A Domain Contributing to the Ion Channel of ATP-Gated P2X₂ Receptors Identified by the Substituted Cysteine Accessibility Method

Terrance M. Egan, William R. Haines, and Mark M. Voigt

Department of Pharmacological and Physiological Sciences, St. Louis University Health Sciences Center, St. Louis, Missouri 63104

P2X receptors are a family of ATP-gated ion channels thought to have intracellular N and C termini and two transmembrane segments separating a large extracellular domain. We examined the involvement of the second putative transmembrane domain (TM2) of the P2X₂ subunit in ion conduction, using the substituted cysteine accessibility method (SCAM). This method tests the ability of hydrophilic reagents such as Ag⁺ or the methanethiosulfonates to modify covalently the sulfhydryl side chains exposed to aqueous environments. ATP-gated current was measured in HEK293 cells transiently expressing either wild-type or functional mutant P2X₂ receptors containing a cysteine substitution in or around TM2. Application of Ag⁺ to gating channels had no sustained effect on wild-type P2X₂ (WT) but irreversibly altered whole-cell currents in 15 mutants. By contrast, bath application of (2-aminoethyl)methanethio-

sulfonate (MTSEA) to closed channels inhibited 8 of the 15 residues affected by Ag⁺ when the channel was gating. Inhibition of the closed channel was prevented in seven of eight mutants when membrane-permeant MTSEA was scavenged by 20 mM intracellular cysteine, indicating that these seven mutants lie on the intracellular side of the channel gate. Further, MTSEA inhibited current through G342C in the absence of intracellular cysteine but augmented the current when cysteine was present, suggesting that this residue may be part of the gate. Taken together, the data help to identify a functional domain of the channel pore by mapping residues on either side of the channel gate.

Key words: ATP receptor; P2X subtype; scanning cysteine mutagenesis; sulfhydryl-modifying reagents; ion channel; transmembrane domain

The ability of extracellular ATP to modulate cellular functions involves the activation of a large family of purinergic (P2) receptors that fall into two discrete subclasses (Burnstock, 1996). Members of the first class are the metabotropic P2 receptors (P2U, P2Y, and others) that belong to the heptahelical G-protein-coupled receptor superfamily. Members of the second class are ligand-gated ion channels, designated P2X receptors (Fredholm et al., 1994). Functional studies of these ionotropic receptors identify them as cationic channels that possess appreciable Ca²⁺ permeability (Benham and Tsien, 1987; Bean, 1992; Evans et al., 1996). Recent molecular cloning studies suggest that the P2X receptors are architecturally distinct from other known ionotropic receptor proteins such as nicotinic–cholinergic, glutamatergic, and GABAergic receptors (Surprenant et al., 1995; Buell et al., 1996a; North, 1996). They are thought to have two transmembrane domains (TM1 and TM2) separated by a large extracellular region containing 10 positionally conserved cysteine residues and variable numbers of consensus sites for N-linked glycosylation, with both the N and C termini located inside the cell (Brake et al., 1994; Valera et al., 1994). This topology is novel

for ligand-gated channels (Ortells and Lunt, 1995) but is similar to that of some K⁺ channels (Aldrich, 1993; Rossier et al., 1994; Suzuki et al., 1994; Krapivinsky et al., 1995; Ortells and Lunt, 1995), amiloride-sensitive sodium channel subunits (Canessa et al., 1993; Jentsch, 1994), a proton-gated cation channel (Waldmann et al., 1997), and putative mechanosensitive channel subunits of nematode (Hong and Driscoll, 1994; Huang and Chalfie, 1994). Although experimental evidence from our laboratories (Torres et al., 1998) and others (Buell et al., 1996b; Collo et al., 1996) supports this model, the functional domains of the protein that form the ion conducting pore are poorly understood. The goal of the work reported here was to map such a domain. We chose to study TM2 because it resembles similar domains of other ion channels in its potential to form an amphipathic α -helix having a hydrophilic face that lines part of the water-filled ion-conducting pore (Brake and Julius, 1996).

Pore-lining domains of other channel proteins have been identified by the substituted cysteine accessibility method (SCAM) (Jakes et al., 1990; Akabas et al., 1992). This method involves replacing individual amino acids with cysteine and then testing the ability of sulfhydryl-reactive reagents to modify the side chains of the substituted residues. It assumes that the modifying reagents react only with sulfhydryls exposed to aqueous environments and that modifications of hydrated residues within the water-filled pore of the ion channel lead, in some cases, to a change in current. We measured the ability of Ag⁺ and (2-aminoethyl)methanethiosulfonate (MTSEA) to alter current through wild-type (WT) and 27 individual cysteine-substituted mutants. Our results suggest that TM2 is long enough to traverse the lipid membrane and forms part of the ion-conducting pore of

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Correspondence should be addressed to Dr. Mark M. Voigt, Department of Pharmacological and Physiological Sciences, St. Louis University Health Sciences Center, 1402 South Grand Boulevard, St. Louis, MO 63104.

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P2X₂. Further, the data predict that TM2 is unlikely to form either a stable α -helix (Surprenant et al., 1995) or β -sheet (Rassendren et al., 1997), as previously suggested.

MATERIALS AND METHODS

Construction of cysteine-substituted mutants. Site-directed mutagenesis of the P2X₂ receptor was performed by the overlap primer extension method (Ausubel et al., 1995). PCR was performed with VENT polymerase (New England Biolabs, Beverly, MA), and all mutations were verified by sequencing with the de-aza-GTP Sequenase kit from Amersham (Arlington Heights, IL).

Cell culture and transfection. Human embryonic kidney (HEK293) cells were plated 1 d before transfection at a density of 4×10^5 cells per 35 mm culture dish and placed in a humidified atmosphere containing 5% CO₂ at 37°C. A mixture of 1 μ g of cDNA and 6 μ l of Lipofectamine (Life Technologies, Gaithersburg, MD) in 1 ml of Opti-MEM (Life Technologies) was added to each plate for 2–5 hr, after which the medium was replaced with Minimal Essential Medium with Earle's salts supplemented with 10% fetal calf serum, glutamine, penicillin, and streptomycin. The cells were returned to the 5% CO₂ incubator for 24–72 hr, at which time they were used for electrophysiology assays. Transfection efficiency, judged by the presence of ATP-gated currents, ranged from ~80% for WT and many of the mutants to ~16% for others. Only S340C and D349C failed to respond to ATP (see below).

Electrophysiology. A suspension of transiently transfected cells was made by agitating the solution bathing the cells attached to the bottom of a single culture dish with a fire-polished Pasteur pipette. An aliquot of this cell suspension was transferred to a recording chamber mounted on the stage of an inverted microscope. Whole-cell current was recorded from single cells (average whole-cell capacitance, 55 ± 2 pF; mean $\pm$ SEM) with an AxoPatch 200A amplifier (Axon Instruments, Foster City, CA) and low-resistance electrodes. The average uncompensated series resistance was 4 ± 0.2 M Ω , of which up to 80% was nullified by using the internal circuitry of the amplifier. The typical holding voltage was –40 mV, although holding voltages of –20 to –80 mV were used occasionally to compensate for relatively large or small currents, respectively. We saw no unusual voltage-dependent behavior of the ATP-gated currents or the ability of sulfhydryl-reactive reagents to alter these currents in this voltage range. In most experiments the pipettes were filled with the following intracellular solution (in mM): 140 CsCl, 10 tetraethylammonium-Cl, 5 EGTA, and 10 HEPES, pH 7.3 with CsOH. The concentration of CsCl was reduced to 130 mM when 20 mM cysteine was included in the filling solution. The composition of the extracellular solution was determined by the nature of the sulfhydryl-specific modifying reagent. When Ag⁺ was used, the extracellular solution was (in mM): 150 NaNO₃, 1 Ca(NO₃)₂, 1 Mg(NO₃)₂, 10 glucose, and 10 HEPES, pH 7.3 with NaOH. When MTSEA was used, the extracellular solution was (in mM): 140 NaCl, 0.5 CaCl₂, 1 MgCl₂, 10 glucose, and 20 HEPES, pH 7.0 with NaOH. This latter solution was designed to slow hydrolysis of MTSEA and to prevent changes in pH caused by hydrolytic byproducts of MTSEA. Stabilizing pH is particularly important because ATP-gated current through P2X₂ is augmented by acidification (King et al., 1996). Drugs were applied by manually moving the electrode and attached cell into the line of flow of solutions exiting one of an array of inlet tubes. Every other tube contained a test solution separated by a tube containing the normal bath solution. Short applications (1–5 sec) of ATP were accomplished by moving cells in a linear manner from control solution through an ATP-containing solution to another control solution. Successive applications were separated by 2–5 min to minimize receptor desensitization. Modifying reagents were applied either in the presence or absence of ATP. The percentage of change, measured from the averages of an equal number (three or more) of steady-state responses before and after application of a modifying reagent, was calculated as $[(I_{ATP, after}/I_{ATP, before} - 1) \times 100]$. Each experiment was repeated at least three times, and the results are displayed as the mean $\pm$ SEM. Data were analyzed by one-way ANOVA. Significance was determined from the Tukey's protected multiple comparison test by using GB-STAT (Dynamic Microsystems). $p \leq 0.01$ was considered significant. Inhibitions and potentiations were analyzed separately because the large potentiations (up to 500%; see Results) made even the largest inhibitions (which can be no more than 100%) appear insignificant. Solutions of MTSEA were made from solids immediately before the start of application. A stock solution of 1 mM Ag(NO₃) was made fresh daily and kept in the dark in the refrigerator; an aliquot of this stock was

336			341				336
SLIPT	IINLA	TALTS	IGVGS	FLCDW	ILLTF	MNKNK	
CCCC	CCCCC	CCCCC	CCCCC	CC*CC	CCC	C	

Figure 1. Amino acid sequence around putative transmembrane region TM2 of P2X₂. The region thought to lie within the membrane is underlined. Each residue mutated to cysteine is marked with a C. The asterisk indicates the position of endogenous C348.

added to the solution in one barrel of the inlet array at the time of each experiment. MTSEA was purchased from Toronto Research Chemicals (Downsview, Ontario, Canada). All other reagents were purchased from Sigma (St. Louis, MO).

RESULTS

The P2X receptor family is composed of at least seven subunits that, although conserved at the structural level, have unique pharmacological and electrophysiological profiles. Of these seven, the P2X₂ subunit was chosen for these studies for the following two reasons. First, transient transfections of HEK293 cells with cDNA encoding this subunit result in large ATP-gated currents in most cells. Second, P2X₂ shows little desensitization during short drug applications and therefore is amenable to the repetitive drug application protocol used in this study.

Wild-type P2X₂ and 27 mutants were characterized in voltage-clamped HEK293 cells. Cysteine mutations (L327C–L347C, D349C–L353C, and M356C) were made throughout the predicted length of the putative TM2 (Fig. 1). Every mutant except S340C and D349C generated robust inward currents in response to ATP (1–100 μ M) applied to cells voltage-clamped at negative transmembrane potentials. This lack of functional expression contrasts with recent findings by Rassendren et al. (1997), who reported that ATP evoked small currents from these two mutants. We cannot explain this difference. We did not attempt a detailed comparison of WT and mutant receptors, however, currents through all of the responsive mutants superficially resembled those through WT in their current densities, kinetics, desensitization, resensitization, and current–voltage relationships, with one exception; currents through S345C decayed at a slower rate than WT on washout of ATP. In 40 cells transfected with either S340C or D349C, ATP (10–1000 μ M) failed to evoke membrane currents in cells voltage-clamped at membrane holding potentials between –100 and 60 mV.

The SCAM was used to identify potential pore-lining residues. Functional homomeric and heteromultimeric WT and mutant receptors were tested by using both ionic Ag⁺ and MTSEA as modifiers. Ag⁺ is a small inorganic monovalent cation that forms a strong covalent S–Ag bond with thiolates exposed to aqueous environments (Dance, 1986). MTSEA forms a disulfide bond and attaches –SCH₂CH₂NH₄⁺. Our experiments were designed to answer three questions. First, which residues are modified by Ag⁺ when the channel is gating? We assume that Ag⁺ permeates the channel and modifies accessible residues on both sides of the channel gate. Second, which residues are modified by MTSEA when the channel is not gating? We assume that MTSEA enters the cell by passive diffusion and labels both intracellular and extracellular residues of the closed channel state(s). Third, are the residues modified by MTSEA in the extracellular and/or the intracellular environments? We assume that MTSEA modifies

Figure 2. Nonspecific and reversible effects of Ag⁺. Both examples are from HEK293 cells transiently transfected with cDNA encoding WT. In this and all figures of raw data, the approximate times of ATP application are shown as solid lines. Holding voltage was -40 mV. *A*, Ag⁺ increased leak current in a reversible manner when it was applied for longer than ~ 10 sec. *B*, Coapplication of Ag⁺ and ATP (open bar) reversibly potentiated current through WT.

only extracellular residues when a high concentration of free intracellular cysteine is present (Holmgren et al., 1996).

Identification of mutated residues exposed to the aqueous environment during gating

Coapplications of ATP (10 or 30 μ M) and 500 nM Ag⁺ were used to identify amino acid residues exposed during gating. The atomic radius of Ag⁺ is close to K⁺ and Na⁺, and Ag⁺ permeates other types of cation nonselective channels [Lü and Miller (1995) and references therein], so it seems likely that P2X₂ is also permeable. This hypothesis was difficult to test because long (more than ~ 10 sec) applications of Ag⁺ increased leak current, and this prevented the accurate measurement of current through P2X₂ (Fig. 2*A*). This increase was seen in both transfected and nontransfected HEK293 cells and was not related to expression of the constructs used in this study. Shorter applications seldom evoked nonspecific currents but did not allow enough time to measure relative ionic permeabilities accurately.

Wild-type P2X₂ contains 13 endogenous cysteines, 11 of which are predicted to be located either in the large extracellular loop or in TM2 (Surprenant et al., 1995; Brake and Julius, 1996). Coapplication of ATP and 500 nM Ag⁺ to WT reversibly potentiated ATP-gated currents (Fig. 2*B*). A similar potentiation by other transitional metals was reported previously (Li et al., 1993, 1996). Of greater importance was the finding that no irreversible modification of ATP-gated current was seen, suggesting that the native cysteines, including C348 in TM2, either were not accessible to Ag⁺ or that, if modified, did not affect current flow through the pore. The lack of an irreversible effect on ligand-gated current through WT permits a clearer interpretation of results obtained via the cysteine-substituted mutant receptors.

Ten of the mutants (P329C, T330C, I331C, I332C, A335C, I341C, F346C, W350C, I351C, and M356C; for example, see Fig. 3*A*) similarly were unaffected by single 5 sec applications of 500 nM Ag⁺ and ATP. By contrast, ATP-gated currents through 12

mutants (L327C, I328C, N333C, L334C, T336C, A337C, L338C, T339C, V343C, S345C, L352C, and L353C; for example, see Fig. 3*B*) were reduced significantly by concurrent administration. In most cases a reduction in peak amplitude was the only irreversible effect seen when Ag⁺ inhibited current. However, in some cases (T336C, A337C, V343C, and S345C) coapplication of ATP and Ag⁺ resulted in both a reduction in peak current and the production of a small sustained inward current (see Fig. 3*B*). The production of sustained inward current may reflect a population of ATP-gated channels that have been locked open by an action of Ag⁺. We favor this explanation because we never saw irreversible induction of inward currents in WT or most mutant receptors and because the sustained inward current was reversed by applications of 5 mM DTT (see Fig. 3*B*). Currents through three mutants (G342C, G344C, and L347C; for example, see Fig. 3*C*) were augmented irreversibly. In all cases the inhibitions or potentiations began immediately after the start of coapplication and progressed during the course of a 5 sec application. The time course of the irreversible modification was obscured by the concurrent reversible potentiation, precluding reliable measurement of reaction rates of Ag⁺ at the various sites. Neither the sustained augmentations nor inhibitions reversed during long (up to 45 min) washouts of Ag⁺, suggesting that a covalent modification at the site of the substituted cysteine was responsible for the change in the size of the current through the pore. A summary of the irreversible effects of Ag⁺ on gating WT and mutant P2X₂ receptors is presented in Figure 4. Longer applications of Ag⁺ may have produced greater effects, and the augmentations and inhibitions that we detail here therefore must be considered to be the lower limits of these effects.

A change in current amplitude can reflect modification of a residue within the pore. Another possibility is that modification of a residue located at a distance from the pore changes current by affecting agonist affinity or efficacy. To exclude this

Figure 3. Irreversible effects of Ag^+ applied to gating channels. ATP (30 μM) was applied once every 3 min to cells expressing mutant receptors. Each trace shows three successive applications of ATP, followed by coapplication of ATP and 500 nM Ag^+ , and then three more ATP applications after the washout of Ag^+ . Coapplication of Ag^+ and ATP (open bars) had no irreversible effect (A), irreversibly inhibited (B) or irreversibly potentiated (C) ATP-gated responses. DTT (5 mM; hatched bar) partially reversed both the sustained inward current and the inhibition of current through V343C by Ag^+ (B). See Results for details. Calibration: 1000 pA, 10 sec.

possibility, we measured the dose–response relationship before and after covalent modification of four mutants (I328C, G342C, V343C, and L353C) having substitutions spaced throughout TM2. Irreversible modification by Ag^+ increased (G342C) or decreased (I328C, V343C, and L353C) the maximum response to a high concentration of ATP but did not alter the EC_{50} values significantly (Fig. 5), suggesting that the addition of Ag^+ did not change agonist efficacy or affinity.

Identification of mutated residues exposed to the aqueous environment when the channel is closed

Some residues that are accessible when the channel is gating may not be accessible when the channel is closed. This is because the channel moves among several conformations (open, closed, and desensitized) in the presence of agonist but exists only in the closed state in the absence of agonist. The 5 sec applications of 500 nM Ag^+ applied in the absence of ATP were used to label extracellular residues accessible in the closed state. This protocol did not result in significant ($p < 0.01$) modification of current through any of the residues tested (I328C, N333C, L334C, T336C,

Figure 4. Average data for wild-type and mutant P2X₂ receptors. Average data for the number of experiments are indicated. Results significantly different ($p < 0.01$) from wild-type (WT) are marked with solid bars. Inhibitions and potentiations are shown in the top and bottom graphs, respectively.

A337C, L338C, T339C, G342C, V343C, S345C, L352C, and L353C) (data not shown), indicating either that these residues are entirely inaccessible in the closed state or that access is limited. Longer applications of Ag^+ may have produced more significant effects but caused the nonspecific leak currents described above.

MTSEA (1 mM), another sulfhydryl-reactive reagent, in our hands did not activate leak currents when it was applied for up to 5 min in the absence of ATP. An added advantage of MTSEA is that it exists at neutral pH as both cationic and uncharged species. The cation is hydrophilic and readily reacts with sulfhydryls exposed to water. The uncharged species is lipophilic and easily crosses cell membranes by passive diffusion (Holmgren et al., 1996). Once it is inside the cell, equilibrium between the charged and uncharged species is reestablished, resulting in a significant intracellular concentration of reactive MTSEA⁺. This means that it can attack the protein from both sides of the membrane, resulting in the modification of residues on both sides of the channel gate even when the channel is closed.

Baseline ligand-gated current was measured by applying ATP for 1 sec every 5 min for a total of three to five applications. Then, MTSEA was applied for 5 min in the absence of ATP, the cells were washed with normal bath solution, and periodic ATP applications were restarted. ATP-gated current through WT was unaffected by MTSEA applied in this way. We then tested 22 cysteine-substituted mutants of TM2, including all 15 mutants accessible to Ag^+ when the channel was gating. Of these, current through eight (I328C, L334C, L338C, T339C, G342C, S345C, L352C, and L353C) were inhibited significantly (Fig. 6), and the rest were unchanged. These inhibitions persisted in an irrevers-

Figure 5. ATP dose-response curves before and after Ag^+ . Dose-response curves were generated before and after a single 5 sec coapplication of 30 μM ATP and 500 nM Ag^+ to cells expressing the indicated mutants.

ible manner for at least 30 min, in keeping with the predicted covalent modification of the cysteinyl side chain. As expected, all of the mutants modified by MTSEA when the channels were in the closed state also were modified by Ag^+ when the channels were gating (compare Figs. 4 and 7). The seven mutants (L327C, N333C, T336C, A337C, V343C, G344C, and L347C) that were not modified by MTSEA apparently do not face an accessible aqueous environment when the channel is closed. This may be because these residues are buried when the channel is closed and swing out into water when the channel opens, or it may be because they occupy a position accessible to Ag^+ but relatively inaccessible to the larger MTSEA.

Sidedness of closed-state reactive residues

The simplest model of TM2 is one that places I328C and L353C near opposite ends of a membrane-spanning α -helix with L353C close to the intracellular surface of the membrane. To test the orientation of TM2 in the membrane, we used the method of Holmgren et al. (1996) to scavenge intracellular sulfhydryl-reactive compounds. This approach uses high levels of free cysteine in the electrode-filling solution to inactivate MTSEA that enters the cell either through the patch leak or diffusion through

the membrane. If the free intracellular cysteine prevents the change in current seen in its absence, then it is reasonable to assume that the mutated residue lies on the intracellular side of the channel gate. Although we assume that cysteine acts as a scavenger, another possibility is that it protects by covalently modifying accessible intracellular residues, making them unsusceptible to subsequent MTSEA applications. In either case, only intracellular residues would be affected when the channel is closed, and our interpretation of the results would remain the same. An additional concern is that extracellular residues are modified covalently by cysteine that flows out of the cell through the pore of the channel during establishment of baseline responses to ATP. Again, residues modified in such a manner would not react with subsequent applications of MTSEA and thus would appear to be intracellular. If cysteine flows out through the channel (and against the electrical gradient), then we expect to see a change in the size and/or shape of the first few ATP-gated currents as cysteine modifies accessible residues. No such change was evident.

The ability of 5 min applications of 1 mM MTSEA to modify current through I328C, L334C, L338C, T339C, G342C, S345C,

Figure 6. MTSEA inhibits some, but not all, cysteine-substituted P2X₂ receptors. MTSEA (1 mM) was applied alone for 5 min after baseline responses to 10 μ M ATP were established. ATP was applied once every 5 min. Current through unmodified mutants (*left-hand sweeps* of each pair of traces) resembled that of WT except that current through S345C took longer to return to baseline on the withdrawal of ATP. ATP-gated currents were reduced after application of MTSEA (*right-hand sweeps* of each pair). Calibration: 500 pA, 3 sec.

L352C, and L353C was reinvestigated with 20 mM cysteine in the pipette solution. Lower concentrations of cysteine gave variable results. The only residue significantly inhibited by MTSEA in the presence of intracellular cysteine was I328C (Fig. 8A), positioning this residue on the extracellular side of the gate. L334C, L338C, T339C, S345C, L352C, and L353C were not inhibited significantly, which suggests that they are intracellular. Of greatest interest was the effect on G342C. MTSEA potentiated current through G342C in the presence of cysteine (Fig. 8B) but inhibited current when cysteine was absent (see Fig. 6). These findings suggest that G342C can be attacked from either side of the membrane. The effect of intracellular cysteine on the ability of MTSEA to modify current through mutant receptors is summarized in Figure 9.

Heteromerization allows nonfunctional homomeric mutants to be tested

Substitution of the -COOH of D349 or the -CH₂OH of S340 with the -SH of cysteine appears in our hands to result in the loss of channel function. There are four possible explanations for this result. First, these mutations might result in a low efficiency in protein translation/translocation. Second, substitutions at these residues change agonist affinity. This idea is not supported by our finding that even very high concentrations of ATP (up to 1 mM)

Figure 7. Average data for wild-type and mutant P2X₂ receptors. Average data for the number of experiments are indicated. Results significantly different ($p < 0.01$) from wild-type (WT) are marked with solid bars.

Figure 8. MTSEA inhibits I328C and potentiates G342C in the presence of 20 mM intracellular cysteine. Three consecutive traces before (*left side*) and after (*right side*, under the open bar) a 5 min application of 1 mM MTSEA are shown for I328C (*A*) and G342C (*B*). Note that the potentiation of G342C was reversed by a 5 min application of 5 mM DTT.

Figure 9. Average data for MTSEA in the presence and absence of 20 mM intracellular cysteine. *Solid bars* are significant data from Figure 7 obtained without cysteine and are shown again here for direct comparison. *Hatched bars* are results obtained in the presence of cysteine. Only I328C and G342C were significantly different (*asterisks*) from WT during cysteine perfusion. Note that G342C is potentiated by MTSEA in the presence of intracellular cysteine but is inhibited in its absence.

did not evoke current in any cells. Third, side chains important for cation conduction have been deleted. Fourth, disulfide bonds form between adjoining cysteinyl side chains of homomeric complexes of S340C and D349C, and these disulfide bonds disrupt current through the mutants. The latter two problems might be overcome by studying heteromultimeric complexes of WT and S340C or D349C in which some, but not all, of the subunits contain substituted residues. These complexes might retain channel activity and at the same time acquire susceptibility to sulfhydryl-specific reagents. We tested this hypothesis by measuring the ability of MTSEA to alter ATP-gated current in cells cotransfected with a mixture of either S340C/WT or D349C/WT cDNAs. Both mixtures produced robust cation currents that resembled those of WT in response to 10 μ M ATP. MTSEA alone or in combination with ATP had no irreversible effect on cells transfected with a mixture of WT and S340C. Because we saw no effect, we draw no conclusion about the accessibility of S340C. By contrast, MTSEA did produce a substantial and irreversible block of current through D349C/WT when it was applied along with ATP (Fig. 10) but had no effect on subsequent ATP applications if it was applied to closed channels. The data argue that D349C is on the intracellular side of the channel gate, but it is less accessible than other intracellular residues (L334C, L338C, T339C, S345C, L352C, and L353C) to the MTSEA that enters the cell by passive diffusion through the lipid membrane when the channel is closed. Coapplication of ATP and MTSEA had no irreversible effect on WT alone (data not shown).

WT/D349C

Figure 10. Heteromultimers of D349C and WT are blocked by MTSEA when the channel is gating. Data are from a cell transfected with a 2:1 mixture of D349C/P2X₂ cDNAs. ATP (10 μ M) was applied once every 5 min to establish a baseline response. A sustained application of ATP (*filled bar*) was begun, during which time some receptor desensitization was apparent, followed immediately by a 20 sec coapplication of ATP and MTSEA (*open bar*). MTSEA caused an immediate decrease in whole-cell current that was easily distinguished from the slower decrease in current caused by receptor desensitization. In control experiments the desensitization of WT followed an exponential time course. Assuming this is also true of D349C/WT, we have indicated the expected progression of whole-cell current in the absence of MTSEA with a *fine-dotted line*. Short ATP applications were restarted 5 min after application of MTSEA. The peak current amplitude never returned to its pre-MTSEA level. Holding voltage was -50 mV.

DISCUSSION

We used scanning cysteine mutagenesis to identify and map a domain of P2X₂ involved in ion conduction. This method carries with it certain assumptions that influence how results are interpreted (Akabas et al., 1992). The guiding principles are that functional mutants do not have unduly perturbed secondary structure, that channels are water-filled pores lined in part by transmembrane-spanning domains, and that Ag⁺ and MTSEA⁺ react much more readily with ionized thiols exposed to water than with nonionized thiols exposed to lipid. Thus, residues that project into the lumen of the channel might be accessible, whereas residues buried in the lipid membrane are not. A change in current indicates that the residue in question is accessible and modified. The SCAM has been used to refine models of topology for numerous proteins in which the pore domain was identified previously by other site-directed mutagenic approaches (Akabas et al., 1992; Lü and Miller, 1995; Cheung and Akabas, 1996; Kuner et al., 1996; Sun et al., 1996), and under these conditions it is reasonable to conclude that reactive residues line the channel pore. It is harder to justify this conclusion when the general location of the pore domain is unknown, because it is also possible that modification of a nonpore-lining residue alters current, perhaps by changing receptor efficacy. In the case of P2X₂, this possibility must be considered carefully, because the locations of the pore and the ATP-binding site are mainly unknown.

The hypothesis that TM2 does, indeed, line the pore of P2X₂ is supported by six lines of evidence. First, hydropathy-plot analysis of P2X₂ predicts two putative transmembrane spanning domains, and one or both of these must constitute part of the pore. Second, cysteine substitution within TM2 results in mutants sensitive to hydrophilic modifying reagents, and a part of TM2 must face water. Third, agonist potency was unchanged in all four mutants (I328C, G342C, G344C, and L353C) in which ATP dose-response curves were generated before and after modification by Ag⁺, arguing against the possibility that current is changed by

modifying agonist EC₅₀. Fourth, some mutants (L334C, L338C, S345C, L352C, and L353C) fail to react with MTSEA in the presence of intracellular cysteine; these sites must be inside the cell and therefore cannot contribute to an extracellular ATP-binding site. By contrast, I328C reacts equally well in the presence and absence of cysteine perfusion and therefore must be outside. Taken together, these data confirm that TM2 spans the membrane. Fifth, G342C is attacked from both sides of the membrane, suggesting that it is exposed to water and contacts both the extracellular and intracellular environments. This can happen only if G342C resides in a water-filled transmembrane pore. Sixth, D349C is modified only when the channel opens, implying that it lies at a restricted site on the intracellular side of a channel gate.

Our initial strategy was to investigate gating channels with Ag⁺ as the modifying reagent. We observed two reversible effects that were unrelated to the presence of an engineered cysteine, and these nonspecific effects somewhat limited the scope of the experiment. Specifically, the transient potentiation of the ATP-gated current prevented accurate measurement of the reaction times of accessible mutants, and induction of a background current restricted the exposure time to Ag⁺. However, these nonspecific effects disappeared on washout of Ag⁺, and in 15 mutants (L327C, I328C, N333C, L334C, T336C, A337C, L338C, T339C, G342C, V343C, G344C, S345C, L347C, L352C, and L353C) an irreversible change in ATP-gated current remained. These data are remarkable for the sheer number of reactive positions. Indeed, it is somewhat surprising that the endogenous cysteine at position 348 was unreactive in WT. It is possible that some of the effects that we assign to cysteine-substituted receptors are attributable to altered positioning of C348 in the mutants. However, our data suggest that this is unlikely, because current through most of the mutant receptors (S345C being the exception) resembles WT and because it is hard to understand how modification of a single residue could account for the variable effects seen with the sulfhydryl-reactive reagents. This issue could be resolved by making cysteine substitutions of a mutant receptor in which C348 is changed to another amino acid. The present results are also remarkable for the lack of a discernible pattern in the reactive sites. For example, all (excluding S340C, which was nonfunctional) but three positions in the span from N333C to L347C reacted strongly, including two tracts of four contiguous sites (T336C–T339C, G342C–S345C). This contiguity seems to rule out stable α -helical and β -sheet secondary structures for TM2 during gating. It does not, however, eliminate the possibility that the channel assumes either of these conformations at some time, for example, when the channel is closed.

The closed channel was studied by applying the modifying reagent in the absence of ATP, and only eight (I328C, L334C, L338C, T339C, G342C, S345C, L352C, and L353C) of the 15 residues that reacted with Ag⁺ during gating were sensitive to MTSEA. Although it is tempting to postulate that Ag⁺ reacts with more residues during gating because additional sites are exposed when the channel opens, an equally plausible explanation is that the smaller Ag⁺ experiences less steric hindrance than the larger MTSEA⁺, thereby gaining access to more residues. We attempted to address this problem by studying the effect of MTSEA on gating channels, but we found that coapplication of ATP and MTSEA caused nonspecific effects in some (but not all) mutants, which made interpretation of the results unreliable. These effects were not unexpected, because studies investigating other ligand-gated ion channels also have reported nonspecific effects of the methanethiosulfonates applied to open channels

(Kuner et al., 1996; Sun et al., 1996). We did not observe such effects when MTSEA was applied in the absence of ATP. Regardless, a pattern of reactivity does seem to emerge from the closed channel data in that all of the reactive sites lie on one face of an α -helical model of TM2 having 3.6 amino acids per turn. If so, then the protein must undergo a major conformation change when the channel opens, because the pattern of reactivity is unordered during gating. Another possibility is that the apparent helical pattern of reactivity in the closed state is deceptive. This appears to be the case as ascertained in experiments that used intracellular cysteine.

We determined the transmembrane orientation of reactive residues of the closed state. Mapping extracellular residues was straightforward; they were detected by bath application of MTSEA and intracellular perfusion of cysteine. Mapping the intracellular vestibule of P2X₂ was problematic. One approach is to measure single-channel current from inside-out membrane patches, using electrodes filled with an extracellular solution containing ATP and an intracellular (bath) solution containing MTSEA. However, the continued presence of ATP in the pipette leads to significant and progressive receptor desensitization, making the absolute measurement of the effect of the modifying reagent tenuous. As an alternative approach, we took advantage of the fact that uncharged MTSEA easily crosses cell membranes and modifies residues on the opposite side from which it is applied and that intracellular perfusion of cysteine can be used to scavenge the permeant MTSEA (Holmgren et al., 1996). We assume that residues that fail to react with MTSEA in the presence of cysteine are contiguous with the intracellular compartment, whereas those that do react are extracellular. From these experiments we learned that, first, I328C is on the extracellular side of the channel gate because it reacts equally well with MTSEA in the presence and absence of cysteine. Second, six mutants (L334C, L338C, T339C, S345C, L352C, and L353C) are intracellular because MTSEA was ineffective when cysteine was present. Third, G342C resides at or near the gate because it is accessible from both sides of the membrane. It is difficult to envision how either an α -helix or a β -sheet could give this pattern of reactivity. A topology that fits the data is an outwardly facing loop with G342C at its apex (Fig. 11). Loop structures recently have been proposed for other ionotropic receptors (Kuner et al., 1996; Sun et al., 1996). The role of the loop in P2X₂ is unknown, but it is tempting to speculate that it forms part of a channel gate that moves when the channel opens. Indeed, G342C is one of three amino acids in TM2 completely conserved in all members of the P2X₂ receptor family (I328C and D349C are the others), and it may play a key role in channel function. Further, it is interesting to note that modification by Ag⁺ of residues at and around G342C produced some of the greatest changes in current when the channel was gating. For example, the largest inhibitions were seen after modification of T339C and V343C, whereas currents through G342C, G344C, and L347C were strongly potentiated. G342C itself must be capable of positioning its cysteinyl side chain in either an inwardly or outwardly facing direction, because MTSEA causes qualitatively polar effects (e.g., inhibition or potentiation), depending on whether the side chain is attacked from the inside or the outside. We do not know how MTSEA causes these two effects, and indeed the mechanism does not influence how the results presented here are interpreted. What is important is that G342C is accessible from both sides.

Our data suggest that residues in TM2 line the pore. A similar conclusion was reported recently in a paper published while this

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