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Thiocyanate Equally Increases Affinity for Two DL-α-Amino-3-hydroxy-5-methylisoxazolepropionic Acid (AMPA) Receptor States

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SUMMARY

The effects of potassium thiocyanate on the binding of DL-α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and L-glutamate to the AMPA receptor complex were studied in rat brain membranes. Thiocyanate increased the affinity of AMPA receptors for both AMPA and L-glutamate and decreased the affinity for CNQX. Displacement of [^{3}H]CNQX binding by AMPA revealed that the affinity for AMPA of both the high and the low affinity states of the receptor increased in the presence of thiocyanate, whereas the proportion of the two states was unchanged. In soluble fractions, which exhibit an apparently homogeneous population of the high affinity state of the receptor, the affinity for AMPA was increased by a magnitude similar to that seen for the high affinity state in the membranes. These results indicate that thiocyanate enhances AMPA binding not by converting low affinity receptors into the high affinity state but by equally increasing the affinities of the high and the low affinity states of AMPA receptors.

AMPA receptors are a subclass of glutamate receptors and are responsible for fast excitatory transmission in the mammalian central nervous system (1, 2). They also appear to be the locus of expression for certain forms of synaptic plasticity, including long term potentiation in the hippocampus (3–5) and long term depression in the cerebellum (6, 7). The recent cloning and expression of a number of AMPA receptor subunits (8, 9) have greatly increased our understanding of AMPA receptor function but leave unanswered a number of questions regarding the properties of AMPA receptors in the brain. Scatchard plots of AMPA binding in brain membranes can typically be resolved by two-site regression analysis into a high affinity component with a K_d of 10–40 nM and a low affinity component with a K_d of 200–1000 nM (10–14). Recent evidence indicates that the high and low affinity AMPA receptors represent two states of the same receptor, rather than two different receptors (14).

In equilibrium binding studies, the affinity of AMPA receptors for AMPA has been shown to be increased severalfold by the presence of chaotropic ions such as thiocyanate (11, 12, 15–17). Although this effect has been widely reported, there has been some disagreement as to the mechanism by which thiocyanate exerts its effects. Murphy *et al.* (11) proposed that the receptor microenvironment was affected by the chaotrope in a manner that resulted in a general increase in affinity. Honore and Drejer (12) and Nielsen *et al.* (16) proposed a different interpretation. They suggested that thiocyanate was causing a change in the equilibrium between the high and the low affinity states of the receptor and that this shift was responsible for the observed effects of thiocyanate. Recent autoradiographic work (18) has also led to the conclusion that thiocyanate causes an interconversion of AMPA receptors from a low to a high affinity state.

In the present set of experiments, we have attempted to reevaluate the effects of thiocyanate on the AMPA receptor complex by measuring the interaction of glutamate, AMPA, and the specific antagonist CNQX with the receptor in the presence and absence of this ion. A major problem with [^{3}H]AMPA binding studies is that sites that have binding affinities higher than a few micromolar (such as might be seen in the absence of thiocyanate) are likely to remain undetected. [^{3}H]CNQX binds to the same population of sites as does [^{3}H]AMPA, but its binding properties are only moderately affected by potassium thiocyanate in the concentration range typically used in binding studies (19); thus, measuring displacement of

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ABBREVIATIONS: AMPA, DL-α-amino-3-hydroxy-5-methylisoxazolepropionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

[^{3}H]CNQX should provide a more accurate means of studying AMPA binding sites in the presence and absence of thiocyanate. Our findings lead us to conclude that thiocyanate does not affect the equilibrium between the high and the low AMPA receptor states. Instead, we propose that thiocyanate nonpreferentially increases the affinity of both the high and the low affinity AMPA receptor states and that the magnitude of the increase is approximately the same for both states.

Materials and Methods

Preparation of membranes. Whole brains were collected from adult male Sprague-Dawley rats that had been anesthetized with ether before decapitation. The brains were kept frozen at −80° for up to 3 months until use. After thawing, the brains were homogenized in 0.32 M sucrose, 1 mM EGTA (20 ml/brain), using a glass/Teflon homogenizer. The homogenate was centrifuged at 800 × *g* for 10 min and the supernatant was recentrifuged at 48,000 × *g* for 30 min. The resultant membrane pellet (P_2) was lysed by resuspension in 20 ml of distilled H_2O containing 1 mM EGTA, followed by a 20-min incubation on ice and centrifugation at 48,000 × *g* for 30 min. The lysis and centrifugation cycle was repeated and the pellet was resuspended in the assay buffer (100 mM Tris acetate, 50 μM EGTA, pH 7.2) to approximately 1 mg/ml; this sample is referred to as "membranes." All procedures for membrane preparation were carried out at 0–4°.

Solubilization. Triton X-100 was added to membranes to achieve a final concentration of 0.4% (w/v). The membrane/detergent mixture was incubated for 20 min at 37° with constant agitation and then centrifuged at 48,000 × *g* for 2 hr. The resultant supernatant was removed and dialyzed for 24 hr against a 100-fold excess of assay buffer containing 0.4% Triton X-100, with one buffer change. This dialysate was termed the "soluble fraction." All soluble fractions were stored at −80° until use.

Receptor binding assays. [^{3}H]CNQX binding was studied using the centrifugation method. Typically, 50–100 μg of protein in a final volume of 100 μl were incubated with [^{3}H]CNQX for the membrane samples; for the soluble samples the final volume was 200 μl, with 10 μl of 25 mg/ml γ-globulin in assay buffer and 90 μl of a 30% polyethylene glycol solution in assay buffer added to produce protein aggregation. These studies were performed in both the presence and the absence of 50 mM potassium thiocyanate. The incubation was for 40–60 min in an ice-water bath (0°). Binding constants were determined at ligand concentrations ranging from 10 to 300 nM. Samples were centrifuged at 48,000 × *g* at 4° for 20 min and then placed back in the ice-water bath for 10–20 min until aspiration. The supernatants were aspirated and the pellets were superficially rinsed with 0.4 ml of 100 mM Tris acetate (containing 15% polyethylene glycol in the case of the soluble samples). The pellets were then dissolved in 10 μl of Beckman Tissue Solubilizer and counted with an efficiency of 0.40 (in the case of the membrane samples) or dissolved in 100 μl of distilled H_2O and counted with an efficiency of 0.35 (in the case of the soluble samples). Results were expressed as specifically bound [^{3}H]CNQX, which equals the difference between the total bound and nonspecifically bound ligand. Nonspecific binding was defined as the binding in the presence of 25 μM unlabeled CNQX. In these experiments, nonspecific binding was typically 20–30% of the total binding.

[^{3}H]AMPA binding was measured for the soluble samples. To determine binding constants, AMPA concentrations were increased between 2 nM and 1.5 μM. Concentrations between 2 and 50 nM were achieved by increasing the concentration of radiolabeled AMPA, whereas those above 50 nM were produced by adding unlabeled AMPA to a fixed [^{3}H]AMPA concentration of 50 nM. These studies were performed in the presence of 50 mM KSCN. Nonspecific binding was defined as the amount of [^{3}H]AMPA bound in the presence of 2.5 mM L-glutamate. In these experiments, nonspecific binding was typically 10–20% of the total binding. Otherwise, these studies were performed as described above.

AMPA and L-glutamate displacement of [^{3}H]CNQX was measured using 50 nM [^{3}H]CNQX, both in the presence and in the absence of 50 mM KSCN. Nonspecific binding was defined as the amount of [^{3}H]CNQX bound in the presence of 25 μM unlabeled CNQX. For these experiments, nonspecific binding was typically 20–30% of the total binding for both the membrane and soluble samples. K_i values were determined by performing the Cheng-Prusoff correction on the raw IC_{50} values, using as an affinity for CNQX the K_d values estimated from the [^{3}H]CNQX binding studies. Corrections for inhibition studies performed in the presence or absence of KSCN used the affinity of CNQX in the presence or absence of KSCN, respectively. Otherwise, these studies were performed as described above. Two-site analysis of binding data was performed by nonlinear regression using the InPlot program (GraphPAD Software, Inc.).

Protein concentrations were determined according to the method of Bradford (20), with the protein assay reagent obtained from Bio-Rad and with bovine serum albumin as standard. Separate standard curves were constructed for samples with and without Triton X-100.

[^{3}H]AMPA and [^{3}H]CNQX were purchased from NEN/DuPont. AMPA was obtained from Tocris and CNQX from Cambridge Research Biochemicals. γ-Globulin, polyethylene glycol, and other reagents were from Sigma.

Results

The binding of 20 nM [^{3}H]AMPA and [^{3}H]CNQX to membranes was measured at KSCN concentrations ranging from 0 to 1000 mM (Fig. 1). AMPA binding reached a plateau at 30–100 mM KSCN and decreased at higher concentrations of the salt. Conversely, CNQX binding was highest in the absence of thiocyanate and was progressively reduced as the thiocyanate concentration was increased. Similar results were obtained with solubilized fractions (data not shown).

Scatchard transformations of [^{3}H]CNQX binding data have been shown in previous studies to be uniformly linear (14, 19). To apply appropriate Cheng-Prusoff corrections in the subsequent displacement experiments, the affinity for [^{3}H]CNQX had to be determined in the presence and absence of KSCN for both the membrane and soluble fractions (Fig. 2). In both preparations, Scatchard transformations of these data were uniformly linear. The B_{max} values were essentially the same with or without thiocyanate in both preparations, but the K_d

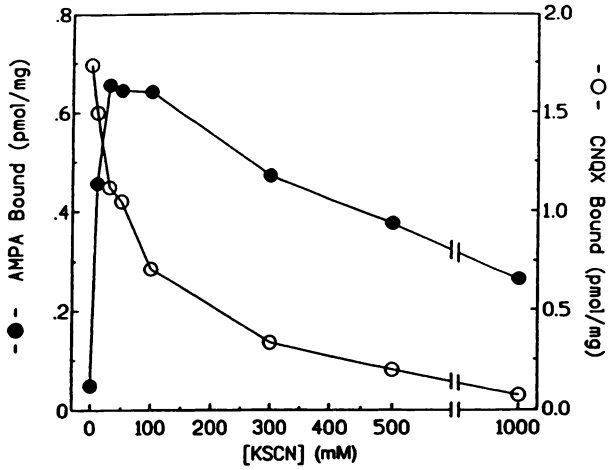

Fig. 1. Optimal concentrations of potassium thiocyanate for 20 nM [^{3}H]AMPA (●) and 20 nM [^{3}H]CNQX (○) binding. The average values of two experiments are shown. AMPA binding was maximal in the range of 30–100 mM KSCN, whereas CNQX binding was highest in the absence of thiocyanate.

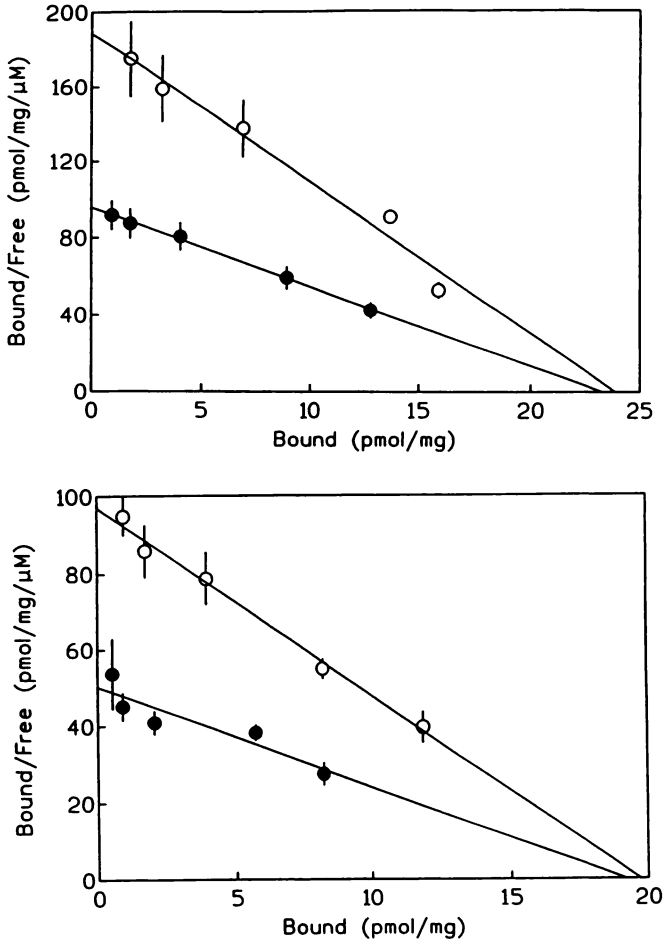

Fig. 2. [^{3}H]CNQX binding to membrane samples (*top*) and soluble samples (*bottom*) in the presence (●) and absence (○) of thiocyanate. Binding of [^{3}H]CNQX was measured at five concentrations between 10 and 300 nM. Shown here are the averaged plots from four independent assays of both the membrane and soluble samples. Data are shown in Table 1.

value was significantly reduced in the presence of the chaotrope. In membranes, the B_{max} and K_d values in the absence of thiocyanate were 23.8 pmol/mg and 126 nM, respectively, whereas in the presence of thiocyanate the values were 23.2 pmol/mg and 226 nM. For the soluble fractions, in the absence of thiocyanate the B_{max} and K_d values were 19.7 pmol/mg and 202 nM, whereas in the presence of KSCN the values were 19.2 pmol/mg and 383 nM, respectively. Thus, the reduction in CNQX binding by KSCN seen in Fig. 1 seems to be due to a decrease in affinity and not a change in the number of receptors available to bind CNQX.

AMPA displacement of [^{3}H]CNQX in membranes in the presence of thiocyanate resulted in an inhibition plot that could best be resolved into two components, i.e., a high affinity component with a K_i of 17 nM, which accounted for 2.6% of the sites occupied by [^{3}H]CNQX, and a low affinity component with a K_i of 696 nM, which accounted for the remaining 97.4% of the sites (Fig. 3; see also Table 1). When displacement was performed in the absence of thiocyanate, two-site regression analysis again provided the best fit, in this case with a high affinity component with a K_i of 446 nM, accounting for 12.3% of [^{3}H]CNQX binding, and a low affinity component with a K_i of 26.9 μM, accounting for the remaining sites. Thus, the affinity for the high affinity site increased 26-fold in the presence of KSCN, whereas the affinity for the low affinity site increased 38-fold. Because the ratio of high to low affinity sites remained similar, it appears most likely that the two types of sites were affected equally by KSCN.

In soluble fractions, AMPA displacement of [^{3}H]CNQX in the presence of 50 mM KSCN resulted in inhibition plots in which the two-site fit was not significantly better than the one-site fit; the K_i value for the average of these plots was 31 nM (Fig. 4). The average K_d of the same four samples for [^{3}H]AMPA binding in the presence of 50 mM KSCN was 32.5 ± 2 nM (mean ± standard error, as determined by linear regression analysis; data not shown). In the absence of thiocyanate the inhibition plots still appeared to have only a single component; the K_i value for AMPA in these samples was 438 nM. Thus, the affinity for AMPA in the soluble fractions increased 14-fold, which is similar to the increases seen for both the high affinity and low affinity components of the membrane samples.

Because L-glutamate is probably the endogenous ligand for AMPA receptors, it was of interest to examine the effects of thiocyanate on glutamate binding. Plots of glutamate inhibition of [^{3}H]CNQX binding were determined for both membrane samples and soluble fractions, in both the presence and the absence of 50 mM KSCN; none of these plots could be effectively resolved into two components (Figs. 5 and 6). The K_i value for the average of the plots for the membranes in the presence of thiocyanate was 7.1 μM, whereas in the absence of the chaotrope the K_i value was 48.6 μM; thus, affinity was increased by a factor of 6.8 in the presence of thiocyanate. In soluble fractions, the averaged K_i values were 0.44 μM with and 1.43 μM without thiocyanate, for a 3.3-fold increase in affinity induced by thiocyanate.

Discussion

Binding studies and autoradiography show that AMPA binds to at least two sites that are distinguishable by their affinities (10–14). Hall *et al.* (14) recently presented evidence that these two types of sites may be interconvertible forms of the same receptor, inasmuch as solubilization of the receptors appeared to convert the normally predominant population of low affinity sites into the high affinity form. This suggests that the affinity of the receptors may be determined in part by interactions with lipids or cytoskeletal elements. In this context, it is of interest to re-examine the suggestion raised in the literature that thiocyanate might produce a similar conversion among two affinity states of the receptor (12, 16, 18). Fig. 3 provides evidence that this is not the case. In the presence of thiocyanate, AMPA displaced most bound [^{3}H]CNQX with an affinity of about 700 nM; an additional component with an affinity of 17 nM accounted for the remaining 3% of the sites. The affinities and the relative abundance of these high and low affinity components are close to the values derived from [^{3}H]AMPA binding studies (10, 13, 14); this validates the assumption that analysis of [^{3}H]CNQX binding provides a means to identify and characterize AMPA receptor sites. When thiocyanate was omitted from the experiment of Fig. 3, the whole displacement curve was shifted to the right, towards higher AMPA concentrations. The predominant site then displayed an affinity for AMPA in the range of 26 μM and, as before, there was a small component with higher affinity (K_i = 446 nM) accounting for the remainder of the sites. Because the high affinity component represented

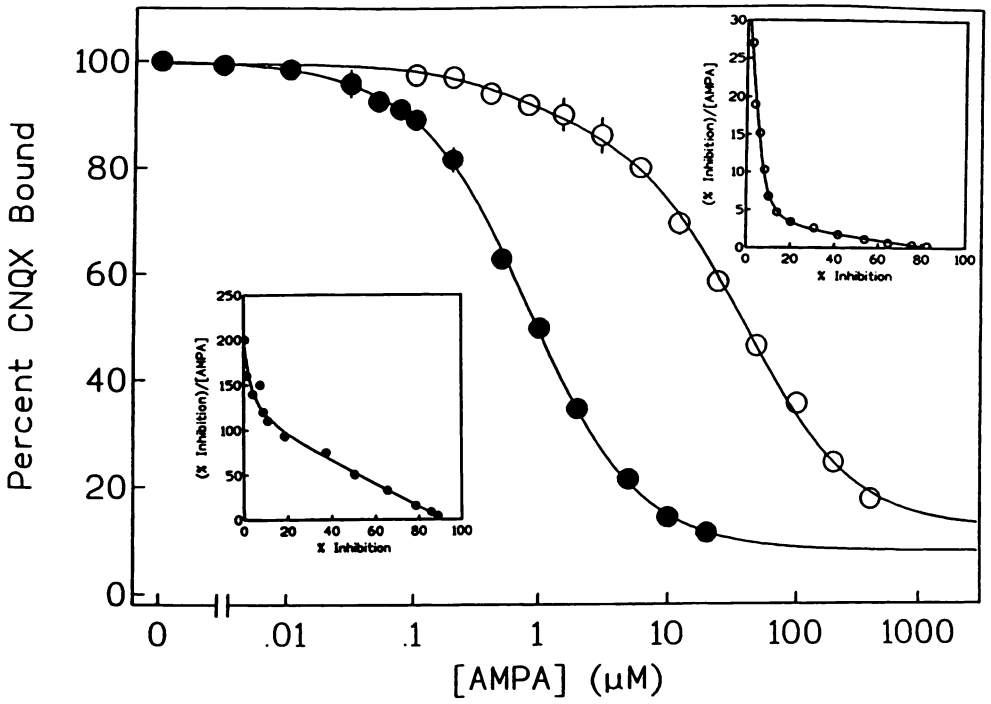

Fig. 3. AMPA inhibition of 50 nM [^{3}H]CNQX binding in membrane samples in both the presence (●) and the absence (○) of 50 mM potassium thiocyanate. Binding of 50 nM [^{3}H]CNQX was measured in the presence of 14 AMPA concentrations between 0 and 400 μM. Each *data point* represents the average of four independent experiments; the *error bars* indicate the standard error. In both cases, the plots were best fit by two-site regression analysis. Hill coefficients for both graphs are 0.6–0.8. Data are shown in Table 1. Data represent Scatchard-type transformations of each inhibition curve.

TABLE 1

Summary of binding constants

Ninety-five percent confidence intervals for all affinity data, as determined by the InPlot program, are within ±40% of each value. The only exceptions are the estimates for the high affinity component in the two-site fits, which have a considerably larger error.

	Membranes		Solubilized proteins	
	−SCN	+SCN	−SCN	+SCN
[^{3}H]CNQX				
K_d (nM)	126	226	202	383
B_{max} (pmol/mg)	23.2	23.8	19.7	19.2
AMPA				
K_i, site 1 (nM)	446	17	438	31
K_i, site 2 (nM)	26,900	696		
% of total for site 1	12.3	2.6	100	100
% of total for site 2	87.7	97.4		
L-Glutamate, K_i (μM)	48.6	7.1	1.43	0.44

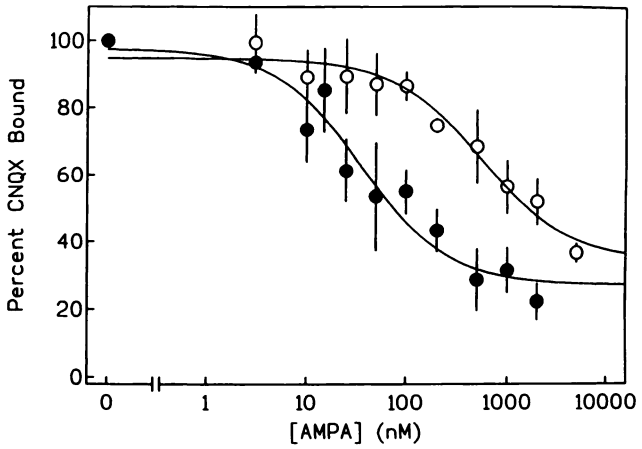

Fig. 4. AMPA inhibition of 50 nM [^{3}H]CNQX binding in soluble samples in both the presence (●) and the absence (○) of 50 mM potassium thiocyanate. Binding of 50 nM [^{3}H]CNQX was measured in the presence of 11 AMPA concentrations between 0 and 5 μM. Each *data point* represents the average of four independent experiments; the *error bars* indicate the standard error. In both cases, two-site fits were not significantly better than one-site fits. Hill coefficients for both graphs are not significantly different from 1. Data are shown in Table 1.

only a small fraction of the total number of binding sites in these samples, the B_{max} and affinity constant values describing it tended to have a large amount of variation. If anything, however, it seemed that the proportion of high affinity sites was actually greater in the absence than in the presence of thiocyanate. These data are best explained by assuming that thiocyanate increases the affinity of all forms of the receptor to a comparable extent, without changing the relative amounts of the high and the low affinity forms.

The previously described conversion to a relatively homogeneous population of high affinity sites upon solubilization (14) can again be seen in the aforementioned experiments done with the solubilized fractions. In this material, binding and displacement data consistently were fitted best by a single type of site; the affinity of this site for AMPA was strongly dependent on the presence of thiocyanate but corresponded closely in all cases to the higher affinity form of the receptor present in membranes. A similar situation was found for glutamate; the single site that could be identified in these studies in the absence of thiocyanate had an affinity of 48.6 μM in membranes and of 1.43 μM in solubilized material. In both preparations, thiocyanate increased the affinity 3–7-fold. This provides additional evidence for our suggestion that thiocyanate enhances

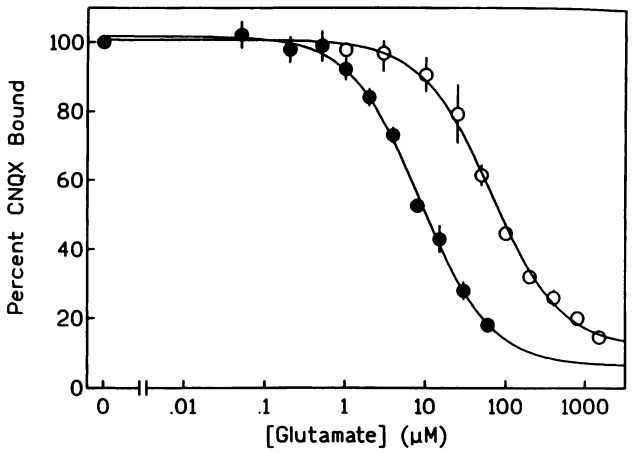

Fig. 5. L-Glutamate inhibition of 50 nM [^{3}H]CNQX binding in membrane samples in both the presence (●) and the absence (○) of 50 mM potassium thiocyanate. Binding of 50 nM [^{3}H]CNQX was measured in the presence of 11 L-glutamate concentrations ranging from 0 to 1.5 mM. Each *point* represents the average of three independent experiments; the *error bars* indicate the standard error. In both cases, two-site fits were not significantly better than one-site fits. Hill coefficients for both graphs are not significantly different from 1. Data are shown in Table 1.

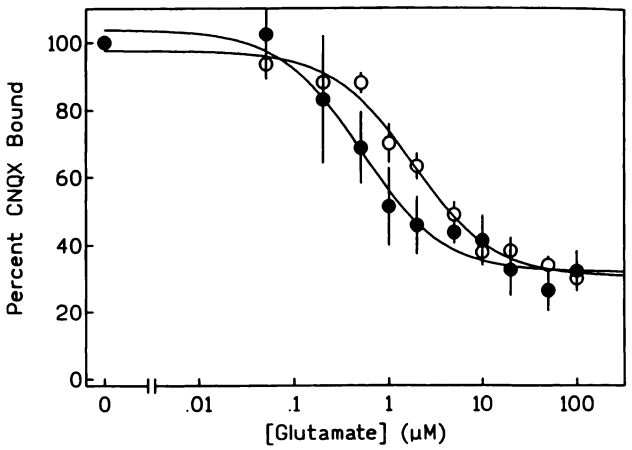

Fig. 6. L-Glutamate inhibition of 50 nM [^{3}H]CNQX binding in soluble samples in both the presence (●) and the absence (○) of 50 mM potassium thiocyanate. Binding of 50 nM [^{3}H]CNQX was measured in the presence of 11 L-glutamate concentrations between 0 and 100 μM. Each *point* represents the average of four independent experiments; the *error bars* signify the standard error. In both cases, two-site fits were not significantly better than one-site fits. Hill coefficients for both graphs are not significantly different from 1. Data are shown in Table 1.

equally the affinity of all receptor states.

The idea that thiocyanate causes a change in the equilibrium between the high and the low affinity states of the receptor was based partly on kinetic data (12). In those studies, however, the B_{max} values were considerably higher in the presence than in the absence of thiocyanate; thus, the idea of a simple redistribution between two affinity states is inadequate to explain the experimental results. Our experiments have shown that a majority of receptors in the absence of thiocyanate have an affinity constant for AMPA in the range of 26 μM. Sites of such low affinity are difficult to detect with any binding technique. In centrifugation assays, the problem arises from an unfavorable ratio of specific to nonspecific binding; in filtration assays, sites are lost because of rapid dissociation. Thus, assays in the absence of thiocyanate are likely to detect only the small population of the higher affinity variant of the receptor. Honore and Drejer (12) recognized this problem and postulated that they might be losing binding sites to dissociation in the absence of thiocyanate. In the same vein, it is difficult to detect this receptor class in assays using [^{3}H]glutamate as ligand. In this case, thiocyanate appears to decrease the dissociation constant of the receptor from 48 μM to about 7 μM, but the latter value is still too high to allow reliable detection in binding assays.

Cha *et al.* (18) have recently published a similar study on the effects of KSCN on [^{3}H]AMPA binding, using the autoradiography technique. Certain of their conclusions appear to be at odds with our work, because their data suggest that it is the B_{max}, not the K_d, of the high affinity site that changes in the presence of thiocyanate. However, the ability to undergo transformation into the high affinity form must be restricted to a select subpopulation of the receptors in their paradigm, because the number of these sites never exceeded 10% of the total receptor population; the remaining majority of sites clearly changed their affinity in the presence of KSCN in the manner described here. Thus, their data are not inconsistent with the idea that AMPA receptors have two distinct forms that are not interconverted by thiocyanate.

It should be pointed out that the low affinity for AMPA of the majority of sites in our experiments accords well with physiological studies that show that concentrations in the micromolar range are needed to produce receptor activation (21, 22). The same holds true for glutamate (22), which presumably is the endogenous ligand for these receptors; computer simulations have shown that association and dissociation rates compatible with the above determined K_d of about 50 μM can provide an adequate description of the waveform of synaptic excitatory post-synaptic potentials (5). Interestingly, studies of AMPA receptors reconstituted in membrane bilayers have found significantly lower EC_{50} values of about 200 nM for AMPA (23), a value similar to the binding affinity of solubilized receptors in the absence of thiocyanate. This suggests that the interconversion between high and low affinity forms observed with solubilization depends on factors other than the mere presence of intact membranes. The similarity of binding constants and EC_{50} values derived from electrophysiological studies on glutamate receptors is in marked contrast to the situation with the nicotinic acetylcholine receptor, where these values can differ by orders of magnitude (24). The reason for this is not clear but may be related to the different desensitization kinetics of these receptor types.

Our determination of the optimal concentration of KSCN for enhancing AMPA binding and the finding that KSCN reduces [^{3}H]CNQX binding are in good agreement with the findings from other laboratories (11, 19). A question of interest is whether the thiocyanate effect is mediated through interaction with a specific site or whether chaotropic ions modulate receptor properties through general changes in protein-solvent interactions. Biphasic activation and inhibition similar to those shown here have been reported for strychnine binding to glycine receptors (25) and have been tentatively linked to the presence of two distinct anion binding sites in the receptor channel. In the case of the cation-conducting AMPA receptor channel, however, such an explanation appears unfounded. The inhibitory effects of high concentrations of KSCN in particular are likely to be a consequence of high ionic strength, because other ionic solutions, such as Tris acetate, produced a similar reduc-

tion in AMPA binding at high concentrations.[1] Because the effects of thiocyanate are observed in soluble fractions, it is likely that the ion acts directly on the receptor to modify its configuration, producing a large increase in affinity for agonists and a small decrease in affinity for antagonists. Beyond this observation, the locus of action for thiocyanate remains obscure, because the effect of thiocyanate does not appear to interact with other manipulations that modulate the properties of the AMPA receptor, including the use of sulfhydryl reagents (13), phospholipase A_2 (26), or phosphatidylserine (27). Finally, recent work indicates that thiocyanate increases the size of synaptic responses mediated by AMPA but not *N*-methyl-D-aspartate receptors (28), an observation that reinforces the assumption that the affinity of the former receptors influences the amplitude of fast excitatory currents at glutamatergic synapses. Accordingly, identifying the mechanisms whereby thiocyanate exerts its effects may provide clues about the regulation of synaptic strength *in situ*.

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[1] R. A. Hall, unpublished observations.

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