PROTEIN KINASES FROM RAT SKELETAL MUSCLE: EVIDENCE FOR SIX DIFFERENT FRACTIONS OF THE ENZYME

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Received 25 November 1971

1. Introduction

It seems generally accepted that cyclic AMP* exerts its diverse effects by stimulation of protein kinases. This concept is supported by the wide distribution of protein kinase in all animals hitherto investigated [1], including bacteria [2] and viruses [3]. Therefore, the question arises whether the different effects of cyclic AMP are mediated by different protein kinases in one and the same tissue. Evidence from several laboratories points to the existence of 2 protein kinase fractions in rabbit skeletal muscle [4] and rat liver [5] and of 3 fractions in rabbit liver [6].

By chromatography on DEAE-cellulose, partially purified extracts (78,000 *g* fractions) prepared from rat skeletal muscle by the method of Walsh et al. [7] resolved into 6 protein peaks. Each of these showed protein kinase activity but they differed from one another in several respects.

2. Experimental

Protein kinase preparations were partially purified from hind leg and chest muscle of normal, fed, male Osborne-Mendel rats (140–200 g) according to the procedure described by Walsh et al. [7] for rabbit skeletal muscle. 100 ml of the 78,000 *g* supernatant (MSE Superspeed 50 ultracentrifuge, rotor no. 59 113) were adsorbed on DEAE-cellulose (column, 3 × 30 cm, DEAE-SS-cellulose from Serva, Heidelberg). The column had been equilibrated and washed with 0.005 M potassium phosphate buffer containing 0.002 M EDTA. The fractions containing the protein peaks (recorded with a Uvicord II LKB Produkter, Bromma, Sweden) appeared on elution with 0.005 M, 0.03 M, 0.1 M, 0.2 M and 0.4 M potassium phosphate buffer (the first 2 buffers containing 0.002 M EDTA) (fig. 1). Protein was precipitated with ammonium sulfate (0.4 g/ml), centrifuged and the precipitate dissolved in a minimal vol (10 ml) of 0.005 M potassium phosphate buffer/0.002 M EDTA, pH 7.0, and dialyzed overnight against the same buffer. The enzyme preparations obtained in this way had a 10–30 times higher specific activity than the crude muscle extract.

Protein kinase activity was measured according to Walsh et al. [7] with slight modifications. 0.5 ml of the assay medium contained: 50 mM sodium glycerol phosphate, pH 6.0; 1 mM potassium phosphate; 30 mM potassium acetate; 3 mg casein (Serva, Heidelberg); 1 mM theophylline; 0.4 mM EDTA; 1.2 mM γ^{32}P-ATP (Amersham, Radiochemical Centre) (0.5–1 × 10^6 cpm (liquid scintillation counter Unilux I, Nuclear Chicago); 3.6 mM magnesium acetate; 0.1 ml protein kinase fraction and cyclic AMP as indicated in the experiments. Protein-bound ^{32}P was determined as described by De Lange et al. [8]; the values were corrected for ^{32}P-incorporation into proteins of the enzyme fractions in the absence of casein. Protein determinations were carried out by the method of Lowry [9].

* *Abbreviations:*
cyclic AMP = adenosine 3',5'-cyclic monophosphate.
cyclic GMP = guanosine 3',5'-cyclic monophosphate.

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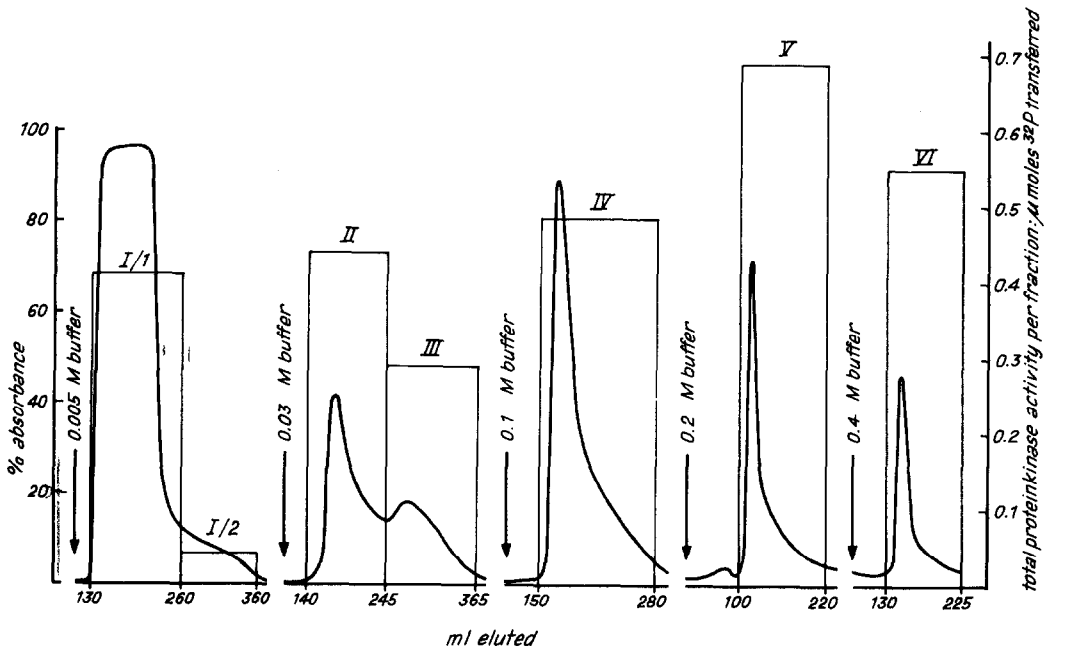

Fig. 1. Elution pattern of the 78,000 *g* supernatant from DEAE-cellulose by potassium phosphate buffers of increasing molarity. Fractions were pooled as indicated by the bars. Further purification steps and the protein kinase assay are described in the text. Incubations were carried out at 30° for 15 min in the presence of 10^{-4} M dibutyryl cyclic AMP. The results of a typical experiment are shown.

3. Results and discussion

Washing the DEAE-cellulose column with potassium phosphate buffers of increasing molarity resulted in the elution of at least 6 protein peaks with protein kinase activity (fig. 1). The first fraction which proved to be independent of cyclic AMP appeared in the 0.005 M potassium phosphate/0.002 M EDTA-wash with the bulk of non-adsorbed proteins and chromogens. The second and the third peak were eluted by 0.03 M potassium phosphate buffer/0.002 M EDTA, pH 7.0; the fourth by 0.1 M potassium phosphate buffer (pH 7.2, no EDTA). After applying 0.2 M buffer (pH 7.2, no EDTA) to the column a fifth peak was eluted, sometimes preceded by a smaller one immediately appearing after the change from 0.1 M to 0.2 M buffer. A sixth peak was eluted with 0.4 M buffer (pH 7.2, no EDTA). Protein kinase activity of each of these peaks was enhanced by cyclic AMP.

Fig. 2 shows the stimulation by cyclic AMP of ^{32}P-incorporation into casein for each of the 5 cyclic AMP-dependent fractions of a typical enzyme preparation. The apparent K_M values estimated from this figure are listed in table 1. When the results of fig. 2 are plotted on a linear scale (not shown) cyclic AMP at 10^{-8} and 10^{-7} M shows a typical cooperative effect with fractions III and IV. This phenomenon is less marked in fraction V and seems to be absent in fractions II and VI.

As can be further seen on fig. 2, each kinase fraction led to a different ^{32}P-incorporation into casein in the absence of cyclic AMP. Fractions II and VI were relatively active and fractions III and IV relatively inactive.

Dibutyryl cyclic AMP stimulated fractions II, III and IV only at concentrations 100 times those of the unsubstituted compound (approx. K_M $2-4 \times 10^{-5}$ M), whereas with fraction VI this difference was less pronounced. With fraction V stimulation by cyclic AMP and dibutyryl cyclic AMP was similar.

The pH-optimum of ^{32}P-incorporation into casein

Table 1

Fraction number	Approximate K_M value* ($\times 10^{-7}$ M)	^{32}P incorporated in the presence of: 10^{-6} M cyclic AMP (nmoles)	10^{-6} M cyclic GMP (nmoles)	10^{-4} M cyclic GMP (nmoles)	In the absence of cyclic nucleotide (nmoles)	Stimulation by 5×10^{-6} M cyclic AMP
I	–	0.84**	–	1.07	0.95	–
II	1.4	3.08	0.88	3.88	0.72	~ 4-fold
III	3.0	1.79	0.32	2.34	0.10	~ 20-fold
IV	2.7	2.51	0.44	3.23	0.20	~ 13-fold
V	1.8	4.21	0.68	4.89	0.34	~ 13-fold
VI	0.5	2.88	1.80	3.40	1.46	~ 2-fold

Experimental conditions are described in the text.

* It must be pointed out that K_M-values are only approximations taken from fig. 2. More accurate determinations did not appear to yield more information before further purification of the fractions.

** In the presence of 10^{-4} M dibutyryl cyclic AMP.

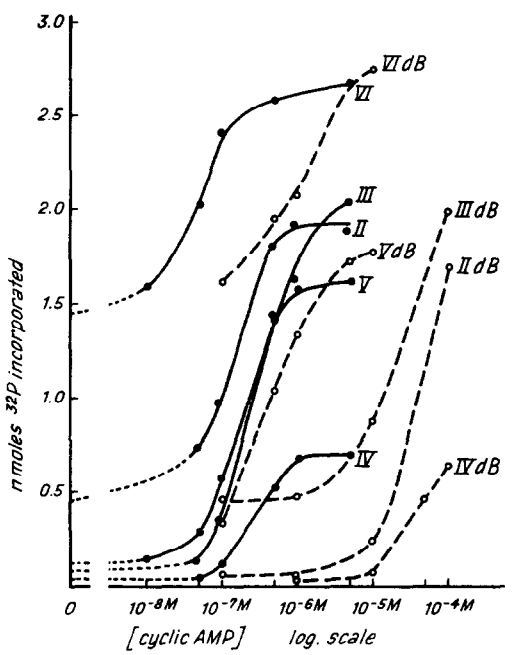

Fig. 2. Protein kinase activity of the partially purified fractions in the absence and in the presence of varying cyclic AMP (closed circles) and dibutyryl cyclic AMP (designated dB, open circles) concentrations. Assay as described under experimental; incubations lasted for 15 min at 30°. Concentrations are given on a logarithmic scale. Protein kinase activity is expressed as nmoles ^{32}P incorporated into 3 mg of casein by 100 ng of partially purified fraction. The reaction was nearly linear over 15 min and the kinetics with enzyme dilutions were linear over a 6-fold range.

in the presence of cyclic AMP lies near 5.8 for all fractions (fig. 3). With increasing pH ^{32}P-incorporation rapidly falls off for fractions II–V, whereas a broad plateau between pH 5.8 and 6.6 is observed for fraction VI. In the absence of cyclic AMP fraction VI has its optimum near pH 6.8; fraction II behaves as in the presence of cyclic AMP and fraction I seems to be only little influenced by pH within the given range.

When tested with cyclic GMP (table 1) all fractions were only little stimulated by concentrations with which maximal stimulation had been obtained with cyclic AMP (5×10^{-6} M). At 10^{-4} M, however, cyclic GMP produced maximal stimulation as also shown by Reimann et al. [4] for their 2 fractions of protein kinase from rabbit muscle.

The foregoing results show that the 6 protein kinase fractions isolated from rat skeletal muscle behave differently: they are eluted at different buffer concentrations and are differently stimulated by cyclic AMP and dibutyryl cyclic AMP; they stimulate ^{32}P-incorporation into casein differently in the absence of cyclic AMP; cyclic AMP has a cooperative effect on some but not on others and the pH-dependence of the kinase reaction varies between the fractions (fractions II to V compared with fractions I and VI). These differences seem to indicate that one is dealing with different enzymes. However, since protein kinases consist of catalytic and regulatory subunits (6, 10–12) and since cyclic AMP-binding proteins have been detected in adrenal cortex [13] and

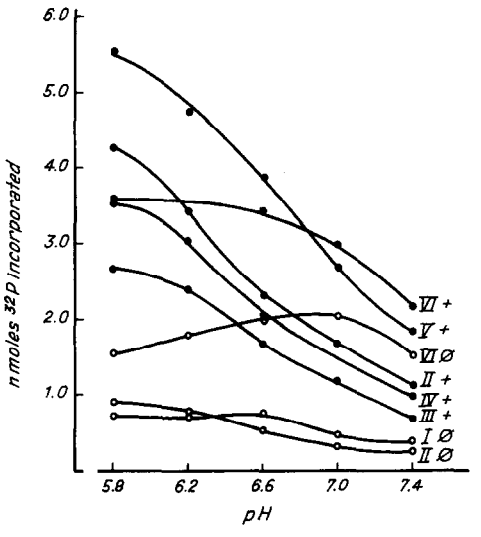

Fig. 3. The pH-dependence of ^{32}P-incorporation into casein in the presence (+) of 10^{-5} M cyclic AMP (fractions II, III, IV, V and VI) and in the absence (ϕ) of cyclic nucleotide (fractions I, II and VI). For assay see under experimental. Incubation for 15 min at 30°.

rat liver [14] 2 other possibilities must also be considered: i) the fractions may derive from the same enzyme but contain different amounts of regulatory subunits; ii) different amounts of different kinds of cyclic AMP-binding proteins which might well be identical with the regulatory subunits, may be present in the various fractions. Further studies will be necessary to examine whether these different isoenzymes may stimulate phosphorylase kinase and hormone-sensitive lipase or inhibit glycogen synthetase in a differential manner. Such a differential regulatory potential of cyclic AMP and various protein kinases might prove to be very important for the regulation by hormones of various cellular processes.

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