA ($n=7$). In further accordance with earlier reports [22, 51], once the steady level of I_2 current was reached, cells responded to subsequent agonist application with monophasic currents (data not shown).

The SS1–SS3 double mutants were functional, but showed only monophasic responses during initial and subsequent BzATP application (Fig. 1a), with the I_1 current amplitude ranging between 5% and 15% of that observed in cells expressing WT receptor (Fig. 1b, Table 1). The lack of I_2 response also indicates that these mutants do not dilate. In further contrast to WT receptor [38, 50], SS1–SS3 double mutants did not show facilitation/sensitization of receptors during repetitive agonist application (data not shown). The SS4 and SS5 double mutants were practically nonfunctional in response to both agonists in 300- μ M concentration (Fig. 1b, Table 1) and increase in the concentration of DNAs from 2 to 4 μ g did not enhance the current amplitude (data not shown). As in controls, the ATP-stimulated current was 4–6-fold lower than BzATP-induced current in SS1–SS3 mutants (Fig. 1b). These results indicated that the selectivity of receptor for agonists was not changed by disruption of SS bonds.

Subcellular localization of the WT and SS1–SS5 P2X7R mutants

To determine if low expression of receptors at the plasma membrane accounted for the decreased or lost responsiveness of mutants, the subcellular expression pattern of WT

Fig. 1 Effect of the disruption of SS1–SS5 bonds of rat P2X7R on receptor function. **a** Time-course of BzATP (100 μ M)-induced current responses recorded from HEK293 cells transfected with WT and SS1–SS3 receptor mutants; I_1 , the initial peak current; I_2 , the sustained current amplitude. Agonist application is indicated by horizontal bars. **b** Maximum I_1 current amplitude for WT and five double mutants of conserved cysteine residues: 119–168 (SS1), 129–152 (SS2), 135–162 (SS3), 216–226 (SS4), and 260–269 (SS5). Data shown are the mean $\pm$ SEM ($n=5$ –15) of responses induced by a short (2–10 s) application of BzATP (black columns) and ATP (white columns), both in 300 μ M

and mutant receptors was analyzed by western blotting. The profiles of the receptor distributions were comparable for all double mutants and distinct from the WT receptor. The WT receptor was present both in the membrane and intracellularly (Fig. 2a). In contrast, the SS1–SS5 double mutants were predominantly localized in the eluate, with very low levels in the plasma membrane. The averaged ratio of P2X7R protein in the membrane and cytosol is shown in Fig. 2b. While this methodology cannot quantify the exact level of receptor within the plasma membrane, it provides a realistic approximation as well as a logical explanation for the electrophysiological results. Thus, the large decrease in BzATP-induced current observed after disruption of all SS

Table 1 Characterization of conserved cysteine-to-threonine mutants of rat P2X7R

Receptor	$I_{\max}$ (nA)	EC ₅₀ (μ M)
WT ($N=61$)	2.9 $\pm$ 0.4	48 $\pm$ 7.0
C119T ($N=34$)	2.2 $\pm$ 0.3	82 $\pm$ 26*
C129T ($N=11$)	0.1 $\pm$ 0.03*	n.d.
C135T ($N=9$)	< 0.1	n.d.
C152T ($N=7$)	< 0.1	n.d.
C162T ($N=5$)	0.1 $\pm$ 0.04*	n.d.
C168T ($N=6$)	< 0.1	n.d.
C216T ($N=6$)	0.1 $\pm$ 0.05*	n.d.
C226T ($N=4$)	< 0.1	n.d.
C260T ($N=9$)	0.2 $\pm$ 0.1*	n.d.
C269T ($N=6$)	< 0.1	n.d.
C119T+C168T ($N=61$)	0.4 $\pm$ 0.1*	207 $\pm$ 230*
C129T+C152T ($N=30$)	0.2 $\pm$ 0.1*	n.d.
C135T+C162T ($N=16$)	0.1 $\pm$ 0.1*	n.d.
C216T+C226T ($N=3$)	< 0.1	n.d.
C260T+C269T ($N=12$)	< 0.1	n.d.

The maximum amplitude of current ($I_{\max}$) was assessed following the application of 1-mM BzATP, and the EC₅₀ values represent the BzATP concentration, producing a half-maximum effect. Data shown are the mean $\pm$ SEM, and N indicates number of experiments. Values that could not be determined are indicated by n.d.

* $p<0.01$ between the wild type (WT) and mutant receptors, estimated by Student's t -test

Fig. 2 Expression of WT and mutant P2X7Rs in HEK293 cells. **a** The expression pattern of biotinylated proteins from WT receptor and five conserved cysteine-to-threonine double mutants (SS1–SS5) in the membrane and the cytosol. Biotinylation data are one representative experiment out of five similar experiments. **b** Relative plasma membrane expression of WT and double mutants presented as a ratio of amount of protein in membrane and cytoplasm

bonds likely reflects attenuated receptor trafficking to the plasma membrane.

Coexpression of cysteine double mutants with WT receptor impairs P2X7R function

The C168A mutation of P2X7R is known to produce a nonfunctional subunit with strong dominant-negative effect on WT receptor in experiments performed in oocytes and HEK293 cells [36]. To examine whether the P2X7R function could be impaired by the SS1–SS5 double mutants, we cotransfected WT (1 μ g) and mutant DNA (1 μ g), and 100- μ M BzATP-induced current was examined 24–72 h after transfection. Control experiments were performed using 1- and 2- μ g DNA of WT receptor. In WT and all cotransfected cells, we consistently observed biphasic current response 1 and 2–3 days after transfection. Representative traces from those experiments are shown in Fig. 3a, and mean values in Fig. 3b. Neither increase in the amount of

DNA for WT receptor nor duration of post-transfection period significantly affected the I_1/I_2 current amplitudes. In contrast, coexpression of WT and mutant subunits had significant effects on I_1/I_2 amplitude in all cases 24 h after transfection. Two to three days after cotransfection, the inhibitory effect on I_1/I_2 amplitude was lost in all cases other than WT+SS1 mutant. These results are consistent with a hypothesis that SS double mutants slowed down trafficking of heteromeric receptor to the cell surface.

The effect of single cysteine mutants on P2X7R function

Like double mutants, the C168T, C129T, C152T, C135T, C162T, C216T, C126T, C260T, and C269T single mutants were also practically nonfunctional (Table 1) and low expressed in the membrane (data not shown). However, contrary to the C119T/C168T double mutant, the C119T single mutant was fully functional. During prolonged (60 s) application of 300- μ M BzATP, the current amplitude increased both in C119T and WT receptors (Fig. 4a) and

Fig. 3 Transient effects of cotransfection of the WT and cysteine double mutants on the receptor function. **a** Example responses to 60-s stimulation with 100- μ M BzATP recorded 20–24 h (black traces) or 48–72 h (gray traces) after transfection. **b, c** Maximum current amplitude in cells transfected with the WT alone (WT, 1 μ g; WT+WT, 2 μ g

DNA) or together with SS1–SS5 double mutants and examined 20–24 h (**b**) and 2–3 days (**c**) after transfection. Cells were stimulated for 40–80 s; I_1 , the initial peak current; I_2 , the sustained current amplitude; ** P <0.01; * P <0.05 between WT and mutants, compared with 1- μ g transfection

Fig. 4 Expression and activation of the C119T mutant. **a** Time-course of BzATP (300 μM)-induced current responses recorded from HEK293 cells transfected with WT-P2X7R (*left*) and C119T mutant (*right*). Both receptors showed an increase in the maximum current amplitude that developed over a period of 60 s, indicating pore dilation. **b** Cell surface expression of biotinylated WT and C119T receptors in HEK293 cells. Bands for both receptors were equally intense in the membrane (*m*) and the eluate (*c*). Data shown are representative experiment out of four similar experiments

their $(I_1 + I_2)/I_1$ ratio was not significantly different (WT = 2.3 ± 0.5 , $n = 12$; C119T = 2.7 ± 0.3 , $n = 6$), indicating that the mechanism necessary for pore dilation [51] was also preserved in this mutant. The biotinylation for C119T in both the eluate and the membrane was comparable with WT levels (Fig. 4b), further indicating that trafficking of this particular mutant was not affected. The C119T mutant also showed typical concentration responses (Fig. 5a), but with the EC_{50} value slightly rightward shifted when compared to WT receptor (WT $EC_{50} = 48 \pm 7$ μM; C119T $EC_{50} = 82 \pm 26$ μM; Fig. 4b). The C119/C168T mutant also showed concentration-dependent increase in the current amplitude, but with much smaller peak amplitude and an estimated EC_{50} value of 207 ± 230 μM (Fig. 5). Collectively, these results suggest that disruption of SS1 bond reduces P2X7R sensitivity for agonist, in addition to affecting trafficking of receptors, and that interaction of liberated Cys168 with another residue probably rescues the trafficking pattern of receptor.

Discussion

The P2XRs are usually found both intracellularly and in the plasma membrane [4], and trafficking to and from the cell surface often regulates the signaling strength of receptors, as documented for P2X4R [3, 40] and P2X3R [47]. For P2X7R, trafficking to the plasma membrane is also of significant physiological relevance. For example, P2X7Rs are abnormally expressed in some types of cancer [16, 31,

Fig. 5 Effects of the disruption of SS1 bond of P2X7R on agonist potency. **a** Representative tracing of BzATP-induced current in cells expressing the WT, C119T, and C119T/C168T receptors. All traces were obtained from different cells stimulated by a short (2–10 s) application of 10-, 30-, 100-, and 300-μM BzATP. **b** BzATP concentration–response curves for WT, C119T, and C119T/C168T receptors. Vertical dashed lines indicate the EC_{50} values. Data shown are mean $\pm$ SEM from 6–30 cells per concentration

42] and participate in the control of cell proliferation [1, 11]. In monocytes and lymphocytes, the receptors are predominantly intracellular [20], whereas when monocytes differentiate into macrophages, there is a large increase in their surface expression and function [21]. This change occurs without a significant increase in receptor mRNA levels, suggesting that the upregulation of surface P2X7R involves a redistribution of intracellular receptors to the plasma membrane.

Based on previous studies, it seems that trafficking of P2XRs to the plasma membrane depends on the C-terminal structure. The conserved YXXXXK motif found in C-terminal of P2XRs other than P2X7R is necessary for their expression at the cell surface [24]. In human P2X7R, a motif

located in the distal region of its C-terminus tail is necessary for proper receptor trafficking; progressive deletions of this tail between residues 551 and 581 are nonfunctional and are not found in the plasma membrane [10, 41]. The same motif is also involved in pore dilation [45, 48].

The N-glycosylation sites within the ectodomain are also essential for trafficking and contribute to the appropriate functionality of P2X1R [37], P2X2R [34, 46], P2X4R [23], P2X6R [25], and P2X7R [29]. For P2X1R, any two out of the four naturally occurring N-glycans are sufficient for robust expression of functional receptors at the cell surface [37]. Deletion of all three glycosylated asparagines of the P2X2 subunits induces an intracellular retention and a complete loss of function of the receptors [34, 46]. Glycosylation is also important for P2X4R localization on the plasma membrane [23]. Transiently expressed P2X6 subunits are not properly trafficked to the cell surface, presumably because of a glycosylation defect [25]. Residue N187 of P2X7R, which belongs to an N-linked glycosylation consensus sequence found in six of the seven P2XR family members, is critical for the cell surface expression of this receptor [29].

The present results reveal for the first time that all five conserved SS bonds in the ectodomain of P2X7R are individually needed for the proper trafficking of receptors to the cell surface, as threonine mutants of all cysteine pairs and nine of ten single-point threonine-to-cysteine mutants responded to agonist with a very low current amplitude and decreased surface expression. Our results also indicate a transient (SS2–SS5) or sustained (SS1) inhibitory effect of double mutants on trafficking of receptors generated by coexpression with WT receptor. Others have also observed that the C168A mutation produces a nonfunctional subunit with a strong dominant-negative effect on WT-P2X7R in oocytes [36]. Disruption of the P2X1R SS5 bond also results in severely reduced expression of the biotinylated protein of mutated receptor at the plasma membrane [15]. In contrast, disruption of SS5 bonds in rat P2X2R and P2X4R does not affect the plasma membrane expression of these receptors [6, 39]. Furthermore, for P2X4R, this bond was suggested to contribute to channel gating rather than trafficking [39]. These data indicate the common and distinct roles of the SS bonds in P2XR function.

At the present time, the reason for altered trafficking of P2X7R cysteine mutants is unknown. Membrane stability of P2XR channels might be promoted by interactions with intracellular proteins that regulate membrane turnover and degradation of the receptor [18, 24]. Another possible mechanism by which the SS mutants could block P2X7R trafficking is a generation of SS bonds between single cysteines and intracellular proteins within the cytoplasm. Interestingly, extracellular SS bonds of P2X7R on phagocytic cells have been shown to play a role in the recognition of epitopes exposed on the surface of apoptotic cells and participate in

their internalization [19], indicating the importance of extracellular cysteines for protein–protein interaction.

We also show that the trafficking of receptors was not affected in single C119T mutant and that this receptor responded to agonist application without changes in the peak amplitude of current, in contrast to the C119–C168 double mutant. This finding further support the conclusion that altered trafficking of receptor accounts for low amplitude current response in double mutant. Most probably liberated Cys168 residue interacted with another residue in the protein molecule to rescue the receptor trafficking. Furthermore, both C119T and C119/C168 mutants showed rightward shifts in the sensitivity of receptors for BzATP, indicating that SS1 bond is also important for the proper ligand binding or transduction of signals from binding to gating receptor domains.

There is a general agreement about the importance of SS1 cysteines for P2XR structure and function, but there is some discrepancy about the extent of the changes observed after SS1 disruption and the relevance of this bond to receptor function. In oocytes expressing the P2X1R, SS1 single and double mutant showed only a 2- to 5-fold decrease in ATP potency, without any change in the peak current amplitude [15]. The EC₅₀ values of P2X2R SS1 single mutants were more right-shifted (30-fold) than those of the other single point cysteine mutants (10- to 15-fold), and the peak current amplitude was reduced by 70–80% [6]. Finally, the P2X4R SS1 single and double mutants were 5- to 10-fold less sensitive than WT receptors and showed no changes in the maximum current amplitude [39]. Based on these data, we suggest that loss of function of the P2X7-SS1 double mutant is comparable with other P2XRs so far investigated; however, the effect on surface expression is unique and represents another specific property of this receptor.

Conclusions

The aims of this study were to understand the role of conserved cysteine residues in rat P2X7R function. So far, studies performed on P2X1, P2X2, and P2X4 subtypes of mammalian P2XRs revealed that no ectodomain SS bond is individually essential for channel function, but that SS1 bond contributes to the proper agonist binding or gating. Our findings are consistent with the second conclusion. More importantly, here, we show for the first time that SS bonds of P2X7R are critical for the proper receptor trafficking from endoplasmic reticulum to the plasma membrane. Clarification of the role of SS1–SS5 bonds in P2X7R trafficking may provide the opportunity to manipulate the receptor function in normal and pathological states.

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