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Molecular size of the kainate binding protein in goldfish brain determined by radiation inactivation

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Abstract—Radiation inactivation analysis was used to estimate the target size of a putative glutamate receptor subtype in goldfish brain. A simple, linear inactivation curve was obtained. The calculated molecular size of the [^{3}H]kainate binding site was 33.8 kDa. The results presented here are comparable to the molecular masses determined for putative glutamate receptors in other lower vertebrates but are markedly different from the sizes of the corresponding glutamate receptor subtypes in mammalian central nervous system.

Mammalian glutamate receptors can be divided into the metabotropic (mGluRs) and the ionotropic (GluRs) classes. Ionotropic receptors have been sub-divided further into the *N*-methyl-D-aspartate (NMDA*), the kainate and the α-amino-3-hydroxy-5-methylisoxazolepropionate (AMPA) subtypes [1]. Likewise, despite the lack of selective ligands, mammalian mGluRs have been sub-divided into two basic groups: those positively coupled to IP3 (mGluR1 and mGluR5) and those that inhibit adenylyl cyclase. Within the latter group mGluR2 and mGluR3 share common sequence motifs as do mGluR4 and mGluR6 [2]. It is important to note, however, that with the exception of quisqualate, agonists at mammalian mGluRs and GluRs do not show cross-reactivity.

In lower vertebrates, on the other hand, the situation is less well defined. Recent reports have suggested that the kainate binding sites present in goldfish brain may represent a novel type of G-protein-linked glutamate receptor. In addition, the kainate binding protein isolated and cloned from the frog *Rana pipiens* [3, 4] shows no channel activity when expressed in Chinese hamster ovary (CHO) cells [4] but it has been proposed that, like the goldfish kainate binding site, this protein may be a G-protein-linked receptor [5].

Mammalian mGluRs are not activated by kainate and have molecular masses in the region of 130 kDa whereas non-NMDA-type ionotropic receptors that are kainate sensitive are ~100 kDa. The differences in the pharmacological characteristics of putative mGluRs in lower vertebrates may correlate to differences in their molecular sizes. So far, the only GluR from lower vertebrates for which function has been shown is the 'unitary' ionotropic receptor complex isolated from *Xenopus* CNS which is composed, at least in part, of 42 kDa subunits [6]. Similarly, purification studies of the goldfish brain [^{3}H]kainate binding site have indicated molecular masses of 45 and 41 kDa and these polypeptides have antigenic epitopes in common with the 48 kDa kainate binding protein from frog brain.

The aim of this study was to estimate the molecular size of the kainate binding protein in goldfish brain by target size analysis. These data are less susceptible to proteolysis artefacts and can provide information on the interaction between molecules which can help in the structural and functional characterization of the native receptor complex.

Materials and Methods

Membrane preparation. Whole goldfish brain membranes were prepared as described previously [7]. Briefly brains were homogenized in 10 vol. of chilled Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA pH 7.4 at 0° with citric acid) in a glass–Teflon homogenizer. The homogenate was centrifuged at 1000 *g* for 15 min, the pellet discarded and the supernatant centrifuged for 35 min at 48,000 *g*. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris–citrate buffer and recentrifuged for 20 min at 48,000 *g*. This freeze–thaw–wash procedure was repeated to a total of three times to ensure complete lysis of membrane vesicles and elimination of endogenous glutamate. The final pellet was resuspended to a volume corresponding to 1 mL per brain. When appropriate, protein assays were performed using the Bio-Rad kit with bovine serum albumin as standards.

Radioligand binding. Goldfish brain homogenate (50 μL) in Tris–citrate buffer was added to test-tubes in triplicate. For standard radiation inactivation dose–response experiments the final [^{3}H]kainate (58 Ci/mmol) concentration was 50 nM. Scatchard analysis was carried out using [^{3}H]kainate concentrations ranging from 1 to 300 nM. Non-specific binding was defined by inclusion of 100 μM unlabelled kainate in the incubation assay. The final assay volume was adjusted to 100 μL with Tris buffer. Binding was allowed to proceed at 0° for 90 min. The reaction was terminated by rapid filtration through GF/B

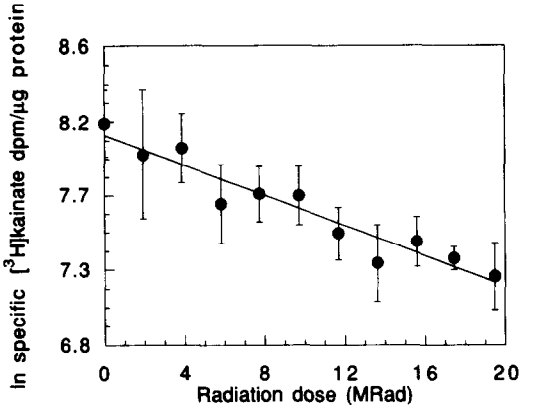

Fig. 1. Radiation inactivation curve for [^{3}H]kainate binding to goldfish brain membranes. The experimental methods and data analysis are as given in the text. The deduced molecular mass is 33.8 kDa. The data are the means and SEMs of at least four independent determinations for each point using membranes prepared from two separately irradiated batches of tissue.

* Abbreviations: AMPA, α-amino-3-hydroxy-5-methylisoxazolepropionate; NMDA, *N*-methyl-D-aspartate.

filters pre-soaked in 0.05% polyethylenimine. The glass filters were washed with three 3-mL aliquots of ice-cold Tris buffer and the total wash time 2–3 sec. Radioactivity remaining on the filters was assayed by liquid scintillation spectroscopy at an efficiency of approximately 48%.

Radiation inactivation. Irradiation of whole frozen goldfish brains was at the 10 MeV linear accelerator at Risø, Denmark. The tissue was irradiated in disposable glass test-tubes (12 mm × 75 mm) as described previously [8]. The tissue was kept on ice bags at −15° during irradiation and was cooled for 2 min at −20° after each dose of 2 Mrad. Membranes from the irradiated tissue were prepared as above.

Results and Discussion

Estimates of the target size of the [^{3}H]kainate binding site were obtained from the plot of the natural log of the residual binding against the radiation dose (Fig. 1). The data yield a radiation inactivation constant (k) of 0.0459. If the empirical proportionality constant ($M_r = k\ 640\ r$) of Kepner and Macey is assumed [9] and r the temperature correction factor is taken as 1.15, as determined by Nielsen and Braestrup [8] for these conditions (−20°), then the calculated molecular mass is 33.8 kDa. It should be noted that there is an inherent variability in the technique of the order of ±20% [8–10]. Thus, these data are in reasonable agreement with previously reported domoate-affinity purification results where the deduced molecular masses were 41 and 45 kDa [11].

Scatchard analyses performed on 0, 9.7 and 21.5 Mrad irradiated tissue confirmed that the affinity (K_D) remained relatively unchanged and that the reduction in [^{3}H]kainate binding with increasing radiation dose is due solely to a decrease in the number of binding sites (B_{max}). Within this set of experiments, the K_D values for 0, 9.7 and 21.5 Mrad irradiated tissue were 130, 148 and 125 nM, respectively and the B_{max} values were 77, 50 and 32 pmol/mg protein, respectively (the values are means of two separate determinations).

The data are best-fit by a simple least-squares linear regression with no evidence of a curvilinear inactivation profile. This is in contrast to the non-linear inactivation profiles for [^{3}H]AMPA in rat [12], chick [13] and *Xenopus* CNS [14]. The situation for [^{3}H]AMPA binding in goldfish CNS is unclear since very little binding can be detected with that radioligand making the experiments difficult to perform and interpret (J.M. Henley, unpublished observations). Nonetheless, the observation that [^{3}H]-kainate displays a simple, linear inactivation profile suggests that the binding site is not subject to modulation by an associated protein with a target size appreciably greater than that estimated for the binding site. If the [^{3}H]kainate binding sites are coupled to G-proteins as has been proposed [15], it would perhaps be expected that the inactivation curve should show some non-linearity. Indeed, theoretical modelling of such multicomponent systems has been shown to produce complex decay curves [16].

To summarize, our experiments yielded a relatively small target size but this was in the same order as the M_r derived from protein purification [11] and are compatible with the M_r values of non-NMDA receptors from various other lower vertebrates (but not mammalian species). The lack of a complex inactivation profile suggests that the [^{3}H]-kainate binding site in goldfish brain is not allosterically coupled to a large molecular mass protein. If these sites are indeed G-protein linked, destruction of the G-protein by radiation inactivation would be expected to decrease [^{3}H]kainate binding. However, since the M_r values of the binding sites and G-proteins are similar it is not possible to address this question using target size analysis alone.

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