Contribution of Transmembrane Regions to ATP-gated P2X2 Channel Permeability Dynamics*

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ATP-gated P2X2 channels undergo activation-depend-ent permeability increases as they proceed from the selective I1 state to the I2 state that is readily permeable to organic cations. There are two main models about how permeability changes may occur. The first proposes that permeability change-competent P2X channels are clustered or redistribute to form such regions in response to ATP. The second proposes that permeability changes occur because of an intrinsic conformational change in P2X channels. In the present study we experimentally tested these views with total internal reflection fluorescence microscopy, electrophysiology, and mutational perturbation analysis. We found no evidence for clusters of P2X2 channels within the plasma membrane or for cluster formation in response to ATP, suggesting that channel clustering is not an obligatory requirement for permeability changes. We next sought to identify determinants of putative intrinsic conformational changes in P2X2 channels by mapping the transmembrane domain regions involved in the transition from the relatively selective I1 state to the dilated I2 state. Initial channel opening to the I1 state was only weakly affected by Ala substitutions, whereas dramatic effects were observed for the higher permeability I2 state. Ten residues appeared to perturb only the I1-I2 transition (Phe131, Arg33, Glu37, Lys82, Ile128, Ile132, Ser130, Gly131, Trp196, Leu226). The data favor the hypothesis that permeability changes occur because of permissive motions at the interface between first and second transmembrane domains of neighboring subunits in pre-existing P2X2 channels.

ATP-gated P2X channels are a large family of transmitter-gated cation channels (1–3). P2X channels are widely expressed in the brain where they mediate, presynaptically facilitate, and postsynaptically modulate fast synaptic transmission (1, 2, 4). P2X subunits (P2X1–P2X7) are the products of seven genes and form at least 11 heteromeric channels (5). P2X channels are likely trimers (6–10), and each subunit possesses two transmembrane domains, both of which are thought to contribute to the pore (11–14). The cytosolic domain of the protein consists of amino and carboxyl termini. P2X channels are cation-selective (2), with some displaying high Ca2+ fluxes (15). P2X2, P2X4, and P2X7 channels also display permeability dynamics, whereby the channel pore dilates in an ATP activation- and time-dependent manner from a fairly selective I1 state to the I2 state that is also permeable to organic cations (16–24). The transition from I1 to I2 typically takes several seconds. Permeability changes have been studied most for recombinant P2X2, P2X4, and P2X7 channels (2, 22), but there is also evidence that permeability changes occur for natively expressed channels in cells throughout the body, including neurons (16, 19, 20, 25–28).

Activation-dependent changes in ionic selectivity for P2X channels imply that the selectivity filter may be dynamic and undergo conformational re-arrangements to switch its preference between ions (22, 29). Two different models are proposed to explain the ability of P2X channels to undergo permeability changes in response to ATP (22). The first, which we term the cluster model, suggests that I1 state-competent P2X channels represent clusters of accreted P2X channels/subunits, perhaps of higher oligomeric state, and that ATP triggers this process (22). The essential features of this hypothesis were first proposed in a pioneering study on mast cells (30). This has recently drawn some support based on the observation of higher molecular weight, presumably P2X sub-unit-containing, bands on protein gels (6, 9) and the finding that permeability changes in P2X2 channels are dependent on channel density (31). First principles also suggest that the pore diameter would increase with the number of helices lining the pore (32). However, in the case of P2X2 channels there is experimental evidence to suggest that channel redistribution and cluster formation may not be needed for pore dilation (23). The second hypothesis, which we term the gating model, proposes that permeability changes occur because of an intrinsic conformational change in the channels. This draws on findings with mutagenesis (16, 17) and ion replacement experiments (33) as well as work providing evidence for a permissive conformational state with FRET1 (34). The gating model also draws indirect support from studies of mechanosensitive channels that are known to dilate from ∼2 to ∼30 Å because of an intrinsic conformational change involving helix tilting (35). Calculations also show that for channels with regular helices and ideal oligomeric symmetry an increase in pore diameter can be achieved with an increase in helical tilt (32). Thus both the cluster and gating models have

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1 The abbreviations used are: FRET, fluorescence resonance energy transfer; TIRF, total internal reflection fluorescence; TM, transmembrane; H3E, human embryonic kidney; NMDG+, N-methyl-D-glucamine; PH, pleckstrin homology; YFP (γ), yellow fluorescent protein; CFP (φ), cyan fluorescent protein; DTT, dithiothreitol; mem-Y, fusion protein between the N-terminal 20 amino acids of neuromodulin and YFP; PH-Y, fusion protein between the pleckstrin homology domain of phospholipase Cγ and YFP.
their merits, but no study has tested the models directly under a fixed set of experimental conditions or in the case of the cluster model, with approaches that have sufficient resolution to provide direct information about P2X2 channels in the plasma membrane.

We tested the cluster model by imaging fluorophore labeled P2X2 channels in the plasma membrane with total internal reflection fluorescence microscopy (34, 36, 37). Our experiments provided no evidence to support the cluster model. We next sought to identify P2X2 subunit regions permissive for permeability changes in an effort to provide insight and constraints on the gating model. Ab initio there was no way to deduce which parts of the protein may be most important because there is no structural information on P2X2 channels, no related channels of known structure, and no channels with comparable primary sequence (1, 2). Because of this quandary we sought to identify regions crucial for the gating model empirically with single site Ala substitutions and analysis of state-specific perturbations. We focused on the transmembrane segments because of their importance for channel function (11–16, 39, 40) and because no detailed study of the TM segments and their role in permeability changes exists. Overall the data are consistent with the gating model and the hypothesis that permeability changes occur because of spatially diverse, and perhaps extensive, molecular rearrangements in the transmembrane, pore, and cytosolic domains (34) of pre-existing and stably expressed P2X2 channels.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**—The cell lines used were HEK-293 cells transiently expressing appropriate wild type (wt), mutant, and fluorescently labeled channels. Some cDNAs were available from previous work (12, 18, 34, 40), some were from R. Alan North (Sheffield University), and others were generated using standard procedures. cDNAs were propagated in DH5α Escherichia coli, and plasmids were purified using standard techniques. Plasmids encoding mem-Y were purchased from Clontech (pEYFP-Mem). For transient expression in HEK-293 cells 0.5–1 μg of plasmid cDNA was used (Effectene, Qiagen).

**TIRF Microscopy**—Briefly, we used an Olympus IX70 scope equipped with a Princeton Instruments cooled I-PentaMAX camera with a High Blue Gen III Intensifier (Roper Scientific). The control of excitation and image acquisition was achieved using MetaMorph software and drivers (Universal Imaging), shutters, filter wheels, and Proscan II control box (Prior). The beams of 488/515 nm argon (150 milliwatt) and 442 nm helium-cadmium (12.5 milliwatt) lasers (Melles Griot, Carlsbad, CA) at <5% power (constant for all experiments reported) were combined and controlled with an IX2-COMBO (Olympus), Uniblitz shutters (Prior), and acoustoptical tunable filter and controller (AA Optoelectronics, Les Ulis, France). The beams of 488/515 nm argon (150 milliwatt) and 442 nm helium-cadmium (12.5 milliwatt) lasers (Melles Griot, Carlsbad, CA) at <5% power (constant for all experiments reported) were combined and controlled with an IX2-COMB (Olympus), Uniblitz shutters (Prior), and acoustoptical tunable filter and controller (AA Optoelectronics, Les Ulis, France) and fed into an optical fiber (VF5-FUR; Olympus) for entry into the TIRF condenser (IX2-RFAEVA-2; Olympus). Cells were plated onto glass-bottom Petri dishes (170 μm thick; Willco Wells BV, Chevreuse, France) and fed into an optical fiber (VF5-FUR; Olympus) for entry into the TIRF condenser (IX2-RFAEVA-2; Olympus). Cells were plated onto glass-bottom Petri dishes (170 μm thick; Willco Wells BV, Amsterdam, Netherlands) 24–48 h before imaging and were viewed with a 60× oil immersion objective lens with a numerical aperture of 1.45 (Olympus). The gain was adjusted for maximum signal-to-noise for each cell and kept constant for all image acquisitions.

**Electrophysiology**—HEK-293 cells were used for recordings 24–48 h posttransfection, gently mechanically dispersed, and plated onto glass coverslips 2–12 h before use. We included this step (11) to ensure adequate voltage clamp during reversal potential measurements from single spherical cells. The extracellular recording solution was comprised of (in mM) NaCl, 150; MgCl2, 1; CaCl2, 1 or 0.1; HEPES, 10; and glucose, 10 (pH 7.4), and the pipette solution was comprised of (in mM) NaCl, 150; MgCl2, 1; CaCl2, 1 or 0.1; HEPES, 10; and glucose, 10 (pH 7.4), and the pipette solution was comprised of (in mM) NaCl, 150; MgCl2, 1; CaCl2, 1 or 0.1; HEPES, 10; and glucose, 10 (pH 7.4), and the pipette solution was comprised of (in mM) NaCl, 150; MgCl2, 1; CaCl2, 1 or 0.1; HEPES, 10; and glucose, 10 (pH 7.4), and the pipette solution was comprised of (in mM) NaCl, 150; MgCl2, 1; CaCl2, 1 or 0.1; HEPES, 10; and glucose, 10 (pH 7.4), and the pipette solution was comprised of (in mM) NaCl, 150; MgCl2, 1; CaCl2, 1 or 0.1; HEPES, 10; and glucose, 10 (pH 7.4), and the pipette solution was comprised of (in mM) NaCl, 150; MgCl2, 1; CaCl2, 1 or 0.1; HEPES, 10; and glucose, 10 (pH 7.4), and the pipette solution was comprised of (in mM) NaCl, 150; MgCl2, 1; CaCl2, 1 or 0.1; HEPES, 10; and glucose, 10 (pH 7.4). If clustering has any relation to permeability changes in the plasma membrane expanse in which they sit. Assuming a uniform distribution of ion channel coordinates, and a membrane protein diffusion coefficient of 10−10 cm2s−1, estimates suggest any two random channels (at three per μm2) will on average stay <0.5 μm apart, but every <100 s they will come within −1 diameter of each other for <3 ms. For mammalian cells these considerations lead to the conclusion that clustering cannot occur randomly simply because there are too few channels for even the highest expression levels where permeability changes have been measured (16, 26, 31, 33, 34). If on the other hand clustering occurs because of specific molecular interactions then regions of high channel density must exist in an ocean of plasma membrane with no or few channels. This is because the total number of channels is too few to support clustering everywhere, which presumably requires several thousand per μm2 (29). If clustering has any relation to permeability changes, then preformed clusters must either exist in a background of plasma membrane relatively devoid of channels, or they must form in response to ATP. We tested these predictions with TIRF microscopy.

We imaged CFP- or YFP-tagged P2X2 channels (18, 34) to...
determine whether they formed clusters in the plasma membrane of HEK cells with TIRF microscopy (Fig. 1A), which affords the excitation and imaging of channels within ~100 nm of the plasma membrane (36). Using TIRF microscopy we found no evidence of P2X2-C or P2X2-Y channel clusters for areas of HEK cells in close apposition to glass, an area called the footprint (Fig. 1, B and C). The higher intensity region in the center of the footprint in Fig. 1C does not represent clustering but rather shows the region of the cell in closest adherence to glass as the edges of the cells curl away out of the evanescent field. Indeed the qualitative appearance of P2X2-Y fluorescence, and quantitative measures of P2X2-Y hot spots, were similar to those observed for two different membrane-targeted YFP proteins (mem-Y and PH-Y) and with T18A P2X2-YFP channels that are known to lack the I2 state (18). In contrast P2X4-Y channels were more clustered in the plasma membrane (Fig. 1, B and D) and showed significantly more hot spots per footprint (Fig. 1B). Examination of line profiles across the highest intensity regions of the footprints (three examples for P2X2-Y and P2X4-Y are shown in Figs. 1, C and D) indicated that P2X2-Y channels displayed a smooth distribution in the plasma membrane with no abrupt peaks corresponding to hot spots that were frequently observed for P2X4-Y-expressing cells (arrows in Fig. 1D). Because we could readily observe and count hotspots for P2X4-Y channels we feel confident in suggesting that P2X2-Y channels do not cluster. Given that all cells expressing P2X2 or P2X2-Y channels undergo permeability changes (114/114 cells; Table I) this implies that cluster formation is not a prerequisite for permeability changes. Seemingly P2X2 channels are quite evenly distributed in the plasma membrane of HEK cells (46).

We next asked whether P2X2-Y channels become more clustered in response to ATP. To this end we imaged P2X2-Y channels and rapidly applied ATP (100 μM). We chose 20-s applications because permeability changes are complete within this time (17, 33). Again we found no evidence that P2X2 channels move in response to ATP, because the images of P2X2-Y footprints were indiscernible before and during ATP, both in terms of appearance (Fig. 2A) and in terms of intensity (Fig. 2D; <5% change, n = 8). The negative data with P2X2-Y channels showing that they do not cluster in response to ATP warranted several controls to be confident that we could measure movement of fluorescence, if it occurred. First, we monitored the access of a fluorescent dye (5 μM Lucifer yellow, n = 4) to the footprint. The dye flowed freely and completely into the area between the cell and glass coverslip within 1 s (Fig. 2, B and D). Second, we imaged footprints from cells expressing PH-Y and hP2Y4 receptors. In response to 50 μM UTP application to cleave phosphatidylinositol 4,5-bisphosphate (47) we measured marked decreases in the intensity of fluorescence within the footprint (Fig. 2, C and D; n = 4). We interpret this to indicate that PH-Y moved into the cytosol due to its greater affinity for phosphatidylinositol 1,4,5-trisphosphate (48). Together, these experiments suggest that access of molecules to the footprint is

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**Fig. 1.** No evidence for P2X2-Y channel clusters in the plasma membrane of HEK cells. A, the schematic illustrates the principle of TIRF (36). Laser light was focused onto the back focal plane of the objective lens, and its position adjusted so that it emerged into the immersion oil at an angle shallower than the critical angle. This created an evanescent field of illumination of depth ~100 nm into the cell, which was adhered to the coverslip. B, summary graph of the numbers of fluorescent hot spots per footprint from HEK cells expressing the various constructs as indicated. C, image of a footprint from a HEK cell expressing P2X2-Y channels. The lower plots show line profiles from 3 footprints. D, as in C but for P2X4-Y channels; the arrows indicate hot spots of fluorescence. The scale bar in C and D is 10 μm.
relatively fast (−1 s). We interpret these data to indicate that P2X2-Y channels do not move in response to ATP to form regions of markedly higher channel density with a time course relevant to permeability changes.

**Ala3 Scanning Mutagenesis and Perturbation Analysis of I1 and I2 States**—Given the lack of evidence in favor of the cluster model for P2X2 channels we next tested the gating model and sought to identify transmembrane regions that may be important for permeability changes. Examination of the first and second TM domain sequences for P2X1–P2X7 subunits provided little further insight (Fig. 3, A and B), a trend reminiscent of findings with Ca\(^{2+}\) flux (15) underscored the necess-

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**TABLE I**

|                | \(E_{rev}\) | \(\Delta E_{rev}\) | \(\rho\) NMDG\(^+\)/pNa\(^+\) | \(\Delta \rho\) NMDG\(^+\)/pNa\(^+\) | \(n\) | Class |
|----------------|------------|---------------------|-----------------------------|-------------------------------------|------|-------|
|                | \(mV\)     | \(mV\)              | \(mV\)                      |                                     |      |       |
| *ut* P2X       | 59.8 ± 0.8 | 17.1 ± 1.0          | 42.2 ± 1.0                  | 0.10 ± 0.004                       | 0.53 ± 0.02 | 5.7 ± 0.2 | 83 |
| P2X2-C/Y       | 65.0 ± 1.1 | 27.1 ± 1.7          | 37.8 ± 2.4                  | 0.08 ± 0.003                       | 0.37 ± 0.02 | 5.1 ± 0.5 | 31 |
| TM1            |            |                     |                             |                                     |      |       |
| G30A           | 53.5 ± 2.4 | 29.3 ± 4.7          | 24.2 ± 3.0                  | 0.13 ± 0.012                       | 0.37 ± 0.073 | 2.8 ± 0.4 | 10 |
| G31A           | 50.0 ± 4.2 | 25.4 ± 2.5          | 23.5 ± 5.7                  | 0.16 ± 0.03                        | 0.13 ± 0.01 | 1.1 ± 0.2 | 9 |
| V32A           | 60.1 ± 2.5 | 33.7 ± 3.1          | 26.4 ± 2.1                  | 0.10 ± 0.01                        | 0.28 ± 0.04 | 2.9 ± 0.2 | 10 |
| H33A           | 64.2 ± 1.3 | 22.3 ± 3.3          | 41.9 ± 3.3                  | 0.08 ± 0.004                       | 0.46 ± 0.05 | 5.8 ± 0.7 | 13 |
| R34A           | 66.0 ± 1.0 | 64.3 ± 3.7          | 1.7 ± 3.5                   | 0.07 ± 0.003                       | 0.09 ± 0.02 | 1.2 ± 0.3 | 11 |
| M35A           | 56.5 ± 2.7 | 13.0 ± 2.1          | 43.5 ± 2.2                  | 0.11 ± 0.02                        | 0.62 ± 0.05 | 5.8 ± 0.8 | 10 |
| V36A           | 56.4 ± 2.3 | 15.2 ± 1.5          | 41.2 ± 2.2                  | 0.11 ± 0.01                        | 0.56 ± 0.03 | 5.3 ± 0.4 | 10 |
| Q37A           | 66.2 ± 1.5 | 57.6 ± 3.9          | 8.6 ± 2.7                   | 0.07 ± 0.005                       | 0.12 ± 0.03 | 1.5 ± 0.2 | 13 |
| L38A           | 52.9 ± 1.9 | 16.6 ± 2.4          | 36.3 ± 3.5                  | 0.13 ± 0.01                        | 0.54 ± 0.05 | 4.6 ± 0.7 | 10 |
| L39A           | 58.0 ± 2.0 | 17.6 ± 2.4          | 40.4 ± 3.3                  | 0.10 ± 0.01                        | 0.52 ± 0.04 | 5.3 ± 0.6 | 10 |
| I40A           | 61.3 ± 1.6 | 14.9 ± 2.6          | 46.4 ± 1.4                  | 0.09 ± 0.01                        | 0.58 ± 0.08 | 6.4 ± 0.4 | 9 |
| L41A           | 69.6 ± 1.0 | 49.7 ± 4.7          | 19.8 ± 3.6                  | 0.02 ± 0.003                       | 0.17 ± 0.03 | 2.5 ± 0.4 | 11 |
| L42A           | 69.2 ± 1.5 | 34.9 ± 3.7          | 34.4 ± 2.9                  | 0.07 ± 0.004                       | 0.28 ± 0.05 | 4.1 ± 0.4 | 10 |
| Y43A           | 81.1 ± 1.1 | 70.2 ± 6.9          | 10.9 ± 6.4                  | 0.04 ± 0.002                       | 0.08 ± 0.03 | 2.0 ± 0.7 | 7 |
| F44A           | 81.0 ± 1.2 | 77.0 ± 1.9          | 4.0 ± 1.4                   | 0.04 ± 0.002                       | 0.05 ± 0.004 | 1.2 ± 0.1 | 10 |
| V45A*          |            |                     |                             |                                     |      |       |
| W46A           | 62.1 ± 3.3 | 26.7 ± 8.9          | 35.3 ± 6.1                  | 0.09 ± 0.01                        | 0.4 ± 0.1  | 4.6 ± 0.8 | 6 |
| Y47A*          |            |                     |                             |                                     |      |       |
| G48A           | 64.4 ± 1.3 | 31.2 ± 5.2          | 33.1 ± 4.5                  | 0.08 ± 0.004                       | 0.35 ± 0.06 | 4.2 ± 0.6 | 10 |
| F49A           | 67.8 ± 1.4 | 30.3 ± 3.4          | 37.3 ± 3.3                  | 0.07 ± 0.004                       | 0.32 ± 0.04 | 4.7 ± 0.6 | 10 |
| I50A           | 67.5 ± 1.7 | 40.3 ± 4.9          | 27.2 ± 1.8                  | 0.07 ± 0.005                       | 0.24 ± 0.04 | 3.4 ± 0.6 | 10 |
| V51A*          |            |                     |                             |                                     |      |       |
| Q52A           | 70.1 ± 1.1 | 53.1 ± 2.7          | 17.0 ± 2.0                  | 0.06 ± 0.002                       | 0.13 ± 0.02 | 2.0 ± 0.2 | 10 |
| K53A           | 66.3 ± 0.8 | 55.0 ± 2.8          | 11.3 ± 2.5                  | 0.07 ± 0.002                       | 0.12 ± 0.02 | 1.6 ± 0.2 | 10 |
| S54A           | 62.6 ± 2.4 | 18.3 ± 2.0          | 44.3 ± 2.2                  | 0.09 ± 0.01                        | 0.50 ± 0.04 | 6.0 ± 0.5 | 10 |
| Variance       |            |                     |                             |                                     | 7.9% | 52.1% |

*indicates no, or negligibly small, ATP-evoked currents; these mutants were not studied further.
We assayed mutants for permeability changes where every amino acid in TM1 and TM2 of P2X2 was exchanged for Ala (40). Four mutants (V45A, Y47A, V51A, and D349A) were expressed in the plasma membrane but were non-functional and not studied further (40). The remaining mutants all produced functional responses in HEK cells when challenged with 100 μM ATP (Table I), a concentration chosen to be near maximal for the mutants and wt P2X2 (40). We tested all the mutants for initial pNMDG+/pNa+ (the I1 state) and for changes in this parameter as they enter the I2 state (Table I). Representative traces for five mutants and wt P2X2 channels are shown in Fig. 3, C–H. For both transmembrane domains the Ala mutants produced only subtle effects on the I1 state measured within 0.2–1 s of applying ATP (Table I; L41A, L42A, Y43A, F44A, Q52A, I328A, I332A, L338A, S340A, G342A, and G344A). More profound effects were observed for the I2 states measured 10–30 s into the ATP application period (Table I; G37A, Q37A, I41A, L42A, Y43A, F44A, Q52A, I50A, K53A, I328A, I332A, I332A, L338A, T339A, S340A, G342A, G344A, S345A, C348A, W350A, L352A, and L353A). The more dramatic effects on I1 are readily apparent from the data presented in Table I and from an analysis of variance: across TM1 and TM2 variance for I1 was 7.9 and 12.4% but 52.1 and 48.0% for I2 (Table I). Clearly I2 is readily perturbed by Ala substitutions, with 12 and 13 residues affected in TM1 and TM2.

To obtain a measure of the mutants that affected the I2 state only, we analyzed the magnitude of the shift in reversal potential (ΔErev) from I1 to I2. In Fig. 4 the residues highlighted in blue are those that showed a ΔErev that was significantly smaller than wt P2X2, whereas the residues in red are those that showed no ΔErev at all. The colored residues (blue and red) thus provide an upper estimate on the number of Ala mutants that may affect the I2 state. We focused on those mutants that totally abolished the I2 state (red in Fig. 4; no ΔErev), because subtle changes in reversal potential may arise because of cell-to-cell variability and expression levels (31, 33). These criteria revealed 6 residues in TM1 and 6 in TM2 that lacked the I2 state (Fig. 4). In TM1 they were F31A, R34A, Q37A, I41A, L42A, Y43A, F44A, V45A, Q52A, I50A, K53A, L338A, T339A, S340A, G342A, G344A, S345A, C348A, W350A, L352A, and L353A. We next tested these mutants for normal membrane expression by comparing the peak ATP-evoked currents in physiological solutions with those measured for wt P2X2 channels. This analysis revealed that ATP-evoked currents for Y43A and F44A were significantly smaller than wt P2X2 channels (−64.8 ± 9.3 and −64.5 ± 2.3 pA/pF, respectively, versus −576.4 ± 116.7 pA/pF for wt P2X2; Fig. 6C). Peak current data for all TM1 and TM2 mutants that showed perturbed I2 states are shown in Fig. 6C and for all the mutants reported in this study in a recent paper by Li et al. (40). Because membrane expression levels are important for permeability changes (31), we excluded these residues from the helical wheel plots in Fig. 4 that emphasize residues that lacked the I2 state but showed membrane expression equal to wt P2X2 channels. All these residues fell on one-half of helical wheel representation of TM1 or TM2 (Fig. 4). The results with S340A should be interpreted with caution because this amino acid is known to affect cation flux through the pore (15, 42). Thus for TM1 and TM2, 4 and 6 residues contribute the most to the I1 to I2 transition, and they fall on one-half of helical wheel representations of TM1 and TM2.

Disulfide Formation between V48C and I328C—Previous studies suggest that channels formed by expressing V48C and I328C mutants form a disulfide bond between these two residues, indicating that these TM1 and TM2 residues lie within ~8.4 Å of each other (14, 55). Biochemical experiments further
suggested that the disulfide was formed between the TM1 and TM2 domains of neighboring subunits (8). Remarkably when interpreted from this perspective all except one of the Ala residues that perturb the I2 state lie on the same half of an helical view of TM1 as V48 and as I328 for TM2 (Fig. 7A). The exception was S340A, and it seems possible that this mutant directly affects the selectivity filter (15, 42) rather than gating to the I2 state. These data suggest that the Ala mutants may perturb the I2 state by hindering rearrangements that occur at the interface between TM1 and TM2 of neighboring subunits (8, 14). We attempted to test this with V48C and I328C mutants.

**A. TM1**

| Suggested | Sequence |
|-----------|----------|
| Disulfide | G V I F R L I Q L V V L V Y V I G W - V F V Y E K G 54 | rP2X1 |
|           | G F V H R M V Q L L I L L Y F V - W Y V F V I Q K S 54 | rP2X2 |
|           | G I N N R A V Q L I L I S Y F V G W - V F L H E R A 49 | rP2X3 |
|           | G L M N R A V Q L L I L I Y V I G W - V F V W E K G 53 | rP2X4 |
|           | G L L Y R V L Q L L I L L Y L I W - V F L I K K S 54 | rP2X5 |
|           | G I S Q R L L Q L C V V Y V I G W - A L L A K K G 55 | rP2X6 |
|           | G T I K W I L H M T V P S Y V S - - F A L M S D K L 50 | rP2X7 |

**B. TM2**

| Suggested | Sequence |
|-----------|----------|
| Disulfide | I P T M T T I G S G I G I F G V A T V L C D L L L L 354 | rP2X1 |
|           | I P T I I L A T I T S G V G S F L C D W I L L 353 | rP2X2 |
|           | I P T I I S S V A A F T S V G V G T V L C D I L L 344 | rP2X3 |
|           | I P T M I N V G S G L A L G G V A T V L C D V I L 358 | rP2X4 |
|           | I P T V I N I G S G L A L M G A A F P C D L V L I 359 | rP2X5 |
|           | I P T A I T V G T G A A W L G M V T F L C D L L L L 352 | rP2X6 |
|           | I Q L V V Y I G S T L S Y F G L A T V C D L L I N 356 | rP2X7 |

**Fig. 3.** Sequence alignment of P2X subunit transmembrane segments and representative traces for Ala mutants. Sequence alignment of all rat P2X subunits for transmembrane domains 1 and 2 (A and B, respectively). C, representative traces for wt P2X2, channels expressed in HEK cells and bathed in NMDG solutions. The upper panels show the steady state current at −60 mV, whereas the lower panels show current-voltage relations determined every 500 ms after the peak response (I1) and finishing at the steady state current-voltage relationship (I2). D–H, as in C for two mutants with normal I1 states but no I2 states (D, E), for two mutants with normal I1 and I2 states (F, G), and one mutant with normal I1 states and impaired I2 states (H).
Consistent with past work (8, 14, 55), the reducing agent dithiothreitol (DTT) (10 mM) produced no effect on ATP-evoked currents recorded from cells expressing wt P2X2 or the single V48C and I328C mutant P2X2 channels (Fig. 5, A–D). However, DTT augmented 6-fold ATP-evoked currents recorded from channels that contained V48C and I328C mutants (Fig. 5, C and D). The magnitude and time course of the DTT effect were consistent with past data (8, 55). We next asked whether V48C and I328C channels undergo permeability changes and display the I2 state. Channels formed by expressing V48C or I328C mutants showed significant increases in NMDG$^+$ permeability and a robust I2 state (Fig. 5, E and F). It is noteworthy that the shift in reversal potential for these single mutants was reduced by about half in relation to wt P2X2 (Table I). We next repeated these experiments for channels formed by co-expressing V48C and I328C double mutants because they should form a disulfide between subunits (8). For these channels the I1 and I2 state were not significantly different from either V48C or I328C mutants (Fig. 5, G and H; Table I). When interpreting this result it is important to consider that co-expression of two subunits is expected to result in a mixed population of channels in the membrane. For these reasons it is problematic to draw comparisons between I1 and I2 states formed from channels upon co-expression of V48C and I328C.

**Fig. 4. I2 state-specific Ala mutants.** A, upper graph shows the effect of single site Ala mutants on the shift in reversal potential measured from cells containing extracellular NMDG$^+$ for TM1, whereas the middle panel shows a helical net representation of TM1 with the colored residues indicated positions where the I2 state was abolished. B, as in A but for TM2. For A and B residues listed in blue showed significantly reduced shifts in reversal potential, those in red showed no shifts in reversal potential (over 30 s), and the remainder were no different from wt P2X2. The lower panels in A and B are helical wheel plots of TM1 and TM2. The highlighted residues are those that lacked I2 states but expressed at levels equal to wt P2X2 channels.
with those of channels formed in cells expressed either mutant alone (Table I). The most reliable test to determine whether a disulfide formed between V48C and I328C upon coexpression is to assay the effect of a reducing agent to break the disulfide. If motions between V48C and I328C were needed to allow permeability changes to occur, then one would expect breaking the disulfide with DTT would affect the ability of V48C and I328C channels to undergo permeability changes. If on the other hand motions at, or near, V48C and I328C are not needed, then breaking the disulfide should have no effect. Remarkably, application of DTT (10 mM for 2–5 min; Fig. 5C) produced significant effects on channels formed by coexpressing V48C and I328C channels. The channels opened to an $I_1$ reversal potential much more depolarized than before DTT (Fig. 5, G–I), as though once the disulfide had been broken the channels readily opened to an $I_2$-like state and then dilated further by $-20$ mV to a larger $I_2$ state. If one assumes that the true $I_1$ $E_{rev}$ is the average measured for V48C, I328C, and V48C and I328C channels without DTT, $I$ and $J$, summary data for experiments like those in $G$ and $H$. 

**FIG. 5. V45C and I328C mutants.** A, whole-cell ATP-evoked currents (100 μM) recorded from HEK cells expressing V45C/I328C mutants. ATP was applied every 2 min for 10 applications, and DTT (10 mM) was applied during the times indicated by the bar. B, representative traces from the panels in A on an expanded time scale. C, average data from experiments like those shown in A; 10 mM DTT was applied for the time indicated. D, summary bar graph for experiments such as those shown in C for the channels as indicated. E, $I_1$, and $I_2$ reversal potentials measured in NMDG+ solutions for V48C mutants. F, same as described for E but for I328C mutants. G, same as in E but for channels formed by coexpressing V45C and I328C mutants. H shows experiments identical to those shown in G but after 5-min application of DTT (10 mM) to the bathing medium. The “true $I_1$, $E_{rev}$” is the average measured for V48C, I328C, and V48C and I328C channels without DTT. I and J, summary data for experiments like those in G and H.
absence of DTT at $20.0 \pm 1.7 \text{ mV}$ ($n = 6$ and 10; $p < 0.05$). The data favor the view that a disulfide forms between V48C and I328C leading to a normal I1 but reduced I2 state. DTT reduces this disulfide leading to a restored I2 state. Presumably V48C/I328C channels in the absence of DTT undergo restricted permeability changes because the permissive rearrangements between TM1 and TM2 are impaired. Our data indicate that permeability changes in P2X2 channels can be impaired by perturbing the interface between TM1 and TM2 by 1) Ala mutants along the interface for TM1 and TM2 (Table I) and 2) by a disulfide formed between residues that span the interface.

Channels That Lack I2 Tend to Desensitize Rapidly—We recently suggested that the P2X2 I2 state gives rise to currents that desensitize slowly over 10 s, whereas channels that lack this state desensitize somewhat more rapidly in physiological solutions (34). This supports previous work on P2X4 and P2X7 channels, which also display permeability changes, and in Na$^+$ solutions show biphasic or slowly decaying currents in response to ATP (2, 16, 26, 56). Collectively, these data imply that one physiological correlate of the I2 state may be enhanced ion flow associated with slow desensitization.

Might the Ala mutants affect the pore directly and complicate our interpretations? There is no way to totally exclude this possibility, but if the Ala mutants do markedly affect the selectivity filter then one expects the relative permeability of another cation to be affected as well. We tested this prediction by examining the $p_{Ca^{2+}}/p_{Na^+}$ of two mutants from each transmembrane domain (G31A, H33A, P329A, and L353A), as well as T18A, which we have shown previously lacks I2 (34). There were no significant differences in $p_{Ca^{2+}}/p_{Na^+}$ as compared with wt P2X2 (Table II). These data provide reassurance that the selectivity filter in the Ala-substituted mutants retains its ability to select Ca$^{2+}$ over Na$^+$ with a fidelity comparable with wt P2X2 channels (15, 42) and support the hypothesis that Ala mutants produce their perturbing effects by affecting channel gating (40).

DISCUSSION

The main finding of the present study is that both transmembrane domains contribute to permeability changes in P2X2 channels and that the interface between transmembrane domains appears important for conformational rearrangements that allow opening to the I2 state.

P2X$_2$ channel permeability changes are known to depend on plasma membrane channel density (31). One interpretation,
consistent with the cluster model for permeability changes (see Introduction), is that clusters of channels are needed in order for P2X<sub>2</sub> channels to undergo permeability changes. Presumably, these hypothetical clusters are either preformed or form in response to ATP. We attempted to test this view by imaging channels formed by CFP- or YFP-labeled channels (P2X<sub>2</sub>-C, P2X<sub>2</sub>-Y). This is a valid approach because the intrinsic fluorescence of the GFP variants provides a measure of channel location in living cells (57) and because tagged P2X<sub>2</sub> channels function in a manner identical to <i>wt</i> P2X<sub>2</sub> channels (18, 58). By using high resolution TIRF microscopy we found no evidence for clusters of P2X<sub>2</sub> channels in the plasma membrane of HEK cells, whereas we could readily detect clusters of P2X<sub>4</sub> channels. The stationary P2X<sub>4</sub> channel clusters may represent channels formed by CFP- or YFP-labeled channels (P2X<sub>2</sub>-C, P2X<sub>2</sub>-Y). We attempted to test this view by imaging P2X<sub>2</sub>-Y clusters before or during ATP, We concluded changes in every HEK cell (Table I), and because we never could readily detect clusters of P2X<sub>4</sub> channels. The previous work was in accord with an intrinsic conformational change that allowed P2X<sub>2</sub> channel permeability changes to occur (34). The previous view encompassed several features that are noteworthy. First, Val<sup>48</sup> and Ile<sup>328</sup> face each other across an interface, as would be expected if they were part of the selectivity filter itself given that S340A is known to affect the gating model for permeability changes. We selected the Ala mutants for I<sub>2</sub> state specificity (see “Results”), in an attempt to minimize direct effects on the pore. The impelling assumption is that the sensitivity of the I<sub>2</sub> state to mutations is informing about gating changes associated with this state (50). Remarkably, the I<sub>2</sub> state was far more readily perturbed by Ala substitutions than the I<sub>1</sub> state, suggesting that these mutants shift the equilibrium in favor of opening to the I<sub>1</sub> state, either by stabilization of this state or destabilization of the I<sub>2</sub> state. Based on the adage that there are more ways to destabilize rather than stabilize protein structures (50) we interpret these data to indicate that there is a greater propensity of mutants to destabilize the I<sub>2</sub> state and favor the I<sub>1</sub> state conformation.

With the exception of S340A, for both TM1 and TM2, the residues that abolished the I<sub>2</sub> state fell on one-half of a helical wheel representations, although there were no contiguous stretches. Our experiments do not provide a precise understanding of Ser<sup>340</sup> because the Ala substitution may affect gating that is thought to occur as the pore dilates (33) or the selectivity filter itself given that S340A is known to affect the ability of P2X<sub>2</sub> channels to select for Ca<sup>2+</sup> ions (15, 42). With the exception of this residue the Ala hits in TM1 and TM2 all fall on one-half of a helical wheel representation of TM1 and TM2. What do the differences between our work and that of Li <i>et al.</i> (40) on an Ala scan across TM1 and TM2 for measures of agonist potency tell us about the motions that occur in P2X channels? The previous work measured EC<sub>50</sub> values for agonist-evoked currents over a time course of 1–2 s (40). In contrast we measured permeability values for agonist applications of ~30 s. This latter time course provides information about the I<sub>2</sub> state, whereas measurements over 1–2 s are too brief to accurately reflect the I<sub>1</sub> state (33). Also, EC<sub>50</sub> values and permeability ratios by definition report distinct aspects of channel function and are not readily comparable. Furthermore, the final data set used in this study is for mutants that abolished the I<sub>2</sub> state and so a close correspondence with the work of Li <i>et al.</i> (40) is not expected. On the other hand if one compares all the Ala hits on I<sub>2</sub> (Table I) to the data of Li <i>et al.</i> (40) there is good agreement for TM1 and TM2 (Leu<sup>41</sup>, Gln<sup>37</sup>, Phe<sup>44</sup>, Ile<sup>50</sup>, Tyr<sup>43</sup>, Ile<sup>328</sup>, Leu<sup>338</sup>, Thr<sup>339</sup>, Ser<sup>340</sup>, Gly<sup>342</sup>, Gly<sup>344</sup>, and Ser<sup>345</sup> are common).

Insight into TM2 residues that contribute to the selectivity filter has been gained by examining the effect of mutations on relative ion permeability and flux (15, 42). The most crucial TM2 residues in determining the ability of P2X<sub>2</sub> channels to select for Ca<sup>2+</sup> over Na<sup>+</sup> were Thr<sup>336</sup>, Thr<sup>339</sup>, and Ser<sup>340</sup>, and these sit on the same half of an α helical representation of TM2 (15). Assuming that the selectivity filter has to project into the central pore these residues allow us to orientate TM2 from the perspective of the present experiments. Using this as a starting point, and the necessity for V48C and I328C to be close to one another (8, 55), we mapped the I<sub>2</sub> state-specific hits onto these helical wheel representations. Our hypothetical view of the simplest possible arrangement that can describe the data presented in this study and elsewhere (8, 15, 42) is shown in Fig. 7 and in Fig. 7B if this unit is repeated around the central axis to form a channel with three subunits. This hypothetical view encompasses several features that are noteworthy. First, Val<sup>48</sup> and Ile<sup>328</sup> face each other across an interface, as would be expected if they formed a disulfide between subunits (8). Second, polar Thr<sup>336</sup>, Thr<sup>339</sup> and Ser<sup>340</sup> residues face the central pore as would be expected if they were part of the selectivity filter (15, 42). Third, many of the residues that face the central cavity have been identified previously in substituted cysteine accessibility mutagenesis experiments (11–14). Fourth, a hydrophobic residue (Val<sup>343</sup> in P2X<sub>2</sub>) always follows a conserved
speculate that relatively more subtle tilting motions could allow the P2X₂ pore to dilate by ~3 Å for entry to the I₂ state. In relation to other transmitter-gated ion channels, such as the Cys loop and glutamate-gated family, our understanding of P2X channels is somewhat limited. For example, although progress has been made, key aspects such as the precise determinants of the ATP binding site and pore remain unclear. Another unresolved question is how the pore of some P2X channels undergoes rearrangements to switch its preference between ions. The present experiments indicate that important motions likely occur at the interface between neighboring subunits and offer a schematized/hypothetical view of the pore and thus several hypotheses that can now be tested with direct structural methods and optical approaches that detect motions of single amino acids and domains (34, 38, 44).

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REFERENCES

1. Khakh, B. S. (2001) Nat. Rev. Neurosci. 2, 165–174
2. North, R. A. (2002) Physiol. Rev. 82, 1013–1067
3. Norenberg, W., and Illes, P. (2000) Naunyn-Schmiedeberg’s Arch. Pharmacol. 362, 324–339
4. Pankratov, Y. V., Lalo, U. V., and Kristal, O. A. (2002) J. Neurosci. 22, 8365–8369
5. Torres, G., Egan, T., and Voigt, M. (1999) J. Biol. Chem. 274, 6653–6659
6. Nicke, A., Baumert, H. G., Rettinger, J., Eichele, A., Lambrecht, G., Mutschler, E., and Schmalzing, G. (1998) EMBO J. 17, 3016–3028
7. Stoop, R., Thomas, S., Rassendren, F., Kawashima, E., Buell, G., Surprenant, A., and North, R. (1999) Mol. Pharmacol. 56, 973–981
8. Jiang, L. H., Kim, M., Spelta, V., Bo, X., Surprenant, A., and North, R. A. (2005) J. Neurosci. 25, 8903–8910
9. Aschcroft, A., Sadler, S., Nicolau, C., Rettinger, J., and Schmalzing, G. (2004) J. Mol. Biol. 342, 333–343
10. Nicke, A., Rettinger, J., and Schmalzing, G. (2003) Mol. Pharmacol. 63, 243–252
11. Egan, T. M., Haines, W. R., and Voigt, M. M. (1998) J. Neurosci. 18, 2350–2359
12. Haines, W. R., Voigt, M. M., Migita, K., Torres, G. E., and Egan, T. M. (2001) J. Neurosci. 21, 5858–5892
13. Rassendren, F., Buell, G., Newbalt, North, R. A., and Surprenant, A. (1997) EMBO J. 16, 3446–3454
14. Jiang, L. H., Rassendren, F., Spelta, V., Surprenant, A., and North, R. A. (2003) J. Biol. Chem. 278, 14992–14998
15. Egan, T. M., and Khakh, B. S. (2004) J. Neurosci. 24, 3434–3440
16. Khakh, B. S., Bao, X., Labara, C., and Lester, H. A. (1999) Nat. Neurosci. 2, 322–330
17. Virginio, C., MacKenzie, A., Rassendren, F. A., North, R. A., and Surprenant, A. (1999) Nat. Neurosci. 2, 315–321
18. Khakh, B. S., Smith, W. B., Chiu, C. S., Ju, D., Davidson, N., and Lester, H. A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5288–5293
19. Schlüchter, R., Rodeau, J. L., and Hugel, S. (2002) FEBS Abstr. 1, A081.017
20. Surprenant, A., Rassendren, F., Kawashima, E., North, R. A., and Buell, G. (1996) Science 272, 755–758
21. MacKenzie, A., Wilson, H. L., Kiss-Toczy, E., Dower, S. K., North, R. A., and Surprenant, A. (2001) Immunity 15, 825–835
22. Khakh, B. S., and Lester, H. A. (1999) Neuron 23, 653–658
23. Smart, M. L., Panchal, R. G., Buell, D. N., Williams, D. A., and Petrou, S. (2002) An. J. Physiol. 283, C77–C84
24. Smart, M. L., Bu, G., Panchal, R. G., Niles, J., Cromer, B., Williams, D. A., and Petrou, S. (2003) J. Biol. Chem. 278, 8853–8860
25. Natile, G. L., and Dubayak, G. R. (1994) J. Biol. Chem. 269, 13988–13996
26. Virginio, C., MacKenzie, A., North, R. A., and Surprenant, A. (1999) J. Physiol. (London.) 519, 335–346
27. Nakazawa, K., Fujimori, K., Takahata, M., and Inoue, K. (1991) J. Biol. Chem. 266, 6653–6659
28. Duan, S., Anderson, C. M., Cheng, E. C., Chen, Y., Chen, Y., and Swanson, R. A. (2003) J. Neurosci. 23, 13190–1328
29. Hille, B. (2001) Ion Channels of Excitable Membranes, Sinauer Associates Inc., Sunderland, MA
30. Cockcroft, S., and Gomperts, B. D. (1979) Nature 279, 541–542
31. Fujimori, K., and Hoshi, T. (2004) J. Physiol. 558, 31–41
32. Spencer, R. H., and Rees, D. C. (2002) Annu. Rev. Biophys. Biomol. Struct. 31, 207–233
33. Kehovertz, V., and Felsenfeld, G. (2004) Annu. Rev. Biochem. 73, 89–115
34. Fisher, J. A., Girdler, G., and Khakh, B. S. (2004) J. Neurosci. 24, 10475–10487
35. Deyo, D. A. (2004) Trends Neurosci. 27, 288–302
36. Steyer, J. A., and Almers, W. (2001) Nat. Rev. Mol. Cell. Biol. 2, 268–275
37. Riven, I., Kalmanzon, E., Segre, L., and Reuveny, E. (2003) Neuron 38, 223–235
38. Leit, J. F., Blanton, M. P., Shahgoli, M., Dougherty, D. A., and Lester, H. A.
39. Haines, W. R., Migita, K., Cox, J. A., Egan, T. M., and Voigt, M. M. (2001) J. Biol. Chem. 276, 32793–32798
40. Li, Z., Migita, K., Samways, D. S., Voigt, M. M., and Egan, T. M. (2004) J. Biol. Chem. 279, 30934–30941
41. Brake, A. J., Wagenbach, M. J., and Julius, D. (1994) Nature 371, 519–523
42. Chaudry, M. I., Terpstra, J., and Lester, H. A. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 10195–10200
43. Eskandari, S., Kreman, M., Kavanagh, M. P., Wright, E. M., Zhang, H., and Zampighi, G. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8641–8646
44. Chaumont, S., Jiang, L. H., Persson, A., North, R. A., and Rassendren, F. (2004) J. Biol. Chem. 279, 29628–29638
45. Kosbrinsky, E., Mirshahi, T., Zhang, H., Jin, T., and Logothetis, D. E. (2000) Nat. Cell Biol. 2, 507–514
46. Hirose, K., Kadowaki, S., Tanabe, M., Takeshima, H., and Iino, M. (1999) Science 284, 1527–1530
47. Kortemme, T., Kim, D. E., and Baker, D. (2004) Science’s STKE 219, 12
48. Yifrach, O., and MacKinnon, R. (2002) Cell 111, 231–239
49. Heeks, D. H., Chang, T. H., and Swartz, K. J. (2002) J. Gen. Physiol. 119, 521–532
50. Li-Smerin, Y., and Swartz, K. J. (2001) J. Gen. Physiol. 117, 205–218
51. Collins, A., Chang, H., Jan, Y. N., and Jan, Y. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1527–1530
52. Roberts, J. A., and Evans, R. J. (2004) J. Biol. Chem. 279, 9043–9055
53. Speliotis, Y., and Surprenant, A. (2003) Nature 423, 321–326
54. Khakh, B. S., Proctor, W. R., Dunwiddie, T. V., Labarca, C., and Lester, H. A. (1999) J. Neurosci 19, 7289–7299
55. Tsien, R. Y. (1998) Annu. Rev. Biochem. 67, 509–544
56. Bobanovic, L. K., Royle, S. J., and Murrell-Lagnado, R. D. (2002) J. Neurosci. 22, 4051–4060
57. Sukharev, S., Betanzos, M., Chiang, C. S., and Guy, H. R. (2001) Nature 409, 92–94
