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Effect of septal kindling on glutamate binding and calcium/calmodulin-dependent phosphorylation in a postsynaptic density fraction isolated from rat cerebral cortex

(*N*-methyl-D-aspartate acid receptors/γ-aminobutyric acid receptors/synaptic cytology)

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ABSTRACT **Postsynaptic density (PSD) fractions were isolated from the cerebral cortices of control and kindled rats and assayed for glutamate and γ-aminobutyric acid-binding capacities and for the Ca^{2+}/calmodulin-dependent protein kinase. Glutamate binding was found to be increased by ≈50% in the PSDs isolated from kindled rats as compared to controls; this increase was almost completely from an increase in B_{max}; K_d decreased only slightly. Studies with inhibitors indicate that the receptors involved were of the *N*-methyl-D-aspartate and quisqualate types. PSDs isolated from control and kindled rats did not differ in γ-aminobutyric acid or flunitrazepam binding. The *in vitro* autophosphorylation of the Ca^{2+}/calmodulin-dependent protein kinase was depressed by 45-76% in PSDs isolated from kindled rats as compared to controls, with little change in amount of the kinase. Therefore, we infer that (*i*) the kindled state is associated with an increase in glutamate activation of postsynaptic sites, allowing Ca^{2+} to enter dendritic spines, (*ii*) a change has occurred in activity of the protein kinase, which is the major cerebral cortex PSD protein, and (*iii*) perhaps major alterations in the PSD are a concomitant to the long-lasting nature of the kindled state.**

Kindling is the term coined by Goddard (1) to describe the progressive development, in response to initially subthreshold electrical stimulation of specific brain sites, of epileptic seizures that progressively spread and increase in intensity. Progression of the kindling phenomenon is characterized by increases in after-discharge duration and amplitude (2-5), a spread to secondary sites (5, 6), and decreases in threshold in distant cortical sites (2). Kindling shows remarkable temporal and spatial specificity as seen in many species from frog (7) to subhuman primates (8) and, once established, may persist without further stimulation for the life of the animal (9). After sufficient training, some animals develop spontaneous seizures (10). Strong pharmacological evidence indicates that this phenomenon is generated through stimulation of identifiable synaptic populations (11). Kindling by stimulation of the medial septal nuclei is associated with a decrease in the activity of the enzyme calmodulin kinase II in cortex and hippocampus (12, 13). However, calmodulin kinase II is a ubiquitous enzyme in brain. It is present in the presynaptic apparatus (14) where it may regulate transmitter biosynthesis by phosphorylation of 5-tyrosine and 5-tryptophan hydroxylases (15) and may regulate transmitter release via synapsin I phosphorylation (16). The enzymatic activity is also present in large quantities postsynaptically, particularly in postsynaptic densities (17), and calmodulin kinase II appears identical to the major postsynaptic density (PSD) protein (18-20). The goal of our study was to investigate whether any biochemical changes in isolated PSDs, such as neurotransmitter binding and phosphorylations, were associated with septal kindling.

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METHODS

Kindling. Male Sprague-Dawley rats were stereotaxically implanted at the medial septal nuclei; the bipolar twisted stainless steel electrodes were located close to midline so that both hippocampi showed after-discharges. After 2 weeks of being handled daily, experimental animals received three daily stimulations (400 μA for 1 sec at 50 Hz) 3 hr apart for 5 days per week through the septal electrode. Kindling criteria included five stage-V seizures, three of them consecutive. Kindled rats were tested for 2 weeks before sacrifice by decapitation without anesthesia. Previous experiments have shown that rest periods of up to 2 months do not reduce either the kindled seizures or the change in protein phosphorylation associated with it (12). Cortices were dissected by free hand in Los Angeles, frozen on dry ice, and shipped to New York.

Isolation of PSD. PSDs were isolated from five to six pooled cortices, as described (21), and checked for purity by electron microscopy, as described (22). Electron microscopic examination of cerebral cortex slices was by the same method (22).

Other Techniques. Phosphorylation was done by using 50 μg of PSD fraction and described methods (21, 23). Electrophoresis and autoradiography were performed as described (22, 23). Bands from dried gels were excised by cutting and soaked in Biofluor (4 ml per band); radioactivity was counted in a Beckman LS-180 scintillation counter. The bindings of L-[^{3}H]glutamate (24) and of [^{3}H]γ-aminobutyric acid (GABA) and [^{3}H]flunitrazepam (25) were assayed by described procedures. Samples were soaked in 4 ml of Hydrofluor each and were counted in a Beckman LS-180 scintillation counter. When used, calmodulin (CaM) was pretreated to remove Ca^{2+} (21). Scatchard binding plots were obtained by the procedure of Rosenthal (26), and protein concentrations were estimated by the method of Lowry *et al.* (27) with bovine serum albumin as standard.

MATERIALS

L-[2,3-^{3}H]Glutamate (25 Ci/mmol; 1 Ci = 37 GBq), [γ-^{32}P]ATP (3000 Ci/mmol) and [*methyl*-^{3}H]flunitrazepam (77 Ci/mmol) were obtained from New England Nuclear, and [^{3}H]GABA (54 Ci/mmol) was from Amersham. L-Glutamate, GABA, *N*-methyl-D-aspartate (NMDA), quisqualate, DL-

Abbreviations: NMDA, *N*-methyl-D-aspartate; GABA, γ-aminobutyric acid; DL-AP4, DL-2-amino-4-phosphonobutyrate; DL-AP5, DL-2-amino-5-phosphonovalerate; CaM, calmodulin; PSD, postsynaptic density.

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2-amino-4-phosphonobutyrate (DL-AP4), DL-2-amino-5-phosphonovalerate (DL-AP5), and diazepam were all obtained from Sigma.

RESULTS

Cytology and Chemistry. Cerebral cortex slices from control and kindled rat brains were examined by electron microscopy, and the number of synapses per field was counted at 10,000 magnification. In 17 fields from each group, number of synapses was 5.4 per field in the control and 5.6 per field in the kindled rat. Also, the two groups did not differ in length and thickness of the PSD or in number of synaptic vesicles in the axon terminals. The isolated PSD fraction from the control and kindled rat cortices were morphologically similar to each other and resembled those obtained from canine cerebral cortex (28) or from canine hippocampus (22). SDS gels of the PSD fractions gave identical profiles of the proteins of PSDs from control and kindled rats.

Ca^{2+}/CaM-Dependent Phosphorylation. Kindling has been shown to reduce phosphorylation of a 50-kDa protein in hippocampal synaptic membrane preparation (29) and of 50-kDa and 58- to 60-kDa proteins in septal-kindled rats by using a *post hoc* assay (12). These two proteins were further identified as the α and β subunits of a Ca^{2+}/CaM-dependent protein kinase II (13). PSDs isolated from cerebral cortex gave similar results. Fig. 1 shows that the *in vitro* Ca^{2+}/CaM-stimulatable phosphorylation of the 51-kDa and the 60-kDa proteins were depressed in the isolated PSD fraction from cerebral cortex of kindled rats as compared to those from control animals. When these bands were excised and counted (Table 1), depression of the Ca^{2+}/CaM-stimulated ^{32}P-incorporation into 50-kDa and 60-kDa bands was from 40–45%; little change was seen in basal phosphorylation. In another experiment, when using PSDs prepared from another set of control and kindled animals, depression ranged from 64–76% (data not shown). Little difference was observed by gel electrophoresis in amounts of the 51- and 60-kDa proteins in PSDs from control and kindled rats (data not shown). Therefore, the marked change in autophosphorylation was not from the much smaller change in amounts of protein. Also noted in the figure is a decrease after kindling in the phosphorylations of two high-molecular-mass (180 and 200 kDa)

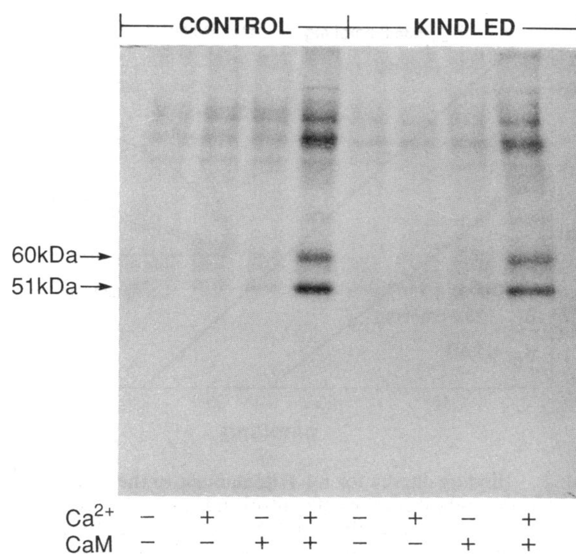

FIG. 1. Ca^{2+}/CaM-dependent phosphorylation of PSD fractions from cerebral cortices of control and kindled adult rats. Fifty micrograms of PSD protein was used for each slot. Additions to the incubation mixture are indicated, where [Ca^{2+}] equals 0.5 mM, and 1.5 μg of CaM is the amount added.

Table 1. Effect of septal kindling on Ca^{2+}/CaM-dependent phosphorylation in PSD fractions

	Phosphorylation, cpm			
	Control		Kindled	
Addition(s)	51 kDa	60 kDa	51 kDa	60 kDa
None (baseline)	77	36	72	33
Ca^{2+}	86	45	96	46
CaM	80	38	73	36
Ca^{2+}/CaM	728	315	430	206
Ca^{2+}/CaM minus baseline	651	279	358	173

The 51-kDa and 60-kDa bands of Fig. 1 were excised from the gel and counted.

PSD proteins; the same decrease after kindling was found earlier (30) for glycoproteins with similar molecular weights from hippocampal and cerebral cortex membranes.

Binding of L-[^{3}H]Glutamate. We next examined, by binding studies, the binding of some neurotransmitters that may be involved in the kindling phenomenon (cf. refs. 31–33). Table 2 shows that at two concentrations of glutamate the binding of this neurotransmitter to PSD fraction was increased by ≈50% in the kindled state as compared to control, whereas no change was seen in the binding of GABA or flunitrazepam. Similar results were obtained by using PSDs obtained from another set of control and kindled rats. When we examined the type of glutamate receptor present in these PSD fractions, it was inferred from the data in Table 3 that our preparation contained a mixture of quisqualate and NMDA types. The inhibition by DL-AP5, but not by DL-AP4, indicated an NMDA type of receptor (34, 35), a situation similar to that previously seen with PSD fractions from canine cerebral cortex and hippocampus (22). Furthermore, no difference was seen in the degree of inhibition between the PSD fractions isolated from the cortex of control and kindled rats (Table 3). To account for the increase in the binding of glutamate in the PSDs from kindled rat, we did concentration curves of the binding, Scatchard plots, and calculated B_{max} and K_d for each. Fig. 2 shows the data from a representative experiment, indicating a slight decrease in K_d and a large increase in B_{max} in the PSD fractions from kindled rats as compared to control ones. This experiment was repeated two more times; B_{max} and K_d from the control rats were 25.8, 23.9, and 22.8 pmol/mg, and 427, 456, and 443 nM, respectively, whereas B_{max} and K_d for the kindled animals were 36.8, 36.8, and 33.9 pmol/mg and 369, 371, and 379 nM, respectively. Averaging these values showed that although the K_d of kindled preparations decreased by 16% compared to control, the B_{max} of kindled preparations increased by 48%, indicating that the kindled state was associated with a large increase in the apparent number of glutamate-binding sites and only a small increase in their affinity for glutamate.

DISCUSSION

Because evidence indicates that kindling involves a trans-synaptic mechanism, we examined properties of PSDs. To obtain sufficient quantities of PSD material, we used cerebral cortex, which undergoes changes very similar to those described in hippocampus during kindling (5, 6, 12).

We examined the receptors for glutamate and GABA because of the many reports of the involvement of those neurotransmitters in kindling (cf. refs. 36–39). Thus, in studies with hippocampal slices, stimulation resulted in an increase in glutamate binding (40) from an apparent increase in glutamate receptors (41). Cytological (42) and electrophysiological experiments (43–48) clearly indicated an involvement of NMDA-type glutamate receptors in kindling. A

Table 2. Specific binding of L-[^{3}H]glutamate, [^{3}H]GABA, and [^{3}H]flunitrazepam to PSD fractions isolated from cerebral cortices of kindled and control rat brains

	Specific binding, % control					
	L-[^{3}H]glutamate		[^{3}H]GABA		[^{3}H]Flunitrazepam	
	50 nM	2 μM	50 nM	2 μM	5 nM	1 μM
Control	100	100	100	100	100	100
Kindled	156	142	91	83	94	88

Cerebral cortex PSD fractions (25 μg per assay) were incubated with L-[^{3}H]glutamate, [^{3}H]GABA, and [^{3}H]flunitrazepam at two concentrations each and processed for binding. Specific binding was obtained from the difference between total and nonspecific binding, the latter being defined as amount of label bound in the presence of 0.5 mM unlabeled L-glutamate for labeled L-glutamate, 100 μM unlabeled GABA for labeled GABA, or 100 μM diazepam for labeled flunitrazepam. Results are the averages of duplicate determinations that varied by <10%.

recent report (49) showed that kindling in a hippocampal membrane preparation increased the number of binding sites of the NMDA-type receptor with little change in affinity. Our current results amplify these earlier experiments and further indicate that in PSDs from cerebral cortex, septal kindling is associated with an increase in glutamate-binding sites of the NMDA and quisqualate types. Because hippocampal stimulation has been postulated (40) to increase the activity of a thiol protease, calpain, which could uncover glutamate receptors through proteolysis, we examined our PSD protein gels for evidence of proteolysis—particularly of the postulated main substrate of calpain, fodrin; we failed to detect any difference between kindled and control rats. Therefore, the observed increase in ligand binding is probably not from an "uncovering" of binding sites in PSDs.

We also found no change in GABA or flunitrazepam binding to the PSD fraction from cerebral cortex in our kindled animals. Previous reports detected an increase in benzodiazepine-binding sites (50–53) and either an increase (51) or no change (50) in GABA-binding sites after kindling, but some of these changes were transient; different results were obtained with washed or unwashed membrane preparations (54), and the pharmacological effects of GABA antagonists on the kindled state were equivocal (55).

Our present study indicates that in animals subjected to septal kindling, autophosphorylation of the Ca^{2+}/CaM-dependent protein kinase in cortical PSDs decreases. A similar change was reported in a synaptic plasma membrane fraction (12, 29), but our study indicates an alteration postsynaptically in PSDs. The alteration in the autophosphorylation of the kinase could lead to change in the structure of spine head and neck because calmodulin kinase is the major protein in both cortical and hippocampal PSDs (22) and probably has a structural role. Structural changes in the PSD have been suggested (56) to regulate postsynaptic responses and to be necessary for long-lasting effects on neurotransmission; the kindled state could be one of these effects. The mechanism of decrease in calmodulin kinase II activity is not clear. Possibly the enzymatic activity is lowered because there is no concomitant decrease in the density of the corresponding protein band. Another explanation is that *in vivo* the enzyme is phosphorylated to a greater degree in the kindled than in the control animal—thus, less phosphate could be added *in vitro*. In this context, autophosphorylation has been shown to render the enzyme less dependent on Ca^{2+}/CaM (57–64), thus raising the baseline Ca^{2+}/CaM-independent activity and maintaining this high activity for a period far beyond that of the transient increase in free intracellular calcium that triggered autophosphorylation. This mechanism has been postulated for long-term storage of information by the kinase in the PSD (65). However, in our kindled animals the enzyme activity in PSD still greatly

Table 3. Effect of quisqualate, NMDA, DL-AP4, and DL-AP5 on specific binding of L-[^{3}H]glutamate to PSD fractions isolated from cerebral cortices of control and kindled adult rats

Compound	Conc., μM	Inhibition of specific binding of L-[^{3}H]glutamate, % Kindled	Control
Quisqualate	0.5	10	15
	5.0	51	ND
	1000.0	68	63
NMDA	2.0	25	30
	20.0	58	ND
	1000.0	71	61
DL-AP4	0.5	2	ND
	100.0	6	9
	1000.0	11	ND
DL-AP5	0.5	23	17
	100.0	51	59
	1000.0	62	ND

PSD fractions (25 μg/250 μl) in 50 mM Tris chloride, pH 7.4, were incubated at 30°C for 10 min. The mixtures were incubated further at the same temperature for 20 min with 0.1 μM L-[^{3}H]glutamate with and without 5 mM L-glutamate and in the presence and absence of various concentrations (Conc.) of quisqualate, NMDA, DL-AP4, or DL-AP5. Specific bindings of radiolabeled L-glutamate were determined as described. Results are averages of duplicate determinations that varied <10% from each other. ND, not determined.

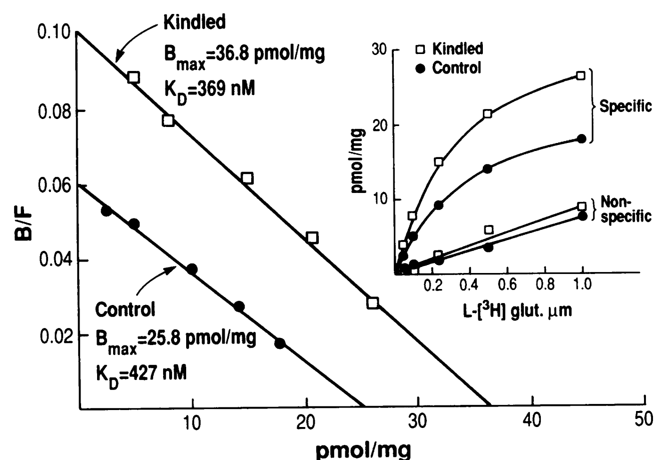

FIG. 2. Binding curves for L-[^{3}H]glutamate to the PSD fractions isolated from cerebral cortices of control and kindled adult rats. Each assay contained 25 μg of PSD in 125 μl of assay buffer. Binding curves and binding parameters were obtained as outlined in text. Nonradioactive L-glutamate (at 1 mM) was used for nonspecific binding; values were subtracted from total binding to give specific binding. B_{max} and K_d were calculated from Scatchard plots. Results are averages of two separate determinations that varied by <10% from each other. (*Inset*) Saturation isotherms. B, bound; F, free; glut., glutamate.

depended on Ca^{2+} plus CaM (Fig. 1 and Table 1). This result, coupled with the lack of increase in Ca^{2+}/CaM-independent activity, makes it unlikely that the changes seen were from increased enzyme autophosphorylation *in vivo*.

The reported increase (66) of a 45-kDa membrane protein after amygdaloid kindling was not found in our work, but both type of kindling and type of membrane preparation assayed differed. Gispen and collaborators (67–69) showed that tetanic stimulation changes phosphorylation in 50- and 52-kDa proteins, but these proteins clearly differ from the calmodulin kinase assayed here in PSDs (12, 13, 29).

Our investigation of the fine morphology of PSDs showed no changes associated with kindling, but this limited study does not invalidate previous results of a swelling of dendritic spines associated with hippocampal stimulation (70–72), of stimulation-associated increases in number of boutons onto dendritic shafts, but not onto spines (73, 74), and of changes in the spine-head morphology (74, 75). However, two other groups (76, 77) found no significant structural changes in the affected areas of hippocampus between control and kindled animals. The selective loss of nonperforated PSDs seen in hippocampal kindling by Geinisman *et al.* (78–80), increasing the ratio of perforated to nonperforated PSDs, is quite compatible with our finding of increased glutamate-binding sites. PSDs from cortex (24, 81) and hippocampus (22) contain glutamate receptors, the activation of which involves the opening of Na^+ and Ca^{2+} channels in the postsynaptic membrane (82–84). The increase in perforated PSDs in kindling (78–80) could reflect an increase in receptor numbers (Table 2) because we postulated (85) that the occurrence of perforation in PSDs is an indication of its division, generating two postsynaptic areas and increasing availability of receptor sites.

The immunocytochemical distribution of NMDA receptors and of calmodulin kinase II in the hippocampus (86, 87) is quite similar; yet, in previous studies of PSD fractions from cortex (24) and hippocampus (22), no evidence was obtained of a direct link between the two occurrences because prior phosphorylation by calmodulin kinase had no effect on subsequent glutamate binding and prior glutamate binding had no effect on subsequent Ca^{2+}/CaM-dependent phosphorylation. Yet both changes could modify neuronal excitability and, therefore, may be intimately involved in the kindling process.

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