Participation of the Lys$^{313}$–Ile$^{333}$ Sequence of the Purinergic
P2X$_4$ Receptor in Agonist Binding and Transduction of
Signals to the Channel Gate*§

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To study the roles of the Lys$^{313}$–Ile$^{333}$ ectodomain sequence of the rat P2X$_4$ receptor in ATP binding and transduction of signals to the channel gate, the conserved Lys$^{313}$, Tyr$^{315}$, Gly$^{316}$, Ile$^{317}$, Arg$^{318}$, Asp$^{320}$, Val$^{323}$, Lys$^{329}$, Phe$^{330}$, and Ile$^{333}$ residues were mutated. Current recordings were done on lifted cells and ATP was applied using an ultrafast solution-switching system. The rates of wild type channel opening and closing in the presence of ATP, but not the rate of washout-induced closing, were dependent on agonist concentration. All mutants other than I317A were expressed in the plasma membrane at comparable levels. The majority of mutants showed significant changes in the peak amplitude of responses and the EC$_{50}$ values for ATP. When stimulated with the supramaximal (1.4 mM) ATP concentration, mutants also differed in the kinetics of their activation, deactivation, and/or desensitization. The results suggest a critical role of the Lys$^{313}$ residue in receptor function other than coordination of the phosphate group of ATP and possible contribution of the Tyr$^{315}$ residue to the agonist binding module. The pattern of changes of receptor function by mutation of other residues was consistent with the operation of the Gly$^{316}$–Ile$^{333}$ sequence as a signal transduction module between the ligand binding domain and the channel gate in the second transmembrane domain.

Purinergic P2X receptors (P2XRs)² are a family of ligand-gated cation channels, termed P2X$_1$ to P2X$_{7}$, which are expressed in the plasma membrane of numerous excitable and nonexcitable cells (1). The native ligand for these receptor-channels is ATP, which is released by cells in a regulated manner (2). Each P2XR subunit is composed of a large extracellular loop connected to the intracellular amino and carboxyl termini through the plasma membrane with two putative transmembrane (TM) segments, which appear to line the pore (3). The homo- or heterotrimeric assembly of subunits organized in a head-to-tail orientation around the central pore probably accounts for the formation of functional P2XRs (4, 5). The occupancy of binding site(s) at the ectodomain region of P2XRs provides the free energy for conformational transitions between resting closed and conducting open states (6). The open gate allows cations to flow down their electrochemical gradients through an internal pore. The resulting depolarization of the plasma membrane and elevation in intracellular calcium concentration underlie the mechanism by which P2XRs control various physiological functions (3, 7). The whole cell current decays progressively and in a receptor-specific mode from an initial peak to a smaller steady current or a complete loss of current, and this process is termed receptor desensitization or channel inactivation. Removal of extracellular ATP leads to reverse conformational changes of the P2XR complex, termed deactivation (of open channels) and resensitization (of desensitized channels) (8).

There is a general agreement that the P2XR complex contains more than one binding site (3). At the present time, however, the exact location of these sites (at the interface of adjoining subunits or at each subunit) is more questionable (6). Based on several site-directed mutagenesis studies (9–13), Evans’ group suggested that Lys$^{68}$, Phe$^{185}$, Phe$^{291}$, Arg$^{295}$, and Lys$^{309}$ residues contribute to ATP binding at P2X$_1$R, with Phe$^{185}$ and Phe$^{291}$ coordinating binding of the adenine ring of agonist (7). In the absence of crystal structure of these proteins, homology modeling represents an alternative method to study the three-dimensional structure of proteins. Among the ATP-binding proteins, the class II aminoacyl-tRNA synthetases has sequence and secondary structure similarities with the ectodomain region of P2XRs (14). We used the x-ray structures of these enzymes as templates for three-dimensional homology modeling of the Lys$^{180}$–Lys$^{326}$ P2X$_4$ ectodomain fragment (15). Experimental results supported the model prediction that the Asp$^{280}$ residue of P2X$_4$R coordinates ATP binding via magnesium ion, the Phe$^{290}$ residue coordinates binding of the adenine ring of ATP, and the Lys$^{190}$, His$^{266}$, and Arg$^{278}$ residues coordinate the action of negatively charged α-, β-, and γ-phosphate groups, respectively. Several lines of evidence also support the hypothesis that the TM2 domain contributes to formation of the channel gate. Among all members of the P2XR family, Gly$^{347}$ (P2X$_4$R numbering) is conserved (Fig. 1B). Scanning cysteine mutagenesis at P2X$_4$R indicates that this residue is accessible from either side of the plasma membrane (16). Mutation of this residue at P2X$_4$R induces changes in the ratio of the sizes of the currents...
through the normal sized and dilated pores. The authors (17, 18) suggest that Gly347 imparts flexibility to a domain of the molecule that may operate as the gate, and that mutations perturb the protein conformations that occur during gating. The corresponding residue at P2X2R was also proposed to form a part of the gating hinge (19).

If ligand binding is distant from the gate, activation of channels must be mediated by allosteric coupling of conformational changes at the binding domain to corresponding changes at the channel gate through the linker region (20). In both intra-subunit models of the ATP-binding domain, there is a stretch of amino acids between the last ectodomain residues that participate in ATP binding and the TM2 domain. The potential role of this fragment in P2X4R activity has not been previously characterized. Furthermore, there was no systematic investigation of this fragment at other P2X subunits. However, several residues in this and nearby sequences were analyzed (10, 12, 19, 21–25). Our three-dimensional P2X4R model predicted that part of this sequence is organized as an α-helix, starting with the Ile317 residue, and could operate as a signal transduction module, whereas Lys313–Tyr315 residues could contribute to the formation of the ligand binding module (Fig. 1A). To test this hypothesis, we mutated the conserved Lys313, Tyr315, Gly316, Ile317, Arg318, Asp320, Val323, Lys329, Phe330, and Ile333 residues (Fig. 1B) and compared such mutants with the wild type P2X4 receptor.

**MATERIALS AND METHODS**

**Site-directed Mutagenesis, Cell Culture, and Transfection**—The COOH-terminal EGFP-tagged P2X4 construct (15) was used as a template for production of plasmids containing specific amino acid residue point mutations of P2X4 cDNA using the QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutagenic oligonucleotide primers were synthesized and polyacrylamide gel electrophoresis purified by Integrated DNA Technology (Coralville, IA). Productions of the correct mutations and absence of coding errors in these constructs were verified by dye terminator cycle sequencing (PerkinElmer Life Sciences; performed by Veritas, Inc., Rockville, MD). Large-scale plasmid DNAs were prepared using a QIAfilter Plasmid Maxi kit (Qiagen, Valencia, CA). Human embryonic kidney (HEK) 293 cells were used for the expression of wild type and mutant P2X4 receptors. HEK293 cells were routinely cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1) containing 10% (v/v) fetal bovine serum (Biofluids, Rockville, MD) and 100 μg/ml gentamicin (Invitrogen) in a water-saturated atmosphere of 5% CO2 and 95% air at 37 °C. For transient transfection, cells were grown on 35-mm dishes at a density of 0.2 × 10^6 cells. Transfection was conducted 24 h after plating the cells using 2 μg of DNA and 5 μl of Lipofectamine 2000 reagent (Invitrogen) in 2 ml of serum-free Opti-MEM. After 4.5 h of incubation, the transfection mixture was replaced with normal culture medium and cells were cultured for an additional 24–48 h. The cells were then mechanically dispersed and re-cultured on 35-mm dishes for 2–10 h before recordings, a procedure that yields numerous single HEK293 cells.

**Current Measurements**—Whole cell patch clamp current recordings were done at room temperature using an Axopatch 200B patch clamp amplifier (Axon Instruments Inc., Union City, CA) and records were filtered at 2 kHz using a low-pass Bessel filter. Patch electrodes, fabricated from borosilicate glass (type 1B150F-3; World Precision Instruments, Inc., Sarasota,
patch electrodes were filled with a solution containing 142 mM NaCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM EGTA, and 10 mM HEPES; the pH was adjusted with 10 mM NaOH to 7.35. The osmolarity of the internal solutions was 306 mosm. The bath solution contained 142 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, and 10 mM HEPES; the pH was adjusted to 7.35 with 10 mM NaOH. The osmolarity of this solution was 295 to 305 mosm. ATP was daily prepared in bath buffer with pH properly readjusted. For peak amplitude dose-response studies, ATP was applied using a fast gravity-driven microperfusion system (BPS-8; ALA Scientific Instruments, Westbury, NY), as previously described (15). Data were collected from recordings of a range of ATP concentration buffers applied to single cells with a washout interval of 3–5 min between each application, and the corresponding currents were normalized to the highest current measured. For studies on activation, deactivation, and desensitization properties of receptors, we used the Ultrafast Solution Switching System application equipment (LSS-3200; EXFO Burleigh Products Group Inc., Victor, NY). ATP solution was applied through the two-barrel glass theta tubing (type TST150–6; World Precision Instruments, Inc., Sarasota, FL) with a ~250 μm diameter tip, which was mounted on the PZS-200 PZT Microstage driven by analog command from pClamp 8.0 software through a PZ-150M amplifier. Voltage applied to the Piezoelectric Actuator produced a rapid lateral displacement (~100 μm) of the theta tubing tip to move the interface between control and test solutions over the whole cell within 1 ms, as measured by the junction potential change in an open pipette tip from a 10-fold dilution of the NaCl solution (for details see supplemental Fig. S1 and its legend). Current responses were always recorded from single spherical cells attached to the bottom of the dish or from lifted cells. For lifted cell recording, after the whole cell was established the cell was carefully lifted from the bottom of the dish above the focus plane of the bottom edge of the theta tube opening. Recording was always done on cells clamped at ~60 mV. Prior to immersing the electrode in bath solution for gigaohm seal, cells expressing P2X₄-fused EGFP were optically detected by an emission signal at 520 nm when excited by 488-nm ultraviolet light.

Modeling—A three-dimensional model of the rat P2X₄ Lys₁₈₀–Lys₃₂₆ ectodomain region was generated using the DeepView/Swiss PdbViewer version 3.7 software package and the SWISS-MODEL server (26, 27). The crystal structure of seryl-tRNA synthetase (Protein Data Bank code 1SES) was used as a template (28). Sequence and secondary structure homology between P2XR and the class II aminoacyl-tRNA synthetases (14) was used to build a three-dimensional model of the rat P2X₄-Lys₁₈₀–Lys₃₂₆ ectodomain region. Disulfide bridges Cys²¹⁷–Cys²²⁷ and Cys²⁶¹–Cys²⁷⁰ were created using the O program (29). The ligand docking started with manual docking of the ATP molecule into the predicted binding site of rat P2X₄ Lys₁₈₀–Lys₃₂₆ ectodomain model on the basis of homology with class II aminoacyl-tRNA synthetases. Subsequently, the resulting complex was energy minimized using the GROMACS 3.1.2 package of programs with the parameters set ffgmx (30). The ATP topology file, as needed in Gromacs, was prepared using the PRODRG server (31). Automated docking of ATP molecule into the predicted P2X₄ agonist binding site using the AutoDock 3.0 program (32) was used to refine the structure of P2X₄-ATP complex.

Confocal Microscopy—The distribution of EGFP-tagged receptors within live cells was examined by laser scanning confocal microscopy. Cells were cultured on poly-L-lysine-coated coverslips, transfected, and imaged the next day. The culture medium was replaced with phenol red and ATP-free Krebs-Ringer buffer, and coverslips with cells were mounted on the stage of a Zeiss LSM 510 Inverted Meta System. Images were collected under >63 objective lens, and further zoom (×2) was also applied. The same detector gain and settings were used for all images.

Calculations—Whenever appropriate, the data were presented as mean ± S.E. values. The time course of the current was fitted by one- or two exponential decay function (ae⁻ᵗ/⁻⁺ + be⁻ᵗ/⁻⁺ + c) using the pClamp 8 program (Axon Instruments). Significant differences, with p < 0.01 or 0.001, were determined by Mann-Whitney test using GraphPad InStat 3.05. Concentration-response data were fitted by a Prism 4 sigmoidal dose-response equation using a nonlinear curve-fitting program that derives EC₅₀ values (GraphPad Software Inc., San Diego, CA). Linear regression analysis and correlation coefficients were generated using Kaleidograph program (Reading, PA).

RESULTS

Characterization of Wild Type P2X₄Rs—The resolution time of a commonly used gravity-driven perfusion system is about 200 ms (33), which limits the analysis of gating properties of wild type and mutant P2XRs. In studies with other ligand-gated receptor channels, the ultrafast perfusion systems, with a resolution time of about 1 ms, were commonly used (34), whereas such analysis was not done for P2XRs. Here we used the LSS-3200 ultrafast solution-switching system to gain quantitative information about P2XR gating. We initially tested the ultrafast solution-switching system using two experimental protocols: patching the cells attached at the bottom of dish (hereafter attached cells) and recording from de-attached cells (hereafter lifted cells), as described under “Materials and Methods” and shown in supplemental Fig. S1. Under both experimental conditions, the response consisted of three phases: a rapid rising phase of inward current induced by application of ATP, a slowly developing decay phase in the presence of agonist, and a relatively rapid decay of current after washout of agonist (supplemental Fig. S2A). The peak current response amplitude was dependent on the ATP concentration, and the estimated EC₅₀ value in wild type receptors was about 3 μM (Table 1). The peak current response was reached within 100 ms (Fig. 2A, and supplemental Fig. S2A). Because none of the commonly used fitting curves describes well this portion of current, we used the 10–90% peak amplitude rise time as a measure of the activation phase.
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TABLE 1
Characterization of wild type (WT) and mutant P2X$_4$R receptors

To determine $EC_{50}$ values, attached cells were stimulated with increasing concentrations of ATP ($n = 5–15$ per mutant), using a fast gravity-driven microperfusion system. The peak amplitude (PA) of current response to the first ATP (1.4 mM) pulse was estimated in a separate series of experiments ($n = 6–12$ per receptor). The activation (AT) and deactivation time (DeaT) values were estimated in response to 1.4 mM ATP applied for 0.1–2 s ($n = 7$). A low affinity agonist for these receptors, than after removal of 2 $\mu$M ATP (126 $\pm$ 6 ms; $n = 6$) (Fig. 2A, inset). This is in accordance with the literature (35). During sustained stimulation with 100 $\mu$M ATP, the inward current decayed toward zero, but with a slower rate compared with the washout time (Fig. 2A). In both preparations, the rate of receptor desensitization was dependent on agonist concentration. 

Removal of agonist was followed by a decay of current to baseline within 500 ms (supplemental Fig. S2A, right bottom). However, the initial fraction of current was not possible to fit well. For this reason, the effects of ATP washout on the kinetic of decay of current were also expressed as 10–90% deactivation time. Deactivation of receptors was agonist-specific; it was faster after removal of 100 $\mu$M 2-MeS-ATP (10–90% deactivation time = 48 $\pm$ 3 ms, $n = 7$), a low affinity agonist for these receptors, than after removal of 2 $\mu$M ATP (126 $\pm$ 6 ms; $n = 6$) (Fig. 2A, inset). This is in accordance with the literature (35).

During sustained stimulation with 100 $\mu$M ATP, the inward current decayed toward zero, but with a slower rate compared with the washout time (Fig. 2A). In both preparations, the rate of receptor desensitization was dependent on agonist concentration (Fig. 2B and supplemental Fig. S2D). Because the one- and two-exponential fittings were not satisfactory for any of the

| PA | $EC_{50}$ | 10–90% AT | 10–90% DeaT | 10% DesT | 90% DesT |
|----|----------|-----------|-------------|----------|----------|
| 10 | 276 $\pm$ 29 | 3.1 $\pm$ 0.5 | 8.2 $\pm$ 0.5 | 113 $\pm$ 6.0 | 58 $\pm$ 4.4 |
| 20 | 284 $\pm$ 66 | 77 $\pm$ 13 || 49.6 $\pm$ 52 || 65 $\pm$ 2.3 || 177 $\pm$ 16 || 18 $\pm$ 1.1 |
| 30 | 280 $\pm$ 33 | 52 $\pm$ 5.9 || 38.7 $\pm$ 2.4 || 234 $\pm$ 11 || 161 $\pm$ 15 || 18 $\pm$ 1.2 |
| 40 | 234 $\pm$ 28 | 28 $\pm$ 4.8 || 27.3 $\pm$ 2.5 || 273 $\pm$ 8.6 || 231 $\pm$ 18 || 13 $\pm$ 0.5 |
| 50 | 93 $\pm$ 8.1 | 3.8 $\pm$ 0.8 | 99.5 $\pm$ 3.9 | 2014 $\pm$ 124 | 193 $\pm$ 11 | 14 $\pm$ 0.5 |
| 60 | 37 $\pm$ 9.1 | 5.1 $\pm$ 1.1 | 216.1 $\pm$ 1.6 | 95 $\pm$ 3.7 | 143 $\pm$ 13 | 15 $\pm$ 1.1 |
| 70 | 227 $\pm$ 32 | 59 $\pm$ 9.5 || 5.6 $\pm$ 0.3 || 28 $\pm$ 2.0 || 27 $\pm$ 1.8 || 12 $\pm$ 0.7 |
| 80 | 104 $\pm$ 12 $\pm$ | 0.6 $\pm$ 0.1 || 8.1 $\pm$ 0.5 || 131 $\pm$ 109 || 196 $\pm$ 14 || 13 $\pm$ 0.8 |
| 90 | 63 $\pm$ 10 | 11 $\pm$ 1.1 | 10.3 $\pm$ 0.6 | 73 $\pm$ 0.9 | 66 $\pm$ 4.2 | 16 $\pm$ 1.5 |
| 100 | 75 $\pm$ 13 | 1.6 $\pm$ 0.4 | 7.5 $\pm$ 1.2 | 309 $\pm$ 28 | 185 $\pm$ 20 | 21 $\pm$ 1.6 |
| 110 | 144 | 5.2 $\pm$ 0.6 | 7.5 $\pm$ 0.9 | 108 $\pm$ 1.6 | 66 $\pm$ 4.6 | 13 $\pm$ 1.0 |
| 120 | 154 | 5.6 $\pm$ 1.3 | 5.0 $\pm$ 0.2 | 446 $\pm$ 19 | 68 $\pm$ 2.6 | 7.1 $\pm$ 0.1 |
| 130 | 309 | 1.5 $\pm$ 0.3 | 7.0 $\pm$ 0.9 | 132 $\pm$ 1.5 | 59 $\pm$ 6.4 | 13 $\pm$ 0.4 |
| 140 | 235 | 1.2 $\pm$ 0.2 | 7.5 $\pm$ 0.8 | 128 $\pm$ 5.3 | 48 $\pm$ 3.5 | 14 $\pm$ 1.0 |
| 150 | 312 | 0.7 $\pm$ 0.1 | 8.3 $\pm$ 0.8 | 194 $\pm$ 7.6 | 50 $\pm$ 3.0 | 14 $\pm$ 0.5 |

FIGURE 2. Characterization of ATP-induced current in lifted HEK293 cells expressing the wild type P2X$_4$R. A, the pattern of current response. Main panel, desensitization pattern in response to application of 100 $\mu$M ATP. Notice the lack of bi-exponential function to fit the peak response. Inset, deactivation kinetics of P2X$_4$R after removal of 2 $\mu$M ATP and 100 $\mu$M 2-MeS-ATP. B, dose-dependent effects of ATP on the desensitization of receptor recorded in the lifted cells. C, independence of 10–90% deactivation time values of ATP concentration. The horizontal line illustrates the mean value for deactivation time. D, inverse relationship between 10 and 90% activation time and ATP concentration. E and F, main panels, inverse relationship between the values for 10 (E) and 90% (F) desensitization time and ATP concentration. Inset, correlation between 10 and 90% activation time and 100 (E) or 90% (F) desensitization time. R, coefficient of correlation. Data points represent mean $\pm$ S.E. from 5 to 12 experiments per dose. When not visible, the error bars were within the circles.
responses (shown by the inability of two-exponential fitting to include the initial decay of current in Fig. 2A, main panel), the results were summarized as 10 and 90% desensitization time values (Table 1). Under both experimental conditions, the peak amplitudes of current responses to 100 μM ATP application were comparable. In contrast, the mean values of 10–90% activation time, 10–90% deactivation time, and 10% desensitization time differed significantly in two protocols (supplemental Fig. S3A).

We also studied the relationship between ATP concentration and activation, deactivation, and desensitization time values. In lifted cells, the deactivation efficiency was independent of ATP concentration and did not correlate with activation and desensitization kinetics of receptor (Fig. 2C). In contrast, the activation efficiency increased with an increase in ATP concentration and the 10–90% activation time and ATP concentration were inversely correlated (Fig. 2D). Like with receptor activation, there was an inverse relationship between the 10 and 90% desensitization time values and ATP concentration (Fig. 2, E and F, main panels). Consequently, there was a strong linear correlation between the 10–90% activation time and 10 and 90% desensitization time values (Fig. 2, E and F, insets). Highly comparable results were also observed in attached cells (supplemental Fig. S4).

These results suggest that rates of activation and desensitization but not deactivation of receptor are dependent on agonist concentration. At low agonist concentrations, the kinetics of receptor activation and desensitization estimated by the whole cell current probably reflects cumulative activation of receptors and the timing of transition between activated and desensitized states, whereas deactivation time could correspond to the mean open time of the channel. To get more synchronous responses and better estimates of the timing of transitions between closed, open, and desensitized states, we further stimulated with multi-maximal ATP concentrations.

Characterization of Mutant Receptors—Electrophysiological measurements indicated that all receptors were functional. However, channels exhibited significant variations in the peak amplitude of currents, the EC50 values, and/or the time values for activation, deactivation, and desensitization when compared with wild type receptors (Table 1). The EC50 values for receptors were estimated in attached cells. K313A, Y315A, G316A, G316S, R318A, and Y323A mutants showed a rightward shift in the sensitivity to ATP, whereas D320A and K329A showed a significant leftward shift in sensitivity. Because of the variations in the EC50 values, the peak current response and the gating kinetics of all mutant receptors were analyzed using 1.4 mM ATP, a concentration that was supramaximal for all receptors other than K313A and K313R.

We initially tested the validity of the LSS-3200 ultrafast solution-switching system in experiments with two ATP concentrations: 100 μM and 1.4 mM. In attached cells expressing wild type receptors, there were obvious differences in the kinetics of receptor activation in response to the two ATP concentrations. Furthermore, in cells treated with 1.4 mM ATP the pattern of deactivation was irregular (data not shown). On the other hand, in lifted cells there were not significant differences in the estimate of receptor activation, desensitization, and deactivation time values in response to 0.1 and 1.4 mM ATP, indicating that the delaying effects of perfusion were minimized (supplemental Fig. S3B). To avoid the impact of high agonist concentration on accuracy of measurements of the gating properties of channels, all measurements of the receptor activity were done using the lifted cell configuration. These experiments are summarized in Table 1.

Our three-dimensional model indicates that Lys313 and Tyr315 residues are integral parts of β strand b6 in close proximity of the ATP binding pocket and thus could participate in ATP binding. Furthermore, Lys313 could be important for the stability of this region due to possible salt-bridge interaction with Glu183 or Glu245 (15). To test the relevance of Lys313 in ATP binding, we generated K313A and K313R mutants. Confocal microscopy indicated that both mutants were expressed at the plasma membrane at comparable levels as the wild type receptor (Fig. 3A). The K313A mutant did not respond to 320 μM ATP application, but generated an inward current in response to 1 and 3.2 mM ATP (Fig. 3B). The response was receptor-mediated, because ATP in this concentration range was ineffective in control cells. When stimulated with 3.2 mM ATP, the peak amplitude of current responses by this mutant was 2.3% of that observed in cells expressing wild type receptors, indicating a shift in ATP potency of about 10,000 times. Similar magnitudes of shifts were observed for several residues identified to
be involved in agonist binding at P2X₄R (15). However, in contrast to these residues, the rescue K313R mutant showed low sensitivity to ATP (Fig. 3C); in response to 3.2 mM ATP the peak amplitude of current responses by this mutant was 4.9% of that observed in cells expressing wild type receptors. These results indicate that only a negligible rescue effect was achieved by preserving the positively charged residue at this position of the molecule, a finding inconsistent with the role of this residue in coordinating the phosphate group of ATP. To test the salt-bridge hypothesis, we generated three mutants: E183A, E245A, and E183A/E245A. As shown in Table 1, all three mutants exhibited enhanced sensitivity to ATP receptors, also arguing against this hypothesis.

The Y315A mutant was functional but showed about 25-fold decreased sensitivity to ATP when compared with the wild type receptor (Fig. 4A). The mutant also showed a 25-fold shift in the EC₅₀ value for αβ-meATP, a partial agonist for this receptor (Fig. 4B). A rightward shift in the sensitivity of this mutant for ATP was accompanied with a delay in activation and desensitization in response to 1.4 mM ATP (Fig. 4C). Both the rapid and slow components of desensitization were affected (Table 1). In contrast, the deactivation time value for this mutant was significantly reduced (Fig. 4D and Table 1).

In our three-dimensional model, the Gly316 residue is located in turn between the β6 strand and the α-helix segment, the latter including Ile317, Arg318, Asp320, and Val323 residues. As shown in Table 1, the majority of mutants in this region responded to 1.4 mM ATP concentration with significantly lower amplitudes of peak responses to the first application of agonist. Confocal microscopy indicated that the low amplitude responders G316P, D320A, V323A, and K329A exhibited comparable plasma membrane expression 24 h after transfection, whereas the plasma membrane expression of the I317A mutant was lower (data not shown). Furthermore, the majority of mutants showed significant changes in the mean values for activation, desensitization, and deactivation.

To test the possibility that changes in the gating values for these mutants were influenced by the lower density of receptors, we compared activation, deactivation, and desensitization properties of the wild type receptor in cells exhibiting normal and lower intensity of fluorescence. Cells with faint fluorescence consistently showed lower amplitude of peak response (in the range of 28 to 65 pA/pF), confirming that intensity of EGFP fluorescence correlates with the level of receptor expression, but the mean values for current activation, desensitization, and deactivation were not significantly different from those shown in Table 1.

The 16-fold decreased sensitivity of G316A mutant for ATP (Fig. 5A, inset) was accompanied with slower activation (Fig. 5A, main panel) and desensitization (Fig. 5B) rates in response to supra-maximal ATP concentration. This mutant also exhibited a slower deactivation property than the wild type receptor even though the potency of ATP for this mutant receptor was significantly reduced (Fig. 5C). The changes in the receptor properties for G316A mutant were not a result of substitution of the polar/uncharged residue with the nonpolar/hydrophobic residue, because the G316S mutant also showed a rightward shift in the sensitivity for ATP and prolonged activation and deactivation time (Fig. 5, A–C). The G316P mutant showed normal sensitivity to ATP, but exhibited similar pattern changes in activation, deactivation, and desensitization kinetics (Fig. 5), clearly indicating that changes in the receptor kinetics induced by mutation of this residue were independent of changes in the EC₅₀ values.

Two other mutants, I317A and I333A, also showed significant changes in the gating properties, although their EC₅₀ values did not differ from controls (Fig. 5D, inset). The I317A mutant exhibited prolonged activation (Fig. 5D, main panel) and desensitization (Fig. 5E, main panel) kinetics, and deactivated with rates highly comparable with the wild type receptor (Fig. 5F). In contrast, the I333A mutant activated (Fig. 5D, main panel) and desensitized (Fig. 5E, inset) more rapidly than the wild type receptor, but deactivation of this mutant was prolonged (Fig. 5F). This mutant clearly showed the existence of two steps in desensitization of receptor, the rapid phase, which was indistinguishable from that observed in the wild type receptor, and the slow phase, which was accelerated (Fig. 5E, main panel and inset).

In a further study, we compared the R318A mutant, which exhibited a rightward shift in the sensitivity to ATP (15), with D320A and K329A mutants, which showed a leftward shift (Fig. 6A, inset). The Arg318 mutant deactivates more rapidly than...
wild type receptors, whereas the K329A and D320A mutants deactivated with a significant delay (Fig. 6A, main panel). In addition, the R318A mutant desensitized more rapidly, whereas K329A (Fig. 6, B and C) and D320A mutants (Table 1) desensitized more slowly than wild type receptors. Two-step desensitization was evident in the K329A mutant in response to 1.4 mM ATP (Fig. 6C) and 100 μM ATP (Fig. 6D).

**DISCUSSION**

In the absence of the crystal structure of P2XRs and selective agonists and antagonists, the molecular recombinant techniques were commonly used for identification of functional P2XR domains. However, the interpretation of these results is not simple and straightforward. One of the principal questions in studies with P2XR mutants is related to the impact of variation in the peak amplitude of the current response on gating. Specifically, it was found that properties of P2X2R are sensitive to changes in receptor density (36, 37). In our experimental conditions, there was no difference in the gating properties of wild type P2X4R in cells responding to 1.4 mM ATP with 30–300 pA/pF current amplitude, which is in the range of changes in the peak amplitude of currents observed in mutants. Experiments with wild type receptor also indicated a positive correlation between EGFP fluorescence and the size of the current. To reduce the impact of the level of receptor expression, all recordings were...
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done in cells exhibiting comparable fluorescence. The confocal analysis also showed comparable plasma membrane expression levels of all mutant receptors other than I317A, which was less expressed. These observations do not exclude variations in the plasma membrane expression of mutants, but support the conclusion that in our experiments changes in the receptor density are not in the range affecting gating.

The other major question is how to distinguish between residues participating in binding from those participating in other receptor functions. Specifically, if the residue is an integral part of the binding pocket, mutation should result in changes of agonist potency. However, modification of the structures that participate in transduction of signals to the channel gate could also produce the shift in EC$_{50}$ by changing the efficacy of coupling to the gate, or by changing the equilibrium between closed-open states (38). In our study that was not the case with the G316P, I317A, F330A, and I333A mutants, because their EC$_{50}$ values were comparable with controls, indicating the role of these residues in functions other than ATP binding. On the other hand, K313A, K313R, Y315A, R318A, D320A, V323A, and K329A mutants showed significant changes in the sensitivity to ATP and gating kinetics, clearly indicating that interpretation of results with these mutants is more complex.

Over a 1000-fold rightward shift in the sensitivity of the K313A mutant resembled the behavior of several other residues previously identified as critical for ATP binding at P2X$_4$R (15). Mutation of the corresponding residue in P2X$_2$R (10), P2X$_3$R (12), and P2X$_7$R (22) produced non-functional channels. Evans’ group (9) has suggested that this residue is an integral part of the ligand binding pocket contributing to the coordination of the phosphate groups of ATP. Consistent with this view, receptor function was partially rescued by K309R-P2X$_4$R and K308R-P2X$_4$R mutants, which exhibited about 25- and 30-fold rightward shifts in the sensitivity to ATP, respectively (10, 12). In contrast, our experiments with the K313R mutant did not show a significant rescue effect, arguing against such a role of this residue in P2X$_4$R.

We also tested the potential relevance of this residue using the three-dimensional modeling of P2X$_4$R. Modeling based on sequence alignment of rat P2X$_4$ fragment Lys$^{180}$–Lys$^{326}$ and seryl-tRNA synthetase (PDB code ISES) fragment Leu$^{246}$–Asn$^{292}$ (see “Materials and Methods” and supplemental Fig. S5) could integrate this residue in the binding pocket. However, it could not accommodate the role of this residue in coordination of the $\alpha$-phosphate, because it is close to the $2'\text{-}$hydroxyl group of the ribose portion of ATP (Fig. 7A). Furthermore, the K313R mutant exhibited only a minor leftward shift in sensitivity to ATP when compared with K313A, a finding inconsistent with the role of positively charged residues in coordination of the phosphate group. Finally, residue Lys$^{190}$ in the model shown in Fig. 7A is far from the phosphate group of ATP, which is in contrast with our earlier results showing a rightward shift in the sensitivity of this mutant for ATP and a rescue of receptor function by the K190R mutant (15). These results suggest that the three-dimensional model of the P2X$_4$ ectodomain does not provide a rationale for the critical role of the Lys$^{313}$ residue in ATP binding. This prompted us to continue investigations on its role in ATP action. Specifically, we tested the potential importance of this residue for the stability of the binding domain due to a possible salt-bridge interaction with Glu$^{183}$ or Glu$^{245}$ predicted by the model of Yan et al. (15), by generating E183A, E245A, and E183A/E245A mutants. However, these experiments revealed a leftward shift in the sensitivity of mutant receptors for ATP, a finding that argues against this hypothesis.

In the absence of other experimental data, we may speculate that the Lys$^{313}$ residue has an important role in the intersubunit organization or action of receptor. This is well documented for the residues contributing to the formation of the zinc binding site at P2X$_3$R (39), as well as for the 25 amino acid residues before the TM2 domain, which are essential for proper protein assembly (40). It has also been suggested that Lys$^{313}$ and Lys$^{67}$ residues (P2X$_2$R numbering) from two different subunits interact in agonist binding or channel opening in heteromeric P2X$_{2/3}$ receptors (41). Thus, it is possible that Lys$^{313}$ residue of P2X$_4$R also participates in the formation of the intersubunit ATP binding pocket, but does not directly contribute to the recognition of ATP molecule.

The Y315A mutant of P2X$_4$R also exhibited a rightward shift in the sensitivity to ATP, accompanied with an increase in the activation and desensitization time values and a faster rate of receptor deactivation. Evans and collaborators (9) used partial agonists as a tool to dissociate between residues contributing to ligand binding from those participating in the signal transduction. Our experiments with partial agonist, $\alpha$-B-meaATP, are consistent with the hypothesis that this residue contributes to the binding domain. In glutamate receptor channels with well defined ligand binding domains, mutations in this region caused shifts in agonist sensitivity, and there was a reverse correlation between the EC$_{50}$ and deactivation values (42). In that respect, a decrease in deactivation time for the Y315A mutant also supports the view that this residue contributes to ATP binding. Only minor changes in the model of Yan et al. (15) were required to accommodate such a role of this residue. In the revised model shown in Fig. 7B, the hydroxyl group of Tyr$^{315}$ is within hydrogen bond distance from the $2'\text{-}$hydroxyl group of the ribose and the Lys$^{190}$ residue accounts for the coordination of the $\alpha$-phosphate.

Glycine and alanine usually substitute with each other in mutagenesis (43). In contrast, the P2X$_4$-G316A mutant showed a 17-fold rightward shift in the sensitivity for ATP, accompanied with an increase in activation time and a delay in receptor desensitization, whereas deactivation of this mutant was delayed. It is interesting that mutation of the P2X$_4$-R-Gly$^{312}$ residue to alanine induced a 3-fold increase in ATP potency with no effects on the time course of the response (24). The G316S mutant also showed a rightward shift in sensitivity to ATP and a delay in activation, desensitization, and deactivation of the receptor, arguing against the relevance of polarity of this residue for channel functions. Because G316P showed no changes in the EC$_{50}$ value, and exhibited changes in activation, deactivation, and desensitization kinetics in the same direction as two other mutants, it is secure to conclude that this particular residue has a role in signal transduction rather than ATP binding.

Glycine residues are often found in sites of sharp bends, turns, or close packing of adjacent secondary structure ele-
ments, where they serve as elements with enhanced conformational freedom due to unrestrained angular range and minimal side chain (44). The Gly316 residue has a unique position in our homology model of P2X4R (Fig. 7B), because it is located in the turn between the β6 strand and the α-helix segment preceding TM2. Thus, this residue could play an essential role in switching proteins between distinct functional states by affecting the flexibility of receptor to the open-closed conformational transition (45, 46). In potassium channels, the glycine residue has a critical role in gating as a hinge (47). In the 5-HT3 receptor, cis-trans isomerization of proline can mimic the role of a hinge in opening the pore (48). In our hands, the G316P mutant rescued the EC50 value but not the activation-deactivation-desensitization properties of the receptor. These findings indicate that Gly316 does not have such a critical role in gating as the glycine hinge in potassium channels, but that the flexibility of Gly316 is important for functional coupling of the ligand binding domain with the α-helix.

The K329A and D320A mutant exhibited about 2- and 5-fold leftward shifts in the sensitivity for ATP, a finding inconsistent with the potential role of these residues in the coordination of the negatively charged phosphate groups and coordination of ATP binding via the magnesium ion, respectively. Our published experiments with the partial agonist, αβ-meATP, are also consistent with the hypothesis that Arg318 contributes to the transduction of signaling rather than agonist binding (15). It is interesting that the increase in sensitivity of the K329A and D320A mutants for ATP was not accompanied by faster desensitization of receptors, as one may predict based on observations that the highly sensitive P2X1R and P2X3R desensitize rapidly, medium sensitive P2X4R and P2X2R desensitize slowly, and the low sensitive P2X7R does not desensitize (3). All three mutants clearly showed the existence of rapid and slow phases in receptor desensitization. Together with the finding that only the fast component was significantly affected in the majority of mutants, these observations are consistent with a hypothesis that conformation changes of P2XRs associated with desensitization occur in two steps.

FIGURE 7. Two models of P2X4R receptor, with (A) and without (B) the Lys313 residue included in the binding pocket. A, model of rat P2X4 ectodomain fragment Lys180–Lys326 with bound ATP is based on homology with aminoacyl-tRNA synthetases (see supplemental Fig. S2). The ectodomain adopts a six-stranded antiparallel β-pleated sheet structure. Residues of rat P2X4 predicted to be involved in ATP binding are shown as sticks. Two disulfide bridges of rat P2X4, Cys217–Cys227 and Cys261–Cys270, are shown in orange. B, location of Tyr315 within the ATP-binding site. The hydroxyl group of Tyr315 is in hydrogen bond distance from the 2′-hydroxyl group of the ribose. For details on modeling, see Ref. 15.
Role of Lys$^{313}$–Ile$^{333}$ Sequence in P2X$_4$R Activity

mutants not involved in ligand binding could reflect the relative stability of open and closed conformations. In the case of P2X$_4$ transmembrane domain mutants, the majority of receptors exhibited enhanced sensitivity to ATP, leading the authors to conclude that leftward shifts in EC$_{50}$ arise predominantly from destabilization of the closed conformation (25). In our experiments with α-helix mutants, both leftward and rightward shifts in receptor sensitivity were observed in an alternating way. Finally, our experiments clearly indicate that mutation of residues in the Gly$^{316}$–Lys$^{329}$ sequence produced larger changes in activation (up to 12-fold) and deactivation (up to 17-fold) kinetics than in the desensitization kinetic (up to 4-fold). Consistent with this conclusion, numerous findings have indicated the major role of intracellular domains in control of receptor desensitization (reviewed in Ref. 3).

Certainly, the first half of the ectodomain and the TM1 domain are also involved in signal transduction and gating (19, 25, 49). Furthermore, the residues nearby the TM1 domain could participate in ATP binding (7). However, at the present time our model does not provide an explanation for the role of this region in binding and signal transduction. The three-dimensional model of P2X$_4$R is strictly based on alignment between P2XR sequences with sequences of class II aminoacyl-tRNA synthetases (14). This in turn limits modeling of the second half of the extracellular domain. In the absence of an appropriate template, an extension of the model to the first half of the ectodomain and the two TM domains would be pure speculation. On the other hand, the development of other homology models could be helpful to test our model and to overcome its limits.

Taken together, these results provide additional information for understanding the structure-function relationship in transduction of signals from the binding pocket of P2X$_4$R toward the TM2 domain. Experimental and modeling data suggested that Tyr$^{315}$ could be the last COOH-terminal residue contributing to the ligand binding pocket. The results further indicate a critical role of Lys$^{313}$ in receptor function but the three-dimensional model of the P2X$_4$ ectodomain did not offer a rationale for the direct role of this residue in ATP binding. Experiments also argued against the model prediction for the relevance of salt-bridge interaction between Lys$^{313}$ and Glu$^{183}$ or Glu$^{245}$. The results further indicated that Gly$^{316}$ provides the flexibility needed for transduction of signals from the ligand binding pocket to the linker region. In addition, mutation-induced changes in the linker region affect agonist potency and the rates of channel closing in the presence of agonist and after its washout. These experimental observations support the model of Yan et al. (15) for the organization of the linker region as an α-helix that acts as an elastic spring, providing the tension needed to open and close the gate hinge and to affect the stability of open and/or closed conformations, which in turn affect the agonist sensitivity of the receptors. The role of the Gly$^{316}$–Ile$^{333}$ sequence in transduction of signaling from the ligand binding pocket to the gate hinge is also consistent with the positions of the ATP binding residues in Roberts (3, 7) and Wilkinson et al. (41) models of the ATP binding pocket, as well as with Khakh and Lesters(17) suggestion that Gly$^{347}$ functions as a gating hinge.

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