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Calcium/calmodulin-dependent protein kinase II: role in learning and memory

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Abstract

Numerous studies over the past decade have established a role(s) for protein phosphorylation in modulation of synaptic efficiency. This article reviews this data and focuses on putative functions of Ca^{2+}/calmodulin-dependent protein kinase II (CaM-kinase II) which is highly concentrated at these synapses which utilize glutamate as the neurotransmitter. Evidence is presented that CaM-kinase II can phosphorylate these glutamate receptor/ion channels and enhance the ion current flowing through them. This may contribute to mechanisms of synaptic plasticity that are important in cellular paradigms of learning and memory such as long-term potentiation in the hippocampus. (Mol Cell Biochem **127/128:** 93–101, 1993)

Key words: protein phosphorylation, learning and memory, synaptic plasticity

Introduction

Neural tissues are the most abundant sources for many protein kinases and phosphatases. Indeed, calcium/dalmodulin-dependent protein kinase II (CaM-kinase II, reviewed in 1 and 2) constitutes 2% of protein in certain regions of the brain such as hippocampus. Major emphasis has been on characterization of the multifunctional protein kinases cAMP-kinase (PKA), protein kinase C (PKC) and CaM-kinase II, and many neuronal systems regulated by protein phosphorylation have been identified over the past ten years. Studies on the Aplysia gill withdrawal reflex, a simple model of associative learning, identified PKA-mediated phosphorylation of a K^+ channel or its associated regulatory protein as the pivotal event in prolonging presynaptic depolarization with resultant increased neurotransmitter release [3]. An intensely studied form of usage-dependent mammalian learning and memory is long-term potentiation (LTP, reviewed in [4]) in region CA1 of rat hippocampus. Since postsynaptic influx of Ca^{2+} is essential for the induction of LTP, it was logical, in analogy to the Aplysia system, to determine if Ca^{2+}-dependent protein kinases might be involved. Indeed, early studies indicated this is the case [5], and more recent work (see below) has strengthened this hypothesis. CaM-kinase II is an attractive candidate for involvement in LTP since 1) its unique regulatory properties make it ideal as a molecular sensor of synaptic usage [6], and 2) it is the most abundant protein in the postsynaptic densities [7] of these excitatory synapses in hippocampus. This minireview will focus on these unique regulatory properties of CaM-kinase II and its neuronal substrates that appear to be important in models of learning and memory such as LTP.

CaM-kinase II

CaM-kinase II is an oligomeric multifunctional isozyme family present in most tissues but particularly abundant

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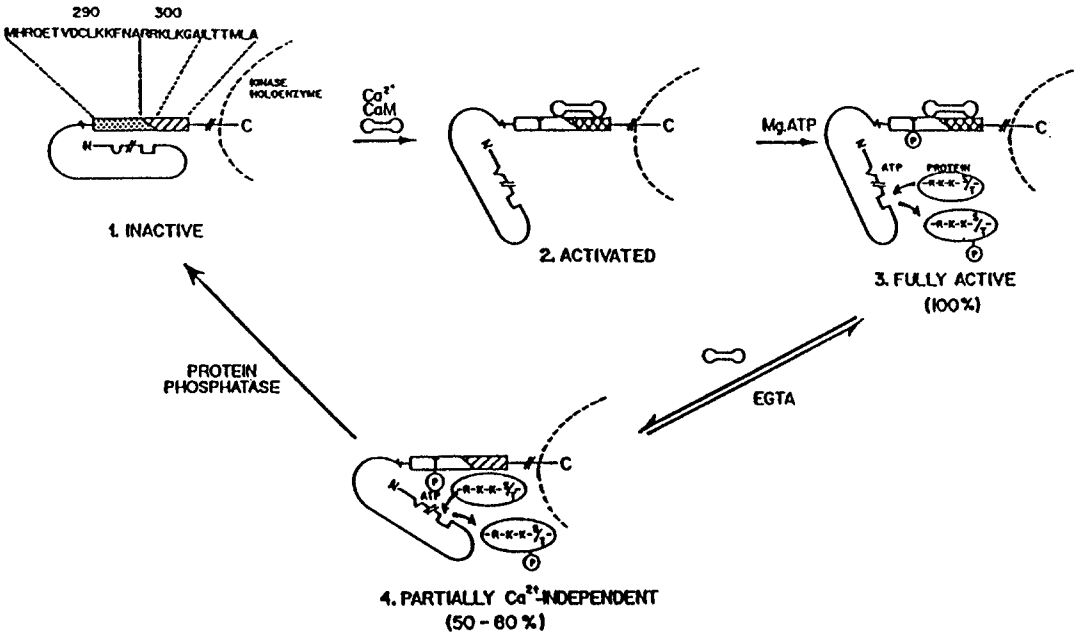


Fig. 1. Regulatory model for CaM-kinase II. The amino acid backbone of an α subunit of the brain kinase is represented by the solid line. The catalytic sites which bind Mg^{2+}/ATP and protein substrate are designated by ⌐/¬, near the NH_2-terminus. The COOH-terminus is thought to be involved in subunit assembly of the oligomeric protein. See text for details.

in brain [1, 2]. The adult rat forebrain enzyme (650 kDa) has been most thoroughly studied and is comprised of 10–12 subunits (50–62 kDa each) of three types, α:β/β′, in molar ratios of 4:1. The α and β subunits are encoded by separate genes, whereas the β′ subunit is a splice variant of the β subunit. Additional subunits, expressed in brain and other tissues, show high sequence conservation in the catalytic and regulatory domains. Domain localization has been determined by analyses of the deduced amino acid sequences [8, 9]: the NH_2-terminal half of the subunits contain the highly conserved kinase motifs; the central portion has a regulatory domain; and the COOH-terminal portion is thought to be involved in subunit assembly and perhaps subcellular localization. Electron microscopy suggests that the COOH-terminal association domains form a central core in the holoenzyme surrounded by globular catalytic domains that are arranged like flower petals [10].

CaM-kinase II is activated by binding of Ca^{2+}/CaM and subsequent autophosphorylation, and the molecular mechanisms of these activations have been extensively studied [1, 2]. Our current understanding of the regulation of CaM-kinase II will be summarized using the model of Fig. 1. The regulatory domain of CaM-kinase II is comprised of two motifs which overlap [11, 12], a CaM-binding domain (residues 296–309) and a bisubstrate-directed autoinhibitory domain (residues 281–302, all residue numbering will refer to the α subunit unless indicated otherwise) which occupies the catalytic domain and inactivates the kinase (form 1, Fig. 1). Binding of Ca^{2+}/CaM to residues 296–309 neutralizes the inhibitory potency of the autoinhibitory domain [12], presumably by a conformational change, promoting its dissociation from the catalytic domain (form 2, Fig. 1). The activated kinase binds its first substrate, Mg^{2+}/ATP, in an ordered reaction mechanism [13]. There is extremely rapid (seconds at 30°) autophosphorylation of Thr^{286} which precedes phosphorylation of exogenous proteins (form 3 of Fig. 1) [14]. Although this intramolecular autophosphorylation of Thr^{286} is not obligatory to phosphorylation of exogenous proteins, it does convert CaM-kinase II into a partially (50–80%) Ca^{2+}/CaM-independent form (form 4, Fig. 1) [15–17]. A model has been proposed whereby formation of this constitutively active Ca^{2+}-independent CaM-kinase II may function as a molecular sensor of synaptic usage [6]. The general concept is that the frequency of excitatory synapse usage would give rise to graded levels of postsynaptic intracellular Ca^{2+}. These various levels of postsynaptic Ca^{2+} would generate different amount of Ca^{2+}-independent CaM-kinase II due to autophosphorylation. Thus, rapidly firing synapses would generate higher levels of constitutively active CaM-kinase II which could then phosphorylate unique substrates (e.g., glutamate receptors, see below) in the postsynaptic density. Another consequence of autophosphorylation of Thr^{286} is that the dissociation rate

for Ca^{2+}/CaM decreases by about 100-fold [18]. Thus, it has been proposed that the autophosphorylated CaM-kinase II can trap CaM for short periods of time and may act as a unique Ca^{2+} spike frequency detector [18]. Protein phosphatases 1, 2A and 2C can dephosphorylate CaM-kinase II and regenerate the original Ca^{2+}/CaM-dependency (form 1, Fig. 1).

The primary consensus phosphorylation sequence [1, 2] in substrates of CaM-kinase II is -R-X-X-S/T- (X can be any residue). The presence of a second Arg two residues NH_2-terminal of the Ser/Thr, which is a strong positive determinant for cAMP-kinase, can be a negative determinant for CaM-kinase II [19]. This presumably explains why CaM-kinase II phosphorylates the Thr whereas cAMP-kinase phosphorylates the adjacent Ser (R-R-A-S-T-I) in cardiac phospholamban [20]. A secondary phosphorylation sequence determinant appears to be an Asp two residues COOH-terminal of the Ser/Thr (-S/T-X-D-) [21]. It should be noted that the Thr^{286} autophosphorylation site in CaM-kinase II contains both determinants (-R-Q-E-T-V-D-). CaM-kinase II is considered a multifunctional protein kinase, like cAMP-kinase and protein kinase C, since it phosphorylates *in vitro* a large number of proteins [1, 2] including numerous metabolic enzymes (e.g., tyrosine and tryptophan hydroxylases), structural proteins (e.g., synapsin, MAP-2 and tau), ion channels (e.g., Cl^- channel and glutamate receptor/channel), hormone receptors (e.g., EGF receptor), and nuclear transcription factors (e.g., CREB and C/EBPβ). There is accumulating evidence in the past several years for phosphorylation of many of these substrates in intact tissues or cells. For example, CaM-kinase II catalyzed phosphorylation of synapsin 1 in the presynaptic terminus is involved in mobilization of synaptic vesicles for exocytosis [22].

An intriguing aspect of CaM-kinase II is the observation that it constitutes the major forebrain postsynaptic density (PSD) protein [7]. This conclusion is based on comparisons between the purified soluble CaM-kinase II 50 and 60 kDa subunits and the isolated 50 and 60 kDa major PSD proteins with regard to 1) immunoreactivity against several polyclonal and monoclonal antibodies, and 2) peptide maps using either ^{32}P- or ^{125}I-labeling. Additionally, soluble and PSD CaM-kinase II are regulated similarly by autophosphorylation [23] and by proteolytic activation with calpain [24]. We are particularly interested in identifying important substrates of CaM-kinase II in the PSD, and glutamate receptors (GluRs) are a good candidate since they are also present in the PSD [25] and glutamate is the major excitatory neurotransmitter in forebrain. GluRs are intimately involved in LTP [4], and there is accumulating evidence that phosphorylation in GluRs may be an important regulatory mechanism.

Protein kinases and synaptic plasticity

There is strong evidence for the involvement of protein kinases in LTP [26], a model of usage-dependent synaptic plasticity in mammalian forebrain [4]. Brief, high-frequency stimulation of hippocampal excitatory synapses results in a substantial increase in subsequent synaptic strength that can last for hours or longer. Initiation of the LTP requires influx of Ca^{2+} into the postsynaptic dendritic spine through activation of N-methyl-D-aspartate (NMDA) GluRs. Multiple lines of evidence strongly implicate postsynaptic serine/threonine and perhaps tyrosine protein kinases in the induction of LTP [26]. For example, postsynaptic microinjection of peptide inhibitors of CaM-kinase II or PKC block induction of LTP [27]. Because of the high concentration of CaM-kinase II in the PSD of these excitatory synapses, it is an especially attractive candidate for a regulatory role. In fact, mice lacking the major isoform of CaM-kinase II do not exhibit LTP [28] or another form of hippocampal learning, the Morris water test [29]. This observation reinforces the conclusion that CaM-kinase II is important for hippocampal learning and memory mechanisms. Expression of LTP appears to involve both increased presynaptic release of glutamate [30] and postsynaptic responsiveness of AMPA-type GluRs [31]. Therefore, we wanted to address two questions: 1) does Ca^{2+}-influx through NMDA channels activated Ca^{2+}-dependent protein kinases such as CaM-kinase II and PKC, and 2) do CaM-kinase II and PKC phosphorylate and regulate AMPA-type GluRs. The first question will be addressed in this section and the second question in the last section.

Elevation of intracellular Ca^{2+} can generate the autophosphorylated, Ca^{2+}-independent form of CaM-kinase II. In cultured cerebellar granule cells [32], PC12 cells [33], and GH_3 cells [34] K^+ depolarization rapidly produces partially Ca^{2+}-independent CaM-kinase II which then slowly decays back to the basal value. The extent and duration of CaM-kinase II activation is potentiated but not stabilized by okadaic acid [32], indicating that multiple endogenous protein phosphatases play an important role in limiting the extent and duration of kinase activation. In the PC12 and GH_3 cell studies, agonists which trigger the phosphatidylinositol (IP_3) pathway

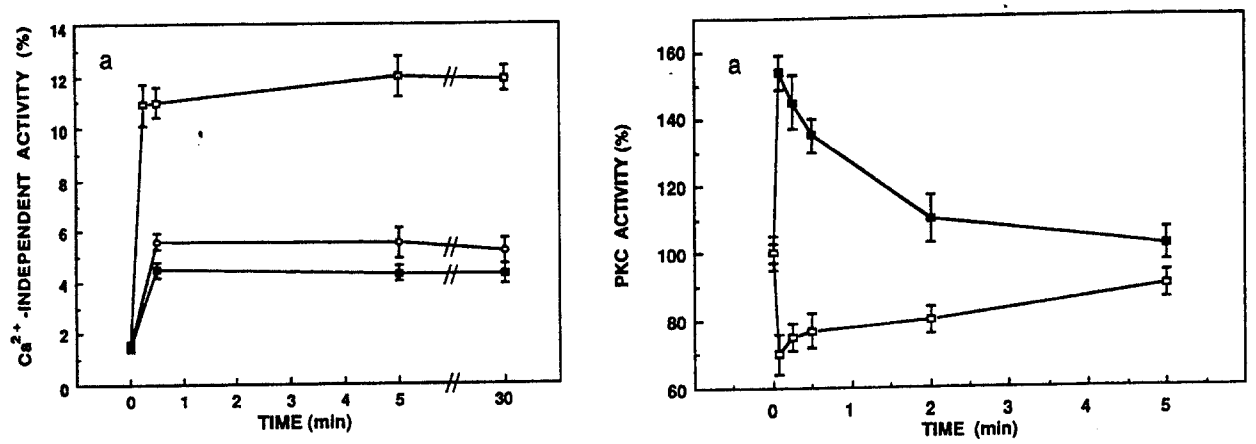
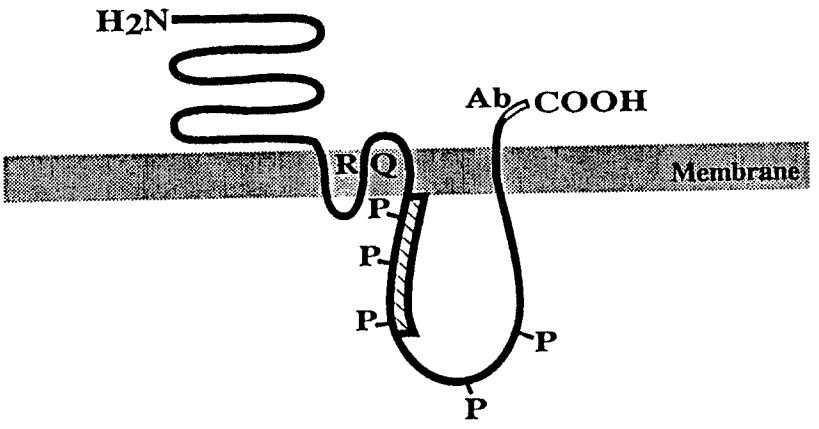


Fig. 2. Activation of CaM-kinase II (left panel) and PKC (right panel) by glutamate in hippocampal neurons. Cultured hippocampal neurons were incubated in Krebs Ringer Hepes (KRH) buffer without Ca^{2+} (left panel) or with Ca^{2+} (righ panel) for one hour. At time 0 the following additions were made: *left panel.* Mg^{2+}-free KRH plus Ca^{2+} (2.7mM) alone (Θ) or with 10μM glutamate/1μM glycine (□) and 100μM APV (an NMDA antagonist) (■); *right panel.* normal KRH plus 10μM glutamate. Ca^{2+}-dependent activity of CaM-kinase II (left panel) or PKC activity (right panel) in the particulate (■) and soluble (□) cell homogenate were determined. Reproduced by permission of J. Biol. Chem. from ref. 39.

give a small, transient activation of CaM-kinase II. Glutamate (10μM) activates CaM-kinase II in cultured cerebellar granule cells, and this response is mediated by NMDA receptors [35]. In cerebellar granule cells [36] PKC translocation and phosphorylation of several membrane proteins is mediated by NMDA receptors (i.e., requires Ca^{2+} influx and is blocked by NMDA antagonists), whereas in cortical neurons [37] NMDA, AMPA and/or metabotropic receptors appear to be involved.

The above results clearly demonstrate that glutamate can elicit activation of both PKC and CaM-kinase II in some neuronal preparations, but effects in hippocampus are of particular interest since synaptic plasticity has been intensively studied in this tissue. Activation of CaM-kinase II and PKC through the NMDA-receptor would be consistent with the body of evidence that one or both of these protein kinases is involved in initiation and/or expression of LTP [26]. In cultured hippocampal neurons glutamate (and phorbol esters) transiently elevate the ^{32}P-labeling of three specific proteins, one of which has been identified as the PKC substrate MARCKS, in the absence or presence of extracellular Ca^{2+} [38]. We have recently investigated regulation of CaM-kinase II and PKC in hippocampal neurons [39]. Glutamate partially activates CaM-kinase II (i.e., increases Ca^{2+}-independence) and causes translocation of PKC (Fig. 2). CaM-kinase II activation is prolonged and mediated through the NMDA receptor whereas the PKC translocation is very transient (1–2min) and apparently requires metabotropic receptor stimulation.

Regulation of protein kinases has also been studied in hippocampal slices. Organotypic cultures of hippocampal slices have high basal CaM-kinase II activation which can be modulated by altering extracellular Ca^{2+} or by addition of a protein kinase inhibitor (H7), a calmodulin inhibitor (W7), or the protein phosphatase inhibitor okadaic acid [40]. However, CaM-kinase II activation is not consistently increased by K^+ depolarization or by NMDA even through these treatments do elevate cytosolic Ca^{2+} [40] and apparently activate the CaM-dependent phosphatase calcineurin [41]. It is interesting that NMDA application to hippocampal slices in normal extracellular Ca^{2+} also fails to induce stable synaptic potentiation. However, if the extracellular Ca^{2+} is increased to 6–10mM then NMDA application does give a long-lasting potentiation [31]. It will be interesting to determine if these conditions also activate CaM-kinase II. Importantly, it has recently been reported that 45–60min after induction of LTP in hippocampal CA1 region there is increased Ca^{2+}-independent PKC activity [42]. It is not know if this stabilized activation of PKC occurs presynaptically. PKC [43] or CaM-kinase II [44] activation can stimulate glutamate release from isolated synaptosomes, and such a mechanism could be involved in the enhanced release of glutamate which probably occurs in LTP [30]. Lastly, induction of LTP or application of NMDA to hippocampal slices can elevate cAMP [45], and NMDA application to hippocampal neurons appears to trigger a protein kinase cascade involving both tyrosine and serine/threonine protein kinases since MAP-2 kinase is activated via phosphorylation on both tyrosine and threonine residues [46].

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GluR1   NLAAFLTVERMVSPIESAEDLAKQTEIAYGTLEAGSTKEFFRRSKIAVFEKMWTYMKSAEPSVFVRTT
GluR5                E          K E    R  S M T          S RQ S
GluR6                E          K E       A M T          S RR S
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Fig. 3. The top diagram is a schematic of the general topology of the ionotropic GluRs. The sequence of the stippled part of GluR1 loop 3 is given below the diagram with putative phosphorylation sites underlined and designated as follows: *PKC phosphorylation site; +CaM-kinase II phosphorylation site; °cAMP kinase phosphorylation site. There are no nonconservative substitutions in GluR1–4 in this sequence, but non-conservative substitutions in GluR5 and R6 are shown, some of which generate new putative phosphorylation sites. Additional phosphorylation sites lie COOH-terminal of this sequence in loop 3. The interaction site of the antibody (Ab) is also designated.

Glutamate receptors

Many members of the GluR family have been cloned in the past several years. They can be categorized into several families: α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA)-type (GluR1-4) [47, 48], kainate-type (GluR5–7) [49] and N-methyl-D-aspartate (NMDA)-type [50] are glutamate-activated ion channels. Metabotropic receptors [51] are coupled through G proteins to signal transduction systems. GluR1–4 have about 70% amino acid sequence homology, and GluR5–7 are 80% homologous with each other but only 40% homologous to GluR1. The NMDA receptor is 20–25% homologous to GluR1. There is evidence that native AMPA ion channels in hippocampus are heteromeric complexes of GluR1-4 [52]. Consistent with this is the observation that coexpression of certain combinations (e.g., GluR1 plus GluR2) of GluRs more closely resemble native AMPA ion channels [47]. GluR1–6 all have a large intracellular loop (loop 3) between transmembrane segments 3 and 4. Of primary relevance to this review, loop 3 has several consensus putative phosphorylation sites for the three multifunctional protein kinases. The metabotropic receptor and the NMDA receptor also have consensus phosphorylation sites on intracellular loops. The putative phosphorylation sites on intracellular loop 3 of GluR1–6 are shown diagrammatically in Fig. 3.

One of the important differences between AMPA-type and NMDA-type ion channels is the higher permeability of the NMDA channels to Ca^{2+}. It should be remembered that Ca^{2+}-influx through the NMDA channel is essential for the induction of LTP in hippocampal region CA1. Once LTP has been induced by a high frequency stimulation of the afferent pathway, expression does not require NMDA channel activity. The enhanced synaptic response during expression of LTP appears to be due to both increased presynaptic release of glutamate [30] plus an enhanced postsynaptic responsiveness of AMPA-type channels [31]. We are interested in whether phosphorylation of AMPA channels may account for this enhanced postsynaptic responsiveness.

Phoshorylation and regulation of glutamate receptors by protein kinases

There is increasing evidence for regulation by GluRs by protein phosphorylation. In white perch retinal cone cells, elevation of cAMP or microinjection of PKA enhances kainate-evoked currents by increasing the frequency of channel opening and channel open time [53]. Using whole cell patch recordings in hippocampal neu-

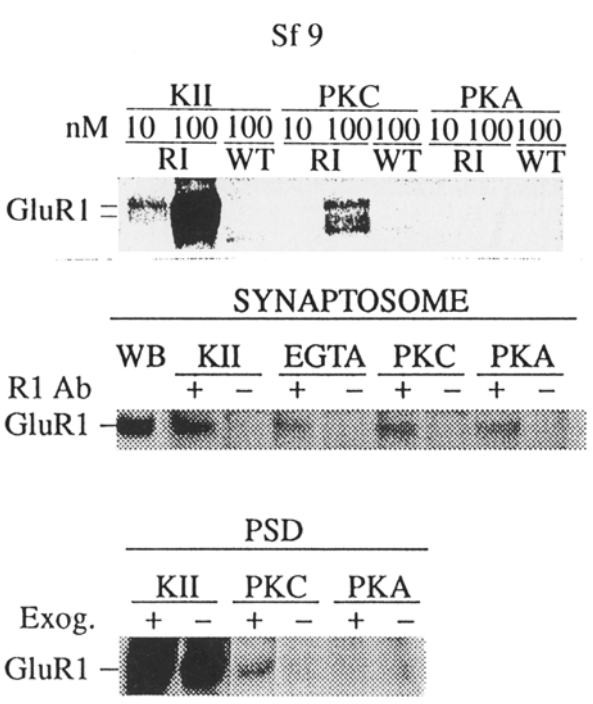

Fig. 4. Phosphorylation of glutamate receptors by protein kinases. Top panel: Homogenates of wild-type (WT) Sf9 cells or cells expressing GluR1 (R1) were immunoprecipitated with a specific antibody to GluR1 [52]. ^{32}P-labeling of the GluR1 in the immunoprecipitate was determined in the presence of 10 or 100 nM purified CaM-kinase II (KII), PKC or PKA. Middle panel. Endogenous protein kinases were selectively activated to phosphorylate endogenous native GluRs in isolated, lysed synaptosomes. Control phosphorylation is shown by the EGTA condition. The far left lane is a Western blot of GluR1. Bottom panel. Native GluRs in isolated PSDs were phosphorylated by endogenous (Exog. -) plus 100 nM exogenous (Exog. +) kinases. After phosphorylation, the synaptosomes and PSD were solubilized with SDS buffer and immunoprecipitated with GluRI antibody (R1 Ab+) or control serum (R1 Ab-). All samples were run on SDS/PAGE and subjected to autoradiography. Reprinted by permission from Nature (ref. 60). Copyright (1993) Macmillian Magazines Limited.

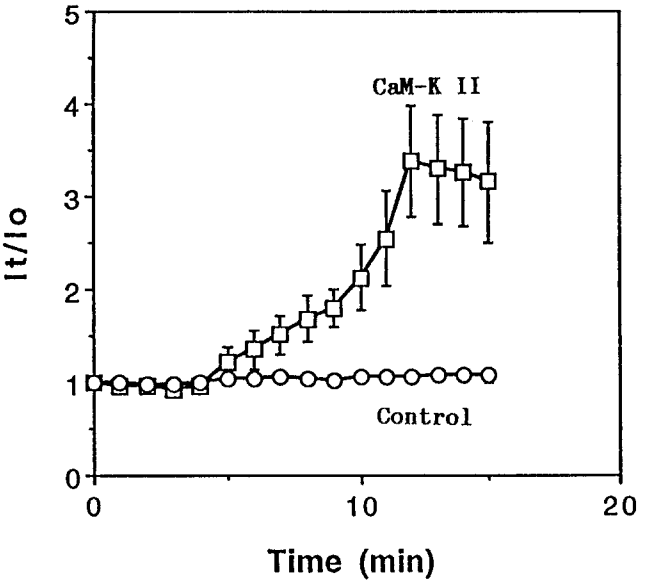

Fig. 5. Enhancement of AMPA ion channels by CaM-kinase II. Whole cell patch clamp recordings were performed on cultured hippocampal neurons with the recording pipette containing 200 nM activated CaM-kinase II (CaM-kinase II) or heat-inactivated CaM-kinase II (control). The CaM-kinase II was activated by autothiophosphorylation to give 60–70% Ca^{2+}-independence. AMPA currents were determined by perfusion with 100 μM kainate for 2–4 sec. The ratio of the maximum kainate-induced current at the indicated times (I_t) was divided by the maximum kainate current attained shortly after patch formation (I_o). Reprinted by permission from Nature (ref. 60). Copyright (1993) Macmillian Magazines Limited.

rons Wang *et al.* [54] showed that 1) the normal rundown of the AMPA current can be prevented by an ATP-regenerating system, 2) this effect of ATP to prevent rundown is enhanced by cAMP analogs or the catalytic subunit of PKA, and 3) the protein phosphatase inhibitor okadaic acid strongly potentiates the AMPA current. Greengard *et al.* [55] also observed an enhancement by forskolin in hippocampal whole cell recordings of AMPA current. Using single-channel analysis of excised patches they further demonstrated that PKA increases the opening frequency and mean open time of the non-NMDA type GluR channels. However, GluR1–5 do not contain a consensus PKA phosphorylation site in loop 3, and we have been unable to phosphorylate GluR1 by PKA (see below). PKA does phosphorylate Ser^{684} in GluR6 and enhances kainate current by 155 ± 19% [56]. Thus, these PKA effects may be due to phosphorylation of GluR6, or they may be indirect due to phosphorylation of a regulatory protein associated with GluRs. For example, oocytes expressing GluR1 and GluR3 give enhanced ion currents upon treatment with a permeable cAMP analog [57], even though these GluRs do not contain PKA consensus phosphorylation sites. Lastly, intracellular injection of PKC in dorsal horn neurons enhances NMDA-gated ion currents [58] by increasing the probability of channel opening and by reducing the Mg^{2+} block [59].

We have recently investigated the phosphorylation of non-NMDA glutamate receptor-ion channels by protein kinases in several systems [60]. We first expressed a clone (GluR1) of one of the AMPA type receptors in the baculovirus/Sf9 cell system and purified it by immunoprecipitation with a specific antibody. Phosphorylation was studied in the immunoprecipitate upon addition of purified protein kinases. As seen in Fig. 4, GluR1 was strongly phosphorylated by CaM-kinase II, weakly phosphorylated by PKC and not phosphorylated by PKA. We next examined phosphorylation of native

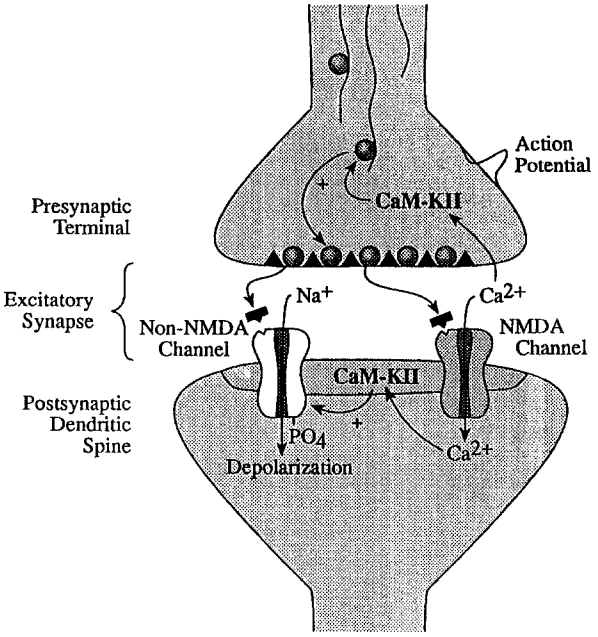

Fig. 6. Model for synaptic regulation by CaM-kinase II. A presynaptic function of CaM-kinse II to phosphorylate synapsin and mobilize synaptic vesicles (see ref. 22) is given in the top half. The bottom half illustrates the activation of CaM-kinase II in the PSD by Ca^{2+} influx through stimulated NMDA glutamate receptor/ion channels [39]. This would result from high frequency usage of the synapse. The activated (i.e., autophosphorylated, Ca^{2+}-independent) CaM-kinase II can then phosphorylate AMPA (non-NMDA) channels which enhances their subsequent responsiveness to released glutamate [60].

GluRs by selective activation of endogenous protein kinases in isolated rat forebrain synaptosomes and postsynaptic densities (Fig. 4) [60]. Again, CaM-kinase II was a good catalyst, PKC was a weaker catalyst and PKA was ineffective. Using cultured hippocampal neurons and agonists which selectively activate each of the three multifunctional protein kinases, we have observed increased ^{32}P-labeling of GluRs by CaM-kinase II and PKC but not PKA (S.E. Tan and T.R. Soderling, J. Neurosci., in press).

Since CaM-kinase II is a strong catalyst for phosphorylation of GluRs, we wanted to determine if this phosphorylation had a regulatory effect on ion channel activity. Whole cell patch clamp recording from cultured hippocampal neurons was used. The recording pipette contained Mg^{2+}/ATP and CaM-kinase II which was 60–70% Ca^{2+}-independent and resistant to protein phosphatases due to its prior autothiophosphorylation. After a five min lag, due to diffusion of CaM-kinase II into the neuron, there was a 3–4 fold enhancement of AMPA current compared to the controls in which the kinase had been heat inactivated (Fig. 5) [60].

Our studies on cultured hippocampal neurons demonstrate that 1) NMDA receptor activation and Ca^{2+} influx can activate CaM-kinase II through autophosphorylation and generation of the constitutively-active Ca^{2+}-independent form, 2) activated CaM-kinase II in isolated postsynaptic density or cultured neurons can phosphorylate GluRs, and 3) phosphorylation of GluRs by CaM-kinase II enhances AMPA current. These results suggest that CaM-kinase II may act as a postsynaptic sensor of synaptic activity as proposed [6], and perhaps could account for the increased postsynaptic non-NMDA responsiveness observed in paradigms of learning and memory such as LTP. This hypothesis is summarized in Fig. 6.

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