ACTIVATION OF RABBIT SKELETAL MUSCLE ADENOSINE-3':5'-MONOPHOSPHATE-DEPENDENT PROTEIN KINASE BY AGITATION[x]

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SUMMARY

Protein kinase activity in a preparation from rabbit skeletal muscle (Wastila,W.B.,Stull,J.T.,Mayer,S.E.,and Walsh,D.A.(1971)J.Biol.Chem. 246,1996-2003.) was increased appr. 15-fold after a 30 to 40 sec agitation of the incubation mixture on a Vortex mixer in either the presence or absence of the protein substrate,histone.Saturating concentrations of adenosine-3':5'-monophosphate (cAMP) stimulated the activity appr. 23-fold. 0.1% Triton X-100,present during the agitation,completely prevented agitation-induced activation,but left cAMP-dependent activation unaffected.

Adenosine-3':5'-monophosphate (cAMP)[1]-dependent protein kinases [EC.2.7.1.37]have been shown to be composed of two dissimilar subunits.While the holoenzyme (RC) is inactive,binding of cAMP to the regulatory subunit (R) effects the release of the active catalytic subunit (C),which catalyzes the transfer of the γ-phosphate of ATP to the hydroxyl groups of seryl or threonyl residues of substrate protein (see reviews 1,2). Highly sensitive and specific methods for the assay of cAMP in biological tissues have been developed,which are based on the activation by the cyclic nucleotide of partially purified protein kinases from bovine heart or rabbit skeletal muscle(3,4).

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[1]The abbreviations used are:cAMP,adenosine-3':5'-monophosphate; DEAE-,diethylaminoethyl-;EDTA,ethylenediaminotetraacetic acid.

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Activation of cAMP-dependent protein kinases by interaction with their substrate proteins has been reported for kinases from various sources (5-7). The substrate proteins (histone, protamine)cause the dissociation of the cAMP-dependent protein kinase resulting in a concomitant increase of cAMP-independent protein kinase activity.

In this communication we wish to report on the novel observation that protein kinase activity in a partially purified preparation from rabbit skeletal muscle can be greatly stimulated in the absence of cAMP and histone simply by mechanical agitation of the protein kinase in the test buffer.

MATERIALS AND METHODS

The protein kinase preparation used in this study was prepared from rabbit skeletal muscle essentially as described by Wastila et al.(8)with adjustment of the buffer and ion exchange column volumes to the smaller scale of the preparations(500 g muscle for the first,300 g for the second preparation).DEAE-cellulose DE-52(Whatman,Maidstone)was used for ion-exchange chromatography.Protein kinase activity was assayed according to Kuo and Greengard(3) using histone(Type III-S,lysine-rich,from calf thymus,Sigma,St.Louis) as substrate. All assays were carried out in 15 ml conical test tubes.γ-[^{32}P]-ATP was synthesized according to Penefsky(9) with a specific activity of 20 Ci/mmol. Inorganic [^{32}P]-phosphate was obtained from the Radiochemical Centre,Amersham.Biochemical reagents were from Boehringer,Mannheim,except for the protein kinase inhibitor from beef heart, which was from Sigma.Triton X-100,analytical grade,was from Serva,Heidelberg, and all other chemicals in analytical grade from Merck,Darmstadt.

RESULTS AND DISCUSSION

Fractions eluted from the DEAE-cellulose column in the final step of the partial purification of protein kinase from rabbit skeletal muscle(8) with 30 mM potassium phosphate buffer, containing 2 mM EDTA, showed low protein kinase activity in the absence of cAMP,but was stimulated 15 to 20 fold,when 10 pmoles of cAMP were included in the incubation mixture(Figure 1). Due to slightly enhanced background activity, in the absence of ad-

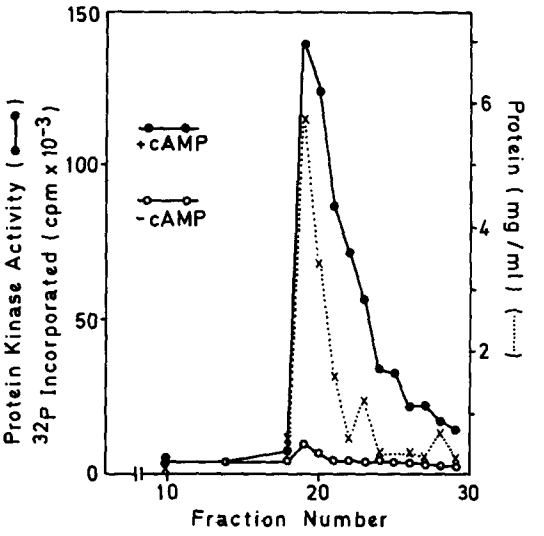

Figure 1. Elution profile of protein kinase from DEAE-cellulose column. Protein kinase from rabbit skeletal muscle was adsorbed to a DEAE-cellulose column, equilibrated with 5 mM potassium phosphate buffer, pH 7.0, containing 2 mM EDTA, and, after washing, eluted with 30 mM potassium phosphate buffer, pH 7.0, again containing 2 mM EDTA (see ref. 8 for details). 6 ml fractions were collected, and 10 µl of each fraction were tested for protein kinase activity in the absence and presence of 10 pmoles cAMP. Protein content of the fractions was determined according to (12). See Table I for details of kinase assay.

ded cAMP, in the first fraction with protein kinase activity (No. 19 in Figure 1) relative stimulation of the kinase activity by cAMP was higher in the subsequent three or four fractions.

High activities in the absence of cAMP with concomitant poor stimulation of the kinase by added cAMP were repeatedly encountered in our work with the kinase preparation and were found to vary with the individual experimentator who performed the assays in this laboratory. These puzzling variations were finally traced back to differences in the mixing of the components of the incubation mixture: the more rigorously the mixing

TABLE I

Activation of protein kinase by cAMP and agitation

Incubation mixtures contained the following components (in the order of addition): 10 µl 0.5 M sodium acetate buffer, pH 6.0, 10 µl 0.1 M Mg-acetate , 70 µl H_2O or appropriate cAMP standard. 10 µl histone (2 mg/ml), 10 µl of fraction 19(Fig.1),containing 58 µg protein determined according to (12),and 10 µl γ-[^{32}P]-ATP(500 pmoles,0.5 µCi). Incubation was for 5 min at 30°C,and phosphorylated histone was isolated according to (3). Agitation experiments (right column) were carried out in the absence of cAMP,and test tubes were agitated on a Vortex mixer for the periods indicated prior to the addition of ATP. Kinase activity in the absence of cAMP and without agitation was 1.2 pmoles ^{32}P incorporated per min into histone. One pmole of incorporated ^{32}P represents about 2200 cpm.Reactions were linear for at least 15 min.

cAMP pmoles/assay tube	relative kinase activity	agitation period (sec)	relative kinase activity
0	1	0	1
5	6	5	9
10	11.5	10	9
20	16	15	12
50	23	30	13
100	21	40	15
1000	23	60	15
		120	15

was done the higher was the kinase activity in the absence of cAMP.When incubation mixtures were subjected to increasing periods of agitation on a Vortex mixer,prior to the addition of the ATP,with which the reaction was started(3), it was found that the kinase activity could be maximally stimulated 15 to 16 fold by a 30 to 40 sec agitation, as compared to an appr. 23 fold stimulation obtained with saturating concentrations of cAMP in the assay mixture(TableI). The agitation effect was found to be fully reproducible with a protein kinase preparation from another rabbit.

TABLE II

Factors affecting agitation-induced activation of protein kinase

Conditions of the assay are described in the legend of Table I. Incubation mixtures were completed with all components prior to the addition of ATP, with which the reaction was started. The term "incubation mixture" refers to the complete mixture without ATP.

Experiment	relative kinase activity
control(-cAMP,-agitation)	1
50 sec agitation of incubation mixture	15
50 sec agitation of incubation mixture containing 100 pmoles cAMP	26
50 sec agitation of incubation mixture without histone	16
50 sec agitation of incubation mixture without kinase	1
50 sec agitation of incubation mixture containing 0.1% Triton X-100	0.4
50 sec agitation of incubation mixture containing 0.1% Triton X-100 + 100 pmoles cAMP	25
50 sec agitation of incubation mixture without histone,followed by 4 min incubation with 40 µg of protein kinase inhibitor,The incubation mixture was then completed by addition of histone and the kinase reaction started by addition of ATP.	6.4

When protein kinase,which had been activated by agitation, was subsequently assayed in the presence of cAMP the total activity did not exceed the activity observed in the presence of saturating cAMP concentrations(Table II),which indicated that mechanical agitation and cAMP produce the same effect,i.e. dissociation of the holoenzyme into the regulatory and catalytic subunits. This was corroborated by experiments in which a heat-stable protein kinase inhibitor,which is known to bind to the free catalytic subunit of protein kinase (10),was added to the

TABLE III

Relative activation of protein kinase by cAMP and by agitation in the fractions of the eluate from the DEAE-cellulose column

	Fraction No.	ratio of agitation-induced activity to cAMP-induced activity
Protein kinase activity in fractions 19-25(Fig.1) was determined either in the presence of 100 pmoles cAMP or after a 50 sec agitation of the incubation mixture prior to the addition of ATP. Conditions of the assay were as described in the legends of Fig.1 and Table I.	19	0.69
	20	0.31
	21	0.21
	22	0.22
	23	0.17
	24	0.20
	25	0.19

agitation-activated kinase prior to the assay (Table II): inhibition of the kinase activity indicates that the catalytic subunit had been released by the agitation.

Activation of the kinase by agitation is independent of the presence of histone, since agitation of the kinase in acetate-buffer and Mg-acetate alone resulted in the same activation that was obtained in the presence of histone (Table II). Agitation of histone alone was without effect (Table II). The degree of activation was similar at 4°C and at room temperature and under aerobic as well as anaerobic conditions (data not shown). Inclusion of 0.1% Trition X-100 in the incubation mixture during the agitation and subsequent assay totally prevented the activation by agitation, but left the activation by cAMP unaffected (Table II). Background activity of the kinase in the presence of 0.1% Trition X-100 and in the absence of added cAMP was reduced, thus leading to a higher relative stimulation of the kinase ac-

tivity by cAMP (Table II).When the other fractions (No.20-25,Figure 1) of the eluate from the DEAE-cellulose column were checked for activation by agitation as well as by cAMP (Table III), is was found that the relative activations achieved by agitation were smaller than that achieved with the peak fraction No.19,Figure 1).This might indicate that the effect of agitation on the kinase activity is not a direct one,but might be mediated by a factor, which is more concentrated in the earlier fractions.On the other hand, the fact that agitation never produced the full activation of the kinase that was obtained in the presence of saturating cAMP concentrations, combined with the knowledge that rabbit muscle contains at least two cAMP-dependent protein kinases(11),may indicate that only one kinase species is preferentially dissociated by agitation.Dissimilar behaviour of the two kinases that have been isolated from rabbit skeletal muscle has been described, for example, for their activation by casein or histone(11). High surface tension of the test solution may be required for the agitation effect, as a surface tension reducing agent,such as Triton X-100,completely suppresses the agitation-induced activation. It is, therefore, suggested to include 0.1% Triton X-100 in the standard protein kinase activation assay for cAMP to ensure low, reproducible background activities in the absence of added cAMP.

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