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CHANGES IN THE SPECIFIC ACTIVITY OF [γ-^{32}P]ATP DURING PROTEIN KINASE ASSAYS OF CRUDE LYMPHOCYTE EXTRACTS

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Summary

Freshly prepared crude extracts of human lymphocytes catalyze the exchange of phosphate between the γ and β positions on ATP which causes a reduction in the specific activity of the γ-phosphate of [γ-^{32}P]ATP. The exchange reaction was time dependent and inhibited by KCl (150 mM). Adenylate kinase is suggested as the most likely enzyme to catalyze this reaction.

Protein phosphorylations catalyzed by ATP:protein phosphotransferase enzymes [EC 2.7.1.37] have been extensively studied. Enzyme activity is usually assayed by measuring the transfer of label from [γ-^{32}P]ATP to an acid-stable alkali-labile product. This paper reports that crude extracts of human lymphocytes catalyze the exchange of ^{32}P between the γ and β positions of ATP which leads to a reduction in the specific activity of the [γ-^{32}P]ATP and an underestimate of the protein kinase activity.

This work arose from a study of the KCl stimulation of lymphocyte casein kinase activity. The initial rate of casein kinase activity in extracts stored at −15°C or in partially purified preparations is stimulated by KCl [1]. Although KCl does not stimulate the initial rate of casein kinase in freshly prepared extracts there is a time dependent development of KCl stimulation with prolonged assay times (See Fig. 1). It was initially thought that the time dependent effect of KCl on fresh extract resulted from an inhibition of

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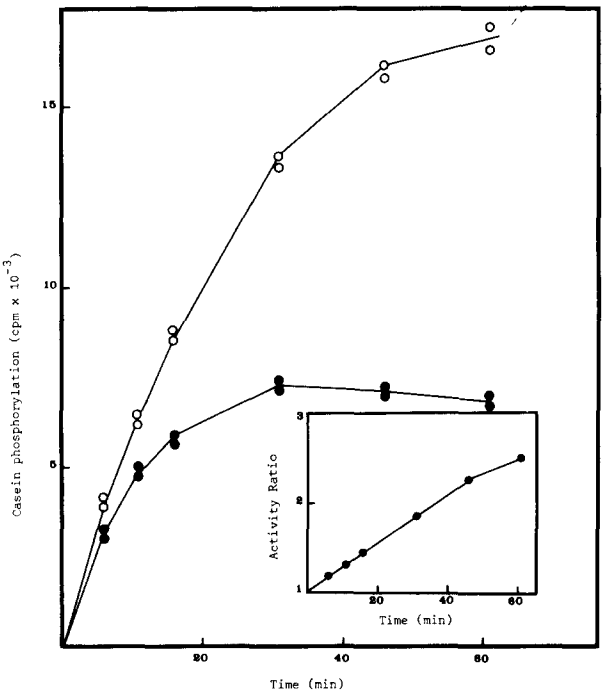

Fig. 1. Time-dependent KCl stimulation of casein kinase activity in freshly prepared lymphocyte extracts Assays were carried out in the presence (○) or absence (●) of 150 mM KCl. The insert shows the ratio of casein kinase activity in the presence of KCl to activity in the absence of KCl (activity ratio).

substrate depletion, possibly ATP, However, subsequent experiments indicated that a contributing factor was the effect of KCl on the equilibration of label between the β and γ positions of ATP which lowered the specific activity of the γ phosphate of ATP.

The preparation of human peripheral blood lymphocytes, their extraction and assay of casein kinase activity were carried out as described before [1, 2]. Isotope exchange between the β and γ positions of ATP was demonstrated by isolating labelled ATP from protein kinase assays (in which the protein substrate was omitted), digesting with myosin ATPase [3] and measuring the amount of label associated with ADP. Following deproteinizing by boiling (3 min) and centrifugation, assay samples were subjected to electrophoresis in 0.05 M citrate—Tris buffer, pH 3.4 [4]. The region corresponding to ATP was identified under ultraviolet light and eluted with water. The isolated ATP was treated with myosin ATPase in reaction mixtures (final volume 100 μl) containing 10 μmoles of glycine buffer pH 9.1, 1 μmole $CaCl_2$, myosin (285 μg protein). Reactions were incubated at 30°C for 10 min and terminated by boiling for 2 min; precipitated protein was removed by centrifugation.

Thin-layer chromatography on polyethyleneimine cellulose with 0.8M LiCl as solvent as used to separate ADP, P_i and residual ATP [5]. The propor-

TABLE I

THE EFFECT OF INCUBATION OF [γ-^{32}P]ATP WITH LYMPHOCYTE EXTRACT ON THE DISTRIBUTION OF LABEL BETWEEN THE γ AND β POSITIONS OF ATP

[^{32}P]ATP was isolated from bulk reaction mixtures both with and without incubation for 15 min. Reaction mixtures contained 25 μmoles *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes) buffer, pH 7.4, 55 nmoles [γ-^{32}P]ATP (0.38×10^6 cpm/nmole), 10 μmoles $MgCl_2$ and extract (480 μg protein) in a total volume of 0.7 ml. The isolated ATP was treated with myosin and fractionated on polyethyleneimine thin-layer plates.

Incubation time (min)	Distribution of radioactivity (%)		
	ATP	ADP	P_i
Zero	38.3	5.0	56.7
15	49.2	23.1	27.7

tion of ^{32}P corresponding to each of the markers was quantitated by liquid scintillation counting of the appropriate areas on the thin-layer plates.

Incubation of [γ-^{32}P]ATP with crude lymphocyte extract for 15 min caused nearly complete equilibration of label between the β and γ position (Table I). The addition of 150 mM KCl to the lymphocyte extract inhibited the exchange of label (Table II). In the experiment reported in Table II the labelled ATP was treated with myosin ATPase without prior isolation by electrophoresis. The exchange of label between the β and γ positions of ATP can be explained by adenylate kinase activity in lymphocyte extracts. This enzyme catalyzes the interconversion of ADP and ATP as shown below.

$$2\ ADP \rightleftharpoons ATP + AMP$$

Thus, any ADP formed during the incubation with extract can give rise to AMP. However, in the presence of [γ-^{32}P]ATP, AMP will generate [^{32}P]ATP. The net effect of this reaction is to equilibrate ^{32}P label between the β and γ positions of ATP and to label any ADP in the β position. Significantly, the equilibration would require only small amounts of ADP, either formed as a result of protein kinase activity or by endogenous ATPase activity. From a practical viewpoint where impure tissue preparations must necessarily be

TABLE II

THE EFFECT OF KCl ON THE EQUILIBRATION OF THE β AND γ PHOSPHATES OF [^{32}P]ATP INCUBATED WITH LYMPHOCYTE EXTRACT

[γ-^{32}P]ATP was incubated with lymphocyte extract and samples fractionated by thin-layer chromatography both before and after treatment with myosin. Reaction mixtures contained 5 μmoles Hepes buffer, pH 7.4, 5.4 nmoles [γ-^{32}P]ATP (0.39×10^6 cpm/nmole), 1 μmole $MgCl_2$ and extract (33 μg protein) in a total volume of 100 μl.

Incubation time (min)	Subsequent treatment	Distribution of radioactivity (%)		
		ATP	ADP	P_i
Zero	—	96	2	2
	myosin	9	5	86
20	—	58	14	28
	myosin	4	49	47
20 plus 150 mM KCl	—	76	7	17
	myosin	6	26	68

employed (for example see Ref. 6) the effects of the exchange reaction in protein kinase assays can be minimized by using low extract protein concentrations and insuring that initial rates are measured.

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