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Absence of calponin phosphorylation in contracting or resting arterial smooth muscle

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We have tested the hypothesis of Winder and Walsh [(1990) J. Biol. Chem. 265, 10148] that the contractile state of smooth muscle is regulated by calponin phosphorylation. Porcine carotid arterial muscles were highly labeled with ^{32}P, then contracted with four different agents for various times. No radioactivity was detected in calponin isolated by 2D or 1D gel electrophoresis from the muscles. Similarly, resting muscles showed no [^{32}P]phosphate in calponin. Apparently the sites of calponin available for phosphorylation in vitro are rendered unavailable in the intact muscle.

Calponin phosphorylation; Smooth muscle contraction; Carotid artery

1. INTRODUCTION

It is generally accepted that phosphorylation of the 20-kDa myosin light chain is the primary regulator of smooth muscle contraction [1,2]. However, regulation through other proteins located in the thin filaments such as leiotonin [3] and caldesmon [4] has also been emphasized. Recently, Takahashi and collaborators purified a heat stable, basic, 34-kDa protein from chicken gizzard [5] and bovine aorta [6]. This protein, named calponin, interacted with actin, tropomyosin, and calmodulin, and possessed the ability to inhibit the actin-activated MgATPase activity of smooth muscle myosin [7,8]. Futhermore, calponin could be phosphorylated by protein kinase C or Ca^{2+}/calmodulin-dependent protein kinase II [8,9]; and most important, upon phosphorylation, calponin lost its ability to inhibit the actin-activated myosin MgATPase [8]. This finding led Winder and Walsh to postulate that smooth muscle contraction may be regulated by calponin phosphorylation [8].

We were searching for the equivalent of the in vitro calponin phosphorylation in intact smooth muscle. Porcine carotid arteries were contracted with various agents for a short or prolonged time but showed no evidence for calponin phosphorylation. Also, no phosphorylation of calponin was found in resting arterial muscles. It appears that the sites of calponin available for phosphorylating enzymes in vitro are unavailable in vivo.

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Abbreviations: PSS, physiological salt solution; 1D, one-dimensional; 2D, two-dimensional; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-amino-ethyl ether)-*N,N,N',N'*-tetraacetic acid; NE, norepinephrine; PDBu, phorbol dibutyrate; DTT, dithiothreitol

2. EXPERIMENTAL

2.1. *Muscle preparation*

Porcine carotid arteries were obtained from the local abatoir and were carried to the laboratory in ice cold PSS (130 mM NaCL, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 0.03 mM CaEDTA, 14.9 mM $NaHCO_3$ and 5.5 mM glucose). After cleaning the arteries, the isolated smooth muscles (approximately 4.5 cm long and 0.4–0.5 cm wide) were attached at one end to Grass FT03 force transducers and stretched to simulate 100 mm Hg mean arterial pressure as described in detail [10]. The arterial muscles were then equilibrated with 70 ml of PSS bubbled with a gas mixture of 95% O_2/5% CO_2 (pH 7.3–7.4) at 37°C for 15 min. Carrier-free [^{32}P]orthophosphate, 2–4 mCi, was added to the bath and the muscles were further equilibrated for 90 min. The muscle strips were then washed 15 times with PSS in 30 min to remove ^{32}P from the extracellular space of the muscles. Subsequently, the muscles were stimulated with various agents and the tension was monitored on a Grass polygraph. At various times in contraction, the muscles were rapidly immersed, while still mounted in muscle chambers, into Dewar flasks containing liquid nitrogen. Resting, unstimulated, muscles were treated identically.

2.2. *Protein preparation*

The frozen muscle strips were pulverized to a powder by percussion using liquid nitrogen-chilled mortars and pestles in the cold room at 4°C. The frozen powder was extracted with 3% perchloric acid and after centrifugation the residue was washed three times with a solution containing 2% trichloracetic acid and 5 mM KH_2PO_4. The final residue was solubilized in 0.25 M Na_2HPO_4 and 0.5% SDS, and the proteins were dialyzed against 2000 volumes of 0.02% SDS and 1.0 mM $(NH_4)HCO_3$ at 25°C overnight. After clarification in the ultracentrifuge, the protein content of the supernatant was determined by the modified biuret method [11], and the proteins were separated by 2D and 1D gel electrophoresis [10].

2.3. *Purification of calponin*

Following the procedure of Abe et al. [12], porcine aorta muscle was heated in a boiling water bath for 2 min, chilled, minced, and homogenized in a solution containing 300 mM KCl, 50 mM histidine buffer (pH 6.9), 1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride and 10 μg leupeptin/ml. After centrifugation at 12000 × g, the supernatant was centrifuged at 150000 × g, and the supernatant was brought to 30% saturation with ammonium sulfate. From this step on our procedure differs from that of Abe et al. [12]. The precipitate of the 0–30% ammonium sulfate fractionation was collected and redissolved by dialysis against the homogenization solution. After centrifugation at 150000 × g, the supernatant was refractionated with ammonium sulfate, 0–30% saturation. The pellet was dissolved by dialysis against 0.1% SDS, 10 mM $(NH_4)HCO_3$ and 1 mM DTT at 25°C clarified by ultracentrifugation. The supernatant was subjected to SDS-PAGE analysis and was found to contain about 75% of the total protein as calponin.

Pure calponin was isolated from this partially purified calponin by preparative SDS-PAGE using 15% polyacrylamide gels, 10.5 cm long. A guide strip was cut from the slab gels, stained and destained for the localization of the calponin band. Calponin was eluted from the unstained gels with a solution containing 0.1% SDS, 200 mM $(NH_4)HCO_3$ and 10 mM DTT at 37°C, dialyzed against distilled water exhaustively, and freeze-dried. This calponin preparation appeared to be a single band on 1D gels as shown on Fig. 1. On 2D gels it also migrated as a single band, focusing in the alkaline pH range, in agreement with the finding of Takahashi et al. [13] that calponin has several isoelectric variants in the pH region of 8.4–9.1.

3. RESULTS

3.1. *Analysis of calponin phosphorylation by 1D gel electrophoresis*

When electrophoresed on SDS-polyacrylamide gels, calponin migrates slightly faster than tropomyosin [7]. Since tropomyosin is not phosphorylated in vertebrate smooth muscle, and no other protein is phosphorylated in this molecular weight range, SDS-PAGE is a useful method for detecting possible calponin phosphorylation. Fig. 1 shows a typical experiment. The purified calponin migrates as a single band and it serves as a marker for the identification of calponin in the total muscle extract. The figure compares phosphorylation in arterial muscles stimulated with 100 mM KCl [10] for 1 and 60 min versus the unstimulated, resting, muscle. No radioactivity is detected in calponin of either contracting or resting muscle. Under these conditions, the myosin light chain, the 28-kDa protein and desmin are markedly phosphorylated. The phosphorylation in the high molecular weight region is due to proteins and nucleic acids. The figure shows the known changes in myosin light chain phosphorylation accompanying contraction and also changes in the phosphorylation of other proteins.

Furthermore, calponin phosphorylation was not detected by 1D gel electrophoresis in muscles contracted with NE, histamine, PDBu, or in resting muscles.

3.2. *Analysis of calponin phosphorylation by 2D gel electrophoresis*

Fig. 2 illustrates the staining profile of a 2D gel and the corresponding autoradiogram of the total muscle

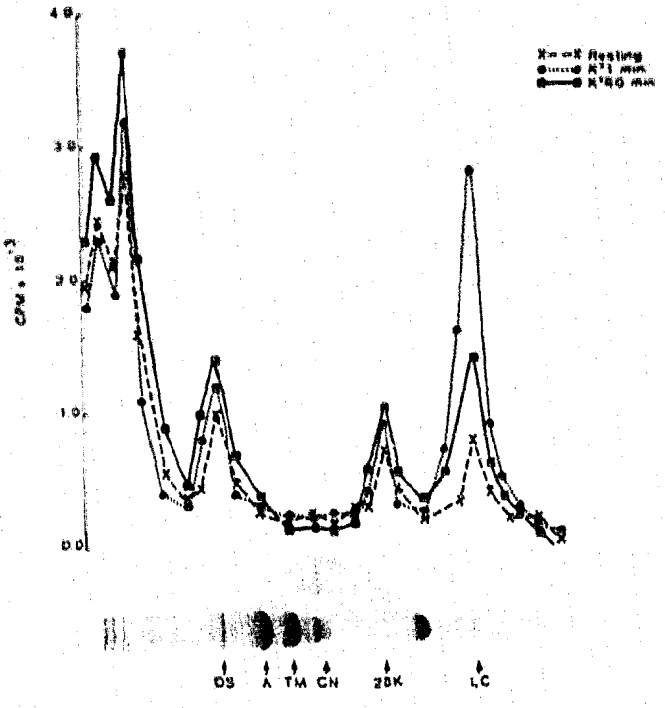

Fig. 1. SDS-PAGE (15% gels) of purified calponin (upper gel) and of total protein extract (lower gel) of ^{32}P-labeled arterial muscles, resting and contracting. For radioactivity determination, the gel with the total protein extract was sliced, digested with H_2O_2, and counted in a Triton-based liquid scintillation fluid. DS, desmin; A, actin; TM, tropomyosin; CN, calponin; LC, 20-kDa myosin light chain.

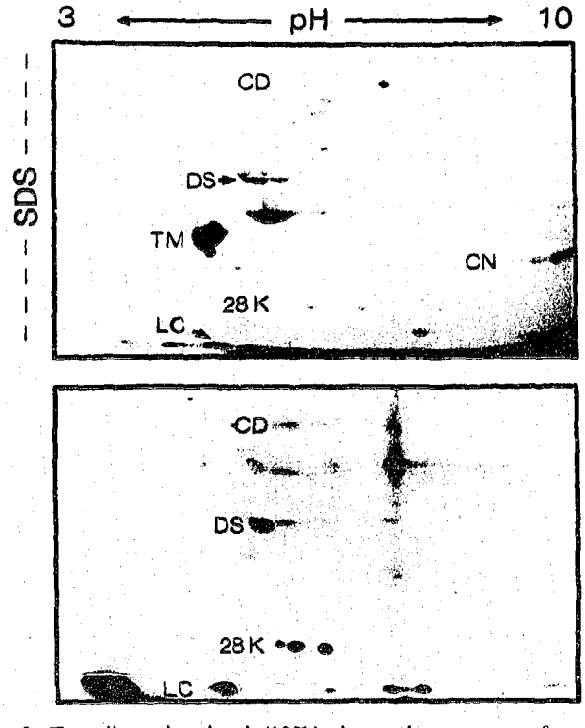

Fig. 2. Two-dimensional gel (10%) electrophoretogram of arterial muscle proteins. (Top) Staining profile. (Bottom) Corresponding autoradiogram. For abbreviations see the legend to Fig. 1.

extract from an arterial muscle contracted with KCl for 60 min. The stained gel shows the typical elongated band for calponin at the molecular weight level of 34 000 in the alkaline pH region. However, the autoradiogram shows no radioactivity for calponin. In contrast, several proteins which appear as strong (light chain and desmin) or weak (28-kDa protein and caldesmon) spots on the staining pattern exhibit intensive (light chain, 28-kDa protein, and desmin) or marked (caldesmon) radioactivity.

Table I lists the conditions of the muscles we have been using in the 2D electrophoretic studies. Four different stimulating agents were employed and in one case subsequent addition of two agents. The time of stimulation varied from 1.5 to 60 min. The active tension produced by these muscles ranged from 0.4 to 0.9 × 10^5 N/m^2. A total of 28 stimulated and 10 resting muscles were analyzed for possible calponin phosphorylation but it could not be found in any case.

3.3. *Phosphorylation of other proteins than calponin*

Fig. 2 reveals several ^{32}P-labeled proteins in porcine arterial muscles. Previously, Takuwa et al. [14] resolved by 2D electrophoresis a large number of [^{32}P]proteins in homogenates from contracted and relaxed bovine carotid arterial muscle. We have found a 28-kDa protein which is rapidly labeled and exists in three different phosphorylated forms. Desmin was identified by its apparent molecular mass (determined as 55 kDa, cf. with [2]) and by its multiplet pattern on 2D gels in the neutral pH range [15]. Caldesmon was identified with aid of the authentic protein [16].

We semiquantitated the incorporation of [^{32}P]phosphate into the 28-kDa protein and desmin based on their staining intensities relative to the 20-kDa myosin light chain and on the concentration, 112 μM, of the light chain in arterial muscle [2]. For caldesmon a 20 μM concentration was assumed [2]. After prolonged stimulation, muscles contained around 1 mol [^{32}P]-phosphate per mol desmin or caldesmon and somewhat less per mol of 28-kDa protein.

Table I

Conditions for studying calponin phosphorylation in porcine arterial muscle

Stimulating agent	Time of stimulation (min)	Active tension (N/m^2 × 10^{-5})	*n*
None	-		10
100 mM KCl	1.5–2	0.8 ± 0.3	6
100 mM KCl	30	0.9 ± 0.3	4
100 mM KCl	60	0.8 ± 0.2	6
200 μM NE	2	0.4	2
200 μM NE	60	0.5	2
100 μM histamine	10	1.4	2
100 μM histamine	60	1.0	2
0.8 μM PDBu	60	0.4	2
0.8 μM PDBu + 100 mM KCl	60 + 2	1.1	2

4. DISCUSSION

The high concentration of calponin, 80 μM [9], suggests a functional role for this protein in smooth muscle. In vitro studies indicate that calponin can inhibit smooth muscle actomyosin MgATPase [7,8], the contractile enzyme, and this inhibition is reversed when isolated calponin is phosphorylated by protein kinase C or Ca^{2+}/calmodulin-dependent protein kinase II [8]. As it turns out from this work, no phosphorylation of calponin takes place in contracting or resting arterial smooth muscle, clearly indicating that the sites of calponin free for phosphorylation in vitro are blocked in the intact muscle. The other alternative that the enzymes required for calponin phosphorylation are not available in arterial muscle is unlikely because protein kinase C is phosphorylating the myosin light chain in this muscle [17], thus in PDBu-treated muscles (Table I) calponin phosphorylation could have occurred. The phenomenon of protein phosphorylation in vitro but not in vivo is not unique for calponin, since it has been shown that phospholamban and troponin I are substrates for protein kinase C in vitro but not in intact beating guinea pig heart [18]. There is a possibility that calponin is phosphorylated in smooth muscles other than porcine carotid arteries, although the general pattern of contractile behavior appears to be uniform in a variety of smooth muscle types [2].

This work confirms the phosphorylation of caldesmon, desmin [19,20] and the 28-kDa protein [21] in contracting smooth muscle. It is possible that these protein phosphorylations are involved in the regulation of smooth muscle contraction.

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