Identification of amino acid residues contributing to the pore of a P2X receptor

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P2X receptors are ion channels opened by extracellular ATP. The seven subunits currently known are encoded by different genes. It is thought that each subunit has two transmembrane domains, a large extracellular loop, and intracellular N- and C-termini, a topology which is fundamentally different from that of other ligand-gated channels such as nicotinic acetylcholine or glutamate receptors. We used the substituted cysteine accessibility method to identify parts of the molecule that form the ionic pore of the P2X₂ receptor. Amino acids preceding and throughout the second hydrophobic domain (316–354) were mutated individually to cysteine, and the DNAs were expressed in HEK293 cells. For three of the 38 residues (I328C, N333C, T336C), currents evoked by ATP were inhibited by extracellular application of methanethiosulfonates of either charge (ethyltrimethylammonium, ethylsulfonate) suggesting that they lie in the outer vestibule of the pore. For two further substitutions (L338C, D349C) only the smaller ethylamine derivative inhibited the current. L338C was accessible to cysteine modification whether or not the channel was opened by ATP, but D349C was inhibited only when ATP was concurrently applied. The results indicate that part of the pore of the P2X receptor is formed by the second hydrophobic domain, and that L338 and D349 are on either side of the channel 'gate'.

Keywords: cysteine scanning mutagenesis/ligand-gated channel/methanethiosulfonate/P2X receptors/substituted cysteine accessibility method

Introduction

P2X receptors are multimeric membrane proteins that incorporate an integral ion channel (Surprenant *et al.*, 1995). The channel opens in response to the binding of extracellular ATP; it is selectively permeable to small cations. Seven cDNAs have been isolated which encode P2X receptor subunits, from neuronal and other tissues (Buell *et al.*, 1996a; North, 1996a). The deduced proteins, 379–575 amino acids, are 35–48% identical between any pair of subunits. The proteins lack a signal sequence and have two hydrophobic domains of ~20 residues each. This has led to the suggestion that they have intracellular N- and C-termini, and two membrane-spanning segments

which are separated by a large (~270 amino acids) extracellular domain. Evidence that some of this domain is extracellular include the observations that point mutations can alter the action of antagonists (E249K in P2X₄; Buell *et al.*, 1996b) or prevent glycosylation (N184S in P2X₁; our unpublished observations). All subunits except P2X₆ express readily in heterologous systems and are presumed to form channels as homomultimers (Collo *et al.*, 1996). In the case of P2X₂ and P2X₃ receptors, heteromeric channel assembly has also been shown (Lewis *et al.*, 1995).

This overall topology of the P2X channel appears to be very different from that established for the other two major families of ligand-gated channels (nicotinic acetylcholine family and glutamate family); those are pentamers, in which each subunit has four or three membrane-spanning segments (Numa, 1989; Karlin, 1993; Hollman *et al.*, 1994). The P2X receptors more closely resemble in structure that proposed for the epithelial sodium channels (ENaC), which also have a large extracellular loop flanked by two hydrophobic regions (Canessa *et al.*, 1994a,b; Rossier *et al.*, 1994; North, 1996b). Epithelial sodium channels belong to a larger family related by sequence homology which includes presumed channel proteins (degenerins) from *Caenorhabditis elegans* (Hong and Driscoll, 1994; Huang and Chalfie, 1994) and a recently described ligand-gated channel from *Helix aspersa* (Lingueglia *et al.*, 1995). There is no detectable similarity of sequence between P2X receptors and the ENaC-based family (North, 1996b). The stoichiometry is not yet known for any of these channels with two hydrophobic domains per subunit.

Our current understanding of the structure and function of P2X receptors remains primitive, and specifically we do not know which regions of the molecule contribute to the ligand-binding site or to the ion-conducting pore. The purpose of the present experiments was to identify residues lining the pore. We chose the second hydrophobic domain for investigation for three reasons. First, the channel lumen must be lined by residues from one or other membrane-spanning segments. Second, in the case of ENaC, degenerins and the peptide-gated channels there is evidence to implicate this domain (Huang and Chalfie, 1994; Lingueglia *et al.*, 1995; Waldmann *et al.*, 1995). Third, if this domain crosses the membrane and is α -helical, then it could expose mostly hydrophobic residues on one face and small or polar residues on the other (Brake *et al.*, 1994; North, 1996a); the hydrophilic face would be a possible candidate for a pore-lining surface.

We have used the approach introduced by Akabas and Karlin for the nicotinic acetylcholine receptor (Akabas *et al.*, 1992, 1994; Stauffer and Karlin, 1994; Akabas and Karlin, 1995); residues are mutated individually to cysteine, and the accessibility of the side-chain in the expressed channel is probed with a water-soluble sulfhydryl

Fig. 1. Cysteine-scanning mutagenesis experiments on P2X₂ receptor expressed in HEK 293 cells. (A) Schematic representation of proposed topology of P2X₂ receptors. Grey segment in putative transmembrane domain (TM2) and pre-TM2 regions indicates region into which cysteines were introduced in the present study (from V316 to T354). (B) Summary of time course of action of MTSET and MTSEA on wild-type P2X₂ receptor. ATP (30 μM) was applied for 1 or 2 s at 90 s intervals; MTSEA or MTSET was applied during the period indicated by the horizontal bar (8 min). Cysteine (10 mM) was included in the patch pipette for experiments labelled 'MTSEA + internal cysteine'; this largely prevented the strong inhibition of the wild-type P2X₂ current. MTSET and MTSEA were tested in HEK cells transiently transfected with wild-type P2X₂ receptor ($n = 36$) as well as in HEK cells stably expressing the P2X₂ receptor ($n = 14$); no significant differences were found and the results shown are pooled from all cells.

reagent. In this study we changed each residue to cysteine from V316 to T354 of the P2X₂ receptor (Figure 1A). We chose the P2X₂ receptor because it expresses robust, non-desensitizing currents when expressed in human embryonic kidney (HEK293) cells (Evans *et al.*, 1995; Collo *et al.*, 1996), and thus allows repeated applications of ATP to the same cell. We examined the effects of three methanethiosulfonates applied extracellularly; these were the ethylamine (MTSEA; small positively charged), ethyltrimethylammonium (MTSET; larger, permanently positively charged) and ethylsulfonate (MTSES; intermediate size, negatively charged) derivatives.

Results

There are ten cysteine residues in the presumed extracellular loop of the P2X₂ receptor, and these are conserved among all the members of the family (Collo *et al.*, 1996; North, 1996b). It was therefore important to ensure that the wild-type receptor was unaffected by extracellular MTS reagents. In HEK293 cells expressing the wild-type receptor, repeated applications of ATP (30 μM, for 2 s) at

intervals of 30–90 s evoked inward currents that were essentially reproducible for up to 25 min (Figure 1B and Figure 2A and B). MTSET (1 mM) or MTSES (1 mM), continuously applied for 8 min, did not cause any significant inhibition of the currents in wild-type channels. In contrast, MTSEA (1 mM, 8 min) caused a progressive inhibition of wild-type currents, particularly after the end of its application (Figure 1B). This inhibition was not generally observed when recordings were made with recording pipettes (intracellular solution) containing cysteine (5 or 10 mM); we interpret this to indicate that MTSEA is entering the cell during prolonged applications and acting from within (Holmgren *et al.*, 1996). In subsequent experiments, we limited the duration of application of MTSEA (at 1 mM) to 8 min and included cysteine in the recording pipette.

The P2X receptors are cation-selective channels, with appreciable permeability to organic cations as large as dimethylammonium. We therefore determined the relative permeability of the cations MTSEA and MTSET, by measuring the reversal potentials in conditions in which these were the predominant extracellular cation. The reversal potentials were (mV) -0.40 ± 1.03 ($n = 5$), -20.3 ± 1.69 ($n = 6$) and -61.0 ± 2.33 ($n = 7$) when the permeant extracellular cation was sodium, MTSEA and MTSET, respectively. These were converted to relative permeabilities (see Materials and methods); $P_{\text{MTSEA}}/P_{\text{Na}}$ was 0.70 ± 0.05 ($n = 6$) and $P_{\text{MTSET}}/P_{\text{Na}}$ was 0.14 ± 0.02 ($n = 7$).

Thirty-eight cDNAs carrying single cysteine substitutions were transfected in these experiments (from V316C to T354C). All expressed currents in response to ATP (30 mM) within the range observed for wild-type channels (1–4 nA); the only exceptions were S340C and D349C in which case the peak currents were reduced (see below). In all transfections, cells were fixed for immunohistochemistry using the C-terminal EYMPME epitope tag. This provides only a qualitative indication of membrane expression; however, there was no apparent difference between the staining of cells expressing S340C and D349C and those transfected with wild-type receptors or receptors with other cysteine substitutions.

Residues accessible to MTSET, MTSEA and MTSES

Figure 2 illustrates and Figure 3 summarizes the effect of applying MTSES, MTSET and MTSEA (1 mM) to cells expressing each of the 38 mutant channels. MTSET and MTSES had very small effects in all except three cases (I328C, N333C and T336C). The actions of MTSEA were strikingly more variable (Figure 3); in this case, there were seven substitutions in which the current evoked by ATP was significantly altered with respect to the wild-type (analysis of variance, Dunnett's post-hoc test; $P < 0.01$). The residues were I328C, N333C, T336C, and four further residues where MTSEA but not MTSET or MTSES were effective (see below).

In addition to the inhibition of the current evoked by ATP, the MTS derivatives also caused a sustained inward current. This developed as soon as the MTS-containing solution reached the bath solution, and it only slowly declined after washout (Figure 2B). This current was only observed for those mutations in which the ATP-evoked current was also reduced; its amplitude varied considerably

Fig. 2. Irreversible inhibition of ATP-evoked current by MTSET. (A) Representative traces of ATP-induced currents recorded from cells transfected with I328C (left) or T336C (right) P2X₂ receptor cDNA. Traces are superimposed currents recorded in response to ATP application (2 s duration, indicated by solid bar above traces) at 90 s intervals; in each panel four consecutive control responses are shown and four consecutive responses during MTSET (1 mM) are shown. (B) Representative traces of ATP-induced currents (ATP applied for 2 s at 90 s intervals throughout) shown on slower time scale. MTSET (1 mM, applied for duration indicated by grey bars) is without effect on the wild-type P2X₂ receptor and on I331C (chosen to represent a typical negative mutation), but inhibits ATP-evoked current in I328C and T336C transfected cells. Note the fast onset of a sustained inward current in the case of I328C and T336C. (C) Time course of inhibition by MTSET at I328C, N333C and T336C. Results are from experiments as illustrated in (A) and (B). ATP applications (30 μ M, 2 s) were at intervals of 30, 60 or 90 s. Lines are best-fit exponentials to data with time constants of 58 s for I328C, 156 s for N333C and 195 s for T336C. Each point is mean $\pm$ s.e.m. of 4–12 experiments.

among cells (5–25% of the peak current evoked by 30 μ M ATP). Current–voltage relations determined during the sustained current showed that it reversed close to 0 mV. This quickly developing, sustained current elicited by MTS reagents occurred even when the P2X₂ receptor had been blocked by the receptor antagonist pyridoxal-5-phosphate-6-azophenyl-2',4'-disulfonic acid (30 μ M, which caused >90% inhibition of the ATP-evoked current) (see Evans *et al.*, 1995). This suggests that the MTS

reagents are not acting directly as receptor agonists; it is possible that their interaction with the receptor cysteines leads to a variable degree of constitutive channel opening.

The inhibition of the ATP-evoked current by MTS reagents occurred over an exponential time course (Figure 2C). For MTSET (1 mM, –60 mV), modification of I328C (time constant 59 ± 6 s, $n = 5$) occurred more rapidly than modification of N333C (230 ± 21 s, $n = 5$) and T336C (221 ± 17 s, $n = 9$) (Figure 2C). Comparable

Fig. 3. Summary of actions of MTSES, MTSET and MTSEA on wild-type and cysteine-substituted P2X₂ receptors. Results are plotted as the peak amplitude of the current evoked by ATP (30 μ M, 2 s application, 90 s intervals) measured 8 min after adding the MTS reagent, relative to its value before adding the MTS reagent. For MTSES and MTSET, $n = 12$ –25 for wild-type, I328C, N333C, T336C, L338C and D349C and $n = 4$ for other residues. For MTSEA, $n = 8$ –34 for all residues, and cysteine (2–10 mM) was present in the intracellular (pipette) solution. Shaded bars identify residues which showed significant inhibition by MTS reagent.

differences in the kinetics of inhibition were seen in the case of MTSES (1 mM) (I328C: 67 ± 8 s, $n = 65$; N333C: 315 ± 18 s, $n = 5$; T336C: 272 ± 8 s, $n = 9$) and MTSEA (1 mM) (I328C: 74 ± 4 s, $n = 3$; T336C: 225 ± 20 s, $n = 6$). With concentrations lower than 1 mM, the onset of the inhibition was slower; however, the rate constant for onset of the effect [$k_{+1} = 1/(\text{time constant} \times \text{MTSET concentration})$] was itself dependent on concentration. For I328C, k_{+1} ranged from $240 \text{ M}^{-1}\text{s}^{-1}$ at 30 mM to $17 \text{ M}^{-1}\text{s}^{-1}$ at 1 mM; for N333C, k_{+1} ranged from $98 \text{ M}^{-1}\text{s}^{-1}$ at 30 mM to $6 \text{ M}^{-1}\text{s}^{-1}$ at 1 mM; and for T336C, k_{+1} ranged from $79 \text{ M}^{-1}\text{s}^{-1}$ at 30 mM to $10 \text{ M}^{-1}\text{s}^{-1}$ at 1 mM. Thus, for all concentrations tested, the rate of reaction at I328C was 3-fold faster than the rates observed for N333C or T336C.

The steady-state level of inhibition observed with MTSET, MTSEA and MTSES was not different in the case of I328 ($P > 0.05$) (Figure 3). In contrast, for N333C and T336C, the positively charged MTSET and MTSEA were significantly more effective than the negatively charged MTSES ($P < 0.01$) (Figure 3). This was most striking in the case of T336C, at which MTSET and MTSEA gave close to 100% block, whereas MTSES inhibited by only 50%.

We sought to determine whether the rate of modification by the charged MTS analogues was dependent on the membrane potential, because this might indicate the position of the substituted cysteines with respect to the membrane electric field. For T336, the inhibition of outward currents (+30 mV) occurred three times faster than the inhibition of

inward current (−60 mV) (Figure 4); however, this finding applied to all three MTS derivatives. The time constants for inhibition of outward current were 62 ± 4 s ($n = 7$) for MTSET (compare with 221 s for inward current, see above); 106 ± 90 s ($n = 6$) for MTSES (compare with 315 s), and 78 ± 7 s ($n = 6$) for MTSEA (compare with 225 s). The observation that the rate of blockade was voltage-dependent for all the reagents is not consistent with a simple effect of the membrane field on k_{+1} . Indeed, the same effect was observed in further experiments when the membrane potential was held at −60 mV between ATP applications (30–90 s intervals) and depolarized to +30 mV only for the ATP application and effect (10–15 s) (Figure 4), or when the membrane potential was set to +30 mV and then adjusted to −60 mV only for the period (10–15 s) required to test the action of ATP. Whole-cell currents at P2X₂ receptors show inward rectification (Evans *et al.*, 1996) and this was also observed for the T336C (Figure 4A); thus, the more rapid block of outward currents compared with inward currents represents an increase in this rectification. No such increased rectification was observed in the case of I328C and N333C. For I328C, the time constants for inhibition by MTSET (1 mM) were 59 ± 6 s ($n = 5$) for inward current (−60 mV) and 55 ± 7 s ($n = 3$) for outward current (+30 mV) and for N333C, the corresponding values were 230 ± 21 s ($n = 5$) and 186 ± 11 s ($n = 4$).

Residues accessible only to MTSEA

MTSEA is significantly smaller in cross-sectional area than MTSET, and it is much more permeant through the wild-

Fig. 4. MTSET increases rectification in T336C P2X₂ receptors. (A) Example of ATP-evoked currents in one cell evoked -60 mV (upper) and in a different cell evoked at +30 mV (lower), before and 30, 60, 90 and 120 s after application of MTSET as indicated. ATP (30 μ M, 1 s) was applied at 30 s intervals throughout; control shows three superimposed consecutive traces immediately before MTSET. In the cell shown at +30 mV, potential was held at -60 mV throughout and then stepped to +30 mV from 3 s before to 10 s after the ATP application. Note the 5-fold difference in calibration: 1 nA applies to upper traces and 0.2 nA applies to lower traces. (B) Summary of results from experiments as shown in (A); line is best-fit exponential with time constant of 57 s at +30 mV (data at -60 mV is same as in Figure 2C with time constant of 195 s).

type P2X receptor. It might therefore be expected to detect residues situated more deeply within the ionic pore (Akabas *et al.*, 1992). In fact, MTSEA unequivocally inhibited the current in two further cases (L338C and D349C) (Figure 3) at which MTSET had no effect (Figure 5A). The inhibition at D349C was particularly complete, and this occurred rapidly (as fast as that seen for I328C) (Figure 5C). The rate of inhibition was the same for inward and outward currents evoked by ATP; time constants were (MTSEA 1 mM) 66 ± 7 s ($n = 7$) at -60 mV and 59 ± 8 s ($n = 4$) at +30 mV. In the case of D349C, application of MTSEA did not cause the quickly developing, sustained current that was observed with the other mutations. Finally, MTSEA significantly increased currents in cells expressing S340C and G342C mutations (Figure 3).

Requirement for channel opening

The degree of inhibition of the ATP-evoked current by the MTS reagents was compared between cells in which the

Fig. 5. P2X₂ receptor with D349C substitution is blocked by MTSEA, but this requires channel opening. (A) ATP-induced currents (2 s application, 90 s intervals) at D349C are unaffected by MTSET (1 mM). (B) ATP-induced currents at T336C are inhibited by MTSET (1 mM) whether (upper panel) or not (middle panel) ATP is applied during the MTSET application. Lower panel shows a control experiment in which ATP but no MTSET was applied. (C) A similar experiment to that shown in (B), but from D349C mutation. The upper trace shows repeated applications of ATP (90 s intervals) during the presence of MTSEA reagent and the lower trace shows experiments in which ATP applications were not made during the MTS application or for 3 min afterwards. (D) Summary of all experiments as illustrated in (B) and (C). The test pulse of ATP was applied after 6 or 8 min in the presence of MTSET or MTSEA; +ATP indicates experiments in which ATP applications were continued throughout the MTS application, and -ATP indicates experiments in which they were not.

ATP was applied repeatedly during the MTS application (as described above) and cells in which the ATP applications were discontinued during (up to 8 min) and for 3 min after the MTS exposure (Figure 5). For D349C, extracellular MTSEA caused no inhibition of the ATP-evoked current if ATP was not applied repeatedly during its presence (Figure 5C and D). For I328C, N333C, T336C and L338C, the degree of inhibition was almost as great as when ATP had been applied repeatedly during the presence of the MTS reagent; these are illustrated for T336C in Figure 5B and D). These results indicate that I328C, N333C and T336C and L338C are accessible to extracellular methanethiosulfonates without the requirement for significant channel opening, whereas D349C is not.

Fig. 6. Agonist and antagonist concentration–response curves in cysteine mutants, and poor reversibility of MTS inhibition by reducing agents. (A) ATP concentration–response curves for wild-type, I328C, N333C, T336C, S340C and D349C. Results are plotted as peak currents recorded; each point is mean $\pm$ s.e.m. for 4–6 experiments. Note the lower maximum currents for S340C and D349C. EC_{50} values calculated from individual cells (see Materials and methods) for wild-type, I328C, N333C, T336C, D349C and S340C were 13 ± 2 ($n = 6$), 15 ± 3 ($n = 4$), 11 ± 3 ($n = 4$), 15 ± 4 ($n = 4$), 20 ± 3 ($n = 6$) and 17 ± 3 ($n = 6$), respectively. (B) Inhibition of the current evoked by 30 μ M ATP by the P2X receptor antagonist pyridoxal-5-phosphate-6-azophenyl-2',4'-disulfonic acid; no differences were found. (C) Summary of actions of dithiothreitol and bis(2-mercaptoethylsulfone) (1 mM, 6 min) applied 5 min after washout of MTSET (T336C, I328C, N333C) or MTSEA (L338C, D349C) ($n = 4$).

Further observations

In the absence of MTS reagents, there were no marked differences in the ATP concentration–response for any of the single point cysteine substitutions; this is illustrated in Figure 6A for I328C, N333C and T336C. The substitutions

S340C and D349C (Figure 6A) were the only ones of 38 which resulted in less than normal peak currents; they ranged from 35–55% (S340C) and 10–35% (D349C) of the values seen in wild-type cells. The reduced current was not associated with any marked change in the effective concentrations of ATP (Figure 6A). The effectiveness of the antagonist pyridoxal-5-phosphate-6-azophenyl-2',4'-disulfonic acid to inhibit the ATP-evoked current was not different for any mutations providing accessible cysteines (Figure 6B).

The effect of the MTS reagents was in all cases only poorly reversible on washing (Figure 2). It was also only partly reversible on the addition of reducing agents dithiothreitol (DTT) or its more potent analogue bis(2-mercaptoethylsulfone) (BMS) (Figure 6C).

Discussion

The wild-type P2X₂ receptor behaves as a cation-selective ionic pore. When measured under bi-ionic conditions, the permeability of the pore declines as the cross-sectional area of the permeant ion increases. Thus, the permeability of dimethylammonium ($3.8 \times 4.2 \times 6.1$ Å) is 0.5 relative to that of sodium, and for tris(hydroxy-methyl)aminomethane ($5.5 \times 5.6 \times 6.4$ Å), tetraethylammonium ($5.8 \times 7.0 \times 7.0$ Å) and *N*-methyl-D-glucamine ($5.0 \times 6.4 \times 12.0$ Å) the corresponding values are 0.16, 0.04 and 0.03 (Evans *et al.*, 1996). For comparison, both MTSEA and MTSET are ~ 10 Å in length, with maximum diameters of ~ 4.8 Å (the $\text{CH}_3\text{-SO}_2\text{-S-CH}_2\text{-CH}_2\text{-}$ moiety) and 5.8 Å [the $-\text{N}^+(\text{CH}_3)_3$ head group] respectively (see Kuner *et al.*, 1996). The present estimates of relative permeability for MTSEA (0.70) and MTSET (0.14) therefore fit within this range; they indicate that MTSEA is readily permeant but MTSET much less so.

Several assumptions attach to the interpretations of cysteine mutagenesis. The first is that the cysteine does not dramatically alter the protein structure, such as exposing a side-chain which is normally buried. Second, inhibition of the current by MTS reagents implies that the cysteine in that position is exposed to a water-accessible part of the receptor. This might be within the channel or its vestibules, or within the ligand-binding site (see McLaughlin *et al.*, 1995). When no effect on the current is observed, this may be because cysteines are not accessible, are accessible but do not react, or are modified but the modification does not lead to an altered macroscopic current (e.g. in a wide part of the pore). A third assumption is that the reagents do not significantly enter the cell through pathways other than the open channel. In the case of MTSEA, this assumption must be questioned for applications of several minutes because we saw a clear inhibition of wild-type currents which could be prevented by intracellular free cysteine (Figure 1). It is known that MTSEA can cross the membrane in its uncharged form (see Holmgren *et al.*, 1996), and internal actions might contribute to the much greater variability seen in the results of experiments with MTSEA than with MTSET or MTSES. There is also one naturally occurring cysteine within the region examined (C348), but this seems not to play a role because none of the extracellular MTS reagents affected wild-type or C348A channels. We tried to reduce these difficulties by limiting MTSEA applications to

8 min and also including free cysteine in the intracellular solution. A complete analysis of the residues contributing to the pore would require exposure of the cytoplasmic aspect of the channel to the MTS reagents. This proved to be difficult. With inside-out membrane patches (ATP in the recording pipette) the currents ran down within 30–60 s of patch excision, which was too short a period of time in which to study cysteine modification.

Three residues were accessible to all three reagents (I328C, N333C and T336C). The observation that I328 is modified by MTSES at a similar rate to that seen with MTSEA and MTSET implies that this residue does not lie within the cation-selective region of the pore. It may lie within the outer vestibule, or alternatively it could be involved in the ligand-binding site. Some evidence against any effect on ligand binding is provided by the finding the ATP dose–response curves were not different among wild-type and mutant. Cysteines at positions N333 and T336 were also modified by all three reagents but, with 8 min applications, much less by MTSES than by MTSET and MTSEA. This would be consistent with their location in a relatively wide part of the pore, but in a region which shows a degree of charge selection. The finding that cysteines introduced into positions on the N-terminus side of I328 could not be detectably modified by any MTS reagent suggests that the residues in this region do not participate directly to the aqueous pore. This includes a sequence which is highly conserved in P2X receptors (DVIVHGQAGKF), and which had been suggested to contribute to the pore (Brake *et al.*, 1994).

At low concentrations, the rate of reaction of all three MTS reagents at N333C and T336C was $\sim 90 \text{ M}^{-1}\text{s}^{-1}$, very much slower than for a diffusion-limited reaction; similarly low values have been reported for engineered cysteines in other channels (Kurz *et al.*, 1995; Yang and Horn, 1995). Although the rate of inhibition increased as the concentration was increased, it did not increase linearly as would be expected for a simple bimolecular reaction. One explanation might be that the dissociation cannot be neglected, although the inhibition was only slowly reversible in the present conditions (Figure 2). Another explanation might be that the MTS reagents must bind to several subunits in order to block the channel; these reactions may not be independent, such that the modification on one or more subunit reduces the rate of modification of further subunits.

In the case of T336C, though not I328C or N333C, outward currents were blocked considerably faster than inward currents. This does not indicate voltage-dependence in the association of MTS reagent because the same effect was found for the positively charged MTSET and the negatively charged MTSES. Furthermore, it is not likely to reflect a voltage-dependent exposure of T336C resulting from a conformational change (see Yang and Horn, 1995), because the increased rate of block did not require the membrane to be maintained at +30 mV between ATP applications. The result therefore implies that the first detectable effect of MTS binding at T336C is an increased rectification. One possible explanation might be that modification of a cysteine in one subunit introduces rectification into the channel, and only as further cysteines are modified on additional subunits does the channel become completely blocked. Irrespective of the molecular mechanism, this

result implies that T336C must lie within, or at the outer edge of, the ion-conducting pathway.

Four further residues were significantly modified by MTSEA, although not by MTSET and MTSES. In general, the effects of MTSEA are less reliably interpreted than those of MTSET and MTSES because of the much greater variability observed (Figure 3) but, nevertheless, in the case of L338C and D349C there was a clear reduction in current. D349C was exceptional among the mutant channels in that it did not express whole-cell currents as large as wild-type cells, although immunohistochemistry provided no evidence that the expression at the membrane was less than that seen for any other mutation. The inhibition of current at D349C by MTSEA was notable in several respects. First, it was complete. Second, it was fast; the blocking rate constant at 1 mM ($k_{+1} 50 \text{ M}^{-1}\text{s}^{-1}$) was comparable with that seen for the most externally located I328C. Third, it did not occur, or at least occurred only to a negligible extent, if the channel was not opened by ATP during the presence of MTSEA (Figure 5). These findings strongly imply that D349 is located on the intracellular side of the channel ‘gate’; presumably it is not accessible to MTSET and MTSES because of their much lower permeability through the open channel. Although channel opening was required for inhibition by MTSEA at D349C, it was not needed for inhibition at L338C. This places the ‘gate’ between these residues. A fourth unique feature of the action of MTSEA on cells expressing D349C was the absence of the quickly developing, sustained inward current that was seen for the other ‘accessible’ substitutions.

The results with cysteines at S340 and G342 were surprising. It is difficult to conceive of reasons why the current should be increased by sulfhydryl modification within the pore. One possible explanation might be that these modifications remove the site of action of an endogenous blocking particle. Another possibility is that these substitutions lead to significant structural perturbations within or among the subunits; this would seem surprising for the relatively minor replacement of an oxygen atom by a sulfur atom in the case of S340C, but clearly it will be important to introduce other residues at these positions and carry out further experiments on the permeability of the mutated channels.

The present experiments have identified three sites within the outer vestibule of the P2X₂ receptor which are accessible to water-soluble, sulfhydryl-modifying reagents of either charge. These residues are located near the N-terminal boundary of the second hydrophobic region of the molecule (I328, N333, T336), and this therefore provides strong support for this aspect of the proposed topology (Figure 1A). I328 is completely conserved among seven P2X receptors, whereas N333 and T336 are substituted by other polar residues in some other receptors. The rectification seen for the modification at T336 suggests a location at the outer mouth of the pore. Four further residues, deeper within the same hydrophobic segment, were also accessible but only to the smaller permeant reagent MTSEA. Among other P2X receptors, there is poor conservation of amino acids at the positions equivalent to L338 (which can be I, L, F or A) and S340 (which can be I, S, L, W or Y) which might be unexpected for a residue in a critical part of the permeation path. However,

G342 and D349 are conserved among all seven P2X receptors. The alternation of accessible with unaffected residues from T336 to G342 might be expected from β -sheeted secondary structure. Secondary structure prediction algorithms such as Chou and Fasman (1974) predict a β sheet for this segment.

In summary, these results suggest that side-chains of certain residues in the second hydrophobic segment of the receptor are exposed within the ionic pore, but they do not support the hypothesis that the pore is formed as the polar face of an amphipathic helix (such as was found for the nicotinic receptor; Akabas *et al.*, 1994). The experiments have succeeded in identifying a residue lying internal to the channel 'gate'. The relatively small number of cysteine substitutions which provided 'hits' with MTS reagents is suggestive that other parts of the P2X receptor molecule, as yet unknown, also contribute to the lining of the pore. These could be in the nature of re-entrant loops as found in potassium channels (MacKinnon, 1995).

Materials and methods

P2X₂ receptor cDNA

A P2X₂ cDNA carrying a C-terminus epitope was used in all experiments. Briefly, the entire coding region of P2X₂ (gift from Dr D.Julius, University of California at San Francisco) was amplified by PCR. Two modifications were introduced through the amplification primers. The nucleotide sequence surrounding the initiation ATG was changed to match the Kozak sequence, and this resulted in the first two amino acids being Met-Gly in place of Met-Val. The 3' end of the reading frame was modified to incorporate 11 additional amino acids (DPGLNEYMPME) at the C-terminus. This antigenic tag (EYMPME) was used for protein detection (Grussenmeyer *et al.*, 1985). The amplified product was subcloned in pcDNA3 (Invitrogen) and fully sequenced on both strands.

Mutagenesis

A silent *Xho*I site was introduced so as to create a 188 bp *Clal*-*Xho*I cassette for mutagenesis; this changed the codon for Ser377 (after the second hydrophobic domain) from TCA to TCG. Mutations were introduced through the amplification primers using *Pfu* DNA polymerase (Stratagene). Mutagenized plasmids were digested with *Clal* plus *Xho*I and the 188 bp fragments were subcloned into the wild-type P2X₂ plasmid. All mutations were sequenced on both strands.

Electrophysiology

Methods of transient transfection of HEK293 cells with P2X₂ receptor cDNAs have been described in detail previously (Evans *et al.*, 1995, 1996; Buell *et al.*, 1996b). Each set of transfections consisted of four mutations and one wild-type receptor. Whole-cell recordings were obtained 12–24 h after transfection. Patch pipettes (5–7 M Ω) contained 154 mM NaCl, 10 mM EGTA and 10 mM HEPES except for experiments with MTSEA in which case 5–10 mM cysteine was added to the internal solution. External solution was: 154 mM NaCl, 2 mM CaCl₂, 2 mM KCl, 1 MgCl₂, 10 mM glucose and 10 mM HEPES. Osmolarity and pH of solutions were 300 mosmol/l and 7.3 respectively. ATP was applied by the fast-flow U-tube delivery system; the methanethiosulfonates were added to both superfusate and fast-flow delivery system except where indicated in results. (2-Aminoethyl)methanethiosulfonate hydrobromide (MTSEA); [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET); and sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES) were obtained from Toronto Research Chemical Inc. (Ontario, Canada); they were dissolved in external solution immediately before (2–5 min) use. Results are shown as mean $\pm$ s.e.m. throughout. ATP concentrations [ATP] evoking half-maximal currents (EC₅₀) were estimated on individual cells by least-squares fitting to $E = [\text{ATP}]^n / (\text{EC}_{50}^n + [\text{ATP}]^n)$ (unconstrained n) where E is current as a fraction of the maximum current; data are presented as the mean these EC₅₀ values $\pm$ s.e.m.

The relative permeability of the wild-type channel to MTSEA ($P_{\text{MTSEA}}/P_{\text{Na}}$) was determined from the reversal potential of the ATP-induced current (E_r) and the previously determined value of $P_{\text{NMDG}}/P_{\text{Na}}$ (0.03; Evans *et al.*, 1996) from $(P_{\text{MTSEA}}/P_{\text{Na}})[\text{MTSEA}]_o + (P_{\text{NMDG}}/P_{\text{Na}})$

$[\text{NMDG}]_o - [\text{Na}]_i \exp(E_r F/RT) = 0$, where the extracellular solution contained MTSEA (100 mM), NMDG (20 mM), histidine (20 mM) and glucose (10 mM). A similar expression was used for the MTSET, and in those experiments the NMDG concentration was 30 mM.

Immunohistochemistry

At 48 h after transfection, coverslips containing transfected cells were fixed (4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 6.9) for 20 min, washed and stored in physiological saline solution. Cells were placed in the primary antibody (mouse monoclonal anti-EYMPME, 1:10 dilution) overnight at room temperature, washed and incubated in the secondary antibody (fluorescein isothiocyanate-conjugated donkey anti-mouse IgG) at 1:1000 dilution for 2 h, washed and viewed with a Zeiss Axiovert fluorescence microscope.

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