Identification of Amino Acid Residues Contributing to the ATP-binding Site of a Purinergic P2X Receptor*

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P2X receptor subunits have intracellular N and C termini, two membrane-spanning domains, and an extracellular loop of about 280 amino acids. We expressed the rat P2X2 receptor in human embryonic kidney cells, and used alanine-scanning mutagenesis on 30 residues with polar side chains conserved among the seven rat P2X receptor subunits. This identified a region proximal to the first transmembrane domain which contained 2 lysine residues that were critical for the action of ATP (Lys69 and Lys71). We substituted cysteines in this region (Asp57 to Asp71) and found that for S65C and I67C ATP-evoked currents were inhibited by methanethiosulfonates. At I67C, the inhibition by negatively charged ethylsulfonate and pentylsulfonate derivatives could be overcome by increasing the ATP concentration, consistent with a reduced affinity of ATP binding. The inhibitory action of the methanethiosulfonates was prevented by pre-exposure to ATP, suggesting occlusion of the binding site. Finally, introduction of negative charges into the receptor by mutagenesis at this position (I67E and I67D) also gave receptors in which the ATP concentration-response curve was right-shifted. The results suggest that residues close to Ile67 contribute to the ATP-binding site.

P2X receptors are ligand-gated ion channels in the plasma membrane (1). They are homomeric or heteromeric proteins, formed by assembly of subunits named P2X1-P2X7. Current evidence suggests that three subunits form a channel (or a trimer, two membrane-spanning domains, and an extracellular loop of about 280 amino acids. We expressed the rat P2X2 receptor in human embryonic kidney cells, and used alanine-scanning mutagenesis on 30 residues with polar side chains conserved among the seven rat P2X receptor subunits. This identified a region proximal to the first transmembrane domain which contained 2 lysine residues that were critical for the action of ATP (Lys69 and Lys71). We substituted cysteines in this region (Asp57 to Asp71) and found that for S65C and I67C ATP-evoked currents were inhibited by methanethiosulfonates. At I67C, the inhibition by negatively charged ethylsulfonate and pentylsulfonate derivatives could be overcome by increasing the ATP concentration, consistent with a reduced affinity of ATP binding. The inhibitory action of the methanethiosulfonates was prevented by pre-exposure to ATP, suggesting occlusion of the binding site. Finally, introduction of negative charges into the receptor by mutagenesis at this position (I67E and I67D) also gave receptors in which the ATP concentration-response curve was right-shifted. The results suggest that residues close to Ile67 contribute to the ATP-binding site.

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introduced through the amplification primers using *Pfu* DNA polymerase (Stratagene) and digestion of the parental template DNA by DpnI (New England Biolab). Fragments of DNA carrying the mutation were excised with appropriate restriction enzymes and subcloned into the wild type P2X<sub>2</sub> plasmid. All mutants were sequenced on both strands.

**Electrophysiology**—Figure 1 shows the alignment of HEK293 cells transiently transfected with P2X<sub>2</sub> receptor cDNAs have been described (11, 15, 16). Whole cell recordings were obtained 12–36 h after transfection. Patch pipettes (5–7 MΩ) contained (mM) 145 NaF, 10 EGTA, and 10 HEPES. External solution was made: 147 NaCl, 2 CaCl<sub>2</sub>, 2 KCl, 1 MgCl<sub>2</sub>, 13 glucose, and 10 HEPES; the solution used for the experiments shown in Fig. 6 contained 1 mM EGTA. Osmolarity and pH of solutions were 300–315 and 7.3, respectively. ATP and MTS compounds were applied by fast-flow using the RSC 200 solution delivery system (Biologic Science Instruments, Grenoble, France). The MTS reagents used were obtained from Toronto Research Chemicals (Ontario, Canada): they were (2-aminoethyl) methanethiosulfonate hydrobromide (MTSEA), (2-trimethylammonium)ethyl methanethiosulfonate (MTSET), sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES), sodium (3-sulfonatopropyl)methanethiosulfonate (MTSPS), sodium (5-sulfonatopentyl)methanethiosulfonate (MTSPES), and propyl methanethiosulfonate (MTSP). MTSP (a liquid) was used from a 1 M stock solution in dimethyl sulfoxide; stock solutions (100 mM) for the other MTS compounds were made daily by dissolving the solid in control external solution, kept at 4°C, and diluted to 1 mM immediately prior (2–5 min) to their application.

Results are shown as mean ± S.E. throughout. ATP concentrations ([ATP]) evoking half-maximal currents (EC<sub>50</sub>) were estimated on individual cells by least-squares fitting to $E = [\text{ATP}] / ([\text{ATP}] + [\text{ATP}]^n)$ (unconstrained $n$), where $E$ is current as a fraction of the maximum current; data are presented as the means of these EC<sub>50</sub> values ± S.E. In these experiments, ATP was applied briefly (2 s) to avoid the possible complications of permeability changes that sometimes occurs with longer applications (17, 18). The usual protocol for measuring inhibition by MTS compounds was as follows: ATP (30 μM) was applied at 2 min intervals throughout the experiment; when the current amplitude was stable (~±5%) the MTS reagent was applied (1–8 min as indicated in text and Abbreviations); repeated applications (μM, 2 min intervals); the currents evoked typically decline over a period of 10–20 min by 10–15% (7). We corrected for this decline when calculating ATP dose-response curves before and in the presence of the MTS reagents (e.g. Fig. 5). Untransfected HEK cells were tested for their sensitivity to micromolar concentrations of ATP; concentrations ≥5 mM evoked no inward currents ($n > 40$). 10 and 30 mM ATP evoked currents of 104 ± 22 pA ($n = 8$; range 20–300 pA) and 350 ± 83 pA ($n = 10$; range 40–540 pA), respectively. Maximal currents in HEK cells transfected with wild-type and functional mutated P2X<sub>2</sub> receptors ranged from 1 to 12 nA. Therefore, currents in response to 10 or 30 mM ATP which were less than 1 nA were considered not to arise from the expressed P2X<sub>2</sub> receptor and are not included in the results. ATP was applied as the sodium salt in all experiments described, but initial experiments showed no significant differences in response to ATP (1 μM to 10 mM) when applied as the magnesium salt. Tests of significance were by Student’s t test or non-parametric Mann-Whitney test using GraphPad InStat software (GraphPad, San Diego, CA). Results were considered significant for $p < 0.01$.

**Immunohistochemistry**—Immunohistochemical methods were described previously (7). The mouse monoclonal anti-EYMPME antibody (used at 1:1000 dilution) was obtained from BabCo (Richmond, CA), secondary antibody was TRITC-conjugated anti-mouse IgG (1:100 dilution) (Sigma). We quantitated membrane-localized immunofluorescence using NIH Image software by measuring fluorescence intensity density (to 30 mM ATP) were not significantly different from currents evoked by 30 μM ATP at the wild-type receptor; for all the mutant channels membrane expression of the channels appeared normal when assessed by immunohistochemistry. Positively charged residues might be expected to participate in ATP binding, so we focussed our attention on these six posi-
tions at which alanine substitution had a marked effect (Fig. 2B, left).

**Lysine/Arginine Substitutions**—We tested the relative importance of charge and structure at these positions by testing lysine to arginine, and arginine to lysine, substitutions (Fig. 2). For K188R the ATP sensitivity was close to wild-type; K71R, R290K, and R304K showed up to 10-fold shifts in sensitivity to ATP. For K308R and K69R the shifts were larger, about 30- and 300-fold; for these mutations maximal currents (to 10 or 30 mM ATP) were not significantly different from currents to 30 μM ATP at the wild-type receptor (n = 5–11).

We also tested the sensitivity of the mutated receptors which exhibited a significant shift in the ATP dose-response curve (Figs. 1B and 2B; K69R, K71A, Q108A, T184A, K188A, N288A, R290A, R304A, and K308R) to αβ-methylene-ATP, βγ-methylene-ATP, AMP-PNP (each at 300 μM), GTP, ITP, and ADP (each at 1 μM); no agonist-evoked currents were observed in any instance.

Results obtained from both the alanine scanning, the Lys to Arg mutations, and the Arg to Lys mutations, allowed us to arrange the positively charged residues in the order in which they tolerated alanine or arginine substitutions from Lys69 (least tolerant), through Lys 308, Arg290, Arg304, Lys71, Lys188, to Lys53 and Lys324 (most tolerant). We therefore selected the region around Lys69 for further study.

**Substituted Cysteine Accessibility from Asp 57 to Lys 71**—All the P2X receptors have glycine in the position corresponding to Gly72 of P2X2, but the region further downstream is poorly conserved among the seven subunits and we reasoned that it was unlikely to be involved in ATP binding. Therefore, we studied the conserved region to the N-terminal side of Lys71. We changed each residue individually to cysteine, and expressed the cDNAs in HEK293 cells. In the case of K69C no currents were evoked by ATP (even at 30 mM), although subsequent immunohistochemistry showed good membrane expression. ATP elicited currents in K71C but the EC50 was shifted to the right by approximately 1000-fold (7300 ± 840 μM, n = 7) compared with the wild-type receptor (8 ± 1 μM, n = 6). For the other 13 cDNAs, cells expressed currents in response to ATP (30 μM) which were not significantly different from wild-type (n = 3–14 for each mutation). Fig. 3A summarizes the results with MTSET at each of the positions; in 11 positions there was little (<30%) or no effect on the current evoked by ATP.

The two positions that were the most sensitive to inhibition by MTSET were Ser65 and Ile67, close to the lysines identified previously. We examined them further with MTS analogs of differing charge and size, using ethylamine-MTS (MTSEA;
small, positively charged, head group) and the sulfonatoethyl- MTS (MTSES; intermediate size, negatively charged head group) in addition to the MTSET (ethyltrimethylammonium-MTS; MTSET; larger, permanently positively charged, head group). As a control, we also included cells transfected with the T336C mutation, which gives 70–95% inhibition with all three reagents (7). Fig. 3 shows the results for applying MTSEA, MTSET, and MTSES. In the case of I67C, the MTSES caused about 80% inhibition, and MTSET caused about 50% inhibition; it was striking that MTSEA had much less effect (20%). This result suggested that both size and charge might be involved in the inhibitory action of the attached methanethiosulfonate side chain at this position, and we therefore extended these studies to further MTS derivatives.

**Methanethiosulfonate Inhibition at I67C**—In the case of MTSEA, the inhibition was so small that onset kinetics could not be reliably measured (Fig. 4). However, the failure to block seems unlikely to result from slow onset kinetics, because MTSEA (1 mM) applied for 4 min completely prevented the subsequent application of MTSES. B, summary data for kinetics of onset of inhibition by the MTS derivatives. Solid symbols are positively charged or neutral analogs, open symbols are negatively charged. Lines are fitted exponentials (see text for time constants), but these are obviously unreliable for MTSEA and MTSP (n = 3–14 cells for each case).

These findings might suggest that the methanethiosulfonate association rate might be influenced by the proximity of positive charge on the receptor, such as Lys69 or Lys71. Therefore, we extended the observations to a neutral derivative MTSP; in this case the block was even more rapid, being essentially complete at the first time tested (30 s) (Fig. 4B).

We considered the possibility that the cysteine side chain at position 67 lies within the membrane electric field; however, this seems unlikely because the degree of inhibition by MTSES measured at 8 min was not significantly different when the cell was held at −100 mV (n = 3), −60 mV (n = 14), or at −20 mV (n = 4). The current-voltage relation was also measured with voltage ramps (from −120 to 40 mV) at different times during the development of the block by MTSES; the current was inhibited without change in its voltage dependence.

Three general classes of mechanism might be considered for the inhibition of the ATP-evoked current by a methanethiosulfonate moiety attached at I67C. First, the MTS side chain might occupy and therefore occlude the ATP-binding site. Second, the I67C might be remote from the ATP-binding site, but the attachment of the MTS moiety might impair the conformational change leading from ATP binding to channel opening. Third, the MTS side chain might enter and occlude the permeation pathway. This seems to be unlikely because the fastest and most effective blockers were the neutral and negatively charged MTS derivatives, and these would not be expected to enter the cation-selective channel (7).

We carried out experiments that might help to distinguish between the first two mechanisms. For these experiments we used primarily MTSES (negatively charged) and MTSP (neutral), because at 1 mM they gave a rapid and almost complete inhibition of the current (Fig. 4B). First, we asked whether the inhibition by the attached methanethiosulfonate could be surmounted by increasing the ATP concentration. Concentration-response curves for ATP were determined after the inhibition by the MTS derivative had become maximal. The results were different for MTS derivatives of different charges. For the neutral compound (MTSP), the ATP concentration-response curve had a clearly depressed maximum (Fig. 5). A similar result was obtained also for the positively charged analogs (Fig. 5). This type of non-surmountable inhibition was also very clearly seen for the T336C mutation (Fig. 5), which we have previously shown to be close to the permeation pathway (7). In contrast, for the negatively charged MTSES (and MTSPS and MTSPES), increasing the concentration of ATP was able to restore the initial current (Fig. 5). This parallel shift in the concentration-response curve is most simply interpreted as a reduction in the affinity of the closed channel for ATP.

Second, we asked whether co-application of ATP could prevent MTSPES from exerting its inhibitory effect. We chose MTSPES for these experiments because the onset of inhibition is sufficiently rapid that a 1-min application could be used. We used 100 μM ATP in order to maximize receptor occupancy. When ATP was applied before and during the MTSPES, there was no inhibition of the current (Fig. 6).

**Other Charged Substitutions at Ile67**—Our observation that the introduction of a negatively charged MTS derivative (MTSES, MTSPS, and MTSPES) at I67C results in a right-shifted ATP concentration-response curve is most simply interpreted as a decrease in ATP binding affinity. In this case, one might expect the same result by altering the protein to incorporate permanently a negative charge at this position, by substitution of aspartate or glutamate. Fig. 7A shows that I67D and I67E had concentration-response curves that were also more than 10-fold right-shifted with respect to the wild-type receptor. In contrast, no right shift was observed with I67K (which most
closely resembles attached MTSEA) or I67R. HEK cells expressing the I67R and I67K mutations had consistently smaller peak current density than wild-type and approximately similar to the degree of steady-state inhibition observed for I67C in the presence of MTSET and MTSEA (Fig. 5), whereas I67D and I67E had peak current density not different from wild-type values (Fig. 7B).

**DISCUSSION**

Charged and Polar Residues—Hydrogen bonding with charged or polar residues is expected to play a key role in the binding of ATP to the P2X receptor. The results of the alanine scanning draw attention to some residues that might be important and others which are probably not (Fig. 1). For seven of the 30 positions (Gln56, Lys69, Glu85, Thr100, Asn202, Asp261, Lys308: asterisks in Fig. 1B), no current could be evoked by ATP; in each case, immunohistochemical testing indicated that there was good receptor expression at the cell membrane. For 15 of the 30 positions, the sensitivity to ATP was not greatly different from those of the wild-type channels (Lys53, Glu59, Asp82, Ser94, Thr105, Glu115, Asp127, Thr144, Thr157, Glu159, Glu167, Asn189, Lys293, Asp315, Lys324: open bars in Fig. 1B). There is variability in the EC50 for ATP of the wild-type receptor, and we have allocated to this group those positions in which the means differ by less than an order of magnitude; in one position (Asp315) the substitution of alanine actually increased the sensitivity to ATP. The remaining eight positions include Lys 71, Gln108, Thr184, Lys188, Asp259, Asn288, Arg290, and Arg304: cells expressing these receptors showed a larger right shift in the ATP concentration-response curve ranging from 30–1000-fold (filled bars in Fig. 1B). Negatively charged side chains are often involved in coordinating the magnesium in proteins which bind MgATP. They are also found in the vestibules and selectivity filters of some cation-selective channels (19). The positions Glu85, Asp259, and Asp261 might be worth further study in these respects.

Positively charged residues usually contribute to the binding of the α and γ phosphates of ATP, and these are most commonly lysine (20, 21). The observation that the P2Xr receptor tolerates substitution with alanine at Lys53, Lys254, and Lys293 suggest that these positions are not critical for ATP recognition. On the other hand, Lys69, Lys71, Lys188, and Lys308 are possible candidates. Maximal current amplitudes in all the lysine substitutions which caused a reduced sensitivity to ATP (K188A, K71A, K71R, K308R, and K69R: Fig. 2) were not significantly different from wild-type cells. Our immunohisto-
chemical observations showed little difference in the membrane-localized immunofluorescence.

Modifications at Ile<sup>67</sup>—The substituted cysteine accessibility method has the advantage that it allows one to introduce side chains of varying charges and head group sizes. The disadvantages are of two sorts. First, a negative result cannot be interpreted; the methanethiosulfonate may bind and give no detectable alteration of properties, or the cysteine may be inaccessible to methanethiosulfonates in aqueous solution. Second, a positive result cannot be interpreted in a functional sense without further experiments. In particular, studies are needed to determine whether the modified cysteine lies within the permeation pathway, at the ligand-binding sites, or simply at one of many sites other sites where it may inhibit the coupling from ligand binding to channel opening.

Direct introduction of cysteine was unhelpful at Lys<sup>69</sup> and Lys<sup>71</sup>. These mutations gave results similar to the K69A and K71A forms; no detectable currents for K69C and a 1000-fold right shift in the ATP concentration-response curve for K71C. The large ATP concentrations required for K71C made it impractical to study the actions of methanethiosulfonates. The simple experiment of trying to modify the residue to resemble a lysine, by applying MTSEA, produced only a small left shift in the concentration-response curve (data not shown). The sequence to the C-terminal side of Lys<sup>71</sup> is poorly conserved; furthermore, secondary protein structure prediction algorithms indicate that this section of the protein has high probability of forming a β-strand. Such a conformation would be consistent with the pattern of “hits” that was observed; both S65C and I67C were strongly inhibited by methanethiosulfonates.

Two kinds of information might be obtained from the inhibition of current by methanethiosulfonates. The first relates to the rate of block. With 2 lysine residues close to the cysteine side chain in I67C it is possible that coulombic forces will influence the association between the MTS derivative and the side chain at I67C it is possible that coulombic forces will influence the association between the MTS derivative and the side chain in I67C and 167E as indicated; currents plotted as percent maximum in each cell. B, concentration-response curve plotted as current density (μA/pF). These experiments were performed at the same time after simultaneous transfections so as to allow direct comparison of functional expression. Each point is the mean ± S.E. of four to eight experiments.

MTSEA, the inhibition was so small that onset kinetics could not be determined; however, it appears that the MTSEA has substantially attached within a 4-min application, because this exposure protected the receptor from inhibition by a subsequent application of MTSES (Fig. 4). The block with all the analogs was irreversible within the ensuing 8–10 min. This provides the opportunity to obtain the second kind of information, from the properties of the “blocked” receptor. The difference between the steady-state inhibition of the current observed with MTSEA and MTSET on the one hand, and MTSP, MTSES, and MTSPES on the other, indicates that both steric factors and charge might play a role in the actions of the MTS moieties.

If this region forms as a β-sheet, then the distance between the two α carbon atoms of Lys<sup>69</sup> and Lys<sup>71</sup> would be about 7 Å. This is less than the Debye length in physiological solution (about 9 Å) (22), implying significant interaction between the lysine head groups. Thus, the introduction of a charged side chain at Ile<sup>67</sup> might be expected to influence the local charge distribution. A negatively charged side chain was introduced either by mutation of aspartate or glutamate, or by exposure to MTSES, MTSPS, or MTSPES. In each case, this resulted in a parallel right shift in the ATP concentration-response curve with little or no inhibition of the peak current (Figs. 5 and 7). The finding of an essentially similar effect by introducing five different negatively charged side chains at this position either by mutagenesis (-CH<sub>2</sub>-COOH and -(CH<sub>2</sub>)<sub>2</sub>-COOH) or cysteine modification (CH<sub>2</sub>-S-(CH<sub>2</sub>)<sub>2</sub>-SO<sub>3</sub>, CH<sub>2</sub>-S-(CH<sub>2</sub>)<sub>3</sub>-SO<sub>3</sub>, and CH<sub>2</sub>-S-(CH<sub>2</sub>)<sub>5</sub>-SO<sub>3</sub>) strongly supports the interpretation that negativity rather than size is important. Conversely, a positively charged side chain was introduced by mutation to lysine or arginine (-CH<sub>2</sub>-NH<sub>2</sub> and -(CH<sub>2</sub>)<sub>2</sub>-NH-C(NH)<sub>2</sub>) or exposure to MTSEA or MTSET (-CH<sub>2</sub>-S-(CH<sub>2</sub>)<sub>2</sub>-NH<sub>2</sub> and -(CH<sub>2</sub>)<sub>2</sub>-S-(CH<sub>2</sub>)<sub>2</sub>-N(CH<sub>3</sub>)<sub>2</sub>). This caused no right shift in the dose-response curves, but a reduced maximum current. A similar picture was observed with the neutral analog MTSP (side chain: -CH<sub>2</sub>-S-(CH<sub>2</sub>)<sub>5</sub>-CH<sub>3</sub>.)

The protection experiment, in which pre-application of ATP prevented the inhibition by the MTS reagent, is admittedly imperfect. The ATP had, of course, opened (and even desensitized) the channel; this conformational change may have buried the cysteine at position 67 even if were far removed from the ATP-binding site. In this experiment, ideally one would prefer to use a structural analog of ATP that is known to be a competitive antagonist, but none exist. On the other hand, the
inhibition by MTSPEs was completely blocked by pre-exposure to ATP; this is certainly consistent with occlusion of the MTSPEs-binding site (Fig. 6).

The Binding-gating Problem—It is notoriously difficult to determine whether a mutation or modification of a channel or receptor protein affects the binding of ligand to the closed state (often termed affinity) or alters the succession of conformational changes that lead from this binding to channel opening (thought of as efficacy). With studies at the whole cell level it is first necessary to show that the membrane expression of the protein is unaffected by the mutation. The majority of the present work reports cysteine modifications; in this case the expression density is not a problem, because each cell serves as its own control. The experiments with mutations I67D, I67E, I67K, and I67R were done by parallel transfection at the same time, and we will assume that equivalent peak currents (pA/pF) reflect equivalent membrane expression. We cannot interpret the results of the alanine substitutions or Lys to Arg and Arg to Lys exchanges in a mechanistic sense without further evidence for unaltered expression, although our immunohistochemical studies did show that even cells expressing channels at which ATP elicits no current, or small currents, still exhibit normal membrane immunofluorescence.

There are two main lines of evidence that the region around Ile67 is involved in binding rather than gating. The first is that the effect of some modifications could be overcome by increasing the ATP concentration. This is true for the mutations I67D and I67E, and for the attachment of any negatively charged MTS compounds to I67C. All these rather different manipulations might increase the surface negativity of the binding pocket. Other modifications at these positions, such as the introduction of neutral or positively charged side chains, led to an inhibition of the current that could not be surmounted by increasing the ATP concentration. Whereas a depression of peak response clearly implies a modification of gating, it is not the case that a direct effect on binding is proved by a right-ward shift in the ATP concentration-response curve. Colquhoun (23) has recently drawn attention to this in his review of the binding-gating problem. This is a particular problem in the case of ion channel agonists which evoke close to maximum channel opening during each occupancy of the binding site (i.e. large βα, they are highly efficacious). The available information suggests that ATP is not a particularly efficacious agonist at P2X2 receptors; Ding and Sachs (24) show that the maximum value of pα at saturating ATP concentrations is about 0.6 (24). The ambiguity in interpreting parallel shifts in concentration-response curves is more serious for monomeric than multimeric receptors (23, 25); strong evidence suggests that channel opening in P2X2 receptors requires the binding of at least two ATP molecules (24). Both of these general considerations tend to support the interpretation that the present observations indicate an impairment of binding. More specifically, the findings that quite different effects on the ATP dose-response curves are observed to result from an alteration of charge at a single position are difficult to reconcile with an effect on gating. It is conceivable, for example, that the introduction of a positive charge (MTSEA, MTSET, I67K, I67R) leads to salt bridge formation, and that this must be broken for the channel to open. This would inhibit gating. In such a scenario, introduction of negative charge would be expected to have no effect, rather than the surmountable inhibition that was observed.

The second line of evidence for binding site involvement follows from the observation that MTS attachment to the cysteine at I67C does not occur when a high concentration of ATP is present; the limitations of this experiment have been noted. In summary, the present experiments have identified polar residues in the P2X2 receptor ectodomain that are necessary for normal function, and our results with mutagenesis and methanthiosulfonates strongly support the interpretation that the region close to Ile67 is involved in forming the ATP binding pocket.

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Note Added in Proof—Similar results with respect to the mutation of the Arg and Lys residues have recently been reported for the P2X receptor (Ennion, S., Hagan, S., and Evans, R. J. (2000) J. Biol. Chem. 275, 29361–29367.

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