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QUANTITATION AND SIGNIFICANCE OF ^{125}I-CALMODULIN BINDING TO MYOSIN LIGHT CHAIN KINASE AND PHOSPHORYLASE DISTRIBUTED ON POLYACRYLAMIDE GELS

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SUMMARY: Glycogen phosphorylase (a or b) binds ^{125}I-calmodulin in a Ca^{2+}-dependent manner, in the ^{125}I-calmodulin overlay technique. This binding is quantitatively identical to ^{125}I-calmodulin binding to myosin light chain kinase. In an *in vitro* assay, calmodulin stimulates phosphorylase activity at limiting concentrations of either glucose-1-phosphate or glycogen, but the K_a is 1000 fold higher than for the kinase, and is not Ca^{2+}-dependent. Activation of phosphorylase, but not myosin light chain kinase, by calmodulin can be mimicked by troponin C or bovine serum albumin. These results demonstrate that the properties of calmodulin interaction with proteins can vary between the ^{125}I-calmodulin technique and a functional assay of calmodulin effect on the same protein.

Calmodulin has been implicated as a primary mediator of Ca^{2+} actions in diverse systems and processes including activation of enzymes, glycogen metabolism, cytoskeletal regulation, and secretion (1). Understanding the exact mechanism by which Ca^{2+}-calmodulin orchestrates these effects, will involve identifying proteins that can interact with calmodulin and defining how this association affects subsequent function. Identification of potential calmodulin-binding proteins has been accelerated by the development of a technique known as the ^{125}I-calmodulin overlay (2,3,4). Binding of ^{125}I-calmodulin to specific proteins can be detected following its incubation with an SDS-polyacrylamide gel on which purified proteins or cell fractions are separated by electrophoresis. One calmodulin binding protein serendipitously identified by laboratories using this technique was glycogen phosphorylase, present in

The abbreviations used are: EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N^1,N^1-tetraacetic acid; SDS, sodium dodecyl sulfate; ACTH, adrenocorticotrophic hormone; BSA, bovine serum albumin; MLCK, myosin light chain kinase.

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many molecular size marker kits (5,6). We demonstrate that this binding is in fact quantitatively indistinguishable from that of binding to a known physiologically relevant calmodulin binding protein, myosin light chain kinase (MLCK). In addition calmodulin can activate both phosphorylase a and b. However the K_{CaM}, Ca^{2+} dependence and protein specificity are markedly different than those relating to the activation of myosin light chain kinase. We conclude that detection and quantitation of proteins by the ^{125}I-CaM gel overlay procedure is not sufficient to categorize a molecule as a physiologically relevant calmodulin binding protein.

MATERIALS AND METHODS

AMP, rabbit liver glycogen, phosphorylase a and b, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. Avidin, soybean trypsin inhibitor, and S. aureus micrococcal nuclease were purchased from Worthington. Glucose-1-phosphate was from Boehringer Mannheim. Fatty acid free bovine serum albumin was from Miles Laboratories, Inc. [^{14}C]glucose-1-phosphate was supplied by New England Nuclear. Orion 0.100 M $CaCl_2$ was from Fisher Scientific. Rabbit skeletal muscle troponin C and carp muscle parvalbumin were generous gifts from Dr. J.D. Potter (Univ. of Miami). The M_r = 20,000 light chain of myosin and MLCK were purified from chicken gizzard by H.L. Foyt.

Phosphorylase assays: The phosphorylase used in this study was tested for the appropriate kinetic behavior. The K_m for rabbit liver glycogen was 0.017% for phosphorylase a and 0.014% for phosphorylase b. The specific activity, determined from initial reaction rates at optimal substrate concentrations, was 49.8 U/mg for phosphorylase a (-AMP) and 77.5 U/mg assayed in the presence of AMP, while the activity for phosphorylase b (+AMP) was 59.2 U/mg. These data agree to within 10% with those determined for purified phosphorylase a and b (7).

Phosphorylase a or b was dialyzed against 50 mM β-glycerophosphate, pH 6.8. Optimal activity was obtained when the phosphorylase was dialyzed at 0.1 mg protein/ml. Phosphorylase activity was determined by measuring the incorporation of [^{14}C]-glucose-1-phosphate into glycogen (8). Phosphorylase was assayed in 50 mM β-glycerophosphate, pH 6.8, 75 mM [^{14}C]-glucose-1-phosphate, 1% (w/v) rabbit liver glycogen, and 60 mM mercaptoethanol. Assays of phosphorylase b contained 1.0 mM AMP (9).

The effects of calmodulin on phosphorylase activity were determined after incubating the proteins for 16 h, the same incubation time used for the overlays. Calmodulin and phosphorylase were dialyzed separately against 50 mM β-glycerophosphate, pH 6.8. Calmodulin (0-35 µg) or another protein was added to 3.5 µg phosphorylase a or b in a total reaction volume of 125 µl. Ca^{2+} was added from a 0.100 M Orion standard in an amount calculated to give the desired free [Ca^{2+}] at a final EGTA concentration of 2 mM (10). Binding of Ca^{2+} by calmodulin, β-glycerophosphate or glucose-1-phosphate (in the assay) was considered in the calculation. Unless otherwise noted the free [Ca^{2+}] was 9 µM. Samples were incubated for 1 h at 30°C, then for 15 h at 4°C. There was no loss in activity or detectable proteolysis during this incubation period. Samples were then assayed with one of the substrates in limiting concentration; i.e. 7.5 mM glucose-1-phosphate or 0.03% rabbit liver glycogen.

^{125}I-calmodulin overlay technique: Calmodulin was purified from rat testes

(11) and iodinated with Bolton Hunter reagent (12). The ^{125}I-calmodulin had a specific activity of 2400 Ci/mmole.

MLCK, purified from chicken gizzard (13), was diluted to 0.1 mg/ml into 1 mg/ml BSA, 1% SDS in plastic tubes to prevent loss due to sticking (14). The diluted MLCK and phosphorylase were prepared in Laemmli sample buffer (15) and heated to 95°C for 5 min, then electrophoresed on 10% polyacrylamide slab gels (16).

After electrophoresis the gels were fixed for 30 min in 40% methanol, 7% acetic acid, washed with water and incubated for 15 h in 10% ethanol. The gels were then washed for 1 h in 10% ethanol and rinsed with water. Gels were first incubated with 100 mM imidazole, pH 7.0 for 30 min, then 20 mM imidazole buffer pH 7.0, 0.1% BSA followed by ^{125}I-calmodulin (1 x 10^6 cpm/gel track) in the same BSA buffer for 16 h at 4°C as described by Nelson et al. (5). KCl (0.2M) was included in all the solutions and washes to prevent nonspecific interactions. Either 1 mM Ca^{2+} or 1 mM EGTA was included in all buffered solutions. After the unbound ^{125}I-calmodulin was removed by washing (10 washes of 400 mls of 20 mM imidazole, 0.1% BSA 0.2M KCl), the gels were rinsed with water, and then washed in 40% methanol, 7% acetic acid at room temperature for 15 h to remove excess BSA. Accurate quantitation of protein in a band requires elimination of the BSA. This wash does not alter ^{125}I-calmodulin binding. The gels were then stained for 2 h in Coomassie Brilliant Blue G250 in 40% methanol, 7% acetic acid and destained in 7% acetic acid, scanned for protein at 560 nm, dried, and subjected to autoradiography at -70°C.

Autoradiogram scanning: The autoradiograms were scanned with a 560 nm filter with a Kontes Fiber Optic Scanner coupled to a Hewlett Packard Integrator 3390A supplied by Dr. D. Bullock. The peak area in cm^2 was multiplied by the band length to compensate for variability due to differences in track width or amount of protein. This value was designated "densitometric signal". This same correction factor was used to calculate the protein intensity signal.

Protein determinations: Protein concentrations were determined using the OD_{280} and the extinction coefficient for each protein, provided by the supplier.

RESULTS AND DISCUSSION

^{125}I-calmodulin binding to MLCK and phosphorylase

Figure 1 depicts the binding of ^{125}I-calmodulin to phosphorylase (panel A) or MLCK (panel B) as a function of enzyme concentration. Some ^{125}I-calmodulin binding was also observed in the region of the gel where carrier BSA and a M_r = 67,000 degradation product of MLCK comigrated (Fig. 1, panel B). Densitometric scans of the radioautograms produced the data replotted in Figure 2. The binding of ^{125}I-calmodulin was quantitatively similar to both enzymes and was directly proportional to the amount of protein in a band. Similar data were obtained for phosphorylase a (not shown). Other reports of difficulty in correlating the amount of protein applied to a gel with the ^{125}I-calmodulin binding did not employ autoradiography (2) or report insufficient information to allow comparison to our results (17). These data imply that phosphorylase and MLCK bind calmodulin with comparable affinity. In

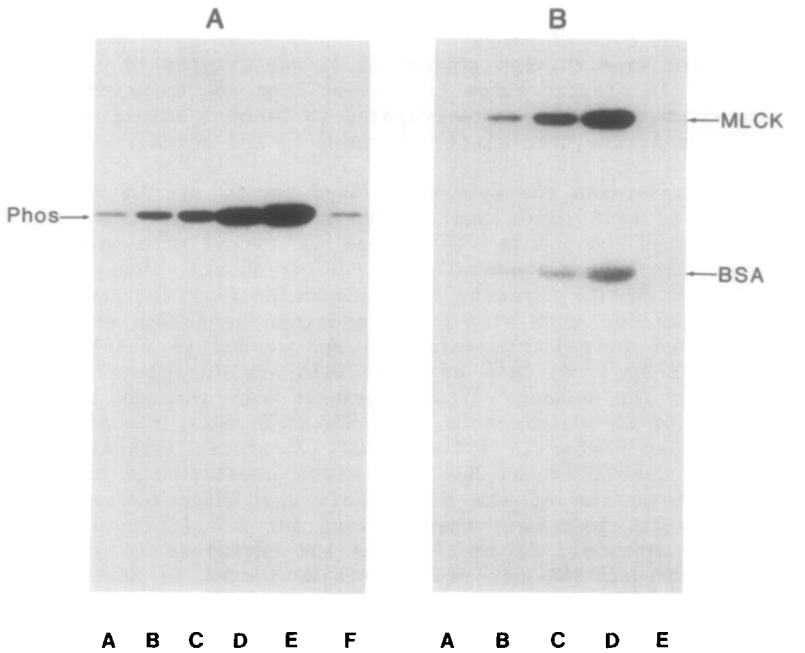

Figure 1: ^{125}I-calmodulin binding to phosphorylase b and MLCK. Panel A. Phosphorylase (0.2-4.0 µg, lanes a-e) or Panel B.MLCK (0.2-2.0 µg, lanes a-d) was applied to a 10% Porzio and Pearson gel and electrophoresed. The gel was incubated with ^{125}I-calmodulin at 1 mM Ca^{2+}, washed, and stained with Coomassie Blue as described in Methods. The tracks were scanned for protein. The gel was dried and an autoradiogram exposed for 120 hrs and scanned. The Ca^{2+} dependence of binding is shown by incubating 2.0 µg of each protein with ^{125}I-calmodulin and 1mM EGTA (panel A, lane f; panel B, lane e).

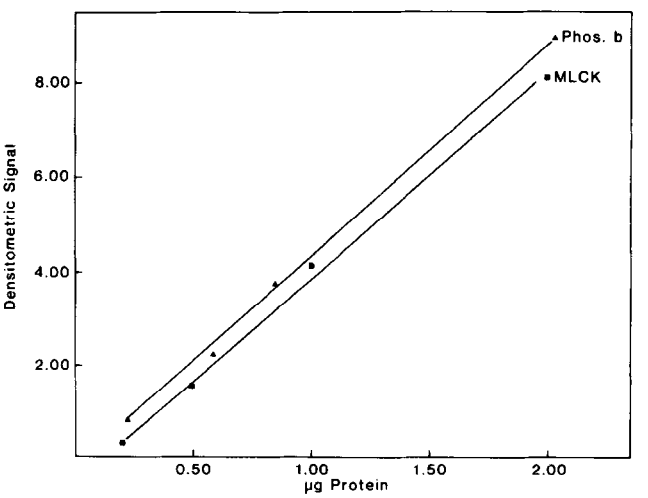

Figure 2: Quantitation of ^{125}I-calmodulin binding to proteins. The autoradiograms from Figure 1 were scanned as described in Methods and the densitometric signal calculated and plotted as a function of the protein present in each band.

order to determine whether differences in phosphorylase and MLCK affinity would be apparent at calmodulin concentrations other than the standard 1 nM concentration, the ^{125}I-calmodulin concentration was varied from 0.05-2.5 nM. The amount of ^{125}I-calmodulin that bound increased by 60 fold (data not shown) but the ratio of ^{125}I-calmodulin-phosphorylase/^{125}I-calmodulin-MLCK varied by only 20%. Finally, binding of ^{125}I-calmodulin to MLCK and phosphorylase was Ca^{2+}-dependent as shown in Fig. 1. Together these data suggest the possibilities that 1) the K_a of calmodulin binding to phosphorylase and MLCK are similar; and 2) that calmodulin should interact directly with phosphorylase in a Ca^{2+}-dependent manner in solution.

Effects of calmodulin on phosphorylase activity

Incubating either phosphorylase a or b in the presence of calmodulin resulted in activation of both enzymes if they were assayed at a limiting concentration of either substrate. The results shown in this paper were obtained at limiting glycogen concentrations, although similar data were obtained at a sub-optimal glucose-1-phosphate concentration. As Fig. 3 shows quantitating the concentration dependence of calmodulin activation allows one to calculate the K_{CaM} (18,19,20). The Hill coefficient was 1.1±0.1 for phos-

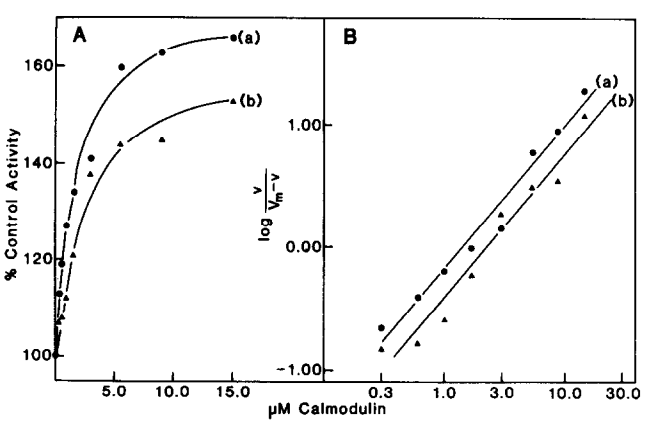

Figure 3: Calmodulin activation of phosphorylase. A. 3.5 μg phosphorylase a (●) or b (▲) were incubated with varying concentrations of calmodulin and subsequently assayed with 0.03% rabbit liver glycogen in the assay as described in Methods. The data are plotted relative to the activity in the absence of calmodulin. B. Data from Panel A were graphed as Eadie-Hofstee plots to derive the V_m (data not shown). The data were then converted to a Hill plot to determine the Hill coefficient and the K_a for calmodulin (K_{CaM}).

phorylase a and b, indicating that there was no cooperative binding. The K_{CaM} of phosphorylase a was 1.9±0.9 μM and for phosphorylase b was 3.1±1.9 μM. Calmodulin did not significantly activate phosphorylase b in the absence of AMP. The affinity constants are 1000 fold lower than that reported for MLCK (21, 22) or for phosphorylase kinase (23) or phosphodiesterase (24, 25). ACTH, β-endorphin, glucagon, and substance P bind calmodulin with dissociation constants in the μmolar range, but no physiological relevance has been demonstrated for this binding (26). But fodrin (27) and spectrin (28) appear to be calmodulin binding proteins with a similar affinity whose interaction with calmodulin may be physiologically important.

The Ca^{2+}-dependence of the calmodulin-phosphorylase interaction was determined by varying the free Ca^{2+} concentration from 10^{-8} to 10^{-4} M (Fig. 4). Ca^{2+} did not affect the basal phosphorylase activity. In contrast to the results obtained using the gel overlay technique, there was no effect of Ca^{2+} on the ability of calmodulin to activate phosphorylase, even in solutions containing 1 mM EGTA with no added Ca^{2+} or 1 mM $CaCl_2$ and no EGTA (data not

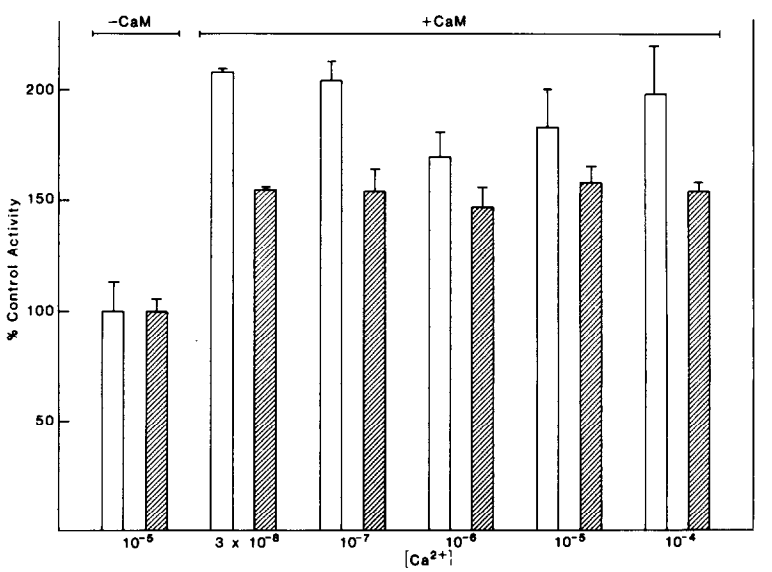

Figure 4: Ca^{2+} effect on calmodulin activation of phosphorylase. 1.7 μg phosphorylase a (open bars) or b (crosshatched) were incubated with a 20 fold molar excess of calmodulin (6 μM) at the indicated Ca^{2+} concentration and assayed with the same concentration of Ca^{2+} in the assay. Data are expressed relative to the activity in the absence of calmodulin.

TABLE 1: PROTEIN SPECIFICITY OF PHOSPHORYLASE ACTIVATION

Protein	Subunit $M_r \times 10^{-3}$	Ca^{2+} Binding	Relative pI	Phosphorylase Activity Ratio (+/- protein)
Phosphorylase *a*	97	-	5.8	1.00
Calmodulin	17	+	3.9-4.3	1.63
Troponin C	18	+	4.2	1.71
Parvalbumin	13	+	4.9	0.99
Myosin light chain	20	+	5.0	1.21
S. a. micrococcal nuclease	16	+	9.6	1.04
Soybean trypsin inhibitor	22	-	4.5	1.12
Avidin	18	-	10.5	0.96
Bovine serum albumin	68	-	5.8-6.1	1.97

3.5 µg phosphorylase *a* were incubated with a 20 fold molar excess of effector protein for 16 h and assayed as described in Methods.

shown). There are other examples of Ca^{2+} independent calmodulin binding to proteins. The constitutive calmodulin subunit of phosphorylase kinase binds independently of Ca^{2+} (29) as do some calmodulin-antibodies (12).

Considering the low affinity of phosphorylase for calmodulin we decided to investigate the protein specificity of activation. Table 1 demonstrates that troponin C could activate the enzyme. Activation of MLCK by calmodulin is highly specific, there is no activation by troponin C (30). However, another Ca^{2+} binding protein, parvalbumin, could not substitute for calmodulin. Fatty acid free BSA not only activated phosphorylase, but the K_{BSA} of 1.1 µM was very similar to that of calmodulin (data not shown). This activation by BSA may explain why another laboratory that included 1 mg/ml BSA in their assays found no calmodulin effect on activity, although they could demonstrate ^{125}I-calmodulin binding (31), at very high phosphorylase concentrations, where the nonphysiological tetramer form of the enzyme would predominate (32).

The low affinity, lack of Ca^{2+}-dependence and lack of protein specificity make it difficult to propose a major physiological role for direct calmodulin

activation of phosphorylase in vivo in response to hormonal induced changes in intracellular [Ca^{2+}]. There may be a role for the calmodulin subunit of phosphorylase kinase to bind phosphorylase. Such an interaction would not be precluded by the proposed arrangement of phosphorylase kinase subunits within the enzyme complex (33). It is not known whether phosphorylase interacts with any of the subunits of phosphorylase kinase other than the catalytic subunit.

The ^{125}I-calmodulin-overlay technique is useful for a preliminary screening of fractions for calmodulin binding proteins. We have shown that binding to some proteins is proportional to the amount of protein in a band. The ability to relate binding to protein concentration would permit analysis of many samples or treatments for changes in a calmodulin binding protein. However, the results of this study demonstrate that calmodulin-protein binding detected by the ^{125}I-calmodulin overlay technique must be interpreted with caution. The properties predicted from observing binding to an electrophoresed protein in a gel matrix may be quite different than those found when the interaction is assessed in solution by other independent techniques. Consequently the ability of a purified protein or an identified protein on an SDS gel to bind ^{125}I-calmodulin in a Ca^{2+}-dependent manner, does not necessarily indicate that a Ca^{2+} stimulated activation of that protein involves a direct calmodulin interaction with the protein.

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