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Thiocyanate stabilizes AMPA binding to the quisqualate receptor

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Calcium and chloride ions stimulated [^{3}H]glutamate binding to quisqualate-sensitive [^{3}H]glutamate binding sites 4-fold, as measured by quantitative autoradiography, whereas 100 mM potassium thiocyanate had no additional effect. In contrast, calcium and chloride had little effect on the binding of [^{3}H](RS)-α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid ([^{3}H]AMPA), but 100 mM thiocyanate stimulated binding 4-fold. AMPA displaced little [^{3}H]glutamate binding from quisqualate-sensitive binding sites in the molecular layer of the cerebellum in the absence of thiocyanate. However, in the presence of thiocyanate AMPA became a more effective displacer, but still displaced only 44% of the quisqualate-sensitive [^{3}H]glutamate binding. The distribution of [^{3}H]glutamate binding to quisqualate-sensitive sites was similar to but not identical with that of [^{3}H]AMPA binding. However, the distribution of AMPA-displaceable [^{3}H]glutamate binding correlated highly ($r = 0.97$, $P < 0.0005$) with that of [^{3}H]AMPA binding. The results suggest that AMPA binds to a subclass of quisqualate-sensitive [^{3}H]glutamate binding sites that are highly influenced by ionic environment and that quisqualate-sensitive binding sites exist in several states.

[^{3}H]Glutamate binding; [^{3}H]AMPA binding; Thiocyanate; (Autoradiography, Receptor, Rat)

1. Introduction

Based on conventional electrophysiological techniques, AMPA ([RS]-α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid) has been shown to be a potent agonist in the mammalian central nervous system at the quisqualate subtype of glutamate receptor (Krogsgaard-Larsen et al., 1980; 1985). Binding studies of the quisqualate receptor using [^{3}H]glutamate and [^{3}H]AMPA have yielded conflicting results, however. [^{3}H]Glutamate binding to quisqualate-sensitive binding sites is highly chloride- and calcium-dependent whereas [^{3}H]AMPA binding is relatively insensitive to calcium and chloride (Greenamyre et al., 1984; Rainbow et al., 1984; Cha et al., 1988). Autoradiograms of [^{3}H]AMPA binding in the absence of thiocyanate consistently demonstrate low levels of binding (Monaghan et al., 1984; Rainbow et al., 1984; Olsen et al., 1987). Recent studies of [^{3}H]AMPA binding have shown a marked sensitivity to thiocyanate (Honoré and Nielsen, 1985; Murphy et al., 1987). In order to investigate the relationship between [^{3}H]AMPA binding sites and quisqualate-sensitive [^{3}H]glutamate binding sites, we have studied the binding of both ligands under various ionic conditions in the rat brain. Based on these data, we propose a model of quisqualate-sensitive binding sites which will account for these various ionic and ligand binding effects.

2. Materials and methods

2.1. Materials

L-[^{3}H]Glutamic acid (specific activity 53 Ci/mmol) was obtained from Amersham (Arling-

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ton Heights, IL) and [^{3}H]AMPA (24.5-27.6 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Non-radioactive AMPA was a gift from Dr. Povl Krogsgaard-Larsen. All other compounds were purchased from Sigma (St. Louis, MO).

2.2. Tissue preparation

Male Sprague-Dawley rats (175-250 g) were decapitated, and the brains were quickly removed, mounted with Lipshaw embedding matrix on a cryotome chuck, and frozen under powdered dry ice. Sections, 20 μm, were cut on a Lipshaw cryostat and thaw-mounted onto gelatin-coated slides. Sections were stored for less than 24 h at $-20\,^\circ$C. In order to remove endogenous glutamate, all sections underwent a wash for 30 min at $2\,^\circ$C in either 50 mM Tris-HCl buffer (Tris-HCl) containing 2.5 mM $CaCl_2$, pH 7.20 or 50 mM Tris-acetate buffer (Tris-Ac), pH 7.20. Sections were blown dry under a stream of room temperature air.

2.3. Receptor autoradiography

A detailed description of the method for glutamate binding autoradiography has been published (Greenamyre et al., 1984). Briefly, in competition studies tissues were incubated for 45 min at $2\,^\circ$C with various competitors in the presence of 200 nM [^{3}H]glutamate (specific activity 4.2-7.3 Ci/mmol) in a total volume of 8 ml. In the presence of 100 μM NMDA, greater than 95% of [^{3}H]glutamate binding was displaceable by 2.5 μM quisqualate. All solutions were adjusted to pH 7.2 with either Tris base, acetic acid, or hydrochloric acid prior to use. Non-specific binding was defined as that [^{3}H]glutamate binding occurring in the presence of 1 mM unlabelled glutamate and represented less than 10% of the total binding of [^{3}H]glutamate. For [^{3}H]AMPA binding, the tissue was incubated for 45 min at $2\,^\circ$C with 20-75 nM of [^{3}H]AMPA. Non-specific binding was determined in the presence of 100 μM unlabelled AMPA or 1 mM glutamate and, in the presence of potassium thiocyanate, represented less than 5% of total binding. In the absence of thiocyanate, the non-specific binding represented less than 15% of total binding. In experiments examining the effects of calcium and chloride, these ions were added as acetate and Tris salts, respectively. Thiocyanate ions were added as potassium thiocyanate. Non-specific binding was determined under each ionic condition.

After the incubation, sections were rinsed quickly 3 times with cold buffer, then rinsed with cold 2.5% (v/v) glutaraldehyde in acetone. Sections were blown dry with warm air. The rinse/drying procedure took no more than 10 s. Dried sections were placed in X-ray cassettes with appropriate radioactive standards (Pan et al., 1983) and apposed to LKB Ultrofilm ^{3}H. The film was exposed to the tissue sections for 14-21 days at $4\,^\circ$C, then developed, fixed and dried. The optical densities of the resultant film images were determined using a computer-assisted microdensitometer (Dauth et al., 1984). Sixteen to twenty-five readings were averaged from each area of interest. The radioactivity was determined by a computer-generated polynomial regression analysis which compared film densities produced by the tissue sections to those produced by the radioactive standards. All data presented were analyzed densitometrically from autoradiographic images.

K_I values were generated by the non-linear regression program LIGAND (Munson and Rodbard, 1980).

3. Results

3.1. Regional distribution of quisqualate-sensitive glutamate binding sites and [^{3}H]AMPA binding sites

Overall, [^{3}H]AMPA binding had a distribution similar to that of quisqualate-sensitive [^{3}H]glutamate binding, although differing somewhat in hippocampus and cerebellum (table 1) (fig. 1). In hippocampus, binding was higher in stratum radiatum and molecular layer of dentate gyrus for [^{3}H]AMPA as compared to quisqualate-sensitive [^{3}H]glutamate binding. In cerebellar molecular layer, binding was relatively less for [^{3}H]AMPA than it was for quisqualate-sensitive [^{3}H]gluta-

TABLE 1

Regional localization of quisqualate-sensitive [^{3}H]glutamate binding and [^{3}H]AMPA binding in 11 regions of rat brain. Values represent the average ± S.E.M. in readings from four rats. Percentages represent binding as compared to binding in the dentate gyrus.

Region	Bound radioligand in pmol/mg protein	
	Quisqualate-sensitive binding [a]	[^{3}H]AMPA binding [b]
Cerebral cortex		
Somatosensory		
Layers I, II	2.26 ± 0.55 (81.6%)	3.71 ± 0.04 (71.6%)
Layers V, VI	0.89 ± 0.44 (32.1%)	2.21 ± 0.05 (42.7%)
Anterior cingulate	1.68 ± 0.44 (60.6%)	2.97 ± 0.07 (57.3%)
Hippocampal formation		
Dentate gyrus	2.77 ± 0.49 (=100%)	5.18 ± 0.04 (=100%)
Stratum radiatum of CA1	1.96 ± 0.48 (70.8%)	4.78 ± 0.55 (92.3%)
CA3	1.40 ± 0.42 (50.5%)	4.10 ± 0.06 (79.2%)
Striatum	1.12 ± 0.36 (40.4%)	2.05 ± 0.10 (39.6%)
Thalamus	0.60 ± 0.25 (21.7%)	1.09 ± 0.02 (21.0%)
Inferior colliculus	0.75 ± 0.21 (27.1%)	1.09 ± 0.01 (30.3%)
Cerebellum		
Molecular layer	1.73 ± 0.32 (62.5%)	2.64 ± 0.21 (51.0%)
Granule cell layer	0.50 ± 0.20 (18.1%)	1.23 ± 0.08 (23.8%)

[a] Quisqualate-sensitive [^{3}H]glutamate binding was carried out in 200 nM [^{3}H]glutamate (specific activity 6.72 Ci/mmol) in 50 mM Tris-HCl, 2.5 mM $CaCl_2$, 100 mM KSCN and 1 mM NMDA. [b] [^{3}H]AMPA binding was carried out in 20 nM [^{3}H]AMPA (specific activity 25 Ci/mmol) in 50 mM Tris-HCl, 2.5 mM $CaCl_2$ and 100 mM KSCN.

mate binding. In the cerebellar molecular layer, specific [^{3}H]AMPA binding in Tris-HCl buffer with 2.5 mM $CaCl_2$ comprised 87% of total binding, with other areas demonstrating equal or higher percentages of specific binding. Similarly, specific [^{3}H]glutamate was greater than 90% in all areas tested. All of the [^{3}H]AMPA binding was displaceable by quisqualate (2.5 μM) and also by kainate (100 μM).

3.2. *Effects of chloride and calcium on ligand binding*

Chloride and calcium exerted a marked stimulation of quisqualate-sensitive [^{3}H]glutamate binding as has been previously reported (Greenamyre et al., 1984; 1985). Levels of binding in the cerebellar molecular layer were increased 4-fold in 40 mM chloride and 2.5 mM calcium versus Tris-Ac buffer (table 2). The increase in [^{3}H]glutamate binding effected by calcium and chloride was due entirely to an increase in the quisqualate-sensitive binding sites and not to a change in N-methyl-D-aspartate-sensitive or kainate-sensitive binding sites (Cha et al., 1988). The cerebellar molecular layer is the brain region which possesses both the highest absolute levels and highest proportion of quisqualate-sensitive [^{3}H]glutamate binding sites (versus quisqualate-insensitive sites) (Cha et al., 1988). In the absence of thiocyanate, chloride ions slightly enhanced [^{3}H]AMPA binding, but the stimulation observed was much less than chloride's stimulation of [^{3}H]glutamate binding. In the absence or presence of calcium and chloride [^{3}H]AMPA binding was present only at low levels in all brain regions investigated.

3.3. *Effects of thiocyanate on ligand binding*

Quisqualate-sensitive [^{3}H]glutamate binding was not affected by the presence of 100 mM thiocyanate (table 2). Thiocyanate increased the ability of AMPA to displace [^{3}H]glutamate binding (Cha et al., 1988). In the absence of

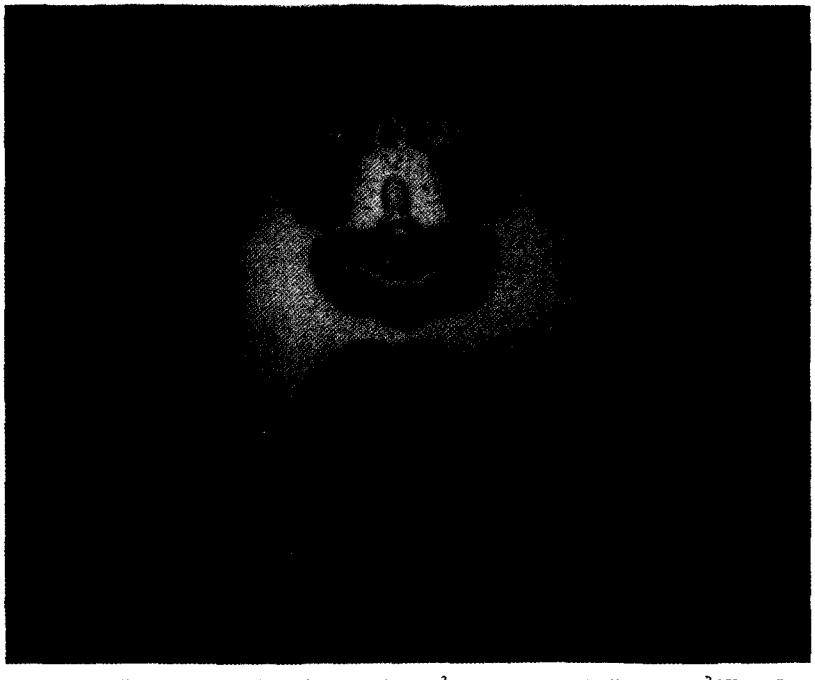

Fig. 1. Digitized images of autoradiograms of quisqualate-sensitive [^{3}H]glutamate binding and [^{3}H]AMPA binding in horizontal sections of rat brain. Upper left: total [^{3}H]glutamate binding (200 nM) in 50 mM Tris-HCl + 2.5 mM $CaCl_2$, 100 mM thiocyanate and 100 μM NMDA. Lower left: same as in upper left, with the addition of 100 μM AMPA. Upper middle: total [^{3}H]AMPA binding (37 nM) in 50 mM Tris-HCl + 2.5 mM $CaCl_2$ and 100 mM thiocyanate. Upper right: total [^{3}H]AMPA binding (37 nM) in 50 mM Tris-HCl + 2.5 mM $CaCl_2$. Lower middle: [^{3}H]AMPA binding as in upper middle panel but in the presence of 100 μM kainate. Lower right: [^{3}H]AMPA binding as in upper middle panel but in the presence of 100 μM NMDA. The upper and lower left images are serial sections digitized from the same film under identical conditions. The four images on the right are also serial sections exposed to the same film and digitized under the same conditions. The results are representative of findings in four different rats.

TABLE 2

Effects of calcium chloride and thiocyanate on quisqualate-sensitive [^{3}H]glutamate and [^{3}H]AMPA binding in cerebellar molecular layer. Values represent the average of readings in four rats that varied less than 20% (pmol/mg protein).

Condition	Quisqualate sensitive [^{3}H]Glutamate binding [a]	[^{3}H]AMPA binding [b]
50 mM Tris-Ac	0.89	0.94
50 mM Tris-Ac + 100 mM KSCN	–	3.67
50 mM Tris-HCl	2.70	1.15
50 mM Tris-HCl + 100 mM KSCN	–	4.11
50 mM Tris-Ac + 2.5 mM Ca-acetate	0.92	0.65
50 mM Tris-Ac, 2.5 mM Ca-acetate + 100 mM KSCN	–	3.33
50 mM Tris-HCl + 2.5 mM $CaCl_2$	3.60	1.24
50 mM Tris-HCl, 2.5 mM $CaCl_2$ + 100 mM KSCN	3.40	3.60

[a] Quisqualate-sensitive [^{3}H]glutamate binding was carried out at 200 nM [^{3}H]glutamate (specific activity 6.1 Ci/mmol). [b] [^{3}H]AMPA binding was carried out at 60 nM [^{3}H]AMPA (specific activity 27.6 Ci/mmol).

thiocyanate, AMPA (up to 100 μM) displaced less than 20% of [^{3}H]glutamate binding in molecular layer of cerebellum whereas in the presence of 100 mM thiocyanate, concentrations of AMPA up to 100 μM displaced as much as 44% of glutamate binding. Thus, in the presence of thiocyanate, AMPA displaced a portion, but not all of the quisqualate-sensitive [^{3}H]glutamate binding, even at the relatively high concentration of 100 μM. Thiocyanate had similarly dramatic effects on [^{3}H]AMPA binding. In the presence of 100 mM thiocyanate, [^{3}H]AMPA binding increased 5-fold in the molecular layer of cerebellum. The stimulatory effects of thiocyanate did not require the presence of chloride or calcium ions, with 100 mM KSCN exerting as much stimulatory effect in Tris-Ac buffer as in Tris-HCl buffer with 2.5 mM $CaCl_2$. The Pearson correlation coefficient of the regional distribution of [^{3}H]AMPA binding in the presence of thiocyanate compared to quisqualate-sensitive [^{3}H]glutamate binding was 0.94 when examined in 11 brain regions. However, when AMPA-displaceable [^{3}H]glutamate binding in the presence of calcium, chloride, and thiocyanate was compared to [^{3}H]AMPA binding, the two correlated even more significantly ($r = 0.97$, $P < 0.0005$) (fig. 2).

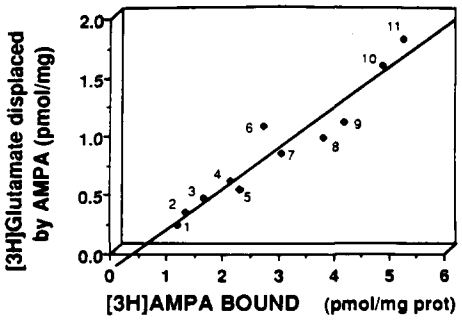

Fig. 2. Correlation between the distribution of AMPA displaceable quisqualate-sensitive [^{3}H]glutamate binding and [^{3}H]AMPA binding in 11 regions of rat brain. AMPA displaceable quisqualate-sensitive [^{3}H]glutamate binding was determined by subtracting binding of [^{3}H]glutamate (200 nM) in 50 mM Tris-HCl, 2.5 mM $CaCl_2$, 100 mM KSCN, 100 μM NMDA and 100 μM AMPA from total [^{3}H]glutamate binding under the same conditions in the absence of AMPA. [^{3}H]AMPA binding (37 nM) was carried out in 50 mM Tris-HCl, 2.5 mM $CaCl_2$ and 100 mM KSCN. Regions: 1, thalamus; 2, granule layer of cerebellum; 3, inferior colliculus; 4, striatum; 5, inner layers of cerebral cortex; 6, molecular layer of cerebellum; 7, cingulate cortex; 8, outer layers of the cerebral cortex; 9, CA_4 region of hippocampus; 10, stratum radiatum of CA_1; 11, stratum moleculare of the dentate gyrus. The Pearson correlation coefficient was $r = 0.97$ ($P < 0.0005$).

4. Discussion

According to the most common classification scheme, excitatory amino acid receptors are classified into at least three subclasses, named for the glutamate analogs which preferentially act at them: NMDA receptors, kainate receptors and quisqualate receptors (Watkins and Evans, 1981). While quisqualate acts preferentially at one class of receptors, it possesses actions at non-quisqualate receptors (Olverman et al., 1984; Greenamyre et al., 1985; Foster and Fagg, 1987; Cha et al., 1988). Much of the lack of specificity of quisqualate's actions may be attributable to contamination of quisqualate by glutamate (Olverman et al., 1984; Cha et al., 1987). The non-selective actions of quisqualate mediated by contaminants are accentuated when high concentrations of quisqualate are employed. We have previously demonstrated that many commercially available preparations of quisqualic acid possess contaminant glutamic acid. However, we have employed quisqualate which is greater than 99% free from contaminant glutamate, as determined by high performance liquid chromatography (HPLC) analysis (Cha et al., 1987). While the prospect of spurious effects due to contaminants must be considered, at the concentrations of quisqualate which we employ to define 'quisqualate-sensitive' [^{3}H]glutamate binding (2.5 μM), the expected contribution from contaminant glutamate would be minimal.

In addition, quisqualate has been shown to interact with various other sites, including an astrocytic membrane binding site (Bridges et al., 1987), glutamate uptake processes (Pin et al., 1984; Zaczek et al., 1987; Kessler et al., 1987), and the enzyme which metabolizes the dipeptide N-acetyl-aspartyl-glutamate (NAAG) (Robinson et al., 1987). We have previously shown that quisqualate's displacement of [^{3}H]glutamate from quisqualate-sensitive binding sites has a K_I value which is at least 10-fold lower than those found

for any of the above mentioned processes (Cha et al., 1988). Thus, at the relatively low concentrations we have employed, quisqualate is likely to be a selective agent.

Nonetheless, AMPA may be a more selective ligand for the quisqualate subtype of glutamate receptor. Electrophysiological studies have demonstrated that AMPA has properties very similar to those of quisqualate in depolarizing membranes (Krogsgaard-Larsen et al., 1985), and has powerful pharmacological effects after local injection into various brain areas in the rat (Arnt, 1981a,b). AMPA also has similar neurotoxic effects to quisqualate (Morgan, 1987). In single channel studies, AMPA opens channels with similar ionic conductances and open channel times as does quisqualate (Christiansen and Nowak, 1987).

The regional correlation between levels of quisqualate-sensitive [^{3}H]glutamate binding and [^{3}H]AMPA binding was excellent, with $r = 0.94$, suggesting that these two binding sites are the same or closely related. Some regional differences existed, however. The binding of [^{3}H]AMPA was relatively higher in the hippocampus as compared to [^{3}H]glutamate binding and relatively lower in the molecular layer of the cerebellum. Secondly, even in the presence of thiocyanate ions, AMPA displaced only a portion of [^{3}H]glutamate bound to quisqualate-sensitive sites. One possible interpretation of these differences is that [^{3}H]AMPA binds to a subpopulation of quisqualate-sensitive glutamate binding sites (Honoré and Nielsen, 1985). The studies reported here investigated that possibility.

The distribution of [^{3}H]AMPA binding sites correlated most highly with the distribution of quisqualate-sensitive [^{3}H]glutamate displaced by unlabelled AMPA. These results suggest that AMPA may be binding to a subset of quisqualate-sensitive [^{3}H]glutamate binding sites, and its binding to this subset is dramatically increased by the presence of thiocyanate.

Honoré and Nielsen (1985) and Murphy et al. (1987) have shown that the addition of 100 mM thiocyanate increases AMPA binding in homogenates several fold. In this autoradiographic study, [^{3}H]AMPA binding was also increased dramatically in the presence of thiocyanate. The stimulatory effects of thiocyanate did not require the presence of chloride or calcium ions. In addition, thiocyanate increased the ability of unlabelled AMPA to displace quisqualate-sensitive [^{3}H]glutamate binding.

The binding of [^{3}H]glutamate to quisqualate-sensitive sites is of high affinity and unaffected by thiocyanate. Whereas calcium and chloride had only small effects on [^{3}H]AMPA binding, these ions substantially increased the binding of [^{3}H]glutamate to quisqualate-sensitive sites. Despite the fact that the two ligands, [^{3}H]AMPA and [^{3}H]glutamate, are differentially affected by these ions, the correlation of regional distributions of [^{3}H]AMPA and quisqualate-sensitive [^{3}H]glutamate binding sites argues strongly that these sites are the same or closely related. The extremely close regional correlation of [^{3}H]AMPA binding and [^{3}H]glutamate binding displaced by AMPA argues that it is AMPA which interacts with a subset of the quisqualate-sensitive sites labelled by [^{3}H]glutamate and not vice versa. AMPA's ability to displace only a portion of quisqualate-sensitive [^{3}H]glutamate binding sites suggests that some of these sites are AMPA-sensitive while others are AMPA-insensitive. These results suggest an hypothesis in which the quisqualate receptor exists in a low affinity quisqualate binding state which is converted in the presence of calcium and chloride to a second state which has high affinity for quisqualate. These first two affinity states have low affinity for AMPA. Finally, in this hypothesis, in the presence of thiocyanate, these low affinity AMPA sites are in equilibrium with a third state which possesses high affinity for AMPA.

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