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Autophosphorylation of type II Ca^{2+}/calmodulin-dependent protein kinase in cultures of postnatal rat hippocampal slices

(synaptic regulation/molecular switch)

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ABSTRACT **Autophosphorylation of Thr^{286} on type II Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) *in vitro* causes kinase activity to become partially independent of Ca^{2+}. Here we report that Thr^{286} is the major CaM kinase autophosphorylation site occupied *in situ* in "organotypic" hippocampal cultures. Measurement of Ca^{2+}-independent CaM kinase activity revealed that approximately one-third of the kinase is autophosphorylated *in situ* when the basal Ca^{2+} concentration is 15–43 nM. This proportion was substantially reduced 30 min after removal of extracellular Ca^{2+} or treatment of the cultures with protein kinase inhibitors and was increased by treatment with okadaic acid. Therefore, the high proportion of autophosphorylated kinase at basal Ca^{2+} concentrations appears to be maintained by Ca^{2+}-dependent autophosphorylation. Homogenates of intact hippocampi also contain a high proportion of Ca^{2+}-independent type II CaM kinase, 13–23% depending on developmental age. Thus, in hippocampal neurons, an important function of the autophosphorylation mechanism may be to produce a relatively high level of CaM kinase activity, even at basal Ca^{2+} concentrations, permitting both upward and downward local regulation by physiological agents.**

Brief tetanic stimulation of certain hippocampal synapses produces long-term potentiation, a long-lasting increase in the strength of synaptic transmission that may play a role in memory formation (1–3). In synapses of the perforant path and Schaffer collateral pathway, activation of *N*-methyl-D-aspartate (NMDA) receptors causes an increase in postsynaptic Ca^{2+} that is necessary for induction of long-term potentiation (4–6). Potentiation of transmission appears to result from enhanced release of presynaptic transmitter (7–9) and/or enhanced current through quisqualate-type glutamate receptors (10, 11). The molecular mechanisms that link postsynaptic Ca^{2+} influx to the enduring increase in synaptic strength are unknown.

One event thought necessary for induction of long-term potentiation is activation of type II Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase II) (12, 13). This kinase constitutes as much as 2% of total protein in neurons of the hippocampus (14). It is distributed throughout the neuronal cytosol (15) and is also the major component of the postsynaptic density (16, 17), where it appears to be positioned to respond to increases in Ca^{2+} concentration produced by activation of NMDA receptors. The kinase is a multimeric holoenzyme composed of ≈12 catalytic subunits encoded by at least two distinct genes (18–20). When the kinase is activated by Ca^{2+}/calmodulin, each subunit is rapidly autophosphorylated at a threonine residue (Thr^{286} in α and Thr^{287} in β) near the calmodulin-binding domain. Autophosphorylation of this residue in as few as one to three of the subunits in a holoenzyme causes all the subunits to remain partially active beyond the duration of the initial activating Ca^{2+} signal (21–23). Ca^{2+}-independent activity can be reversed by dephosphorylation of Thr^{286} and Thr^{287} (22).

The lack of appropriate model systems has limited the study of CaM kinase regulation in neurons of the central nervous system. Neuronal cell lines and primary cultures have been useful for physiological studies of synaptic transmission; however, they are not ideal for study of the biochemical events associated with synaptic transmission and its regulation. Neuronal cell lines usually display phenotypes more typical of embryonic neurons than of adult neurons. They contain low concentrations of type II CaM kinase (data not shown), and the density of synapses that they form in culture is relatively low. Primary cultures of embryonic hippocampal pyramidal cells begin to express adult levels of CaM kinase II subunits after several weeks in culture (24), but the density of synapses in the cultures is still low (25). Physiologists have successfully used acutely prepared slices of hippocampus to study long-term potentiation, but the slices contain a layer of dying tissue on either side that would be included in biochemical samples. "Organotypic" cultures of slices of rat hippocampus live for 8 weeks or more and have been shown to develop the cytoarchitecture, principal neuronal cell types, and major synaptic pathways found in the adult hippocampus (17, 26). In this study, we examined regulation of autophosphorylation of the kinase and its consequent Ca^{2+}-independent activity in these cultures.

METHODS

Reagents. Sprague–Dawley albino rats were obtained from Simonsen Laboratories (Gilroy, CA). Rat tail collagen was from D. McDowell and P. Patterson (California Institute of Technology). Rat type II CaM kinase, bovine synapsin I, and calmodulin were purified as described (27, 28). Protein kinase inhibitor (PKI) 14–24 amide (Walsh inhibitor) was purchased from Peninsula Laboratories. Radiochemicals were purchased from ICN. Pharmacological agents were obtained from Sigma or Tocris Nuramin (London). BAY K8644 was a gift from A. Scriabine (Miles Pharmaceutical Division, West Haven, CT). H7, HA1004, W7, and W5 were purchased from Seikagaku America (St. Petersburg, FL).

Preparation of Organotypic Cultures. Organotypic cultures of rat hippocampal slices were prepared by a modification of the roller-tube method of Gähwiler (29). Hippocampi from 4- to 6-day-old Sprague–Dawley albino rat pups were sliced into 400-μm parasagittal sections with a wire-grid slicer fabricated at the California Institute of Technology. Freshly cut slices were incubated at 4°C in Gey's balanced salt solution for 30

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Abbreviations: CaM kinase, type II Ca^{2+}/calmodulin-dependent protein kinase; NMDA, *N*-methyl-D-aspartate.

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min and then placed on glass coverslips coated with rat-tail collagen. Slices were immobilized in a clot of polymerized Vitrogen (Collagen Corp.) applied in 9:1 dilution with 10× minimum essential medium (MEM) and then placed in tubes containing 1 ml of medium (29) buffered with 25 mM Hepes, pH 7.4. For most experiments, cultures were treated with a mixture of antimitotic agents (0.33–1 μM each of uridine, 5-fluorodeoxyuridine, and cytosine β-D-arabinofuranoside) for 20 hr on the fourth or fifth day to limit proliferation of glial cells. Cultures grown in this way are two- to four-cell layers thick.

Assay for CaM Kinase Activity. Before treatments, medium was replaced with a physiological saline solution containing 125 mM NaCl, 2 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose (PS buffer) buffered either with 25 mM Hepes (pH 7.4) or by equilibration with 5% CO_2/95% O_2. Results were identical with either buffer. After treatment, slices were frozen on 60% propylene glycol (Lacco, South Gate, CA) precooled on dry ice, scraped from coverslips, homogenized on ice in 100 μl of H buffer per culture (20 mM Tris·HCl, pH 8/1 mM imidazole/2 mM EDTA/20 mM $Na_4P_2O_7$/soybean trypsin inhibitor at 25 mg/liter/leupeptin at 1 mg/liter/2 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride) in a glass/Teflon homogenizer, and immediately assayed for kinase activity. H buffer inhibited phosphatase and kinase activity, thus preserving the autophosphorylation state of endogenous and exogenously added CaM kinase (data not shown).

For assays, homogenate protein (2–4 μg) was added to a solution at 30°C containing synapsin I at 0.2 mg/ml, calmodulin at 50 μg/ml, 0.5 μM protein kinase inhibitor, 13 mM dithiothreitol, 6.6 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (2–3 × 10^3 cpm/pmol), 0.4 mM EGTA and, for assays in the presence of Ca^{2+}, 0.6 mM $CaCl_2$ in a final volume of 30 μl. Reactions were terminated after 15 s by adding 15 μl of $NaDodSO_4$ stop solution, and incorporation of ^{32}P into synapsin was measured as described (27, 28).

To determine the rate of Ca^{2+}-independent CaM kinase II activity in homogenates in which 100% of the kinase is in the autophosphorylated state, autophosphorylation of the kinase was initiated as described above, except that synapsin I was excluded and, in some cases, ATP was replaced by ATP[γ-thio]triphosphate. After 5–90 s of autophosphorylation, synapsin I or synapsin I plus EGTA (final concentration, 2 mM) was added directly to each tube, and phosphorylation was terminated after 15 s.

Labeling of CaM Kinase in Organotypic Cultures. Five- to 10-slice cultures were rinsed twice with phosphate-free Eagle's basal medium with Earle's Salts (BME/E)/1.8 mM $CaCl_2$/0.8 mM $MgSO_4$, supplemented as described for the culture medium (29), and then incubated for 12–24 hr at 37°C in phosphate-free BME/E with 2–3 mCi of carrier-free $H_3{}^{32}PO_4$ per ml (1 Ci = 37 GBq). At the end of incubation, cultures were rinsed twice with phosphate-free BME/E, frozen, and homogenized. Homogenates were brought to 1% Nonidet P-40, cleared by centrifugation at 10,000 × g for 1 min, and incubated for 8–12 hr at 4°C with monoclonal antibodies against type II CaM kinase [4A4, 4A11, and 6E9 (14); 10–20 μg/ml]. Immune complexes were precipitated by incubation for 2 hr with rabbit anti-mouse immunoglobulin and protein A-Sepharose CL-4B (20 mg/ml). The beads were collected by centrifugation at 10,000 × g for 2 min, washed six times with NET buffer (18) supplemented with 1% (vol/vol) Nonidet P-40 and twice with NET buffer, resuspended in $NaDodSO_4$ stop solution, and fractionated by $NaDodSO_4$/PAGE. Labeled subunits were detected by autoradiography.

Other Procedures. Tryptic phosphopeptide maps were prepared as described (22, 30). Protein concentrations were measured by the method of Wallace and Partlow (31), which is insensitive to collagen. $NaDodSO_4$/PAGE was performed by the method of Laemmli (32). Calcium measurements with Fura-2 were performed by W. Regehr and D. Tank as described (6).

RESULTS

Autophosphorylation of Thr^{286} *in Situ*. Cultures were incubated overnight in $^{32}PO_4$ and then homogenized (*Methods*). The α and β subunits of the kinase were immunoprecipitated and then separated by $NaDodSO_4$/PAGE (Fig. 1*B; Inset*). The α subunit was cut from the gel and digested with trypsin. Tryptic peptides were fractionated by reverse-phase HPLC to generate a phosphopeptide map (Fig. 1). The mobilities of the four phosphopeptides that contain Thr^{286} are known from previous studies of purified kinase (Fig. 1 *A* and *C*; refs. 22

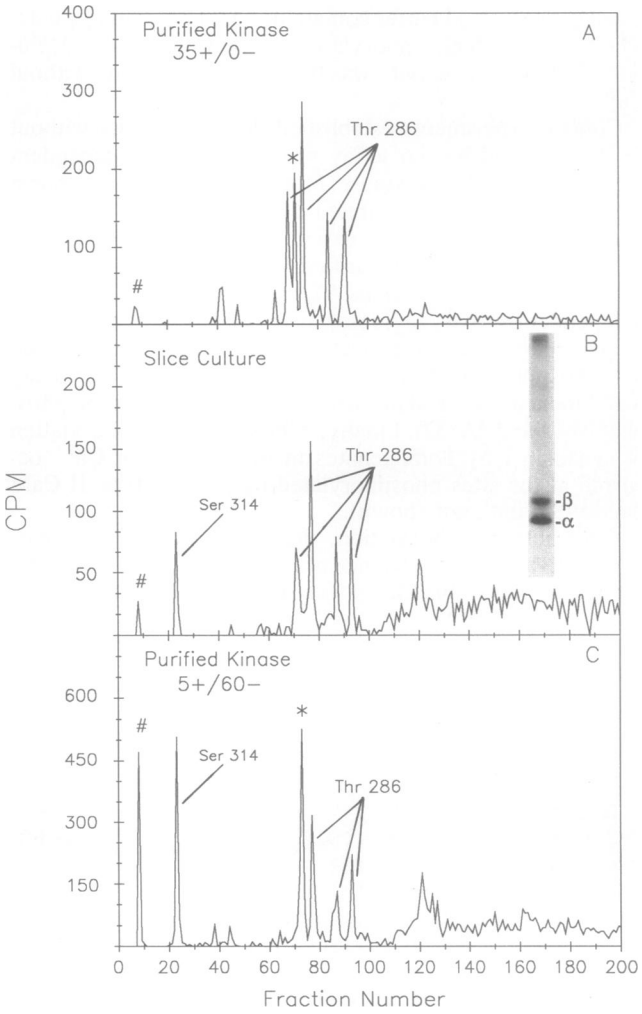

FIG. 1. Tryptic phosphopeptide map of the α subunit of type II CaM kinase phosphorylated *in situ* in organotypic hippocampal cultures. Purified kinase holoenzyme was autophosphorylated *in vitro* as described (22, 30) either with Ca^{2+} for 35 s (*A*) or with Ca^{2+} for 5 s and then without Ca^{2+} for an additional 60 s after Ca^{2+} had been chelated by EGTA (*C*). Labeled α subunit was isolated from culture homogenates. An autoradiogram of labeled kinase immunoprecipitated from the homogenate is shown as an inset in *B*. Tryptic peptide maps were prepared as described. In all maps, phosphopeptides containing Thr^{286} appear as a pair of split peaks (22). The N-terminal peptide, which contains a slowly autophosphorylated threonine residue, is labeled with an asterisk (30). In *C*, this peak has obscured the leading peak of the first Thr^{286} doublet. Also in *C*, several peaks are visible that contain sites autophosphorylated only after Ca^{2+} removal. These sites include Ser^{314}, Thr^{305}, and a peptide containing an unidentified site that elutes in the void volume (#; ref. 30). Similar results were obtained in three experiments.

and 30). Two peaks are generated when a portion of the amino-terminal glutamine cyclizes to pyroglutamate. Two additional peaks arise from these parent peaks by an unknown reversible reaction (22). Most labeled phosphate incorporated into the α subunit *in situ* was located on Thr^{286} (Fig. 1*B*). Some labeled phosphate was also recovered in the peptide containing Ser^{314}, a site autophosphorylated *in vitro* only when Ca^{2+} is removed after autophosphorylation of Thr^{286} (30).

Ca^{2+}-Independent Kinase Activity *in Situ*. The extent of phosphorylation of Thr^{286} *in situ* cannot be quantified reliably because of losses of material during the chemical procedures used to generate the peptide map. Therefore, we determined the proportion of the kinase in the Ca^{2+}-independent state by measuring the level of Ca^{2+}-independent CaM kinase activity in culture homogenates. Sets of five cultures were homogenized in an ice-cold buffer containing sodium pyrophosphate, which inhibits dephosphorylation of the CaM kinase (*Methods*). CaM kinase activity was then assayed with and without Ca^{2+}.

Control experiments established that the activity without Ca^{2+} measured by our assay represents Ca^{2+}-independent activity of type II CaM kinase. Monoclonal antibodies against the kinase specifically inhibited Ca^{2+}-independent activity in homogenates to the same extent that they inhibited Ca^{2+}-independent activity of purified kinase with synapsin I as substrate (88 ± 5% versus 97 ± 1%). In addition, Ca^{2+}-independent activity in homogenates decayed in the absence of pyrophosphate, as expected if the activity depends on autophosphorylation of Thr^{286} and Thr^{287} (Fig. 2). The decay was blocked by okadaic acid, a potent inhibitor of phosphatases 1 and 2A (33). Finally, >90% of the phosphorylation of synapsin I by homogenates in the absence of Ca^{2+} occurred at the sites phosphorylated by purified type II CaM kinase (34; data not shown).

After autophosphorylation, the CaM kinase is only partially activated in the absence of Ca^{2+}. Therefore, to convert measured ratios of Ca^{2+}-independent to Ca^{2+}-stimulated activity to actual percentages of CaM kinase molecules in the autophosphorylated state, we determined the maximum proportion of Ca^{2+}-independent activity that would be detected in our assay if all of the CaM kinase molecules in a homogenate were autophosphorylated. Aliquots of homogenates were incubated in the presence of Ca^{2+}/calmodulin and ATP for sufficient time to fully autophosphorylate endogenous CaM kinase at Thr^{286} and Thr^{287}. After autophosphorylation, the rate of synapsin phosphorylation without Ca^{2+} reached a maximum of 45 ± 1% of the rate with Ca^{2+}. This maximum percentage was not changed by substitution of ATP [γ-thio]-triphosphate for ATP in the autophosphorylation reaction. [Thiophosphorylated proteins are resistant to dephosphorylation by cellular phosphatases (18, 35)]. In subsequent experiments, the measured percentage of Ca^{2+}-independent activity to Ca^{2+}-stimulated activity in each homogenate was divided by 0.45 to convert it to the actual percentage of CaM kinase molecules in the autophosphorylated state.

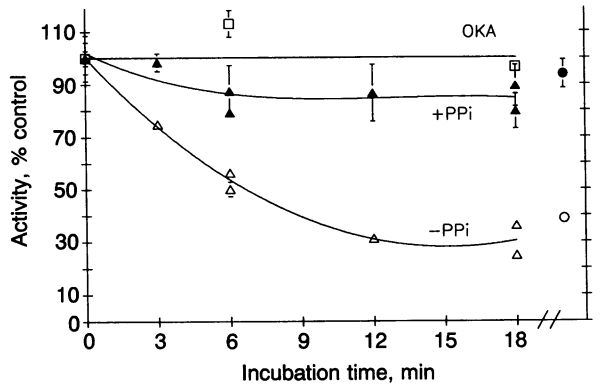

FIG. 2. Effect of phosphatase inhibition on the decay of Ca^{2+}-independent CaM kinase activity in homogenates of organotypic hippocampal cultures. Four sets of five hippocampal cultures were frozen and homogenized as described. Sodium pyrophosphate was omitted from the homogenization buffer for two sets, one of which contained 1 μM okadaic acid. CaM kinase activity was determined immediately with and without calcium. Aliquots of each homogenate were incubated at 20°C for the times indicated and then placed on ice; CaM kinase activity was then determined again. ▲, Ca^{2+}-independent activity in homogenates with sodium pyrophosphate; △, activity in homogenates without sodium pyrophosphate; and □, activity in homogenates with okadaic acid; error bars indicate SD. Ca^{2+}-independent activity also decayed more rapidly in the absence of sodium pyrophosphate in homogenates of 35-day-old rat hippocampi at 0°C. ●, With sodium pyrophosphate; ○, without sodium pyrophosphate. Total CaM kinase activity did not change during incubations. Each point represents quadruplicate assays.

Substantial levels of Ca^{2+}-independent activity were detected in homogenates of slice cultures, of adult forebrain and hippocampus, and of superfused acute slices of adult hippocampus (Table 1). The amount of CaM kinase in the Ca^{2+}-independent state was highest in homogenates of slice cultures (34%). The mean level in homogenates of forebrains from animals <25 days old was ≈23%, whereas in animals older than 25 days it was ≈13%. Thus, there appears to be a reduction in the level of autophosphorylation *in situ* of Thr^{286} and Thr^{287} between 5 and 7 weeks of age.

Basal Ca^{2+} concentrations in neurons in hippocampal cultures, measured in PS buffer equilibrated with 5% CO_2/95% O_2 as described under *Methods*, ranged from 15 to 43 nM (mean 31 ± 2 nM, n = 15). Therefore, the high proportion of autophosphorylated CaM kinase appears to be maintained even at the usual low basal cytosolic Ca^{2+} concentrations.

Stability of Ca^{2+}-Independent Kinase *in Situ*. The level of Ca^{2+}-independent activity in the cultures was not altered after incubation in 1 mM kynurenic acid for 1 hr to 2.5 days to block excitatory amino acid receptors, in 100–200 μM 5-phosphonoaminovaleric acid (AP5) to inhibit NMDA receptors, in 1 μM tetrodotoxin for 2 hr to inhibit any sponta-

Table 1. Proportion of CaM kinase autophosphorylated *in situ* in cultures and in tissues

Tissue homogenate	n	Ca^{2+}-independent activity, %	CaM kinase in Ca^{2+}-independent state, % total
Maximally autophosphorylated homogenates	10	45 ± 1	100
Hippocampal cultures (2–8 weeks in culture)	44	15.5 ± 0.6	34 ± 1
Forebrain (4–24 days of age)	12	10.5 ± 0.3	23.3 ± 0.9
Hippocampus (5–6 days of age)	2	9.0 ± 0.2	19.8 ± 0.3
Forebrain (27–72 days of age)	11	6.0 ± 0.3	13.2 ± 0.7
Hippocampus (25–57 days of age)	4	5.8 ± 0.5	13 ± 1
Acutely prepared hippocampal slices (40–70 days of age)	8	4.0 ± 0.7	9 ± 2

Cultures and tissues were homogenized, and type II CaM kinase activity was measured in the homogenates as described. Percent Ca^{2+}-independent activity was calculated for each homogenate and then divided by percent Ca^{2+}-independent activity in maximally autophosphorylated homogenates (45%) to calculate percent of total CaM kinase in the Ca^{2+}-independent state.

neous electrical activity, or in serum-free medium for 12 days. Similarly, growth of cultures for 2.5 weeks in 100 μM 5-phosphonoaminovaleric acid/20 mM Mg^+, beginning immediately after their preparation, did not reduce the level of Ca^{2+}-independent activity.

Depolarization of neurons in the cultures by adding 60 mM K^+ to the bathing medium produced an average 38% increase (range, 14–78%) in Ca^{2+}-independent activity in approximately half of the experiments (8 of 18) and no detectable change in the other experiments. Application of 100 μM NMDA to the cultures had no effect on the level of Ca^{2+}-independent kinase activity after 10–15 s (n = 11), although the same treatment consistently increased cytosolic Ca^{2+} concentrations, which peaked at ≈10 s (data not shown). After incubation of cultures for 30 min in 1–2 μM okadaic acid, a phosphatase inhibitor (33), addition of 100 μM NMDA only slightly increased Ca^{2+}-independent kinase activity (9.6 ± 6.3%, n = 9). The addition of glutamate, BAY K8644, nifedipine, picrotoxin, or carbachol, in normal or depolarizing salt solution, did not alter the proportion of Ca^{2+}-independent kinase.

In contrast to treatments that affect membrane channels and receptors, application of the membrane-permeant protein kinase inhibitor H7 and the calmodulin antagonist W7 reduced the steady-state proportion of Ca^{2+}-independent kinase *in situ*. Exposure of cultures to 300 μM H7, which has a K_i for the CaM kinase of 30–50 μM (data not shown), reduced the proportion of Ca^{2+}-independent kinase in homogenates by 35% (range, 10–52%; n = 7) after 30 min. HA1004 (300 μM), which has a K_i for CaM kinase of 16 μM but is less membrane permeable, reduced the proportion by 26% (range, 22–28%; n = 3). W7 (100 μM) produced an average 60% reduction (range, 48–72%; n = 2), whereas W5 (100 μM), a much less potent antagonist, produced a 29% reduction (range, 28–29%; n = 2). None of these treatments affected total Ca^{2+}-stimulated kinase activity. The proportion of Ca^{2+}-independent kinase was also substantially reduced by removal of Ca^{2+} from the external medium. Two hours after Ca^{2+} removal, the proportion of Ca^{2+}-independent kinase in homogenates was reduced by 60–70% (Fig. 3*A*). The decline was complete after 30 min with a half time of ≈5–7 min (Fig. 3*B*). Removal of external Ca^{2+} also dramatically reduced autophosphorylation of Thr^{286} *in situ*, as evidenced by reduced incorporation of $^{32}PO_4$ into tryptic peptides containing Thr^{286} (data not shown). Removal of Ca^{2+} had no significant effect on incorporation of $^{32}PO_4$ into tryptic peptides containing Ser^{314}. Taken together, these results suggest that continuing Ca^{2+}-stimulated autophosphorylation of the kinase at Thr^{286} is required to maintain the level of Ca^{2+}-independent activity for >20–30 min *in situ*.

The steady-state proportion of Ca^{2+}-independent kinase was increased by okadaic acid, a membrane-permeant inhibitor of phosphatases 1 and 2A. Incubation of the cultures with 1–2 μM okadaic acid for 30 min increased the proportion of Ca^{2+}-independent kinase by 40 ± 9% (n = 8). Thus, the proportion of Ca^{2+}-independent kinase is dynamic and depends upon the balance between rates of autophosphorylation and phosphatase activity.

DISCUSSION

We have shown that Thr^{286} is the major site autophosphorylated on type II CaM kinase *in situ* in organotypic cultures of hippocampal neurons. Approximately one-third of the CaM kinase molecules are in the Ca^{2+}-independent state when the basal Ca^{2+} concentration in the neurons is 15–45 nM. This surprisingly high proportion of Ca^{2+}-independent kinase appears to be maintained by a dynamic steady-state between autophosphorylation and dephosphorylation of Thr^{286}. Treatment of the neurons with the protein kinase

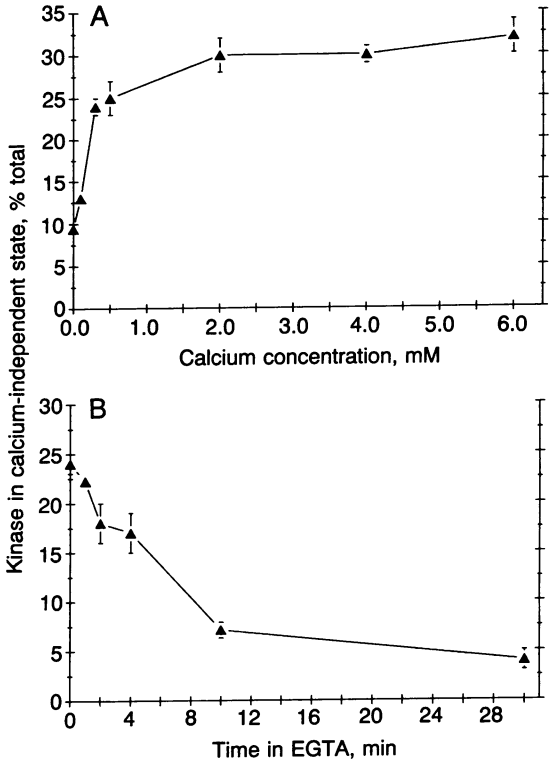

FIG. 3. Effect of external calcium concentration on Ca^{2+}-independent CaM kinase activity *in situ*. (*A*) Cultures were equilibrated in a salt solution with the indicated concentrations of added calcium for 2–3 hr at 37°C, then frozen and homogenized as described. Ca^{2+}-independent kinase was determined as percentage of total kinase as described. (*B*) Cultures were placed into a salt solution containing 200 μM EGTA at 37°C for the indicated times, after which each culture was frozen and homogenized. Percentage of CaM kinase in the Ca^{2+}-independent state was determined as described. Changes in external Ca^{2+} concentration had no effect on total CaM kinase activity. Each point represents mean ± SEM of two to four separate experiments.

inhibitor H7 or the calmodulin antagonist W7 reduces the basal proportion of Ca^{2+}-independent kinase, whereas treatment with the phosphatase inhibitor okadaic acid increases it. Removal of external Ca^{2+} also reduces basal Ca^{2+}-independent kinase, implying that continuous Ca^{2+}-dependent autophosphorylation is necessary to maintain the basal Ca^{2+}-independent activity for >20–30 min (Fig. 3*B*).

The high basal proportion of Ca^{2+}-independent activity is also found in homogenates of freshly dissected rat forebrain and hippocampus. Dissected brain regions were homogenized rapidly under conditions that preserve the endogenous protein-phosphorylation state, and the homogenates were assayed immediately. Therefore, the measured levels of Ca^{2+}-independent activity probably reflect accurately the autophosphorylation state of the kinase *in vivo*, although we cannot rule out changes in the levels during dissection. The drop from 23% Ca^{2+}-independent kinase to 13% between postnatal days 23 and 26 suggests a developmental change in expression of a protein phosphatase or perhaps in Ca^{2+} mobilization or buffering systems. Similarly, the higher level of Ca^{2+}-independent kinase in cultured hippocampal neurons compared to intact tissue may reflect, in part, a difference in the balance of processes that affect the equilibrium autophosphorylation state.

Phosphorylation of CaM kinase II in intact cells or synaptosomes has been demonstrated previously. Addition of serum to a fibroblast cell line stimulated phosphorylation of type II CaM kinase at serine residues (36). Depolarization of isolated synaptosomes with high K^+ enhanced phosphory-

lation of threonine in the CaM kinase and generated transient Ca^{2+}-independent activity (37). Fukanaga *et al.* (38) reported that, in cultured cerebellar granule neurons, under basal conditions, 4–7% of the total kinase molecules are in the Ca^{2+}-independent state. In contrast to our results with hippocampal neurons, brief depolarization of the cerebellar neurons with high K^+ reliably produced a transient increase in the proportion of Ca^{2+}-independent CaM kinase to ≈13% of total kinase molecules. Thus, not only do cerebellar neurons in culture have a much lower basal proportion of Ca^{2+}-independent kinase than hippocampal neurons, but this proportion is more readily altered by brief changes in Ca^{2+} concentration.

The high, stable proportion of Ca^{2+}-independent CaM kinase in hippocampal neurons indicates that previous hypotheses about the function of the CaM kinase and its activation by autophosphorylation may require revision. For example, the bulk of CaM kinase within hippocampal neurons does not behave as predicted by the model for memory storage proposed by Lisman and Goldring (18, 39, 40). However, it is possible that the neurons contain a small pool of CaM kinase that is sensitive to regulation by NMDA receptors and behaves in a switch-like fashion, as predicted by their model. We may have been unable to detect large changes in autophosphorylation of such a pool with our present methods because of the high background of Ca^{2+}-independent kinase. Nevertheless, our results suggest that a different but important physiological role for the autophosphorylation mechanism in hippocampal neurons may be to keep a substantial portion of the CaM kinase molecules (13–34%) active at low Ca^{2+} concentrations. The level of basal CaM kinase activity in the cytosol of hippocampal neurons predicted by kinetic equations is considerably less than 1% in the absence of activation by autophosphorylation, assuming a basal Ca^{2+} concentration of 50 nM, a calmodulin concentration of 50 μM, four Ca^{2+}-binding sites on calmodulin with K_d values of 1 μM, and a CaM kinase concentration of 30 μM. The high concentration of CaM kinase in hippocampal neurons (14) may enhance its rate of activation by the few molecules of calmodulin with four bound Ca^{2+} ions at basal Ca^{2+}. Furthermore, a low concentration of basal phosphatase activity may allow each activation event to produce a long-lived autophosphorylated kinase holoenzyme. This mechanism would permit rapid regulation of kinase activity, both downward by activation of phosphatases and upward by elevations in Ca^{2+} concentration. However, our inability to induce an increase in the overall proportion of Ca^{2+}-independent kinase by application of pharmacological agents to hippocampal neurons suggests that if such regulation occurs in these neurons, it is likely to be highly localized.

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1. Bliss, T. V. P. & Lomo, T. (1973) *J. Physiol. (London)* **232**, 331–356.
2. Morris, R. G. M., Anderson, E., Lynch, G. S. & Baudry, M. (1986) *Nature (London)* **319**, 774–776.
3. Kennedy, M. B. (1989) *Cell* **59**, 777–787.
4. Lynch, G., Larson, J., Kelso, S., Barrionuevo, G. & Schottler, F. (1983) *Nature (London)* **305**, 719–721.
5. Malenka, R. C., Kauer, J. A., Zucker, R. S. & Nicoll, R. A. (1988) *Science* **242**, 81–84.
6. Regehr, W. G. & Tank, D. W. (1990) *Nature (London)* **345**, 807–810.
7. Bliss, T. V. P., Douglas, R. M., Errington, M. L. & Lynch, M. A. (1986) *J. Physiol. (London)* **377**, 391–408.
8. Malinow, R. & Tsien, R. W. (1990) *Nature (London)* **346**, 177–180.
9. Bekkers, J. M. & Stevens, C. F. (1990) *Nature (London)* **346**, 724–729.
10. Kauer, J. A., Malenka, R. C. & Nicoll, R. A. (1988) *Neuron* **1**, 911–917.
11. Muller, D., Joly, M. & Lynch, G. (1988) *Science* **242**, 1694–1697.
12. Malenka, R. C., Kauer, J. A., Perkel, D. J., Mauk, M. D., Kelly, P. T., Nicoll, R. & Waxham, M. N. (1989) *Nature (London)* **340**, 554–557.
13. Malinow, R., Schulman, H. & Tsien, R. W. (1989) *Science* **245**, 862–866.
14. Erondu, N. E. & Kennedy, M. B. (1985) *J. Neurosci.* **5**, 3270–3277.
15. Ouimet, C. C., McGuinness, T. L. & Greengard, P. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5604–5608.
16. Kennedy, M. B., Bennett, M. K. & Erondu, N. E. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 7357–7361.
17. Kennedy, M. B., Bennett, M. K., Bulleit, R. F., Erondu, N. E., Jennings, V. R., Miller, S. G., Molloy, S. S., Patton, B. L. & Schenker, L. J. (1991) *Cold Spring Harbor Symp. Quant. Biol.*, in press.
18. Miller, S. M. & Kennedy, M. B. (1986) *Cell* **44**, 861–870.
19. Bennett, M. K. & Kennedy, M. B. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1794–1798.
20. Lin, C. R., Kapiloff, M. S., Dugrain, S., Tatemoto, K., Russo, A. F., Hanson, P., Schulman, H. & Rosenfeld, M. G. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5962–5966.
21. Bulleit, R. F., Bennett, M. K., Molloy, S. S., Hurley, J. B. & Kennedy, M. B. (1988) *Neuron* **1**, 63–72.
22. Miller, S. G., Patton, B. L. & Kennedy, M. B. (1988) *Neuron* **1**, 593–604.
23. Hanson, P. I., Kapiloff, M. F., Lou, L. L., Rosenfeld, M. G. & Schulman, H. (1989) *Neuron* **3**, 59–70.
24. Scholz, W. K., Baitinger, C., Schulman, H. & Kelly, P. T. (1988) *J. Neurosci.* **8**, 1039–1051.
25. Bartlett, W. P. & Banker, G. A. (1984) *J. Neurosci.* **4**, 1954–1965.
26. Gähwiler, B. H. (1988) *Trends Neurosci.* **11**, 484–489.
27. Bennett, M. K., Erondu, N. E. & Kennedy, M. B. (1983) *J. Biol. Chem.* **258**, 12735–12744.
28. Miller, S. G. & Kennedy, M. B. (1985) *J. Biol. Chem.* **260**, 9039–9046.
29. Gähwiler, B. H. (1984) *Experientia* **40**, 235–308.
30. Patton, B. L., Miller, S. G. & Kennedy, M. B. (1990) *J. Biol. Chem.* **265**, 11204–11212.
31. Wallace, L. J. & Partlow, L. M. (1978) *Anal. Biochem.* **87**, 1–10.
32. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
33. Bialojan, C. & Takai, A. (1988) *Biochem. J.* **256**, 283–290.
34. Huttner, W. B. & Greengard, P. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5402–5406.
35. Gratecos, D. & Fischer, E. H. (1974) *Biochem. Biophys. Res. Commun.* **58**, 960–967.
36. Ohta, Y., Ohba, T., Fukunaga, K. & Miyamoto, E. (1988) *J. Biol. Chem.* **263**, 11540–11547.
37. Gorelick, F. S., Wang, J. K. T., Lai, Y., Nairn, A. C. & Greengard, P. (1988) *J. Biol. Chem.* **263**, 17209–17212.
38. Fukanaga, K., Rich, D. P. & Soderling, T. R. (1989) *J. Biol. Chem.* **264**, 21830–21836.
39. Lisman, J. E. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3055–3057.
40. Lisman, J. E. & Goldring, M. A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5320–5324.