A Mechanism for Synaptic Frequency Detection through Autophosphorylation of CaM Kinase II

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ABSTRACT A model for the regulation of CaM kinase II is presented based on the following reported properties of the molecule: 1) the holoenzyme is composed of 8–12 subunits, each with the same set of autophosphorylation sites; 2) autophosphorylation at one group of sites (A sites) requires the presence of Ca^{2+} and causes a subunit to remain active following the removal of Ca^{2+}; 3) autophosphorylation at another group of sites (B sites) occurs only after the removal of Ca^{2+} but requires prior phosphorylation of a threshold number of A sites within the holoenzyme. Because B-site phosphorylation inhibits Ca^{2+}/calmodulin binding, we propose that, for a given subunit, phosphorylation of a B site before an A site prevents subsequent phosphorylation at the A site and thereby locks that subunit in an inactive state. The model predicts that a threshold activation by Ca^{2+} will initiate an "autophosphorylation phase." Once started, intra-holoenzyme autophosphorylation will proceed, on A sites during periods of high [Ca^{2+}] and on B sites during periods of low [Ca^{2+}]. At "saturation," that is when every subunit has been phosphorylated on a B site, the number of phosphorylated A sites and, therefore, the kinase activity will reflect the relative durations of periods of high [Ca^{2+}] to periods of low [Ca^{2+}] that occurred during the autophosphorylation phase. Using a computer program designed to simulate the above mechanism, we show that the ultimate state of phosphorylation of an array of CaM kinase II molecules could be sensitive to the temporal pattern of Ca^{2+} pulses. We speculate that such a mechanism may allow arrays of CaM kinase II molecules in postsynaptic densities to act as synaptic frequency detectors involved in setting the direction and level of synaptic modification.

INTRODUCTION

The temporal pattern of synaptic activity can be an important determinant in long term modification of synaptic efficacy. For example, in the CA1 region of the hippocampus, Dudek and Bear (1992) established that afferent stimulation consisting of a few hundred pulses delivered at 1–3 pulses per second induces long term depression (LTD), whereas the same number of pulses delivered at 50 pulses per second induces long term potentiation (LTP). It is proposed that both phenomena require a postsynaptic increase of [Ca^{2+}] via NMDA receptors (For reviews see Malenka and Nicoll, 1993; Bear and Malenka, 1994). Again, in the same region of the hippocampus, prior synaptic activity of a certain pattern can inhibit induction of LTP (Huang et al., 1992). These observations indicate the presence of a molecular system for the detection of temporal patterns.

A mechanism by which Ca^{2+} transients induce long term changes in the nervous system may be through the regulation of Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) activity. CaM kinase II is a Ca^{2+}-activated enzyme that becomes Ca^{2+}-independent upon autophosphorylation (Miller and Kennedy, 1986). It is expressed at high levels in the brain, particularly in the hippocampus (Ouimet et al., 1984; Erondu and Kennedy, 1985; Fukunaga et al., 1988). The enzyme occurs in soluble form throughout neurons and is a major component of postsynaptic densities (Kennedy et al., 1983; Kelly et al., 1984; Dosemeci and Reese, 1993). Numerous studies indicate that the induction of LTP requires CaM kinase II activation (Malenka et al., 1989; Malinow et al., 1989; Ito et al., 1991; Fukunaga et al., 1993; Pettit et al., 1994). In addition, gene knockout studies show that both LTP and LTD are either absent or attenuated in mice lacking the gene for α-CaM kinase II (Silva et al., 1992; Stevens et al., 1994).

A hypothesis to explain the bidirectional effect of Ca^{2+} in inducing both potentiation and depression is based on a model by Lisman (1989) that proposes that high levels of Ca^{2+} will result in increased autophosphorylation of CaM kinase II, whereas intermediate levels of Ca^{2+} will cause its dephosphorylation by selective activation of phosphatases. The hypothesis assumes that low frequency and high frequency stimulation patterns will produce intermediate and high levels of Ca^{2+}, respectively. Although this hypothesis is consistent with numerous findings, Malenka (1994) has pointed out 1) that experimental manipulations expected to give rise to intermediate Ca^{2+} levels do not necessarily produce LTD, and 2) that this model does not explain the need for "prolonged and patterned afferent stimulation" to achieve homosynaptic LTD.

The forgoing hypothesis is based on the assumption that the frequencies of presynaptic firing patterns determine the levels of postsynaptic Ca^{2+}. It is also likely that the presynaptic firing pattern is conveyed postsynaptically as a

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Abbreviations: Long Term Potentiation (LTP); Long Term Depression (LTD); Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II)

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pattern or "frequency" of postsynaptic Ca^{2+} transients; that is, the presynaptic firing frequency may influence not only the amplitude but also the frequency of the postsynaptic Ca^{2+} signal. In this case, a mechanism for the detection of postsynaptic Ca^{2+}-pulse frequency is needed to translate the temporal pattern of activity into synaptic modification. The "calmodulin trapping" model proposed by Meyer et al. (1992) suggests that CaM kinase II may discriminate temporal patterns of Ca^{2+} under certain conditions.

The calmodulin trapping model for the detection of Ca^{2+}-pulse frequency by CaM kinase II (Meyer et al., 1992; Hanson et. al., 1994) is based on the observation that autophosphorylation on Thr-286 increases the enzyme's affinity for calmodulin. Because of this, when calmodulin concentration is limiting, repetitive Ca^{2+} pulses will have a cooperative effect in promoting autophosphorylation and consequent kinase activity. However, because the dissociation time of calmodulin from a subunit phosphorylated on Thr-286 is on the order of seconds, trains of Ca^{2+} pulses with intervals <1 s could not be differentiated by this mechanism. More importantly, according to this model, prolonged exposure of the molecule to Ca^{2+} pulses, even at low frequency, will eventually lead to maximal Ca^{2+}-dependent autophosphorylation and thus to maximal autonomous activity. That is, prolonged low frequency and high frequency Ca^{2+} pulses will have identical effects.

In this article we propose a different model which takes into account the experimental observations that CaM kinase II has at least two distinct classes of autophosphorylation sites: phosphorylation of one class occurs in the presence of Ca^{2+} and confers Ca^{2+}-independent activity; phosphorylation of the other class occurs in the absence of Ca^{2+} and inhibits binding of calmodulin. The model predicts that arrays of the enzyme can detect the duration of postsynaptic Ca^{2+} pulses, as well as the duration of the intervals between pulses, through permutations of the autophosphorylation states. This "interval detection" model differs from the "calmodulin trapping" model (Meyer et al., 1992; Hanson et al., 1994) in that 1) the enzyme does not require a rate-limiting concentration of free calmodulin for frequency detection, 2) the enzyme may respond to shorter intervals between pulses, and 3) the final autonomous activity of the enzyme following prolonged or saturation exposure to Ca^{2+} pulses will not necessarily be maximal but will depend on the frequency of the pulses.

MODEL AND METHODS

Properties of CaM kinase II relevant to an interval detection model

Although CaM kinase II is a holoenzyme composed of 8–12 equivalent subunits, oligomeric structure is not needed either for activity or Ca^{2+}-dependence because monomeric forms of the enzyme, obtained by truncating the association domain, show Ca^{2+}/calmodulin-dependent kinase activity (Hanson et al., 1994). Oligomeric structure, on the other hand, appears to be important for regulation of the enzyme through autophosphorylation as will be explained below. There are several isoforms of CaM kinase II subunits; in neurons the main types are α and β. Because the α form appears to predominate in the forebrain (Kanaseki et al., 1991), we will discuss our model in terms of the α-isoform. However, it should be noted that the properties of the β-isoform are also compatible with the requirements of the model.

A particular threonine residue (Thr-286) is phosphorylated in the presence of Ca^{2+}. This process of phosphorylation is intraholoenzyme and intersubunit (Hanson et al., 1994; Mukherji and Soderling, 1994). We have designated these residues as A sites in this paper. Experiments using various autonomous forms of the enzyme indicate that, even when Ca^{2+}/calmodulin is not needed for enzymatic activity, A-site phosphorylation cannot occur unless Ca^{2+}/calmodulin binds to the regulatory portion of the subunit, presumably to "expose" the site (Hanson et al., 1994; Brickey et al., 1994; Colbran et al., 1988). Thus, A-site phosphorylation cannot happen in the absence of Ca^{2+}. Phosphorylation of an A site generates Ca^{2+}-independent activity, which is, however, less than the maximal activity in the presence of Ca^{2+} (Miller and Kennedy, 1986; Hanson et al., 1989). According to a recent study, this Ca^{2+}-independent kinase activity toward exogenous substrates increases with the number of A sites being phosphorylated (Ikeda et al., 1991).

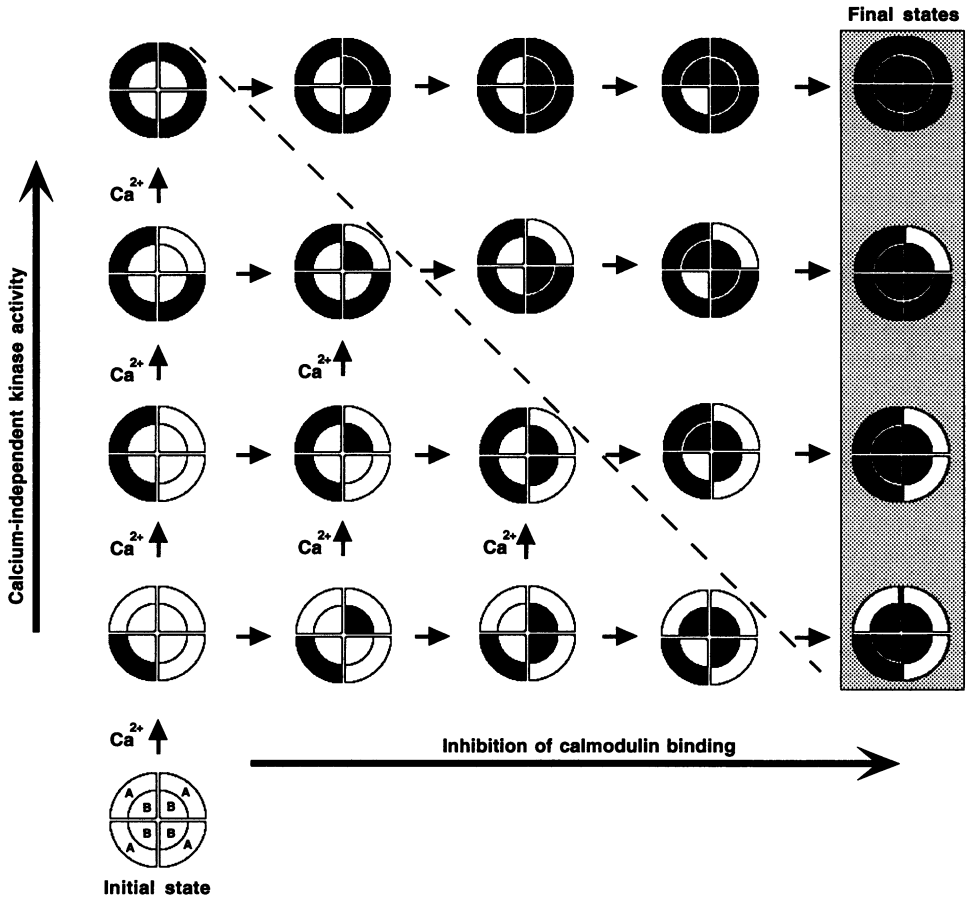
Following the A-site phosphorylation of a threshold number of subunits per holoenzyme, another set of sites becomes subject to autophosphorylation. Phosphorylation of particular residues located within the calmodulin-binding domain (Thr-305/306) occurs only after calmodulin dissociates from the enzyme upon removal of Ca^{2+} (Patton et al, 1990). We have designate these residues as B sites. Phosphorylation of B sites inhibits binding of Ca^{2+}/calmodulin and thus blocks Ca^{2+} -dependent activity (Lickteig et al., 1988; Patton et al., 1990; Hanson and Schulman, 1992).

Both intra- and intersubunit phosphorylation of B sites appears to occur. Hanson et. al. (1994) presented data indicating that Ca^{2+}-independent phosphorylation can occur by an intrasubunit reaction. Mukherji and Soderling (1994) observed that although basal autophosphorylation at Thr-306 is intrasubunit in a nonautonomous enzyme, an intersubunit phosphorylation of the same residue occurs when the enzyme is first made autonomous (Ca^{2+}-independent) by Ca^{2+}/calmodulin-dependent autophosphorylation (see note added in proof in Mukherji and Soderling (1994)). Also, a substochiometric level of Ca^{2+}/calmodulin-dependent autophosphorylation of CaM kinase II can trigger maximal Ca^{2+}-independent autophosphorylation (Miller and Kennedy, 1986; Lou and Schulman, 1989). The above data imply that phosphorylation of a relatively small number of subunits on their A sites permits phosphorylation of B sites throughout the holoenzyme by inter- and intrasubunit reactions. The number of A sites per holoenzyme that need to be phosphorylated to trigger maximal Ca^{2+}-independent phosphorylation appears to be even smaller for the postsynaptic density-associated CaM kinase II compared with the soluble enzyme (A. Dosemeci, unpublished observations). In the model presented below, this number is assumed to be 1.

A model for the regulation of CaM kinase II activity through autophosphorylation

Based on the above properties of the enzyme, the following model for its regulation is proposed (Fig. 1). If all subunits of the holoenzyme are in the unphosphorylated form (Fig. 1, *initial state*), no phosphorylation of either A or B sites occurs in the absence of Ca^{2+}. A Ca^{2+} influx is required to initiate autophosphorylation at A sites. If high Ca^{2+} levels persist for a sufficient time, all subunits will become phosphorylated on A sites first (Fig. 1, *left column*) The holoenzyme, when maximally phosphorylated on A sites, can exhibit maximal autonomous kinase activity. After [Ca^{2+}] falls and calmodulin dissociates, subunits already phosphorylated on their A sites, will become phosphorylated on their B sites as well (Fig. 1, *upper row*). This will not change the level of autonomous kinase activity but will prevent those subunits from binding Ca^{2+}/calmodulin.

If, however, Ca^{2+} is removed at any time before complete A site phosphorylation, some subunits will become phosphorylated on their B sites first (Fig. 1, phosphorylation events shown by horizontal arrows below the diagonal line). Phosphorylation of a subunit on a B site will



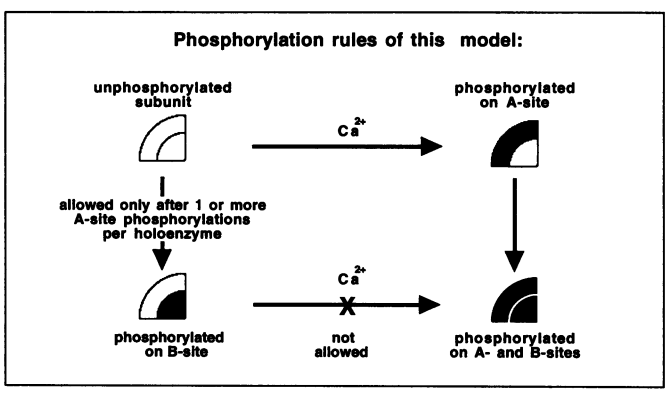


FIGURE 1 Model for the regulation of CaM kinase II by autophosphorylation. For reasons of simplicity, the CaM kinase II holoenzyme is shown to contain only four subunits instead of ten. Every circle corresponds to one autophosphorylation state of the holoenzyme. Because a random order of subunit phosphorylation is assumed, each autophosphorylation state of the holoenzyme is defined only by the number of its subunits at each of the four possible phosphorylation states (phosphorylated at A and B; A only; B only; none). Phosphorylation events on A sites in the presence of Ca^{2+} are depicted by vertical arrows and phosphorylation events on B sites in the absence of Ca^{2+} are depicted by horizontal arrows. Note that once every subunit in a holoenzyme is phosphorylated on at least one site (any one of the states immediately below the diagonal line), no more A-site phosphorylation can occur and Ca^{2+}-independent kinase activity of the tetramer becomes fixed at one of the four possible levels. For a decamer, the number of phosphorylation events needed for saturation and the number of possible activity levels would be ten each, instead of four.

prevent it from binding Ca^{2+}/calmodulin. Because an A site can become available for phosphorylation only when Ca^{2+}/calmodulin binds to the the regulatory portion of the subunit to "expose" it, blocking calmodulin binding due to prior B-site phosphorylation will prevent phosphorylation on an A site during future high Ca^{2+} phases; that is, it will block the subunit's potential to become autonomous. Also, failure to bind Ca^{2+}/calmodulin will block the potential of the subunit to be activated in the presence of Ca^{2+}. Therefore, phosphorylation on a B site before an A site will lock the subunit into an inactive state that can only be reversed by dephosphorylation.

A Ca^{2+} pulse capable of causing phosphorylation of at least one A site on a holoenzyme will initiate an autophosphorylation phase. When all subunits on the holoenzyme are phosphorylated on their B sites, a saturation or "final state" will be reached. In this state, the enzyme will be unable to bind calmodulin and, therefore, further phosphorylation of vacant A sites will not be possible. At this stage, the fraction of subunits with their A sites phosphorylated will reflect the pattern of synaptic activity that followed the initiating Ca^{2+} pulse. If an initial "trigger Ca^{2+}" is simply followed by a "quiet phase" with no further rises in Ca^{2+}, all the remaining subunits will be phosphorylated on their B sites first and the holoenzyme will be locked in a low activity state (Fig. 1, *lower row*). Different levels of A-site phosphorylation will be attained depending on the duration of the Ca^{2+} pulses compared with the duration of intervals between pulses (Fig. 1, *final states*). The number of possible final states would be determined by the number of subunits making up the holoenzyme. Thus, whereas the tetramer in Fig. 1 has four possible final states, the decamer would be able to carry graded information from 1 to 10. An important consequence of the model is that the effect of a long Ca^{2+} pulse is not equal to the combined effects of a series of short, discrete Ca^{2+} pulses of the same total duration. The duration of the intervals following Ca^{2+} pulses is an important factor in determining the pattern of autophosphorylation and, therefore, the final activity of the enzyme.

Description of the simulation program

The simulation program can model an array of up to 1000 holoenzymes (molecules) with 10 subunits per molecule. Each subunit is assigned one A site and one B site, initially unphosphorylated. Adjustable parameters include the probabilities of phosphorylation per unit time of A sites and of B sites; the probability of hydrolysis per unit time (nonselective for phosphorylated A and B sites); the width of Ca^{2+} pulses in ms; and the number of calcium pulses per second.

The algorithm implements the following rules: unphosphorylated A sites may be phosphorylated only in the presence of Ca^{2+}; unphosphorylated B sites may be phosphorylated only if 1) Ca^{2+} is absent, and 2) one or more A sites are phosphorylated on subunits of the same molecule. The parameters determine the probability of a phosphorylation event occurring on a given A site in the presence of Ca^{2+} or on a B site in the absence of Ca^{2+}. A simulation cycle consists of generating a random number for each subunit that is eligible for phosphorylation by the above rules, Phosphorylation is determined by comparison of this number with the calculated probability. The phosphorylation pattern of each molecule is recorded as a function of time and number of pulses.

The program output records the progressive changes in 1) number of holoenzymes with one or more phosphorylated A sites, 2) the total number of phosphorylated A sites, 3) the total number of phosphorylated B sites, and 4) the number of subunits phosphorylated on B sites only. Fig. 2 illustrates an output of the program for an array of 100 holoenzymes, each containing 10 subunits and other parameters as defined in the legend. In this and all other simulations, the probabilities of A-site and B-site phosphorylations on one subunit, under their respective permissive conditions were assigned to be 0.05 per second based on the initial rates of Ca^{2+}-dependent and Ca^{2+}-independent phosphorylation in Miller and Kennedy (1986) and Lou and Schulman (1989). In both studies the initial rate of Ca^{2+}-independent autophosphorylation was about the same as the initial rate of Ca^{2+}-dependent autophosphorylation. These particular references were chosen because they contain rate information for Ca^{2+}-dependent and Ca^{2+}-independent autophosphorylations that were obtained in parallel experiments, and therefore are directly comparable.

Flow diagram of the simulation program

```
enter parameters
start program
main loop:
calculate program parameters from input parameters
reset arrays and time to zero
loop to alternate between Ca2+ ON and Ca2+ OFF
  loop to scan each molecule
    if Ca2+ OFF
      then if molecule is autonomous
        then continue
        else abort loop and scan next molecule
    seed new random number series
    loop to scan each subunit
      assign a state of reactivity (from random number series)
      if not already phosphorylated
        calculate if state of reactivity is sufficient for phosphorylation
        if so record state of this site
      if phosphorylated
        calculate if state of reactivity is sufficient for hydrolysis
        if so record state of this site
    until all subunits are tested
  until all molecules are tested
switch Ca2+ state
continue until manually stopped or 3000 events are recorded.
```

The program was written in FutureBASIC (Macintosh version; Zedcor, Inc.). The source code and the compiled program are available from the authors.

RESULTS

The phosphorylation of a particular subunit at a particular site during a finite time interval is probabilistic and thus the degree of accuracy by which a group of CaM kinase II molecules is able to reflect the temporal pattern of Ca^{2+} pulses is limited by the size of the group. As pointed out by Lisman and Goldring (1988) ~100 CaM kinase II molecules in a typical postsynaptic density may constitute a functional group. Simulation allows us to test the ability of a group of CaM kinase II molecules to differentiate pulse frequencies.

The predicted pattern of autophosphorylation of 100 CaM kinase II molecules in response to Ca^{2+} pulses of varying frequencies was examined using the simulation shown in Fig. 3. The number of phosphorylated A sites, and thus the activity of the enzyme at saturation, increases in correlation with the frequency of Ca^{2+} pulses. The values obtained in multiple simulations for each frequency fall within a relatively narrow range (Fig. 3, see also values for standard deviations in legend) indicating that, with the parameters used, the levels of A-site phosphorylation corresponding to the frequencies tested are significantly different from each other.

The simulations in Fig. 3 were carried out assuming that autophosphorylation activity of the kinase is the only factor determining its state of phosphorylation. Under physiological conditions, however, phosphatase activity that will reverse autophosphorylation is also expected to be present. Indeed, protein phosphatase 1 remains tightly bound and capable of dephosphorylating CaM kinase II in isolated postsynaptic densities, suggesting the presence of a functional kinase-phosphatase complex (Dosemeci and Reese, 1993). Using the same conditions as in Fig. 3, the simulations in Fig. 4, illustrate the effect of constant levels of phosphatase activity that act at equal rates on phosphorylated A and B sites. In the presence of phosphatase, the A-site phosphorylation still reaches a steady state, but the steady-state levels are lower. More importantly, the presence of phosphatase activity allows the resetting of the system. In the absence of phosphatase activity, the kinase remains set following saturation of autophosphorylation sites. In the presence of phosphatase, the enzyme will eventually readjust its state of phosphorylation according to changing pulse frequency (Fig. 4).

Huang et al. (1992) have observed that prior synaptic activity, in the form of a series of 30 Hz tetani of 150-ms duration each and applied at 2-min intervals, can block induction of LTP. In the simulation in Fig. 5, the temporal patterns of Ca^{2+} pulses were designed to mimic patterns of afferent stimulation used in the above physiological experiment, with each 150-ms stimulus assumed to give rise to a postsynaptic Ca^{2+} pulse of 400-ms duration. As illustrated in Fig. 5 *A*, a long-lasting rise in [Ca^{2+}] levels (starting time shown by arrow) results in the phosphorylation of all subunits on A sites. However, prior exposure of the molecules to a series of 20 Ca^{2+} pulses of 400-ms duration each at

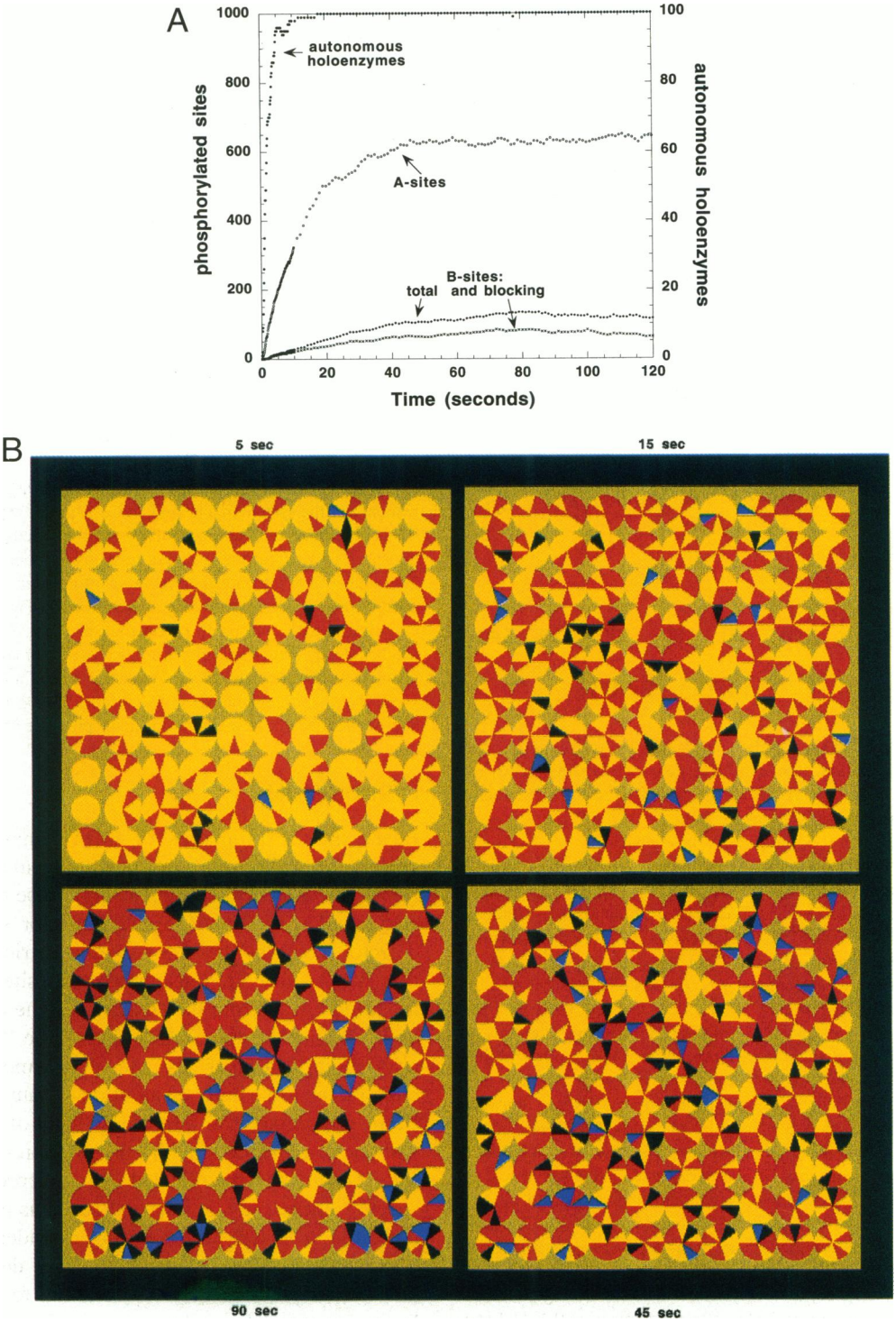


FIGURE 2 *a*: Graph showing four different outputs of the program as a function of time. The adjustable parameters were as follows. Number of holoenzymes: 100; Probability of A-site phosphorylation: 0.05/s; Probability of B-site phosphorylation: 0.05/s; Probability of hydrolysis: 0.025/s; Frequency: 9 Hz; Width of Ca^{2+} pulses: 100 ms. Program outputs are: 1) autonomous holoenzymes, which refers to the number of holoenzymes with at least one subunit phosphorylated on an A site; 2) A sites, which refers to the number of subunits phosphorylated on the A site, i.e., subunits that are active in the absence of Ca^{2+}; 3) B sites (total), which refers to the number of subunits phosphorylated on the B site, i.e., subunits which cannot bind calmodulin 4) B sites (blocking), which refers to the subunits phosphorylated on B sites only, i.e., subunits blocked in an inactive state. *b*: Results of a simulation illustrating the progressive change in the autophosphorylation of an array of 100 CaM Kinase II molecules. All adjustable parameters were as in *a*. Each circle represents one CaM Kinase II holoenzyme, composed of 10 subunits (slices). Naive holoenzymes are shown by the smaller circles and autonomous holoenzymes by bigger circles. Different phosphorylation states of individual subunits are depicted by different colors, with yellow corresponding to naive, red phosphorylated on the A site only, black phosphorylated on the B site only, and blue phosphorylated on both A and B sites. The four panels, arranged clockwise, show the patterns attained after 5-, 15-, 45-, and 90-s exposure to 9-Hz Ca^{2+} pulses.

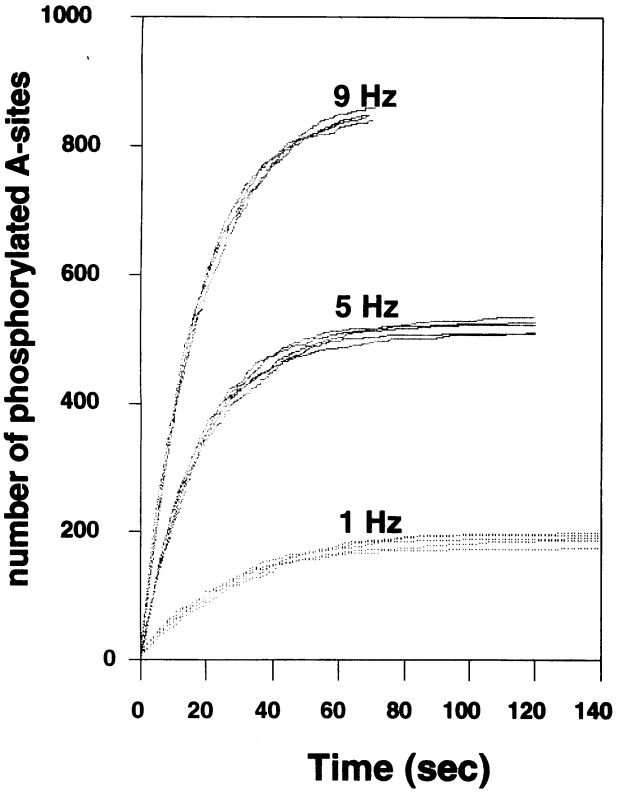


FIGURE 3 Frequency of Ca^{2+} pulses determines the fraction of A sites phosphorylated at saturation. Results of five consecutive simulations, recording the time course of total A-site phosphorylations in 100 CaM kinase II molecules, exposed to a continuous train of 100-ms Ca^{2+} pulses, comparing frequencies of 1, 5, and 9 Hz. The probabilities of A-site and B-site phosphorylation on one subunit, under their respective permissive conditions were assigned to be 0.05 per second (appproximated from initial rates of Ca^{2+}-dependent and Ca^{2+}-independent autophosphorylation in Miller and Kennedy (1986) and Lou and Schulman (1989)). The simulation program permits specification of up to 1000 holoenzymes with up to 10 subunits per holoenzyme. Each subunit has one A site and one B site, all initially unphosphorylated. The enzyme molecules are uniformly exposed to square pulses of Ca^{2+} of specified width and frequency. The rates of phosphorylation of A and B sites are each adjustable in terms of probability per unit time. In the above model, sequential phosphorylation is not obligatory at either A or B sites. The algorithm implements the following rules: an unphosphorylated A site may be phosphorylated only in the presence of Ca^{2+} and only if the subunit is not phosphorylated on a B site; an unphosphorylated subunit may be phosphorylated on a B site only if 1) Ca^{2+} is absent, and 2) one or more A sites are phosphorylated on any subunit of the same molecule. The program output includes, as a function of time, the number of molecules with one or more phosphorylated A sites; the total number of subunits phosphorylated on an A site; and the total number of subunits phosphorylated only on a B site. The average standard deviations of the trials for each of the last 50 time points were 9.4, 11.1 and 8.1, for 1 Hz, 5 Hz, and 9 Hz respectively.

100-s intervals (Fig. 5 *B*, *arrowheads*) blocks any effect of a later continuous Ca^{2+} pulse. This is due to extensive B-site phosphorylation during the low frequency Ca^{2+} pulses that prevent A-site phosphorylation in the same subunits. In the simulation, 20 Ca^{2+} pulses were necessary to block A-site phosphorylation, whereas 6 stimuli were needed in the actual experiment to block LTP.

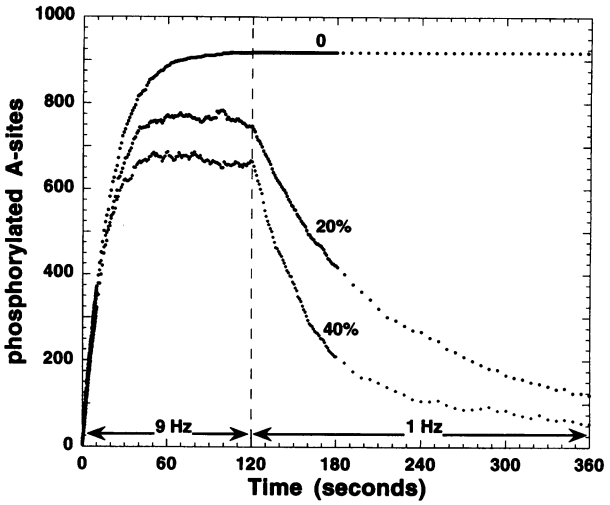


FIGURE 4 Phosphatase activity allows resetting of the system. The response of the system to a change in Ca^{2+}-pulse frequency was evaluated in the presence and absence of phosphatase activity. Simulation with phosphatase was carried out assuming dephosphorylation rates that are 20% and 40% of the phosphorylation rate for either an A or a B site. Other conditions and parameters were as described in Fig. 2. During the initial exposure to Ca^{2+} pulses at a frequency of 9 Hz, A-site phosphorylation reaches steady-state levels either in the presence or absence of phosphatase activity. At this stage, when the frequency of the Ca^{2+} pulse is switched to 1 Hz, the system remains set in the absence of phosphatase activity but is altered to reflect the new conditions in the presence of phosphatase activity.

DISCUSSION

Based on the properties of CaM Kinase II, the model demonstrates the feasibility of a molecular mechanism for frequency detection through a single type of self-modifying enzyme. The essential requirements for such a frequency detector would be that 1) every unit carries two "modification sites"—one site (designated as A site in the model) is modified only during the "event" and the other (designated as B site in the model) only during the "interval"; and 2) prior modification of one site blocks modification of the other on the same unit. An additional feature of CaM Kinase II, the requirement for a threshold level of A-site phosphorylation to trigger B-site phosphorylation in the absence of Ca^{2+} ensures that the molecule starts recording only with an "event" (increased Ca^{2+} levels) and thus prevents its being locked in an inactive state during extended quiet phases.

According to the model, frequency detection by CaM kinase II is possible if [Ca^{2+}] can fall to levels that allow B-site phosphorylation between consecutive phases of synaptic activity. The threshold afferent stimulation frequency below which intervals of sufficiently low [Ca^{2+}] can be obtained between consecutive stimuli would depend on the effectiveness of postsynaptic mechanisms for removal of Ca^{2+}. The geometry of postsynaptic dendritic spines seems ideally designed to allow rapid changes of local free Ca^{2+} concentration and thus to reflect the temporal pattern of presynaptic glutamate release. Even based on a diffusion model, the Ca^{2+} pulse can be dissipated very quickly within a spine (Gamble and Koch, 1987).

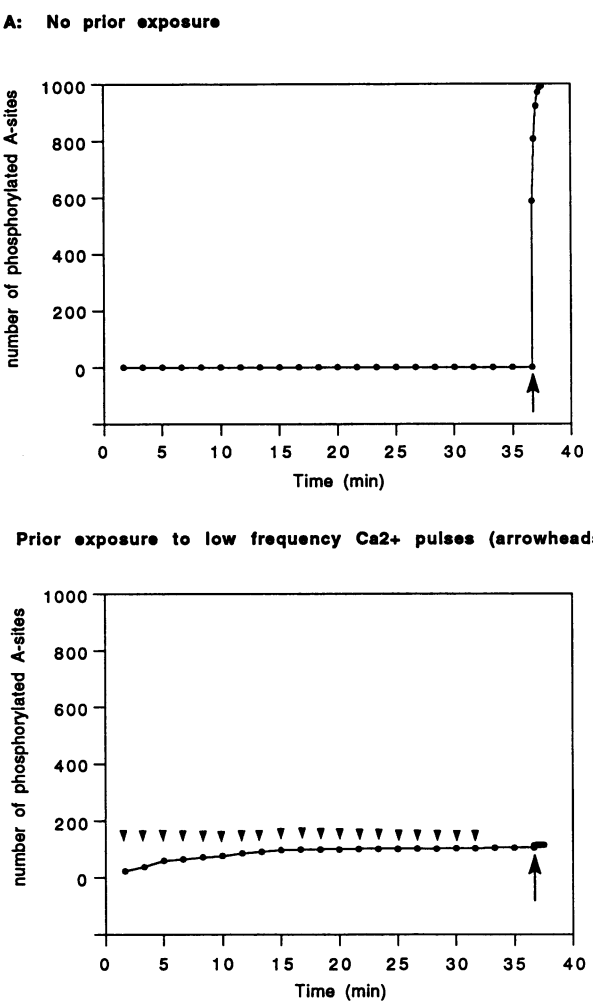


FIGURE 5 Previous exposure to low-frequency Ca^{2+} pulses can block the activation because of sustained exposure to Ca^{2+}. The number of A sites phosphorylated was evaluated following exposure of 100 CaM kinase II molecules to sustained high [Ca^{2+}], with or without prior exposure to 20 Ca^{2+} pulses of 0.4-s duration each, with 100-s intervals between pulses. These temporal patterns of Ca^{2+} were designed to simulate patterns of afferent stimulation in physiological experiments, which show inhibition of LTP by prior synaptic activity (Huang et al., 1992). Arrowheads indicate 0.4-s pulses and arrows indicate the beginning of the sustained high Ca^{2+} phase. The simulation program described in Fig. 3 was employed using the same values for phosphorylation probabilities. The upper plot simulates exposure of naive (unphosphorylated) enzymes to a sustained increase in Ca^{2+} levels. The sustained Ca^{2+} phase that starts at 37 min causes phosphorylation of nearly all subunits on their A sites. In the lower plot, the series of short Ca pulses shown by arrowheads cause a small degree of A-site phosphorylation, but almost maximal B-site phosphorylation. Prior B-site phosphorylation of unphosphorylated subunits blocks A-site phosphorylation by sustained high Ca^{2+}.

Because B-site phosphorylation cannot proceed while calmodulin remains attached to the enzyme, dissociation of calmodulin after [Ca^{2+}] returns to basal levels may cause a delay in the onset of Ca^{2+}-independent phosphorylation. This factor was omitted in our evaluation for reasons of simplicity. However, because the dissociation of dansylated calmodulin from unphosphorylated subunits takes only 170 ms (Meyer et al, 1992) and that of native calmodulin is expected to be even less (K_D is three times larger), the delay in B-site phosphorylation due to undissociated calmodulin is expected to be <100 ms. On the other hand the slow dissociation of calmodulin from subunits phosphorylated on A sites (Meyer et al., 1992) is not relevant for the model because subsequent phosphorylation of these subunits on B sites does not influence the outcome. It is proposed that interval detection occurs through the phosphorylation of a subunit on a B site first, thus locking it into an inactive state.

Although CaM kinase II has multiple autophosphorylation sites on each subunit, in the present model, autophosphorylation of only two of these, Thr-286 (A site) and Thr-305/306 (B site), has been considered. Phosphorylation of either Thr-305 or Thr-306 is sufficient to block calmodulin binding (Hanson and Schulman, 1992) and it is not clear whether both sites become phosphorylated upon the removal of Ca^{2+}. Because phosphorylation of the second "inhibitory" site on the same subunit is not expected to effect the functional status, we have chosen to include phosphorylation of only one B site per subunit into the model. However, it must be pointed out that, in case two sites become phosphorylated, both phosphates will have to be hydrolyzed to reverse the blockage of calmodulin binding; that is, it will take longer to reset the system. It is interesting to note that a third autophosphorylation site, Ser-314 in the α-subunit and Ser-315 in the β-subunit, is insensitive to phosphatases 1 and 2A (Patton et al., 1990). This site becomes phosphorylated under the same conditions as the B site, but unlike the B site phosphorylation, the phosphorylation of Ser-314 does not totally prevent Ca^{2+}-calmodulin binding, but only decreases the enzyme's sensitivity for the complex (Patton et al., 1990). Another site, T-253, is autophosphorylated both in the presence and absence of Ca^{2+} following a brief exposure of postsynaptic densities to Ca^{2+} (Dosemeci et al., 1994).

The above considerations suggest that the autophosphorylation of CaM kinase II is even more complex than assumed for the purposes of the model and may carry additional types of information storage capacity not anticipated by the present model. Although the model clearly implies that CaM kinase II can act as a molecular frequency detector, the range of frequencies that can be effectively discriminated by the kinase is expected to be influenced by factors such as the speed of postsynaptic Ca^{2+} removal, the dissociation rate of calmodulin from the kinase and the actual rates of phosphorylation, and dephosphorylation. Also, it should be noted that our simulations were done assuming maintained frequencies that imply a regularity of synaptic activity. Under physiological conditions where such regular activity may not occur, the enzyme would still be sensitive to the average duration of intervals relative to Ca^{2+} pulses within the autophosphorylation phase. In this case, the term "interval detection" would be more appropriate than "frequency detection" in describing the function of the kinase.

In the CA1 region of the hippocampus, CaM kinase II appears to be a necessary initial factor for the induction of

long term synaptic modification. Both LTP and LTD are dependent on postsynaptic elevation of Ca^{2+} (Mulkey and Malenka, 1992; Malenka and Nicoll, 1993; Bear and Malenka, 1994), and mice lacking α-CaM kinase II show either absent or attenuated LTP as well as LTD (Silva et al., 1992; Stevens et al., 1994). A recent study using transgenic mice that express a Ca^{2+}-independent form of CaM kinase II suggests an involvement of the kinase in determining the direction of the modification as a function of stimulation frequency (Mayford et al., 1995).

Based on our model for CaM Kinase II, the hypothesis may be put forward that the ratio of A-site to B-site phosphorylation of CaM kinase II establishes the direction of a potential long-term synaptic modification. Thus, a high ratio, that is a high level of Ca^{2+}-independent activity would set up conditions permissive to LTP and a low ratio, would block LTP and set up conditions permissive to LTD. This could explain observations such as the failure of low frequency stimulation to produce LTP and the inhibitory effects of previous synaptic activity on LTP induction (Huang et al., 1992). According to the model, these conditions would tend to produce a low ratio of A-site versus B-site phosphorylation and thus lock the enzyme in a low activity state, as illustrated in Fig. 3 (1 Hz) and Fig. 5 *B*. However, it should be noted that, because of the lack of experimental data on certain parameters such as the actual levels and dynamics of phosphatase activity in spines and the duration of individual postsynaptic Ca^{2+} pulses in response to particular stimulation protocols, the usefulness of the present simulations is more in exploring the implications of the new model rather than to provide quantitative predictions.

Homosynaptic LTD can be produced in the CA1 region of the hippocampus by afferent stimulation at low frequency (Dudek and Bear, 1992). An involvement of CaM kinase II, with autophosphorylation properties as in the model, would explain the requirements both for Ca^{2+} and for a specific temporal pattern of synaptic activity for the induction of homosynaptic LTD. In addition to a low ratio of A-site to B-site phosphorylation of CaM kinase II, other factors such as phosphatase activation are probably needed for the induction of LTD. As mentioned in the Introduction, it has been proposed that moderate, but not high, levels of Ca^{2+} cause an increase in phosphatase activity, thus allowing bidirectional regulation of of CaM kinase II by Ca^{2+} levels (Lisman, 1989). An involvement of various phosphatases in LTD is supported by recent experimental evidence (Mulkey et al., 1993, 1994). It is possible that selective phosphatase activation at intermediate levels of Ca^{2+} is part of a mechanism for the detection of the amplitude of the Ca^{2+} signal, whereas permutation of the autophosphorylation states of CaM kinase II, as described in our model, is a mechanism for the detection of the frequency of the Ca^{2+} signal. Because both mechanisms are factors in regulating the autonomous activity of CaM kinase II, the two properties of the signal, amplitude and frequency, may thus be integrated to decide on the direction and degree of synaptic modification. An involvement of phosphatase may also be important for 1) further reducing the activity of CaM kinase II following exposure to low frequency Ca^{2+} pulses (in this context, it will be important to determine the relative specificities of the phosphatases for A and B sites), and 2) reducing phosphorylation levels of substrate proteins of CaM Kinase II below basal or existing levels.

As mentioned in the Results section, phosphatase activity will tend to reset the kinase. This implies that, unless phosphatases are permanently inhibited in the particular compartment where the CaM Kinase II pool is located, long term synaptic modification cannot be "maintained" through the autophosphorylation state of CaM Kinase II. Thus, if the kinase is involved in long term synaptic modification it must be only during the "induction" phase. In fact, autophosphorylation of CaM Kinase II may be a "trigger" or initiating step that does not have to be maintained even through the entire induction phase. Physiological experiments that show that consecutive application of low frequency and high frequency stimulation patterns to the Schaffer collateral-CA 1 pathway can cause consecutive potentiation and depression. (Dudek and Bear, 1993) would argue for the presence of such a temporal frequency detection system that can constantly be regenerated.

Existing models and hypotheses on the mechanism of CaM kinase II involvement in synaptic modification have been based on autophosphorylation at the A sites only. The interval detection model presented here differs from previous models by the crucial role of B-site phosphorylation. Future experimental studies should be able to test our hypothesis based on this criterion. The hypothesis put forward predicts that induction of potentiation should be accompanied by a high ratio of A-site to B-site phosphorylation, whereas synaptic activity that causes depression or blockage of potentiation should result in a low ratio of A-site to B-site phosphorylation. Using antibodies that can discriminate between different phosphorylated forms, it may be possible to monitor the levels of A-site and B-site phosphorylations following protocols that lead to LTP/LTD. Because, according to the model, B-site phosphorylation is necessary for frequency discrimination by CaM kinase II, specific inhibition of B-site phosphorylation should block the effects of low frequency stimulation. Similarly, mutants that lack capability to regulate calmodulin binding through autophosphorylation at B sites, would be able to express LTP but should fail to exhibit frequency dependent bidirectional synaptic modification.

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REFERENCES

Bear, M. F. and R. C. Malenka. 1994. Synaptic plasticity: LTP and LTD. *Curr. Opin. Neurobiol.* 4:389–399.

Brickey, D. A., J. G. Bann, Y.-L. Fong, L. Perrino, R. G. Brennan, and T. R. Soderling. 1994. Mutational analysis of the autoinhibitory domain of calmodulin kinase II. *J. Biol. Chem.* 269:29047–29054.

Colbran, R. J., Y. L. Fong, C. M. Schworer, and T. R. Soderling. 1988. Regulatory interactions of the calmodulin-binding, inhibitory, and autophosphorylation. *J. Biol. Chem.* 263:18145–18151.

Dosemeci, A., N. Gollop, and H. Jaffe. 1994. Identification of a major autophosphorylation site on postsynaptic density-associated Ca^{2+}/calmodulin-dependent protein kinase. *J. Biol. Chem.* 269:31330–31333.

Dosemeci, A., and T. S. Reese. 1993. Inhibition of endogenous phosphatase in postsynaptic density fraction allows extensive phosphorylation of the major postsynaptic density protein. *J. Neurochem.* 61: 550–555.

Dudek, S. M., and M. F. Bear. 1992. Homosynaptic long-term depression in area CA1 of hippocampus and effects of N-methyl-D-aspartate receptor blockade. *Proc. Natl. Acad. Sci. USA.* 89:4363–4367.

Dudek, S. M., and M. F. Bear. 1993. Bidirectional long-term modification of synaptic effectiveness in the adult and immature hippocampus. *J. Neurosci.* 13:2910–2918.

Erondu, N. E., and M. B. Kennedy. 1985. Regional distribution of type II Ca^{2+}/calmodulin-dependent protein kinase in rat brain. *J. Neurosci.* 5:3270–3277.

Fukunaga, K., S. Goto, and E. Miyamoto. 1988. Immunohistochemical localization of Ca^{2+}/calmodulin-dependent protein kina and various tissues. *J. Neurochem.* 51:1070–1078.

Fukunaga, K., L. Stoppini, E. Miyamoto, and D. Muller. 1993. Long-term potentiation is associated with an increased activity of Ca^{2+}/calmodulin-dependent protein kinase II. *J. Biol. Chem.* 268:7863–7867.

Gamble, E., and C. Koch. 1987. The dynamics of free calcium in dendritic spines in response to repetitive synaptic input. *Science.* 236:1311–1315.

Hanson, P. I., M. S. Kapiloff, L. L. Lou, M. G. Rosenfeld, and H. Schulman. 1989. Expression of a multifunctional Ca^{2+}/calmodulin-dependent protein kinase and mutational analysis of its autoregulation. *Neuron.* 3:59–70.

Hanson, P. I., T. Meyer, L. Stryer, and H. Schulman. 1994. Dual role of calmodulin in autophosphorylation of multifunctional CaM kinase may underlie decoding of calcium signals. *Neuron.* 12:943–956.

Hanson, P. I., and H. Schulman. 1992. Inhibitory autophosphorylation of multifunctional Ca^{2+}/calmodulin-dependent analyzed by site-directed mutagenesis. *J. Biol. Chem.* 267:17216–17224.

Huang, Y. Y., A. Colino, D. K. Selig, and R. C. Malenka. 1992. The influence of prior synaptic activity on the induction of long-term potentiation. *Science.* 255:730–733.

Ikeda, A., S. Okuno, and H. Fujisawa. 1991. Studies on the generation of Ca^{2+}/calmodulin-independent activity of calmodulin-dependent protein kinase II by autophosphorylation. Autothiophosphorylation of the enzyme. *J. Biol. Chem.* 266:11582–11588.

Ito, I., H. Hidaka, and H. Sugiyama. 1991. Effects of KN-62, a specific inhibitor of calcium/calmodulin-dependent protein kinase II, on long-term potentiation in the rat hippocampus. *Neurosci. Lett.* 121:119–121.

Kanaseki, T., Y. Ikeuchi, H. Sugiura, and T. Yamauchi. 1991. Structural features of Ca^{2+}/calmodulin-dependent protein kinase II revealed by electron microscopy. *J. Cell Biol.* 115:1049–1060.

Kelly, P. T., T. L. McGuinness, and P. Greengard. 1984. Evidence that the major postsynaptic density protein is a component of a Ca^{2+}/calmodulin-dependent protein kinase. *Proc. Natl. Acad. Sci. USA.* 81: 945–949.

Kennedy, M. B., M. K. Bennett, and N. E. Erondu. 1983. Biochemical and immunochemical evidence that the major postsynaptic density protein is a subunit of a calmodulin-dependent protein kinase. *Proc. Natl. Acad. Sci. USA.* 80:7357–7361.

Lickteig, R., S. Shenolikar, L. Denner, and P. T. Kelly. 1988. Regulation of Ca^{2+}/calmodulin-dependent protein kinase II by Ca^{2+}/calmodulin-independent autophosphorylation. *J. Biol. Chem.* 263:19232–19239.

Lisman, J. 1989. A mechanism for the Hebb and the anti-Hebb processes underlying learning and memory. *Proc. Natl. Acad. Sci. USA.* 86: 9574–9578.

Lisman, J., and M. Goldring. 1988. Feasibility of long-term storage of graded information by the Ca^{2+}/calmodulin-dependent protein kinase molecules of the postsynaptic density. *Proc. Natl. Acad. Sci. USA.* 85:5320–5324.

Lou, L. L., and H. Schulman. 1989. Distinct autophosphorylation sites sequentially produce autonomy and inhibition of the multifunctional Ca2+/calmodulin-dependent protein kinase. *J. Neurosci.* 9:2020–2032.

Malenka, R. C. 1994. Multiple forms of NMDA receptor-dependent synaptic plasticity in the hippocampus. *In* Long-Term Potentiation, vol. 2. M. Baudry and J. L. Davis, editors. MIT Press, Cambridge, MA. 121–141.

Malenka, R. C., J. A. Kauer, D. J. Perkel, M. D. Mauk, P. T. Kelly, R. A. Nicoll, and M. N. Waxham. 1989. An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. *Nature.* 340:554–557.

Malenka, R. C., and R. A. Nicoll. 1993. NMDA-receptor-dependent synaptic plasticity: multiple forms and mechanisms. *Trends Neurosci.* 16: 521–527.

Malinow, R., H. Schulman, and R. W. Tsien. 1989. Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science.* 245:862–866.

Mayford, M., J. Wang, E. R. Kandel, and T. J. O'Dell. 1995. CaMKII regulates the frequency-response function of hippocampal synapses for the production of both LTD and LTP. *Cell.* 81:891–904.

Meyer, T., P. I. Hanson, L. Stryer, and H. Schulman. 1992. Calmodulin trapping by calcium-calmodulin-dependent protein kinase. *Science.* 256: 1199–1202.

Miller, S. G., and M. B. Kennedy. 1986. Regulation of brain type II Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation: a Ca^{2+}-triggered molecular switch. *Cell.* 44:861–870.

Mukherji, S., and T. R. Soderling. 1994. Regulation of Ca^{2+}/calmodulin-dependent protein kinase II by inter- and intrasubunit-catalyzed autophosphorylations. *J. Biol. Chem.* 269:13744–13747.

Mulkey, R. M., S. Endo, S. Shenolikar, and R. C. Malenka. 1994. Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. *Nature.* 369:486–488.

Mulkey, R. M., C. E. Herron, and R. C. Malenka. 1993. An essential role for protein phosphatases in hippocampal long-term depression. *Science.* 261:1051–1055.

Mulkey, R. M., and R. C. Malenka. 1992. Mechanisms underlying induction of homosynaptic long-term depression in area CA1 of the hippocampus. *Neuron.* 9:967–975.

Ouimet, C. C., T. L. McGuinness. and P. Greengard. 1984. Immunocytochemical localization of calcium/calmodulin-dependent protein kinase II in rat brain. *Proc. Natl. Acad. Sci. USA.* 81:5604–5608.

Patton, B. L., S. G. Miller, and M. B. Kennedy. 1990. Activation of type II calcium/calmodulin-dependent protein kinase by Ca^{2+}/calmodulin is inhibited by autophosphorylation of threonine within the calmodulin-binding domain. *J. Biol. Chem.* 265:11204–11212.

Pettit, D. L., S. Perlman, and R. Malinow. 1994. Potentiated transmission and prevention of further LTP by increased CaMKII activity in postsynaptic hippocampal slice neurons. *Science.* 266:1881–1885.

Silva, A. J., C. F. Stevens, S. Tonegawa, and Y. Wang. 1992. Deficient hippocampal long-term potentiation in α-calcium-calmodulin kinase II mutant mice. *Science.* 257:201–206.

Stevens, C. F., S. Tonegawa, and Y. Wang. 1994. The role of calcium-calmodulin kinase II in three forms of synaptic plasticity. *Curr. Biol.* 4:687–693.