DISTINGUISHING CHARACTERISTICS BETWEEN GLUTAMATE AND KAINIC ACID BINDING SITES IN BRAIN SYNAPTIC MEMBRANES

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1. Introduction

The demonstration of strong neuroexcitatory activity produced by extracellular application of L-glutamic and L-aspartic acid [1–4] has led to biochemical studies aimed at defining the membrane sites with which these putative neurotransmitters interact [5–10]. The pursuit of the biochemical characterization of the excitatory amino acid receptor sites has been conducted along two lines:

(1) Determination of membrane binding activity through the use of radioactively labeled glutamate or aspartate [5–8]; or
(2) Measurement of the receptor recognition function through the use of radioactively labeled kainic acid (2-carboxy-4-isopropenyl-3-pyrrolidine acetic acid) [9,10], a potent neuroexcitatory agent which is presumed to act on the excitatory amino acid receptors [11,12].

We have shown that brain synaptic membranes are enriched in high affinity L-[^{3}H]glutamic acid binding sites which have a number of the pharmacologic characteristics of the receptor for this excitatory amino acid [5,13,14]. It was also shown that this high affinity glutamate binding activity of synaptic membranes is associated with a small molecular weight glycoprotein [15]. Glutamate binding to this protein exhibits a similar pattern of sensitivity to various glutamate analogs as does the binding of L-glutamate to the synaptic membranes [5,14,15]. However, the binding of L-[^{3}H]glutamic acid either to the synaptic membranes [6,14] or to the purified binding protein [14] is not affected by the presence of a 10^3-fold greater concentration of kainic acid. Evidence is provided here which is strongly suggestive of the distinct nature of the membrane macromolecules which function as the binding sites for L-glutamic acid and those for kainic acid.

2. Materials and methods

2.1. *Membrane preparation and cholate extraction of membranes*

Synaptic plasma membranes were obtained after osmotic rupturing of the isolated rat brain synaptosomes in a hypotonic buffer medium as in [16]. These membranes were suspended in 0.32 M sucrose–5 mM Tris–SO_4–1 mM $MgSO_4$–0.5 mM EDTA (pH 7.4) at final protein conc. 6–12 mg/ml. The membrane suspension was divided into small aliquots, quickly frozen in liquid nitrogen, ans stored at –80°C. Each membrane suspension was used within 2–3 weeks of its preparation. Na-Cholate extraction of the synaptic membranes was accomplished by rapidly thawing an aliquot of membrane suspension at 37°C in sufficient volume of a phosphate buffer medium (135 mM K-phosphate–0.11 M sucrose–1 mM $MgSO_4$ (pH 7.4)) to bring to final protein conc. 2–3 mg/ml. This mixture was incubated at 37°C for 5 min, allowed to stand at room temperature for 10 min, and subsequently centrifuged at 39 000 × g for 25 min. The membrane pellets were resuspended in either 50 mM Tris–HCl (pH 7.4) or in 50 mM Tris–HCl buffer which contained 0.5% (w/v) of Na-cholate. The volume used for this resuspension was equal to that used during the thawing steps. Following a 45 min incubation at room temperature these suspensions were centrifuged at 100 000 × g for 60 min (4°C). The final supernatant (cholate extract) was dialyzed (Spectrapore membranes M_r cutoff 6000–8000) against a 200-fold vol. 50 mM Tris–HCl buffer. The membrane pellets were resuspended in 50 mM Tris–HCl at final protein conc. 1–3 mg/ml. The protein concentration of all samples was measured by the Lowry procedure [17].

2.2. *Binding assays*

The L-glutamate binding activity of all particulate

subcellular fractions was measured by a microfuge centrifugation assay as in [18]. *p*-Chloromercuriphenyl sulfonate was not routinely included in the binding assays. Glutamate binding to the soluble extract from synaptic membranes was monitored by a Millipore filtration assay employing L-[^{3}H]glutamic acid (40–50 Ci/mmol, New England Nuclear Corp.) as in [14]. The binding of [^{3}H]kainic acid (2.4–2.6 Ci/mmol, Amersham/Searle Corp.) to either the synaptic membranes or to the cholate extract was measured by a Millipore filtration assay. The binding assay involved the incubation of 450 μl membrane suspension or of cholate extract in 500 μl final vol. medium containing 50 mM Tris–HCl (pH 7.6) with variable amounts of [^{3}H]kainic acid. Non-specific binding was determined by measuring [^{3}H]kainic acid binding in the presence of 10^{-5} M non-labeled kainic acid. All samples were incubated at room temperature for 25 min, the reaction was stopped with 0.5 ml cold 50 mM Tris–HCl buffer, and the samples were immediately filtered through Millipore filters (HAWP, 0.45 μm). The filters were washed with 0.5 ml Tris–HCl buffer. Non-specific binding of [^{3}H]kainic acid to synaptic membranes consituted 25–50% of the total binding and was subtracted from all samples in the calculations of the specific binding.

3. Results and discussion

The same membrane preparations used for the study of glutamate binding activity were found to possess [^{3}H]kainic acid binding sites which were quite sensitive to the presence in the incubation medium of either non-labeled kainic acid or of non-labeled L-glutamic acid (fig.1). The addition of unlabeled kainic acid caused a concentration-dependent displacement of [^{3}H]kainic acid from its binding sites with an estimated concentration for 50% displacement IC_{50} = 1.1 × 10^{-8} M (fig.1). This value is quite similar to the K_I (1.2 × 10^{-8} M) for kainic acid inhibition of [^{3}H]-kainic acid binding to rat brain cerebellar membranes measured by a centrifugation assay [10]. L-Glutamic acid displaced [^{3}H]kainic acid from the membranes with an estimated IC_{50} = 7.2 × 10^{-8} M (fig.1). Despite the fact that both kainic acid and L-glutamic acid were active in displacing bound [^{3}H]kainic acid, the displacement produced by non-labeled kainate showed no cooperativity (Hill coefficient (n_H) 0.89) whereas that which was brought about by L-glutamate exhibited negative cooperativity (n_H 0.61). These findings are very similar to the demonstration [19] that kainic acid displacement of [^{3}H]kainic acid which was bound to rat cerebellar membranes had n_H 0.92 whereas displacement of [^{3}H]kainic acid by L-glutamic acid had n_H 0.62. These results are suggestive of two different but interacting binding sites for glutamate and kainic acid.

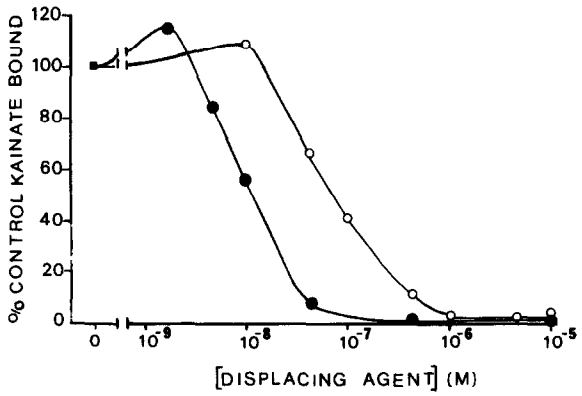

Fig.1. [^{3}H]Kainic acid binding to synaptic membranes. Displacement of specifically bound [^{3}H]kainic acid (17 nM) by non-labeled kainic acid (•) and L-glutamate (○). Each data point is the mean of 6–15 determinations. Standard error of the mean varied from 4–12%.

The probable non-identity of the glutamate and kainic acid binding sites in synaptic membranes was further substantiated by treating the synaptic membranes with 0.5% (w/v) Na-cholate (table 1). Treatment of these membranes with this concentration of Na-cholate did not cause a loss of L-[^{3}H]glutamate binding activity, but, on the contrary, it led to a moderate increase in glutamate binding. However, membranes treated with Na-cholate lost ~50% of kainic acid binding activity (table 1). The lost binding activity was traced to the supernatant obtained following the cholate extraction and the 100 000 × *g* centrifugation of the cholate-extracted membranes, while <1% of the glutamate binding activity was recovered in this supernatant fraction (table 1).

When these results are considered with our findings that the purified glutamate binding protein from brain synaptic membranes does not have any level of interaction with kainic acid [14], then, they indicate that the glutamate binding entity is distinct from the macromolecular species which binds kainic acid in the synaptic membranes. These observations offer an explanation at the molecular level for the previously determined

Table 1
Effects of cholate treatment of synaptic membranes on [^{3}H]kainic acid and [^{3}H]glutamate binding

Preparation	Protein (mg)	[^{3}H]Kainic Acid[a] binding (pmol/mg^{-1} protein)	[^{3}H]Glutamate binding (pmol/mg^{-1} protein)
Control membranes	3.15	0.173 ± 0.057 (7)	5.26 ± 0.90 (4)
0.5% Na-Cholate-treated membranes	2.39	0.084 ± 0.055 (8)	17.08 ± 2.17 (4)
Control membrane supernatant	0.98	0	0
0.5% Na-Cholate soluble supernatant	1.93	0.065 ± 0.026 (7)	0.10 ± 0.02 (4)

[a] Binding of [^{3}H]kainic acid (17 nM) to all fractions was measured by the Millipore filtration assay. [^{3}H]Glutamate (92 nM) binding to membranes was measured by the microfuge centrifugation assay. All values are the mean (± SE) of the no. determinations shown in parentheses

differences in electrophysiologic actions and in neuropharmacologic characteristics of L-glutamic acid and kainic acid excitation in the central nervous system [20–23]. The methods described here for the physical separation of these two membrane binding sites for glutamate and kainic acid open up the possibility for further characterization of the kainic acid binding sites and for further exploration of the possible endogenous molecules which normally interact with these sites.

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