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Modulation of DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/quisqualate receptors by phospholipase A_2: A necessary step in long-term potentiation?

(glutamate/hippocampus)

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ABSTRACT **The effects of kainate (KA)-induced epileptic seizures on the binding properties of hippocampal glutamate receptors, on the modulation of DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/quisqualate receptor by phospholipase A_2 (PLA_2), and on the formation of long-term potentiation (LTP) were studied in hippocampal membranes and hippocampal slices. Systemic administration of KA (10 mg/kg; 15 hr survival) produced specific changes in the binding properties of the AMPA/quisqualate receptors and its regulation. Whereas the binding of various ligands to the *N*-methyl-D-aspartate receptors was not modified by KA treatment, there was a significant decrease in the maximal number of binding sites for [^{3}H]AMPA. In addition, the increase in [^{3}H]AMPA binding elicited by PLA_2 treatment of hippocampal, but not cerebellar, membranes was markedly decreased after KA injection. LTP was also substantially reduced in area CA_1 of hippocampal slices from KA-treated animals. The loss of LTP was not due to changes in postsynaptic responses elicited by the bursts that trigger the potentiation effect, thus suggesting that KA treatment disrupts processes that follow *N*-methyl-D-aspartate receptor activation. Systemic administration of KA was associated with calpain activation as the amount of spectrin breakdown products was increased severalfold in hippocampus but not in cerebellum. Pretreatment of telencephalic membranes with calpain greatly reduced the PLA_2-induced increase in [^{3}H]AMPA binding. The results provide evidence in favor of an essential role of PLA_2 in the development of LTP and suggest that the order of activation of different calcium-dependent processes is critical for producing the final changes underlying LTP.**

Although substantial progress has been made in describing the initial events required for the induction of long-term potentiation (LTP), the steps involved in the translation of the initial trigger into long-lasting changes in synaptic efficacy continue to be a subject of intense controversy (1). In field CA_1 of hippocampus, activation of the *N*-methyl-D-aspartate (NMDA) receptor (2, 3) and an increase in intracellular calcium in postsynaptic structures (4, 5) are critical in producing the selective increase in the synaptic currents mediated by the DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/quisqualate subtype of glutamate receptors that maintain LTP (6–8). Thus, it is reasonable to propose that expression of LTP is due to the activation of one or several calcium-dependent processes that modify the properties of the AMPA/quisqualate receptors. The duration of hippocampal LTP (weeks to months) indicates that the critical calcium-dependent processes produce very long-lasting changes in synaptic function. Among these processes, calcium-dependent lipases and proteases are particularly attractive candidates since the modifications produced by these enzymes are more likely to be very long-lasting. The involvement of calcium-dependent proteases in LTP has been extensively documented (9). Several arguments indicate that phospholipase A_2 (PLA_2) might also play an important role in LTP. Stimulation of NMDA receptors causes an increased release of arachidonic acid, probably as a result of the activation of endogenous PLA_2 (10), whereas inhibitors of this enzyme block the formation of LTP (11–14). Moreover, treatment of telencephalic membranes with exogenous phospholipases increases the affinity of the AMPA/quisqualate receptor for the radioligand [^{3}H]AMPA, without changing the binding properties of agonists for the NMDA receptor complex (15, 16). If several calcium-dependent processes participate in LTP, it is important to determine whether a sequential or parallel order of activation is required for producing the final changes underlying LTP.

Previous studies have shown that epileptiform activity in the limbic system disrupts the mechanisms that induce LTP (17–19). We reasoned that such a situation might provide a unique opportunity to establish the roles of different calcium-dependent processes in LTP induction and especially that of calcium-dependent phospholipases. We report here that epileptic seizures produced by systemic administration of kainic acid (KA) prevent the induction of LTP in field CA_1 of hippocampal slices. Moreover, the increase in [^{3}H]AMPA binding elicited by PLA_2 treatment of hippocampal membranes is lost after KA administration. These results suggest that PLA_2-induced modification of AMPA/quisqualate receptors is a necessary step in the development of LTP.

MATERIALS AND METHODS

Tissue Preparation and Binding Assays. Male Sprague–Dawley rats (200–250 g) were injected i.p. with saline or KA (10 mg/kg in Tris-buffered saline). Animals were sacrificed by decapitation 12–16 hr after injection and the hippocampus and cerebellum were quickly removed and homogenized in chilled (4°C) 0.32 M sucrose (containing 1 mM EGTA, pH 7.4) with a glass/Teflon homogenizer. Membranes were prepared as described by differential centrifugation, osmotic lysis, and detergent treatment (15, 20). Membrane suspensions (0.5–1.0 ml) were incubated with 4.0 mM $CaCl_2$ in the

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Abbreviations: NMDA, *N*-methyl-D-aspartate; CPP, [(±)-2-carboxypiperazine-4-yl]propyl-1-phosphoric acid; TCP, *N*-(1-[thienyl]cyclohexyl)piperidine; AMPA, DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; PLA_2, phospholipase A_2; LTP, long-term potentiation; EPSP, excitatory postsynaptic potential; STP, short-term potentiation; IPSP, inhibitory postsynaptic potential; KA, kainic acid; TBS, θ-burst stimulation.

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presence or absence of 1.0 μg of PLA_2 per mg of protein for 45 min at 37°C (PLA_2 from porcine pancreas; 600 units per mg of protein; Sigma). The membranes were then washed by a cycle of dilution/centrifugation (48,000 × g for 20 min) with 10 ml of cold Tris acetate buffer and were resuspended in the same buffer. All standard binding assays for glutamate receptors were performed as described (15). In some experiments, telencephalic membranes (1.0 mg of protein per ml) were preincubated for 30 min at 35°C with 2.0 μg of purified calpain I per ml in Tris acetate buffer containing 4.0 mM $CaCl_2$ in the presence or absence of 500 μM leupeptin. Control and treated membranes were washed free of drugs by a cycle of dilution/centrifugation (48,000 × g for 20 min) with 10 ml of cold Tris acetate buffer and were resuspended in the same buffer before determining the effect of PLA_2 on [^{3}H]AMPA binding (16).

Hippocampal Slice Preparation for Electrophysiology. Hippocampal slices were prepared from control and KA-injected rats as described (21). Briefly, transverse slices (450 μm thick) were placed in an interface chamber and perfused at 1.0 ml/min with artificial cerebrospinal fluid containing 124 mM NaCl, 3 mM KCl, 1.25 mM KH_2PO_4, 2.5 mM $MgSO_4$, 3.4 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose, and 2 mM L-ascorbate. They were maintained at 35°C and oxygenated with 95% O_2/5% CO_2. After a 1-hr equilibration period, a glass recording electrode (1–5 MΩ; filled with 2 M NaCl) was positioned in stratum radiatum of field CA_1 to record population excitatory postsynaptic potentials (EPSPs) evoked by a bipolar stimulating electrode (twisted 60 μM nichrome) activating fibers of the Schaffer commissural system. EPSPs evoked by single and θ-burst stimulation (TBS) [10 trains of four 100-Hz pulses given at 5 Hz (22)] were stored on a computer for later analysis.

Western Blot for Spectrin Proteolysis. The hippocampus and cerebellum were dissected in ice-cold homogenization buffer consisting of 0.32 M sucrose, 10 mM Tris, 2 mM EDTA, 1 mM EGTA, 100 μM leupeptin, 1 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone per ml (pH 7.4). An aliquot was added to 1/3 vol of 3× SDS/PAGE sample buffer and placed in a boiling water bath for 5 min. The procedures for SDS/PAGE, electrophoretic transfer, immunodetection, and densitometric quantification were as described (23, 24). Calpain I was purified from erythrocytes as described (24).

RESULTS

Effects of KA Treatment on Excitatory Amino Acid Receptor Binding Sites. The binding of different glutamate receptor-related ligands was determined in hippocampal membranes prepared from control and KA-injected rats. Evaluation of the binding saturation kinetics for [^{3}H]AMPA in hippocampal membranes produced a nonlinear Scatchard plot that was best fitted by a two-site model with high- and low-affinity binding sites (Fig. 1*A*). In control rats, the two-site analysis provided apparent dissociation constants of 16 and 660 nM and a B_{max} of 0.36 and 16.2 pmol per mg of protein for the high- and low-affinity binding sites, respectively (Table 1). No changes in binding affinity for either component were observed in KA-treated animals. However, the maximum number of binding sites for the low-affinity component of the binding, which accounted for >95% of the [^{3}H]AMPA binding sites, decreased by ≈35% after injection of KA. The B_{max} value for the high-affinity component of [^{3}H]AMPA binding was not modified by the treatment (Table 1).

We also measured the binding of different NMDA receptor ligands in membranes prepared from control and KA-treated rats (Fig. 1*B*). In the presence of micromolar concentrations of KA, >90% of [^{3}H]glutamate binding represents binding to NMDA receptors (20). Treatment of rats with KA did not modify the binding of [^{3}H]glutamate under these conditions, nor did it modify the binding of the tritiated NMDA antagonist [(±)-2-carboxypiperazine-4-yl]propyl-1-phosphoric acid ([^{3}H]CPP) to the NMDA receptor or of [^{3}H]glycine to the glycine site associated with this receptor complex. In addition, binding of tritiated *N*-(1-[thienyl]cyclohexyl)piperidine ([^{3}H]TCP), a ligand for the NMDA receptor-associated ionic channels, was not significantly affected by KA treatment when the channel was fully activated by high concentrations of glutamate and glycine.

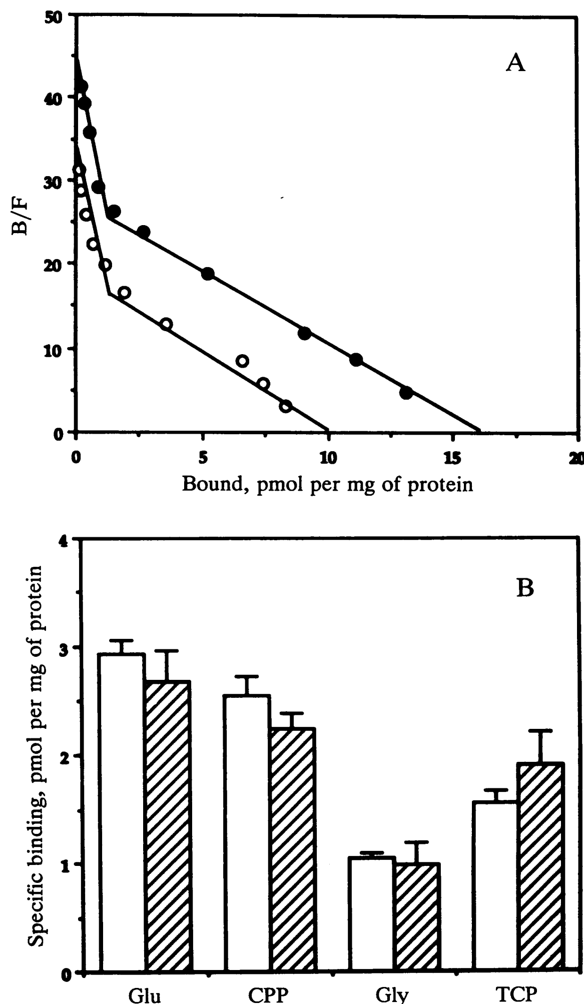

FIG. 1. Effect of KA treatment on the binding of different glutamate receptor-related ligands. (*A*) The binding of increasing concentrations (4–3000 nM) of [^{3}H]AMPA to hippocampal membranes from control and KA-treated animals was performed at 0°C for 40 min. Data were calculated as pmol per mg of protein and were plotted as a Scatchard plot (B/F, bound/free; pmol per mg of protein); the experiment was replicated three times with similar results. •, Control membranes; ○, KA-treated membranes. Data were analyzed by the LIGAND program (25) to generate K_d and B_{max} (Table 1). (*B*) Hippocampal membranes (1 mg/ml) were prepared from control and KA-treated animals. The binding of [^{3}H]glutamate (Glu; 100 nM), [^{3}H]glycine (Gly; 50 nM), [^{3}H]CPP (CPP; 100 nM), and [^{3}H]TCP (TCP; 30 nM) to the NMDA receptor complex was determined as described (15). The binding of [^{3}H]TCP to the phencyclidine site of the NMDA receptor was measured by incubating hippocampal membranes in Tris/Hepes buffer at 24°C for 50 min in the presence of 10 μM glutamate and 10 μM glycine (15). Results are means ± SEM of three or four experiments.

Modulation of [^{3}H]AMPA Binding by PLA_2 Treatment. Treatment of telencephalic membranes with exogenous phospholipases has been shown to produce an increase in the affinity of the AMPA/quisqualate receptor for the radioli-

Table 1. Effect of KA treatment on characteristics of [^{3}H]AMPA binding to rat hippocampal membranes

	High affinity		Low affinity	
	K_d, nM	B_{max}, pmol per mg of protein	K_d, nM	B_{max}, pmol per mg of protein
Control	16 ± 10	0.36 ± 0.20	660 ± 44	16.2 ± 0.83
KA treated	19 ± 13	0.38 ± 0.20	637 ± 107	10.4 ± 1.39*

Data from Fig. 1*A* were analyzed by the LIGAND program (25). Results are means ± SEM of three experiments.
*$P < 0.05$ (Student's *t* test).

gand [^{3}H]AMPA, without changing the binding properties of agonists for the NMDA receptor complex (15, 16). In control rats, [^{3}H]AMPA binding to hippocampal and cerebellar membranes was increased by 40% and 60%, respectively, after treatment with exogenous PLA_2 (1 μg per mg of protein) (Fig. 2). Stimulation of [^{3}H]AMPA binding by PLA_2 treatment was markedly reduced in hippocampal membranes prepared from KA-injected rats (Fig. 2*A*); in contrast, KA treatment did not reduce the effect of PLA_2 on [^{3}H]AMPA binding to cerebellar membranes (Fig. 2*B*).

Activation of the calcium-dependent protease calpain and the proteolytic degradation of spectrin is a common event in various neuropathological conditions. Systemic injection of the excitatory amino acid KA in adult rats produced a calpain-mediated proteolysis of spectrin in the hippocampus, but not in the cerebellum, as indicated by an immunodetection assay of spectrin breakdown products (Fig. 3*A*). In hippocampus, the amount of spectrin breakdown products measured 15 hr after the injection of KA was increased >10-fold compared to control rats. No changes in spectrin breakdown values were observed in cerebellar homogenates from KA-treated versus control animals. The increase in [^{3}H]AMPA binding to the AMPA/quisqualate receptor produced by PLA_2 treatment of telencephalic membranes was significantly reduced by prior incubation of the membranes (30 min; 35°C) with purified calpain I (Fig. 3*B*). Addition of the protease inhibitor leupeptin (500 μM) during the preincubation totally prevented the effect of calpain on the increase in [^{3}H]AMPA binding induced by PLA_2.

LTP in Hippocampal Slices from KA-Treated Rats. Hippocampal slices were prepared from control and KA-injected rats (12–15 hr survival) and perfused for 2–3 hr with artificial cerebrospinal fluid before applying high-frequency stimulation to the Schaffer commissural pathway. Four evoked responses were averaged 2.0 and 32 min after TBS to determine the possible effect of KA treatment on short-term potentiation (STP) and LTP, respectively. As shown in Fig. 4*A*, KA-treated rats exhibited the same amount of STP as control rats but a greatly reduced magnitude of LTP. It should be noted that the size and shape of the evoked potentials during the baseline period were not detectably different in slices prepared from control and KA rats. In some of the latter cases, however, large dendritic field potentials evoked multiple (two) population spikes, which became superimposed on the field EPSPs. Previous studies have shown that during TBS, postsynaptic responses to the bursts that follow the initial burst become enhanced, in part because the first burst suppresses feedforward inhibitory postsynaptic potentials (IPSPs). The second and later burst responses exhibit an AP-5 sensitive component, thereby indicating that the temporal summation of EPSPs is of sufficient magnitude and duration to overcome the voltage-dependent block of the NMDA receptor channel (26). The area measured beneath the response envelope of bursts (bursts 2, 4, 6, 8, and 10) was calculated as a percentage of the value of the first burst response. KA treatment did not reduce the percentage increase in burst responses occurring during TBS (Fig. 4*B*).

DISCUSSION

The present study indicates that systemic administration of KA produced specific changes in AMPA/quisqualate receptor binding and in their regulation. Whereas the binding of various ligands to the NMDA receptors was not modified by the treatment, the AMPA/quisqualate receptors exhibited a significant decrease in the number of binding sites. The increase in [^{3}H]AMPA binding elicited by PLA_2 treatment of hippocampal membranes was also markedly decreased after KA injection; incubation of control membranes with calpain similarly reduced the effect of PLA_2 on AMPA/quisqualate receptor binding. Finally, KA treatment also markedly decreased the amount of LTP induced in CA_1 of hippocampal slices without affecting the magnitude of STP or detectably changing the response of postsynaptic cells recorded during LTP-inducing stimulation.

The specific decrease in [^{3}H]AMPA binding observed in KA-treated rats could be due to the down-regulation of the

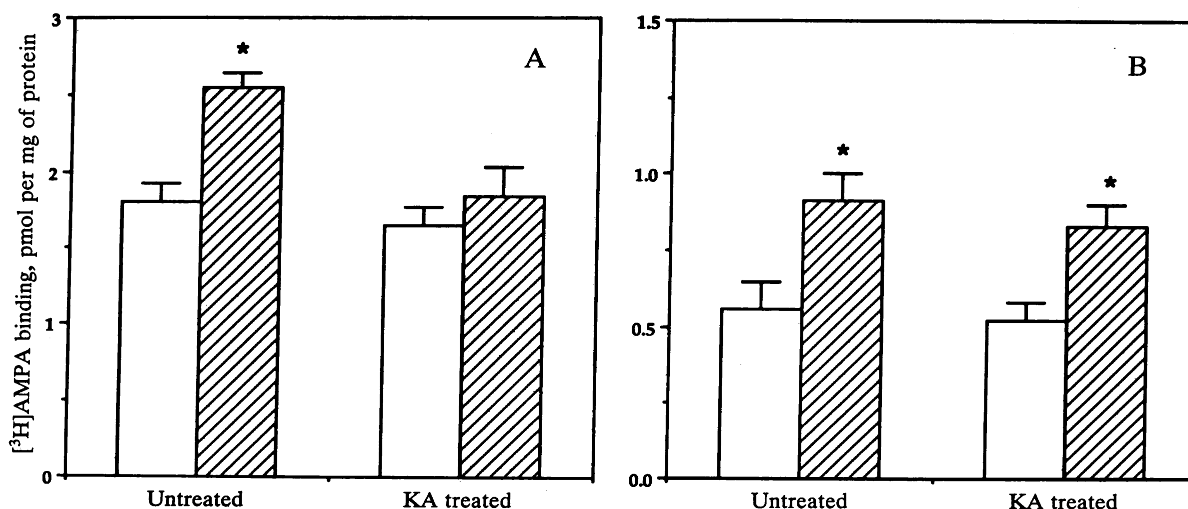

FIG. 2. Effect of KA treatment on the stimulation of [^{3}H]AMPA binding elicited by PLA_2. Hippocampal (*A*) and cerebellar (*B*) membranes were incubated with 4.0 mM $CaCl_2$ in the absence (open bars) or presence (hatched bars) of 1.0 μg of PLA_2 per mg of protein for 30 min at 37°C. After centrifugation, the binding of [^{3}H]AMPA (50 nM) to the quisqualate receptor was determined. Results are means ± SEM of six experiments and are expressed as pmol per mg of protein. *, $P < 0.05$ (Student's *t* test) in PLA_2-treated versus control membranes.

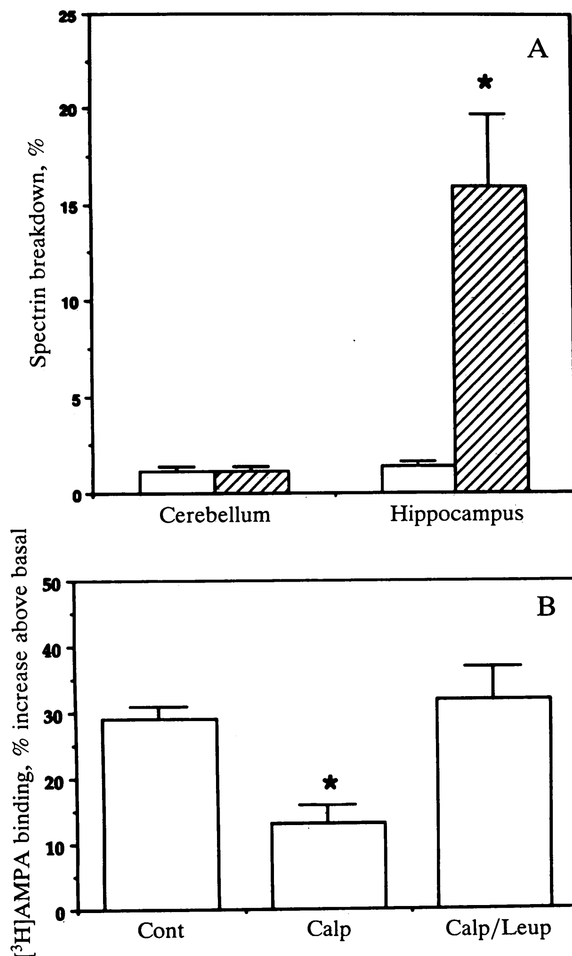

FIG. 3. KA-induced spectrin proteolysis and effect of calpain treatment on the stimulation of [^{3}H]AMPA binding elicited by PLA_2. (*A*). Animals were injected with KA (10 mg/kg; i.p.) and sacrificed 15 hr later. Levels of breakdown products 1 and 2 were quantified by reflective scanning densitometry as described (23). Results are expressed as the percentage of total spectrin immunoreactivity and are means ± SEM of four experiments. *, $P < 0.05$; Student's *t* test. (*B*) Telencephalic membranes were pretreated with 2.0 μg of purified calpain I per ml as described. After centrifugation, the membranes were resuspended in Tris acetate buffer and incubated with or without PLA_2. The binding of [^{3}H]AMPA (50 nM) was then determined. Results represent the PLA_2-induced stimulation of [^{3}H]AMPA binding (expressed as percentage increase over basal binding) under the conditions indicated (control, Cont; calpain, Calp; calpain and leupeptin, Calp/Leup) and are means ± SEM of six experiments. *, $P < 0.05$; Student's *t* test.

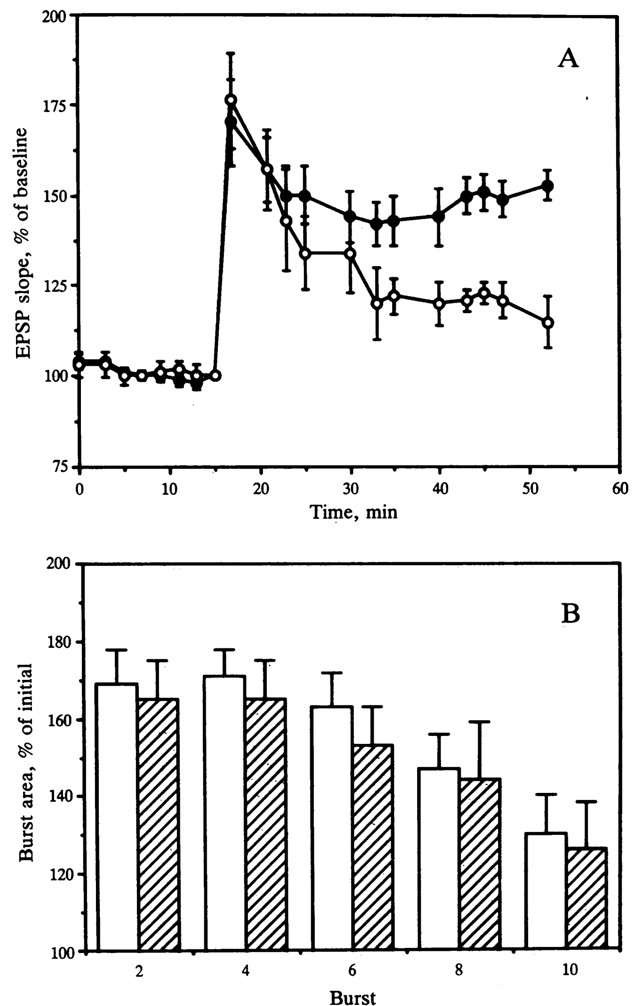

FIG. 4. Effect of KA treatment on LTP and on the burst responses evoked by TBS in hippocampal slices. (*A*) Field EPSPs were recorded in stratum radiatum of CA_1 pyramidal cells of hippocampal slices in control (●) and KA-treated (○) rats. The initial slope of each EPSP was calculated and the values of four successive responses were averaged. The baseline value represents the average of all the values obtained during the 15-min interval preceding the TBS. The data were then expressed as percentage of the baseline value and each point represents the mean ± SEM of five different experiments. (*B*) Field EPSPs were recorded in stratum radiatum of CA_1 pyramidal cells and TBS was delivered in slices from control (open bars) and KA-treated (hatched bars) rats. The net depolarization (i.e., area of total negative responses) produced by each burst was determined and is expressed as a percentage of the first burst response (the figure displays results only for bursts 2, 4, 6, 8, and 10 in the train). Results are means ± SEM of five experiments.

AMPA/quisqualate receptors, as a result of an increased release of excitatory amino acids elicited by seizure activity. A decrease in receptor number could also be due to a loss of hippocampal neurons but this is not likely as the binding of several ligands to the NMDA receptor complex was not modified in KA-treated rats. Moreover, neuronal cell loss in the hippocampus 15 hr after KA administration is quite small (27). Finally, it is possible that irreversible damage to structural elements such as membrane lipids and cytoskeletal proteins and/or altered turnover of regulatory proteins are associated with KA-induced seizures. In particular, alterations in lipid environment produced by treatment of membranes with various phospholipases have been shown to produce differential modifications of the AMPA/quisqualate and the NMDA receptor complex (15).

As previously reported in other models of epileptic activity (17–19), we observed that the induction of seizure activity as a result of systemic KA administration is followed by a loss of LTP in field CA_1 of hippocampal slices. The loss of LTP does not appear to be due to major alterations in the physiological characteristics of the NMDA receptors. Short-term potentiation was not modified in slices from KA-treated rats and the burst responses evoked by high-frequency stimulation exhibited the typical buildup of depolarization, which is due to both a reduction of IPSPs and the activation of NMDA receptors (26). Thus, the loss of LTP maintenance is more likely the result of the disruption by KA treatment of processes that normally follow the activation of the ionic currents that are the initial trigger for LTP. In particular, the PLA_2-induced increase in affinity of the AMPA/quisqualate receptors is markedly diminished after KA treatment. It is thus reasonable to assume that the PLA_2-induced modification of the AMPA/quisqualate receptor is an important if not necessary step for LTP maintenance. This assumption is

strengthened by recent results from our laboratory indicating that the PLA_2-induced increase in [^{3}H]AMPA binding is also absent in membranes prepared from neonatal animals at a developmental stage when LTP is not induced by high-frequency stimulation (M.B. *et al.*, unpublished data).

Some clues concerning the possible mechanisms responsible for the loss of the PLA_2 effect on the AMPA/quisqualate receptor after KA treatment are provided by the results of the experiments with calpain pretreatment of the membranes. First, we replicated the results of Seubert *et al.* (28) showing that systemic KA administration is associated with calpain activation as the amount of spectrin breakdown products was markedly increased in hippocampus but not in cerebellum. Pretreatment of telencephalic membranes with calpain markedly decreased the PLA_2-induced increase in [^{3}H]AMPA binding. Thus, it is conceivable that the selective activation of calpain in hippocampus after KA treatment results in the impairment of PLA_2 to produce an increase in [^{3}H]AMPA binding to hippocampal membranes. As calpain does not appear to be activated in cerebellum, the PLA_2 effect on [^{3}H]AMPA binding to cerebellar membranes remains unaffected. The reason why pretreatment of membranes with calpain or the *in situ* activation of calpain produces a reduction in the PLA_2-induced increase in [^{3}H]AMPA binding to membranes remains unclear. One possible explanation could be that, as in blood platelets and erythrocytes, calpain activation is associated with phospholipid redistribution in the lipid bilayer (29), thus modifying the effect of PLA_2 on the lipid environment of the AMPA/quisqualate receptor. Independent of the precise mechanisms involved, it remains that our results provide evidence in favor of a necessary role of PLA_2 in LTP possibly mediated through a shift in the affinity of the AMPA/quisqualate receptor. Moreover, our results also suggest that the order of activation of different calcium-dependent processes is a critical factor in producing long-lasting alterations in synaptic efficacy.

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