Amino Acid Residues Involved in Gating Identified in the First Membrane-spanning Domain of the Rat P2X<sub>2</sub> Receptor* 

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P2X receptors are a family of multimeric membrane proteins that function as ion channels gated by extracellular ATP. Hydrophobicity 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 P2X<sub>2</sub> receptor, of Leu<sup>29</sup> to Val<sup>51</sup> and of Ile<sup>331</sup> to Leu<sup>353</sup>. Considerable experimental evidence now supports the view that the N and C termini of a second form functional channels (5).

The first hydrophobic segment of the rat P2X<sub>2</sub> receptor extends from residue Leu<sup>29</sup> to Val<sup>51</sup>. In the rat P2X<sub>2</sub> receptor, we mutated amino acids in this segment and adjoining flanking regions (Asp<sup>15</sup> through Thr<sup>69</sup>) 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 Val<sup>48</sup> at the outer end of the first hydrophobic segment takes part in the gating movement of channel opening.

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1 The abbreviations used are: MTSES, (2-sulfonatoethyl) methanethiosulfonate; αβmeATP, αβ-methylene-ATP; MTs, methanethiosulfonate; MTsE, (2-aminoethyl) methanethiosulfonate; MTSET, (2-(trimethylammonium)ethyl) methanethiosulfonate; MTBSE, butyl methanethiosulfonate; MTS, methyl methanethiosulfonate; EC<sub>50</sub>, concentration evoking half-maximal response.
this suggests that Asp^{349} 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 P2X2 receptor, and we therefore began our cysteine substitutions at Asp^{57}. We have recently reported the effects of MTS compounds on the region Asp^{57} to Lys^{71} (9); in the present experiments we extended the cysteine substitutions at Thr^{90} (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 <sub>2</sub> Receptor cDNA and Mutagenesis—A P2X <sub>2</sub> 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 generated by PCR.

Electrophysiology—Transfected human embryonic kidney 293 cells using Lipofectin or LipofectAMINE 2000 was as described previously (10). Whole-cell recordings and fast-flow perfusion (see ref. 11) were performed on the cultured cells using the patch clamp and fast-flow perfusion system of L. T. Smith et al. (12) and A. L. T. Smith et al. (13) modified to use Fast-Flow-1. We have found that the ATP concentration-response curves at wild-type and cysteine-substituted receptors are qualitatively similar (10). We used fast-flow perfusion with a flow rate of 0.5 ml/min. Currents were measured using an Axopatch-200B amplifier with the Pclamp software (Axon Instruments) and were low-pass filtered at 5 kHz. The effects of MTSM were tested by perfusing the cells for 7–10 min with 100 mM MTSM in control solution. The effects of point mutations to cysteine were tested by introducing the point mutation and then testing the ATP concentration-response curve. In some cases the ATP concentration-response curves were obtained using 100 mM MTSM after the resting currents had decayed. In these cases the cells were perfused with 10 mM HEPES, pH 7.5, containing 0.2 mM CaCl <sub>2</sub> and 5 mM MgCl <sub>2</sub>. The resting currents were then recorded and the cells were perfused with ATP (1–100 mM) and the ATP concentration-response curves were obtained. The concentrations of MTSM and other reagents are given in parentheses at the appropriate point in the text.

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<sub>50</sub> 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 = 9; 3 μM ATP) or 1.6 ± 0.5 s (n = 29; 30 μM ATP) for F44C. Second, there was a 10-fold increase in sensitivity to ATP (EC<sub>50</sub> 0.72 ± 0.1 μM; n = 4). Third, there was a remarkable increase in effectiveness of αβmeATP. The wild-type P2X<sub>2</sub> receptor is essentially insensitive to αβmeATP (13); indeed, we found that 100 and 300 μM αβmeATP evoked currents in cells expressing wild-type P2X<sub>2</sub> receptors were, 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<sub>50</sub> 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 during 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 were 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 MTSES (10). At 8 of 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, MTB. At 1 mM (for 8 min), the inhibitions by MTB were as follows: V15C, 90 ± 3% (n = 3); P19C, 89 ± 7.8% (n = 4); V23C, 82 ± 4.1% (n = 3); and V24C, 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 MTSES on the positions on the C-terminal side of D349C (W350C, I351C, 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 I351C (76.5 ± 6.2%; n = 3) and L352 (69.8 ± 5.1%; n = 3).

Effects of MTS Compounds with Positively or Negatively...
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 (similar time constant) 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 all 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. 4). 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 EC₅₀. 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 Val²⁴ 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, Val¹⁴ 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. The
Fig. 5. V24C is accessible to MTSET when the channel is open, and V48C is more accessible to MTSET and MTSM when the channel is opened. A, left, at V24C MTSM (1 ms; 8 min) completely inhibits whether ATP is applied during the MTSM application (upper panel) or not (lower panel). Right, in the case of V48C the MTSM application is fully effective when ATP is applied during the MTSM application (upper panel) but much less effective when ATP is not applied (lower panel). B, left, V24C is sensitive to inhibition by MTSET if ATP is repeatedly applied (presumably, because MTSET enters the cell through the P2X channel when it opens). Right, at V48C inhibition by MTSET was much less when ATP was not applied repeatedly. Calibrations apply to all records. C, summary of experiments shown in A and B. *, p < 0.05; **, p < 0.01 (n = 3–10 in each case).

The 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/T336C. 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 mV 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 P2X receptor channel was constitutively open in this mutant 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). ATP-evoked currents for V48C/N333C and V48C/T336C were similar to those observed for single cysteine mutants V48C, N333C, or T336C (range 1–8 nA), and application of dithiothreitol at 10 mM for 20 min had no effect on the currents (see Fig. 6C).

**DISCUSSION**

**Effects of Introducing Cysteines**—The rat P2X 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 completely conserved among all mammalian P2X receptors. R34C also did not express channels; arginine is found in all

![Image](https://example.com/image.png)

**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$_2$, where it is replaced by tryptophan. In four positions the introduction of cysteine led to an obviously altered phenotype. In the case of T18C and L29C, the response to ATP declined markedly when ATP was applied more than once. Thi$_{18}$ in the P2X$_2$ receptor has been shown by Boué-Grabot et al. (16) to be phosphorylated by protein kinase C, and this alters the desensitization kinetics. Leu$_{29}$ has not previously been mutated, but we note that it lies very close to the inner end of the second transmembrane domain (Trp$_{350}$ to Thr$_{355}$) have previously been shown to be unreactive to methyl, ethyltrimethylammonium, and ethylsulfonate derivatives of methanethiosulfonates. After modification of a cysteine with methanethiosulfonates, Shaded residues indicate positions at which cysteine substitution results in strong inhibition. Open letters indicate positions where cysteine substitution results in non-functioning channels. Bold circles indicate two positions at which functional channels were expressed, but methanethiosulfonates could not be studied because of profound run-down of response when ATP application was repeated. Val$_{48}$ and Ile$_{328}$ can be disulfided. The present work reports results from V48C (Fig. 4) all directly affect permeation, but independent reduction in open channel current would not. It is conceivable that mutations P19C, V23C, V24C, F44C, and Ile$_{350}$ to Thr$_{355}$ have previously been shown to be unreactive to 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$_{50}$ 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
cases was there, for example, any obvious effect on the rectification of the whole-cell current after cysteine modification.

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 during channel opening. Moreover, the currents in cells expressing the V48C/I328C combination were very much reduced in amplitude (Fig. 6). At the same time, the currents in cells expressing the doubly mutated receptors V48C/I328C, V48C/N333C, and V48C/T336C, the latter two combinations resulted in currents that were not different from wild-type receptors, but the currents in cells expressing the V48C/I328C combination were very much reduced in amplitude (Fig. 6). At the same time, these cells exhibited large steady inward currents at −60 mV. Application of either dithiothreitol or bismercapt ethanol greatly increased the ATP-induced current and concomitantly decreased the persistent inward current. Taken together, these observations suggest that a disulfide formed between V48C and I328C results in a channel that is constitutively open and cannot be opened by applying ATP. The small currents that we observed prior to adding the reducing agent might indicate that the disulfide bond had not formed in all the channels or that the channels could operate, though poorly, with the disulfide bond in place. The impairment of channel opening by the V48C/I328C disulfide is quite consistent with the conclusion reached above that Val\(^{48}\) moves during channel opening. Moreover, the finding that the V48C channel does not open when this position is “tethered” to I328C suggests that the movement of Val\(^{48}\) is not “fortuitous” at some incidental part of the protein, but is a necessary component of the gating mechanism.

It is not possible to conclude from the present work whether the disulfide is formed between V48C and I328C on the same receptor subunit or on different receptor subunits that contribute to the multimeric channel. However, the results do put constraints on models for the channel. In Fig. 7 the two membrane-spanning domains are depicted as α-helices. There is no evidence for this, except to say that it is strongly favored by secondary structure prediction algorithms (20, 21). The accessible residues in the first transmembrane domain are located at one side of the helix. The proximity of Val\(^{48}\) and Ile\(^{328}\) indicated by the present results is fully consistent with this structure. For both transmembrane segments virtually all of the accessible residues mapped by cysteine scanning can be aligned along 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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