Protein Kinase \( \mu \) (PKM) is a newly described form of PKC that is necessary and sufficient for the maintenance of hippocampal long term potentiation (LTP) and the persistence of memory in Drosophila. PKM is the independent catalytic domain of the atypical PKC isoform and produces long term effects at synapses because it is persistently active, lacking autoinhibition from the regulatory domain of PKC\( \zeta \). PKM has been thought of as a proteolytic fragment of PKC. Here we report that brain PKM is a new PKC isoform, synthesized from a PKM mRNA encoding a PKC\( \zeta \) catalytic domain without a regulatory domain. Multiple \( \zeta \)-specific antisera show that PKM is expressed in rat forebrain as the major form of \( \zeta \) in the near absence of full-length PKC\( \zeta \). A PKC\( \zeta \) knockout mouse, in which the regulatory domain was disrupted and catalytic domain spared, still expresses brain PKM, indicating that this form of PKM is not a PKC\( \zeta \) proteolytic fragment. Furthermore, the distribution of brain PKM does not correlate with PKC\( \zeta \) mRNA, but instead with an alternate \( \zeta \) transcript thought incapable of producing protein. In vitro translation of this RNA, however, generates PKM of the same molecular weight as that in brain. Metabolic labeling of hippocampal slices shows increased \textit{de novo} synthesis of PKM in LTP. Because PKM is a kinase synthesized in an autonomously active form and is necessary and sufficient for maintaining LTP, it serves as an example of a link coupling gene expression directly to synaptic plasticity.

LTP is a persistent enhancement of synaptic transmission widely studied as a physiological model of memory (1). LTP can be divided into two phases: induction, which triggers the potentiation, and maintenance, which sustains it over time. Many molecules have been implicated in LTP induction, which is initiated by the activation of \( N \)-methyl-D-aspartate (NMDA) receptors and involves several protein kinases (2). In contrast, very little is known about the molecular mechanism of maintenance. Recently, however, a specific, autonomously active form of the atypical PKC\( \zeta \) isozyme (3, 4), PKM, has been found both necessary and sufficient for maintaining LTP (5–7). Overexpression of PKM also prolongs memory in \textit{Drosophila} mela-
nogaster, suggesting it is part of an evolutionarily conserved molecular mechanism for memory storage (8).

The unique role of PKM in LTP maintenance is due, in part, to its unusual structural and enzymatic properties as an autonomously active kinase. PKM is a newly described form of PKC\( \zeta \), consists of the independent catalytic domain of a PKC isoform (5). PKC isoforms are divided into three classes: conventional, novel, and atypical (re-
viewed in Refs. 9–11). Each isoform is a single polypeptide consisting of an N-terminal regulatory domain and a C-terminal catalytic domain linked by a hinge (Fig. 1A, left). The regulatory domain contains binding sites for second messengers and an autoinhibitory pseudosubstrate sequence, which interacts with and blocks the active site of the catalytic domain. Second messengers stimulate PKC by binding to the regulatory domain, translocating the enzyme to the cytosol, and releasing the autoinhbition. In vitro studies have shown that PKC may then be cleaved at its hinge, permanently removing the regulatory domain to form the independent catalytic domain of PKM (Fig. 1A, left) (12, 13). Lacking autoinhibition from a regulatory domain, PKM can persistently phosphorylate substrates in the absence of second messengers. Indeed, once formed in LTP, the autonomous activity of PKM maintains synaptic potentiation for at least several hours (7).

Because PKM is usually thought of as a proteolytic fragment of PKC (12, 13), our early work had assumed that PKM was formed from PKC\( \zeta \) by calpains (5), \( \zeta \)–dependent proteases that become active during LTP (14) and can generate PKM \textit{in vitro} (12). Our subsequent studies showed, however, that the increase of PKM in LTP, like the persistence of synaptic potentiation, requires new protein synthesis (6). These results, together with the observation that PKC\( \zeta \) is the only PKC isoform with a stable PKM form in hippocampus (5, 15), suggested that PKM may be generated by a molecular mechanism unique to \( \zeta \).

One possible mechanism for generating PKM might be that a product of protein synthesis regulates PKC\( \zeta \) proteolysis. To characterize this mechanism the amount of PKC\( \zeta \), the putative

\* This work was supported by National Institutes of Health Grants MH057068 and MH53576 (to T. C. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

To whom correspondence should be addressed: Dept. of Pharmacology, Box 29, SUNY Downstate Medical Center, 450 Clarkson Ave., Brooklyn, NY 11203. Tel.: 718-270-3933; Fax: 718-270-8974; E-mail: tsacktor@downstate.edu.

The abbreviations used are: LTP, long term potentiation; PKM, protein kinase \( \mu \); PKC\( \zeta \), protein kinase \( \zeta \); PKC\( \alpha \), protein kinase \( \alpha \); NMDA, \( N \)-methyl-D-aspartate; \( \alpha \)-PKC, atypical protein kinase \( \zeta \); \( 5^\prime \)-RACE, \( 5^\prime \)-rapid amplification of cDNA ends; EPSP, excitatory postsynaptic potential; CA1, cornus ammonus 1; HT, reverse transcription; RARP, rat acidic ribosomal protein; UTR, untranslated region; CRE, cAMP-response element; CREB, cAMP-response element-binding protein; C/EBP, CCAAT/enhancer-binding protein; ORF, open reading frame; CaMKII, Ca\(^{2+} \)/calmodulin-dependent protein kinase II.

**IMPLICATIONS FOR THE MOLECULAR MECHANISM OF MEMORY**

A. Ivan Hernandez‡, Nancy Blace‡, John F. Crary‡§, Peter A. Serrano‡, Michael Leitges‡, Jenny M. Libien‡, Gila Weinstein‡, Andrew Tcherapanov‡, and Todd Charlton Sacktor‡

From the Departments of Physiology, Pharmacology, and Neurology, the Graduate Program in Neural and Behavioral Science, State University of New York Downstate Medical Center, Brooklyn, New York 11203 and Max-Planck-Institut für Experimentelle Endokrinologie, Fedor-Ly- Lenn-Strasse 7, D-30625 Hannover, Germany.
PKM1 Synthesis from a Brain mRNA

precursor, needs to be established. Vertebrates, however, express two atypical PKCs, ζ and η; traditional antisera to the C terminus of PKCζ recognize proteins from both genes (16, 17). (PKCζ (16) and η (17) are human and mouse orthologues, respectively.) Thus the relative amounts of PKCζ and PKCη in brain have not been determined.

Alternatively, PKMζ could be formed by a novel mechanism as a ζ gene product distinct from PKCζ. A ζ RNA containing a partial C-terminal PKCζ sequence has been identified (18, 19), which is produced by an internal promoter active in certain rat prostate tumor lines and in brain (20). This alternative PKCζ RNA, however, is thought not to produce a protein and has been referred to as an untranslatable “ζ pseudogene” RNA (21).

Here we show PKMζ synthesis from this alternate ζ RNA, which we now call PKMζ mRNA. Because it is a gene product distinct from PKCζ, PKMζ is a new atypical PKC isoform, and the protein synthesis-dependent mechanism for generating PKMζ in brain may be a core molecular mechanism for the maintenance of LTP.

EXPERIMENTAL PROCEDURES

Experimental animals were used in accordance with the State University of New York Downstate Medical Center Institutional Animal Use Committee.

Antiserum Production—Peptides were synthesized by Quality Control Biochemicals (Hopkinton, MA) and correspond to N-terminal, hinge, catalytic, and C-terminal regions of PKCζ (see Fig. 1A for sequences). The peptides were coupled to bovine serum albumin (Pierce), mixed with Titermax Gold (CytRx Corp., Norcross, GA), and injected intramuscularly into female New Zealand rabbits. After 1–3 boosts at 4-week intervals, the antisera were affinity-purified on peptide conjugated with Titermax Gold (CytRx Corp., Norcross, GA), and injected intra-

400

followed by centrifugation, as above. The pellet was washed 3 times with 50 ml of IP buffer without aprotinin and boiled at 95 °C for 5 min with 40 μl of 5× sample buffer with 400 μl of IP buffer without aprotinin and boiled at 95 °C for 5 min

with 400 μl of IP buffer without aprotinin and boiled at 95 °C for 5 min with 40 μl of 5× sample buffer with 400 μl of IP buffer without aprotinin and boiled at 95 °C for 5 min with 40 μl of 5× sample buffer

Calpain Cleavage—A mixture (200 μl) containing calpain (1.25 units of either porcine calpain I or II, Calbiochem), baculovirus-Sf9 expressed PKCζ (1 μg), prepared as previously described (7), 20 μm Tris-HCl (pH 7.5), and 2 mM dithiothreitol was prewarmed for 2 min at 20 °C, and the proteolytic reaction was started with the addition of 20 μl of CaCl2 (1.5 mM final concentration). After 1 h, the reaction was stopped with the addition of 12.5 μl of 25 mM EDTA and placed on ice. Proteolytic fragments were analyzed with C-terminal antisera by Western blot, as described above.

Total mRNA Isolation—For reverse transcription (RT)-PCR, 60 μg of various tissues were used for the single-step method of total RNA isolation by guanidinium thiocyanate/phenol/chloroform extraction (22). Total RNA sample for RT was treated with DNase I and repurified by the same method. The quality of the total RNA was measured by formaldehyde gel electrophoresis and spectrophotometry. For RNase protection, Northern blotting, and 5′-RACE with oligo-capping, the cesium chloride RNA isolation method was used. Briefly, 200 μg of tissue from a 3-month old male Sprague-Dawley rat was homogenized in 2 ml of guanidinium thiocyanate homogenization buffer (4.0 M guanidinium thiocyanate, 0.1 M Tris-HCl, 1% β-mercaptoethanol, 0.5% N-laurylsarcosine) for 30 s. The homogenate was centrifuged for 10 min at 3,000 × g. The supernatant was transferred to a cushion of 2 ml of 5.7 M CsCl and 10 mM EDTA (pH 7.5) and centrifuged for 16 h at 81,000 × g. The pellet containing total RNA was resuspended in 300 μl of diethyl pyrocarbonate-treated water by heating to 70 °C for 10 min, 33 μl of 3 M sodium acetate (pH 5.2), and 825 μl of 100% ETOH was added, and the sample was stored at −80 °C.

RT-PCR—Total RNA was used to synthesize cDNA with the SuperScript Preamplification System for First Strand cDNA Synthesis kit (Invitrogen). Two hundred ng of cDNA was used in a 100-μl final volume PCR. Amplification was for 34 cycles with 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min as cycle parameters, with a final step of 72 °C for 10 min. For amplification of PKCζ and PKMζ cDNAs, specific forward primers were F5′-CATGCGCCAGGAGCACC3′ and F5′-CTCTTATAGATGCTGCTTCC-3′, respectively, and R5′-TGAAGCCTCATGACACAGCTC-3′ was the reverse staple primer. As a control we used glyceraldehyde-3-phosphate dehydrogenase primers, F5′-ACATGGTCCTAGTTCG-3′ and R5′-CAGATCCACACGGAATA-3′.

RNase Protection—RNase protection was performed using the RPAII kit (Ambion, Austin, TX). The probes for PKCζ, PKMζ, and RABP were gifts from T. Powell (Cleveland Clinic, Cleveland, OH) (23).

Northern Blot—Total RNA (30 μg) was electrophoresed and transferred to nitrocellulose, rinsed, and UV cross-linked. Digestion with EcoRI-SphI and KpnI-EcoRI gave a 457- and 227-bp specific fragment for PKCζ and PKMζ, respectively. The fragments were radiolabeled with [32P]dCTP using a Stratagene random octamer protocol (Stratagene Clon- ing Systems, La Jolla, CA). Hybridization conditions were performed according to instructions for Stratagene QuickHyb hybridization. Blots were developed overnight by film exposure at −70 °C or by PhosphoImager (Storm 860 gel and blot imaging system, Amersham Biosciences).

Oligo-capping and 5′-RACE—Oligo-capping and 5′-RACE (24) were performed with a GeneRacer Kit (Invitrogen), using rat hippocampus and kidney RNA as templates, according to the manufacturer’s instructions. The GeneRacer Kit 5′-primer was used as a forward primer and 5′-CCGCGCCACATCTACTACAACTAGA-3′ as a reverse primer specific for PKMζ. For screening of oligo-capping 5′-RACE clones, PCR was performed using the GeneRacer 5′-nested primer and 5′-CTCTTCCTTACACAGCACC3′ as nested primers. The PCR conditions were 20 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, with an initial step of 94 °C for 3 min and a final step of 72 °C for 10 min. 5′-RACE without tobacco alkaline phosphatase yielded no clones.

Plasmid™ accession numbers of the published human and mouse PKMζ, cDNAs obtained by oligo-capping and 5′-RACE are as follows: AL514298, AL534124, AL535166, AL535303, AL5358613, AL5358826, and AL5358967 (human), and AU051559, AU067133, AU078875, and BB585441 (mouse). Throughout the text, the first nucleotide in the longest rat PKMζ mRNA, shown in Fig. 4D, is referred to as the first nucleotide of rat PKMζ mRNA.

In Vitro Transcription-Translation—in vitro transcription-translation was performed with the T7-coupled wheat germ extract system kit, according to the manufacturer’s instructions (Promega, Madison, WI). For the T7-PKMζ clone (sequence 48–1982), we used a PKMζ clone (gift from T. Powell, Cleveland Clinic, Cleveland, OH). Briefly, PKMζ

...
PKM<sub>z</sub> Synthesis from a Brain mRNA

PKM<sub>z</sub> was digested with AatII and XhoI and the pBlueScript SK-( ) vector with HindIII and XbaI. The XbaI sites were ligated, and the HindIII site of the vector was filled in and ligated with a polished AatII site from the PKM<sub>z</sub>. The T7-PKM<sub>z</sub>-(347–1982) clone was created by digesting PKM<sub>z</sub>-(48–1982) with EcoRI and XhoI and subcloned into pGEM-ZZ (Promega, Madison, WI). The SP6-PKM<sub>z</sub>-(587–1982) clone was created by digesting PKM<sub>z</sub>-(48–1982) with SalI and XbaI, and subcloning into pGEM-Z (Promega, Madison, WI). The experiment with the T7 clone was performed with linearized plasmid digested with XhoI. The experiment with the SP6 clone was performed both with and without linearization at the XbaI site with identical results.

**Hippocampal Slice Electrophysiology—** Transverse hippocampal slices (450 μm) were prepared with a McIlwain tissue slicer from 16- to 22-day-old Sprague-Dawley rats. Slices were placed in a slice chamber filled initially with a saline solution containing 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 2.6 mM NaHCO<sub>3</sub>, 11 mM glucose, 10 mM MgCl<sub>2</sub>, and 0.5 mM CaCl<sub>2</sub> (pH 7.4), equilibrated with 95% O<sub>2</sub>, 5% CO<sub>2</sub>. Test stimuli (duration 100 μs) were delivered every 15 s through bipolar tungsten electrodes placed across the Schaffer collateral/commissural fibers. Field EPSPs were recorded using glass microelectrodes filled with the saline solution (resistance 2.5–5.0 megohms) and placed in CAI stratum radiatum. The current intensity of test stimuli (25–50 μA) was set to produce one-third maximal EPSPs. Analysis of the initial 10–50% of the field EPSP slope was performed with Superscope (GW Instruments, Somerville, MA). Following 15 min of stable recordings, LTP was induced with two 100-Hz 1-s tetanic trains, 20 s apart. Control slices received test stimulation for equivalent periods of time as LTP recordings.

**Metabolic Labeling of Hippocampal Slices—** Following stimulation, hippocampal slices were transferred to a 8-well culture dish in a 32.5 °C water bath. Each slice was submerged in a well containing 8 ml of resting buffer, continuously perfused with 95% O<sub>2</sub>, 5% CO<sub>2</sub>, and 1.2 mM MgCl<sub>2</sub> and 1.7 mM CaCl<sub>2</sub> (resting buffer) (5). Test stimuli (duration 100 μs) were delivered every 15 s through bipolar tungsten electrodes placed across the Schaffer collateral/commissural fibers. Field EPSPs were recorded using glass microelectrodes filled with the saline solution (resistance 2.5–5.0 megohms) and placed in CAI stratum radiatum. The current intensity of test stimuli (25–50 μA) was set to produce one-third maximal EPSPs. Analysis of the initial 10–50% of the field EPSP slope was performed with Superscope (GW Instruments, Somerville, MA). Following 15 min of stable recordings, LTP was induced with two 100-Hz 1-s tetanic trains, 20 s apart. Control slices received test stimulation for equivalent periods of time as LTP recordings.

**RESULTS**

PKM<sub>z</sub> Is Expressed Exclusively in Brain—Vertebrates express two atypical isoforms, PKC<sub>z</sub> and PKC<sub>α</sub> (16, 17). Because PKC<sub>α</sub> is nearly identical to ζ in its C terminus, antisera raised against this region of PKC<sub>z</sub> recognize both atypical isoforms (16) (Fig. 1A, right). We used this antisem to screen a panel of antibody against this region of PKC<sub>z</sub> recognized by the C-terminal antiserum, but only a trace amount of PKM<sub>α</sub> was detectable in brain. These results suggest that the major form of atypical PKM in brain is PKM<sub>z</sub>.

To confirm that the 55-kDa protein recognized by the C-terminal PKC<sub>z</sub> antiserum is PKM<sub>z</sub> and to determine the level of expression of PKC<sub>z</sub>, we developed a battery of antisera raised against different isozyme-specific epitopes in the N-terminal, hinge, and catalytic domains of PKC<sub>z</sub> (Fig. 1A, right). By Western blot, all the antisera to ζ, but not to PKC<sub>α</sub>, recognized baculovirus-overexpressed PKC<sub>z</sub> in Sf9 cells (Fig. 1C, left). In kidney, antisera to both ζ and α detected a 72-kDa band (Fig. 1C, center), indicating the expression of both full-length aPKCs. In hippocampus, however, the PKC<sub>α</sub> and the aPKC C-terminal antisera both recognized a 72-kDa band, but none of the ζ-specific antisera detected full-length PKC<sub>z</sub> (Fig. 1C, right). Overdevelopment of Western blots with the ζ-specific antisera showed only trace amounts of PKC<sub>z</sub> in hippocampus (data not shown). The full-length aPKC recognized by the aPKC C-terminal antiserum in hippocampus is thus predominantly PKC<sub>α</sub>, and the expression of PKC<sub>z</sub> is very low.

In contrast, PKM<sub>z</sub> was abundantly expressed in hippocampus (Fig. 1C, right). The antisera to the ζ hinge, ζ catalytic, and aPKC C-terminal all recognized PKM<sub>z</sub> in the hippocampus, but, as expected, the η N-terminal antiserum did not. We confirmed that the protein detected by the aPKC C-terminal antiserum was PKM<sub>z</sub> by showing that this antisem immunoprecipitated a 55-kDa protein from hippocampal extracts that was recognized by the ζ-specific catalytic domain antiserum on Western blot (Fig. 1D).

We next determined whether the relative abundance of PKM<sub>z</sub> and the paucity of PKC<sub>z</sub> that we observed in hippocampus was found in other brain regions (Fig. 1E). The ζ N-terminal and ζ catalytic domain antisera (Fig. 1E, left and middle) showed PKC<sub>z</sub> expression in kidney and cerebellum but did not detect PKC<sub>z</sub> in either neocortex or hippocampus. In contrast, the ζ catalytic and aPKC C-terminal antisera (Fig. 1E, middle and right) demonstrated PKM<sub>z</sub> expression in all brain regions. Therefore, PKM<sub>z</sub> and PKC<sub>z</sub> show differential regional expression in brain, with both PKM<sub>z</sub> and PKC<sub>z</sub> in cerebellum, but only PKM<sub>z</sub> in hippocampus and neocortex.

Brain PKM<sub>z</sub> Is Not a Proteolytic Fragment of PKC<sub>z</sub>—The expression of PKM<sub>z</sub> in the near absence of PKC<sub>z</sub> in hippocampus and neocortex suggests that PKM<sub>z</sub> may not be produced by PKC<sub>z</sub> proteolysis. It is possible, however, that the cleavage of PKC<sub>z</sub> in these regions was complete. As our original hypothesis for the formation of PKM<sub>z</sub> was proteolysis by calpain (5), we compared the size of brain PKM<sub>z</sub> with the PKM<sub>z</sub> fragment generated by calpain proteolysis of recombinantly expressed PKC<sub>z</sub>. Both calpains I and II produced PKM<sub>z</sub> fragments that appeared smaller than endogenous brain PKM<sub>z</sub> (Fig. 2A).

Although these results suggest that calpain proteolysis does not produce PKM<sub>z</sub> of the appropriate size, other proteases might cleave PKC<sub>z</sub> at alternative sites (25). Therefore, to determine whether brain PKM<sub>z</sub> is a proteolytic fragment of PKC<sub>z</sub>, we examined mice in which PKC<sub>z</sub> was eliminated by genetic disruption of its regulatory domain (PKC<sub>z</sub>-reg /−/−) (26). In mice, PKC<sub>z</sub> is a single copy locus at chromosome 4, 83.0 centimorgans. In the PKC<sub>z</sub> knockout characterized by Leitges et al. (26), a Lac/Neo cassette was inserted into the sequence encoding amino acids 112–140 of the regulatory domain (exon 5), thereby disrupting the ζ regulatory domain but sparing its catalytic domain. Western blots with the ζ-specific catalytic domain antisem showed that although the homozgyous PKC<sub>z</sub>-reg knockout completely lacked PKC<sub>z</sub> in cerebellum and kidney, PKM<sub>z</sub> in brain was preserved (Fig. 2B). A second knockout line showed identical results (data not shown). Com-
PKMζ is a brain-specific form of PKCζ prominent in hippocampus and neocortex. A, left, models of the inactive and active conformations of PKC and the proteolytic formation of PKM. PKC consists of a C-terminal catalytic domain (green) tethered by a hinge (yellow) to an N-terminal regulatory domain (red), which contains an autoinhibitory pseudosubstrate sequence. PKC is maintained in an inactive state by the interaction between the pseudosubstrate and the catalytic site. PKC is activated by second messengers, which produce a conformational change that releases the autoinhibition. PKM is the independent catalytic domain of PKC, usually thought of as a product of proteolysis at the hinge. Because PKM lacks a regulatory domain, it is constitutively active. Right, diagram showing epitopes used to generate antisera in the regulatory, hinge, and catalytic domains of PKCζ and PKCζ/λ. The antiserum to the C terminus of ζ (aPKC C term) recognizes both atypical isoforms. PKCζ-specific antisera are to the N-terminal (N term), hinge, and catalytic domain. A PKCζ/λ-specific antiserum is to its catalytic domain. B, left, PKMζ Synthesis from a Brain mRNA
pared with wild-type, heterozygous PKCζ-reg knockout animals (PKCζ-reg +/−) expressed diminished levels of PKCζ, but also no loss of PKMζ (Fig. 2B). These results demonstrate that brain PKMζ is expressed in the complete absence of PKCζ, and therefore proteolysis or other post-translational modification of PKCζ is not the mechanism of PKMζ formation.

The Distribution of an Alternate PKMζ mRNA, Encoding an Independent ζ Catalytic Domain, Correlates with PKMζ Protein—A novel mechanism for the formation of PKMζ could be its direct synthesis as a ζ gene product other than PKCζ. The ζ gene produces two sets of RNAs: a full-length PKCζ mRNA and a second potential PKMζ mRNA, previously referred to as ζ' (23) or the “ζ pseudogene transcript” (21) (Fig. 3A, top). The 5’ end of this RNA is a unique sequence not present in PKCζ mRNA, whereas its 3’ end is identical to the 3’ end of PKCζ mRNA, consisting of a partial ζ regulatory domain and a complete hinge, catalytic domain, and 3’-UTR (18, 20, 21, 23, 27). In contrast to PKCζ mRNA, however, the 5’-terminal of PKMζ mRNA does not contain an AUG that could initiate translation of the partial ζ regulatory domain. Instead, the open reading frame (ORF) of the ζ kinase sequence in PKMζ mRNA begins in the hinge and extends to the C terminus of the kinase domain (Fig. 3A, top). To determine whether the patterns of expression of the PKCζ and PKMζ mRNAs correlate with the PKCζ and PKMζ proteins, we analyzed their distribution by RT-PCR, RNase protection, and Northern blot analysis (Fig. 3).

By using specific forward primers that distinguish between PKCζ catalytic antisera and PKMζ catalytic antisera, Western blots of rat tissues with aPKC C-terminal antiserum shows atypical PKM is specifically expressed in brain. High levels of full-length aPKC are in lung, liver, kidney, testis, and brain. Right, Western blot with the PKCζ catalytic antiserum shows expression of full-length PKCζ in lung, liver, kidney, testis, and brain but only trace amounts of PKM in brain. C, PKMζ is the major form of ζ in hippocampus. Western blots of baculovirus-overexpressed PKCζ in Sf9 cells (bac-Sf9 PKCζ, left), rat kidney (center), and hippocampus (right). All ζ antisera, but not the PKCζ antisera, recognize overexpressed PKCζ. Kidney expresses both PKCζ and PKCζ. In contrast, the only detectable form of ζ in hippocampus is PKMζ. The 72-kDa band detected with the aPKC C-terminal antiserum in hippocampus is PKCζ. D, Western blot with ζ-specific catalytic antisera shows the 55-kDa protein immunoprecipitated (IP) by the aPKC C-terminal antiserum is PKMζ. Lane 1, hippocampal homogenate prior to immunoprecipitation. Lanes 2–4, immunoprecipitation pellets: lane 2, normal goat serum control; lane 3, aPKC C-terminal antisera immunoprecipitates PKMζ; lane 4, preincubation of aPKC C-terminal antisera with immunizing peptide blocks immunoprecipitation. Lanes 5 and 6, supernatants of experiments of lanes 2 and 3, respectively, showing most of the PKMζ was immunoprecipitated. Because of differences in volumes after the immunoprecipitation, less supernatant (S) than pellet (P) protein was loaded on the gel. E, Western blot shows PKMζ and PKCζ expression in various brain regions and kidney. Left, ζ N-terminal antisera shows strong expression of PKCζ in kidney, low expression in cerebellum, and no expression in hippocampus and neocortex. Center, the ζ catalytic antisera shows identical results for PKCζ, but also expression of PKMζ in brain tissues but not kidney. Right, the aPKC C-terminal antisum shows aPKC in all tissues and PKMζ only in brain. All experiments in the figure were performed in triplicate with equivalent results.

Fig. 2. Brain PKMζ is not formed by proteolysis of PKCζ. A, Western blot with aPKC C-terminal antiserum shows calpain I and II proteolysis of recombinantly expressed PKCζ produces a cleaved PKMζ fragment smaller than endogenous brain PKMζ. B, PKMζ is expressed in a PKCζ knockout mouse in which the ζ regulatory domain was targeted, but the catalytic domain was spared (PKCζ-reg −/−). Western blot with ζ-specific catalytic domain antisem shows PKCζ expression in wild-type (PKCζ-reg +/+ ) and heterozygous (PKCζ-reg +/− ) mice in both kidney and cerebellum but not in the knockout (PKCζ-reg −/− ). Brain PKMζ is expressed in all animals. Experiments were performed in triplicate with equivalent results.
lung, testis, and cerebellum but not in neocortex or hippocampus (Fig. 3A, bottom). Thus the distributions of PKCζ and PKMζ mRNAs strongly correlate with PKCζ and PKMζ proteins, with both PKMζ protein and mRNA the exclusive forms of ζ expressed in forebrain (compare Fig. 1E).

We confirmed and quantified these results using RNase pro-
PKM₃ mRNA formation from an internal promoter within the PKCζ gene. A, the intron-exon structure of the human PKCζ gene shows two exon clusters separated by a large intron: exons 1–4, encoding the PKCζ 5’-UTR (light blue) and regulatory domain (red), and exons 5–18, encoding the remaining regulatory domain, hinge (yellow), catalytic domain (green), and 3’-UTR (gray). The unique 5’-PKM₃ mRNA sequence is in a single exon (exon 1, dark blue) within the large intron. PKCζ mRNA transcription begins at exon 1. Transcription initiated from exon 1 and alternative splicing to exon 5 generates PKM₃ mRNA. B–D, analysis of the transcription start site of PKM₃ mRNA. B, top, diagram of PKM₃ mRNA showing specific primer used in oligo-capping with 5’-RACE to determine the 5’ sequence of capped PKM₃ mRNAs. Bottom, PCR products of 5’ end clones obtained by oligo-capping with 5’-RACE of PKM₃ mRNA, showing expression of multiple length 5’ ends in rat hippocampus (left) and a single length 5’-terminal in kidney (right). C, frequency of rat brain PKM₃ mRNA clones obtained with the numbered start sites shown in D. The PKM₃ mRNA clones from kidney all began at start site 3. D, alignment of the complete rat, mouse, and human PKM₃ exon 1 with adjacent 5’-intron sequence. Small asterisks denote identical sequences. Colored arrows show the 5’-PKM₃ terminal obtained by oligo-capping and 5’-RACE for rat (red), mouse (blue), and human (green) PKM₃ mRNAs. Black arrow with large asterisk denotes the start site of PKM₃ mRNA (ζ) from the rat Dunning G prostate tumor cell line (20). A canonical CRE (bar) is conserved in all three species. Humans have a partial duplication of the CRE as an adjacent insert.
Fig. 5. In vitro translation of PKM\(\zeta\) mRNA produces PKM\(\zeta\). Top, diagram of PKM\(\zeta\) mRNA shows the six miniature ORFs and the large kinase ORF. Numbers correspond to start sites of the ORFs and the length of the arrows denotes their size. The unique PKM\(\zeta\) mRNA sequence is represented by a dashed line; the sequence in common with PKC\(\xi\) mRNA begins at bp 381. Bottom, top panel, autoradiogram of PKM\(\zeta\) synthesized from PKM\(\zeta\) mRNA in the wheat germ in vitro transcription/translation system. Lanes 1 and 2, PKM\(\zeta\) mRNA containing full-length 5'-UTR; lane 3, truncation of the unique PKM\(\zeta\) sequence, which includes the first four miniature ORFs, increases PKM\(\zeta\) expression; lane 4, truncation of the PKM\(\zeta\) 5'-UTR to 9 bp upstream of the kinase ORF shows no further increase in expression; lane 5, no template. Bottom panel, Western blot with C-terminal antiserum for PKM\(\zeta\) using probes specific to their unique 5'-UTRs; lane 1, PKM\(\zeta\) mRNA (lanes 1–4) has the same molecular weight as endogenous brain PKM\(\zeta\) from hippocampal homogenate (lane 6). Experiments were performed in triplicate with equivalent results.

We then determined the sizes of the PKC\(\xi\) and PKM\(\zeta\) mRNAs in different brain regions and kidney by Northern blot, using probes specific to their unique 5' ends (Fig. 3C, top). The PKM\(\zeta\) mRNA is found as a 2.3- and 4.7-kb species in brain but is not expressed in kidney (Fig. 3C, left). These sizes are similar to those reported previously (27). We determined the relative levels of the two \(\zeta\) mRNAs by comparing them to an mRNA of the housekeeping gene, rat acidic ribosomal protein (RARP, Fig. 3B, center and bottom). The expression of PKM\(\zeta\) mRNA in brain was higher than that of PKC\(\xi\) mRNA in any tissue examined.

We next examined the promoter region of PKM\(\zeta\) mRNA by determining the 5' terminus of PKM\(\zeta\) mRNA. Powell et al. (23) had used 5'-RACE to sequence PKM\(\zeta\) mRNA, which may not yield the complete 5' terminus. Therefore, we employed the oligo-capping and 5'-RACE method that enriches for capped mRNAs, which has been used to determine the transcriptional start site of mRNAs (24). By using a reverse primer specific to PKM\(\zeta\) mRNA (Fig. 4B, top), we found that the 5' termini from all 32 clones obtained from hippocampus by oligo-capping and 5'-RACE exhibited unique sequences within exon 1 but no PKC\(\xi\) sequence (Fig. 4, B–D). This result confirms that PKM\(\zeta\) mRNA is produced by an internal promoter followed by alternative splicing and not by alternative splicing of PKC\(\xi\) mRNA (which would have resulted in a PKC\(\xi\) 5'-terminal exon in PKM\(\zeta\) mRNA). Heterogeneity of start sites was observed by oligo-capping 5'-RACE for PKM\(\zeta\) mRNA produced in brain (Fig. 4B, left, and C–D), but only a single start site was observed for the low abundant PKM\(\zeta\) mRNA from kidney (Fig. 4B, right). Although the 5' terminus of the longest rat brain PKM\(\zeta\) mRNA clone was upstream of the site of transcription initiation reported for the alternate \(\zeta\) RNA of prostate tumor cells (20), the majority of the brain PKM\(\zeta\) mRNA start sites were downstream of this site (Fig. 4D). Analysis of the 5' ends of the published oligo-capped 5'-RACE products from human and mouse PKM\(\zeta\) mRNAs also showed only PKM\(\zeta\) exon 1 and no PKC\(\xi\) sequences and were heterogeneous in length (Fig. 4D).

Analysis of the promoter region of the PKM\(\zeta\) mRNA showed a conserved canonical cAMP-response element (CRE) (Fig. 4D),
first identified in the rat (20). We find the human PKMζ promoter contains an additional partial CRE duplication in an insert immediately 3' to the canonical CRE. Other putative transcription factor binding sites found in the PKMζ mRNA promoter region in all three species include those for nuclear factor-κB (NF-κB, −74 to the longest rat PKMζ transcript) and CCAAT/enhancer-binding protein (C/EBP, −272).

In Vitro Translation of PKMζ mRNA—Although the AUG in the hinge of PKMζ mRNA (corresponding to methionine 184 of PKCζ (27)) is within a putative Kozak sequence (28), a previous attempt to translate PKMζ mRNA did not produce detectable protein in the rabbit reticulocyte system (21), a result which we confirmed (data not shown). However, the sequence 5' to the kinase ORF is long (595 bp) and contains 6 short ORFs, which may serve to inhibit, and thus regulate, the translation of the kinase ORF (Fig. 5, top). We therefore examined PKMζ mRNA expression in the wheat germ in vitro translation system, which may contain fewer inhibitory factors, and varied the length of the 5'-UTR (Fig. 5, bottom). PKMζ mRNA with a full-length 5'-UTR (lanes 1 and 2) expressed PKMζ, as shown by both autoradiography of [35S]methionine/cysteine-labeled proteins and Western blot with aPKC C-terminal antiserum. Consistent with a role in translational regulation, truncation of the 5'-UTR sequence containing the first four short ORFs increased PKMζ expression (lane 3). Interestingly, additional shortening of the 5'-UTR to within 9 nucleotides of the putative start AUG, which removes the two short ORFs with the strongest Kozak sequences, did not further significantly increase expression (lane 4). In contrast to the short PKM fragment produced by calpain cleavage of PKCζ (Fig. 2A), PKMζ produced by PKMζ mRNA was identical in size to endogenous brain PKMζ (Fig. 5, lane 6).

**LTP Increases de Novo Synthesis of PKMζ**—Finally, we examined the synthesis of PKMζ in LTP. LTP in the CA1 region of hippocampal slices was induced by two 100-Hz 1-s trains, 20 s apart, and followed for 10 min to ensure stable synaptic potentiation (Fig. 6, A and B). The tetanized slices and control slices that received test stimulation for equivalent periods were then transferred to a bath containing [35S]methionine/cysteine for 30 min. In parallel experiments we found that this manipulation did not disturb synaptic responses, because slices that were returned to the recording chamber after incubation showed stable field excitatory postsynaptic potential (EPSP) responses (data not shown). After labeling, the slices were homogenized, PKMζ immunoprecipitated with C-terminal antiserum, and new synthesis determined by incorporation of label into immunoprecipitated PKMζ. The level of de novo synthesis of PKMζ in tetanized slices was 246 ± 75% of that in control slices that received test stimulation alone (set at 100%, n = 6, p < 0.01, paired t test, Fig. 6, C and D). Thus there is a large increase in PKMζ synthesis during LTP.

**DISCUSSION**

**PKMζ, a New Brain-specific Atypical PKC Isoform**—PKMζ was first described by Nishizuka and colleagues (13) as a constitutively active proteolytic fragment of PKC. Because proteolysis is irreversible, PKM formation was recognized early on as a potential mechanism for the persistent activation of PKC, in contrast to its transient activation by lipid second messengers, which are usually rapidly metabolized (12). Although an attractive mechanism to sustain long term cellular functions (9), physiological PKM formation was not detected for many years.

In a 1993 study of multiple PKC isozymes in LTP, however, we observed a PKM form specific to the atypical PKCζ isoform in rat hippocampus (5). This PKM was not generated artifac-
Finally during homogenization (5) and was, moreover, the specific form of PKC to persistently increase in LTP maintenance (5, 6). Because proteolysis was the only mechanism known to produce PKM, our initial studies had assumed that PKM was generated by cleavage of PKC. We subsequently observed, however, that PKM formation required new protein synthesis during LTP, a result that was not easily explained by proteolysis (6).

Here we have characterized the mechanism of brain PKM formation, which is a novel protein synthesis-dependent pathway for the persistent activation of a kinase. We first determined the relationship between PKM and PKC by examining their distribution in brain and other tissues with newly designed ζ-specific antisera. PKM, but not PKC, was found exclusively in brain. The only other PKC isoforms expressed specifically in nervous tissue are the conventional PKC isozymes (9–11), are separate PKC in all and PKM isoforms to be segregated in distinct cell types. This pattern of expression suggested that PKCζ and PKM might not have a precursor-product relationship. Analysis of PKCζ regulatory domain knockout mice confirmed that brain PKM was expressed in the complete absence of full-length PKCζ.

These results indicated that brain PKM was not formed by a proteolytic mechanism but perhaps as a distinct ζ gene product. The ζ gene produces two RNAs (18, 23, 27) from separate dedicated promoters (20). The first promoter produces PKCζ mRNA and the second, an internal promoter within the PKCζ gene, produces an RNA in certain rat prostate tumor cell lines and in brain (20). This alternate ζ RNA was thought to be incapable of translation (21). We observed, however, that the distribution of PKCζ mRNA and this alternate ζ RNA correlated well with PKCζ and PKM proteins, respectively. Furthermore, although the alternate ζ RNA did not produce detectable protein in the rabbit reticulocyte translation system, in agreement with a previous study (21), in vitro translation in the wheat germ translation system produced PKMζ that was identical in size to endogenous brain PKMζ, demonstrating that the alternate ζ RNA was PKMζ mRNA (Fig. 5). The long 5′-UTR of PKMζ mRNA, which contains multiple short ORFs, may have contributed to the difficulty in observing translation in all in vitro systems. Indeed, truncation of the PKMζ mRNA 5′-terminus that eliminated the first four short ORFs greatly increased the translation of the message. Brain PKMζ and PKCζ are therefore distinct ζ gene products and, by the standard nosology of the PKC family (9–11), are separate PKC isoforms. Alternative names for PKCζ and brain PKMζ would be PKCζ1 and PKCζ2, respectively.

PKMζ from PKMζ mRNA—PKM formation from its own mRNA allows for mechanisms of regulation not possible by proteolysis. Separate mRNAs, for example, permit the PKC and PKM isoforms to be segregated in distinct cell types. This may be important because a constitutively active PKMζ, lacking regulation by second messengers, could have a dominant effect over full-length PKCζ, disrupting the important cellular functions of the latter that include insulin and growth factor signaling (3, 4, 31, 32). The catalytic domains of PKCζ and PKCζζ, however, are extremely similar (16, 17), and PKMζ might also have a dominant effect over PKCζζ. This suggests that the two atypical isoforms might be differentially compartmentalized within neurons.

Although not as common a mechanism as alternative splicing, separate dedicated promoters have been found to increase the diversity of products from several genes, including two other PKCs, Caenorhabditis elegans PKC1 (33) and mouse PKCθ (34). In addition to providing a mechanism for tissue-specific expression, separate promoters may allow different sets of transcription factors to regulate each mRNA. The promoter region of PKMζ mRNA contains several putative binding sites for activity-dependent transcription factors. The presence of a conserved canonical CRE in the mouse, rat, and human PKMζ promoter suggests regulation by the transcription factor CREB (cAMP-response element binding protein), which has been implicated in memory formation in a variety of species (35). CREB and PKMζ may thus lie in the same signaling pathway, a result consistent with experiments showing that overexpression of CREB (36) and PKMζ (8) produce similar memory enhancement phenotypes in Drosophila. Interestingly, the human PKMζ mRNA promoter has an additional partial CRE insert that might enhance the binding of CREB and the activity-dependent transcription of PKMζ mRNA. The PKMζ mRNA promoter region also contains putative binding sites for other transcription factors implicated in memory, including NF-kB and C/EBP (37–39).

Alternative promoters also provide each ζ mRNA with a distinct 5′-UTR that may allow for differential mechanisms of translational regulation. Exon 1, which contributes the unique sequence to the PKMζ 5′-UTR, is strongly conserved in mouse, rat, and human, and results in a 5′ terminus that is much longer than that of PKCζ mRNA (Fig. 4). Exon 1 may thus contribute to translational regulation specific to PKMζ mRNA. Indeed, this sequence of 5′-UTR, rather than the shorter sequence shared by the two ζ mRNAs, was the major inhibitory constraint on PKMζ translation (Fig. 5). By oligocapping and 5′-RACE, the 5′ terminus of brain PKMζ mRNA appears to be

**Fig. 7. Model of LTP maintenance by PKMζ synthesis.** Our previous work has shown that activation by the excitatory neurotransmitter glutamate (Glu) of the NMDAR receptor (NMDAR) during tetanus triggers the induction of PKMζ formation (5, 6), and that PKMζ phosphorylation potentiates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPA)-mediated synaptic transmission in LTP maintenance (7). We now show the formation of PKMζ in LTP is through increased de novo protein synthesis from a PKMζ mRNA, produced by a dedicated internal promoter within the PKCζ gene that is nested between clusters of regulatory domain exons (reg, red) and catalytic domain exons (cat, green). Potential activity-dependent regulatory mechanisms for PKMζ formation include enhanced transcription by CREB and increased local translation.
heterogeneous, consisting of different lengths of exon 1. This suggests multiple transcriptional start sites, which is characteristic of transcription without a TATA box (40–43). Although this heterogeneity could possibly be due to artifacts of the oligocapping and 5'-RACE method, the 5' terminus of the low abundant PKM mRNA in kidney, obtained in parallel experiments, was only a single length (Fig. 4B). The functional significance of the apparent heterogeneity of brain PKM mRNA is not clear, but one possibility is that variation in the length of the 5'-UTR regulates the translational efficiency of the message. Different patterns of transcription factors may produce these distinct PKM mRNA sizes, providing a mechanism to regulate the capacity of a neuron to translate PKM in response to synaptic stimulation.

Implications for LTP Maintenance—Our present findings have important implications for the molecular mechanism of LTP. Our original model for PKM formation was based upon observations with C-terminal antisera (5), which recognize both aPKC isoforms, z and d/λ. We found a sequential activation of aPKC in LTP, rapid translocation of full-length aPKC in induction followed by a sustained increase of PKM in maintenance, and we therefore assumed that the two kinases were related as proteolytic precursor and product (5). Whereas a proteolytic mechanism may be important for PKM formation in other forms of synaptic plasticity (44, 45), our current results indicate that the two aPKCs activated in LTP induction and maintenance are distinct isoforms: PKCλ/δ, the full-length aPKC expressed in hippocampus, rapidly translocates during induction, whereas PKM is synthesized de novo from its own mRNA in maintenance (Fig. 7).

Future work will be required to determine the relative contributions of translational and transcriptional regulation to PKM synthesis in LTP. However, because the size of the PKM mRNA is ~100 kb and the rate of transcription is thought to be 1–2 kb/min (46), new transcription of PKM mRNA may require many minutes to hours. Therefore, the de novo synthesis of PKM we observed 10–40 min after tetanization in hippocampal slices (Fig. 6, C and D) is likely due to increased translation of pre-existing PKM mRNA. Release from the translational block mediated by the long 5'-UTR of the PKM mRNA by rapamycin-sensitive pathways or an internal ribosomal entry site (47) are potential mechanisms for the increase in PKM synthesis that we observed.

PKM, a Cognitive Kinase for Long Term Synaptic Memory Storage—Autonomously active kinases have long been attractive candidates for maintaining long term memory because they provide a cogent molecular mechanism for the persistence of synaptic plasticity (48–50). When transiently stimulated by second messengers, several kinases can produce a short term enhancement of synaptic transmission (49). Following the intense synaptic stimulation that induces long term synaptic plasticity, however, a few serine-threonine kinases may also be converted from second messenger-dependent into second messenger-independent forms through persistent post-translational modifications. Two well known examples in LTP are the autophosphorylation of Ca2+/calmodulin-dependent protein kinase II (CaMKII) (48) and PKC oxidation (51). Both modifications result in a conformational change that displaces the autoinhibitory pseudosubstrate of the enzymes, rendering the kinases autonomously active. Because they may then persistently enhance synaptic transmission, these autonomously active forms might serve as molecular memory stores and thus have been called “cognitive kinases” (49).

However, because most cognitive kinases are generated by post-translational modifications of second messenger-dependent enzymes, their persistent action is fundamentally limited by protein turnover, because they are eventually replaced by their non-autonomously active precursors (52). It is therefore generally assumed that cognitive kinases serve mainly in the non-protein synthesis-dependent, early decremental phase of LTP, but serve only a transitional role in the late phase of LTP that requires new protein synthesis and is thought to mediate long term memory. Consistent with this view, inhibitors of CaMKII and conventional novel PKCs block LTP induction but do not reverse LTP maintenance when applied after potentiation has been stably established (7, 53).

PKM, however, has a structure and function different from other cognitive kinases that allows it to couple new protein synthesis directly to the mechanism of synaptic enhancement. PKM is not produced by a post-translational modification of a second messenger-dependent enzyme but is synthesized de novo by transcriptional and translational mechanisms that completely eliminate the autoinhibitory pseudosubstrate of PKC from its catalytic domain (Fig. 7). Thus in contrast to other cognitive kinases, PKM is an autonomous kinase that is generated and maintained by new protein synthesis. Furthermore, the persistent increase in PKM activity, unlike that of CaMKII and conventional/novel PKCs, is critical for maintaining the late phase of LTP, as demonstrated by the reversal of established LTP by PKM inhibitors (7). Thus the synthesis of PKM provides the missing link between gene expression and the mechanism of synaptic enhancement in LTP. These properties may allow PKM to serve as a molecular substrate of long term memory.

REFERENCES
1. Martin, S. J., Grimwood, P. D., and Morris, R. G. (2000) Annu. Rev. Neurosci. 23, 649–711.
2. Sanes, J. R., and Lichtman, J. W. (1999) Nat. Neurosci. 2, 597–604.
3. Zhou, G., Wooten, M. W., and Coleman, E. S. (1994) Exp. Cell Res. 214, 1–11.
4. Hira, T., and Chida, K. (2003) J. Biochem. (Tokyo) 133, 1–7.
5. Sacktor, T. C., Osten, P., Valsamis, H., Jiang, X., Naik, M. U., and Sublette, E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8342–8346.
6. Osten, P., Valsamis, L., Harris, A., and Sacktor, T. C. (1996) J. Neurosci. 16, 2444–2451.
7. Ding, L. S., Benardo, L. S., Serrano, P. A., Blace, N., Kelly, M. T., Crary, J. F., and Sacktor, T. C. (2002) Nat. Neurosci. 5, 295–306.
8. Drier, E. A., Tello, M. K., Cowan, M., Wu, P., Blace, N., Sacktor, T. C., and Yin, J. C. (2002) Nat. Neurosci. 5, 316–324.
9. Nishizuka, Y. (1995) FASEB J. 9, 484–496.
10. Dekker, L. V., Palmer, R. H., and Parker, P. J. (1995) Curr. Opin. Struct. Biol. 5, 396–402.
11. Newton, A. C. (2003) Biochem. J. 370, 361–371.
12. Kishimoto, A., Kajikawa, N., Shiota, M., and Nishizuka, Y. (1983) J. Biol. Chem. 258, 1156–1164.
13. Takai, Y., Kishimoto, A., Inoue, M., and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7603–7609.
14. Vanderklish, P., Saito, T. C., Gall, C., Arai, A., and Lynch, G. (1995) Brain Res. Mol. Brain Res. 28, 25–35.
15. Naik, M. U., Benedikz, E., Hernandez, L., Libien, J., Hrabe, J., Valsamis, M., Dow-Erwin, D., Osman, M., and Sacktor, T. C. (2000) J. Comp. Neurosci. 426, 243–256.
16. Selbie, L. A., Schmitz-Peiffer, C., Sheng, Y., and Biden, T. J. (1993) J. Biol. Chem. 268, 24296–24302.
17. Akimoto, K., Mizuno, K., Osada, S., Hira, S., Tanuma, S., Suzuki, K., and Ohno, S. (1994) J. Biol. Chem. 269, 12677–12683.
18. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Iiguchi, K., and Nishizuka, Y. (1988) J. Biol. Chem. 263, 6927–6932.
19. Chihara, J., Kanazawa, M., Blumberg, P. M., Mushinski, J. F., and Mischak, H. (1992) Gene (Amst.) 122, 305–311.
20. Marshall, B. S., Price, G., and Powell, C. T. (2000) DNA Cell Biol. 19, 707–719.
21. Andrea, J. E., and Walsh, M. P. (1995) Biochem. J. 310, 835–843.
22. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
23. Powell, C. T., Fair, W. R., and Heston, W. D. (1994) J. C. Comp. Neurol. 252, 7603–7609.
24. Maruyama, K., and Sugano, S. (1994) Gene (Amst.) 138, 171–174.
25. Frutos, S., Moscat, J., and Diaz-Meco, M. T. (1999) J. Biol. Chem. 274, 10765–10770.
26. Leitges, M., Sanz, L., Martin, P., Duran, A., Braun, U., Garcia, J. F., Camacho, F., Diaz-Meco, M. T., Bennert, P. D., and Moscat, J. (2001) Mol. Cell 8, 771–780.
27. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Iiguchi, K., and Nishizuka, Y. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3099–3103.
28. Kozak, M. (1987) FEBS Lett. 212–216.
29. Kozak, M. (1987) FEBS Lett. 212–216.
30. Sekiguchi, K., Igarashi, K., and Nishizuka, Y. (1987) FEBS Lett. 212–216.
31. Kozak, M. (1987) FEBS Lett. 212–216.
32. Dobrinski, K., Igarashi, K., and Nishizuka, Y. (1987) FEBS Lett. 212–216.
33. Dobrinski, K., Igarashi, K., and Nishizuka, Y. (1987) FEBS Lett. 212–216.
34. Dobrinski, K., Igarashi, K., and Nishizuka, Y. (1987) FEBS Lett. 212–216.
PKMζ Synthesis from a Brain mRNA

175–178

31. Farese, R. V. (2002) Am. J. Physiol. 283, E1–E11
32. Wooten, M. W., Seibenhener, M. L., Nedigh, K. B., and Vandenplas, M. L. (2000) Mol. Cell. Biol. 20, 4494–4504
33. Land, M., Islas-Trejo, A., and Rubin, C. S. (1994) J. Biol. Chem. 269, 14820–14827
34. Nino, Y. S., Irie, T., Takaishi, M., Hosono, T., Huh, N., Tachikawa, T., and Karski, T. (2001) J. Biol. Chem. 276, 36711–36717
35. Silva, A. J., Kogan, J. H., Frankland, P. W., and Kida, S. (1994) J. Biol. Chem. 269, 14820–14827
36. Yin, J. C., Del Vecchio, M., Zhou, H., and Tully, T. (1995) Cell 81, 107–115
37. Alberini, C. M., Ghirardi, M., Metz, R., and Kandel, E. R. (1994) Cell 76, 1099–1114
38. Roeder, R. G. (1996) Trends Biochem. Sci. 21, 327–335
39. Merle, E., Freudenthal, R., and Romano, A. (2002) Neuroscience 112, 161–172
40. Roeder, R. G. (1996) Trends Biochem. Sci. 21, 327–335
41. Smale, S. T. (1997) Biochim. Biophys. Acta 1331, 73–88
42. Suzuki, Y., Ishihara, D., Sasaki, M., Nakagawa, H., Hata, H., Tsunoda, T., Watanabe, M., Komatsu, T., Ota, T., Isogai, T., Suyama, A., and Sugano, S. (2000) Genomics 64, 286–297
43. Suzuki, Y., Taira, H., Tsunoda, T., Mizushima-Sugano, J., Sese, J., Hata, H., Ota, T., Isogai, T., Tanaka, T., Morishita, S., Okubo, K., Sakaki, Y., Nakamura, Y., Suyama, A., and Sugano, S. (2001) EMBO Rep. 2, 388–393
44. Powell, C. M., Johnston, D., and Sweatt, J. D. (1994) J. Biol. Chem. 269, 27858–27863
45. Shilatifard, A. (1998) PNAS 12, 1437–1446
46. Yeh, S. H., Lin, C. H., Lee, C. F., and Gean, P. W. (2002) J. Biol. Chem. 277, 46720–46729
47. Roeder, R. G. (1996) Trends Biochem. Sci. 21, 327–335
48. Chang, Y. Y., and Thiels, E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8310–8313
49. Malinow, R., Madison, D. V., and Tsien, R. W. (1988) Nature 335, 820–824
50. Klann, E., and Thiels, E. (1999) Prog. Neuropsychopharmacol. Biol. Psychiatry 23, 359–376
51. Roberson, E. D., and Sweatt, J. D. (1999) Learn. Mem. 6, 381–388
52. Otmakhov, N., Griffith, L. C., and Lisman, J. E. (1997) J. Neurosci. 17, 5357–5365