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Involvement of Ectodomain Leu 214 in ATP Binding and Channel Desensitization of the P2X4 Receptor

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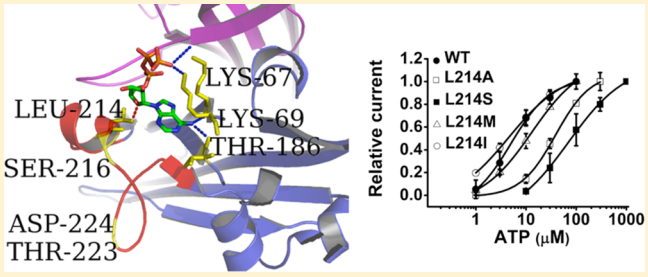
Supporting Information

ABSTRACT: P2X receptors are trimeric ATP-gated cation permeable ion channels. When ATP binds, the extracellular head and dorsal fin domains are predicted to move closer to each other. However, there are scant functional data corroborating the role of the dorsal fin in ligand binding. Here using site-directed mutagenesis and electrophysiology, we show that a dorsal fin leucine, L214, contributes to ATP binding. Mutant receptors containing a single substitution of alanine, serine, glutamic acid, or phenylalanine at L214 of the rat P2X4 receptor exhibited markedly reduced sensitivities to ATP. Mutation of other dorsal fin side chains, S216, T223, and D224, did not significantly alter ATP sensitivity. Exposure of L214C to sodium (2-sulfonatoethyl) methanethiosulfonate ($MTSES^-$) or (2-aminoethyl) methanethiosulfonate hydrobromide in the absence of ATP blocked responses evoked by subsequent ATP application. In contrast, when $MTSES^-$ was applied in the presence of ATP, no current inhibition was observed. Furthermore, L214A also slightly reduced the inhibitory effect of the antagonist 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP, and the blockade was more rapidly reversible after washout. Certain L214 mutants also showed effects on current desensitization in the continued presence of ATP. L214I exhibited an accelerated current decline, whereas L214M exhibited a slower rate. Taken together, these data reveal that position L214 participates in both ATP binding and conformational changes accompanying channel opening and desensitization, providing compelling evidence that the dorsal fin domain indeed has functional properties that are similar to those previously reported for the body domains.



P2X receptors (P2XRs) are trimeric, cation permeable ion channels activated by adenosine 5′-triphosphate (ATP).[1] When ATP binds, the receptor undergoes large conformational changes that lead to the opening of the transmembrane ion permeable pore.[2] This process allows entry of Na^+ and Ca^{2+}, thus causing membrane depolarization and affecting various downstream Ca^{2+}-dependent signaling processes.[3] The seven known P2XR subtypes are widely distributed in mammalian tissues and involved in physiological functions as diverse as pain signaling, inflammation, synaptic transmission, taste, and bone formation.[4]

Crystal structures of the zebrafish P2X4 receptor (zfP2X4R) have been determined in both the closed ligand-free state and open ATP-bound state.[5,6] In the absence of the intracellular domains, which were not resolved in the crystal structures, the overall shape of the P2XR subunit resembles a dolphin: the tail forming the transmembrane domain and the extracellular head, body, left flipper, and dorsal fin contributing to the ATP binding pocket (Figure 1 of the Supporting Information). The head and dorsal fin domains are predicted to move closer to each other following ATP binding, closing like a jaw over the ligand and producing the initial conformational movement required to gate the channel.[6,7] Functional studies had previously identified many key amino acids critical to ATP sensitivity that the structures show to be located within the putative binding pocket. For example, mutations of conserved positive, polar, and aromatic side chains in the upper and lower bodies of adjacent subunits have been shown to markedly reduce ATP potency in P2X1R,[8−11] P2X2R,[12,13] P2X3R,[14,15] and P2X4R.[13,16,17] These include the positively charged and conserved lysines K67 and K69 (rat P2X4 numbering) in the lower body, which are shown in the open channel structure interacting with the negatively charged phosphate groups of ATP. Adjacent to these is the conserved polar residue T186, mutation of which has been shown to markedly reduce ATP potency, and which the open channel crystal structure suggests interacts with the adenine ring.[6] In the upper body region are an additional conserved lysine, K313, and an NFR motif, the aromatic F294 side chain, that have been proposed to interact

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with the adenine ring of ATP[9] but also appear in the crystal structure interacting with the charged phosphates of ATP. More recently, side chains within the head domain[18] and left flipper domain[19] have been confirmed to regulate ligand binding and selectivity.

To date, however, there are scant functional data confirming a role of the dorsal fin in ligand binding. The dorsal fin region of each subunit is shown to come into the proximity of the bound ATP molecule in the open channel structure. On the basis of our homology model of the rat P2X4 receptor (rP2X4R), we sought to test the hypothesis that ATP makes hydrophobic contact with uncharged side chains in the dorsal fin. We used site-directed mutagenesis, electrophysiology studies, methanethiosulfonates (MTS), ATP analogue 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (TNP-ATP), and ivermectin (IVM) to investigate the effects of noncharged amino acids in the dorsal fin domain on ATP binding in rP2X4R. Our results provide compelling evidence that the dorsal fin domain indeed has functional properties that are similar to those previously reported for the body domains, a result that highlights the implication of this domain in agonist binding.

MATERIALS AND METHODS

DNA Constructs. Point mutations were engineered using the KOD-Plus-Mutagenesis Kit (TOYOBO) and verified by Beijing Genomics Institute (BGI, Shenzhen, China).

Cell Culture. HEK-293 cells were cultured and transfected with the wild-type (WT) or mutant receptor cDNA (1 μg/mL). GFP cDNA (1 μg/mL) was cotransfected as a transfection reporter. The patch clamp was performed after transfection for 24 h. Transfected cells were trypsinized and plated on coverslips 1 h before the patch clamp recording.

Patch Clamp Recordings. The perforated patch clamp was performed using an Axopatch 200B amplifier (Axon Instruments), a Digidata 1440A interface, and Clampfit version 10.2 (Molecular Devices). The data were low-pass filtered at 2 kHz and digitized at 5 kHz. Electrophysiology was performed at room temperature voltage clamped at −60 mV using standard voltage clamp techniques as we previously described.[20] Patch electrodes had resistances of 2−4 MΩ when filled with a solution containing 154 mM CsCl, 11 mM EGTA, 10 mM HEPES, and 200 μg/mL amphotericin B (pH 7.2 adjusted with CsOH), and recordings were made only when the access resistance was <25 MΩ. The external solution contained 154 mM NaCl, 10 mM D-glucose, 10 mM HEPES, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ (pH 7.4 adjusted with NaOH). The averaged cell capacitance was 20.3 ± 3.5 pF. The sodium (2-sulfonatoethyl)methanethiosulfonate ($MTSES^-$) and (2-aminoethyl) methanethiosulfonate hydrobromide ($MTSEA^+$) were obtained from Toronto Research Chemicals (Toronto, ON) and used as previously described.[12] TNP-ATP trisodium salt was from Invitrogen (Eugene, OR). IVM was dissolved in dimethyl sulfoxide, stored in stock solutions at 10 mM, and diluted to 3 μM in the external solution prior to experiments. All drugs were applied using an RSC-200 Rapid Solution Changer (BioLogic). Solution exchange occurred in 20 ms/tube. All other chemicals were purchased from Sigma.

Data Analysis. Concentration−response data were fit to the Hill equation (SigmaPlot version 10.0, SPSS Inc.): $I = I_{max}/[1 + (EC_{50}/[A])^{n_H}]$, where I and I_{max} are the peak current of a given ATP concentration and the maximal current, respectively, [A] is the concentration of ATP, and n_H is the Hill coefficient. The concentrations evoking half-maximal currents (EC_{50}) were obtained. The time between ATP applications was at least 120 s depending on the recovery of the cell from desensitization. The time constants corresponding to the decay of current from the steady state to the baseline evoked by washout of ATP were fit to a standard single-exponential function (Clampfit version 10.2). Tests for differences were made using the Independent-Samples T Test for a two-group comparison or one-way analysis of variance for a muiltiple-group comparison (IBM SPSS statistics 19, SPSS Inc.). Pooled data are presented as means ± the standard error of the mean for n cells.

Homology Modeling and ATP Docking. Sequence alignment of rP2X4R and zfP2X4R was performed using ClustalW.[21] We used the ATP-bound open state (Protein Data Bank entry 4DW1)[6] as a template for homology models of rP2X4R. The model was built and refined by using Discovery Studio 3.0 (Accelrys, Inc., San Diego, CA). All figures were prepared with PyMOL (Schrödinger, LLC, Portland, OR).

RESULTS

L214 Substitutions Alter ATP Sensitivity. We decided to select rP2X4R as a receptor model because of large and reproducible ATP-gated currents and the sensitivity to the polycyclic lactone, ivermectin (IVM). The latter property provides an effective means of studying mutants exhibiting substantially impaired current amplitudes.[22,23] To test the prediction of parts of the dorsal fin that come into the proximity of ATP during agonist binding, we investigated whether the introduction of alanine substitutions within this domain would affect ATP potency.

The perforated patch method was used for its reproducible responses to ATP without a progressive loss of peak current amplitude when more than two drug applications were needed. HEK-293 cells expressing the WT rP2X4Rs responded to ATP with a robust inward current (Table 1 and Figure 1A) in the perforated patch configuration [EC_{50} = 5.0 ± 1.2 μM (n = 7)]. Mutant receptors S216A, T223A, and D224A had EC_{50} values (Table 1) comparable to that observed for the WT, suggesting that these side chains are not critical determinants of ATP sensitivity. However, compared to the WT, the L214A mutant displayed a marked decrease in ATP sensitivity [EC_{50} = 38.8 ±

Table 1. Summary of Functional Data for the rP2X4 WT and Mutant Receptors[a]

	ATP EC_{50} (μM)	I_{max} (pA/pF)	tested cells	n_H
WT	5.0 ± 1.2	75 ± 7	7	1.4 ± 0.2
L214A	38.8 ± 3.6***	67 ± 9	4	1.3 ± 0.1
S216A	5.3 ± 1.4	65 ± 24	3	1.0 ± 0.3
T223A	8.1 ± 2.0	107 ± 38	4	1.3 ± 0.2
D224A	4.5 ± 1.0	75 ± 24	5	1.0 ± 0.2
L214S	70.2 ± 18.1***	42 ± 13	7	1.2 ± 0.2
L214C	26.5 ± 8.2*	83 ± 18	8	1.2 ± 0.2
L214I	4.5 ± 0.8	44 ± 17	5	1.3 ± 0.5
L214M	13.1 ± 0.7	79 ± 16	5	1.1 ± 0.1
WT with IVM	1.3 ± 0.1	250 ± 30	7	1.4 ± 0.1
L214E with IVM	12.6 ± 0.2***	90 ± 32	4	1.7 ± 0.1
L214F with IVM	12.2 ± 3.2***	150 ± 74	4	1.1 ± 0.2

[a] I_{max} represents the response to the 100 μM ATP application for all receptors except L214A (to 300 μM) and L214S (to 1 mM). The last three rows list functional data in the presence of 3 μM IVM. Significant differences from WT or WT with IVM are denoted with three asterisks (p < 0.001) or one asterisk (p < 0.05), respectively.

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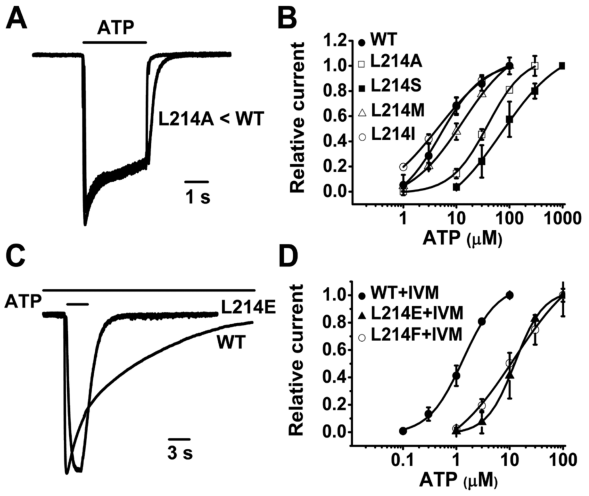

Figure 1. L214 substitutions alter ATP sensitivity. (A) Representative recordings (a 3 s ATP application) of WT rP2X4R and L214A mutant currents evoked by 100 μM ATP and time course of receptor deactivation after removal of ATP. (B) Concentration−response relationship for the ATP-activated peak current through WT P2X4R and L214 mutants. (C) Representative recordings (a 3 s ATP application) of WT P2X4R and L214E mutant currents evoked by 10 μM ATP in the presence of 3 μM IVM (3 min preincubation) and time course of receptor deactivation after removal of ATP. (D) Concentration−response relationship for the ATP-activated peak current through WT P2X4R and L214E and L214F mutants in the presence of 3 μM IVM.

3.6 μM (n = 4)] (Table 1 and Figure 1B). The deactivation time constant upon removal of ATP was independent of ATP concentration [10 μM, 333 ± 9 ms; 30 μM, 394 ± 38 ms; 100 μM, 385 ± 64 ms (n = 3)], with an average constant of 371 ± 24 ms. On average, for the L214A mutant, the time constant was decreased significantly [95 ± 14 ms (n = 4; p < 0.001)] (Figure 1A). In further studies, we focused on this residue.

The observation that substitution of L214 with an alanine resulted in a right-shifted ATP concentration−response curve indicated a significant decrease in ATP potency. The mutation of L214 to a small polar (L214S) residue caused a larger right shift in the ATP concentration−response curve [EC_{50} = 70.2 ± 18.1 μM (n = 7)] with respect to WT (Table 1 and Figure 1B). Introducing amino acids with similar structures (L214I and L214M) did not significantly change the EC_{50} values (Table 1 and Figure 1B). These data suggest that hydrophobicity is important for normal ligand binding.

Substituting the bulky nonpolar Phe or a negatively charged Glu failed to yield reasonable current amplitudes in response to 100 μM ATP, rendering accurate determination of the EC_{50} unfeasible. In this instance, the sensitivity of rP2X4R to be potentiated by IVM was exploited. This was done to rescue function for these mutants, as has been successfully achieved for other P2X4 mutants.[22] Preincubation with 3 μM IVM increased the current amplitude of both L214F and L214E. For the WT receptor in the presence of IVM, the EC_{50} value for ATP was 1.3 ± 0.1 μM (n = 7). The L214F and L214E mutants exhibited EC_{50} values of 12.2 ± 3.2 μM (n = 4) and 12.6 ± 0.2 μM (n = 4), respectively (Table 1 and Figure 1D), indicating a significant decrease in ATP sensitivity upon substitution of L214 with phenylalanine or glutamate. These data suggest that the size and charge at position 214 are also essential for receptor function.

In addition to causing a potentiation of the ATP-gated current amplitude through P2X4Rs, IVM also prolongs the deactivation upon agonist removal. Interestingly, the effect of IVM on the deactivation time of L214E [3.9 ± 0.4 s (n = 4)] was markedly reduced compared to that of WT [26.5 ± 3.9 s (n = 5; p < 0.001)] (Figure 1C).

Effects of MTS Reagents on ATP Responses at the Cysteine 214 Mutant. A decrease in ATP potency could result from an effect on agonist binding and/or gating of the channel. We hypothesized that if the side chain of position 214 occupies the ATP binding site, then an introduced cysteine here should be accessible to covalent modification by water-soluble MTS reagents in the absence of ATP, as previously shown.[11,12] In contrast, we would predict that when there is a high probability of ATP occupying the binding site, the side chain of L214C would no longer be accessible to the same MTS reagents. Therefore, we generated a mutant receptor, L214C, and tested the ability of $MTSES^-$ to modify ATP-evoked responses through this mutant. $MTSES^-$ is negatively charged and was used for an initial investigation because the binding domain is lined mainly with positively charged residues that are suitable for interaction with ATP. The WT P2X4Rs were not activated by MTS reagents (Figure 2 of the Supporting Information). Then we performed the following two experiments.

In the first, we tested whether $MTSES^-$ could covalently modify L214C in the absence of ATP. Here, cells were exposed to repeated applications of ATP alone to demonstrate reproducible ATP-evoked currents. Then, in the absence of ATP, cells were pre-exposed to $MTSES^-$ (1 mM) for a very brief period of 2 s, followed by additional applications of ATP and $MTSES^-$ together. Consistent with our prediction, the ATP-evoked current was significantly suppressed [85 ± 1% blocked by $MTSES^-$ (n = 10; p < 0.001)] after exposure to $MTSES^-$, indicating that $MTSES^-$ can access and modify L214C (Figure 2A). In addition, a positively charged MTS derivative, $MTSEA^+$ (1 mM, 2 s), also caused 82 ± 4% inhibition (n = 3; p < 0.001) (Figure 2B).

However, the inhibitory effect of $MTSES^-$ was reversible (showing a near 100% recovery following a 15 min washout). Previous investigations found that the partial recovery of current after washout of $MTSES^-$ with an external solution may be related to agonist-induced trafficking.[13] Therefore, we decided to see whether a longer incubation with the MTS reagents had an irreversible effect. $MTSES^-$ caused a near complete block of ATP-gated current following a 20 min preincubation and also a 59 ± 1% (n = 3) block following a 15 min washout (Figure 2C). The reducing agent, DTT (10 mM, 5 min pretreatment), partially increased ATP-induced currents as compared with the responses before DTT application (Figure 2C).

Responses were inhibited by 99 ± 1% after a 20 min $MTSEA^+$ preincubation (Figure 2C). The ATP-evoked current was still inhibited significantly by 71 ± 6% (n = 3) following a 15 min washout with an external solution and was immediately and fully reversed by DTT (Figure 2C), indicating that the majority of the plasmalemmal L214C receptors were covalently modified by $MTSEA^+$. These results show that longer incubations with MTS reagents are required to covalently modify the receptors, suggesting that washout following short applications of $MTSES^-$ (2 s) results from trafficking of unmodified mutant P2X4Rs to the cell surface. Furthermore, the similar modulatory effects of positively and negatively

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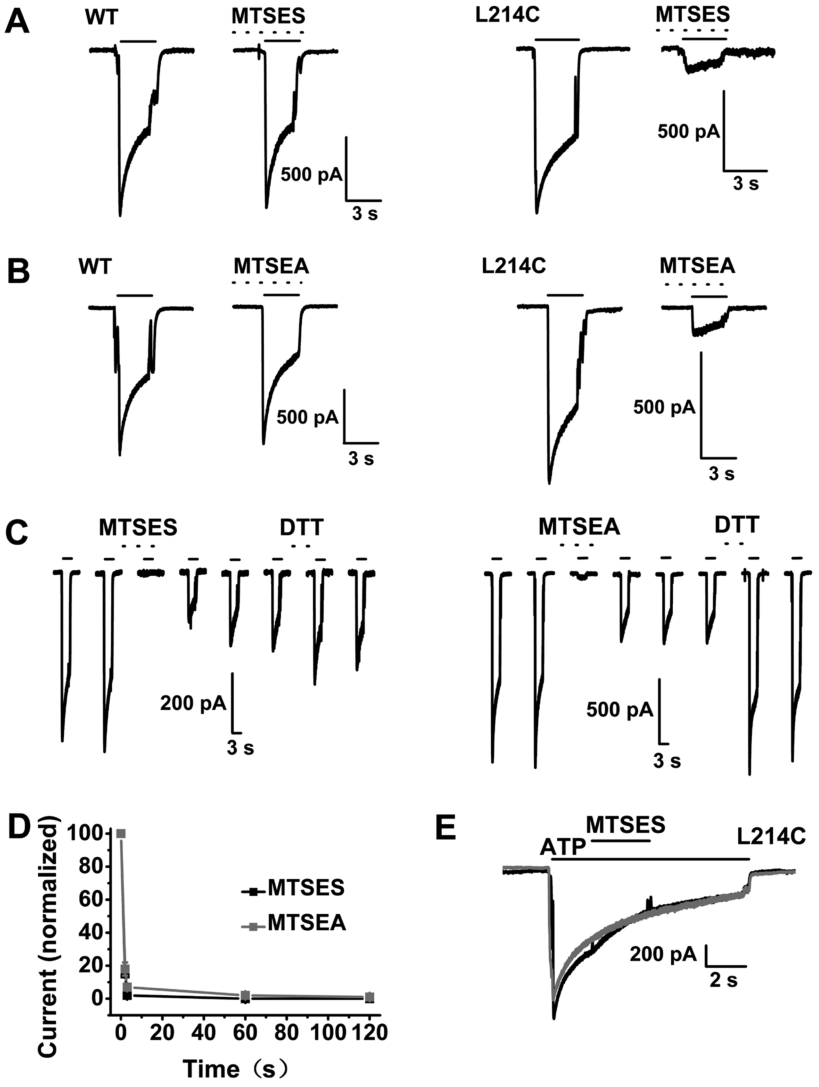

Figure 2. Effects of MTS reagents on ATP responses of the cysteine mutant. Representative recordings of currents observed before and during the addition of $MTSES^-$ (A) and $MTSEA^+$ (B) on the WT P2X4R and L214C mutant. ATP (100 μM, 3 s duration) application is indicated by the solid line. The application of MTS reagents (1 mM, 2 s preincubation) is indicated by the dotted line. (C) Representative recordings of currents observed before, during, and after the addition of $MTSES^-$ (left) and $MTSEA^+$ (right) to the WT P2X4R and L214C mutant. The application of ATP (100 μM, 3 s duration) is indicated by the solid line. The application of MTS reagents (1 mM, 20 min preincubation) or DTT (10 mM, 5 min preincubation) is indicated by the dotted line. (D) Summary of the average data of individual measurements from three or more cells for the kinetics of onset of inhibition by $MTSES^-$ (black) and $MTSEA^+$ (gray). (E) Sequential overlaid traces of the ATP-gated current, separated by 180 s, are shown before (gray) and during (black) an application of $MTSES^-$.

charged MTS compounds suggest that it is the introduction of the bulk of the side groups rather than the charge that modifies ATP′ action. It should be noted that the different reduction effects of DTT may be related to the different membrane permeability of $MTSES^-$ (membrane impermeant) and $MTSEA^+$ (membrane permeant). $MTSEA^+$ could modify more of the pool of P2X4 receptors.

In parallel control experiments, the application of $MTSES^-$ or $MTSEA^+$ had no significant effect [93 ± 5 or 103 ± 12% of the control, respectively ($p > 0.05$; $n = 5–7$)] on the amplitude of ATP-evoked responses at WT P2X4Rs (Figure 2A,B), consistent with previous studies.[24]

In the second experiment, we tested whether $MTSES^-$ could still effectively modify L214C in the presence of a saturating concentration of ATP. Because the onset of inhibition by the $MTSES^-$ was rapid (Figure 2D), a 3 s application could be used. Here, cells were exposed first to a binding site saturating concentration of ATP (1 mM) and then, in the continued presence of ATP, additionally exposed to a 3 s application of $MTSES^-$ (Figure 2B). Again, consistent with our prediction, $MTSES^-$ had little effect on the current amplitude in the presence of ATP ($n = 4$), suggesting that $MTSES^-$ cannot access or modify L214C when ATP is occupying its binding site.

L214 Plays a Critical Role in the Slowly Reversible Block by TNP-ATP. Our data suggest that L214 is involved in ATP binding. We then attempted to determine whether the L214 mutation was also involved in the interaction with TNP-ATP, a known P2XR competitive antagonist that is thought to occupy the ATP binding pocket.[25−27] An ATP concentration that was close to the EC_{50} value of the ATP concentration−response curve was used in these experiments for WT and L214A mutant receptors. TNP-ATP (30 μM) largely inhibited ATP currents after a 4 min preincubation [89 ± 1% ($n = 9$) for

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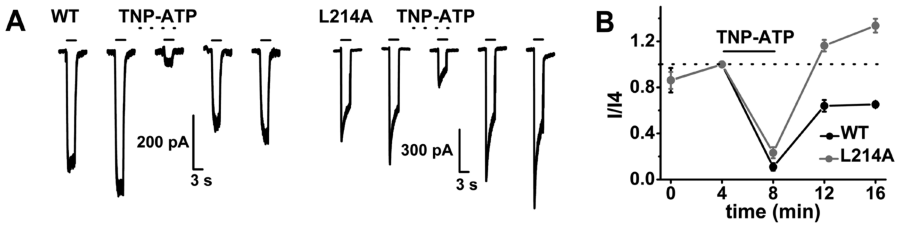

Figure 3. Residue 214 plays a role in the slowly reversible block by TNP-ATP. (A) Representative recordings of currents observed before, during, and after the addition of TNP-ATP to the WT P2X4R and L214A mutant. The application of ATP (5 μM for WT P2X4R, 40 μM for L214A, 3 s duration) is indicated by the solid line. The application of TNP-ATP (30 μM, 4 min preincubation) is indicated by the dotted line. (B) Time course of inhibition by TNP-ATP (30 μM) on WT P2X4R (black line) and L214A (gray line). Results are plotted as averaged values of individual measurements from four or more cells. Peak amplitudes of ATP-gated currents were normalized to the peak current of the ATP-gated current that immediately preceded the application of TNP-ATP.

WT and 77 ± 2% (n = 4) for L214A]. TNP-ATP caused a slightly weaker but significant (p < 0.001) inhibition of the current magnitude through L214A (Figure 3A). Interestingly, the inhibition rapidly reversed within 4 min of TNP-ATP washout in the mutant receptor [116 ± 3% remaining (n = 4)] compared to WT P2X4Rs [64 ± 2% remaining (n = 7; p < 0.001)] (Figure 3A,B). This suggests that Leu is essential for the irreversible component of the block, but it is not the sole residue contributing to P2X4R in binding the antagonist.

Molecular Modeling Predicts That L214 Occupies the ATP Binding Pocket of rP2X4R. A comparative analysis revealed that the amino acid sequences of rP2X4R and zfP2X4R are 61% identical. We generated a homology model of rP2X4R based on the crystal structures of zfP2X4R. The dorsal fin appeared to contribute part of the binding pocket (Figure 4A, structure, and Figure 4B). It comprised an α-helix and a β-sheet, connected by a short random coil (red in the structure in Figure 4A). Positions mutated into a single alanine are colored yellow in the three-dimensional model of rP2X4R and red in amino acid sequences of rP2X4 (Figure 4A). L214 was located in the α-helix of the dorsal fin domain and projects into the ATP binding cavity, close to the ATP molecule (labeled LEU-214, yellow in the structure in Figure 4). Neighboring residues, K215 (not labeled) and S216 (labeled SER-216, yellow in the structure in Figure 4), were also near the cavity. In contrast, T223 and D224, dorsal fin residues in the random coil, were distant from the putative location of the bound ATP molecule (labeled THR-223 and ASP-224, respectively, yellow in the structure in Figure 4). On the basis of the model, the shortest distance measured between the backbone carbonyl oxygen atom of L214 and the oxygen atom of the ATP ribose ring was approximately 2.2 Å (red dotted line between ATP and L214 in Figure 4A). An interaction between the side chain of L214 and ATP is quite feasible on the basis of this proximity, assuming the model is accurate.

L214 Mutants Display Altered Desensitization Patterns. We have found that IVM reduced the current decline during a 3 s application of ATP on the L214E mutant (Figure 5A). WT currents declined to a value that was 36.2 ± 6.8% (n = 4) of their initial peak amplitude, whereas for L214E, the value was 4.7 ± 0.8% (n = 3; p < 0.05). We were therefore interested in further investigating the extent to which position L214 was involved in regulating desensitization during prolonged ATP exposure. The specific identity of the amino acid at position 214 differs among P2X family members, although the hydrophobic nature of the side chain is consistent. Leu is present at this position in P2X2R, P2X4R, and P2X5R, Ile in P2X1R, and Met in P2X3R (Figure 4). Given that P2X1R and

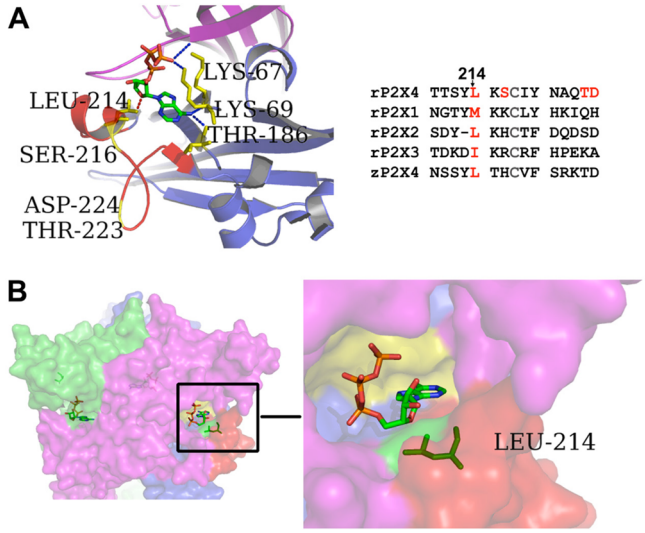

Figure 4. Molecular modeling predicts that L214 occupies the ATP binding pocket of rP2X4R. (A) Residues L214, S216, T223, and D224 are colored yellow from the homology model of rP2X4R. The dorsal fin domain except for residues L214, S216, T223, and D224 is colored red, and the other residues of the subunit are colored blue. The neighboring subunit is colored light magenta. The shortest distance measured between L214 and the ATP ribose ring is labeled and indicated using red dashed lines. L214 and three other highly conserved residues (including K67, K69, and T186 of the same subunit) contacted with the triphosphate chain or adenine base of the ATP molecule are shown as sticks. Amino acid sequences of four rP2XR subtypes and zfP2X4R are shown using single-letter code. The conserved cysteines are colored gray. Four amino acids that are colored red in rP2X4R have been studied. (B) The left panel is a surface representation of the ATP binding pocket. Each subunit is shown in different colors. The dorsal fin of one subunit is colored red. The right panel shows a detailed view of the relative position of L214 and the ATP binding pocket. Residue L214 and the ATP molecule are shown as sticks.

P2X3R are desensitized very rapidly, we hypothesized that substituting the Leu present at position 214 in P2X4 with Ile or Met would enhance desensitization. They did not significantly differ in terms of their EC_{50} values (Figure 1B and Table 1) compared to WT, and an ATP concentration that was close to the EC_{80} value of the ATP concentration−response curve was used in these experiments. In cells expressing these WT or mutant receptors, 30 μM ATP was applied for 60 s, and the time taken for the current to decline to 20% of the maximal amplitude was measured. Substitution of Ile for Leu produced a marked increase in the rate of desensitization compared with

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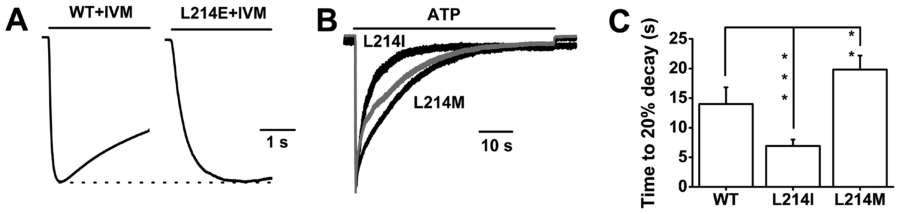

Figure 5. Residue 214 plays a role in channel desensitization. (A) Normalized current traces showing currents decline in WT and L214E during ATP application (10 μM for WT P2X4R and 100 μM for L214E, 3 s) in the 3 min preincubation and continue in the presence of IVM (3 μM). (B) Normalized current traces illustrating the desensitization of currents mediated by the L214I and L214M mutants and WT P2X4R in the presence of 30 μM ATP for 60 s. (C) Histograms showing the time from the maximum to 20% current decay during the continued presence of 30 μM ATP for 60 s.

that of the WT receptor [$t_{100\%-20\%}$ = 14 ± 3 s (n = 7)]. The time to 20% current decay for L214I [$t_{100\%-20\%}$ = 7 ± 1 s (n = 5)] was ~2-fold shorter. In contrast, and unexpectedly, substitution with Met slowed the rate of desensitization [$t_{100\%-20\%}$ = 20 ± 2 s (n = 5)] compared to WT receptor (Figure 5B,C). Although rates of desensitization were different for these mutants, there was no difference in the total extent of desensitization, with ATP-gated currents declining to the same approximate minimum after a 60 s application of agonist (<7% of the total current amplitude) (data not shown).

DISCUSSION

In this paper, we present data confirming the involvement of the dorsal fin domain in ligand binding and signal transduction, providing further experimental validation of the ATP binding domain structure as predicted by X-ray crystallography.

Amino acid substitutions introduced at L214 that markedly changed the size, hydrophobicity, or charge of this residue were observed to markedly attenuate ATP potency. In contrast, substitution of Leu with the similarly sized hydrophobic amino acid, Ile, at this position had little effect on ATP potency. These data are consistent with the prediction of the ATP-bound open structure, which suggests L214 has a direct, hydrophobic interaction with the ribose ring of ATP. When substituted with Cys, side chain 214 was found to be accessible to rapid covalent modification by the water-soluble sulfhydryl-reactive agent, $MTSES^-$, but in a manner that was competitively inhibited by ATP. This provides direct evidence that L214 is oriented in the ATP-accessible binding cavity proper. Importantly, the structure indicated that L214 is the only residue within the dorsal fin oriented toward, and in close proximity of, the bound ATP. The flanking residue in the small dorsal fin α-helix, S216, is twisted almost a full 180° from the binding cavity, which likely explains why substitution of this residue had no significant effect on ATP potency in our study. Previous studies of P2X1R have shown that substitution of the largely conserved Lys residue analogous to K215 in rP2X4R also has no effect on ATP potency,[8] consistent with the prediction that this side chain is also oriented away from the binding cavity. We also mutated dorsal fin residues predicted to lie even more distant from the binding cavity, T223 and D224, and observed no effect on ATP potency, confirming that these residues do not interact with ATP in the binding site. They also do not appear to be important for maintaining the stability of the dorsal fin domain in the same way that has been shown for nonbinding residues in the left flipper.[19]

When an agonist binds, the head and dorsal fin domains come closer to promote pore opening; when a larger antagonist binds in the pocket, just as "a foot in the door", the closure of the site is impaired and the closed pore is stabilized.[7,18,28] TNP-ATP is a close structural analogue of ATP with only a trinitrophenyl group added to the ribose moiety. Because substitution of Ala at position 214 did not simply remove the sensitivity to TNP-ATP, the position of Leu is probably not a critical requirement for TNP-ATP's function as a "foot" to stabilize the conformation of closed jaws. One straightforward interpretation would be that several residues participated in the initial inhibition. However, the reversible block, along with the slightly but significantly changed inhibition effect, implies that L214 is recognized by TNP-ATP and further indicates L214 is located in the ATP binding pocket.

Therefore, our data suggest that (1) L214 is indeed oriented into the ATP binding cavity and (2) this residue, and the dorsal fin generally, is likely involved in making a hydrophobic contact with ATP during agonist binding. This is entirely consistent with the structural model, which suggests that the dorsal fin domain contacts the ribose ring of ATP and is not involved in making electrostatic contacts with the phosphate groups.

In addition to its clear involvement in ligand binding, position L214 appears to influence receptor desensitization and IVM sensitivity. This is not entirely unexpected given that ligand binding residues are commonly involved in the initial steps of signal transduction linking agonist binding to channel gating. For example, in P2X3R, the single residue, S275, in the left flipper has been implicated in both agonist binding and desensitization.[19] We investigated whether the residue at this position might in part confer the differences in desensitization kinetics between P2X4R and the more rapidly desensitizing receptors, P2X1R and P2X3R. However, although substituting L214 with the Ile present in P2X1R increased the rate of desensitization in P2X4R, substitution with the Met of P2X3R actually slowed desensitization. It is interesting that merely substituting Leu with Ile markedly affected the desensitization of P2X4R, as Leu and Ile have the same molecular weight and differ only in the position of one methyl group within the side chain seen from the chemical formula. Previous studies have implicated numerous residues in the regulation of desensitization, particularly within the intracellular domains,[29,30] and clearly, L214 alone is not a major contributor to this process. Recently, an interesting report speculated that the desensitization process may result from exchange of agonist orientations in the binding site, with bound ATP initially stabilizing an open state before flipping around and stabilizing a closed, potentially desensitized conformation.[31] In this respect, mutation of L214 might alter desensitization by influencing the ability for ATP to make this switch in the orientation in the binding cavity. Notably, the hydropathy index (HI) of Ile (4.5) is greater than that of Leu (3.8), whereas the HI of Met is smaller (1.9). The

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increased hydrophobic surface may change the interactions between residue 214 and other amino acids involved in signal transduction and then stabilize the desensitization state.

The L214A mutant exhibited a faster deactivation rate after agonist withdrawal. Privious studies suggested that changes in conformation associated with gating represent the rate-limiting step for current deactivation for P2XR.[32,33] In that respect, a faster deactivation rate indicated a faster transition from the open to closed state. We also found that introduction of a glutamate or a phenylalanine at position 214 reduced the sensitivity of P2X4R to the effect of IVM on current deactivation. Our data are similar to those obtained for the effects of the lateral portal mutant of P2X4R, E51Q,[34] which also specifically reduced the effect of IVM on deactivation without necessarily preventing the effect of IVM on potentiation.[35] The mechanism by which IVM alters P2X4 channel function has yet to be elucidated, but these data provide further evidence of L214's importance in the gating characteristics of the P2X4 channel.

Taken together, our data represent the first comprehensive functional investigation focusing on the involvement of a specific side chain in the dorsal fin domain of the P2X4R subunit in ATP binding. Importantly, findings from this investigation also suggest that this residue plays a role in the conformational changes accompanying channel opening and desensitization.

■ ASSOCIATED CONTENT

Supporting Information

Homology model of rP2X4R (Figure S1) and representative recordings of current traces observed during the addition of $MTSES^-$ and $MTSEA^+$ to WT P2X4R and L214C (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

EC_{50}, ATP concentration giving the half-maximal current; I_{max}, maximal current amplitude; IVM, ivermectin; $MTSES^-$, sodium (2-sulfonatoethyl) methanethiosulfonate; $MTSEA^+$, (2-aminoethyl) methanethiosulfonate hydrobromide; DTT, dithiothreitol; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP; HEK, human embryonic kidney.

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