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Autophosphorylation reversibly regulates the Ca^{2+}/calmodulin-dependence of Ca^{2+}/calmodulin-dependent protein kinase II

(phosphorylation/dephosphorylation/thermal stability)

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ABSTRACT **Ca^{2+}/calmodulin-dependent protein kinase II contains two subunits, α (M_r 50,000) and β (M_r 60,000/58,000), both of which undergo Ca^{2+}/calmodulin-dependent autophosphorylation. In the present study, we have studied the mechanism of this autophosphorylation reaction and its effect on the activity of the enzyme. Both subunits are autophosphorylated through an intramolecular mechanism. Using synapsin I as substrate, Ca^{2+}/calmodulin-dependent protein kinase II, in its unphosphorylated form, was totally dependent on Ca^{2+} and calmodulin for its activity. Preincubation of the enzyme with Ca^{2+}, calmodulin, and ATP, under conditions where autophosphorylation of both subunits occurred, converted the enzyme to one that was only partially dependent on Ca^{2+} and calmodulin for its activity. No change in the total activity, measured in the presence of Ca^{2+} and calmodulin, was observed. The nonhydrolyzable ATP analog adenosine 5′-[β,γ-imido]triphosphate did not substitute for ATP in the preincubation. Moreover, dephosphorylation of autophosphorylated Ca^{2+}/calmodulin-dependent protein kinase II with protein phosphatase 2A resulted in an enzyme that was again totally dependent on Ca^{2+} and calmodulin for its activity. We propose that autophosphorylation and dephosphorylation reversibly regulate the Ca^{2+} and calmodulin requirement of Ca^{2+}/calmodulin-dependent protein kinase II.**

Numerous effects of hormones and neurotransmitters are believed to be mediated by changes in intracellular Ca^{2+} concentration (1–4). Many of the effects of Ca^{2+} are in turn believed to be mediated by the Ca^{2+}-binding protein calmodulin (CaM, ref. 5). One of the most prominent enzymes activated by the Ca^{2+}/CaM complex is Ca^{2+}/CaM-dependent protein kinase II (Ca/CaM kinase II). Ca/CaM kinase II has been found to phosphorylate a variety of substrate proteins and is believed to be involved in regulation of neurotransmitter release (6) and synthesis (7–9) and microtubule assembly–disassembly (10, 11).

Ca/CaM kinase II has been purified to homogeneity using synapsin I (12–14), myosin light chain (15), glycogen synthase (16–18), microtubule-associated protein 2 (19), and tubulin (20) as substrates. Ca/CaM kinase II, purified from rat forebrain, has a native molecular weight of 630,000 and is composed of α (M_r 50,000) and β (M_r 60,000/58,000) subunits (12–14, 19, 20). While the individual subunits appear to be identical in preparations of Ca/CaM kinase II from different brain regions or different tissues, the ratio of α to β has been found to vary (14, 21–23). Both the α and β subunits of Ca/CaM kinase II undergo rapid autophosphorylation in a Ca^{2+}/CaM-dependent manner (12–14, 16, 18–20). Autophosphorylation of several protein kinases has been found to lead to changes in activity and/or changes in the dependence of the enzymes on their activators. For example, autophosphorylation of phosphorylase kinase causes an increase in enzymatic activity (24); autophosphorylation of the insulin receptor, a tyrosine protein kinase, gives rise to an activated enzyme that is no longer dependent on insulin for its activity (25). Autophosphorylation of Ca/CaM kinase II may therefore be a potentially important regulatory mechanism. In the present study, the effect of autophosphorylation on the Ca/CaM-dependence of Ca/CaM kinase II activity has been investigated.

MATERIALS AND METHODS

Materials. Ca/CaM kinase II was purified as described (14) from rat forebrain. Synapsin I was prepared from bovine brain by modification of the procedure of Ueda and Greengard (26). CaM was prepared from bovine brain by the method of Grand *et al.* (27). Protein phosphatase 2A was purified by the method of Resink *et al.* (28) and was a gift from H. Hemmings of this laboratory.

Adenosine 5′-[β,γ-imido]triphosphate (Ado*PP*[NH]*P*) was purchased from Boehringer Mannheim. All other chemicals used were obtained as described (14).

Measurement of Ca/CaM Kinase II Autophosphorylation. In all experiments, Ca/CaM kinase II (0.2–0.5 mg/ml) was diluted into 5 mM Tris·HCl, pH 7.5/5 mM 2-mercaptoethanol containing bovine serum albumin (1 mg/ml), phenylmethylsulfonyl fluoride (0.1 mM), and leupeptin (10 μg/ml). Aliquots (5–10 μl) of Ca/CaM kinase II (0.023–2.3 μg) were incubated in an assay mixture (50–100 μl) containing 50 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 0.4 mM EGTA, in the absence or presence of 0.9 mM $CaCl_2$ and 1.5–3.0 μg of CaM, unless otherwise indicated. The reaction was initiated by the addition of [γ-^{32}P]ATP (final concentration 3–50 μM, specific activity 0.1–2 $\times$ 10^4 cpm/pmol) and was allowed to continue for various times at 0°C. The reaction was terminated by the addition of 25–50 μl of $NaDodSO_4$-"stop" solution (14), and the mixture was boiled for 2 min and subjected to $NaDodSO_4$/PAGE. Labeled Ca/CaM kinase II subunits were localized by autoradiography and excised from the gel. The radioactivity was quantitated by liquid scintillation spectrometry. The molecular weights of the holoenzyme and the α and β subunits were taken to be 630,000, 50,000, and 60,000, respectively. An α/β subunit ratio of 3:1 was used for calculation of stoichiometry of phosphorylation for the subunits (12, 14).

Measurement of the Effect of Autophosphorylation of Ca/CaM Kinase II on Enzyme Activity. The effect of autophosphorylation on Ca/CaM kinase II activity was measured by a two-step assay. In the first step (preincubation), the autophosphorylation reaction was carried out as described above, except that nonradioactive ATP was used and the autophosphorylation reaction was terminated by the

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Abbreviations: CaM, calmodulin; Ca/CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; Ado*PP*[NH]*P*, adenosine 5′-[β,γ-imido]triphosphate.

addition of 10–20 μl of 90 mM EDTA/5 mM EGTA. In the second step (assay), the activity of Ca/CaM kinase II was measured using synapsin I as substrate. Aliquots (5–10 μl) of the "stopped" autophosphorylation mixture were immediately added to tubes containing the standard reaction mixture (100 μl), composed of 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 0.4 mM EGTA, 5 mM 2-mercaptoethanol, 10 μg of synapsin I, 5 μg of bovine serum albumin, 50–100 μM [γ-^{32}P]ATP (200–1600 cpm/pmol), in the absence (Ca^{2+}/CaM-independent activity) or presence of 0.9 mM $CaCl_2$ and 3 μg of CaM (total activity, the Ca^{2+}/CaM-independent activity plus Ca^{2+}/CaM-dependent activity). The reaction was initiated by the addition of the enzyme. All reaction mixtures were incubated for 15 sec at 30°C to ensure initial rates. Incorporation of ^{32}P into synapsin I was quantitated either by using phosphocellulose paper or by using $NaDodSO_4$/PAGE as described (14). The two assays gave identical results.

Dephosphorylation of Autophosphorylated Ca/CaM Kinase II. Ca/CaM kinase II (360 ng in 100 μl) was preincubated with nonradioactive ATP (for measurement of kinase activity) or radioactive ATP (for quantitation of ^{32}P incorporation into kinase subunits) at 0°C as described above. After termination of the reaction with 20 μl of 90 mM EDTA/5 mM EGTA, aliquots of the stopped phosphorylation reaction were added to tubes containing either H_2O or protein phosphatase 2A (10 units/ml) and were incubated at 0°C for another 5 min. Aliquots (5 μl) of the incubation mixture (containing 12 ng of kinase) were then immediately assayed for enzyme activity as described above.

Protein Determination. Protein concentrations were measured by the method of Peterson (29), with bovine serum albumin as standard.

RESULTS

Mechanism of Autophosphorylation of Ca/CaM Kinase II. In order to examine the mechanism of autophosphorylation of the α and β subunits, different amounts of Ca/CaM kinase II (0.023–2.3 μg) were incubated with Ca^{2+}, CaM, and 10 μM ATP at 0°C. At all concentrations of Ca/CaM kinase II examined, the phosphorylation of α and β subunits was approximately linear up to 10 sec. The time course of phosphorylation of 2.3 μg of Ca/CaM kinase II is shown in Fig. 1*A*. Under conditions where the autophosphorylation of both α and β subunits of Ca/CaM kinase II was at initial rates, the total amount of phosphate incorporated per mol of subunit was constant over a 100-fold range of enzyme concentration (Fig. 1*B*), indicating an intramolecular mechanism of autophosphorylation (30). The plot of the log initial rate versus the log subunit concentration (Van't Hoff plot) gave slopes (the orders of the reaction) of 1.07 and 1.06 for α and β subunits, respectively (data not shown).

Effect of Preincubation of Ca/CaM Kinase II with Ca^{2+}, CaM, and ATP. When Ca/CaM kinase II was assayed without preincubation with Ca^{2+}, CaM, and ATP, enzyme activity and autophosphorylation were totally dependent on Ca^{2+} plus CaM. In order to examine the effects of autophosphorylation, Ca/CaM kinase II was preincubated with Ca^{2+}, CaM, and 3 μM ATP for various times. Since the total activity of Ca/CaM kinase II decreases following preincubation at 30°C in the presence of Ca^{2+}/CaM alone (data not shown), preincubation was carried out at 0°C. A progressive increase in Ca^{2+}/CaM-independent kinase activity (i.e., activity that was not dependent on exogenously added Ca^{2+}/CaM) was observed with increasing time of preincubation (Fig. 2), reaching a plateau after 2.5 min. When 50 μM ATP was present in the preincubation mixture, Ca^{2+}/CaM-independent activity reached the same plateau point after 10 sec of preincubation (data not shown). When

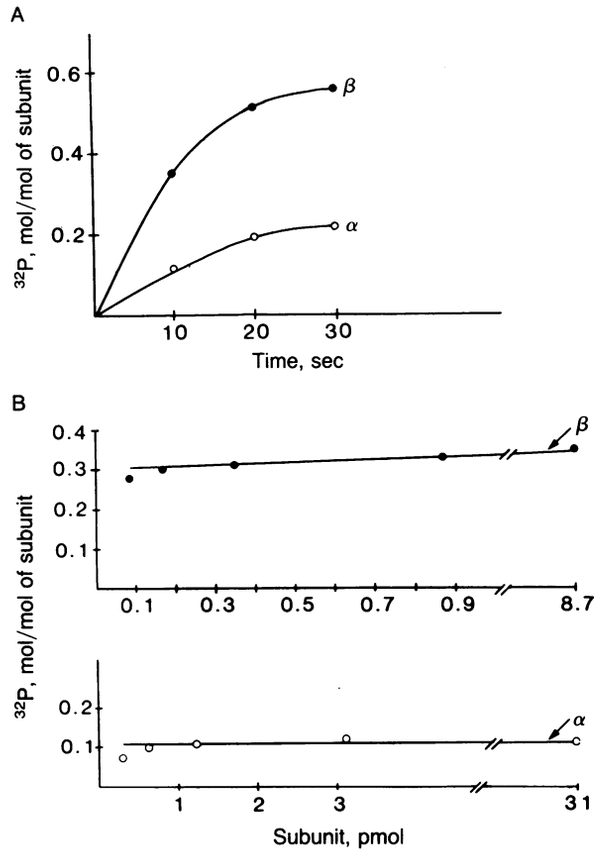

FIG. 1. Effect of time (*A*) and enzyme concentration (*B*) on autophosphorylation of α and β subunits of Ca/CaM kinase II. Ca/CaM kinase II was incubated with 10 μM [γ-^{32}P]ATP at 0°C. The reaction was terminated by the addition of 50 μl of $NaDodSO_4$-"stop" solution, and the proteins were subjected to $NaDodSO_4$/PAGE. The phosphorylated subunits were visualized by autoradiography, the bands were excised, and the radioactivity was quantitated by liquid scintillation counting. For *A*, 2.3 μg of Ca/CaM kinase II was used. For *B*, a 10-sec incubation period was used.

either Ca^{2+}, CaM, or ATP was omitted and the preincubation was prolonged up to 5 min, no Ca^{2+}/CaM-independent activity was observed (Fig. 2). Very little change in the total activity was observed regardless of preincubation conditions or preincubation time. Thus, Ca^{2+}, CaM, and ATP were all required for the generation of Ca^{2+}/CaM-independent enzyme activity, suggesting that autophosphorylation of Ca/CaM kinase II may be involved. Similar results were obtained when smooth muscle myosin light chain was used as substrate for Ca/CaM kinase II (data not shown).

Autophosphorylation should occur with the same time course and with significant stoichiometry, if it is to be responsible for the generation of the Ca^{2+}/CaM-independent kinase activity. To examine this, Ca/CaM kinase II was preincubated at 0°C with Ca^{2+}, CaM, and 3 μM [γ-^{32}P]ATP for various times. Autophosphorylation of the α and β subunits paralleled the time course of the generation of the Ca^{2+}/CaM-independent activity (compare Figs. 2 and 3). In agreement with previous studies (12–14, 31), no phosphorylation of the α or β subunits was observed when the enzyme was preincubated with only Ca^{2+} and ATP or CaM and ATP. At 2.5 min of preincubation, the stoichiometry of phosphorylation was 0.2, 0.7, and 3.7 mol/mol of α and β subunits and holoenzyme, respectively (Fig. 3). When Ca/CaM kinase II was incubated at 30°C, the maximum stoichiometry of phosphorylation was 1.7, 3.0, and 24.3 mol/mol of α and β subunits and holoenzyme, respectively (unpublished obser-

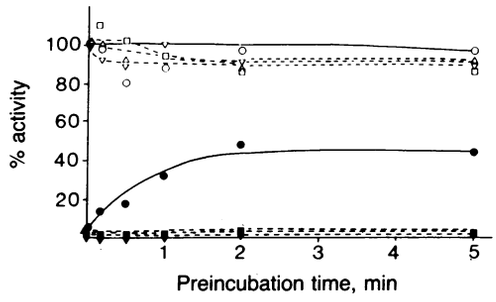

FIG. 2. Time course of generation of Ca^{2+}/CaM-independent kinase activity upon preincubation with Ca^{2+}/CaM and 3 μM ATP. Ca/CaM kinase II (180 ng) was preincubated at 0°C, with Ca^{2+}/CaM/ATP (●, ○), Ca^{2+}/ATP and no CaM (▼, ▽), EGTA/CaM/ATP (▲, △), or Ca^{2+}/CaM and no ATP (■, □). At the indicated preincubation times, aliquots of the reaction mixture were removed for measurement of kinase activity at 30°C in the presence of either EGTA (●, ▼, ▲, ■) or Ca^{2+}/CaM (○, ▽, △, □). Results are expressed as percent of kinase activity in the presence of Ca^{2+}/CaM when there was no preincubation. This activity did not vary significantly with different preincubation conditions. Every data point represents a different preincubation. Total activity was measured in duplicate. Five other experiments gave similar results.

vations). The total number of α and β subunits has been estimated to be 9 and 3, respectively, for the forebrain kinase (12). Thus, only a few of the potential 27 sites (2 on each α subunit and 3 on each β subunit) in the holoenzyme were phosphorylated under the preincubation conditions used in the present studies. In several different experiments, the maximum Ca^{2+}/CaM-independent activity varied between 50 and 80% of the total activity. Despite this variability, the extent of Ca^{2+}/CaM-independence was found to be proportional to the amount of phosphate incorporated per mol of subunits (data not shown).

If the transfer of the γ-phosphate of ATP is necessary for the observed effect on Ca/CaM kinase II activity, then nonhydrolyzable ATP analogs should not be able to substitute for ATP in the preincubation mixture. Preincubation of Ca/CaM kinase II for 30 sec at 0°C with various concentrations of ATP yielded a kinase that was almost totally independent of exogenously added Ca^{2+}/CaM for its activity (Fig. 4). However, Ado*PP*[NH]*P*, a nonhydrolyzable, competitive analog of ATP was ineffective in generating a Ca^{2+}/CaM-independent kinase when substituted for ATP in the preincubation mixture. No change in total activity was

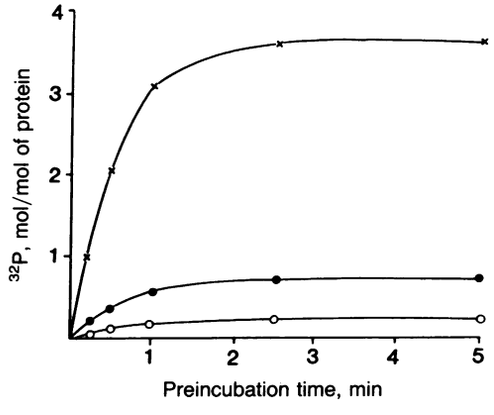

FIG. 3. Time course of autophosphorylation of Ca/CaM kinase II at 0°C. Ca/CaM kinase II (180 ng) was incubated with Ca^{2+}, CaM, and 3 μM [γ-^{32}P]ATP. At the indicated times, aliquots were removed and subjected to $NaDodSO_4$/PAGE as described in the legend to Fig. 1. Stoichiometry of ^{32}P incorporation is shown for holoenzyme (×), α subunit (○), and β subunit (●).

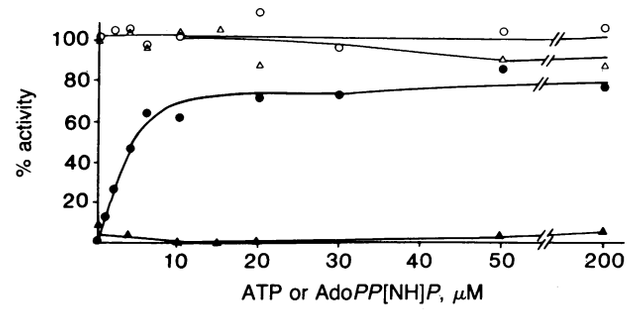

FIG. 4. Dependence of generation of Ca/CaM-independent kinase activity on ATP concentration in the preincubation mixture. Ca/CaM kinase II was preincubated, at 0°C for 30 sec, with various concentrations of ATP (●, ○) or Ado*PP*[NH]*P* (▲, △) and assayed for activity in the presence of EGTA (●, ▲) or Ca^{2+}/CaM (○, △). See legend to Fig. 2 for definition of 100% activity. Ca^{2+}/CaM-independent activity was measured in duplicate. Two other experiments gave similar results. The change in specific activity of [^{32}P]ATP, brought about by the dilution of nonradioactive ATP from the preincubation assay into the synapsin I assay, was corrected in the data presented for ATP. No adjustment was made in the data presented for Ado*PP*[NH]*P*.

observed when up to 200 μM ATP was used in the preincubation mixture (Fig. 4). The small decrease observed at higher concentrations of Ado*PP*[NH]*P* was probably due to the carryover of the analog into the Ca/CaM kinase II assay and its competition with ATP in the assay (data not shown).

Effect of Dephosphorylation of Autophosphorylated Ca/CaM Kinase II on Enzyme Activity. To test whether the effect of autophosphorylation on enzyme activity was reversible, Ca/CaM kinase II was first autophosphorylated to generate a Ca^{2+}/CaM-independent enzyme and then incubated in the presence or absence of protein phosphatase 2A at 0°C for 5 min. Under such conditions, protein phosphatase 2A removed 90% of the ^{32}P incorporated into both α and β subunits (Fig. 5). Measurement of the activity of the dephosphorylated kinase showed that enzyme activity was

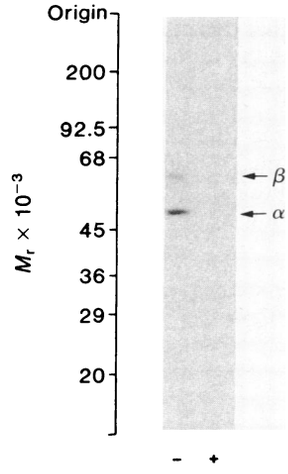

FIG. 5. Dephosphorylation of autophosphorylated Ca/CaM kinase II by protein phosphatase 2A. Ca/CaM kinase II (360 ng in 100 μl) was phosphorylated with Ca^{2+}, CaM, and 50 μM [γ-^{32}P]ATP for 30 sec. The stoichiometry of phosphorylation was 3, 0.2, and 0.5 mol/mol for holoenzme and α and β subunits, respectively. After the reaction was stopped with 20 μl of 90 mM EDTA/5 mM EGTA, 25-μl aliquots of the phosphorylated kinase were removed and incubated in the absence (−) or presence (+) of protein phosphatase 2A (10 units/ml). After 5 min of incubation at 0°C, the reaction was terminated with $NaDodSO_4$-"stop" solution and autophosphorylation of the kinase subunits was analyzed as described in the legend to Fig. 1. Arrows indicate the α and β subunits of Ca/CaM kinase II.

Table 1. Reversal of Ca^{2+}/CaM-independence by dephosphorylation of Ca/CaM kinase II

Ca/CaM kinase II	Phosphatase 2A	Ca/CaM kinase II activity, μmol per min per mg	
		+ EGTA	+ Ca^{2+} and CaM
Unphosphorylated	−	0.05	5.14
	+	0.05	5.38
Autophosphorylated	−	4.04	5.13
	+	0.07	5.55

Ca/CaM kinase II was preincubated with Ca^{2+} plus CaM, in the absence (unphosphorylated) or presence (autophosphorylated) of 50 μM nonradioactive ATP for 30 sec at 0°C, and then incubated in the absence (−) or presence (+) of protein phosphatase 2A as described in the legend to Fig. 5. After 5 min at 0°C, 5-μl aliquots (containing 12 ng of kinase) were removed and assayed for enzyme activity in the absence (+ EGTA) or presence of Ca^{2+}/CaM, using synapsin I as substrate. Ca^{2+}/CaM-independent activity (+ EGTA) was measured in duplicate. Three different experiments gave similar results.

again totally dependent on Ca^{2+}/CaM (Table 1). Moreover, the recovery of Ca^{2+}/CaM-dependence paralleled the extent of dephosphorylation of Ca/CaM kinase II by protein phosphatase 2A (data not shown). No change in ^{32}P incorporation into the subunits was observed in the absence of protein phosphatase 2A, and the phosphorylated kinase remained Ca^{2+}/CaM-independent. There was also very little change in enzyme activity when unphosphorylated kinase was incubated at 0°C for 5 min with protein phosphatase 2A (Table 1). When protein phosphatase 2A was added directly to the kinase assay, no change in the Ca^{2+}/CaM-independent or total activity was detected.

Thermal Instability of Autophosphorylated Ca/CaM Kinase II. In the present study, no change in total activity of Ca/CaM kinase II was observed when the enzyme was preincubated with Ca^{2+}, CaM, and ATP at 0°C. However, in a number of other studies, autophosphorylation has been reported to inhibit total Ca/CaM kinase II activity (32–34). In most of these studies, preincubation was carried out at 30°C. It seemed possible that this discrepancy was due to a decrease in the thermal stability of the autophosphorylated enzyme at 30°C. To investigate this possibility, Ca/CaM kinase II was preincubated at 0°C with Ca^{2+}, CaM, and 3 μM ATP for 0, 10, 30, and 120 sec (the Ca^{2+}/CaM-independent activities were 0, 10, 14, and 33% of the total activity, respectively, under these conditions). After termination of the autophosphorylation reaction by the addition of EDTA/EGTA, the reaction mixture was incubated at 30°C for various times. No change in ^{32}P incorporation into the subunits was observed during incubation at 30°C, suggesting that no further phosphorylation/dephosphorylation occurred after the phosphorylation was terminated by addition of EDTA/EGTA (data not shown). However, loss of total activity that paralleled the degree of autophosphorylation was observed (Fig. 6). The unphosphorylated enzyme lost 10% of its total activity after 150 sec of incubation at 30°C. In contrast, autophosphorylated Ca/CaM kinase II lost up to 70% of its total activity after 150 sec of incubation at 30°C. Under the conditions used in the experiment shown in Fig. 5, this loss of activity of autophosphorylated enzyme could not be reversed by dephosphorylation with protein phosphatase 2A (data not shown).

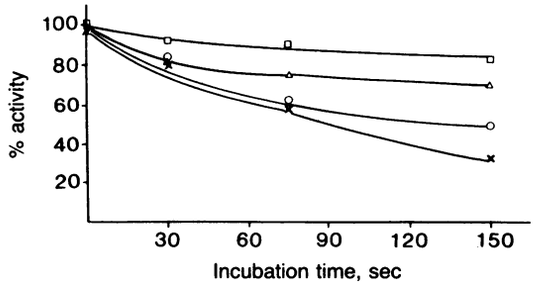

FIG. 6. Comparison of the thermal stability of nonphosphorylated and phosphorylated Ca/CaM kinase II. Ca/CaM kinase II (540 ng in 150 μl) was preincubated at 0°C with Ca^{2+}, CaM, and 3 μM ATP for 0 (□), 10 (△), 30 (○), or 120 sec (×). After addition of 30 μl of 90 mM EDTA/5 mM EGTA, the reaction mixtures were transferred to 30°C. At the indicated incubation time, aliquots were removed and assayed for activity in the presence of Ca^{2+} and CaM; 100% activity is defined as in Fig. 2. Total activity was measured in duplicate. Two other experiments gave similar results.

DISCUSSION

In the present study, we examined the effect of autophosphorylation of Ca/CaM kinase II on enzyme activity. Preincubation of Ca/CaM kinase II with Ca^{2+}, CaM, and ATP generated an enzyme that was partially independent of added Ca^{2+} and CaM for its activity. The maximum Ca^{2+}/CaM-independent activity obtained was 50–80% of the total activity. A nonhydrolyzable ATP analog could not substitute for ATP in the preincubation mixture, suggesting that the transfer of γ-phosphate had to occur for the effect to be observed. Thus, autophosphorylation is most likely to be responsible for the generation of Ca^{2+}/CaM-independent kinase activity. This is further supported by the observation that dephosphorylation of autophosphorylated Ca/CaM kinase II regenerated an enzyme that was again totally dependent on exogenously added Ca^{2+} and CaM for activity.

We studied the mechanisms and stoichiometry of autophosphorylation. The initial phosphorylation sites on the α and β subunits were phosphorylated through an intramolecular mechanism. Ca^{2+}/CaM-independent activity was observed after incorporation of only 2–4 mol of phosphate per mol of holoenzyme, as measured at the end of the autophosphorylation assay. Autophosphorylation of Ca/CaM kinase II continues during the synapsin I assay and contributes less than 2 mol of phosphate per mol of holoenzyme (data not shown). Thus phosphorylation of only a few of the 27 available sites would appear to be sufficient for Ca^{2+}/CaM-independent activity to occur. An intramolecular mechanism for the autophosphorylation reaction was also proposed by Kuret and Schulman (33); however, in their study, autophosphorylation of Ca/CaM kinase II was not performed under initial-rate conditions. A preliminary report (34) also suggested an intramolecular mechanism for the autophosphorylation reaction.

There have been several conflicting reports concerning the effect of autophosphorylation on the activity of Ca/CaM kinase II. Autophosphorylation of Ca/CaM kinase II has been reported to activate (35), inhibit (32–34), or have no effect (36) on kinase activity and to increase (35), decrease (37), or have no effect (32) on the affinity of Ca/CaM kinase II for CaM. Recently, Saitoh and Schwartz (36) reported that phosphorylation changed the subcellular distribution of an enzyme in an *Aplysia* neuronal membrane/cytoskeletal complex that appeared to be an isozyme of Ca/CaM kinase II. In addition, the phosphorylated enzyme became independent of added Ca^{2+}/CaM for its activity (36). A preliminary report (34) also suggested that although autophosphorylation reduced enzyme activity to 20–30% of controls, most of this activity no longer depended on Ca^{2+}/CaM. In the present study, autophosphorylation of Ca/CaM kinase II was found to alter the Ca^{2+}/CaM requirement of the enzyme with no change in the total activity. One of the main differences between this and previous studies was that in the present

study the autophosphorylation reaction (preincubation) was at 0°C. Since autophosphorylated Ca/CaM kinase II was found to have a decreased thermal stability compared to that of the unphosphorylated kinase, an apparent loss of activity would be observed if autophosphorylation were carried out at 30°C. In any case, under certain conditions (preincubation at 0°C), autophosphorylation did not lead to a loss of activity. It will be important to determine whether the autophosphorylated enzyme becomes independent of its activators (Ca^{2+} and CaM) *in vivo*. It seems likely that after an initial short rise in intracellular Ca^{2+} concentration, the enzyme becomes autophosphorylated and remains activated even after the Ca^{2+} concentration returns to basal levels.

Both α and β subunits, in their phosphorylated form, have been found to bind CaM in a Ca^{2+}-dependent manner by use of an ^{125}I-labeled CaM gel-overlay technique (14, 35). Thus, although autophosphorylated Ca/CaM kinase II may not require Ca^{2+}/CaM for its activity, it may still bind CaM in a Ca^{2+}-dependent manner. It is not known whether either one or both subunits are responsible for the loss of Ca^{2+}/CaM-dependence. Both subunits were phosphorylated with the same time course, both subunits have been shown to be CaM-binding proteins (12–14, 20), and both subunits covalently bind 8-azidoadenosine 5′-triphosphate (13, 35). Several different isozymes of Ca/CaM kinase II exist that contain different ratios of α and β subunits. By using isozymes that contain only α or only β subunit, it may be possible to determine which subunit is responsible.

Note Added in Proof. After this manuscript was submitted, a more detailed report of ref. 34 was published (38). The results presented in that report were in general agreement with the results of the present study. That report suggested, however, that the loss of enzyme activity observed when autophosphorylation was carried out at 30°C could be partially reversed by dephosphorylation of Ca/CaM kinase II by a cerebellar phosphatase preparation. The apparent reversal of the loss of enzyme activity was probably a consequence of the thermal instability of the autophosphorylated kinase, caused by the conditions used for dephosphorylation (5 min at 30°C). Incubation of autophosphorylated kinase at 30°C would result in a large decrease in enzyme activity. If dephosphorylation of the autophosphorylated kinase occurred in less than 5 min, the loss of activity caused by thermal instability would be less than in the control, since the length of time the kinase was in its autophosphorylated form would be less than in the control. We have obtained results suggesting that this is indeed the case. Thus, autophosphorylation primarily regulates the Ca^{2+}/CaM-dependence of Ca/CaM kinase II. The decrease in activity of the autophosphorylated kinase is the consequence of its thermolability and presumably is not physiologically relevant.

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