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Effects of thiocyanate and AMPA receptor ligands on (*S*)-5-fluorowillardiine, (*S*)-AMPA and (*R,S*)-AMPA binding

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Abstract

AMPA receptors can be labeled using the agonist radioligands [^{3}H](*R,S*)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid ([^{3}H](*R,S*)-AMPA), [^{3}H](*S*)-AMPA or [^{3}H](*S*)-5-fluorowillardiine. In the presence of KSCN, [^{3}H](*R,S*)-AMPA and [^{3}H](*S*)-AMPA bind to a single population of sites in rat brain membranes, whereas [^{3}H](*S*)-5-fluorowillardiine binds with two affinity components. KSCN increased the affinity of the low affinity [^{3}H](*S*)-5-fluorowillardiine component > 4-fold and increased the density of both components 1.5–1.7-fold, arguing against KSCN-induced interconversion of low to high affinity states. KSCN, which promotes receptor desensitization, increased the potency of AMPA isomers, (*S*)-5-fluorowillardiine, quisqualate and cyclothiazide for inhibition of [^{3}H](*S*)-5-fluorowillardiine binding suggesting that these ligands discriminate desensitized and nondesensitized receptors. In contrast, KSCN did not greatly affect the potency of glutamate, kainate, or competitive antagonists suggesting that these ligands do not discriminate desensitized and nondesensitized receptors. In the presence of KSCN, the rank order potency for agonists and antagonists was similar or identical in all assays indicating that the three radioligands bind identical glutamate recognition sites, a conclusion supported by their identical total receptor density. However, AMPA isomers displayed 6–10-fold higher potency for displacement of [^{3}H](*S*)- or (*R,S*)-AMPA relative to [^{3}H](*S*)-5-fluorowillardiine binding. This finding, coupled with the marked two component binding by [^{3}H](*S*)-5-fluorowillardiine but not [^{3}H](*S*)- or (*R,S*)-AMPA, suggests qualitative differences between the interaction of these ligands with the agonist recognition site. © 1997 Elsevier Science B.V.

Keywords: AMPA receptor; Thiocyanate; (*S*)-5-Fluorowillardiine; (*S*)-AMPA ((*S*)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid); (*R,S*)-AMPA ((*R,S*)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid)

1. Introduction

AMPA and kainate receptors constitute the non-NMDA subclass of ionotropic excitatory amino acid receptors (for review see Fletcher and Lodge, 1996; Bettler and Mulle, 1995). AMPA receptors mediate the fast excitatory postsynaptic potentials mediated by glutamate in the central nervous system, allowing subsequent NMDA receptor activation. Although the subunit composition of native AMPA receptors has not been established, cloning and expression studies indicate that they are comprised of GluR1–4 (GluRA–D) subunits. Receptor complexes containing the edited GluR2 subunit form Ca^{2+}-impermeable cation channels, whereas those lacking the GluR2 subunit are permeable to Ca^{2+} ion. Ca^{2+} permeability is an important property determining physiological as well as pathological sequelae of AMPA receptor activation. AMPA receptor antagonists may be useful in the treatment of cerebral ischemia, neurodegenerative diseases and epilepsy (Fletcher and Lodge, 1996; Bettler and Mulle, 1995).

AMPA receptors are selectively activated by α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) itself, the active isomer having the *S*-configuration. Substituted (*S*)-willardiines are also AMPA receptor agonists, with the 5-fluoro analog being the most selective for AMPA over kainate receptors (Wong et al., 1994). Quisqualate is a potent AMPA receptor agonist with poor selectivity over metabotropic glutamate receptors (Fletcher and Lodge, 1996; Bettler and Mulle, 1995). Numerous substituted quinoxalinedione competitive antagonists have been developed which range from AMPA-selective (e.g., 6-nitro-7-sulphamoylbenzo[*f*]quinoxaline-2,3-dione (NBQX)) (Sheardown et al., 1990), to relatively nonselective (e.g., 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 6,7-dinitroquinoxaline-2,3-dione (DNQX)) (Keana et al.,

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1995), to NMDA receptor glycine site-selective (e.g., 6,7-dichloro-5-nitroquinoxaline-2,3-dione (ACEA 1021)) (Keana et al., 1995). Noncompetitive 2,3-benzodiazepine antagonists (e.g., GYKI 52466) are selective AMPA receptor antagonists (Fletcher and Lodge, 1996). AMPA receptor potentiators have been described (e.g., cyclothiazide) which block receptor desensitization (Fletcher and Lodge, 1996).

Until recently, the only agonist radioligand available for AMPA receptors was [^{3}H](*R,S*)-AMPA. The development of enantiomerically pure [^{3}H](*S*)-AMPA (Hawkins et al., 1995a) and [^{3}H](*S*)-5-fluorowillardiine (Hawkins et al., 1995b) have provided additional tools to study AMPA receptors; however, a direct comparison of these radioligands has not been made. In addition, the high affinity of [^{3}H](*S*)-5-fluorowillardiine allows the evaluation of the effect of KSCN on ligand interactions at AMPA receptors.

2. Materials and methods

2.1. Compounds

[^{3}H](*S*)-5-Fluorowillardiine (45 Ci/mmol), [^{3}H](*S*)-AMPA (45 Ci/mmol), [^{3}H](*R*)-AMPA (45 Ci/mmol), (*S*)-5-fluorowillardiine, (*S*)-AMPA, (*R*)-AMPA and CNQX were obtained from Tocris Cookson (St. Louis, MO, USA). [^{3}H](*R,S*)-AMPA (52.3 Ci/mmol) was obtained from NEN (Boston, MA, USA). (*R,S*)-AMPA, quisqualate, cyclothiazide and DNQX were from Research Biochemicals International (Natick, MA, USA). Kainate was from Sigma (St. Louis, MO, USA). Glutamate was from Fluka (New York, NY, USA). NBQX was prepared according to Jacobsen et al. (1989) and ACEA 1021 was synthesized as described previously (Keana et al., 1995) by Chemsyn Science Laboratories (Lenexa, KS, USA).

2.2. Membrane preparation

Rat brain membranes were prepared using the method of Hall et al. (1993) with modifications. Whole brains from male Sprague-Dawley rats obtained frozen (Pel-Freez, Roger, AK, USA) and stored at −80°C were thawed and homogenized in 0.32 M ice-cold sucrose/1 mM EGTA, pH 7.0 (20 ml/brain) using a glass/Teflon homogenizer. All following procedures were at 0–4°C except where indicated. The homogenate was centrifuged at 800 × *g* for 10 min. The supernatant was centrifuged at 48 000 × *g* for 30 min. The resultant P2 pellet was resuspended in 20 ml 0.04% Triton X-100/1 mM EGTA, pH 7.0 and incubated at 37°C for 20 min. The suspension was centrifuged at 48 000 × *g* for 30 min. The pellet was resuspended in 20 ml 1 mM EGTA, pH 7.0 and incubated on ice for 20 min. The suspension was centrifuged at 48 000 × *g* for 30 min and the pellet was stored at −80°C. On the day of the assay, the pellets were thawed, resuspended in 20 ml assay buffer (50 μM EGTA/100 mM Tris-acetate, pH 7.2), and centrifuged at 48 000 × *g* for 30 min. This washing procedure was repeated twice for a total of three wash steps. The final pellet was resuspended in assay buffer at 1 mg protein/ml.

2.3. AMPA receptor binding assays

Aliquots of the membrane suspension containing 200 μg protein were incubated with the appropriate radioligand in a final volume of 0.5 ml in a 96-well titer plate (1 ml well capacity) (Beckman, Fullerton, CA, USA). Nonspecific binding was determined in the presence of 1 mM glutamate. Assay buffer was supplemented with 50 mM KSCN in all assays using [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA and for some assays using [^{3}H](*S*)-5-fluorowillardiine as indicated in the tables and figures. For saturation experiments, membranes were incubated with increasing concentrations of radioligand ('hot' only) at the concentrations indicated in the figures. For competition experiments, the concentrations of [^{3}H](*S*)-5-fluorowillardiine in the absence of KSCN, [^{3}H](*S*)-5-fluorowillardiine in the presence of KSCN, [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA were 20, 5, 10 and 10 nM, respectively, and the percent specific binding under these conditions was about 70, 90, 85 and 85%, respectively. Inhibitors were added in 5 μl dimethyl sulfoxide (DMSO) or assay buffer. The assays were incubated for 60 min on ice and filtered on GF/B 96-well filter plates (Packard, Meriden, CT, USA), and rinsed 3 times with approx. 1 ml ice-cold assay buffer (50 mM KSCN present in rinse if present in assay buffer). Microscint scintillation cocktail (50 μl; Packard) was added to each well of the dried filter plates, which were then sealed, shaken vigorously for 5 min, and counted for 10 min/well on a TopCount 6-detector scintillation counter (Packard).

2.4. Data analysis

For saturation experiments, non-linear curve fitting of the specific binding as a function of free radioligand concentration on the overall data set for each radioligand (all individual experiments combined) was done using the one and two component hyperbolic functions in Prism 2.0 (GraphPad, San Diego, CA, USA). The two component hyperbola was chosen if the sum of squares was significantly lower than the one component hyperbola by *F*-test. The individual experiments were then analyzed using the selected hyperbolic model to obtain dissociation constant (K_d) and receptor density (B_{max}) values. For the one component hyperbolic data, Scatchard transformations were constructed and plotted with a linear regression, whereas the two component hyperbolic fit was transformed into Scatchard format and plotted with the transformed data. In all cases, K_d and B_{max} values were obtained from hyperbolic fits and means ± S.E.M. values were calculated from

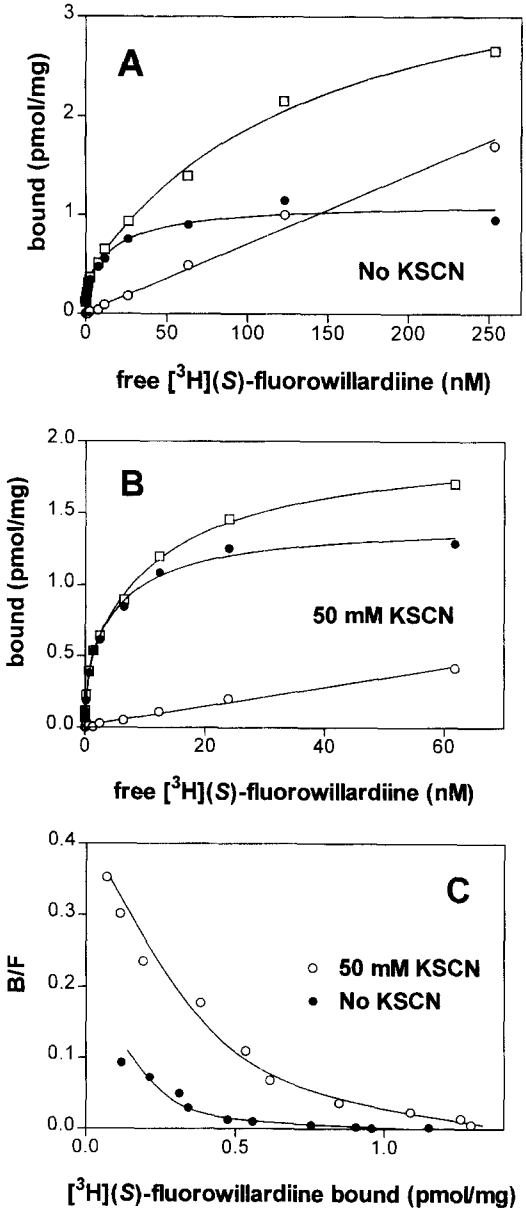

Fig. 1. Effect of KSCN on [^{3}H](*S*)-5-fluorowillardiine binding in rat brain membranes. (A) Saturation plot in the absence of KSCN. Total (□), specific (●) and nonspecific (○) binding from a single representative experiment ($K_{d1} = 0.33$ nM, $B_{max1} = 0.28$ pmol/mg; $K_{d2} = 21$ nM, $B_{max2} = 0.85$ pmol/mg). (B) Saturation plot in the presence of 50 mM KSCN. Total (□), specific (●) and nonspecific (○) binding from a single representative experiment ($K_{d1} = 0.31$ nM, $B_{max1} = 0.42$ pmol/mg; $K_{d2} = 6.8$ nM, $B_{max2} = 1.0$ pmol/mg). (C) Scatchard transformation of the same data in the absence (●) or presence (○) of 50 mM KSCN. [^{3}H](*S*)-5-Fluorowillardiine was incubated with rat brain membranes for 60 min at 4°C. See Table 1 for mean ± S.E.M. K_d and B_{max} values.

the individual experiments. Significant differences between binding parameters in the absence and presence of KSCN were evaluated by *t*-test using SPSS 6.1 (SPSS, Chicago, IL, USA). For competition experiments, non-linear curve fitting of the overall data for each drug averaged for each concentration was done using the sigmoidal equation in Prism. The data were fit to a full inhibition, Hill coefficient = 1.0 model unless the sum of squares for a partial inhibition or variable Hill coefficient model was significantly lower by *F*-test. The concentration of test compound producing 50% inhibition (IC_{50}) of specific binding and the maximal extent of inhibition (I_{max}) were determined for the individual experiments with the same model used for the overall data and then the means ± S.E.M. values of the individual experiments were calculated.

3. Results

3.1. Effect of KSCN on [^{3}H](*S*)-5-fluorowillardiine binding

Saturation data of [^{3}H](*S*)-5-fluorowillardiine binding in both the absence (Fig. 1A) and presence (Fig. 1B) of 50 mM KSCN fit a two component hyperbolic model which is more easily viewed in Scatchard transformations of the data (Fig. 1C). The K_d and B_{max} values calculated from the two component hyperbolic model (Table 1) indicated that KSCN did not affect the affinity of the high affinity component, but increased the affinity of the low affinity component over 4-fold. KSCN also increased the apparent

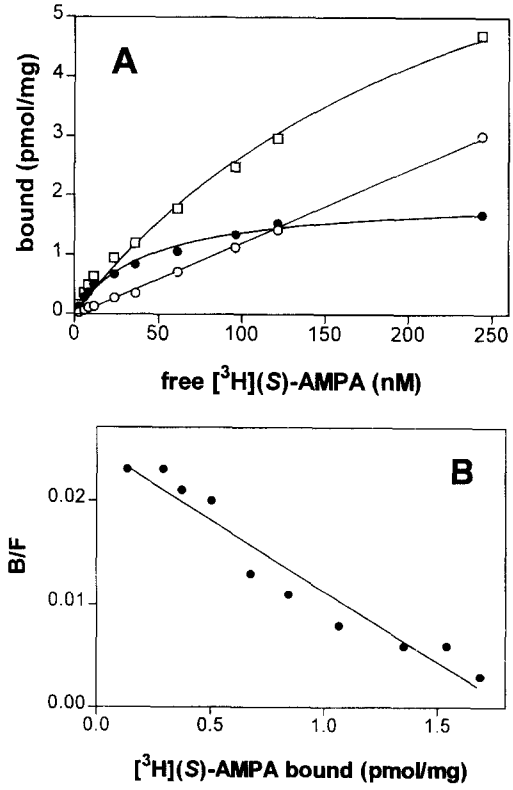

Fig. 2. Saturation analysis of [^{3}H](*S*)-AMPA binding in rat brain membranes. (A) Total (□), specific (●) and nonspecific (○) binding from a single representative experiment ($K_D = 44$ nM, $B_{max} = 2.0$ pmol/mg). (B) Scatchard transformation of the same data. [^{3}H](*S*)-AMPA was incubated with rat brain membranes in the presence of 50 mM KSCN for 60 min at 4°C. See Table 1 for mean ± S.E.M. K_d and B_{max} values.

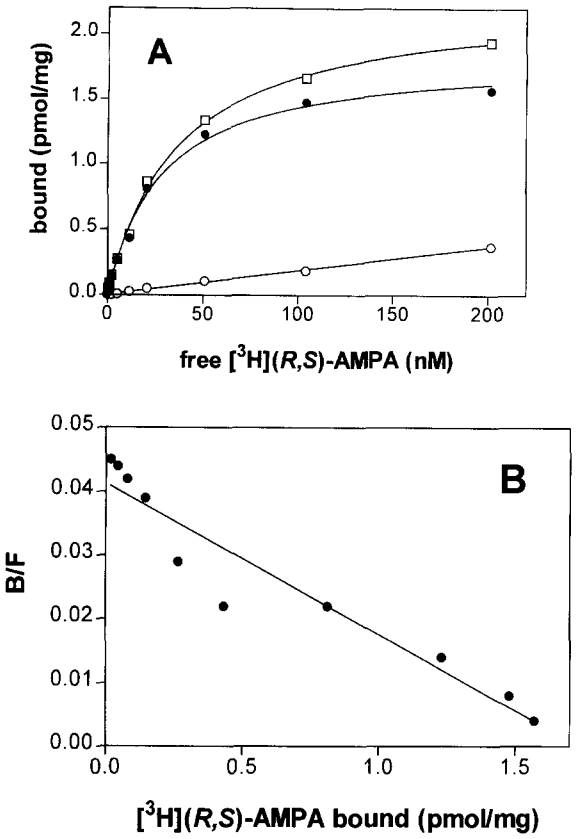

Fig. 3. Saturation analysis of [^{3}H](*R,S*)-AMPA binding in rat brain membranes. (A) Total (□), specific (●) and nonspecific (○) binding from a single representative experiment (K_d = 28 nM, B_{max} = 1.8 pmol/mg). (B) Scatchard transformation of the same data. [^{3}H](*R,S*)-AMPA was incubated with rat brain membranes in the presence of 50 mM KSCN for 60 min at 4°C. See Table 1 for mean ± S.E.M. K_d and B_{max} values.

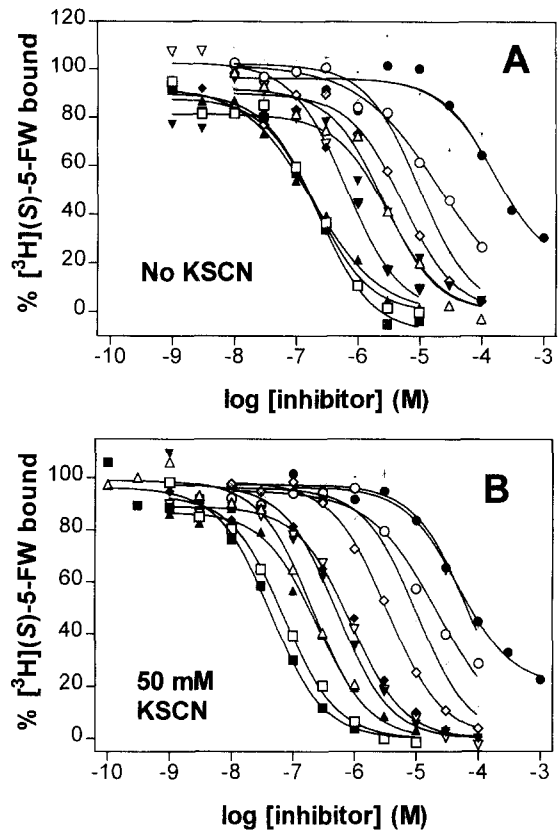

Fig. 4. AMPA receptor modulator inhibition of [^{3}H](*S*)-5-fluorowillardiine binding in rat brain membranes. (A) No KSCN. (B) 50 mM KSCN. ACEA 1021 (×), (*S*)-AMPA (△), (*RS*)-AMPA (▼), CNQX (◆), (*R*)-AMPA (*), DNQX (▽), (*S*)-5-fluorowillardiine (■), cyclothiazide (●), glutamate (◇), kainate (○), NBQX (▲), quisqualate (□). Inhibitors were incubated with 20 or 5 nM [^{3}H](*S*)-5-fluorowillardiine and rat brain membranes for 60 min at 4°C in the absence or presence of 50 mM KSCN, respectively. See Table 2 for mean ± S.E.M. IC_{50}, I_{max} and Hill slope values.

density of both low and high affinity binding sites 1.5- and 1.7-fold, respectively.

3.2. Comparison of [^{3}H](S)-5-fluorowillardiine, [^{3}H](S)-AMPA, [^{3}H](R,S)-AMPA binding in the presence of KSCN

In the presence of KSCN, [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA displayed one component saturation and Scatchard plots (Figs. 2 and 3). Although two component saturation was apparent in some individual experiments with both ligands, the overall data did not fit a two component significantly better than a one component hyperbola for either ligand. The affinities of [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA were similar to each other (K_d approx. 30–40 nM) (Table 1), and are > 4- and 90-fold lower than the low and high affinity [^{3}H](*S*)-5-fluorowil-

Table 1
Saturation parameters for AMPA receptor radioligands

Radioligand	KSCN	High affinity		Low affinity	
	(mM)	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)
[^{3}H](*S*)-5-Fluorowillardiine	0	0.31 ± 0.01	0.23 ± 0.03	32 ± 7	0.84 ± 0.01
[^{3}H](*S*)-5-Fluorowillardiine	50	0.31 ± 0.01	0.39 ± 0.04 [a]	7.2 ± 1.1 [a]	1.3 ± 0.2 [a]
[^{3}H](*S*)-AMPA	50	41 ± 2	1.7 ± 0.2	–	–
[^{3}H](*R,S*)-AMPA	50	28 ± 2	1.8 ± 0.2	–	–

Radioligands were incubated with rat brain membranes for 60 min at 4°C in the absence or presence of 50 mM KSCN as indicated. See Figs. 1–3 for representative saturation curves. Values represent means ± S.E.M. of at least three independent experiments.
[a] Significant difference ($P < 0.05$) in the presence of KSCN by two-tailed *t*-test.

lardiine affinities, respectively. All three radioligands shared virtually identical receptor densities (approx. 1.7 pmol/mg protein) if the high and low affinity [^{3}H](*S*)-5-fluorowillardiine B_{max} values are summed.

3.3. Effect of KSCN on AMPA receptor modulator inhibition of [^{3}H](*S*)-5-fluorowillardiine binding

KSCN increased the potency of certain AMPA receptor agonists for displacement of [^{3}H](*S*)-5-fluorowillardiine binding (Table 2, Fig. 4AB). KSCN had the greatest effect on (*S*)-AMPA (11-fold increase), lesser effects on (*R,S*)-AMPA, (*S*)-5-fluorowillardiine and quisqualate (3–5-fold increase), and little effect ($\leq$ 1.6-fold increase) on glutamate and kainate. In contrast, KSCN had no effect on all antagonists examined, including CNQX, DNQX, NBQX and ACEA 1021 (Table 2, Fig. 4AB). The potentiator cyclothiazide was 3-fold more potent in the presence of KSCN.

Table 2
Effect of potassium thiocyanate (KSCN) on AMPA receptor modulators in the [^{3}H](*S*)-5-fluorowillardiine assay in rat brain membranes

Inhibitor	No KSCN		50 mM KSCN	
	IC_{50} (μM)	I_{max} (%)	IC_{50} (μM)	I_{max} (%)
Agonists				
(*S*)-AMPA	2.5 ± 0.5	93 ± 7	0.22 ± 0.03	99 ± 5
(*R,S*)-AMPA	2.9 ± 0.6	84 ± 3	0.57 ± 0.08	94 ± 4
(*R*)-AMPA	> 100	–	72 ± 14	98 ± 5
(*S*)-5-Fluorowillardiine	0.23 ± 0.05	97 ± 8	0.048 ± 0.011	96 ± 4
Glutamate	5.5 ± 0.7	90 ± 4	3.5 ± 0.4	98 ± 8
Kainate	21 ± 3	103 ± 6	24 ± 3	95 ± 4
Quisqualate	0.20 ± 0.03	90 ± 7	0.072 ± 0.006	94 ± 7
Antagonists				
CNQX	0.79 ± 0.05	97 ± 6	1.0 ± 0.2	89 ± 2
DNQX	0.67 ± 0.14	103 ± 4	0.85 ± 0.13	90 ± 3
NBQX	0.20 ± 0.04	93 ± 11	0.25 ± 0.02	87 ± 4
ACEA 1021	9.8 ± 0.8	102 ± 5	10 ± 1	96 ± 4
Potentiator				
Cyclothiazide	167 ± 37	79 ± 5	53 ± 13	77 ± 3

Inhibitors were incubated with 20 or 5 nM [^{3}H](*S*)-5-fluorowillardiine and rat brain membranes for 60 min at 4°C in the absence or presence of 50 mM KSCN, respectively. Hill values were not different from 1.0 except for kainate (0.68 ± 0.11) and NBQX (0.85 ± 0.06) in the absence of KSCN and kainate (0.79 ± 0.08) in the presence of KSCN. Values represent means ± S.E.M. of at least three independent experiments.

Table 3
AMPA receptor modulator inhibition of [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA binding in the presence of potassium thiocyanate (KSCN) in rat brain membranes

Inhibitor	[^{3}H](*S*)-AMPA		[^{3}H](*R,S*)-AMPA	
	IC_{50} (μM)	I_{max} (%)	IC_{50} (μM)	I_{max} (%)
Agonists				
(*S*)-AMPA	0.022 ± 0.003	93 ± 4	0.030 ± 0.009	100 ± 6
(*R,S*)-AMPA	0.097 ± 0.020	99 ± 4	0.067 ± 0.023	95 ± 2
(*R*)-AMPA	9.3 ± 1.8	97 ± 6	9.1 ± 2.0	90 ± 2
(*S*)-5-Fluorowillardiine	0.023 ± 0.004	89 ± 2	0.022 ± 0.003	92 ± 4
Glutamate	0.82 ± 0.22	89 ± 5	0.82 ± 0.13	99 ± 1
Kainate	5.6 ± 0.66	88 ± 4	12 ± 2	92 ± 2
Quisqualate	0.021 ± 0.003	101 ± 4	0.027 ± 0.001	98 ± 5
Antagonists				
CNQX	0.39 ± 0.07	104 ± 10	0.54 ± 0.01	93 ± 1
DNQX	0.48 ± 0.07	98 ± 2	0.51 ± 0.04	95 ± 1
NBQX	0.16 ± 0.02	102 ± 9	0.12 ± 0.02	94 ± 4
ACEA 1021	7.2 ± 0.3	98 ± 6	4.8 ± 0.5	92 ± 5
Potentiator				
Cyclothiazide	55 ± 10	88 ± 2	55 ± 8	82 ± 6

Inhibitors were incubated with 10 nM [^{3}H](*S*)-AMPA or [^{3}H](*R,S*)-AMPA and rat brain membranes for 60 min at 4°C in the presence of 50 mM KSCN. Hill values were not different from 1.0 except for cyclothiazide (0.64 ± 0.06) and quisqualate (0.89 ± 0.11) in the [^{3}H](*S*)-AMPA assay and glutamate (0.81 ± 0.03) in the [^{3}H](*R,S*)-AMPA assay. Values represent means ± S.E.M. of at least three independent experiments.

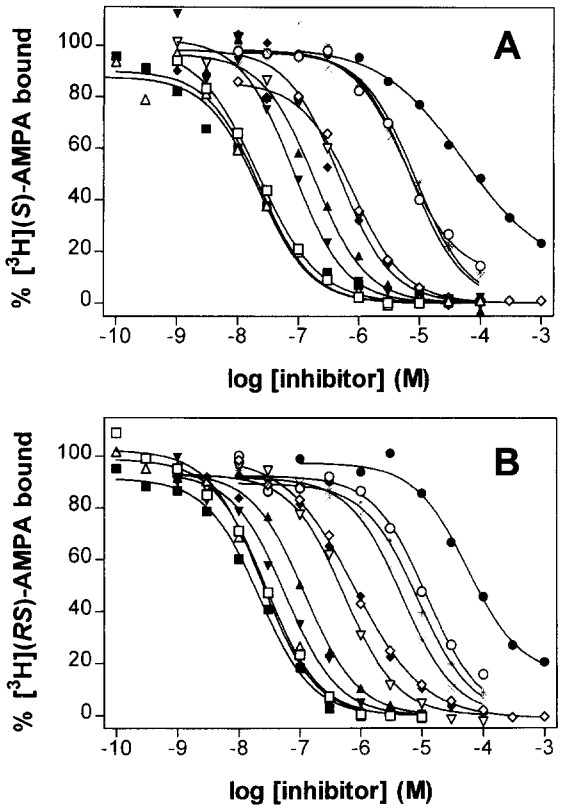

Fig. 5. AMPA receptor modulator inhibition of [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA binding in rat brain membranes. (A) [^{3}H](*S*)-AMPA. (B) [^{3}H](*R,S*)-AMPA. ACEA 1021 (×), (*S*)-AMPA (△), (*R,S*)-AMPA (▼), CNQX (◆), (*R*)-AMPA (*), DNQX (▽), (*S*)-5-fluorowillardiine (■), cyclothiazide (●), glutamate (◇), kainate (○), NBQX (▲), quisqualate (□). Inhibitors were incubated with 10 nM [^{3}H](*S*)-AMPA or [^{3}H](*R,S*)-AMPA and rat brain membranes for 60 min at 4°C in the presence of 50 mM KSCN. See Table 3 for mean ± S.E.M. IC_{50}, I_{max} and Hill slope values.

3.4. Inhibition of [^{3}H](S)-5-fluorowillardiine, [^{3}H](S)-AMPA and [^{3}H](R,S)-AMPA binding by AMPA receptor modulators in the presence of KSCN

The rank order potency for agonist inhibition of [^{3}H](*S*)-5-fluorowillardiine binding in the presence of KSCN was: (*S*)-5-fluorowillardiine > quisqualate > (*S*)-AMPA > (*R,S*)-AMPA > glutamate > kainate > (*R*)-AMPA (Table 2, Fig. 4B). The same or similar rank order potency for these agonists was observed using [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA as radioligand (Table 3, Fig. 5AB). However, the AMPA isomers were 6–10-fold more potent in the [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA assays than in the [^{3}H](*S*)-5-fluorowillardiine assay in the presence of KSCN, whereas the other agonists were about 2–4-fold more potent in the [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA assays (Tables 2 and 3, Fig. 4B and Fig. 5AB). The rank order potency for antagonist inhibition of [^{3}H](*S*)-5-fluorowillardiine binding in the presence of KSCN was: NBQX > DNQX ≥ CNQX > ACEA 1021 (Table 3, Fig. 4B). As for the agonists, the same or similar rank order potency for antagonists was observed using [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA as radioligand (Table 3, Fig. 5AB). The antagonists examined were 1.4–2.6-fold less potent in the [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA assays than the [^{3}H](*S*)-5-fluorowillardiine assay. The allosteric modulator cyclothiazide showed no consistent potency differences between assays.

4. Discussion

Several agonist and antagonist radioligands have been used to label the AMPA receptor agonist recognition site. In addition to the well studied [^{3}H](*R,S*)-AMPA, [^{3}H](*S*)-5-fluorowillardiine (Hawkins et al., 1995b) and [^{3}H](*S*)-AMPA (Hawkins et al., 1995a) are now available. In the presence and absence of KSCN, [^{3}H](*S*)-5-fluorowillardiine displayed two component Scatchard plots in the present filtration study consistent with the previous centrifugation report (Hawkins et al., 1995b). In contrast, data from saturation experiments in the presence of thiocyanate did not fit a two component better than a one component model for [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA. This contrasts with many (Hall et al., 1992, 1993; Honore and Drejer, 1988) studies which have reported two component Scatchard plots. It may be that the filtration technique employed did not detect significant numbers of low affinity sites, although most (e.g., Honore and Drejer, 1988) but not all (e.g., Murphy et al., 1987) previous filtration studies found two component saturation. Thus, the clear two component binding by [^{3}H](*S*)-5-fluorowillardiine may be due to its much higher affinity at both AMPA receptor sites than [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA. However, the total receptor density was virtually identical for all three radioligands suggesting that [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA did not underestimate the receptor level under these conditions. Alternatively, it may be that [^{3}H](*S*)-5-fluorowillardiine possesses a much higher differential affinity for the two AMPA receptor affinity states than does [^{3}H](*S*)-AMPA.

The two component Scatchard plots for [^{3}H](*R,S*)-AMPA have been interpreted to represent high and low affinity states of a single interconvertible receptor population (Honore and Drejer, 1988; Hall et al., 1992). Subunit selectivity apparently cannot explain the high and low affinity sites for AMPA receptors as is the case for kainate receptors (Fletcher and Lodge, 1996; Bettler and Mulle, 1995) since recombinant AMPA receptors have been reported to bind [^{3}H](*R,S*)-AMPA with similar affinities. Early studies indicated that homomeric recombinant receptors displayed only high affinity binding (12–40 nM) (Kawamoto et al., 1994; Keinanen et al., 1990; Kuusinen et al., 1995), consistent with the suggestion that the low affinity state is only favored in the synaptic milieu (Hall et al., 1992). However, two component saturation has re-

cently been observed in certain homomeric receptor complexes (GluR2) providing further support for high and low affinity states existing in a single receptor population (Hennegriff et al., 1996).

Potassium thiocyanate markedly enhances the binding of AMPA receptor agonists. Originally, thiocyanate was proposed to shift low affinity to high affinity forms of the receptor (Honore and Drejer, 1988). More detailed studies suggested that thiocyanate increases the affinity of both high and low affinity AMPA receptors in parallel (Hall et al., 1993). In electrophysiological experiments, thiocyanate inhibits AMPA peak responses and increases the decay rate (Arai et al., 1995). The binding and electrophysiological data suggest that the receptor favors a high affinity desensitized conformation in the presence of this chaotropic agent (Arai et al., 1995).

In the present study, thiocyanate increased [^{3}H](*S*)-5-fluorowillardiine binding by increasing the affinity of the low, but not the high, affinity component and by increasing the apparent density of both low and high affinity components to about the same extent. Consistent with previous studies using [^{3}H](*S*)-5-fluorowillardiine (Hawkins et al., 1995b) or AMPA displacement of [^{3}H]CNQX binding (Hall et al., 1993), the effect of thiocyanate on [^{3}H](*S*)-5-fluorowillardiine is not due to interconversion of receptors from a low to a high affinity conformation. However, in contrast to these reports (Hall et al., 1993; Hawkins et al., 1995b) which observed an increase in the affinity of both affinity components with no change in receptor density or proportion, the present [^{3}H](*S*)-5-fluorowillardiine data indicate that the effect of thiocyanate is to unmask approximately equal proportions of high and low affinity receptors as well as to increase the affinity of only the low affinity receptor conformation. Although the reasons for these differences are not known, [^{3}H](*S*)-5-fluorowillardiine displayed substantially higher affinity and lower densities in the present filtration study compared to the previous centrifugation study (Hawkins et al., 1995b). In this regard, thiocyanate has been observed to increase [^{3}H](*R,S*)-AMPA binding density in an autoradiography study (Cha et al., 1992).

The high affinity of [^{3}H](*S*)-5-fluorowillardiine allows sufficient binding in the absence of KSCN, providing the possibility of evaluating the effect of thiocyanate on unlabeled ligands. As expected, thiocyanate increased the potency of (*S*)-5-fluorowillardiine and (*S*)- and (*R,S*)-AMPA for inhibition of [^{3}H](*S*)-5-fluorowillardiine binding. Quisqualate displayed increased potency in the presence of thiocyanate suggesting that it binds AMPA receptors in a similar manner. In contrast, thiocyanate had no effect on glutamate and kainate potencies suggesting that these agonists bind AMPA receptors in a qualitatively different manner than quisqualate, (*S*)-5-fluorowillardiine and (*S*)-AMPA. Since thiocyanate is thought to promote AMPA receptor desensitization (Arai et al., 1995), the lack of effect of thiocyanate on kainate and glutamate potency suggests that these agonists do not discriminate between desensitized and nondesensitized receptors.

Interestingly, two component displacement of [^{3}H](*S*)-5-fluorowillardiine by unlabeled (*S*)-5-fluorowillardiine was not observed even though [^{3}H](*S*)-5-fluorowillardiine displays marked two component Scatchard plots in saturation experiments. This can be explained in terms of the IC_{50} values expected for homologous displacement of radioligand binding to two affinity states (or populations) of receptors. Using the Cheng-Prusoff equation (Cheng and Prusoff, 1973) to calculate the expected IC_{50} values for unlabeled (*S*)-5-fluorowillardiine displacement of [^{3}H](*S*)-5-fluorowillardiine binding to the two components observed in saturation experiments and assuming $K_i = K_d$, there is only a 2.6- and 2.3-fold difference between calculated IC_{50} values for homologous displacement from the high and low affinity components in the absence and presence of KSCN, respectively. This 2–3-fold difference in IC_{50} values would not be detected in standard competition experiments.

KSCN also increased the potency of cyclothiazide inhibition of [^{3}H](*S*)-5-fluorowillardiine binding about 3-fold. Cyclothiazide is thought to noncompetitively inhibit [^{3}H](*R,S*)-AMPA binding by decreasing receptor desensitization, thereby reducing agonist ligand affinity (Kessler et al., 1996). The reduced potency of cyclothiazide for inhibition of [^{3}H](*S*)-5-fluorowillardiine binding in the absence of thiocyanate is consistent but less dramatic than that observed for [^{3}H](*R,S*)-AMPA (Kessler et al., 1996).

KSCN did not have consistent effects on the potency of the antagonists CNQX, DNQX, NBQX and ACEA 1021 for displacement of [^{3}H](*S*)-5-fluorowillardiine binding suggesting that these quinoxalinedione ligands do not discriminate between desensitized and nondesensitized AMPA receptors. This finding is consistent with the little or no effect of KSCN on [^{3}H]NBQX and [^{3}H]NS 257 binding (Dev et al., 1996; Nielsen et al., 1995), but contrasts with the reported thiocyanate inhibition of [^{3}H]CNQX binding (Hall et al., 1993; Nielsen et al., 1990). [^{3}H]CNQX, [^{3}H]NBQX and [^{3}H]NS 257 bind single populations of receptors suggesting that these antagonists also do not discriminate between high and low affinity receptors (Dev et al., 1996; Nielsen et al., 1990, 1995). The main practical limitation of these radioligands is their rapid off-rate, precluding their use in filtration assays.

The same or similar rank order potencies for agonists and antagonists in the [^{3}H](*S*)-5-fluorowillardiine and the [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA assays suggest that these ligands bind identical agonist recognition sites on the AMPA receptor. This is supported by the virtually identical B_{max} values and the similar modulatory effect of cyclothiazide as previously reported for [^{3}H](*R,S*)-AMPA (Kessler et al., 1996). The IC_{50} values for the agonists and antagonists examined (excluding AMPA isomers) were about 2–4-fold higher in the [^{3}H](*S*)-5-fluorowillardiine assay than the [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA as-

says. The consistently lower potency of agonists and antagonists for displacement of [^{3}H](*S*)-5-fluorowillardiine relative to [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA binding is probably due to the higher affinity of [^{3}H](*S*)-5-fluorowillardiine which requires higher concentrations of the inhibitor to 'out-compete' the radioligand. However, the possibility that the inhibitors display reduced potency due to displacement of [^{3}H](*S*)-5-fluorowillardiine from low as well as high affinity sites, whereas [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA are displaced only from high affinity sites, cannot be ruled out.

In contrast to the 2–4-fold difference in potency observed with other ligands, the AMPA isomers display 6–10-fold higher potency for displacement of [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA relative to [^{3}H](*S*)-5-fluorowillardiine binding. This marked potency difference suggests that (*S*)-AMPA and (*S*)-5-fluorowillardiine do not bind the AMPA receptor identically. They may bind overlapping sites and/or interact differentially with residues located in the agonist binding pocket. However, the alternative possibility that (*S*)-AMPA and (*S*)-5-fluorowillardiine bind with differential affinities to subtypes of AMPA receptor populations present in brain membranes cannot be excluded.

In conclusion, the radioligands [^{3}H](*S*)-5-fluorowillardiine, [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA provide similar information about the AMPA receptor agonist recognition site. [^{3}H](*S*)-5-Fluorowillardiine has relatively high affinity, high percent specific binding, displays clear two component saturation presumably corresponding to two AMPA receptor affinity states, and can be used in filtration assays in the absence of thiocyanate. [^{3}H](*S*)-AMPA and [^{3}H](*R,S*)-AMPA have essentially identical profiles, indicating that the [^{3}H](*R*)-AMPA enantiomer of the racemic radioligand has no obvious effect in binding assays. This is supported by the lack of specific binding of [^{3}H](*R*)-AMPA up to a concentration of 200 nM (S.A. Espitia and J.E. Hawkinson, unpublished observations). Thiocyanate markedly increases the potency of the AMPA isomers, (*S*)-5-fluorowillardiine and quisqualate, but has minimal effects on other agonists and all antagonists examined.

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