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CALCIUM ENHANCED INACTIVATION OF CALMODULIN DEPENDENT PROTEIN KINASE FROM SYNAPTOSOMES

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SUMMARY: Calcium-calmodulin dependent protein kinase from synaptosomal cytosol rapidly loses activity upon storage at 4°C. In the presence of calcium, the loss of activity is greatly enhanced with only trace levels remaining after two hours. Calcium-calmodulin dependent protein kinase, purified by affinity chromatography on calmodulin-Sepharose, is also quite labile and the loss of enzyme activity in the partially purified preparation is similarly accelerated in the presence of calcium. Removal of calcium improves stability somewhat, whereas calmodulin itself apparently has no protective effect on the enzyme.

INTRODUCTION

It is becoming apparent that various protein kinase enzymes share a general property of pronounced instability. For example, Ca^{2+}, CaM-dependent tubulin kinase activity is decreased by a process which apparently involves proteolytic degradation (1), and the calcium activation of cerebral cortical kinase is decreased with preincubation at 37°C by a process which is stimulated by calcium (2,3). There also appears to be specific, limited proteolysis of a cAMP-dependent protein kinase from rat brush border cells which may be involved in *in vivo* regulation of enzyme activity (4). Although our initial attempts at purification of a Ca^{2+}, CaM-dependent kinase from the cytosol of brain synaptosomes were promising (5), we soon encountered extreme variability in recovering enzyme activity from CaM-Sepharose affinity columns, which prompted us to study the stability of CaM-dependent protein kinase in more detail.

MATERIALS AND METHODS

Acrylamide, N,N'-methylene-bis-acrylamide, glycine, ammonium persulfate, N,N,N',N'-tetramethylethylenediamine and Coomassie brilliant blue R250 were all of electrophoresis grade quality and obtained from Bio-Rad Laboratories. Ultrapure sodium dodecyl sulfate (SDS) was from BDH Chemicals Ltd. Sepharose 4B and cyanogen bromide-activated Sepharose 4B were obtained from Pharmacia, and [γ -

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^{32}P]ATP (10-40 Ci/mmol) from New England Nuclear. XRP film was obtained from Kodak, and Cronex Lighting-Plus intensifying screens from DuPont.

Whole brains (less cerebellum) were rapidly dissected from male Sprague-Dawley rats and homogenized (within 20 seconds after decapitation) in 7 volumes of 0.32 M sucrose, 50 mM Tris-HCl, pH 7.4. Synaptosomal cytosol was prepared as follows: a crude mitochondrial pellet (P_2) was prepared by differential centrifugation (6), and after three washes, was subjected to hypotonic shock in 5 mM Tris-HCl, pH 8. The resulting lysate was centrifuged at 100,000 x g for 60 minutes and the supernatant was used as the source of protein kinase and its substrates. All procedures were carried out at 4°C.

Protein kinase was assayed by measuring ^{32}P (from ^{32}P[ATP]) incorporation into endogenous acid precipitable proteins. Each assay tube contained: Hepes, 50 mM, pH 7.0; ATP 5 µM (containing aproximately 1 x 10^6 dpm of [γ -^{32}P]ATP); $MgCl_2$, 10 mM; and EGTA, 400 µM in a final volume of 300 µl. Enzyme activity in the absence of calcium is referred to hereafter as basal activity. Activation of the enzyme by calcium and CaM was measured in the presence of 100 µM calcium and 1 µg of CaM. The reaction was initiated by addition of 100 µg of the tissue extracts (containing both enzyme and substrates) and incubation was carried out for 2 minutes at 30°C. The reaction was terminated by addition of 1 ml of ice-cold 20% trichloroacetic acid (TCA). Protein was separated from the reaction mixture by filtration over Millipore filters (0.45 µm) and each filter was rinsed twice with 2 ml of ice-cold 10% TCA. The filters were then dissolved in 15 ml of Filtron-X and the amount of ^{32}P incorporation was quantitated by liquid scintillation spectrometry. Incubations were carried out in a similar manner for SDS polyacrylamide gel electrophoresis (SDS-PAGE) samples, except that the final incubation volume was 200 µl. These reactions were terminated with 100 µl of an SDS "stop" solution containing 9% (w/v) SDS, 30 mM Tris-HCl, pH 8.0; 6% (v/v) 2-mercaptoethanol and 27% (w/v) sucrose (7). SDS-PAGE, autoradiography, and protein staining of gels was carried out as previously described (8). Protein was measured by the method of Bradford (9).

Calmodulin was prepared essentially as described by Charbonneau and Cormier (10) with minor modifications (7). CaM-Sepharose was prepared as described by Klee and Krinks (11).

RESULTS

The chromatographic patterns of freshly prepared synaptosomal lysate on CaM-Sepharose are presented in Figure 1. Most protein passes through in the effluent but a small amount of protein, containing the major substrates for the kinase and most of the protein kinase activity, bound to CaM-Sepharose and was eluted with EGTA. The protein kinase in the eluate was activated approximately 20 fold when assayed in the presence of Ca^{2+} and CaM (Figure 1A). Upon storage of the lysate at 4°C, prior to chromatography, decreasing amounts of basal and CaM-activated protein kinase activity were subsequently eluted from CaM-Sepharose. After 24 hours of storage (Figure 1B), the amount of enzyme activity eluted from the gel was approximately 50% of that which could be eluted at the zero time point and after 48 hours (Figure 1C), only negligible kinase activity

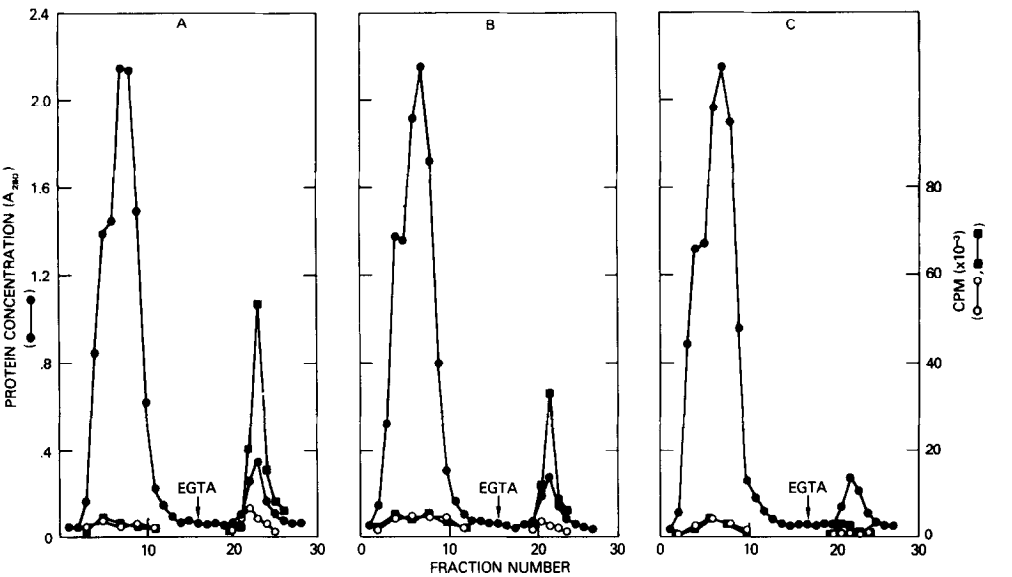

Figure 1 Elution pattern of cytosol proteins from CaM-Sepharose and Ca^{2+}-CaM-dependent protein kinase activity. $CaCl_2$ was added to extracts to a concentration of 1.0 mM which were immediately loaded onto a CaM-Sepharose column (1 x 8 cm) equlibrated with 20 mM Hepes, pH 7.0, 1.0 mM 2-mercaptoethanol, 0.02% NaN_3, 1.0 mM $CaCl_2$ and 300 mM NaCl. The column was washed with equilibrating buffer until the effluent was devoid of protein. The column was eluted with equilibration buffer containing 1.0 mM EGTA in place of calcium. Protein kinase activity was assayed as described in Methods. Arrow denotes change to buffer containing EGTA. Absorbance at 280 nm (●), ^{32}P incorporation in the absence (○) and presence (■) of calcium and calmodulin. A: cytosol chromatographed on the day of preparation. B: cytosol chromatographed after 24 hours at 4°C. C: cytosol chromatographed after 48 hours at 4°C.

(basal or CaM-activated) was eluted. The amount of protein bound to CaM-Sepharose after prolonged storage deceased slightly after 24 hours as compared to fresh preparations. At no time was protein kinase activity recovered in the effluent of the CaM-Sepharose column under the present chromatographic conditions. In some experiments synaptosomes were lysed into buffer containing 1 mM Ca^{2+} and then subsequently chromatographed on CaM-Sepharose as described above for the zero time point preparation. Under these conditions (approximately 2 hours of exposure to Ca^{2+} during lysis and centrifugation), only a trace amount of basal or CaM-activated protein kinase activity was eluted by the column (data not shown). Thus, the rapid loss of enzyme activity upon storage is accelerated further by Ca^{2+}.

The stability of the protein kinase which had been partially purified on CaM-Sepharose was also investigated. Figure 2 shows the effects of storage of

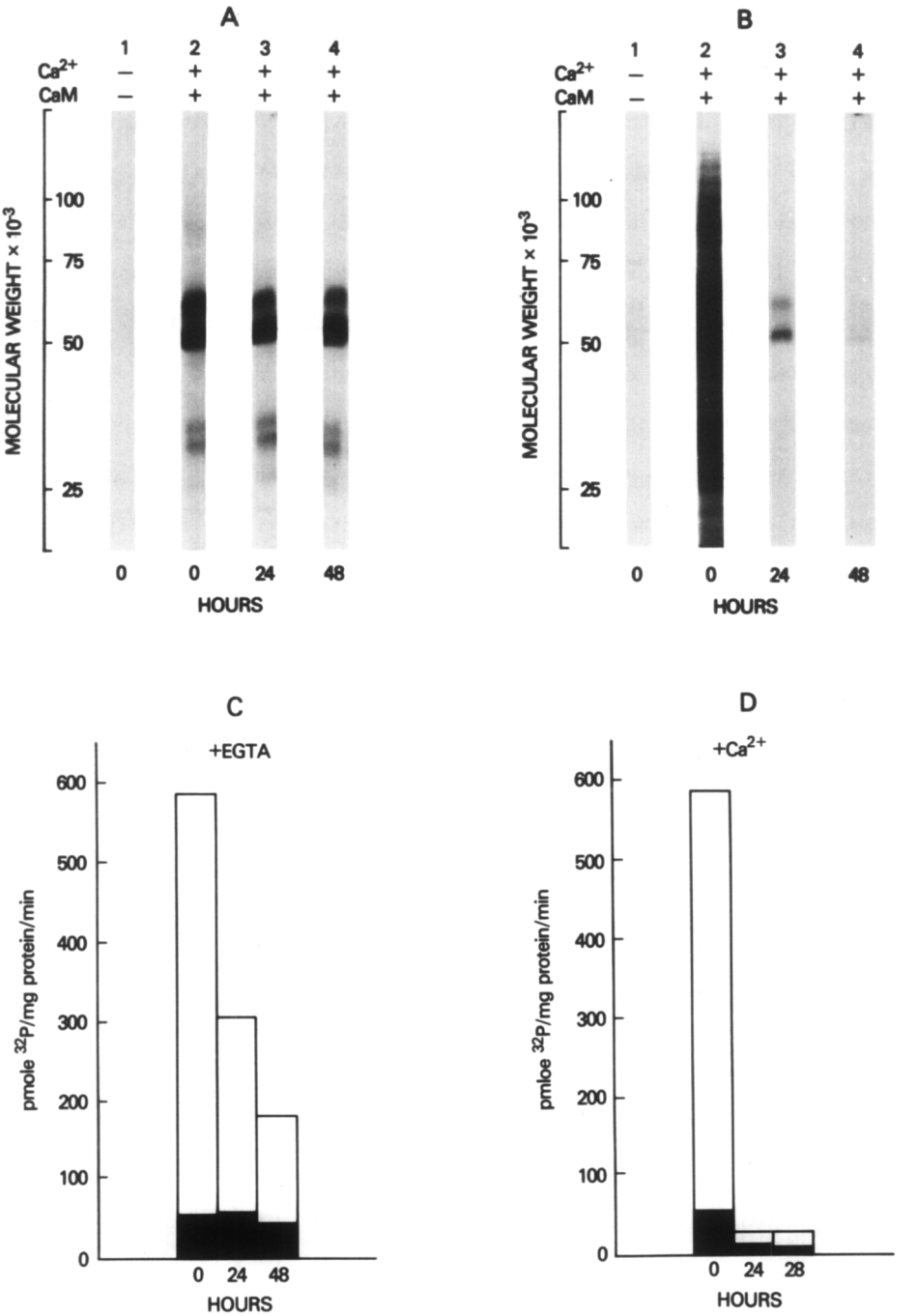

Figure 2 Incorporation of ^{32}P into endogenous substrates in a CaM-Sepharose eluate containing either 1 mM EGTA or 1 mM $CaCl_2$. Ca^{2+}, CaM-dependent protein phosphorylation was examined by SDS-PAGE and autoradiography after 0 (lanes 2), 24 (lanes 3), or 48 hours (lanes 4) at 4°C. Lanes 1 show protein phosphorylation of the zero point eluate in the absence of calcium and calmodulin. A: eluate stored at 4°C in the absence of calcium (1 mM EGTA). B: eluate stored at 4°C in the presence of 1 mM $CaCl_2$. C and D: protein kinase activity measured by acid precipitation assay; eluates stored under same conditions as A and B respectively. Protein kinase activity measured in the absence (■) and presence (□) of calcium and calmodulin.

the zero time point CaM-Sepharose eluate (corresponding to enzyme preparation in Figure 1A) in the presence or absence of Ca^{2+} at 4°C, on protein kinase activity. Figure 2A shows autoradiographs of a CaM-Sepharose eluate stored in the absence of Ca^{2+} (1 mM EGTA). CaM-activation of the protein kinase decreases slightly after 24 and 48 hours but the pattern of protein phosphorylation is unaffected. The ^{32}P incorporation into a similar preparation, measured by precipitation, is shown in Figure 2C. Basal protein kinase activity remains constant for 48 hours after elution, whereas CaM-activation of the protein kinase is reduced to approximately 50% of its initial activity after 24 hours and to 30% after 48 hours of storage. If the CaM-Sepharose eluates are immediately adjusted to 1 mM Ca^{2+}, the partially purified protein kinase rapidly loses activity, much like the crude preparation lysed in the presence of Ca^{2+} (see above). Autoradiographs of CaM-Sepharose eluates containing Ca^{2+} are shown in Figure 2B. Storage of the eluate for 24 hours drastically reduces the amount of protein phosphorylation and protein kinase activity is non-existent at 48 hours. The preparation shown in Figure 2B is the same as that in Figure 2A, except that the film has been exposed for a longer period of time due to the low level of phosphorylation at the 24 and 48 hour time points. Again, ^{32}P incorporation was measured by precipitation as well (Figure 2D) and it can be seen that after only 24 hours of storage at 4°C the amount of CaM-activated protein kinase activity was reduced to less than 10% of the fresh preparations. The addition of CaM (10 µg/mg protein) to the eluate fraction did not enhance the stability of the enzyme in either the presence or absence of Ca^{2+} (data not shown). The results indicate that the partially purified protein kinase is also quite unstable and that the destabilization is enhanced by Ca^{2+}.

DISCUSSION

The Ca^{2+}, CaM-dependent protein kinase found in the soluble compartment of central nervous system synaptosomes appears to be highly unstable. When the cytosol is stored at 4°C for 24 to 48 hours and then chromatographed on CaM-Sepharose, the amount of protein kinase activity in the eluate is markedly decreased relative to enzyme chromatographed immediately after tissue preparation.

This is not entirely due to a decrease in the amount of protein bound to the CaM-Sepharose (the protein binding is decreased after 24 hours of storage), but also to a decrease in protein kinase activity. After 48 hours of storage, protein binding is the same as at 24 hours, whereas protein kinase activity (both basal and CaM-activated) is negligible. Since we measured only the phosphorylation of endogenous substrates, we cannot rule out the possibility that the substrates, and not the enzyme, are undergoing degradation during storage. However, the observation that an extract which had been stored for 48 hours (devoid of activity on endogenous substrates) did not phosphorylate histone IIa, whereas fresh preparations did, argues against this interpretation. It appears that the loss of enzyme activity in cytosol preparations (lysate not chromatographed on CaM-Sepharose) is not due to a loss in sensitivity to Ca^{2+}-CaM stimulation since (as shown in Figure 1), there is a decrease in basal protein kinase activity, and the ratio of basal to Ca^{2+}-CaM activated activity remains relatively constant. This suggests that there is a loss of total enzyme activity, rather than a loss of enzyme sensitivity to calcium and calmodulin.

Calcium plays an important role in the degradation of the enzyme. The change in activity in a CaM-Sepharose eluate in the presence of calcium is similar to that seen in cytosol. The basal activity is reduced by approximately 80% and, in this case, the Ca^{2+}, CaM-stimulated activation is also reduced. In the absence of calcium (1 mM EGTA) the basal protein kinase activity remains relatively constant over a 48-hour period. However, the Ca^{2+}, CaM-stimulated activity is reduced to 52% of the zero time point at 24 hours and 31% at 48 hours. Although it appears that loss of total enzyme activity can be alleviated somewhat by addition of various protease inhibitors (unpublished observation), further studies must be done to determine the mechanism of loss of activation by calcium and calmodulin. The present studies confirm and extend those of Dunkley and Robinson (2,3) and indicate that the inactivation of Ca^{2+}-CaM dependent protein kinase from synaptosomes begins immediately upon extraction of the enzyme from brain.

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