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Role of ectodomain lysines in the subunits of the heteromeric $P2X_{2/3}$ receptor

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Running Title: Ectodomain lysines in heteromeric $P2X_{2/3}$ receptors

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ABBREVIATIONS: αβmeATP, αβ-methylene-ATP

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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 αβ-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.

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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 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., 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:

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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 $P2X_2$ homomeric receptor, two conserved ectodomain lysine residues 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), with the evidence being 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 $P2X_1$ recepor (Lys^{68} and Lys^{309}; Ennion et al., 2000). These lysines are situated about 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} to Lys^{326}) 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 $P2X_{2/3}$ channel their importance in channel function depends greatly on whether they are in the $P2X_2$ or the $P2X_3$ subunit.

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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. $P2X_2$ plasmid (0.2 μg) was co-transfected 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 terminus EYMPME epitope (Jiang et al., 2000, 2003).

Electrophysiology Whole-cell patch clamp recordings were made at 19 - 22 ^{O}C 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 NaC1, 2 KC1, 2 $CaC1_2$, 2 $MgCl_2$, 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.

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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 αβmeATP (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 μM) 23 ± 9 pA, *n* = 8; αβmeATP (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 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.

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

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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 αβmeATP 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 αβmeATP up to 100 μM. At such high concentrations $P2X_2$ 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 $P2X_2$ subunits with $P2X_3$ [K299A] gave no currents in response to αβmeATP (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. Co-expression of $P2X_2$ [K69A,K308A] with $P2X_3$ produced no sustained currents to αβmeATP (*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 co-expressed, and the results of the functional studies, is shown in Fig. 4.

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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 $\alpha\beta$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

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$P2X_3$ 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 differing 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

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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 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 $P2X_3$ (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 found surprising the observation that cells co-transfected 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 with homomeric $P2X_2$

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receptors, that ATP can elicit currents when $P2X_2$[K69A] and $P2X_2$[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 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 αβmeATP binding sites (Fig. 4B, *left* and *middle*), but a 'double-dead' $P2X_2$ 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, $P2X_2$ 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 αβmeATP 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; Burgaard 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, which are likely to have value as pain-relieving drugs.

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This article has not been copyedited and formatted. The final version may differ from this version.

References

Agboh KC, Webb TE, Evans RJ, Ennion SJ (2004) Functional characterization of a P2X receptor from Schistosoma mansoni. *J Biol Chem* **279**:41650-41657.

Barclay J, Patel S, Dorn G, Wotherspoon G, Moffatt S, Eunson L, Abdel'al S, Natt F, Hall J, Winter J, Bevan S, Wishart W, Fox A, Ganju P (2002) Functional downregulation of $P2X_3$ receptor subunit in rat sensory neurons reveals a significant role in chronic neuropathic and inflammatory pain. *J Neurosci* **22**:8139-8147.

Barrera NP, Ormond SJ, Henderson RM, Murrell-Lagnado RD, Edwardson JM (2005) Atomic force microscopy imaging demonstrates that $P2X_2$ receptors are trimers but that $P2X_6$ receptor subunits do not oligomerize. *J Biol Chem* **280**:10759-10765.

Beato M, Groot-Kormelink PJ, Colquhoun D, Sivilotti LG (2002) Openings of the rat recombinant a1 homomeric glycine receptor as a function of the number of agonist molecules bound. *J Gen Physiol* **119**:443-466.

Burgard EC, Niforatos W, van Biesen T, Lynch KJ, Kage KL, Touma E, Kowaluk EA, Jarvis MF (2000) Competitive antagonism of recombinant $P2X_{2/3}$ receptors by 2', 3'-*O*-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP). *Mol Pharmacol* **58**:1502-1510.

Chaumont S, Jiang LH, Penna A, North RA, Rassendren F (2004) Identification of a trafficking motif involved in the stabilization and polarization of P2X receptors. *J Biol Chem* **279**:29628-29638.

Cockayne DA, Hamilton SG, Zhu QM, Dunn PM, Zhong Y, Novakovic S, Malmberg AB, Cain G, Berson A, Kassotakis L, Hedley L, Lachnit WG,

Burnstock G, McMahon SB, Ford AP (2000) Urinary bladder hyporeflexia and reduced pain-related behaviour in $P2X_3$-deficient mice. *Nature* **407**:1011-1015.

Cockayne DA, Dunn PM, Zhong Y, Rong W, Hamilton SG, Knight GE, Ruan HZ, Ma B, Yip P, Nunn P, McMahon SB, Burnstock G, Ford AP (2005) $P2X_2$ knockout mice and $P2X_2/P2X_3$ double knockout mice reveal a role for the $P2X_2$ receptor subunit in mediating multiple sensory effects of ATP. *J Physiol* **567**:621-639.

Ding S, Sachs F (1999) Single channel properties of $P2X_2$ purinoceptors.*J Gen Physiol* **113**:695-720.

Ennion S, Hagan S, Evans RJ (2000) The role of positively charged amino acids in ATP recognition by human $P2X_1$ receptors. *J Biol Chem* **275**:29361-29367.

Finger TE, Danilova V, Barrows J, Bartel DL, Vigers AJ, Stone L, Hellekant G, Kinnamon SC (2005) ATP signalling is crucial for communication from taste buds to gustatory nerves. *Science* **310**:1495-1499.

Freist W, Verhey JF, Stuhmer W, Gauss DH (1998) ATP binding site of P2X channel proteins: structural similarities with class II aminoacyl-tRNA synthetases. *FEBS Lett* **434**:61-65.

Gourine AV (2005) On the peripheral and central chemoreception and control of breathing: an emerging role of ATP. *J Physiol* **568**:715-724.

Honore P, Kage K, Mikusa J, Watt AT, Johnston JF, Wyatt JR, Faltynek CR, Jarvis MF, Lynch K (2002) Analgesic profile of intrathecal $P2X_3$ antisense oligonucleotide treatment in chronic inflammatory and neuropathic pain states in rats. *Pain* **99**:11-19.

Jarvis MF et al. (2002) A-317491, a novel potent and selective non-nucleotide antagonist of $P2X_3$ and $P2X_{2/3}$ receptors, reduces chronic inflammatory and neuropathic pain in the rat. *Proc Natl Acad Sci U S A* **99**:17179-17184.

Jarvis MF (2003) Contributions of $P2X_3$ homomeric and heteromeric channels to acute and chronic pain. *Expert Opin Ther Targets* **7**:513-522.

Jarvis MF, Bianchi B, Uchic JT, Cartmell J, Lee CH, Williams M, Faltynek C (2004) [^{3}H]A-317491, a novel high-affinity non-nucleotide antagonist that specifically labels human $P2X_{2/3}$ and $P2X_3$ receptors. *J Pharmacol Exp Ther* **310**:407-416.

Jiang L-H, Rassendren F, Surprenant A, North RA (2000) Identification of amino acid residues contributing to the ATP-binding site of a purinergic P2X receptor. *J Biol Chem* **275**:34190-34196.

Jiang L-H, Kim M, Spelta V, Bo X, Surprenant A, North RA (2003) Subunit arrangement in P2X receptors. *J Neurosci* **23**:8903-8910.

Kucenas S, Li Z, Cox JA, Egan TM, Voigt MM (2003) Molecular characterization of the zebrafish P2X receptor subunit gene family. *Neuroscience* **121**:935-945.

Lewis C, Neidhart S, Holy C, North RA, Buell G, Surprenant A (1995) Coexpression of $P2X_2$ and $P2X_3$ receptor subunits can account for ATP-gated currents in sensory neurons. *Nature* **377**:432-435.

Nagaya N, Tittle RK, Saar N, Dellal SS, Hume RI (2005) An intersubunit zinc binding site in rat $P2X_2$ receptors. *J Biol Chem* **280**:25982-25993.

Nicke A, Baumert HG, Rettinger J, Eichele A, Lambrecht G, Mutschler E, Schmalzing G (1998) $P2X_1$ and $P2X_3$ receptors form stable trimers: a

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

North RA (2002) Molecular physiology of P2X receptors. *Physiol Rev* **82**:1013-1067.

Roberts JA, Evans RJ (2004) ATP binding at human $P2X_1$ receptors. Contribution of aromatic and basic amino acids revealed using mutagenesis and partial agonists. *J Biol Chem* **279**:9043-9055.

Rosenmund C, Stern-Bach Y, Stevens CF (1998) The tetrameric structure of a glutamate receptor channel. *Science* **280**:1596-1599.

Sine SM (2002) The nicotinic receptor ligand binding domain. *J Neurobiol* **53**:431-446.

Vial C, Roberts JA, Evans RJ (2004) Molecular properties of ATP-gated P2X receptor ion channels. *Trends Pharmacol Sci* **25**:487-493.

Virginio C, Robertson G, Surprenant A, North RA (1998) Trinitrophenyl-substituted nucleotides are potent antagonists selective for $P2X_1$, $P2X_3$, and heteromeric $P2X_{2/3}$ receptors. *Mol Pharmacol* **53**:969-973.

Vlaskovska M, Kasakov L, Rong W, Bodin P, Bardini M, Cockayne DA, Ford AP, Burnstock G (2001) $P2X_3$ knock-out mice reveal a major sensory role for urothelially released ATP. *J Neurosci* **21**:5670-5677.

Yan Z, Liang Z, Tomic M, Obsil T, Stojilkovic SS (2005) Molecular determinants of the agonist binding domain of a P2X receptor channel. *Mol Pharmacol* **67**:1078-1088.

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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 $P2X_1$ αβmeATP (300 μM); for $P2X_4$ ATP (1 mM); and for $P2X_7$ BzATP (300 μM); each 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 (αβmeATP, 300 μM).

Fig. 2. ATP elicits significant currents when lysine-mutant $P2X_2$ subunits are co-expressed (*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). 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 $P2X_2$ and $P2X_3$ subunits. ***A***, currents elicited by 2 s application of 30 μM αβmeATP. $P2X_2$[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 co-expressed with wild type $P2X_3$. However, the rapidly-desnsitizing homomeric $P2X_3$ responses were typically observed on the first application of αβmeATP. ***B***, concentration-response curves for some of the

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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 $P2X_2$ 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), so long as only one of the $P2X_2$ lysines is substituted by alanine (*left, middle*) but would have only one such contact when $P2X_2$ subunit has both lysines changed to alanine.

Notes: [1]tested with ATP; [2]tested with αβmeATP; [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 αβmeATP.

Figure 1

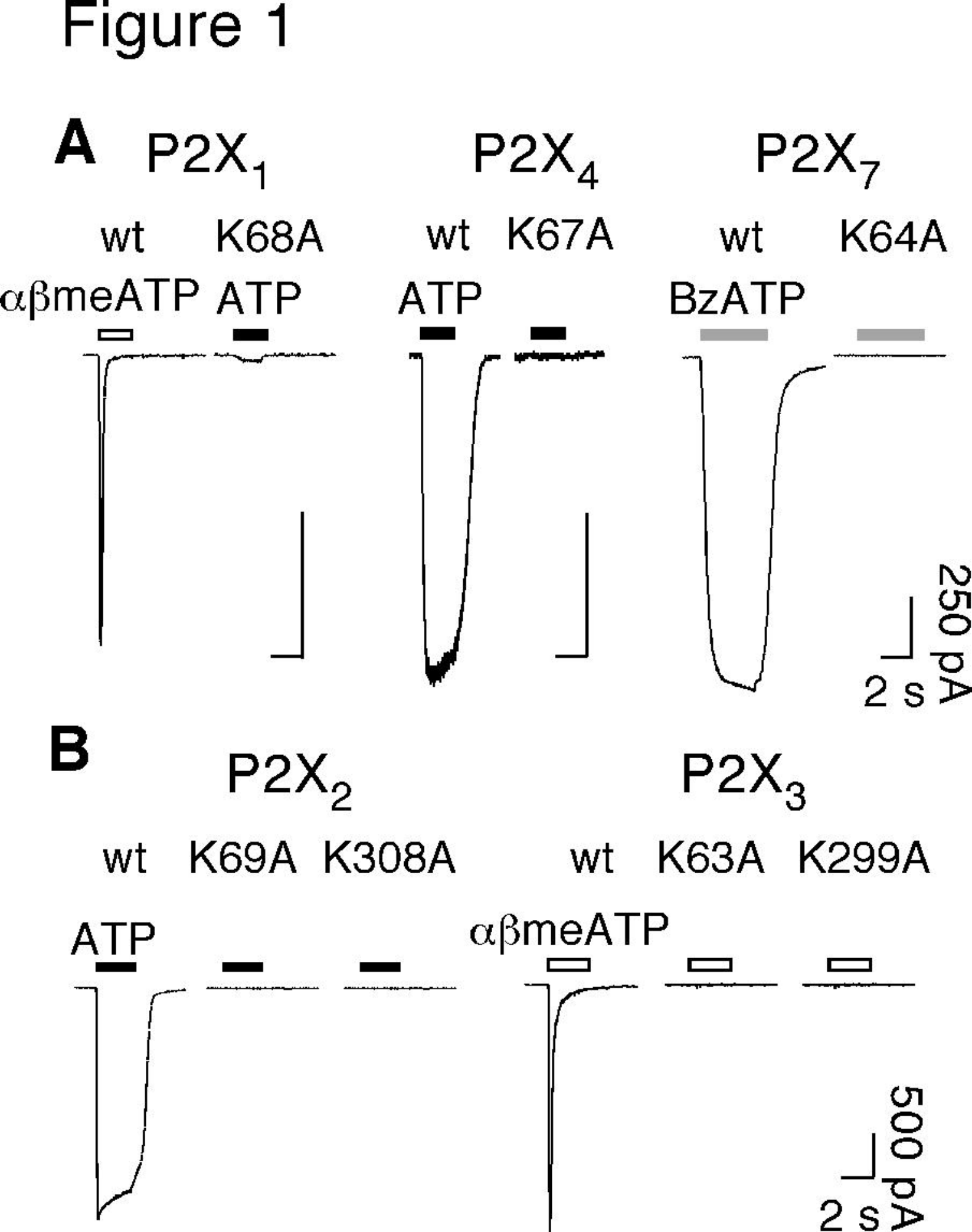

A
P2X1
P2X4
P2X7
wt
K68A
wt
K67A
wt
K64A
αβmeATP
ATP
ATP
BzATP
250 pA
2 s
B
P2X2
P2X3
wt
K69A
K308A
wt
K63A
K299A
ATP
αβmeATP
500 pA
2 s

Figure 2

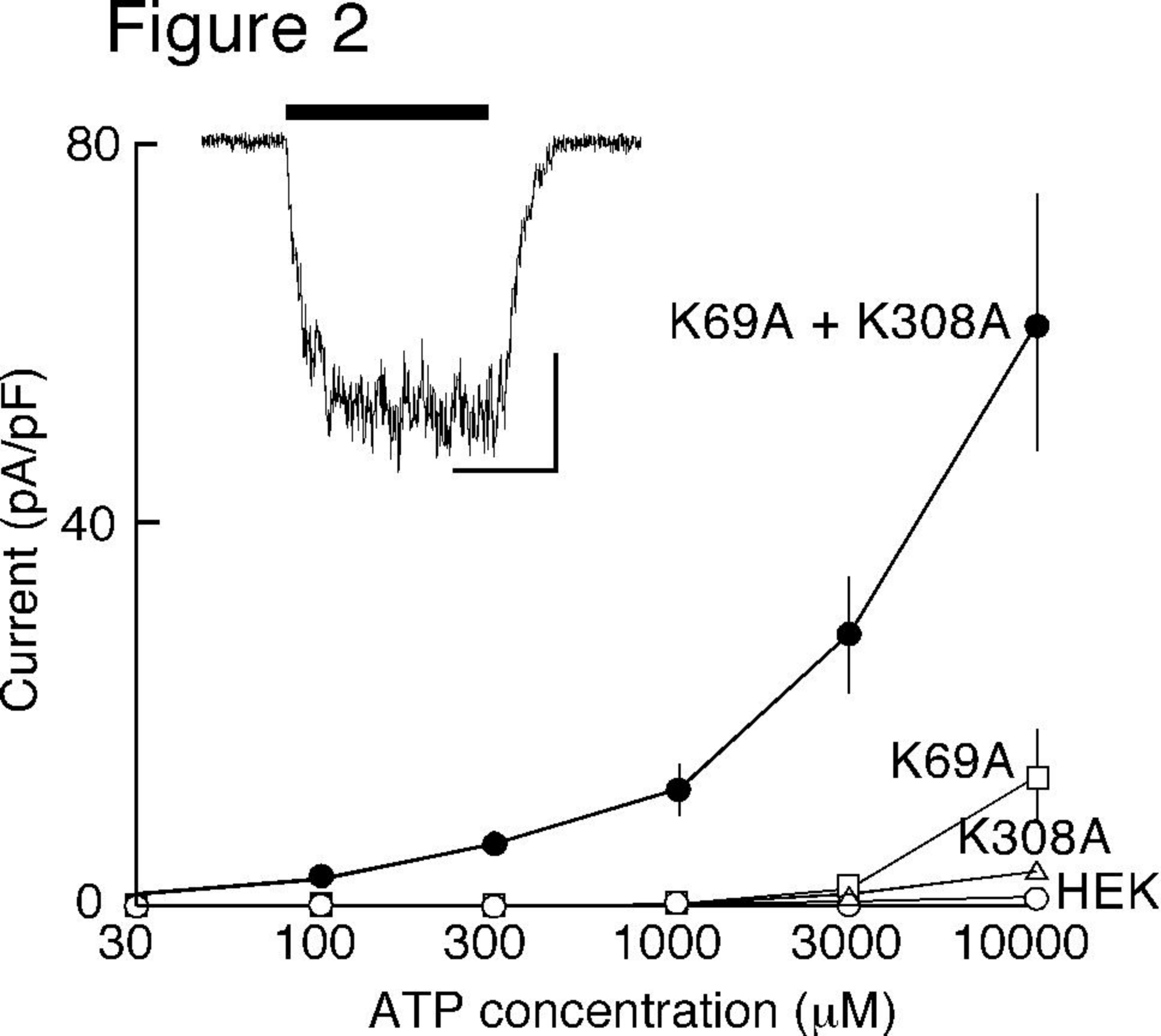

80
40
0
Current (pA/pF)
K69A + K308A
K69A
K308A
HEK
30
100
300
1000
3000
10000
ATP concentration (μM)

Figure 3

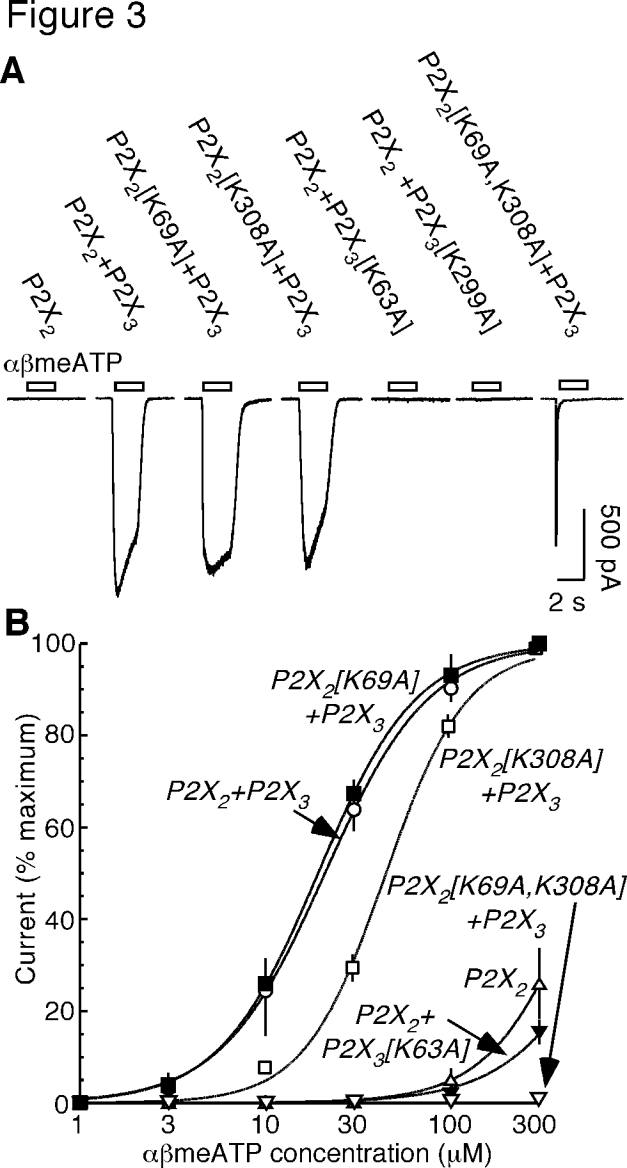
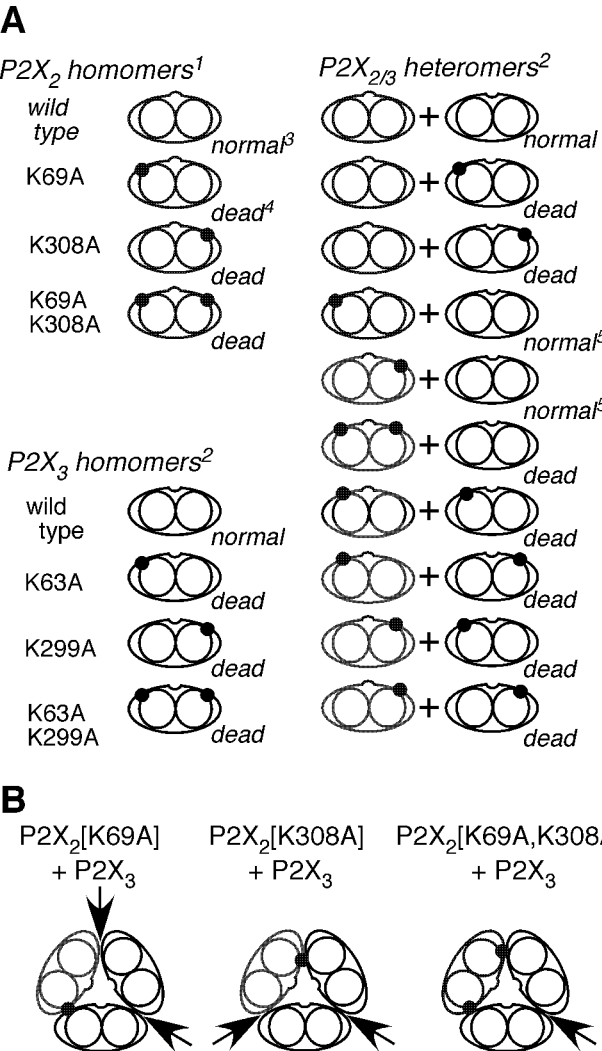

A
P2X2
P2X2+P2X3
P2X2[K69A]+P2X3
P2X2[K308A]+P2X3
P2X2+P2X3[K63A]
P2X2 +P2X3[K299A]
P2X2[K69A,K308A]+P2X3
αβmeATP
500 pA
2 s
B
Current (% maximum)
αβmeATP concentration (μM)
P2X2[K69A] +P2X3
P2X2+P2X3
P2X2[K308A] +P2X3
P2X2[K69A,K308A] +P2X3
P2X2
P2X2+ P2X3[K63A]

Figure 4


A
P2X2 homomers1
wild type
normal3
K69A
dead4
K308A
dead
K69A K308A
dead
P2X3 homomers2
wild type
normal
K63A
dead
K299A
dead
K63A K299A
dead
P2X2/3 heteromers2
normal
dead
dead
normal5
normal5
dead
dead
dead
dead
dead
B
P2X2[K69A] + P2X3
P2X2[K308A] + P2X3
P2X2[K69A,K308A] + P2X3