Autophosphorylation of calmodulin kinase II: functional aspects

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Autophosphorylation of purified calmodulin kinase II dramatically inhibited protein kinase activity and enhanced substrate selectivity. Inhibition was observed over a wide range of calmodulin concentrations but calmodulin binding was unaffected. Autophosphorylation of calmodulin kinase II may be a mechanism for limiting phosphorylation to physiological substrates and terminating some of calcium's actions in synaptic events.

Autophosphorylation *Calmodulin kinase II* *Synapsin I* *Ca^{2+}* *Protein kinase*

1. INTRODUCTION

Calmodulin kinase II (Calm-k II) is a calcium- and calmodulin-dependent protein kinase which is highly concentrated in brain [1,2]. Calm-k II may mediate some of calcium's actions in neurons, including transmitter synthesis and release, and is ideally located to mediate some of calcium's postsynaptic actions as well [2–6]. Calm-k II is composed of subunits of apparent molecular mass 50 and 60 kDa which autophosphorylate [7,8]. We report that autophosphorylation dramatically inhibits kinase activity and that this inhibition may enhance substrate selectivity.

2. MATERIALS AND METHODS

Calm-k II was isolated from Sprague-Dawley rat forebrains as described by Goldenring et al. [7] with minor modifications. The purified enzyme had properties similar to those reported [7,8]. On SDS-PAGE its subunits had apparent molecular masses of 50 and 60 kDa and were in a ratio of approximately 3:1. The K_m for ATP (1.8 μM), EC_{50} for calcium (1.0 μM), IC_{50} for diazepam (25.0 μM) and substrate specificity were also very similar to those reported for Calm-k II.

Protein phosphorylation was carried out in vitro using [γ-^{32}P]ATP. In a volume of 75 μl, final concentrations were: 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.5 mM EGTA, 0.6 mM $CaCl_2$ (final calcium concentration was approx. 0.006 mM) and 3 units calmodulin unless specified otherwise (1 calmodulin unit stimulates 0.016 activated units of phosphodiesterase). To determine Calm-k II activity at different states of autophosphorylation, purified enzyme (2.0 μg) was first preincubated for varied times in the presence of 5 μM cold ATP. After this pretreatment, exogenous substrate and [^{32}P]ATP were added, final ATP concentration was 80 μM and specific activity was 0.3–1.0 μCi/nmol. The reaction was stopped after 1 min by the addition of SDS and mercaptoethanol (each at 4% final concentrations).

The effect of calmodulin concentration on Calm-k II activity was determined using phosphorylated or unphosphorylated enzyme and synapsin I as substrate. Calm-k II (0.4 μg) was preincubated in the presence of 13.5 nM calmodulin and 5 μM ATP for 10 min. Synapsin I

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(3.0 μg) (a generous gift from Dr M.B. Kennedy) was then added with 80 μM ATP (specific activity 0.3–1.0 μCi/nmol) and calmodulin (CalBiochem-Behring, final concentrations ranging from 13.5 to 1000.0 nM). The reactions were stopped after 30 s as described above. These assays were also performed in the presence of 5 μM ADP or 5 μM AMP.

The time course of phosphorylation was carried out using 0.5 μg purified enzyme, 10 μM ATP and excess protein substrate. Phosphoproteins were separated by electrophoresis on 10% polyacrylamide gels [9]. Quantitation of incorporated ^{32}P was done by excising from the gels the appropriate bands which were then subjected to liquid scintillation counting.

^{125}I-calmodulin binding to Calm-k II (6.0 μg) was accomplished in SDS-PAGE gels by using the gel overlay technique [10] and on nitrocellulose paper [11]. Calmodulin binding to Calm-k II on nitrocellulose paper was linear between 1.0 and 15.0 μg purified enzyme.

3. RESULTS

Autophosphorylation of Calm-k II resulted in a dramatic inhibition of enzymatic activity for all substrates tested (fig.1). There was a strong correlation between preincubation time (i.e. degree of autophosphorylation) and loss of kinase activity. The degree of autophosphorylation was estimated by subtracting the amount of ^{32}P incorporated into 50 and 60 kDa subunits after preincubation from maximal incorporation without preincubation. The correlation coefficient for the degree of autophosphorylation vs radioactivity incorporated into substrate protein was found to be −0.874 for glycogen synthase and −0.982 for Lys-histone. Preincubation for 5 min resulted in a decrease of ^{32}P incorporation of approx. 12.6-fold for glycogen synthase, 7.1-fold for Lys-histone and 3.4-fold for synapsin I. Inhibition of Calm-k II by autophosphorylation was observed over a wide range of calmodulin concentrations above 13.5 nM (fig.2).

Calmodulin binding was studied to determine if autophosphorylation influences the binding of calmodulin and therefore possibly enzymatic activity. Calmodulin binding was observed predominantly in the 50 and 60 kDa regions, thus in agreement with previous investigations [5]. There was no difference between calmodulin binding to the prephosphorylated Calm-k II compared to binding to the untreated enzyme.

The accumulation of ADP and/or AMP during pretreatment cannot account for Calm-k II inhibition. Neither the addition of 5 μM ADP nor AMP

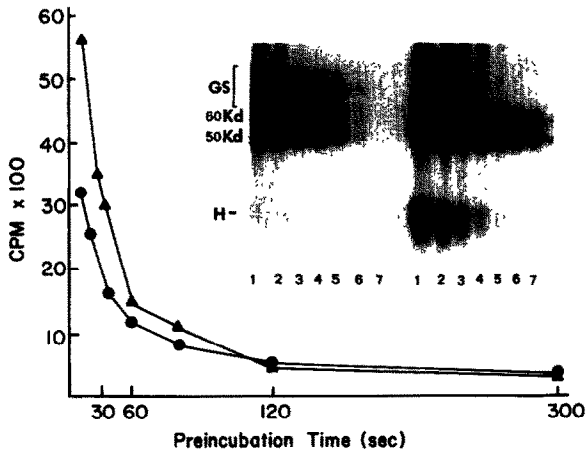

Fig.1. Decrease of Calm-k II activity with autophosphorylation. Calm-k II was incubated for various times in 5 μM ATP before either 5 μg glycogen synthase (●) or 10 μg lysine-rich histone (▲) was added together with 80 μM [^{32}P]ATP. The graph represents radioactivity incorporated into substrates as a function of preincubation time for a typical experiment. Inset: autoradiogram of the SDS-PAGE gel from this experiment.

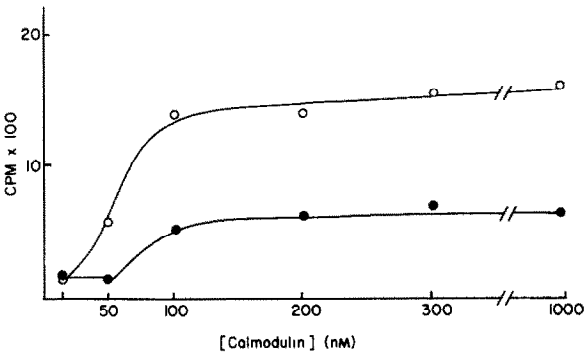

Fig.2. Decrease of Calm-k II activity after pretreatment (●) and in untreated enzyme (○) in the presence of calmodulin concentrations ranging from 13.5 to 1000 nM. The graph illustrates ^{32}P incorporation into synapsin I as a function of calmodulin concentration.

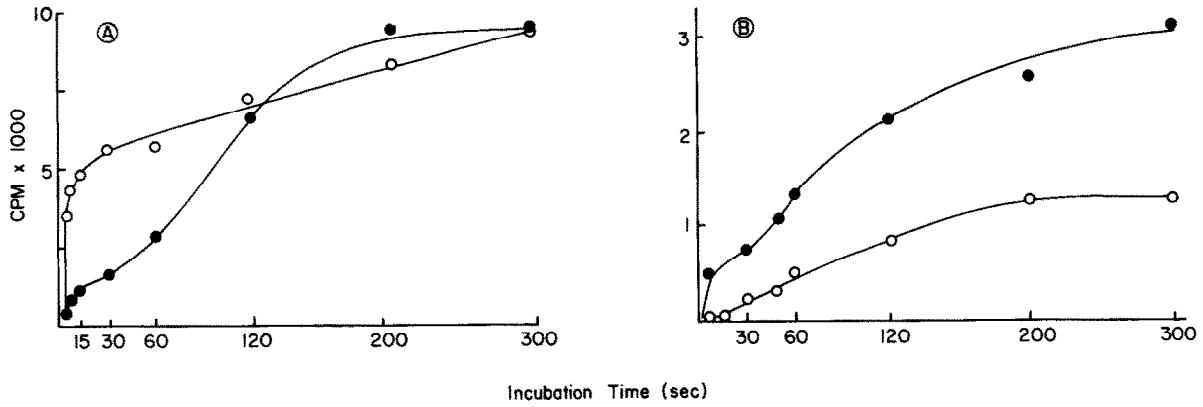

Fig.3. Time course of phosphorylation. Calm-k II (0.5 μg) was incubated in the presence of either glycogen synthase (5.0 μg) or Lys-histone (10.0 μg) for the times designated. The graphs illustrate ^{32}P incorporation into Calm-k II (●) and either glycogen synthase (A) or Lys-histone (B) (○).

to the phosphorylation reaction affected kinase activity.

If autophosphorylation inhibits protein kinase activity, then substrate specificity might be determined by the time sequence of phosphorylation. For example, if a protein is rapidly phosphorylated prior to Calm-k II autophosphorylation, it will incorporate large amounts of ^{32}P before the enzyme turns itself off. This is the case for glycogen synthase (fig.3A) and synapsin I (not shown). The majority of phosphate is incorporated into these substrates within the first 15 s of incubation prior to autophosphorylation of the enzyme, when kinase activity tails off. Lysine-rich histone is a less favorable substrate for Calm-k II and significant kinase autophosphorylation occurs before Lys-histone is substantially phosphorylated (fig.3B). After Calm-k II is highly phosphorylated, kinase activity is low for all substrates.

4. DISCUSSION

Here we have investigated the effect that autophosphorylation has on the activity and substrate selection of purified Calm-k II. The degree of enzyme inhibition was found to be closely correlated to the degree of autophosphorylation over a wide range of calmodulin concentrations. These findings differ from those of Shields et al. [12]. Working with synaptic junctions, they reported that autophosphorylation of Calm-k II stimulates kinase activity at low calmodulin concentrations and calmodulin binding. The source of these discrepancies may be related to differences between synaptic junction and purified enzyme preparations.

Calmodulin binding to purified Calm-k II in polyacrylamide gels and on nitrocellulose paper appeared to be unaffected by autophosphorylation. Other investigators have reported an increase in calmodulin binding with autophosphorylation using the gel overlay technique [12,13] and a decrease in affinity (at low calcium concentrations) and number of binding sites using a native cytoskeletal preparation [13]. Phosphorylation of Calm-k II decreases its mobility and can cause some smearing in SDS-polyacrylamide gels. Increases in calmodulin binding using the gel overlay method may be due to smearing of protein in the gels which might increase accessibility for binding. Unaltered calmodulin binding in our purified preparation using high calcium concentrations suggests that autophosphorylation does not alter binding, but also could reflect an offsetting combination of this smearing effect (although little smearing was observed) and a true decrease in binding site number.

Calm-k II phosphorylates many proteins in vitro but few are likely to be physiological substrates. Inhibition of Calm-k II by autophosphorylation has recently been reported using casein [14,15], a poor substrate for this kinase [1,7,8]. We have found that kinase activity is inhibited by autophosphorylation for preferred substrates as

well, such as synapsin I. This mechanism limits phosphorylation to rapidly phosphorylated substrates; when incubated with several substrates, Calm-k II phosphorylated glycogen synthase, then itself, and in the process dramatically reduced its activity in regard to histone phosphorylation. This mechanism should enhance substrate specificity in vivo and limit phosphorylation to selected proteins.

Calm-k II appears to play an important role in transmitter release [2,4] and is also ideally located to mediate some of calcium's postsynaptic actions [2,6]. Stimulus induced elevations in calcium concentrations in the presynaptic terminal would be expected to increase the state of phosphorylation of synapsin I and other preferred substrates, before leading to extensive autophosphorylation of Calm-k II which would then reduce its activity. Autophosphorylation may therefore be a rapid means of terminating some of calcium's actions. The active dephosphorylated form of Calm-k II would presumably be regenerated by a phosphatase. Recently a synaptosomal extract has been reported to contain a phosphatase which is capable of dephosphorylating synaptic junction proteins including Calm-k II [16]. It remains to be seen if Calm-k II can be reactivated by this phosphatase. Since increased phosphorylation of synapsin I may lead to an increase in transmitter release, these changes in kinase activity might alter the synaptic properties of the cell. In view of this, it is interesting to note that a decrease in Calm-k II activity has been reported in two pathological conditions associated with increased calcium influx; the kindling model of epilepsy and cerebral ischemia [9,17–19]. These speculations illustrate the potential importance of changes in Calm-k II activity associated with autophosphorylation for brain function and pathology.

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