Desensitization Masks Nanomolar Potency of ATP for the P2X<sub>1</sub> Receptor

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ATP-gated P2X<sub>1</sub> receptors feature fast activation and fast desensitization combined with slow recovery from desensitized states. Here, we exploited a non-desensitizing P2X<sub>2</sub>/P2X<sub>1</sub> chimera that includes the entire P2X<sub>1</sub> ectodomain (Werner, P., Seward, E. P., Buell, G. N., and North, R. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15485–15490) to obtain a macroscopic representation of intrinsic receptor kinetics without bias arising from the overlap of channel activation and desensitization. From the stationary currents made amenable to analysis by this chimera, an EC<sub>50</sub> for ATP of 3.3 nM was derived, representing a >200- and >7000-fold higher ATP potency than observed for the parental P2X<sub>1</sub> and P2X<sub>2</sub>, receptors, respectively. Also, other agonists activated the P2X<sub>2</sub>/P2X<sub>1</sub> chimera with nanomolar EC<sub>50</sub> values ranging from 3.5 to 73 nM in the following rank order: 2-methylthio-ATP, 2′,3′-O-(4-benzoylbenzoyl)-ATP, α,β-methylene-ATP, adenosine 5′-O-(3-thiotriphosphate). Upon washout, the P2X<sub>2</sub>/P2X<sub>1</sub> chimera desensitized more quickly at high ATP concentration (ranging from 63 to 2.5 s) that is inversely related to the EC<sub>50</sub> value for the corresponding agonist. This suggests that deactivation time courses reflect unbinding rates, which by themselves define agonist potencies. The P2X<sub>2</sub>/P2X<sub>1</sub> chimera and the P2X<sub>2</sub>, receptor possess virtually identical sensitivity to inhibition by the P2X<sub>2</sub>, receptor-selective antagonist NF279, a suramin analog. These results suggest that the P2X<sub>2</sub>, ectodomain confers nanomolar ATP sensitivity, which, within the wild-type P2X<sub>2</sub>, receptor, is obscured by desensitization such that only a micromolar ATP potency can be deduced from peak current measurements, representing an amalgam of activation and desensitization.

Extracellular ATP is a ubiquitous signaling molecule that exerts fast effects by directly gating cation-conducting channels designated P2X receptors, which exist on a large variety of cells, including many excitable cells and different leukocytes (1). Seven genes encoding P2X subunits (P2X<sub>1</sub>–7) are known in mammals. All P2X subunits share a common membrane topology, with two hydrophobic membrane-spanning segments (M1 and M2) separated by a large extracellular loop of ~300 amino acid residues, which comprise the ATP-binding domain. At least part of the pore of the P2X receptor is lined by M2 (2), which includes a conserved glycine residue that is likely to constitute the channel gate (3), but also residues of the C-terminal cytoplasmic tail appear to play a role in controlling channel permeability (4). The cytoplasmic N-terminal tails of the various subunit isoforms are approximately the same length (~30 amino acid residues), whereas the cytoplasmic C-terminal tails are highly variable in length. Both chemical cross-linking studies and blue native polyacrylamide gel electrophoresis analysis of wild-type and concatenated P2X<sub>1</sub> and P2X<sub>2</sub>, receptors indicate that P2X receptors feature a trimeric architecture, which is unique among ligand-gated ion channels (5–7). Further details of the structure of P2X receptors are unknown because neither high resolution electron micrographs nor x-ray or NMR analyses have been published.

Various homo- and heteromeric P2X receptors have been characterized in heterologous expression systems. Based on their sensitivity to the ATP analog α,β-MeATP and the rate of current desensitization, homomeric P2X receptors are generally divided into at least two categories: rapidly desensitizing (P2X<sub>1</sub>, and P2X<sub>3</sub>, receptors) and slowly or non-desensitizing (P2X<sub>2</sub>A, P2X<sub>4</sub>, and P2X<sub>7</sub>, receptors). The term P2X<sub>2</sub>A distinguishes the non-desensitizing full-length P2X<sub>2</sub>A subunit from the desensitizing C-terminal splice variant termed P2X<sub>2</sub>B, which lacks a stretch of 69 amino acids C-terminal to M2 (8). The different rates of desensitization have been attributed by mutational analysis to various structural motifs, including N- and C-terminal domains (9, 10) and a highly conserved protein kinase C site (11).

We have demonstrated recently that nanomolar ATP concentrations drive significant fractions of the rapidly desensitizing P2X<sub>1</sub>, receptor pool into a long lasting refractory closed state, from which it recovers only slowly with a time constant of ~12 min (12). A 50% steady-state P2X<sub>1</sub>, receptor desensitization was achieved by the sustained exposure of P2X<sub>1</sub>, receptor-expressing oocytes to as little as 3 nM ATP. Several lines of experimental evidence suggest that the P2X<sub>1</sub>, receptor enters the desensitized state both at low and high ATP concentrations exclusively through the open conformation. Such behavior can be adequately described by a minimal three-state model, closed-open-desensitized, according to which P2X<sub>1</sub>, receptor activation and desensitization follow the same reaction pathway, i.e. without significant closed-to-desensitized transition. Recovery from desensitization occurs without channel opening, i.e. exclusively from desensitized to closed.

One conclusion of our previous desensitization study (12) is that the nanomolar potency of ATP for receptor desensitization...
can be attributed to the large ratio between the fast activation rate and the slow recovery rate from desensitization. The affinity for binding of ATP to the resting state of the P2X<sub>1</sub> receptor could not be deduced from our experiments, but model calculations based on simulated data allowed us to estimate an upper limit of 100 nM for the ATP affinity of this step. Up to this concentration, the ATP binding affinity seems to have no influence on the EC<sub>50</sub> values for receptor activation and desensitization. This suggests that the resting P2X<sub>1</sub> receptor state possesses a higher affinity for ATP than revealed by peak current measurements, which provide only an E<sub>C50</sub> value determined under non-steady-state conditions that is significantly biased by the rapid rate of P2X<sub>1</sub> receptor desensitization.

Desensitization can be fully eliminated in the P2X<sub>1</sub> receptor by simply replacing the first 47 amino acids of the P2X<sub>1</sub> subunit with a complementary portion of the P2X<sub>2</sub> subunit corresponding to the cytoplasmic N-terminal tail and almost the entire first transmembrane domain (9). Because these regions comprise only intracellular portions of the subunit and ATP interacts exclusively with the ectodomain (13, 14), the ATP binding properties of such a P2X<sub>2</sub>/P2X<sub>1</sub> chimera can be expected to be identical to that of the genuine P2X<sub>1</sub> receptor. In this study, we exploited this non-desensitizing P2X<sub>2</sub>/P2X<sub>1</sub> chimera for a more rigorous evaluation of agonist and antagonist potencies under steady-state conditions without the bias of desensitization. We found that elimination of desensitization unmasks nanomolar ATP potency for the P2X<sub>1</sub> receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP (disodium salt) was purchased from Roche Applied Science (Mannheim, Germany). α,β-MeATP, 2-MeSATP, ATP-S<sub>γ</sub>S, and BaATP were from Sigma (München, Germany). The suramin analog NF279 (8,8′-Carbonylbis[iminodi-(4-1-phenyle)carboxamido] bis(1,5-naphthalenylsulfonic acid) hexa- dium salt) was kindly provided by Günter Lambrecht (Pharmakologisches Institut für Naturwissenschaftler, Universität Frankfurt am Main, Frankfurt am Main, Germany). P2X<sub>1</sub> Receptor Expression in Xenopus Oocytes—Plasmids encoding the rP2X<sub>2</sub>, subunit (GenBank TM accession number Z80477 (15)) and the wild-type rP2X<sub>2A</sub> subunit (accession number U14414 (16)) with an N-terminal hexahistidinetag (His<sub>6</sub>-rP2X<sub>2A</sub>) have been described previously (5). To generate an rP2X<sub>2</sub>/P2X<sub>1</sub> chimera comprising the first 47 N-terminal and amino acids of the rP2X<sub>2</sub> subunit joined in-frame with Val<sup>1</sup>-Ser<sup>80</sup> of the 399-amino acid rP2X<sub>2</sub> subunit (9), Snal sites were introduced by site-directed mutagenesis, cutting just before the corresponding Val<sup>1</sup> codons of the pNKS2-rP2X<sub>1</sub> and pNKS2-His<sub>6</sub>-rP2X<sub>2</sub> plasmids, respectively. The N-terminal His<sub>6</sub>-rP2X<sub>2A</sub> sequence was excised with HindIII and SnaBl and ligated in-frame between the HindIII site of the vector and the inserted SnaBl cleavage site of pNKS2-rP2X<sub>1</sub>, yielding pNKS2-rP2X<sub>1</sub>/P2X<sub>2</sub>. Capped cRNAs were synthesized from linearized plasmids, and 50-μl aliquots were injected into follicle cell-free Xenopus oocytes (17) at 0.5 μg/μl (P2X<sub>1</sub>, cRNA), 2.5 μg/μl (rP2X<sub>2A</sub>, cRNA), and 25 μg/μl (rP2X<sub>2</sub>/P2X<sub>1</sub>, cRNA). Prior to electrophysiological experiments, the injected oocytes were incubated at 18 °C in sterile oocyte Ringer’s solution (90 mM NaCl, 1 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 10 mM Hepes, pH 7.4) supplemented with 50 μg/ml gentamicin.

**Electrophysiology**—Two-electrode voltage clamp recordings were performed 1–3 days after cRNA injection. Microelectrodes were pulled from borosilicate glass, filled with 3.3 M KCl, and broken at the tips to achieve electrical resistances below 1.0 and 1.5 megohms for the current and potential electrode, respectively. Currents were recorded with a Turbo TEC-05 amplifier (npi electronic, Tamm, Germany), low-pass-filtered at 200 Hz, and sampled at 500 Hz (INT-10 AD/DA converter, npi electronic) using commercially available software (CellWorks, npi electronic). To avoid activation of endogenous Cl<sup>−</sup>-free oocyte Ringer’s solution (MgCl<sub>2</sub> substituted for CaCl<sub>2</sub> and the potential electrode, respectively). Currents were recorded with a Turbo TEC-05 amplifier (npi electronic, Tamm, Germany), low-pass-filtered at 200 Hz, and sampled at 500 Hz (INT-10 AD/DA converter, npi electronic) using commercially available software (CellWorks, npi electronic). To avoid activation of endogenous Cl<sup>−</sup>-free oocyte Ringer’s solution (MgCl<sub>2</sub> substituted for CaCl<sub>2</sub> and the potential electrode, respectively). Currents were recorded with a Turbo TEC-05 amplifier (npi electronic, Tamm, Germany), low-pass-filtered at 200 Hz, and sampled at 500 Hz (INT-10 AD/DA converter, npi electronic) using commercially available software (CellWorks, npi electronic). To avoid activation of endogenous Cl<sup>−</sup>-free oocyte Ringer’s solution (MgCl<sub>2</sub> substituted for CaCl<sub>2</sub> and the potential electrode, respectively). Currents were recorded with a Turbo TEC-05 amplifier (npi electronic, Tamm, Germany), low-pass-filtered at 200 Hz, and sampled at 500 Hz (INT-10 AD/DA converter, npi electronic) using commercially available software (CellWorks, npi electronic). To avoid activation of endogenous Cl<sup>−</sup>-free oocyte Ringer’s solution (MgCl<sub>2</sub> substituted for CaCl<sub>2</sub> and the potential electrode, respectively). Currents were recorded with a Turbo TEC-05 amplifier (npi electronic, Tamm, Germany), low-pass-filtered at 200 Hz, and sampled at 500 Hz (INT-10 AD/DA converter, npi electronic) using commercially available software (CellWorks, npi electronic).
of the rP2X2/P2X1 chimera is indicative of a high ATP potency (Fig. 2B). In contrast to the micromolar EC50 values of ATP for the parental P2X receptors, the rP2X2/P2X1 chimera was half-maximally activated by 3.3 ± 0.1 nM ATP (nH = 1.6 ± 0.1) (Fig. 2A and B), corresponding to almost 4 orders of magnitude higher potency than for the rP2X2A receptor and still >200-fold higher potency than for the rP2X1 receptor. Strikingly, virtually the same concentration of 3.2 nM ATP was previously found to half-maximally desensitize the wild-type rP2X1 receptor by sustained exposure to low levels of ATP (12).

The nanomolar potency of ATP for the rP2X2/P2X1 chimera could be observed only with completely defolliculated oocytes, which are virtually devoid of ecto-ATPase activity (19). Expression of the chimera in incompletely defolliculated oocytes resulted in a profound reduction of ATP potency (Fig. 2C) in a manner attributable to rapid ATP breakdown by ecto-ATPases located on residual follicle cells (20).

**Deactivation Rates Define Agonist Potencies for the rP2X2/P2X1 Chimera**—The stable ATP analog α,β-MeATP is a high potency agonist for the rapidly desensitizing P2X1 and P2X2 receptors, but a low potency agonist for the P2X2A receptor (EC50 > 300 μM) (21). Accordingly, α,β-MeATP can be used to distinguish between P2X1- and P2X2-like agonist properties. Consistent with P2X1-like agonist properties, the rP2X2/P2X1 chimera was efficiently activated at low concentrations of α,β-MeATP (Fig. 3G). Virtually equal currents could be elicited by maximally effective ATP and α,β-MeATP concentrations of 1 μM each (Fig. 3A). Upon α,β-MeATP washout, the current declined also monoexponentially (τ = 2.5 ± 0.3 s, n = 8), but at a 25-fold higher rate than after ATP washout (Fig. 3F), although still slow enough to be resolved unbiased by the rate of solution exchange in our system. Strikingly, a similar 20-fold ratio difference existed also between the EC50 values for α,β-MeATP and ATP, 67 ± 3 nM (nH = 1.7 ± 0.1) and 3.3 ± 0.1 nM (nH = 1.6 ± 0.1), respectively (Figs. 2B and 3G). This led us to hypothesize that deactivation rates are inversely related to EC50 values.
To examine this issue further, we determined the deactivation time constants (Fig. 3, B–F) and EC₅₀ values (Fig. 3G) for three additional well known P₂X receptor agonists: 2-MeSATP, BzATP, and ATP·γS. Upon washout, currents declined monoexponentially to the base-line level in response to all of the agonists (Fig. 3, B–F), yet with markedly different rates. A similar slow deactivation as with ATP was observed after washout of 2-MeSATP (τ₂₅⁰ = 63 ± 2 s versus τ₂-MeSATP = 53 ± 2 s), which activated the rP₂X₂/P₂X₁ chimera as potently as ATP (EC₅₀ = 3.5 ± 0.1 and 3.3 nM, respectively) (Fig. 3G). In contrast, washout of the significantly less potent agonists ATP·γS and α,β-MeATP (EC₅₀ = 73 ± 4 and 67 ± 3 nM, respectively) was followed by a much faster deactivation (τ₂-MeATP = 3.9 ± 0.2 s and τₐ,β-MeATP = 2.5 ± 0.3 s). BzATP showed intermediate potency (EC₅₀ = 16.6 ± 0.7 nM) and, upon washout, resulted in an intermediate deactivation rate (τ₂-BzATP = 23.8 ± 0.5 s). Plotting the EC₅₀ values for the various agonists against the corresponding deactivation rate constants revealed a linear relationship (Fig. 3H), thus lending support to the hypothesis that deactivation rates define agonist potencies for the rP₂X₂/P₂X₁ chimera.

Antagonist Binding Properties of the rP₂X₂/P₂X₁ Chimera—In a further attempt to characterize the ligand binding properties of the rP₂X₂/P₂X₁ chimera, we took advantage of NF279, a derivative of the classical P₂X antagonist suramin. The P₂X antagonist selectivity profile of NF279 is different from that of suramin. In particular, NF279 allows one to distinguish between recombinant rP₂X₁ and rP₂X₂A receptors, which are blocked at 1 and 10 μM ATP with IC₅₀ values for NF279 of 19 nM and 0.76 μM, respectively (22) (see also Fig. 4B). To examine the inhibitory potency of NF279 for the rP₂X₂/P₂X₁ chimera, we co-applied 1–100 nM NF279 and 5 μM ATP (Fig. 4A), a concentration that elicited an approximately half-maximal response in the sustained presence of ATP (Fig. 4A). Fig. 4B shows the corresponding concentration-inhibition curves. A fit of the Hill equation to the data yielded an IC₅₀ value of 16.4 ± 0.8 nM (nH = 2.0 ± 0.2) compared with an IC₅₀ value of 19 ± 0.8 nM for the wild-type rP₂X₁ receptor (22). The almost identical IC₅₀ values and the virtually overlapping concentration-inhibition curves indicate that NF279 produces a equipotent inhibition of the rP₂X₂/P₂X₁ chimera and the rP₂X₂A receptor, an ~50 times higher NF279 concentration is required.

To elucidate the mechanism of antagonism by NF279 for the rP₂X₂/P₂X₁ chimera, ATP concentration-response curves were established in the absence and presence of increasing concentrations of NF279. Concentration-response curves were shifted to the right without altering the maximal ATP response (Fig.

![Image](http://www.jbc.org/abs/images/6429fig2.jpg)
The inset BzATP (\(\text{H9251}\)), was activated with high potency by \(\text{H9251}\) NF279 blocked the rP2X2/P2X1 chimera with virtually the same activated by saturating concentrations (1 IC50 value as for the wild-type rP2X1 receptor, whereas receptor (23, 24). (iv) The rP2X2/P2X1 chimera does not include agreement with KD receptor. (i) Like the rP2X1 receptor, the rP2X2/P2X1 chimera data to Equation 3 (see '4C'), indicating that the antagonism is competitive. A fit of the ATP-binding site, which is located just extracellular to the extracellular portions of the P2X2 receptor and hence no part of rP2X2A receptor. (iii) The EC50 values of 3.3 and 67 nM for ATP higher NF279 concentrations were required for blocking the antagonist binding properties as the wild-type rP2X1 receptor based on monoexponential function to the receptor currents after agonist washout. The gray curves represent nonlinear least-squares fits of the Hill equation to the data. H, EC50 values of these fits plotted versus the respective rate constants of deactivation \(k_{dA} = 1/r\). The straight line represents a linear fit to the data \(r = 0.96; p < 0.05\). The inset shows the region near the origin on an expanded scale to allow two overlapping data points to be seen separately.

FIG. 3. Correlation between potencies and deactivation rate constants for various agonists of the rP2X2/P2X1 chimera. A, currents activated by saturating concentrations (1 \(\mu\)M) of \(\alpha,\beta\)-MeATP (black bar) and ATP (gray bar) demonstrating that both agonists elicited virtually the same maximal response. B-F, original current traces recorded in response to ATP, 2-MeSATP, BzATP, ATP-\(S\), and \(\alpha,\beta\)-MeATP, respectively. Agonists were applied for the periods indicated by the horizontal bars. Currents that deactivated slowly are shown on an expanded time scale. Note the markedly different rates of current decline. The gray curves underlying the original current traces (black curves) represent fits of a single monoeXponential function to the receptor currents after agonist washout. G, agonist concentration-response curves for ATP (\(\blacktriangle\), 2-MeSATP (\(\blacklozenge\), BzATP (\(\triangle\), \(\alpha,\beta\)-MeATP (\(\ast\)), and ATP-\(S\) (\(\bullet\)). Lines represent nonlinear least-squares fits of the Hill equation to the data. H, EC50 values of these fits plotted versus the respective rate constants of deactivation \(k_{dA} = 1/r\). The straight line represents a linear fit to the data \(r = 0.96; p < 0.05\). The inset shows the region near the origin on an expanded scale to allow two overlapping data points to be seen separately.

4C), indicating that the antagonism is competitive. A fit of the data to Equation 3 (see “Experimental Procedures”) yielded a \(K_{a}\) value for NF279 of 12.5 \(\pm\) 1.2 nM \((n_H = 2.3 \pm 0.2)\) and an EC50 value for ATP of 3.3 \(\pm\) 0.2 nM \((n_H = 1.8 \pm 0.1)\).

DISCUSSION

This study shows that elimination of desensitization un-masks nanomolar ATP potency for the rP2X1 receptor based on the analysis of a non-desensitizing rP2X2/P2X1 chimera described first by others (9). Several lines of evidence strongly indicate that the rP2X2/P2X1 chimera features the same agonist and antagonist binding properties as the wild-type rP2X1 receptor. (i) Like the rP2X1 receptor, the rP2X2/P2X1 chimera was activated with high potency by \(\alpha,\beta\)-MeATP, which is a low potency agonist for P2X3\(_{\alpha2}\) receptors. (ii) The suramin analog NF279 blocked the rP2X2/P2X1 chimera with virtually the same IC50 value as for the wild-type rP2X1 receptor, whereas ~50-fold higher NF279 concentrations were required for blocking the rP2X3\(_{\alpha2}\) receptor. (iii) The EC50 values of 3.3 and 67 nM for ATP and \(\alpha,\beta\)-MeATP, respectively, obtained in this study are in close agreement with \(K_{a}\) values derived in binding studies with \(\alpha,\beta\)-[\(\text{H}\)]MeATP and [\(\text{32P}\)]ATP as ligands for the recombinant P2X1 receptor (23, 24). (iv) The rP2X2/P2X1 chimera does not include extracellular portions of the P2X2 receptor and hence no part of its ATP-binding site, which is located just extracellular to the transmembrane domains in P2X channels (13, 14).

Interestingly, transplantation of the first transmembrane domain of the P2X1 subunit to the P2X2 subunit has been shown to confer \(\alpha,\beta\)-MeATP sensitivity to the corresponding receptor chimera, indicating that the first transmembrane domain participates in \(\alpha,\beta\)-MeATP-induced channel gating (21). However, the high \(\alpha,\beta\)-MeATP sensitivity of the rP2X2/P2X1 chimera studied here appears to result from the rP2X2 ectodomain because the first transmembrane segment originated entirely from the \(\alpha,\beta\)-MeATP-insensitive rP2X2 subunit. It must be mentioned that, in the original study, the EC50 of ATP for an apparently identical rP2X2/P2X1 chimera was found to be not different from the control value (9). Because it is known that the true potency of ATP to activate P2X receptors in native tissue can be profoundly reduced by ectonucleotidas, we consider degradation of ATP by the ecto-ATPase in native Xenopus oocytes, as used in this study, do not show significant extracellular hydrolysis of ATP (19). Indeed, we could artificially render the rP2X2/P2X1 chimera non-responsive to low ATP concentrations using incompletely defolliculated oocytes for expression.

For theoretical reasons (25), a significantly lower EC50 for
ally all activated P2X 1 receptors close rapidly by desensitiza-

tion and not by direct transition to the reactivable closed state,
fits of the Hill equation to the data. Note that the fast desensitization of the rP2X 1 receptor to ATP and the comparably slow binding of NF279
required a 10-s pre-equilibration of the rP2X1 receptor with NF279 before stimulation with ATP. This period is sufficient to ensure that a binding
equilibrium between NF279 and the rP2X1 receptor is reached (22). In contrast, the steady-state inhibition of the non-desensitizing receptors can
be directly deduced from the current plateaus attained in the simultaneous presence of ATP and NF279. C, the rightward shift of the ATP
concentration-response curves at increasing concentrations of NF279 (0, 30, 100, and 300 nM) combined with the absence of depression of maximal
current indicates that NF279 acts as a competitive antagonist with ATP for the rP2X2/P2X1 chimera. A simultaneous fit of all the data to Equation
3 yielded a $K_{i}$ value for NF279 of 12.5 ± 1.2 nM ($n_{H} = 2.3 ± 0.2$ nM, $n = 5-6$).

the non-desensitizing rP2X2/P2X1 chimera than for the fast
desensitizing rP2X1 receptor is not surprising. Because virtu-
ally all activated P2X1 receptors close rapidly by desensitiza-
tion and not by direct transition to the reactivable closed state,
peak current measurements provide no more than an EC$_{50}$
determination under non-steady-state conditions (12). By elim-
ination of desensitization, stationary currents become amena-
table to analysis that allows for a determination of the EC$_{50}$
under steady-state conditions. The nanomolar ATP sensitivity
of the rP2X2/P2X1 chimera is compatible with our previous
calculations based on a kinetic model of the rP2X1 receptor
showing that any intrinsic affinity value below 100 nM can
be directly deduced from the current plateaus attained in the simultaneous presence of ATP and NF279. C, the rightward shift of the ATP
concentration-response curves at increasing concentrations of NF279 (0, 30, 100, and 300 nM) combined with the absence of depression of maximal
current indicates that NF279 acts as a competitive antagonist with ATP for the rP2X2/P2X1 chimera. A simultaneous fit of all the data to Equation
3 yielded a $K_{i}$ value for NF279 of 12.5 ± 1.2 nM ($n_{H} = 2.3 ± 0.2$ nM, $n = 5-6$).

only when apyrase is added to metabolize extracellular ATP
(26), suggesting that the low level of endogenous ATP
secretion is sufficient to desensitize this receptor (10). More-
over, a chimera containing the extracellular domain of the
rP2X3 receptor flanked by the transmembrane and cytosolic
domains of the rP2X2A receptor exhibits a 30-fold lower EC$_{50}$
value compared with the rP2X2A receptor (26). A leftward shift
of the concentration-response curve is also observed when the
native rP2X2A ectodomain is substituted with the rP2X3
ectodomain, suggesting that transmembrane domain-flanking
sequences can also affect agonist potency (27).

Deactivation of the P2X/P2X Channel-mediated Stationary Current Reflects ATP Unbinding—The kinetic behavior of
the rP2X3 receptor can be adequately described by a minimal
three-state reaction model: $C + ATP \leftrightarrow O_{ATP} \leftrightarrow D_{ATP}$, where
C is closed, O is open, and D is desensitized. Given that the
N-terminal part and the ectodomain of the P2X3 receptor
determine desensitization and ligand binding, respectively,
the rP2X2/P2X3 chimera corresponds to a non-desensitizing
rP2X3 receptor represented by the following reaction dia-
gram: $C + ATP \leftrightarrow O_{ATP}$. The experimentally determined
agonist potency is given by $EC_{50} = K_{A}(1 + E)$, where $K_{A}$
denotes the agonist equilibrium dissociation constant for the
binding step, and $E$ is the equilibrium constant for the closed-
to-open conformation change (28). As apparent from this equation, $E_{\text{C50}}$ approximates $K_A$ solely under the restricted condition that $E$ is $\ll 1$. To discuss our data in terms of $K_A$ and $E$, an expanded reaction diagram is necessary, given by Reaction 1.

$$
\text{Reaction 1}
$$

The observed slow deactivation of the rP2X$_2$/P2X$_1$ chimera after ATP washout could then be due to (i) a channel-inherent slow transition from OATP to CATP, followed by a faster dissociation of ATP from C$_{\text{ATP}}$ to C, or, alternatively, (ii) a slow ATP dissociation after fast channel closure. In both cases, the time course of deactivation reflects the time course of ATP dissociation, although not necessarily the microscopic dissociation rate.

A possible clue that allows one to distinguish between these two possibilities comes from the observation that the potency of several agonists to activate the rP2X$_2$/P2X$_1$ chimera correlates well with the time constant of deactivation after washout of the corresponding agonist. Hence, the deactivation after agonist washout appears to reflect the unbinding properties of the agonist, $C_{\text{agonist}} \rightarrow C + \text{agonist}$, and not the channel-inherent $O_{\text{agonist}} \rightarrow O_{\text{agonist}}$ transition. A similar relationship has been previously observed for the $E_{\text{C50}}$ values and agonist unbinding rates for nicotinic acetylcholine receptor and $N$-methyl-$d$-aspartate receptors and is thus in line with the widespread view that differences in agonist affinity are predominantly determined by the ligand unbinding rate (29, 30). It should be noted, however, that a time constant of 63 s for ATP unbinding is still too short to account for slow recovery of the rP2X$_2$ receptor from the desensitized state, which occurs with a 10-fold longer time constant of $\sim$12 min (12). In summary, current deactivation after removal of agonist directly reflects dissociation of ATP from the receptor. Circumstantial evidence favors the view that agonist dissociation represents the rate-limiting step for current deactivation. Accordingly, current deactivation may simply correspond to the microscopic dissociation rate of agonist.

**Antagonism by the P2X$_1$-selective Suramin Derivative NF279**—We have previously shown for the desensitizing rP2X$_1$ receptor that ATP concentration-response curves are shifted to the right and exhibit an increasing depression of the maximal response to ATP when oocytes are pre-equilibrated in the presence of increasing concentrations of the P2X$_1$-selective antagonist NF279 (22). This behavior could be assigned to a so-called hemic-equilibrium resulting from the fast rise of the P2X$_1$ receptor-mediated current to its peak, leaving little time for the slowly dissociating NF279 to equilibrate with the fast binding ATP. An apparent $K_D$ value of 14 nM was estimated using a double-reciprocal plot (31), but this analysis did not allow us to confirm or reject a competitive type of antagonism by NF279 for the P2X$_1$ receptor. The present experiments with the non-desensitizing rP2X$_2$/P2X$_1$ chimera clearly indicate that NF279 meets the criteria for competitive antagonism in that it causes parallel rightward shifts in the ATP concentration-response curve without depression of maximal current. The virtually identical IC$_{50}$ and $K_B$ values obtained with the rP2X$_2$/P2X$_1$ chimera suggest that the simplifying assumptions we made previously were reasonable approximations. Because the NF279 binding properties of the rP2X$_2$/P2X$_1$ chimera closely reflect those of the rP2X$_1$ receptor, the use of the chimera can be advantageous in unraveling the exact mechanism of antagonism, which cannot be determined reliably by non-steady-state measurements.

**Physiological Implications**—The present findings suggest that ATP sensitivity and unbinding are, in addition to $\alpha$-MeATP responsiveness and desensitization patterns, additional fundamental features that distinguish P2X receptors. Two principles seem to have evolved. (i) Highly ATP-sensitive P2X receptors such as the P2X$_1$ receptor (and potentially also the P2X$_2$ receptor) feature slow agonist unbinding combined with rapid and sustained desensitization, whereas (ii) moderately or poorly ATP-sensitive P2X receptors feature rapid agonist unbinding and slow desensitization. We postulate that the link of high ATP sensitivity and sustained desensitization is not by coincidence but is the result of a demand of high ATP sensitivity and related slow ATP unbinding; desensitization serves to ensure rapid termination of the P2X$_1$ receptor-mediated current despite the slow rate of ATP unbinding, thus shaping the response. Likewise, enduring elevated low levels of ATP, even if existing only occasionally, would lead without desensitization to cell toxicity problems arising from permanently open cation channels. Low ATP concentrations may, for instance, occur at the border of a synaptic cleft and, due to the high ATP sensitivity of the P2X$_1$ receptor, lead to a silencing of P2X$_1$ receptor responses when this synapse is repeatedly activated. On the other hand, moderately or poorly ATP-sensitive receptors may serve to respond repeatedly to rapid rises to high ATP concentrations as they occur, for instance, under physiological conditions in a synapse, where the transmitter rises and falls rapidly. Due to their low ATP sensitivity, these P2X receptors shut immediately when ATP concentrations fall to the submicromolar level and are thus immediately prepared to respond to the next rise in the ATP level. Altogether, these two receptor principles may complement each other in subserving particular demands in neuronal excitability.

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