

Conserved Cysteine Residues in the Extracellular Loop of the Human P2X₁ Receptor Form Disulfide Bonds and Are Involved in Receptor Trafficking to the Cell Surface

STEVEN J. ENNION and RICHARD J. EVANS

Department of Cell Physiology and Pharmacology, University of Leicester, Leicester, United Kingdom

Received August 10, 2001; accepted October 25, 2001

This paper is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

P2X receptors contain 10 conserved cysteines in the extracellular loop. To investigate whether these residues form disulfide bonds, we created a series of single and double cysteine-alanine mutants in the human P2X₁ receptor. Mutants were expressed in *Xenopus laevis* oocytes and effects on ATP potency, cell-surface expression, and *N*-biotinoylaminoethyl methanethiosulfonate (MTSEA-Biotin) labeling of free cysteines were determined. For the majority of single mutants, only a modest decrease (2- to 5-fold) in ATP potency was recorded. For mutants C261A and C270A, the peak current amplitudes were reduced by 93.6 ± 2.0 and $95.0 \pm 1.0\%$, respectively; this was a result of low cell-surface expression of these mutant receptors. Wild-type receptors showed no labeling with MTSEA-biotin suggesting that all 10 cysteine residues in the ex-

tracellular loop are disulfide-bonded. Mutation of cysteines at positions 126, 132, 149, 159, 217, and 227 resulted in MTSEA-biotinylation of a free cysteine residue created by the disruption of a disulfide bond and provides direct biochemical evidence for at least three disulfide bonds. Based on phenotypic comparisons of single and double cysteine mutants, we propose the following disulfide bond pairs in the human P2X₁ receptor: C117-C165, C126-C149, C132-C159, C217-C227, and C261-C270. None of these bonds are individually essential for channel function. However, trafficking of the receptor to the cell membrane is severely reduced by disruption of the C261-C270 disulfide bond or disruption of C117-C165 together with another bond.

P2X receptors comprise a family of seven (P2X₁₋₇) cation channels that are gated by extracellular ATP. They play an important role in a variety of physiological processes, including control of smooth muscle contractility, activation of sensory nerves, and fast synaptic transmission [for review, see Robertson et al. (2001)]. The receptor subunits associate to form functional homo- or heterotrimeric channels; this, coupled with the existence of splice variants for some subunits, results in a variety of recombinant P2X receptor phenotypes [for review, see North and Surprenant (2000)]. P2X receptors have a membrane topology of two transmembrane domains, intracellular amino and carboxy termini, and a large extracellular loop [for review, see Khakh (2001)]. Recent advances have identified the molecular basis of a number of properties of the channels. These include 1) amino acid residues in the transmembrane domains that are associated with ionic permeation (Rassendren et al, 1997; Egan et al, 1998; Jiang et al, 2001; Migita et al, 2001); 2) a conserved protein kinase C phosphorylation site in the intracellular amino terminus (Boue-Grabot et al, 2000); 3) residues in the intracellular

carboxy terminus that are involved in regulating channel gating (Brandle et al, 1997; Simon et al, 1997; Koshimizu et al, 1998; Smith et al, 1999); 4) residues in the extracellular loop that are involved in ligand binding (Buell et al, 1996; Garcia-Guzman et al, 1997; Ennion et al, 2000; Jiang et al, 2000); and 5) a series of *N*-glycosylation sites that contribute to surface expression levels and ATP potency (Valera et al, 1995; Newbolt et al, 1998; Rettinger et al, 2000).

One feature of the family of P2X receptors that has received little direct attention is the function of 10 cysteine residues within the extracellular loop (Fig. 1). These cysteines are conserved in all known P2X receptors in mammalian species, *Xenopus laevis*, and zebrafish and have often been assumed to form a series of five disulfide bonds. However, no direct experimental evidence for the existence of these bonds has been presented. Disulfide bonds are known to play an important role in the formation and maintenance of ion channel structure. For example, in the Kir2.1 inwardly rectifying potassium channel (Cho et al, 2000) and the nicotinic receptor (Brejc et al, 2001), the disulfide bonds are thought to stabilize individual subunits. However, disulfide bonds can also form between adjacent subunits (e.g., in the

This work was funded by British Heart Foundation grant PG/98128.

ABBREVIATIONS: WT, wild-type; DTT, dithiothreitol; β ME, β -mercaptoethanol; MTSEA-Biotin, *N*-biotinoylaminoethyl methanethiosulfonate; PAGE, polyacrylamide gel electrophoresis; DTSSP, 3,3'-dithio-bis(succinimidyl-propionate); ER, endoplasmic reticulum.

TWIK potassium channel, where an intersubunit disulfide bond is responsible for dimerization of the receptor) (Lesage et al, 1997). Any disulfide bonding that exists between the 10 conserved cysteine residues of the P2X₁ ion channels is likely to be intra- rather than intersubunit; a variety of denaturing agents that do not break disulfide bonds still dissociate P2X₁ multimers into monomeric receptors and demonstrate that P2X₁ receptor multimers are maintained by noncovalent interactions (Nicke et al, 1998).

To investigate the possible existence and role of disulfide bonds between the 10 conserved extracellular cysteine residues in P2X receptors, we have studied the effects of a series of cysteine-to-alanine mutations in human P2X₁ receptors expressed in *X. laevis* oocytes on ATP potency, cell-surface expression, and the existence of free cysteine residues.

Materials and Methods

Site-Directed Mutagenesis of the Human P2X₁ Receptor.

The human P2X₁ plasmid construct used in this study has been described previously (Ennion et al, 2000). Point mutations were introduced using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The 10 conserved cysteine residues at positions 117, 126, 132, 149, 159, 165, 217, 227, 261, and 270 were mutated to alanine. Double mutations in which 2 of the 10 cysteines were both mutated to alanine were created by conducting serial rounds of site-directed mutagenesis as

P2X ₁	-YEKGQYTSGLLI-SSVSVKLGLAVLTQ-----LPGLGPQVWDVADYV	91
P2X ₂	---KSYQDSSETPGESSITTKVKGITMSR-----DKVWDVVEEYV	87
P2X ₃	LHEKAYQVRDTEATSSVVTIKVKGSLYA-----NRVMDVSDYV	81
P2X ₄	---KGYQETDSVV-SSVTKVKGAVNTS-----KLGRVLDVADYV	90
P2X ₅	---KKGYQDVTSLQSAVITKVGVAFTNT-----SDLGQRLWDVADYV	92
P2X ₆	---KKGYQERDLEPQESITKLGKGVSVTQI-----KELGNRLWDVADYV	91
P2X ₇	-SDRLYQRKEPVT-SSVHTKVKGIKAEVKEIVENGVKRLVHVSFDTADYT	94
P2X ₁	FPAQGDNSFVVMTEFVTPKQYQGYGAEHPE-G-GIKEDSGCPGKAKR	139
P2X ₂	KPPEGGSVVSIIITRIEVTSPQTLGIPESMRVHSSTCHSDDUCLAGQLDM	137
P2X ₃	TPPQGTSTVFVITIKMIVTENQMGQCPESR--EKYKIVSDSGC--GPEPL	127
P2X ₄	TPAEGENSLFVMTNVIITMNTQGTGCPRIIP-ATTVCSDASCTAGSAGT	139
P2X ₅	1PAQGENVFVVTNLVITPNQKQNVCAENEGIPDGAQKSDSCGAGEAVT	142
P2X ₆	KPPQGENVFVLTNLTFLVTPAQVQGGCPPEHSPVPLANGVWDEDCEEGGT	141
P2X ₇	FPLQG-NSFFVMTNFTKTEGQQRICPEPYPT-RTTICSSDRGCKKGMMDP	142
P2X ₁	KAQGIKTGKVAE-NDVTKVGLIFGKVEVEVDDIPRFPALLREAEENFTLF	188
P2X ₂	QNGGIRTKGCPYYHGDGSKTCEVSAKCPVEDGT-SDNHFLGKMAENFTIL	186
P2X ₃	PGGGITLTCGRVNY-SSVLTGCTIQCKCPTEVDTP-VETPIM-MEAEENFTIF	174
P2X ₄	HSNGVSTGRCVAE-NGSVKTCVEAAGCPVEDDTHVPQAFILKAAENFTLL	188
P2X ₅	AGNGVKTGKCLKRNGLARGICELFAWCPLETSS-RPEEPFLKEAEDFTIF	191
P2X ₆	HSNGVKTGKCVVFNTHIR-TELWSWCPVESGV-VPSRPLLAQAQNFTEF	189
P2X ₇	QSKGIKTGKCVVH-EGNQKTCVEVSAKCEAEVEEAPRFPALLNSAEENFTVL	191
P2X ₁	1KNSISEPFRKVN-RRNLVEEVAAHM--KTCFLPHKTLHPLCPVFGQYGV	235
P2X ₂	1KNSIHYPKFKFS-KGNISQ-KSDYL--KCTFFDQSDPYCPIFRLGFT	232
P2X ₃	1KNSIRFPFLNFR-KGNILPLNITARDM--KCTRFHFDKDPFCPIIRVGCV	221
P2X ₄	1KNNIWPKEFNFS-KRNILPLNITTYL--KSCLYDAKTDPFCPIIRLGSI	235
P2X ₅	1KNNIWPKEFNFS-KNNVMDWDKRSFL--KSCIFGPK-NHYCPPIIRLGSI	237
P2X ₆	1KNTVTFSEFNFS-KSNALETWDDPTTF--KICRYEPQFSPYCPVFRIGDL	236
P2X ₇	1KNNIDFPGHNYTTR-NIL---PGLNIT--KTFHKTQNPFCPIIRLGDT	234
P2X ₁	VQESGQGNFSTLAEKGVGVGTTDWHCLDLDVHVRHCPPTYRFHGLYF---E	282
P2X ₂	VEKAGENFTLAKKGGVTGVTINNNCLDLSSEFCNPKYSFRRLLDPKYD-	281
P2X ₃	VKEAGQDFAKLARTGGVGLGKLGWCLDLDKAWDCNPKYSETRLDVSSEK	271
P2X ₄	VENAGHSFQDMAVEGGIMGIVNWLCLNLDRAASCLPRYSFRRLDTRDVE	285
P2X ₅	VWAGSDFQDLALRGVGTGTINENNCLDLDKASFCNPKYSFRLDNKLSK	286
P2X ₆	VAKAGCTFDFLAILGGSGVTRVHWDCLDLDGSDCNPKYSF-QLRK---E	280
P2X ₇	FRRTGDNFSDVATCCGIMGTETYNWCLNLDKWFHRCNPKYSFRLDDKTTN	284
P2X ₁	KNLSPGENFRFAHEVE-NGTNYRHLKVFVGIRFDILVDGKAGKEDILP-	330
P2X ₂	-PASSGYNFRFAKYKINGTITTTTTLIKAYGIRFDIVHCGAQKFS---	326
P2X ₃	SSVSFGYNFRFAKYKMGENSEYRTLLKAFGIRFDVLVYCNACKFN-	317
P2X ₄	HNVSFGYNFRFAKYRLLMGNEQRTLLKAYGIRFDILVFGKAGKEDILPT	335
P2X ₅	-SSVSGYNFRFAKYRDAAGVEFRTLLKAYGIRFDIVHCGAQKFS---	331
P2X ₆	-----SYNFRATHWGGGVEATLLKLYGIRFDILVFGGAGKGF---	323
P2X ₇	VSLYPGYNFRFAKYKFR-NNVEKRTLLKVFGRFDILVFGGAGKEDITQL	333

Fig. 1. Ten cysteine residues are conserved in the extracellular loop of P2X receptors. Amino acid sequences of the extracellular loop regions of the human P2X₁₋₇ receptors are aligned. Conserved cysteine residues are boxed.

follows: C217A/C227A, C126A/C132A, C132A/C149A, C149A/C159A, C117A/C165A, C126A/C159A, C126A/C149A, C132A/C159A, C117A/C126A, C117A/C149A, C165A/C217A, C159A/C165A, C126A/C165A, and C149A/C165A. Double mutations of residues close to cysteines 261 and 270 were also constructed by serial site-directed mutagenesis and were as follows: C261A/H260C, C270A/H269C, and C270A/R271C. In all mutants, introduction of the correct mutation(s) and the absence of spontaneous mutations were confirmed by DNA sequencing (Automated ABI sequencing service, Leicester University).

X. laevis Oocyte Expression System. cRNA was transcribed from wild-type (WT) and mutated plasmids as described previously (Ennion et al, 2000). De-folliculated *X. laevis* oocytes were injected with 50 nl of cRNA (1 mg/ml) using an Inject +Matic micro injector (J. Alejandro Gaby, Genève, Switzerland) and stored at 18°C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM sodium pyruvate, and 5 mM HEPES, pH 7.6) before use 3 to 7 days later.

Electrophysiological Recordings. Two-electrode voltage clamp recordings were made from oocytes using a GeneClamp 500B amplifier with a Digidata 1200 analog-to-digital converter and pClamp 8 acquisition software (Axon Instruments, Union City, CA) as described previously (Ennion et al, 2000). External ND96 solution had 1.8 mM BaCl₂ replacing the 1.8 mM CaCl₂ to prevent activation of endogenous calcium activated chloride channels. ATP (magnesium salt; Sigma, Dorset, Poole, UK) was applied from a U-tube perfusion system whereas the antagonist suramin (Bayer, Newbury, Berkshire, UK) and the reducing agents dithiothreitol (DTT) and β-mercaptoethanol (βME) (Sigma) were bath-perfused and also present at the appropriate concentration in the U-tube application of ATP. Reproducible responses to ATP were recorded when applications were separated by 5 min to allow recovery from receptor desensitization. Concentration response data were fitted with the Hill equation: $Y = [X]^{n_H} \times M / ([X]^{n_H} + (EC_{50})^{n_H})$ where Y is response, X is agonist concentration, n_H is the Hill coefficient, M is maximum response, and EC_{50} is the concentration of agonist evoking 50% of the maximum response. pEC_{50} is the $-\log_{10}$ of the EC_{50} value. Data are presented as mean \pm S.E.M. and differences between mean means were tested with the Student's t test.

Cell Surface Biotinylation. Cell-surface proteins were biotinylated (15 oocytes per reaction) with either Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) or *N*-biotinoylaminoethyl methanethiosulfonate (MTSEA-Biotin; Toronto Research Chemicals, Toronto, Canada). Both these reagents are impermeable to the cell membrane and therefore only biotinylate surface localized proteins in intact cells. Sulfo-NHS-LC-Biotin reacts with primary amines and was used to assess membrane levels of P2X₁ receptors as described previously (Ennion et al, 2000). MTSEA-Biotin reacts only with cysteinyl sulfhydryl groups and was used to assess the availability of free cysteines in the extracellular loop of the P2X₁ receptor (i.e., those cysteines not in a disulfide bond and in an accessible area of the protein tertiary structure). Oocytes injected previously with wild-type or mutant P2X₁ cRNA were incubated in biotinylation agent [Sulfo-NHS-LC-Biotin (0.5 mg/ml) or MTSEA-Biotin (0.15 mg/ml)] for 30 min in ND96 buffer. After washing five times in ND96 buffer, oocytes were homogenized in buffer H [100 mM NaCl, 20 mM Tris-Cl, pH 7.4, 1% Triton X-100, 10 μl/ml protease inhibitor cocktail (Sigma P8340)] in a volume of 20 μl/oocyte. After centrifugation at 16,000 g (4°C) for 2 min, a 7.5-μl aliquot of the supernatant was mixed 50:50 with SDS-PAGE sample buffer (50 mM Tris-Cl, pH 6.8, 50 mM DTT, 2% SDS, 10% glycerol, 10% βME), and stored on ice before SDS-PAGE to assess total P2X₁ (surface plus internal receptors). Two hundred microliters of the remaining supernatant was diluted 50:50 with buffer H and 30 μl of streptavidin agarose beads (Sigma) added. Samples were rolled at 4°C for 3 h to allow the streptavidin to bind biotinylated proteins. Beads were washed four times with buffer H with a 2-min centrifugation (16,000g) between washes. After the final wash, buffer H was replaced with 30 μl of SDS-PAGE sample buffer. Protein samples were heated to 80°C for 2 min before sepa-

ration on a 10% SDS-PAGE gel. Gels were transferred to polyvinylidene difluoride membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and processed for anti P2X₁ antibody (1:500 dilution) (Alamone, Israel) staining as described previously (Ennion et al, 2000).

Cross-Linking of Proteins with DTSSP. Oocytes previously injected with wild-type or mutant P2X₁ cRNA were homogenized in phosphate-buffered saline plus 1% Triton X-100 (20 μ l per oocyte; 15 oocytes per reaction) and rolled at 4°C for 15 min. After centrifugation (16,000g at 4°C for 2 min), 20 μ l of the supernatant was mixed with an equal volume of 3,3'-dithio-bis(succinimidyl-propionate) (DTSSP; Pierce) in phosphate-buffered saline plus 1% Triton X-100 to give the appropriate concentration of cross-linking agent. Reactions were incubated at room temperature for 30 min and 2 μ l of 1 M Tris-Cl, pH 7.4, was added to quench the reaction. An equal volume (40 μ l) of SDS-PAGE sample buffer (minus the reducing agents DTT and β ME) was added and the samples heated at 70°C for 2 min before separation on a 10% SDS-PAGE gel. Cross-linked P2X₁ receptors were visualized by Western blotting and anti-P2X₁ antibody staining as described above. The concentration of DTSSP used was titrated from 3 mM to 0.9 μ M for the wild-type P2X₁ receptor and from these results a concentration of 30 μ M DTSSP was chosen for cross-linking of C261A and C270A mutant receptors.

Results

Effects of Individual Cysteine Mutations on the Properties of Human P2X₁ Receptors. ATP evoked concentration-dependent inward currents in oocytes injected with WT P2X₁ receptor cRNA with an EC₅₀ of 1.2 μ M, similar to that reported previously (Ennion et al, 2000) (Fig. 2; Table 1). The functional role of the 10 conserved cysteine residues in the extracellular loop was investigated using a series of individual cysteine-to-alanine point mutations; all the mutants produced functional channels (Fig. 2). Substitution at positions C159A and C261A had no effect on ATP potency. For mutants C117A, C126A, C132A, C149A C165A, and C270A there was a modest 2- to 5-fold decrease in potency ($p < 0.05$; $n = 5$), whereas larger 8- to 45-fold changes ($p < 0.01$; $n = 5$) were recorded for C217A (EC₅₀, 9.8 μ M) and C227A (EC₅₀, 54.4 μ M) (Table 1). For the mutant receptor C227A (the mutant with the largest decrease in ATP potency), there was only a small increase in the affinity for the receptor to the P2 receptor antagonist suramin [pA_2 , 7.0 ± 0.1 ; compare 6.7 ± 0.1 reported previously for P2X₁ WT receptor (Ennion et al, 2000); $p < 0.05$, $n = 4$]; this suggests that there have not been major structural changes in the P2X receptors as a result of mutations. C-to-A mutation had no major effect on the time course of the P2X₁ receptor mediated responses (Table 1). The peak current amplitude to a maximal concentration of ATP was unaffected for the majority of C-to-A mutants (Fig. 3A). However, for mutants C132A, C159A, C261A, and C270A, peak currents were 55.2 ± 9.0 , 53.8 ± 4.0 , 6.4 ± 2.0 , and $4.1 \pm 1.0\%$ of WT responses, respectively ($p < 0.01$, $n = 5-9$) (Fig. 3A; Table 1). These results demonstrate that individual cysteine residues are not essential for the production of functional P2X₁ receptors; however, C132A, C159A, and most noticeably C261A and C270A are associated with a reduction in current amplitudes.

The Effects of C261A and C270A Mutants on P2X₁ Receptor Cell Surface Expression. The substantial reduction in peak current amplitude for C261A and C270A could result from a decrease in cell surface expression and/or a modification of channel gating. The level of cell-surface

expression of P2X₁ receptors was estimated using Sulfo-NHS-LC Biotin as described previously (Ennion et al, 2000) (Fig. 3 B). Robust surface expression was detected for WT and C117A and C217A mutant P2X₁ receptors. The single band with a molecular mass of ~ 57 kDa (Fig. 3B) corresponds to the glycosylated form of the P2X₁ receptor (Valera et al, 1995; Rettinger et al, 2000). In contrast, the mutant C261A and C270A P2X₁ receptors were barely detectable at the membrane surface. The amount of P2X₁ receptor protein produced by the oocytes can be estimated from total cell lysates. For WT, the P2X₁ receptor antibody detects a smeared band that can often be discriminated into two components; corresponding to different levels of post-translational processing/glycosylation (Fig. 3C). For C261A and C270A, there was a single band of ~ 54 to 55 kDa and the higher molecular mass band of the doublet seen for the WT P2X₁ receptor was absent. Because gels were run under reducing conditions, this difference in size does not correspond to differential run properties caused by a disulfide bond. The absence of the second higher molecular mass band is therefore most likely to correspond to a partially processed/glycosylated form of the receptor. The lack of detection of surface P2X₁ receptor biotinylation shows that the C261A and C270A mutants are inefficiently trafficked to the membrane surface.

The decreased surface expression, and similar phenotype,

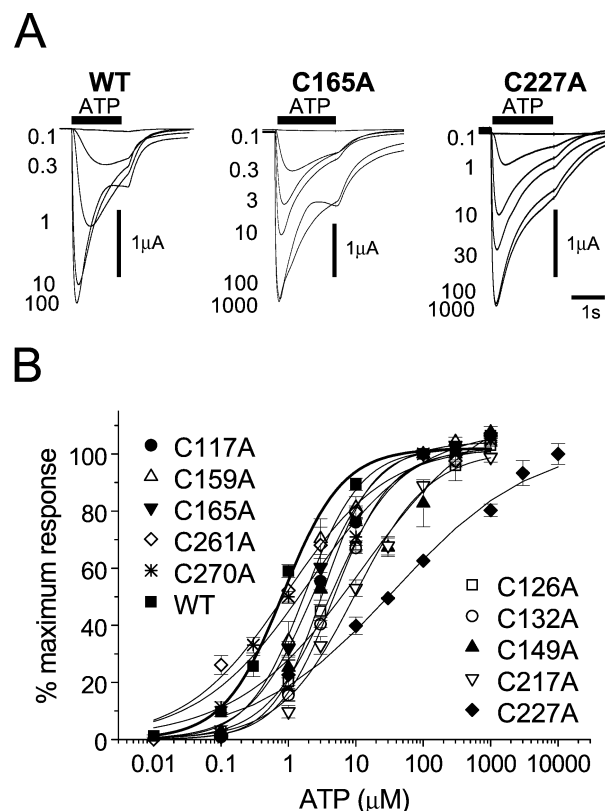


Fig. 2. Effects of cysteine-to-alanine mutations on ATP potency. Two-electrode voltage-clamp recordings were made from oocytes expressing WT or mutant P2X₁ receptors. A, concentration-dependent ATP-evoked (concentration in micromolar) inward currents recorded from WT, C165A, and C227A mutant P2X₁ receptors. B, concentration-response curves for WT and the 10 cysteine-to-alanine point mutations; currents are normalized to the maximal response for a given oocyte ($n = 5-8$ oocytes). The bold line indicates the WT concentration response curve. Holding potential was -60 mV.

Effects of C261A and C270A Mutations on Subunit Assembly. As with all multimeric membrane proteins, the P2X₁ receptor forms its multisubunit composition in the endoplasmic reticulum (ER) (Nicke et al, 1998; Rettinger et al. 2000). One possible explanation for the decreased surface expression observed with the C261A and C270A mutants could have been a reduced ability to form interactions either between P2X subunits or with some as-yet-unidentified accessory protein. To address this possibility, we used the cross-linking agent DTSSP (Nicke et al, 1998). Initial experiments with cytoplasmic extracts from WT P2X₁ expressing oocytes were performed to establish the optimal concentration of cross-linking agent (Nicke et al, 1998) (Fig. 4A). High concentrations of DTSSP (0.3–3 mM) produced a large molecular mass adduct of cross-linked proteins with no detection of P2X₁ receptor subunit monomers. At 90 μ M DTSSP monomeric P2X₁ receptors were apparent along with three specific high molecular mass adducts, the largest of which disappeared at concentrations of 9 to 30 μ M resolving a monomer and higher molecular mass/trimeric form of the receptor [similar to that reported previously (Nicke et al, 1998)]. At concentrations <9 μ M, only monomeric forms of

Detection of Disulfide Bonds in the Extracellular Loop of WT Human P2X₁ Receptors Using MTSEA-Biotin Labeling of Free Cysteines in Mutant Recep-

Properties of currents recorded in *X. laevis* oocytes expressing mutant and WT P2X₁ receptors pEC₅₀ is $-\log 10$ EC₅₀ (molar). Peak I is the current recorded in response to a maximal concentration of ATP. For MTSEA biotinylation, Y indicates MTSEA biotinylation was observed, and N indicates MTSEA biotinylation was not observed. Values are shown \pm S.E.

Mutant	EC ₅₀	pEC ₅₀	Peak I	Half Width	MTSEA Biotinylation
			<i>nA</i>	<i>ms</i>	
WT	1.2	5.97 ± 0.07	−6383 ± 504	1847 ± 262	N
C117A	3.2	5.50 ± 0.04**	−7204 ± 784	1912 ± 510	N
C126A	4.6	5.34 ± 0.05**	−5932 ± 480	1156 ± 256	Y
C132A	4.8	5.32 ± 0.01**	−3523 ± 544**	509 ± 47**	Y
C149A	5.1	5.35 ± 0.11**	−5908 ± 908	1044 ± 172*	Y
C159A	2.0	5.74 ± 0.10	−3436 ± 246**	1747 ± 342	Y
C165A	2.7	5.60 ± 0.07**	−6651 ± 756	1701 ± 198	N
C217A	9.8	5.04 ± 0.07**	−6965 ± 412	1825 ± 366	Y
C227A	54.4	4.37 ± 0.16**	−5031 ± 1191	1402 ± 93	Y
C261A	1.4	5.98 ± 0.17	−406 ± 128**	1167 ± 298	N
C270A	2.3	5.67 ± 0.09*	−262 ± 77**	685 ± 40*	N
C117A/C165A	5.9	5.25 ± 0.07**	−5649 ± 742	841 ± 131*	N
C126A/C132A	105.2	4.06 ± 0.18**	−4007 ± 1078*	597 ± 99**	N
C126A/C149A	2.4	5.62 ± 0.03**	−6600 ± 473	859 ± 135*	N
C126A/C159A	4.6	5.35 ± 0.07**	−6440 ± 309	948 ± 112*	N
C132A/C149A	26.5	4.59 ± 0.05**	−6459 ± 498	560 ± 123**	N
C132A/C159A	2.2	5.67 ± 0.03*	−5887 ± 1007	720 ± 166*	N
C149A/C159A	10.3	5.00 ± 0.04**	−7472 ± 410	1066 ± 246	N
C217A/C227A	4.3	5.42 ± 0.12**	−7017 ± 465	1431 ± 31	N
C117A/C126A	NF				N
C117A/C149A	NF				N
C126A/C165A	NF				N
C159A/C165A	NF				N
C149A/C165A	NF				N
C165A/C217A	NF				N

** $p < 0.01$; different from WT as measured by Student t test.

tors. The methanethiosulfonate compounds react with free cysteine residues and have been used in a variety of applications to investigate protein structure and function. We have used an MTSEA-Biotin assay to detect accessible free cysteine residues in WT and mutant P2X₁ receptors. WT P2X₁ receptors showed no biotinylation (Fig. 5B), demonstrating that all cysteines in the extracellular loop region are unavailable, either from being disulfide-bonded and/or from residing in an area of the molecule that is inaccessible to MTSEA-

Biotin. A cysteine-alanine point mutation of a residue that normally forms a disulfide bond will result in the bond being broken and, providing the residue is in an accessible area of the protein, the availability of a free cysteine residue (Fig. 5A). Biotinylation of a free cysteine residue was detected for the mutants C126A, C132A, C149A, C159A, C217A, and C227A (Fig. 5B), indicating that these residues are normally involved in disulfide bond formation in the WT receptor. It is considered unlikely that the free cysteines result from major structural changes in the protein as in the majority of cases, these mutations resulted only in small 2- to 5-fold changes in ATP potency. MTSEA binding was not detected for the mutants C117A and C165A, suggesting that either 1) these residues are not involved in disulfide bond formation, 2) the partners of these residues in a putative disulfide bond reside in an inaccessible region of the receptor, or 3) the liberated cysteine residue may be in a positively charged "pocket" of the receptor and so repel the positively charged MTSEA-biotin. It was not possible to test the latter possibility, because uncharged biotinylated sulfhydryl compounds are not currently available. No biotinylation for the mutants C261A and C270A was expected, because the level of cell-surface expression for these two mutants was previously shown to be

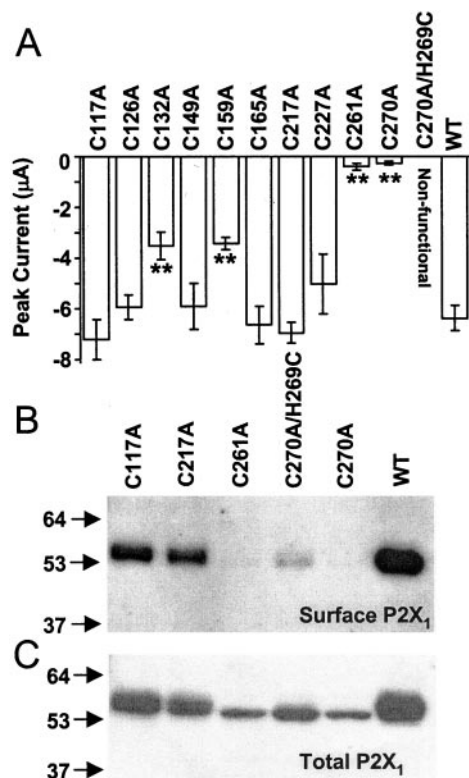


Fig. 3. Reduced current amplitudes for C261A and C270A mutants result from reduced trafficking to the cell membrane. A, Peak current amplitudes recorded in *X. laevis* oocytes in response to 100 μ M ATP for WT, single cysteine-to-alanine mutants, and a double mutation (C270A/H269C) in which the cysteine at position 270 is effectively moved to position 269. **, significantly different from WT currents ($p < 0.01$). B, Western blot of Sulfo-NHS-LC biotin labeled surface P2X₁ receptors. C, Western blot of total (surface localized plus cytoplasmic) P2X₁ receptors. Size markers in kilodaltons.

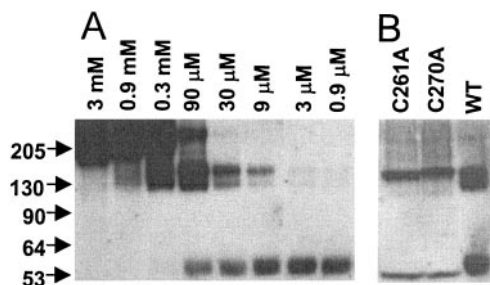


Fig. 4. Cross-linking of P2X₁ receptors with DTSSP. A, concentration dependence of cross-linking; supernatants from homogenates of oocytes expressing WT receptors were incubated with different concentrations of DTSSP, separated by electrophoresis, blotted and probed with P2X₁ antibody. B, supernatants from homogenates of oocytes expressing C261A, C270A mutant, or WT receptors were incubated with 30 μ M DTSSP and processed as above. Eight times the amount of protein was loaded in the C261A and C270A lanes to compensate for the lower expression levels associated with these mutants. Size markers are in kilodaltons.

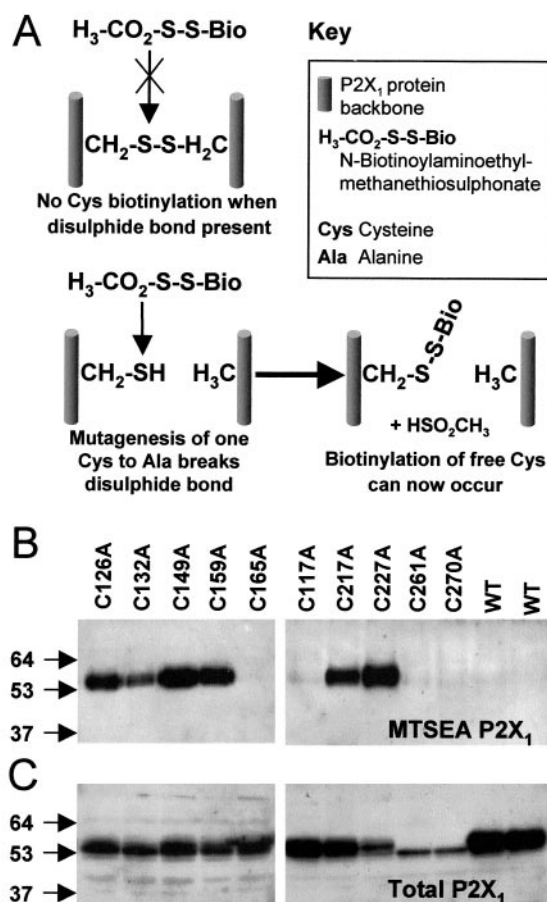


Fig. 5. Detection of free cysteine residues in WT and mutant P2X₁ receptors using MTSEA-biotin. A, schematic of reaction between sulfhydryl group on a free cysteine with MTSEA-biotin. B, P2X₁ antibody probed Western blot of surface biotinylated proteins from supernatants of WT and mutant injected oocytes previously incubated in MTSEA-biotin. C, total protein western blot of the samples shown in B. Size markers are in kDa.

too low for detection by the biotinylation agent Sulfo-NHS-LC-Biotin (Fig. 3B).

Do Adjacent Cysteine Residues Form Disulfide Bonds? In the extracellular loop of P2X receptors, cysteine residues occur in apparent doublets separated by 9 to 17 amino acids (Fig. 1). It was tempting, therefore, to speculate that sequential cysteines formed disulfide bonds as do, for example, the ten cysteine residues of the corticotropin-releasing factor binding protein (Fischer et al, 1994). To test this, we mutated adjacent pairs of cysteine residues to form a series of double-mutants (C117A/C126A, C126A/C132A, C132A/C149A, C149A/C159A, C159A/C165A, C165A/C217A, and C217A/C227A) and examined their effects on ATP potency and free cysteine residues (MTSEA-biotinylation). It was predicted that when the correct pair of cysteines in a disulfide bond had been mutated, there would be no or a minimal further change in ATP potency (because the disulfide bond had already been broken with the point mutant) and that MTSEA-biotinylation would be lost. In contrast, a double mutation corresponding to incorrect partners may result in a decrease in ATP potency (because of the breaking of two disulfide bonds) and an increase in biotinylation because of the formation of a further free cysteine residue.

The double mutants were expressed in oocytes and peak

current amplitude, ATP potency, and levels of surface MTSEA-biotinylation were determined. The mutants C126A/C132A, C132A/C149A, C149A/C159A, and C217A/C227A expressed functional channels with peak current amplitudes similar to those of WT P2X₁ receptors, C126/C132 had an ~40% decrease in current amplitude (Figs. 6A and 7A). For the double mutant C217A/C227A, there was a smaller decrease in ATP potency compared with the WT (<4 fold) than for either of the single point mutants C217A (~8 fold) or C227A (~45 fold; $p < 0.05$). In contrast, for C149A/C159A and C132A/C149A and C126A/C132A, there was an additional decrease in ATP potency compared with the individual point C-to-A mutations of ~3-, 5-, and 21-fold or ~10-, 26-, and 100-fold compared with the WT, respectively ($p < 0.01$) (Table 1). ATP application (up to 1 mM), however, failed to evoke inward currents from the double mutants incorporating either C117A or C165A (i.e., C117A/C126A, C117A/C149A, C159A/C165A, and C165A/C217A).

Surface MTSEA-biotinylation was not detected for any of these double mutations demonstrating that no free cysteine residues were available (Fig. 6B, as a positive control MTSEA-biotinylation was still seen for C149A). For the double mutants C117A/C126A, C117A/C149A, C159A/C165A, and C165A/C217A, this is not surprising because these receptors were barely detected at the cell surface in Sulfo-NHS biotinylation studies (Fig. 7, B and C).

Effects of the Remaining Double Cysteine Mutations at Residues C126, 132, 149, and 159 on ATP Potency and MTSEA-Biotin Binding at P2X₁ Receptors. The majority of adjacent double mutants that formed functional channels produced a further decrease in ATP potency indicating that these may have resulted in the breaking of two disulfide bonds. Residues C261 and C270 reduce surface expression, C117 and C165 do not liberate a free cysteine, and C217–C227 are likely to form a disulfide bond as the double mutation did not give an additional decrease in ATP potency. To investigate the pairings of the remaining conserved cysteine residues, we generated a second series of mutants containing the remaining combinations of C126, 132, 149, and 159 mutations (C126A/C149A, C126A/C159A, and C132A/C159A). These all formed functional channels with WT levels of P2X₁ receptor currents (Fig. 7A); none of these showed MTSEA-biotinylation (data not shown). For C126/C149A, ATP was more potent than for either of the individual point mutants; for C132A/C159A, ATP potency was either increased (C132A, $p < 0.01$) or unaffected (C159A) compared with individual mutants; and for C126A/C159A, ATP potency was either unaffected (C126A) or decreased (C159A, $p < 0.05$) compared with the single point mutants (see Table 1).

Double Mutants Incorporating C117 or C165 Result in Poor Surface Expression. The results from the “adjacent” double cysteine mutants indicated that double mutants incorporating C117A or C165A failed to produce functional channels and were barely detected at the cell surface (Fig. 7, A and B). We therefore made a series of further mutants incorporating C117A or C165A to determine whether these would form functional channels and a mutant that incorporated both these residues (C117A/C165A). Characterization of these mutants demonstrated that receptors incorporating C117A or C165A and either C126A, C149A, C159A, or C217A resulted in barely detectable levels of surface expression and

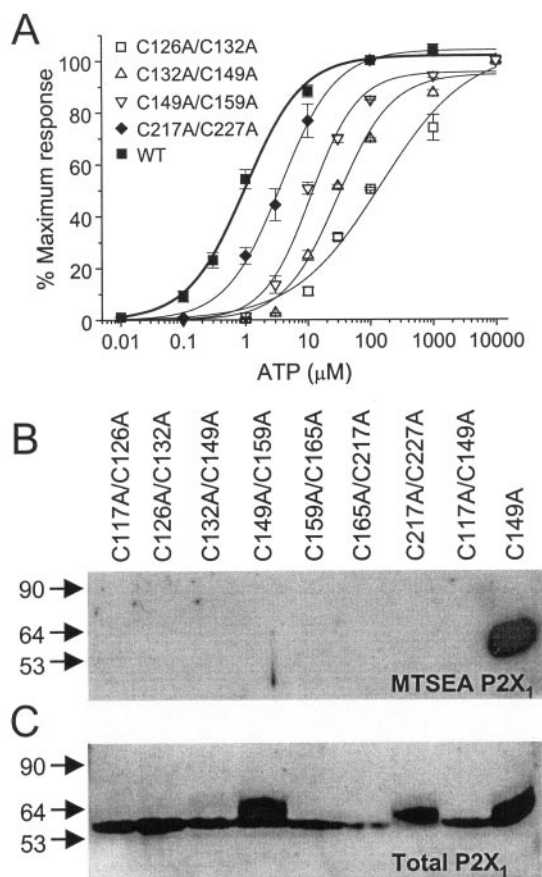


Fig. 6. Effects of double cysteine mutation on ATP potency and MTSEA-Biotin binding. A, ATP dose-response curves for functional double cysteine mutations. B, P2X₁ antibody probed Western blot of surface biotinylated proteins from supernatants of C149A and double-mutant-injected oocytes previously incubated in MTSEA-Biotin. No biotinylation occurs in any of the double cysteine mutations whereas the control single mutant C149A is biotinylated. C, total protein Western blot of the same samples as in B probed with anti P2X₁. Size markers in kilodaltons.

nonfunctional channels. However, in contrast, the double mutant C117A/C165A resulted in the formation of functional channels with normal current amplitude and only a decrease of ~5-fold in ATP potency compared with the WT receptor.

Discussion

The conservation of 10 cysteine residues in the extracellular loop of P2X receptors has led to speculation that these are involved in disulfide bond formation. The lack of effect of the reducing agents (β MME and DTT) on the properties of P2X₁ (this study) or P2X₂ (Rassendren et al, 1997) receptors suggested that disulfide bonds are either not present or play no major role in maintaining P2X receptor structure. However, disulfide bonds of mature proteins are often inaccessible to reducing agents unless the protein is denatured (Tatu et al, 1993); for example, the Kir2.1 channel (Cho et al, 2000) contains two interchain disulfide bonds that are essential for function; however, these receptors are unaffected by reducing

agents. To study directly the role of disulfide bonds, we used site-directed mutagenesis and electrophysiological and biochemical methods to show that cysteine residues do form disulfide bonds in the extracellular loop of the human P2X₁ receptor and that some of these bonds are essential for normal trafficking of the receptor to the cell surface.

The most striking effect of point mutation was observed for C261A and C270A receptors (>90% decrease in current peak amplitude). These mutant receptors are synthesized by oocytes but are inefficiently trafficked to the cell surface resulting in low levels of cell-surface receptor (Fig. 3) and low current amplitudes (Table 1). P2X₁ receptors form trimers in the ER, are glycosylated, and transported to the cell surface (Nicke et al, 1998; Rettinger et al, 2000). One possibility was that C261A and C270A mutation could have interfered with subunit assembly and this impaired membrane trafficking. Our studies with the cross-linking agent DTSSP, however, show that these mutants form complexes that probably correspond to P2X₁ homotrimers (Nicke et al, 1998). The relative proportion of monomer to trimer is the same as for WT channels and indicates that the reduced trafficking is not caused by inefficient formation of trimers. This is consistent with the finding that disulfide bonds between subunits are not required for subunit association (Nicke et al, 1998).

C261A and C270A mutants have an effect on the apparent molecular mass of P2X₁ subunits. The P2X₁ receptor can be glycosylated (Valera et al, 1995) at four sites, with each site increasing the apparent molecular mass by 2 to 3 kDa (Rettinger et al, 2000). For WT P2X₁ receptors, a smeared doublet band corresponding to fully (~57 kDa) and partially (~54 kDa) glycosylated forms of the receptor (Figs. 3 and 4) was detected. The apparent molecular mass of ~54 kDa for the C261A and C270A mutants suggests that they are missing a single N-linked oligosaccharide side chain. Glycosylation has been shown to play a role in trafficking of proteins from the ER to the cell surface (Fiedler and Simon, 1995). It is unlikely that the decrease in cell surface expression can be accounted for solely by the reduction in glycosylation as P2X₁ mutants lacking a single glycosylation site did not display a similar phenotype (Rettinger et al, 2000). The C261A and C270A mutations had little or no effect on the functional properties of the channels (ATP potency and time course of response) and suggest that there is no gross protein misfolding. Single C-to-A mutations would break individual disulfide bonds and mutation of either cysteine in the bond would be expected to have a similar effect on channel function. The almost identical phenotype of C261A and C270A mutants (decreased surface expression, reduction in glycosylation, and minimal changes in ATP potency) suggests that these residues normally form a disulfide bond that is essential for normal trafficking of the P2X₁ receptor to the cell surface.

Direct biochemical evidence for the existence of disulfide bonds in the extracellular loop of P2X receptors was provided using MTSEA-biotin. MTSEA-biotin did not bind to WT P2X₁ receptors, indicating that there are no free cysteine residues in the extracellular loop. Point mutation of a cysteine that normally forms a disulfide bond would break the bond leaving an accessible "free" cysteine residue. MTSEA-biotin binding was detected for six of the cysteine residues in the extracellular loop (C126, 132, 149, 159, 217, and 227) demonstrating that these residues are normally disulfide-bonded to partner cysteines.

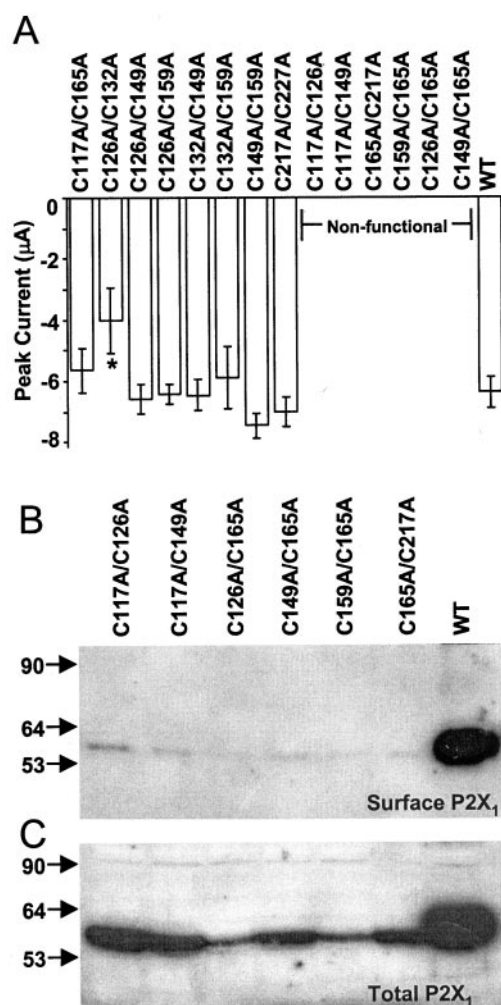


Fig. 7. NHS-LC-Biotin labeling of non functional double cysteine mutants shows inhibition of cell surface trafficking. **A**, peak current amplitudes recorded in cRNA injected *X. laevis* oocytes in response to a 1 mM application of ATP for WT and double cysteine mutations. **B**, Western blot of supernatants from oocytes previously incubated in NHS-LC-Biotin, which biotinylates primary amine groups on surface proteins. **C**, Western blot of total protein from samples shown in **B**. Note nonfunctional double mutations containing either C117A or C165A are not trafficked efficiently to the cell surface.

Double mutants were generated to investigate the pairing of cysteine residues by analysis of MTSEA binding and ATP potency. It was predicted that double mutation of cysteine residues that normally form disulfide bonds would result in loss of MTSEA-biotinylation and conversely that incorrect pairs of mutations would lead to an increase in MTSEA-biotinylation. However, MTSEA-biotinylation was not detected for any of the double mutants tested and this approach could not be used to assign disulfide bond pairing. There is some redundancy in the combination of double mutants that can be produced; for example, there are four ways that double-bond mutants can be generated in which two defined disulfide bonds can be broken. Because of the lack of discriminatory ability of this approach to determine disulfide pairings with the 14 double mutants we already made, we did not proceed with other combinations. Loss of binding could be accounted for in some of the mutants by the removal of normally paired residues; in the remaining cases, it is possible that the two freed cysteine residues form a new promiscuous disulfide bond; hence, no free cysteine residues are available for MTSEA-biotinylation. The spontaneous formation of disulfide bonds between cysteines that are not normally bonded in the mature protein configuration is a common feature during the folding process of proteins that have nonsequential cysteine pairing for disulfide bonds (Freedman, 1995). Analysis of ATP potency at the double mutants suggests that any new promiscuous bonds result in only a minor conformational change in protein structure and indicate that the residues that form new bonds are normally in relatively close proximity (see Fig. 8). The apparent close spatial proximity of cysteine residues C117, C126, C132, C149, C159, and C165 (Fig. 8B) raises the intriguing possibility that several of these residues may form a metal ion binding site, because P2X receptors are known to be sensitive to metal ions such as zinc (e.g., Michel and Humphrey, 1994). Cysteines form essential components of metal ion binding sites in many proteins (e.g., zinc finger motif transcription factors and metalloenzymes). However, none of the known consensus sequences for such motifs are present in the P2X receptors.

Phenotypic comparisons of single- and double-cysteine mutations have allowed us to speculate on the pattern of disulfide bonding. It was predicted that single point mutants corresponding to a disulfide pair would have similar properties and that when the correct pair of cysteines in a disulfide bond had been mutated, there would be no or a minimal further change in properties (because the disulfide bond had already been broken with the point mutant). In contrast, a double mutation corresponding to incorrect partners may result in a further change in phenotype (because of the breaking of two native disulfide bonds). The mutants C117A and C165A have essentially identical phenotypes: 1) a modest, ~2-fold decrease in ATP potency, 2) no effect on current amplitudes, 3) no detection of MTSEA-biotinylation, and 4) C117A/C165A mutants were functional but those incorporating C117A or C165A with other cysteine point mutations did not produce functional responses and showed poor surface expression levels. These results suggest that residues C117-C165 normally form a disulfide bond, however we have no direct evidence to support this. The lack of MTSEA-biotinylation for C117A or C165A mutants indicates that this disulfide bond is in a region of the receptor that is inaccessible to

this reagent (Fig. 8). The functional expression of the double mutant C117A-C165A showed that this putative disulfide bond is not essential; however, breaking this bond and an additional disulfide bond lead to intracellular retention of the P2X receptor protein.

Analysis of the double mutant C217A/C227A suggests that C217-C227 normally forms as a disulfide bond. Single point mutants of these residues produced the largest decrease in ATP potency (8- and 45-fold, respectively). In contrast, the C217A/C227A double mutant has a lesser effect on ATP potency (~4-fold decrease) and suggests that C217-C227 forms a disulfide bond in the WT receptor. One possible explanation for the double mutant phenotype compared with single mutants is that removal of the free cysteine residue and replacement of the polar side group (which could interact with negatively charged ATP) with nonpolar alanine partially recovers the WT phenotype. The attribution of remaining paired residues (C126, C132, C149, and C159) was made

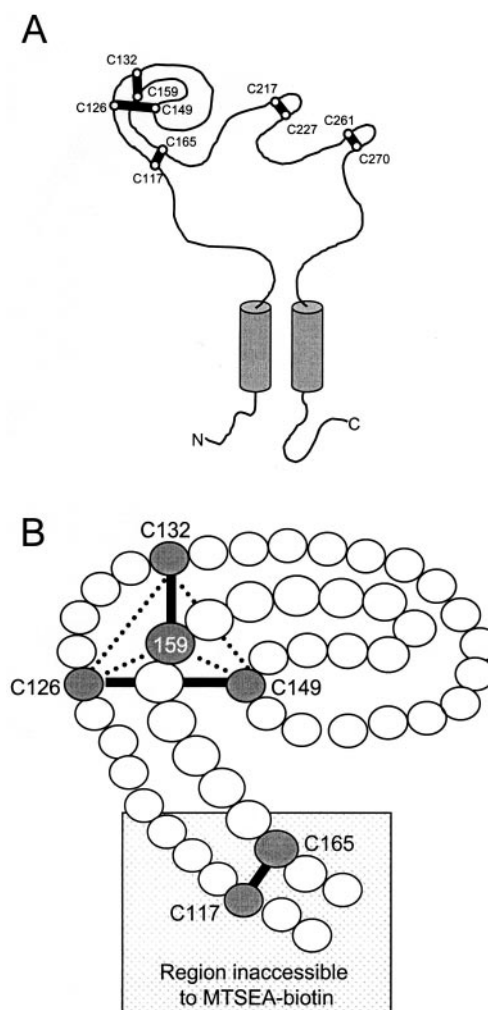


Fig. 8. Model of proposed disulfide bonds in the human P2X₁ receptor. A, line representation of the P2X₁ receptor showing the relative positions of disulfide bonds (thick, short black lines) between C117-C165, C126-C149, C132-C159, C217-C227, and C261-C270. Transmembrane domains are represented as shaded cylinders. B, protein "snake" diagram representing proposed binding of cysteines in the area between residues C117 to C165. Thick black lines represent proposed disulfide bonds in the wild-type human P2X₁ receptor. Dotted lines indicate spontaneous "incorrect" bonds that may form when two of the four cysteine residues C126, C132, C149, and C159 are mutated to alanine in the double mutations studied.

by analysis of the properties of the mutants. The ~50% reduction in peak current amplitude for both C132A and C159A coupled with the lack of additional effect on ATP potency of the double mutant C132A/C159A suggests that these residues normally form a disulfide bond. By deduction, this leaves C126-C149 forming a disulfide bond. This is further supported by the fact that the C126A/C149A double mutant did not produce an additional decrease in ATP potency compared with the single mutants. Other combinations of these four residues forming the WT complement of disulfide bonds are unlikely based on the results from double mutants. The double mutants C126A/C132A, C132A/C149A, and C149A/C159A produced an additional decrease in ATP potency (2- to 25-fold compared with single mutants and 10- to 100-fold compared with WT). This suggests that these mutations result from the breaking of two native disulfide bonds and possible promiscuous bonds, and that these residues do not correspond to paired disulfide bonded cysteine residues in the WT receptor.

In summary, we have provided biochemical evidence to demonstrate the existence of at least three disulfide bonds in the extracellular loop of the human P2X₁ receptor that are most likely to form in the pairs C126-149, C132-159, and C217-227. Our results also suggest that the remaining four cysteine residues conserved in the family of P2X receptors form two additional disulfide bonds (C117-165 and C261-270). Disruption of these bonds (either C261-270 alone or C117-165 and another bond) prevents efficient trafficking of P2X₁ receptors to the cell surface, suggesting that these bonds confer important tertiary structure. A model of the proposed disulfide bonds is shown in Fig. 8. This work provides the first insight into the folding/structural organization of the extracellular loop of this novel family of ligand-gated ion channels and provides essential constraints for modeling of the receptor structure.

Acknowledgments

We thank Jonathan Ritson for help in performing electrophysiological recordings.

References

- Boue-Grabot E, Archambault V, and Seguela P (2000) A protein kinase C site highly conserved in P2X subunits controls the desensitization kinetics of P2X₂ ATP-gated channels. *J Biol Chem* **275**:10190-10195.
- Brandle U, Spielmanns P, Osteroth R, Sim J, Surprenant A, Buell G, Ruppersberg JP, Plinkert PK, Zenner H-P, and Glowatzki E (1997) Desensitization of the P2X₂ receptor controlled by alternative splicing. *FEBS Lett* **404**:294-298.
- Brejc K, van Dijk WJ, Klassen RV, Schuurmans M, van der Oost J, Smit AB, and Sixma TK (2001) Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature (Lond)* **411**:269-276.
- Buell G, Lewis C, Collo G, North RA, and Surprenant A (1996) An antagonist-insensitive P2X receptor expressed in epithelia and brain. *EMBO (Eur Mol Biol Organ) J* **15**:55-62.
- Cho HC, Tsushima RG, Nguyen T-T, Guy HR, and Backx PH (2000) Two critical cysteine residues implicated in disulfide bond formation and proper folding of Kir2.1. *Biochem* **39**:4649-4657.
- Egan TM, Haines WR, and Voigt MM (1998) A domain contributing to the ion channel of ATP-gated P2X₂ receptors identified by the substituted cysteine accessibility method. *J Neurosci* **18**:2350-2359.
- Ennion S, Hagan S, and Evans RJ (2000) The role of positively charged amino acids in ATP recognition by human P2X₁ receptors. *J Biol Chem* **275**:29361-29367.
- Fiedler K and Simon K (1995) The role of N-glycans in the secretory pathway. *Cell* **81**:309-312.
- Fischer WH, Behan DP, Park M, Potter E, Lowry PJ, and Vale W (1994) Assignment of disulfide bonds in corticotropin-releasing factor-binding protein. *J Biol Chem* **269**:4313-4316.
- Freedman RB (1995) The formation of protein disulphide bonds. *Curr Opin Struct Biol* **5**:85-91.
- Garcia-Guzman M, Soto F, Gomez-Hernandez JM, Lund P-E, and Stuhmer W (1997) Characterization of recombinant human P2X₄ receptor reveals pharmacological differences with the rat homologue. *Mol Pharmacol* **51**:109-118.
- Michel AD and Humphrey PP (1994) Effects of metal cations on [³H]alpha, beta-methylene ATP binding in rat vas deferens. *Naunyn-Schmiedeberg's Arch Pharmacol* **350**:113-122.
- Jiang L-H, Rassendren F, Spelta V, Surprenant A, and North RA (2001) Amino acid residues involved in gating identified in the first membrane-spanning domain of the rat P2X₂ receptor. *J Biol Chem* **276**:14902-14908.
- Jiang L-H, Rassendren F, Surprenant A, and North RA (2000) Identification of amino acid residues contributing to the ATP binding site of a purinergic P2X receptor. *J Biol Chem* **275**:34190-34196.
- Khakh BS (2001) Molecular physiology of P2X receptor and ATP signalling at synapses. *Nat Rev Neurosci* **2**:165-174.
- Koshimizu T, Tomic M, Koshimizu M, and Stojilkovic SS (1998) Identification of amino acid residues contributing to the desensitization of the P2X₂ receptor channel. *J Biol Chem* **273**:12853-12857.
- Lesage F, Lauritzen I, Duprat F, Reyes R, Fink M, Heurteaux C, and Lazdunski M (1997) The structure, function and distribution of the mouse TWIK-1 K⁺ channel. *FEBS Lett* **402**:28-32.
- Migita K, Haines WR, Voigt MM, and Egan TM (2001) Polar residues of the second transmembrane domain influence cation permeability of the ATP-gated P2X₂ receptor. *J Biol Chem* **276**:30934-30941.
- Newbolt A, Stoop R, Virginio C, Surprenant A, North RA, Buell G, and Rassendren F (1998) Membrane topology of an ATP-gated ion channel (P2X receptor). *J Biol Chem* **273**:15177-15182.
- Nicke A, Baumert H, Rettinger J, Eichele A, Lambrecht G, Mutschler E, and Schmalzing G (1998) P2X₁ and P2X₃ receptors form stable trimers: a novel structural motif of ligand-gated ion channels. *EMBO (Eur Mol Biol Organ) J* **17**:3016-3028.
- North RA and Surprenant A (2000) Pharmacology of cloned P2X receptors. *Annu Rev Pharmacol Toxicol* **40**:563-580.
- Rassendren F, Buell G, Newbolt A, North RA, and Surprenant A (1997) Identification of amino acid residues contributing to the pore of a P2X receptor. *EMBO (Eur Mol Biol Organ) J* **16**:3446-3454.
- Rettinger J, Aschrafi A, and Schmalzing G (2000) Roles of individual N-glycans for ATP potency and expression of the rat P2X₁ receptor. *J Biol Chem* **275**:33542-33547.
- Robertson SJ, Ennion SJ, Evans RJ, and Edwards FA (2001). Synaptic P2X receptors. *Curr Opin Neurobiol* **11**:378-386.
- Simon J, Kidd EJ, Smith FM, Chessell IP, Murrell-Lagnado R, Humphrey PPA, and Barnard EA (1997) Localization and functional expression of splice variants of the P2X₂ receptor. *Mol Pharmacol* **52**:237-248.
- Smith FM, Humphrey PPA, and Murrell-Lagnado RD (1999) Identification of amino acids within the P2X₂ receptor C-terminus that regulate desensitization. *J Physiol* **520**:91-99.
- Tatu U, Braakman I, and Helenius A (1993) Membrane glycoprotein folding, oligomerization and intracellular transport: effects of dithiothreitol in living cells. *EMBO (Eur Mol Biol Organ) J* **12**:2151-2157.
- Valera S, Talabot F, Evans RJ, Gos A, Antonarakis SE, Morris MA, and Buell G (1995) Characterization and chromosomal localization of a human P(2X) receptor from the urinary bladder. *Recept Channels* **3**:283-289.

Address correspondence to: Dr. Richard Evans, Department of Cell Physiology and Pharmacology, University of Leicester, PO Box 138, Leicester, LE1 9HN UK. E-mail: rje6@le.ac.uk