RESEARCH COMMUNICATION

Phosphorylation by casein kinase II alters the biological activity of calmodulin

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Calmodulin is the major intracellular Ca^{2+} -binding protein, providing Ca^{2+} -dependent regulation of numerous intracellular enzymes. The phosphorylation of calmodulin may provide an additional mechanism for modulating its function as a signal transducer. Phosphocalmodulin has been identified in tissues and cells, and calmodulin is phosphorylated both *in vitro* and in intact cells by various enzymes. Phosphorylation of calmodulin on serine/threonine residues by casein kinase II decreases its ability to activate both myosin-light-chain kinase and cyclic nucleotide phosphodiesterase. For myosin-light-chain kinase the primary effect is an inhibition of the V_{max} of the reaction, with no apparent change in the concentration at which half-maximal velocity is attained ($K_{0.5}$) for either Ca^{2+} or calmodulin. In contrast, for phosphodiesterase, phosphorylation of calmodulin significantly increases the $K_{0.5}$ for calmodulin without noticeably altering the V_{max} or the $K_{0.5}$ for Ca^{2+} . The higher the stoichiometry of phosphorylation of calmodulin, the greater the inhibition of calmodulin-stimulated activity for both enzymes. Therefore the phosphorylation of calmodulin by casein kinase II appears to provide a Ca^{2+} -independent mechanism whereby calmodulin regulates at least two important target enzymes, myosin-light-chain kinase and cyclic nucleotide phosphodiesterase.

INTRODUCTION

Calmodulin, an intracellular regulator of Ca^{2+} signals, modifies a diverse array of essential intracellular physiological processes by binding to enzymes [1,2]. The mechanism of differential activation of enzymes by calmodulin is unknown, but post-translational modification (e.g., phosphorylation) may represent a biologically important step in regulating calmodulin function. Phosphocalmodulin has been detected in chicken brain and muscle [3] and rat brain [4]. Calmodulin is phosphorylated *in vitro* by the insulin-receptor tyrosine kinase from a variety of tissues [5–8]. Mitogenic stimuli, such as insulin ([9]; D. B. Sacks, H. W. Davis, D. L. Crimmins & J. M. McDonald, unpublished work) and serum (J. Traugh, personal communication) are reported to stimulate calmodulin phosphorylation in intact rat adipocytes, rat hepatocytes (insulin) and 3T3-L1 cells (serum). In addition, serine/threonine kinases from a variety of tissues phosphorylate calmodulin *in vitro* [3,4,10–12] and in intact cells [3,4,13].

Casein kinase II is a multipotential serine/threonine kinase which phosphorylates proteins in the cytosol, nucleus and membranes *in vitro* and *in vivo* [14]. Recent evidence implicates a role for casein kinase II in the mechanism of action of certain growth factors. For example, insulin [15–17], epidermal growth factor [15,18] and insulin-like growth factor [17] increase the activity of casein kinase II. The observations that casein kinase II stimulates the phosphorylation of calmodulin *in vitro* [4,10] and in intact 3T3-L1 cells (J. Traugh, personal communication) and that insulin enhances serine/threonine phosphorylation of calmodulin in hepatocytes (D. B. Sacks, H. W. Davis, D. L. Crimmins & J. M. McDonald, unpublished work) led us to examine the effects of phosphorylation on calmodulin function. Here we demonstrate that phosphorylation of calmodulin *in vitro*

by casein kinase II alters the activation of two calmodulin-dependent enzymes, namely myosin-light-chain kinase and cyclic nucleotide phosphodiesterase.

EXPERIMENTAL

Materials

Ca^{2+} -free porcine brain calmodulin was obtained from Ocean Biologics (Edmond, WA, U.S.A.). By using a micro phosphate method [19] the maximum phosphate detected in this calmodulin is 1.33 nmol of phosphate/mg of calmodulin, which is equivalent to approx. 0.02 mol of phosphate/mol of calmodulin. All other reagents were from standard suppliers.

Protein phosphorylation assays

Phosphorylation was performed by preincubating 8.5 μM -calmodulin for 10 min at 30 °C in a reaction mixture containing 50 mM-Tris/HCl, pH 7.2, 10 mM- MgCl_2 , 500 μM -ATP, 1.7 μM -poly(L-lysine) (average molecular mass 41 kDa), 1 mM-EGTA and 0.4 mM- CaCl_2 (free Ca^{2+} concentration of 0.5 μM) in a final volume of 70 μl . The reaction was initiated by adding 150–225 enzyme units of casein kinase II (1 enzyme unit is the amount of enzyme that catalyses the incorporation of 1 μmol of ^{32}P into casein/min at 30 °C). After 90 min at 30 °C, the reaction was terminated by quick freezing. Phosphocalmodulin was separated from the other reagents by phenyl-Sepharose chromatography [20]. Briefly, 1 ml of phenyl-Sepharose was pre-equilibrated with 5 mM- CaCl_2 and 1.5 M-NaCl in 50 mM-Tris/HCl, pH 7.4 (buffer A). The Ca^{2+} concentration of the sample was adjusted to 2 mM and the sample was applied to the column. The column was washed extensively with buffer A (to remove polylysine), followed by 5 mM- CaCl_2 in 50 mM-Tris/HCl, pH 7.4 (buffer B). Calmodulin was eluted from the column with modified buffer B (the

Abbreviation used: $K_{0.5}$, concentration at which half-maximal velocity is obtained.

CaCl₂ being replaced by 2 mM-EGTA). Samples were desalted on a Bio-Rad 10 DG column and concentrated under vacuum. Control calmodulin was processed identically, except that casein kinase II was omitted from the phosphorylation assay.

Stoichiometry of calmodulin phosphorylation was determined by performing phosphorylation, as described above, in the presence of [γ -³²P]ATP. After the reaction had been stopped, 10% (w/v) trichloroacetic acid at 4 °C and 0.1% (w/v) BSA were added to the thawed sample, which was processed by SDS/PAGE as previously described [6]. The calmodulin was excised from the gel, solubilized and ³²P incorporation was determined by liquid-scintillation spectrometry [6]. The stoichiometry of phosphorylation is expressed as a percentage, with 100% representing 1 mol of phosphate/mol of calmodulin, and values ranged from 25 to 100%. The stoichiometry of the preparations used here are included in the Figure legends. The sites of casein kinase II-stimulated phosphorylation of calmodulin are Thr-79 (14%), Ser-81 (50%), Ser-101 (25%) and Thr-117 (11%) (values in parentheses are the relative amounts of phosphorylation at each site, expressed as percentage of total ³²P incorporation) [21]; D. B. Sacks, H. W. Davies, D. L. Crimmins & J. M. McDonald, unpublished work). Although in theory one could anticipate incorporation of 4 mol of phosphate/mol of calmodulin, we have been unable to achieve stoichiometries above 1 mol/mol.

Enzyme assays

Myosin-light-chain kinase activity assay mixtures contained 20 mM-Tris/HCl, pH 7.5, 5 mM-MgCl₂, 20 μ M bovine skeletal-muscle myosin light chain, 0.1–0.2 μ g of bovine platelet myosin-light-chain kinase, 0.1 mM-CaCl₂ and 0.125 mM-[γ -³²P]ATP and various concentrations of calmodulin or phosphocalmodulin, as indicated in the Figure legends, in a final volume of 200 μ l [22]. After 10 min at 22 °C, reactions were terminated by adding 50% (w/v) trichloroacetic acid/10% (w/v) sodium pyrophosphate, and ³²P incorporation was determined by spotting duplicate samples on to nitrocellulose squares (2 cm $\times$ 2 cm), washing extensively and performing liquid-scintillation counting.

Phosphodiesterase activity was measured by monitoring the fluorescence change of a methylantraniloyl derivative of cyclic GMP [23]. Standard assay mixtures contained 90 mM-KCl, 5 mM-MgCl₂, 10 mM-Mops, pH 6.8, 10 μ M-cyclic GMP, 4 nM- (of the 63 kDa isoenzyme of) cyclic nucleotide phosphodiesterase (generously provided by Dr. Rajendra Sharma, University of Calgary, Calgary, Alberta, Canada) and various concentrations of Ca²⁺, calmodulin or phosphocalmodulin, as indicated in the Figure legends, in a final volume of 2 ml. Excitation was at 280 nm and fluorescence changes were monitored at 450 nm at 25 °C.

Other methods

Casein kinase II, prepared from rabbit reticulocytes [24], was purified to apparent homogeneity (kindly provided by Dr. Jolinda Traugh, University of California, Riverside, CA, U.S.A.). The enzyme is inhibited by low doses of heparin, uses GTP as a substrate, exhibits β -subunit autophosphorylation and phosphorylates calmodulin exclusively on serine/threonine residues (D. B. Sacks, H. W. Davies, D. L. Crimmins & J. M. McDonald, unpublished work). Myosin-light-chain kinase was purified to apparent homogeneity from bovine platelets by a modification of the method of Hathaway & Adelstein [22]. After purification on the Sephacryl S-200-column, fractions containing myosin-light-chain kinase activity were applied to a column (1 cm $\times$ 10 cm) of phosphocellulose, and purification was continued as described in [22]. Protein content was determined by the method of Bradford [25], with BSA as standard. In five

preparations protein concentrations were also measured by amino acid analysis with a Waters Pico Tag column. All results obtained in the two assays agreed within 10%. Results were identical for both native calmodulin and phosphocalmodulin, demonstrating that phosphate incorporation does not alter protein binding to Coomassie Blue. Free Ca²⁺ concentrations were measured with an Orion Research Ca²⁺ ion electrode 93-20 [26].

RESULTS

Non-phosphorylated control calmodulin (incubated and purified on a phenyl-Sepharose column as described in the Experimental section) stimulates myosin-light-chain kinase activity in a concentration-dependent manner up to a maximum of 10.9 ± 2.5 -fold (mean $\pm$ S.E.M., $n = 3$) (Fig. 1). The $K_{0.5}$ (concentration at which half-maximal velocity is attained) is obtained at approx. 4 nM-calmodulin. This pattern of activation is virtually identical with that produced by untreated, non-phosphorylated, native calmodulin (results not shown). Calmodulin phosphorylated by casein kinase II (98% stoichiometry), however, not only fails to activate, but also slightly inhibits, myosin-light-chain kinase activity at high concentrations (Fig. 1). The relative stoichiometry of the phosphorylation of calmodulin by casein kinase II affects the degree of inhibition. In contrast with calmodulin phosphorylated to a stoichiometry of 98%, calmodulin phosphorylated to 50% is able to produce a slight dose-dependent increase in myosin-light-chain kinase activity (Fig. 1). However, maximum stimulation is only 24% of that achieved by control calmodulin. Phosphocalmodulin with stoichiometries between 50 and 98% exhibit intermediate effects on myosin-light-chain kinase activity, all being less effective stimulators than control calmodulin (results not shown).

The Ca²⁺-dependencies of the ability of calmodulin and casein kinase II-phosphorylated calmodulin to activate myosin-light-

Fig. 1. Activation of myosin-light-chain kinase by calmodulin and phosphocalmodulin

Kinase assays were performed in the presence of 100 μ M-Ca²⁺ and control calmodulin (□) or two different preparations of phosphocalmodulin (■, ◆) at the indicated concentrations. Phosphocalmodulin with a stoichiometry of 98% (■) or 50% (◆) was used. Kinase activity is expressed as a percentage of the maximal activity attained with control calmodulin. '0' on this scale is obtained in the absence of calmodulin. High concentrations (98%) of phosphocalmodulin resulted in measurements below this value, producing apparently negative activity. For control calmodulin each point represents the mean $\pm$ S.E.M. for three separate determinations each performed in duplicate. For each phosphocalmodulin the result of a representative experiment is shown, with each determination performed in duplicate. The absence of error bars indicates that the range is smaller than the size of the symbol.

Fig. 2. Ca^{2+} -dependence of myosin-light-chain kinase activation by calmodulin and phosphocalmodulin

Kinase assays were performed in duplicate in the presence of 28.2 nM-calmodulin (□) or 28.2 nM-phosphocalmodulin (50 %, ◆) at the Ca^{2+} concentrations indicated. A representative experiment is shown.

Fig. 3. Activation of phosphodiesterase by calmodulin and phosphocalmodulin

Phosphodiesterase assays were performed in the presence of 33 μM - Ca^{2+} and control calmodulin (□) or two different preparations of phosphocalmodulin (■, ▲) at the indicated concentrations. Phosphocalmodulin with a stoichiometry of 98 % (■) or 67 % (▲) was used. For control calmodulin each point represents the mean $\pm$ S.E.M. of two or three separate determinations. The absence of error bars indicates that the range is smaller than the size of the symbol. For each phosphocalmodulin, the result of a representative experiment is shown.

chain kinase were determined (Fig. 2). In the presence of control calmodulin, half-maximal and maximal activation of myosin-light-chain kinase is observed at approx. 90 μM - and 250–500 μM - Ca^{2+} respectively. With phosphocalmodulin at a stoichiometry of 50 %, the corresponding Ca^{2+} concentrations are virtually identical. This suggests that phosphorylation does not change the $K_{0.5}$ of calmodulin for Ca^{2+} . The activation of myosin-light-chain kinase by phosphocalmodulin is significantly less than that produced by control calmodulin at all Ca^{2+} concentrations studied (Fig. 2). This is consistent with the observation (see Fig. 1) that a change in V_{max} is the only alteration induced by phosphorylation.

Phosphorylation of calmodulin by casein kinase II also alters the interaction of calmodulin with cyclic nucleotide phosphodiesterase. Activation of phosphodiesterase by phosphocalmodulins with stoichiometries of 98 and 67 % is markedly inhibited (Fig. 3). Fig. 3 shows that the V_{max} for the enzyme appears relatively unchanged, but the $K_{0.5}$ values for both phosphocalmodulin preparations are markedly increased. For

Fig. 4. Ca^{2+} -dependence of phosphodiesterase activation by calmodulin and phosphocalmodulin

Phosphodiesterase assays were performed in the presence of 7 nM-calmodulin (□) or 7 nM-phosphocalmodulin (98 %, ■). The free Ca^{2+} concentrations indicated were obtained with Ca^{2+} /EGTA buffers. A representative experiment is shown.

calmodulin phosphorylated to 98 and 67 % the approximate $K_{0.5}$ values are 8.75 and 4.0 nM respectively, compared with 2.8 nM for non-phosphorylated calmodulin.

The Ca^{2+} -dependencies for calmodulin and phosphocalmodulin stimulation of phosphodiesterase are shown in Fig. 4. The dependencies on Ca^{2+} concentration for activation are similar for calmodulin and phosphocalmodulin. For both preparations maximal activity is obtained at 17 μM , with half-maximal activation occurring between 3 and 17 μM free Ca^{2+} . These data do not exclude the possibility of a subtle or minor shift in the $K_{0.5}$ for Ca^{2+} . The differences observed in the V_{max} in Fig. 4 are due to the fact that the concentrations of control and phosphocalmodulin, although the same (7 nM), give maximal and submaximal stimulation of phosphodiesterase respectively (see Fig. 3).

DISCUSSION

We have demonstrated that phosphorylation of calmodulin by casein kinase II inhibits the ability of calmodulin to activate myosin-light-chain kinase and cyclic nucleotide phosphodiesterase, albeit by apparently different mechanisms. The V_{max} for myosin-light-chain kinase is decreased in the presence of phosphocalmodulin without an apparent alteration in the $K_{0.5}$ for calmodulin. In contrast, with phosphodiesterase, the $K_{0.5}$ for phosphocalmodulin is decreased, whereas the V_{max} is essentially unchanged. The $K_{0.5}$ values for Ca^{2+} for both enzymes are unchanged, implying that these effects are independent of Ca^{2+} . In contrast with these observations, calmodulin phosphorylated on Tyr-99 by the insulin-receptor kinase increases phosphodiesterase activity and has increased affinity for Ca^{2+} [27].

The biological effects of other post-translational modifications of calmodulin are analogous to our observations. For example, acetylation of lysine residues of calmodulin decreases the tightness of binding to calcineurin, with multiple acetylations further decreasing the affinity [28]. In addition, post-translational methylation of calmodulin decreases the capacity of calmodulin to activate cyclic nucleotide phosphodiesterase, and the extent of this diminution in capacity correlates with the level of the methylation [29]. Similarly, in our studies, a higher stoichiometry of phosphorylation results in a greater decrease in the ability of calmodulin to activate both myosin-light-chain kinase and phosphodiesterase (see Figs. 1 and 3).

The specific regions of calmodulin involved in the binding and activation of calmodulin-dependent enzymes is the subject of considerable speculation. Recent evidence suggests that, in addition to the *N*- and *C*-terminal lobes of calmodulin, the central helix is in contact with myosin-light-chain kinase [30]. Replacement of residues Glu-82–Glu-84 with lysine [31] and phosphorylation of residues Thr-79, Ser-81, Ser-101 and Thr-117 of calmodulin by casein kinase II ([21]; D. B. Sacks, H. W. Davies, D. L. Crimmins & J. M. McDonald, unpublished work) produce identical effects. Both these forms of calmodulin increase the $K_{0.5}$ for calmodulin for phosphodiesterase and decrease the V_{max} for myosin-light-chain kinase. These remarkable similarities suggest that identical molecular mechanisms are responsible for the altered interaction with substrates. Since substitution with lysine residues and the covalent linkage of phosphate molecules both alter the charge in this region, electrostatic potential is probably responsible for the modified calmodulin activity. Consistent with this, when residues Ser-81–Glu-84 inclusive are deleted, but not replaced, the effect on biological activity is different [32]. Electrostatic potential is reported to be an important factor in the formation of calmodulin-target complexes [28]. Approx. 75% of casein kinase II-stimulated phosphate incorporation into calmodulin occurs on residues Thr-79, Ser-81 and Thr-117, which are adjacent to localized regions of high electrostatic potential [33], lending further credence to this hypothesis. The alteration of charge in the central helix could directly affect the interaction of calmodulin with its target proteins, or could do so indirectly by reducing the flexibility of the hinge region. Other possible explanations for the decreased biological activity of phosphocalmodulin include an alteration in the tertiary conformation of calmodulin by phosphate, steric hindrance of the calmodulin–enzyme interactions by the phosphate groups, or an alteration in Ca^{2+} binding to calmodulin.

Our data demonstrate that the casein kinase II-stimulated incorporation of phosphate groups into the central helix and a third Ca^{2+} -binding pocket alters the interaction of calmodulin with two important target enzymes. Since calmodulin is phosphorylated on four residues by casein kinase II, different forms of phosphocalmodulin are present in the preparations we examined. Work is required to separate these different forms and determine the effect of each on myosin-light-chain kinase and phosphodiesterase activity. Recently we demonstrated that sequential phosphorylation of calmodulin, initially by casein kinase II followed by the insulin receptor, both enhances insulin-receptor-mediated phosphorylation and alters the specificity of the receptor kinase ([21]; D. B. Sacks, H. W. Davies, D. L. Crimmins & J. M. McDonald, unpublished work). Calmodulin would be an ideal locus for the convergence of multiple kinases. Phosphorylation by casein kinase II and other kinases may represent a critical mechanism for differentially regulating the activity of calmodulin independently of Ca^{2+} .

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