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MOLECULAR PHARMACOLOGY
Vol. 66, No. 6
3038/1186560
Mol Pharmacol 66:1738–1747, 2004
Printed in U.S.A.

Divergent Effects of the Purinoceptor Antagonists Suramin and Pyridoxal-5′-phosphate-6-(2′-naphthylazo-6′-nitro-4′,8′-disulfonate) (PPNDS) on α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptors

Erika Suzuki, Markus Kessler, Kyle Montgomery, and Amy C. Arai

Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, Illinois

Received May 19, 2004; accepted September 23, 2004

ABSTRACT

Suramin is a large naphthyl-polysulfonate compound that inhibits an array of receptors and enzymes, and it has also been reported to block currents mediated by glutamate receptors. This study shows that suramin and several structurally related compounds [8,8′-[carbonylbis(imino-3,1-phenylenecarbonylamino)]bis-(1,3,5-naphthalenetrisulfonic acid), 6Na (NF023), 8,8′-(carbonyl-bis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino))bis-1,3,5-naphthalenetrisulfonic acid, Na (NF279), and 4,4′,4″,4‴-[carbonyl-bis[imino-5,1,3-benzenetriyl-bis-(carbonylimino)]]tetrakis-benzene-1,3-disulfonic acid, 8Na (NF449)] reduce binding of [^{3}H]α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and [^{3}H]fluorowillardiine to rat brain membranes and homomeric GluR1–4 receptors, with IC_{50} values in the range of 5 to 180 μM. Inhibition often was less than complete at saturating drug concentrations and thus seems to be noncompetitive in nature. Pyridoxal-5′-phosphate-6-(2′-naphthylazo-6′-nitro-4′,8′-disulfonate) (PPNDS) is a potent antagonist of purinoceptors that shares some structural elements with suramin yet is smaller than the latter. PPNDS also had potent effects on AMPA receptors (EC_{50} value of 4 μM) but of a kind not seen with the other compounds in that it increased binding affinity for radioagonists severalfold. In addition, PPNDS slowed association and dissociation rates more than 10 times. In physiological experiments with GluR2 receptors, PPNDS at 50 μM reduced the peak current by 30 to 50% but had only small effects on other waveform aspects such desensitization and steady-state currents. This pattern of effects differentiates PPNDS from other compounds such as thiocyanate and up-modulators, which increase agonist binding by enhancing desensitization or slowing deactivation, respectively. Receptor model simulations indicate that most effects can be accounted for by assuming that PPNDS slows agonist binding/unbinding and stabilizes the bound-closed state of the receptor. By extension, suramin is proposed to stabilize the unbound state and thereby to reduce affinity for agonists. These drugs thus act through a novel type of noncompetitive antagonism.

Suramin is an extended molecule with a symmetrical backbone of amide-linked aromatic rings and with three negatively charged sulfonate substituents on each of the terminal naphthyl elements (Fig. 1). As such, it shares many structural similarities with a broader class of polysulfonated compounds that include the azo-dyes trypan blue, Evans blue, Chicago sky blue, and basilen blue (also called reactive blue 2). Like many of the latter, suramin has a remarkably diverse range of pharmacological actions, yet the mechanisms underlying these effects are in most instances still obscure. Suramin was developed 80 years ago and is still occasionally used for the treatment of trypanosomiasis and onchocerciasis (Wang 1995). In vitro, suramin affects a bewildering array of molecular and cellular processes. It inhibits numerous signaling proteins, including growth factors and interleukins (Voogd et al., 1993); among its many other targets are G proteins (Freissmuth et al., 1996) and enzymes such as reverse transcriptase (De Clercq, 1987) and ectonucleotidases

This work was supported by National Institutes of Health grant NS41020.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.104.003038.

ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, *N*-methyl-D-aspartate; PPADS, pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline; PPNDS, pyridoxal-5′-phosphate-6-(2′-naphthylazo-6′-nitro-4′,8′-disulfonate); HEK, human embryonic kidney; HBS, HEPES-buffered saline; SCN, thiocyanate anion; GluR, glutamate receptor; FW, fluorowillardiine; NF023, 8,8′-[carbonylbis(imino-3,1-phenylenecarbonylimino)]bis-(1,3,5-naphthalenetrisulfonic acid), 6Na; NF279, 8,8′-(carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino))bis-1,3,5-naphthalenetrisulfonic acid, Na; NF449, 4,4′,4″,4‴-[carbonyl-bis[imino-5,1,3-benzenetriyl-bis-(carbonylimino)]]tetrakis-benzene-1,3-disulfonic acid, 8Na; GYKI52466, 4-(8-methyl-9*H*-1,3-dioxolo[4,5-*h*][2,3]benzodiazepin-5-yl)-benzeneamine dihydrochloride.

(Lambrecht, 2000). However, suramin seems particularly potent in blocking purinergic receptors with low micromolar inhibition constants (Charlton et al., 1996; Lambrecht, 2000).

There are also reports that suramin inhibits glutamatergic synaptic transmission (Motin and Bennett, 1995; Gu et al., 1998) and whole-cell currents mediated by AMPA receptors (Nakazawa et al., 1995; Zona et al., 2000) and NMDA receptors (Ong et al., 1997; Peoples and Li, 1998). Suramin was observed to inhibit binding of radioligands to NMDA receptors (Balcar et al., 1995) but not kainate receptors (Ong et al., 1997), which suggested some selectivity among glutamate receptors. Apart from these studies, there have been few efforts to characterize the molecular actions of suramin and the nature of the impact on glutamate receptor function. Even less is known about possible interactions with more recent P2X receptor antagonists, many of which possess structural elements similar to those in suramin. Figure 1 shows examples of three such compounds with terminal naphthyl-polysulfonate groups (Freissmuth et al., 1996; Lambrecht, 2000; Rettinger et al., 2000). A somewhat distinct structural family of purinoceptor antagonists was established some time ago with the development of PPADS (Lambrecht et al., 1992), which has since been widely used as a highly selective P2X receptor blocker. This compound does not have mirror symmetry, is much smaller than suramin, and was indeed found to have no or weak effects on glutamate receptor responses (Motin and Bennett, 1995; Gu et al., 1998; Zona et al., 2000). PPNDS, another member of this drug family, has recently been identified as having even greater affinity for P2X receptors and a marked selectivity toward the P2X1 receptor subtype with a nanomolar inhibition constant (Lambrecht et al., 2000). Yet the two sulfonate groups and the nitro substituent in this compound occupy the same positions as the sulfonate groups in suramin (Fig. 1) and thus the possibility of an interaction with glutamate receptors cannot be dismissed.

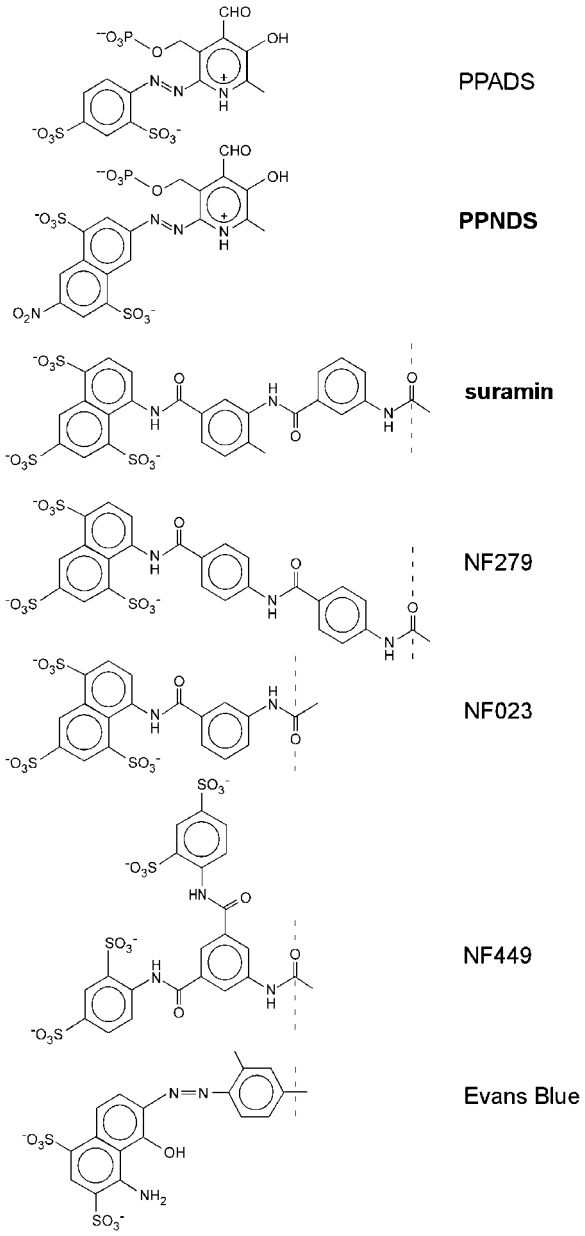

Fig. 1. Drug structures. For drugs that possess mirror symmetry, only one-half the molecule is shown; the symmetry axis is indicated with a dashed line. PPADS and PPNDS are derivatives of pyridoxal-5-phosphate and do not have mirror-symmetry. Evans blue has been reported to inhibit AMPA receptor currents (Keller et al., 1993). All compounds are normally supplied as sodium salts. The formulas show the anionic forms prevailing in neutral aqueous solutions.

The present study was intended to examine whether suramin inhibits binding to AMPA receptors, to clarify the nature of such inhibition, and to test whether the structurally related compounds have similar actions. Binding tests with radiolabeled agonists were used initially because they can readily distinguish between different subclasses of antagonists and their mode of inhibition. For instance, if suramin were to act as a competitive antagonist then it should completely displace radiolabeled agonists such as [^{3}H]AMPA from their binding site, as it is the case for quinoxaline compounds such as CNQX (Honore and Drejer, 1988). In contrast, 2,3-benzodiazepines such as GYKI52466 block AMPA receptor currents through a distinct site because they do not inhibit the binding of agonists (Kessler et al., 1996), except under specific circumstances (Arai et al., 2002b). Guanine nucleotides constitute a third group of AMPA receptor blockers that also reduce agonist binding but through an as yet unidentified mechanism (Monahan et al., 1988; Baron et al., 1989; Dev et al., 1996). Last, if suramin were to act as a channel blocker then binding of agonists would presumably remain unaffected. During examination of the structural analogs described above, it was also found that PPNDS affected AMPA receptors in ways that differed substantially from those of suramin, both in physiological and binding experiments. Thus, the second goal of this project was to identify which aspects of receptor kinetics are most likely influenced by PPNDS. The findings with both suramin and PPNDS have been incorporated into a working model that suggests that both drugs bind to a domain near the agonist-binding site yet with opposite consequences for the stability of the agonist-bound state.

Materials and Methods

Binding Assays. Binding tests were carried out with membranes from rat brain and from HEK293 cells expressing one of the AMPA receptor subunits. Animals were anesthetized with halothane before decapitation according to an institutionally approved protocol and in observation of the guidelines of the National Institutes of Health.

Membranes from rat brain were prepared according to conventional procedures (Kessler et al., 1996) involving homogenization in isotonic sucrose and differential centrifugation to obtain a P_2 pellet fraction, followed by an osmotic lysis and repeated washing by centrifugation. For binding tests, membranes were suspended in HEPES-buffered saline (HBS; 150 mM NaCl, 20 mM HEPES, and 0.1 mM EGTA, pH 7.4). Binding studies with recombinant receptors used a preparation of permeabilized and washed cells. Most receptor subunits examined here are stably expressed in HEK293 cells (Hennegriff et al., 1997; Arai et al., 2000) and periodically confirmed by Western blots. HEK293 cells were collected into HBS containing in addition 2 mM EGTA and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The cells were centrifuged at least four times (2000*g*; 10 min) and resuspended in HBS without additions; after the first centrifugation, 0.1% saponin was included in the HBS to permeabilize the membranes, and the cell suspension was left at 25°C for 3 min. All other steps were carried out at 0 to 4°C. The cells were stored on ice for up to 1 month without evident change in receptor properties and washed on each test day before use. For some tests, the permeabilized cells were extensively sonicated with an ice-cooled tip, and the resulting membrane fragments were collected by high-speed centrifugation (15 min; 60,000*g*).

Unless mentioned otherwise, binding tests were conducted using the following protocols. Aliquots of rat brain membranes or permeabilized cells (10–20 μg of protein) were incubated at 0°C with radioligand and appropriate additions in 50-μl volume. Where indicated (+SCN), incubation media contained 50 mM KSCN (potassium thiocyanate), which increases affinity for [^{3}H]AMPA about 10-fold. Incubations were terminated after 30 to 60 min by filtration through GF/A or GF/C filters after diluting the sample in 5 ml of ice-cold buffered saline containing 50 mM KSCN (wash buffer); the filters were rapidly rinsed with 15 ml of additional wash buffer. In some assays with brain membranes, incubations were terminated by 15-min centrifugation in a Beckman JA 18.1 rotor with rotor temperature calibrated to match incubation temperature. The pellet was quickly rinsed with ice-cold wash buffer. Stock solutions of test drugs were prepared in HBS. Nonspecific binding was determined with 5 mM glutamate and subtracted from total binding. The drugs in general had no significant effect on nonspecific binding and did not cause quenching of scintillation counting. Protein content was determined according to Bradford (1976) with serum albumin as standard. Binding data were usually normalized and averaged across experiments and plotted as mean and S.E.M. Binding curves were fitted through nonlinear regression using the Prism program from GraphPad Software Inc. (San Diego, CA).

Excised Patch Currents Mediated by GluR2 Receptors. Currents mediated by homomeric GluR2(R607Q) flop or flip receptors were measured in HEK293 cells transiently transfected with the respective cDNAs in a pRK5 vector, using LipofectAMINE (Invitrogen, Carlsbad, CA). Cells were submitted to experimentation 72 to 96 h after transfection. Patch pipettes had a resistance of 2 to 5 MΩ and were filled with a solution of 130 mM CsF, 10 mM EGTA, 2 mM ATP Mg^{2+} salt, and 10 mM HEPES, pH 7.3. The extracellular solution contained 140 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES, pH 7.3. Patches were excised from the cells and positioned in front of a double pipet delivering a constant flow of background medium (without or with test drug) in one flow line and medium containing 1 mM L-glutamate in the second flow line. The patch was initially placed in the background flow line. A piezo device then moved the double pipet in a fraction of a millisecond into the glutamate flow line (Arai et al., 1996). In general, 10 responses were collected at 5-s intervals and averaged to give one trial. When testing PPNDS, several control trials were collected and then the background flow line was changed to medium containing 50 μM PPNDS. After three to four drug trials, the background medium was switched back to control medium. In tests with suramin, trials were repeatedly alternated between control condition and the various drug concentrations. The holding potential was −70 mV. Data were acquired with a patch amplifier (AxoPatch-1D) at a filter frequency of 5 kHz and digitized at 10 kHz with PClamp/Digidata 1322A (Axon Instruments Inc., Union City, CA).

Materials. *S*-[^{3}H]AMPA (40.8 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{3}H]Fluorowillardiine (36 Ci/mmol), PPNDS, PPADS, NF023, NF279, and NF449 were obtained from Tocris Cookson Inc. (Ballwin MO). Suramin and common laboratory reagents were from Sigma-Aldrich.

Results

Suramin reliably inhibited the binding of all radiolabeled ligands examined in this study. However, inhibition constants and extent of inhibition varied considerably between assay configurations (Fig. 2). In rat brain membranes, binding of [^{3}H]AMPA (+SCN; 25°C) was inhibited almost completely with an IC_{50} value of about 100 μM (Fig. 2A) and a Hill slope near 1. In contrast, binding of the agonist [^{3}H]fluorowillardiine (FW) was inhibited by only 70 to 80% (95% confidence intervals for lower asymptote, 12–32% at 25°C and 17–47% at 0°C), and Hill slopes were shallower (0.6–0.7; Fig. 2B). The less than complete inhibition was not caused by residual binding to another receptor because [^{3}H]FW binding was completely displaced by AMPA and CNQX (inset). Less than complete inhibition was also observed with [^{3}H]CNQX (IC_{50} value of 43 μM; lower asymptote, 20 ± 6%; $n = 2$; not shown). Similar differences between the radioagonists were seen in tests with GluR2-flop receptors stably expressed in HEK293 cells (Fig. 2C). In this preparation, [^{3}H]AMPA binding (without SCN; 0°C) was again inhibited by more than 95%, whereas [^{3}H]FW binding reached a plateau at 21% (95% confidence interval, 17–24%). IC_{50} values were generally smaller than in brain (18 μM for [^{3}H]AMPA), but Hill slopes were steeper, in particular for FW ($n_H = -1.6$). Similar inhibition profiles and IC_{50} values (17–49 μM for [^{3}H]AMPA binding without SCN) were obtained in tests with four other AMPA receptor subunits of both the flip and flop type (not shown). A number of observations rule out nonspecific drug effects or interference with the assay method, a possibility that was considered carefully given the massed negative charges and high aromaticity. For instance, inhibition was the same when incubations were terminated by centrifugation and filtration (Fig. 2A) and was only weakly dependent on ionic strength (not shown). With both assay techniques, values for nonspecific binding were not significantly altered except at the highest suramin concentrations (1–3 mM), at which they were reduced by maximally 20%. Identical inhibition curves were obtained if incubation times were extended from 30 to 90 min or beyond (not shown).

The less than complete inhibition seen in some assays suggested that the interaction between suramin and agonists is not competitive. In the remainder of Fig. 2 (D–F), we examined the nature of these interactions in more detail for [^{3}H]AMPA binding. These tests were carried out in the presence of KSCN so that near saturating concentrations of [^{3}H]AMPA could be reached. Figure 2D shows that suramin progressively shifted saturation curves for [^{3}H]AMPA to the right with only insignificant changes (<12%) in the B_{max} value. A similar right-shift was seen in inverse concentration-effect experiments in which suramin inhibition curves were constructed for different [^{3}H]AMPA concentrations (IC_{50} values of 44, 109, and 531 μM at 2, 30, and 180 nM

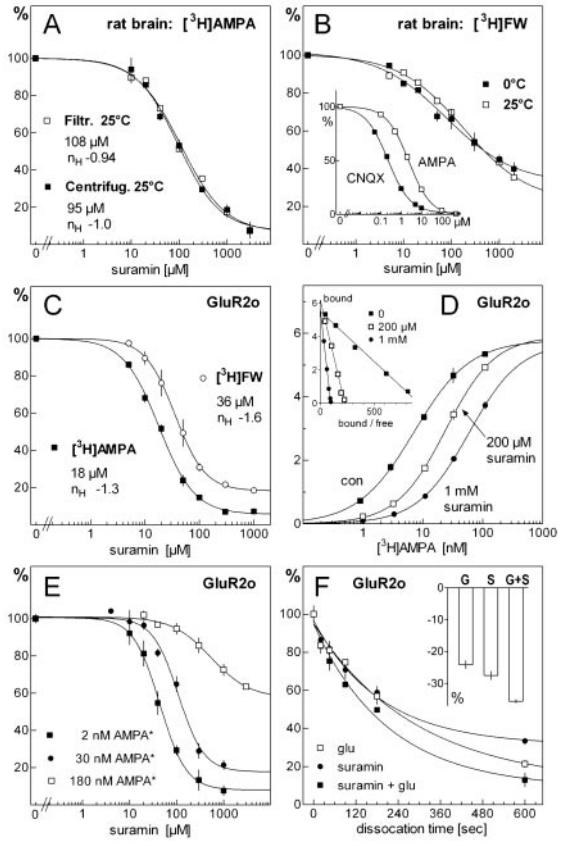

Fig. 2. Suramin inhibits binding to native (A and B) and homomeric (C–F) AMPA receptors. A, binding of [^{3}H]AMPA (50 nM) at 25°C to rat brain membranes in the presence of 50 mM potassium thiocyanate (SCN), using either filtration or centrifugation to terminate the incubation. Nonspecific binding was determined with 5 mM glutamate and subtracted from total binding. For each experiment, binding at each concentration of suramin was normalized to that in its absence. The data shown are means and S.E.M. from three experiments conducted with each assay type. The averaged data points were fitted with four-point logistic equations to obtain the IC_{50} and n_H values shown in the figure. Binding without suramin was 0.5 to 0.8 pmol/mg protein; specific binding was 80% (filtration) or 50% (centrifugation) of total binding. B, binding of [^{3}H]FW (50 nM, no SCN, filtration assay) at 0°C (IC_{50} value of 77 μM; n_H value of -0.61; bottom asymptote, 32%) and at 25°C (IC_{50} value of 178 μM; n_H value of -0.65; bottom asymptote, 23%; $n = 3$ each). The inset shows inhibition by CNQX and AMPA at 0°C in the same preparation (CNQX: IC_{50} value of 0.22 μM; n_H value of -0.80; bottom asymptote, 1%; AMPA: IC_{50} value of 0.24 μM; n_H of -0.80; bottom asymptote, -1%; both $n = 2$). Binding at 0 μM suramin was 2.4 (25°C) and 2.9 (0°C) pmol/mg; specific binding was >85% of total binding. C–F, tests with GluR2 flop receptors (GluR2o) stably expressed in HEK293 cells. C, binding of [^{3}H]AMPA (50 nM; $n = 7$) and [^{3}H]FW (5 nM; $n = 3$), both measured in the absence of SCN. Control binding was 1.6 (AMPA) and 1.7 (FW) pmol/mg; specific binding was 80 and 97% of total binding, respectively. D, effect of 0.2 and 1 mM suramin on [^{3}H]AMPA saturation curves ($n = 2$) measured in the presence of SCN. Specific binding was >90% of total binding under all conditions. Curve-fitting to the data points with four-point logistic equations yielded K_D values of 7.1, 24, and 59 nM; B_{max} values of 5.8, 5.9, and 5.7 pmol/mg; and n_H values of 0.93, 1.06, and 1.01, respectively. The inset shows an Eadie-Hofstee transformation of the same binding data and a linear regression fit to those data; B_{max} values without and with suramin differed by less than 12%. E, inhibition curves for suramin at three different [^{3}H]AMPA concentrations. Assays were conducted in the presence of SCN at 2, 30, and 180 nM [^{3}H]AMPA. IC_{50} values (95% confidence intervals) for suramin were 44 μM (37–51) at 2 nM [^{3}H]AMPA, 109 μM (89–132) at 30 nM [^{3}H]AMPA, and 531 μM (262–1077) at 180 nM [^{3}H]AMPA; n_H values were -1.55, -1.59, and -1.05, and lower asymptotes were 8.0 ± 2.2, 18 ± 3, and 57 ± 5%, respectively. F, effects of suramin and/or glutamate on dissociation (0°C) of bound AMPA; representative experiment (from $n = 3$). GluR2o receptors were equilibrated for 90 min with 10 nM [^{3}H]AMPA in the presence of SCN. To force dissociation, 2 μl of 50 mM glutamate, 20 mM suramin, or a combination of both, were added to 20 μl of the equilibrated aliquots. Inset, percentage dissociated at 90 s ($n = 6$).

[^{3}H]AMPA, respectively; Fig. 2E). Both right-shifts are basically compatible with a competitive interaction. However, a second notable change in Fig. 2E was that the lower asymptote progressively increased with the result that less than 50% of binding was inhibited at 180 nM [^{3}H]AMPA. The obvious similarity with [^{3}H]FW inhibition curves thus makes it seem likely that the nature of the inhibition is the same for both radioagonists, i.e., that suramin reduces affinity for both agonists through an allosteric mechanism, the main difference being that the reduction in affinity is larger for AMPA than for FW. The last panel of Fig. 2 shows dissociation of bound [^{3}H]AMPA upon adding high concentrations of glutamate, suramin, or a combination of both. If suramin reduces agonist affinity through an independent site, then dissociation would be expected to be accelerated by the drug. This was not the case, however, because dissociation time courses were rather similar for all three conditions, which in essence suggests that suramin can only occupy its site after glutamate has dissociated from *its* site. Although this may seem to be typical for competitive interactions, other interpretations are possible, as discussed later.

Polysulfonate analogs of suramin have recently been developed that possess orders of magnitude higher affinity for specific subtypes of purinergic receptors. The experiments of Fig. 3 examined the effects of three of these analogs, NF023, NF279, and NF449 on AMPA receptor binding. All compounds inhibited agonist binding to GluR2o and the IC_{50} values were similar to that of suramin, with NF023 being the most potent (4.5 μM). The most unusual finding was that inhibition by NF279 was governed by a Hill coefficient of a magnitude not previously seen in binding tests with any other AMPA receptor ligands (Fig. 3A). The coefficient ranged between 2.5 and 5.3 with an average of 4.1 and was similar across subunits (not shown). Tests with native AMPA receptors in rat brain membranes (Fig. 3B) similarly revealed a larger n_H value for NF279 (1.8) than for NF023 (0.9). In these experiments, maximal inhibition was again far from complete with lower asymptote values near 50%.

PPADS has been widely used as a P2X receptor antagonist but is now increasingly supplanted by more potent and selective inhibitors, one of them being PPNDS. Both of these

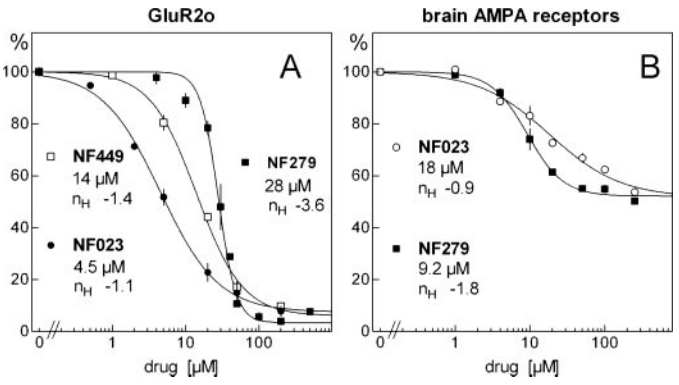

Fig. 3. Suramin analogs. A, binding of [^{3}H]AMPA (50 nM) to GluR2o receptors in the absence of thiocyanate (0°C); averages (with S.E.M.) from three (NF023), seven (NF279), and two (NF449) experiments. The 95% confidence interval of n_H for NF279 was from -2.9 to -4.2. B, binding of [^{3}H]FW (50 nM) to rat brain membranes (0°C); data are from two (NF023) and five (NF279) experiments. The n_H value, but not the bottom asymptote, differed significantly between the two curves [$F(1,10) = 8.912$; $p = 0.013$].

compounds contain a polysulfonated ring element, are much smaller than suramin, and lack mirror-symmetry (Fig. 1). PPNDS, however, possesses a naphthyl-disulfonate component that is almost identical to the terminal ring elements in suramin. As shown in Fig. 4A, PPADS was a weak inhibitor of [^{3}H]FW binding, which accords with the reported lack on AMPA receptor currents. In contrast, PPNDS produced a severalfold increase in [^{3}H]FW binding to rat brain membranes (Fig. 4A) and thus had effects that are fundamentally different from those of the other compounds. The drug was potent with an EC_{50} of 4.1 μM at 0 and 25°C, and the effect was highly reproducible over a wide range of incubation times (not shown). PPNDS also enhanced [^{3}H]fluorowillardiine binding if thiocyanate was included in the assay (2.3-fold; Fig. 4A); thiocyanate itself had only modest effects on [^{3}H]FW binding (60% increase at the near saturating concentration of 50 mM), in accordance with Hawkins et al. (1995). These observations allow us to dismiss the possibility that PPNDS mimicked the action of thiocyanate. Effects of PPNDS on recombinant GluR2 flop receptors (Fig. 4B) were similar to those on brain receptors with EC_{50} values of 3 to 5 μM. Scatchard tests showed that binding affinity for [^{3}H]FW was increased 8-fold after addition of 40 μM PPNDS (Fig. 4B, inset). An increase in agonist affinity was also observed in equivalent Scatchard tests with [^{3}H]AMPA (without SCN), but the magnitude of the increase was smaller (2.9-fold; not shown; $n = 2$). Binding to other AMPA receptor subunits (GluR1 flip, GluR1 flop, and GluR4 flip) was increased in similar manners by PPNDS (not shown).

Unusual results were also obtained when measuring association and dissociation of [^{3}H]FW. In the absence of drug, binding to rat brain membranes at 25°C reached a level close to equilibrium within seconds (rate constant estimated at >15 min^{-1}; Fig. 4C). When 100 μM PPNDS was added together with the radioagonist, binding again reached 85% of its plateau value within the first minute (rate constant 6 min^{-1}). However, when membranes were first preincubated with PPNDS, the subsequent association of [^{3}H]FW was drastically slowed and equilibrium was not reached for almost 1 h, the rate constant for the dominant component (66%) being 0.07 min^{-1}. Dissociation of [^{3}H]FW was similarly slowed more than 10 times if membranes had been equilibrated with the radioagonist in the presence of 100 μM PPNDS (Fig. 4D). These observations suggest that PPNDS can greatly slow association and dissociation of agonists and also that some aspects of drug action may change with longer exposure to PPNDS.

Figures 5 and 6 show the effects of PPNDS and suramin on AMPA receptor-mediated responses to glutamate pulses. These tests were carried out with outside-out patches excised from HEK293 cells transiently transfected with GluR2(R607Q) flip or flop receptors and used a piezo-driven application system to allow submillisecond resolution of the receptor current. The effects of 50 μM PPNDS on these responses were remarkably small and consisted mainly in a reduction in the amplitude (Fig. 5). This reduction was most prominent in the first trial after infusion of the drug and amounted to about 20 to 40%. Continued infusion of the drug reduced the peak amplitude further but at a lower rate. Other response parameters such as the rise time, the decay time constant and the steady-state current usually showed an increase during drug infusion but typically by a factor of

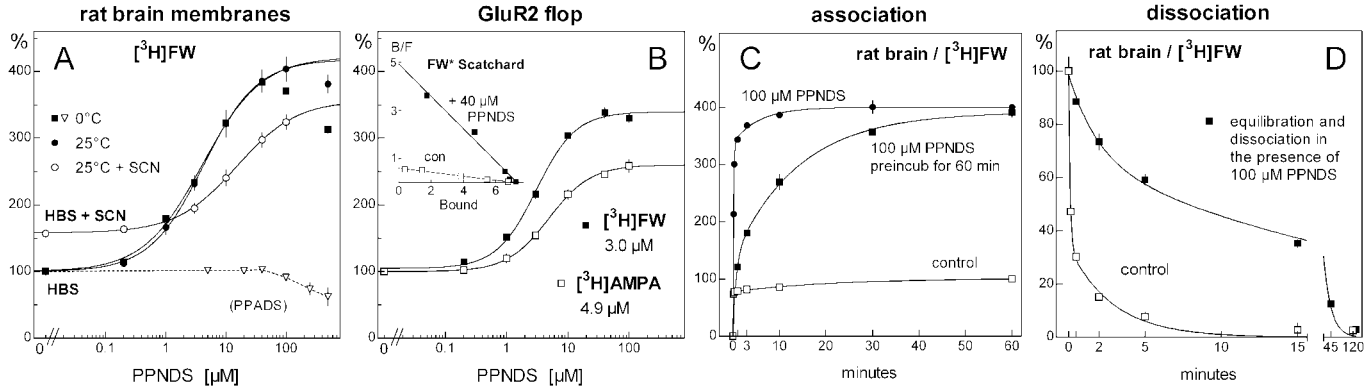

Fig. 4. Effect of PPNDS on agonist binding to AMPA receptors. A, binding of [^{3}H]FW (50 nM) to rat brain membranes at 0 or 25°C, without and with 50 mM SCN. For all data, binding was normalized to that without drug and without SCN. Percent values from three to five experiments for each assay condition were averaged and expressed as mean and S.E.M. Data points were fitted with logistic equations; data points obtained for the highest two drug concentrations (100 and 500 μM) were excluded from this fit because of a possibility of nonspecific effects. Control binding (0 PPNDS) was 2.7 pmol/mg protein at 25°C, or about 10% of the B_{max} value according to saturation tests. A also includes data obtained with PPADS (0°C; −SCN; $n = 3$). B, binding of [^{3}H]AMPA and [^{3}H]FW to GluR2o receptors. Membranes were incubated for 30 min at 0°C with 20 nM [^{3}H]AMPA (no SCN) or 5 nM [^{3}H]FW and the PPNDS concentrations indicated on the *x*-axis. Inset, Scatchard transformation of [^{3}H]FW saturation experiments in the absence of PPNDS ("con") and at the near saturating PPNDS concentration of 40 μM (averaged values from three experiments for each condition). Binding was measured at 0°C at 0.5, 2, 10, 30, and 100 nM [^{3}H]FW. K_D values for FW derived from the slope of the regression lines are 11.7 nM in the absence of drug and 1.5 nM in the presence of 40 μM PPNDS. C, association of [^{3}H]FW to rat brain membranes at 0 and 100 μM PPNDS (25°C; representative experiment). Membranes were mixed at t = 0 with 50 nM [^{3}H]FW and filtered at the time indicated. PPNDS was added either together with [^{3}H]FW (circles) or 60 min before (squares). Data were normalized to control binding at 60 min. Curve fittings with two-exponential functions were in all cases statistically preferred over single-exponential fits ($p < 0.05$; extra-sum-of-squares F-tests by Prism). Rate constants (in min^{-1}) were simultaneous application, 6.7 (85%) and 0.18 (15%); and PPNDS preincubation, 1.5 (34%) and 0.07 (66%). Association in controls was too fast to be accurately determined (>15 min^{-1} for the dominant component). Similar results were obtained in six other experiments with both rat brain and GluR2o receptors. D, dissociation at 25°C from brain membranes pre-equilibrated for 90 min with 50 nM [^{3}H]FW, either without or with 100 μM PPNDS (representative experiment). Dissociation was initiated by adding 2 μl of 50 mM glutamate (2 mM final). Data were normalized to the respective binding at t = 0. Two-exponential fits were again statistically preferred over one-site fits, which may be in part a result of the presence of high- and low-affinity binding sites in brain membranes. Rate constants were (in min^{-1}): control 10 (66%) and 0.35 (34%), with PPNDS 0.5 (32%) and 0.04 (68%). Similar results were obtained in four other experiments with rat brain and GluR2o receptors.

less than 2. Steady-state currents in some experiments increased by a larger factor, but they always remained small compared with the respective peak currents (left) and hence responses with both receptor subtypes continued to exhibit strong desensitization even after infusion of the drug, in clear distinction from the effects of typical AMPA receptor upmodulators such as CX546, which essentially abolished desensitization (Arai et al., 2002b). PPNDS effects were not readily reversible and most response parameters did not return to baseline after drug offset, even after washout times of several minutes. The effects of suramin in the same recording system differed substantially from those of PPNDS in that currents were completely blocked (Fig. 6) and readily reversed after drug washout. However, like PPNDS, suramin did not have a significant effect on the waveform of the response (Fig. 6, inset). The IC_{50} value was on the order of 20 to 60 μM and thus agreed with those obtained from binding tests.

Figure 7 illustrates one possible interpretation for the effects of PPNDS in the framework of a previously used elementary receptor model (Patneau and Mayer, 1991; Ambros-Ingerson and Lynch, 1993; Arai et al., 2002b). The equation given in the figure caption shows how binding affinity is related to rate constants in this particular model. A plausible first scenario would be that PPNDS slows both association and dissociation of the agonist without affecting channel gating and desensitization. As shown by the simulations of receptor currents (Fig. 7, center), a 20-fold slowing of these rate constants (condition 2) leads to a reduction in the peak current similar to that observed with modest or no changes in steady-state currents, rise times, and decay time constants. With these modifications, however, binding affinity is not altered and hence there must be additional changes in receptor kinetics. One such possibility is that PPNDS causes a stabilization of the bound-closed state with the consequence that dissociation from this state is slowed more than the association (condition 3 in Fig. 7). This would lead to a commensurate increase in binding affinity because the K_D value is in first approximation given by the ratio $k_{-1}/k_1 \times k_{-2}/k_2$. If we assume that the rate of dissociation is slowed 200 times, then the response wave form remains similar to that of condition 2, but the binding K_D is greatly reduced and hence binding measured at any fixed [^{3}H]agonist concentration would be increased; small changes in other rate constants may account for the observed minor changes in the steady-state current. These properties are reminiscent of those observed with PPNDS and thus we tentatively propose that the primary effect of this drug is to slow agonist association and dissociation and that it either stabilizes the bound-closed receptor state or destabilized the unbound state.

Discussion

This study has shown that suramin-type compounds and PPNDS have major effects on AMPA receptors at low micromolar concentrations and that due caution should be exercised when interpreting experiments in which such drugs are used to block purinergic receptors. Of greater interest, how-

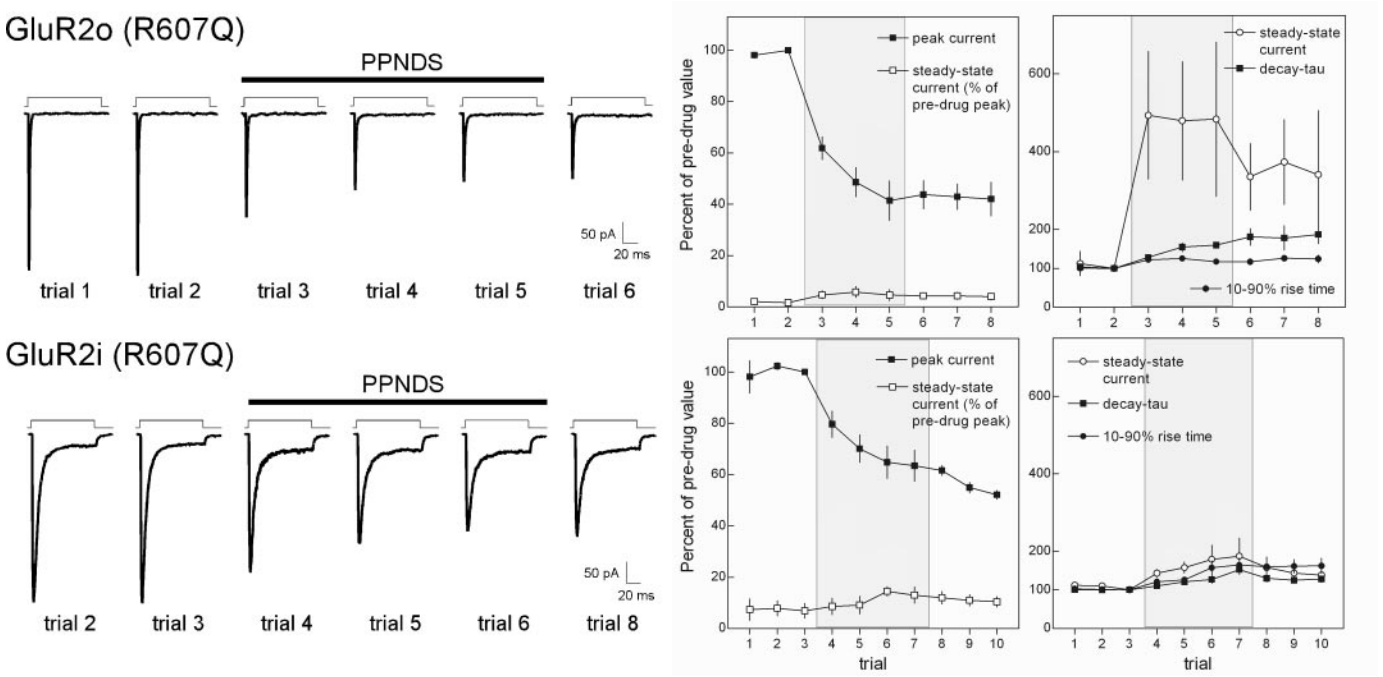

Fig. 5. Effects of PPNDS on currents through GluR2 flip and flop receptors. Left, recordings collected before, during, and after application of 50 μM PPNDS. Outside-out patches were excised from HEK293 cells transiently transfected with GluR2(R607Q) flip or flop. Traces are averages of ten consecutive responses to 100-ms pulses of 1 mM L-glutamate. Right, averaged data for the peak current (left) and steady-state current, 10 to 90% rise-time and decay time constant (right) before, during, and after exposure to 50 μM PPNDS. The drug was applied in the background flow solution to which the patch was exposed between the glutamate pulses; the shaded area indicates the duration of drug exposure. For each trial, 10 recordings collected at 5-s intervals were averaged. Response parameters were extracted for each trial and normalized to those of the trial immediately preceding PPNDS application. Data are means and S.E.M. from seven (flip) and nine (flop) patches. The decay time constants and steady-state values used for this graph were obtained from single-exponential fittings using a window of about 50 ms; with longer glutamate applications, steady-state currents tended to decay further toward baseline but at a very slow rate (not analyzed). In the graphs on the left, steady-state currents are also shown as a percentage of the peak current immediately before drug.

ever, are questions concerning the nature of the drug action and the finding that effects on AMPA receptors differed substantially between drugs. Thus, suramin and the NF analogs consistently inhibited agonist binding, whereas PPNDS increased agonist binding to an extent rarely seen with other drugs. The effects of suramin on [^{3}H]AMPA binding on first inspection seemed competitive with essentially complete inhibition at high drug concentrations and a right-shift in the saturation concentration-effect curve for AMPA. However, the only partial inhibition of [^{3}H]fluorowillardiine binding and of [^{3}H]AMPA binding when tested at high [^{3}H]AMPA concentrations makes it in the end seem more likely that suramin instead acted on a site distinct from that for agonists and that it thereby lowered the affinities for the latter. The inhibition by suramin can thus be distinguished from the antagonistic effects produced by drugs like CNQX and the 2,3-benzodiazepines.

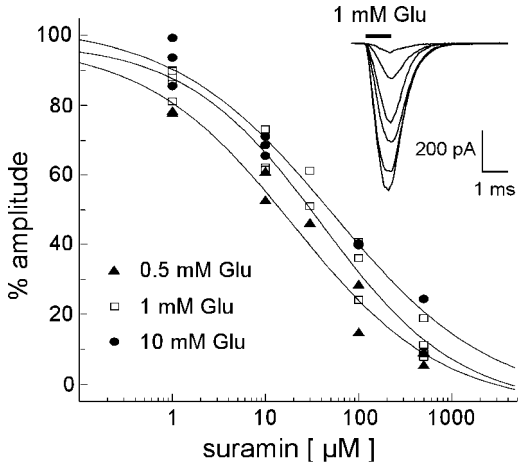

Fig. 6. Inhibition by suramin of GluR2 receptor currents. Currents through GluR2(R607Q) flop receptors in response to 1-ms pulses of 0.5, 1, or 10 mM glutamate in outside-out patches. Suramin was present at 1 to 500 μM in the background medium between glutamate pulses. Data were collected from nine patches and fitted with logistic curves for each glutamate concentration. IC_{50} values were 21, 31, and 66 μM, respectively, and Hill slopes were between 0.5 and 0.6. Inhibition was complete when extrapolated to saturating suramin concentrations. The inset shows a typical set of responses from one patch in the presence of 0, 1, 10, 30, 100, and 500 μM suramin.

The effects of PPNDS were unexpected yet highly consistent across preparations and assay conditions. Binding affinity for FW was increased 8-fold and accompanied by a slowing of both association and dissociation. In physiological tests, PPNDS reduced the peak amplitude of responses to glutamate but had conspicuously weak impact on other response parameters. This pattern of effects is unique and clearly different from that previously reported for other modulatory factors. Thus, thiocyanate, which also increases binding affinity for [^{3}H]AMPA, accelerated desensitization in patch experiments (Arai et al., 1995) and lowered the steady-state current in oocyte recordings (Bowie and Smart, 1993), both of which pointed to the conclusion that it facilitates transition into the desensitized state. PPNDS did not promote desensitization and its effects on binding were not occluded by thiocyanate. Agonist binding is also increased in the presence of many drugs referred to as up-modulators. Although structurally and functionally diverse, these compounds augment the current passed through AMPA receptors by increasing the peak current and/or slowing the decay of the current after glutamate pulses (Arai and Lynch, 1998; Arai et al., 2002a,b). Increases in agonist binding produced by these drugs were usually less than 100%, with only a few exceptions (Arai et al., 2002a,b). The latter compounds, however, also caused a dramatic slowing of response deactivation and desensitization, and it has been argued that binding increase and response prolongation are related (Arai et al., 2002b). These effects are again clearly distinct from those of

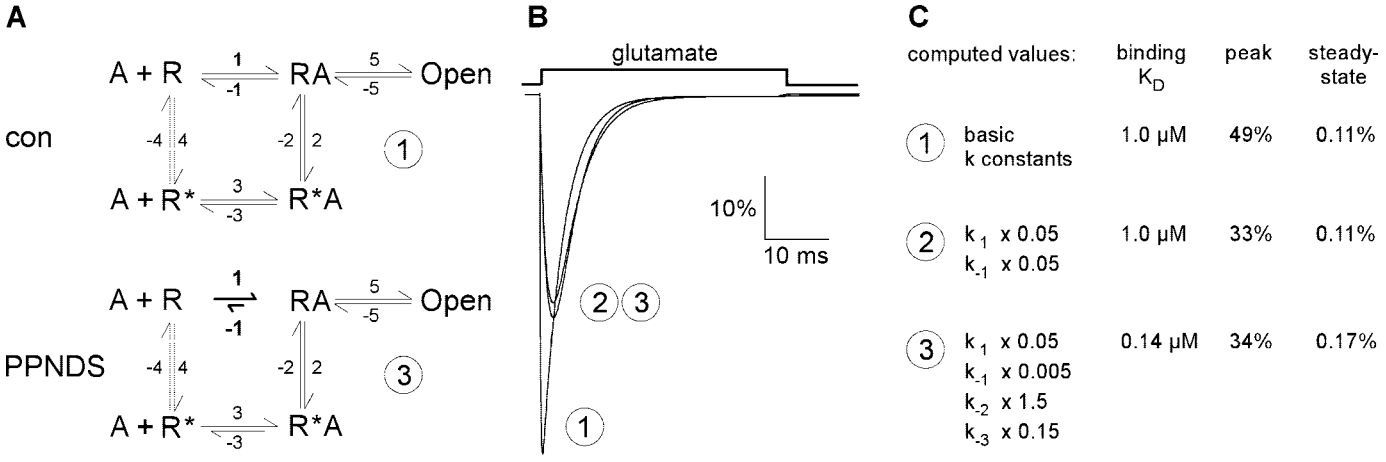

Fig. 7. Simulations with a five-state receptor model. A, scheme of five-state receptor model. Rate constants suggested to be reduced by PPNDS are indicated by shortened arrows in the lower scheme. R, receptor; A, agonist; R*, desensitized states; numbering of rate constants is indicated next to arrows. Basic rate constants were selected as follows: k_1,7.5 μM^{-1} s^{-1}; k_3, 50 μM^{-1} s^{-1}; k_{-1}, 10000 s^{-1}; k_{-3}, 4 s^{-1}; k_2, 1500 s^{-1}; k_{-2}, 1.2 s^{-1}; k_4, 3 s^{-1}; k_{-4}, 40 s^{-1}; k_5, 10,000 s^{-1}; and k_{-5}, 2000 s^{-1}. These rate constants are similar to those previously used to simulate brain receptors (Arai et al., 2002) but were adjusted in k_1, k_{-2}, k_3, k_{-3}, k_5, and k_{-5} to reproduce the faster response kinetics, smaller steady-state/peak ratios, and lower binding K_D values of homomeric receptors such as GluR2 flop. B, responses to 60-ms pulses of glutamate for three selected conditions: 1, with the basic set of rate constants; 2, with the assumption that association and dissociation rates are slowed 20×; and 3, that association is slowed 20× and dissociation is slowed 200×; in the latter case k_{-2} and k_{-3} were adjusted by small factors to maintain microreversibility and to account for the observation that the steady-state was slightly increased. The y-axis unit represents the percentage of receptors in the open state. C, factors by which the rate constants were multiplied, and associated values for binding K_D, peak current, and steady-state current. The binding K_D was computed from the equation $K_D = (k_{-1}/k_1 + k_2 \times k_{-3}/k_{-2} \times k_3)\,(1 + k_2/k_{-2} + k_5/k_{-5})^{-1}$ (Ambros-Ingerson and Lynch, 1993) and represents the expected binding affinity for glutamate. Actual binding affinity constants of homomeric receptors for glutamate are on the order of 0.6 to 6 μM, depending on the subunit (E. Suzuki, M. Kessler, and A.C. Arai, unpublished data).

PPNDS. Based on such considerations, it seems unlikely that the primary effect of PPNDS is to influence receptor functions involving desensitization (k_2/k_{-2} in Fig. 7) or channel gating (k_5/k_{-5}). Simulations with the five-state kinetic receptor model indicated instead that most observed effects can be reproduced if association and dissociation rates for agonists are reduced with the latter being more extensive. Similar conclusions were obtained when using the two-ligand glutamate receptor model of Jonas et al. (1993) (not shown). The marked slowing of association shown in Fig. 2 has indeed been a distinctive feature of the PPNDS effect. It should also be noted, however, that the associations and dissociations measured in those binding tests involve multiple kinetic transitions, including those related to desensitization, and thus the magnitude of changes in k_1 and k_{-1} can not be directly inferred from those measurements.

A further question is whether the actions of PPNDS and suramin involve separate sites and mechanisms. One plausible way to explain the slowing of association and dissociation caused by PPNDS would be to assume that the drug restricts passage to the agonist site. The latter is indeed buried deep within a cleft between the S1 and S2 domains that form the two lobes of the glutamate receptor "flytrap" mechanism (Stern-Bach et al., 1994; Mano et al., 1996). By binding to the outer part of the cleft, PPNDS could thus potentially reduce the rate with which agonists move into and out of the cleft, without occupying the agonist-binding site itself. It is of interest in this regard that guanine nucleotides also seem to bind in this region. In the chicken kainate binding protein, mutation of a lysine residue at the outer edge of the upper lip of the flytrap reduced the potency of GDP to inhibit kainate binding (Paas et al., 1996). Given that guanine nucleotides inhibit [^{3}H]AMPA binding (Dev et al., 1996) and that suramin and PPNDS inhibit proteins with nucleotide-binding sites (see Introduction), we conjecture that the compounds examined in this study mimic guanine nucleotides and that suramin and PPNDS bind to the same receptor domain yet differ in the specifics of their docking mode and the impact on receptor kinetics. The proposed modes of action are sketched in Fig. 8. PPNDS, by binding to the outer lips of the S1-S2 domain, stabilizes the receptor in a "closed-cleft" conformation that is characteristic for the "agonist-bound" state. If agonists and PPNDS are applied together, the agonist presumably binds first and then becomes stabilized when PPNDS associates with the receptor. As a consequence, equilibrium binding affinity is increased and dissociation of the agonist is slowed. If PPNDS is applied before the agonist, as in some association experiments, the receptor is shifted into the closed-cleft conformation even if the agonist site is not occupied. This greatly slows subsequent penetration of the agonist into the cleft and hence association with the receptor. In physiological tests, responses to glutamate pulses are accordingly reduced in amplitude because glutamate is not able to effectively reach its site in short time. According to this scenario the downstream conformational changes associated with channel opening and desensitization kinetics would not be influenced in a major way by PPNDS.

Binding of suramin would have opposite consequences. Perhaps because of its larger molecular dimensions and the presence of two polyanionic domains, it may act like a wedge that prevents the S1 and S2 domains to close around the bound glutamate (Fig. 8, right). It would thus hold the receptor in a conformation nearer that of the unbound state and thereby lower the affinity for agonist in binding tests. In studies using an S1-S2 model system, cleft-closure upon agonist binding was shown to involve a rotation of the two lobes relative to each other. The degree of this rotation was smaller for FW than for AMPA because of the larger molecular size of FW (Jin et al., 2003). It is thus conceivable that the "open-cleft" state stabilized by suramin can still bind FW with only modest loss of affinity, whereas the smaller AMPA molecule may not be able to make sufficient contact with the two lobes. This could explain the much larger reduction in affinity seen with AMPA as radioligand (a similar, although inverse, rationale may explain why PPNDS produced a larger gain in affinity for [^{3}H]FW than for [^{3}H]AMPA). In addition, if suramin binds to the open-cleft state, it may not be able to

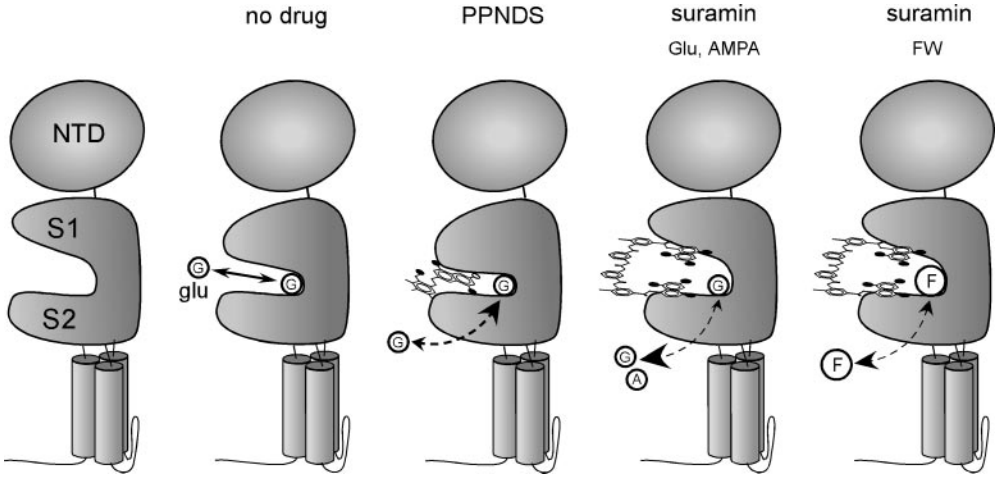

Fig. 8. Suggested mode of action for PPNDS and suramin. The scheme represents a single subunit of the tetrameric receptor complex. The indicated structural elements of the subunit are the S1-S2 domain, which contains the agonist binding site in the depth of a cleft, the N-terminal domain (NTD) of unknown function, and the three transmembrane helices connected to S1-S2. G, glutamate; A, AMPA; and F, fluorowillardiine. Far left, receptor in the conformation without bound agonist. The other diagrams show various extents of S1-S2 domain closure after binding of the agonist and the proposed influence of the drugs. Arrow size indicates the shift in the association-dissociation equilibrium relative to the situation without drug. Drugs are represented by simplified structures with negatively charged substituents indicated with black dots; only one of the possible suramin conformations is shown. The structures are not drawn to scale. For suggested mode of drug action, see text.

bind as long as glutamate is holding the receptor in a closed-cleft conformation. This could explain the apparent competitive behavior observed in the dissociation experiments, which suggested that glutamate must leave before suramin can bind.

An assumption underlying many of the above-mentioned deliberations has been that the polysulfonated ring elements represent the critical pharmacophore. However, several polysulfonated azo-dyes also inhibit AMPA receptor currents, and it has been argued that their action mainly involves hydrophobic interactions mediated by the aromatic backbone. This was based on observations such as that currents were almost completely blocked by 1 μM Evans blue (Fig. 1), whereas Chicago sky blue, which differs only in a methoxy substituent on the central benzidine moiety, was ineffective at this concentration (Keller et al., 1993; Price and Raymond, 1996). In our binding tests, however, Evans blue and Chicago sky blue exhibited comparable potencies for inhibiting [^{3}H]FW binding, and the IC_{50} values were in the 100 μM range (not shown). Thus, the purported "nanomolar" physiological actions of Evans blue, which are of a kind that is evidently not detectable in our binding tests, perhaps involve different pharmacophores than our "micromolar" actions of suramin, PPNDS, and Evans blue, which we believe to depend rather on the polysulfonated naphthyl structures.

In conclusion, the drugs examined here produced stimulatory and inhibitory effects on agonist binding that are unique and differ in major ways from those produced by other groups of AMPA receptor modulators. The drug effects exhibit mixed competitive and noncompetitive traits that can be adequately understood if one assumes that they do not physically occupy the binding domain of the agonists yet control access for agonists and compete for, or stabilize, certain receptor conformations. This would lead to an effective functional antagonism in the case of suramin yet perhaps to a more controlled and partial response attenuation in the presence of PPNDS. Although PPNDS is unlikely to cross the blood-brain barrier, compounds acting like it might offer new avenues to treat conditions such as epilepsy in which a limited reduction of phasic AMPA receptor activation is desired. This highlights further the need to determine the pharmacophores responsible for its action.

Acknowledgments

We thank Dr. K. M. Partin (Colorado State University, Ft. Collins, CO) for generously providing plasmids for GluR2(R607Q) flip and flop.

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Address correspondence to: Dr. M. Kessler, Department of Pharmacology, Southern Illinois University School of Medicine, MC 9629, 801 N. Rutledge St., Springfield, IL 62702. E-mail: mkessler@siumed.edu