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P2X receptor channels show threefold symmetry in ionic charge selectivity and unitary conductance

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In the closed structure of the P2X cation channel, three α-helical transmembrane domains cross the membrane obliquely. In rat P2X2 receptors, these intersect at Thr^{339}. Replacing Thr^{339} by lysine in one, two or three subunits progressively increased chloride permeability and reduced unitary conductance. This implies that the closed-open transition involves a symmetrical separation of the three subunits and that Thr^{339} from each subunit contributes symmetrically to the open channel permeation pathway.

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The conducting pore of ligand-gated ion channels is typically formed as a passage along the central axis of several subunits. ATP-gated channels (P2X receptors) are among the simplest such channels; in this case, the central cation-selective channel is formed by the second of two transmembrane domains (TM2) from each of three subunits[1]. In the crystal structure of the closed zebrafish P2X4.1 receptor, these TM2 helices (from N334 to L361) cross the membrane at an oblique angle such that the narrowest part of the channel is delimited by two helical turns from L340 to A347 (ref. 2). The corresponding region of the rat P2X2 receptor (I332 to T339) is an important determinant of the conducting properties of the open channel[3–8]. We introduced a lysine residue at this position (T339K) in one, two or three of the TM2 helices of the rat P2X2 receptor (**Supplementary Methods**) and found that the open channel functions as a symmetrical trimer, in which each TM2 helix contributes equally to the permeation pathway.

The current evoked by ATP (**Supplementary Methods**) at wild-type P2X2 receptors showed marked inward rectification[9]. In contrast, outward currents through P2X2[T339K] receptors were larger at positive holding potentials[7] (**Fig. 1a**). The concatemer with three wild-type subunits (TTT) showed inward rectification similar to channels formed by the expression of single wild-type subunits and the rectification of the three-lysine concatemer (KKK) resembled that of the homotrimeric channel formed from single P2X2[T339K] subunits (**Fig. 1a**). Channels containing one or two lysine residues showed intermediate inward rectification (**Fig. 1a** and **Supplementary Table 1**). There was no obvious position dependence among forms KTT, TKT and TTK. Concatemers containing two T339K subunits all showed enhanced outward currents, although this was less for KKT than for KTK and TKK (**Fig. 1a**). Of all the constructs, only KKT showed evidence of partial breakdown (**Supplementary Fig. 1**) and it is possible that wild-type monomers were also formed.

Wild-type P2X2 channels have negligible chloride permeability[1,10], but substitution by lysine at T339 converted the P2X receptor channel from being cation selective to anion preferring; the ratio of the permeability of chloride to that of sodium (P_{Cl}/P_{Na}) increased from <0.1 for the wild-type channel to ~2 for the T339K channel (**Supplementary Table 1**). For concatemeric channels, the increase in chloride permeability was progressive with the number of lysines at this position (**Fig. 1b**). There was a strong correlation between the increase in outward current measured at 150 mV and the increase in chloride permeability (**Fig. 1c**). In other words, the large outward currents in P2X2[T339K] are the result of the increased inward movement of chloride ions when the cell is strongly depolarized. This suggests that the electrostatic environment around T339 is critical for the charge selectivity of the permeating ions.

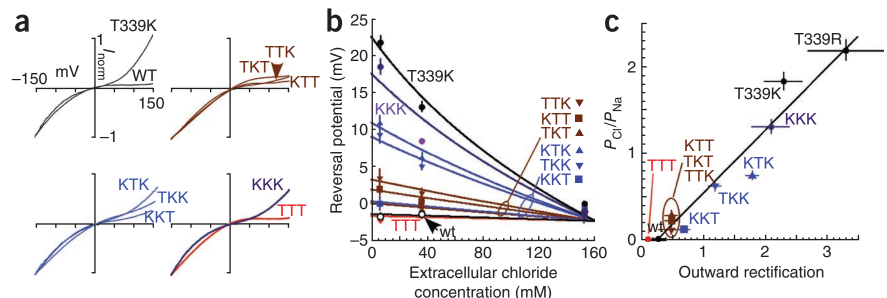

Figure 1 Lysine at 339 progressively increases chloride permeability and outward current. (**a**) Current-voltage plots for ATP-induced currents in ten cells expressing concatenated trimeric P2X2 receptors with one, two or three lysines at position 339. Currents are normalized and scale bars apply to all panels (actual currents at −150 mV: wild type (WT), 2,000 pA; T339K, 700 pA; KTT, 3,100 pA; TKT, 2,900 pA; TTK, 2,700 pA; KKT, 230 pA; KTK, 800 pA; TKK, 1,800 pA; TTT, 3,300 pA; KKK, 1,900 pA). ATP concentrations were 10 or 30 μM (close to EC_{50}). (**b**) Reversal potential for ATP-evoked currents became dependent on the chloride concentration as lysines were introduced at position 339. Data are mean ± s.e.m. (**c**) P_{Cl}/P_{Na} (determined from **b**) increased according to the number of lysines at position 339 and outward rectification increased proportionately (Pearson's $r = 0.97$).

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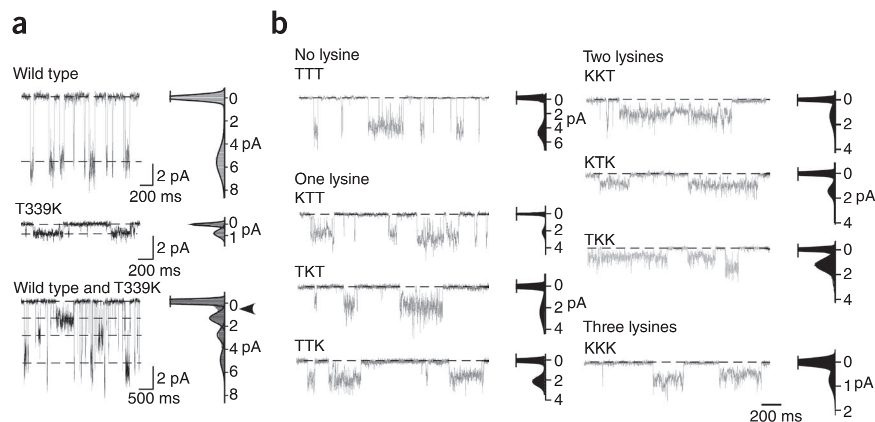

Figure 2 Lysine at 339 reduces single channel currents. (**a**) ATP (0.3 μM or 1 μM) activated single channels in outside-out patches from cells expressing cDNAs encoding wild-type P2X2 (top), P2X2[T339K] (middle) or both (bottom) subunits. The bottom trace shows the intermediate current amplitudes: zero current/closed channel peak is truncated and the arrowhead indicates the position of the third level (<1 pA). Holding potential = –120 mV. (**b**) Outside-out recordings of single-channel activity in patches from cells expressing concatenated cDNAs. The amino acid at position 339 in each subunit of the trimer is indicated above each trace. ATP concentrations were 1 to 10 μM. Recordings on the right are all-points histograms used to estimate unitary current amplitudes; zero level peaks are truncated.

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Single-channel recording revealed that wild-type rat P2X2 receptors open to a single conducting level in ATP (27.3 ± 1.3 pS, n = 12; **Fig. 2a**). P2X2[T339K] had much reduced unitary currents (6.1 ± 0.6 pS, n = 7). The corresponding values when potassium was the main internal ion were 41.1 ± 3.3 pS (n = 7) and 6.1 ± 0.3 pS (n = 8), so we used internal potassium in subsequent experiments to more easily discriminate levels intermediate between wild type and T339K. Outside-out patches from cells transfected with both wild-type and T339K cDNAs usually showed multiple conductance levels (**Fig. 2a**). In 9 of 44 patches, a single open level was observed at 44 ± 1.6 pS; in 8 of 44 patches, a single open level occurred at 7.2 ± 0.1 pS. In 11 patches, we observed three open levels (that is, four peaks in the all points histogram), which corresponded in amplitude to wild-type level, and two new intermediate levels (II, 14.4 ± 0.9 pS; III, 24.5 ± 1.1 pS). In 16 patches, we observed a single intermediate conductance level, corresponding in amplitude to either level II or III (**Supplementary Fig. 2**).

Concatemers with only wild type or only T339K in each of the three subunits provided channels with unitary conductances similar to those observed with the corresponding monomers (**Fig. 2b** and **Supplementary Fig. 2**). Concatemers that contained one or two T339K subunits had unitary conductances that were not different from the intermediate levels observed with coexpression of monomers (**Supplementary Fig. 2**).

The progressive increase in positivity of the electrostatic field strength at this position by introduction of one, two or three positive charges converts the P2X2 receptor from being tenfold cation selective to being a channel that prefers anions, which accounts for the rectification that was previously reported[1,9]. Similar conversion of ion selectivity from cationic to anionic by mutagenesis is reported for some other ion channels (for example, nicotinic receptors[11] and cyclic nucleotide–gated channels[12]). Introduction of a single lysine into the trimeric concatemer reduced the unitary current by about 50% and further lysine additions caused further stepwise reductions (**Fig. 2** and **Supplementary Fig. 2**). There was no obvious change in open probability in T339K as compared with wild-type channels, indicating that the T339K substitution did not change gating (for example, by endowing the protein with a new voltage dependence).

It is currently thought that the P2X receptor pore opens by a separation[13] and counter-clockwise rotation of each of the three TM2 helices, driven by forces transmitted through connecting rods passing through the ectodomain from three intersubunit binding sites[14].

Our findings with concatenated channels strongly suggest that opening of P2X2 channels occurs by equivalent and symmetrical rearrangement of the TM2 helices. We conclude that the side chain of the residue that occludes the permeation pathway of the closed P2X receptor also contributes to the selectivity filter of the open channel (T339 in the P2X2 receptor; **Supplementary Fig. 3**). This result is consistent with a symmetrical iris-like separation of the three TM2 helices; other recent evidence suggests that this is accompanied by a steepening and rotation of these helices[14,15].

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS
R.A.N., L.E.B. and L.C. conceived and designed the experiments and analyzed the data. H.E.B., L.B. and W.J.W. generated the constructs and carried out western blotting. L.E.B. and L.C. performed the single-channel and whole-cell electrophysiology. L.E.B. constructed molecular models. R.A.N. wrote the paper with contributions from the other authors.

COMPETING FINANCIAL INTERESTS
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