ATP Binding at Human P2X₁ Receptors

CONTRIBUTION OF AROMATIC AND BASIC AMINO ACIDS REVEALED USING MUTAGENESIS AND PARTIAL AGONISTS

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P2X receptors comprise a family of ATP-gated ion channels with the basic amino acids Lys-68, Arg-292, and Lys-309 (P2X₁ receptor numbering) contributing to agonist potency. In many ATP-binding proteins aromatic amino acids coordinate the binding of the adenine group. There are 20 conserved aromatic amino acids in the extracellular ligand binding loop of at least 6 of the 7 P2X receptors. We used alanine replacement mutagenesis to determine the effects of individual conserved aromatic residues on the properties of human P2X₁ receptors expressed in Xenopus oocytes. ATP evoked concentration-dependent (EC₅₀ = 1 μM) desensitizing currents at wild-type receptors and for the majority of mutants there was no change (10 residues) or a 6-fold decrease in ATP potency (6 mutants). Mutants F185A and W259A failed to form detectable channels at the cell surface. F185A and F291A produced 10- and 160-fold decreases in ATP potency. The partial agonists 2β,3′,5′-O-(4-benzoyl)-ATP (BzATP) and P²⁺,P³⁻di(adenosine 5′)-pentaphosphate (Ap₅Pₐ₅) were tested on a range of mutants that decreased ATP potency to determine whether this resulted predominantly from changes in agonist binding or gating of the channel. At K68A and K309A receptors BzATP and Ap₅Pₐ₅ had essentially no agonist activity but antagonized, or for R292A potentiated, ATP responses. At F185A receptors BzATP was an antagonist but Ap₅Pₐ₅ no longer showed affinity for the receptor. These results suggest that residues Lys-68, Phe-185, Phe-291, Arg-292, and Lys-309 contribute to ligand binding at P2X receptors, with Phe-185 and Phe-291 coordinating the binding of the adenine ring of ATP.

P2X receptors for ATP are a novel family of ligand-gated cation channels comprised of seven receptor subunits (P2X₁-7) that associate most likely as homo- and heterotrimeric channels to give a range of properties (for review see Ref. 1). They have been implicated in a variety of actions, as diverse as mediating fast synaptic transmission (2) to regulating interleukin release (3). There is considerable interest in developing P2X receptor subtype selective drugs, for example, P2X₁ receptor-targeted drugs may have applications in the regulation of smooth muscle contraction (4, 9) and platelet function (10, 11). We have previously identified positively charged amino acids that may coordinate the action of negatively charged phosphate groups of ATP (12) as well as investigating the role of disulfide bonds (13) and conserved negatively charged residues (14) (see Fig. 1).

The site(s) of recognition of the adenine group that gives nucleotide selectivity to the P2X receptors and what residues are associated with the adenine and ribose moieties of ATP are unclear. Some insight into the amino acids responsible for drug binding has come from studies on other ATP-binding proteins where work based on mutagenesis and crystal structures has identified the contribution of aromatic amino acids. For example, phenylalanine (F) coordinates the recognition of the adenine group of ATP by DEAD box helicases (15) and tyrosine (Y) may form hydrogen bonds with the ribose of ATP by UvrB DNA helicase (16). In addition conserved aromatic amino acids may also contribute to cation-π interactions (17, 18). For P2X receptors it is possible that the electrostatic potential on the face of the aromatic ring coordinates the binding of magnesium complexed to the phosphate groups of ATP or other cations as co-factors of ATP action.

In this study we have used alanine replacement mutagenesis of conserved aromatic amino acids in the extracellular loop of the P2X₁ receptor (Fig. 1) to determine their role in the functional properties of the receptor. Our prediction is that if a residue is important for ATP action then replacement of the conserved aromatic amino acid with alanine would result in change in the properties of the receptor. Similarly a lack of effect of the mutation would indicate that a conserved aromatic amino acid was not essential for normal receptor function. The mutants correspond to residues that are completely conserved in the P2X₁ receptor family (P2X₁ receptor residues Tyr-55, Tyr-274, Trp-164, Trp-259, Phe-185, Phe-243, Phe-276, and Phe-291), positions where there is a conserved aromatic group (Tyr, Trp, or Phe: Tyr-90, Phe-195, Phe-230, Phe-293, Phe-315, and Phe-326) (Fig. 1). We have expressed mutant receptors in Xenopus oocytes and determined the effects on agonist and antagonist action as well as receptor expression. Mutants F185A and F291A resulted in decreases in the EC₅₀.
The amino acid sequence of human P2X<sub>1</sub> is shown aligned to equivalent residues of rat P2X<sub>1</sub> and human P2X<sub>2</sub>. Conserved aromatic residues across the P2X family are highlighted (boxed). Previously reported mutants important for ATP potency are highlighted for P2X<sub>1</sub> (shaded box), P2X<sub>2</sub> (bold), and non-important residues (light gray). Numbered residues refer to the P2X<sub>1</sub> sequence. SwissProt accession numbers for sequences are P51575, P49653, P56373, Q99571, Q95036, O15547, and Q99572 for P2X<sub>1</sub>—<sub>7</sub>, respectively.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—The human P2X<sub>1</sub> receptor construct, described previously (12), was used as a template for production of plasmids containing conserved aromatic residue mutations to alanine. Point mutations were introduced using the QuickChange<sup>®</sup> mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Phenylalanine residues at positions 100, 185, 195, 198, 230, 243, 276, 289, 291, 293, 297, 311, 315, and 326 were mutated to alanine. Tryptophan residues at positions 85, 164, and 259 were mutated to alanine and tyrosine residues at positions 55, 90, and 274 were mutated to alanine. Production of the correct mutations and absence of coding errors in the P2X<sub>1</sub> mutant constructs was verified by DNA sequencing (Automated ABI Sequencing Service, University of Leicester).

**P2X<sub>1</sub> Mutant and Wild-type Receptor Expression in Xenopus laevis Oocytes**—Mutant and wild-type plasmids were transcribed to produce sense strand cRNA (mMessage mMachine<sup>®</sup>, Ambion) as described previously.

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1 The abbreviations used are: BzATP, 2',3'-O(4-benzoyl)-ATP; Ap<sub>a</sub>, P<sup>3</sup>,P<sup>5</sup>-di(adenosine 5')-pentaphosphate; Ap<sub>p</sub>, P<sup>3</sup>,P<sup>5</sup>-di(adenosine 5')-pentaphosphate.
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Mutually defolliculated stage V-X. laevis oocytes were injected with 50 nl (50 ng) of cRNA using an Inject<sup>®</sup>-Matric microinjector (J. Alejandro Gaby, Genève, Switzerland) and stored at 18 °C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM sodium pyruvate, 5 mM HEPES, pH 7.6). Media was changed daily prior to recording 3–7 days later.

Electrophysiological Recordings—Two-electrode voltage clamp recordings were made from X. laevis oocytes using a GeneClamp 800B amplifier with a Digidata 1322 analog to digital converter and pClamp 8.2 acquisition software (Axon Instruments, Union City, CA) essentially as described previously (12). External ND96 bath solution contained 1.8 mM Ba<sup>2+</sup> replacing 1.8 mM Ca<sup>2+</sup> to prevent activation of endogenous calcium-activated chloride channels. ATP (Mg salt, Sigma) was applied via a U-tube perfusion system as was the P2 receptor antagonist suramin (Bayer) was bath-perfused and also co-applied with ATP from the U-tube. Reproducible responses to agonists were recorded when there was a 5-minute interval between applications. Concentration response curves were fitted with the Hill equation: 
\[ Y = \frac{[X]^H \times M}{[X]^H + ([EC_{50}]^H)} \]
where [X] is agonist concentration, Y is response, X is agonist concentration, M is the Hill coefficient, H is the Hill coefficient, M is maximum response, and EC<sub>50</sub> is the half maximal response.

The concentration ratio for suramin (3 μM) was calculated (EC<sub>50</sub> response in the presence of suramin/EC<sub>50</sub> response in the absence of suramin) and used to estimate antagonist potency; pK<sub>B</sub> estimate is the-log<sub>10</sub>(EC<sub>50</sub>) concentration ratio - 1 - log<sub>10</sub>(EC<sub>50</sub>) suramin concentration). Relative efficacy (e) for comparison of wild-type and mutants was calculated using the equation: 
\[ e = \frac{I_{max}}{I_{max} (WT)} \]
All data are shown as mean ± S.E. with significant differences calculated using the Student’s t test. n corresponds to the number of oocytes tested.

Western Blotting—Expression levels of wild-type and mutant receptors were estimated by Western blot analysis of total cellular protein and cell surface proteins. Oocytes injected with wild-type or mutant receptor cRNA were homogenized in buffer (100 mM NaCl, 20 mM Tris-Cl, pH 7.4, 1% Triton X-100, and 10 μl/ml protease inhibitor mixture) at 20 μl/oocyte. Total cellular protein samples were prepared as described previously (12). Sulfo-NHS-LC-Biotin (Pierce) was used to label the cell surface proteins and estimate the level of wild-type or mutant receptor trafficked to the cell surface membrane. Sulfo-NHS-LC-Biotin is impermeable to the cell membrane and can only biotinylate proteins available at the cell surface. Oocytes previously injected with wild-type or mutant receptor cRNA were treated with Sulfo-NHS-LC-Biotin (0.5 mg/ml) in ND96, for 30 min. Oocytes were homogenized in buffer H and the spin-cleared supernatant was mixed with streptavidin-agarose beads (Sigma) and treated as described previously (13). All samples were heated to 95 °C for 5 min prior to loading. Samples were run on a 10% SDS-PAGE gel, transferred to nitrocellulose, and screened for immunoreactivity for the anti-P2X<sub>1</sub> antibody (1:500) (Alomone, Israel).

RESULTS

Effects of Mutation of Conserved Aromatic Amino Acids on the Response to ATP—ATP evoked concentration-dependent desensitization inward currents with an EC<sub>50</sub> of ~1 μM at wild-type (WT) P2X<sub>1</sub> receptors similar to those reported previously (12) (Fig. 2, Table I). Of the 20 conserved aromatic amino acid mutants tested alanine substitution was tolerated and ATP-evoked responses were evoked from all of them except F195A and W259A (Fig. 2, Table I). Mutants F100A, F230A, F276A, F297A, and W164A had no effect on ATP potency or the time course of the response. Mutants F198A, F289A, F293A, Y274A, and W85A had no effect on ATP potency but there was a small (less than 2-fold) change in the time course of the current. Mutants F291A and Y55A had no effect on ATP potency or the time course of the current. Mutants F198A, F289A, F315A, and Y55A had reduced peak current amplitudes (Fig. 3, Table I). These properties could result from either reduced receptor expression and/or an impairment of the channel to open and pass current in response to the binding of ATP. To address this we have determined the levels of both total (Western blot using a crude cell lysate) and cell surface (Western blotting to detect P2X<sub>1</sub> receptor labeled with membrane impermeant sulfo-NHS-LC biotin) P2X<sub>1</sub> receptors for WT and mutants where there was a reduction in peak current amplitude (Fig. 3). WT P2X<sub>1</sub> receptors were detected in both total cell lysates and following cell surface biotinylation and migrated as a single band of ~53 Kd corresponding to a glycosylated monomeric receptor subunit. The equivalent levels of total and cell surface expression for WT, F198A, and F291A indicate that the modest decrease in peak current amplitude (50 and 20%, respectively) may result from a change in channel opening. Both F198A and Y55A mutants resulted in an ~50% decrease in peak current amplitude, however, for the Y55A there is a marked decrease in both surface and total levels of receptor expression compared with WT suggesting that the reduction in expression accounts for the reduction in peak current amplitude. The mutants that failed to form functional channels (F195A and W259A) were below the limit of detection at the cell surface. However, when we mutated these residues to tyrosine functional channels that were indistinguishable from WT were produced (with peak currents of 7644 ± 313 and 8406 ± 597 nA and EC<sub>50</sub> values of 6.22 ± 0.15 and 5.81 ± 0.15 for F195Y and W259Y, respectively, n = 3–8) indicating that the conservative aromatic substitution with tyrosine can rescue channel function. For the F195A mutant we were unable to detect any receptor expression in total cellular lysates indicating that this receptor is inefficiently translated and/or selectively degraded; this suggests that this conserved aromatic residue is required for the normal folding and expression of the protein. In contrast the W259A mutant receptor was detected in total cell lysates, albeit at a reduced level (~22% of P2X<sub>1</sub>, WT) indicating that insufficient trafficking of the receptor to the cell surface contributes significantly to the lack of functional response of this mutant. A similar effect was seen for the equivalent mutation in a P2X<sub>2</sub> receptor background (21). These results show that alanine cannot functionally substitute for the conserved amino acids at positions Phe-195 and Trp-259 and the role of these conserved aromatic amino acids in ATP action remains to be determined.

Conserved Aromatic Amino Acids and Agonist Selectivity—In a range of nucleotide binding enzymes the nucleotide specificity is governed by aromatic amino acids, for example, in the P<sub>1</sub>-ATPase it is suggested that tyrosine 368 in the α-subunit confers adenine nucleotide specificity, whereas the β-subunit binding site that lacks a tyrosine residue also binds GTP and ITP (22). We therefore tested whether point mutations to remove conserved tyrosine residues or other conserved aromatic amino acids that had the greatest effect on ATP potency (F185A and F291A) resulted in any change in nucleotide action at P2X<sub>1</sub> receptors. The nucleotides UTP, GTP, CTP, and ITP (all 100 μM) were weak partial agonists at the WT P2X<sub>1</sub> receptor with an order of efficacy CTP > UTP > ITP > GTP (0.31, 0.06, 0.04, and 0.02, respectively). Mutants F291A and Y274A resulted in increased efficacy to GTP, UTP, and GTP, respectively, but not to the other nucleotides tested. This suggests...
that these residues may contribute to nucleotide selectivity at the P2X\textsubscript{1} receptor. Mutants F185A, Y55A, and Y90A either had no effect or a reduction in agonist efficacy for nucleotides UTP, GTP, CTP, and ITP (Fig. 4).

**Fig. 2. Effect of P2X\textsubscript{1}, conserved aromatic mutations to alanine on ATP potency.** P2X\textsubscript{1}, wild-type (WT) or aromatic mutants were expressed in oocytes and ATP potency was tested by two-electrode voltage clamp. A, currents recorded in response to ATP (micromolar unless otherwise indicated) at P2X\textsubscript{1}, wild-type and aromatic mutants F185A and F291A. B, concentration response curves for P2X\textsubscript{1} WT (bold) and phenylalanine mutants. C, concentration response curves for P2X\textsubscript{1} WT (bold) and tyrosine and tryptophan mutants. Mean currents were normalized to the maximum response (n = 3–7). Holding membrane potential was −60 mV.

**Use of the Partial Agonist BzATP to Investigate Binding Versus Gating Effects**—Our work has identified a number of mutations in the P2X\textsubscript{1} receptor, including the aromatic amino acids Phe-185 and Phe-291 (this study) as well as the positively
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TABLE I
Summary of data for ATP action at P2X<sub>1</sub>, WT and aromatic mutants

| Mutant  | ATP EC<sub>10</sub> | ATP pEC<sub>10</sub> | Peak I | Time to peak | Half-time | Suramin |
|---------|---------------------|----------------------|--------|--------------|-----------|---------|
| WT      | 0.87                | 6.06 ± 0.10          | nA     | 680 ± 21     | 801 ± 130 | 6.71 ± 0.15 |
| F100A   | 1.51                | 5.82 ± 0.24          | -24 ± 15<sup>a</sup> | NF<sup>b</sup> | NF<sup>b</sup> | >7.5 |
| F185A   | 8.66                | 5.06 ± 0.19<sup>a</sup> | -8708 ± 297 | 821 ± 23<sup>a</sup> | 1409 ± 166<sup>d</sup> | 7.16 ± 0.22 |
| F195A   | NF<sup>b</sup>      | NF<sup>b</sup>       | 566 ± 16<sup>b</sup> | 515 ± 27<sup>b</sup> | 6.85 ± 0.19 |
| F198A   | 1.39                | 5.86 ± 0.20          | -5160 ± 660<sup>b</sup> | 638 ± 42<sup>b</sup> | 5.35 ± 0.6<sup>d</sup> |
| F230A   | 1.3                 | 5.89 ± 0.11<sup>a</sup> | -10148 ± 1090 | 101 ± 22 | 1.2 ± 22<sup>a</sup> | 1.2 ± 0.12<sup>d</sup> |
| F243A   | 2.29                | 5.64 ± 0.16<sup>a</sup> | -8629 ± 727 | 629 ± 17<sup>c</sup> | 850 ± 145 | 7.17 ± 0.18<sup>d</sup> |
| F276A   | 1.41                | 5.85 ± 0.08          | -5887 ± 566 | 640 ± 26 | 918 ± 158 | 7.02 ± 0.04 |
| F289A   | 0.88                | 6.18 ± 0.05          | -7619 ± 997 | 566 ± 16<sup>b</sup> | 515 ± 27<sup>b</sup> | 6.85 ± 0.19 |
| F291A   | 140.93              | 3.85 ± 0.17<sup>a</sup> | -7203 ± 732<sup>b</sup> | 708 ± 33 | 1545 ± 179<sup>b</sup> | >7.5 |
| F293A   | 1.36                | 5.87 ± 0.12          | -10247 ± 735 | 570 ± 21<sup>c</sup> | 1000 ± 150 | >7.5 |
| F297A   | 0.78                | 6.11 ± 0.12          | -9432 ± 735 | 617 ± 43 | 713 ± 169 | 6.48 ± 0.18 |
| F311A   | 3.82                | 5.42 ± 0.22<sup>c</sup> | -12165 ± 1142<sup>b</sup> | 779 ± 19<sup>c</sup> | 2249 ± 270<sup>c</sup> | >7.5 |
| F315A   | 3.12                | 5.51 ± 0.20<sup>a</sup> | -7081 ± 654<sup>b</sup> | 621 ± 35 | 493 ± 112<sup>b</sup> | 6.77 ± 0.04 |
| F326A   | 1.53                | 5.82 ± 0.06<sup>a</sup> | -8026 ± 1353 | 636 ± 44 | 458 ± 68<sup>d</sup> | 6.67 ± 0.11 |
| Y55A    | 1.94                | 5.71 ± 0.11<sup>a</sup> | -4572 ± 231<sup>c</sup> | 596 ± 17<sup>c</sup> | 462 ± 146<sup>c</sup> | 7.46 ± 0.02<sup>d</sup> |
| Y96A    | 1.76                | 5.75 ± 0.07<sup>a</sup> | -10718 ± 739 | 760 ± 51 | 1403 ± 300 | <7.5 |
| Y274A   | 1.13                | 5.95 ± 0.09          | -7794 ± 683 | 612 ± 29<sup>b</sup> | 719 ± 116 | 6.56 ± 0.13 |
| W55A    | 1.35                | 5.87 ± 0.09          | -8871 ± 462 | 650 ± 21 | 1779 ± 290<sup>d</sup> | >7.5 |
| W164A   | 1.35                | 5.87 ± 0.07          | -8891 ± 400 | 752 ± 130 | 756 ± 139 | 7.34 ± 0.04<sup>d</sup> |
| W259A   | NF<sup>b</sup>      | NF<sup>b</sup>       | -18 ± 7<sup>c</sup> | NF<sup>b</sup> | NF<sup>b</sup> | NF<sup>b</sup> |

<sup>a</sup> p < 0.001, different from WT as measured by Student’s t test.
<sup>b</sup> p < 0.05, different from WT as measured by Student’s t test.
<sup>c</sup> NF, non-functional receptor, therefore data not collected.
<sup>d</sup> p < 0.01, different from WT as measured by Student’s t test.

**Fig. 3.** Effect of aromatic mutations on mean peak current and cell surface expression. The majority of conserved aromatic residues gave maximum peak current values similar to P2X<sub>1</sub> wild-type (WT). A, aromatic mutants F291A, F198A, Y55A, F195A, and W259A all exhibited significantly reduced peak current values compared with P2X<sub>1</sub> WT (n = 5–12). B, to further investigate the cause for this reduction Western blot analysis was carried out using P2X<sub>1</sub> antibody on total and cell surface-expressed protein. Cell surface proteins were extracted by sulfo-NHS-LC-Biotin labeling and isolated with streptavidin-agarose beads. Immunoreactivity was observed at the correct Mr for P2X<sub>1</sub> protein and was not observed for non-injected oocytes (data not shown).
charged residues Lys-68, Arg-292, and Lys-309, that result in a decrease in agonist potency (12, 13). The interpretation of the underlying mechanism for these changes in ATP potency (a measure of the amount of agonist required to induce channel opening), however, raises a number of issues about agonist action. In particular whether the shift is because of a change in binding affinity at the receptor (including any conformational change in the protein associated with ligand binding, e.g. the glutamate induced closure of the ligand binding clam shell (23)) and/or the functional translation of agonist binding to the opening of the channel; i.e. gating. The substituent components of ATP: adenosine, AMP, ADP, and polyphosphates (24, 25), show no agonist or antagonist action at P2X1 receptors. This demonstrates that both an adenine ring and a triphosphate chain are required for high affinity binding at the P2X1 receptor and suggests that a conformational change in the protein is associated with high affinity binding of ATP (see Fig. 9). Recent work on ionotropic glutamate receptors has revealed that partial agonists result in different ligand-binding core conformational states (degree of closure of the clam shell) that in turn control the open probability of the channel (20) and suggest that partial agonists may be able to discriminate between mutations that interfere with agonist binding versus channel opening.

At WT P2X1 receptors BzATP evokes concentration-dependent desensitizing responses with an EC_{50} of \(0.5 \mu M\) (PEC_{50} 6.31 ± 0.11), similar to the potency of ATP (Fig. 5, A and C). However, the maximum response to BzATP is reduced compared with that of ATP indicating that BzATP is a partial agonist with an efficacy of 0.55 ± 0.06 (full agonist efficacy of 1) (as reported previously (26)) (Table II), in addition, the time course of maximal responses was also slower than those of ATP (time to peak was increased to 922 ± 47 ms and the half-time of the response was prolonged to 2756 ± 130 ms, \(n = 6\)). We have used the partial agonist BzATP to investigate further the decrease in ATP potency associated with aromatic amino acid mutants as well as a range of other mutants we have identified in previous studies that resulted in a decrease in ATP potency (12, 13) (Fig. 5, B and C, Table II). For mutants F185A, F291A, R292A, K68A, and K309A with less than a 10-fold decrease in ATP potency the point mutations had no, or less than a 2-fold effect on the partial agonist activity of BzATP (Fig. 5C) suggesting that these residues do not make a major contribution to agonist binding to the P2X1 receptor. In contrast the partial agonist activity of BzATP was reduced to 5% for mutants F185A, R292A, F291A, K68A, and K309A (these mutants resulted in a 10 to 1400-fold decrease in ATP potency) (Fig. 6A). However, for mutant C227A that resulted in a 45-fold decrease in ATP potency there was a smaller reduction in the efficacy of BzATP. To determine whether the decrease in efficacy at mutant receptors was because of BzATP no longer having significant affinity for the receptor we co-applied ATP and BzATP. For mutants F185A, F291A, R292A, K68A, and K309A, BzATP reduced the response to co-applied ATP (Fig. 6A) demonstrating that BzATP was still capable of binding to these mutant receptors.

2 J. A. Roberts and R. J. Evans, unpublished observations.

Fig. 4. Agonist selectivity of conserved aromatic residues. The potency of nucleotides CTP, UTP, ITP, and GTP were tested at P2X1 wild-type (WT), phenylalanine mutants F291A and F185A, which displayed decreased ATP potency, and tyrosine mutants Y55A, Y90A, and Y274A, which may couple to nucleotide sensitivity. Sample traces are shown with 3 s agonist application (bar). A, Y274A shows increased sensitivity to UTP. B, F291A and Y274A both show increased efficacy to GTP. C, F185A and Y90A show significant reduction in CTP efficacy. D, F185A and Y90A show a significant reduction in ITP efficacy (\(n = 3–7\)).
Fig. 5. Partial agonism of BzATP at P2X, WT and conserved aromatic mutants. A, concentration response curves for ATP (■) and BzATP (▲) at P2X, wild-type (WT) receptors expressed in oocytes normalized to the maximal response (n = 5). B, concentration response curves for ATP (▼) and BzATP (▼) at F185A receptors expressed in oocytes normalized to the maximal response (n = 3). C, BzATP efficacy at P2X, WT and mutant receptors that show similar ATP potency and mutants that show significant shifts in ATP potency reported in this paper and previously. Efficacy is calculated using maximum activating ATP concentrations versus maximum activating BzATP concentrations (n = 3–6).
Estimation of the Affinity of BzATP at P2X<sub>1</sub> Receptors—The mutation F185A changed BzATP from being a partial agonist to an antagonist at the P2X<sub>1</sub> receptor. We were therefore interested to characterize in more detail the antagonist properties of BzATP at this mutant receptor. When co-applied with ATP, BzATP resulted in a concentration-dependent parallel rightward shift in the ATP concentration response. Schidling analysis gives an estimate for the affinity of BzATP at the mutant F185A P2X<sub>1</sub> receptor with a pA<sub>2</sub> value of 6.14. At the WT P2X<sub>1</sub> receptor ATP and BzATP have the same EC<sub>50</sub> value. If the 10-fold shift in ATP potency at the F185A receptor is mirrored in the change in sensitivity to BzATP this would give an estimate of the pA<sub>2</sub> of BzATP at the P2X<sub>1</sub> receptor of 6.14. The slope of the Schidling plot is −1.75 and suggests that more than a single molecule of BzATP is required to block the actions of ATP and that the above estimate is inaccurate. This is perhaps not surprising as the above experiment was not performed under equilibrium conditions (given the desensitizing nature of the responses) and at least two, possibly three molecules of ATP bind to the P2X<sub>1</sub> receptor to activate it. In addition if ATP binding shows cooperativity (i.e. binding of one ATP molecule increases the affinity of the receptor to subsequent ATP binding) analysis of the results will be further complicated. A recent study has shown that ATP has a K<sub>1/2</sub> for receptor desensitization of −3 nM at the P2X<sub>1</sub> receptor compared with an EC<sub>50</sub> of ~0.7 μM for receptor activation (27). This was interpreted as reflecting the nanomolar affinity of ATP at the P2X<sub>1</sub> receptor in binding studies (28). However, given the possibility of positively cooperative binding of at least two agonist molecules this nanomolar estimate may refer to the binding of the final ligand required to activate/desensitize the channel, and not the earlier lower affinity binding step(s).

Characterization of the Effects of the Partial Agonist Ap<sub>A</sub> at Mutant P2X<sub>1</sub> Receptors—Diadenosine polyphosphates are two adenosine molecules linked by a polyphosphate chain and Ap<sub>A</sub> acts as a partial agonist at P2X<sub>1</sub> receptors with an efficacy of 0.35 ± 0.02 and an EC<sub>50</sub> of ~0.7 μM (pEC<sub>50</sub> 6.15 ± 0.06, n = 4) as reported previously (26) (Fig. 7A, Table II). We therefore tested this compound to determine whether this had a similar change to BzATP in activity at mutant P2X<sub>1</sub> receptors. For mutants F198A and K70A interestingly the efficacy of Ap<sub>A</sub> increased, for F293A and Y55A the efficacy was reduced by 2–3-fold, and for the remainder of the mutants agonist activity was almost abolished (Fig. 7B, Table II). When Ap<sub>A</sub> was co-applied with ATP there was a reduction in the amplitude of the response compared with ATP alone for F291A, K68A, and K309A indicating that Ap<sub>A</sub> is still binding to the receptor and now acting as an antagonist. Interestingly for the R292A mutant co-application of ATP and Ap<sub>A</sub> potentiated the response (by −30%) compared with ATP alone suggesting that in this case although Ap<sub>A</sub> has no agonist effect it may act as an allosteric regulator. In contrast for mutant F185A co-application of Ap<sub>A</sub> has no effect on the response to ATP indicating that Ap<sub>A</sub> no longer has significant affinity for this mutant receptor.

Antagonist Effects—Previous studies have suggested that the amino acids that ATP and antagonists bind to are different. For example, the rat P2X<sub>1</sub> receptor is activated by ATP but suramin and PPADS are ineffective antagonists (29, 30) and we have shown for the P2X<sub>1</sub> receptor that it is possible to decrease ATP potency by over 1400-fold with relatively little effect on suramin affinity (12). It was therefore of interest to determine the antagonist action of suramin at the mutant receptors to determine whether conserved aromatic amino acids contributed to suramin sensitivity. To test whether there was an effect on the antagonist binding properties of the receptor we determined the extent of antagonism by suramin (3 μM) of a response to an EC<sub>90</sub> concentration of ATP (Fig. 8, Table I). Mutants F185A, F276A, F289A, F297A, F315A, F326A, and Y274A had no effect on suramin action indicating that these residues are not essential for the action of suramin. There were small increases in suramin action for F230A, F243A, Y55A, and W164A. Larger shifts in antagonism were reported for mutants F100A, F291A, F293A, F311A, Y90A, and W85A where the response to an EC<sub>90</sub> concentration of ATP was reduced >90% by suramin (3 μM) indicating that the pA<sub>2</sub> value was >7.5. It is interesting that the mutants that had the greatest effect to increase the antagonist action of suramin were grouped “close” to the transmembrane domains (W85A, Y90A, F100A and F291A, F293A and F311A). F198A has a marked effect reducing by ~23-fold the affinity of suramin for the receptor, interestingly this mutation has no effect on ATP potency (Fig. 8B). These results are consistent with previous studies that suggest that the sites of ATP and suramin binding are different.

### DISCUSSION

This work has shown that the majority of conserved aromatic amino acids are not essential for normal P2X<sub>1</sub> receptor channel function, Phe-195 and Trp-259 are required for normal trafficking of the receptor to the cell surface, and mutants F185A and...
FIG. 6. BzATP is an antagonist at aromatic mutants with a lowered BzATP efficacy. A, F185A, F291A, R292A (100 μM ATP) and K68A and K309A (3 mM ATP) all show a reduced response to ATP in the presence of 100 μM BzATP compared with P2X₃ wild-type (WT) receptors (100 μM ATP) (n = 3). B, concentration response curves to ATP with increasing concentrations of BzATP (■, 0 BzATP; ○, 10 μM BzATP; ▲, 30 μM BzATP; and △, 100 μM BzATP) at the F185A receptor. C, Schild analysis for F185A antagonism with BzATP (n = 3–4).
Fig. 7. Partial agonism and antagonism of $Ap_5A$ at $P2X_1$ WT and conserved aromatic mutants. A, concentration response curves for ATP (■) and $Ap_5A$ (▲) at $P2X_1$ wild-type (WT) receptors expressed in oocytes normalized to the maximal response ($n = 4$). B, $Ap_5A$ efficacy at $P2X_1$ WT, mutant receptors that show similar ATP potency, and mutants that show significant shifts in ATP potency reported in this paper and previously. Efficacy is calculated using maximum activating ATP concentrations versus maximum activating $Ap_5A$ concentrations ($n = 4–6$). C, $Ap_5A$ as an antagonist (100 μM ATP) at mutant receptors with reduced $Ap_5A$ efficacy, F185A (100 μM ATP) shows no effect, whereas F291A (100 μM ATP), K68A and K309A (3 mM ATP) are all reduced significantly, R292A (100 μM ATP) shows potentiation of the ATP response by $Ap_5A$ ($n = 3–8$).
F291A result in a decrease in ATP potency suggesting they may be involved in agonist action at the receptor. We have used partial agonists to investigate the underlying mechanism(s) of a shift in agonist potency at mutant P2X₁ receptors and suggest that residues Lys-68, Phe-185, Phe-291, Arg-292, and Lys-309 are directly involved in agonist binding to the receptor.

In the P2X₁ receptor background removal of conserved aromatic amino acids Phe-195 or Trp-259 abolished responses to ATP and we failed to detect P2X₁ receptor protein at the cell surface for either of these mutants, however, function was rescued with the conservative substitution of tyrosine residues at these positions. This suggests that these aromatic residues play an essential role in channel processing and function. This is supported by studies on the P2X₂ receptor that demonstrated similar findings for the Trp-256 mutant (equivalent to Trp-259 in the P2X₁ receptor) where substitution with the aromatic amino acid tyrosine but not with leucine, serine, or phenylalanine was tolerated (21). These results, and the lack of effects of removal of the phenylalanine at position 243 in the P2X₁ receptor (240 in P2X₂) (21) suggest that, like for mutagenesis on conserved positively charged amino acids, equivalent mutants in different P2X receptor subtype backgrounds give similar results (12, 31).

The F185A mutant gave an ~10-fold decrease in ATP potency. This is unlikely to result from a major conformational change in the receptor as this mutation had no effect on the actions of the P2 selective antagonist suramin and suggests that this conserved residue contributes to the action of ATP. Previous mutagenesis studies on P2X receptors have identified residues close to Phe-185 that when mutated also result in a significant decrease in ATP potency (Lys-190 in P2X₁, ~5-fold decrease (12); and Thr-184 and Lys-188 in P2X₂, ~100-fold decrease (31)). These results suggest the conserved region corresponding to residues 185–190 in the P2X₁ receptor (Phe-I/L/V/I/V/I/K contributes to ATP action at P2X receptors. Whether the asparagine, at position 184 in the P2X₁ receptor, which is conserved in all the P2X receptors except P2X₅ also contributes to ATP action as it does when it precedes a phenylalanine at position 291 (see below) remains to be determined.

Removal of the conserved phenylalanine at position 291 resulted in an ~160-fold decrease in ATP potency at the P2X₁ receptor. It has also been previously shown that mutation of

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**Fig. 8. Effect of suramin at P2X₁ WT and conserved aromatic mutations.** The effect of the P2X₁ antagonist suramin at 3 μM was tested on P2X₁ WT receptors and the conserved aromatic mutants were generated. A, trace data for the P2X₁ WT receptor using 100 μM ATP in the presence and absence of 3 μM suramin. B, trace data for the F198A receptor using 100 μM ATP in the presence and absence of 3 μM suramin. C, trace data for the F291A receptor using 100 μM ATP in the presence and absence of 3 μM suramin.

**Fig. 9. Model of agonist binding and gating at P2X₁ receptors.** The figure shows a schematic representation of the P2X₁ receptor and changes in conformations associated with ATP binding. With no ligand bound the channel is closed, the receptor undergoes a conformational change associated with ATP binding (signified by a change in the shape of the extracellular region of the protein). If the binding energy is sufficient then the channel can open: the gating event. In addition, the channel can enter a ligand bound desensitized state (not shown). The figure also shows the amino acids residues that we propose interact with ATP. The two aromatic amino acids Phe-185 and Phe-291 sandwich the adenine ring (Ad) and Lys-68 and Lys-309 coordinate the binding of the phosphate chain (P-P-P), Rib corresponds to the ribose group. The contribution of the intracellular amino- and carboxyl-terminal domains to channel gating remains to be determined.
the adjacent conserved asparagine or arginine residues also results in an 100-fold decrease in ATP potency (12, 31). Mutation of other conserved amino acids in this region (F299A and F293A, this study, and Lys-293 in P2X1 (31)), however, had no effect on ATP potency. These results suggest that the conserved NFR sequence is involved in ATP activation of P2X receptors.

The reduction in ATP potency at mutant P2X1 receptors indicates that these amino acids contribute to agonist action at the receptor. The interpretation of these data is complicated as ATP potency is a measure of the ability of the agonist to evoke ion channel opening and constitutes two interconnected steps: ligand binding to the receptor (this includes likely conformational changes in the receptor structure) and the subsequent opening of the liganded channel (model of P2X action see Fig. 9). Partial agonists have been shown recently to result in different ligand bound forms of the agonist-gated channels (differing degrees of conformational change associated with agonist binding) and this influences the opening of the channel (20). This supports previous studies on ligand gated ion channels where the single channel conductance is the same regardless of the agonist (partial or full) used; it is the properties of channel opening that change (reviewed in Ref. 32). Thus changes in the properties of partial agonists can reveal effects on agonist binding/conformational changes.

In the present study we have shown that for the P2X1 receptor mutants with a decrease in ATP potency (K68A, F185A, F291A, R292A, and K309A) the activity of the partial agonist BzATP was essentially abolished, however, BzATP still binds to the channel and acts as an antagonist. The results for these mutants suggest that the lack of agonist activity results from the fact that BzATP does not induce sufficient conformational change in the P2X1 receptor to mediate gating and indicate that the decrease in ATP potency at these mutants results from a reduction in the affinity of ATP at the receptor to mediate the normal conformational changes associated with binding. However, it cannot be ruled out that these effects result from co-incidental alterations in the structure of the extracellular domain of the receptor associated with the mutagenesis. The P2X receptor most likely forms from the trimeric assembly of subunits and at least 2 molecules of ATP, if not three (33) are required to activate P2X receptors. Given the trimeric nature of the functional channel it seems likely there will be three ATP binding sites, however, whether these correspond to one binding site per subunit or whether the ATP binding site is formed between subunits, like the agonist binding site of other ligand gated channels, remains to be determined. Based on models of agonist binding and gating at N-methyl-D-asparatate receptors (reviewed in Ref. 32), a 150-fold reduction in ATP potency for the F299A mutant would require a 22,000-fold (for two binding sites, square root approximation) reduction in the gating constant that is unlikely given only the modest 20% decrease in the peak current amplitude of the response. For the F185A mutant with a 10-fold decrease in potency a 100-fold decrease in the gating constant may seem plausible to account for the effect of the mutation. However, for the mutant F185A further support for a direct effect on agonist binding is provided by the loss of action of Ap5A (as a partial agonist or to antagonize the actions of ATP) at these receptors. Similarly the possible allosteric potentiation by Ap5A of ATP responses at R292A mutant receptors suggests that Arg-292 is involved in agonist binding. In addition, Jiang et al. (31) showed that cysteine substitution at isoleucine 67 in the P2X1 and subsequent incorporation of methylthiosulfonate compounds reduced ATP potency suggesting that nearby conserved lysine residues (corresponding to Lys-68 and Lys-70 in the P2X1 receptor) were involved in ATP binding (however, an effect of the methylthiosulfonate compounds on the ability of ATP to evoke a conformational change in the protein cannot be discounted). Taken together these results suggest that the reductions in ATP potency and partial agonist effects at F185A, F291A, R292A, and most likely the K68A and K309A mutant P2X1 receptors arise predominantly from a change in agonist binding to the receptor. These substantiate previous studies on the role of conserved positively charged amino acids as well as showing for the first time the role of Phe-185 and Phe-291 in coordinating ATP binding at P2X receptors.

The use of BzATP and Ap5A on mutant receptors has revealed that the conserved phenylalanine residues at positions 185 and 291 are likely to contribute to ATP binding to the P2X1 receptor. For Phe-185 the conservative amino acid substitution to tyrosine to maintain the aromatic group produced channels that were indistinguishable from WT receptors (peak current amplitude 8292 ± 610 nA, ATP pEC50 6.35 ± 0.08, and partial agonist efficacy for BzATP and Ap5A of 0.68 ± 0.05 and 0.45 ± 0.02, respectively, n = 3–10). A similar rescue of the response was seen for F291Y with no effect on ATP potency (ATP pEC50 5.92 ± 0.1, n = 3), a small −10% decrease in peak current amplitude (−5706 ± 275 nA, n = 7), and interestingly, addition of the hydroxyl group to the aromatic ring (F-Y mutant) reduced by 50% the partial agonist actions of BzATP and Ap5A at the receptor (efficacy of 0.30 ± 0.06 and 0.19 ± 0.06, respectively, n = 3). These results suggest that aromatic amino acids at positions 185 and 291 in P2X1 receptors are involved in ATP binding to the receptor. We suggest that the adenine moiety of an ATP molecule is sandwiched between these two aromatic residues similar to that demonstrated in the crystal structure of 5’-nucleotidase where the adenine ring of ATP is stacked between two phenylalanine residues (34).

In summary these results have highlighted residues in the P2X1 receptor (i) Lys-68, (ii) Lys-309, (iii) Phe-185, and (iv) Phe-291 (Arg-292 that are important for determining the actions of ATP at P2X receptors). The loss of agonist action of the partial agonists BzATP and Ap5A revealed that these mutations are most likely to result from a change in agonist binding at the receptor and not directly the gating of the channel. Whether these effects result from direct modification of the initial interaction of the ATP molecule with the P2X1 receptor or the ability to evoke a conformational change in the protein associated with agonist binding awaits the solving of the crystal structure of these receptors. However, based on studies on other ATP-binding proteins we speculate that regions i and ii are involved in recognition of the phosphate moiety and regions iii and iv regulate binding of the adenine ring. Given the trimeric nature of the P2X receptors, and modeling of agonist activation, it seems likely that there are three such ATP binding sites on the receptor.

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