Kindling alters the calcium/calmodulin-dependent phosphorylation of synaptic plasma membrane proteins in rat hippocampus
(epilepsy/plasticity/phosphoproteins/protein kinase)

Claude G. Wasterlain*† and Debra B. Farber§

*Epilepsy Research Laboratory, Sepulveda Veterans Administration Medical Center, Sepulveda, CA 91343; and †Brain Research Institute and Departments of *Neurology and §Ophthalmology, University of California, Los Angeles, School of Medicine, Los Angeles, CA 90024

Communicated by H. W. Magoun, November 14, 1983

ABSTRACT Septal kindling was associated with an inhibition of the post hoc phosphorylation of several synaptic plasma membrane proteins of rat hippocampus. In control rats, the [32P]P incorporation into proteins of molecular weights 50,000, 58,000, and 60,000 was markedly stimulated by combined calcium/calmodulin, whereas in kindled animals, the response to combined calcium/calmodulin was reduced. Calcium alone, cAMP, or cGMP modulated [32P]P incorporation into several synaptic plasma membrane proteins but did not differentiate control from kindled tissues. Both control and kindled rats showed nonspecific inhibition of calcium/calmodulin-stimulated phosphorylation in the post hoc assay by corticosteroid and by [Leu]enkephalin. The differences between control and kindled animals were most striking in hippocampus and in the amygdaloid-entorhinal area; less pronounced in cortex, basal ganglia, and brain stem; and not significant in cerebellum, a region where kindling cannot be elicited. An 8-wk period of rest after kindling did not reduce these changes, suggesting that they may be as persistent as the kindling behavior itself.

Combined calcium and calmodulin stimulate the phosphorylation of many brain proteins, and several recent studies suggest that this process plays an important role in central synaptic neurotransmission (1-5). The state of phosphorylation of synaptic vesicle membrane proteins modulates the amount of several neurotransmitters released from these vesicles in vitro (6). Some anticonvulsants inhibit the calcium/calmodulin-dependent phosphorylation of synaptic vesicle proteins, and this inhibition may mediate their effects on synaptic function (7). Kindling is a model of epilepsy in which repeated stimulation of some brain sites with small amounts of electrical current (8) or of agonists of excitatory neurotransmitters (9) results in a progressive buildup of response, culminating in full-fledged epileptic seizures upon every stimulation. Kindling can be most easily obtained from the limbic brain but cannot be elicited from some brain regions such as cerebellum, no matter how many times stimulation is repeated. Once established, this phenomenon is persistent and may last a lifetime. Recent studies indicate that it occurs transynaptically, so that kindling induced by carbachol or muscarine is completely prevented by blockage of muscarinic synapses in the area of stimulation (9). Extensive histological and ultrastructural investigations have disclosed no morphological change associated with kindling. A number of modifications in synaptic biochemistry have been reported in kindled animals, but most of them are too short-lived to account for the lasting change in behavior. For example, β-adrenergic and muscarinic receptors undergo transient decreases (10-12), the number of benzodiazepine binding sites may be reduced (13), and some enzymes involved in neurotransmitter metabolism may be affected (14-16).

We recently reported that kindling by medial septum stimulation was associated with a highly significant, long-lasting inhibition of the phosphorylation of hippocampal synaptic plasma membrane proteins in vitro (17). This phosphorylation was unaffected by cAMP, cGMP, or calcium alone. We now report that (i) the calcium/calmodulin-enhanced phosphorylation of some synaptic plasma membrane proteins is markedly reduced in some brain regions of kindled rats, (ii) this reduction is most marked in brain areas most susceptible to kindling and absent in some regions incapable of it, and (iii) this difference persists for weeks in the absence of any further electrical stimulation of the animals.

METHODS

Male Holtzman rats were stereotaxically implanted into the medial septal area with bipolar twisted stainless steel electrodes. Some animals received both septal and dorsal hippocampal electrodes and were used for electrographic recording only. Septal electrodes were located close to the midline, so that both hippocampi showed after discharges. After 2 wk of daily handling, experimental animals received three stimulations (400 μA for 1 sec at 60 Hz) 3 hr apart, 5 days per week, through the septal electrode. Controls were handled in a similar fashion but received sham stimulation. Full kindling was defined as three consecutive stage 5 seizures. Kindled rats were rested for 2 wk before sacrifice by decapitation without anesthesia. Previous experiments have shown that rest periods up to 2 months do not reduce either the kindled seizures or the change in protein phosphorylation associated with it (17).

Brains were rapidly chilled in ice-cold 0.32 M sucrose, and cortex, hippocampus, brain stem, and cerebellum were dissected by the method of Glowinski and Iversen (18). Thalamus, caudate-putamen, and hypothalamus were kept as a block and called "basal ganglia." Spinal cords also were dissected from some animals. Synaptic plasma membranes were obtained from each brain region by the method of DeRobertis (19), with minor modifications. The phosphorylation assay was carried out in vitro with [γ-32P]ATP (specific activity, 2.4 Ci/μmol; 1 Ci = 37 GBq) as described (17). In a volume of 75 μl, final concentrations were: Tris-HCl, 20 mM (pH 7.4); 500 μM EGTA; 10 μM ATP; 10 mM MgCl2; and, if needed, calmodulin and CaCl2, 3 units and 600 μM, respectively. The reaction was stopped by the addition of NaDodSO4 and mercaptoethanol (final concentration, 4% each). Phosphorylated proteins were separated by electrophoresis on 15% polyacrylamide gels. The following molecular weight standards were used: phosphorylase B, 94,000; bovine serum albumin, 67,000; ovalbumin, 45,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,000; ribonuclease, 14,400. Gels were stained with Coomassie blue, de- stained, dried, and exposed to DuPont Cronex 6+/x-ray film for autoradiography, using intensification screens.
Quantitation of the amount of incorporated $^{32}$P was done by spectrophotometric scanning of the autoradiogram. Comparisons were made only between samples of the same gel. Ratios of $^{32}$P incorporation between controls and experimental animals were calculated from the ratios of optical density above background of the respective peaks of the spectrophotometric scannings.

RESULTS

Behavioral and Electrographic Responses. No seizures were observed on the first stimulation. A prominent hippocampal afterdischarge was recorded after the first stimulation in every animal (Fig. 1). A septal afterdischarge was also seen in the majority of rats. Subsequent stimulations produced a progressive growth in duration and complexity of both afterdischarges, which became biphasic, as wet-dog shakes appeared, then shortened as behavioral seizures began to develop. Seizures were very similar to those induced by amygdaloid kindling and were classified according to Racine (20).

Biochemical Effects. The patterns of hippocampal protein bands separated by NaDodSO$_4$/PAGE, as visualized with Coomassie blue staining, were similar in control and kindled rats. Autoradiograms showed phosphorylation of a large number of proteins. $^{32}$P incorporation into some bands was stimulated by cAMP, into others by cGMP, but neither situation showed a difference between kindled and control animals. $^{32}$P incorporation into synaptic plasma membrane proteins in both control and kindled rats was unaltered by the addition to the phosphorylation medium of calcium concentrations ranging from 3 $\mu$M to 1 mM. Concentrations above 1 mM were inhibitory. Control and kindled tissues did not differ. Addition of calmodulin alone (3 units) or of calcium (0.6 mM), calmodulin (3 units), and EGTA (1 mM) did not alter protein phosphorylation. However, addition of calcium (0.1 mM)/calmodulin (3 units) markedly stimulated incorporation of $^{32}$P into synaptic plasma membrane proteins of approximate molecular weights 50,000, 58,000, and 60,000 (Fig. 2). This stimulation was much less effective in membranes of kindled animals than in controls (Table 1 and Fig. 2). Phosphorylation of a protein of molecular weight 80,000 and of three proteins of molecular weight higher than 100,000 was also enhanced by calcium/calmodulin, but the decrease in $^{32}$P incorporation into these proteins in kindled rats was not significant (Fig. 2).

Studies of the time course of $^{32}$P incorporation in the pres-
Different from

Ca2+/Cm/

105 Ca2+

after similar periods

tact

100 100 100 100

mals and three paired

the

behavioral

to

Table

1. Effects of kindling on \(^{32}\)P incorporation in hippocampal synaptic plasma membrane proteins

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Synaptic plasma membrane proteins, % of baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(M_i 58,000-60,000)</td>
</tr>
<tr>
<td>Baseline</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Kindled</td>
</tr>
<tr>
<td>Ca(^{2+}) (100 (\mu M))</td>
<td>149 ± 42</td>
</tr>
<tr>
<td>Cm</td>
<td>105 ± 27</td>
</tr>
<tr>
<td>Ca(^{2+}/Cm)</td>
<td>1106 ± 216</td>
</tr>
<tr>
<td>Ca(^{2+}/Cm) / EGTA</td>
<td>82 ± 13</td>
</tr>
</tbody>
</table>

Values represent means ± SEM; \(n = 3\) except for Ca\(^{2+}/Cm\) (Cm), where \(n = 6\) (controls) and 8 (kindled).

* Different from controls, \(P < 0.02\).

Different from controls, \(P < 0.01\).

Fig 2. Autoradiograms of hippocampal synaptic plasma membrane phosphoproteins of control and kindled rats separated by NaDodSO\(_4\)/PAGE. The approximate molecular weights of the phosphoproteins most prominently altered in kindling are shown \(\times 10^{-3}\). Baseline conditions of phosphorylation included 1 mM Mg\(^{2+}\) and 10 \(\mu M\) \(^{32}\)P-ATP; 0.1 mM calcium chloride and 3 units of calmodulin (Cm) were added to stimulate \(^{32}\)P incorporation.

ence of calcium/calmodulin indicated that it was nearly maximal at 15 sec and maximal at 30 sec and remained at the same level after incubation times of 1, 2, and 3 min. \(^{32}\)P incorporation was decreased by 13% at 5 min and by 28% at 10 min. Calcium/calmodulin stimulation was maximal with low concentrations (3 \(\mu M\)) of calcium ions in the phosphorylation medium. Additions of calcium chloride and EGTA, yielding free calcium concentrations ranging between 3 \(\mu M\) and 500 \(\mu M\), were equally effective in both control and kindled tissues.

Changes in protein phosphorylation patterns and response to calcium/calmodulin stimulation were observed in rats that did not receive electrical stimulation for 2 months after the establishment of kindling. Previous studies had shown that the behavioral manifestations of kindling persisted nearly intact after similar periods of rest (8). Three fully kindled animals and three paired controls were rested for 8 wk. The reduction of calcium/calmodulin-stimulated \(^{32}\)P incorporation into hippocampal proteins of molecular weights 50,000, 58,000, and 60,000 observed in Fig. 2 for the kindled rats was also present after this longer rest. Expressed as the percentage of baseline control (\(n = 3\)), mean values for three rested kindled rats were 73% and 77%, respectively, for unstimulated phosphorylation of the proteins of molecular weights 58,000–60,000 and 50,000. Calcium/calmodulin stimulation increased \(^{32}\)P incorporation to 93% and to 8% of baseline control for the proteins of molecular weights 58,000–60,000 and 50,000, respectively, in control hippocampi but only to 334% and 357% in kindled hippocampi.

Studies of brain regions revealed differences between kindled and control rats in the phosphorylation pattern of synaptic plasma membrane proteins. These differences were most striking in the hippocampus, which receives its relatively large excitatory muscarinic projection from medial septum and where early and prominent afterdischarges occur, and in the amygdala-entorhinal region. It should be noted that this latter region includes, in addition to amygdaloid nuclei, a large amount of surrounding tissues and overlying cortex. Differences between kindled and control animals were also observed in neocortex, with a pattern of \(^{32}\)P incorporation into proteins similar to that of other regions. Basal ganglia and brain stem showed the kindling effect in the majority of animals. Cerebellum and spinal cord showed no difference between kindled and control animals. The protein of molecular weight 50,000, which is clearly phosphorylated in a calcium/calmodulin-dependent manner in all brain regions, was incorporated \(^{32}\)P in low amounts in cerebellum (Fig. 3). Thus, the most striking effects were seen in areas most prone to kindling, such as the hippocampus and the amygdaloid region, and no effects were observed in regions incapable of it, such as the cerebellum.

Bovine corticotropin inhibited calcium/calmodulin-stimulated \(^{32}\)P incorporation into hippocampal synaptic plasma membrane proteins to the same extent in controls (ID\(_{50} = 8\) units/ml for the proteins of molecular weights 58,000–60,000 and 8.7 units/ml for the protein of molecular weight 50,000) and in kindled rats (ID\(_{90} = 5.6\) units/ml and 8.5 units/ml, respectively). [Leu]Enkcephalin had similar inhibitory effects (ID\(_{50}\) in controls = 10 \(\mu M\) for the proteins of molecular weights 58,000–60,000 and 9 \(\mu M\) for the protein of molecular weight 50,000; ID\(_{50}\) in kindled rats = 8 \(\mu M\) and 10 \(\mu M\), respectively).

Preliminary data indicate that diazepam administered to rats in concentrations sufficient to prevent the development of kindled seizures also blocks the appearance of changes in protein phosphorylation described in this paper. Furthermore, diazepam concentrations effective in vivo (10 \(\mu M\) and more) inhibit calcium/calmodulin-stimulated \(^{32}\)P incorporation into the proteins of molecular weights 50,000, 58,000, and 60,000 in vitro (21).

DISCUSSION

The results described above suggest that several proteins of hippocampal synaptic plasma membranes are phosphorylated by calcium/calmodulin-dependent kinase and, for at least three of those proteins, the stimulation of this process by calcium/calmodulin is reduced in the hippocampi of rats kindled from medial septum as compared to controls. This could modify the basic properties of synaptic membranes in kindled animals, including their response to calcium influx, resulting in a change in excitability, and may therefore, part of the engram of kindling or one of its aftereffects. The reduced response to calcium/calmodulin observed in kindled animals actually could reflect (i) a persistently higher endogenous level of phosphorylation of these proteins in vivo, leaving fewer sites available for phosphorylation in vitro, (ii) a decrease in protein kinase activity or (iii) an increase in phosphoprotein phosphatase activity. Prelimi-
nary studies to identify the proteins affected by kindling suggest that the 50,000, 58,000, and 60,000 molecular weight proteins may be subunits of a calcium/calmodulin-dependent protein kinase. Several proteins of similar molecular weight and properties have been found previously in synaptic plasma membranes. These include bands DPH-M and DPH-L and the subunits of calcium/calmodulin kinase of DeLorenzo (6), the subunits of synapsin I kinase of Kennedy and Greengard (22), the subunits of tubulin, or the B50 protein of Zwiers et al. (23, 24), which has a higher level of phosphorylation in long-term potentiation.

The persistence of the changes in the phosphoproteins associated with kindling is as remarkable as the persistence of the kindling behavior itself. In view of the long period separating epileptic seizures and sacrifice, it is unlikely that the alterations in biochemical response are postictal. It is also unlikely that they are the result of surgical trauma or implantation because, in this model, the electrodes are implanted into the septum at a considerable distance from the hippocampus, where the most striking changes take place and which itself is never surgically traumatized.

The decrease in $^{32}$P incorporation into synaptic plasma membrane proteins after 5 and 10 min of incubation was seen in both kindled and control tissues, suggesting that phosphoprotein phosphatases are active in our preparations. The rapid establishment of a plateau must reflect a balance of kinase and phosphatase activities, with the former predominant at early times. Because the difference in amount of phosphorylation between kindled and control animals is already present at 15 sec, it appears more likely to be due to a modification of substrate or kinase activity than to a change in phosphatase activity.

In kindled rats, changes in calcium/calmodulin-dependent phosphorylation of synaptic plasma membrane proteins were most prominent in the areas most prone to kindling, such as the hippocampus and the amygdaloid region. Cortex, basal ganglia, and brain stem, where moderate inhibition was observed, are large heterogeneous regions that include both kindling-prone and kindling-resistant areas. In the cerebellum, an area that is totally resistant to kindling, no biochemical change was observed, and one of the proteins affected by kindling, the protein of molecular weight 50,000, was present in low concentrations. Therefore, there is a rough parallel between the regional variations in kindling and susceptibility and regional variations in calcium/calmodulin-dependent protein phosphorylation of synaptic plasma membranes.

In summary, our data suggest that kindling is associated with a reduced response of some synaptic plasma membrane proteins to calcium/calmodulin-stimulated phosphorylation as tested in vitro. This effect is remarkable by its persistence, by its close association with the kindling behavior, and by a regional distribution reminiscent of that of the behavioral thresholds themselves.

We are indebted to Ms. Eva Csiszar and Ms. Sheryl Weens for skilful technical help and to Ms. Ethel Mason for assistance in the preparation of the manuscript. This work was supported by the Research Service of the Veterans Administration and by Research Career Development Award 1 KO4 EY 144 to D.B.F. from the National Institutes of Health.