EPINEPHRINE ACTIVATION OF PHOSPHOFRUCTOKINASE IN PERFUSED RAT HEART

Regulation via α-adrenergic receptor mechanism independent of phosphorylation

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1. Introduction

Epinephrine treatment of the perfused rat heart leads to the activation of phosphofructokinase (PFK) via an α-adrenergic receptor mechanism, independent of changes in the intracellular concentration of cyclic AMP [1]. The activation is characterized by a loss in sensitivity to the inhibitors, ATP [1] and citrate [2], and is stable to gel-filtration [2,3]. Reconversion of the activated form to the non-activated form is catalyzed in heart extracts but does not appear to involve phosphoprotein phosphatase [2]. Here, the enzyme assay from [1,4] and SDS–polyacrylamide gel electrophoresis of immunoprecipitates of PFK were used to examine the relationship between α-receptor-mediated activation and phosphorylation.

2. Materials and methods

Rabbit skeletal muscle PFK (Sigma, 185 units/mg) was labelled with ^{14}C by using iodo[2-^{14}C]acetic acid. The reaction mixture contained 100 μg salt-free PFK and 1 mM iodo[2-^{14}C]acetic acid (4 μCi) in 0.6 ml 100 mM Tris–HCl (pH 7.4). After 3 h at 30°C 2 mM dithiothreitol was added to stop the reaction. The resulting specific radioactivity was ~500 cpm/μg PFK.

Goat antiserum to rabbit skeletal muscle PFK was prepared, fractionated and stored as a powder at −20°C [5,6]. Hearts were perfused with low-phosphate medium containing [^{32}P]phosphate (0.1 mCi/ml for 30 min) [7], agonists were present for 5 min, then the hearts were freeze-clamped in liquid N_2-cooled tongs. Frozen heart powders were homogenized in 5 vol. 100 mM Tris–HCl (pH 7.4) containing 1 mM dithiothreitol, 100 mM phosphate and 30 mM NaF. Supernatants (100 000 × *g* for 15 min) were mixed with an equal volume of reconstituted antiserum (100 mg/ml Tris–HCl–dithiothreitol) and allowed to stand at 4°C for 17 h. Immunoprecipitates (8000 × *g* for 10 min) were washed twice with isotonic saline and solubilized by incubation for 10 min at 95°C in 200 μl 63 mM Tris–HCl (pH 7.0), 1% SDS, 2.5% 2-mercaptoethanol, 5% glycerol and 0.02% bromophenol blue. Electrophoresis on 6% gels (10 cm) was done as in [8]. The specific radioactivity of [γ-^{32}P]-ATP in heart powders was determined as in [9]. The activity ratio of PFK [1] was determined in hearts to which [^{32}P]phosphate had not been added. Other experimental details were as in [1,3,4].

3. Results

Goat antiserum to rabbit skeletal muscle PFK was found to inactivate control and epinephrine-activated PFK in heart extracts (fig.1). Each form reacted in a similar manner; ~20 μl (2.0 mg) of antiserum inactivated all of the enzyme activity in 100 μl 1:5 heart extract. Densitometric scans of the stained PFK (band II, fig.2) on the polyacrylamide gels indicated that the amount of antiserum required to precipitate all of the PFK in 100 μl extract was ~20 mg or 10-fold that required to inactivate the enzyme. Serum from non-immunized goats did not inactivate PFK.

SDS–Polyacrylamide gels of the rabbit skeletal muscle PFK indicated several minor contaminants (fig.2). Phosphorylase was one of these and thus immunoprecipitates from [^{32}P]phosphate-labelled extracts contained significant radioactivity in the

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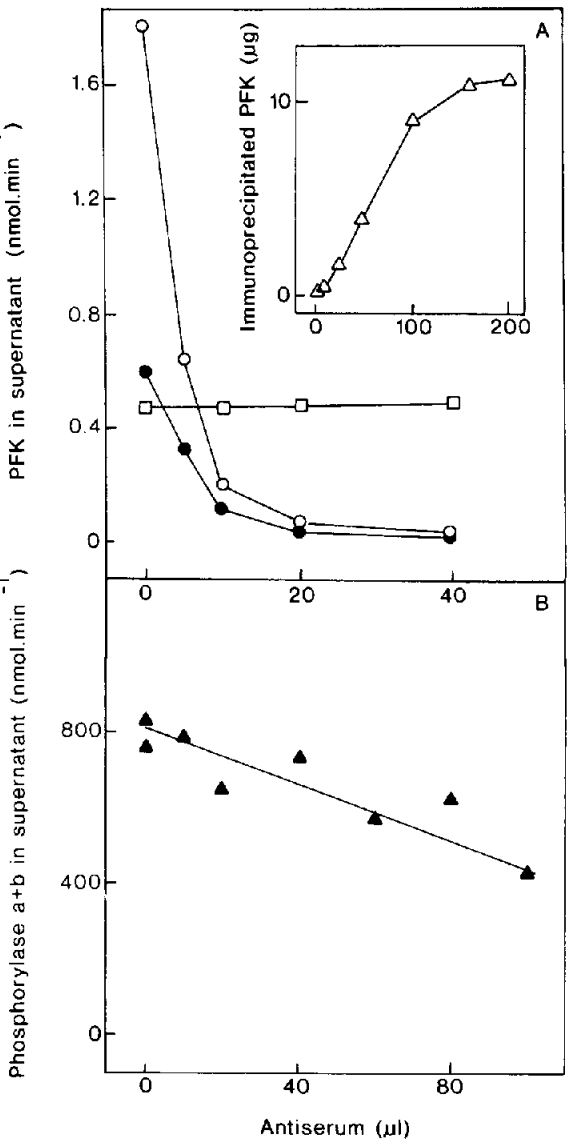

Fig.1. Characterization of the antiserum prepared against rabbit skeletal muscle PFK. Heart extracts (1:5) from either control (●) or epinephrine-treated hearts (○) were prepared as in section 2. Supernatants (100 μl) were added to the indicated volumes of antiserum (100 mg/ml) and allowed to stand at 4°C for 17 h. (A) The immunoprecipitates were collected by centrifugation for 10 min at 8000 × *g*, washed twice in isotonic saline, solubilized and applied to SDS–polyacrylamide gels. Gels were stained (Coomassie brilliant blue) and the band corresponding to the subunit of PFK (see fig.2) scanned using a densitometer; estimation of the amount of protein in this region (insert, △) was made from comparisons with known quantities of PFK applied to separate gels. Corrections were made for the slight amount of protein in the region of the PFK subunit that derived from the antiserum. The remaining activity of PFK inthe supernatants following removal of the immunoprecipitates was assayed at 100 μM ATP [4] (○,●). Control serum was also tested for its ability to inactivate control heart extract PFK (□). (B) The remaining activity of phosphorylase in the supernatants following removal of the immunoprecipitates was assayed [4] (▲). Values in (A,B) are for the original 100 μl heart extract supernatant.

region of the phosphorylase subunit (band I, fig.2; ~95 000 M_r). Antiserum to PFK also inactivated phosphorylase in heart extracts (fig.1) but ~24 mg was required to inactivate all of the phosphorylase in 100 μl extract. In addition when heart extracts were prepared in buffer containing a large excess of exogenous unlabelled phosphorylase (30 × extract content) the radioactivity in band I was reduced to background (fig.2). Commercial phosphorylase (Sigma, 30 units/mg) contained no apparent contaminants (fig.2).

Gel electrophoresis profiles of solubilized immunoprecipitates from either control or epinephrine-treated hearts were similar. Three major protein bands were apparent. Band I (fig.2) corresponding to the subunit of phosphorylase contained ^{32}P radioactivity. Band II corresponding to the subunit of PFK contained ^{14}C radioactivity corresponding to the *S*-carboxy[^{14}C]-methylated PFK but did not contain any significant ^{32}P radioactivity. The third band (III) was attributable to the immunoglobulin subunit.

In table 1 the effects of α- and β-adrenergic agonists on [^{32}P]phosphate incorporation into rat heart PFK were examined and compared with the activity ratio for the same enzyme. As reported in [1], activation of PFK was mediated by catecholamines possessing α-adrenergic receptor activity. Thus naphazoline and epinephrine treatment led to activation of the enzyme but isoproterenol was without effect. Regardless of the agonist used there was no detectable radioactivity in the region corresponding to the subunit of PFK (band II). As a result of the contaminating phosphorylase in the commercial preparation of PFK, radioactively labelled phosphorylase was precipitated by the antiserum to PFK. Thus radioactivity was detected in band I, corresponding to the subunit of phosphorylase. Labelling of this enzyme by [^{32}P]-phosphate was increased by β-adrenergic agonists.

The limit of detection was ~5 cpm above background (per gel slice). From estimation of the amount

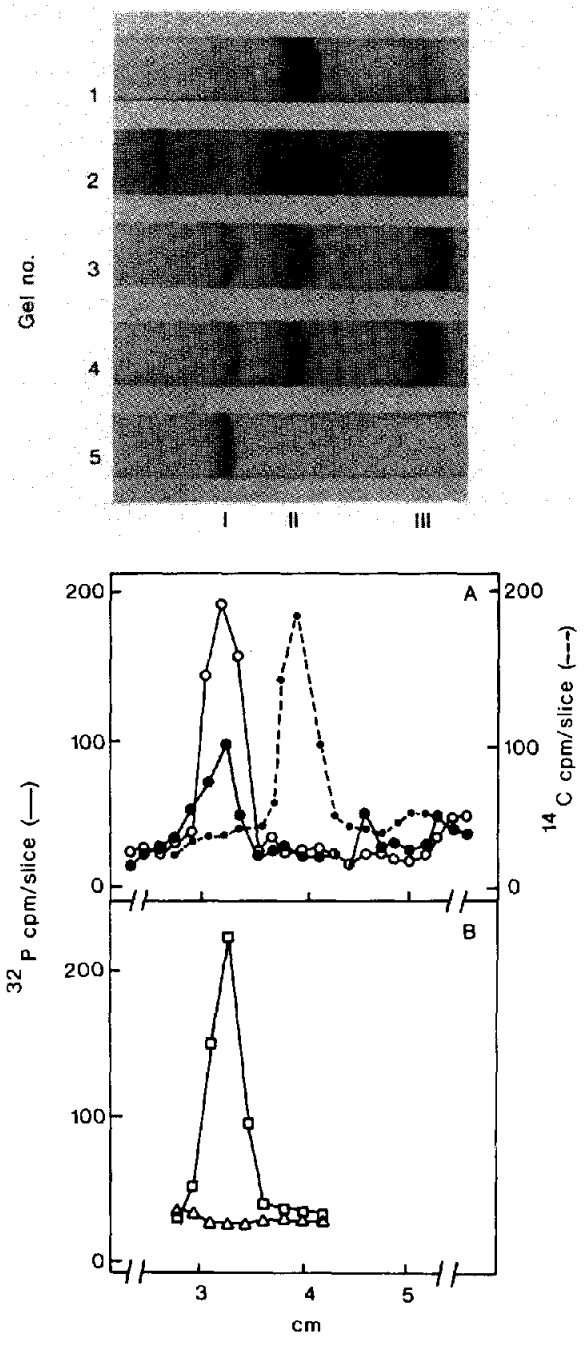

Fig.2. SDS–Polyacrylamide gels of commercial rabbit skeletal muscle PFK (1), antiserum of PFK (2), solubilized immunoprecipitate from control heart extract (3), solubilized immunoprecipitate from epinephrine-treated heart extract to which 1 μg *S*-carboxy[^{14}C]methylated-PFK had been added before mixing with antiserum (4), and rabbit skeletal muscle phosphorylase *a* (Sigma) (5). The gels (10 cm) were run from left to right, stained with Coomassie brilliant blue and scanned using a densitometer. Sections of the gels between 2–5.5 cm have been cut for scanning and photography. Bands (I–III) correspond to the subunits of phosphorylase, PFK and immunoglobulin, respectively. (A) Gel 3 (•) and gel 4 (○) derived from [^{32}P]phosphate-perfused control and epinephrine-treated hearts, respectively, were sliced into 1.6 mm sections and counted [17] for ^{32}P. Since gel 4 also contained exogenous *S*-carboxy[^{14}C]methylated-PFK, slices were counted for ^{14}C to locate the PFK subunit. (B) ^{32}P Radioactivity from two additional gels is shown: (▫) solubilized immunoprecipitate from epinephrine-treated, [^{32}P]phosphate-perfused heart extract; (△) solubilized immunoprecipitate from the same epinephrine-treated [^{32}P]phosphate-perfused heart extract that had been prepared in the presence of 500 μg exogenous phosphorylase *a*. Experimental details are in section 2 or fig.1.

of PFK protein in band II and from the determination of the specific radioactivity of [γ-^{32}P]ATP in the heart extracts which was 18 ± 5.3 cpm/pmol, it was possible to calculate the extent of phosphorylation of PFK that could be detected. On average each gel contained 8.5 μg endogenous PFK protein in band II.

Thus phosphorylation to the extent of 0.02 mol/mol enzyme (360 000 M_r) (10 cpm above background) would have been detected. Similar calculations applied to phosphorylase and using protein estimations from densitometric scans and the determined specific radioactivity of [γ-^{32}P]ATP indicated that epinephrine increased the phosphorylation from 0.27–0.53 mol/mol enzyme subunit (94 000 M_r).

Table 1
Effect of α- and β-adrenergic agonists on [^{32}P]phosphate incorporation into rat heart phosphofructokinase

Additions to perfusate (10^{-5}M)	Net radioactivity in gel (cpm)[a]		Activity ratio of extract PFK
	Phosphorylase (band I)	PFK (band II)	
None	262	2 (2)	0.21 ± 0.01 (4)
Naphazoline	150	7 (2)	0.42 ± 0.02 (3)
Epinephrine	515	1 (4)	0.60 ± 0.10 (3)
Isoproterenol	611	16 (1)	0.24 ± 0.04 (3)

[a] The limit of detection was 5 cpm above background

Experimental conditions were as in fig.2 and the text. Mean values are shown. Where appropriate SEM have been calculated with no. perfusions in parentheses

4. Discussion

Evidence has accumulated from other laboratories to indicate that muscle PFK is phosphorylated both in vivo [10,11] and in vitro [12]. Although most reports acknowledge that the extent of phosphorylation does not alter the catalytic or regulatory properties of the skeletal muscle enzyme (e.g., [11]), in [13,14] the liver enzyme was altered following phosphorylation and in [15] skeletal muscle enzyme was affected. There have been no reports describing the phosphorylation of the heart enzyme in vivo. These findings suggest that α-adrenergic-mediated activation of heart PFK does not involve phosphorylation of the enzyme. The sensitivity of the technique involving SDS–polyacrylamide gel electrophoresis of immunoprecipitates permitted the detection of changes in phosphate content of the order of 0.02 mol/mol tetramer. Since values for the phosphorylation of phosphorylase obtained using the same techniques were of the order expected [16], it appeared unlikely that failure to detect phosphorylation of PFK resulted from extract phosphatase activity.

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