Cellular/Molecular

Cysteine Substitution Mutants Give Structural Insight and Identify ATP Binding and Activation Sites at P2X Receptors

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P2X receptors for extracellular ATP are a distinct family of ligand-gated cation channels involved in physiological processes ranging from synaptic transmission to muscle contraction. Common ATP binding motifs are absent from P2X receptors, and the extent of the agonist binding site is unclear. We used cysteine-scanning mutagenesis, radiolabeled 2-azido ATP binding, and methanethiosulfonate (MTS) compounds to identify amino acid residues involved in ATP binding and gating of the human $P2X_1$ receptor. The pattern of MTSEA [(2-aminoethyl)methanethiosulfonate hydrobromide] biotinylation was also used to determine the accessibility of substituted cysteine residues and whether this changed on addition of ATP. Analysis of cysteine-substituted mutants of the last 44 amino acid residues (S286–I329) in the extracellular loop before the second transmembrane segment showed that N290, F291, R292, and K309 mutants had reduced ATP potency and 2-azido ATP binding. MTS reagents produced additional shifts in ATP potency at these residues, suggesting that they are directly involved in ATP binding; the effects were dependent on the charge of the MTS reagent at K309C; one explanation for this is that K309 interacts directly with the negatively charged phosphate of ATP. The remainder of the cysteine substitutions had little or no effect on ATP potency. However, at the mutants D316C, G321C, A323C, and I328C, MTS reagents did not change ATP potency but modified agonist-evoked responses, suggesting that this region may contribute to the gating of the channel.

Key words: ATP; P2X receptor; mutagenesis; binding; ion channels; agonist

Introduction

P2X receptors for ATP are ligand-gated cation channels and form from the homotrimeric or heterotrimeric assembly of seven different receptor subunits ($P2X_{1-7}$) to give a range of phenotypes (North, 2002). They are expressed widely in the nervous system and contribute to gut peristalsis (Ren et al., 2003), neuronal hypoxia reflexes (Rong et al., 2003), mechanotransduction (Cockayne et al., 2000), and pain sensation (Cockayne et al., 2000; Souslova et al., 2000; Tsuda et al., 2003; Chessell et al., 2005). P2X receptors do not contain common consensus ATP binding sequences [e.g., the Walker motif (Walker et al., 1982)] and the structure and organization of the binding site is unclear. A model incorporating four coordinates for ATP action at the $P2X_1$ receptor with positively charged lysines K68 and K309 coordinating binding of the phosphate tail of ATP and the adenine ring sandwiched between the domains incorporating phenylalanine (F) residues F185, T186 and N290, F291, and R292 with R292 coordinating the binding of the α-phosphate has been proposed (Vial et al., 2004b). This model is supported by mutagenesis data from $P2X_2$ (Jiang et al., 2000; Clyne et al., 2002) and $P2X_7$ (Worthington et al., 2002) receptors. In the model, the residues associated with ATP binding are at least 18–20 amino acids from the TM2

(second transmembrane segment) region that forms part of the ion-conducting transmembrane pore of the channel. The model predicts that the region between K309 and the second transmembrane segment contributes to the transduction of agonist binding to channel gating. However, to substantiate models of the receptor, it is also important to consider the contribution of adjacent nonconserved residues. The variation in agonist potency and other pharmacological properties between P2X receptor subtypes indicates that nonconserved amino acid residues contribute to drug action at the receptor and may lead to subunit-specific localized changes in the agonist binding environment or channel properties. To further address the role of N290, F291, R292, K309, and adjacent nonconserved amino acid residues and the contribution of the 20 amino acid region before the second transmembrane segment, we have undertaken substituted cysteine-scanning mutagenesis of the region S286–I329 of the human $P2X_1$ receptor. This approach has been used to study the structure of a range of ion channels and proteins (Akabas et al., 1992) including P2X receptors (Rassendren et al., 1997; Egan et al., 1998; Jiang et al., 2000, 2001; Haines et al., 2001) and the ATP binding site of Kir6.2 (Trapp et al., 2003). Analysis of effects of $P2X_1$ receptor-substituted cysteine mutants on the time course, ATP potency, binding of radiolabeled 2-azido ATP, regulation by charged methanethiosulfonate (MTS) compounds (Akabas et al., 1992), and determination of the accessibility of residues in an *N*-biotinylaminoethyl methanethiosulfonate (MTSEA-biotin) assay has allowed us to identify amino acid residues involved in ATP binding, channel properties, and information on the secondary structure of this section of the extracellular segment.

Materials and Methods

Site-directed mutagenesis. Cysteine point mutations for residues S286–I329 were introduced via the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) using a human P2X$_1$ receptor plasmid as the template, as described previously (Ennion et al., 2000). Production of the correct mutations and absence of coding errors in the P2X$_1$ mutant constructs was verified by DNA sequencing (Automated ABI Sequencing Service, University of Leicester).

Expression in Xenopus laevis *oocytes.* Wild-type (WT) and mutant constructs were transcribed to produce sense-strand cRNA (mMessage mMachine, Ambion, Austin, TX) as described previously (Ennion et al., 2000). Manually defolliculated stage V *X. laevis* oocytes were injected with 50 nl (50 ng) of cRNA using an Inject+Matic microinjector (J. A. Gaby, Inject+Matic, Genéva, Switzerland) and stored at 18°C in ND96 buffer (in mM: 96 NaCl, 2 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 5 sodium pyruvate, and 5 HEPES, pH 7.6). The medium was changed daily before recording 3–7 d later.

Electrophysiological recordings. Two-electrode voltage-clamp recordings (at a holding potential of −60 mV) were performed on cRNA-injected oocytes using a GeneClamp 500B amplifier with a Digidata 1322 analog-to-digital converter and pClamp 8.2 acquisition software (Molecular Devices, Menlo Park, CA), as described previously (Ennion et al., 2000). Native oocyte calcium-activated chloride currents in response to P2X receptor stimulation were reduced by replacing 1.8 mM CaCl$_2$ with 1.8 mM BaCl$_2$ in the ND96 bath solution. ATP (Mg salt; Sigma, Poole, UK) was applied via a U-tube perfusion system. Because of the large size of oocytes, it is difficult to apply solutions rapidly. For desensitizing responses, this results in the true time course of the response being underestimated as a result of the relatively slow solution exchange and activation of responses. Experiments on nondesensitizing P2X$_2$ receptors expressed in oocytes indicate that 10–90% solution exchange takes ∼300–500 ms and means the rise times of P2X$_1$ currents (∼100 ms for wild type) only give an indication of the speed of activation. The slower decay time of ∼1 s for P2X$_1$ WT currents is less likely to be affected by solution exchange and can be used to discriminate mutants with a change in time course. ATP (0.01 μM to 10 mM) was applied at 5 min intervals; using this regimen, reproducible ATP-evoked responses were recorded. Individual concentration–response curves were fitted with the Hill equation: $Y = [(X)^H \times M]/[(X)^H + (EC_{50})^H]$, where Y is the response, X is the agonist concentration, H is the Hill coefficient, M is the 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. Mutants that had considerably shifted ATP potency were tested with ATP concentrations up to 10 mM. For the calculation of EC_{50} values, individual concentration–response curves were generated for each experiment, and statistical analysis was performed on the pEC_{50} data generated. In the figures, concentration–response curves are fitted to the mean normalized data.

Characterization of the effects of MTS compounds. To study the effect of MTS compounds on ATP activation at WT and cysteine mutants, ATP (approximate EC_{90-95} concentration) was applied, and either MTSEA or sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES) (Toronto Research Chemicals, Toronto, Ontario, Canada) was bath perfused (for at least 5 min, the recovery time required between application to see reproducible responses) before coapplication with ATP via the U-tube. This procedure should allow MTS reagents to bind to free cysteine residues in either the nonactivated (absence of ATP) or ligand-bound activated (presence of ATP) state. MTS reagents (1 mM) were made in ND96 solution immediately before use. The effect of MTS reagents on the concentration responses to ATP was investigated by application of ATP in the presence of MTS reagents, or after incubating oocytes overnight in MTS reagents, and the following day, ATP concentrations were applied via the U-tube with ND96 bathing solution (no MTS reagents present); the same results were found with either method.

2-Azido ATP and P2X$_1$ receptors; patch-clamp recordings from human embryonic kidney 293 cells expressing P2X$_1$ receptors. Human embryonic kidney 293 (HEK293) cells and HEK293 cells subcloned after transfection with the human WT P2X$_1$ receptor (P2X$_1$cl-1 cells) were maintained in minimal essential medium with Earle's salts (with GlutaMAX I) sup-

plemented with 10% fetal bovine serum and 1% nonessential amino acid (Invitrogen, Paisley, UK) at 37°C in a humidified atmosphere of 5% CO$_2$ and 95% air as described previously (Vial et al., 2004a). HEK293 cells were transiently transfected with mutant P2X$_1$ receptors using Lipo-fectAMINE 2000 reagent/Opti-MEM (Invitrogen). For patch-clamp studies, as a control to identify cells that were efficiently transfected, the pEGFP-N1 (Clontech, Cambridge, UK) vector (10% of total plasmid transfected) was cotransfected with P2X$_1$ plasmids, and recordings were made only from fluorescent cells expressing the pEGFP. After 24 h, the cells were plated onto glass coverslips and kept in culture for a maximum of 3 d for use in patch-clamp experiments. HEK293 cells expressing WT or mutant P2X$_1$ receptors were incubated in the presence of 1 μM 2-azido ATP (Affinity Labeling Technologies, Lexington, KY). They were then placed 7.5 cm from a UV light source (Uvitech LF206.MS; Flowgen, Ashby Park, Leicestershire, UK) on the 312 nM setting and illuminated three times for 2 min with gentle mixing by swirling between exposures. Any effects of UV illumination in the presence of 2-azido ATP on subsequent ATP-evoked P2X$_1$ receptor-mediated currents were determined in whole-cell patch-clamp experiments as described previously (Vial and Evans, 2002). In preliminary studies, we showed that the UV irradiation protocol, in the presence or absence of ATP (1 μM), had no effect on the peak amplitude of ATP (1 μM)-evoked P2X$_1$ receptor currents.

^{32}P 2-Azido ATP radiolabeling of the P2X$_1$ receptor. To assess the effects of mutations on agonist binding, we used a ^{32}P 2-azido ATP binding assay. We used this for wild type, F289C, a mutant with no change in ATP potency, and the five mutants with the greatest decrease in ATP potency (G288C, N290C, F291C, R292C, and K309C). Five oocytes injected with WT or mutant receptor cRNA were incubated in the presence of 1 μM ^{32}P 2-azido ATP (Affinity Labeling Technologies). They were then placed 7.5 cm from a UV light source (Uvitech LF206.MS; Flowgen) on the 312 nM setting and illuminated three times for 2 min with gentle mixing by swirling between exposures. Oocytes were washed in ice-cold PBS, lysed in 100 μl of buffer H [100 mM NaCl, 20 mM Tris-Cl, pH 7.4, 1% Triton X-100, and 10 μl/ml protease inhibitor mixture (P8340; Sigma)], and centrifuged for 2 min at 16,000 × g. Supernatant (85 μl) was removed, and 0.9 μg of anti-P2X$_1$ antibody (Alomone Labs, Jerusalem, Israel) was added and incubated for 1 h on ice (it is unlikely that 2-azido ATP binding to the extracellular segment interferes with anti-P2X$_1$ receptor binding, because the epitope for this antibody is the final 16 amino acid residues of the intracellular C terminus of the receptor). Thirty microliters of protein A-Sepharose (40 mg/ml; Amersham Biosciences, Buckinghamshire, UK) were added and incubated overnight with rolling at 4°C. The Sepharose/antibody/protein complex was centrifuged for 2 min, and the beads were washed four times with buffer H. After the final wash, the Sepharose pellet was resuspended in SDS sample buffer and heated to 95°C for 5 min. Eluted P2X$_1$ receptors were loaded onto a 10% SDS-PAGE gel and run at 120 V. After electrophoresis, the gel was dried overnight and exposed to Hyperfilm film (Amersham Biosciences) with an intensifying screen at −80°C for 1 week, after which the film was developed. Densitometry analysis was performed on the resultant films with data corrected by background subtraction calculated for each lane and expressed as a percentage of wild type. We have assumed that the surface expression of wild type and the mutants used in the binding assay are equivalent because there were (1) no differences in the peak amplitude of ATP-evoked currents and (2) no differences in levels of total protein expression (see Fig. 2).

Western blotting. MTSEA-biotin was used to probe the various cysteine mutants to map the extracellular loop region S286–I329 of the P2X$_1$ receptor. MTSEA-biotin reacts covalently with sulfhydryl groups provided by accessible cysteine residues. The WT P2X$_1$ receptor contains no "free" cysteine residues (Ennion and Evans, 2002), and therefore an introduced cysteine would be free to bind MTS compounds if accessible.

Six oocytes injected with WT or mutant receptor cRNA were preincubated at room temperature for 30 min in 0.5 ml of ND96 solution containing either 15 U/ml apyrase (type III, A7646; Sigma) or 1 mM ATP. After the preincubation period, an additional 0.5 ml of ND96 was added containing either 15 U/ml apyrase and 2 mM MTSEA-biotin or 1 mM ATP and 2 mM MTSEA-biotin. Oocytes were incubated for an additional 30 min, after which they were washed twice in ND96 and lysed in buffer H at

20 μl per oocyte, as described previously. Lysates were centrifuged at 16,000 × g for 5 min, and the cleared lysate was removed from the cell debris pellet. Ten microliters of cleared lysate (1/12th sample) were taken for total P2X$_1$ protein analysis, and the remainder was mixed with 30 μl of 4% streptavidin–agarose beads (S1638; Sigma) and rolled overnight at 4°C. Beads were washed four times with buffer H, after which 20 μl of SDS-PAGE sample buffer was added to the beads and heated at 95°C for 5 min before loading on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose, probed with P2X$_1$ primary antibody (1:1000; Alomone Labs) and secondary goat anti-rabbit antibody (A6154, 1:2500 dilution; Sigma), developed using ECL Plus (Amersham Biosciences), and exposed to Hyperfilm film (Amersham Biosciences). Densitometry analysis was performed on scanned images of the resultant films (ImageJ, http://rsb.info.nih.gov/ij/). Analysis of P2X$_1$ WT and mutant MTSEA-biotinylated protein levels and the corresponding total P2X$_1$ protein band were determined (corrected for background gel levels). The level of biotinylation was calculated for apyrase and ATP treatment and corrected for the total P2X$_1$ receptor. For wild type, this was 0.58 ± 0.09% and reflects not only the level of biotinylation but also the efficiency of the streptavidin purification steps. A threshold value of biotinylation was set as three times the WT level of biotinylation, and data are shown as fold biotinylation relative to this threshold. The level of biotinylation in the presence of ATP was also expressed as a percentage of the biotinylation in the presence of apyrase in paired samples, and any effects were determined using a Student's paired two-tailed t test.

Analysis. All data are shown as mean ± SEM. Significant differences between WT and mutant current amplitudes, time courses, agonist potency, and MTS reagent modification were calculated by one-way ANOVA, followed by Dunnett's test for comparisons of individual mutants against control using the SPSS 12.0 for Windows package (SPSS, Chicago, IL). The significance of any changes in ATP potency by MTS compounds and the effects of ATP on MTSEA biotinylation at particular mutants were determined with the appropriate Student's t test. *n* corresponds to the number of oocytes tested for electrophysiological data and the number of experiments for biochemical studies.

Results

The effect of cysteine-scanning mutagenesis on human P2X$_1$ receptor ATP-mediated responses

To characterize the role of amino acid residues S286–I329 on ATP action at the P2X$_1$ receptor, we generated individual cysteine mutants and determined their effect on ATP potency (Fig. 1). WT human P2X$_1$ receptors responded to ATP in a concentration-dependant manner (EC$_{50}$ = ~0.9 μM). Oocyte recordings provide only an approximation of the true EC$_{50}$ because of the speed of solution exchanges and the non-steady-state nature of the desensitizing P2X$_1$ receptor current. Cysteine substitution was well tolerated, and functional responses were recorded from all of the mutant channels. Cysteine substitution at positions R295 and R305 produced small currents to ATP (10 mM) of 16 ± 4 and 94 ± 36 nA, respectively. R295C and R305C were poorly expressed at the cell surface, and they were unaffected by reducing agents (data not shown), suggesting the small amplitude of responses did not result from the formation of disulphide bonds; these mutants were not studied further. The point mutation R295A, however, had no effect on peak current amplitude or ATP potency (pEC$_{50}$ = 6.3 ± 0.1), and we have previously shown that R305A has normal ATP potency (Ennion et al., 2000). The majority (33 of 44) of cysteine substitution mutants showed no significant change in ATP potency (Table 1). A modest twofold to threefold increase in ATP potency was seen for two mutants, L318C and G321C (EC$_{50}$ values of ~0.3 μM); previously, we have shown that the G321A mutation has no effect on ATP potency (Digby et al., 2005). Approximate 5-fold decreases in ATP potency were recorded for F297C (EC$_{50}$ = 4.8 μM) and F311C (EC$_{50}$ = 6.2 μM), with ~10- and ~20-fold decreases in potency
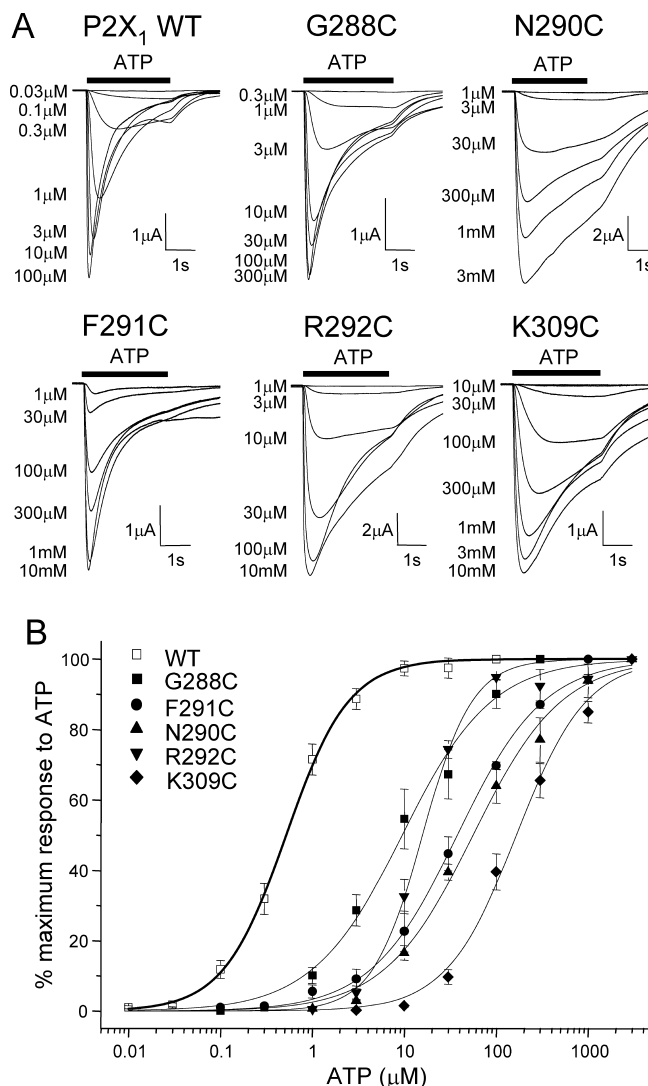

Figure 1. Effect of point cysteine P2X$_1$ mutations on ATP potency. ATP potency was tested on WT and P2X$_1$ receptor mutants expressed in oocytes by two-electrode voltage clamp (holding potential, −60 mV). ATP application is indicated by bar. ***A***, Trace data representative of currents observed for WT and mutant receptors in response to ATP applications at 5 min intervals. ***B***, Concentration–response curves to ATP for WT and mutants G288C, F291C, N290C, R292C, and K309C (*n* = 3–8). Error bars indicate SEM.

for G288C (EC$_{50}$ = 9.3 μM) and R292C (EC$_{50}$ = 15 μM). The largest change in ATP potency was at the mutants N290C, F291C, and K309C with a >50-fold decrease in agonist potency (EC$_{50}$ values of 64, 44, and 175 μM, respectively) (Table 1, Fig. 1). For the mutants N290C and F291C, this reduction in potency was associated with a decrease in Hill slope (1.3 ± 0.1, 0.9 ± 0.1, and 0.7 ± 0.1 for wild type and mutants, respectively; *p* < 0.01); however, in contrast, the Hill slope was increased for the R292C mutant (1.8 ± 0.2; *p* < 0.05).

A decrease in ATP potency could result from an effect on agonist binding and/or gating of the channel. To address this issue, we used a cross-linking assay with 2-azido ATP; this contains a photo-active azido (-N$_3$) group that is chemically inert until activation by UV light when a nitrene group is formed that reacts according to free-radical rules with any nearby part of the receptor. 2-Azido ATP is an agonist at the P2X$_1$ receptor, and on irradiation, a covalent linkage between the compound and the active site of the receptor is formed (given the desensitization of

Table 1. Summary of the effects of cysteine substitution

	pEC$_{50}$	Peak (nA)
P2X1 WT	6.1 ± 0.1	−8600 ± 740
S286C	6.1 ± 0.1	−10,600 ± 430
P287C	5.8 ± 0.1	−12,000 ± 310*
G288C	5.0 ± 0.1***	−10,600 ± 380
F289C	6.3 ± 0.2	−11,000 ± 350
N290C	4.2 ± 0.1***	−8100 ± 1400
F291C	4.4 ± 0.1***	−9500 ± 1200
R292C	4.8 ± 0.1***	−9700 ± 2200
F293C	5.7 ± 0.1	−10,000 ± 580
A294C	6.0 ± 0.1	−10,700 ± 1200
R295C	ND	−16 ± 4***
H296C	6.1 ± 0.2	−5400 ± 810*
F297C	5.3 ± 0.1***	−8030 ± 610
V298C	6.2 ± 0.02	−10,040 ± 1200
E299C	6.3 ± 0.1	−9700 ± 760
N300C	5.9 ± 0.1	−10,100 ± 450
G301C	5.8 ± 0.1	−4900 ± 480**
T302C	6.3 ± 0.1	−10,300 ± 730
N303C	6.3 ± 0.01	−14,000 ± 390***
Y304C	6.3 ± 0.1	−10,200 ± 1100
R305C	ND	−94 ± 36***
H306C	6.2 ± 0.02	−8400 ± 470
L307C	6.2 ± 0.04	−7700 ± 460
F308C	6.1 ± 0.2	−7600 ± 400
K309C	3.8 ± 0.1***	−7800 ± 810
V310C	6.0 ± 0.2	−10,400 ± 870
F311C	5.2 ± 0.1***	−9900 ± 750
G312C	5.7 ± 0.2	−9800 ± 810
I313C	5.9 ± 0.1	−9300 ± 940
R314C	5.8 ± 0.2	−8700 ± 420
F315C	6.1 ± 0.2	−9200 ± 690
D316C	5.7 ± 0.1	−10,900 ± 1200
I317C	6.2 ± 0.1	−7300 ± 880
L318C	6.5 ± 0.1*	−7900 ± 360
V319C	6.2 ± 0.1	−8300 ± 780
D320C	5.7 ± 0.01	−9500 ± 720
G321C	6.5 ± 0.1*	−3200 ± 960***
K322C	6.4 ± 0.1	−7000 ± 390
A323C	5.9 ± 0.2	−7700 ± 540
G324C	5.9 ± 0.1	−6900 ± 250
K325C	6.4 ± 0.04	−7600 ± 500
F326C	6.0 ± 0.2	−4800 ± 330*
D327C	6.1 ± 0.04	−5400 ± 310*
I328C	6.1 ± 0.1	−5800 ± 360
I329C	6.2 ± 0.1	−6500 ± 270*

pEC$_{50}$ values are shown as calculated from experimental data. pEC$_{50}$ is −log$_{10}$ of the EC$_{50}$. Peak values were taken on first application of a maximal concentration of ATP [for mutants with no change in ATP potency, this was 100 μM, a saturating concentration (see Fig. 1); for mutants with decreased ATP potency, this was 10 mM]. Conserved residues in five of seven P2X subtypes are highlighted in bold. ND, Not determined. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. $n = 3$–12.

Figure 2. Effect of P2X$_1$ cysteine mutants on ^{32}P 2-azido ATP binding. **A**, Total P2X$_1$ protein levels determined by Western blot analysis with P2X$_1$ antibody for WT and mutant receptors were similar. **B**, Mean peak currents to ATP for WT and mutant receptors showing no significant differences in amplitude. **C**, Autoradiograph depicting radiolabeled ^{32}P 2-azido ATP cross-linked to WT and mutant receptors [mean EC$_{50}$ for ATP (in micromolars) is shown in parentheses]. **D**, Densitometric analysis of autoradiographs for ^{32}P 2-azido ATP cross-linked to WT and mutant receptors. Mutant F289C (no change in EC$_{50}$) shows no change in the level of cross-linked ^{32}P 2-azido ATP compared with wild type. **$p < 0.01$; ***$p < 0.001$; $n = 6$. Error bars indicate SEM.

agonist-evoked responses in the continued presence of the agonist within 1–2 s, it is likely that 2-azido ATP cross-links to desensitized receptors). In ongoing studies on P2X$_1$ WT receptors expressed in HEK293 cells, we showed that UV irradiation, or incubation with 1 μM 2-azido ATP followed by a >5 min washout, has no effect on the amplitude of ATP-evoked currents (data not shown). However, UV irradiation in the presence of 1 μM 2-azido ATP, followed by a >5 min washout, reduced subsequent ATP currents (1 μM) by ~60% (2277 ± 588 and 770 ± 270 pA for control and UV irradiation in the presence of 2-azido ATP, respectively; $n = 7$ for each; $p < 0.05$). This indicates that the 2-azido ATP had cross-linked to the receptors, most likely locking them in an agonist-bound desensitized state, and blocked subsequent receptor activation. We therefore tested the effects of
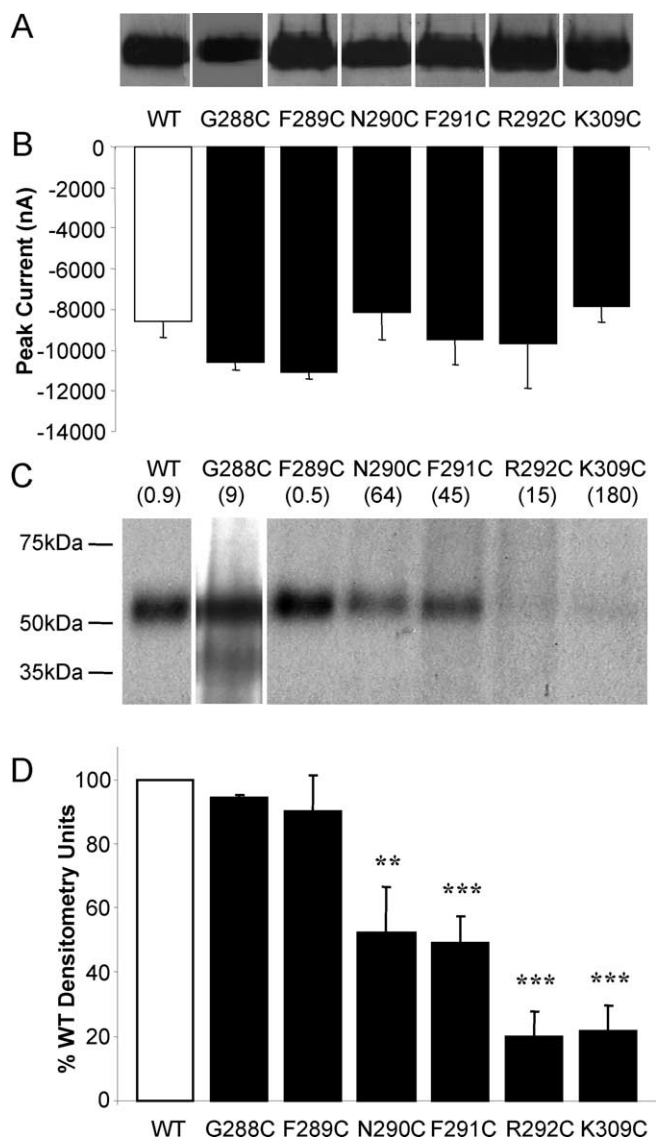
1 μM 2-azido ATP on ATP (EC$_{90}$ concentration)-evoked responses at the mutants G288C, N290C, F291C, R292C, and K309C. Irradiation in the presence of 2-azido ATP reduced by ~55% the ATP response at G288C (2524 ± 405 and 1158 ± 308 pA for control and 2-azido ATP irradiation; $n = 9$ for each; $p < 0.05$); this is a similar reduction as that of the WT and indicates there is little effect on 2-azido cross-linking to the G288C mutant channel (and consistent with the lack of effect of MTS reagents at this mutant; see below, Effects of MTS compounds on ATP responses at cysteine mutants). Irradiation in the presence of 2-azido ATP had no effect on the amplitude of subsequent responses to ATP at N290C, F291C, R292C, and K309C mutants (data not shown). These results suggest that there is a reduction in cross-linking of 2-azido ATP to the mutants N290C, F291C,
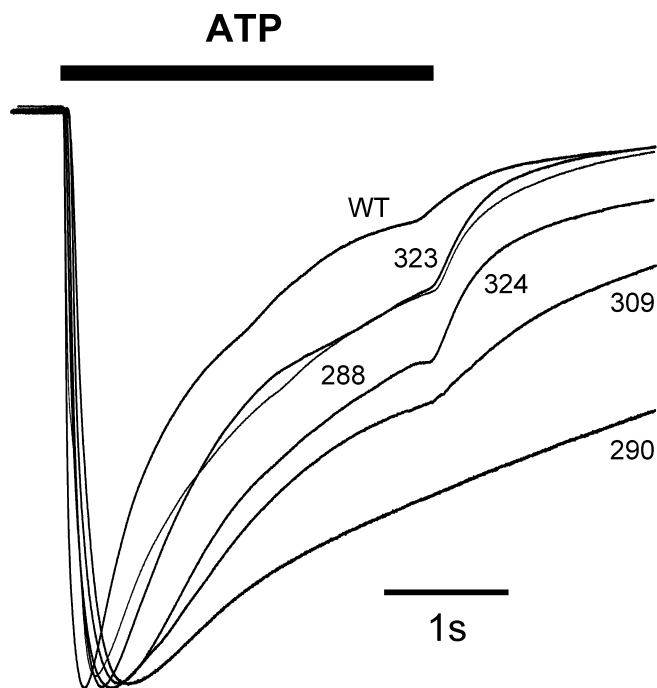
Figure 3. Effect of P2X$_1$ mutations to cysteine on the time course of ATP action. Representative recordings depicting the time course of P2X$_1$ receptor currents evoked in response to a maximal concentration of ATP (application period indicated by bar) for WT and mutants with an increased EC$_{50}$ (G288C, N290C, and K309C) or an unchanged EC$_{50}$ (A323C and G324C) are shown.

R292C, and K309C and would be consistent with a decrease in agonist binding at these mutants. We therefore sought to test whether this could be measured directly using radiolabeled ^{32}P 2-azido ATP.

WT and mutant P2X receptors expressed in *Xenopus* oocytes were incubated with radiolabeled ^{32}P 2-azido ATP and exposed to UV light. Oocytes were lysed, and P2X$_1$ receptors were immunoprecipitated (to remove background nonspecific binding), run on a gel, and exposed to film. Levels of 2-azido ATP binding were compared with wild type for the five mutants with the greatest shifts in ATP potency. The autoradiographs show a single band at ~55 kDa corresponding to P2X$_1$ receptors. Wild type and the mutants had the same level of total P2X$_1$ receptor and peak amplitude currents (Fig. 2), indicating that the receptors are expressed at equivalent levels and a reduction in intensity of the band corresponds to a decrease in binding. Radiolabeling at wild type and the mutants G288C (10-fold decrease in ATP potency) and F289C (no change in ATP EC$_{50}$) was detected at similar levels; in contrast, binding to the mutants N290C and F291C was reduced by >50% and by ~80% for R292C and K309C (Fig. 2). The lack of effect of the G288C mutation on radiolabeling suggests that this cysteine substitution reduced ATP potency by an effect on the gating of the receptor (and this is consistent with the lack of modification by MTS reagents compared with adjacent the residues N290C, F291C, and R292C; see below). For the mutants N290C, F291C, R292C, and K309C, there is no simple relationship between the reduction in the amount of 2-azido ATP binding and the decrease in agonist potency at the receptors. The ~80% reduction for R292C and K309C suggests that the change in potency at these receptors results predominantly from a decrease in agonist binding. The increase in Hill slope for the R292C mutant also indicates a contribution from effects on conformational changes and gating of the receptor. At N290C and F291C, although these showed a greater decrease in agonist potency than
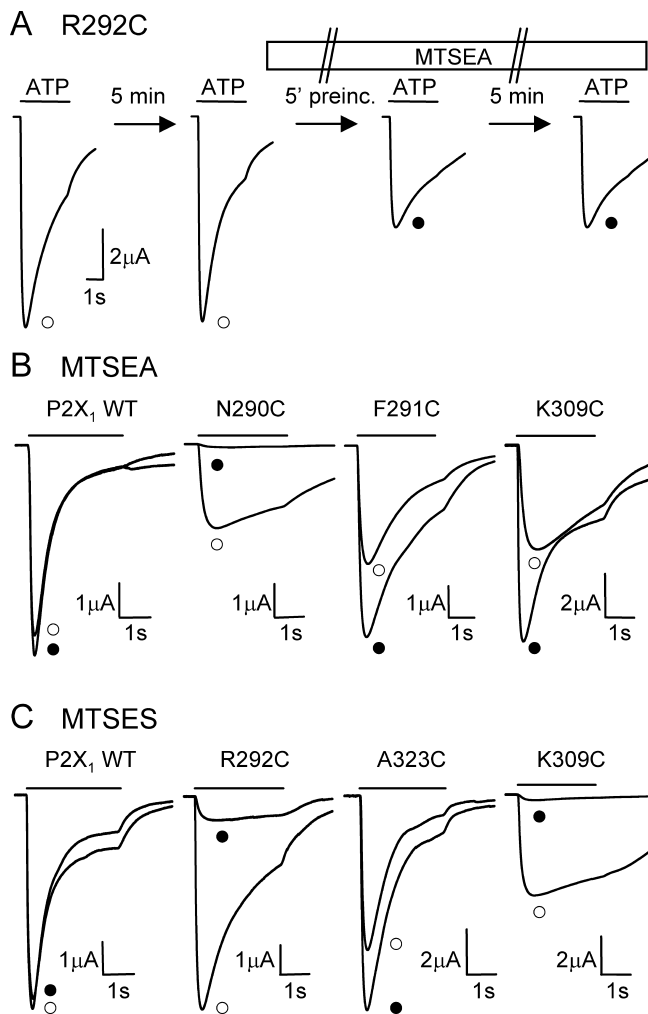

Figure 4. Effect of MTS compounds on ATP responses of P2X$_1$ cysteine mutants. *A*, Protocol for testing of MTS compounds; at 5 min intervals, reproducible responses to ATP were recorded (open circles). The oocytes were then perfused with MTS reagent (1 mM), and at 5 min intervals, ATP was coapplied with the MTS reagent (filled circle). The traces are shown for the R292C mutant and the application of MTSEA. *B*, Representative recordings of ATP-evoked responses from P2X$_1$ wild type and the mutants N290C, F291C, and K309C under control conditions (open circles) and after application of MTSEA (filled circles; 1 mM for 5 min in the absence of ATP then coapplied with ATP). *C*, Representative recordings collected for P2X$_1$ WT and mutants R292C, A323C, and K309C under control conditions (open circles) and following application of MTSES (filled circles; 1 mM for 5 min in the absence of ATP then coapplied with ATP).

R292C, they did not show as great a reduction in radioligand binding. The ~40% reduction in 2-azido ATP cross-linking and the decrease in Hill slope for the mutants N290C and F291C suggest that the reduction in their potency results from an ~50% decrease in agonist binding and with the additional decrease in potency attributable to an effect on channel gating. These results show directly for the first time that mutation of residues N290, F291, R292, and K309 decreased agonist binding at P2X receptors.

WT P2X$_1$ receptor channels open rapidly and desensitize during the continued presence of ATP (time for the peak response to decay by 50% is 986 ± 56 ms for responses to 100 μM ATP, a maximal concentration). The time course of the response gives a measure of the transduction steps after agonist binding (i.e., channel gating), as does the deactivation step on washout of ATP. For the majority of cysteine substitutions, there was no change in the time course of decay of the response during continued ATP
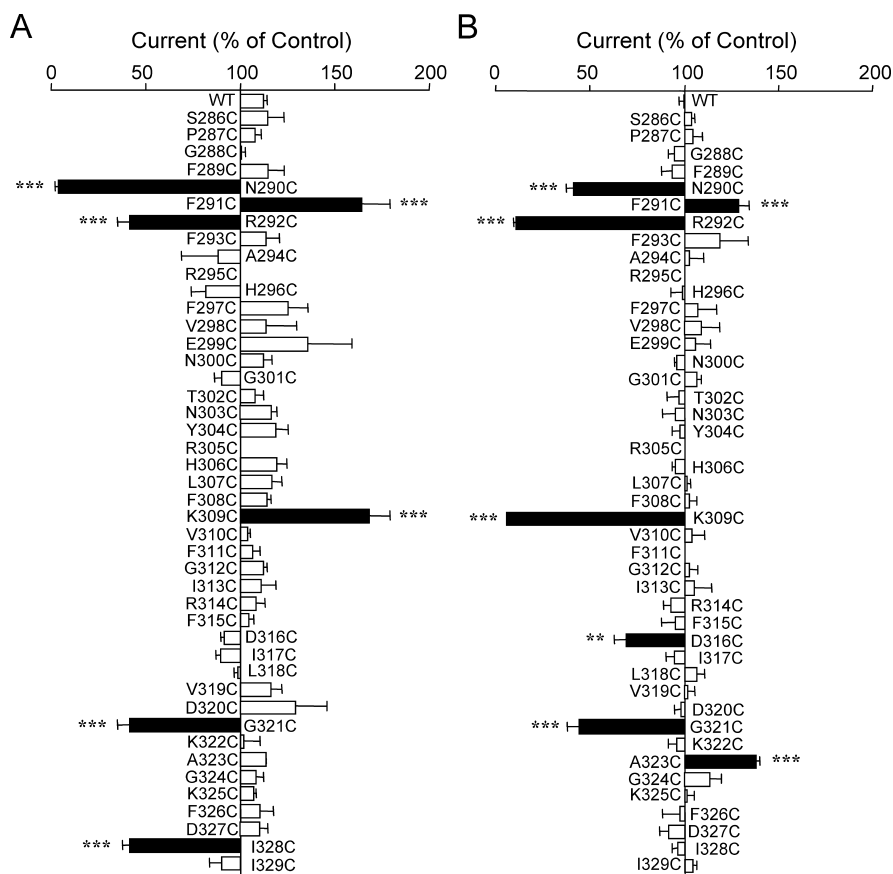
Figure 5. Summary of effects of MTS compounds on ATP responses of P2X$_1$ cysteine mutants. **A**, Effect of MTSEA (1 mM) on cysteine-scanning mutants S286 –I329 of the human P2X$_1$ receptor ($n = 3–4$). **B**, Effect of MTSES (1 mM) on cysteine-scanning mutants S286 –I329 of human P2X$_1$ ($n = 3–4$). **$p < 0.01$; ***$p < 0.001$. Error bars indicate SEM.

application (maximal ATP concentration). Mutants with a change in time course could be divided into two categories: (1) mutants G288C, N290C, and K309C with decreased ATP potency showed a prolongation in the decay of the evoked current (2669 ± 165, 2907 ± 158, and 2198 ± 353, respectively); and (2) mutants A323C and G324C had no effect on ATP potency but doubled the decay time (2125 ± 281 and 2022 ± 212, respectively) (Fig. 3). These results suggest that A323 and G324 contribute to the gating of the P2X$_1$ receptor with mutation of these residues to cysteine leading to prolonged channel opening before entering the desensitized state.

Effect of MTS compounds on ATP responses at cysteine mutants

For P2X receptors, the ability to introduce charge using MTS reagents may be a useful tool because (1) the ATP molecule has negative charge associated with the phosphate tail; (2) in physiological solutions, magnesium is complexed with ATP, resulting in localized positive charge; and (3) the channel is selective for cations. We have therefore tested the ability of positively charged MTSEA and negatively charged MTSES to modify ATP-evoked responses at WT and mutant P2X$_1$ receptors. For these protocols following establishment of reproducible control responses, MTS reagents (1 mM) were applied for the 5 min between ATP applications as well as being coapplied with ATP (5 min is required between ATP applications to allow for recovery from receptor desensitization). This should allow the effects of MTS reagents at

any accessible cysteine residues either in the nonliganded (MTS reagent application in the absence of ATP) or ATP-bound (MTS reagent in the presence of ATP) receptors to be determined.

At WT P2X$_1$ receptors, ATP (30 μM)-evoked responses were potentiated slightly by positively charged MTSEA (1 mM; $112 \pm 2\%$ of the control response) (Figs. 4, 5). This effect is unlikely to be at a free cysteine residue in the receptor, because the 10 cysteine residues in the extracellular segment are considered to form five disulphide bonds (Clyne et al., 2002; Ennion and Evans, 2002), and is most likely to be a nonspecific effect. At the majority of cysteine mutants, MTSEA had no effect on the amplitude of responses compared with WT channels (Fig. 5). A reduction in the amplitude of ATP responses after MTSEA (1 mM) treatment was observed for the mutants N290C, R292C, G321C, and I328C (MTSEA reduced the responses to 4 ± 2, 42 ± 7, 41 ± 7, and $41 \pm 4\%$ relative to the control ATP response, respectively) (Figs. 4, 5). In contrast, at mutants F291C and K309C, the addition of MTSEA potentiated ATP-evoked responses to 164 ± 15 and $168 \pm 10\%$ relative to control, respectively. In all cases, the maximal effects of MTSEA on the amplitude of ATP responses were seen in 5–10 min (one to two ATP applications; ATP was applied at 5 min intervals).

Negatively charged MTSES (1 mM) had no effect on the amplitude of ATP-evoked responses at WT P2X$_1$ receptors ($100 \pm 3\%$ of control response) and the majority of individual cysteine-substituted mutants (Figs. 4, 5). However, ATP responses at the mutants N290C, R292C, K309C, D316C, and G321C were all significantly inhibited by 1 mM MTSES (41 ± 4, 10 ± 1, 6 ± 0.2, 69 ± 6, and $44 \pm 6\%$ relative to the control ATP response, respectively). F291C and A323C were both significantly potentiated (129 ± 5 and $138 \pm 2\%$ relative to control, respectively). In all cases, the maximal effects of MTSES on the amplitude of ATP responses were seen in 5–10 min of MTSES application (ATP was applied at 5 min intervals).

The modification of some mutant P2X$_1$ receptor responses highlights a contribution of these residues to ATP action at the receptor. The amplitude of P2X$_1$ responses could be regulated by a change in the ATP potency of the receptor and/or an effect on channel gating and ionic permeation. To establish whether a change in ATP potency was involved, we determined the effect of MTS reagents on the concentration–response curves. For this, MTS compounds were present in the superfusion solution and concomitantly applied with ATP. For mutants D316C (pEC$_{50}$ = 5.7 ± 0.07 and 5.9 ± 0.22 for control and MTSES, respectively), G321C (pEC$_{50}$ = 6.5 ± 0.07, 6.8 ± 0.20, and 6.4 ± 0.18 for control, MTSEA, and MTSES, respectively), A323C (pEC$_{50}$ = 5.9 ± 0.17 and 6.1 ± 0.05 for control and MTSES, respectively), and I328C (pEC$_{50}$ = 6.1 ± 0.15 and 6.2 ± 0.13 for control and MTSEA, respectively), the MTS compounds had no effect on ATP potency, demonstrating that at these mutants, the MTS

compounds were not acting to interfere with ATP binding to the receptor but were having an effect on channel gating or ionic permeation. In contrast, at residues N290C, F291C, R292C, and K309C, MTS compounds changed the potency of ATP at the receptors. At N290C and R292C, MTSES and MTSEA decreased ATP potency by ~18- and 5-fold, respectively ($p < 0.005$ for pEC_{50} values), whereas MTSEA potentiates at F291C by 5-fold ($p < 0.005$). The similar modulatory effects of positively charged MTSEA and negatively charged MTSES at N290C (inhibition), F291C (potentiation), and R292C (inhibition) receptors suggest that they result from the introduction of the bulk of the side groups and that localized charges at these residues do not contribute to ATP action. At K309C, the effects of the MTS compounds were dependent on the charge. ATP potency was increased ~20-fold after incubation with positively charged MTSEA (the MTSEA-modified cysteine residue is of similar size and charge to the lysine residue and has been used to rescue function of a lysine mutation in the intracellular domain of the P2X$_4$ receptor (Fountain and North, 2006) ($p < 0.0005$ for pEC_{50} values), and responses even to a maximal concentration of ATP (10 mM) were abolished by negatively charged MTSES. This suggests that a charge at position K309 is important for mediating the binding of ATP. MTS reagents react irreversibly with cysteine residues, and overnight incubation with MTS compounds had the same effects on ATP potency as bath application (Fig. 6) and can be used to reduce the amount of costly MTS reagents used.

MTSEA-biotin accessibility at P2X$_1$ WT and cysteine mutants

MTS compounds have identified substituted cysteine residues that contribute to ATP action at the P2X$_1$ receptor. For residues in which MTS compounds have no effect, it is unclear whether this is because the residue does not contribute to the actions of ATP at the receptor or whether the residue is inaccessible. To help discriminate between these two possibilities, and gain some experimental evidence for the secondary structure of the extracellular loop, we have determined directly whether MTSEA-biotin reacts with individual cysteine point mutants of the P2X$_1$ receptor. The MTSEA-biotin molecule is like a ball and chain with an ~0.5 × 0.7 nm planar head group, and the reactive disulphide is near the end of a flexible chain (~1.7 × 0.5 nm) incorporating the MTSEA (~1.0 × 0.5 nm; estimated using SwissPDBViewer). Residues that react with MTSEA-biotin must therefore be accessible through an opening at
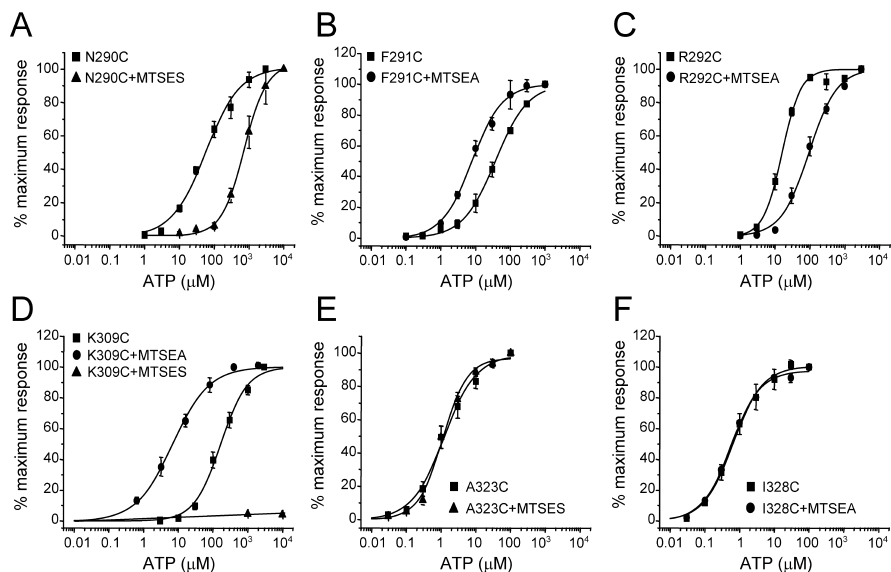

Figure 6. Effect of MTS compounds on ATP potency at cysteine mutants. *A*, At N290C, MTSES decreased ATP potency. *B*, At F291C, MTSEA (1 mM) application increases ATP potency. *C*, At R292C, ATP potency was decreased by overnight incubation with MTSEA (1 mM). *D*, Overnight incubation with MTSEA (positive charge, 1 mM) increased ATP potency at K309C, whereas overnight incubation with MTSES (negative charge, 1 mM) abolished the response. *E*, *F*, At A323C and I328C, ATP potency is unaffected by incubation with MTSES and MTSEA, respectively (1 mM for each; $n = 3–6$). Data are shown for overnight incubations with MTS reagent that gave the same results as bath-applied MTS reagents. Error bars indicate SEM.
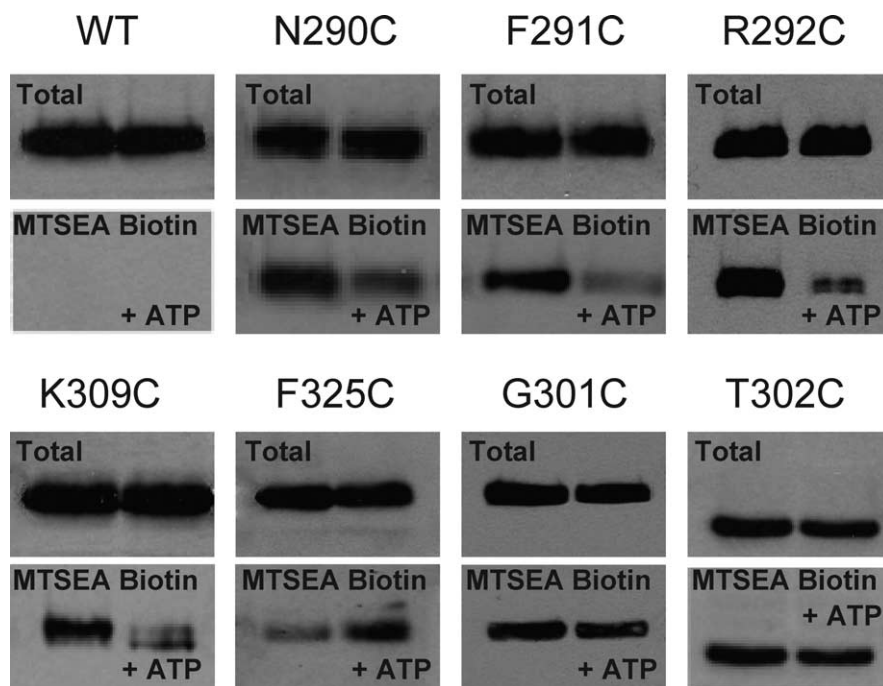

Figure 7. MTSEA-biotin labeling of mutant and WT P2X$_1$ receptors. Oocytes expressing WT and mutant P2X$_1$ receptors were incubated in MTSEA-biotin, which biotinylates free cysteine residues. Biotin-labeled proteins were isolated with streptavidin–agarose beads, and the resultant proteins were run on SDS-PAGE, blotted for P2X$_1$ receptor, and compared with total levels of receptor expression. In control conditions, apyrase was added to break down any endogenously released ATP. The degree of MTSEA biotinylation was also determined in the presence of ATP. There is a reduction in the molecular mass of T302C because this mutation removes a consensus sequence for glycosylation (N300G301T302); a similar reduction was also seen for N300C (not shown).

least 0.5 nm in diameter and >2.2 nm deep widening to at least 0.7 nm (to incorporate the biotin). MTSEA-biotin can be used to differentiate water-exposed cysteines from cysteines buried within a protein structure, because only the water-exposed cysteines react at an appreciable rate.

MTSEA biotinylation was detected by Western blotting with an anti-P2X$_1$ receptor antibody after isolation of biotinylated proteins with streptavidin–agarose beads (Fig. 7); any endogenous ATP released from the oocytes was broken down by incubations in the presence of apyrase. At WT P2X$_1$ receptors, very low levels of biotinylated protein were detected as reported previously (Ennion and Evans, 2002), consistent with the 10 cysteine residues in the extracellular loop forming five disulphide bonds (Clyne et al., 2002; Ennion and Evans, 2002). A threshold for positive biotinylation was set at more than three times the WT background level (Figs. 7, 8). For the majority of substituted cysteine residues (36 of 42), MTSEA biotinylation was detected above threshold, demonstrating that these substituted cysteine residues are accessible at the receptor surface. For N300C–T302C, this corresponds to a site of golgi-mediated glycosylation consistent with surface accessibility (Rettinger et al., 2000; Roberts and Evans, 2006). The degree of biotinylation was dependent on the position of substitution, indicating that the local environment or accessibility is variable across this region of the receptor. We also determined whether the level of biotinylation was changed by stimulation with ATP (Figs. 7, 8).

ATP reduced MTSEA biotinylation for cysteine-substituted residues in the regions S286–E299, Y304, F308C–D316C, and L318–D320. In these cases (with the exception of N290F291R292 and K309 cysteine mutants), there was little or no effect on ATP potency. One explanation for these changes in MTSEA biotinylation could be that localized charge in the binding pocket associated with the bound ATP interfered with MTSEA biotinylation. However, when either positively (MTSEA) or negatively charged (MTSES) reagents are applied to these mutants, they had no effect on ATP potency (supporting that they do not contribute directly to the agonist binding site) and suggest that at these positions, ATP does not directly interfere with MTSEA biotinylation. An alternative is that the decrease in MTSEA biotinylation at these residues results from a conformational change in the extracellular loop of the P2X$_1$ receptor associated with agonist binding making the substituted cysteine residues less accessible. A conformational change is also supported by the increase in MTSEA biotinylation in response to ATP for K325C, a residue close to the predicted pore region of the P2X receptor channel. Such a conformational change associated with agonist binding could be similar to the "clam shell" closure of the agonist-binding pocket of ionotropic glutamate receptors (Jin et al., 2003). If the decrease in MTSEA biotinylation does result from a conformational change in the P2X$_1$ receptor, this gives us a first glimpse of how agonist binding leads to structural changes in the extracellular ligand-binding loop of the receptor. It is tempting to speculate that the lack of change by ATP application to MTSEA biotinylation in the region N300C–N303C while the adjacent regions showed marked decreases suggests that this region could act as a stable point or hinge around which conformation changes occur bringing together or stabilizing the proximity of the N290F291R292 and K309 residues that we suggest contribute to the ATP binding site.

Barely above threshold levels of biotinylation were detected for H306C, R314C, G321C, A323C, and F326C (either in the presence or absence of ATP), suggesting that there is restricted accessibility of MTSEA-biotin at these positions. Very low levels equivalent to wild type were detected for K322C (either in the presence or absence of ATP), indicating that this residue is buried within the protein or MTSEA is unable to bind. Overall, the MTSEA biotinylation results indicate regions of the extracellular loop that are readily accessible on the surface of the protein can be used to develop models of the extracellular segment of the recep-
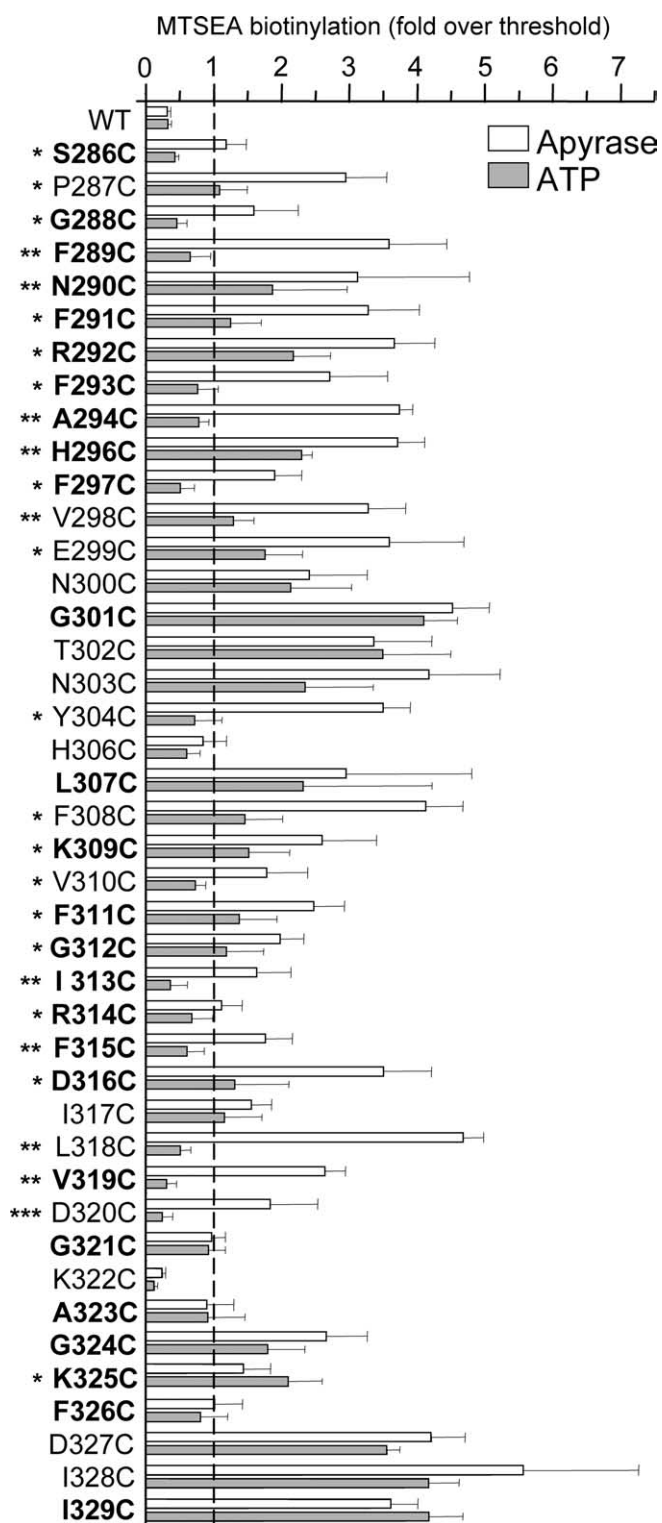

Figure 8. Summary of MTSEA-biotin labeling of P2X$_1$ mutants and WT receptor and the effects of ATP stimulation. A summary of the level of MTSEA biotinylation in the presence of apyrase and ATP is shown. MTSEA biotinylation was calculated as the biotinylation intensity expressed as the percentage of total P2X$_1$ receptor levels (for wild type or the particular mutant) from expression in the same batch of oocytes. A threshold for biotinylation is shown (dashed line) at more than threefold the basal WT level. Significant changes in the level of biotinylation in response to ATP application were determined with the paired Student's t test. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $n = 3–5$. Residues in bold are conserved in at least five of the human P2X receptor subunits. Error bars indicate SEM.

tor and give an insight into conformational changes after receptor activation.

Discussion

The majority of cysteine mutants of the 44 amino acids before the second transmembrane segment had little (4 mutants) or no (33 mutants) effect on ATP potency and were unaffected by MTS reagents. Several of these mutants react with MTSEA-biotin, indicating their accessibility, but MTSEA had no effect on ATP responses (Fig. 9). This allows us to exclude an essential role of these residues in mediating ATP action at the P2X$_1$ receptors. We identified residues where cysteine substitution (1) reduced currents and expression at the cell surface (R295C and R305C); however, mutants R295A and R305A (Ennion et al., 2000) had no effect on ATP potency, indicating that individual positive charges at these residues are not essential; (2) decreased ATP potency, 2-azido ATP binding, and modification by MTS reagents (K309C, R292C); (3) decreased ATP potency, modification by MTS reagents, a modest reduction in 2-azido ATP binding, and a reduction in Hill slope (N290C and F291C); or (4) had little or no effect on ATP potency and modification of amplitude by MTS reagents (D319C, G321C, A323C, and I328C).

Mutations in the extracellular domain could have effects on the binding of agonist to the receptor and/or the conformation change after agonist binding that controls activation of the channel (Colquhoun, 1998). The ~200-fold decrease in ATP potency at K309C is consistent with previous alanine-scanning mutagenesis of this residue in P2X$_1$ (Ennion et al., 2000), P2X$_2$ (Jiang et al., 2000), and P2X$_7$ (Worthington et al., 2002) receptors. Several features of this mutation are indicative of an effect on agonist binding. For the 200-fold reduction in potency to result from a gating effect, this would require an ~40,000-fold reduction in gating constant with two molecules of ATP, and considerably higher if three bind to activate the channel. Because there is no reduction in the maximal response, gating would have to be highly efficacious at the WT receptor, which is unlikely given the low probability of single P2X channel openings (Ding and Sachs, 1999). Similarly, there is no detectable reduction in the Hill slope that would be expected if there were a change in gating. The ~80% reduction in the amount of radiolabeled 2-azido ATP cross-linked to the mutant K309C receptor further supports a decrease in agonist binding. The rescue of potency with positively charged MTSEA suggests that K309 is involved in binding of the negatively charged phosphate group of the ATP molecule. Finally, the
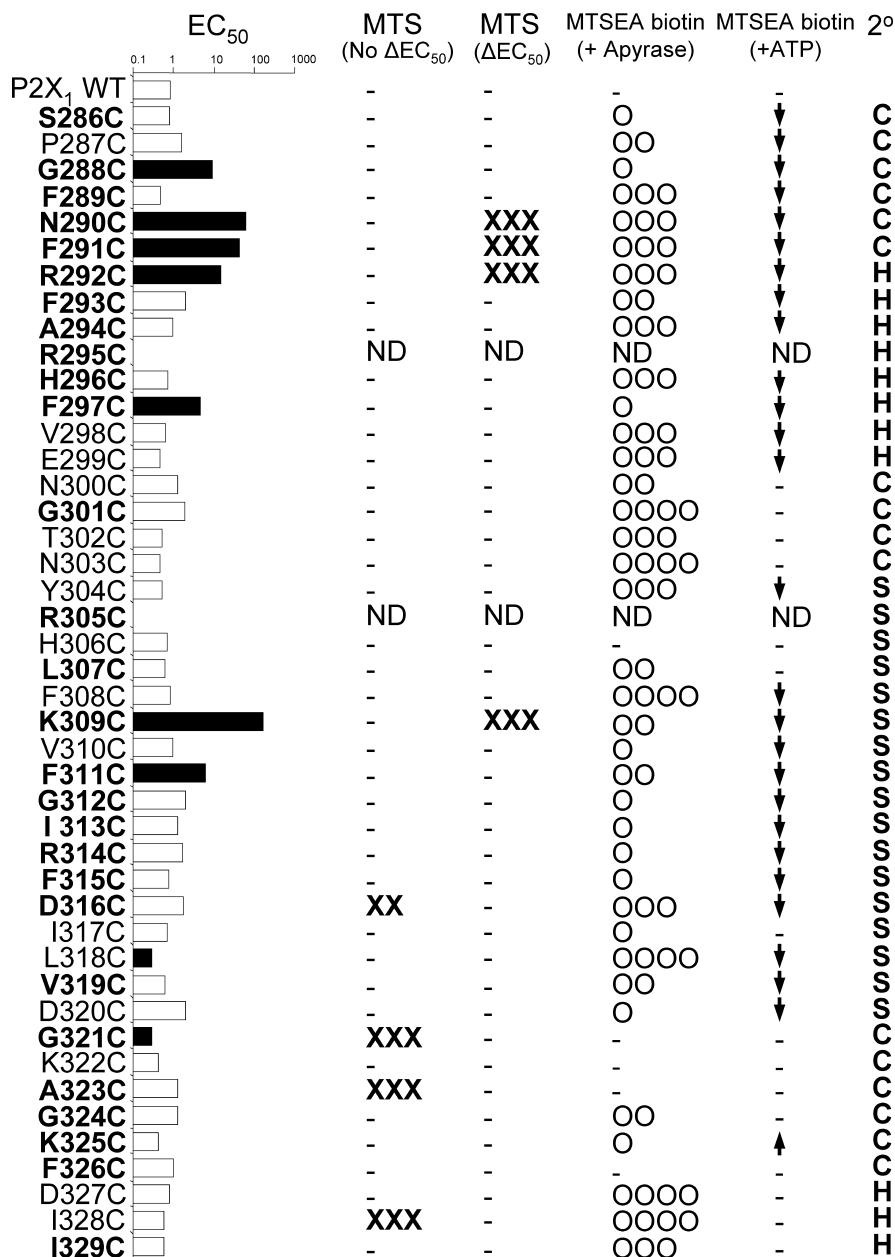

Figure 9. Summary of the effects of cysteine mutants on ATP potency, modification by MTS reagents, and MTSEA biotinylation. Mutants with a significantly changed EC$_{50}$ compared with wild type are highlighted (black bar). Those mutants affected by MTS compounds are depicted as XX (25–50% change) and XXX (>50% change) and are divided into mutants with ATP potency that was affected by MTS reagents and mutants that were not. Relative levels of MTSEA biotinylation are shown in the presence of apyrase: ○, one to two times threshold; ○○, two to three times threshold; ○○○, three to four times threshold; ○○○○, more than five times threshold. n = 3–5. Significant decreases and increases in MTSEA biotinylation on ATP application are shown. ND, Not determined. Structural predictions for the region S286 –I329 [PSIPRED (McGuffin et al., 2000)] are labeled C (coil), H (helix), and S (strand). Residues in bold are conserved in at least five of the human P2X receptor subunits.

binding of MTSEA-biotin to the free cysteine residue introduced in the K309C mutant receptor was reduced by ATP application. Together, these results support the idea that lysine residue 309 forms part of the ATP binding site of the P2X receptor.

The decreased ATP potency of the cysteine mutations N290C, F291C, and R292C is consistent with previous alanine-scanning mutagenesis studies (Ennion et al., 2000; Jiang et al., 2001; Roberts and Evans, 2004, 2006). The decrease in 2-azido ATP cross-linking and MTSEA biotinylation by ATP application indicate that these residues contribute to the ATP binding site. At N290C

and R292C, ATP potency was further decreased by MTSEA or MTSES. In contrast, at F291C, ATP potency increased toward the WT levels after treatment with MTS reagents. The lack of discrimination between the effects of positive and negatively charged MTS compounds suggests that it is the localized disruption associated with binding of the MTS reagent, and not the charge, that modifies ATP potency. This indicates that it is unlikely that the positive charge of R292 coordinates binding of negatively charged phosphate on ATP, as suggested previously (Ennion et al., 2000; Vial et al., 2004b). The asparagine-phenylalanine-arginine (NFR) motif is totally conserved in P2X receptors, and we have suggested that the aromatic phenylalanine is involved in binding of the adenine ring of ATP (Roberts and Evans, 2004); this is consistent with residues involved in ATP binding at other proteins (see below). In addition, the current results suggest that this motif is involved in agonist-induced conformational changes at the P2X$_1$ receptor.

A model of the C-terminal half of the extracellular segment of the P2X$_4$ receptor was proposed based on similarity to the tRNA synthetases (Freist et al., 1998), and this has been supported by some mutagenesis studies (Yan et al., 2005). However, the residues suggested to form the P2X$_4$ receptor binding site are different from those identified for the P2X$_1$ receptor (Stojilkovic et al., 2005), suggesting that there may be differences in ATP action between P2X receptor subunits.

Analysis of the local ATP binding environment in a number of crystal structures identified a similar pattern of residues involved in ATP binding to P2X$_1$ receptors, as at rat synapsin II (Roberts et al., 2006) with an asparagine-aromatic-positive charge [asparagine-tryptophan-lysine (NWK)] motif with the aromatic group and positive charge coordinating the binding of the adenine and ribose groups of ATP [Protein Data Bank code li7I (Berman et al., 2000)]. An additional aromatic residue in synapsin II was associated with the adenine ring, and this is consistent with our previous work showing that F185 mutants decrease ATP potency (Roberts and Evans, 2004). Three positively charged lysine residues are also key components of the ATP binding environment at synapsin II, and these could correspond to K68, K70, and K309 that we have proposed to coordinate ATP binding (Ennion et al., 2000). The interface between subunits has been shown to be important for zinc binding in P2X$_2$ receptors (Nagaya et al., 2005) and ATP binding in P2X$_{2/3}$ receptors (Wilkinson et al., 2006), and recent work on P2X$_1$ receptors shows that a disulphide bond can be formed between adjacent subunits of cysteine residues introduced at positions K68 and F291 (Marquez-Klaka et al., 2007). Based on these findings, we suggest a revised working model of the binding site at the P2X$_1$ receptor (Fig. 10).

MTSEA biotinylation has provided a useful assay to determine the surface accessibility of introduced cysteine residues. The lack (K322C) or threshold levels of MTSEA biotinylation (S286C, H306C, R314C, G321C, A323C, and F326C) suggests that these residues are either inaccessible (K322C) or give restricted access to MTSEA-biotin. In contrast, the majority of substituted cysteine residues showed MTSEA biotinylation with the relative levels giving an indication of relative accessibility. Of the MTSEA-biotinylated cysteine residues, only the N290F291R292 motif and K309 cysteine mutants showed changes in ATP potency after MTS application. MTSEA biotinylation at these residues was also reduced by ATP application. This would be consistent with ATP competing with the MTSEA-biotin for occupation of the agonist binding site and further supports the close association of the NFR motif and K309 with the ATP binding site.

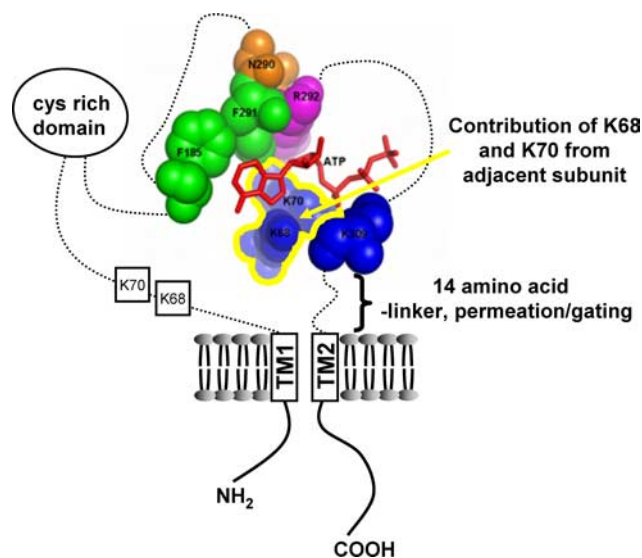The cysteine mutants D316C, G321C, A323C, and I328C were

Figure 10. Model of ATP action at P2X$_1$ receptors. A schematic model of the P2X$_1$ receptor shows the two transmembrane domains (TM1 and TM2), intracellular N termini (NH$_2$) and C termini (COOH), and the large extracellular ligand-binding loop. The P2X$_1$ receptor ATP-binding domain is based on mutagenesis data and similarity to the binding environment of ATP in the crystal structure of rat synapsin II [Protein Data Bank code 1i7l (Berman et al., 2000)]. Residues N290, F291, and R292 form a highly conserved motif across the P2X family, and mutagenesis studies confirm its role in ATP action (Ennion et al., 2000; Roberts and Evans, 2004; Roberts and Evans, 2006; present study). A similar motif (NWK) is found in rat synapsin II and also forms part of the ATP binding environment. Positively charged lysine residue K309 is thought to interact with the negatively charged phosphates of ATP in the P2X$_1$ receptor, and lysine is also present in the ATP binding environment of rat synapsin II. There are two additional lysine residues in the local binding environment of synapsin II that could correspond to K68 and K70 identified previously as having a role in ATP action at P2X$_1$ receptors (Ennion et al., 2000). A recent study (Marquez-Klaka et al., 2007) indicates that these are likely to come from an adjacent subunit, and these residues are shown outlined in yellow. The cysteine-rich (cys rich) domain C117–C165 is likely to contain three disulphide bonds: C117–C165, C126–C149, and C132–C159 (Ennion and Evans, 2002). The ATP binding region of the receptor is linked to opening of the channel by a 14 amino acid gating linker; mutagenesis of this region regulates the time course of the response with little or no effect on ATP potency.

sensitive to MTS reagents. At P2X$_2$ receptors (Rassendren et al., 1997), MTS reagents had no effect on residues equivalent to the conserved residues G321C and A323C, suggesting that constraints imposed by nonconserved parts of the receptor contributed to actions of MTS compounds at the P2X$_1$ receptor. At D316C and G321C, both MTSEA and MTSES inhibited responses indicating that introduction of bulk and not charge mediated the inhibition. At A323C, only negatively charged MTSES had an effect, and at I328C, only positively charged MTSEA was effective and suggests that the adjacent charged K322 and D327 residues contribute to efficacy of the MTS compounds at these residues. The MTSEA biotinylation of residues G324C, K325C, and D327C–I329C also gives an insight into the minimum dimensions and accessibility of this region of the channel (see above) that has been suggested to contribute to the outer vestibule of the channel pore (Rassendren et al., 1997).

The second transmembrane segment forms part of the P2X receptor channel pore and starts at about residue P330. Individual cysteine mutants in the region D316–I329 had little or no effect on ATP potency. MTS reagents modified responses at D316C, G321C, A323C, and I328C mutants with no effect on ATP potency; in addition, the mutants A323C and G324C showed a slowing in the time course of desensitization (Fig. 3). These results suggest that this region of the receptor does not contribute directly to ATP binding and reduction in amplitude of

ATP responses at D316C, G321C, and I328C mutants by MTS compounds results from a decrease in ATP efficacy. This reduction in efficacy suggests that these residues are likely to be involved in regulation of ionic permeation or channel gating (Fig. 10). This is similar to the cys-loop ligand-gated ion channels and glutamate ion channels where the ligand binding site is distant from the transmembrane segments and residues close to the transmembrane segments link agonist binding to channel opening (Czajkowski, 2005) and supports recent work on P2X$_4$ receptors (Yan et al., 2006).

In summary, this study has provided new insight into the molecular basis of ATP action at P2X receptors, the direct experimental evidence for the ATP binding site, and the accessibility of the C-terminal portion of the extracellular segment and has aided in the refining of a model of agonist action at this novel family of ATP-binding proteins.

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