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Comparative study of [^{3}H]glutamate binding sites in rat retina and cerebral cortex

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It has been believed that the high affinity and Na^+-independent binding of radiolabeled L-glutamic acid (Glu*), a potential candidate for a central excitatory neurotransmitter, is indeed a biochemical measure for the association of this acidic amino acid with its physiologically relevant synaptic receptors [1–3]. The binding is affected significantly by the inclusion of some physiological inorganic ions such as Cl^-, Ca^{2+} and Na^+ [4]. These biochemical binding studies together with electrophysiological investigations have revealed the multiplicity of receptors for central excitatory acidic amino acid neurotransmitters: *N*-methyl-D-aspartic acid (NMDA) sensitive receptors (A1), quisqualic acid (QA) sensitive receptors (A2), kainic acid (KA) sensitive receptors (A3), and L-2-amino-4-phosphonobutyric acid (AP4) sensitive receptors (A4) [4, 5].

On the other hand, relatively little attention has been paid to the binding of [^{3}H]Glu in the retina which is supposed to contain Glu-ergic synapses in its structure [6, 7]. It has been demonstrated that [^{3}H]Glu really binds to membrane fractions from the chick retina with a high affinity [8]. In the present study, we have attempted to analyze the retinal [^{3}H]Glu binding in comparison with cerebral [^{3}H]Glu binding in terms of the modulation by various ions.

Materials and methods

Materials. QA, NMDA, KA, DL-AP4 and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{3}H]Glu (L-[3,4-^{3}H]glutamic acid, 46.6 Ci/mmole) was obtained from New England Nuclear (Boston, MA, U.S.A.). Other chemicals used were all of commercially guaranteed grade.

Membrane preparation. Groups of three male albino Wistar rats weighing 200–250 g were housed together in a metallic breeding-cage at a room temperature of 25 ± 2° and a humidity of 55 ± 5%, in a room with a 12-hr light–dark cycle. Animals were decapitated between 10:00 and 11:00 a.m. in the light cycle. Retinas were dissected out and placed into ice-cold deionized distilled water for homogenization within 1 min after the animals were killed. Cerebral cortex was dissected out on a chilled plastic plate according to the procedures described by Glowinski and Iversen [9]. The retina and cerebral cortex were homogenized individually in 50 vol. glass-distilled deionized water using a Polytron homogenizer at setting No. 6 for 30 sec at 4°. The homogenates were centrifuged at 50,000 *g* for 30 min, and the resultant pellets were suspended in 50 mM Tris-acetate buffer (pH 7.4). The suspensions were again centrifuged as above. These washing procedures were repeated three times. The final pellets thus obtained were suspended in 0.32 M sucrose and the suspensions were frozen at −80° for 19–20 hr [10]. The frozen suspensions were thawed at room temperature and washed twice by centrifuging at 50,000 *g* for 30 min with 50 mM Tris-acetate buffer (pH 7.4) before each use.

Binding assay for [^{3}H]*Glu.* Each membranous homogenate suspension was incubated with 10 nM [^{3}H]Glu in 500 μl of 50 mM Tris-acetate buffer (pH 7.4) at 2° or 30° for 60 min in the presence and absence of various compounds. Incubation was terminated by the addition of 3 ml of ice-cold buffer and subsequent filtration through a Whatman GF/B glass filter under a constant vacuum of 15 mm Hg. After washing the filter four times with 3 ml of ice-cold buffer, the radioactivity trapped on the filter was measured by a liquid scintillation spectrometer (LSC 900, Aloka, Japan) using 5 ml of modified Triton–toluene scintillant [11] at a counting efficiency of 40–42%. The radioactivity found in the presence of 1 mM nonradioactive Glu was subtracted from each experimental value to obtain the specific binding of [^{3}H]Glu [12]. The specific binding increased linearly with incubation time and reached a plateau within 30 min independently of the incubation temperature. Thin-layer chromatography on cellulose-coated plates with phenol–H_2O (75:25, at 22 ± 2°) as a solvent system revealed that no significant degradation of the radioactive ligand occurred during the incubation with cerebral and retinal preparations at 30° for 60 min.

Binding assays were always carried out in triplicate with a variation of less than 10%. Protein content was measured by the method of Lowry *et al.* [13]. The protein concentration usually employed was between 200 and 250 μg per assay. Results were usually expressed as the mean ± SE, and the statistical significance was determined by Student's *t*-test.

Results and discussion

As shown in Fig. 1, neither Cl^- nor Cl^-/Ca^{2+} elicited a significant alteration in the retinal binding despite the occurrence of a profound augmentation of the cerebral binding by these ions. Chloride as well as Cl^-/Ca^{2+} induced a significant increment of the density of the binding sites without altering their affinities in the cerebral synaptic membranous preparations [12]. In addition, Na^+ remarkably facilitated the binding of [^{3}H]Glu to cerebral preparations with a concomitant suppression of the retinal binding. A 100 mM concentration of sodium acetate exerted about a 5-fold elevation of the cerebral binding, while inducing a 50% reduction of the retinal binding (Fig. 1). These results suggest the possible difference in the ionic modulatory mechanisms of the binding between the retina and cerebral cortex. Since sodium ions are known to affect differentially the opiate receptor binding of agonists and antagonists [14], it seems possible that Na^+ may provide a useful tool for the differentiation and/or subclassification of the Glu binding sites. Similarly significant suppression of [^{3}H]Glu binding occurs in the peripheral endocrine organs such as the pituitary [10] and adrenal [15].

* Abbreviations: AP4, L-2-amino-4-phosphonobutyric acid; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; Glu, L-glutamic acid; KA, kainic acid; NMDA, *N*-methyl-D-aspartic acid; and QA, quisqualic acid.

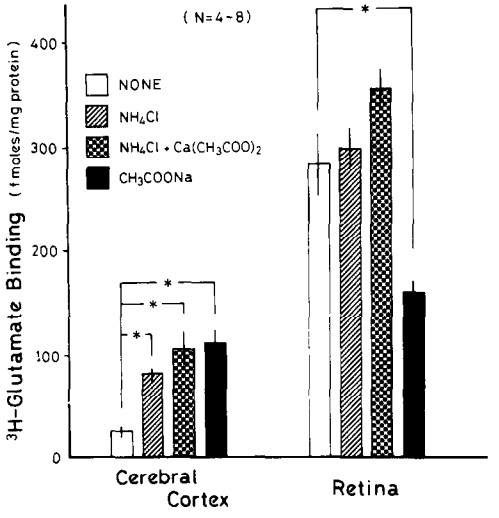

Fig. 1. Effects of various ions on [^{3}H]Glu binding. Each membranous homogenate preparation was incubated with 10 nM [^{3}H]Glu in 50 mM Tris-acetate buffer (pH 7.4) at 30° for 60 min in the presence of either 20 mM NH_4Cl, 20 mM NH_4Cl + 2.5 mM $Ca(CH_3COO)_2$ or 100 mM CH_3COONa. Each value represents the mean ± SE obtained from four to eight independent experiments. Key: *$P < 0.05$, compared with each control value.

In vitro addition (10^{-5}–10^{-3} M) of QA resulted in a significant inhibition of the retinal binding to a slightly greater extent than that of the cerebral binding (Fig. 2). Inclusion of 1 mM QA produced almost complete abolition of the retinal binding while decreasing the cerebral binding to 30%. Neither NMDA nor KA had such a significant effect on [^{3}H]Glu binding in either central structure. In addition, QA exhibited a similarly potent elimination of the Cl^--dependent and Ca^{2+}-stimulated bindings to a significantly greater extent than that of the basal binding, with a concurrent, considerably less potent, inhibition of the Na^+-dependent binding in the cerebral preparations [12]. These results suggest that QA-sensitive receptors may be predominantly detected among three distinctly different subclasses of the central acidic amino acid receptors in these two excitable tissues under the experimental conditions employed. The present results, along with the fact that QA elicits no prominent action on the uptake of the latter neurotransmitter [4], again support the proposal that the retinal binding indeed corresponds to the association with the QA-sensitive receptors, rather than reflects the uptake of Glu in this central structure. It is conceivable, however, that the autoradiographic study may reveal other subclasses of the receptors in the retina as observed in the case of cerebral Glu receptors [16].

It has been suggested that Cl^--dependent [^{3}H]Glu binding may be attributable to the anion-driven uptake of this excitatory amino acid into the resealed membrane vesicles [4, 17], or may be due to a linkage of the receptors to the anion channels [15]. Therefore, the effect of an anion transport inhibitor, DIDS, on the retinal binding was examined. Reduction of the incubation temperature from 30° to 2° induced a drastic elimination of the retinal binding while affecting the cerebral binding less (Fig. 3). These results clearly indicate the strict dependence of retinal binding on incubation temperature. *In vitro* addition of DIDS produced a prominent and concentration-dependent diminution of the temperature-dependent portion of the binding in the cerebral cortex without significantly altering that in the retina (Fig. 3). DIDS at 1 mM caused an almost complete abolition of the temperature-dependent binding in the cerebral cortex with no significant change of that in the retina. These results suggest a possible difference between the cerebral cortex and the retina in the putative interaction of the Glu receptors with the anion transport carriers.

It has been proposed that photoreceptor cells release a neurotransmitter which depolarizes the horizontal cells in the dark and that the light induces a reduction of the rate of transmitter release, which in turn results in hyperpolarization of the horizontal cells [18]. Although removal of Cl^- originates in an abolition of the action of the natural neurotransmitter released from the photoreceptor cells in the mud puppy retina [19], presence of the DIDS-sensitive band 3 [20] in the rodent retina is uncertain at present. This may explain the discrepancies between the above-mentioned previous observations and the present results.

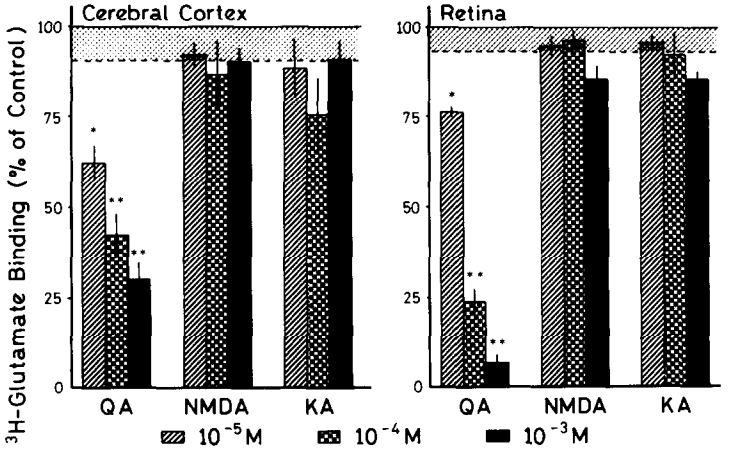

Fig. 2. Effects of various agonists on [^{3}H]Glu binding. Membranous homogenate suspensions were incubated with 10 nM [^{3}H]Glu in 50 mM Tris-acetate buffer (pH 7.4) at 30° for 60 min in the presence of various concentrations of either QA, NMDA or KA. Each value represents the mean ± SE obtained from six separate experiments. Key: *$P < 0.05$ and **$P < 0.01$, compared with each control value. Cerebral cortex, 49.8 ± 12.8 fmoles/mg protein; retina, 296 ± 21.0 fmoles/mg protein.

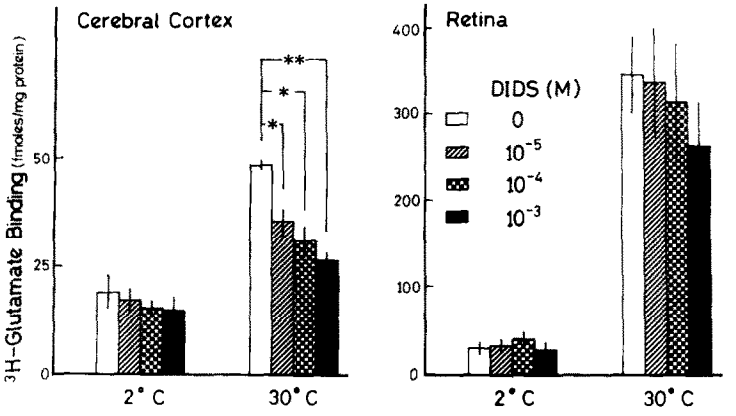

Fig. 3. Effect of DIDS on [^{3}H]Glu binding. Each membranous preparation was incubated with 10 nM [^{3}H]Glu in 50 mM Tris-acetate buffer (pH 7.4) at 2° or 30° for 60 min in the presence of various concentrations of DIDS. Each value represents the mean ± SE obtained from four to six independent experiments. Key: *$P < 0.05$ and **$P < 0.01$, compared with each control value.

In summary, it appears that the retinal Glu receptors may be lightly linked to the anion transport carriers, which is distinctly different from the coupling in the cerebral cortex. Further biochemical and pharmacological characterizations of the retinal Glu receptors are now under way in our laboratory.

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Effect of hypolipidemic drugs on the metabolism of lauric acid and dimethylaminoazobenzene by rat liver microsomes

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Hepatic responses to clofibrate and other hypolipidemic drugs have been studied for many years [1, 2]. Aside from frank toxicity and carcinogenicity, prominent effects include hepatomegaly, associated with both hyperplasia and hypertrophy of hepatocytes, and proliferation of peroxisomes and endoplasmic reticulum [1, 2]. Enzymic activity associated with these organelles is often, but not always, induced. Liver enlargement occurs after clofibrate feeding.