ANALYSIS OF [^{3}H]KAINIC ACID BINDING WITH RAT AND FROG BRAIN MEMBRANES

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Kainic acid (KA), a structural analog of glutamate, if injected directly into various brain structures, injures the neutron bodies and causes their degeneration, while leaving axons running close to the site of injection intact [10, 11]. Because of its ability to injure neuron bodies selectively, KA is widely used in neurobiological research [1-3, 10, 11]. It has been suggested that lesions caused by KA may simulate changes in the brain observed in Huntington's chorea and certain forms of epilepsy [6, 10]. The mechanism of the neurotoxic action of KA is not completely clear. It has been claimed that KA exerts its effect through the glutamatergic system, by releasing glutamate, or that it acts directly on glutamate receptors, and so causes long-lasting membrane depolarization of the neurons, leading to their death [3]. It was found in 1976 that tritium-labeled [^{3}H]-KA binds selectively with brain membranes with high affinity [12]. However, it has not yet been absolutely proved that the binding sites of [^{3}H]-KA are identical with glutamate receptors.

The aim of this investigation was to analyze binding of [^{3}H]-KA with brain membranes in vitro and the effect of various neuroactive amino acids, suggested an endogenous ligands for binding sites of [^{3}H]-KA, on binding.

EXPERIMENTAL METHOD

Experiments were carried out on noninbred male albino rats weighing 220-270 g and on winter frogs. Choice of the frog's brain as an experimental object was determined by the high density of high-affinity binding sites of [^{3}H]-KA, so that the working concentration of [^{3}H]-KA could be fixed close to the dissociation constant. The animals were decapitated and the brain removed in the cold. The brain was divided into parts: cerebellum, frontal zones of the cortex, striatum, and hippocampus. A suspension of membranes was prepared by the method in [9] in the writers' modification. Fresh brain tissue was homogenized in a glass homogenizer with Teflon pestle in 0.32 M sucrose (10% homogenate) and centrifuged at 1000g for 10 min. The supernatant was diluted with bidistilled water, and centrifuged at 48,000g for 30 min. The coarse fraction of synaptic membranes thus obtained was washed by centrifugation three times: first in bidistilled water, then in 100 volumes in 50 mM Tris-citrate buffer, pH 7.35, and the final residue was homogenized in a "Virtis" homogenizer. Binding was carried out on polypropylene tubes. The incubation medium consisted of 100 μl of [^{3}H]-KA (specific radioactivity 4 Ci/mmole, Amersham Corporation, England), 100 μl of displacing substances or buffer, and 800 μl of membrane suspension (0.25-0.5 mg protein for rats, 0.1-0.2 mg for frogs). The tubes were incubated at 0°C for 60 min. Binding was stopped by centrifugation at 10,000g for 10 min in a Microcentrifuge-12 (from Beckman, USA). The residue was washed twice and solubilized in an NCS tissue solubilizer (Amersham Corporation) at 40° C for 4 h, after which radioactivity was determined in Ready-Solv scintillator in an LS-6800 counter (Beckman), with counting efficiency of 39-41%. Specific binding was determined as the difference between binding in the absence and presence of 10^{-3} M L-glutamate. The following substances were used: kainic acid (from Sigma, USA), quinolinic acid, kynurenine, L-aspartate, and taurine (generously provided by Professor I. P. Lapin), glycine (from Reanal, Hungary), L- and D-glutamate, folic acid, and methyltetrahydrofolic acid (from Sigma), and gamma-aminobutyric acid (GABA) (from Serva, West Germany). The experimental results were analyzed by plotting curves between Scatchard, Hill, and Lineweaver–Burk coordinates.

The concentrations of substances inhibiting binding [^{3}H]-KA by 50% (IC_{50}) were calculated by logit-probit analysis, and inhibition constants (K_I) were calculated by the method in [5].

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TABLE 1. Characteristics of Binding of [^{3}H]-KA with Rat and Frog Brain Membranes (M±m)

Test object	High-affinity regions		Low-affinity regions	
	B_{max}, fmoles/mg protein	K_d, nM	B_{max}, fmoles/mg protein	K_d, nM
Rat brain:				
cortex	390±24	9,1±3,2	1 560±260	71±13
striatum	430±36	9,0±4,0	1 100±220	69±8
hippocampus	390±25	11,2±3,8	1 025±120	60±12
cerebellum	—	—	530—12	48,2—6,0
Frog brain	2400±340	4,3±1,2	14 200±421	55,6±8,8

Legend. Mean results of 3-4 experiments in triplicate are shown. B_{max}) Minimal binding, K_d) dissociation constant.

TABLE 2. Effect of Substances on Binding on [^{3}H]-KA (5 and 50 nM) with Rat and Frog Brain Membranes

Substance	High-affinity sites (frog brain)		Low-affinity sites (rat cerebellum)	
	IC_{50}, nM	K_I	IC_{50}, nM	K_I
KA	12,4±0,6	5,7	68±12	33,3
L-Glutamate	98,4±14,0	45,5	700±140	334,8
Folate	12000±1 300	5 556	950±210	467,9
D-Glutamate	>10 000	—	>10 000	—
Tetrahydrofolate	>10 000	—	>10 000	—
Quinolinic acid	>10 000	—	>1 000	—
Kynurenine	>10 000	—	—	—
L-Aspartate	>10 000	—	—	—
Taurine	>10 000	—	>1 000	—
GABA	>10 000	—	>1 000	—

Legend. Mean results of three experiments in triplicate are shown.

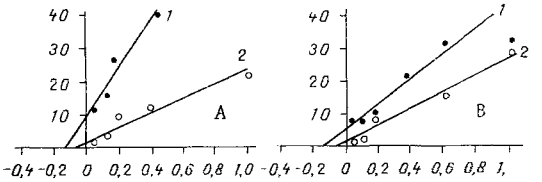

Fig. 1. Kinetics of [^{3}H]-KA binding with frog brain membranes in presence (1) and absence (2) of 10 μM L-glutamate (A) and folate (B) between Lineweaver–Burk coordinates. Abscissa, reciprocals of concentration of free acid (in mM); ordinate, reciprocals of specific binding of [^{3}H]-KA (in fmoles/mg protein).

EXPERIMENTAL RESULTS

Specific binding of [^{3}H]-KA in the rat and frog brain was saturating, and reached a plateau in concentrations of 100 nM. Saturation isotherms were found in all parts of rat brain except the cerebellum, and in the frog brain there were two binding sites: with high and low affinity (Table 1). In the rat brain there were clear regional differences in the distribution of these binding sites. For instance, the greatest density of high-affinity binding sites was observed in the striatum, and of low-affinity sites in the cortex. In the cerebellum, high-affinity binding sites were virtually absent, and for that reason, to analyze the action of the substances on low-affinity binding sites only, the cerebellum was subsequently used. The number of binding sites in the frog brain was five to 10 times greater than in the rat brain, so that it was possible to use concentrations of [^{3}H]-KA close to the dissociation constant of the high-affinity sites, namely 5 nM (Table 1). Among the substances studied, KA, L-glutamate, and folic acid displaced [^{3}H]-KA most actively from the binding sites (Table 2). L-Glutamate was more active against high-affinity binding sites, folic acid against low-affinity sites. D-Glutamate and the active folic acid metabolite methyltetrahydrofolic acid, which has a neurotoxic action [14], and also convulsants of endogenous origin [7], namely quinolinic acid and kynurenine, did not affect binding of [^{3}H]-KA (Table 2). Analysis of displacement of [^{3}H]-KA from binding sites by L-glutamate and folic acid, in Hill's coordinates, showed gently sloping lines with Hill's coefficients of 0.5 for

L-glutamate and 0.6 for folic acid, evidence of negative cooperativeness. However, the gentle slope of the lines in Hill's coordinates does not enable the character of displacement to be judged accurately, because such a slope may be observed when several binding sites with different affinity are present. Accordingly, for more accurate analysis of the character of inhibition of [^{3}H]-KA binding by L-glutamate and folic acid, the kinetics of binding was studied with frog brain membranes in the presence and in the absence of these substances. The experimental results are shown between Lineweaver–Burk coordinates in Fig. 1. The results show that both L-glutamate and folic acid inhibited binding of [^{3}H]-KA noncompetitively.

The experimental results thus show that although L-glutamate and folic acid inhibit binding of [^{3}H]-KA, they do not satisfy the criteria to be met by endogenous ligands, and this inhibition of binding is noncompetitive in character. This suggests that KA binding sites and glutamate receptors are not identical, although they may perhaps be subunits of a single complex. This hypothesis is supported by the fact that KA has been shown experimentally to be a very weak antagonist of [^{3}H]glutamate binding [9].

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