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Phosphorylation of caldesmon

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The phosphorylation of caldesmon was studied to determine if kinase activity reflected either an endogenous kinase or caldesmon itself. Titration of kinase activity with calmodulin yielded maximum activity at substoichiometric ratios of calmodulin/caldesmon. The sites of phosphorylation on caldesmon for calcium/calmodulin-dependent protein kinase II and endogenous kinase were the same, but distinct from protein kinase C sites. Phosphorylation in the presence of Ca^{2+} and calmodulin resulted in a subsequent increase of endogenous kinase activity in the absence of Ca^{2+}. These results suggest that caldesmon is not a protein kinase and that kinase activity in caldesmon preparations is due to calcium/calmodulin-dependent protein kinase II.

Phosphorylation; Caldesmon; Calmodulin (Ca^{2+})-dependent kinase II; (Smooth muscle)

1. INTRODUCTION

Phosphorylation of myosin is accepted as an important regulatory component in smooth muscle [1]. However, with a single mechanism it is difficult to account for the variety of responses seen under physiological conditions and other regulatory mechanisms have been implicated to provide a greater flexibility. Caldesmon is the most cited possibility. This suggestion is based on its inhibition of superprecipitation [2] and Mg^{2+}-ATPase of actomyosin [3]. A problem with caldesmon as a potential regulatory mechanism is its relatively weak binding to Ca^{2+}-CaM. Most K_ds are reported to be in the μM range [4], considerably weaker than the Ca^{2+}-CaM-MLCK complex. Thus it is unlikely that the Ca^{2+}-CaM-caldesmon complex could exist under conditions where MLCK is inactive. Caldesmon can be phosphorylated and it was suggested that phosphorylated caldesmon does not inhibit ATPase activity [3] nor bind to myosin [5]. The former point is controversial [6,7]. However, if phosphorylation of caldesmon is involved in physiological function, it is important to identify the caldesmon kinase since this will determine the Ca^{2+}-sensitivity of this system. It is established that caldesmon can be phosphorylated by protein kinase C [8,9], casein kinase II [9] and CaM-kinase II [9]. The intriguing possibility also was raised that caldesmon itself is a kinase subject to autophosphorylation [10]. Our objectives were to characterize the kinase activity associated with caldesmon preparations and to evaluate whether caldesmon itself is a kinase and if not, to identify the kinase.

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Abbreviations: CaM, calmodulin; MLCK, myosin light chain kinase; CaM-kinase II, calcium-calmodulin-dependent protein kinase II; EGTA, ethylene glycol *bis*(β-aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid

2. MATERIALS AND METHODS

Caldesmon was prepared by the methods of Bretscher [11] (involving a heat treatment) and Lash et al. [6] (not involving a heat treatment). For the latter procedure the caldesmon following chromatography on DEAE-Sephacel was concentrated by ammonium sulfate precipitation. The concentration of ammonium sulfate used was important and influenced the level of kinase activity retained with caldesmon. Kinase activity was reduced in caldesmon preparations subject to a prior fractionation at 30% ammonium sulfate saturation. (Caldesmon precipitates between 30% and 50% saturation.)

Calmodulin was isolated from frozen bull testes [12], MLCK from frozen turkey gizzard [12] and protein kinase C was partially purified from bovine brain [13]. CaM-kinase II, isolated from rat brain [14] was a generous gift from Dr H. Schulman (Stanford University). A polyclonal antibody to CaM kinase II was generated in rabbits using a synthetic peptide, corresponding to residues 39–70 [15], coupled to keyhole limpet hemocyanin. Electrophoresis and Western blots were carried out as described previously [16]. Conditions for phosphorylation assays [12] are given in the figure legends. Protein concentrations were determined with the BCA protein reagent (Pierce).

3. RESULTS AND DISCUSSION

3.1. *Titration of caldesmon kinase activity with calmodulin*

The endogenous kinase activity present in caldesmon preparations is dependent on Ca^{2+} and CaM [3]. Either caldesmon is a kinase or the caldesmon preparations

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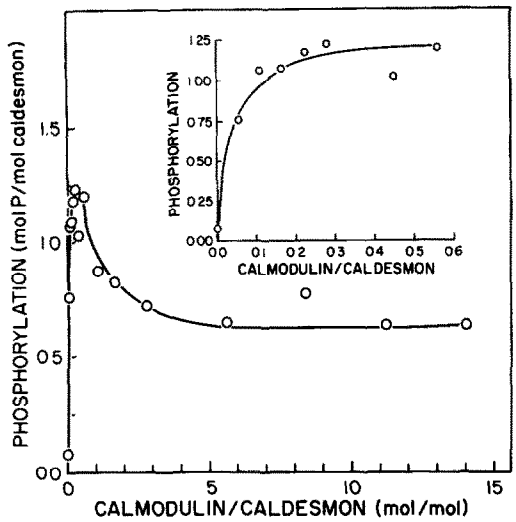

Fig.1. Titration of kinase activity with calmodulin. Assay conditions: 1 mM [γ-^{32}P]ATP, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 50 mM KCl, 30 mM Tris-HCl (pH 7.5), 0.25 mg/ml caldesmon, 25°C, and varying amounts of calmodulin. Phosphorylation level determined at 1 h. Inset shows expanded scale at low calmodulin concentrations.

contain a CaM-dependent protein kinase. (MLCK was eliminated as one of the latter [3,17].) To distinguish between these possibilities the kinase activity was titrated with CaM as shown in fig.1. Maximum kinase activity was obtained at a ratio of CaM/caldesmon of considerably less than one. The inset of fig.1 shows an expanded scale at low CaM levels and maximum activity was obtained at a molar ratio of CaM/caldesmon of between 0.1 and 0.2. These results suggest that caldesmon is not a kinase and that the affinity of the unknown kinase for CaM is stronger than the affinity of the CaM-caldesmon complex. Activation constants for CaM and CaM-kinase II are in the range of 20–100 nM [18].

3.2. *Phosphorylation of caldesmon by CaM-kinase II and protein kinase C*

In fig.2A are shown time courses of phosphorylation of caldesmon by CaM-kinase II and the endogenous kinase. Phosphorylation of heat-treated caldesmon by CaM-kinase II showed a faster initial phase and a slower second phase, each accounting for about 1 mol P/mol caldesmon. Vorotnikov et al. [9] reported that two sites on caldesmon are phosphorylated by CaM-kinase II, located in the N- and C-terminal domains. Phosphorylation of caldesmon by endogenous kinase also is shown in fig.2A. The addition (at the arrow) of purified CaM-kinase II does not increase the phosphorylation level. These results suggest that the sites of phosphorylation for CaM-kinase II and the endogenous kinase are the same.

The inset of fig.2A shows protein staining patterns and Western blots of caldesmon and purified CaM-kinase II. Components consistent with the CaM-kinase II subunits were detected by the CaM-kinase II antibody in the caldesmon preparation containing kinase activity. Some reaction of the antibody with caldesmon was observed. Comparison of the CaM-kinase II peptide with caldesmon [19] indicates about 50% match (for identity and conservative substitutions) with the sequence 224–248.

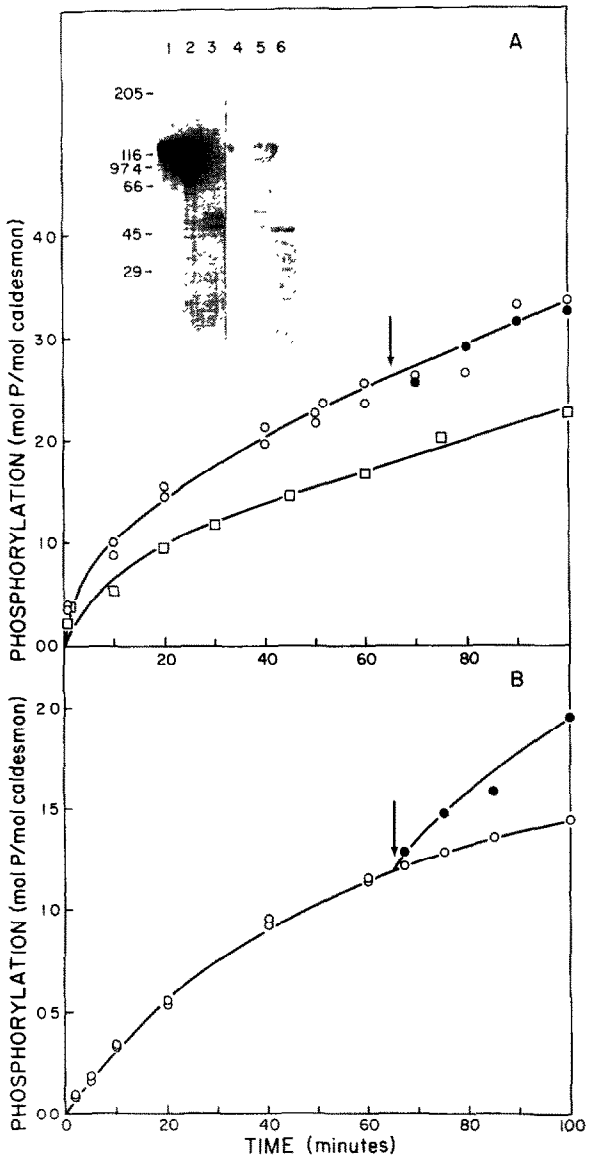

Fig.2. Phosphorylation and sequential phosphorylation of caldesmon by CaM-kinase II and protein kinase C. (A) □, phosphorylation of heat-treated caldesmon (0.28 mg/ml) by CaM-kinase II (0.15 μg/ml); ○, phosphorylation of non-heat treated caldesmon (0.28 mg/ml) by endogenous kinase plus calmodulin (0.1 mg/ml); ●, at the arrow CaM-kinase II (0.15 μg/ml) added. Inset shows protein staining patterns (1–3) and Western blots (4–6) of heat-treated caldesmon (1,4), caldesmon containing kinase activity (2,5) and purified CaM-kinase II (3,6). (B) ○, phosphorylation of caldesmon (0.28 mg/ml) by endogenous kinase plus calmodulin (0.1 mg/ml). At arrrow (●) 20 μl of partially purified protein kinase C, 100 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate and 150 μg/ml phosphatidylserine added. Other conditions as in fig.1.

In fig.2B are shown time courses of sequential phosphorylation by endogenous kinase and protein

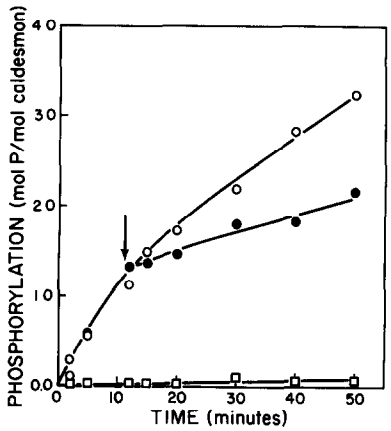

Fig.3. Ca^{2+}-dependence of endogenous kinase. Assay conditions: 1.5 mM [γ-^{32}P]ATP, 7.5 mM $MgCl_2$, 75 mM KCl, 30 mM Tris-HCl (pH 7.5), 0.38 mg/ml caldesmon, 0.08 mg/ml calmodulin. Reaction started in presence of 1.5 mM EGTA (□); reaction started in presence of Ca^{2+} (○); EGTA added to 1.5 mM at arrow (●).

kinase C. The addition of protein kinase C resulted in an increase in the level of caldesmon phosphorylation. This indicates that the phosphorylation sites for endogenous kinase and protein kinase C are distinct.

3.3. *Ca^{2+}-dependence of endogenous kinase*

CaM-kinase II shows a unique feature in that autophosphorylation results in the generation of a partially Ca^{2+}-independent kinase [18]. It was of interest to determine if this property is observed for the endogenous kinase. Caldesmon was phosphorylated by endogenous kinase in the presence of Ca^{2+} and CaM (i.e. conditions suitable for autophosphorylation of CaM-kinase II). After 13.5 min excess EGTA was added and the subsequent rate of phosphorylation monitored. The phosphorylation rate of caldesmon initiated in the presence of EGTA was approximately 2 mmol P transferred/mol caldesmon/min (fig.3). For the reaction started in the presence of Ca^{2+} followed by the addition of EGTA this rate increased approximately 10-fold (fig.3). These results are consistent with the autophosphorylation of the endogenous kinase, occurring in the presence of Ca^{2+} and CaM, and the subsequent reduction of Ca^{2+}-sensitivity.

Autoradiograms of aliquots withdrawn 10 min after the addition of EGTA show the incorporation of ^{32}P into the caldesmon bands plus a component of approximately 60 kDa (data not shown). This could be the β-subunit of CaM-kinase II [18,20].

The above results indicate that caldesmon itself is not a kinase and that the CaM-dependent kinase activity associated with caldesmon probably is due to CaM-kinase II. Conserved kinase sequences were not detected in the caldesmon sequence [19]. If phosphorylation of caldesmon is involved in any physiological process its Ca^{2+}-dependence would reflect the interaction of CaM with CaM-kinase II rather than that of the CaM-caldesmon complex. The role of CaM-kinase II in smooth muscle activity is not established and whether caldesmon is linked to its physiological function is a priority for future research.

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