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ACCELERATED COMMUNICATION

Role of Ectodomain Lysines in the Subunits of the Heteromeric $P2X_{2/3}$ Receptor

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ABSTRACT

Lysine residues near each end of the receptor ectodomain (in rat $P2X_2$ 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 $P2X_3$ 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 coexpression 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, coexpression 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 as 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 knockouts (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 hetero-oligomeric 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., 1995). $P2X_2$ receptors are activated by ATP but not by the analog $\alpha\beta$-methylene-ATP ($\alpha\beta$meATP), and the current is largely sustained through a 2-s agonist application. $P2X_3$ receptors are activated by both ATP and $\alpha\beta$meATP, and the current desensitizes very rapidly (<100 ms). The current through heteromeric $P2X_{2/3}$ channels can be identified as the sustained component when $\alpha\beta$meATP is the agonist (Lewis et al., 1995).

The molecular operation of P2X subunits is not well understood. Each receptor subunit has intracellular amino and carboxyl termini and two transmembrane domains (TM1: approximately residues 30–50; TM2: approximately residues 330–354) that are joined by an ectodomain of approximately 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 $P2X_2$ homomeric receptor, two conserved ectodomain lysine resi-

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ABBREVIATIONS: $\alpha\beta$meATP, $\alpha\beta$-methylene-ATP

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dues have been suggested to play a role in binding the negatively charged phosphate moiety of ATP (Lys^{69} and Lys^{308} in rat P2X receptor), but the evidence is most complete for Lys^{69} (Jiang et al., 2000). A similar role for the corresponding lysines has also been identified in the case of the $P2X_1$ receptor (Lys^{68} and Lys^{309}; Ennion et al., 2000). These lysines are situated approximately 20 amino acids from the outer end of each TM. Experiments on the $P2X_1$ receptor using partial agonists also suggest a role for aromatic residues Phe^{185} and Phe^{291} 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 $P2X_4$ receptor. This is based on secondary structure homology between part of the receptor (Lys^{180}–Lys^{326}) and class II aminoacyl-tRNA synthetases (Freist et al., 1998).

The experiments reported herein focused on the two juxtamembrane lysines proposed to take part in ATP binding. We have found that, for the heteromeric $P2X_{2/3}$ channel, their importance in channel function depends greatly on whether they are in the $P2X_2$ or the $P2X_3$ subunit.

Materials and Methods

Molecular and Cell Biology. Rat P2X receptor cDNAs were used as described previously (Jiang et al., 2000). Mutations were introduced using QuikChange site-directed mutagenesis (Stratagene, La Jolla CA) and confirmed by sequencing. Wild-type and mutated receptors were transiently expressed in human embryonic kidney 293 cells using Lipofectamine 2000 (Invitrogen, San Diego CA) according to the manufacturer's instructions. Transfected cells were harvested and replated on glass coverslips 24 to 48 h after transfection and kept at 37°C for 18 to 48 h before electrophysiological recording. $P2X_2$ plasmid (0.2 μg) was cotransfected with $P2X_3$ 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-terminal EYMPME epitope (Jiang et al., 2000, 2003).

Electrophysiology. Whole-cell patch-clamp recordings were made at 19 to 22°C using a HEKA EPC9 amplifier (Heka, Lambrecht, Germany). The holding potential was −60 mV. Patch pipettes (3–6 MΩ) contained 145 mM NaCl, 10 mM HEPES, and 10 mM EGTA, pH adjusted to 7.3 with NaOH. The external solution contained 147 mM NaC1, 2 mM KC1, 2 mM $CaC1_2$, 2 mM $MgCl_2$, 10 mM HEPES, and 13 mM glucose, pH adjusted to 7.3 with NaOH. Both internal and external solutions were 290 to 310 mOsM. 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 −20°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 nonfunctional 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. Figure 1 also shows that equivalent mutations in other rat P2X receptors also prevented any response to agonist [$P2X_1$[K68A]: ATP (300 μM) 23 ± 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, coexpression of these two mutated receptors clearly did (Fig. 2). The currents were small compared with wild-type $P2X_2$ currents (>600 pA/pF) but not obviously different in other properties (Fig. 2). It was striking that coexpression of two "dead" subunits resulted in formation of a channel that was responsive to 100 or 300 μM 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.

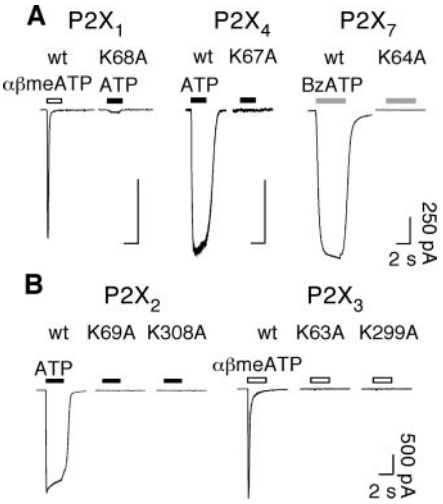

Fig. 1. A, mutation of conserved ectodomain lysines causes loss of function at rat P2X receptors. Pairs of traces show currents at wild-type and lysine mutant receptors. Agonists were: for $P2X_1$, $\alpha\beta$meATP (300 μM); for $P2X_4$, ATP (1 mM); and for $P2X_7$, BzATP (300 μM). Each was applied for 2 s. B, mutation of Lys^{69} or Lys^{308} at $P2X_2$ causes a loss of function (ATP, 1 mM), and equivalent mutations to Lys^{63} and Lys^{299} in $P2X_3$ have a similar effect ($\alpha\beta$meATP, 300 μM).

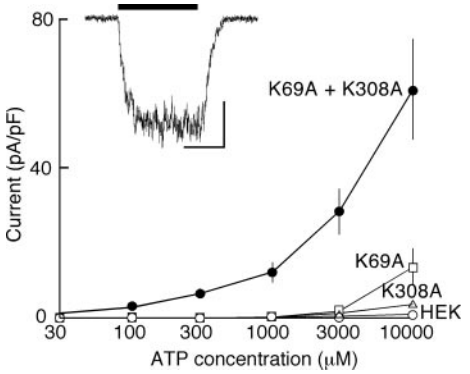

Fig. 2. ATP elicits significant currents when lysine-mutant $P2X_2$ subunits are coexpressed ($n = 9$). However, receptors $P2X_2$[K69A] and $P2X_2$[K308A] expressed separately do not respond to ATP at concentrations up to 3 mM ($n = 12$). Human embryonic kidney, nontransfected cells ($n = 3$). Inset shows a representative trace elicited by 1 mM ATP when lysine mutants are coexpressed; scale bars, 50 pA and 1 s.

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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 $P2X_2$ or $P2X_3$ subunit. The currents in cells expressing mutant $P2X_2$ receptors with wild-type $P2X_3$ subunits were essentially the same as those observed in wild-type heteromeric receptors (Fig. 3). The $\alpha\beta$meATP concentrations giving half-maximal currents (EC_{50}) were: for $P2X_{2/3}$ wild-type, 27 ± 3.3 μM (n = 5); for $P2X_2$[K69A] + $P2X_3$, 22 ± 2.6 μM (n = 5), and for $P2X_2$[K308A] + $P2X_3$, 44 ± 2.6 μM (n = 8) (the last two values are significantly different, $p < 0.0001$). In contrast, cells expressing wild-type $P2X_2$ with $P2X_3$[K63A] subunits showed no currents in response to $\alpha\beta$meATP up to 100 μM. At such high concentrations, $P2X_2$ homomeric receptors are sensitive to $\alpha\beta$meATP (n = 5) (Fig. 3) (see also Jiang et al., 2003), and the small current observed was presumably passing through such receptors. Likewise, coexpression of wild-type $P2X_2$ subunits with $P2X_3$ [K299A] gave no currents in response to $\alpha\beta$meATP (up to 100 μM, n = 6) (Fig. 3). These results indicate that a wild-type $P2X_3$ subunit can rescue function when it enters a heteromer with a mutated $P2X_2$ subunit, but not vice versa.

This rescue did not occur when *both* ectodomain lysines were mutated in the same $P2X_2$ subunit. Coexpression of $P2X_2$ [K69A,K308A] with $P2X_3$ produced no sustained currents to $\alpha\beta$meATP (n = 9) (Fig. 3). However, a fast desensitizing current typical of homomeric $P2X_3$ receptors was usually observed (Fig. 3). A schematic summary of the expression of the further $P2X_2$ and $P2X_3$ subunits (either wild-type or mutant) that were coexpressed, and the results of the functional studies, is shown in Fig. 4.

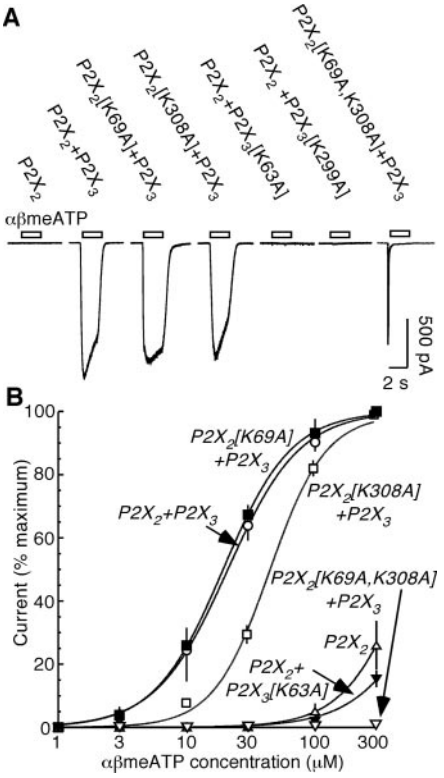

Fig. 3. Coexpression of $P2X_2$ and $P2X_3$ subunits. A, currents elicited by a 2-s application of 30 μM $\alpha\beta$meATP. $P2X_2$[K69A] and $P2X_2$[K308A] can be rescued to form functional channels by expression with wild-type $P2X_3$. The corresponding mutations in the $P2X_3$ subunits are not rescued by wild-type $P2X_2$ subunits. The double-mutant $P2X_2$[K69A,K308A] subunit does not function when coexpressed with wild-type $P2X_3$. However, the rapidly desnsitizing homomeric $P2X_3$ responses were typically observed on the first application of $\alpha\beta$meATP. B, concentration-response curves for some of the combinations shown in A. Responses are expressed as a percentage of the maximal response (mean ± S.E.M., five to eight cells at each point).

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 laevis*, one bullfrog, nine zebrafish, and six pufferfish); the zebrafish $P2X_2$ receptor has Asp at this position, and it cannot 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; it is found in all the above 44 sequences and in two of the five related *Dictyostelium discoideum* 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

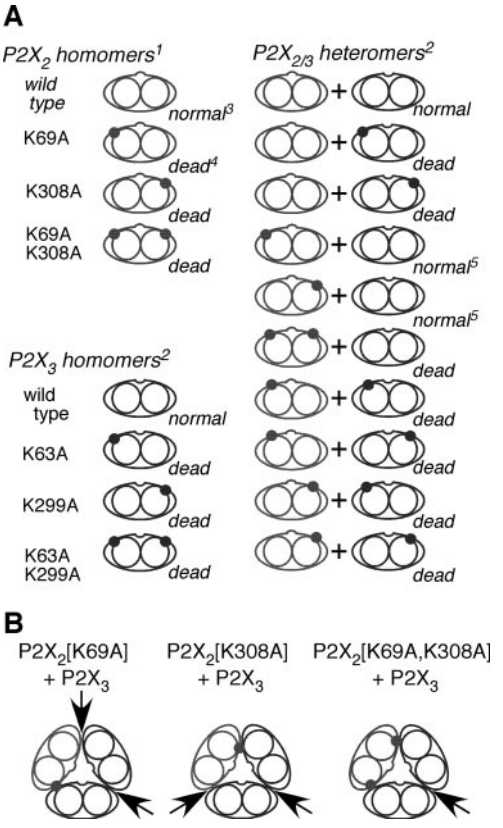

Fig. 4. A, schematic illustration of the $P2X_2$ and $P2X_3$ subunits used, and summary results. Small solid circles indicate positions of lysine-to-alanine substitution near outer end of TM1 or TM2. B, schematic interpretation. Channels with two $P2X_3$ subunits and one $P2X_2$ subunit might provide two subunit contacts with two lysine residues (arrows, putative binding site) if only one of the $P2X_2$ lysines were substituted by alanine (left, middle) but would have only one such contact when $P2X_2$ subunit has both lysines changed to alanine. 1, tested with ATP; 2, tested with $\alpha\beta$meATP; 3, normal wild-type currents were 522 ± 74 pA/pF (n = 9; measured 200 ms after beginning superfusion); 4, dead indicates no detectable current (<2 pA/pF); 5, ATP also evoked a sustained current, suggesting that the involvement of these residues is not specific to $\alpha\beta$meATP.

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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 $\alpha\beta$meATP have two copies of $P2X_3$ and one copy of $P2X_2$. In other words, a functional channel can be formed and activated by $\alpha\beta$meATP 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 $P2X_3$ was complete in the case of $P2X_2$[K69A] and slightly less so for $P2X_2$[K308A] (Fig. 3), indicating that the two $P2X_2$ 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 $P2X_2$ and $P2X_3$ receptors for retention in the plasma membrane. In that case, wild-type $P2X_3$ subunits could restore the appearance and retention in the membrane of mutant $P2X_2$ subunits ($P2X_2$[K366A]), and this rescue was fully reciprocal because wild-type $P2X_2$ also restored the appearance of $P2X_3$[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 ($P2X_3$) subunits are needed for channel function. The conclusion that the functional heteromer contains two $P2X_3$ subunits and one $P2X_2$ 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 $P2X_{2/3}$ receptor is close to two, whereas for the homomeric $P2X_3$ and homomeric $P2X_2$ 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 different degrees of intersubunit cooperativity in the heteromeric versus the homomeric channels.

For the homomeric $P2X_1$ and $P2X_2$ receptors, there is evidence that Lys^{69} and Lys^{308} 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 $P2X_2$ subunit may be interpreted in this context. This would imply that a heterotrimeric P2X receptor could open from a less than fully liganded state. For example, the tetrameric α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid 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 $P2X_3$ (though not $P2X_2$) 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 $P2X_2$ subunit of the trimer might provide insufficient energetic impediment, whereas two such independent substitutions in the $P2X_3$ subunits might prevent it completely.

We were surprised to find that cells cotransfected with $P2X_2$[K69A,K308A] subunit and wild-type $P2X_3$ subunit produced no detectable heteromeric current (Fig. 3). In other words, the wild-type $P2X_3$ subunits can rescue function in a channel containing a P2X subunit with a single mutation ([K69A] or [K308A]) (a dead $P2X_2$) but not in a channel containing both those mutations (a "double-dead" $P2X_2$). This could have a simple energetic explanation of the type introduced above; namely, both $P2X_3$ subunits become liganded by $\alpha\beta$meATP, 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 Lys^{69} and Lys^{308}. This would be consistent with the finding that in homomeric $P2X_2$ receptors, ATP can elicit currents when $P2X_2$[K69A] and $P2X_2$[K308A] are coexpressed, 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 Ding and Sachs, 1999; Jiang et al., 2003), then these observations can be explained if both Lys^{69} of one subunit and Lys^{308} of another (or equivalent for the $P2X_3$ subunits) are needed at one ATP binding site. The presence of a dead $P2X_2$ subunit would still provide for two $\alpha\beta$meATP binding sites (Fig. 4B, left and middle), but a double-dead $P2X_2$ subunit would prevent $\alpha\beta$meATP 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, $P2X_2$ 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 $\alpha\beta$meATP binding sites forms at the $P2X_3/P2X_3$ and $P2X_2/P2X_3$ interfaces is consistent with the observations that both nucleotide (Virginio et al., 1998; Burgard et al., 2000) and non-nucleotide antagonists (Jarvis et al., 2002, 2004) mostly fail to discriminate between $P2X_3$ homomeric receptors and $P2X_{2/3}$ 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 $P2X_{2/3}$ heteromeric receptors; such molecules are likely to have value as pain-relieving drugs.

Acknowledgments

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