Possible role for calmodulin and the Ca\(^{2+}\)/calmodulin-dependent protein kinase II in postsynaptic neurotransmission

PHILIP SIEKEVITZ
Rockefeller University, New York, NY 10021

Contributed by Philip Siekevitz, March 20, 1991

ABSTRACT The theory presented here is based on results from in vitro experiments and deals with three proteins in the postsynaptic density/membrane—namely, calmodulin, the Ca\(^{2+}\)/calmodulin-dependent protein kinase, and the voltage-dependent Ca\(^{2+}\) channel. It is visualized that, in vivo in the polarized state of the membrane, calmodulin is bound to the kinase; upon depolarization of the membrane and the intrusion of Ca\(^{2+}\), Ca\(^{2+}\)-bound calmodulin activates the autophosphorylation of the kinase. Calmodulin is visualized as having less affinity for the phosphorylated form of the kinase and is translocated to the voltage-dependent Ca\(^{2+}\) channel. There, with its bound Ca\(^{2+}\), it acts as a Ca\(^{2+}\) sensor, to close off the Ca\(^{2+}\) channel of the depolarized membrane. At the same time, it is thought that the configuration of the kinase is altered by its phosphorylated states; by interacting with Na\(^{+}\) and K\(^{+}\) channels, it alters the electrical properties of the membrane to regain the polarized state. Calmodulin is moved to the unphosphorylated kinase to complete the cycle, allowing the voltage-dependent Ca\(^{2+}\) channel to be receptive to Ca\(^{2+}\) flux upon the next cycle of depolarization. Thus, the theory tries to explain (i) why calmodulin and the kinase reside at the postsynaptic density/membrane site, and (ii) what function autophosphorylation of the kinase may play.

The following theory is based on in vitro experimental findings concerning the postsynaptic density (PSD), a structure attached to the postsynaptic membrane, occurring at chemical synapses in the central nervous system (see ref. 1). The theory is derived from speculations (2–9) concerning the possible regulatory activity of a protein kinase found in the PSD. More particularly, it is based on recent observations from the literature concerning the ubiquitous Ca\(^{2+}\)-binding protein calmodulin (CaM) (see ref. 10).

In concise form I present a proposal, based on properties of the PSD (1), in which the movement of CaM from one protein to another (i) can regulate Ca\(^{2+}\) flow in the dendritic spine; (ii) can regulate the protein kinase activity of the major protein of cerebral cortex and hippocampus PSDs, the Ca\(^{2+}\)/CaM-dependent protein kinase, and in doing so can change the ionic properties of the postsynaptic membrane; and (iii) may thus be involved in the overall regulation of synaptic transmission. These ideas could explain why the protein kinase exists in such large amounts at the synapse: it acts as a structural protein with ties to the Ca\(^{2+}\) and possibly to Na\(^{+}\) and to K\(^{+}\) channels. It could explain the functional reasons for the phosphorylations and dephosphorylations of this protein, in that different conformational states result therefrom. And finally, it could explain the occurrence of CaM at this important site of the synapse. All of the above speculation depends on the idea that the phosphorylation states of the kinase and the ionic milieu in which it acts change the conformation of the protein in its interactions with the ionic channels in the postsynaptic membrane that are embedded in the structure of the PSD. Ca\(^{2+}\)-bound CaM is postulated to be a necessary cofactor in the changes by activating the kinase and by controlling Ca\(^{2+}\) movements through interactions with voltage-dependent Ca\(^{2+}\) channels.

The experimental observations are as follows. (i) CaM is found in PSDs isolated from cerebral cortex (11), cerebellum (12), and hippocampus (13), as confirmed by immunocytochemical methods (14). (ii) CaM is necessary, together with Ca\(^{2+}\), for the activation of a two-subunit (50 kDa and 60 kDa) postsynaptic protein kinase, appropriately called Ca\(^{2+}\)/CaM-dependent protein kinase II, also found in PSDs isolated from cerebral cortex (15), cerebellum (12), and hippocampus (13). (iii) Together the two subunits of the kinase make up the major protein in PSDs isolated from cerebral cortex (15–17); indeed, this major protein accounts for 30–50% of the mass of the PSD (16–18) and thus probably has a structural role in the PSD (ref. 1). (iv) CaM, with its bound Ca\(^{2+}\), seems to be part of a voltage-dependent Ca\(^{2+}\) channel complex in synaptic membrane and in PSD fractions from cerebral cortex and cerebellum (19) and from hippocampus (13). (v) In vitro activity of the protein kinase is only manifest when Ca\(^{2+}\) and CaM are added, as first shown for a brain membrane fraction (20). Subsequent work, by laboratories too numerous to mention here, on the activity of the enzyme in various types of isolated brain fractions showed in every instance that exogenous Ca\(^{2+}\) plus CaM is necessary to give maximal activity. (vi) The peculiarity of this observation is indicated by finding in cerebral cortex PSDs (15) enough CaM to maximally activate the enzyme, even when Ca\(^{2+}\) is added, which evidently is not available to the kinase in the isolated preparations. Also, it is probable that enough Ca\(^{2+}\) is present in these preparations to bind to CaM, since the initial homogenization solution contained 0.5 mM Ca\(^{2+}\). The relevant experiment, performed with either a cerebral cortex PSD (15) or with a synaptic junction (21) fraction, was to remove the inactive CaM from the fractions by EGTA-complexing of Ca\(^{2+}\) and then to add back Ca\(^{2+}\)-bound CaM; indeed the same CaM molecules in the synaptic junction fraction were added back to the CaM-depleted fraction (21). In both cases (15, 21), addition of Ca\(^{2+}\) plus CaM activated the kinase; furthermore, maximal activity was obtained by the addition of CaM in the amount originally in the fraction (15). This lack of accessibility of PSD CaM to PSD kinase was also found in hippocampal fractions (13). These results imply that in the isolated fractions, while endogenous CaM could not interact with the kinase, exogenous CaM could reach the kinase and interact with it. (vii) The endogenous Ca\(^{2+}\)-bound CaM in isolated synaptic membrane and PSD fractions from cerebral cortex (19) and hippocampus (13) is sufficient to give maximal binding of nitrendipine, a specific antagonist of the voltage-dependent Ca\(^{2+}\) channel; removal of Ca\(^{2+}\)-bound CaM by EGTA treatment practically obliterated the binding, while subsequent addition of Ca\(^{2+}\) plus CaM to the depleted preparation restored the binding to almost control levels (13, 19). These latter findings would indicate that in the isolated

Abbreviations: PSD, postsynaptic density; CaM, calmodulin.
fractions, Ca\textsuperscript{2+}-bound CaM is either bound to the Ca\textsuperscript{2+} channel protein or to a protein closely linked to it, and conversely, it is not bound to the Ca\textsuperscript{2+}/CaM-dependent protein kinase.

The above experiments were performed with isolated preparations, but can the results be carried over to the in vivo situation? What I propose is that in vivo, CaM exists in either one of two binding sites in the PSD and that it moves from one to the other with changes in the physiological states of the postsynaptic membrane. The idea is similar to the proposal (22) of a “flip-flop” movement of CaM-binding proteins, which bind to CaM or to some other protein depending on the Ca\textsuperscript{2+} concentration (22). Fig. 1 is a much simplified diagram of the postulated events, simplified in that it describes a particular type of synapse that contains postsynthetically both the CaM-dependent kinase and the voltage-dependent Ca\textsuperscript{2+} channel; it may or may not also have the Ca\textsuperscript{2+} channel gated by glutamate-receptor binding. What is shown is the contiguity of the Ca\textsuperscript{2+}/CaM-dependent protein kinase to the voltage-dependent Ca\textsuperscript{2+} channel. For clarity, the various ion channels (except for the Ca\textsuperscript{2+} channels) believed to be attached to the PSD and to extend through the postsynaptic membrane (cf. 1), which here are theorized to be also adjacent to the protein kinase in the PSD, are not included in the diagram. The two states visualized here are the polarization conditions of the postsynaptic membrane: in the polarized state (in the presence of low amounts of Ca\textsuperscript{2+}; see below), CaM is bound to the protein kinase (Fig. 1a); whereas in the final depolarized state (in the presence of higher levels of Ca\textsuperscript{2+}; see below), CaM is bound to the voltage-dependent Ca\textsuperscript{2+} channel (Fig. 1d).

Is there any evidence for this assertion? All in vitro experiments with synaptic junctions, synaptic membranes, or PSD fractions have membranes that are depolarized or were conducted where no membranes are evident, as in the PSD fraction. As mentioned above, in these experiments Ca\textsuperscript{2+} plus CaM had to be added to show maximal kinase activity, indicating that the endogenous CaM is not available to the kinase. But it does not have to be added to give nitrendipine binding (13, 19), indicating that the endogenous Ca\textsuperscript{2+}-bound CaM is available to the nitrendipine-binding component. The isolated PSD has a CaM-binding component of 165 kDa in addition to one of 50 kDa (23), and the nitrendipine-binding component of the purified channel is a 170-kDa protein, at least in muscle (24). Thus, the 165-kDa PSD protein could be the nitrendipine-binding protein of the

---

Fig. 1. Movements of calmodulin, phosphorylation and dephosphorylation, and polarization and depolarization. PM, postsynaptic membrane; K, Ca\textsuperscript{2+}/CaM-dependent protein kinase II; K-P and K-PP, phosphorylation states of the kinase; V.D.Ch, voltage-dependent Ca\textsuperscript{2+} channel; Ca-Ch, ATP-Ca\textsuperscript{2+} pump or Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange channel; Glu-R, glutamate receptor complex. The Na\textsuperscript{+}, K\textsuperscript{+}, and Cl\textsuperscript{−} ion channels involved in the changes of membrane potential are not shown.
Ca\(^{2+}\) channel and could be the protein to which CaM is bound in the isolated synaptic membrane and PSD fractions. On the other hand, experiments with tissue slices (25) or with intact synapsosomal preparations (26), which undoubtedly have polarized membranes, do not require the addition of Ca\(^{2+}\)/CaM to give phosphorylation of the protein kinase. One objection to this idea is that readdition of Ca\(^{2+}\)/CaM to a preparation depleted by EGTA of the Ca\(^{2+}\)-bound CaM should restore the CaM only to the postulated site from which it was removed, namely the voltage-dependent Ca\(^{2+}\) channel. However, while the readdition does restore nitrendipine binding to this channel (13, 19), it also restores the activation of the protein kinase (15, 21), implying a binding of the exogenous Ca\(^{2+}\)-bound CaM to the kinase, a situation postulated not to occur in the isolated preparations. A possible rejoinder is that the CaM affinity for the kinase is only half that for the voltage-dependent Ca\(^{2+}\) channel (19), but in the readdition experiment, enough Ca\(^{2+}\)/CaM is added to saturate the kinase site.

What is the physiological event that moves Ca\(^{2+}\)/CaM from one binding site to the other? I propose that upon depolarization of the postsynaptic membrane, Ca\(^{2+}\) enters certain dendritic terminals through voltage-sensitive glutamate receptor (probably NMDA receptor) channels (see refs. 27 and 28) and through voltage-sensitive Ca\(^{2+}\) channels (refs. 29–31; see ref. 32), increasing the intracellular level of Ca\(^{2+}\) (Fig. 1 b and c). Both glutamate receptors (13, 33, 34) and voltage-dependent Ca\(^{2+}\) channels (13, 19) have been found in PSD fractions from cerebral cortex and hippocampus. Indeed, another receptor, the GABA\(_A\)/GABA\(_B\) receptor (35–38), and another channel, a Ca\(^{2+}\)-dependent K\(^+\) channel (39), are also found in isolated PSD fractions. All of these findings imply (cf. 1) that in vivo the active part of the receptor and channel molecules extends to the extracellular surface of the postsynaptic membrane, but that another segment of these protein molecules is firmly anchored within the PSD on the internal surface of the membrane.

Inside the terminal, a low concentration of Ca\(^{2+}\) rapidly activates the CaM bound to the kinase (15–18), causing a low level of autophosphorylation of the kinase (depicted as K-P in Fig. 1c). The assumption here is that, since CaM has four Ca\(^{2+}\)-binding sites that exhibit positive cooperativity (40–42) and since three of these sites need to be occupied for the activation of another PSD protein [a cyclic nucleotide phosphodiesterase (40)], in the initial state the CaM species with only two bound Ca\(^{2+}\) sites (K\(_A\) = 10\(^{-6}\) M) is sufficient to bind CaM to the unphosphorylated kinase, but the CaM species with three or four Ca\(^{2+}\) sites occupied (K\(_A\) > 10\(^{-5}\) M) may be necessary for the activation of the kinase. Indeed, it has been shown (43) that binding of CaM to different CaM-binding proteins is dependent on the different K\(_A\) values for the binding of Ca\(^{2+}\) to the complexes of CaM with its binding proteins.

Ca\(^{2+}\)-bound CaM is proposed to have less affinity for the phosphorylated kinase; it immediately comes off and is translocated to the adjacent nitrendipine-binding component of the Ca\(^{2+}\) channel (Fig. 1 c and d). The resultant intermediate depolarized state has the CaM bound to the Ca\(^{2+}\) channel protein complex (Fig. 1 d and e). Upon membrane repolarization the Ca\(^{2+}\)-bound CaM is translocated back to the kinase, restoring the state shown in Fig. 1a. However, in the isolated fractions, repolarization does not occur, and the Ca\(^{2+}\)-bound CaM remains bound to the Ca\(^{2+}\) channel protein (Fig. 1g). This hypothesis assumes that the binding of the Ca\(^{2+}\)-bound CaM to the channel is the unique property of the kinase. The dissociation of the kinase is a reflection not only of the phosphorylation state of the kinase but also of the influence of the polarization state of the membrane upon the conformations of these two proteins. The Ca\(^{2+}\)-CaM complex has a higher affinity for the kinase when the membrane is polarized (Fig. 1a) and a higher affinity for the voltage-dependent Ca\(^{2+}\) channel protein when the membrane is depolarized (Fig. 1 d, e, and g). That is, the affinities of CaM for the two proteins depend not only on the intrinsic affinities (see ref. 19) but also on the configurations of the two proteins as modified by the polarization state of the membrane.

There is some evidence for the type of translocation mentioned above. In the case of other CaM-binding proteins, it has been shown that phosphorylation by protein kinase C of neuromodulin (also known as protein P-57, GAP-43, B-50, or F-I) results in the release of CaM from its binding site on this protein (44), while another protein kinase substrate in brain tissue (called MARCKS protein) also releases CaM upon phosphorylation (45). In the case of the Ca\(^{2+}\)/CaM-dependent protein kinase, at first glance the evidence seems to be negative. Both in a synaptic junction (46) and a cytoskeletal (47) preparation, autophosphorylation of the kinase seemed to result in an increase in CaM binding to the kinase, as measured by comparing the binding of [\(^{125}\)I]-labeled CaM to the protein on SDS gels between phosphorylated and nonphosphorylated preparations. However, when the binding of [\(^{125}\)I]-labeled CaM was performed on a native cytoskeletal preparation and assayed by a filtration method and not by binding to denatured and renatured proteins on gels, autophosphorylation of the kinase was found to give a decrease in CaM binding (47). This latter event occurred at a Ca\(^{2+}\) concentration of 0.5 \(\mu\)M, which is about the EC\(_{50}\) Ca\(^{2+}\) concentration for autophosphorylation of the kinase in particulate fractions (48–50). Indeed, the EC\(_{50}\) Ca\(^{2+}\) concentration for nitrendipine binding in the presence of saturating CaM in isolated PSD fractions was higher than 0.5 \(\mu\)M, at about 1.0 \(\mu\)M (19), and at high Ca\(^{2+}\) concentrations, CaM was more effectively bound to the nitrendipine binding site than to the protein kinase (19). As mentioned above, CaM has four binding sites for Ca\(^{2+}\) that show positive cooperativity (40–42). It could be that changes in the quaternary structure of CaM effected by higher concentrations of Ca\(^{2+}\) (43, 51, 52) caused not only the autophosphorylation of the kinase but also removal of CaM from the kinase and its translocation to the Ca\(^{2+}\) channel protein (Fig. 1 c, d, and e).

But does the shutting of CaM, proposed above, have any meaning for neurophysiological function in vivo? First, a 51-kDa protein, most likely the \(\alpha\) subunit of the kinase, does become phosphorylated in vivo (53). Also, depolarization by high K\(^+\) of intact synaptosomes activated a Ca\(^{2+}\)/CaM-dependent protein kinase (54), resulting in an increase in tissue slices (25), synaptosomes (26), and intact cerebellar cells (55) of the phosphorylation of the 51-kDa subunit of the kinase, indicating a connection between the polarization state of the membrane and the phosphorylation state of the kinase. One objection to this postulated in vivo shuttling is that CaM may not be rate-limiting in vivo—that sufficient CaM is available for all of the sites in the postsynaptic membrane or PSD. A possible rejoinder is that, although soluble CaM may be abundant in vivo, there exists a sequestered domain where the exchange of CaM between soluble and bound forms is limited, and this domain is the tight structure of the PSD.

But why have this elaborate CaM shuttle? Does it serve any purpose? A recapitulation and elaboration of my scheme is as follows. Upon depolarization, there occurs a rapid movement of Ca\(^{2+}\) into the dendritic spine (Fig. 1b). At low concentration, Ca\(^{2+}\) activates CaM, which is still bound to the kinase. This phosphorylated kinase would tend to keep the Ca\(^{2+}\) channels open, allowing a continuing flow of Ca\(^{2+}\) into the spine head (Fig. 1a). As the Ca\(^{2+}\) concentration increases, Ca\(^{2+}\)-bound CaM is removed from the phosphorylated kinase and translocated to the voltage-dependent Ca\(^{2+}\) channel site (Fig. 1d). The binding of Ca\(^{2+}\)-CaM to the Ca\(^{2+}\) channel protein changes the latter's configuration, which event is proposed to block the further entry of Ca\(^{2+}\).
Concomitant with the CaM shuttle cycle is a phosphorylation cycle, necessitating the action of a protein phosphatase (Fig. 1f). Thus, it is noteworthy that whereas calcineurin, a Ca\(^{2+}\)/CaM-requiring protein phosphatase, is now thought not to be localized at the PSD (59), another phosphatase, Ca\(^{2+}\)/CaM-nondependent protein phosphatase 1 (60), has been localized at synaptic junctions (61), where it is capable of dephosphorylating the phosphorylated protein kinase that is present there (60).

No one as yet knows of any specific neurochemical function that is modified according to the phosphorylated state of the kinase, although theories exist (see below). I suggest that the phosphorylation/dephosphorylation changes of the kinase so alter the channel properties of the postsynaptic membrane as to regulate the movements of \(\text{Na}^+\), \(\text{K}^+\), and \(\text{Cl}^-\) ions and to restore the polarized state of the membrane (Fig. 1a). Thus, the states in Fig. 1e, f, and a depict rapid events of the phosphorylation cycle leading to the depolarized state seen in Fig. 1a. The movement of Ca\(^{2+}\)/CaM from the voltage-dependent Ca\(^{2+}\) channel to the kinase (Fig. 1f) then renders the Ca\(^{2+}\) channel responsive to Ca\(^{2+}\) influx upon membrane depolarization (Fig. 1b). The energy for the process could come from the ATP phosphorylating the kinase, thus changing its configuration. Furthermore, it is known that protein phosphorylation in general is a mechanism for the regulation of various ion channels (see ref. 62). More specifically, a purified voltage-dependent Ca\(^{2+}\) channel from muscle is activated by phosphorylation by a Ca\(^{2+}\)-dependent protein kinase (63), the latter enzyme also being found in PDS (64). As a corollary, it should be mentioned that the purified autophosphorylated form of the Ca\(^{2+}\)/CaM-dependent protein kinase phosphorylates a subunit of the Ca\(^{2+}\)/CaM-dependent cyclic-nucleotide phosphodiesterase (65), the latter enzyme also being found in PDS (66), and this phosphorylation decreases the affinity of CaM for the diesterase, inhibiting it, and is blocked by CaM binding to the diesterase (65). Thus, it could be that these PSD-bound proteins act in concert to keep the inward Ca\(^{2+}\) channels open. As long as the CaM-dependent phosphorylation system is allowed to operate, the channel remains open, and this system is kept in this state by the Ca\(^{2+}\)/CaM-activated kinase inhibiting the phosphodiesterase, which is responsible for the breakdown of cAMP. Also in this regard, what happens in the kindled state has some relevance. In kindling, low-intensity electrical stimulation in any of many cerebral cortex areas leads to increases in after-discharge duration and amplitude and a decrease in threshold (see ref. 67), all properties of the synaptic membrane. It is noteworthy that the kindled state has been found to be associated with a decrease in the in vitro autophosphorylation of the PSD Ca\(^{2+}\)/CaM-dependent protein kinase and with an increase in the number of PSD glutamate-binding sites (68). Thus, there is a correlation between the phosphorylation state of the PSD protein kinase and the condition of the ionic channels in the synapse, probably postsynaptic, membrane.

Finally, even in the absence of added Ca\(^{2+}\)/CaM, further autophosphorylation of the protein kinase can occur (K-P-P in Fig. 1e). This assertion is supported by results from many laboratories that the membrane-bound (69, 70) or purified (71–77) kinase, once phosphorylated to a low extent by the addition of Ca\(^{2+}\) plus CaM, can undergo further phosphorylation that is independent of the presence of Ca\(^{2+}\) and CaM. Indeed, the enzyme in the PSD has the same property (78). This property of the protein kinase has been postulated (2–6) to be a factor in the neurophysiology of long-term events in the central nervous system, such as long-term potentiation and memory. Through the use of kinase inhibitors on cells, it has been demonstrated (3, 79) that persistent kinase activity is associated with long-term potentiation. Also, a translocation of CaM has been observed (79) from a membrane-bound form to a soluble form in hippocampus during long-term potentiation. Based on changes in Ca\(^{2+}\) levels and on the autophosphorylation of the kinase under these conditions, a thorough theoretical thesis has been postulated (7–9) and a model has been presented (7, 8) in which the phosphorylated kinase can store information or synaptic weight, a necessary condition for the memory process. A further exploration of the model (9) involves changes primarily in internal Ca\(^{2+}\) concentration and in the activities of protein phosphatases and of a cAMP-dependent protein kinase as further regulators of synaptic weight. As I mentioned before, the biochemical function of the phosphorylated kinase may reside in its ability in the phosphorylated state to interact with ion-channel proteins in the postsynaptic membrane because of changes in its conformation upon phosphorylation. Alterations in the Na\(^+\) and K\(^+\) channels may maintain the depolarized state of the membrane, increasing the synaptic weight of that junction. Thus, the activity of the kinase at any particular junction may keep the membrane potential larger than that of other, less active junctions.

In summary, this proposal cites many in vitro and some in vivo findings and, based on properties of some PSD proteins, attempts to combine them into a somewhat coherent theory of synaptic events—that may be involved in the regulation of synaptic transmission.

I thank Dr. John Lisman, Brandeis University, for many helpful and critical comments on two earlier versions of the paper; he straightened me out on some aspects of some of the postulates and drove me to examine more deeply my thoughts on the whole theory.