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CALMODULIN LIGANDS

THE INTERACTION OF MUSCLE PHOSPHORYLASE KINASE WITH PHOSPHODIESTERASE

COMPARISON OF CALMODULIN LIGANDS IN MUSCLE EXTRACTS FROM NORMAL AND PHOSPHORYLASE KINASE-DEFICIENT MICE *

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Summary

Interactions between phosphorylase kinase (ATP:phosphorylase-*b* phosphotransferase, EC 2.7.1.38) and calmodulin were studied with pure preparations of muscle phosphorylase kinase, and with crude extracts from muscles of control (C_{57} Black) and deficient (ICR/IAn) mice, which lack muscle phosphorylase kinase activity.

Calmodulin was determined by its ability to stimulate a calmodulin-depleted phosphodiesterase.

The amount of calmodulin bound to phosphorylase kinase in muscle extract was estimated to a maximum of 30% of the total amount of calmodulin.

In the muscle of the deficient strain a decrease of 35% in the total amount of calmodulin was observed. This correlates with the absence of the calmodulin fraction specifically bound to phosphorylase kinase.

From sucrose gradient studies we demonstrated that in the presence of Ca^{2+} the amount of calmodulin bound to phosphorylase kinase was enhanced, compared to the control in the presence of EGTA. This observation was made both in crude extracts and in pure phosphorylase kinase preparations.

Sucrose gradient also showed that muscle phosphorylase kinase can be dissociated to low molecular species when extracts are made in the presence of Ca^{2+}; this dissociation was found to be related to a Ca^{2+}-dependent proteolytic effect.

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* Part of this work has been presented in an abstract form [28].

Introduction

Calmodulin (or Ca^{2+}-dependent regulatory protein) is a small heat-stable Ca^{2+}-binding protein which was first shown to activate the cyclic nucleotide phosphodiesterase (3′:5′-cyclic nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) in the presence of Ca^{2+} [1,2]. Calmodulin was later shown to activate other enzymes such as adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) [3,4], membrane bound Ca^{2+}-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) [5], myosin light chain kinase [6—8], NAD kinase (ATP:NAD^+ 2′-phosphotransferase, EC 2.7.1.23) in higher plants [9] and human platelet phospholipase (phosphatide 2-acylhydrolase, EC 3.1.1.4) [10].

Recently, Cohen et al. [11] demonstrated that purified rabbit skeletal muscle phosphorylase kinase (ATP:phosphorylase-*b* phosphotransferase, EC 2.7.1.38) which is Ca^{2+} activated, contains, in addition to the previously described α, β and γ chains, a δ subunit the characteristics of which are similar to calmodulin.

It has been shown [12,13] that phosphorylase kinase is able to bind two molecules of calmodulin per (α β γ) unit, the binding being tighter for the first than for the second molecule.

Since the muscular concentration of phosphorylase kinase is high (nearly 1% of soluble proteins) and that of calmodulin was reported to be low [7], phosphorylase kinase was expected to bind most of the skeletal calmodulin; but the situation in muscle tissue was not known.

In the present work we studied the calmodulin ligand properties of skeletal muscle phosphorylase kinase attempting to answer the following questions.

(a) Is calmodulin, bound to phosphorylase kinase, able to stimulate another calmodulin activated enzyme like phosphodiesterase?

(b) Is phosphorylase kinase the main calmodulin ligand in skeletal muscle?

(c) Considering skeletal muscle calmodulin level, what are the consequences of the absence of phosphorylase kinase in the ICR/IAn strain of mice whose skeletal muscle is deficient in phosphorylase kinase activity [14] and phosphorylase kinase protein [15,16]?

(d) Is calmodulin tightly bound to the phosphorylase kinase molecule?

Our approach was to investigate the interactions between phosphorylase kinase, phosphodiesterase, and calmodulin in freshly purified phosphorylase kinase preparations as well as in crude muscle extracts. Calmodulin activity was studied in whole preparations and after sucrose gradient centrifugation, in muscle extracts from normal and phosphorylase kinase-deficient mice.

Materials and Methods

Materials

Mice of the ICR/IAn strain carrying the gene for skeletal muscle phosphorylase kinase deficiency and control strain C57 Black were obtained from our own colonies.

Methods

Preparation of mouse muscle extracts. Hind leg muscles were removed from adult mice following decapitation, rapidly minced and homogenized in a

Potter-Elvehjem homogenizer with 4 vol. of 20 mM Tris-HCl/1 mM dithiothreitol, pH 7.9.

When extracts were used for sucrose gradient analysis, either 100 μM $CaCl_2$ or 1 mM EGTA (ethyleneglycol bis(β-aminoethyl)-*N,N'*-tetraacetic acid) was added with or without additional antiproteolytic agents: 1 mM phenylmethanesulfonyl fluoride, 0.5 mM *N*-α-*p*-tosyllysylchloromethyl ketone), 0.5 mM *p*-tosylamido-2-phenylethylchloromethyl ketone), 0.05 mM cathepsin D inhibitor (pepstatin) and 2.5 μg/ml cathepsin B inhibitor (leupeptin).

The homogenates were centrifuged for 10 min at 33 000 $\times g$ and the supernatants were used for experimentation.

Purification procedures. Muscle phosphorylase *b* kinase was purified from rabbit muscle according to Cohen [17] except that 0.5 phenylmethanesulfonyl fluoride, 0.05 mM *N*-α-*p*-tosyllysylchloromethyl ketone and 0.05 mM *p*-tosylamido-2-phenylethylchlorimethyl ketone were included in all buffers. This technique allows the simultaneous preparation of its substrate, phosphorylase *b* [18], starting from the supernatant of the pH 6.1 precipitation.

Calmodulin was purified either from bovine brain according to Lin et al. [19] or by boiling a purified muscle phosphorylase kinase preparation for 5 min at 90°C [11].

Calmodulin-depleted cyclic-AMP phosphodiesterase was partially purified from bovine brain according to Cheung [20].

Preparation of antibodies against muscle phosphorylase kinase. Antiserum to pure rabbit muscle phosphorylase kinase was prepared in chicken as previously described [16] and was further fractionated to obtain the γ-globulin fraction according to the procedure of Koch (unpublished results). The γ-globulin fraction from non-immunized chicken sera was prepared by the same procedure.

Antigen-antibody precipitation in muscle extracts. 1 ml C 57 Bl muscle extract (12 mg proteins/ml) was made 0.5 M NaCl/1% Triton and then allowed to stand at 4°C in the presence of a previously determined amount of anti-muscle phosphorylase kinase γ globulins, which leads to the precipitation of the whole phosphorylase kinase (solution A). A control experiment was done with an equal amount of γ globulins purified from the serum of non-immunized chicken (solution B).

After standing overnight at 4°C, a precipitate was only visible in solution A. After a 10 min 33 000 $\times g$ centrifugation, the supernatants were decanted; the precipitate obtained from solution A was washed in 0.5 ml of the initial buffer, centrifuged again and then resuspended in 0.5 ml of the same buffer.

Enzyme assays. Unless otherwise, noted, phosphorylase kinase was routinely assayed according to Cohen and Cohen [21] and cyclic nucleotide phosphodiesterase activity was measured by the standard radioactive isotope procedure [22] slightly modified [23].

Assay of calmodulin activity. Calmodulin activity was measured according to Lin et al. [19] by its ability to stimulate, in the presence of calcium, the activity of a partially purified calmodulin-depleted cyclic-AMP phosphodiesterase.

Prior to assay, each fraction was heated for 5 min at 90°C, rapidly cooled and then centrifuged for 10 min at 10 000 $\times g$. Calmodulin activity was assayed in the presence of 50 μM calcium or 100 μM EGTA.

The level of calmodulin in mouse muscle extracts was determined in refer-

ence to a standard curve obtained using purified bovine brain calmodulin.

Sucrose density gradient analysis. 0.5 ml mouse muscle 33 000 × *g* supernatant was layered on 32.5 ml linear sucrose gradients (5—20%), containing 20 mM Tris-HCl, 1 mM dithiothreitol, pH 7.9, and either 100 μM Ca^{2+} or 1 mM EGTA, in the presence or in the absence of antiproteolytic agents (see above), but used at 5-times less concentration than in the extracts. Centrifugation was performed for 2 h at 50 000 rev./min at 4°C in a TV 850 vertical rotor (Sorvall ultracentrifuge, model OTD 65).

Results

Validity of the experimental procedure

During the preparation of this article. Shenolikar et al. [24] reported that the heat treatment which is used prior to calmodulin estimation by phosphodiesterase activation did not release most of the calmodulin from calmodulin-binding proteins in a crude muscle extract. Prior to this work we controlled the validity of the method used: that is, we determined the calmodulin level starting either by boiling an extract containing about 12 mg protein/ml prior to dilution or we previously diluted the extract and then boiled the preparation. The dilutions were from 1 : 1 to 1 : 100 in both cases. The amount of calmodulin was, respectively, 16 μg/g muscle and 55 μg/g muscle. An underestimation could be made when muscle extracts are boiled under their concentrated form. This inconvenience can be overcome by heating more diluted extracts. Our maximal estimation is about twice that measured by Yagi et al. [7] but very close to that estimated by Shenolikar et al. [24].

Calmodulin concentration in normal and phosphorylase kinase-deficient mice

In the determination of calmodulin level in mouse muscle extract a variability was observed from day to day in the same strain. This was due to the many variables involved in the test system. We, therefore, chose the method of paired animals, using in each experiment one or several control and one or several kinase-deficient mice. For each animal calmodulin levels were determined using 10 protein concentrations (from 12 mg/ml to 120 μg/ml before heating) to determine the optimal level of specific stimulation.

The calmodulin level was constantly lower in the kinase-deficient mice (35 μg/g muscle) than in the control (55 μg/g muscle). The calmodulin concentration in ICR/IAn mouse muscle extracts was about 65% of that in control C 57 Black mice.

Determination of the amount of calmodulin bound to phosphorylase kinase in C 57 Black mouse muscle extracts, by specific precipitation of the whole phosphorylase kinase in the presence of anti-phosphorylase kinase γ globulins

Phosphorylase kinase assays were performed on A and B supernatants before and after incubation with antiserum (see Methods); while no phosphorylase kinase activity was found in A supernatant (not more than 0.5% of the initial activity found in the starting muscle extract), the phosphorylase kinase activity was found unchanged in B supernatant (in reference to the starting muscle extract).

The calmodulin level determined in A and B supernatants and in the resuspended antigen-antibody precipitate obtained from solution A were compared: the calmodulin amount in B supernatant (without antibody) was taken as 100%; we found about 70 and 20%, respectively, in A supernatant and in the phosphorylase kinase antibody precipitate.

Analysis of the antigen-antibody precipitate by electrophoresis on SDS-polyacrylamide gel [15] showed that phosphorylase kinase had been specifically precipitated in these conditions.

We could conclude that at least 20% of the total soluble muscle calmodulin is bound to phosphorylase kinase.

Interaction of muscle phosphorylase kinase, cyclic-AMP phosphodiesterase and calmodulin in pure phosphorylase kinase preparations

Phosphorylase kinase preparations. To determine whether calmodulin could stimulate phosphodiesterase activity when bound to phosphorylase kinase, as well as in the free form, calmodulin-deficient phosphodiesterase activity was assayed by adding increasing amounts of purified phosphorylase kinase either native or after heating for 5 min at 90°C.

Fig. 1 shows that in the free form, a δ subunit obtained from a boiled purified phosphorylase kinase (the other denatured subunits being eliminated by centrifugation) activated phosphodiesterase. The apparent cooperativity and the extent of activation were the same as those elicited by purified calmodulin from bovine brain. In contrast, when bound to phosphorylase kinase, calmodulin was less effective and the kinetics of stimulation did not show any apparent cooperativity. The curves presented here allowed us to calculate the ratio between δ subunits and α, β, γ, in our preparation. This ratio was found equal

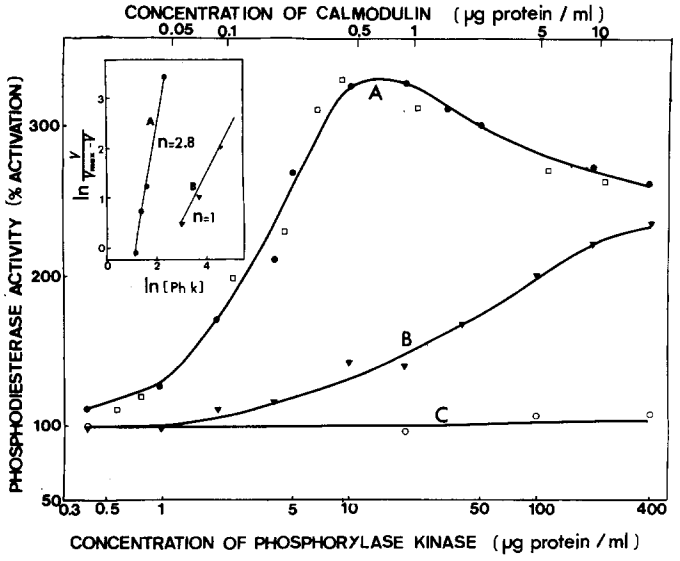

Fig. 1. Stimulation of phosphodiesterase activity by calmodulin. Curve A in the presence of purified bovine brain calmodulin (□) or pure phosphorylase kinase (●) after 5 min at 90°C. Curve B in the presence of pure native phosphorylase kinase. Curve C in the presence of EGTA. In the insert the Hill plots corresponding to curve A and B are given.

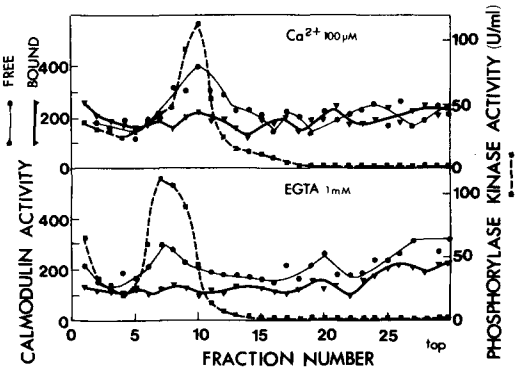

Fig. 2. Stimulation of phosphodiesterase activity by calmodulin from phosphorylase kinase. Sucrose density gradients.

to 0.8. Although only freshly purified muscle phosphorylase kinase was used in these experiments, we could not eliminate the possibility that the activation of phosphodiesterase observed with native phosphorylase kinase was due to free calmodulin released from phosphorylase kinase.

Sucrose gradients. The presence or the absence of free or loosely-bound calmodulin in the purified preparation of phosphorylase kinase was checked on a linear sucrose gradient (Fig. 2). EGTA is known to release calmodulin from its ligand, therefore we included either 1 mM EGTA or 100 μM Ca^{2+} in the buffer.

In the presence of Ca^{2+} no free calmodulin activity was detected at the top of the gradient. It seems, therefore, that calmodulin remained bound to the undissociated phosphorylase kinase molecule. Conversely, in the presence of EGTA, only part of the calmodulin was found in the phosphorylase kinase peak. Some free calmodulin was detected at the top of the gradient. In addition, some calmodulin migrated between the peak of phosphorylase kinase activity and that of free calmodulin (fraction 20) suggesting that calmodulin was, in part, bound to an inactive degraded form of phosphorylase kinase or to subunits other than the catalytic one. Finally these curves confirm that calmodulin bound to the native enzyme is much less able to activate phosphodiesterase than free calmodulin.

Sucrose gradient study in complete extracts: relative repartitions of phosphorylase kinase and calmodulin activities in control and ICR/IAn mouse muscle extracts (Figs. 3 and 4)

Phosphorylase kinase. In the presence of calcium (100 μM in the extract and in the gradient, Fig. 3A and C) in addition to a small peak at the top of the tube, one major peak of phosphorylase kinase was observed with an apparent molecular weight of 850 000—900 000 (peak I). A minor peak with a molecular weight of about 100 000 was constantly observed (peak II); it represented 10—20% of the total activity. The proportion of peak II was greatly increased (up to 70% of the total phosphorylase kinase activity) when antiprotease agents were omitted in the extract and the gradient buffer (Fig. 3A).

In the absence of calcium (that is, in the presence of 1 mM EGTA) whether

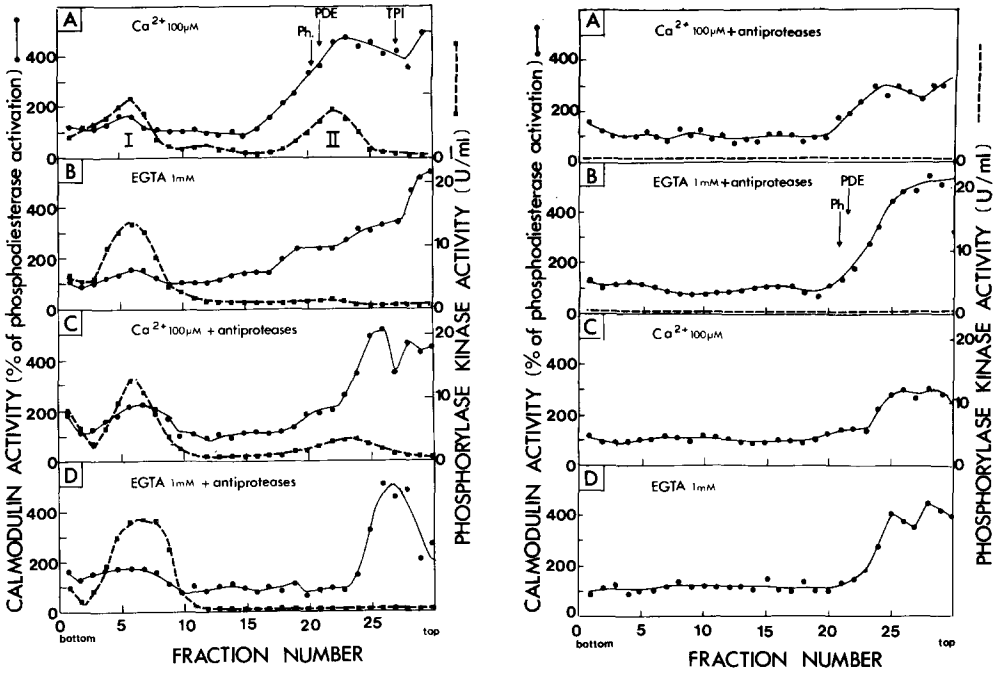

Fig. 3. Sucrose gradient analysis of C_{57} Black mouse muscle extracts. The extracts and the gradient were made in the presence of either; A, 100 μM Ca^{2+} plus antiproteolytic agents or B, 1 mM EGTA plus antiproteolytic agents. Pure calmodulin (10 μg) was run simultaneously in the presence of; C, 100 μM Ca^{2+} or D, 1 mM EGTA.

Fig. 4. Sucrose gradient analysis of ICR/IAn mouse muscle extracts. The extract and the gradient were made in the presence of either; A, 100 μM Ca^{2+} plus antiproteolytic agents or B, 1 mM EGTA plus antiproteolytic agents. Pure calmodulin (10 μg) was run simultaneously in the presence of; C, 100 μM Ca^{2+} or D, 1 mM EGTA.

antiproteolytic agents were present or not (Fig. 3B and 3D), only peak I was observed.

Calmodulin. In the presence of Ca^{2+} a peak of calmodulin activity could be detected associated with the peak I of phosphorylase kinase in the presence or in the absence of antiproteolytic agents. The absence of this peak in ICR/IAn mouse muscle extracts which lack phosphorylase kinase (Fig. 4A B) confirmed the association of calmodulin with phosphorylase kinase.

In the absence of Ca^{2+}, calmodulin is still associated with the phosphorylase kinase activity, but to a lesser extent (Fig. 3B and D).

A second peak of calmodulin was constantly found at the top of the gradient in a position which, under these conditions, could not be absolutely distinguished from that of free calmodulin (Fig. 4C and D) (probably as a dimer M_r 34 000) or that of an association with other binding proteins.

There was no evidence for a binding to endogenous phosphodiesterase although some overlapping was observed.

Despite the lack of a rigourous quantification of calmodulin in these conditions in all cases the calmodulin peak associated with phosphorylase kinase was smaller than the second peak.

Discussion

The present work gives additional confirmation that muscle phosphorylase kinase binds calmodulin. It allows one to estimate the percentage of muscle calmodulin that is bound to phosphorylase kinase by a method different from that of Shenolikar et al. [24]. Our estimation of phosphorylase kinase-bound calmodulin, based on specific immunoprecipitation, is between 20 and 30%. This estimation is lower than that of Shenolikar et al. [24]. They found 35—40% of calmodulin bound to phosphorylase kinase in a 35% $(NH_4)_2SO_4$ precipitate. The discrepancy between the results may be due to the difference in the technique used, we feel that the precipitation with antibodies is more specific.

Based on amino acid analysis [11] and on the amount of δ subunit (in terms of protein), released by heating a known amount of purified phosphorylase kinase [24], it has been established that the δ subunit is present in stoichiometric amounts with the α, β and γ subunits. We obtained similar results in estimating the δ subunit by its ability to stimulate phosphodiesterase. Since, in our conditions, phosphorylase kinase represents about 0.7% of the soluble muscle protein, 16 μg calmodulin/g muscle would bind to phosphorylase kinase. Consequently, if phosphorylase kinase binds 20—30% of calmodulin in muscle, the total amount of calmodulin expected in muscle would be of 53—80 μg/g muscle, in fact, our experimental values was 55 μg calmodulin/g muscle. This result is in agreement with our calculated estimation and that of Shenolikar et al. [24] (50 mg/1000 g skeletal muscle) but should be taken as the minimal amount of calmodulin present in muscle extract.

A decrease in calmodulin concentration was observed in the ICR/IAn mouse strain deficient in phosphorylase kinase. Nevertheless the decrease remained moderate. This observation correlates with the idea that the fraction of calmodulin specifically bound to phosphorylase kinase is absent. A difference of calmodulin level due only to strain differences is ruled out by the results obtained in the brain where the same amount of calmodulin was detected in the two different strains; brain was chosen as control since calmodulin level is much higher than in muscle, whereas phosphorylase kinase activity is much lower. Moreover, a similar calmodulin decrease was recently reported [24] in the muscle of kinase-deficient mice using another control strain (C_3H/He-mg).

The complex between phosphorylase kinase and calmodulin was further demonstrated in the study of the phosphorylase kinase and calmodulin profiles, on linear sucrose gradients. The high molecular weight form of kinase and the minor peak of calmodulin comigrated.

Nevertheless, one should note that a large peak of calmodulin was independent of phosphorylase kinase activity and migrated to a position of lower molecular weight. Whether this peak represents free polymers of calmodulin or calmodulin complexed to other proteins (myosin light chain kinase or phosphodiesterase) is not demonstrated. Furthermore the position of this calmodulin peak was constantly dependent on the presence or the absence of Ca^{2+} or EGTA: while in EGTA the position of the calmodulin peak could not be distinguished from that of free calmodulin, in the presence of calcium, this peak was extended to an area of higher molecular weight. This suggested that in the presence of calcium calmodulin was bound to other proteins and that

EGTA induced a dissociation of the complexes. This result is in a good agreement with the observation [24] that in muscle extracts calmodulin was visualized, after polyacrylamide gel electrophoresis in 8 M urea, as a fast migrating band only in the presence of EGTA, but not in the presence of Ca^{2+}; calmodulin was then tightly bound to other proteins.

The proportion of calmodulin comigrating with phosphorylase kinase was lower when the experiment was made in the presence of EGTA than in the presence of Ca^{2+}. This indicates that part of the calmodulin but not all was released from phosphorylase kinase in the presence of EGTA. Therefore, phosphorylase kinase as well as other ligands of the calmodulin [2] binds part of this protein tightly. The other part is more labile and can be released by EGTA. It has been suggested recently [8,24] that phosphorylase kinase could bind two calmodulin molecules per $\alpha\ \beta\ \gamma$ units: a weakly-bound molecule which activates the enzyme and a tightly-bound molecule referred as subunit δ, responsible for the dependence of phosphorylase kinase activity on calcium.

The results, however, obtained with pure phosphorylase kinase may lead to a reappreciation of these conclusions: purified phosphorylase kinase, containing 1 subunit per ($\alpha\ \beta\ \gamma$) unit, should contain only strongly-bound calmodulin. Nevertheless, the complex phosphorylase kinase calmodulin could be partially dissociated in the presence of EGTA (Fig. 2).

Sucrose gradients also showed, that in the presence of calcium, phosphorylase kinase in muscle extracts underwent a dissociation, since in addition to the expected high molecular weight peak an activity could be detected at the top of the gradient. A similar active phosphorylase kinase with a molecular weight of 110 000 was recently described in rabbit liver [25]. This low molecular weight phosphorylase kinase peak was markedly diminished but not abolished by the addition of antiproteolytic agents and did not exist when the extract and the centrifugation were effected in the presence of EGTA. As the antiproteolytic agents included inhibitors for serine proteases a trypsin-like activation, which is known to lead to the formation of low molecular weight species [26], is unlikely. The fact that, despite antiprotease, there remains a partial dissociation only in the presence of calcium is in favor of an action of the Ca^{2+}-dependent kinase activating factor (KAF of Huston and Krebs [27]). Moreover, this observation raised the question of the subunit nature of this low molecular weight active species. This problem is actually under study in our laboratory.

Partial dissociation of the phosphorylase kinase molecule in the presence of Ca^{2+} was very small or completely absent when the purified enzyme was studied in sucrose gradient (Fig. 2).

Finally it is clear from the results depicted in Figs. 1 and 2 that bound calmodulin is not very efficient in activating phosphodiesterase, as compared to free calmodulin. When present as phosphorylase kinase δ subunit calmodulin is not directly available to activate other enzymatic systems.

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