Secondary Structure and Gating Rearrangements of Transmembrane Segments in Rat P2X4 Receptor Channels

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Abstract

P2X receptors are cation selective channels that are activated by extracellular nucleotides. These channels are likely formed by three identical or related subunits, each having two transmembrane segments (TM1 and TM2). To identify regions that undergo rearrangement during gating and to probe their secondary structure, we performed tryptophan scanning mutagenesis on the two putative TMs of the rat P2X4 receptor channel. Mutant channels were expressed in Xenopus oocytes, concentration–response relationships constructed for ATP, and the EC50 estimated by fitting the Hill equation to the data. Of the 22 mutations in TM1 and 24 in TM2, all but one in TM1 and seven in TM2 result in functional channels. Interestingly, the majority of the functional mutants display an increased sensitivity to ATP, and in general these perturbations are more pronounced for TM2 when compared with TM1. For TM1 and for the outer half of TM2, the perturbations are consistent with these regions adopting α-helical secondary structures. In addition, the greatest perturbations in the gating equilibrium occur for mutations near the outer ends of both TM1 and TM2. Surface biotinylation experiments reveal that all the nonfunctional mutants traffic to the surface membrane at levels comparable to the WT channel, suggesting that these mutations likely disrupt ion conduction or gating. Taken together, these results suggest that the outer parts of TM1 and TM2 are helical and that they move during activation. The observation that the majority of nonconducting mutations are clustered toward the inner end of TM2 suggests a critical functional role for this region.

Key words: purinergic receptors • point mutation • scanning mutagenesis • oocytes • ligand-gated channel

Introduction

P2X receptors are cation channels that are gated by extracellular nucleotides. These widely distributed ion channels are highly permeable to calcium (Egan and Khakh, 2004), and perform diverse physiological functions (for a comprehensive recent review see North, 2002). Over the past decade, seven mammalian P2X receptor subunits have been cloned (P2X1–P2X7) that form both homomeric and heteromeric channel assemblies (Brake et al., 1994; Valera et al., 1994; North, 2002). P2X receptor channels have structural and functional properties distinct from other ligand-gated channels. They are likely formed by three identical or related subunits (Nicke et al., 1998; Stoop et al., 1999; Jiang et al., 2003a; Aschrafi et al., 2004), each having a large extracellular segment of ~280 amino acids that is flanked by two transmembrane segments (TM1 and TM2), placing both the NH2 and COOH termini intracellular (Newbolt et al., 1998) (Fig. 1 A). In contrast, ion channels of the Cys-loop family (e.g., nicotinic acetylcholine receptor channels) contain five subunits, with each subunit having four transmembrane segments (Lester et al., 2004), whereas glutamate-gated ion channels are tetramers in which each subunit has three segments that cross the membrane (Wollmuth and Sobolevsky, 2004). P2X receptor channels are also distinct in the ability to produce a large conductance pathway. Cells expressing homomeric P2X2, P2X4, or P2X7 receptor channels, as well as P2X2/3 heteromeric receptor channels, become permeable to molecules as large as YO-PRO-1 dye (523 D for the free cation) following long exposures to extracellular ATP, suggesting that the channels undergo pore dilation (Khakh et al., 1999; Virginio et al., 1999). The molecular mechanism for the increase in permeability is still not known, nor is it clear how the TMs are arranged around the pore.

In most channels of known structure, the membrane-spanning segments adopt α-helical secondary structures (Walz et al., 1997; Chang et al., 1998; Doyle et al., 1998; Dutzler et al., 2002; Jiang et al., 2002b, 2003b; Kuo et al., 2003; Miyazawa et al., 2003). However, scanning cysteine accessibility experiments on the two TMs of rat P2X4 receptor channels did not resolve their secondary structure. Using methanethiosulfonate (MTS) reagents on TM2, Rassendren et al. (1997) concluded that their results do not support the hypothesis that the pore is
formed as the polar face of an amphipathic helix. A separate MTS and silver accessibility study on TM2 of the P2X$_2$ receptor channel discusses the secondary structure of the TM but seems to argue against helical structure (Egan et al., 1998). Two studies were performed on TM1, both of which make no assessment of helical structure (Haines et al., 2001b; Jiang et al., 2001).

An alternate approach to examining secondary structure is alanine or tryptophan scanning mutagenesis. The rationale behind this approach has been previously discussed (Monks et al., 1999; Li-Smerin et al., 2000a). The underlying assumption is that side chains exposed to the lipid membrane in both closed and open conformations would be relatively tolerant to mutation, as long as the substituted amino acid is hydrophobic. In contrast, side chains involved in protein packing in either closed or open conformations would be relatively sensitive to mutation. For an α-helix with a significant lipid-exposed surface, a pattern might emerge in which every third or forth residue exhibits a similar sensitivity to the mutation. This approach has been used successfully for inward rectifier potassium channels (Choe et al., 1995; Cukras et al., 2002), voltage-activated potassium channels (Monks et al., 1999; Hong and Miller, 2000; Li-Smerin et al., 2000a,b; Li-Smerin and Swartz, 2001; Hackos et al., 2002), nicotinic acetylcholine receptor channels (Tamamizu et al., 2000; Guzman et al., 2003), glutamate receptor channels (Panchenko et al., 2001), and GABA$_A$ receptor channels (Jenkins et al., 2002). More recently, while the present manuscript was in writing, an alanine scan of both TMs in P2X$_2$ was published (Li et al., 2004). This alanine scan is consistent with helical structure for TM1 and is inconclusive for TM2.

In the present study, we used scanning mutagenesis on the rat P2X$_4$ receptor channel to probe the secondary structure of the TMs and to identify regions that undergo rearrangements during gating. We individually replaced each residue in the two TMs with tryptophan, and in one case mutated a tryptophan to alanine, expressed each mutant channel in oocytes, and then examined the concentration dependence for activation of the channel by ATP. The results have important implications for the structure of P2X receptor channels and for the movements of the TMs during gating. A preliminary report of some of these observations has appeared (Silberberg and Swartz, 2004).

**Materials and Methods**

**Mutagenesis**

Rat P2X$_4$ cDNA in pcDNA3 (provided by F. Soto, Max-Planck Institute for Experimental Medicine, Göttingen, Germany) was subcloned into pGEM-HE vector (Liman et al., 1992) in order to improve expression in oocytes. Mutant P2X receptor channels were generated by PCR using the P2X$_4$ DNA as template. The generation of the various mutations was confirmed by DNA sequencing. Mutant and wild-type (WT) P2X receptor channel cRNA was synthesized using T7 polymerase from DNA linearized with NotI.

**Electrophysiological Recording**

The WT and mutant channels were expressed in *Xenopus* oocytes by injecting ~50 nl of cRNA per oocyte at different dilutions. Membrane currents were recorded under voltage clamp using an OC-725C oocyte clamp amplifier (Warner Instrument Corp.) and digitized online using a Digidata 1321A interface board and pCLAMP 9 software (Axon Instruments). The sampling frequency was set to at least two times the corner frequency of the low-pass filter. To minimize voltage-clamp errors, the maximal current recorded from each mutant was limited by adjusting the amount of cRNA injected, by the time between cRNA injection and current recording, and by the holding voltage during current recording. The recording solution contained (in mM): 100 NaCl, 5 Hapes, 0.3 CaCl$_2$, pH 7.6. Solutions containing ATP were prepared freshly each day and the pH of the solution was readjusted.

Concentration–response relationships were constructed for the WT channel and for each of the mutant channels. In the early experiments, each tested concentration of ATP was bracketed by a measurement with a reference concentration of ATP. The currents were then corrected for desensitization by normalizing the current activated by a given concentration of ATP to the current activated by the preceding reference concentration of ATP. At least four individual concentration–response relationships were then averaged. In most experiments, however, each oocyte was used to test a single concentration of ATP. A reference concentration of ATP was applied first, followed 2 min later by a test concentration. Responses to the test concentrations of ATP were then normalized to the reference concentration. Each concentration was tested on at least four oocytes and all oocytes used to generate a concentration–response relationship were from the same batch.

**Analysis**

The Hill equation was fit to concentration–response relationships using SigmaPlot 2000 software (SPSS Inc.) according to

$$\frac{1}{I_{\text{max}}} = \frac{[\text{ATP}]^n}{[\text{ATP}]^n + \text{EC}_{50}^n},$$

where $I$ is the normalized current at a given concentration of ATP, $I_{\text{max}}$ is the maximum normalized current, $\text{EC}_{50}$ is the concentration of ATP ([ATP]) yielding a current half the maximum, and $n$ is the Hill coefficient. The results are reported as the mean ± SEM where SEM represents the goodness of fit of the Hill equation.

Fourier transform methods (Cornette et al., 1987; Komiya et al., 1988; Rees et al., 1989a,b; Li-Smerin et al., 2000a,b; Li-Smerin and Swartz, 2001) were used to evaluate the periodicity of the perturbations in $\text{EC}_{50}$ for mutations in various regions of TM1 according to

$$P(\omega) = |X(\omega)^2 + Y(\omega)^2|,$$

where

$$X(\omega) = \sum_{n=1}^{N} (E_n - \bar{E}) \sin(n\omega),$$

where

$$Y(\omega) = \sum_{n=1}^{N} (E_n - \bar{E}) \cos(n\omega).$$
and

\[ Y(\omega) = \sum_{i=1}^{n} \left( E_i - \bar{E} \right) \cos(\omega i) \]

\( P(\omega) \) is the Fourier transform power spectrum as a function of the angle between adjacent side chains (\( \omega \)), \( n \) is the number of residues of a segment, \( E_i \) is the fold change in \( EC_{50} \) relative to WT at a given position \( i \), and \( \bar{E} \) is the average value of the fold change in \( EC_{50} \) for the segment.

The ratio of the mean power in the range of 100 ± 15° over the quantifiable helical character from power spectra according to

\[ \alpha-PI = \left[ \frac{1}{30} \int P(\omega) d\omega \right] \left[ \frac{1}{180} \int P(\omega) d\omega \right] \]

Values of \( \alpha-PI > 2 \) have been considered indicative of \( \alpha \)-helical secondary structure (Cornette et al., 1987; Rees et al., 1989b).

**Detection of Surface Protein**

Surface protein was detected using the procedure of Ennion and Evans (2002) with slight modifications. 3 d after injection of cRNA, oocytes were incubated for 30 min at room temperature with Sulfo-NHS-LC-Biotin (0.5 mg/ml; Pierce Chemical Co.) in ND96 buffer containing (in mM) 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 Na-Hepes, pH 7.6. After washing six times in ND96 buffer, oocytes were homogenized in 400 μl of buffer H containing (in mM) 100 NaCl, 20 Tris.Cl, pH 7.4, 1% Triton X-100, 5 μl/ml protease inhibitor cocktail (EMD Bioscience). Homogenization and all subsequent steps were performed at 4°C. After centrifugation at 16,000 g for 2 min, a 20-μl aliquot of the supernatant (total protein) was mixed with equal volume of 2X LDS sample buffer plus reducing agent: 50% 4X LDS sample buffer (Invitrogen), 20% 2-mercaptoethanol, 100 mM DTT. The remaining supernatant was diluted 1:1 with buffer H plus 50 μl of streptavidin agarose beads (Pierce Chemical Co.) and then tumbled gently overnight at 4°C. The streptavidin agarose beads were washed six times with buffer H with a 2-min centrifugation (16,000 g) between each wash. At the end of the final wash, 40 μl of 1X LDS sample buffer plus reducing agent was added to the beads and samples were heated at 95°C for 10 min. Following a 2-min centrifugation (16,000 g), the supernatant (surface protein) and total protein (collected earlier) were separated in a 4–12% NuPage Bis-Tris gel (Invitrogen) using a running buffer containing (in mM) 50 MOPS, 50 Tris base, 1 EDTA, and 20% methanol. The nitrocellulose membrane was probed with rabbit anti-P2X₄ antibody (Alomone Labs) diluted 1:100 in PBS-T containing (in mM) 137 NaCl, 2.7 KCl, 10 Na₂HPO₄, 2 KH₂PO₄, 0.05% Tween-20, and P2X₄ expression was detected using ECL Western blotting reagents (Amersham Biosciences). The mutant Shaker potassium channels used as a control lacked residues 6–46 and contained c-myc epitopes inserted at both termini (for details see Hackos et al., 2002). For the Shaker Kv gels, the nitrocellulose membrane was probed with anti-myc antibody (Invitrogen).

**RESULTS**

The objective of this study was to use tryptophan scanning mutagenesis to probe the secondary structure of the TMs in P2X receptor channels and to identify regions that undergo rearrangement during gating. Fig. 1 B shows the regions of the rat P2X₄ receptor channel that were mutated, aligned either with P2X₄ receptor channel subunits cloned from other species (top), or with the other cloned rat P2X receptor subunits (bottom). Each residue was individually mutated to tryptophan, except for W46, which was mutated to alanine. Following expression in Xenopus oocytes, two-electrode voltage clamp techniques were used to measure ATP-activated membrane currents and construct concentration–response relationships for each mutant channel. For most mutations, each oocyte was used to test a single concentration of ATP in order to circumvent potential errors in the concentration–response relationship arising from incomplete recovery from desensitization between applications of ATP. A reference concentration of ATP was applied first, followed 2 min later by a test concentration (Fig. 2 A). Responses to the test concentrations of ATP were then normalized to the reference concentration. Superimposed normalized current

**Figure 1.** Putative transmembrane regions of P2X receptor channels. (A) Schematic representation of the general topology of a P2X receptor channel subunit. (B) Sequence alignment of TM1 (left) and TM2 (right) of P2X₄ receptor channels from different species (top) and of rat P2X₄-P2X₇ (bottom). Bold residues are identical across all known P2X receptor channels (except for a Y to F substitution in Xenopus P2X₇). The major findings of this study are summarized above the sequences. X indicates mutations to tryptophan that result in nonconducting channels. Empty circles and bars indicate positions and regions of large perturbations in EC₅₀ induced by the mutations, respectively.
traces, activated by a range of ATP concentrations, for the WT receptor channel and for Y42W are shown in Fig. 2 B. The mean (±SEM) normalized current for a range of ATP concentrations are plotted in Fig. 2 C for the WT channel (black circles) and for Y42W (white squares). A Hill equation was fit to the data (solid lines through the data in Fig. 2 C), giving an EC$_{50}$ of 11.8 ± 0.7 μM and (1.5 ± 0.1), and 1.4 ± 0.1 μM and (1.3 ± 0.1), for the WT and Y42W receptor channels, respectively. This analysis reveals that the Y42W mutant channel is significantly more sensitive to ATP when compared with the WT channel.

**Tryptophan Scanning TM1**

Of the 22 mutants studied in TM1, all except V28W give rise to ATP-gated currents when expressed in *Xenopus* oocytes. Concentration–response relationships can readily be constructed for all of the conducting mutants aside from L40W, which requires several minutes to fully deactivate following the application of ATP. Table I summarizes the estimated EC$_{50}$ and $n$ for the mutations in TM1. Because changes in $n$ are not easily interpretable and since no obvious trend in $n$ is observed, we focus on the estimated EC$_{50}$ values, which are presented in a histogram in Fig. 3 A. The vertical solid and dashed lines represent the EC$_{50}$ ± SEM of the WT receptor channel. Although the sensitivity of some mutants to ATP is similar to the WT channel, it is immediately apparent that many of the mutant channels display a perturbed sensitivity to ATP. Remarkably, with only one exception (L40W, see Table I), these latter mutants all display an increased sensitivity to ATP, as seen by a leftward shift in EC$_{50}$. It is unlikely that so many residues would directly contribute to the agonist binding site for such a small ligand. ATP binding promotes opening of the gate in P2X receptors, and thus the leftward perturbations in EC$_{50}$ probably reflect changes in the relative stability of the open and closed conformations. By shifting this equilibrium toward the open state, the mutations in TM1 bias the ATP binding step toward the bound state. The interesting observation that so many mutations shift the close–open equilibrium toward the open state will be addressed further in the Discussion.

Visual examination of the perturbations in EC$_{50}$ (Fig. 3 A) reveals that for every third or forth residue (N32W, G45W, I39W, V49W, L38W, and F48W) the estimated EC$_{50}$ and $n$ for the Mutations in TM1

| Mutation | EC$_{50}$ (μM) | $n$ | Fold change in EC$_{50}$ |
|----------|----------------|-----|-------------------------|
| WT       | 11.8 ± 0.7     | 1.5 ± 0.1 | 1.0                    |
| V49W     | 8.2 ± 1.9     | 1.3 ± 0.3 | 1.4                    |
| F48W     | 5.5 ± 0.2     | 1.5 ± 0.1 | 2.2                    |
| V47W     | 1.4 ± 0.3     | 1.0 ± 0.2 | 8.4                    |
| W46A     | 2.0 ± 0.1     | 1.6 ± 0.1 | 5.9                    |
| G45W     | 0.5 ± 0.04   | 1.4 ± 0.1 | 23.6                   |
| H44W     | 1.5 ± 0.1     | 1.9 ± 0.3 | 7.9                    |
| V43W     | 1.6 ± 0.2     | 1.3 ± 0.1 | 7.4                    |
| Y42W     | 1.4 ± 0.1     | 1.3 ± 0.1 | 8.4                    |
| A41W     | 11.3 ± 0.2    | 1.6 ± 0.05 | 1.0                    |
| L40W     | >30           | ND     | <0.4                   |
| I39W     | 2.0 ± 0.2     | 1.4 ± 0.1 | 5.9                    |
| L38W     | 7.6 ± 0.6     | 1.2 ± 0.1 | 1.6                    |
| L37W     | 13.5 ± 0.7    | 1.4 ± 0.1 | 0.9                    |
| Q36W     | 2.7 ± 0.1     | 1.6 ± 0.1 | 4.4                    |
| V35W     | 14.5 ± 3.5    | 1.1 ± 0.1 | 0.8                    |
| A34W     | 8.7 ± 0.9     | 1.2 ± 0.1 | 1.4                    |
| R33W     | 6.1 ± 0.4     | 1.4 ± 0.1 | 1.9                    |
| N32W     | 2.9 ± 0.2     | 1.4 ± 0.1 | 4.1                    |
| M31W     | 6.1 ± 1.3     | 1.0 ± 0.1 | 1.9                    |
| L30W     | 12.2 ± 1.6    | 1.0 ± 0.1 | 1.0                    |
| G29W     | 9.1 ± 0.8     | 1.2 ± 0.1 | 1.3                    |
| V28W     | ND            | ND     | ND                     |

*Fold change in EC$_{50}$ was calculated as EC$_{50}$(WT)/EC$_{50}$(mutant).  
**ND, not determined.*
Q36W, I39W, Y42W, and G45W; bold typeface and shaded bars) the perturbation in EC$\text{}_{50}$ is greater than those of the neighboring residues. This is more clearly seen when the fold change in EC$\text{}_{50}$ relative to WT is examined (Fig. 3 B). For these “high impact” positions, the magnitude of the perturbation progressively increases from inside to outside. When mapped onto a helical projection of TM1, the five residues labeled in Fig. 3 are located on one side of the wheel, pointing to a face of an $\alpha$-helix that is likely to be involved in protein–protein interactions (Fig. 4 A). Mapping these residues on a helical net diagram of TM1 shows that the sensitive face of TM1 spans most of the length of the segment (Fig. 4 B). To quantify the observed pattern in TM1, we used Fourier analysis, which can identify periodicity in the data (see MATERIALS AND METHODS). The periodicity for an $\alpha$-helix residing in a bimodal environment (e.g., an amphipathic helix) is $\sim$100° ($360°/3.6$ residues per turn). For the region between G29W and V43W, the strongest peak in the power spectrum of the fold change in EC$\text{}_{50}$ is observed at 102° and the calculated periodicity index ($\alpha$-PI) is 2.3 (Fig. 4 C), suggesting that this region adopts an $\alpha$-helical secondary structure. For longer segments of TM1, the power spectra are more complex, resulting from an increased ATP sensitivity observed for each consecutive position between Y42W and V47W (black bar in Fig. 3 A). However, the clear helical pattern in EC$\text{}_{50}$ perturbations (Figs. 3 and 4) encompasses the entire length of TM1, suggesting that this entire segment is an $\alpha$-helix.

Tryptophan Scanning TM2

In the region of the second TM, we mutated each residue between T335 and L358 to tryptophan (Fig. 1 B). Table II summarizes the estimated EC$\text{}_{50}$ and $n$ for the mutations in TM2, and the estimated EC$\text{}_{50}$ values are also shown graphically in Fig. 5 A. The results for TM2 are clearly more complex than for TM1. 7 out of the
24 mutated residues (S341W, A349W, L352W, C353W, D354W, V357W, and L358W) located primarily toward the inside of TM2, do not give rise to measurable currents when exposed to 300 μM ATP, while three residues (N338W, G340W, and V351W) give small currents. A concentration–response relationship could not be determined for V351W, which like L40W in TM1 requires several minutes to fully deactivate following the application of ATP. Aside from G347W and I356W, all of the remaining mutants are more sensitive to ATP relative to WT. Large perturbations in EC₅₀ are observed toward the outside of TM2, similar to TM1, however, the magnitude of perturbations in TM2 is considerably greater than for TM1. In TM2, 5 out of 17 functional mutations result in a >10-fold increase in sensitivity to ATP, whereas in TM1, only 1 out of 21 functional mutants results in a similar change in sensitivity (see Tables I and II). Finally, for the outer half of TM2, the largest perturbations in EC₅₀ are at every fourth position (M336W, G340W, A344W, and V348W; bold typeface and shaded bars), as most clearly seen when the fold change in EC₅₀ relative to WT is plotted (Fig. 5 B).

Of the seven mutations that result in nonfunctional channels, six are located near the inner end of TM2, significantly restricting an examination of helical periodicity. However, for the outer half of TM2, the four mutations that cause the largest perturbations in EC₅₀ are distributed in a helical pattern, mapping to one side of a helical wheel representation and lying on a single face of a helical net representation (Fig. 6). Fourier analysis was not performed on the limited data for TM2 since the longest uninterrupted region is seven residues long. Hence, while the pattern in ATP sensitivity suggests that the outer region of TM2 is an α-helix, the pattern is not as unambiguous as for TM1.

**Trafficking of the Nonfunctional Mutants to the Surface Membrane**

Although at many positions tryptophan substitution amounts to a rather radical mutation, the majority of mutants in this study (38 out of 46), and in previous tryptophan scanning mutagenesis studies (Collins et al., 1997; Monks et al., 1999; Hong and Miller, 2000; Li-Smerin et al., 2000a,b; Tamamizu et al., 2000; Panchenko et al., 2001; Hackos et al., 2002; Jenkins et al., 2002; Guzman et al., 2003), result in channels that

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**TABLE II**

| Mutation | EC₅₀ (μM) | n  | Fold change in EC₅₀* |
|----------|----------|----|---------------------|
| WT       | 11.8 ± 0.7 | 1.5 ± 0.1 | 1.0 |
| T335W    | 1.6 ± 0.01 | 2.0 ± 0.01 | 7.4 |
| M336W    | 0.8 ± 0.1  | 1.2 ± 0.1  | 14.8 |
| I337W    | 1.5 ± 0.1  | 1.4 ± 0.2  | 9.1 |
| N338W    | 1.5 ± 0.3  | 1.4 ± 0.3  | 7.9 |
| V339W    | 1.1 ± 0.1  | 2.2 ± 0.6  | 10.7 |
| G340W    | 0.3 ± 0.1  | 0.9 ± 0.2  | 39.3 |
| S341W    | ND        | ND          | ND |
| G342W    | 2.2 ± 0.2  | 1.7 ± 0.2  | 5.4 |
| L343W    | 6.3 ± 0.4  | 1.4 ± 0.1  | 1.9 |
| A344W    | 0.3 ± 0.06 | 1.0 ± 0.2  | 39.3 |
| L345W    | 0.7 ± 0.01 | 1.8 ± 0.04 | 16.9 |
| L346W    | 1.8 ± 0.3  | 1.3 ± 0.2  | 6.6 |
| G347W    | 21.8 ± 3.4 | 1.1 ± 0.1  | 0.5 |
| V348W    | 0.5 ± 0.1  | 1.2 ± 0.2  | 23.6 |
| A349W    | ND        | ND          | ND |
| T350W    | 7.0 ± 0.3  | 1.4 ± 0.04 | 1.7 |
| V351W    | ND        | ND          | ND |
| L352W    | ND        | ND          | ND |
| C353W    | ND        | ND          | ND |
| D354W    | ND        | ND          | ND |
| V355W    | 6.4 ± 0.1  | 1.8 ± 0.01 | 1.8 |
| I356W    | 11.6 ± 1.8 | 2.0 ± 0.6  | 1.0 |
| V357W    | ND        | ND          | ND |
| L358W    | ND        | ND          | ND |

*Fold change in EC₅₀ was calculated as EC₅₀(WT)/EC₅₀(mutant).
ND, not determined.
fold properly, traffic to the plasma membrane, and form functional channels. Do the eight mutations (one in TM1 and seven in TM2) that fail to give rise to membrane currents disrupt folding and/or trafficking, or are the native residues vital for channel function? To address this question, we estimated the surface protein expression level for each of the nonfunctional mutants and compared it to that of the WT channel.

To this end, oocytes were injected with equal amounts of cRNA encoding WT or mutant P2X4 channels, and after 3 d to allow for expression, the surface membrane proteins of intact oocytes were biotinylated using a membrane-impermeant, primary amine–specific, biotinylation reagent. Biotinylated oocytes were then washed, homogenized, and centrifuged to isolate a crude protein fraction (see materials and methods). A small aliquot of the crude fraction (equivalent to one oocyte) was used to evaluate the quantities of P2X4 receptor channel protein in total oocyte membranes. The remaining membrane sample was purified using streptavidin agarose beads. Following PAGE separation (20 oocytes per lane), proteins were transferred to nitrocellulose membrane, probed with rabbit anti-P2X4 antibody, and detected using ECL. We also estimated the amount of WT P2X4 channel protein from oocytes that were not treated with the biotinylation reagent in order to evaluate the specificity of the streptavidin agarose bead fractionation for biotinylated protein. No P2X4 receptor channel protein is detected in the bead fraction of nonbiotinylated oocytes (Fig. 7 A, top), confirming the specificity of the fractionation procedure. When the levels of P2X4 protein in total and surface membranes are compared for the WT and nonfunctional mutants, a rather surprising result is seen: the protein levels in the surface membrane fractions for each of the nonfunctional mutants are indistinguishable from the WT P2X4 receptor channel (Fig. 7 A, top).

To confirm that the biotinylation reagent selectively labels surface proteins, we performed parallel experiments with the Shaker Kv channels, where specific mutants have been documented to be retained in the ER. Two c-myc–tagged Shaker Kv channel mutants, a nonfunctional mutant that is retained in the ER (I477D), and a weakly conducting mutant with surface membrane expression comparable to the WT Shaker Kv channel (F481W), were expressed in oocytes and isolated in parallel with P2X4 receptor channels. As previously reported (Hackos et al., 2002), two dominant forms of F481W are detected in the crude membrane preparation: a core-glycosylated species (70 kD), corresponding to ER-retained protein, and a more heavily glycosylated form (100 kD) that can traffic to the surface membrane. In contrast, for I477D, only the core-glycosylated species is observed in the crude membrane preparation (Fig. 7 B). If the biotinylated surface membrane fraction is contaminated with protein from internal membranes (in the unlikely event that biotin was to gain access into the oocytes), we would expect to observe both core-glycosylated Shaker protein and the heavily glycosylated form in the biotinylated fraction. However, for the mutant that traffics to the surface membrane (F481W), only the heavily glycosylated form is detected in the biotinylated surface membrane fraction, and for the ER-retained mutant (I477D), no Shaker protein is observed in this fraction (Fig. 7 B). These control experiments clearly demonstrate that the surface protein preparation is not contaminated by internal membranes (in the unlikely event that biotin was to gain access into the oocytes), we would expect to observe both core-glycosylated Shaker protein and the heavily glycosylated form in the biotinylated fraction. However, for the mutant that traffics to the surface membrane (F481W), only the heavily glycosylated form is detected in the biotinylated surface membrane fraction, and for the ER-retained mutant (I477D), no Shaker protein is observed in this fraction (Fig. 7 B). These control experiments clearly demonstrate that the surface protein preparation is not contaminated by the intracellular membrane fraction. Taken together, these results suggest that all of the nonfunctional P2X4 channel mutants fold properly and efficiently traffic to the surface membrane. This contrasts with nonfunc-
tional mutants in Kv channels, where nonfunctional mutants are often retained in the ER (Papazian et al., 1995; Tiwari-Woodruff et al., 1997; Hackos et al., 2002; Kitaguch et al., 2004).

**Coassembly of WT and Nonconducting Mutant Subunits**

The surface expression of the nonfunctional mutant channels indicates that the mutations interfere with channel function and not with the maturation or trafficking of the protein. Thus, these results identify the inner part of TM2 as serving a critical functional role in either the processes of gating or ion conduction in P2X4 receptor channels. To further substantiate this conclusion and to examine the dominance of mutant subunits in causing this nonconductive phenotype, we tested whether mutant and WT subunits could coassemble to form functional channels. Oocytes were co-injected with 1:1 mixtures of mRNA for the WT and nonconducting mutant channel subunits, and the current in response to 10 μM ATP was measured (Fig. 7 C). Oocytes injected with a 1:1 dilution of WT mRNA served as a control. If the mutant and WT subunits do not interact, then the expression level and conduction properties of the functional channels should be the same in oocytes injected with WT alone when compared with oocytes co-injected with mutant mRNA. At the other extreme, if WT and mutant subunits can randomly coassemble as trimers and a single mutant subunit is sufficient to silence the channel, then the current should be attenuated by 87.5% relative to the control (dashed line in Fig. 7 C). Current levels that do not fall into these two categories are more complicated to interpret, since coassembled channels might have altered unitary conductance and/or open probability in accordance with the number of mutant subunits per channel. Nevertheless, reduced or enhanced currents indicate coassembly if the differences from WT are statistically significant. Six of the nonconducting mutants (V28W, A349W, L352W, C353W, D354W, and V357W) significantly attenuated the current by 50–89%, suggesting that these mutant subunits coassemble with WT subunits. In three of these instances, the current was inhibited by an amount close to 87.5%, suggesting that these mutants may exhibit a dominant-negative phenotype, with a single copy of the mutation disrupting channel function.

**DISCUSSION**

In the present study, we investigated the secondary structure and movements of the TMs in rat P2X4 receptor channels using tryptophan scanning mutagenesis, estimating changes in ATP sensitivity as a gauge for whether the substitution is well tolerated. A number of previous studies on rat P2X2 receptor channels were undertaken to determine whether TM1 and TM2 line the pore, to locate the regions forming the gate, and to identify residues that account for the selectivity of the channel (Rassendren et al., 1997; Egan et al., 1998; Nakazawa et al., 1998a; Khakh et al., 1999; Virginio et al., 1999; Haines et al., 2001a,b; Jiang et al., 2001; Migita et al., 2001; Egan and Khakh, 2004). In addition, an alanine scanning mutagenesis study on the two TMs of the rat P2X4 receptor channel was published while this manuscript was in preparation (Li et al., 2004). In the following we discuss the implications of the present results for the structure and gating of P2X receptor channels, referring to these earlier findings where pertinent.
Secondary Structure of the TMs

The tryptophan scan of TM1 shows a clear pattern in ATP sensitivity (Figs. 3 and 4). Five residues, spanning the majority of TM1, are more sensitive to ATP than the neighboring residues, and all these residues map to one face of an α-helix. A helical secondary structure for TM1 is also supported by Fourier transform analysis (Fig. 4 C). A pattern consistent with helical structure was also seen for the outer half of TM2 (Figs. 5 and 6), however, Fourier transform analysis was not informative for this TM given the large number of nonconducting mutations. From these results, we conclude that the entire length of TM1 and probably at least the outer part of TM2 are α-helices, as depicted in Fig. 8 A where the residues on the sensitive face of the helices are indicated in red. The assignment of helical secondary structure from scanning mutagenesis studies has been validated by X-ray or atomic resolution EM structures in at least three instances, including inward rectifier K+ channels (Choe et al., 1995; Cukras et al., 2002), voltage-activated K+ channels (Monks et al., 1999; Hong and Miller, 2000; Li-Smerin et al., 2000a; Li-Smerin and Swartz, 2001; Jiang et al., 2003b), and nicotinic ACh receptor channels (Guzman et al., 2003; Miyazawa et al., 2003). An α-helical structure for TM1 has also been proposed in a recent alanine scanning mutagenesis study on the TMs of the P2X2 receptor channel (Li et al., 2004). It is reassuring to note that all the positions in TM1 labeled in red in Fig. 8 A, except for G45, were also sensitive to alanine substitution in P2X2. The alanine scan did not reveal an interpretable pattern for any part of TM2, possibly due to the more conservative nature of the alanine substitution for many positions in TM2. We have also made a number of alanine substitutions in TM2 and find that these mutants typically display a less severe phenotype than for tryptophan substitutions (unpublished data).

Why Do Tryptophan Substitutions Disproportionately Increase ATP Sensitivity?

One of the striking observations in the present study is that tryptophan substitutions most commonly increase the ATP sensitivity of the P2X4 receptor channel (Figs. 3 and 5; Tables I and II). Whereas only 1 out of 38 mutations that give rise to functional ATP-activated channels (L40W) causes a decrease in ATP sensitivity of at least twofold, six mutations display more than a 10-fold increase in sensitivity to ATP (Tables I and II). A total of 20 mutations increase the sensitivity to ATP at least fourfold, while only two mutations display a reduced sensitivity to ATP. Because it is unlikely that mutations in TM1 and TM2 directly contribute to the ATP binding site (Nakazawa et al., 1998b; Ennion et al., 2000; Jiang et al., 2000; Roberts and Evans, 2004), we propose that these high-impact mutations increase the sensitivity to ATP by altering the closed–open equilibrium of the channel in favor of the open state. An introduced tryptophan could exert this effect on the closed–open equilibrium by either stabilizing the open state or destabilizing the closed state. It is improbable that introduction of tryptophan at so many positions would stabilize any particular conformation because this would require an improvement or optimization in packing. Thus, we favor the view that the large perturbations...
in \( \text{EC}_{50} \) arise predominantly from destabilization of the closed conformation. Single channel experiments might be able to resolve this issue. However, P2X<sub>4</sub> receptor channels are difficult to study on the single channel level. These channels have a small conductance (12 pS at ~150 mV), desensitize, and rapidly rundown in excised patches of membrane (Priel and Silberberg, 2004).

A disproportionate disruption of the closed conformation can be rationalized by proposing that the closed conformation of the transmembrane region of the channel is intrinsically more stable than the open conformation, and thus more readily perturbed by mutation. This idea has support from both structural and functional investigations on the transmembrane gate regions in \( K^+ \) channels. Inspection of the X-ray structures of KcsA, which is thought to be closed, and MthK, which is thought to be open, gives the impression that KcsA is a more optimally packed structure than MthK (Doyle et al., 1998; Jiang et al., 2002a,b). From functional studies, mutations in the gate region of the Shaker Kv channel also most commonly shift the closed–open equilibrium toward the open state (Hackos et al., 2002; Yifrach and MacKinnon, 2002), consistent with the notion that the closed gate is more stable. Thus, the type of perturbation observed here in P2X<sub>4</sub> is very consistent with an effect on the closed–open equilibrium, and with a more stable closed conformation of the transmembrane domain of the channel.

**Arrangement of TMs around the Ion Conduction Pore**

The ion conduction pore in P2X receptor channels is likely to be positioned at the central axis of the protein given that they have only two TMs per subunit and most likely assemble as trimers (Nicke et al., 1998; Stoop et al., 1999; Jiang et al., 2003a; Aschrafi et al., 2004), which provides too few helices to form more than one pore (Spencer and Rees, 2002). Two aspects of the present results are consistent with an arrangement of the TMs around the pore in which TM1 is more peripheral than TM2, as depicted in Fig. 8 B. First, there is a marked disparity between the two TMs in the number of mutations that give rise to nonconducting channels; TM1 contains one such mutant, whereas TM2 contains seven. Although we do not understand the underlying mechanism for the nonconducting mutants, the rather drastic nature of this phenotype suggests that these residues are likely to be buried in the protein. Second, the residues that tolerate mutation to tryptophan with only minor effects on the gating equilibrium are good candidates for positions that are exposed to the surrounding lipid. For TM1, 10 out of 22 mutations result in a less than twofold change in \( \text{EC}_{50} \), whereas only 5 out of 24 mutations have similar weak effects in TM2.

The present results also suggest that the arrangement of the TMs around the pore is somewhat different for the outer and inner parts of the channel. In TM1, most of the tolerant positions are located in the inner half of the TM (below I39), suggesting that there is extensive lipid exposure in this region. The high impact residues in TM1 (Fig. 8 A) identify a face of the helix that is likely to be involved in protein–protein interactions that stabilize the closed conformation of the channel. Toward the outside of TM1 there is a stretch of six residues that show large changes in \( \text{EC}_{50} \) (shaded area in Fig. 8 A), suggesting that interactions between TM1 and other parts of the protein are more extensive in the outer region. This is portrayed as a more tightly packed arrangement of the TMs in the outer region relative to the inner region (Fig. 8 B), but could well involve interactions with other parts of the protein within the TM1–TM2 linker. In addition, the clustering of nonconducting mutants toward the inner part of TM2 also raises the possibility that the secondary structure is different for the inner and outer regions.

**Where Is the Gate Region in P2X Receptor Channels?**

The gate in an ion channel can be operationally defined as the region of the protein that serves to minimize the flow of ions in the closed conformation. In potassium channels, for example, the transmembrane helices form a physically occluding barrier to the movement of ions at the inner end of the pore (del Camino and Yellen, 2001; Armstrong, 2003). Opening of the gate in these channels is thought to involve movement of the transmembrane helices, although the nature and extent of the movements remains unresolved (Jiang et al., 2002b; Swartz, 2004; Webster et al., 2004). In the present study, the effects of tryptophan substitution on the closed–open equilibrium indicate that the local environment of the mutated residue is different in the open and closed states. Thus, the perturbations observed here strongly imply that the TMs move with channel opening. The distribution of perturbed positions throughout the TMs gives the impression of rather extensive rearrangements of the TMs during gating, rather than being restricted to a focal region. The clear bias for large perturbations toward the outer half of TM1 and TM2 (Y42-V47 and T335-L346; shaded areas in Fig. 8) indicates that these regions undergo significant environmental changes between open and closed states, although large perturbations do not necessarily indicate that the movements are correspondingly large. The notion of gating motions in the outer regions of the transmembrane domain is consistent with studies examining the differential sensitivity of P2X<sub>1</sub> and P2X<sub>2</sub> receptor channels to the partial agonist \( \alpha_2 \text{meATP} \) (Haines et al., 2001a) and with state dependence for MTS reaction (Jiang et al., 2001). One
The present results also draw attention to the inner regions of the transmembrane domain of the channel, where seven nonconducting mutants are located, six of which are clustered within the inner part of TM2. The sensitivity of this inner region may reflect a tightly packed and relatively delicate structure that poorly tolerates substitution. However, it is worth keeping in mind that none of these mutants are ER retained, arguing against gross alterations in the protein fold. Most of the nonconducting mutants remain competent to assemble with WT subunits, also arguing against a general disruption of oligomerization. It is tempting to speculate that this inner region of TM2 has an important role in either the gating or ion selectivity of P2X receptor channels. Although the results discussed above point to movements in the outer regions of the TMs, they do not exclude the involvement of the sensitive inner region in gating. In much the same way, the previous studies implicating outer regions of TM2 in ion conduction and selectivity (Nakazawa et al., 1998a; Migita et al., 2001; Egan and Khakh, 2004) do not rule out an important contribution of the inner region.

**Conclusions**

The results presented here provide evidence that TM1 and the outer region of TM2 adopt α-helical secondary structures in P2X receptor channels. The majority of mutations within the transmembrane domain enhance the sensitivity of the channel for ATP, suggesting that the closed conformation is more optimally packed. The distribution of perturbations in the closed–open equilibrium indicates that the outer regions of both TMs undergo rearrangements during gating. Finally, the distribution of mutations giving rise to nonconducting channels identifies an inner region of TM2 that may play a critical role in channel function.

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