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Thr^{339}-to-Serine Substitution in Rat $P2X_2$ Receptor Second Transmembrane Domain Causes Constitutive Opening and Indicates a Gating Role for Lys^{308}

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$P2X_2$ receptors are ATP-gated ion channels widely expressed by neurons. Thr^{339} lies in the second of the two transmembrane domains of the rat $P2X_2$ receptor protein, and is likely to be close to the narrowest part of the pore. Single-channel and whole-cell recording after expression in human embryonic kidney 293 cells showed that $P2X_2$[T339S] receptors had pronounced spontaneous channel openings that were never seen in wild-type $P2X_2$ receptors. $P2X_2$[T339S] receptors were 10-fold more sensitive than wild type to exogenous ATP, and $\alpha\beta$meATP also increased channel opening. Two conserved ectodomain lysine residues (Lys^{69} and Lys^{308}) are critical for function and have been proposed to contribute to the ATP binding site of P2X receptors. The spontaneous opening of $P2X_2$[K69A/T339S] receptors was not different than that seen in $P2X_2$[T339S], but for $P2X_2$[K308A/T339S] the spontaneous activity was absent. Suramin, which is a noncompetitive antagonist at wild-type $P2X_2$ receptors, had a pronounced agonist action at both $P2X_2$[T339S] and $P2X_2$[K69A/T339S] receptors but not at $P2X_2$[K308A/T339S]. 2′,3′-*O-O*-(2,4,6-Trinitrophenyl)-ATP (TNP-ATP), which is a competitive agonist at wild-type receptors, was also an agonist at $P2X_2$[T339S] receptors, but not at either double mutant. The results indicate that the T339S mutation substantially destabilizes the closed channel and suggest an important role in channel gating. The correction of this gating defect, in the absence of any agonist, by the second mutation K308A shows that Lys^{308} is also involved in channel gating. A similar interpretation can account for the results with suramin and TNP-ATP.

Key words: P2X receptors; ATP; binding site; channel gating; mutations; lysine

Introduction

P2X receptors are membrane ion channels that serve as one class of receptor for extracellular ATP. In the nervous system, they have been ascribed roles in neuronal synaptic transmission and its modulation (North and Verkhratsky, 2006; Sim et al., 2006) as well as in the function of microglia (Tsuda et al., 2005). The $P2X_2$ receptor is widely expressed in nervous tissue, and experiments using $P2X_2$ knock-out mice show functional roles at the presynaptic side of excitatory synapses on to stratum radiatum interneurons (Khakh et al., 2003), the postsynaptic aspect of synapses on some myenteric plexus neurons (Ren et al., 2003), and on primary afferent nerves in taste buds (Finger et al., 2005), bladder, and other sites (Cockayne et al., 2005).

P2X receptors assemble and function as trimers. The individual subunits appear to have internal N and C termini, two membrane-spanning (TM) segments, and an extracellular domain of ~280 aa (North, 2002). There has been considerable progress in understanding the molecular operation of P2X receptors, mostly thus far by inferences from biophysical experiments on expressed receptors that contain engineered mutations. Such experimental approaches have indicated, for example, that parts of the TM domains contribute to the permeation pathway, and that residues around Thr^{339}, Ser^{340}, and Gly^{342} in TM2 are near the narrowest part of the pore (Migita et al., 2001; Egan and Khakh, 2004; Egan et al., 2006). They also indicate that the outer ends of the TMs (Val^{48} and Ile^{328}) of different subunits move relative to each other when the channel opens (Jiang et al., 2001).

Mutagenesis in the extracellular domain of the receptor has drawn attention to the role of two conserved lysine residues (Lys^{69} and Lys^{308} in rat $P2X_2$ receptors). It has been suggested that the positive charges provided by these residues might interact with the negatively charged phosphate moiety of the ATP molecule (Ennion et al., 2000; Jiang et al., 2000; Roberts et al., 2006; Wilkinson et al., 2006; Yan et al., 2006). However, attractive as such an interpretations may be, it is not possible on the basis of measurements of macroscopic channel activity to distinguish between a primary alteration in agonist binding and a change in the mechanism by which a liganded closed channel undergoes the conformational change into an open channel (Colquhoun, 1998, 2006).

One way to distinguish binding from gating is to eliminate the binding step altogether: that is, to study spontaneous openings of the channel in the absence of any agonist. In the present approach, we have used a $P2X_2$ receptor that differs from wild type only by the removal of a methyl group at Thr^{339} (i.e., side chain is $–CH_2OH$ instead of $–CH(OH)CH_3$). These $P2X_2$[T339S] chan-

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nels showed spontaneous channel openings. They could also be opened by suramin and by 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (TNP-ATP), which are antagonists at wild-type P2X receptors. By studying the combined effects of the T339S substitution with changes to the lysines, we conclude that Lys^{308} plays a key role in channel gating.

Materials and Methods

Molecular and cell biology. Mutations in rat $P2X_2$ receptors were made as described previously (Rassendren et al., 1998; Jiang et al., 2000, 2001). The wild-type and mutant $P2X_2$ subunits were transiently coexpressed together with GFP vector in HEK293 cells (by Lipofectamine 2000; Invitrogen, San Diego, CA), using 0.1 and 1 μg per transfection, respectively. Transfected cells were seeded on glass coverslips coated with poly-L-lysine.

Western blot and surface expression. Cell surface protein was labeled using EZ-link sulfo-NHS-LC-biotin (Pierce, Cramlington, UK). Confluent cells (one 35 mm culture dish per sample) were washed three times in PBS, pH 8.0, and incubated in 1 ml of PBS containing 0.5 mg/ml biotin for 30 min at 4°C. After washing, cells were centrifuged, and pellets lysed in PBS containing 2% Triton X-100 and antiproteases (complete EDTA; Roche, Lewes, UK) for 1 h at 4°C. After centrifugation (16,000 × *g*; 2 min) to remove debris, total protein samples were removed and assayed for protein content using a protein assay kit (Bio-Rad, Hemel Hempstead, UK). Biotinylated surface protein in the cell lysate was bound to immuno-pure immobilized streptavidin beads (Pierce) overnight at 4°C. After washing in 0.2% Triton X-100, SDS-PAGE sample buffer was added and the samples were boiled (5 min; 100°C) to release cell surface protein. Samples were separated on 4–12% Nu-PAGE gels (Invitrogen) and transferred to PVDF membranes. Western blotting was performed according to standard protocols and proteins were visualized using anti-EYMPME primary antibody (Universal Biologicals, Cambridge, UK) and HRP-conjugated secondary antibody (both at 1:5000 dilution), followed by detection using the ECL-plus kit (Amersham Biosciences, Buckinghamshire, UK) and Kodak BioMax MS film. Band densities were quantified using GeneSnap/GeneTools software (Syngene, Frederick, MD), and densities were compared using analysis of variance.

Electrophysiological recording. Recordings were made at room temperature 24–72 h after transfection, using outside-out and whole-cell configurations of the patch-clamp technique (Hamill et al., 1981). For single-channel recording, coverslips were removed from the recording chamber after the outside-out configuration was obtained. Recording pipettes were pulled from borosilicate glass (World Precision Instruments, Stevenage, UK) and had resistances of 10–20 MΩ for single-channel recording and 2–4 MΩ for whole-cell recording. The usual holding potential was −60 mV for whole-cell and −100 mV for single-channel recording. The extracellular solution contained the following (in mM): 147 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 13 glucose. When a high concentration of Na_2ATP was used, the pH was carefully corrected with NaOH and the NaCl content was adjusted to maintain its normal value. The intracellular (pipette) solution comprised the following (in mM): 147 NaF, 10 HEPES, and 10 EGTA. All solutions were maintained at pH 7.3 and 300–315 mOsm/L. Chemicals were purchased from Sigma (Poole, UK).

Currents were recorded with a patch-clamp amplifier (Axopatch 200B) using pClamp 9 software (Molecular Devices, Palo Alto, CA). The data were low-pass filtered at 3 kHz with an eight-bessel filter (LHBF-48X; NPI, Tamm, Germany) and digitized at 10 kHz (single channel) or 2 kHz (whole cell). We generated dose–response curves for ATP and other compounds by applying these drugs through an RSC200 rapid perfusion system (Biologic, Claix, France). Agonists were applied for 2 s or, in the case of lower concentrations, until a steady-state current was recorded. Current–voltage relationships were obtained from the currents measured during a linear voltage ramp, which consisted of one step to −150 mV and a 400 ms linear voltage ramp to +100 mV; this was applied every 1 s from a holding potential of −60 mV.

Data analysis. Electrophysiological measurement data were analyzed using Clampfit 9 software (Molecular Devices) and Prism 4.0 (GraphPad Software, San Diego, CA). Pooled data are given as the mean ± SEM. Tests for statistical significance were performed using nonparametric ANOVA. Concentration–response curves for individual cells were fit to $I = I_{max}/[1 + (EC_{50}/[A])^n]$, where *I* is the current, and [*A*] is the agonist concentration. The fit was used to estimate the maximum response (I_{max}), the concentration of agonist needed to evoke a half-maximal response (EC_{50}), and the Hill slope (*n*). The change in free energy (in joules per mole) required to open the channel ($\Delta(\Delta G)$) was calculated from the differences in pEC_{50} values of mutant and wild-type receptors, using $(\Delta(\Delta G)) = -RT \times \ln(EC_{50,mut}/EC_{50,wt})$, where *R* is the molar gas constant, *T* is the temperature (in degrees kelvin), and $EC_{50,mut}$ and $EC_{50,wt}$ are the half-maximal ATP concentrations for the mutant and wild-type receptors, respectively (Maksay et al., 1999; Möttig et al., 2001; Li et al., 2004).

Results

Spontaneous channel openings in $P2X_2$[T339S]

Outside-out patch recordings from cells expressing wild-type rat $P2X_2$ receptors showed no activity in the absence of applied agonist (up to 30 min) (Fig. 1*A*). ATP (1–30 μM) evoked discrete inward currents with unitary conductance of 19.1 ± 0.5 pS ($n = 22$; measured at −100 mV) (Fig. 1*B*). Patches from cells expressing $P2X_2$[T339S] receptors showed marked spontaneous channel activity (Fig. 1*C*). The unitary conductance of the spontaneously opening channels was 18.4 ± 0.6 pS ($n = 6$), which is not different from the value for wild-type channels in the presence of ATP ($p > 0.05$) (Fig. 1*E*,*F*). We observed similar spontaneous activity in $P2X_2$[T339G], but the unitary currents were of lower amplitude (12.9 ± 0.8 pS; $n = 9$; $p < 0.001$).

Many cells release ATP, and we were concerned that the spontaneous activity observed in $P2X_2$[T339S] receptors might result from trace amounts of ATP present in the external solution. For example, HEK293 cells expressing $P2X_{1/5}$ heteromeric receptors have a standing inward current (up to 50 pA) that results from the release of ATP from the same or neighboring cells (Surprenant et al., 2000). We incubated the cells expressing $P2X_2$[T339S] receptors with apyrase (4 U/ml; 1 h) and we included apyrase (10 U/ml) in the perfusion solution for up to 30 min. Neither treatment altered the spontaneous activity that we observed (Fig. 1*D*). In most experiments, we removed all the other cells by taking the coverslips from the recording chamber after we had established the outside-out configuration. This would effectively remove all possible sources of extracellular ATP. This also did not alter the spontaneous activity.

The spontaneous channel activity was also apparent at the whole-cell level: cells expressing $P2X_2$[T339S] receptors showed a large inwardly rectifying current (Fig. 2*B*) that was absent from cells expressing wild-type receptors. We quantified the difference in the whole cell currents by measuring inward current at −100 mV, from the voltage ramps (see Materials and Methods) (Fig. 2*B*,*C*). The values were as follows: wild type, 2.2 ± 0.2 pA/pF ($n = 33$); T339S, 23.7 ± 5.2 ($n = 26$); and T339G, 17.9 ± 7.4 ($n = 8$). Spontaneous openings were not observed with T339C or T339A receptors.

Application of ATP further increased the channel activity in $P2X_2$[T339S] and $P2X_2$[T339G] receptors, without changing the unitary conductance of the elementary channel openings. The peak whole-cell currents evoked by ATP (10–100 μM; pA/pF; at −60 mV) for wild-type, $P2X_2$[T339S] and $P2X_2$[T339G] receptors were 172 ± 19 ($n = 18$), 194 ± 31 ($n = 7$), and 222 ± 62 ($n = 8$), suggesting that the point mutation had resulted in a channel that was open ∼10% relative to that evoked by a maximal concentration of ATP. The concentration–response curve for ATP as an agonist at the $P2X_2$[T339S] and $P2X_2$[T339G] receptors was

shifted ~10-fold to the left when compared with parallel experiments on wild-type receptors (Fig. 3*A*). From the ratio of the pEC_{50} values, the value of $\Delta(\Delta G)$ between wild type and $P2X_2$[T339S] was $-2.33RT$ J/mol.

$P2X_2$[T339S] receptors were also more sensitive to $\alpha\beta$meATP, which is a very weak partial agonist at wild-type $P2X_2$ receptors (Fig. 3*A*). The EC_{50} for wild-type receptors was ~400 μM, whereas it was only 50 μM for $P2X_2$[T339S]. For $\alpha\beta$meATP, the value of $\Delta(\Delta G)$ between wild-type and $P2X_2$[T339S] receptors was $-2.15RT$ J/mol. This indicates that the difference in free energy for channel activation introduced by the T339S mutation is independent of agonist.

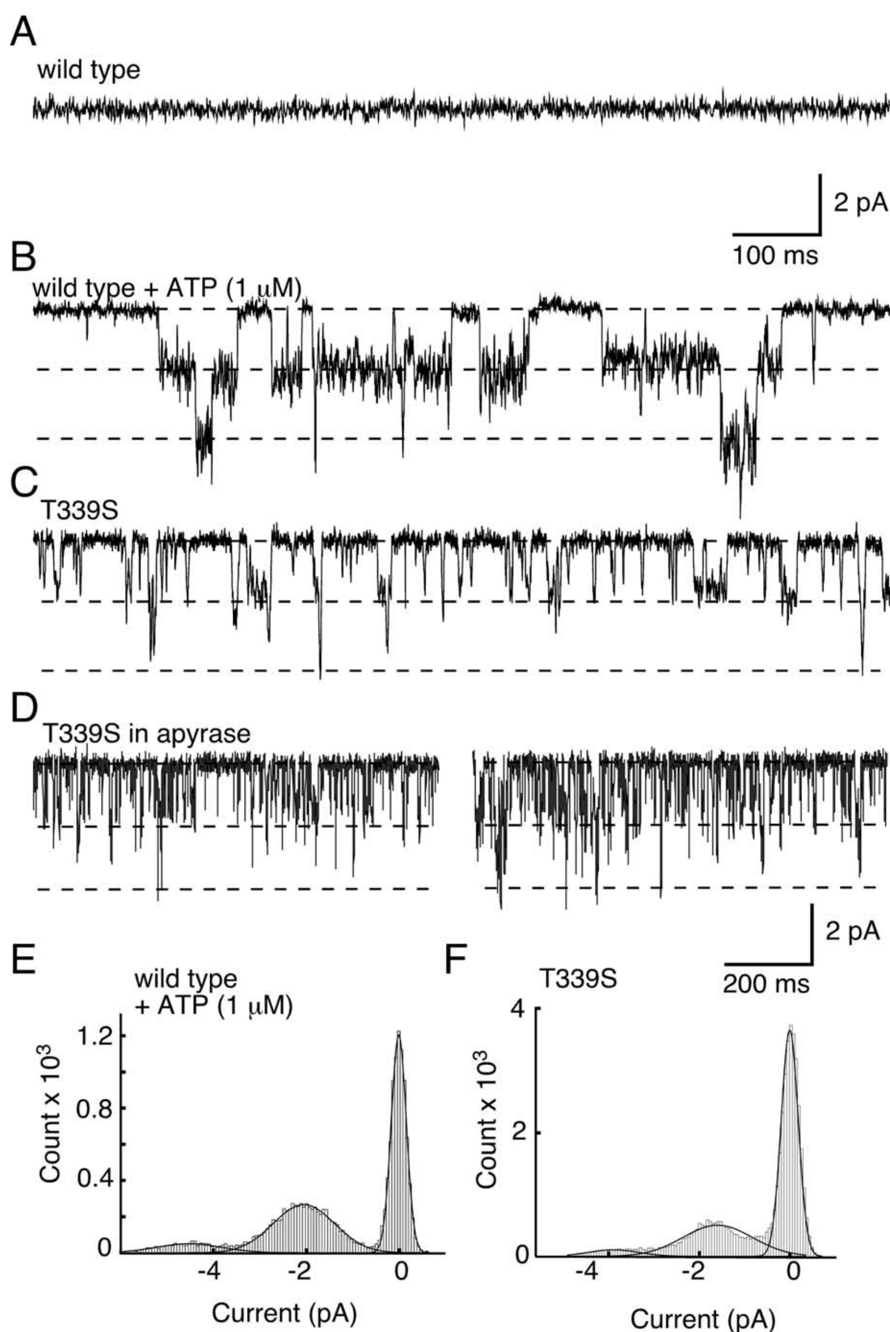

Figure 1. $P2X_2$[T339S] receptors are spontaneously active. ***A***, No spontaneous openings are observed in outside-out patches from cells expressing wild-type receptor. ***B***, ATP (1 μM) activates single channels in outside-out patch from cell expressing wild-type $P2X_2$ receptors. ***C***, $P2X_2$[T339S] receptors show spontaneous opening in the absence of applied ATP. ***D***, Spontaneous activity in $P2X_2$[T339S] was still observed after prolonged treatment in apyrase. Left, Cells had been incubated in apyrase (4 U/ml; 60 min). Right, After additional application of apyrase (10 U/ml; 30 min) to perfusing solution during outside-out recording. All records were low-pass filtered at 3 kHz and digitized at 10 kHz: holding potential, −100 mV. ***E***, All-points histogram for wild-type receptors activated by ATP (1 μM). ***F***, All-points histogram for $P2X_2$[T339S] receptors with no added ATP.

Effect of Lys[69] and Lys[308] mutations on spontaneous activity in $P2X_2$[T339S]

Lys[69] has been implicated in ATP binding to P2X receptors (Jiang et al., 2001; Roberts and Evans, 2004). Outside-out patch recording showed that spontaneous channel activity in $P2X_2$[K69A/T339S] was not different from that observed in $P2X_2$[T339S] (Fig. 2*A*). Basal whole-cell currents at −100 mV in $P2X_2$[K69A/T339S] were 14.8 ± 2.3 pA/pF ($n = 19$). These findings further support the view that spontaneous channel activity was not attributable to the activation by trace ATP, in the sense that the removal of a probable ATP binding site does not alter the basal channel activity. We confirmed previous findings that $P2X_2$[K69A] was essentially insensitive to ATP (Fig. 3*B*). Cells transfected with $P2X_2$ subunits carrying both mutations [K69A/T339S] provided substantial currents in response to ATP, although the concentration response curve was shifted ~10,000 times rightward relative to that for $P2X_2$[T339S] (Fig. 3*B*).

The results for Lys[308] were very different. In this case, we observed that cells expressing the double mutation [K308A/T339] showed very little spontaneous activity in outside-out patches (Fig. 2*A*). Whole-cell recordings gave basal currents for $P2X_2$[K308A/T339S] of 2.7 ± 0.3 pA/pF ($n = 13$) (Fig. 2*C*), which was not different from wild type ($p > 0.05$). Substitution of Lys[308] by cysteine gave a similar result as K308A: spontaneous activity was not observed (2.5 ± 0.4 pA/pF; $n = 14$; $p > 0.05$). We tested whether modification of the cysteine side chain by methanethiosulfonate ethyltrimethylammonium (MTSET) would rescue the spontaneous channel activity seen in the wild-type channel; however, in cells expressing $P2X_2$[K308C/T339S] receptors, there were only very rare spontaneous channel openings, and these were not changed by application of MTSET (1 mM; 10 min). Application of ATP to cells transfected with $P2X_2$[K308A] receptors evoked no current, in confirmation of the study by Jiang et al. (2001) (Fig. 3*C*). ATP also did not activate any current at $P2X_2$[K308A/T339S] receptors (Fig. 3*C*).

We tested whether the positive charge of arginine might substitute for lysine in the context of blocking the spontaneous activity introduced by the $P2X_2$[T339S] mutation. Figure 2 shows that $P2X_2$[K308R/T339S] channels also exhibited considerable spontaneous channel activity, with unitary events similar in amplitude to that observed in $P2X_2$[T339S]. Basal whole-cell cur-

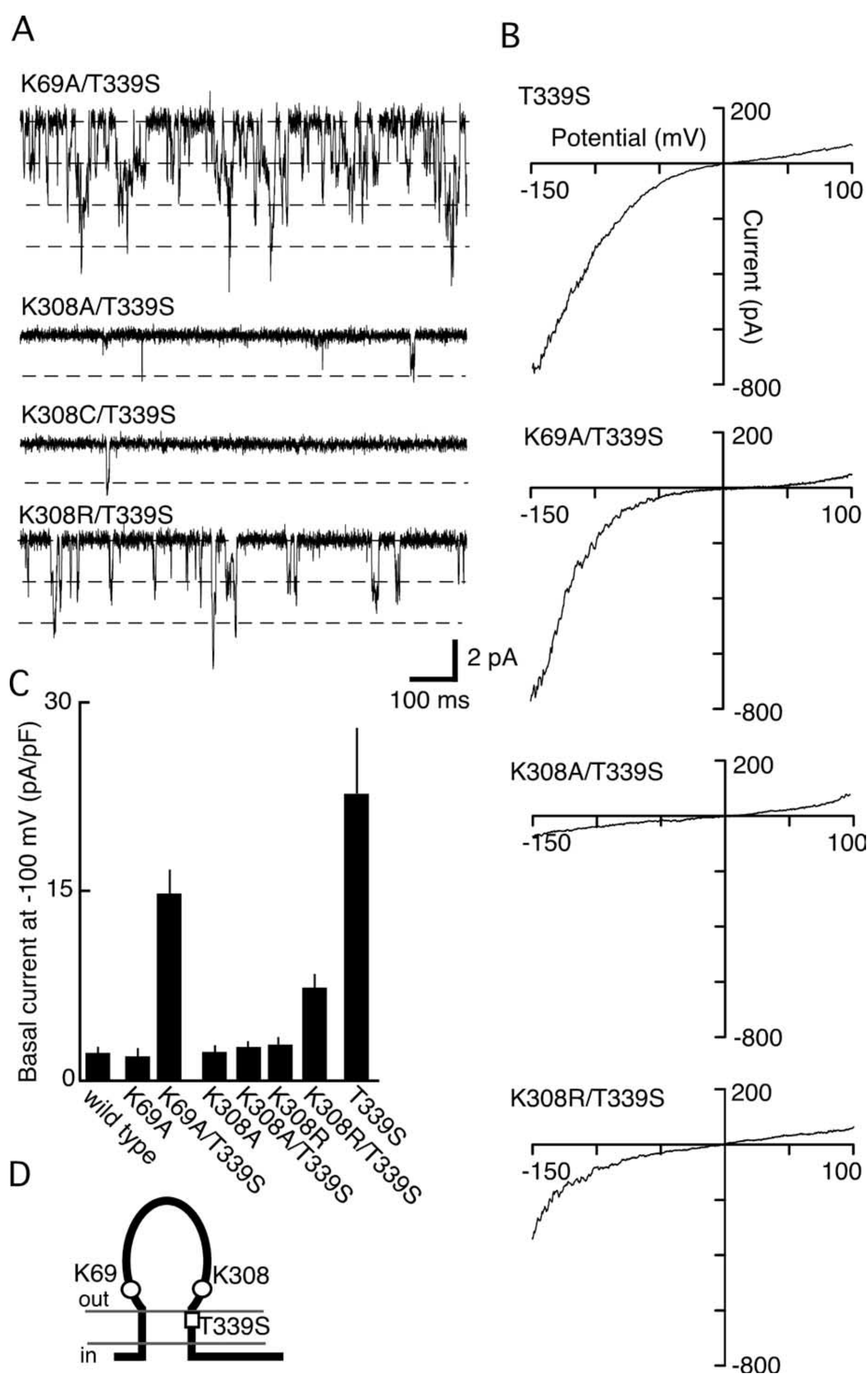

Figure 2. Spontaneous channel activity in P2X$_2$[T339S] does not require Lys[69] but does require Lys[308]. ***A***, Outside-out recording shows that spontaneous channel opening persists when Lys[69] is mutated ([K69A/T339S]), but not when Lys[308] is mutated ([K308A/T339S], [K308C/T339S]): holding potential, −100 mV. [K308R/T339S] gives limited spontaneous activity. ***B***, Current–voltage plots for whole-cell currents in typical cells expressing the point mutations indicated, in the absence of ATP. ***C***, Summary data for spontaneous inward current (−100 mV) recorded in the eight groups of cells indicated ($n = 9$–33). ***D***, Schematic to illustrate relative positions of Lys[69], Lys[308], and Thr[339].

rents were 7.4 ± 3.2 pA/pF ($n = 19$). This indicates that arginine can only partially substitute for lysine in the context of maintaining the spontaneous activity seen with the [T339S] mutation.

As shown by Jiang et al. (2001), we found that currents could be evoked by ATP at P2X$_2$[K308R] receptors but the effective ATP concentrations were very much increased (Fig. 3*C*). However, P2X$_2$[K308R/T339S] receptors were more sensitive to ATP than P2X$_2$[K308R], with an EC$_{50}$ of ~100 μM (Fig. 3). The difference in free energy associated with the K308R mutation was the same whether in a wild type (−2.33 *RT* J/mol) or [T339S] background (−2.38 *RT* J/mol). Likewise, the difference in free energy associated with the T339S mutation was the same whether in wild type (−4.04 *RT* J/mol) or [K308R] background (−4.00 *RT* J/mol). These results suggest that the substitutions at the two positions Lys[308] and Thr[339] may each inhibit gating by independent mechanisms.

The expression at the plasma membrane of the mutated receptors was not different from that of the wild-type P2X$_2$ receptor ($p > 0.05$). The relative cell surface expression ratios determined by Western blot and densitometry were as follows: wild type, 0.94 ± 0.1 ($n = 14$); [T339S], 1.2 ± 0.6 ($n = 4$); [K69A], 1.0 ± 0.19 ($n = 4$); [K69A/T339S], 1.1 ± 0.11 ($n = 4$); [K308A], 0.84 ± 0.23 ($n = 5$); [K308A/T339S], 0.50 ± 0.15 ($n = 4$); [K308C/T339S], 1.0 ± 0.06 ($n = 3$); and [K308R/T339S], 0.96 ± 0.14 ($n = 4$). In view of this evidence that there are not marked differences among various mutated receptors in terms of expression at the plasma membrane, we may seek to interpret the absolute values of the currents evoked by ATP. In the original studies on Lys[69] and Lys[308], it was found that substitution of either lysine by alanine produced a channel that was insensitive to ATP up to 3 mM (Jiang et al., 2000). The increased sensitivity to ATP that we find in the [T339S] mutant allows the effect of each of the lysine-to-alanine substitutions to be examined more closely, and this showed a qualitative difference between their contributions. In the background of the T339S substitution, the substitution [K308R] caused a clear reduction in the maximum current (Fig. 3*C*), whereas the effect of removing Lys[69] (in K69A) was more consistent with a rightward shift in the ATP concentration–response curve. As Colquhoun (1998) has pointed out, a primary change in a binding site might be expected to cause a right-shift in agonist concentration–response curve, whereas a primary effect at a subsequent step (such as gating) would be expected to reduce the maximal response.

Partial agonist actions of suramin and TNP-ATP at P2X$_2$[T339S]

Suramin is an antagonist at most P2X receptors (Khakh et al., 2001), including rat P2X$_2$ (Brake et al., 1994; Evans et al., 1995). However, we found that suramin strongly increased channel activity when applied to P2X$_2$[T339S] receptors (Fig. 4*A*,*B*) (also

$P2X_2$[T339G]) (data not shown): it had no such agonist activity at wild-type receptors in concentrations up to 1 mM (Fig. 4*D*). The unitary conductance of channels activated by suramin (18 ± 1 pS; $n = 6$; $p > 0.05$) was not different from that which occurred spontaneously. At the whole-cell level, the suramin EC_{50} for $P2X_2$[T339S] was ~100 μM (Fig. 4*B*) (150 μM for $P2X_2$[T339G]) (data not shown). The current–voltage relationship for the suramin-activated currents was not strikingly different from that for ATP-activated currents at wild-type receptors (Fig. 4*C*), although the maximal current amplitudes were approximately one-fifth of those activated by ATP (100 μM) (Fig. 5). Suramin retained its activity to block ATP-evoked currents in $P2X_2$[T339S] receptors (Fig. 4*D*). The IC_{50} was ~10 μM, similar to that observed for wild-type receptors (Fig. 4*D*), and to the values previously reported for rat $P2X_2$ receptors (Evans et al., 1995). These results indicate that suramin is a partial agonist at $P2X_2$[T339S] receptors.

A similar agonist action of suramin was observed in $P2X_2$[K69A/T339S] (Fig. 5*A*). These data are consistent with suramin binding to a site distinct from that of ATP, in agreement with other studies suggesting that suramin does not act as a competitive antagonist (Evans et al., 1995; Trujillo et al., 2006). In contrast, mutation of Lys[308] (i.e., $P2X_2$[K308A/T339S]) resulted in the loss of the agonist action of suramin. This is consistent with Lys[308] playing a key role in gating, independent of the agonist used to open the channel.

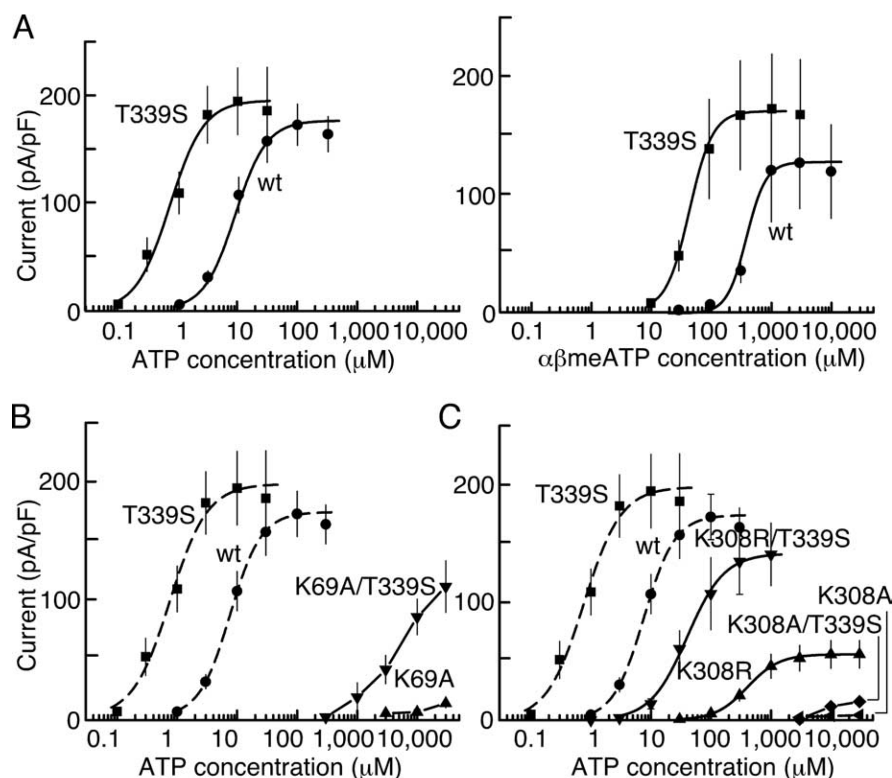

Figure 3. Agonist-activated currents in $P2X_2$[T339S] alone and in combination with substitutions at Lys[69] and Lys[308]. ***A***, ATP (left) and αβmeATP (right) concentration–response curves for cells expressing wild-type or $P2X_2$[T339S] receptors. ***B***, ATP concentration–response curves for double mutations [K69A/T339S] and [K69A] alone. The broken lines show same data as in ***A***. ***C***, ATP concentration–response curves for double mutations [K308A/T339S] and [K308A] alone, and for double mutations [K308R/T339S] and for [K308R] alone. The broken lines show same data as in ***A***. Peak currents were measured at −60 mV.

TNP-ATP is a competitive antagonist at P2X receptors, with a 1000-fold selectivity for $P2X_1$, $P2X_3$, $P2X_{2/3}$ receptors relative to others (Virginio et al., 1998; Khakh et al., 2001). We found that TNP-ATP activated $P2X_2$[T339] receptors (Fig. 5*B*). This agonist action required a very high concentration (EC_{50}, ~200 μM), but wild-type $P2X_2$ receptors were not activated by such concentrations of TNP-ATP (up to 3 mM). In both the double mutants $P2X_2$[K69A/T339S] and $P2X_2$[K308A/T339S], TNP-ATP lost its agonist action (Fig. 5*B*). If TNP-ATP binds to the same site(s) as ATP, as is suggested for the homomeric $P2X_3$ receptor (Burgard et al., 2000) and for the homomeric $P2X_2$ receptor (Trujillo et al., 2006), then these results indicate that Lys[69] is involved in agonist binding. PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid) (3 mM; 5 min application) had no discernible agonist action at $P2X_2$[T339S] receptors.

Discussion

This is the first report of single-channel recordings in conjunction with mutations in a P2X receptor. We found that replacement of Thr[339] by either serine or glycine produced a channel with considerable spontaneous activity. However, replacement by alanine or cysteine did not result in significant spontaneous channel opening. This suggests that this part of the protein is critically involved in the molecular rearrangements of channel gating, but it does not yet allow a simple interpretation in terms of the precise side-chain requirements. In any event, the Thr residue at position 339 of the rat $P2X_2$ receptor is not completely conserved among P2X subunits [in mammalian sequences, it is Gly in $P2X_1$; Ala in $P2X_4$, $P2X_5$, and $P2X_6$; Ser in $P2X_7$; and Thr in $P2X_2$ and $P2X_3$; and in the protist *Dictyostelium discoideum* sequence, it is Gly (Fountain et al., 2007)]. Therefore, the atomic detail of the change introduced by [T339S] is quite subunit specific. Previous mutagenesis experiments on rat $P2X_2$ receptors in which whole-cell currents were measured indicate that Cys (Egan et al., 1998; Rassendren et al., 1998) and Ala (Li et al., 2004) are well tolerated at this position in the rat $P2X_2$ receptor (Egan et al., 1998; Rassendren et al., 1998), although replacement of Thr[339] by Ala was reported to slightly increase ATP sensitivity (Li et al., 2004). In rat $P2X_4$ receptors, the substitution of the equivalent Ala[344] by Trp renders the channel ~30-fold more sensitive to ATP (Silberberg et al., 2005).

The destabilization of a closed state of the channel that occurs with the [T339S] substitution allows one also to study the agonist actions of αβmeATP. This ATP analog is generally considered to be without agonist action at homomeric $P2X_2$ receptors, and it is therefore a valuable tool in distinguishing $P2X_1$ receptors, and $P2X_3$ subunit-containing receptors (Lewis et al., 1995; North, 2002). Wild-type $P2X_2$ receptors are indeed activated by αβmeATP, but only at concentrations exceeding 30–100 μM (Wilkinson et al., 2006). Other point mutations been described in $P2X_2$ receptors that result in an increased sensitivity to αβmeATP (e.g., Phe[44] in the first TM of $P2X_2$ receptors) (Jiang et al., 2001). We note that Phe[44] and Thr[339] are situated at approximately the same relative distance through the membrane in most models of the P2X receptor (North, 2002) and could both contribute to a channel gate.

A role in permeation for Thr[339] comes from the work by Egan

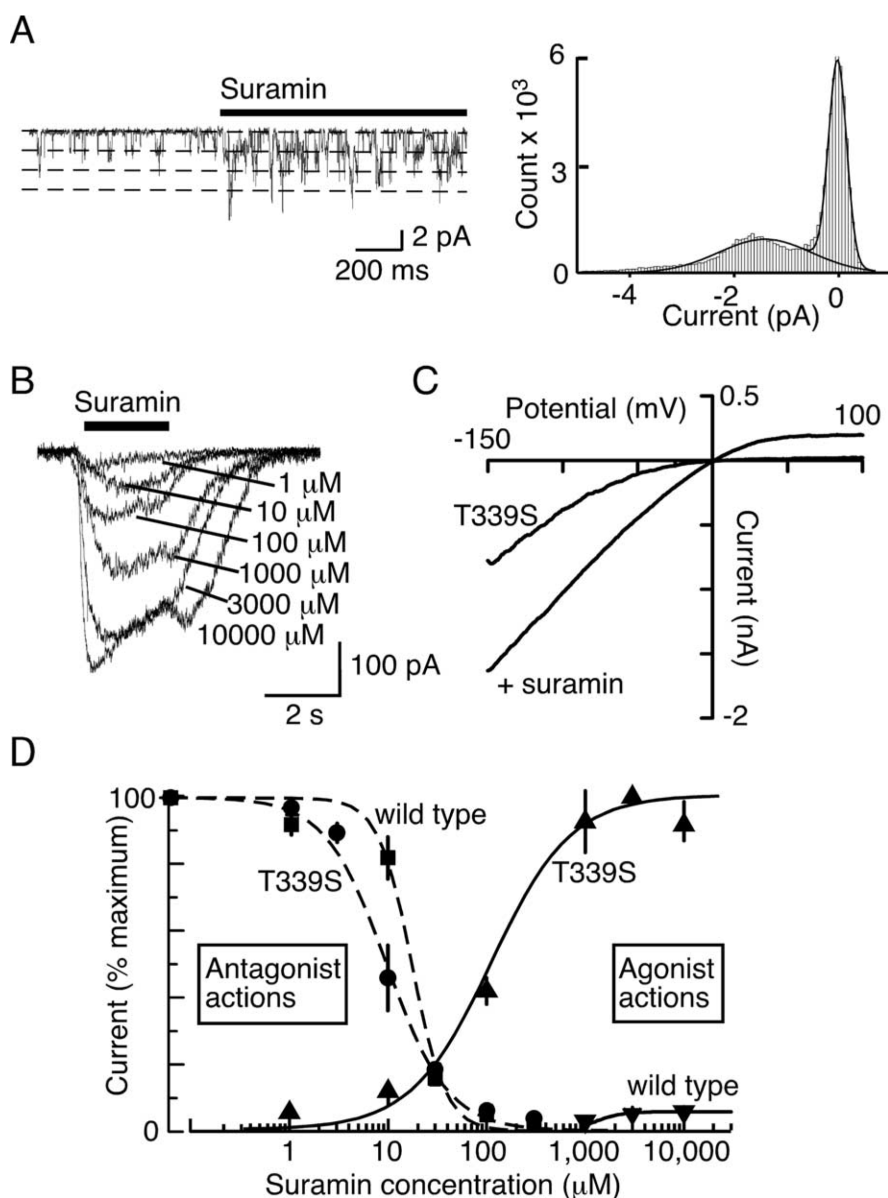

Figure 4. Agonist actions of suramin. ***A***, Outside-out patch recordings (left) and all-points histogram (right) from cell expressing $P2X_2$[T339S] receptors: suramin concentration was 30 μM. ***B***, Whole-cell recordings show currents evoked by different concentrations of suramin. ***C***, Current–voltage relationship for current activated by suramin (100 μM). ***D***, Concentration–response curve for suramin as agonist at $P2X_2$[T339S] receptors (continuous line), and as an antagonist of ATP action at $P2X_2$[T339S] and wild-type receptors (broken line). ATP concentration was 5 μM for wild type and 1 μM for T339S.

and Khakh (2004). They measured both membrane currents and the change in intracellular calcium concentration, and were thus able to calculate the fraction of the inward current carried by calcium. They found that the introduction of amino acids with larger side chains (Val, Tyr) at this position led to a marked reduction in the permeability of calcium relative to sodium ions. They concluded that this residue therefore lies close to the selectivity filter of the channel.

An important consideration of the present work was to use the spontaneous activity of the mutant P2X receptor channel to distinguish inferences regarding the effects of mutations on agonist binding or on channel gating (Colquhoun, 1998, 2006). However, one must expect that a wide range of mutations could destabilize a channel protein so as to produce spontaneous activity. Thus, several ligand-gated channels have been reported to open spontaneously when (relatively minor) mutations have been introduced into their structure. Some examples for homomeric channels are GABA ρ receptors (Pan et al., 1997; Torres and Weiss, 2002), glycine $\alpha1$ receptors (Dupre et al., 2007), nicotinic $\alpha7$ receptors (Bertrand et al., 1997), and glutamate $\delta2$ receptors (Zuo et al., 1997). The approach is of course subject to the criticism that the open state of the mutated channel is surely not identical with that of the liganded channel. It is for this reason that we have focused in the present study on the relatively striking change observed with a rather minor side chain alteration (Thr to Ser), at a position in the receptor at which such an alteration is tolerated in other closely homologous subunits.

With these caveats, we proceed to discuss the additional effect of mutating either of the lysine residues previously inferred to be involved in ATP binding. Taking first Lys^{69}, we found no evidence that the replacement of Lys^{69} by Ala had any effect on the spontaneous channel activity introduced by T339S; that is, there was no difference between [T339S] and [K69A/T339S] in the degree of spontaneous activity observed in the absence of agonist. It was difficult to measure accurately the relative change in effectiveness of applied ATP caused by the substitution of T339S because of the very weak response to ATP observed in $P2X_2$[K69A] channels ($pEC_{50} \ll 1.5$) (Fig. 3*B*) (Jiang et al., 2000). This made it impossible to estimate the $\Delta(\Delta G)$ associated with the substitution of Ala for Lys^{69} in the wild-type channel. ATP did have a clear agonist action at the [K69A/T339S] channel ($pEC_{50} \approx 2$) (Fig. 3*B*), which is consistent with the 10-fold leftward shift of the dose–response curve for ATP that was observed when this substitution was introduced alone into the wild-type channel.

Whether Lys or Ala was present at position 69 made no difference to the agonist action of suramin that was observed in the $P2X_2$[T339S] background. On the other hand, the nucleotide analog TNP-ATP lost its agonist action at $P2X_2$[T339S] when the additional [K69A] mutation was introduced. These results are consistent with suramin acting allosterically to open the $P2X_2$ channel that has been destabilized by the T339S substitution. They are in accord with the interpretation that the competitive blocker TNP-ATP likely shares key binding residues with ATP, including Lys^{69}. A simple conclusion from these results is that Lys^{69} does not play a significant role in channel gating (at least for the [T339S] receptor): the large effect of removing the lysine at this position remains consistent with a direct involvement in agonist binding.

Turning next to Lys^{308}, the most surprising finding was that the double-mutant channel $P2X_2$[K308A/T339S] did not show

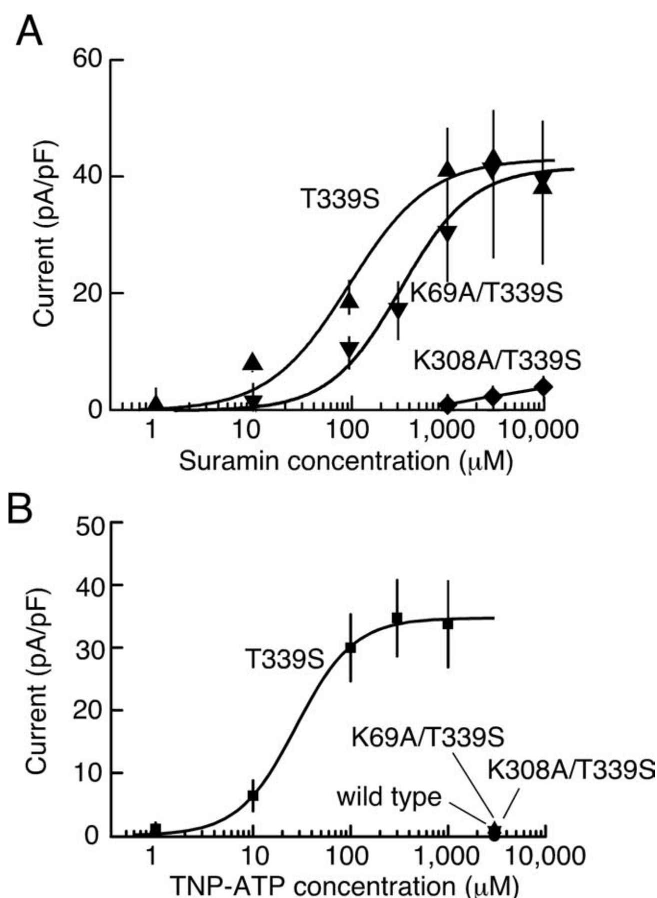

Figure 5. Agonist actions of suramin and TNP-ATP at $P2X_2$[T339S] receptors. ***A***, Maximal currents activated by suramin at the receptors indicated. ***B***, Maximal currents activated by TNP-ATP, at the receptors indicated. Note the difference in the current scales compared with Figure 3. Error bars indicate SEM for 6–11 cells in each case.

the spontaneous opening observed with $P2X_2$[T339S]. In other words, lysine at position 308 is required for the conformational change associated with channel opening. If we proceed on the assumption that the open state(s) observed with the T339S mutation are essentially similar to those normally induced by ATP (they have the same unitary conductance), then it follows that Lys^{308} is critical for channel opening. With respect to this effect, arginine can only partially substitute for lysine. The K308R substitution did not cause such a large shift in ATP potency as K308A, and this allowed us to compare the change in free energy associated with the introduction of the T339S in the wild-type ($-2.33RT$) and the K308R ($-2.38RT$) channels. This similarity suggests that each mutation has an independent effect on channel gating.

At the $P2X_2$[T339S] receptor, suramin behaved as a partial agonist. In the absence of ATP, suramin activated currents with an EC_{50} of ~100 μM. The properties of the currents (unitary conductance, current–voltage relationship) were not obviously different from those evoked by ATP. Suramin also blocked the effect of added ATP with an IC_{50} of ~10 μM; this concentration is similar to that required to block wild-type $P2X_2$ receptors (Fig. 4) (Evans et al., 1995). Removal of the lysine residue at position 69 did not alter the agonist action of suramin, consistent with its action at an allosteric site, distinct from that used by ATP. Removal of the lysine residue at position 308 did prevent the agonist action of suramin. This would be consistent with Lys^{308} being critical for channel opening in the $P2X_2$[T339S] receptor (see above), although a direct involvement of Lys^{308} in suramin binding cannot be excluded.

The interpretation of the experimental findings with TNP-ATP are complicated by the need to use rather high concentrations [at least, relative to those that block $P2X_1$, $P2X_3$, and $P2X_{2/3}$ receptors (Virginio et al., 1998)]. However, the agonist action of TNP-ATP at $P2X_2$[T339S] receptors was completely lost when either lysine residue was additionally mutated to alanine (Fig. 5*B*). Taken with the previous results, the simplest interpretation of these findings is that TNP-ATP loses its agonist action in $P2X_2$[K69A/T339S] because Lys^{69} is needed for binding, and it loses its effect at $P2X_2$[K308A/T339S] because the P2X receptor cannot open without an intact Lys^{308}.

The principal finding of the present work is that Lys^{308} is similarly important for opening of the $P2X_2$ receptor channel whether this is driven by ATP binding or by a relatively minor amino acid substitution within the second TM [T339S]. Although this does not exclude a role for Lys^{308} in ATP binding, it clearly implies a role in channel gating. The additional finding that Lys^{308} is also important for the agonist action of suramin is most consistent with a primary role in gating. These findings refocus attention on the very highly conserved region of all P2X receptors between Lys^{308} and the extracellular end of the second TM domain (likely in the region of Ile^{328}). This could imply that this part of the receptor forms a crucial "linker" that transduces binding in the ectodomain to movement of the transmembrane regions (Yan et al., 2006). However, another simple model that would position the lysine closer to the middle of the second TM would be a reentrant loop of the kind that is found in potassium channels, and that was initially proposed for P2X receptors when their sequences were first disclosed (Brake et al., 1994; Valera et al., 1994).

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