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Surface protein phosphorylation by ecto-protein kinase is required for the maintenance of hippocampal long-term potentiation

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ABSTRACT **During the induction of long-term potentiation (LTP) in hippocampal slices adenosine triphosphate (ATP) is secreted into the synaptic cleft, and a 48 kDa/50 kDa protein duplex becomes phosphorylated by extracellular ATP. All the criteria required as evidence that these two proteins serve as principal substrates of ecto-protein kinase activity on the surface of hippocampal pyramidal neurons have been fulfilled. This phosphorylation activity was detected on the surface of pyramidal neurons assayed after synaptogenesis, but not in immature neurons nor in glial cells. Addition to the extracellular medium of a monoclonal antibody termed mAb 1.9, directed to the catalytic domain of protein kinase C (PKC), inhibited selectively this surface protein phosphorylation activity and blocked the stabilization of LTP induced by high frequency stimulation (HFS) in hippocampal slices. This antibody did not interfere with routine synaptic transmission nor prevent the initial enhancement of synaptic responses observed during the 1–5 min period immediately after the application of HFS (the induction phase of LTP). However, the initial increase in the slope of excitatory postsynaptic potentials, as well as the elevated amplitude of the population spike induced by HFS, both declined gradually and returned to prestimulus values within 30–40 min after HFS was applied in the presence of mAb 1.9. A control antibody that binds to PKC but does not inhibit its activity had no effect on LTP. The selective inhibitory effects observed with mAb 1.9 provide the first direct evidence of a causal role for ecto-PK in the maintenance of stable LTP, an event implicated in the process of learning and the formation of memory in the brain.**

The long-term potentiation (LTP) of synaptic strength in hippocampal pyramidal neurons is a neurophysiological process implicated in the formation of memory traces in the brain (1, 2). While the initial chain of events that triggers this process is well-known, the mechanisms that determine the duration and stability of LTP have not yet been fully elucidated. Although it is generally accepted that the phosphorylation of proteins by several different kinases is involved in this stabilization, their exact localization and roles are still obscure (2, 3). Protein phosphorylation, a ubiquitous step in intracellular pathways that produce transient changes in neuronal activity, was found to serve also as a key mechanism of molecular adaptation in processes underlying the induction of long-lasting alterations in synaptic function (for reviews, see ref. 4), including learning and memory formation (5–7). The first study that implicated protein phosphorylation in the process of learning (5) identified a synaptic phosphoprotein that was later shown to be a major neuronal substrate of protein kinase C (PKC), that plays a role in the maintenance-phase of LTP (8, 9). The specific PKC-isozyme involved in the maintenance of LTP was reported to be PKC ζ, a member of the atypical class (not stimulated by diacylglycerol or phorbol esters) of PKCs (10).

The discovery of neuronal ecto-PK (11) has revealed that the powerful regulatory machinery of protein phosphorylation operates in the extracellular environment of the brain, where it can use adenosine triphosphate (ATP) secreted from synaptic vesicles of stimulated neurons to regulate various neuronal functions (12). Recent studies have demonstrated the activity of several types of ecto-PK on the surface of intact brain neurons, including activity with catalytic properties of atypical PKCs (13), such as the PKC ζ implicated in the maintenance of LTP. The accumulation of increased ATP concentrations in the synaptic cleft that is induced by a train of repetitive stimuli delivered at high frequency (14, 15), and its utilization by an ecto-PK with a relatively high K_m for ATP (16) were proposed as biochemical events that are unique to the process of LTP and do not occur during routine synaptic transmission (14, 16). Indeed, exocytosis of vesicular ATP induced by high frequency stimulation (HFS) that causes LTP has been demonstrated directly in hippocampal slices (17), and low-frequency presynaptic stimulation delivered in hippocampal slices together with exogenously added ATP has been reported to induce LTP (18). The results of experiments conducted to determine the effects of several nonhydrolyzable and slowly hydrolyzable analogs of ATP on LTP induced by HFS (19) have provided evidence that the removal of the gamma phosphate of extracellular ATP is associated with the maintenance phase of LTP, and that this activity has properties consistent more with the phosphotransferase action of a kinase than with the hydrolytic activity of ATPase. We thus hypothesized that the maintenance of stable LTP may involve the activity of ecto-PK. The present study was designed to determine whether hippocampal pyramidal neurons have ecto-PK activity, whether selective inhibition of this activity interferes with LTP, and whether the induction and maintenance phases of hippocampal LTP are regulated differently by ecto-PK activity. The results have provided evidence that ecto-PK is a physiological activity operating on the surface of pyramidal neurons, and that this activity plays a causal role in the maintenance of stable LTP in the hippocampus. An initial report of this study has been presented (20).

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Abbreviations: LTP, long-term potentiation; PK, protein kinase; KRB, Krebs–Ringer buffer; DIV, days *in vitro*; EPSP, excitatory postsynaptic potential; HFS, high frequency stimulation.

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MATERIALS AND METHODS

Culture of Hippocampal Pyramidal-Neurons and Glial Cells. Mouse embryonic hippocampal neurons were isolated from fetuses at the gestational age of 16 days, before emergence of granule neurons. Pyramidal neurons were maintained in serum-free medium without adding cocultures of glial cells. During dissection procedures the tissues were maintained in Hanks' balanced salt solution (HBSS) containing 25 mM Hepes buffer (pH 7.4). Embryonic hippocampi were triturated with a 10 ml pipette in Basal medium Eagle's medium (BME) with 15% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS). After passage through a sterile cell strainer (40 μm, Falcon), cells were counted and plated at the density of 1.2×10^5 cells per cm^2 in 48-well plates previously coated for 12 hr with poly-L-lysine (4 mg/100 ml) and rinsed with sterile phosphate-buffered saline (PBS). After a 2-hr incubation at 37°C, the medium was replaced with Neurobasal medium (GIBCO) containing B-27 supplement (GIBCO) and 500 μM freshly dissolved glutamine. The cells kept in this chemically defined medium maintained over 95% viability without further changing of medium. Routine assays of the present study were conducted with neurons maintained in culture for 18 days. A mixed glial primary culture was prepared from hippocampi of newborn mice (1 day postpartum). The tissue was kept in HBSS containing 25 mM Hepes buffer during the dissection. Hippocampi were mechanically dissociated with a 10 ml pipette in BME with 15% FBS and 1% PS. After passing through a 40 μm sterile cell strainer, cells were plated at a high cell density (2×10^5/cm^2) in serum-supplemented medium (BME + 15% FBS + 1% PS) that was changed every 4 days.

Protein Phosphorylation Assays. All the procedures used in experiments involving phosphorylation of proteins were carried out as detailed in refs. 11 and 13. Adult mouse hippocampal slices were prepared according to standard procedures used in our LTP studies (see below). Both hippocampi were dissected, placed in cold Ringer's solution containing: 124 mM NaCl, 3.1 mM KCl, 1.3 mM KH_2PO_4, 1.3 mM $MgSO_4$, 3.1 mM $CaCl_2$, 25.5 mM $NaHCO_3$, 10.0 mM glucose, and were cut into 400-μm thick slices that were preincubated for at least 1.5 hr before experimentation. For labeling phosphoproteins under the conditions in which ATP induces LTP, 15 μCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP was added, and to terminate the phosphorylation reaction "SDS-stop solution" was added (11). In experiments conducted with cultured neurons, we used cells attached to the bottom of 48-well plates (13, 21). The medium from each well was removed and the intact cells were rinsed twice and covered with 125 μl of freshly prepared Krebs–Ringer buffer (KRB; pH 7.4) prewarmed to 37°C. Phosphorylation reactions were initiated by adding 5 μl of KRB containing 15 μCi of [γ-^{32}P]ATP or ^{32}Pi, and nonlabeled ATP to make the initial total ATP concentration in each reaction mixture 0.1 μM. Reactions were terminated by addition of 26 μl of 6× SDS-stop solution (making 2% SDS and 2% 2-mercaptoethanol, final concentrations). Before SDS/PAGE, the samples were heated for 5 min at 95°C. Proteins were separated in a 7–16% linear gradient of PAGE under reducing conditions. Bio-Rad high- and low-molecular mass standards were used to estimate the molecular mass of each protein band. Gels were stained with Coomassie brilliant blue (G-250), destained, and dried. Incorporation of radiolabeled phosphate was detected by exposure of dried gels to x-ray film (XRP) that was developed in a Kodak X-Omat processor. Autoradiographs were quantitated by computerized image analysis with Microscan 1000-gel analysis software (Technology Resource, Nashville, TN).

Measurement and Analysis of LTP. Hippocampal slices were prepared according to standard procedures (17–19). After 1-hr preincubation, each slice was transferred to an interface recording chamber (volume = 1.0 ml) maintained at 33°C. A bipolar, stimulating electrode was placed on Schaffer collateral-commissural fibers and recording electrodes were guided into pyramidal or radiatum layers of CA1 to record population spike or excitatory postsynaptic potential (EPSP), respectively. The magnitude of the population spike was measured as an average distance between the highest negativity and preceding and following positivities. The initial slope of the negative wave was taken as a measure of field EPSP. The strength of the stimulation in all tested slices was in the range of 0.01–0.05 mA. The pulse duration was fixed at 150 μS. The potentials were monitored for 15–20 min, and experiments were performed only on slices with stable responses to low frequency (0.03 Hz) stimulation. To allow equilibrated diffusion into the slice, monoclonal antibodies (mAb 1.9 and mAb 1.12, both purchased from GIBCO) were added directly to the chamber (20 μg/ml final concentration) with a Hamilton syringe in a volume of 10 μl, 2 hr before the induction of LTP. All responses were evoked at 25% of the maximal response and monitored every 30 sec throughout the entire experiment, except for the brief periods of high-frequency stimulation (100 Hz for 1 sec repeated three times in 10-sec intervals) required to induce LTP. The data were stored in an IBM computer for subsequent analysis as described (18, 19).

RESULTS

Extracellular Protein Phosphorylation in Hippocampal Slices and Neurons. To examine directly whether the long-term increase of synaptic strength observed during the incubation of hippocampal slices with 0.5 μM extracellular ATP involves protein phosphorylation by ecto-PK (18, 19), we have incubated in the present study hippocampal slices with 0.5 μM radiolabeled [γ-^{32}P]ATP. The proteins that were phosphorylated by the exogenously added ATP in these reactions can be seen in the right lane of the representative autoradiogram depicted in Fig. 1*A*. Conclusive demonstration that the extracellular phosphorylation of these proteins plays a role in synaptic plasticity requires evidence that these proteins are substrates of ecto-PK on the surface of mature hippocampal pyramidal neurons, and then to identify a selective, membrane-impermeable inhibitor of this activity and determine its effects on LTP. Accordingly, we have implemented and improved conditions for culturing near-pure populations of pyramidal neurons from mouse hippocampus that undergo neuritogenesis and synaptogenesis in culture, and can be maintained for over 3 weeks in a serum-free, chemically defined medium. The appearance of these mature cells 18 days after plating [18 days *in vitro* (DIV)] is shown in Fig. 1*B*. These cells were incubated with [γ-^{32}P]ATP in a medium with the ionic composition of a physiological extracellular environment used both in studies of ecto-PK and of LTP. The left lane of the autoradiogram shown in Fig. 1*A* demonstrates that the two main proteins phosphorylated in pyramidal neurons by extracellular ATP comigrate with two of the proteins phosphorylated by [γ-^{32}P]ATP in hippocampal slices. These proteins migrated in the gels used here with apparent molecular mass of 48 kDa and 50 kDa.

Ecto-PK Activity on the Surface of Pyramidal Neurons. The criteria required as evidence for the presence of ecto-PK on the surface of the neurons studied here were fulfilled with experiments conducted as described in our previous reports (11–13, 21, 22). Results from three of these experiments are shown here in Fig. 2 and in Table 1. Pyramidal neurons at 18 DIV were incubated for 20 min with 0.1 μM [γ-^{32}P]ATP (15 μCi per well; see lanes marked "A") or with 15 μCi per well of inorganic [^{32}P]PO_4^{-3} (^{32}Pi; see lanes marked "P"). Both extracellular and intracellular phosphorylation of proteins are detected under these conditions, because radiolabeled ^{32}Pi formed by ecto-ATPase from added [γ-^{32}P]ATP penetrates the cells, whereas in cells labeled with ^{32}Pi, intracellularly

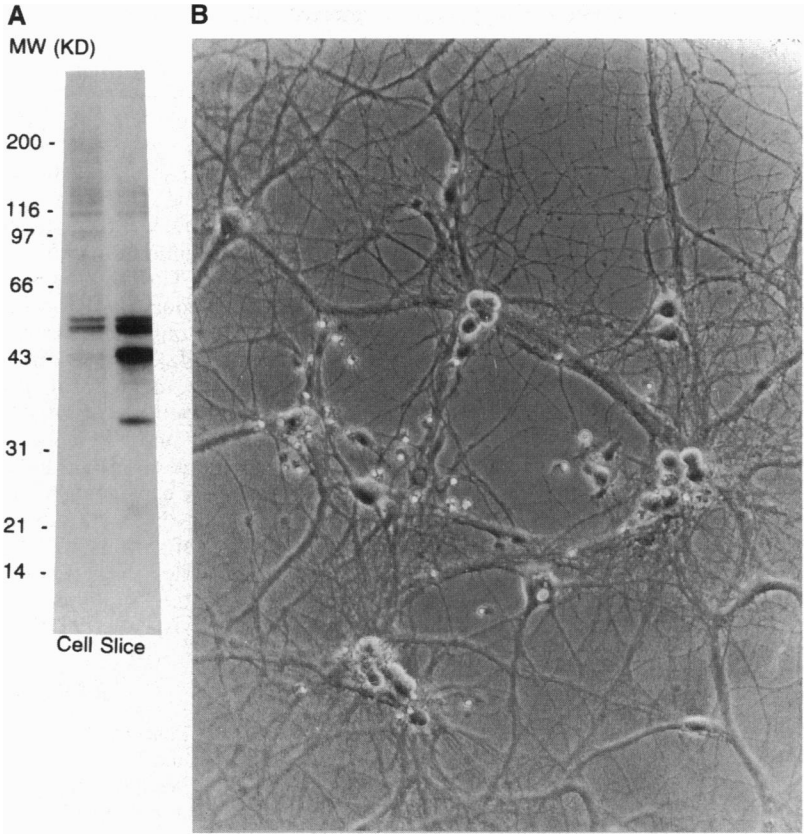

FIG. 1. (*A*) Phosphorylation of proteins by extracellular [γ-^{32}P]ATP in mouse hippocampal slices and in mouse hippocampal primary neurons. Hippocampal neurons (*Left*) or slices (*Right*) were incubated at 37°C with 15 μCi of [γ-^{32}P]ATP for 20 min in KRB as detailed. Final ATP concentration for hippocampal slices was 0.5 μM and for hippocampal neurons it was 0.1 μM, as these were the standard ATP concentrations used in our previous studies with these preparations (12, 13, 19). Solubilized reaction products were separated by 7–16% SDS/PAGE, and the autoradiogram depicted here is representative of five separate experiments. Reactions with cultured pyramidal neurons using final ATP concentration of 0.5 μM, and with slices under conditions identical to those used for the induction of LTP by ATP (18, 19) gave results very similar to those shown here in the left and right lanes, respectively. (*B*) Phase contrast photomicrograph (×400) of cultured hippocampal pyramidal neurons used in the reaction of *A*. Neurons from the hippocampus of mice embryos at embryonic day 16 were plated in 48-well plates at $1.2 \times 10^5/cm^2$ and maintained in a serum-free medium for 18 days until maturation as detailed.

produced [γ-^{32}P]ATP can be slowly released to the medium (12, 13, 21). However, the labeling of phosphoproteins that is detected after addition of ^{32}Pi is prevented when a 10,000 excess of nonlabeled ("cold") Pi is co-added with the label (see lane "P+" in Fig. 2*A*). Addition of "cold"-Pi with the [γ-^{32}P]ATP (lane marked "A+" in Fig. 2*A*) prevents the radiolabeling of intracellular proteins, but not the phosphorylation of extracellular sites in protein substrates of ecto-PK. Fig. 2*A* demonstrates that both of these requirements were fulfilled with pyramidal neurons. If the proteins phosphorylated by [γ-^{32}P]ATP in the presence of 1 mM "cold" Pi, as seen in lane "A+" of Fig. 2*A*, are indeed substrates of ecto-PK, their phosphorylation under these conditions should be prevented by adding the ATP consuming enzyme apyrase to the medium (see lane "A+" in Fig. 2*B*). However, intracellular phosphorylation of proteins by added ^{32}Pi (lane "P+" in Fig. 2*B*) should not be affected by the addition of apyrase. These requirements were also fulfilled as seen in Fig. 2*B*.

The endogenous protein substrates of ecto-PK on the surface of pyramidal neurons. Phosphorylation of the 48 kDa and 50 kDa protein substrates of ecto-PK in pyramidal neurons incubated with extracellular [γ-^{32}P]ATP as shown in lane "A+" of Fig. 2*A* could be detected within the shortest reaction time tested (1 min), and then increased gradually and reached plateau levels within 30–40 min. Assays conducted under the same conditions during the differentiation of these pyramidal neurons in-culture revealed that surface phosphorylation of the 48/50 kDa proteins is not detectable during the onset of neuritogenesis (1–4 DIV), becomes prominent during synaptogenesis (8–12 DIV) and peaks upon synaptic maturation (14–18 DIV) (data not shown; ref. 23). The same reaction conditions were used to determine that the 48 kDa/50 kDa proteins are phosphorylated on the surface of mature hippocampal neurons but not on hippocampal glial cells, as shown in Fig. 2*C*. Taken together, the results shown in Figs. 1 and 2 demonstrate that the 48/50 kDa proteins phosphorylated by extracellular [γ-^{32}P]ATP in hippocampal slices are neuronal-specific substrates of ecto-PK activity on the surface of mature hippocampal pyramidal neurons. These neurons form the synapses in which HFS and ATP induce LTP in the CA1 region of the hippocampus (1–3, 8–10, 18, 19).

Inhibition of ecto-PK activity by an anti-PKC antibody. Several studies provided direct evidence for a functional role of a specific protein phosphorylation system in synaptic activity by using an antibody that interacts with a specific component of this system (4). Among them, in a study related to the mechanisms of LTP, the authors used an antibody that interferes with the phosphorylation of protein B50 to demonstrate directly "a causal relationship between the PKC substrate B50 and the release of neurotransmitter" (24). Using the same approach, we tested here the effects of a monoclonal antibody termed mAb 1.9 on ecto-PK activity in pyramidal neurons. mAb 1.9 is an antibody developed by Mochly-Rosen and Koshland (25, 26) that interacts specifically with the catalytic domain of PKC and inhibits its enzymatic activity. We have found that in brain neurons cultured from chicken telenceph-

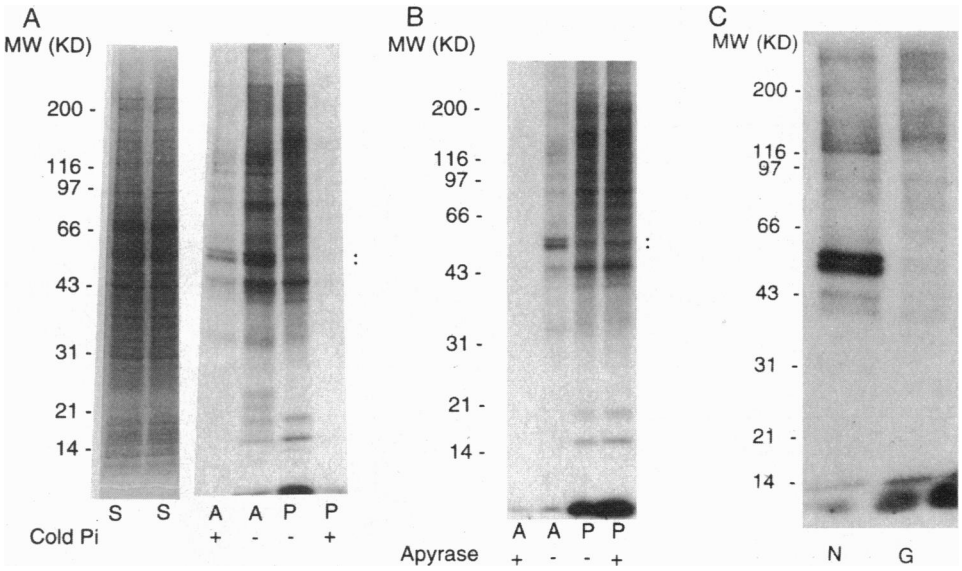

FIG. 2. Extracellular and intracellular protein phosphorylation in hippocampal neurons. (*A*) Effect of unlabeled inorganic phosphate. Cultured cells were incubated for 20 min at 37°C with 15 μCi of [γ-^{32}P]ATP (A) or ^{32}Pi (P) in KRB prepared without phosphate ions. Final ATP concentration was 0.1 μM. Two lanes of Commassie blue staining pattern of proteins obtained from cells under these two conditions (*Left*) showed no differences. In this autoradiogram, the lanes marked with (+) are those in which 1.0 mM unlabeled inorganic phosphate was included in the reaction buffer. (*B*) Effect of the ATP hydrolyzing enzyme apyrase. Cultured pyramidal neurons were incubated at 37°C for 20 min with 15 μCi of [γ-^{32}P]ATP in KRB with 1 mM unlabeled inorganic phosphate (A) or with 15 μCi of ^{32}Pi in KRB without unlabeled inorganic phosphate (P). Final ATP concentration was 0.1 μM. Apyrase (5 units/ml, Sigma) was added 30 sec before addition of the label. The lanes with apyrase are marked (+). Proteins were separated by 7–16% SDS/PAGE and autoradiograms are presented. Molecular mass standards (kDa) are shown on the left, and the asterisks indicate the positions of the endogenous protein substrates of ecto-PK referred to in the text as the 48 kDa/50 kDa duplex. (*C*) Extracellular protein phosphorylation by [γ-^{32}P]ATP in mouse hippocampal primary neurons and in mouse hippocampal glial cells. Cultured hippocampal neurons and glia were incubated at 37°C with 15 μCi of [γ-^{32}P]ATP (0.1 μM) for 20 min as in lane A+ of *A*. Proteins were separated by PAGE and a representative autoradiogram is depicted. Molecular mass standards (kDa) are shown on the left.

alon, addition of mAb 1.9 to the medium selectively inhibited the surface phosphorylation of specific substrates of an ecto-PK activity with catalytic properties of atypical PKCs (13). When mAb 1.9 was added to the reaction medium of hippocampal pyramidal neurons assayed for ecto-PK activity at 18 DIV, it inhibited selectively and significantly the surface phosphorylation of the 48 kDa and 50 kDa proteins (see Table 1), without affecting the surface phosphorylation of the 43 kDa protein labeled under these reaction conditions (seen in Fig. 1). Thus, the surface phosphorylation of the 48 kDa and 50 kDa proteins on hippocampal pyramidal neurons is carried-out by the enzymatic activity we have termed ecto-PKC (13). The acidic PK inhibitor K-252b was used in several previous studies to implicate extracellular protein phosphorylation in neuritogenesis, in synaptogenesis and in LTP (21, 23, 27–29). While questions raised recently concerning the membrane impermeability of K-252b (30) are yet to be resolved, the IgM molecule of mAb 1.9 represents a most appropriate membrane-impermeable probe for determining the involvement of ecto-PK activity in LTP.

Table 1. A monoclonal antibody directed to the catalytic region of PKC (mAb 1.9) inhibits the phosphorylation of specific protein substrates of ecto-PK on the surface of hippocampal pyramidal neurons

Phospho-protein, kDa	Control	mAb 1.9, % of control	*P*
50	100 ± 6.22	51.66 ± 5.11	0.0039
48	100 ± 10.29	47.72 ± 5.71	0.0113

mAb 1.9 (10 μg/ml; GIBCO) was added to the wells 1 hr before the initiation of the reactions by adding [γ-^{32}P]ATP, as detailed for lane "A+" in Fig. 2*A*. Phosphorylation of individual protein bands was quantified by computerized image analysis (13). Data shown are means ± SEM values from three separate experiments, and *P* values were obtained with Student's *t* test. Three identical experiments conducted with another batch of mAb 1.9 (GIBCO) produced very similar results.

Inhibition of Ecto-PK Prevents the Stabilization of LTP. LTP was induced in hippocampal slices by high frequency electrical stimulation (HFS; refs. 17–19). As expected, an increase was recorded in the slope of the EPSP (Fig. 3*A*) and in the amplitude of the population spike (Fig. 3*C*), and both remained stable for over 30 min. The slope of the EPSP (Fig. 3*B*) and the magnitude of the population spike (Fig. 3*D*) measured just before the application of HFS were not altered during long preincubation with mAb 1.9, and remained the same as those recorded before adding the antibody, which did not differ from those seen in the control slices (see Fig. 3 *A* and *C*, "a" trace). Thus, mAb 1.9 does not influence routine synaptic transmission. In contrast, mAb 1.9 had a time-dependent effect on the duration of the amplification of hippocampal evoked potentials induced by HFS, and prevented the stabilization of the LTP (Fig. 3 *B* and *D*). The slope of the EPSP (Fig. 3*B*) and the magnitude of the population spike (Fig. 3*D*) were initially elevated even in the presence of mAb 1.9. However, both parameters declined steadily to reach prestimulus values when HFS was applied to slices preincubated with mAb 1.9. The size and shape of the potentials recorded 30 min after HFS returned to the same values observed before HFS (Fig. 3 *B* and *D*, compare traces "a" to "c"), again confirming that mAb 1.9 does not interfere with basal activity. The identical time-dependence patterns of the attenuation by mAb 1.9 of the slope of the EPSP and of the magnitude of the population spike (compare Fig. 3 *B* and *D*) indicate that mAb 1.9 diminishes both the HFS-induced enhancement of synaptic efficiency and the increase in number of activated neurons, respectively. A control antibody, mAb 1.12, did not exert any effects on LTP (see Fig. 3 legend). The transient elevation in the hippocampal evoked potentials observed in the presence of mAb 1.9 was much longer than the 2–3 min period characteristic of posttetanic potentiation

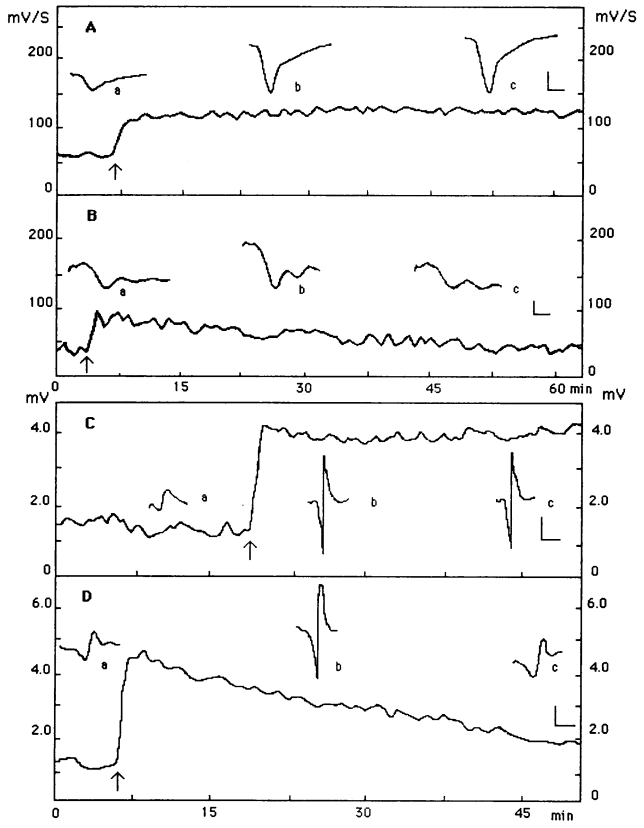

FIG. 3. The influence of mAb 1.9 on electrically induced LTP in hippocampal slices: Time-course from representative experiments. *A* and *B* represent an increase in the slope of EPSP following application of HFS in control slices and in slices preincubated with mAb 1.9, respectively. The amplification of the population spike induced by HFS in control slices and in the slices incubated with mAb 1.9 is depicted in *C* and *D*, respectively. The potentials shown at the upper part of each panel (marked as a, b, and c), represent the average of 10 potentials recorded 5 min before and 10 and 30 min after application of HFS, respectively. The lower part of each panel depicts the change in the EPSP slope (*A* and *B*) and magnitude of the population spike (*C* and *D*) during the entire recording period. *A* and *C* are representative of two types of control experiments that produced the same results: (*i*) slices incubated without adding any antibody, and (*ii*) slices incubated with the control antibody mAb. 1.12, added at the same time and concentration as mAb 1.9. mAb 1.12 binds to brain PKC but does not inhibit its activity. The arrow in each panel indicates the time-point of HFS application. Calibration: *A* and *B*, −2 mS, 0.8 mV; *C*, −2 mS, 1.0 mV; *D*, −2 mS, 1.25 mV.

(PTP). The gradual decline over 30–40 min thus represents interference by mAb 1.9 with the maintenance phase of LTP. As the early increase in evoked potentials recorded immediately after HFS application was identical in the presence and absence of mAb 1.9 (Fig. 4, 1 min bars), it is concluded that mAb 1.9 has no effects on the initial steps in the induction of LTP. However, in numerous experiments conducted with mAb 1.9 as exemplified in Fig. 3, we have never observed a persistent, stable elevation of the hippocampal evoked potentials (Fig. 4, 30 min bars). In summary, while mAb 1.9 has no effects on routine synaptic transmission nor on the initial induction of LTP, in the presence of this antibody the LTP induced by high frequency stimulation cannot be maintained.

DISCUSSION

The main new finding of the present study is that hippocampal pyramidal neurons have an ecto-PK that plays a role in the maintenance of stable LTP. This result complements our

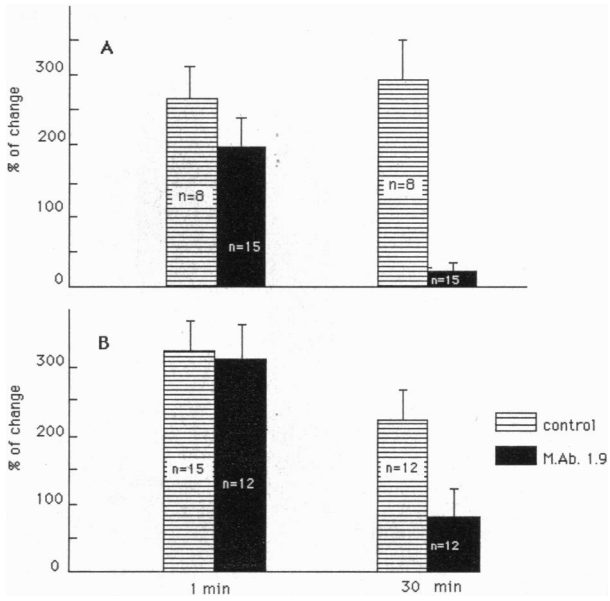

FIG. 4. The influence of mAb 1.9 on electrically induced LTP: average results recorded from the number of slices indicated by "n," using two separate batches of antibody. (*A*) The changes in EPSP. (*B*) The changes in population spike. The experiments were performed as described in the legend to Fig. 3. The results are expressed as the means ± SEMs of the percent of change from prestimulation values (observed 10 min before HFS), as recorded 1 min and 30 min after the application of HFS.

previous finding that extracellular ATP participates in the maintenance phase of LTP rather than in its initial induction (19). Furthermore, the results obtained here *in situ* are in complete agreement with studies of LTP in the hippocampus *in vivo,* in which the use of PK inhibitors revealed a phase of initial potentiation (0–5 min) that does not require PKC activity, and a PKC-dependent persistence phase between 5–60 min (9). This later phase of LTP requires the catalytic activity of PKC but not its regulatory domain (3, 9). The activity of an ecto-PK in pyramidal neurons, which is inhibited by an antibody that binds to the catalytic domain of PKC (mAb 1.9) but is not stimulated by phorbolesters, fulfills this prediction as well. The murine mAb, mAb 1.9, used in our studies was prepared by Mochly-Rosen and Koshland (25, 26) against a highly purified preparation of rat brain PKC. mAb 1.9 was found to bind near the active site of PKC and strongly inhibited the enzymatic activity of intact PKC as well as the enzymatic activity of the proteolytically prepared catalytic fragment of PKC called PKM (25, 26). The evidence that this antibody is directed specifically against PKC included removal of antibodies by immobilized purified PKC, inhibition of PKC activity and lack of inhibition of cAMP-dependent PK or calcium/calmodulin-dependent kinase (25, 26). Accordingly, we have termed the surface-located PK that is inhibited specifically by mAb 1.9 as ecto-PKC (13). As a control antibody we used in the present study mAb 1.12, which did not inhibit LTP (see Fig. 3 legend). Since mAb 1.12 also binds to neuronal PKC but does not inhibit its enzymatic activity (25, 26), this result supports the conclusion that inhibition of the catalytic activity of ecto-PKC is required for the inhibition of LTP by mAb 1.9.

All neurons express PKC, but addition of the IgM molecules of mAb 1.9 to the medium cannot inhibit intracellular PKC in intact cells. On the other hand, addition of mAb 1.9 to the medium can inhibit PKC activity in membrane-fragments from broken cells that may adhere to intact cultured neurons, and are always present in fresh tissue slices. However, interaction of an antibody with broken membrane fragments cannot inhibit LTP. The manner in which mAb 1.9 inhibits LTP thus

provides evidence that: (*i*) ecto-PK is a physiological activity operating on the surface of pyramidal neurons, and (*ii*) ecto-PKC activity is required for the maintenance of stable LTP. The period at which the 48 kDa/50 kDa protein substrates of ecto-PKC become phosphorylated for the first time on the surface of developing pyramidal neurons appears to coincide with the onset of synaptogenesis (23). If the extracellular phosphorylation of this surface protein duplex indeed provides a signal for triggering the formation of synapses during development, it could fulfill the same function in the adult brain during the maintenance phase of LTP.

Recent results have demonstrated the presence of the PKC-epitope that is recognized by mAb 1.9 on the external surface of the plasma membrane by immunofluorescent rim-staining in platelets (31), and visualization of the interaction of mAb 1.9 with an externally oriented epitope on the surface membrane of unfixed brain synaptosomes (carried-out by immunoelectron microscopy) has determined that ecto-PKC is concentrated in the region of junctional contacts, within the synaptic cleft (ref. 32, unpublished data). Ecto-PK operating on the surface of cells that store ATP within secretory vesicles and release it by exocytosis (neurons and platelets) is an activity-dependent enzyme, namely, it can play special roles in the chain of events involved in cell activation subsequent to cellular stimulation (12, 22). Furthermore, the phosphorylation of certain surface proteins by secreted ATP may be an event triggered uniquely by neuronal activity associated with processes underlying synaptic plasticity (14, 16). The finding that mAb 1.9 interferes with LTP but has no effect on routine transmission, supports the notion that phosphorylation of surface proteins by ecto-PKC is such a unique HFS-dependent biochemical event.

The requirement of ecto-PK for the maintenance of LTP indicates a role for this enzyme in mechanisms underlying synaptic plasticity. This conclusion opens a new line of investigation: the mechanisms by which the extracellular phosphorylation of surface proteins may regulate memory formation. The potential mechanisms include, but are not limited to, regulation by ecto-PK of neuronal cell-adhesion (11), dendritic-sprouting and/or synaptogenesis (21, 27–29), and the modulation of receptors (12, 33), uptake-carriers (34), and ion-fluxes (16) by secreted ATP. Due to the extracellular location of ecto-PK, peptides and antibodies that inhibit this enzyme may prove useful also for studies in which specific biochemical events can be successfully correlated with actual behavioral memory consolidation (6, 7, 35). Finally, taken together with the finding that Alzheimer β-amyloid peptides at relevant pathophysiological concentrations regulate neuronal ecto-PKC (13), our results suggest that aberrant surface protein phosphorylation activity may be a potential etiological factor in dementia, neurodegenerative disorders, and certain developmental disabilities.

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