Effect of Kainate-Induced Experimental Epilepsy on NADPH-Diaphorase and Calcium-Binding Proteins in Rat Hippocampal Neurons

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Experimental epilepsy induced in rats by infusion of kainic acid into the lateral cerebral ventricles decreased the number of NADPH-diaphorase-positive neurons in the hippocampal formation by 55-79% and increased activity of this enzyme in CA1 and CA3 pyramidal neurons. All parvalbumin-immunoreactive cells were highly resistant to the cytotoxic effects of kainate in contrast to calbindin- and calretinin-positive interneurons, whose amount decreased by 50%.

Key Words: *nitric oxide; calcium-binding proteins; epileptogenesis*

The development of epileptiform activity results from imbalance between the inhibitory and excitatory neurotransmitter systems [3,4,9,10]. Since NO is involved in the regulation of these processes, changes in NO-ergic neurotransmission is considered as an important factor of epileptogenesis [2,5,13]. The involvement of this or that types of cortical neurons into the formation of epilepsy is little studied. Our aim was to study the reaction of GABA- and NO-ergic hippocampal neurons in rats during experimental epilepsy induced by kainate.

MATERIALS AND METHODS

The experiments were carried out on the brain of random-bred rats (n=9) weighing 150-200 g. The animals were maintained under standard vivarium conditions. Hyperexcitability of cortical neurons was induced by intraventricular infusion of kainic acid, a selective agonist of glutamate receptors. The rats were anesthetized with sodium thiopental (40 mg/kg). Using a microsyringe and stereotaxic apparatus, 0.5 μl kainic acid solution (Sigma) on 0.2 M phosphate buffer (pH 7.4) was slowly infused into the lateral ventricles transcranially over 15-30 min. The final concentration of kainic acid was 2 μg per 1 μl solution.

Group 1 rats (n=3) received kainate unilaterally; in group 2 rats (n=3) the preparation was infused into both ventricles simultaneously. One rat of group 2 was injected with 1 μl N-nitro-L-arginine (L-NNA, Sigma), a selective blocker of nitric oxide synthase (NOS) into the temporal lobe region. Two days after infusion, the rats were anesthetized, the brain was isolated and analyzed by histochemical methods for activity of NADPH-diaphorase (NADPH-d, EC 1.6.99.1) and calcium-binding proteins. Control rats received no proconvulsant.

To measure NADPH-d activity, the brain was fixed for 2 h at 4°C in 4% paraformaldehyde solution prepared on 0.1 M sodium phosphate buffer (pH 7.4) and washed in 15% sucrose solution for 24 h. Cryostat sections (25 μ) were thermally stabilized for 1 h at 37°C in a medium containing 50 mM Tris buffer, 0.2% triton X-100, 0.8 mg/ml β-NADPH (Sigma), 0.4 mg/ml HCT, pH 8.0.

To detection of calcium-binding proteins we used primary rabbit polyclonal antisera (Swant) against calretinin (CR) and calbindin (CB) and mouse monoclonal antibodies (Sigma) against parvalbumin (PA). The sections were incubated in 0.1 M phosphate buffer (pH 7.4) containing biotinylated goat serum against

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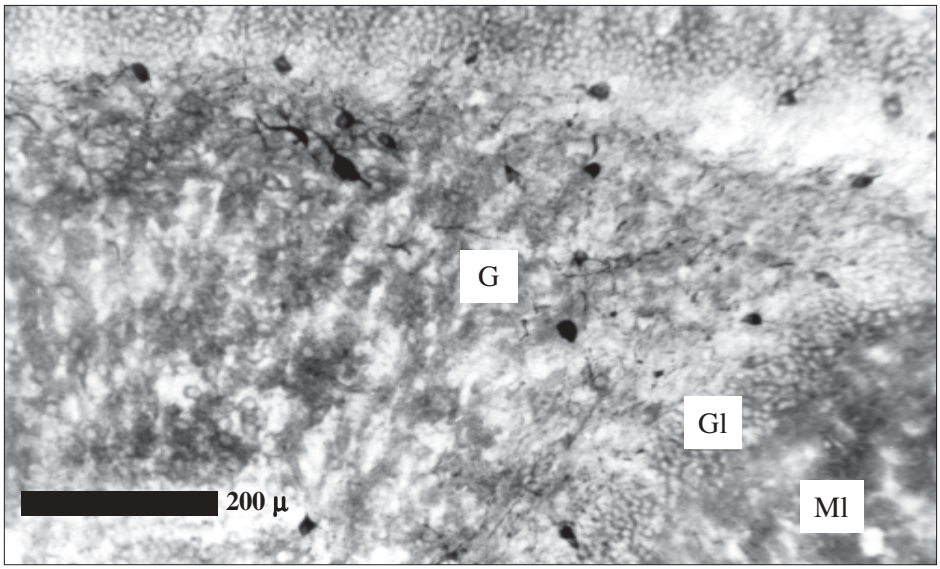

Fig. 1. Effect of kainate on NADPH-diaphorase in rat fascia dentata. Single large multipolar cells are responsive to kainate. Ml, molecular layer; Gl, granular layer; G, gate (*hilus*).

rabbit IgG (1:200, Vector Laboratories) for 1 h at room temperature and then with avidin-biotinylated horseradish peroxidase complex (1:100 Vectastain Elite ABC kit, Vector) for 1 h at room temperature. After incubation, the sections were washed in phosphate buffer and placed for 5-10 min into a medium containing 0.05% solution of 3,3'-tetrahydrochloride diaminobenzidine (Sigma) on phosphate buffer and 0.01% H_2O_2. Then the sections were thoroughly washed in phosphate buffer, mounted on slides, dehydrated, and covered with balsam.

The preparations were examined under a videosystem coupled to computer and to a Vickers M-85 microdensitometer. NADPH-d activity was evaluated by the density of histochemical precipitate. The data were processed statistically using Student's *t* test.

RESULTS

The study of activity of NADPH-d and immunoreactive calcium-binding proteins revealed ambiguous reaction of different neurons to kainate infusion (Table 1). On day 2 after kainate infusion, the number and density of NADPH-d-positive neurons in the hippocampal formation decreased by 55-79% compared to control sections. In kainate-treated rats NADPH-d-positive neurons were sparse. The upper region of the hippocampus was characterized by lower enzyme activity compared to the lower region. The maximum activity of the enzyme was focused in supra- and subpyramidal layers of the hippocampus, and in the subgranular and polymorphous layers of fascia dentata. Solitary NADPH-d-positive neurons were observed in *alveus* and *fimbria*.

Dense clusters of NADPH-d-positive neurons characteristic of infragranular level of the fascia dentata in the control rats were not detected on the experimental sections. The granular and molecular layers also demonstrated pronounced decrease in the number of positive-stained neurons (Fig. 1).

Reduction of the number of NADPH-d-positive neurons correlated with the duration and severity of seizures. The intensity of diaphorase staining of the neuropil in the hippocampus and fascia dentata increased, while after injection of L-NNA this parameter decreased. Changes in NADPH-d activity in *hilus* and

TABLE 1. Effect of Kainate-Induced Epilepsy on Distribution of NADPH-d and Calcium-Binding Proteins in Rat Hippocampal Formation

Marker	Neurons of *hilus*	Interneurons of granular layer of fascia dentata	CA1 pyramidal neurons	CA3 pyramidal neurons	CA1-CA3 interneurons
NADPH-d	↓	↓	↑	↑	↓
CR	↓	↓	—	—	↓
CB	—	↓	↓	↓	↓
PA	—	↓	—	—	↓

Note. The arrows ↓ and ↑ mark decrease and increase in the number of stained cells, respectively; "—" means "no marker".

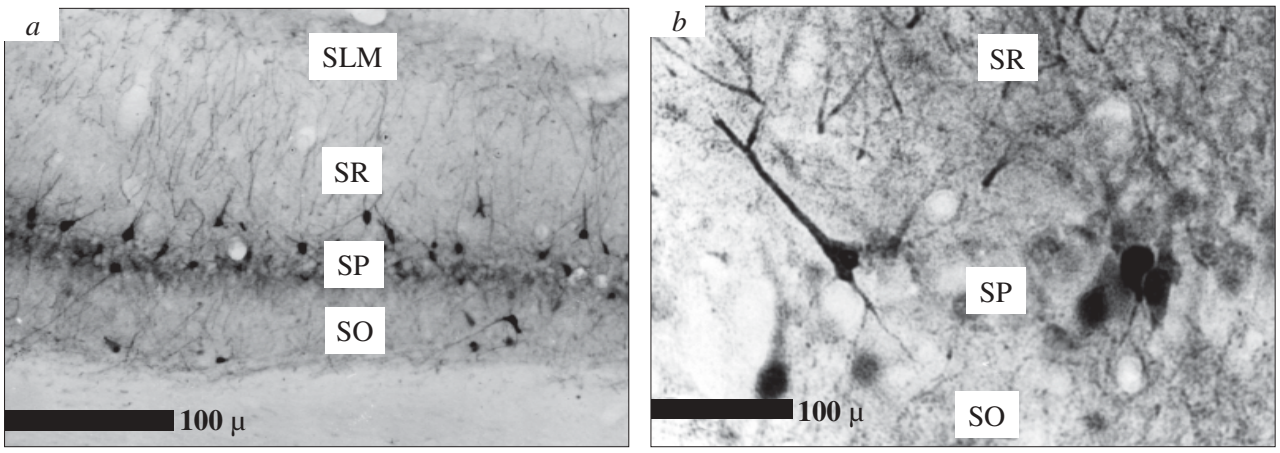

Fig. 2. Effect of kainate on calcium-binding proteins in rat hippocampal neurons. *a*) parvalbumin-immunoreactive interneurons in CA3 field; *b*) reduction of calbindin-containing neurons in stratum pyramidale (SP) of CA1 field by kainate. SLM, *stratum lacunosum-moleculare*; SR, *stratum radiatum*; SO, *stratum oriens*.

CA1 and CA3 neurons were similar (Table 2). The decrease in the number of NO-ergic interneurons was paralleled by the appearance of NADPH-d activity in the cytoplasm of CA1 and CA3 pyramidal cells. They were detected by the presence of large dark-blue diformazan granules in the apical processes of the cells.

NADPH-d-positive cells of the hippocampal formation are inhibitory neurons expressing CR, CB, and PA. The maximum density of PA-immunopositive neurons was found in CA3 and CA2 fields and in the fascia dentata (Fig. 2, *a*). PA was absent in CA1 field. Under conditions of experimental epilepsy, all PA-immunoreactive cells demonstrated high tolerance to the cytotoxic effects in contrast to CB-positive neurons, whose number decreased by 47.3% (Fig. 2, *b*). In the fascia dentata we also found a pronounced decrease in the density of CR-interneurons in the gate region. The neurochemical heterogeneity of neurons determines uneven neuronal damage in different subdivisions of the hippocampal formation. Among projection cells, CA3 pyramidal neurons were most tolerant, CA2 cells were less resistant, and CB-immunoreactive granular cells and CA1 pyramidal cells were most susceptible to kainane kindling. However, interneurons expressing CR demonstrated the least resistance to the cytotoxic effects of kainic hyperexcitation.

The seizure syndrome, provoked by intracerebroventricular infusion of kainic acid, develops by the mechanism of experimental kindling-epilepsy, which changes plastic properties of synapses in the neuronal networks and activity of the corresponding receptors [11,12]. According to our observations, injection of L-NNA at the peak of the epileptic attack moderates convulsions, but does not eliminate them. This fact makes it possible to consider NO not only as a factor provoking seizures, but also as an endogenous anticonvulsant. It can be hypothesized that the character of effector action of NO is determined by the degree of excitations of neurons, the state of Ca^{2+} channels coupled to glutamate receptors, and by corresponding activity of neuronal NOS isoform [6,7].

Injection of kainate into hippocampal formation caused death of some cells in the gate region and some pyramidal neurons in the CA3 field. That is why excitation of CA1 field pyramidal cells attains a critical level, where it cannot be counterbalanced by the inhibitory mechanisms. Reduction of the inhibitory protection of principal cells provokes the appearance of

TABLE 2. Effect of Intracerebroventricular Injection of Kainate on NADPH-d Activity in Hippocampal Formation

Structure	Control	Kainate	L-NNA
Neurons *hilus*	47.0±1.2	57.0±3.3	45.0±1.1*
Interneurons of granular layer of fascia dentata	31.0±2.3	36.0±2.3	24.0±2.4*
Stratum pyramidale CA1	71.0±1.3	88.0±2.3	64.0±3.1*
Stratum pyramidale CA3	65.0±1.1	75.0±2.1	67.0±2.2*
CA1-CA3 interneurons	38.0±2.1	42.0±2.1	34.0±2.7*

Note. Activity is given in optical density units. *$p<0.05$ compared to kainate.

local hyperexcitation foci, which leads to neurodestructive consequences [1,8].

Thus, the established non-uniform lesion to inhibitory NO-ergic interneurons during epileptic status is underlain by their neurochemical and mediator heterogeneity. During experimental epilepsy, NO exerts opposite effects on target cells, which relate to its cytotoxic proconvulsant and neuroprotective anticonvulsant action.

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