Amino Acid Residues Involved in Gating Identified in the First Membrane-spanning Domain of the Rat P2X2 Receptor

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The first hydrophobic segment of the rat P2X2 receptor extends from residue Leu29 to Val51. In the rat P2X2 receptor, we mutated amino acids in this segment and adjoining flanking regions (Asp15 through Thr66) individually to cysteine and expressed the constructs in human embryonic kidney cells. Whole-cell recordings were used to measure membrane currents evoked by brief (2-s) applications of ATP (0.3–100 μM). Currents were normal except for Y16C, R34C, Y43C, Y55C, and Q56C (no currents but normal membrane expression by immunohistochemistry), Q37C (small currents), and F44C (normal current but increased sensitivity to ATP, as well as αβ-methylene-ATP). We used methanethiosulfonates of positive, negative, or no charge to test the accessibility of the substituted cysteines. D15C, P19C, V23C, V24C, G30C, Q37C, F44C, and V48C were strongly inhibited by neutral, membrane-permeant methanethiosulfonates. Only V48C was also inhibited by positively and negatively charged methanethiosulfonates, consistent with an extracellular position; however, accessibility of V48C was increased by channel opening. V48C could disulfide with I328C, as shown by the large increase in ATP-evoked current caused by reducing agents. The results suggest that Val48 at the outer end of the first hydrophobic segment takes part in the gating movement of channel opening.

P2X receptors are a family of multimeric membrane proteins that function as ion channels gated by extracellular ATP. Hydropathy plots for P2X receptors suggest that two parts of the protein are sufficiently long and hydrophobic to cross the plasma membrane (1). These are the regions, in the P2X2 receptor, of Leu29 to Val51 and of Ile331 to Leu353. Considerable experimental evidence now supports the view that the N and C termini are intracellular, and the region between Val51 and Ile331 faces the extracellular aspect. First, antibodies against N- and C-terminal epitopes work only in permeabilized cells (2). Second, the proteins can be glycosylated at both natural and artificially introduced consensus sequences (NX(S/T)) at several positions in the extracellular domain from Pro62 to Lys324 (in the P2X2 receptor), though not at such positions in the N terminus (positions 9, 16, or 26) (2–4). Third, concatenated cDNAs in which the C terminus of one construct is joined to the N terminus of a second form functional channels (5).

There is now biochemical evidence that the P2X receptors form channels as trimers (6, 7). However, the parts of the individual subunits that contribute to different functions of the receptor are little understood. Mutations of several positively charged residues have been shown to decrease the effectiveness of ATP as an agonist at the P2X2 (8) and P2X3 (9) receptors, and these residues occupy corresponding positions (e.g. Lys69, Lys71, Lys188, Arg290, Arg304, and Lys308 in rat P2X2, numbering). The region around Lys69 and Lys71 is of particular interest with regard to a possible ATP binding site. The P2X2 receptor functions normally when Ile67 is mutated to cysteine (I67C). However, the attachment of a negatively charged methanethiosulfonate (2-sulfonatoethyl) methanethiosulfonate; MTSES) led to a parallel rightward shift in the ATP concentration-response curve that was not seen with neutral (methyl methanethiosulfonate; MTSM) or positively charged methanethiosulfonate (2-(trimethylammonium)ethyl methanethiosulfonate; MTSET). Point mutations that introduced a negative charge (I67E and I67D), but not those that introduced a positive charge (I67R and I67K), also caused inhibition of the current that could be overcome by increasing the ATP concentration. Together these results provide strong evidence that this region of the receptor contributes to the ATP binding site (9).

When ATP binds to the P2X2 receptor the protein undergoes a conformational change that results in the opening of a cation-permeable channel. The substituted cysteine accessibility method has also been used to implicate residues in and around the second transmembrane domain in the formation of the ion-conducting pathway (10, 11). In particular, P2X2-T336C is almost completely blocked by exposure for 8 min to MTSET and MTSES; both negatively and positively charged MTS reagents were effective, suggesting that Thr336 is located outside the membrane electric field, but the finding that outward currents were blocked more rapidly than inward currents indicates that the attached side chain might directly interfere with permeation (10). Another residue within the second hydrophobic segment (Asp349) showed prominent block by MTSEA, but not by MTSET and MTSES. This block did not occur if the MTSEA was applied without opening, and in view of the fact that MTSEA is quite permeable through the P2X2 receptor channel,
this suggests that Asp\textsuperscript{449} is situated internal to the “gate” of the channel (10).

The purpose of the present experiments was to ascertain whether residues in and around the first hydrophobic segment might also contribute to the ATP binding site or to the permeation pathway. P2X receptors are not well conserved in the regions corresponding to the first 14 amino acids of the P2X\textsubscript{2} receptor, and we therefore began our cysteine substitutions at Asp\textsuperscript{15}. We have recently reported the effects of MTS compounds on the region Asp\textsuperscript{57} to Lys\textsuperscript{71} (9); in the present experiments we ended the cysteine substitutions at Thr\textsuperscript{60} (see Fig. 1). As a first approach we used MTSM, a small, neutral methanethiosulfonate, in conjunction with point mutations to cysteine. We reasoned that this might provide a picture of cysteines accessible to the aqueous environment on both the intracellular and extracellular aspects of the receptor. We followed this with tests of positively and negatively charged methanethiosulfonates for those positions at which MTSM caused a large inhibition. In an effort to understand further the mechanism of the inhibition we studied the effect on the ATP concentration-response curve and asked whether the inhibition required channel opening. Finally, we sought to determine whether substituted cysteines in the two transmembrane domains were sufficiently close to form disulfide bonds.

**EXPERIMENTAL PROCEDURES**

**P2X\textsubscript{2} Receptor cDNA and Mutagenesis—**A P2X\textsubscript{2} subunit cDNA carrying a C terminus epitope was used; its source and the methods used for introducing point mutations were as described previously (10). All mutants were sequenced on both strands.

**Electrophysiology—**Transient transfection of human embryonic kidney 293 cells using Lipofectin or LipofectAMINE 2000 was as described previously (10, 12–14). Standard whole-cell recordings and fast-flow agonist applications were made as previously described (10, 12, 13).

**Internal solution contained (in mM):** 145 NaF, 10 EGTA, and 10 HEPES. Current-voltage relations were obtained by ramp voltages (1-s duration) from −120 to 40 mV. The following MTS reagents were used obtained from Toronto Research Chemicals (Ontario, Canada): MTSEA, MTSET, MTSES, MTSM, and butyl methanethiosulfonate (MTSB). MTSM and MTSB were used from a 1 M stock solution (in HEPES), and from a 25 mM stock solution (in MOPS). The electric field at the membrane in these cells. For Q37C, ATP-evoked currents were smaller (0.3 to 2 nA), and the EC\textsubscript{50} was about three times higher than for the wild-type receptor (29 ± 5.7 μM; n = 4).

**RESULTS**

**Effects of Cysteine Substitutions—**We introduced cysteine into each position individually and studied the actions of ATP on human embryonic kidney 293 cells expressing the mutated receptors. ATP (30 μM) elicited currents not distinguishable from those in cells expressing wild-type receptors (1–8 nA) for all the mutated receptors except Y16C, R34C, Q37C, Y43C, F44C, Y55C, and Q56C. Cells expressing Y16C, R34C, Y43C, Y55C, and Q56C showed no responses to ATP (up to 3 or 10 mM); immunohistochemistry showed staining of the plasma membrane in these cells. For Q37C, ATP-evoked currents were smaller (0.3 to 2 nA), and the EC\textsubscript{50} was about three times higher than for the wild-type receptor (29 ± 5.7 μM; n = 4).

Cells expressing two further mutations (T18C and L29C) responded well to an initial application of ATP, but the current declined steeply with repeated applications, and they could therefore not be usefully studied. At T18C, the current also declined during the ATP application more rapidly than seen at wild-type channels; at the end of a 2-s application (30 μM) the current was 43 ± 7% (n = 5) of its peak for T18C and 89 ± 2.8% (n = 8) for the wild-type receptor. This is similar to the finding of Boué-Grabot et al. (16) for T18A.

At F44C, cysteine substitution caused three distinct changes in the properties of the receptor. First, ATP-evoked currents returned back to the baseline level more slowly than normal after a brief (2-s) application. The times required to return to half the peak current at the end of ATP application were 0.44 ± 0.04 s (n = 15) for wild-type receptors (30 μM ATP) and 1.2 ± 0.8 s (n = 12) for ATP. Second, there was 10-fold increase in sensitivity to ATP (EC\textsubscript{50} 0.72 ± 0.1 μM; n = 4). Third, there was a remarkable increase in effectiveness of αβmeATP. The wild-type P2X\textsubscript{2} receptor is essentially insensitive to αβmeATP (13); indeed, we found that 100 and 300 μM αβmeATP evoked currents in cells expressing wild-type P2X\textsubscript{2} receptors, respectively, 1.1 ± 0.2 and 8.1 ± 1.3% (n = 5) of the currents evoked by 100 μM ATP. In contrast, at F44C receptors αβmeATP activated currents with an EC\textsubscript{50} value of 10.8 ± 0.5 μM (n = 4), and the maximum current evoked by αβmeATP (100 μM (1.7 ± 0.2 nA; n = 5) was similar to that evoked by a maximal concentration of ATP (3 μM (1.2 ± 0.2 nA; n = 5). There was no difference in the holding current between cells expressing F44C and wild-type receptors.

**Accessibility to MTS—**We used MTSM for an initial screen of this segment of the receptor; it is small and uncharged, and we expected that it would cross the membrane readily and react with accessible residues on either the cytoplasmic or the extracellular part of the receptor protein. Fig. 1 illustrates representative ATP-evoked currents prior to and after an 8-min application of MTSM at 1 mM and after washing for 4 min. The effects of MTSM are summarized in Fig. 2. There was no significant effect on ATP-evoked currents at wild-type receptors. We included T336C as a control mutation, and found that the inhibition (>80%) was similar to that previously reported for MTSEA, MTSET, and MTSSES (10). At 8 of the 39 cysteine-substituted receptors that responded to ATP, MTSM caused a large (≥60%) inhibition of the current that was significantly different from the wild-type (p < 0.001); these were D15C, P19C, V23C, V24C, G30C, Q37C, F44C, and V48C (Fig. 3). This inhibition did not reverse on washing out the MTSM for up to 10 min (Fig. 1). These effects of MTSM were mimicked closely by another neutral MTS derivative, MTSB. At 1 mM (for 8 min), the inhibitions by MTSB were as follows: V15C, 90 ± 3% (n = 3); P19C, 89 ± 7.8% (n = 4); V23C, 82 ± 4.1% (n = 3); and V42C, 97 ± 1.1% (n = 4). The inhibition of the current by MTSM was not obviously dependent on membrane potential as judged from ramp current-voltage plots.

Previous studies on the second transmembrane domain found no effects by MTSEA, MTSET, and MTSSES on the positions on the C-terminal side of D349C (W350C, A351C, L352C, L353C, and T354C). These positions are clearly at the inner aspect of the second transmembrane domain, and we re-examined them using MTSM. We found that MTSM (1 mM, 8 min) gave rise to significant inhibition (p < 0.001) at I350C (76.5 ± 6.2%; n = 3) and L352 (69.8 ± 5.1%; n = 3).

**Effects of MTS Compounds with Positively or Negatively Charged Conjugates**
Charged Head Groups—Val24 and Gly30 are believed to be on the intracellular aspect of the receptor. The speed of the reaction of MTSM at V24C (time constant, 2 min; see Fig. 3) and G30C (2 min; see Fig. 3) indicates that MTSM crosses the cell membrane rapidly. This implies that the slower rates of reaction observed for some other positions such as P19C indicate a slower forward reaction rate rather than slower access to the intracellular soluble compartment.

MTSET is not expected to cross the membrane, although it can permeate the open P2X receptor channel (10). It is therefore a useful probe of residues accessible to the extracellular aspect or intracellular residues if ATP has been applied to open the channel while MTSET is in the extracellular solution (see Ref. 10). MTSES, being negatively charged, is not expected to enter the cell whether the channel is open or not. Fig. 3 compares the effects of MTSM with MTSES and MTSET on ATP-evoked currents in cells expressing these eight mutated receptors. Val48 is believed to be on the extracellular aspect of the receptor (see the Introduction). Both MTSET and MTSES caused large inhibition of the current at V48C but had much lesser effect at any other position; MTSEA (1 mM; 8 min) also caused inhibition by 88.8 ± 7.4% (n = 4). The inhibition at V48C by all four MTS reagents was relatively slow (time constant about 3 min at 1 mM MTSET; see Fig. 4). After treatment with MTSET or MTSEA, the ATP-evoked currents did not return completely to initial holding current. The residual holding current was 483 ± 680 pA (n = 3) for MTSEA (1 mM) and 411 ± 127 pA (n = 3) for MTSET (1 mM) when measured 15 s after the first application of ATP (30 μM; 2 s) in the presence of methanethiosulfonate. This was in marked contrast to the effect of MTSM or MTSES, where the currents declined quickly and completely to the baseline level. It suggests that channel closure is impaired when a positively charged methanethiosulfonate attaches at Val48.

Taken together, the results with MTSM, MTSES, and MTSET are consistent with the topology currently proposed for the P2X2 receptor. Introduction of cysteine at positions Asp15, Pro19, Val23, Val24, and Gly30 (before the first hydrophobic segment) and Ile351 and Leu352 (end of the second hydrophobic segment) led to significant inhibition by the membrane permeant MTSM but little or no inhibition by charged MTS derivatives. Conversely, cysteine substitution at Val48 (at the outer edge of the first hydrophobic segment) resulted in strong inhibition by MTSM, MTSES, and MTSET as we have previously described for three residues (Ile328, Asn333, and Thr336) at the beginning of the second hydrophobic domain (10).

Effects of MTSM Modification on the ATP Concentration-response Curve—The shape of the concentration-response curve for ATP might provide information on the mechanism by which the current is inhibited (9, 15). Before treatment with MTSM, the EC50 values for D15C, P19C, V23C, V24C, and V48C were 3.1 ± 0.4 (n = 3), 6.9 ± 0.2 (n = 3), 7.3 ± 0.8 (n = 4), 10.6 ± 1.0 (n = 6), and 3.4 ± 0.2 μM (n = 3), respectively.

FIG. 1. MTSM inhibition of cysteine-substituted P2X2 receptors. Currents at wild-type and the indicated mutated receptors were elicited by ATP (30 μM; 2 s) applied at 2-min intervals. When stable currents were achieved, MTSM (1 mM) was applied for 8 min and followed by a 4-min washing.

FIG. 2. Cysteine scanning with MTSM in region Asp15 to Thr40 of the P2X2 receptor. Effect of MTSM (1 mM; 8 min) on currents evoked by ATP (30 μM) in cells expressing each of the single point mutations (n = 3–10 for each mutation) is shown. MTSM was not tested at seven positions at which cysteine substitution resulted in either non-functional channels (*, Y16C, B34C, Y43C, Y55C, and Q56C) or channels at which the ATP-evoked currents declined strongly with repeated applications (**, T18C and L29C). Black bars indicate those positions at which the effect of MTSM was significantly different from that at the wild-type P2X2 receptor (p < 0.001). TM1, first transmembrane domain.
After treatment they were not different (4.8 ± 0.4, n = 3; 9.3 ± 1.1, n = 3; 10 ± 1.3, n = 4; 9.4 ± 1.4, n = 6; and 4.0 ± 0.4 μM, n = 3, respectively). These values are close to those for the wild-type receptor (7.9 ± 1.1 μM; n = 8). In other words, MTSM modification at these positions results in a simple depression of the maximum current evoked by ATP, with little change in the EC_{50}. This is similar to the result observed with T336C (see Fig. 4 and Refs. 9 and 10).

Dependence of MTS Inhibition on Channel Opening by ATP—V24C was rapidly and completely inhibited by MTSM (Figs. 1, 3, and 5). This inhibition was essentially the same even when ATP applications were discontinued during the presence of the MTSM (Fig. 5A, left). On the other hand, Fig. 5B (left) shows that the positively charged MTSET produced little or no inhibition of the currents at V24C unless ATP was repeatedly applied. We interpret this to indicate that Val^{24} is situated on the intracellular aspect of the receptor, but it can be accessed by MTSET entering through the open channel. This is the same result, and the same conclusion, as we made previously for inhibition at D349C by MTSEA (10).

In the case of V48C, inhibition was observed with MTSM, MTSET, and MTSES (Fig. 3). However, the effectiveness of MTSM and MTSET was considerably greater when the ATP was repeatedly applied than when it was not applied during the presence of the MTS derivative (Fig. 5, A and B, right). This result implies that conformational changes associated with ATP binding and channel opening moves V48C into a position in which it is much more readily accessible to reaction with MTS derivatives. In other words, Val^{48} moves as a result of channel opening, and by moving it becomes more accessible to MTS derivatives.

Disulfide Formation between V48C and I328C—We have previously presented evidence that T336C is located in the outer vestibule of the ionic channel; the evidence for this was that outward currents were inhibited more rapidly than inward currents as the MTSET reacted with the cysteine.

(TM1 of P2X<sub>2</sub> Receptor)
present work indicates that Val48 is situated at the outer edge of the membrane, and we therefore asked whether these residues were sufficiently close to form disulfides that altered the properties of the channel. We expressed the double mutants V48C/I328C, V48C/N333C, and V48C/T336. The current elicited by ATP (30 μM) at the V48C/I328C receptor was much smaller (243 ± 70 pA; n = 11) than wild-type, V48C, or I328C receptors (Fig. 6). We also observed relatively large inward currents when the cells were held at −60 mV; it normally required less than −50 pA to hold a human embryonic kidney 293 cell at −60 mV, but for V48C/I328C this was −235 ± 52 pA (n = 8). This suggested that the P2X2 receptor channel was constitutively open in this mutated receptor. Dithiothreitol (10 mM) greatly increased the amplitude of the current evoked by ATP (about 6-fold) over 20 min and progressively reduced the sustained holding current in the absence of ATP (Fig. 6, A and C). The ATP-evoked current increased exponentially with time constant (τ) of 5.9 ± 0.8 min (n = 8) with 10 mM dithiothreitol, whereas the sustained inward holding current declined rather more slowly (τ = 19.3 ± 7.4 min; n = 6) and had reached −76 ± 41 pA at 20 min. A further reducing agent, bismercaptoethanol (5 mM), also potentiated the ATP-evoked currents (Fig. 6, B and C). Its action was somewhat more rapid than that of dithiothreitol (τ = 1.2 ± 0.5 min; n = 4). Bismercaptoethanol also reduced the inward holding current from −299 ± 115 to −48 ± 17 pA (n = 4) during a 20-min application (τ = 4.3 ± 1.6 min; n = 3).

Effects of Introducing Cysteines—The rat P2X2 receptor was tolerant of cysteine introduced in all but 5 of the 46 positions examined between Asp15 and Thr60 (Figs. 2 and 7). Y16C, Y43C, Y55C, and Q56C were non-functional; these residues are more slowly (τ = 5.7 ± 0.9 min; n = 7) than wild-type, V48C, or I328C receptors. All currents were normalized to that measured prior to application of dithiothreitol or bismercaptoethanol (n = 3–8 cells for each case). BMS, bismercaptoethanol.

Fig. 6. V48C/I328C disulfide alters channel opening. A, P2X2 receptor with double mutation V48C/I328C showed very small responses to ATP, but these increased 6-fold after applying dithiothreitol (10 mM; solid bar). The effect declined when dithiothreitol application was discontinued, and was repeatable. ATP (30 μM; 2 s) was applied at the times indicated at the top of each current trace (min). B, bismercaptoethanol (5 mM) had a similar effect. C, summary of potentiation of ATP-evoked current in P2X2-V48C/I328C by dithiothreitol (filled diamonds) and bismercaptoethanol (open squares). Also shown are the lack of any effect of dithiothreitol of wild-type (open circles), V48C (filled triangles), I328C (filled triangles), and V48C/T336C (filled squares) receptors. All currents were normalized to that measured prior to application of dithiothreitol or bismercaptoethanol (n = 3–8 cells for each case). BMS, bismercaptoethanol.
subunits except P2X\textsubscript{7}, where it is replaced by tryptophan. In four positions the introduction of cysteine led to an obviously profound run-down of response when ATP application was repeated.

The effect of mutating Phe\textsubscript{44} to cysteine was surprising in that it resulted in a 10-fold increase in sensitivity to ATP and an even larger increase in sensitivity to \(\alpha\beta\text{meATP}\). Insensitivity to \(\alpha\beta\text{meATP}\) has come to be regarded as a major distinguishing feature among the different subtypes of P2X receptor, with those containing P2X\textsubscript{1} and P2X\textsubscript{3} subunits sensitive (P2X\textsubscript{1} and P2X\textsubscript{3} homomers and P2X\textsubscript{1,3} heteromers) and those not containing these subunits several hundred-fold less sensitive (17). There have not been previous reports of point mutations conferring sensitivity to \(\alpha\beta\text{meATP}\) in P2X receptors of the insensitive subclasses (P2X\textsubscript{2}, P2X\textsubscript{4}, P2X\textsubscript{5}, and P2X\textsubscript{7}). phenylalanine is found in this position in the P2X\textsubscript{2} and P2X\textsubscript{3} subunits, but in the others the residue is leucine, valine, or isoleucine. One interpretation of the increased effectiveness is that this position contributes to the ATP binding site. This seems somewhat unlikely in view of the fact that it is situated well within the first hydrophobic domain. A more likely explanation might be that \(\alpha\beta\text{meATP}\) normally can bind to the P2X receptor in much the same way as ATP but that it has very low efficacy to induce the conformational change leading to channel opening. From the results for the wild-type channel, the EC\textsubscript{50} for \(\alpha\beta\text{meATP}\) can be very crudely estimated as a little more than 1 mM, but this is difficult to verify experimentally because of doubts that the \(\alpha\beta\text{meATP}\) might contain small amounts of ATP. In F44C, the EC\textsubscript{50} value for ATP shifted about 10-fold (from 8 to 0.7 \(\mu\text{M}\)); the EC\textsubscript{50} value for \(\alpha\beta\text{meATP}\) must then have shifted about 100-fold (from about 1000 to 10 \(\mu\text{M}\)). A direct effect of this mutation on channel gating was also indicated by the observation that the currents evoked by ATP took longer to decline to the baseline in F44C than in wild-type channels; this is consistent with slowed channel closing. Further analysis of this position with other substitutions, and single channel recordings, is likely to provide insight into the mechanisms of gating. One corollary of this interpretation is that \(\alpha\beta\text{meATP}\) should act as a competitive antagonist of ATP at wild-type P2X\textsubscript{2} receptors. Although this has not been reported, there is evidence that \(\alpha\beta\text{meATP}\) is an antagonist of ATP action at P2X receptors in sympathetic neurons (18).

Effects of Methanethiosulfonates—We used principally the methyl, ethyltrimethylammonium, and ethylsulfonate derivatives of methanethiosulfonates. After modification of a cysteine these would result in a neutral \([-\text{CH}_2\text{S-S-CH}_3]\), positive \([-\text{CH}_2\text{S-S-CH}_2\text{N}^+(\text{CH}_3)_2]\), or negative \([-\text{CH}_2\text{S-S-CH}_2\text{CH}_2\text{SO}_3^-]\) side chain on the receptor protein. MTSM has had no significant effect on the wild-type P2X\textsubscript{2} receptors but gave strong inhibition of ATP-evoked currents in eight of the cysteine-substituted receptors (Figs. 2 and 7). Where MTSM has no effect, we cannot say whether the cysteine is not accessible to an aqueous solution or whether the cysteine is modified, but this modification does not change the channel properties in any way that we have studied. The rapid rate at which MTSM reacts with some cysteines at intracellular locations (e.g., G30C and V24C) indicates that there is little obstacle to its passage across the plasma membrane. Other methanethiosulfonates did not have significant effects on cysteines at intracellular positions in the N-terminal region of the receptor, except for V24C. In this case, the action of MTSET was dependent on ATP application, suggesting that it entered the cell through the open channel. We have shown previously that MTSET is about 16% as permeable as sodium through P2X\textsubscript{2} receptors (10); we have not used MTSEA, because we (10) and others (19) have found that it can enter the cell quite readily, presumably in its uncharged state. Cysteine substitutions at positions in the inner edge of the first hydrophobic domain (Trp\textsubscript{350} to Thr\textsubscript{354}) have previously been shown to be unreactive to MTSM and MTSET; the present work showed that two of them were accessible to MTSM, and this is consistent with an intracellular location. In general, the results with MTSM and MTSET are as would be expected on the basis of the topological models currently proposed for the receptor (Fig. 7).

For all the modified cysteines, the reduction in the ATP-evoked current occurred without change in the EC\textsubscript{50} value. In other words, increasing the ATP concentration could not overcome the inhibition of the current resulting from methanethiosulfonate application. One can distinguish broadly between a reduced affinity of the closed channel for ATP (i.e. binding), an impaired ability of the channel to open and stay open when ATP is bound (gating), and a reduced current through the open channel (permeation) (14). Impairment of binding or gating would usually produce a rightward parallel shift in the concentration-response curve before the maximum is reduced, whereas reduction in open channel current would not. It is conceivable that mutations P19C, V23C, V24C, F44C, and V48C (Fig. 4) all directly affect permeation, but independent direct measurements would be required to show this. In no...
Movement of Val$_{48}$ with Channel Opening—The inhibition of current observed in V48C closely resembled that which we have previously found for I328C, N333C, and T336C (10), all of which are located close to the outer end of the second transmembrane domain. The finding that all three methanethiosulfonates (positive, neutral, and negative) cause strong inhibition indicates that this position is situated outside the membrane electric field. We were surprised therefore to observe that the reaction at V48C occurred much more rapidly when ATP was repeatedly applied than when it was not applied (Fig. 5). This result implies that the cysteine in the position of Val$_{48}$ moves to one face of an α-helix (Fig. 7). Key residues involved in gating the mechanosensitive channel of Escherichia coli (which also has two membrane-spanning domains per subunit) have a similar relative orientation (22). The proposed model suggests several opportunities for future experiments to increase our understanding of the modus operandi of P2X receptors.

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