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Commentary

Cognitive kinases

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Kant proposed that Space and Time underlie all knowledge; he further proposed that their mental representations are not derived empirically but are *a priori* or innate (1). For present-day neuroscience, the physical basis of Kant's representation of Space might be understood as localization of function, both sensory and motor, to specific brain regions, often somatotypic but always mapped in some topologically coherent pattern (for a review, see ref. 2). What would embody the concept of Time? One of the principal ways Time is apprehended is through memory, a connection between innate brain structure and temporal aspects of the empirical world. A fair case could be made that Time is represented in the brain by a family of Ser/Thr protein kinases—the cAMP-dependent protein kinase, protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinases II (Ca^{2+}/CaM kinase) and closely related family members, for example, CDC2-regulated cyclins (3).

The prevailing hypothesis guiding cognitive neurobiological research is that persistent changes in synaptic efficacy underlie the formation of learning and memory (4–8). Altered synaptic efficacy can be produced presynaptically and postsynaptically. Any process that changes the amount of neurotransmitter released would serve as a presynaptic mechanism: modulation of ion channels is the most thoroughly studied; changes in the mobilization of synaptic vesicles for exocytosis appear to be the other common presynaptic process (for a review, see ref. 8). Any process that alters receptor action would serve as a postsynaptic mechanism. Molecular events that underlie later stages of learning are changes in synaptic structure that result in making these pre- and postsynaptic processes endure (9). These changes, which are brought about by gene expression, are the favored explanation for the persistence of memory (10–12).

Long-term potentiation (LTP) is currently the predominant vertebrate model for the production of explicit memory (for reviews, see refs. 13–15). Originally, LTP was described in intact animals, where brief high-frequency stimulation of afferent pathways to the hippocampus causes transmission to be enhanced for hours to days. The synapses enhanced are between the afferent pathway stimulated and its postsynaptic neurons in the hippocampus (16, 17). Since LTP can be demonstrated *in vitro* (18, 19), it now is routinely examined in hippocampal slices, which remain healthy for several hours in the hands of most investigators. The experimental accessibility of the slice preparation permits versatile combinations of electrophysiological, pharmacological, and biochemical analyses, which reveal that the enhancement of synaptic transmission in LTP involves both postsynaptic and presynaptic processes (see ref. 8).

Many pharmacological studies with hippocampal slices have implicated members of the Ser/Thr family of protein kinases in mediating the induction and maintenance of LTP. Earlier experimental and theoretical work in both invertebrates and vertebrates had revealed that the enzyme molecules of this family of protein kinases have a special mnemonic characteristic: post-translational modification of a kinase—autophosphorylation, specific proteolytic cleavage, and changes in subcellular location—caused by activation of that kinase by its second messenger produces an enzyme that can be activated subsequently by decreased concentrations of the original second messenger. The modification thus makes the enzyme less dependent on the second messenger, thereby prolonging its action well beyond the initial stimulating event. In the limit, this recursive mechanism can result in a persistently active (or autonomous) protein kinase that is largely independent of the original second messenger stimulus (for a review, see ref. 20; for cAMP-dependent protein kinase, see ref. 21; and for Ca^{2+}/CaM kinase II, see refs. 13 and 22).

In this issue of the *Proceedings*, Klann *et al.* (23) and Sacktor *et al.* (24) confirm that induction of LTP in slices of rat hippocampus results in the immediate activation of PKC and report that kinase activity persists during the maintenance phase (long after the initial high-frequency stimulation to the CA1 region). These results provide direct evidence that PKC is activated in LTP. They also hold out the promise of a finer resolution of the temporal aspects of LTP.

The temporal pattern of stimulation is important for determining the duration of the synaptic enhancement. Brief low-frequency stimulation causes release of the neurotransmitter glutamate producing transient depolarization of postsynaptic hippocampal neurons; depolarization is brought about largely by conventional ligand-gated ionophoric receptors for glutamate. A single 1-sec train of high-frequency stimuli causes the release of enough glutamate to activate these conventional receptors and a critical number of *N*-methyl-D-aspartate (NMDA) receptors as well. NMDA receptors have the special property of being gated by both glutamate and by voltage. This form of stimulation, used by Sacktor *et al.* (24), induces LTP within seconds, which is then maintained for several hours. Information is also transmitted in the retrograde direction from the postsynaptic target neuron back to presynaptic terminals, augmenting the release of glutamate (for a review, see ref. 8). Three or more sets of high-frequency stimulation, separated by periods of 5–10 min, in addition to causing depolarization and the activation of NMDA receptors, which produces the induction and maintenance phases, also initiate an additional late-phase form of LTP. This late phase extends the synaptic enhancement of the hippocampal synapses for much longer periods of time: many hours in slices and, presumably, weeks to months in intact animals. The late-phase process begins only an hour or two after the initial course of stimulation (for a review, see ref. 25). These are the conditions of stimulation used by Klann *et al.* (23).

LTP is thus characterized by three phases. Induction marks the transference of the process from ionic to biochemical—the increased influx of Ca^{2+} ion through NMDA receptors activating two of the three major Ser/Thr protein kinases, Ca^{2+}/CaM kinase II and PKC. Prior to the two papers that appear in this issue, almost all of the evidence for the participation of PKC in induction was pharmacological (for reviews, see refs. 23 and 24). The results presented in these papers now show directly that PKC is activated during induction of LTP. Previously, it was controversial whether PKC also participates in the maintenance phase of LTP. The two papers under discussion now show convincingly not

only that PKC is activated persistently in the maintenance phase but also that the activated kinase is autonomous. Before discussing the molecular mechanisms by which persistently active PKC is produced and some of the apparent discrepancies between the results reported in the two papers, the late phase needs to be mentioned. This phase, which has been difficult to examine experimentally because of the transient viability of hippocampal slices, now appears to depend on the activation of cAMP-dependent protein kinase, the third major member of the Ser/Thr protein kinase family, and to require newly synthesized protein (25).

Consistent with the idea that the entry of Ca^{2+} through NMDA receptors initiates LTP, Ser/Thr protein kinases that are Ca^{2+}-dependent (Ca^{2+}/CaM kinase II and the Ca^{2+}-activated isoforms of PKC) had been implicated in the induction phase. Several lines of evidence made Ca^{2+}/CaM kinase II an attractive enzyme for initiating the expression of LTP: in addition to its dependence on Ca^{2+}, it is one of the most abundant proteins in dendrites, constituting a large proportion of the postsynaptic density (see ref. 13). Furthermore, selective inhibitors of the regulatory domain of the kinase (for example, calmidazolium and synthetic peptides), as well as the isoquinoline H7, a general inhibitor of Ser/Thr protein kinases, block induction of LTP in hippocampal slices. Still further, experimental data on Ca^{2+}/CaM kinase II were the first to become available that appeared to support Crick's (26) and Lisman's (27) theoretical models of molecular memory involving second messenger-induced persistent kinase activity through intermolecular autophosphorylation (for a review, see ref. 22). Finally, inferential support was provided by the observations that spatial learning is impaired in mice made mutant in α Ca^{2+}/CaM-dependent protein kinase II (28) and that hippocampal slices from these transgenic mutant mice are deficient in their ability to produce LTP (29). But the evidence for PKC was unclear.

Most difficult to interpret were the pharmacological observations that inhibitors specific to PKCs—for example, sphingosine and polymyxin B—block induction but not maintenance, whereas others block both induction and maintenance. To make matters worse, results from different laboratories were inconsistent. The results also depended on how the drugs were administered; for example, injection of H7 into the postsynaptic neuron blocks induction alone, while application in the bath blocks maintenance as well. While this may be the first evidence for the involvement of a presynaptic protein kinase in the maintenance of LTP, the ineffectiveness of the specific PKC inhibitors was still unexplained (for reviews, see refs. 23 and 24). Tentatively, the general opinion was that PKC is required for postsynaptic induction but not for maintenance. To clarify these issues and to discuss the significance of the results presented by Klann *et al.* (23) and by Sacktor *et al.* (24) in this issue of the *Proceedings*, it is necessary to review current information on the structure of the PKCs (Fig. 1).

To date, 10 distinct isoforms of PKC have been characterized; 7 are expressed in vertebrate nervous tissue. Like other members of the Ser/Thr protein kinase family, PKCs are composed of two general regions, one regulatory and the other catalytic. With PKC, regulation is inhibitory: in the absence of extrinsic activators (second messengers and other factors—for example Ca^{2+}, specific proteins, lipids, and membrane), the kinase molecule is inactive because the regulatory portion of the enzyme molecule masks the catalytic site. A domain in the regulatory portion of the kinase contains a sequence of amino acid residues that are similar or identical to the sequence that is required in substrate proteins for phosphorylation to occur. Unlike the sequence in substrate proteins, however, the serine or threonine residue to which a phosphoryl group would be transferred is absent. In its place, the corresponding sequence in the regulatory domain contains a residue that lacks a hydroxyl moiety, usually alanine. Because it binds to the catalytic site with great affinity, the regulatory sequence (indicated by ψ in Fig. 1) behaves as a pseudosubstrate. Binding of the second messenger (together with other factors) to the regulatory portion of the kinase changes the conformation of the inactive enzyme, causing dissociation of the regulatory and catalytic portions of the kinase, thereby releasing the catalytic site for action. In PKC, the catalytic portion is located at the carboxyl-terminal part of the molecule and is connected to the amino-terminal regulatory portion by a hinge region: the inactive kinase can be pictured as being folded at the hinge; the activated kinase is extended as shown in the figure. In all 10 isoforms, the structure of the catalytic part of the molecule is highly conserved; in contrast, Nishizuka (30) recognizes three subspecies of the enzyme that differ markedly in their regulatory regions. Nishizuka (31) originally isolated the catalytic portion presumably of several PKC isoforms, believing it to be a new type of unregulated protein kinase. These fragments, which require Mg^{2+} but are active in the absence of any second messenger, were produced as a result of proteolytic cleavage at the hinge region by the abundant Ca^{2+}-dependent protease, calpain, during extraction of the kinase from brain for purification. Fragments of PKC containing active catalytic regions are now called PKM—M for Mg^{2+}.

The PKC-specific inhibitors used in these pharmacological studies affect domains in the regulatory part of the kinase molecule, interacting with sites required for activating the catalytic portion. For example, sphingosine and polymyxin B interact with the C1 domain, which is thought to be the regulatory region that binds the lipid activator diacylglycerol or its analog, phorbol ester, and to mediate much of the kinase's translocation to membrane (or to phosphatidylserine *in vitro*). This domain, which contains two cysteine-rich zinc-finger motifs in typical

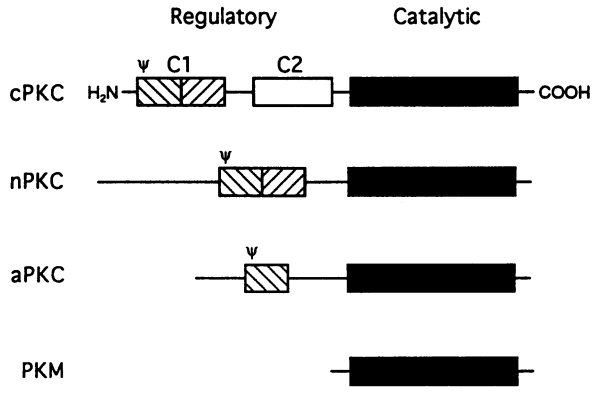

FIG. 1. Structure and classification of PKCs. Nishizuka (30) classifies the 10 known isoforms of PKC into three subspecies on the basis of the constant domains, C1 and C2, in the regulatory portion of the kinase: c or classical (PKCα, βI, βII, γ), n or new [PKCδ, ε, η (L), θ], and a or atypical (PKCz and λ). c- and nPKCs contain a C1 domain with two cysteine-rich zinc-finger motifs, the most amino-terminal one containing the regulatory pseudosubstrate sequence of amino acids (ψ) (see text). The lack of C2, the domain responsible for activation by Ca^{2+} ion, distinguishes PKCs that are Ca^{2+}-activated (cPKCs) from those that are Ca^{2+}-independent (nPKCs and aPKCs). PKMs are fragments of PKCs that contain active catalytic regions; they are generated by proteolytic cleavage at the hinge region connecting the amino-terminal regulatory portion of a PKC molecule and the carboxyl-terminal catalytic portion.

PKCs (cPKCs and nPKCs), also contains the pseudosubstrate sequence situated toward the amino-terminal part of the first cysteine-rich domain. Two isoforms, PKCζ and PKCλ, contain only the first cysteine-rich domain and are categorized as atypical PKCs (aPKCs); while aPKCs are translocated to membrane (and require phosphatidylserine), they are not affected by diacylglycerol or phorbol esters. C2, the regulatory domain that confers sensitivity to Ca^{2+}, enhances the affinity by which the lipid activator binds. Subspecies of PKCs containing a C2 domain are Ca^{2+}-activated and were the first to be characterized. When PKCs lacking the C2 domain were discovered and found to be Ca^{2+}-independent, they were called minor forms. As Ca^{2+}-independent isoforms are now known to be neither minor in number nor in abundance, Nishizuka (30) proposed a new classification: PKCs with C1 domains are conventional or classical (cPKCs) and PKCs lacking C2 are new (nPKCs). Both cPKCs and nPKCs are considered typical, however.

Klann *et al.* (23), using a selective substrate in enzyme assays, and Sacktor *et al.* (24), using isoform-specific antisera, now show that both Ca^{2+}-activated and Ca^{2+}-independent isoforms of PKC are activated after the high-frequency stimulation, but only transiently. Furthermore, they show that PKC is active during the maintenance phase 30–180 min after induction. Unexpectedly, they both find a persistently active form of the kinase with atypical properties: it is independent of diacylglycerol or phorbol ester, it is not activated by Ca^{2+} and, most surprising of all, it is present in the cytoplasm and independent of phosphatidylserine. Sacktor *et al.* (24) present strong evidence that this atypical activity is PKMζ. Specific antibodies raised against a carboxyl-terminal peptide sequence of PKCζ were used to detect three major proteins in the CA1 region of the hippocampus with molecular weights of 51,000, 70,000, and 160,000. While the larger protein is not yet identified, the M_r 70,000 protein corresponds to intact PKCζ. Cyanogen bromide peptide mapping indicates that the M_r 51,000 component is PKMζ. In addition, the PKCζ-specific antiserum immunoprecipitates only one autophosphorylated polypeptide with a molecular weight of 51,000.

Although there was some previous indication that PKM forms of PKC are not always artifacts of extraction but sometimes might have physiological significance (32), the idea was not widely accepted. Sacktor *et al.* (24) now show that generation of PKMζ occurs in the neuron and is not due to extraction: the increase in PKMζ is only observed in stimulated slices and no increase is seen if NMDA receptors (and LTP) are blocked. Formation of PKMζ would account for the failure of PKC-specific agents to affect maintenance, since their targets in the intact PKC molecule are absent in PKMζ and, therefore, no longer can affect PKC activity. To the contrary, H7 would be expected to interfere with PKMζ's function, since it blocks the ATP-binding site (the C3 domain), which is still present at the amino-terminal end of the catalytic portion of the molecule. H7 is not a specific inhibitor of PKC, however, since it is also an effective inhibitor of other Ser/Thr protein kinases.

Klann *et al.* (23) assayed PKC activity at various times beginning 3 h after stimulation and show that the atypical kinase peaks at 45 min and persists for at least 2 h more. (The slow decrease in activity observed after 45 min probably reflects declining viability of the slice preparation.) As with Sacktor *et al.* (24), activation of NMDA receptors and the induction of LTP are necessary for this persistence to occur. In contrast, however, Klann *et al.* (23) find no evidence for the formation of PKM and suggest that proteolytic activation is not responsible for the increase in PKC activity. They suggest that, since neither of the two well-known mechanisms—proteolysis and membrane insertion—can account for the persistence, activation of PKC must occur by another mechanism. They present some evidence that autophosphorylation or phosphorylation by other protein kinases might be involved; phosphorylation is known to occur in both the regulatory and the catalytic portions of PKC (33–37). Thus, it might be possible that phosphorylation in the regulatory portion of a PKC might endow that enzyme with its atypical behavior, a suggestion akin to the mechanism proposed for Ca^{2+}/CaM kinase II (22). Phosphorylation of regulatory domains could also account for the failure of PKC-specific inhibitors to affect maintenance: in a PKM, the targets of the inhibitors are absent; in a phosphorylated PKC, the targets could be blocked. Nonetheless, neither modification would account for the difference in sensitivity to the inhibitors, which depend on how the agents are administered. These differences probably can best be explained by the locations of the PKC affected. Thus, injection of inhibitors into the postsynaptic neuron would be expected to act selectively on postsynaptic PKCs, while bath-applied inhibitors would act on kinases in presynaptic terminals as well.

What might account for the major differences between the two papers in explaining the molecular mechanisms of persistence? The simplest explanation would appear to be the antibody Klann *et al.* (23) used to search for a PKM. This antibody was raised by Huang *et al.* (35) against partially purified cPKCs and does not detect abundant nPKCs (compare figure 4*A* of ref. 23 with figure 1*B* of ref. 24). The antibody therefore probably is not capable of recognizing PKMζ. Even so, Klann *et al.* (23) present some tentative evidence for their phosphorylation mechanism as well as a suggestion for the synthesis of PKC. Is it possible that the generation of PKMζ by proteolysis and the regulation of persistence by protein phosphorylation both occur during the maintenance phase of LTP and that increased PKC synthesis occurs at the transition between early-phase and late-phase LTP? Some of the differences seen between the two papers may thus be explained by the difference in stimulation protocol: the protocol of Sacktor *et al.* (24) producing the early phase and the protocol of Klann *et al.* (23) producing both phases of LTP.

Both of these papers present results that illustrate how learning and memory might be reflected in the molecular properties of PKC. In a metaphorical sense, PKC and the other Ser/Thr protein kinases behave as if they were taught, memorizing temporal connections between events in the empirical world. Further suggestion that these kinases are Time's molecular representation is their involvement in neural mechanisms that set circadian pacemakers (for a review of circadian rhythm in both vertebrates and invertebrates, see ref. 38). Furthermore, Ser/Thr protein kinases are crucial regulators of the timing and coordination of the eukaryotic cell cycle (39, 40). To modern neuroscientists, Kant's idea that knowledge begins with experience but must conform to innate concepts of Space and Time is a prescient philosophical formulation of post-Darwinian ideas of brain and behavior (41). Time will tell if there is any scientific future in thinking that the complex biochemical pathways underlying LTP have a Kantian beat.

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