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Phosphorylation sequences in h-caldesmon from phorbol ester-stimulated canine aortas

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The high molecular weight form of caldesmon (h-caldesmon) is phosphorylated in vascular smooth muscle. The stoichiometry of caldesmon phosphorylation increases in response to stimulation of the muscle by several contractile agonists; however, the responsible kinase has not been identified. In this study, we have sequenced the phosphopeptides prepared from h-caldesmon phosphorylated in vitro by protein kinase C (PKC) as well as the phosphopeptides prepared from caldesmon phosphorylated in intact canine aortas that were stimulated to contract with PDBu. PKC phosphorylated three sites located in the C terminus: GSS*LKIEE, AEFLNKS*VQK and NLWEKQS*VDK, while h-caldesmon from intact tissue was phosphorylated at two separate sites also in the C terminus: VTS*PTKV and S*PAPK. By comparison to known substrate consensus sequences for various protein kinases these data suggest that h-caldesmon is directly phosphorylated by a proline-directed protein kinase and not by PKC.

Caldesmon; Smooth muscle; Protein phosphorylation; Protein kinase C; Proline-directed protein kinase

1. INTRODUCTION

Caldesmon is an actin binding protein that was first identified in gizzard smooth muscle as a 140,000 Da band on polyacrylamide gels (h-caldesmon) [1]. h-Caldesmon has since been found in all types of smooth muscle and a lower molecular weight variant of approximately 80,000 Da (l-caldesmon) has been identified in non-muscle cells [2,3]. Moreover, cDNA's for both forms have been cloned and sequenced [4,5]. h-Caldesmon is known to inhibit actomyosin ATPase activity, in vitro [6–9] and to increase the binding of actin to myosin [8]. The latter effect may be the result of 'tethering', that is, binding of the carboxyl terminus of caldesmon to actin and the amino terminus to myosin [9,10]. Because of these effects and complex interactions, h-caldesmon is postulated to modulate the contractile properties of vascular smooth muscle [2,3,11].

Caldesmon is a phosphoprotein in vascular smooth muscle [12–14] and in non-muscle cells. In non-muscle cells, 'caldesmon kinase' has been identified as $p34^{cdc2}$ and phosphorylation leads to dissociation of l-caldesmon from actin during mitosis [15–18]. In the vasculature, h-caldesmon is phosphorylated in response to several contractile agonists that induce sustained isometric tension, including PDBu and KCl [12,13]. However, while several kinases are known to phosphorylate h-caldesmon, in vitro [19,20–22], the native 'caldesmon kinase' and the proximate second messengers required for activation have not yet been identified.

In this study, we directly addressed the question of whether h-caldesmon, in intact tissue stimulated to contract with PDBu, is phosphorylated by PKC. Our results demonstrate that the caldesmon phosphopeptides prepared from intact, stimulated aortas are different from those prepared from caldesmon phosphorylated in vitro by PKC. We suggest that phosphorylation of h-caldesmon in response to PDBu may result from a cascade that ultimately leads to activation of a proline-directed protein kinase.

Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; MOPS, morpholinopropane sulfonic acid; PKC, protein kinase C; PDBu, phorbol-12,13-dibutyrate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, *Nα-p*-tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride.

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2. EXPERIMENTAL

2.1. *Materials*

Most chemicals and reagents were purchased from Sigma. [^{32}P]orthophosphate and [^{32}P]ATP were from New England Nuclear. *S. aureus* protease was obtained from Pierce, trypsin-TPCK treated was from Worthington and endoproteinase Lys-C was from Boehringer Mannheim. All HPLC columns were purchased from Bio-Rad.

2.2. *Phosphorylation of caldesmon by protein kinase C*

Porcine stomach h-caldesmon was maximally phosphorylated by protein kinase C, in the presence of [^{32}P]ATP by a method we have described previously [12]. h-Caldesmon was digested with trypsin and the phosphopeptides separated using a combination of iron-chelate affinity chromatography [23] and reverse-phase HPLC. Phosphopeptides were sequenced with the Applied Biosystems Model 477 Protein Sequencer using procedures described previously [24]. The identity of the phosphorylated residues was deduced from several experimental

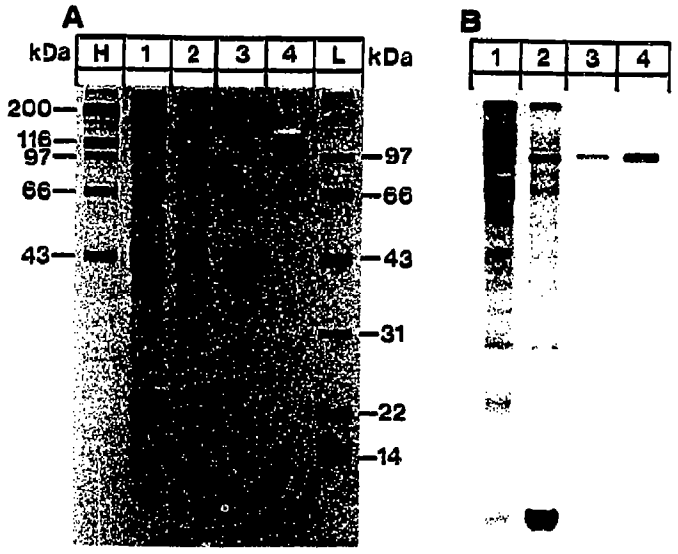

Fig. 1. Purification of caldesmon from canine aorta. $^{32}PO_4$-labeled caldesmon was purified from canine aorta as described under experimental procedures. Panel A is a Coomassie blue-stained polyacrylamide gel (7.5%) and panel B is an autoradiogram of the gel in panel A. Lane 1 is the original boiled homogenate; lane 2 shows the proteins after 30–50% ammonium sulfate precipitation; lane 3 is after Sepharose-4B chromatography; and lane 4 is after DEAE purification. Lanes H and L contain molecular weight markers.

protocols including phosphoamino acid analysis, a comparison of the ratio of DTT-serine/PTH-serine in the sequencing cycle [25,26] and a correlation of the temporal sequence of [^{32}P]phosphate liberation with amino acid sequence [27].

2.3. h-Caldesmon purification from intact tissue and sequencing of the phosphopeptides

Tissue strips were dissected from canine aortas and stimulated to contract for 30 min by 1 μM PDBu at 36°C. PDBu was chosen as the agonist because phosphopeptide maps of caldesmon from arteries that are unstimulated (relaxed) or stimulated to contract with either KCl or PDBu are identical [12]. The only difference upon agonist stimulation, is the level of phosphate incorporated into caldesmon. In order to label caldesmon, some tissue strips were pre-incubated for 90 min in a solution containing [^{32}P]orthophosphate (5 mCi/4 ml) in place of unlabeled phosphate. At the end of PDBu stimulation, tissue strips were freeze-clamped with liquid N_2-cooled tongs and ground to a fine powder. The ground tissue (58.7 g) was added to 6 vols of boiling extraction buffer that consisted of 300 mM KCl, 5 mM EGTA, 1 mM EDTA, 25 mM NaF, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 mM imidazole, pH 7.0, 10 mM dithiothreitol, 1 μg/ml leupeptin and 0.1 mM each of TPCK, TLCK and PMSF. h-Caldesmon was purified from this solution by centrifugation, differential ammonium sulfate precipitation, and chormatography using Sepharose-4B and DEAE-Sephacel. The chromatographic separations were performed using a buffer that consisted of 20 mM MOPS, pH 7.0, 10 mM NaF and 1 mM each of EDTA, EGTA, dithiothreitol, sodium orthovanadate and sodium pyrophosphate.

Purified h-caldesmon was dialyzed against NH_4HCO_3, lyophillized and then subjected to proteolysis using 30 μg *S. aureus* protease. Peptides were purified by HPLC using a combination of reverse-phase

Table I

Sequences of phosphorylation sites on h-caldesmon from stimulated canine aorta. Peaks A and B (Fig. 2b and 2c) contained the canine aorta phophopeptides listed under 'original sequence(s)'. Also listed are the caldesmon sequences from human fibroblast [5], rat liver [18] and chicken gizzard [4] that most closely match the canine aorta sequence. Differences in primary sequence are indicated by lower case letters and gaps in the sequence are denoted by a dash

	Peak A sequences	
	Original sequences	Limit phosphopeptide[a]
Canine aorta	**NTFSRPGAGARASEE**	
Human	**NTFSRPG**--g**RAS**vd	
Rat	**NaFS**psrsgg**RAS**gd	
Chicken	Unknown	
Canine aorta	**KQSVDKVTS*PTKV**	**VTS*PTKV**
Human	**KQSVDKVTS PTKV**	
Rat	**KQSVDKVTS PTKV**	
Chicken	**KQSV**eKpaa sssk	
	Peak B sequences	
	Original sequence	Limit phosphopeptide[b]
Canine aorta	**GLTKTPDGNKS*PAPKPSDLRPGDVSGK**	**S*PAPK**
Human	**wLTKTPDGNKS PAPKPSDLRPGDVSsK**	
Rat	**wLTKsPDGNKS PAPKPSDLRPGDVSGK**	
Chicken	**wLTKTPeGNKS PAPKPSDLRPGDVSGK**	

[a]This phosphopeptide contained all the radioactivity in peak A and was purified after subjecting peak A to tryptic digestion. A phosphoamino acid analysis of this peptide yielded only phosphoserine.

[b]This phosphopeptide contained all the radioactivity in peak B and was purified after subjecting peak B to digestion with trypsin and endoproteinase Lys-C.

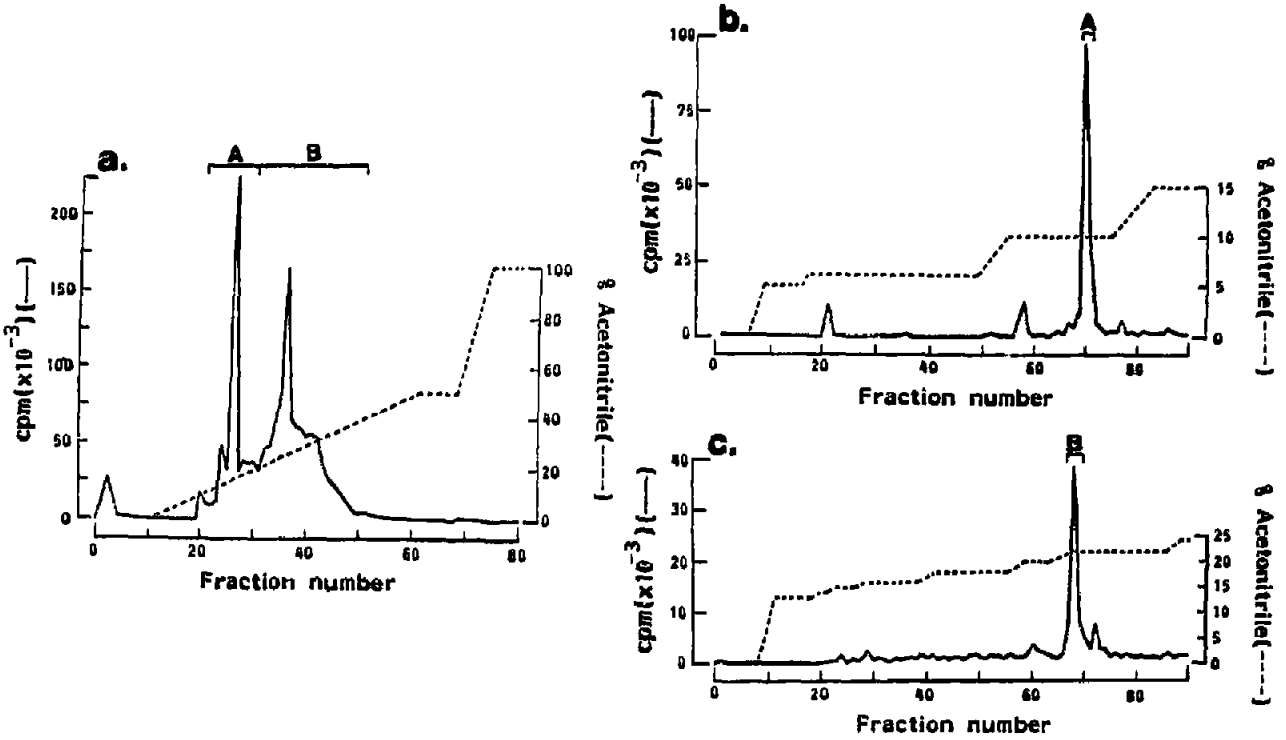

Fig. 2. Purification of caldesmon phosphopeptides. The sample shown in lane 4 of Fig. 1 was digested, and the phosphopeptides purified, as described in section 2. Panel a shows phosphopeptide elution from a C18 reverse-phase column. Fractions containing peaks A and B were pooled, purified by ion-exchange chromatography and then eluted from the same C18 column as shown in panels b and c, respectively.

and ion-exchange columns, and then sequenced as above. Where indicated, further digestion of phosphopeptides was carried out using trypsin in 100 mM NH_4HCO_3 or endoproteinase Lys-C in a buffer consisting of 25 mM Tris, pH 8.5 and 1 mM EDTA.

3. RESULTS

The results of caldesmon purification from phorbol ester-stimulated canine aortas are shown in Fig. 1. Importantly, the $^{32}PO_4$/caldesmon ratio remained constant throughout purification, verifying the absence of concurrent phosphorylation or dephosphorylation. Passage of the protease-digested caldesmon over a C18 column resolved two major radioactive peaks (Fig. 2a). These two initial peaks were further purified by HPLC to yield peaks A and B (Fig. 2b and c), that were present in approximately equal amounts after taking into account losses incurred throughout purification. In the original sample, 58% of the radioactivity was incorporated into caldesmon; the remainder was distributed evenly among proteins on polyacrylamide gels. Of the total radioactivity in caldesmon, 81% was accounted for by the $^{32}PO_4$ in peaks A and B. No other radioactive peaks were detected in any of the various HPLC runs.

Upon sequence analysis, peak A (Fig. 2b) contained two peptides possessing several serine or threonine residues (Table I). This sample was further digested with

Table II

Major sequences on porcine stomach caldesmon phosphorylated by protein kinase C. Porcine stomach caldesmon was phosphorylated by PKC and the phosphopeptides were purified as described under experimental procedures. The upper sequence in each set is the PKC phosphorylation site in porcine stomach caldesmon. The lower sequences are the closest matching sequences in caldesmon from human fibroblast [5], rat liver [18] and chicken gizzard [4]. Percent of total phosphate is equal to the amount of phosphate incorporated into the site divided by the total amount of phosphate incorporated into caldesmon

Source	Sequence	Percent of total phosphate in caldesmon
Porcine	**GSS*LKIEE**	**50**
Human	GSS LKIEE	
Rat	GSS LKIEE	
Chicken	GSS LKIEE (592)	
Porcine	**AEFLNKS*VQK**	**35**
Human	AEFLNKS VQK	
Rat	AEFLNKS VQK	
Chicken	AEFLNKS AQK (603)	
Porcine	**NLWEKQS*VDKVTSPTK**	**15**
Human	NLWEKQS VDKVTSPTK	
Rat	NLWEKQS VDKVTSPTK	
Chicken	NLWEKQS VEKPAASSS (735)	

trypsin and the resulting phosphopeptide purified by HPLC. Sequencing of this limit phosphopeptide yielded the sequence, VTSPTKV (Tabel I), that is not found in gizzard caldesmon. Phosphoamino acid analysis confirmed that phosphate was incorporated into a serine residue (data not shown). Peak B (Fig. 2c) consisted of only one peptide that began with a sequence corresponding to position 692 in gizzard caldesmon. The length of this original peptide and its termination site could not be determined. Treatment of peak B with trypsin gave the sequence Thr^{695}-Lys^{718} (Table I) of gizzard caldesmon and further digestion with endoproteinase Lys-C gave the sequence SPAPK (Table I). Table II summarizes the phosphopeptide sequences prepared from caldesmon phosphorylated in vitro by PKC.

4. DISCUSSION

In earlier studies, we reported that caldesmon is phosphorylated during isometric contraction of vascular smooth muscle [12,13]. Moreover, we observed that phosphopeptide maps prepared from ^{32}P-labeled caldesmon immunoprecipitated from unstimulated muscles or muscles stimulated with KCl or PDBu were indistinguishable from one another but clearly different from maps prepared from caldesmon phosphorylated in vitro with Ca^{2+}/calmodulin kinase II or PKC [12]. From this we concluded that 'caldesmon kinase' was neither PKC nor Ca^{2+}/calmodulin kinase II. Of course, it is possible that in response to stimulation by other agonists, these kinases might catalyze caldesmon phosphorylation in intact muscle.

In the present study, we selected PDBu as the agonist for investigation because it is known to bind directly to and activate PKC. Activated PKC can phosphorylate a number of contractile, cytoskeletal and ion channel/antiporter proteins any or all of which may lead to the increase in isometric tension that is observed in PDBu-treated vascular muscle [28]. PKC could also activate other protein kinase systems through a phosphorylation cascade or via changes in intracellular Ca^{2+}.

From the present study it is clear that caldesmon is phosphorylated in PDBu-stimulated muscles by a proline-directed protein kinase and not by PKC. In non-muscle cells, one kind of proline-directed protein kinase, $p34^{cdc2}$, has been implicated as a 'caldesmon kinase' and recently this enzyme has been shown to phosphorylate h-caldesmon in vitro [17,19]. However, another family of proline-directed protein kinases, the MAP kinases (i.e. MAP, microtubule-associated protein) is also present in many mammalian tissues [29]. Recently, we have identified two members of this family, $p42^{mapk}$ and $p44^{mapk}$, in bovine aortic smooth muscle and in cultured bevine aortic cells (Adam and Hathaway, in preparation). Thus, considerably more work remains to identify 'caldesmon kinase' with certainty. However, since the MAP kinases and $p34^{cdc2}$ have been implicated in growth, differentiation and proliferation of cells [29] and vascular smooth muscle has been shown to contract in response to stimulation with several growth factors [30], further investigation of the problem of caldesmon phosphorylation in vascular muscle may elucidate additional links between growth and contractile regulation.

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