Vol. 108, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1982 Pages 534-540

THE ATPMg-DEPENDENT PHOSPHATASE IS PRESENT IN MAMMALIAN VASCULAR SMOOTH MUSCLE

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Received August 12, 1982

An ATPMg-dependent phosphorylase phosphatase was identified in vascular smooth muscle from bovine aorta. The smooth muscle enzyme, like the corresponding enzyme from striated muscle, exists as an inactive phosphatase (F_C-enzyme) which can be activated by a protein, F_A, in the presence of ATP and Mg^{2+}. Moreover, smooth muscle F_C is activatable by skeletal muscle F_A and skeletal muscle F_C can be activated by smooth muscle F_A. The mode of activation of aortic F_C by aortic F_A is similar to that reported for the skeletal muscle proteins. In accord with earlier findings obtained with the skeletal muscle system, the activity of the aortic phosphatase is inhibited by a specific heat-stable modulator protein (previously called phosphatase inhibitor-2). Thus, the fundamental properties of arterial ATPMg-dependent phosphatase appear to be identical to those of its skeletal muscle counterpart which purportedly represents the major phosphorylase phosphatase in that tissue. Since glycogen phosphorylase is activated when vascular smooth muscle contracts, ATPMg-dependent protein phosphatase may participate in coordinating arterial metabolism and contractility.

INTRODUCTION

Isometric contraction is associated with activation of phosphorylase in a variety of smooth muscles including aorta (1), coronary artery (2), uterus (3), and trachealis (4). Temporal studies of relations between development of isometric force and activation of phosphorylase in coronary artery (2), and trachealis (4) showed that phosphorylase activity peaked before isometric force, but enzymic activity subsequently declined while force was maintained. These findings suggest that dephosphorylation of phosphorylase by phosphorylase phosphatase occurs during the late phase of isometric contraction. Accordingly, regulation of phosphorylase phosphatase may participate in coordinating contractility and metabolism in smooth muscle.

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0006-291X/82/180534-07$01.00/0

A multifunctional ATPMg-dependent protein phosphatase has been identified in several mammalian tissues including liver, heart and skeletal muscle (5-9). This phosphatase, effective in dephosphorylating phosphorylase a, glycogen synthase, phosphorylase kinase and other proteins, is apparently the inactive form of the major physiologically relevant phosphatase involved in coordinating glycogen synthesis and breakdown (10). However, whether or not the ATPMg-dependent phosphatase is present in mammalian smooth muscle is unknown.

Potentially, the ATPMg-dependent phosphatase is a prime target for regulation because it consists of several components. In tissues examined, the enzyme exists as an inactive F_C form which can be activated by a protein, F_A, in the presence of ATP and Mg^{2+} (5-10). Moreover, the extent of phosphatase activation can be increased or decreased in a concentration dependent manner by a specific modulator protein previously identified as phosphatase inhibitor-2 (11).

In this study, we found that the major identified components of ATPMg-dependent phosphorylase phosphatase (i.e., F_C, F_A, and modulator protein) are also present in vascular smooth muscle of bovine aorta. The occurrence of ATPMg-dependent phosphorylase phosphatase in smooth muscle is consistent with the hypothesis that the enzyme participates in coordinating arterial glycogen metabolism and contractility.

METHODS

Methods used for the identification of ATPMg-dependent phosphatase in aortic preparations were similar to those described previously for skeletal muscle (7,8). Briefly, bovine aortic muscularis (500-750g) was homogenized at 4°C in 4 vols of 50mM Tris pH 80, 0.5mM dithiothreitol (DTT), 1mM ethylenediamine tetraacetic acid (EDTA), 0.5mM bennamide, 0.1mM Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK), 0.1mM L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), 0.1mM phenylmethylsulfonyl fluoride (PMSF) and 2mM ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetraacetic acid (EGTA). The homogenate was centrifuged (10,000 g, 20 mins) and the resulting supernatant was filtered through glass wool and interacted with DEAE Sephadex A-50 equilibrated in 20mM Tris pH 7.0 and 0.5mM DTT (Buffer A). The nonadsorbed or breakthrough protein was processed for purification of F_A, whereas protein bound to the gel was processed for F_C.

The DEAE Sephadex was washed thoroughly with 0.2M NaCl in Buffer A and bound protein was eluted with a linear gradient of 0.2-0.4M NaCl in Buffer A (800ml total vol), followed by an additional 200ml of 0.4M NaCl-Buffer A. Collected fractions (10ml) were assayed for protein (A_{280}) and phosphorylase phosphatase (see below). Appropriate fractions containing F_C (ATPMg-dependent phosphatase) were pooled, precipitated with 30-60% $(NH_4)_2SO_4$, dissolved in a minimal vol of Buffer A (3-5ml) and extensively dialyzed against the same buffer. The resulting fraction was applied to a column of polylysine Sepharose 4B (0.9x6cm) which had been equilibrated with Buffer A. The column was washed with 50ml of 0.2M NaCl in the same buffer and bound protein was eluted with 0.2-0.5M NaCl buffer gradient (100ml, total vol). Fractions (1.5ml) were assayed for both F_C and modulator protein. The active fractions were pooled, concentrated and stored at -20°C in Buffer A containing 50% glycerol.

The breakthrough fraction containing F_A from the initial DEAE step was interacted with CM Sephadex C-50. After washing the column with 0.1M NaCl-Buffer A, bound protein was eluted with a 0.1-0.3M NaCl-buffer gradient (600ml, total vol). Fractions (10ml) showing F_A activity (measured as an activator for F_C) were pooled, precipitated with 30-60% $(NH_4)_2SO_4$, and after extensive dialysis against Buffer A, the fraction was applied to Blue Sepharose CL-6B (1.5x12cm) equilibrated in Buffer A. The column was washed with 100ml of 0.2M NaCl-Buffer A and bound protein was eluted with 100ml of 0.4M NaCl-Buffer A. Fractions active for F_A were pooled, concentrated and stored in 50% glycerol-Buffer A at -20°C.

Rabbit skeletal muscle F_A (100,000 U/mg), and F_C (6000 U/mg) purified as described previously (3,4) were used to assay column fractions for aortic F_C and F_A respectively. $(\gamma^{32}P)$Phosphorylase a, which was used as substrate, was prepared using cyclic AMP, phosphorylase kinase and (γP)ATP as described by Krebs et al. (12). One unit of phosphatase is that amount of enzyme which releases 1nmol of ^{32}P per min at 30°C.

To assay for ATPMg dependent phosphatase 10µl of an appropriately diluted fraction was incubated with 10µl of a saturating concentration of skeletal muscle F_A in 20mM Tris pH 7.0, 0.5mM DTT, 1mg/ml bovine serum albumin (Sigma), 5mM caffeine, 0.1mM ATP and 0.5mM Mg acetate for 10 mins at 30°C. The reaction was started by adding 10µl of ice cold 20% trichloroacetic acid and 100µl of bovine serum albumin (6mg/ml). Following centrifugation, 200µl of the supernatant was used for liquid (Aqualuma) scintillation spectroscopy. Assays for spontaneous phosphatase activity were performed in the absence of F_A, ATP and Mg acetate. A saturating concentration of skeletal muscle F_C was substituted for F_A in assaying fractions for F_A activity.

Previous studies with skeletal muscle showed that heat-stable modulator protein copurified with F_C (11). Accordingly, fractions from aortic smooth muscle were heated (90-95°C, 10 mins) to destroy associated phosphatase activity, appropriately diluted and assayed for modulator protein as described previously (11). Assays for histone f2b (Sigma) and phosvitin (Sigma) kinase activity were performed using standard procedures (10,13,14).

RESULTS AND DISCUSSION

The major components of ATPMg-dependent protein phosphatase (F_C, F_A and modulator protein) were demonstrable in aortic preparations (Figs. 1 and 2). It is noteworthy that aortic F_C was detected using F_A from skeletal muscle (Fig. 1A), and that skeletal muscle F_C was used to detect aortic F_A (Fig. 2A). This suggests that the proteins are interchangeable and not specific for different types of muscle. However, this does not preclude the possibility that significant differences in kinetic properties might exist between F_A and F_C proteins from different tissues.

Two patterns of elution for F_C were evident during ion exchange chromatography of different aortic preparations on DEAE Sephadex A-50. That is, either a single peak of activity, or as shown in Fig. 1A, a forked peak eluted between 0.25-0.35M NaCl. The forked profile for F_C activity persisted through chromatography on polylysine-Sepharose 4B (Fig. 1B). This appears to reflect inhibition by the modulator protein which was found to peak at the same location. In this context, it is important to recognize that the well known heat-stable inhibitor-2 of phosphorylase phosphatase (15-17) was recently renamed modulator protein because it was shown to increase and then decrease the activity of ATPMg-dependent

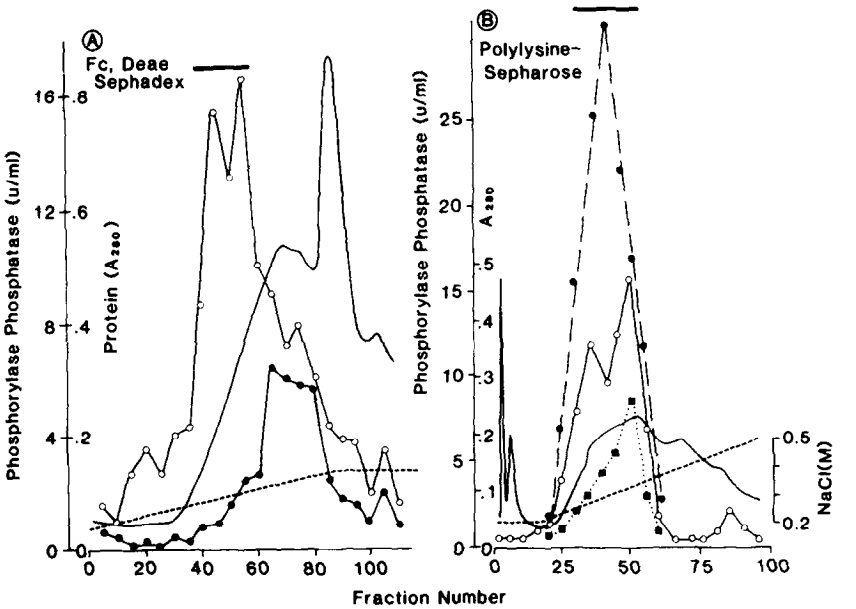

Fig. 1 Panel A shows the elution profile of aortic F_C (ATPMg-dependent phosphorylase phosphatase, —O—) and spontaneously active phosphorylase phosphatase (—●—) from an extract subjected to ion exchange chromatography on DEAE Sephadex A-50 as described in METHODS. Protein (A_{280}) in this and subsequent figures is shown by the continuous curve and the concentration of NaCl is given by the dashed line. Fractions indicated by the horizontal bar denote F_C fractions which were pooled, concentrated and chromatographed on polylysine-Sepharose as shown in Panel B. Fractions were assayed for F_C activity measured in the usual way (—O—), F_C activity measured in the presence of added modulator protein (20ng/ml assay mixture, ···■···) and for the concentration of endogenous modulator protein (—●—). Fractions indicated by the bar at the top of the peak were pooled and concentrated (see RESULTS and DISCUSSION).

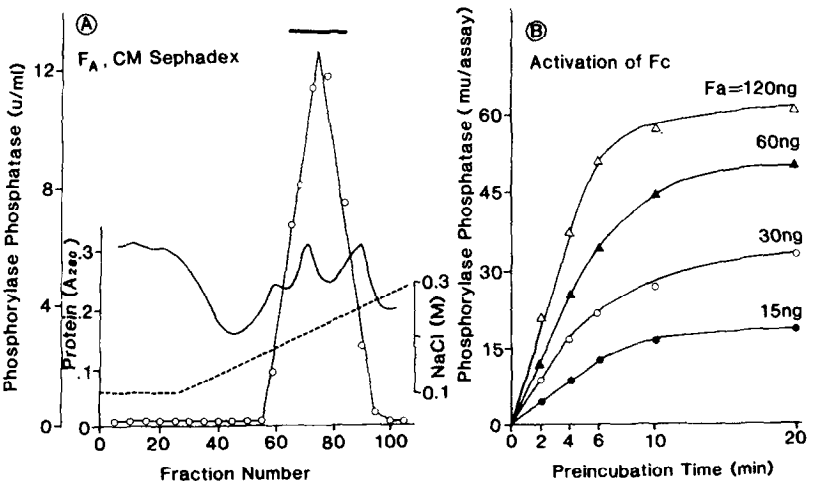

Fig. 2 Panel A shows the elution profile of aortic F_A (—O—, measured as an activator of skeletal muscle F_C) from CM Sephadex C-50 as described in METHODS. Panel B shows the time course for activation of a fixed amount of aortic F_C (160 potential milliunits of ATPMg-dependent phosphorylase phosphatase per assay) by the different amounts of F_A (ng protein per assay) indicated above each curve.

phosphatase in a concentration dependent manner (11). Thus, saturation of F_C with modulator protein is absolutely required for activation of ATPMg-dependent phosphatase by F_A; however, an excess of modulator inhibits the activated phosphatase.

Assays repeated in the presence of added skeletal muscle modulator protein (20ng/ml, Fig. 1B) showed that the ATPMg-dependent phosphatase activity was markedly depressed in the first limb of the F_C peak, whereas reduction of activity in the second limb of the peak was noticeably less pronounced. This strongly suggests that the concentration of modulator protein in the first limb of the aortic peak exceeded its concentration in the second limb, and that all of the F_C enzyme was saturated with modulator protein. Direct assay of the fractions for modulator protein confirmed this inference and showed a several fold excess of modulator over F_C activity. Thus, the data in Fig. 1B shows that the amount of modulator protein present is sufficient to create several fold more F_C activity than was actually contained in the same fractions. Since an excess of modulator protein is inhibitory (11), this results in a forked peak of F_C activity.

In accord with previous findings reported for skeletal muscle (7,8), it was not possible to assess ATPMg-dependent phosphatase activity in the initial aortic extracts because of exactly the same reason: namely, interference by the presence of heat-stable inhibitors of the enzyme. About 35% of the F_C activity present in the DEAE Sephadex pool was recovered following concentration with 30-60% $(NH_4)_2SO_4$ and chromotography on polylysine-Sepharose (Table 1). Ninety percent of the enzymic activity in the partially purified F_C preparation (i.e., after polylysine-Sepharose) was ascribable to ATPMg-dependent phosphatase activatable by F_A. The remaining 10% of the activity was attributable to spontaneously active phosphatase which overlapped with the trailing F_C activity on DEAE Sephadex (Fig. 1A, Table 1).

Almost 30% of the F_A activity initially present in the single peak seen after elution from CM Sephadex C-50 (Fig. 2A) was recovered following chromatography on Blue Sepharose CL-6B (Table 1). The F_A preparation was free of histone kinase activity, but like skeletal muscle F_A (10) showed kinase activity against phosvitin (2nmol ^{32}P incorporated/min/mg). It is likely that smooth muscle F_A, as its skeletal muscle counterpart, is also a glycogen synthase kinase (8). However, further studies are required to establish this point with certainty.

The kinetics for activation of smooth muscle F_C by smooth muscle F_A were similar to the kinetics reported for the skeletal muscle system (7,8). Thus, when a large excess of F_C

TABLE 1. Recovery of Aortic Fc and F_A

	Preparative Step	Total Protein (mg)	Phosphorylase Phosphatase (Units)		
			Total	Spontaneous	ATPMg Dependent
Fc:	1) DEAE pool	52	1200	130	1070
	2) 30-60% $(NH_4)_2SO_4$	25	720	128	592
	3) Polylysine Sepharose	1.4	400	21	379
F_A:	1) CM pool	60			5500
	2) 30-60% $(NH_4)_2SO_4$	20			6000
	3) Blue Sepharose	2			1560

F_C fractions were assayed for total phosphatase activity measured in the presence of saturating skeletal muscle F_A, 0.1mM ATP and 0.5mM Mg acetate whereas spontaneous activity was measured in the absence of F_A, ATP and Mg^{2+}. ATPMg dependent phosphorylase phosphatase activity is the difference between total and spontaneous activity. Aortic F_A activity was measured as an activator for skeletal muscle F_C (see text for details).

(160 potential milliunits of ATPMg-dependent phosphatase per assay) was activated by F_A a discrete level of activated phosphatase was attained for each concentration of F_A tested (Fig. 2B). As previously discussed at length (5,7,18), these data are compatible with a protein-protein interaction mechanism for activation of the phosphatase, or reflect an equilibrium between an activating and inactivating reaction for the F_C-enzyme.

Since ATPMg-dependent phosphorylase phosphatase is a key enzyme involved in the regulation of glycogen metabolism, its demonstrated presence in mammalian vascular smooth muscle is consistent with the hypothesis that it may participate in coordinating arterial glycogen metabolism and contractility. The possibility that this multisubstrate phosphatase (5-10) also is involved in the dephosphorylation of smooth muscle contractile and regulatory proteins (19-21) is under investigation.

ACKNOWLEDGEMENTS

J.V.R. is Onderzoeksleider van het, NFWO. This work was supported by NIH grants HL20196 and HL 22619 and by a grant from the Fonds voor Geneeskundig Wetenschappelijk Onderzoek. We are grateful to Ms. M. Evens and Ms. R. Verbiest for excellent technical assistance, and to Ms. R. Jacobs for typing the manuscript.

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