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Rapid activation of hippocampal casein kinase II during long-term potentiation

(learning/*N*-methyl-D-aspartate/protein phosphorylation/K^+ channel)

C. CHARRIAUT-MARLANGUE*, S. OTANI, C. CREUZET, Y. BEN-ARI, AND J. LOEB

Laboratoire de Neurobiologie et Physiopathologie du Développement, Institut National de la Santé et de la Recherche Médicale U 29, 123 Boulevard de Port-Royal, 75014 Paris, France

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ABSTRACT **Several studies suggest that protein kinase C and type II Ca^{2+}/calmodulin-dependent protein kinase are activated during induction of long-term potentiation (LTP). We now report that casein kinase II (CK-II), which is present in high concentration in the hippocampus, is also activated in the CA1 region during LTP. CK-II activity increased within 2 min after a train of high-frequency electrical stimulations and reached a maximum (2-fold increase) 5 min later before returning to baseline value. The stimulated protein kinase activity, which was blocked by a selective antagonist of *N*-methyl-D-aspartate receptors, exhibited specific properties of CK-II, including phosphorylation of the specific substrates of CK-II, marked inhibition by a low heparin concentration, and the use of GTP as a phosphate donor. CK-II activity was also selectively and rapidly augmented in another form of LTP produced by bath application of tetraethylammonium; this LTP (called LTP_k) is Ca^{2+} dependent but *N*-methyl-D-aspartate independent. Phosphorylation of casein that was not inhibited by heparin (i.e., casein kinase I) remained unchanged. We suggest that an increase in CK-II activity is important in LTP induction.**

Long-term potentiation (LTP) is a form of synaptic plasticity widely investigated as a possible physiological mechanism underlying memory formation (1, 2). Among events that lead to LTP, the activation of multifunctional regulatory protein kinases, such as protein kinase C (PKC) and Ca^{2+}/calmodulin kinase II, is well documented (3–5). Activation of PKC in LTP generation in CA1 and the dentate gyrus has been reported to induce phosphorylation of specific proteins, in particular neuromodulin (B-50, GAP-43, and F1) (6–8).

Recently, the purified neuromodulin was shown to be phosphorylated by casein kinase II (CK-II) on Ser-192 (9), whereas Ser-41 was phosphorylated by PKC (10). Casein kinase I and CK-II are Ca^{2+} and cyclic nucleotide-independent cytosolic and nuclear protein kinases, named so because casein is the best substrate for *in vitro* phosphorylation (11–13). These protein kinases are widely distributed in all eukaryotic tissues. These kinases differ in molecular structures, phosphorylate different substrates, and respond differently to effectors. Within this group, CK-II can be selectively identified because (*i*) it is inhibited by a low heparin concentration, (*ii*) it is stimulated by polycations, and (*iii*) it uses GTP as well as ATP as phosphate donor (11). CK-II activity is 3–4 fold higher in the adult rat brain, including the hippocampus (14, 15), than in most other peripheral tissues (14), suggesting a specific role in neuronal function. Considerable interest in CK-II has arisen recently because its activity rapidly and transiently increases in response to several mitogens, such as the tumor-promoting phorbol 12-myristate 13-acetate (16), hormones (17, 18), and growth factors (18, 19). CK-II activation may result from activation of other protein kinases, and such an interaction between PKC and CK-II was recently described in primary cultures from baby rat kidney epithelial cells (16).

Because of the evidence associating phosphorylation of neuromodulin by PKC and CK-II and involvement of this phosphoprotein in synaptic plasticity, we studied changes in CK-II activity during LTP. We now report a rapid activation of CK-II in response to a high-frequency electrical train. The same results were obtained in a form of Ca^{2+}-dependent, *N*-methyl-D-aspartate (NMDA)-independent LTP (LTP_k), which is induced by the K^+ channel blocker tetraethylammonium (TEA) (20).

MATERIALS AND METHODS

Slice Preparation. Experiments were done in the CA1 area of transverse hippocampal slices prepared from adult male Wistar rats (190–240 g). Slices were incubated for at least 2 hr in continuously oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid of the following composition: 126 mM NaCl/3.5 mM KCl/1.2 mM NaH_2PO_4/1.3 mM $MgCl_2$/2.0 mM $CaCl_2$/10 mM glucose/25 mM $NaHCO_3$, pH 7.3. Each slice (in which connections between CA1 and CA3 were cut to prevent seizure propagation from CA3) was then transferred to a fully submerged chamber and continuously superfused with warm (33°C) oxygenated artificial cerebrospinal fluid at a rate of 650 μl/min. A glass micropipette electrode was placed in the stratum radiatum for recording. The field excitatory postsynaptic potential (EPSP) was evoked by 0.033-Hz test stimulation through a bipolar tungsten-wire-stimulating electrode placed on the Schaffer collateral/commissural pathway. After recording stable baseline responses for at least 15 min, LTP was induced by two trains of high-frequency stimulation (100 Hz, 500-ms train duration) or by 25 mM TEA bath application for 10 min, as described (20). Control slices (unstimulated or low-frequency stimulation at 1 Hz with the same number of pulses as in LTP-inducing trains) were obtained under the same conditions. In separate slices, trains were delivered in the presence of the NMDA receptor antagonist D-2-amino-5-phosphonovalerate (D-APV, 50 μM), and TEA was applied in the presence of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) or the voltage-dependent Ca^{2+} channel blocker flunarizine (30 μM). Both procedures selectively prevented induction of LTP_k (20).

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; D-APV, D-2-amino-5-phosphonovalerate; CK-II, casein kinase II; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EPSP, excitatory postsynaptic potential; LTP, long-term potentiation; LTP_k, K^+ channel blockade-dependent form of LTP; NMDA, *N*-methyl-D-aspartate; TEA, tetraethylammonium; PKC, protein kinase C.

*To whom reprint requests should be addressed.

Each experiment was done with slices from a single animal. At the end of the experiments, CA1 areas were removed and stored at −80°C for later biochemical analysis.

Homogenate Preparations. CA1 areas were homogenized in 50 mM Tris·HCl, pH 7.4/1 mM EGTA/1 mM phenylmethylsulfonyl fluoride/0.2% Triton X-100, sonicated, stirred for 30 min at 4°C, and then centrifuged at 15,000 × *g* for 15 min to remove insoluble material. The supernatants were retained.

Determination of CK-II Activity. The standard assay to measure CK-II activity was done by an adaptation of the Hathaway and Traugh technique (11) with casein as substrate. Because CK-II is specifically inhibited by heparin (5 μg/ml) addition to the assay mix, CK-II activity was measured as the difference between activity with and without heparin. For the standard phosphorylation assay, 5 μg of homogenate proteins was incubated at 33°C with 10 μM [γ-^{32}P]ATP (Amersham, 0.3 μCi; 1 Ci = 37 GBq) in 50 mM Tris·HCl, pH 7.6/10 mM $MgCl_2$/150 mM KCl (buffer A), and casein at 1 mg/ml in a total volume of 35 μl for 2 min with and without heparin. Reactions were stopped by spotting 30 μl of the reaction mixture onto cellulose disks (Whatman), and the filters were immediately immersed for 45 min at 0°C in 10% (wt/vol) trichloroacetic acid/5 mM unlabeled ATP. Then the filters were washed three times (for 20 min each) at 0°C in 5% trichloroacetic acid/10 mM $Na_4P_2O_7$/10 mM Na_3PO_4. After drying, filters were put in a scintillation vial to measure radioactivity by the Cerenkov effect. Data presented here were corrected for endogenous phosphorylation of homogenates.

The activity that was not inhibited by heparin consists principally of casein kinase I activity.

CK-II activity was also assayed by using a specific synthetic peptide (Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu) as substrate (21). The assay was continued for 15 min at 33°C in 35 μl of buffer A containing 4 μg of protein from homogenates, 2.3 mM peptide, and 20 μM [γ-^{32}P]ATP or [γ-^{32}P]GTP (1.5 μCi). The reaction was stopped by adding 10 μl of 125 mM H_3PO_4. Reaction mixtures were spotted onto P81 papers (Whatman). The papers were washed four times with 1 liter of 10 mM H_3PO_4 and dried; the radioactivity was then measured.

Determination of Protein Content. The protein content of the samples was determined according to Lowry (22) with bovine serum albumin as standard.

RESULTS

Characterization of CK-II Activity in the CA1 Region of the Hippocampus. In control slices, relatively high levels of CK-II activity were found in the CA1 region of the hippocampus. As shown in Table 1, total casein kinase activity was 83.1 ± 5 pmol of ^{32}P (min × mg of protein). Using heparin to selectively block CK-II activity, we found that over half of this activity (49.4 ± 5.2) was from CK-II. This kinase activity shared two other characteristic features of authentic CK-II (Table 1): (*i*) it phosphorylated the CK-II-specific substrate peptide (21), and (*ii*) it used GTP as a phosphate donor (11). CK-II was enriched in the particulate fraction (Table 1).

During low-frequency electrical stimulation of the Schaffer collateral (i.e., at 0.03 Hz), CK-II activity remained constant during 30 min; the mean level was 50 pmol of ^{32}P (Fig. 1*B*).

Increase in CK-II Activity During LTP. *LTP induced by high-frequency electrical stimulation.* In accordance with earlier studies, high-frequency trains caused a persistent enhancement of the EPSP slope 50–70% and 20–30% (10 min and 1 hr after the train, respectively) (Fig. 1*A*) as compared to control. This enhancement was associated with a rapid activation of CK-II in comparison with control slices; the time course of this activation is shown in Fig. 1*B*. The latency of enhancement was very short, and a peak was reached 5 min after the train with an activity of 96.7 ± 6 pmol of ^{32}P. A 25% increase (62.7 ± 5 pmol of ^{32}P in CK-II activity was detected as soon as 2 min after induction. It reached a maximum 5 min after the train and declined to basal value 10–15 min after the train (n = 3). Similar observations were made with the synthetic CK-II-specific substrate (n = 3, see Fig. 3).

LTP induced by bath application of TEA (LTP_K). Long-lasting potentiation induced by 25 mM TEA produced a large enhancement of the slope of the field EPSP lasting >2 hr (20). Rapid activation of CK-II was seen between 1 and 10 min after TEA application (Fig. 2); this increase was of 30%, 120%, and 136% 1, 2, and 5 min, respectively, after the end of TEA application. CK-II activity remained increased at a level of 90 pmol 15 min to 1 hr after TEA. This effect is not from a direct interaction between TEA and CK-II activity because in control homogenates a similar activity was found with and without TEA (25 mM for 10 min, n = 2, data not shown).

The protein kinase measured in these experiments shares similar properties with CK-II—i.e., casein phosphorylation, inhibition by heparin, phosphorylation of the specific CK-II peptide, and the use of GTP or ATP as a donor (Table 2). CK-II was also stimulated by polylysine (data not shown). It is interesting to emphasize that the activity due to other kinases that phosphorylate casein but are not inhibited by heparin (principally casein kinase I) was not stimulated during LTP.

Activation of CK-II Is Blocked by Selective Receptor Antagonists. We have also tested the effects of the selective NMDA antagonist (D-APV) (23) on the electrical NMDA-dependent form of LTP. As expected from earlier studies, this procedure blocked potentiation of the field EPSP (data

Table 1. CK-II activity in CA1 region of the hippocampal slice

Substrate	Activity	Phosphate donor	Protein kinase activity, pmol/(min × mg of protein)		
			Homogenate	Cytosol	Particulate
Casein	Total	ATP	83.1 ± 5	41.5 ± 1.2	192 ± 0.2
Casein + heparin	CK-I-like	ATP	33.7 ± 1.3	22.2 ± 1.3	150 ± 6
	CK-II	ATP	49.4 ± 5.2	19.4 ± 1.3	42 ± 3
CK-II peptide	CK-II	ATP	52.4 ± 3.5	ND	ND
		GTP	67.3 ± 2.1	ND	ND

Characterization of CK-II activity in the CA1 region of a hippocampal slice. Protein kinase activity was determined on homogenates (5 μg of protein), as described. Total casein kinase and CK-II activities were measured relying on heparin inhibition and use of a specific substrate peptide for CK-II. Values are expressed in pmol of ^{32}P incorporated (min × mg of protein) ± SE from five different experiments. Cytosolic and particulate fractions were obtained from centrifugation of the homogenate for 1 hr at 100,000 × *g*. The supernatant formed the soluble fraction. The pellet was resuspended in 50 mM Tris·HCl, pH 7.4/0.9 M NaCl, stirred 30 min at 4°C, and centrifuged 1 hr at 100,000 × *g* to separate solubilized proteins from particulate fraction. CK-II activity was measured in 2 μg of protein samples of each fraction after normalization for salt. ND, not determined.

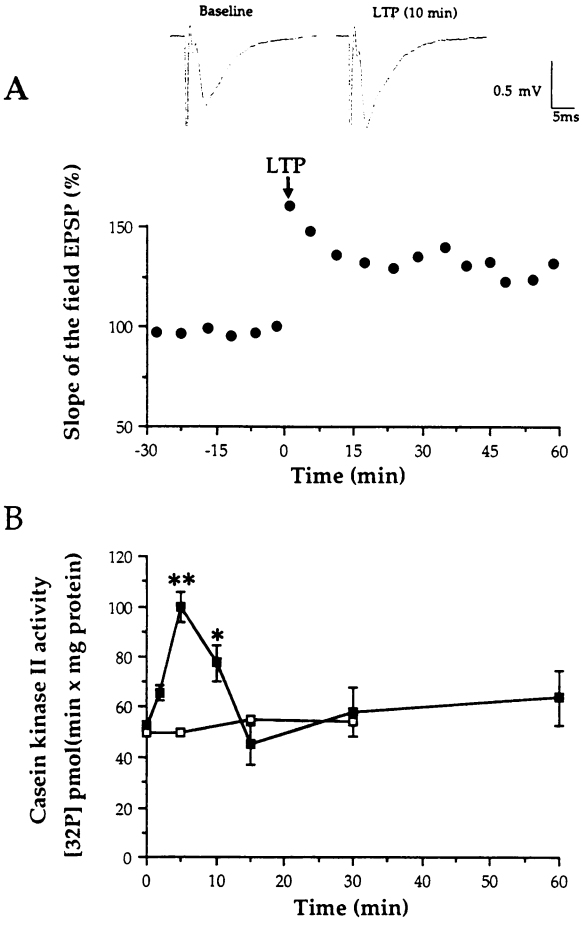

FIG. 1. Time course of CK-II activity in CA1 homogenates during LTP induced by an electrical train. (*A*) LTP of the field EPSP. The increase in slope of the field EPSP evoked by the Schaffer collateral/commissural pathway was represented as a percentage of the baseline; each point represents the mean of 10 EPSPs. Traces represent the field EPSP just before stimulation (baseline) and the potentiated EPSP (LTP) 10 min after stimulation. (*B*) Activity of CK-II (using casein as substrate) in three separate experiments in which LTP was induced by a 100-Hz high-frequency stimulation (■) versus 1-Hz low-frequency stimulation (□). Results are expressed in pmol of ^{32}P incorporated (min × mg of protein). Error bars represent SE; data points without error bars are the means of two values. (Student's *t* test, $**P \leq 0.005$, $*P \leq 0.01$).

not shown). This procedure also fully blocked any enhancement of CK-II activity (Fig. 3, $n = 3$).

In three other slices, LTP_k was prevented either by the selective AMPA receptor antagonist CNQX (24) or by flunarizine, a Ca^{2+}-channel blocker that prevents the burst of Ca^{2+} spikes. Both procedures blocked LTP_k but not LTP_E induced by a train of electric stimulation (data not shown, see ref. 20). Fig. 4 shows that the increase in CK-II activity was

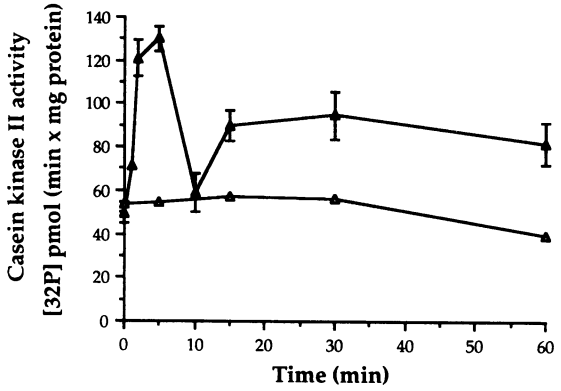

FIG. 2. Time course of CK-II activity in CA1 homogenates during LTP induced by 25 mM TEA. The data represent the activities of CK-II (using casein as substrate) in three separate experiments in which slices were incubated 10 min with (▲) or without (△) 25 mM TEA and then transferred in artificial cerebrospinal fluid and removed for assay subsequently with various delays. Results are presented as in Fig. 1.

abolished by the two types of blockers. In contrast, the CK-II activity was increased in LTP_k, despite the presence of D-APV. These results support the idea that this type of LTP is from a blockade of K^+ channels by TEA and the activation of quisqualate/kainate receptors, which, in turn, induce a Ca^{2+} influx through voltage-dependent Ca^{2+} channels.

DISCUSSION

The principal conclusion of our study is that in CA1, CK-II activity transiently increases during the induction of (Ca^{2+}-dependent) NMDA-dependent and -independent forms of LTP. This increase has a short latency, reaching a peak within 5 min after induction.

On the basis of casein- and CK-II peptide-specific substrate phosphorylation, inhibition by a low concentration of heparin, activation by polylysine, and the ability to use ATP or GTP as phosphate donor, the stimulated protein kinase described here was identified as CK-II (11–13). This activation of CK-II appeared LTP-specific because in electrical LTP it is blocked by the specific NMDA receptor antagonist D-APV, whereas in LTP_k this activation is inhibited by the specific AMPA receptor antagonist CNQX or by flunarizine, a selective blocker of voltage-dependent Ca^{2+} channels (20).

Interestingly, a rapid activation of CK-II has been seen in other experimental conditions in cell cultures. Sommercorn and Krebs (17), Klarlund and Czech (18), and Ackerman and Osheroff (19) have shown that CK-II activity increases during the early phases of insulin, insulin-like growth factor I, and epidermal growth factor actions; responses generally peak within 15 min. This increase could be up to 4-fold control levels. The activation of CK-II by growth factors suggests a role for CK-II as an intermediate step in signal transduction

Table 2. Properties of the stimulated protein kinase activity in LTP experiments

Substrate	Activity	Phosphate donor	Protein kinase activity, pmol/(min × mg of protein)		
			Control	LTP	LTP_k
Casein	Total	ATP	88.1 ± 5	134.2 ± 3.4	156.1 ± 7.5
Casein + heparin	CK-I-like	ATP	38.7 ± 3.3	33.7 ± 1.3	32 ± 0.4
	CK-II	ATP	49.4 ± 5.2	100.5 ± 7.3	124.1 ± 8.4
CK-II peptide	CK-II	ATP	52.4 ± 3.5	115.7 ± 3	ND
		GTP	67.3 ± 2.1	113.3 ± 3.2	ND

Summary of properties of the stimulated protein kinase activity in LTP experiments (at 5 min). CK-II was measured in homogenates from CA1 slices in which LTP was induced by high-frequency train (LTP) or by TEA (LTP_k), using casein or the specific synthetic peptide substrates. Data (means ± SE, $n = 3$) were obtained in three independent LTP experiments. ND, not determined.

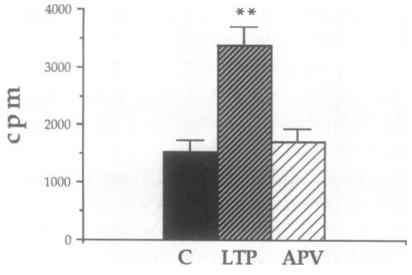

FIG. 3. Activation of CK-II during LTP was blocked by a specific antagonist of NMDA receptors. Bars represent CK-II activity as determined with the CK-II-specific peptide substrate. Note that LTP was associated with a highly significant increase in ^{32}P-labeled phosphate incorporation into casein that was prevented by prior application of D-APV (n = 3, Student's t test, $^{**}P \leq 0.05$). C, control.

from receptors to cellular events. The mechanisms of CK-II activation are still unknown. CK-II could be activated through a protein kinase cascade, as suggested by recent studies in *Xenopus* oocytes (25) and in human cells (26) in which activation of CK-II correlated with the hyperphosphorylation of its β subunit.

In hippocampal area CA1, activation of NMDA receptors has been found necessary to induce electrical LTP (3). Activation of the NMDA receptor–channel complex triggers a rise in intracellular [Ca^{2+}] in the postsynaptic cell (27). Other lines of evidence indicate that LTP_k is due to a rise in intracellular [Ca^{2+}] mediated by the activation of voltage-dependent Ca^{2+} channels (20). This rise in Ca^{2+} is the common feature of the two LTP processes and could be responsible for CK-II activation. In many cellular processes, Ca^{2+} signals act through Ca^{2+}-dependent protein kinases, which, in turn, regulate some other effector protein molecules by phosphorylation. As CK-II is not a Ca^{2+}-dependent protein kinase, only indirect mechanisms may be proposed. A recent study (16) suggests PKC activation (consecutively to a rise in intracellular [Ca^{2+}]) could mediate the activation of CK-II through a protein kinase cascade.

Several papers have suggested that PKC and Ca^{2+}/calmodulin kinase II are involved in the formation and/or maintenance of LTP (28–31). The activation of PKC may lead to phosphorylation of proteins, such as neuromodulin (8). CK-II has been reported to catalyze the phosphorylation of

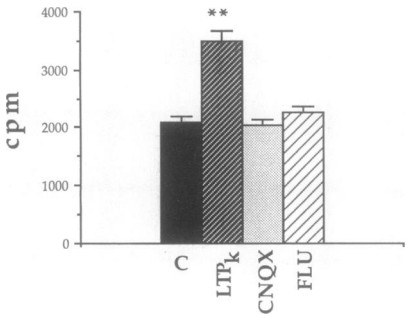

FIG. 4. Activation of CK-II during LTP_k was blocked by specific antagonists. In three experiments, slices were incubated with either CNQX, a specific antagonist of AMPA receptors, or flunarizine (FLU), a Ca^{2+} channel blocker, 15 min before starting TEA-bath application to inhibit LTP_k. Values are compared with those from control (C) slices and from slices after LTP_k. Bars represent CK-II activity, as determined by the incorporation of ^{32}P-labeled phosphate into casein. Note that the LTP induced by TEA (LTP_k) was associated with a significant increase in CK-II; this increase was prevented by CNQX or flunarizine, blockers of AMPA receptors and voltage-dependent Ca^{2+} channels, respectively (Student's t test, $^{**}P \leq 0.05$).

a large number of protein substrates (11, 13). Recently, neuromodulin and other neuronal proteins, including a dopamine- and cAMP-regulated phosphoprotein (DARPP-32) (32) and microtubule-associated protein 1B (33), have been shown to be substrates for CK-II, further indicating a potentially important role for this kinase in neurons. In some cases CK-II may work synergistically with another protein kinase. Thus, the phosphorylation of the dopamine- and cAMP-regulated phosphoprotein by CK-II facilitates its phosphorylation by a cAMP-dependent protein kinase in rat caudate-putamen slices (32). This phosphorylation converts the dopamine- and cAMP-regulated phosphoprotein into a potent inhibitor of protein phosphatase 1 (34). It is tempting to speculate that the phosphorylation of neuromodulin by CK-II may facilitate its phosphorylation by PKC. A synergistic system involving PKC, CK-II, and Ca^{2+}/calmodulin kinase II via the neuromodulin could then occur in LTP (35).

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