The Role of Positively Charged Amino Acids in ATP Recognition by Human P2X<sub>1</sub> Receptors*

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P2X receptors for ATP are a family of ligand-gated cation channels. There are 11 conserved positively charged sites in the extracellular loop of P2X receptors. We have generated point mutants of these conserved residues (either Lys → Arg, Lys → Ala, Arg → Lys, or Arg → Ala) in the human P2X<sub>1</sub> receptor to determine their contribution to the binding of negatively charged ATP. ATP evoked concentration-dependent (EC<sub>50</sub> ~ 0.8 μM) desensitizing responses at wild-type (WT) P2X<sub>1</sub> receptors expressed in Xenopus oocytes. Suramin produced a parallel rightward shift in the concentration response curve with an estimated pK<sub>s</sub> of 6.7. Substitution of amino acids at positions Lys-53, Lys-190, Lys-215, Lys-325, Arg-202, Arg-305, and Arg-314 either had no effect or only a small change in ATP potency, time course, and/or suramin sensitivity. Modest changes in ATP potency were observed for mutants at K70R and R292K/A (20- and 100-fold decrease, respectively). Mutations at residues K68A and K309A reduced the potency of ATP by >1400-fold and prolonged the time course of the P2X<sub>1</sub> receptor current but had no effect on suramin antagonism. Lys-68, Lys-70, Arg-292, and Lys-309 are close to the predicted transmembrane domains of the receptor and suggest that the ATP binding pocket may form close to the channel vestibule.

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1 The abbreviations used are: PCR, polymerase chain reaction; WT, wild-type; TBST, Tris-buffered saline with Tween 20; PAGE, polyacrylamide gel electrophoresis.
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, 5 mM HEPES, pH 7.6) prior to recording for 3–7 days later.

Electrophysiological Recordings—Two-electrode voltage clamp recordings were made from oocytes using a GeneClamp 500A amplifier (Axon Instruments). Microelectrodes were filled with 3 M KCl and the external solution consisted of ND96 with 1.8 mM BaCl₂ replacing the 1.8 mM CaCl₂ in order to prevent activation of endogenous calcium-activated chloride channels (2). Membrane currents were recorded at a holding potential of −60 mV and were acquired using a Digidata 1200 analog to digital converter with pClamp 7 acquisition software (Axon Instruments). ATP (magnesium salt, Sigma) was applied from a nearby U-tube perfusion system (24), whereas the antagonist suramin (Bayer) was bath-perfused and also present at the appropriate concentration in the U-tube application of ATP. Repeated exposures of agonist were separated by 5 min in order to allow recovery from receptor desensitization. Non-injected and water-injected oocytes tested from at least seven separate batches of oocytes (>50 oocytes) gave no detectable currents in response to ATP application (range: 100 μM to 1 mM). Data are presented as mean ± S.E. Differences between means were tested using Student’s paired t test. Concentration response data were fitted with the equation $Y = (\text{E}_{\text{max}} \times \text{X}^H) / (\text{EC}_{50} + \text{X}^H)$ where $Y$ = response, $X$ = agonist concentration, $H$ is the Hill coefficient, $M$ is maximum response, and $\text{EC}_{50}$ is the concentration of agonist evoking 50% of the maximum response. $\text{pEC}_{50}$ is the $-\log_{10}$ of the EC50 value. The concentration ratio for suramin (3 μM) was determined (EC50 value for ATP in suramin/EC50 value for ATP with no suramin) and used to estimate antagonist potency; $\text{pK}_A$ estimate = $-\log_{10}$ concentration ratio $- 1$ $- \log_{10}$[suramin concentration]. For the mutants K68A and K309A, concentration-response curves did not reach a plateau at the highest ATP concentration tested (10 μM).

**Protein Expression Analysis**—Expression levels of mutant receptors, which were either nonfunctional or gave very low peak currents, were estimated by Western blot analysis and biotinylation of cell surface proteins. For Western blot analysis, oocytes injected previously with 50 ng of cRNA were homogenized in buffer H (100 mM NaCl, 20 mM Tris-Cl, pH 7.4, 1% Triton X-100, 10 μM protease inhibitor mixture Sigma P8340) for 15 min. After centrifugation at 16,000 g for 2 min, a 7.5-μl aliquot of the supernatant was mixed 50:50 with gel sample buffer (50 mM Tris-Cl, pH 6.8, 10% glycerol, 2% SDS, 10% glycerol), heated for 2 min at 90 °C, and separated on a 10% SDS-PAGE gel. The gel was transferred to nitrocellulose and blocked in 5% milk powder overnight. The membrane was incubated with anti-P2X 1 antibody (1:500 dilution) (Alamone, Israel) in TBS+ 5% milk powder, washed for 1.5 h, washed three times for 5 min in TBST, and incubated with anti-rabbit horseradish peroxidase secondary antibody (1:1000 dilution) (Sigma) for 40 min. After three 5-min washes in TBST, visualization of the protein bands was achieved with an ECL (Plus) kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. In order to confirm that synthesized mutant receptors had been processed correctly and transported to the cell membrane of the oocyte, biotinylation of all surface proteins with Sulfo-NHS-LC-Biotin (Pierce) was performed. The protein bands were visualized with an ECL (Plus) kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. In order to confirm that synthesized mutant receptors had been processed correctly and transported to the cell membrane of the oocyte, biotinylation of all surface proteins with Sulfo-NHS-LC-Biotin (Pierce) was performed. The protein bands were visualized with an ECL (Plus) kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. In order to confirm that synthesized mutant receptors had been processed correctly and transported to the cell membrane of the oocyte, biotinylation of all surface proteins with Sulfo-NHS-LC-Biotin (Pierce) was performed. The protein bands were visualized with an ECL (Plus) kit (Amersham Pharmacia Biotech).
ability of ATP to evoke functional responses, its potency, is the result of two steps: 1) the binding of ATP to the receptor (affinity) and 2) the ability of bound ATP to open the ion channel (efficacy). Therefore, in the present study, we refer to the potency of ATP and can only make inferences about the effects of the mutations on the affinity of ATP for the receptor.

To investigate the role of conserved lysine residues in the activation of P2X<sub>1</sub> receptors by ATP, lysine to arginine (to maintain the positive charge; Fig. 2) or lysine to alanine (to neutralize the positive charge; Fig. 3) point mutants were generated and tested (Table I). Point mutants at conserved lysines 53, 215, and 325 either had no effect (K215R, K325R (Fig. 2, A and B), K325A) or only a small (~2-fold) increase in ATP potency (K53A/R and K215A). K190R resulted in a small (~2-fold) decrease in ATP potency. However, when the positive charge was removed at this position (K190A), the potency was decreased by ~5-fold (Fig. 3). Mutations at K70 resulted in either an ~5- or ~18-fold decrease in ATP potency for K70A and K70R (EC<sub>50</sub> values for ATP were ~4 and 15 µM, respectively). The conservative substitution K309R (Fig. 2, A and B) resulted in a modest ~25-fold decrease in ATP potency (EC<sub>50</sub> = 20 µM); however, when the positive charge was removed, there was a more substantial ~1400-fold decrease in ATP potency (EC<sub>50</sub> > 1.2 mM, Fig. 3, A and B). This suggests that both the charge and chemical properties of the lysine residue are important at this position. The point mutation K68A resulted in a substantial ~1800-fold decrease in ATP potency (EC<sub>50</sub> > 1.5 mM, Fig. 3, A and B). The K68R P2X<sub>1</sub> receptor did not form functional channels, although the protein was expressed at the membrane (see “P2X Receptor Expression Analysis”)

There are three conserved arginine residues in the extracellular loop of P2X receptors, and at the position equivalent to amino acid 202 there is either an arginine or a lysine residue. These residues have been individually mutated to either lysine or alanine residues (Fig. 4, Table I). Mutations at residues

| Mutant | ATP EC<sub>50</sub> | 50% time | Peak I | Suramin |
|--------|------------------|----------|--------|---------|
| WT     | 6.10 ± 0.05      | 0.8 ± 0.15| 6240 ± 703| 6.72 ± 0.07 |
| K35R   | 5.94 ± 0.06*     | 0.3 ± 0.02*| 5718 ± 594| 6.30 ± 0.13* |
| K68R   | NA               | NA       | NA     | NA      |
| K70R   | 4.83 ± 0.03**    | 0.9 ± 0.06| 5708 ± 817| 7.62 ± 0.44** |
| K190R  | 5.79 ± 0.07**    | 3.2 ± 0.27**| 6796 ± 368| 6.88 ± 0.03  |
| K215R  | 6.11 ± 0.05      | 0.5 ± 0.12| 5471 ± 640| 7.13 ± 0.06** |
| K309R  | 4.70 ± 0.05**    | 6.5 ± 0.77**| 2490 ± 606| 7.47 ± 0.05** |
| K325R  | 6.07 ± 0.09      | 0.5 ± 0.07| 6451 ± 508| 6.63 ± 0.04  |
| K53A   | 5.87 ± 0.13      | 0.5 ± 0.07| 3887 ± 305*| 6.40 ± 0.13* |
| K68A   | 2.86 ± 0.08**    | 74.6 ± 8.99**| 1129 ± 132**| 6.91 ± 0.09* |
| K70A   | 5.40 ± 0.03**    | 1.7 ± 0.22**| 7643 ± 427**| 8.68 ± 0.09  |
| K190A  | 5.37 ± 0.03**    | 2.3 ± 0.17**| 6355 ± 404| 8.63 ± 0.08  |
| K215A  | 5.83 ± 0.07**    | 0.4 ± 0.05| 3726 ± 418*| 6.48 ± 0.06* |
| K309A  | 2.95 ± 0.03**    | 9.7 ± 0.52**| 2431 ± 304**| 6.76 ± 0.04* |
| K325A  | 6.03 ± 0.05      | 0.9 ± 0.12| 5884 ± 328| 6.69 ± 0.10  |
| K202K  | 6.01 ± 0.07      | 1.0 ± 0.10| 5206 ± 530| 6.88 ± 0.10  |
| K292K  | 4.01 ± 0.04**    | 6.6 ± 0.99**| 4123 ± 840| 6.73 ± 0.13  |
| K305K  | 5.77 ± 0.05**    | 0.9 ± 0.20| 1310 ± 125**| NA         |
| K314K  | 5.92 ± 0.09      | 0.5 ± 0.16| 3867 ± 267*| 8.67 ± 0.11  |
| K202A  | 5.65 ± 0.04**    | 1.1 ± 0.13| 7242 ± 741| 6.43 ± 0.03**|
| K292A  | 4.15 ± 0.07**    | 2.8 ± 0.48**| 7142 ± 315| 6.39 ± 0.03* |
| K305A  | 4.53 ± 0.04**    | 0.4 ± 0.02| 104 ± 12**| 6.60 ± 0.08  |
| K314A  | 5.53 ± 0.05**    | 0.4 ± 0.02*| 4601 ± 373| 6.84 ± 0.05  |

* Full concentration-response relationships could not be constructed in the presence of suramin; pK<sub>A</sub> was therefore estimated from concentration ratio of EC<sub>50</sub>.  
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Arg-202, Arg-305, and Arg-314 resulted in either no effect on the EC<sub>50</sub> for ATP (R202K, R314K) or only a small ~2–3-fold decrease in ATP potency (R202A, R305K, R305A, R314A). Mutation of Arg-292 to either lysine or alanine resulted in an ~90–120-fold decrease in ATP potency at the P2X<sub>1</sub> receptor. 

Effects of Mutations on the Time Course of P2X<sub>1</sub> Receptor Responses—Responses of WT P2X<sub>1</sub> receptors to ATP were transient and decayed during continued agonist application; this was particularly noticeable at high agonist concentrations where the response to 10 µM ATP (a concentration that evoked 90% of the maximum response; EC<sub>50</sub>) decayed to 50% of the peak response in 0.85 s (Fig. 5, Table I). When constructing the concentration-response curves for mutant P2X<sub>1</sub> receptors, it was apparent that the time course of the currents evoked by ATP at some of the mutant receptors was affected (Figs. 2–4). To quantify this we determined the time from peak current to 50% decay after an EC<sub>90</sub> concentration of ATP was applied (adjusted for each mutant receptor) (Table I, Fig. 5). The decay of mutant K53R, K305A, and R314A P2X<sub>1</sub> receptor mediated responses was approximately twice as fast as for WT P2X<sub>1</sub> receptors. The mutations K53A, K70R, K215R, K215A, K325R, K325A, R202A, R202K, R305K, and R314K had no effect on the time course of the P2X<sub>1</sub> receptor current. A small ~3-fold decrease in the rate of decay of P2X<sub>1</sub> receptor currents was seen for the point mutants K70A, K190R, and K190A. The mutants K309R, K309A, and K292 showed a modest 8–12-fold increase
in the time course of the P2X1 current. The most marked change was seen for the K68A P2X1 receptor mutant where there was an ~90-fold increase in the time to 50% decay of the response to ~75 s (Fig. 5).

**Effects of Mutations on Antagonism by Suramin**—The P2 receptor antagonist suramin (3 μM) resulted in a parallel shift (16-fold increase in the EC50 for ATP in the presence of suramin) in the concentration response to ATP at recombinant WT P2X1 receptors similar to that reported previously (25) (Fig. 6A). The parallel shift in the concentration response indicates that the suramin antagonism is competitive (see Ref. 26) and yields a pK_B estimate of ~6.7 for suramin. We have estimated the pK_B value for suramin (at 3 μM) on mutant P2X1 receptors to test whether the mutations in the P2X1 receptor have affected the ability of suramin to antagonize the response to ATP. The mutants K68A, K70A, R292K, K309A, R305A, K309R, R314K, R314A, K325R, and K325A had no effect on the pK_B for suramin. For R305K, the antagonism by suramin was not competitive and maximum responses in the presence of suramin were only ~60% of the response in the absence of suramin. Suramin was a less effective antagonist at K53A, K53R, K202A, K215A, and K292A mutant P2X1 receptors resulting in shifts in the EC50 for ATP of between 4- and 8-fold. In contrast suramin was a more effective antagonist at K70R (Fig. 6B) and K215R receptors leading to a 40–120-fold shift in the EC50 for ATP. For K68A and K309A in the presence of suramin, responses to the maximal concentration of ATP tested (10 mM) were 25.8 ± 6 and 35.8 ± 1.9%, respectively, of those in the absence of suramin. We have therefore estimated the pK_B from the concentration ratio at the EC20 and EC30 values, respectively. These results are of particular interest as in general mutants that caused the largest increases in EC50 for ATP either had no effect (K68A, K70A, R292K, K309A) or increased (K309R, K70R) the affinity of suramin,
indicating that these mutations have not had a major effect on the conformation of the P2X1 receptor.

**P2X Receptor Expression Analysis**—Inward currents in response to ATP application were not recorded for the K68R mutant P2X1 receptor, and only small responses were recorded from the mutants K68A, R305A, and R305K (Table I). We have used a P2X1 receptor-selective antibody to investigate the level of receptor expression. In Western analysis bands corresponding to a protein of 60 kDa were detected with an antibody directed to the carboxyl terminus of the P2X1 receptor in extracts from oocytes injected with P2X1 receptor cRNAs but not water-injected controls (Fig. 7A). Sulfo-NHS-LC-Biotin treatment was used to estimate the expression of P2X1 receptors on the cell surface (Fig. 7B). The surface expression of Lys-68 mutant P2X1 receptors demonstrates that the lack of functional response (K68R) or small functional response (K68A) to ATP application does not result from low levels of receptor expression and may result in part from changes in the kinetics of channel opening. In contrast, for the mutants at Arg-305, there was reduced (barely detectable for R305A) expression of biotinylated P2X1 receptors compared with WT controls, and this appears to correlate with the amplitude of the functional response for these mutants (Table I).

**DISCUSSION**

Point mutations of conserved positively charged amino acids in the extracellular loop of the P2X1 receptor have indicated that residues Lys-68, Lys-70, Lys-309, and Arg-292 contribute to the binding of ATP. Mutation of these residues also prolonged the time course of the P2X1 receptor-mediated current, indicating that they may have some effect on the kinetics of channel opening. The comparative lack of effect on antagonism by suramin indicates that these mutations did not substantially modify the structure of the P2X1 receptor.

The majority of mutants of the conserved positively charged residues (Lys-53, Lys-190, Lys-215, Lys-325, Arg-202, Arg-305, and Arg-314) either had no effect or only a small change in ATP potency, time course, and/or suramin sensitivity. As ATP is a negatively charged molecule, positive charge on the surface of the protein may act to attract the negatively charged phosphate group of ATP toward the binding site of the receptor. The small changes in potency could be accounted for by electrostatic effects resulting from changes in the distribution of surface charge on the receptor or from small changes in the conformation of the P2X1 receptor. These results suggest that these lysine and arginine residues do not play an essential role in the formation of the ATP binding pocket of P2X receptors.
Modest effects on ATP potency were recorded for mutations at Lys-70. The potency of ATP was reduced more for K70R with maintenance of positive charge than for K70A, where the charge was neutralized. These results suggest that in terms of determining ATP potency no charge at position 70 is better than the wrong positively charged amino acid. In addition, the mutation K70R resulted in an ~8-fold increase in the effectiveness of suramin as an antagonist (for K70A, there was no effect). The increase in suramin $pK_a$ estimate indicates that this region of the receptor also contributes to the binding of suramin. This is consistent with a previous study on P2X4 receptors that demonstrated that residue 78 can contribute markedly to suramin activity (15).

Mutation of Arg-292 resulted in an ~100-fold decrease in ATP potency following substitution with either the conservative lysine mutation to maintain the positive charge or neutralizing the charge at this residue. This suggests that it is not just the charge but also the arginine residue that is important at this position. Previously arginine residues have been shown to contribute to ATP binding, e.g. at metabotropic P2Y1 receptors (21) and the sarcoplasmic reticulum ATPase (28).

The most substantial decreases on ATP potency were recorded for mutations at residues Lys-68 and Lys-309. Previously lysine residues have been shown to be important in a variety of ATP-binding proteins including kinases (18), the cystic fibrosis transmembrane conductance regulator (29), the multidrug resistance protein (17), $K_{ATP}$ potassium channels (20), and P2Y receptors (21). It has been suggested that the lysine may interact directly with one of the phosphates of ATP (17, 30). In the present study, the lack of effect on suramin indicates that there has not been a substantial change in the conformation of the mutant receptors, and these lysine residues are important for ATP binding but not suramin antagonism. At WT P2X1 receptors, a fully saturated maximal response (presumably 100% receptor occupancy) is achieved at ~100 $\mu$M ATP. For K68A and K309A mutant receptors, <10% of the maximal responses is recorded at this concentration of ATP. The shifts in the concentration-response relationships with these mutants are therefore most likely to correspond to a decrease in the affinity of ATP for the receptor and a concomitant decrease in ATP receptor occupancy at a given agonist concentration.

The kinetics of channel opening of P2X1 receptors following ATP binding are also affected by some of the mutations in positive charge in the extracellular loop of the receptor. In particular for residues Lys-68, Lys-309, and Arg-292, which resulted in a substantial decrease in ATP potency, there is a prolongation of the current consistent with a slowing of the desensitization of the response. It is likely that P2X1 receptor desensitization corresponds to the channel going into a ligand-bound closed state similar to that described for nicotinic acetylcholine receptors (31), where agonist binding leads to change in the structure of the acetylcholine binding site (32). Mutations K68A, K309A/R, and R292K/A may thus result in a stabilized/prolonged open state of the P2X1 receptor channel. The K68R mutant of the P2X1 receptor is expressed at the membrane, although ionic currents were not recorded and it is possible that this may result from an inhibition of channel opening. The residues equivalent to Lys-68, Arg-292, and Lys-309 of the P2X1 receptor are conserved throughout the P2X family, including relatively non-desensitizing forms of the receptor. This suggests that desensitization results from their interaction with some other variant amino acid(s) to affect the time course of the P2X receptor currents. Previous studies have identified other regions of the P2X receptor that contribute to the time course of the response, including the second transmembrane domain (33), the intracellular carboxyl terminus (10–12), and a protein kinase C motif in the intracellular amino terminus (13). Thus, the control of the time course of P2X receptor currents is a complicated multifactorial process.

The residues that we have identified to be involved in ATP binding (Lys-68, Lys-70, Arg-292, Lys-309) are in two clusters close to the predicted transmembrane domains (Fig. 8) and suggest that the ATP binding pocket may form as an extension of the ion channel pore vestibule. P2X receptors form from the multimeric assembly of at least three P2X receptor subunits (8). It is therefore possible that the ATP binding site could be formed from the interaction of residues within a P2X receptor subunit (Fig. 8A) and/or between adjacent subunits (Fig. 8B), as has been demonstrated for other ligand-gated ion channel families, e.g. $\gamma$-aminobutyric acid A (34) and nicotinic acetylcholine receptors (27).

The present study has identified positively charged amino acid residues that are involved in ATP binding and activation of the P2X1 receptor. The conservation of these residues throughout the family of P2X receptors suggests that they contribute to a common binding pocket for the phosphate group(s) of ATP. Given the range of pharmacological properties of P2X receptors, in addition, other regions of the receptor are also likely to contribute to ligand recognition.

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Note Added in Proof—Similar results have been reported for P2X4 receptors (Ennion, S., Hagan, S., and Evans, R. J. (May 25, 2000) J. Biol. Chem. 10.1074/jbc.M003637200).

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