Role of ectodomain lysines in the subunits of the heteromeric P2X$_{2/3}$ receptor

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ABBREVIATIONS: αβmeATP, αβ-methylene-ATP
Abstract

Lysine residues near each end of the receptor ectodomain (in rat P2X2 Lys$^{69}$ and Lys$^{308}$) have been implicated in ATP binding to P2X receptors. We recorded membrane currents from human embryonic kidney cells expressing P2X subunits, and found that lysine to alanine substitutions at equivalent positions in the P2X3 receptor (Lys$^{63}$ and Lys$^{299}$) also prevented channel function. Heteromeric P2X$_{2/3}$ receptors are formed when P2X$_2$ and P2X$_3$ subunits are expressed together; they can be distinguished by their relatively sustained response to $\alpha\beta$-methylene-ATP. By co-expression of wild type P2X$_3$ and mutated P2X$_2$ subunit, we found that the heteromeric P2X$_{2/3}$ channel functioned normally when either lysine in the P2X$_2$ subunit was mutated to alanine (i.e. [K69A] or [K308A]), but not when both lysines were mutated to alanine (i.e. [K69A, K308A]). However, co-expression of wild type P2X$_2$ with a mutated P2X$_3$ subunit ([K68A] or [K299A]) produced no functional heteromers. The rescue of the single lysine mutant P2X$_2$ subunit by wild type P2X$_3$ (but not the converse) suggests that the heteromeric channel contains one P2X$_2$ and two P2X$_3$ subunits, and that the receptor functions essentially normally so long as two subunits are not mutated. The failure to rescue function in the P2X$_2$ subunit with both lysines mutated by wild type P2X$_3$ suggests that these residues from two different subunits interact in agonist binding or channel opening.
The heteromeric P2X$_{2/3}$ receptor is of interest because of its predominant expression on a subset of primary afferent nerves involved in the sensation of chronic noxious damage (Jarvis, 2003), visceral distension (Vlaskovska et al., 2001), hypoxia (Gourine, 2005) and taste (Finger et al., 2005). A key role for these receptor subunits is supported by experiments using P2X$_3$ gene knock-outs (Cockayne et al., 2000; 2005), P2X$_3$ RNA suppression (Barclay et al., 2002; Honoré et al., 2002), and pharmacological antagonists selective for P2X$_3$ subunit-containing receptors (Jarvis et al., 2002).

There is good evidence that P2X subunits form channels as trimers (Nicke et al., 1998; North, 2002; Jiang et al., 2003; Barrera et al., 2005). Like other ligand-gated channels, P2X receptors can form by homo- or heterooligomeric assembly of subunits (for review see North, 2002). Although both P2X$_2$ and P2X$_3$ subunits are able to form homomeric channels, the heteromeric P2X$_{2/3}$ receptor that is also formed can be distinguished by its unique functional properties (Lewis et al., 1996). P2X$_2$ receptors are activated by ATP but not by the analog αβ-methylene-ATP (αβmeATP), and the current is largely sustained through a 2 s agonist application. P2X$_3$ receptors are activated by both ATP and αβmeATP and the current desensitizes very rapidly (<100 ms). The current through heteromeric P2X$_{2/3}$ channels can be identified as the sustained component when αβmeATP is the agonist (Lewis et al., 1996).

The molecular operation of P2X subunits is not well understood. Each receptor subunit has intracellular amino- and carboxy- termini, and two transmembrane domains (TM1: approximately residues 30 to 50; TM2:...
approximately residues 330-354) that are joined by an ectodomain of about
280 residues. The ectodomain is glycosylated and disulfide-bonded. There
are no obvious 'canonical' ATP-binding amino acid sequences in the
ectodomain. In the case of the P2X₂ homomeric receptor, two conserved
ectodomain lysine residues have been suggested to play a role in binding the
negatively charged phosphate moiety of ATP (Lys₆⁹ and Lys³₀⁸ in rat P2X
receptor), with the evidence being most complete for Lys₆⁹ (Jiang et al.,
2000). A similar role for the corresponding lysines has also been identified in
the case of the P2X₁ receptor (Lys₆⁸ and Lys³₀⁹; Ennion et al., 2000). These
lysines are situated about 20 amino acids from the outer end of each TM.
Experiments on the P2X₁ receptor using partial agonists also suggest a role
for aromatic residues Phe¹₈⁵ and Phe²₉¹ to coordinate the binding of the
adenine ring of ATP (Roberts and Evans, 2004; Vial et al., 2004). An
alternative model of ATP binding has been proposed by Yan et al., (2005) in
the case of the P2X₄ receptor. This is based on secondary structure
homology between part of the receptor (Lys¹₈⁰ to Lys³₂⁶) and class II
aminoacyl-tRNA synthetases (Freist et al., 1998).

The present experiments focus on the two juxtamembrane lysines
proposed to take part in ATP binding. We have found that for the heteromeric
P2X₂/₃ channel their importance in channel function depends greatly on
whether they are in the P2X₂ or the P2X₃ subunit.


**Materials and Methods**

**Molecular and cell biology** Rat P2X receptor cDNAs were used as previously described (Jiang et al., 2000). Mutations were introduced using Quickchange site-directed mutagenesis (Stratagene, La Jolla CA) and confirmed by sequencing. Wild type and mutated receptors were transiently expressed in human embryonic kidney (HEK) 293 cells using lipofectamine 2000 (Invitrogen, San Diego CA) according to the manufacturer’s instructions. Transfected cells were harvested and re-plated on glass coverslips 24 - 48 h after transfection and kept at 37º C for 18 - 48 h prior to electrophysiological recording. P2X2 plasmid (0.2 µg) was co-transfected with P2X3 plasmid (1 µg), these ratios having been established to produce equal amounts of protein when detected by Western blot with a common C-terminal epitope tag (Jiang et al., 2003). Enhanced green fluorescent protein cDNA (0.1 µg) was also included. Immunocytochemistry of transfected cells was as described, using a C terminus EYMPME epitope (Jiang et al., 2000, 2003).

**Electrophysiology** Whole-cell patch clamp recordings were made at 19 - 22 ºC using a HEKA EPC9 amplifier (Heka, Lambrecht, Germany). The holding potential was -60 mV. Patch pipettes (3 - 6 MΩ) contained (mM): 145 NaCl, 10 Hepes and 10 EGTA (pH 7.3 with NaOH). The external solution contained (mM): 147 NaCl, 2 KC1, 2 CaCl2, 2 MgCl2, 10 Hepes and 13 glucose (pH 7.3 with NaOH). Both internal and external solutions were 290 - 310 mOsmol/l. Agonists were applied using the RSC 200 rapid solution changer (Biologic Science Instruments, Grenoble, France), with flow-pipe tips approximately 150 µm from cell. All chemicals were purchased from Sigma (Poole, UK), and were made up daily from aliquots stored at -20º C.
Results

Homomeric channels  Previous work on homomeric human P2X$_1$ (Ennion et al., 2000) and rat P2X$_2$ (Jiang et al., 2000) receptors has indicated that Lys$^{69}$ and Lys$^{308}$ (rat P2X$_2$ numbering) are essential for channel function. We found that alanine substitution at the equivalent positions in the P2X$_3$ subunit also led to a non-functional homomeric channel. We observed no current with applications of $\alpha\beta$meATP (up to 1 mM; $n=5$) (Fig. 1) or ATP (up to 1 mM; $n=7$). Immunocytochemistry of transfected cells showed no difference in the pattern or distribution between wild type and mutated subunits. Fig. 1 also shows that equivalent mutations in other rat P2X receptors also prevented any response to agonist (P2X$_1$[K68A]: ATP (300 $\mu$M) $23 \pm 9$ pA, $n=8$; $\alpha\beta$meATP (300 mM) no current, $n=3$; P2X$_4$[K67A]: ATP (1 mM) no current, $n=8$; P2X$_7$[K64A]: ATP (3 mM) no current, $n=3$).

Neither P2X$_2$[K69A] nor P2X$_2$[K308A] gave any detectable current in response to 1 or even 3 mM ATP (Fig. 1). However, co-expression of these two mutated receptors clearly did (Fig. 2). The currents were small compared to wild type P2X$_2$ currents (>600 pA/pF), but not obviously different in other properties (Fig. 2). It was striking that co-expression of two ‘dead’ subunits resulted in formation of a channel that is responsive to 100 or 300 $\mu$M ATP. If we assume that neither mutation affects expression or assembly, then one would expect three-eighths of all trimeric channels to be [K69A]$_2$.[K308A] and three-eighths to be [K69A].[K308A]$_2$. This result therefore indicates that the effects of these two point mutations are not independent.

Heteromeric channels  For the P2X$_{2/3}$ receptors there was a striking difference in the effect of a single point mutation of either of the lysine
residues, depending whether it was present in the P2X2 or P2X3 subunit. The currents in cells expressing mutant P2X2 receptors with wild type P2X3 subunits were essentially the same as those observed in wild type heteromeric receptors (Fig. 3). The αβmeATP concentrations giving half-maximal currents (EC50) were: for P2X2/3 wild type, 27 ± 3.3 µM (n = 5); for P2X2[K69A] + P2X3, 22 ± 2.6 µM (n = 5), and for P2X2[K308A] + P2X3, 44 ± 2.6 µM (n = 8)(the last two values are significantly different, p < 0.0001). In contrast, cells expressing wild type P2X2 with P2X3[K63A] subunits showed no currents in response to αβmeATP up to 100 µM. At such high concentrations P2X2 homomeric receptors are sensitive to αβmeATP (n = 5) (Fig. 3)(also Jiang et al., 2003) and the small current observed was presumably passing through such receptors. Similarly, co-expression of wild type P2X2 subunits with P2X3[K299A] gave no currents in response to αβmeATP (up to 100 µM, n = 6) (Fig. 3). These results indicate that a wild type P2X3 subunit can rescue function when it enters a heteromer with a mutated P2X2 subunit, but not vice versa.

This rescue did not occur when both ectodomain lysines were mutated in the same P2X2 subunit. Co-expression of P2X2[K69A,K308A] with P2X3 produced no sustained currents to αβmeATP (n = 9)(Fig. 3). However, a fast desensitizing current typical of homomeric P2X3 receptors was usually observed (Fig. 3). A schematic summary of the expression of the further P2X2 and P2X3 subunits (either wild type or mutant) that were co-expressed, and the results of the functional studies, is shown in Fig. 4.
Discussion

The two lysines that are the focus of the present study are very highly conserved among P2X subunits. Lys$^{69}$ is present in 41 of 43 vertebrate sequences (including seven human, seven rat, seven mouse, one guinea pig, three chick, two xenopus, one bullfrog, nine zebrafish, six pufferfish); the zebrafish P2X$\_2$ receptor has Asp at this position, and it can not be activated by ATP when heterologously expressed (Kucenas et al., 2003). This lysine is also present in the one invertebrate (schistosome) sequence that is known to operate as an ATP-gated channel (Agboh et al., 2004). Lys$^{308}$ is even more highly conserved, being found in all the above 44 sequences, and also in two of the five related Dictyostelium sequences. Of the eight positively charged residues in the P2X$\_2$ receptor ectodomain, Lys$^{69}$ and Lys$^{308}$ are the two where replacement by alanine causes the greatest loss of channel function: even replacement by arginine results in more than 100-fold reduction in sensitivity to ATP (Jiang et al., 2000). The present experiments confirm that the lysine in the first of these positions is critical also for function of other P2X receptors, including P2X$\_3$.

The heterotrimeric P2X$\_2/3$ receptor must contain either one or two copies of the P2X$\_3$ receptor subunit. The simplest interpretation of the one way rescue of P2X$\_2$ mutants by P2X$\_3$ (but not vice-versa, Fig. 3), is that the P2X$\_2/3$ receptors activated by αβmeATP have two copies of P2X$\_3$ and one copy of P2X$\_2$. In other words, a functional channel can be formed and activated by αβmeATP even though it contains one ‘dead’ P2X$\_2$ subunit (e.g. P2X$\_2[K69A]$) but no opening can be elicited from a receptor comprising two dead P2X$\_3$ subunits with a wild type P2X$\_2$ subunit. The rescue of wild type...
P2X₃ was complete in the case of P2X₂[K69A], and slightly less so for P2X₂[K308A] (Fig. 3), indicating that the two P2X₂ lysines do not have precisely equivalent roles.

This result contrasts with earlier findings in which a highly conserved intracellular C-terminal lysine was mutated (Chaumont et al. 2004). This lysine is required in homomeric P2X₂ and P2X₃ receptors for retention in the plasma membrane. In that case, wild type P2X₃ subunits could restore the appearance and retention in the membrane of mutant P2X₂ subunits (P2X₂[K366A]), and this rescue was fully reciprocal because wild type P2X₂ also restored the appearance of P2X₃[K357A] (Chaumont et al., 2004). Taken together, these results imply that only a single wild type subunit is needed for successful retention in the membrane, but two wild type (P2X₃) subunits are needed for channel function. The conclusion that the functional heteromer contains two P2X₃ subunits and one P2X₂ subunit agrees well with the results of a completely independent approach in which subunits were joined by disulfide bonds between engineered cysteine substitutions at the outer ends of the TMs (Jiang et al., 2003). The finding is also consistent with the observation that the limiting slope of the Hill plot for activation of the heteromeric P2X₂/₃ receptor is close to two, whereas for the homomeric P2X₃ and homomeric P2X₂ receptors it is close to three (Jiang et al. 2003). However, the present analysis can not distinguish between different numbers of ligand binding sites and differing degrees of intersubunit cooperativity in the heteromeric versus the homomeric channels.

For the homomeric P2X₁ and P2X₂ receptors, there is evidence that Lys⁶⁹ and Lys³⁰⁸ contribute to an ATP binding site (Ennion et al., 2000; Jiang...
et al., 2000; Roberts and Evans, 2004). Therefore, our present finding that the heteromeric channel operates normally even though it incorporates a mutated P2X2 subunit may be interpreted in this context. This would imply that a heterotrimeric P2X receptor can open from a less than fully liganded state. For example, the tetrameric AMPA receptor (Rosenmund et al., 1998) and the pentameric glycine receptor (Beato et al., 2002) can open from less than fully liganded states. But other interpretations are possible. For example, the P2X3 (though not P2X2) subunit lysines may be critical for multimeric channel assembly. Or the mutation might impede the conformational change of gating: a lysine to alanine substitution in a single P2X2 subunit of the trimer might provide insufficient energetic impediment, whereas two such independent substitutions in the P2X3 subunits might prevent it completely.

We found surprising the observation that cells co-transfected with P2X2[K69A,K308A] subunit and wild type P2X3 subunit produced no detectable heteromeric current (Fig. 3). In other words, the wild type P2X3 subunits can rescue function in a channel containing a P2X subunit with a single mutation ([K69A] or [K308A]) (a ‘dead’ P2X2) but not in a channel containing both those mutations (a ‘double-dead’ P2X2). This could have a simple energetic explanation of the type introduced above; namely, both P2X3 subunits become liganded by αβmeATP but the mutations provide too great an energy barrier for opening to occur. Or it could indicate that the effects of the lysines are not independent, and that an interaction occurs between Lys69 and Lys308. This would be consistent with the finding with homomeric P2X2
receptors, that ATP can elicit currents when P2X2[K69A] and P2X2[K308A] are co-expressed, but not when either is expressed alone.

If one assumes that at least two agonist binding sites are required for channel activation (see above, and Jiang et al., 2003; Ding and Sachs, 1999), then these observations can be explained if both Lys69 of one subunit and Lys308 of another (or equivalent for the P2X3 subunits) are needed at one ATP binding site. The presence of a ‘dead’ P2X2 subunit would still provide for two αβmeATP binding sites (Fig. 4B, left and middle), but a ‘double-dead’ P2X2 subunit would prevent αβmeATP binding at two sites and therefore prevent channel function (Fig. 4B, right). The interpretation that the agonist site is contributed from two different subunits in a heteromeric channel has parallels with nicotinic acetylcholine receptors (Sine, 2002). Moreover, P2X2 subunits carrying mutations of an allosteric zinc binding site have been covalently joined in tandem from concatenated cDNAs: these experiments indicated that histidines from different subunits contribute to the zinc binding site (Nagaya et al., 2005).

The conclusion that the αβmeATP binding sites forms at the P2X3/P2X3 and P2X2/P2X3 interfaces is consistent with the observations that both nucleotide (Virginio et al. 1998; Burgaard et al., 2000) and non-nucleotide antagonists (Jarvis et al., 2002, 2004) mostly fail to discriminate between P2X3 homomeric receptors and P2X2/3 heteromeric receptors. A more complete understanding of the agonist binding site will inform the further development of small molecules that act as competitive antagonists at the P2X2/3 heteromeric receptors, which are likely to have value as pain-relieving drugs.
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References


novel structural motif of ligand-gated ion channels. *EMBO J* **17**:3016-3028.


Footnotes

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Fig. 1. **A**, Mutation of conserved ectodomain lysines causes loss of function at rat P2X receptors. Pairs of traces show currents at wild type and lysine mutant receptors. Agonists were: for P2X1 αβmeATP (300 µM); for P2X4 ATP (1 mM); and for P2X7 BzATP (300 µM); each applied for 2 s. **B**, Mutation of Lys69 or Lys308 at P2X2 causes a loss of function (ATP, 1 mM), and equivalent mutations to Lys63 and Lys299 in P2X3 have a similar effect (αβmeATP, 300 µM).

Fig. 2. ATP elicits significant currents when lysine-mutant P2X2 subunits are co-expressed (n = 9). However, receptors P2X2[K69A] and P2X2[K308A] expressed separately do not respond to ATP at concentrations up to 3 mM (n = 12). HEK, non-transfected cells (n = 3). Insert shows a representative trace elicited by 1 mM ATP when lysine mutants are co-expressed, scale bars represent 50 pA and 1 s.

Fig. 3 Co-expression of P2X2 and P2X3 subunits. **A**, currents elicited by 2 s application of 30 µM αβmeATP. P2X2[K69A] and P2X2[K308A] can be rescued to form functional channels by expression with wild type P2X3. The corresponding mutations in the P2X3 subunits are not rescued by wild type P2X2 subunits. The double mutant P2X2[K69A,K308A] subunit does not function when co-expressed with wild type P2X3. However, the rapidly-desensitizing homomeric P2X3 responses were typically observed on the first application of αβmeATP. **B**, concentration-response curves for some of the
combinations shown in A. Responses are expressed as a % of the maximal response (mean ± s.e. mean, 5 - 8 cells at each point).

Fig. 4. A, Schematic illustration of the P2X\textsubscript{2} and P2X\textsubscript{3} subunits used, and summary results. Small solid circles indicate positions of lysine to alanine substitution near outer end of TM1 or TM2. B, Schematic interpretation. Channels with two P2X\textsubscript{3} subunits and one P2X\textsubscript{2} subunit might provide two subunit contacts with two lysine residues (arrows, putative binding site), so long as only one of the P2X\textsubscript{2} lysines is substituted by alanine (left, middle) but would have only one such contact when P2X\textsubscript{2} subunit has both lysines changed to alanine.

Notes: \textsuperscript{1}tested with ATP; \textsuperscript{2}tested with αβmeATP; \textsuperscript{3}normal wild type currents were 522 ± 74 pA/pF (n = 9; measured 200 ms after beginning superfusion); \textsuperscript{4}dead indicates no detectable current (<2 pA/pF); \textsuperscript{5}ATP also evoked a sustained current, suggesting that the involvement of these residues is not specific to αβmeATP.
Figure 1

A  P2X₁  P2X₄  P2X₇
   wt  K68A  wt  K67A  wt  K64A
αβmeATP  ATP  ATP  BzATP

B  P2X₂  P2X₃
   wt  K69A  K308A  wt  K63A  K299A
ATP  ATP  ATP  αβmeATP  αβmeATP
Figure 2

The graph shows the current (pA/pF) as a function of ATP concentration (μM). Different lines represent different conditions:
- Solid circles: K69A
- Open squares: K308A
- Triangle: HEK

The inset shows a close-up view of the current response to ATP at low concentration levels. The graph indicates that K69A + K308A shows a significant current response at lower ATP concentrations compared to the other conditions.
Figure 3

A

B

Current (% maximum)

αβmeATP concentration (μM)
Figure 4

A

\(P2X_2\) homomers

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\(P2X_3\) homomers

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B

\(P2X_2[K69A]\) + \(P2X_3\)

\(P2X_2[K308A]\) + \(P2X_3\)

\(P2X_2[K69A,K308A]\) + \(P2X_3\)