tion, transgenic cells are first selected, but then a second round of selection is necessary to identify the correctly targeted cells. The two rounds of selection will require a greater number of cell divisions, and the cells could easily become senescent by the time the correctly targeted cells are identified, and they would certainly be senescent before a homozygous mutant is produced. To address this problem, we generated a 40-day-old fetus using the CL1 cell line at 0.8 population doubling from senescence. The fetus was removed from the uterus, and fibroblasts were derived from it. The number of population doublings until senescence was 31 and 33 for the nuclear transfer and same-age nonmanipulated fetal fibroblasts, respectively. These data suggest that fibroblast life-span can be enhanced by nuclear transfer. This approach could enable us to generate as many gene targeting events as needed by subjecting the cell line to the successive rounds of nuclear transfer.

This somatic cell nuclear transfer procedure could improve the efficiency of producing transgenic cattle and broaden the scope of applications for transgenic cattle. With previous microinjection techniques, about 500 embryos would have to be injected and transferred to recipient cows to get one transgenic offspring (*16*). For the nuclear transfer technique with transgenic somatic cells, the transfer of nine embryos to four cows produced a transgenic offspring, greatly reducing the time and costs involved. With the nuclear transfer approach, an entire herd of the appropriate sex transgenic cattle could be produced in one generation, whereas the traditional microinjection approach would require at least two generations, and likely more, to obtain a production herd. This is a savings of 2 years for each generation. Finally, the somatic cell nuclear transfer approach could broaden the scope of use of transgenic cattle because it allows the targeting of DNA inserts to specific sites in the genome. This is important for deleting or replacing bovine genes that might interfere with human protein isolation or cause rejection of grafted tissues. Inserting genes into a selected site could be used to ensure tissue-specific and consistent expression levels of transgenes. Furthermore, insertion of genes into the same site in multiple lines of animals could be used to quickly generate homozygous lines of animals while avoiding inbreeding.

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10. The plasmid pCMV/βGEO was constructed by inserting the βGEO fusion fragment {Xho I to Xba I [G. Fiedrich *et al.*, *Genes Dev.* **5** (no. 9), 1513 (1991)]} into pcmvβ (Clonotech) between Not I sites.
11. The transgene was detected in transfected cells, fetuses, and tissue from adult animals by PCR with a 21-base sense primer (ACT3βGEO) 5′-CGCTGTGGTACACGCTGTGCG-3′, and a 22-base antisense primer (ACT4βGEO) 5′-CACCATCCAGTGCAGGAGCTCG-3′ (Amitof Biotech). Reactions were run for 35 cycles, with denaturation at 95°C for 30 s, annealing at 65°C for 1 min, followed by extension for 2 min at 72°C. A final extension for 10 min at 72°C was included after the last cycle. The amplified product was a 782–base pair (bp) fragment. The samples were analyzed by separating them by size in a (1%) tris-borate EDTA agarose gel containing ethidium bromide.
12. For nuclear transfer, bovine oocytes were aspirated from slaughter-collected ovaries, put in maturation media [P. Damiani *et al.*, *Mol. Reprod. Devel.* **45**, 521 (1996)], and shipped to the laboratory overnight at 38.5°C. Oocytes were mechanically enucleated at 18 hours after maturation, and chromosome removal was assessed with bisBENZIMIDE (Hoechst 33342; Sigma, St. Louis, MO) dye under ultraviolet light. Successfully enucleated oocytes were fused with actively dividing CL1 fibroblasts by using one electrical pulse of 180 volts/cm for 15 μs (Electrocell Manipulator 200, Genetronics, San Diego, CA). After 2 to 4 hours, oocytes were then chemically activated with calcium ionophore (5 μM) for 4 min (catalog number 407952; Cal Biochem, San Diego, CA) and 2 mM 6-dimethylaminopurine (DMAP, Sigma) in CR2 with bovine serum albumin (BSA) (3 mg/ml) for 3 hours (fatty acid free, Sigma) [J. L. Susko-Parrish *et al.*, *Devel. Biol.* **166**, 729 (1994)]. After activation, eggs were washed in hamster embryo culture medium (HECM)–Hepes five times and placed, for culture, in a 500-μl well of CR2 (Specialty Media, Lavallette, NJ) with BSA (3 mg/ml) (fatty acid free) along with 1×10^6 mouse embryonic fibroblasts (MEFs) per milliliter. Incubation was performed for 6.5 days at 38.5°C and 5% CO_2 in air, and selected embryos were shipped overnight in a portable incubator to the embryo transfer facility.
13. Calf tissue samples (peripheral ear tissue) were prepared for Southern blot analysis according to P. Laird *et al.* [*Nucleic Acids Res.* **19**, 4293 (1991)]. Ten micrograms of genomic DNA was run in a 0.75% agarose gel in tris-acetate buffer, transferred onto Zetabind (Cuno, Meriden, CT), cross-linked by ultraviolet light, prehybridized for 4 hours, then hybridized at 42°C for 8 hours. The blot was washed and exposed to Biomax film (Kodak). A probe of 3881 bp, EcoR I–EcoR I DNA fragment of βGEO was labeled with ^{32}P-deoxycytidine triphosphate (dCTP) by using a random primed labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).
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17. We thank J. Balise, R. Duby, J. Morris, N. Kieser, E. Dickinson, and N. Ward Mackall for their technical assistance; G. Morgan for cell cycle studies; J. Hill, S. R. Bumgardner, and A. J. Russel for attending calves at birth; and C. Looney for embryo transfer work. Supported by a grant from Advanced Cell Technology to the University of Massachusetts.

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A Signaling Complex of Ca^{2+}-Calmodulin–Dependent Protein Kinase IV and Protein Phosphatase 2A

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Stimulation of T lymphocytes results in a rapid increase in intracellular calcium concentration ($[Ca^{2+}]_i$) that parallels the activation of Ca^{2+}-calmodulin–dependent protein kinase IV (CaMKIV), a nuclear enzyme that can phosphorylate and activate the cyclic adenosine monophosphate (cAMP) response element–binding protein (CREB). However, inactivation of CaMKIV occurs despite the sustained increase in $[Ca^{2+}]_i$ that is required for T cell activation. A stable and stoichiometric complex of CaMKIV with protein serine-threonine phosphatase 2A (PP2A) was identified in which PP2A dephosphorylates CaMKIV and functions as a negative regulator of CaMKIV signaling. In Jurkat T cells, inhibition of PP2A activity by small t antigen enhanced activation of CREB-mediated transcription by CaMKIV. These findings reveal an intracellular signaling mechanism whereby a protein serine-threonine kinase (CaMKIV) is regulated by a tightly associated protein serine-threonine phosphatase (PP2A).

Cellular responses to external signals require coordinated control of protein kinases and phosphatases; multiple complexes containing both intracellular signaling enzymes are likely to be important for the regulation and specificity of signal transduction pathways. The targeting of protein kinases and phosphatases to specific subcellular compartments, through association with scaffold proteins such as A-kinase anchoring proteins, may contribute to the specificity of cellular signaling (*1*). However, the enzymes are retained by the anchoring protein in their inactive state (*2*), and potential regulatory interactions within these multiprotein complexes remain unknown. Preexisting com-

plexes containing active protein kinases and phosphatases provide an alternative and conceptually attractive mechanism by which the appropriate phosphorylation state of intracellular substrates is maintained. The α subunit of casein kinase II (CKII), but not the CKII holoenzyme (containing α and β subunits), associates with the catalytic (C) subunit of PP2A (*3*). This association was proposed as a mechanism for growth suppression in which CKIIα-stimulated phosphorylation of the C subunit of PP2A would increase the activity of the phosphatase. However, PP2A exists in cells as a heterotrimeric holoenzyme rather than as a free catalytic subunit (*4*), and phosphorylation of the C subunit of PP2A on Ser-Thr residues in vivo has not been shown.

CaMKIV is important for T cell activation (*5*). Stimulation of the T cell receptor (TCR) causes increases in $[Ca^{2+}]_i$ and activation of CaMKIV. Because CaMKIV phosphorylates the nuclear protein CREB on Ser^{133}, it appears to contribute to increased transcription of immediate early genes containing CRE sequences. The immediate early genes are required for increased expression of the interleukin-2 (IL-2) gene (*6*). Activation of CaMKIV in T cells is transient, rising to 15 times the background amount by 1 min after TCR stimulation and returning to basal activity within 5 min (*7–9*). This decline in CaMKIV activity occurs despite a maintained $[Ca^{2+}]_i$ in excess of that needed for activation of the enzyme. After binding of Ca^{2+}-calmodulin, CaMKIV is phosphorylated on a single Thr residue in the activation loop by a Ca^{2+}-calmodulin–dependent kinase kinase (CaMKK) (*9*, *10*) and, once activated, CaMKIV activity becomes independent of Ca^{2+} and calmodulin (*8*, *9*, *11*, *12*). Because phosphorylation of CaMKIV is required to generate autonomous activity, we reasoned that a protein phosphatase might inactivate CaMKIV even in the presence of increased $[Ca^{2+}]_i$.

Phosphorylated CaMKIV (*8*) [as well as CREB phosphorylated on Ser^{133} (*13*)] is an in vitro substrate for PP2A. To determine whether these enzymes exist as a complex in cells, we immunoprecipitated CaMKIV from Jurkat T cells and analyzed the immune complex by immunoblotting with an antibody specific for the C subunit of PP2A. As shown in Fig. 1A, PP2A coimmunoprecipitated with CaMKIV (*7*). Because both CaMKIV and PP2A proteins are abundant in the brain, we used this tissue to further test whether CaMKIV and PP2A exist as a multiprotein complex. Rat brain soluble extracts were incubated with a glutathione S-transferase–CaMKIV (GST-CaMKIV) fusion protein (*9*). Both the C and Bα regulatory subunits of PP2A were isolated with wild-type GST-CaMKIV (Fig. 1B). Because the B subunit is variable among holoenzyme preparations of PP2A, these results suggested the presence of a CaMKIV-PP2A holoenzyme complex.

To independently assess the presence of a complex, we tested whether PP2A holoenzyme bound calmodulin-Sepharose (an affinity resin for CaMKIV). A small fraction (less than 10%) of PP2A holoenzyme (ABαC) from rat brain soluble extracts bound calmodulin-Sepharose in a Ca^{2+}-dependent manner (Fig. 1C). We attempted to copurify CaMKIV and PP2A from rat brain soluble extract by sequential purification on phenyl-Sepharose, calmodulin-Sepharose, Mono Q, and Superdex-200 gel filtration columns (*14*). Immunoblot analysis of the gel filtration fractions demonstrated that CaMKIV remained associated with the PP2A holoenzyme (Fig. 1D). Calmodulin overlay of the peak gel filtration fraction revealed the presence of a single Ca^{2+}-calmodulin–binding protein that comigrated with CaMKIV (Fig. 1E). The CaMKIV-PP2A complex had an apparent molecular mass of 232 kD (from gel filtration chromatography), and both its size and protein immunoblot analysis (*15*) indicated that the PP2A heterotrimer and kinase were present in a 1:1 ratio. Furthermore, immune complexes obtained from the peak gel filtration fraction with an antibody specific for CaMKIV contained CaMKIV and PP2A in a 1:1 stoichiometry (*7*, *16*).

To determine whether the kinase domain of CaMKIV is sufficient to interact with PP2A, and to test whether kinase activity was necessary for this interaction, we analyzed a series of GST-CaMKIV deletion and point mutants for their ability to interact with PP2A (*9*, *17*). Wild-type and several mutant GST-CaMKIV fusion proteins associated with PP2A, but GST alone (Fig. 2A) or glutathione-Sepharose beads did not (*18*). As evidenced by the interaction of the deletion mutant that contained the entire catalytic domain (residues 1 to 317), association with PP2A did not require the autoinhibitory or calmodulin-binding domains of CaMKIV (Fig. 2A). In contrast, an NH_2-terminal deletion mutant of CaMKIV that contained amino acid residues 306 to 474 did not interact with PP2A. Catalytically inactive GST-CaMKIV mutants and a mutant GST-

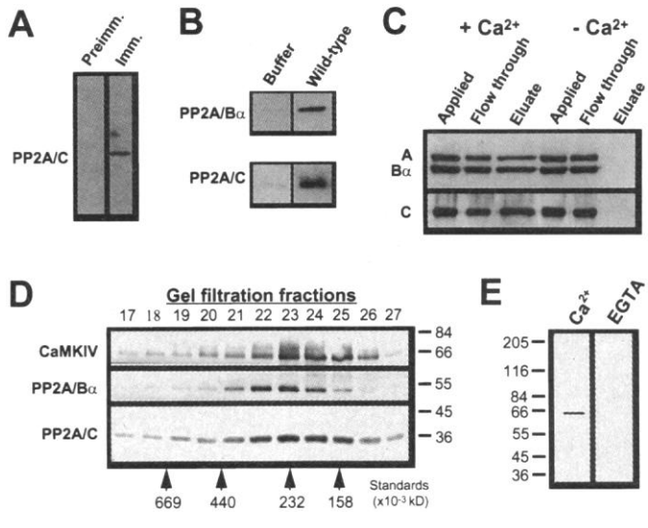

Fig. 1. Identification of a CaMKIV-PP2A complex. (**A**) Coimmunoprecipitation of PP2A with CaMKIV. Immunoprecipitations from Jurkat T cell extracts were performed as described (*7*) with a rabbit polyclonal antibody raised against the COOH-terminal 17 residues of human CaMKIV (Imm.) or preimmune serum (Preimm.). Immune complexes were subjected to immunoblot analysis with a monoclonal antibody to the C subunit of PP2A (*15*). (**B**) Isolation of PP2A with a GST-CaMKIV fusion protein (*9*). Rat brain soluble extracts were incubated with buffer or wild-type GST-CaMKIV fusion protein (10 μg) for 3 hours at 4°C and then purified with glutathione-Sepharose (*17*). The resin was extensively washed and bound proteins were eluted with 20 mM glutathione, resolved by SDS-PAGE, and subjected to immunoblot analysis with antibodies to the C and Bα regulatory subunits of PP2A. (**C**) Binding to calmodulin-Sepharose. Fractions containing proteins of large molecular mass (250 to 700 kD) from gel filtration of rat brain soluble extracts were incubated with calmodulin-Sepharose in the presence (+Ca^{2+}) or absence (–Ca^{2+}) of 5 mM $CaCl_2$ (*36*). Bound proteins were eluted with 15 mM EGTA, resolved by SDS-PAGE, and subjected to protein immunoblotting with antibodies to the indicated PP2A subunits. (**D**) Elution of CaMKIV and PP2A from a Superdex-200 gel filtration column after sequential fractionation of brain extracts on phenyl-Sepharose, calmodulin-Sepharose, and Mono Q columns (*14*). Presented is an immunoblot of fractions from the final gel filtration column showing CaMKIV and the C and Bα subunits of PP2A. Although not shown, the A subunit of PP2A was also present. (**E**) Calmodulin overlay was performed on fraction 23 of the gel filtration column in the presence of 1 mM Ca^{2+} or 1 mM EGTA (*15*).

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CaMKIV (T200A) not activated by CaMKK (9) associated with PP2A; this demonstrated that neither CaMKIV activity nor its phosphorylation by CaMKK is required for interaction with PP2A.

To test whether PP2A catalytic activity was required for association with CaMKIV, we used microcystin-Sepharose to purify PP2A from extracts prepared from rat brain and Jurkat T cells (19). Both PP2A and CaMKIV were isolated from the extracts with the resin (Fig. 2B). Because microcystin is an inhibitor of PP2A that binds to the substrate binding site of the phosphatase catalytic subunit (20), PP2A activity appeared not to be required for association with CaMKIV. Association of CaMKIV and PP2A with microcystin-Sepharose was attenuated by pretreatment of the extracts with free microcystin. Hence, the purification of the proteins by the affinity resin occurred in a microcystin-dependent fashion and was not a result of nonspecific association with the beads.

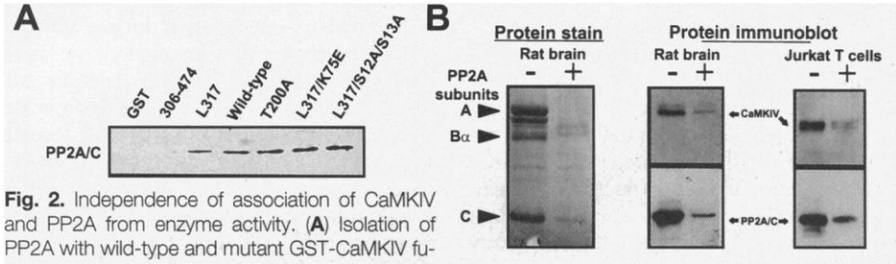

Fig. 2. Independence of association of CaMKIV and PP2A from enzyme activity. (**A**) Isolation of PP2A with wild-type and mutant GST-CaMKIV fusion proteins (9, 17). Rat brain soluble extracts were incubated with the indicated GST fusion proteins (10 μg) and analyzed as described in Fig. 1. Data shown are from a single experiment repeated two to six times with various wild-type and mutant GST-CaMKIV fusion proteins. (**B**) Isolation of CaMKIV with microcystin-Sepharose. Rat brain or Jurkat T cell soluble extracts were incubated with microcystin-Sepharose in the absence (–) or presence (+) of the PP2A inhibitor microcystin (19). Bound proteins were eluted with SDS-PAGE sample buffer and subjected to immunoblot analysis with antibodies to the C subunit of PP2A and to CaMKIV. The rat brain immunoblot (middle) was stained with Ponceau S (left) to compare proteins eluted from microcystin-Sepharose (15). The locations of the A, Bα, and C subunits of PP2A are marked by arrows. Results are representative of three independent experiments.

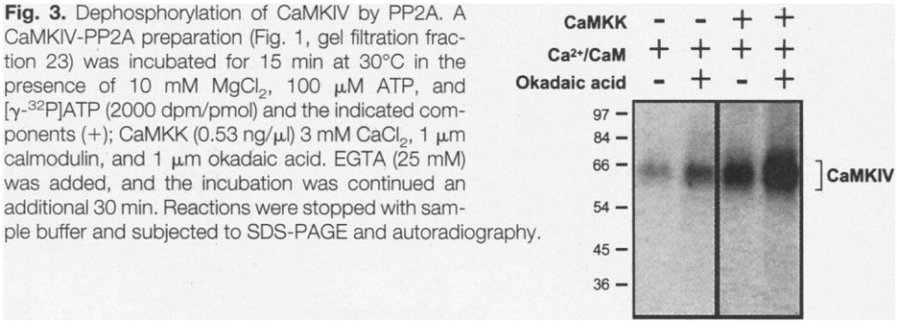

Fig. 3. Dephosphorylation of CaMKIV by PP2A. A CaMKIV-PP2A preparation (Fig. 1, gel filtration fraction 23) was incubated for 15 min at 30°C in the presence of 10 mM $MgCl_2$, 100 μM ATP, and [γ-^{32}P]ATP (2000 dpm/pmol) and the indicated components (+); CaMKK (0.53 ng/μl) 3 mM $CaCl_2$, 1 μm calmodulin, and 1 μm okadaic acid. EGTA (25 mM) was added, and the incubation was continued an additional 30 min. Reactions were stopped with sample buffer and subjected to SDS-PAGE and autoradiography.

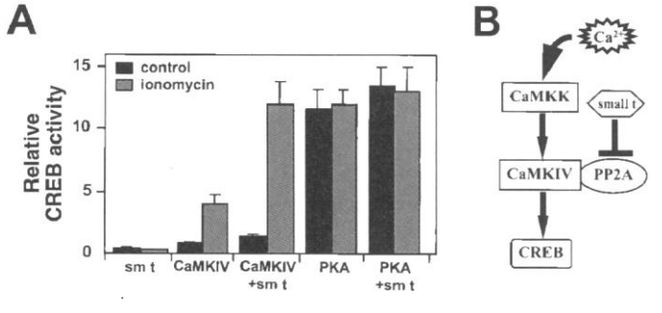

Fig. 4. Regulation of CREB activity in Jurkat T cells. (**A**) Enhancement of CaMKIV-mediated CREB activity by small t antigen, an inhibitor of PP2A activity. Jurkat cells were transiently cotransfected with 5×(Gal4)-luciferase reporter plasmid (5 μg) and Gal4-CREB expression plasmid (5 μg) together with expression plasmids for CaMKIV (3 μg), small t (1 μg), or constitutively active protein kinase A (PKA, 0.1 μg), as indicated (37). Eighteen hours after transfection, cells were stimulated for 5 hours with buffer (control) or calcium ionophore (1 μM ionomycin) and assayed for luciferase activity. A low concentration of PKA expression plasmid cDNA (0.1 μg) was used in these experiments to achieve a small amount of luciferase activity. Increasing the amount of PKA expression plasmid resulted in a large increase in luciferase activity that also was unaffected by expression of small t (16). Bars represent the relative induction over the value for control plasmid alone, normalized for amount of protein and efficiency of transfection. The values are means ± SE (n = 4). (**B**) A model for Ca^{2+}-mediated phosphorylation of CREB in T lymphocytes.

Although association of CaMKIV with PP2A did not require activity of either enzyme, we tested the potential activity of CaMKIV and PP2A toward each other in vitro. The highly enriched complex of CaMKIV-PP2A was incubated in the presence or absence of Ca^{2+}-calmodulin, CaMKK, and the PP2A inhibitor okadaic acid, and the proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE). The only phosphorylated protein detected by autoradiography appeared to be CaMKIV, as indicated by its size and increased phosphorylation after treatment with CaMKK (Fig. 3). Immunoblot analysis confirmed that the only okadaic acid–sensitive phosphatase present in the preparation was PP2A (18). Thus, the enhanced phosphorylation of CaMKIV observed in the presence of okadaic acid (Fig. 3) indicates that CaMKIV is a substrate for PP2A and suggests that PP2A may also regulate CaMKIV activity in vivo.

To determine whether PP2A regulates CaMKIV activity in intact cells, we examined the effects of SV40 small t antigen, a specific inhibitor of PP2A activity (21–24), on CaMKIV-mediated activation of CREB-dependent transcription in Jurkat T cells. We used a Gal4-CREB construct together with a 5×(Gal4)-luciferase reporter plasmid because this assay specifically requires phosphorylation of Ser^{133} of the Gal4-CREB molecule. This chimeric protein binds DNA as a monomer and is therefore not influenced by endogenous CREB or other proteins that can heterodimerize with CREB. Previous studies have established that CaMKIV activates transcription by direct phosphorylation of Gal4-CREB on Ser^{133} (25) and that this assay requires phosphorylation of CaMKIV by CaMKK (9, 25, 26). Expression of small t antigen augmented CaMKIV-mediated Gal4-CREB activity that had been elicited by the Ca^{2+} ionophore ionomycin (Fig. 4) or by the CD3 antibody (16). This effect appeared to be specific for Ca^{2+}-dependent, CaMKIV-mediated activation of Gal4-CREB, as expression of small t antigen had no effect on its own or on Gal4-CREB activity stimulated by constitutively active cAMP-dependent protein kinase, which also specifically and directly phosphorylates Gal4-CREB on Ser^{133} but in a Ca^{2+}-independent manner (25, 26). These results indicate that the CaMKIV-PP2A complex regulates CaMKIV activity and plays a key role in controlling CRE-mediated gene transcription in T cells.

In T cells, the Ca^{2+}-calmodulin–dependent dephosphorylation of nuclear factor of activated T cells ($NFAT_c$) by calcineurin allows its translocation from the cytosol to the nucleus, where it participates in the in-

duction of IL-2 gene transcription (*27–29*). Sustained increases in $[Ca^{2+}]_i$ during T cell activation maintain $NFAT_c$ in the nucleus long enough to induce IL-2 transcription; once $[Ca^{2+}]_i$ returns to basal amounts, NFAT is rapidly redistributed out of the nucleus to the cytosol (*30, 31*). Our experiments show how another Ca^{2+}-initiated regulatory event (CaMKIV-stimulated gene transcription) may be inhibited without a decrease in $[Ca^{2+}]_i$. The CaMKIV-PP2A complex would permit rapid dephosphorylation and inactivation of CaMKIV.

The complex of CaMKIV and PP2A exists in resting T cells and is not altered as a function of time after activation (*16*). How, then, would phosphorylation of CaMKIV and CREB happen at all? Several possible mechanisms may explain the inactivation of CaMKIV in the face of a sustained elevation of $[Ca^{2+}]_i$. First, it is plausible that PP2A is constitutively active toward CaMKIV but that CaMKK transiently outpaces the phosphatase (before becoming inactive itself) to ensure that CaMKIV remains active long enough to phosphorylate CREB. After phosphorylation, a coactivator, phospho-CREB binding protein (CBP), is recruited to the transcription apparatus and CBP binding occurs through the region of CREB that includes Ser^{133} (*32*). A second possibility is that PP2A could be inhibited by a Ca^{2+}-independent posttranslational modification such as phosphorylation (*33*) or by an additional interacting protein (*34*). Finally, it seems possible that the state of phosphorylation of CaMKIV could, by itself, regulate PP2A activity. Once CaMKIV binds calmodulin and is phosphorylated on Thr^{200} by CaMKK, it undergoes autophosphorylation on a number of Ser residues in the extreme NH_2-terminus (*9, 11*). The function of some of these modifications is unknown, but could be to regulate activity of PP2A either directly or by recruitment of another protein to the complex.

Regardless of the additional mechanisms involved, our results may reveal a way in which the duration of immediate early gene expression can be regulated independently of $[Ca^{2+}]_i$. Our findings that CaMKIV-PP2A complexes also occur in the brain suggest that similar regulatory mechanisms may contribute to synaptic plasticity and its role in the molecular basis for memory, where the phosphorylation state of CREB is regulated in a Ca^{2+}-dependent manner by CaMKIV (*35*).

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15. Samples were separated on SDS-polyacrylamide gels (10%) and transferred to a nitrocellulose membrane in 10 mM CAPS (3-cyclohexylamino-1-propane sulfonic acid) containing 10% methanol for 1 hour at 1 A. Proteins on the membrane were visualized with Ponceau S followed by washing in TTBS [25 mM tris-HCl (pH 7.4), 137 mM NaCl, 3 mM KCl, and 0.2% Tween-20]. After incubation with 2% nonfat milk in TTBS for 1 hour, membranes were incubated with affinity-purified polyclonal antibodies to the A and Bα subunits of PP2A (1:500) or with monoclonal antibodies to the C subunit of PP2A (1:5000) or to CaMKIV (1:2000 for 1 hour). Membranes were then incubated with alkaline phosphatase– or horseradish peroxidase–conjugated secondary antibodies for 1 hour, and bound antibodies were visualized by colorimetric detection or chemiluminescence. Calmodulin overlays were done as described for the immunoblot analysis, except that biotinylated calmodulin (1:1500) was substituted for the primary antibody and alkaline phosphatase–conjugated streptavidin (1:10,000) was substituted for the secondary antibody. Assessment of CaMKIV and PP2A molar stoichiometry was performed by preparing a standard curve of purified CaMKIV and PP2A C subunit and comparing the immunoblot intensities with partially purified CaMKIV-PP2A samples.
16. K. Anderson and A. Means, unpublished data.
17. Soluble extract was prepared from one rat brain by homogenization in phosphate-buffered saline (PBS) containing 1 mM PMSF, leupeptin (10 μg/ml), soybean trypsin inhibitor (20 μg/ml), pepstatin (2 μg/ml), and 2 mM benzamidine, and centrifugation at 30,000*g* for 45 min. Approximately 1 mg of extract was incubated with purified GST fusion protein (10 μg), or buffer, for 3 hours at 4°C. Glutathione-Sepharose (40 μl of a 1:1 slurry) was added and the incubation was continued 1 hour. The beads were collected and washed six times (5 min per wash) with PBS. Bound proteins were eluted with 20 mM glutathione in PBS at room temperature. Eluates were subjected to immunoblotting.
18. R. Westphal and B. Wadzinski, unpublished data.
19. C. MacKintosh and R. W. MacKintosh, *Trends Biochem. Sci.* **19**, 444 (1994). Rat brain soluble extracts were prepared as described (*14*). Jurkat T cell extracts were prepared by homogenization in buffer A containing 0.5% Triton X-100 and centrifugation for 15 min at 13,000*g*. Microcystin-Sepharose (40 μl of a 1:1 slurry) in the absence or presence of 1 μM microcystin was added to the supernatant and the samples incubated overnight at 4°C. The beads were washed six times (5 min per wash) with buffer A, and bound proteins eluted with Laemmli sample buffer were subjected to immunoblot analysis.
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36. Soluble extracts were prepared from rat brain as described (*14*), and the proteins precipitating between 25 and 50% ammonium sulfate were fractionated by Superdex-200 gel filtration chromatography. The fractions eluting with an apparent molecular mass of 250 to 700 kD were incubated with calmodulin-Sepharose in homogenization buffer in the presence or absence of 5 mM $CaCl_2$ and 5 mM $MgCl_2$. The calmodulin-Sepharose was extensively washed, and bound proteins eluted in homogenization buffer containing 15 mM EGTA were subjected to immunoblot analysis.
37. Jurkat T cells at log growth phase were collected by centrifugation, resuspended at 10^6 cells per 0.2 ml of RPMI medium with fetal calf serum (10%), mixed with the indicated plasmids, and transfected by electroporation with a Bio-Rad Gene Pulser (250 V, 960 μF).

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