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Chemoreception in *Hydra vulgaris* (*attenuata*): initial characterization of two distinct binding sites for L-glutamic acid

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To elucidate the relationship between L-glutamic acid and the putative chemoreceptor for glutathione, binding of L-[^{3}H]glutamate to a crude membrane fraction from *Hydra vulgaris* (*attenuata*) has been characterized. The binding of L-[^{3}H]glutamate was rapid, reversible and saturable. A Scatchard analysis of the specific binding revealed values of 10 μM for the dissociation constant (K_d) and 170 pmol/mg for the maximal capacity of binding sites (B_{max}). A maximum of 65% of the specific L-[^{3}H]glutamate binding was inhibited by the chemostimulatory peptide, glutathione. This glutathione-sensitive glutamate binding presumably represents the association of glutamate with a putative chemoreceptor which modulates feeding behavior in hydra. The remaining 35% of the specific L-[^{3}H]glutamate binding may be due to a second class of glutamate binding sites which is insensitive to glutathione. The identification of glutathione-insensitive glutamate binding is the first indication of a putative glutamate receptor, which may mediate an action independent of the glutathione-induced feeding response. The glutathione-insensitive and glutathione-sensitive sites must have similar affinities for glutamate since these sites were indistinguishable by Scatchard analysis. A preliminary characterization of the glutathione-insensitive site, performed in the presence of saturating levels of glutathione, revealed inhibition of glutathione-insensitive glutamate binding by kainate and quisqualate, but not by *N*-methyl-D-aspartate. A comparison of glutamate analogue inhibition of total specific L-[^{3}H]glutamate binding with analogue inhibition of glutathione-insensitive L-[^{3}H]glutamate binding suggests that kainate and α-aminoadipate may be selective ligands for the glutathione-insensitive and glutathione-sensitive glutamate binding sites, respectively.

Introduction

Chemoreception plays a central role in the ability of an organism to respond to environmental stimuli. Whether the subsequent response is limited to a single cell, as in bacterial and protozoan chemotaxis, or involves more complex, multicellular processes such as vertebrate taste and olfaction, the initial event involves interaction of a chemical signal with the membrane of a receptive cell [1]. It has been noted that although chemical activation of cellular receptors evolved in single-cell organisms, it is in lower metazoans such as coelenterates that these chemical signals were first transmitted from cell to cell to effect a coordinated organismal response [2,3]. The coelenterate, hydra, has a well-defined sensory induced feeding response [2,4] and is among the simplest multicellular organisms possessing a nervous system.

Reduced glutathione (γ-glutamyl-L-cysteinylglycine, GSH) is a physiological activator of a cascade of behaviors collectively referred to as the feeding response in hydra [5]. During predation, hydras capture and pierce living prey by discharging specialized organelles known as nematocysts. The flow of GSH from the wounded prey initiates a feeding response, which includes coordinated tentacle movements and mouth opening. The development by Lenhoff of a quantitative assay of the mouth opening response [6] led to the elucidation of many physicochemical properties of the putative glutathione chemoreceptor (reviewed in Ref. 2). A dose-response curve of the duration of glutathione-induced mouth opening indicated that a half-maximal response is elicited by 1 μM glutathione at pH 7.0 [7]. The γ-glutamyl residue is essential for the activity of GSH and glutamic acid is reported to be a competitive

Abbreviation: GSH, γ-glutamyl-L-cysteinylglycine.

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inhibitor of this activity [8]. A recent study of L-[^{3}H]glutamate binding to a nematocyst-rich fraction from *Hydra vulgaris* (formerly referred to as *Hydra attenuata*) has demonstrated that glutamate binding is inhibited by glutathione [9]. However, because the specificity, affinity and reversibility of this GSH-sensitive glutamate binding were not evaluated, it is difficult to assess the relationship between L-[^{3}H]glutamate binding and the behavioral effects of applied glutamate. For this purpose, a crude membrane fraction was prepared from *H. vulgaris* and the specific binding of L-[^{3}H]-glutamic acid was characterized in the present study. Crude membrane fractions have been used successfully in initial biochemical analyses of both taste [10] and olfactory [11] tissues where the chemoreceptive mechanisms had been inferred from behavioral and/or electrophysiological studies.

Materials and Methods

Isolation of membranes

Specimens of *H. vulgaris* were cultured in BVC solution [12] at 18°C and fed with *Artemia* nauplii. The animals belong to a single, asexually reproducing clone [13]. After a 2 day starvation period, 1000–2000 hydras were homogenized in cold 50 mM Tris-HCl (pH 7.4) by six hand strokes using a glass-Teflon homogenizer. The homogenate was centrifuged at $1000 \times g$ for 15 min at 4°C. The pellet (P1) was resuspended in 20 vol. of 50 mM Tris-HCl, then further disrupted on ice with six 20-second pulses from a Fisher biohomogenizer. This homogenate was centrifuged at $1000 \times g$ for 15 min. The resulting supernatant was combined with the previous supernatant (S1) and centrifuged at $30000 \times g$ for 15 min, yielding a crude membrane fraction (P1). The membrane fraction was washed once ($30000 \times g$, 15 min) prior to use in binding assays.

Binding assay

L-[^{3}H]Glutamate binding to the membrane preparation was assayed in 0.5 ml of 50 mM Tris-HCl (pH 7.4) containing 100 nM radioligand. The amount of protein added to each incubation was 150–250 μg (15–85 hydra, depending upon size). Membranes were incubated for 15 min with the exception of those samples used in time-course experiments. Following incubation at 25°C, the assays were rapidly filtered under vacuum through MSI cellulose filters (0.45 μm). Filters were washed twice with 4 ml of cold 50 mM Tris-HCl buffer and radioactivity was measured by liquid scintillation spectrometry. Specific binding was operationally defined as the binding obtained in the presence of 100 nM L-[^{3}H]glutamate (total) minus the binding obtained in the presence of 100 nM radioligand plus an excess (200 μM) of unlabelled L-glutamate (nonspecific). 70% of the total L-[^{3}H]glutamate binding to the membrane fraction was represented by specific binding. Under these conditions, the amount of specific binding was directly correlated with the concentration of protein added to the incubation medium. Protein was quantified by the method of Lowry et al. [14] using bovine serum albumin as the standard. A statistical analysis of data from saturation experiments was performed by the computer program, LIGAND [15], as modified by McPherson [16].

Materials

L-[^{3}H]Glutamate was obtained from Amersham (Arlington Heights, IL; 40–60 Ci/mmol) and from New England Nuclear (Boston, MA; 25 Ci/mmol). All other reagents were purchased from Sigma (St. Louis, MO). MSI cellulose filters (0.45 μm) were purchased from Fisher Scientific (Pittsburgh, PA).

Results

The binding of L-[^{3}H]glutamate to the membrane preparation was both rapid and stable at 25°C. Maximal specific binding was observed within 15 min and was sustained for at least 30 min (Fig. 1). The addition of excess unlabelled L-glutamate resulted in a rapid loss of L-[^{3}H]glutamate binding that was consistent with reversible association of the ligand with the membranes (Fig. 1). Specific L-[^{3}H]glutamate binding was saturable (Fig. 2A), and a Scatchard analysis of saturation binding isotherms (Fig. 2B) was linear (correlation coefficient = 0.95), suggesting that glutamate associates with a single class of binding sites. The equilibrium dissociation constant (K_D) was determined to be 10 ± 3 μM and the maximal capacity of binding sites (B_{max}) was 170 ± 50 pmol/mg protein (approx. 0.4 pmol of L-glutamate bound/hydra). A coefficient of 1.0 ± 0.01 generated from a Hill analysis of L-[^{3}H]glutamate binding (Fig. 3) indicated that the binding was not cooperative.

A preliminary characterization of the specificity of glutamate binding (Table I) was obtained by comparing the inhibitory efficacy of selected compounds assayed at a single, high concentration (200 μM). The specific glutamate binding appeared to be stereoselective, with L-glutamate demonstrating a greater inhibitory potency than D-glutamate. L-aspartate inhibited a small percentage of the L-[^{3}H]glutamate binding, while D-aspartate had no effect. Reduced glutathione and the glutathione derivatives, *S*-methylglutathione and oxidized glutathione, were very effective inhibitors of the specific binding. Several glutamate analogues were also observed to inhibit glutamate binding. Of these, the following rank order of inhibitory potency was determined: quisqualate > α-aminoadipate > kainate. Quinolinic acid, *N*-methyl-D-aspartate and glycine had no effect on L-[^{3}H]glutamate binding.

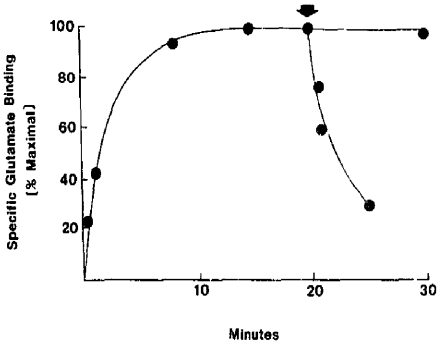

Fig. 1. Time-course of specific L-[^{3}H]glutamate binding at 25°C. The association reaction was measured by incubating membranes (150–250 μg protein) for varying times in 0.5 ml of 50 mM Tris-HCl (pH 7.4) containing 100 nM L-[^{3}H]glutamate. Dissociation was assayed at several time points following the addition of 200 μM unlabelled glutamate (arrow) to membranes pre-incubated with radioligand for 20 min. Duplicate incubations, varying from each other by less than 10% were performed in all experiments. Data presented are from a representative experiment using 1000 hydra.

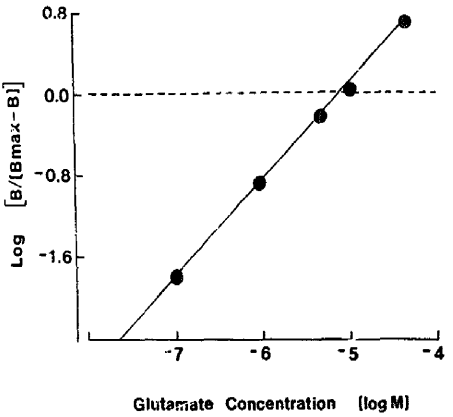

Fig. 3. Hill analysis of L-glutamate binding. Data generated from saturation experiments (Fig. 2A) were subjected to Hill analysis using the computer software program, LIGAND [15]. From the slope of the line, a Hill coefficient of 1.0±0.01 was obtained. B, pmol of L-glutamate bound to the membrane preparation. B_{max} = 170 pmol/mg protein (see legend to Fig. 2B).

L-[^{3}H]glutamate binding was inhibited by concentrations of glutathione exceeding 2 μM. (Fig. 4). In the presence of 7 μM glutathione the GSH-sensitive glutamate binding was reduced by 50%, and maximal inhibition of L-[^{3}H]glutamate binding was effected by 200 μM GSH. No more than 65% of the specific L-[^{3}H]glutamate binding was inhibited by reduced glutathione, even in the presence of 800 μM glutathione (a molar excess of 8000:1 over glutamate). The remaining 35% of the specific glutamate binding might, therefore, be due to the presence of a second class of glutamate binding sites which is insensitive to glutathione. An indirect Hill analysis of L-[^{3}H]glutamate binding in the presence of glutathione (Fig. 5) yields a Hill coefficient which is significantly less than unity, 0.58. This value is

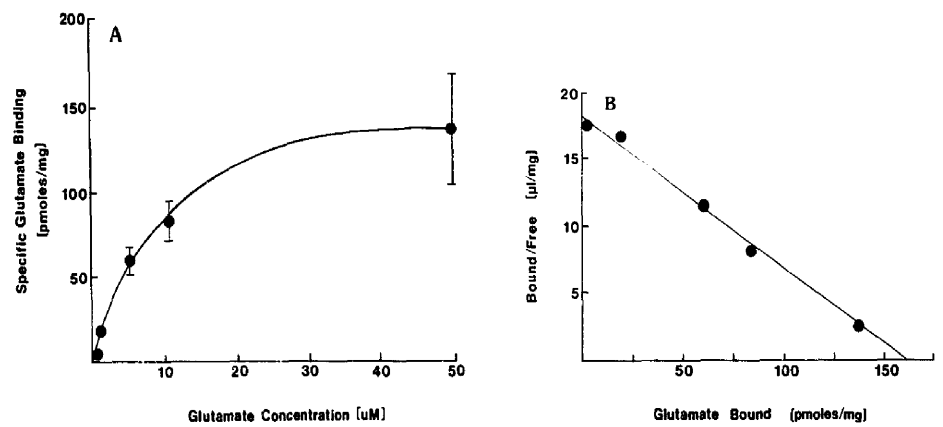

Fig. 2. (A) Saturation curve of specific L-[^{3}H]glutamate binding. The crude membrane fractions were incubated for 15 min at 25°C in the presence of 100 nM radioligand and sufficient concentrations of unlabelled glutamate to bring the final concentration of glutamate to the concentrations shown in the figure. The number of pmol/mg of glutamate bound at each concentration was calculated by adjusting the specific activity of L-[^{3}H]glutamate to account for the addition of unlabelled ligand. Values represent the means of four determinations performed in duplicate, and error bars represent standard error of the mean. The absence of error bars on a data point signifies that the standard error for this point is less than the area of the symbol for the data point. L-[^{3}H]Glutamate binding was also assayed in the presence of 20 μM (n = 2) and 100 μM (n = 2) glutamate. Incubation with 20 μM glutamate resulted in an average of 159 pmol bound/mg and incubation with 100 μM glutamate yielded an average of 164 pmol bound/mg. (B) Scatchard analysis of saturation isotherms. The data generated from saturation experiments (Fig. 2A) were transformed into a Scatchard plot. The K_d and B_{max}, 10 μM and 170 pmol/mg, respectively, were evaluated with the nonlinear least squares curve fitting program, LIGAND [15]. The data best fit a one-site model of ligand binding.

TABLE I

Effects of selected compounds on L-[^{3}H]glutamate binding

Specific binding of L-[^{3}H]glutamate (100 nM) was examined in the presence of a single concentration (200 μM) of each selected compound to determine the relative inhibitory potency of various glutamate and glutathione analogues. Results are expressed as percent inhibition of specific glutamate binding. Values represent the means of two to four duplicate determinations. Individual determinations deviated no more than 15% from the mean.

Compound (200 μM)	% Inhibition of specific L-[^{3}H]Glutamate binding
L-Glutamate	100 [a]
D-Glutamate	62
L-Aspartate	14
D-Aspartate	0
Reduced glutathione	65
Oxidized glutathione	69
S-Methyl glutathione	74
Kainate	19
Quisqualate	73
α-Aminoadipate	61
N-Methyl-D-aspartate	0
Quinolinic acid	0
Glycine	0

[a] 200 μM unlabelled L-glutamate was used to define nonspecific binding, therefore inhibition of L-[^{3}H]glutamate binding by 200 μM glutamate is operationally set at 100%.

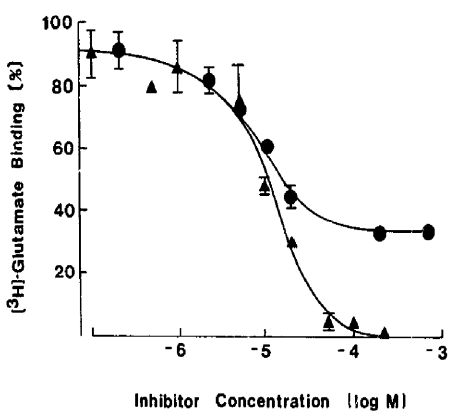

Fig. 4. Inhibition of specific L-[^{3}H]glutamate binding by L-glutamate and reduced glutathione. Membranes were incubated under equilibrium conditions with L-[^{3}H]glutamate (100 nM) and increasing concentrations of either unlabelled L-glutamate (▲) or reduced glutathione (●). Results for glutamate binding in the presence of inhibitor are expressed as a percent of the specific binding in the absence of inhibitor. Values represent the means of four glutamate competition experiments and three glutathione competition experiments. All experiments were performed in duplicate. Error bars represent standard error of the mean. The absence of error bars on a data point signifies that the standard error for this point is less than the area of the symbol for the data point.

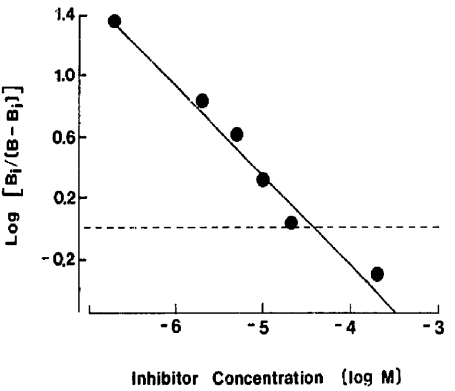

Fig. 5. Indirect Hill analysis of inhibition of L-[^{3}H]glutamate binding by glutathione. The concentration dependence of inhibition of specific glutamate binding by glutathione (Fig. 4) was subjected to indirect Hill analysis. A Hill coefficient of 0.58 was derived from the slope of the line. *B*, pmol of L-glutamate bound in the absence of glutathione; B_i, pmol of L-glutamate bound in the presence of glutathione at concentration, I.

consistent with a model in which glutathione selectively associates with only one of two classes of glutamate binding site.

The glutathione-insensitive glutamate binding site was partially characterized by examining the extent of inhibition of L-[^{3}H]glutamate binding by several glutamate analogues in the presence of 200 μM glutathione, a concentration which eliminates glutamate binding to the GSH-sensitive site (see Fig. 4). A comparison of the relative ability of selected compounds to inhibit GSH-insensitive L-[^{3}H]glutamate binding is shown in Table II. While both kainate and quisqualate were effective inhibitors of glutamate binding when assayed at 200 μM, a significantly greater fraction of the specific binding was displaced by kainate. L-Aspartate, *N*-methyl-D-aspartate, α-aminoadipate and quinolinic acid had no effect on the GSH-insensitive glutamate binding.

Discussion

L-[^{3}H]Glutamate binding to the hydra membrane preparation had many of the characteristics expected of ligand association with a membrane-bound receptor. The specific binding was rapid, reversible, stable, saturable and of reasonably high affinity given the concentrations at which glutamate elicits behavioral responses. A Scatchard analysis of saturation isotherms suggested that glutamate associates with a single class of binding site; however, glutathione competition for L-[^{3}H]glutamate binding revealed the presence of both glutathione-sensitive and glutathione-insensitive glutamate binding sites in the membrane preparation. The

TABLE II

Effects of selected compounds on glutathione-insensitive L-[^{3}H]glutamate binding

Specific binding of L-[^{3}H]glutamate (100 nM) was examined in the presence of a single concentration (200 μM) of each selected compound to determine the relative inhibitory potency of several glutamate analogues. 200 μM glutathione was added to all incubations to eliminate L-[^{3}H]glutamate binding to the glutathione-sensitive binding site. Results are expressed as percent inhibition of specific glutathione insensitive-glutamate binding. Values represent the means of two to four duplicate determinations. Individual determinations deviated no more than 15% from the mean.

Compound (200 μM)	% Inhibition of specific glutathione-insensitive L-[^{3}H]glutamate binding
L-Glutamate	100 [a]
D-Glutamate	70
Kainate	72
Quisqualate	40
α-Aminoadipate	0
N-Methyl-D-aspartate	0
Quinolinic acid	0
L-Aspartate	0

[a] 200 μM unlabelled L-glutamate was used to define nonspecific binding, therefore inhibition of GSH-insensitive L-[^{3}H]glutamate binding by 200 μM L-glutamate is operationally set at 100%.

inability of the Scatchard analysis to distinguish between these two classes of binding site signifies that the sites must have similar affinity for glutamate.

High concentrations of reduced glutathione inhibited a maximum of 65% of the specific L-[^{3}H]glutamate binding. This glutathione-sensitive glutamate binding presumably represents the association of glutamate with a putative chemoreceptor, which mediates the glutathione-induced feeding response in hydra. Behavioral studies have estimated that glutathione-induced mouth opening is inhibited half-maximally by either 10 μM [17] or 20 μM [2] glutamate. A third study [18] of inhibition of glutathione-induced mouth opening by glutamate estimated a dissociation constant for glutamate (K_i) of 7 μM. These values are in excellent agreement with the equilibrium dissociation constant, 10 μM, derived from a Scatchard analysis of data from saturation binding experiments (Fig. 2B). Glutamate has been thought to inhibit the feeding response by competing with glutathione for a single site on the chemoreceptor [8]. This proposed competitive mechanism of inhibition was based upon two behavioral observations: (i) the γ-glutamyl residue of glutathione is essential for activation of the feeding response [8] and (ii) the inhibition of mouth opening by glutamate could be overcome by increasing the GSH:glutamate concentration ratio [8,17]. The observed displacement of L-[^{3}H]glutamate binding by low micromolar concentrations of reduced glutathione is consistent with a competitive mechanism of inhibition; however, initial studies of [^{35}S]glutathione binding to a similar membrane preparation [19] indicate that glutathione binding is not inhibited by glutamate. This finding suggests that glutamate may act allosterically by binding to a site which is distinct from the glutathione-specific binding site, because mutual inhibition would be expected if both ligands associate with the same site. Under certain conditions, the effects of allosteric antagonists may appear to be competitive, since the inhibition of function by an antagonist can be overcome by adding an increased concentration of agonist [20]. The possibility that glutamate acts allosterically was first raised by Hirakawa and Kijima in a study of mouth opening behavior [18]. Although these researchers believed that competitive inhibition was a more likely mechanism of glutamate action based on the importance of the γ-glutamyl moiety in stimulating feeding, it was noted in this study that the effects of applied glutamate were consistent with either a competitive or an allosteric model.

The observed inability of unlabelled GSH or GSH analogues to compete for a significant fraction of L-[^{3}H]glutamate binding indicates the presence of a second, GSH-insensitive, glutamate binding site in the crude membrane preparation. This is the first indication of a putative glutamate receptor in hydra, which may mediate an action independent of the GSH-induced feeding response. Glutamate has been shown to induce microvilli formation in gastrodermal cells of hydra, a process which is presumably linked to the uptake of free amino acids (reviewed in Ref. 4). The glutathione-insensitive L-glutamate binding identified in our preparation could represent the association of glutamate with either a transport protein or with a putative receptor which regulates microvilli formation. It has not been determined if specific L-glutamate binding is a prerequisite for either glutamate transport or microvilli formation. It is also possible that glutamate plays a role in neural signaling in hydra. L-Glutamate is an important excitatory neurotransmitter in both the vertebrate central nervous system and invertebrate neuromuscular junction [21–23]. The classification of glutamate receptor subtypes is based on the observed selectivity of a receptor for various glutamate analogues. At least three glutamate receptor subtypes, kainate, quisqualate and *N*-methyl-D-aspartate, have been identified in the mammalian central nervous system [24]. With one possible exception [25], none of the glutamate receptors identified in invertebrates are of the *N*-methyl-D-aspartate subtype [26–33]. The finding that L-[^{3}H]glutamate binding to the putative hydra receptor is displaced by kainate and quisqualate but is insensitive to *N*-methyl-D-aspartate is consistent with the data from other invertebrates. However, glutamate has not yet been implicated as a neurotransmitter in coelenterates [34], nor has the putative hydra receptor been localized.

A comparison of inhibition of the total specific L-[^{3}H]glutamate binding by glutamate analogues (Table I) with inhibition of GSH-insensitive binding (Table II) by the same analogues suggests that the GSH-sensitive and GSH-insensitive sites have different specificities. The observation that kainate inhibits 73% of the GSH-insensitive binding, and only 19% of the total specific binding (total specific binding = 65% GSH-sensitive binding + 35% GSH-insensitive binding), may reflect the selective association of kainate with the GSH-insensitive site. Kainate appears to have no effect on the GSH-induced feeding response (Bellis, unpublished data), a finding which supports the hypothesis that the function of the GSH-insensitive glutamate site is not related to feeding behavior. Like kainate, quisqualate inhibits GSH-insensitive binding, but is a much more effective inhibitor of the total specific glutamate binding than kainate. Therefore, it is likely that quisqualate associates with both the GSH-sensitive and GSH-insensitive sites. The effect of quisqualate on the feeding response has not been determined. α-Aminoadipate does not inhibit the binding of glutamate to the GSH-insensitive site, although it inhibits total specific glutamate binding by 61%. Because 65% of the total specific binding is represented by GSH-sensitive binding, α-aminoadipate may eliminate GSH-sensitive binding by selectively associating with the GSH-sensitive site. Behavioral studies of the feeding response have indicated that α-aminoadipate, like glutamate, is a strong inhibitor of glutathione-induced mouth opening [2]. A comparison of inhibition of total specific glutamate binding (Table I) with inhibition of GSH-insensitive glutamate binding (Table II), while informative, is a limited means of characterizing the two glutamate binding sites, particularly since the specificity of the GSH-sensitive site is only indirectly examined. A complete characterization of both the GSH-insensitive and GSH-sensitive glutamate binding sites will depend upon either the development of a subcellular fractionation technique which allows the physical separation of these sites, or the identification of selective ligands or labels for each of the two classes of binding site. The present work suggests that α-aminoadipate and kainate are excellent candidates for selective ligands at the GSH-sensitive and GSH-insensitive sites, respectively.

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