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Autophosphorylation of Neuronal Calcium/Calmodulin-Stimulated Protein Kinase II

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Contents

Abstract

A unique feature of neuronal calcium/calmodulin-stimulated protein kinase II (CaM-PK II) is its autophosphorylation. A number of sites are involved and, depending on the in vitro conditions used, three serine and six threonine residues have been tentatively identified as autophosphorylation sites in the α subunit. These sites fall into three categories. Primary sites are phosphorylated in the presence of calcium and calmodulin, but under limiting conditions of temperature, ATP, Mg^{2+}, or time. Secondary sites are phosphorylated in the presence of calcium and calmodulin under nonlimiting conditions. Autonomous sites are phosphorylated in the absence of calcium and calmodulin after initial phosphorylation of Thr-286. Mechanisms that lead to a decrease in CaM-PK II autophosphorylation include the thermolability of the enzyme and the activity of protein phosphatases. A range of in vitro inhibitors of CaM-PK II autophosphorylation have recently been identified. Autophosphorylation of CaM-PK II leads to a number of consequences in vitro, including generation of autonomous activity and subcellular redistribution, as well as alterations in conformation, activity, calmodulin binding, substrate specificity, and susceptibility to proteolysis. It is established that CaM-PK II is autophos-phorylated in neuronal cells under basal conditions. Depolarization and/or activation of receptors that lead to an increase in intracellular calcium induces a marked rise in the autophosphorylation of CaM-PK II *in situ*. The incorporation of phosphate is mainly found on Thr-286, but other sites are also phosphorylated at a slower rate. One consequence of the increase in CaM-PK II autophosphorylation *in situ* is an increase in the level of autonomous kinase activity. It is proposed that the formation of an autonomous enzyme is only one of the consequences of CaM-PK II autophosphorylation *in situ* and that some of the other consequences observed in vitro will also be seen. CaM-PK II is involved in the control of neuronal plasticity, including neurotransmitter release and long-term modulation of postreceptor events. In order to understand the function of CaM-PK II, it will be essential to ascertain more fully the mechanisms of its autophosphorylation *in situ*, including especially the sites involved, the consequences of this autophosphorylation for the kinase activity, and the relationships between the state of CaM-PK II autophosphorylation and the physiological events within neurons.

Index Entries: Calcium/calmodulin-stimulated protein kinase II; autophosphorylation mechanisms; autophosphorylation sites; thermal lability; dephosphorylation; inhibitors; autophosphorylation consequences; autophosphorylation *in situ*.

Introduction

The brain contains many protein kinases whose activities are increased by both calcium and calmodulin (Yamauchi and Fujisawa, 1980; Kennedy and Greengard, 1981; Nairn et al., 1985; Ohmstede et al., 1989). Early studies by a number of groups characterized a particularly abundant kinase, which had a broad substrate specificity and a wide regional and subcellular distribution (Schulman and Greengard, 1978; DeLorenzo et al., 1979; Yamauchi and Fujisawa, 1979; O'Callaghan et al., 1980; Wrenn et al., 1980; Grab et al., 1981; Fukunaga et al., 1982; Bennett et al., 1983; Rostas et al., 1983; Kelly et al., 1984). The kinase investigated was called by a number of names (Rostas et al., 1986b; Schulman, 1988), but in this article, the enzyme will be referred to as calcium/calmodulin-stimulated protein kinase II, abbreviated to CaM-PK II (Rostas and Dunkley, 1992).

Possible Functions

A fundamental property of neuronal CaM-PK II is that it can phosphorylate many proteins (Schulman, 1988; Cohen, 1988; Colbran et al., 1989a). There is convincing evidence that *in situ* CaM-PK II phosphorylates tryptophan hydroxylase and tyrosine hydroxylase, the rate-limiting enzymes in the synthesis of the monoamine neurotransmitters, synapsin I, a presynaptic protein involved in the control of neurotransmitter release, microtubule-associated protein-2 (MAP-2), Tau and tubulin, involved in main-

tenance of the cytoskeleton, which is crucial for a number of neuronal functions both pre- and postsynaptically, and ion channels, which control neuronal function (Schulman, 1988; Nairn, 1990). There are also many other proteins that are phosphorylated by CaM-PK II in vitro, which may turn out to be substrates in intact neuronal tissue, including metabolic enzymes, ATPases, ribosomal proteins, and receptors (Schulman, 1988; Cohen, 1988; Colbran et al., 1989a; Nairn, 1990; Fujisawa, 1990).

The activity of neuronal CaM-PK II is altered in a number of circumstances, which may give further indications as to the function of the enzyme *in situ* (Rostas et al., 1986a,b; Schulman, 1988; Cohen, 1988; Colbran et al., 1989a; Nairn, 1990; Nichols et al., 1990; Rostas, 1991; Kelly, 1992; Rostas and Dunkley, 1992). Thus, the activity of CaM-PK II shifts from being soluble to particulate during development, especially during the maturation phase of neurons, which follows synapse formation (Rostas, 1991). The enzyme is involved in neuronal plasticity, since it appears to be required for the initial phases of long-term potentiation (Malinow et al., 1988; Malenka et al., 1989; Colbran et al., 1989a; Kennedy, 1989; Soderling et al., 1990; Kelly, 1992; Waxham et al., 1992b) and visual stimulation (Schulman, 1988; Hendry and Kennedy, 1986), and has been proposed to be involved in memory formation (Lisman and Goldring, 1988). The enzyme is also modulated in pathological conditions, such as kindling (Goldenring et al., 1986; Schulman, 1988; Wu et al., 1990; Bronstein et al., 1990), hypoxia/ischemia (Taft et al., 1988; Churn et al., 1990; Aronowski et al., 1992), and neurotoxicity (Abou-Donia and Lapadula, 1990).

CaM-PK II is a particularly abundant enzyme in the nervous system and is especially enriched in the hippocampus, where it can represent as much as 1% of brain protein (Erondu and Kennedy, 1985). This level seems unnecessary for a protein that functions only as an enzyme, and it has been suggested that CaM-PK II might also be a structural protein, especially in the cytoskeleton and the postsynaptic density (PSD) (Rostas et al., 1986b; Kelly, 1992; Rostas and Dunkley, 1992).

Properties

CaM-PK II is a member of a family of calcium/calmodulin-stimulated protein kinases that have broad substrate specificity, and are found in both neuronal and nonneuronal tissue. Soluble CaM-PK II is isolated from the cerebral cortex of adult rats as a holoenzyme of approximately 600 kDa with two major subunits, α (50 kDa) and β (60 kDa), in a ratio of 3:1 (Rostas et al., 1988). When cloned separately, the subunits each bind calmodulin and have catalytic activity (Waxham et al., 1989; Yamauchi et al., 1989; Hanson et al., 1989; Ohsako et al., 1990), with the α subunit forming multimers, whereas the β subunit remains monomeric (Yamauchi et al., 1989,1990). The α subunit is not expressed in all rat neurons. It is produced late in development relative to the β subunit, and is enriched in dendrites of hippocampal and cortical neurons, whereas the β subunit is mainly present in the cell bodies (Rostas et al., 1986a; Walaas et al., 1988; Burgin et al., 1990). It is therefore uncertain exactly how many different forms of CaM-PK II exist, but at least in some cells, the multimeric holoenzyme containing both α and β subunits is unlikely to be the in vivo form of the enzyme (Rostas and Dunkley, 1992).

Cloning experiments have made the situation even more complex, since five subunits of CaM-PK II have now been identified, including the α, β, β', δ, and γ subunits (Bulleit et al., 1988; Tobimatsu et al., 1988; Tobimatsu and Fujisawa, 1989). Each subunit has a number of domains that are essentially the same, including the ATP, substrate, and calmodulin-binding domains, and the regulatory (inhibitory) domain (Colbran et al., 1989a,b; Lou and Schulman, 1989; Fujisawa, 1990; Rostas and Dunkley, 1992). In addition, each subunit has an association domain that varies widely between the subunits. It is assumed that this domain is involved in the associations between subunits that occur when holoenzymes are formed, as well as in the binding of CaM-PK II to membranes and the cytoskeleton (Rostas and Dunkley, 1992).

CaM-PK II is enriched in a number of brain regions, especially the forebrain. It is present in

all subcellular locations, but is enriched in the soluble, synaptic membrane, and synaptosomal and cytoskeletal compartments (Rostas et al., 1986b; Schulman, 1988; Dunkley et al., 1988a; Rostas and Dunkley, 1992). It is especially enriched in PSDs, where it represents some 50–60% of the protein (Rostas et al., 1986a,b; Kelly, 1992). CaM-PK II at distinct subcellular sites differs in solubility, and the most insoluble form in the PSD has a number of properties that differ from the soluble enzyme (Rostas and Dunkley, 1992). To date, no sequence or posttranslational differences have been found between the soluble and PSD forms of the enzyme, and it is assumed that the proteins that bind to CaM-PK II and the mode of association between subunits are determining the differences in properties (Rostas and Dunkley, 1992). There is little information available concerning the locations of the β', δ, and γ subunits (Tobimatsu et al., 1988).

CaM-PK II can be autophosphorylated in the presence of calcium and calmodulin. This was initially shown by Fukunaga et al. (1982) and was subsequently confirmed by a number of groups (Goldenring et al., 1982; Kennedy et al., 1983a,b; Bennett et al., 1983; Yamauchi and Fujisawa, 1985). It was found that both subunits contained more than 1 mol P_i/mol, depending on the conditions used (Kennedy et al., 1983a; Bennett et al., 1983). Goldenring et al. (1983) were the first to indicate that phosphothreonine was present along with phosphoserine.

Scope of the Review

Neuronal CaM-PK II has been the subject of large number of studies over the last decade, and many reviews are already available (Rostas et al., 1986b; Cohen, 1988; Schulman, 1988; Colbran et al., 1989a; Kennedy, 1989; Fujisawa, 1990; Nairn, 1990; Kelly, 1992; Rostas and Dunkley, 1992). Each review includes discussion of CaM-PK II autophosphorylation, and many references are included; however, no previous review has focused entirely on this subject. The aim of this article is therefore to provide a review of the mechanisms that increase and decrease neuronal CaM-PK II autophosphorylation in vitro and the consequences of this, to document the evidence that CaM-PK II autophosphorylation occurs in intact neuronal and neuroendocrine cells, and to summarize the evidence that this has a functional consequence(s).

CaM-PK II Autophosphorylation In Vitro

Overview

The autophosphorylation of CaM-PK II is a complex process, and a simplified scheme for the α subunit is therefore provided for orientation (Fig. 1). When the enzyme is dephosphorylated, it has very little activity in the absence of calcium and calmodulin (Schulman, 1988; Colbran et al., 1989a). It is generally found that the either CaM-PK II is not phosphorylated *in situ* (*see below*) or the conditions used for isolation of the kinase have led to almost complete dephosphorylation of CaM-PK II. Addition of calcium and calmodulin in the presence of ATP initiates autophosphorylation of the primary sites on CaM-PK II, especially Thr-286, and the phosphorylation of substrates. Depending on the conditions, continued incubation can lead to the autophosphorylation of secondary sites and a decrease, or little change, in substrate phosphorylation. The phosphorylation of Thr-286 leads to an enzyme that can have activity against substrates in the absence of calcium and calmodulin. This is observed if, after the initial autophosphorylation of Thr-286, EGTA is added to the incubation to remove calcium and therefore dissociate calmodulin from CaM-PK II. In this state, CaM-PK II is referred to as being autonomous. If ATP is then added, a burst of further autophosphorylation occurs, leading to the phosphorylation of the autonomous sites on CaM-PK II that are distinct from those that were labeled with calmodulin. This autonomous autophosphorylation can lead to either no change or a decrease in substrate phosphorylation.

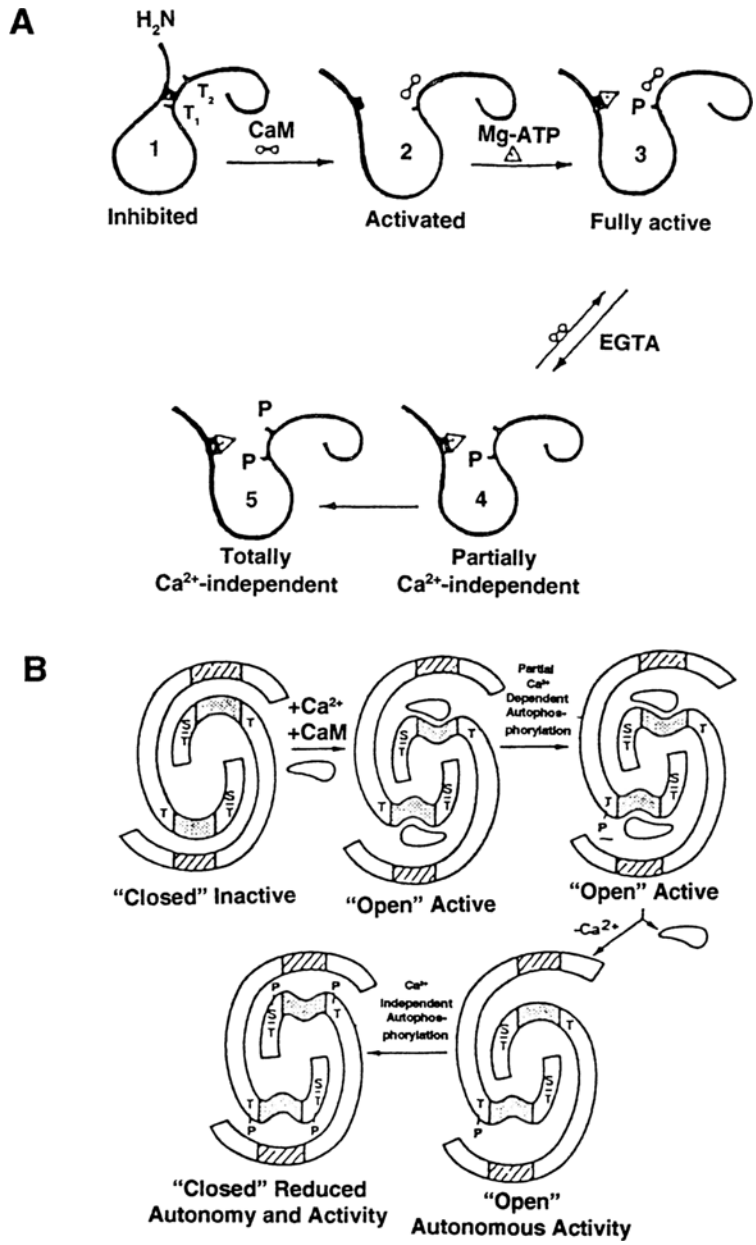

Fig. 1. Models for the autophosphorylation of the α subunit of CaM-PK II. The first model (A, upper panel) assumes that all of the phases depicted can occur with monomeric enzyme (Colbran et al., 1989), whereas the second model (B, lower panel) assumes that autophosphorylation involves interaction between subunits in a multimeric enzyme (Lou and Schulman, 1989). Both models show that inactive CaM-PK II first binds calcium and calmodulin, and changes its conformation. ATP then binds, and the enzyme is able to phosphorylate substrates, as well as to autophosphorylate Thr-286. If EGTA is now added to the enzyme to remove calcium, the calmodulin dissociates, and the enzyme can continue to phosphorylate substrates in an autonomous manner. Other site(s) can become autophosphorylated in the autonomous enzyme. The two models differ in that with model A it is assumed that the extra sites phosphorylated in the autonomous enzyme lead to total calcium independence, whereas in model B, the extra phosphorylation sites lead to reduced autonomous activity. This issue is discussed in the section in the text concerning in vitro consequences.

The autophosphorylation of CaM-PK II occurs by an intramolecular mechanism (Shields et al., 1984; Kuret and Schulman, 1985; Lai et al., 1986). It does not require interaction between the α and β subunits (Kuret and Schulman, 1985; Fong et al., 1989; Hanson et al., 1989; Waxham et al., 1989; Yamauchi et al., 1989; Ohsako et al., 1990) or association of subunits to form multimers (Yamauchi et al., 1989; Waxham et al., 1989; *see below*). Furthermore, the monomers were able to express autonomous activity after incubation in EGTA. Expression in CHO cells of a form of the α subunit of CaM-PK II lacking the association domain provided a monomeric enzyme that was hardly autophosphorylated and did not become autonomous in the presence of EGTA (Yamauchi et al., 1989), suggesting an important role for this domain in maintaining CaM-PK II autophosphorylation. Mutagenesis of Arg-283 blocked autophosphorylation of Thr-286, suggesting that this residue is also essential (Fong and Soderling, 1990). Thus, autophosphorylation of CaM-PK II can occur in a monomeric subunit, and this model for CaM-PK II autophosphorylation is shown in Fig. 1A. When CaM-PK II is in its usual multimeric form (Rostas et al., 1986b; Rostas and Dunkley, 1992), interactions between subunits may occur whereby the phosphorylation of sites in one subunit may be catalyzed by another subunit, and this model is shown in Fig. 1B.

It is clear that interaction between subunits does occur in the multimeric kinase, since autophosphorylation of only a few of the possible sites in the holoenzyme allows full autonomous activity (Rostas et al., 1987; Schulman, 1988; Colbran et al., 1989a; Lou et al., 1986). When CaM-PK II is isolated from rat cerebral cortex, it is normally a holoenzyme with a ratio of α:β subunits of 3:1–4:1, the prominent form being either a dodecamer or decamer (Rostas et al., 1986b; Schulman, 1988; Rostas and Dunkley, 1992). When only two to four subunits in the holoenzyme are autophosphorylated on Thr-286 (approx 0.1 mol P_i/mol subunit), this is sufficient to induce significant autonomous activity, with the extent of the calcium independence depending on the conditions used (Miller and Kennedy, 1986; Lai et al., 1986; Lou et al., 1986). Presumably this occurs because the change in conformation of a few subunits induces a change in conformation of other subunits. This could then lead to substrate activity in the absence of full autophosphorylation (Fong et al., 1989).

Incubation of multimeric CaM-PK II in the presence of calcium and calmodulin allows autophosphorylation, which, depending on incubation conditions, the procedures used for protein estimation, and the assumptions about holoenzyme composition, varies from 0.1–4 mol P_i/mol α subunit (Miller and Kennedy, 1986; Saitoh et al., 1986; Lou et al., 1986; Yamauchi and Fujisawa, 1985; Schworer et al., 1988; Lickteig et al., 1988; Lou and Schulman, 1989; Yamauchi et al., 1991); under the same conditions, the β subunit always had a higher molar ratio of P_i than the α subunit. This represented approx 30 mol P_i/mol holoenzyme and indicates that more than one site can be autophosphorylated on each subunit. It is possible to increase further the extent of autophosphorylation of CaM-PK II by initiating incubations in the presence of calcium and calmodulin, until 2–4 mol P_i are incorporated/mol holoenzyme and then adding EGTA to allow a burst of autonomous autophosphorylation. Under these conditions, up to 42 mol P_i/mol holoenzyme can be incorporated (Lou and Schulman, 1989; Schulman, 1988).

Autophosphorylation Sites

Understanding which sites are labeled under which conditions is essential to understanding the role of autophosphorylation in controlling CaM-PK II function (Table 1).

The autophosphorylation of Thr-286 in the α subunit has been extensively investigated, since phosphorylation of this site is both necessary and sufficient to convert CaM-PK II into an autonomous enzyme (Miller et al., 1988; Schworer et al., 1988; Kwiatkowski et al., 1988; Thiel et al., 1988; Lickteig et al., 1988; Patton et al., 1990; Hanson et al., 1989; Fong et al., 1989; Lou and Shulman, 1989). Thr-286 phosphorylation is not essential

Table 1
CaM-PK II Autophosphorylation Sites

Sites	Phosphorylation conditions: Limiting +Ca/CM	Nonlimiting +Ca/CM	Limiting +Ca/CM then -Ca/CM	References[b]
α Subunit				
Thr-Nter		+		6
Thr-253		+		7
Ser-279		+		1,7
Thr-286	+	+	+	1–10
Thr-305			+	5,6,10
Thr-306?[a]			+	6
Thr-310?[a]			+	6
Ser-314			+	5,6,10
Ser-318?[a]			+	6
β Subunit				
Thr-Nter?[a]		+		6
Thr-254?[a]		+		7
Ser-280		+		1
Thr-287	+	+	+	1
Thr-306			+	6
Thr-307?[a]			+	6
Thr-311?[a]			+	6
Ser-315			+	6,10
Ser-319?[a]			+	6
Ser-343		+		1
Ser-371		+		1
Thr-382	+	+	+	1

[a]In the α subunit, Thr-306, Thr-310, and Ser-318 are possible autophosphorylation sites, whereas the other sites are confirmed directly. In the β subunit, Thr-307, Thr-311, and Ser-319 are possible sites, whereas Thr Nter (a Thr residue found in the peptide containing the N-terminal amino acid) and Thr-254 are included only by analogy with the α subunit.

[b]1, Miller et al., 1988. 2, Schworer et al., 1988. 3, Thiel et al., 1988. 4, Lickteig et al., 1988. 5, Colbran and Soderling, 1990. 6, Patton et al., 1990. 7, Hanson et al., 1989. 8, Fong et al., 1989. 9, Lou and Schulman, 1989. 10, Colbran et al., 1988.

for the autophosphorylation of the secondary sites in CaM-PK II, nor is it necessary to have this site phosphorylated in order for substrates to be phosphorylated (Fong et al., 1989; Hanson et al., 1989). Thr-286 is the first site in CaM-PK II to be phosphorylated (Fig. 2), and its labeling is favored under limiting incubation conditions, such as low temperature, and short times and low levels of magnesium and ATP (Schulman, 1988). Low temperatures and short incubation times favor initial events in enzyme reactions. Autophosphorylation of CaM-PK II at Thr-286 can be observed at 4°C within seconds (Lai et al., 1986) and precedes the autophosphorylation of other residues. Magnesium binds to ATP, the complex serves as a substrate for all protein kinase reactions, and the concentration of Mg required for half-maximal activity of CaM-PK II is 5–10 m*M* $MgCl_2$ (Schulman, 1988). Increasing the concentration of magnesium might therefore be expected to increase CaM-PK II autophosphorylation at all sites, and this was observed with cytoskeletal CaM-PK II (LeVine et al., 1986). Limiting magnesium levels might be expected to favor Thr-286 autophosphorylation. A further reason for using low magnesium concentrations to favor Thr-286

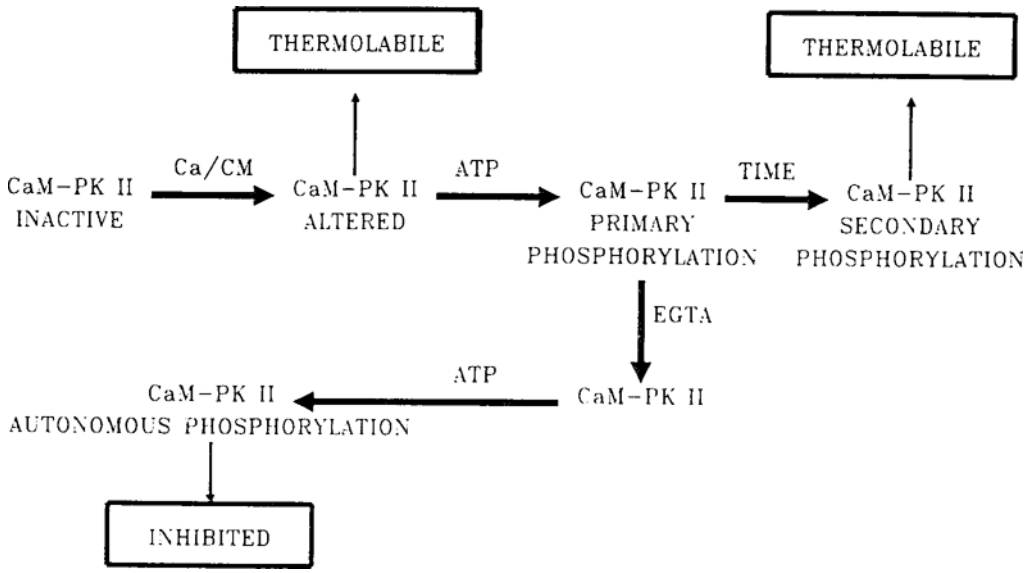

Fig. 2. Inactivation of CaM-PK II When CaM-PK II is isolated, it is inactive. Addition of calcium (Ca) and calmodulin (CM) alters the conformation of the enzyme, and this leads to a major increase in its thermolability. Addition of ATP leads to the phosphorylation of the primary sites and protects the enzyme from thermal denaturation. Under nonlimiting conditions, including longer incubation times, CaM-PK II is phosphorylated on secondary sites, and this can lead to thermal inactivation, especially if Thr-253 is phosphorylated. Addition of EGTA to CaM-PK II that has been even partially phosphorylated at Thr-286 leads to a loss of Ca and CM from the enzyme. If ATP is now added, the autonomous sites become phosphorylated. This can lead to an inhibition of the enzyme's activity.

autophosphorylation is that, with soluble CaM-PK II, a serine residue in both the α and β subunits was phosphorylated at high magnesium concentrations (5 m*M*) causing the enzyme to become thermally unstable (*see below*), leading to loss of activity (Lickteig et al., 1988). Increasing the concentration of ATP from 3 to 50 μ*M* increases the rate at which autonomous CaM-PK II is formed, but also leads to loss of kinase activity (Schulman, 1988). At more physiological levels of ATP (500–1000 μ*M*) CaM-PK II is stabilized. One possible explanation for this anomaly is that, when low ATP is used, an extra site(s) becomes phosphorylated on CaM-PK II, which increases the thermal lability of the enzyme (Lou and Shulman, 1989).

In the β subunit, Thr-287 is equivalent to Thr-286 in the α subunit, and its phosphorylation is also rapid, occurs under the same limiting conditions, and leads to autonomous activity (Miller et al., 1988). However, in addition a further primary site, Thr-382, is rapidly labeled in the β subunit. This site is in the association domain of the β subunit, and is not present in either the α or the β' subunits.

Increasing the time and temperature of the incubation and the concentration of magnesium, while maintaining the calcium and calmodulin concentrations, leads to the phosphorylation of a set of secondary sites in the α subunit that includes Thr-253 (Hanson et al., 1989), Ser-279 (Miller et al., 1988; Hanson et al., 1989), and a threonine residue found in a peptide containing the N-terminal amino acid (Patton et al., 1990). In the β subunit, Ser-280, Ser-343, and Ser-371 (Miller et al., 1988) were all phosphorylated under the same conditions.

After incubation of CaM-PK II under limiting conditions leading to phosphorylation of the primary site(s), EGTA addition allows an

increase in the rate of autophosphorylation, and a number of new (autonomous) sites become phosphorylated. The phosphorylation of these autonomous sites does not occur in CaM-PK II in which Thr-286 has been replaced with an Ala (Fong et al., 1989), indicating that phosphorylation of the autonomous sites is dependent on prior phosphorylation of at least some of the Thr-286 residues in the multimer. In the α subunit, these new sites include Thr-305 and Ser-314 (Colbran et al., 1989b; Colbran and Soderling, 1990; Patton et al., 1990). In addition, a number of other sites become phosphorylated, possibly including Thr-306, Thr-310, and Ser-318 (Patton et al., 1990). Similar sites are labeled in the β subunit, including Thr-306, Ser-315, and possibly Thr-307, Thr-311, and Ser-319 (Patton et al., 1990).

It is clear that the pattern of autophosphorylation sites in CaM-PK II is dependent on the incubation conditions used. However, there are also further complexities. First, there is preliminary evidence to suggest that phosphorylation of one site on CaM-PK II modulates the phosphorylation of other sites (Fong et al., 1989; Hanson et al., 1989). Second, Thr-305 in the α subunit and Thr-306 and Thr-382 in the β subunit are sites that would not have been predicted to be phosphorylated by CaM-PK II, since they do not readily conform to the so-called consensus sequences for this enzyme (Miller et al., 1988). Although this may be expected (Kemp and Pearson, 1990), perhaps these sites are labeled by intersubunit interaction in a constrained holoenzyme (Fig. 1B) and are not labeled when the enzyme exists as a monomer (Fig. 1A). Third, divalent metal ions, when tested for their effects on the autophosphorylation of CaM-PK II, gave unexpected results. Manganese was able to substitute for magnesium, but fewer sites were autophosphorylated (Kuret and Schulman, 1985; Lou and Schulman, 1989). At low concentrations, zinc inhibited autophosphorylation, but at concentrations above 100 μM, zinc produced a concentration-dependent stimulation of CaM-PK autophosphorylation in the absence of calcium and calmodulin (Weinberger and Rostas, 1991). However, the enzyme was now only able to phosphorylate most of its normal substrates poorly, including synapsin I (Weinberger and Rostas, 1991). It was confirmed that the effect of zinc was directly on the enzyme and not on calmodulin, ATP, or protein phosphatases. The unusual effects of zinc on CaM-PK II can be used to detect and identify low levels of CaM-PK II, even in crude tissue extracts (Jeitner et al., 1991). Fourth, CaM-PK II autophosphorylation and substrate phosphorylation are stimulated by gangliosides, but the increased autophosphorylation occurs on Ser residues and not on Thr residues, and no autonomous enzyme is formed (Fukunaga et al., 1990). In summary, activation of CaM-PK II autophosphorylation is extremely complex with multiple sites being labeled and the conditions of incubation having a significant effect on the extent and nature of this labeling.

Thermal Lability

There are a number of factors that decrease CaM-PK II autophosphorylation in vitro. The first is the extent to which the enzyme preparation has retained its in vivo activity, as CaM-PK II is very labile postmortem (Fig. 2).

Thus, brain could not be frozen at –20 or –70°C for any significant period of time or even stored at 4°C for more than 15 min, because loss of CaM-PK II activity would be observed (Kuret and Schulman, 1984). Bovine brain contains little if any CaM-PK II activity, and this is presumably the result of the kinases postmortem lability (Kuret and Schulman, 1984). The enzyme also appears to be degraded during isolation and storage (Burke and DeLorenzo, 1982; Goldenring et al., 1984; Kuret and Schulman, 1984). This can be minimized by addition of protease inhibitors (Burke and DeLorenzo, 1982) and especially by removal of calcium (Kuret and Schulman, 1984; Miller and Kennedy, 1985; McGuinness et al., 1985).

When calcium was added to crude membrane fractions from rat cerebral cortex and then preincubated at 37°C, phosphorylation of known CaM-PK II substrates was rapidly inactivated

(Dunkley and Robinson, 1981a,b). Others have observed thermolability of CaM-PK II. Lai et al. (1986) found that the total activity of CaM-PK II decreased following incubation at 30°C in the presence of calcium and calmodulin alone, but this was not found at 0°C. Rostas et al. (1987) found that incubation of CaM-PK II in the presence of calcium and calmodulin alone led to a loss of activity of enzyme when subsequently measured against a peptide substrate. These data suggest that CaM-PK II has a thermolability that is exaggerated in the presence of calcium and calmodulin. This is presumably because of the change in the conformation of CaM-PK II that occurs on addition of calmodulin (Fig. 1). Exposure to calcium and calmodulin at high temperatures should therefore be avoided during enzyme preparation. Furthermore, preincubation of the enzyme at 37°C with calcium and calmodulin may not be an appropriate procedure, and enzyme reactions should be initiated by addition of the enzyme rather than ATP (Dunkley and Robinson, 1981a,b).

The thermolability of CaM-PK II can be altered by autophosphorylation. Rostas et al. (1987) found that autophosphorylation of CaM-PK II partially protected against inactivation of the enzyme induced by incubation with calcium and calmodulin. This was also seen with site-directed mutants (Hanson et al., 1989). The simplest explanation for these results is that autophosphorylation of Thr-286, plus possibly Ser-279 (Hanson et al., 1989), stabilizes the enzyme by changing its conformation and thereby overcoming the enzyme's thermolability.

Autophosphorylation of CaM-PK II under other conditions, however, appears to increase the thermolability of the enzyme. The data are, in general, consistent with the increased thermolability being the result of the phosphorylation of sites other than Thr-286 that are labeled under nonlimiting conditions and in the absence of EGTA (Fig. 2). Lai et al. (1986) autophosphorylated CaM-PK II to different extents and subsequently measured activity against synapsin 1. They found that the greater the autophosphorylation, the greater the loss of enzyme activity, and dephosphorylation of CaM-PK II could not reverse this loss. Patton et al. (1990) observed a thermolability of CaM-PK II when the time of autophosphorylation was increased, and this change was known to lead to the phosphorylation of secondary sites (Miller et al., 1988). Lou et al. (1986) found a loss of enzyme activity when autophosphorylation was performed at low ATP concentrations (5 μM), but when high concentrations (500 μM) were used, activity was not decreased by autophosphorylation. When low ATP is used, an extra site(s), including Thr-253 (Hanson et al., 1989), becomes phosphorylated on CaMPK II, which increases thermal lability (Lou and Schulman, 1989). CaM-PK II was shown to be phosphorylated at a unique serine in both the α and β subunits at high (5 mM) magnesium concentrations (Lickteig et al., 1988). It was suggested that this was owing to a direct interaction of magnesium with the enzyme and that the phosphorylation of the unique serine site caused the enzyme to be thermally unstable (Lickteig et al., 1988). Perhaps the site labeled with high magnesium is also Thr-253. Yamauchi et al. (1991) found that there was a decreased stability of CaM-PK II after autophosphorylation and that forebrain kinase was more susceptible than cerebellar kinase to inactivation. This suggests that other factors, such as the ratio of α to β subunits, may also have a role in determining the enzyme's thermal stability, or, alternately that the α and β subunits are differentially sensitive to thermal inactivation.

Phosphatases

The second factor that can decrease CaM-PK II autophosphorylation in vitro is the activity of protein phosphatases (PP), including PP1 (Shields et al., 1985; Schworer et al., 1986; Patton et al., 1990) and PP2A (Lou et al., 1986; Lai et al., 1986; Patton et al., 1990), but not alkaline phosphatase (Miller and Kennedy, 1986). Dephosphorylation of CaM-PK II by PP1 or PP2A almost completely reverses autonomy and, therefore, must occur at Thr-286. PP1 only partially dephosphorylated CaM-PK II (Schworer et al., 1986), whereas PP2A

rapidly dephosphorylated Thr-305, and only slowly dephosphorylated Ser-314 and an unidentified site (Patton et al., 1990). Fukunaga et al. (1989) found with cerebellar granule cell CaM-PK II, which is predominately the β subunit, that okadaic acid decreased the rate of loss of autonomy, suggesting involvement of PP1 and/or PP2A. However, there was also an okadaic acid-insensitive phosphatase present whose identity was not known.

Inhibitors

The third factor that can decrease CaM-PK II autophosphorylation in vitro is the presence of inhibitor substances (Table 2). Three classes of inhibitors were previously identified: calcium or calmodulin antagonists, which block enzyme activation; irreversible inhibitors, which modify specific amino acid groups; and the antianxiety/anticonvulsive agents, whose mechanism of action is unknown (*see* Table 3 in Schulman, 1988). Since then, other agents have been shown to inhibit CaM-PK II autophosphorylation in vitro. Sphingosine inhibits CaM-PK II autophosphorylation and substrate phosphorylation by competing for the calmodulin binding site (Jefferson and Schulman, 1988; Jefferson et al., 1991). Synthetic peptides corresponding to sequences within CaM-PK II have been developed that block CaM-PK II autophosphorylation and substrate activity (Colbran et al., 1988,1989b Kelly et al., 1988; Colbran and Soderling, 1990). The peptides each have characteristic patterns of inhibition depending on their size, but they all either block calmodulin binding or mimic the inhibitory domain sequence. At least one of these peptides (CaM-PK II 281–309) has been shown to be nonspecific, and also to inhibit myosin light-chain kinase and protein kinase C almost equally (Smith et al., 1990). A new and more specific inhibitor of CaM-PK II autophosphorylation and substrate phosphorylation has been developed (KN-62) that acts by binding to CaM-PK II and preventing subsequent interaction with calmodulin (Tokumitsu et al., 1990). This inhibitor does not, therefore, affect the autonomous form of the enzyme. Staurosporine inhibits CaM-PK II autophosphorylation and substrate activity by interacting with the catalytic domain at a position distinct from the ATP- and substrate-binding domains (Yanagihara et al., 1991). Arachidonic acid inhibits CaM-PK II autophosphorylation and substrate phosphorylation by reversible binding of the fatty acid to the regulatory region of the kinase (Piomelli et al., 1989). High K buffer, which mimics the intracellular ionic environment, inhibits CaM-PK II autophosphorylation, and although the mechanism for this inhibition is not known, it is not owing to the high ionic strength or tonicity of the buffer (Dunkley et al., 1991). Other agents are able to inhibit CaM-PK II activity against substrates and might be expected to alter its autophos-phorylation. Synthetic peptides developed to inhibit myosin light-chain kinase (MLCK 480–501) and protein kinase C (PKC 19–36) both inhibit CaM-PK II activity against peptides and MAP-2 in vitro (Smith et al., 1990).

Table 2
Inhibitors of CaM-PK II Autophosphorylation

Mechanism	Agents	IC_{50} (μM)
Calcium antagonists	EGTA	
	Fluoride	
Calmodulin antagonists	Antipsychotics	5–100
	Mellitin	4
	Calmidazolium	0.5
	Sphingosine	5
Calmodulin binding antagonist	KN-62	0.9
	CaM-PK II(281–309)	0.075
	CaM-PK II(284–309)	0.075
Catalytic domain blocker	Staurosporine	0.02
Regulatory domain blocker	Arachidonic acid	24
	PKC(19–36)	30
	MLCK(480–501)	1.7
Unknown action	Ergot derivatives	9–31
	Antianxiety\ depressives	8–400
	High K	

Consequences

The consequences of autophosphorylation of CaM-PK II in vitro have previously been reviewed (Schulman, 1988; Colbran et al., 1989a). The production of an autonomous enzyme and increased thermal lability of certain forms of the kinase have been discussed above; however, there are other consequences, and these will be briefly described here in the order in which they were discovered.

Autophosphorylation of CaM-PK II was observed to decrease the mobility of the enzyme on PAGE (Bennett et al., 1983; Goldenring et al., 1983; Rostas et al., 1983; Kuret and Schulman, 1984). It was noted that this mobility shift only occurred when >1 mol of P_i was incorporated/mol of holoenzyme (Bennett et al., 1983), and that both the α and β subunits shifted, with the α subunit showing the greatest shift (Goldenring et al., 1983). Increases in mobility of CaM-PK II occurred after autophosphorylation in the presence of zinc (Weinberger and Rostas, 1991). It is assumed that autophosphorylation of CaM-PK II changes the conformation of individual subunits and alters the binding to SDS, leading to the observed mobility shifts. The sites phosphorylated on CaM-PK II might therefore be expected to influence the changes in mobility of the enzyme. Lou et al. (1986) observed the mobility shift only when the incubation conditions (low ATP) favored the phosphorylation of the sites other than Thr-286 that are normally labeled in the presence of calcium and calmodulin. The increased mobility of CaM-PK II in the presence of zinc was assumed to be the result of the phosphorylation of a unique site(s) on the enzyme, but this has not yet been determined (Weinberger and Rostas, 1991). In contrast, Lickteig et al. (1988) suggested that the appearance of a 53-kDa polypeptide did not appear to result from the selective phosphorylation of specific peptides.

Autophosphorylation of CaM-PK II alters the affinity of the enzyme for calmodulin, and early studies showed that autophosphorylation either increased (Shields et al., 1984) or decreased (LeVine et al., 1985) calmodulin binding. Since then, the calmodulin binding domain in CaM-PK II has been defined as being between residues 295–309 in the α subunit (Bennett and Kennedy, 1987; Hanley et al., 1987,1988; Kelly et al., 1988; Lickteig et al., 1988; Colbran et al., 1989a,b). It appears that autophosphorylation of CaM-PK II at Thr-286 increases the binding of calmodulin, presumably by changing the conformation of other subunits in the multimer leading to greater accessibility of their calmodulin binding domains. However, when autophosphorylation occurs in the presence of EGTA, Thr-305 also becomes phosphorylated and, since this site is in the middle of the calmodulin binding domain, the affinity of CaM-PK II for calmodulin is decreased dramatically (Patton et al., 1990; Colbran and Soderling, 1990). Ser-314 has been proposed to be involved in calmodulin binding, but when this site is autophosphorylated, there is only a small decrease in calmodulin binding (Patton et al., 1990; Colbran and Soderling, 1990). The affinity of soluble CaM-PK II for calmodulin is many-fold less than for other calmodulin binding proteins, with an activation constant of between 20–100 n*M* (Colbran et al., 1989a). The amount of calmodulin bound to the kinase differed between cytoskeletal, or PSD, and soluble enzyme (Suzuki and Tanaka, 1986; Colbran et al., 1989a). Furthermore, CaM-PK II subunits expressed in a mammalian system showed a slight modulation of calmodulin binding (Yamauchi et al., 1989), whereas expression in a bacterial system markedly modified calmodulin binding (Waxham et al., 1989). These unique calmodulin-binding characteristics are likely to be a result of differences in the nature of the association between the subunits of CaM-PK II (Waxham et al., 1989) and other CaM-PK II-binding proteins (Rostas and Dunkley, 1992). These associations are also likely to be altered by autophosphorylation. The effect of autophosphorylation on calmodulin binding is therefore dependent on the site(s) phosphorylated, and hence on the conditions used for the in vitro incubation, as well as the source of the kinase, which determines the nature of CaM-PK II associations.

Autophosphorylation of CaM-PK II causes a rapid translocation of the enzyme from the cytoskeleton to the soluble fraction in Aplysia (Saitoh and Schwartz, 1985). No such translocation was found with synaptic junction preparations (Suzuki and Tanaka, 1986) or in synaptosomes after depolarization (Gorelich et al., 1988). However, Aronowski et al. (1992) stated that "...in preliminary in vitro studies with control brain homogenates we have also found that translocation correlates initially with autophos-phorylation of CaM-PK II." A slower redistribution of CaM-PK II from the soluble to the particulate fraction occurs during development (Rostas et al., 1 986b; Rostas, 1991) after cerebral ischemia (Aronowski et al., 1992) in brain and in hippocampal pyramidal neurons in culture (Scholz et al., 1988). Whether these redistributions have anything to do with autophosphorylation or the rapid translocation events seen in Aplysia is not known, especially since it has been suggested that the redistribution that occurs during development may involve particular associations of CaM-PK II with developmentally regulated binding proteins (Rostas and Dunkley, 1992). However, a possible role for autophosphorylation of Thr-382 in the β subunit was proposed to be the localization of the enzyme to particular subcellular domains (Miller et al., 1988).

Autophosphorylation of CaM-PK II generally decreases the activity of the enzyme. As discussed above, autophosphorylation increases the thermolability of the enzyme, perhaps because of phosphorylation of Thr-253, but in addition formation of an autonomous enzyme also decreases CaM-PK II activity (Fig. 2; Schulman, 1988), although this is not always observed (Patton et al., 1990). Hashimoto et al. (1987) suggested that separate autophosphorylation sites in CaM-PK II are associated with formation of autonomous activity and suppression of total activity. This was confirmed by Lou and Schulman (1989). The sites responsible for this decreased activity may include Thr-305 and/or Thr-314, since phosphorylation of these sites blocks calmodulin binding and would decrease total activity to the level of autonomous activity (Fukunaga et al., 1989).

Autophosphorylation of CaM-PK II can alter the substrate specificity of the kinase. A number of calmodulin-binding proteins, including calcineurin, smooth muscle myosin light-chain kinase, myelin basic protein, cyclic nucleotide phosphodiesterase, and caldesmon, are all substrates of the autonomous CaM-PK II and are not phosphorylated by the enzyme in the presence of calmodulin (Colbran et al., 1989b; Hashimoto et al., 1987,1989; Miyamoto et al., 1990). The reason for this has been suggested to be that calmodulin binding to the substrate blocks the sites phosphorylated by the autonomous enzyme, but this was not the case for myelin basic protein and calcineurin (Miyamoto et al., 1990). Furthermore, the substrate specificity of forebrain and cerebellar CaM-PK II changes upon autophosphorylation even with substrates that are not known to bind calmodulin (Yamauchi et al., 1991).

Autophosphorylation of CaM-PK II is necessary for CaM-PK II to be proteolytically degraded to a constitutively active enzyme (Kwiatkowski and King, 1989). Presumably, the change in conformation of CaM-PK II exposes sites for proteolytic cleavage.

CaM-PK II Autophosphorylation *In Situ*

Overview

A number of studies have attempted to analyze CaM-PK II autophosphorylation directly, by PAGE and autoradiography, after labeling synaptosomes (Dunkley and Robinson, 1986; Dunkley et al., 1986,1988a,1991), or brain slices (Rodnight et al., 1988; Yip and Kelly, 1989), *in situ* with radiolabeled phosphate. Dunkley et al. (1986) found that the α and β subunits of CaM-PK II were "... hardly labeled in intact synaptosomes relative to lysed synaptosomes." When brain slices were used, the level of CaM-PK II seen on two-dimensional PAGE was either too low to be detected directly (Rodnight et al., 1988) or was only seen after enrichment of CaM-PK II by ex-

traction of tissue with detergent (Yip and Kelly, 1989).

In other studies, CaM-PK II was enriched by immunoprecipitation after *in situ* labeling of cells with radiolabeled phosphate. In each study some autophosphorylated CaM-PK II was detected, but not for all the conditions investigated. In none of these studies was the recovery of labeled CaM-PK II, or the proportion of total CaM-PK II that was autophosphorylated ever determined. The original study used the nonneuronal rat embryo fibroblast 3Y1 cells (Ohta et al., 1988). Subsequent studies with neuronal tissue have used synaptosomes (Gorelick et al., 1988), cerebellar granule cells in culture (Fukunaga et al., 1989), hippocampal tissue slices (Yip and Kelly, 1989; Ocorr and Schulman, 1991), and organotypic cultures (Molloy and Kennedy, 1991). Neuroendocrine tissue has also been used, including PC12 cells (MacNicol et al., 1990) and rat pituitary GH3 cells (Jefferson et al., 1991). The CaM-PK II in these various cell types is not identical, but since so few *in situ* studies have been undertaken on the kinases' autophosphorylation, the nonneuronal cell studies are included in the discussion below.

Injection of inorganic phosphate into rats in vivo and subsequent isolation of subcellular fractions enriched for PSDs suggested that CaM-PK II was phosphorylated (Berman et al., 1980; Gurd and Bissoon, 1985). However, the kinase was only tentatively identified by its mobility on a one-dimensional gel. No phosphorylation of CaM-PK II was found in another *in situ* study (Rodnight et al., 1986).

Synaptosomes

It is clear that CaM-PK II is autophosphorylated *in situ*, but the level of incorporation of radiolabel was always low. This was especially the case with synaptosomes where the data show that, even though there are significant quantities of CaM-PK II present, virtually no autophosphorylation could be detected without enrichment of the enzyme (Fig. 3A). The autophosphorylation of CaM-PK II in synaptosomes will be discussed here, because it illustrates a number of issues relevant to all *in situ* studies.

A detailed study revealed that methodological factors made a significant contribution to explaining the low level of CaM-PK II autophosphorylation in synaptosomes. The location of 62% of CaM-PK II on the outside of intact synaptosomes, where it was inaccessible to radiolabeled ATP generated within the synaptosome, was the major factor (Dunkley et al., 1988a). However, thermal inactivation of CaM-PK II occurs *in situ* during the preincubation at 37°C for 45 min, which is used to incorporate the radiolabeled phosphate (Fig. 3B). Furthermore, the high K buffer present within synaptosomes also contributed to a further decrease in CaM-PK II autophos-phorylation (Dunkley et al., 1991). The loss owing to thermolability and high K buffer together accounted for a decrease of some 20% of the total CaM-PK II autophosphorylation. The maximum level of CaM-PK II labeling expected in intact synaptosomes can therefore be calculated to be only 18% of the total found in lysed synaptosomes (Dunkley et al., 1991). However, the actual level of CaM-PK II labeling was even less than 18%, and other factors must be contributing to the low incorporation of radioactivity into CaM-PK II. High levels of protein phosphatase activity within synaptosomes would tend to decrease the autophosphorylation of all the possible sites that were labeled in the lysed synaptosomes (Dunkley et al., 1991). Alternately, a low level of protein phosphatase activity in synaptosomes under basal conditions (Sim et al., 1991; Sim, 1992) would lead to a decrease in the number of vacant sites in CaM-PK II available to be autophosphorylated on addition of radiolabeled ATP. We cannot choose between these mechanisms to date. Furthermore, intracellular conditions that would favor the phosphorylation of a limited number of sites (e.g., high ATP, low Mg, limited calcium or calmodulin), or the phosphorylation of sites, such as Thr-305, which blocks calmodulin binding, would also lead to a decrease in potential CaM-PK II autophosphorylation.

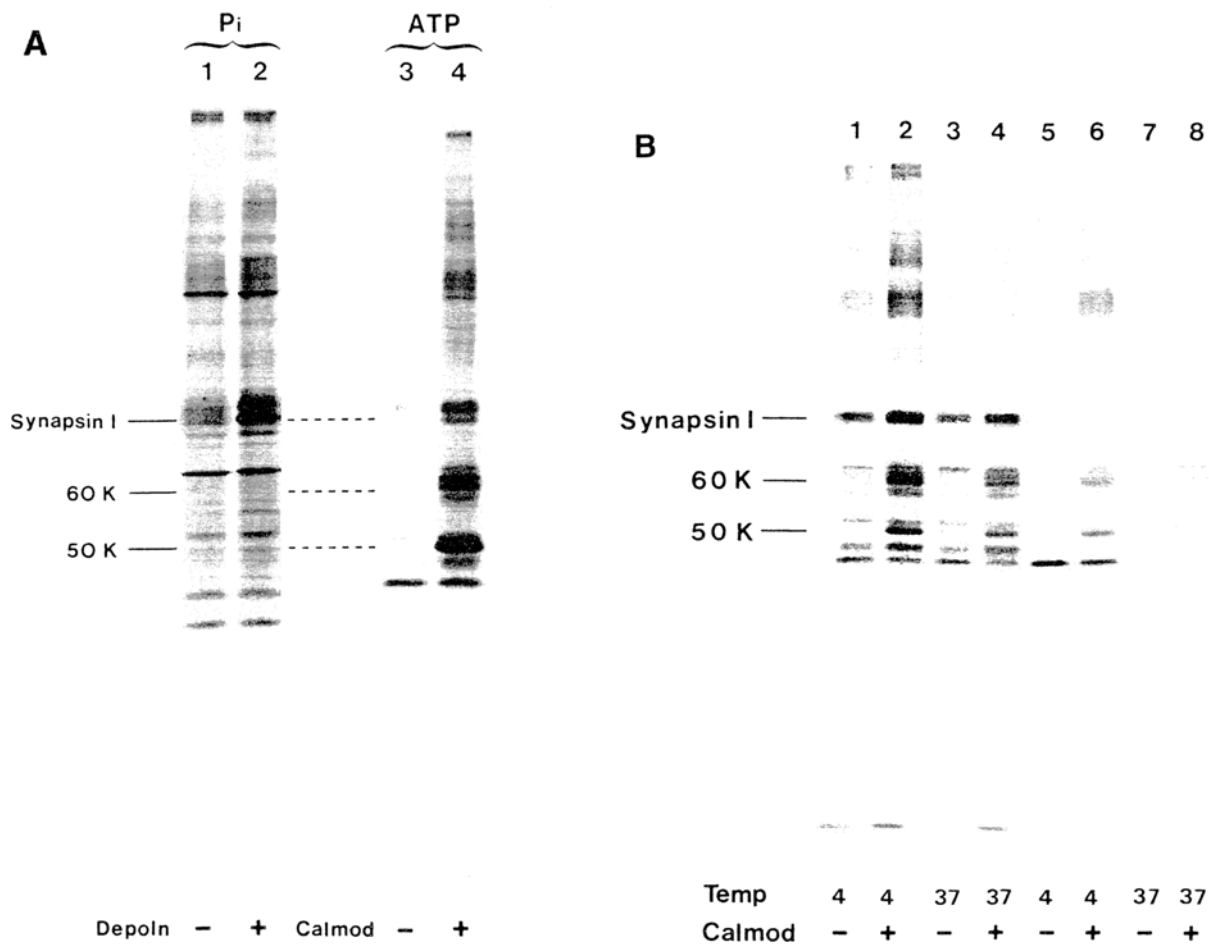

Fig. 3. Autophosphorylation of synaptosomal CaM-PK II. Synaptosomes isolated from rat cerebral cortex (Dunkley et al., 1988b) were either retained intact in isotonic Krebs buffer or lysed in hypotonic Tris buffer (Dunkley et al., 1991). (**A**) Intact synaptosomes were incubated with $^{32}P_i$ and then either retained in Krebs buffer (track 1) or depolarized in high K buffer (track 2). Lysed synaptosomes were incubated in [γ-^{32}P]ATP in the absence (track 3) or presence (track 4) of calmodulin. The α (50K) and β (60K) subunits of CaM-PK II and the major CaM-PK II substrate synapsin I are indicated. It can he seen that relative to the CaM-PK II available for autophosphorylation as seen in the lysed synaptosomes there is little labeled in the intact synaptosomes. (**B**) Lysed (tracks 1–4) or intact (tracks 5–8) synaptosomes were incubated with [γ-^{32}P] ATP, in the presence (+) or absence (–) of calmodulin, after incubating at either 4 or 37°C for 45 min. It can be seen that incubation at 37°C decreased CaM-PK II autophosphorylation and that a significant proportion of CaM-PK II is labeled in intact synaptosomes. This CaM-PK II is on the outside of the synaptosomes, presumably associated with the PSD (Dunkley et al., 1991). The phosphorylated synaptosomal proteins were fractionated by PAGE, the gels were dried, and the radiolabeled proteins were detected by autoradiography.

Autophosphorylation Sites

When intact tissues are incubated with radiolabeled phosphate under basal conditions, CaM-PK II becomes labeled. The sites that were phosphorylated were investigated. Gorelick et al. (1988) found with synaptosomes that most of the labeled phosphate was in Thr, with only 13% being in Ser. However, when peptide maps were produced from the synaptosomal CaM-PK II, very little labeled phosphate was detected in the peptides that were known to contain Thr-286, although other unidentified peptides were seen. A very low level of incorporation of radiolabeled

phosphate into Thr-286-containing peptides under basal conditions was also the case with other cells (Fukunaga et al., 1989; MacNicol et al., 1990; Jefferson et al., 1991). In contrast, Molloy and Kennedy (1991) found with hippocampal cultures under basal conditions that, when CaM-PK II peptides were obtained by digestion with trypsin, those containing Thr-286 predominated, whereas a peptide containing Ser-314 was also clearly labeled with phosphate. Phosphorylation of the autonomous site (Ser-314) normally only occurs in the presence of EGTA in vitro. The reason Thr-286-containing peptides were clearly seen under basal conditions only with the hippocampal cultures is likely to relate to technical factors, including the use of more efficient HPLC procedures to fractionate the peptides, higher levels of radiolabeled phosphate added to the incubations/mg cells, and the longer preincubation times with the radiolabeled phosphate. In addition, the hippocampal cultures had a considerably higher proportion of their CaM-PK II in the autonomous form under basal conditions than is seen with other cells (*see below*). Under basal conditions, the proportion of CaM-PK II that is able to incorporate radiolabeled phosphate is therefore very low, and the site(s) that is autophosphorylated is generally not known; however, for organotypic hippocampal cultures, Thr-286 predominates.

Depolarization of cells with high K buffer increases the level of CaM-PK II autophosphorylation. Gorelick et al. (1988) found that a tryptic peptide containing Thr-286 obtained from the α subunit was phosphorylated to 75% above basal levels within 5 s and that other serine-containing peptides also became phosphorylated at a slower rate over the next 5 min. Similar results were seen with GH3 cells (Jefferson et al., 1991). Fukunaga et al. (1989) found that a peptide containing Thr-287 in the β subunit was phosphorylated within 30 s and that other peptides were also phosphorylated; it was argued that Thr-306 in the α subunit and Thr-307 in the β subunit were unlikely to be phosphorylated under the conditions used. It was noted that the phosphopeptide maps for the *in situ* phosphorylated CaM-PK II differed from those seen in vitro with lysed tissue, suggesting that different sites may be labeled (Yip and Kelly, 1989). Yip and Kelly also noted that the ratio of β/α subunit phosphorylation was greater *in situ* than in vitro, that most of the labeled β subunit was in the Triton X-100 soluble fraction, and that there was very little labeling of CaM-PK II in the Triton X-100 insoluble fraction, which would include CaM-PK II present in the PSD. Depolarization therefore rapidly increases the phosphorylation of Thr-286, whereas other sites are more slowly labeled. The pattern of autophosphorylation *in situ* is not identical to that found in vitro, the sites phosphorylated have not yet been identified, and the degree of stimulation is not equal for CaM-PK II at all cellular locations. In contrast, the nonneuronal 3Y1 cells, when stimulated with fetal calf serum, showed CaM-PK II phosphorylated *in situ* only on serine residues (Ohta et al., 1988), but here too the phosphopeptide maps differed from those seen in vitro.

In all of the studies, the increase in CaM-PK II autophosphorylation on depolarization with high K was dependent on extracellular calcium (Gorelick et al., 1988; Yip and Kelly, 1989; MacNicol et al., 1990; Ocorr and Schulman, 1991; Molloy and Kennedy, 1991; Jefferson et al., 1991) and correlated with an increase in intracellular calcium (MacNicol et al., 1990; Jefferson et al., 1991). Bradykinin increased the level of intracellular calcium in PC12 cells in the absence of extracellular calcium by releasing it from intracellular stores, and this also led to an increase in phosphorylation of a peptide containing Thr-286, as well as the labeling of other peptides (MacNicol et al., 1991). However, when thyrotropin-releasing hormone was added to GH3 cells, there was a transient increase in intracellular calcium to the same level as seen with high K, but no radiolabeled phosphate was found in CaM-PK II (Jefferson et al., 1991); there was, however, an increase in the level of autonomous enzyme, suggesting that autophosphorylation must have occurred, but was below the level of detection.

Consequences

It is established that the phosphorylation of Thr-286 is essential for the formation of autonomous CaM-PK II activity. It has therefore been assumed that, if there is a change in the level of autonomous activity of CaM-PK II, there has been autophosphorylation of Thr-286. However, it must be remembered that the degree of autonomous activity is not directly proportional to autophosphorylation. This is because, in a multimer, autophosphorylation of only some of the available Thr-286 sites changes the conformation of the enzyme, allowing substrate phosphorylation to occur, but for the formation of an autonomous kinase and the phosphorylation of the autonomous sites, removal of the calmodulin is then required.

The level of autonomous CaM-PK II is easier to measure and more sensitive than measuring CaM-PK II autophosphorylation, and in a number of studies, autonomous activity was detected after *in situ* manipulation of cells (Gorelick et al., 1988; Fukunaga et al., 1989; Piomelli et al., 1989; MacNicol et al., 1990; Ocorr and Schulman, 1991; Molloy and Kennedy, 1991; Jefferson et al., 1991). The percent of CaM-PK II in an autonomous form under basal incubation conditions was generally <5%, except in the case of the organotypic hippocampal cultures, where levels of 15% were found with 2–8 wk cultures (Molloy and Kennedy, 1991). It may be that these cells were leakier to calcium, which led to increased autonomous activity. Depolarization of cells with high K generally increased the level of autonomous CaM-PK II activity, except in the case of posterior pituitary cells (Ocorr and Schulman, 1991). The extent of the increases was from 5 to 40% above basal depending on the cell type and the conditions used. Molloy and Kennedy (1991) found that a number of agents that modulate neuronal function did not alter the level of autonomous kinase, perhaps because of the already high basal levels. However, they found that the protein kinase inhibitor H7 and the calmodulin antagonist W7 both decreased autonomous CaM-PK II activity.

The increases in autonomous CaM-PK II activity after depolarization with high K were all dependent on extracellular calcium. Bradykinin also increased autonomous activity by raising intracellular calcium from intracellular stores. The level of autonomous activity and the time that it was maintained for appeared to correlate with the level of intracellular calcium (Fukunaga et al., 1989; MacNicol et al., 1990; Ocorr and Schulman, 1991; Molloy and Kennedy, 1991; Jefferson et al., 1991).

Fukunaga et al. (1989) found that the level of autonomous CaM-PK II activity was critically dependent on protein phosphatase activity and that addition of okadaic acid increased the levels of autonomous kinase. It was suggested that the relative activities of CaM-PK II and protein phosphatases may determine the level of CaM-PK II autophosphorylation in intact cells (Colbran et al., 1988) and that this ratio was dependent on the intracellular level of calcium (Fukunaga et al., 1989). Gorelick et al. (1988) found that total CaM-PK II autophosphorylation continued to rise over a 5-min period of depolarization, whereas the level of phosphorylation of Thr-286 began to decrease by 30 s, but by 5 min was still very high. The phosphorylation of the CaM-PK II substrate synapsin I rose for 15 s and fell to baseline again by 5 min. This suggests that, in depolarized synaptosomes, protein phosphatases are active against substrates (Sim et al., 1991), but do not readily reverse the continued autophosphorylation of CaM-PK II. However, over the same time, the level of autonomous CaM-PK II fell to zero. The reason for this is unknown, but may relate to other consequences of CaM-PK II autophosphorylation, including inactivation, translocation, altered substrate specificity, or proteolysis, none of which have been investigated to date.

The level of autonomous CaM-PK II in synaptosomes was inhibited by arachidonic acid (Piomelli et al., 1989). CaM-PK II autophosphorylation in PC12 cells was inhibited by KN-62 (Tokumitsu et al., 1990). Peptide inhibitors of CaM-PK II modulate synaptic function, including the formation of long-term potentiation after microinjection (Kelly, 1992), but they can also

modulate hippocampal synaptic transmission and synapsin I phosphorylation by bath application (Waxham et al., 1992a). The use of inhibitory agents, such as these *in situ*, together with analysis of autophosphorylation sites will shed more light on the nature and role of CaM-PK II autophosphorylation in living cells.

Summary

A unique feature of neuronal calcium/calmodulin-stimulated protein kinase II (CaM-PK II) is its autophosphorylation. A large number of sites are involved and, depending on the in vitro conditions used, three serine (279, 314, 318) and six threonine (near the N terminus, 253, 286, 305, 306, 310) residues have been tentatively identified as autophosphorylation sites in the a subunit; two extra serine (343, 371) and one extra threonine (382) have been proposed for the β subunit. These sites fall into three categories for the α subunit. Primary sites, including Thr-286, are phosphorylated in the presence of calcium and calmodulin, but under limiting conditions of temperature, ATP, Mg^{2+}, or time. Secondary sites, including a threonine near the N terminus, Thr-253, and Ser-279, are phosphorylated in the presence of calcium and calmodulin under nonlimiting conditions. Autonomous sites, including Thr-305 and Ser-314, are phosphorylated in the absence of calcium and calmodulin after initial phosphorylation of Thr-286 under limiting conditions.

Factors that lead to a decrease in CaM-PK II autophosphorylation in vitro include the thermolability of the enzyme, the activity of protein phosphatases, and the presence of inhibitors. The thermolability of CaM-PK II is often overlooked and should be taken into account more often in designing protocols since it occurs in both in vitro and *in situ* studies. A loss of the kinase activity owing to heat could be misinterpreted as a physiological inactivation, such as occurs with the formation of the autonomous CaM-PK II. This is especially relevant when it is recognized that certain forms of CaM-PK II are more susceptible than others. A range of in vitro inhibitors of CaM-PK II autophosphorylation have recently been identified, the most effective of which include sphingosine, KN-62, staurosporine, and peptides that mimic the regulatory region of the kinase, including CaM-PK II 281–309.

Autophosphorylation of CaM-PK II leads to a number of consequences in vitro, including generation of autonomous (calcium-independent) activity, rapid subcellular translocation, and possibly a slower redistribution, as well as to alterations in conformation, calmodulin binding, activity, substrate specificity, and suceptibility to proteolysis. There is, therefore, considerable potential for modulation of the function of CaM-PK II by autophosphorylation.

It is established that CaM-PK II is autophosphorylated in neuronal and neuroendocrine-derived cells under basal conditions, and the sites labeled are generally unknown. However, the level of autophosphorylation was always very low, suggesting that only a small proportion of the total kinase, or only a few sites, were being phosphorylated. Depolarization and/or activation of receptors that lead to an increase in intracellular calcium induces a marked rise in the autophosphorylation of CaM-PK II *in situ*. The incorporation of phosphate is mainly found on Thr-286, but other sites are also phosphorylated at a slower rate. One consequence of the increase in CaM-PK II autophosphorylation *in situ* that has been investigated is an increase in the level of autonomous kinase activity. The extent of this increase depends on the cell type, the level of intracellular calcium achieved, and the activity of endogenous protein phosphatases.

Whether CaM-PK II autophosphorylation *in situ* leads to any other functional consequences is presently unknown, but the information on in vitro consequences and the range of potential inhibitors reviewed here for the first time suggest a number of potential avenues for further studies. It is proposed that the formation of an autonomous enzyme is only one of the consequences of CaM-PK II autophosphorylation *in*

situ and that some of the other consequences observed in vitro will also be seen. CaM-PK II is involved in the phosphorylation of a range of substrates and in the control of neuronal plasticity, including neurotransmitter release and long-term modulation of postreceptor events. In order to understand the function of CaM-PK II, it will be essential to ascertain more fully the mechanisms of its autophosphorylation *in situ*, including especially the sites involved, the consequences of this autophosphorylation for the kinase activity, and the relationships between the state of CaM-PK II autophosphorylation and the physiological events within neurons.

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