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FURTHER STUDIES ON THE ACTIVATION OF MICROSOMAL ($Na^+ + K^+$)-ATPase BY A LEUKOCYTIC PRODUCT

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Summary

Previous studies showed that microsomal ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) is activated by a proteinaeous material released by polymorphonuclear leukocytes. Investigations on the mode of action of the activator have been conducted by the isolation of ^{32}P-labeled phosphoenzyme intermediates formed in the reaction of ATP and ($Na^+ + K^+$)-ATPase, which has been postulated to occur through the formation and hydrolysis of acyl phosphate intermediates. The activator caused a concentration-dependent decrease in the recovery of phosphoenzyme intermediates that was not quantitatively altered by the Na^+ or K^+ concentration of the reaction mixture or by the presence of 1 mM ouabain. A decline in phosphoenzyme intermediate recovery was promoted by the addition of the activator to preformed phosphoenzyme intermediates but not by activator that had been pretreated with protease or phenol. In addition, the activator caused a concentration-dependent stimulation of the *p*-nitrophenyl phosphatase and acetyl phosphatase activities of microsomal ($Na^+ + K^+$)-ATPase. It was proposed that the activator stimulates the dephosphorylation step of the ($Na^+ + K^+$)-ATPase reaction sequence.

Introduction

Polymorphonuclear leukocytes and other phagocytic cells release a number of mediator substances when properly stimulated. These include endogenous pyrogen, which produces fever by stimulating certain hypothalamic thermoreceptors [1], and leukocytic endogenous mediator, which alters serum zinc and iron levels [2] and promotes hepatic amino acid flux [3], the synthesis of acute-phase proteins [4,5] and the release of neutrophilis from the bone marrow [6]. Although leukocytic endogenous mediator and endogenous pyrogen are heat labile, low-molecular weight (M_r = 10 000—30 000) proteins which

are inseparable by Sephadex chromatography [7] or by polyacrylamide gel electrophoresis [8], they differ in pH stability and species specificity and are thought to be different substances [5,9]. Recently it was shown that rabbit polymorphonuclear leukocytes obtained from glycogen-induced peritoneal exudates released an in vitro activator of microsomal ($Na^+ + K^+$)-ATPase [10] that increased the activity of the whole brain enzyme by an average of 25% ± 6 S.D.

In the present study, investigations of the mode of action of the ATPase activator were conducted by the isolation of ^{32}P-labeled phosphoenzyme (E-P) intermediates formed in the reaction of ATP and ($Na^+ + K^+$)-ATPase, which has been postulated to occur by the reaction sequence [11,12]:

$$E + ATP \overset{Na^+, Mg^{2+}}{\rightleftharpoons} E_1P + ADP \overset{Mg^{2+}}{\longrightarrow} E_2P \overset{K^+}{\rightarrow} E + P_i$$

In this model the enzyme is phosphorylated in a Na^+-dependent step by ATP, undergoes a conformational change, and is dephosphorylated in the presence of K^+. The terminal hydrolytic step of the reaction is manifested independently of the overall ($Na^+ + K^+$)-ATPase reaction as a K^+-dependent phosphatase activity. Our results indicate that the activator does not influence the Na^+-dependent phosphokinase reaction or the Mg^{2+}-dependent conformational change, but instead promotes the dephosphorylation of the E_2P complex. A preliminary report of this investigation has been communicated [13].

Experimental

Materials

[γ-^{32}P]ATP, with a specific activity of 10—30 Ci/mmol, and Aquasol scintillation spectrometry solution were obtained from New England Nuclear Corporation; and disodium ATP, disodium *p*-nitrophenyl phosphate, lithium-potassium acetyl phosphate, ouabain, and Grade IV protease from Sigma Chemical Company. Millipore filters, type HA, 25 mm, 0.45 μm pore diameter were purchased from the Millipore Corporation.

The disodium salt of *p*-nitrophenyl phosphate was converted to the Tris form by the method of Emmelot and Bos [14]. The lithium-potassium salt of acetyl phosphate was converted to the Tris form by shaking it with Dowex-50, 100-200 mesh (Sigma Chemical Co.), in the Tris form.

Microsomal ($Na^+ + K^+$)-ATPase was prepared from rabbit whole brains as previously described [10]. Leukocytic extract was prepared from rabbit polymorphonuclear leukocytes obtained from acute peritoneal exudates induced by the intraperitoneal infusion of 0.15 M NaCl and shellfish glycogen [10].

Measurement of phosphoenzyme complexes

The method of Phang and Weiss [15] was used in the determination of the phosphorylated intermediates formed during the hydrolysis of ATP by ($Na^+ + K^+$)-ATPase. Rabbit brain microsomal ATPase was reacted with [γ-^{32}P]ATP in 1.0 ml of a reaction mixture containing 50 mM Tris · HCl buffer, pH 7.4; 0.1 mM EDTA; and 3 mM $MgCl_2$. After a 0.1—1.0 min period of incubation at 0°C, the reaction was stopped by the addition of 0.2 ml of ice-cold 50%

trichloroacetic acid followed by 0.1 μmol of unlabeled P_i. The reaction mixture was passed through a Millipore filter prewashed with 10% trichloroacetic acid, 0.05 M H_3PO_4, 0.05 M disodium ATP, and 0.1 M Tris · HCl buffer, pH 8.0, to decrease nonspecific adsorption of radioactivity by the filter. The filter was washed with 10% trichloroacetic acid, dissolved in Aquasol scintillation fluid, and measured for radioactivity. In studies with leukocytic extract the yield of E-P intermediates was calculated from the difference in radioactivity recovered in the presence and absence of enzyme. In these calculations it was assumed there was one E-P intermediate per ATPase and that all phosphate bound, after appropriate corrections, was E-P intermediate. The filter itself, or unlabeled protein trapped by the filter, did not quench ^{32}P activity. Each experiment was repeated at least three times although only the results of one experiment are shown.

Enzyme assays

$(Na^+ + K^+)$-ATPase was assayed as described previously [10]. ATPase activity is expressed as μmol of inorganic phosphate released in 10 min per mg of microsomal protein. Protein was measured by the method of Lowry et al. [16].

p-Nitrophenyl phosphatase activity was measured by the production of *p*-nitrophenol. The standard assay medium contained 5 mM Tris/*p*-nitrophenyl phosphate; 20 mM Tris · HCl buffer, pH 7.4; 0.1 mM EDTA; 10 mM KCl; and 5 mM $MgCl_2$. The total volume was 1.0 ml. After a 5 min period of incubation at 37°C the reaction was stopped by the addition of ice-cold 0.5 M NaOH. After centrifugation at 13 000 × *g* for 10 min, the amount of *p*-nitrophenol in the supernatant fluid was determined from the absorbance at 410 nm. *p*-Nitrophenyl phosphatase activity is expressed as μmol *p*-nitrophenol released in 5 min per mg of protein.

Acetyl phosphatase activity was measured by the disappearance of acetyl phosphate, using the method of Lipmann and Tuttle [17]. The assay conditions were identical to those for *p*-nitrophenyl phosphatase, except that an incubation time of 20 min was used. Acetyl phosphatase activity is expressed as μmol acetyl phosphate hydrolyzed in 20 min per mg of protein.

While the results of one experiment are reported, each experiment was repeated at least twice with triplicate assays for enzyme activity.

Results

Effect of leukocytic extract on E-P intermediates

Leukocytic extract produced a concentration-dependent decrease in the recovery of E-P intermediates formed during the reaction of [^{32}P]ATP and $(Na^+ + K^+)$-ATPase (Fig. 1). Although the effect of leukocytic extract was not quantitatively altered by the concentration of Na^+ or K^+, the E-P yield was dependent upon the concentration of these ions; a maximal yield was obtained in the presence of 10 mM Na^+ and a minimal yield in the presence of 0.1 mM K^+. The recovery of radioactivity reached a maximum after a reaction time of 10 s and remained constant for 30 s. The time-course of E-P recovery was not altered by the presence of leukocytic extract.

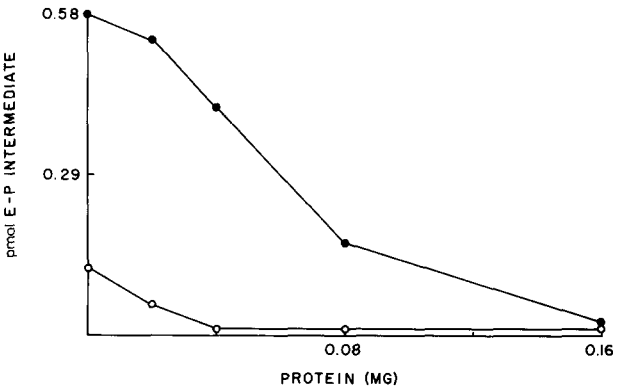

Fig. 1. Effect of leukocytic extract on the yield of E-P intermediates. 30 pmol of [γ-^{32}P]ATP was reacted with ATPase for 1.0 min in a reaction mixture containing either 20 mM NaCl (●) or 20 mM KCl (○). The results represent the mean of three experiments.

Leukocytic extract affected the steady state concentration of E-P intermediates which had been preformed in the presence of Na^+ and Mg^{2+} (Fig. 2). Although the E-P recovery remained relatively constant when neither leukocytic extract nor K^+ were present, the addition of either substance initiated a rapid decline in the E-P yield which attained a constant, minimal level after approximately 10 s. The effect induced by leukocytic extract was concentration-dependent and varied with individual preparations; however, an average loss of 68% ± 10 S.D. of the initial E-P intermediates was observed. The addition of 1 mM K^+ hydrolyzed 77% ± 9 S.D. of the intermediates. A rapid and complete dephosphorylation was initiated by the addition of 10 mM K^+ or by the simultaneous addition of 1 mM K^+ and leukocytic extract.

Treatment of leukocytic extract with protease resulted in a complete loss

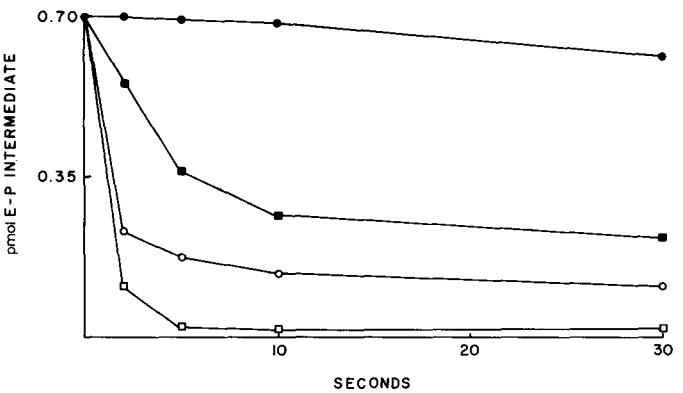

Fig. 2. Effect of 1 mM K^+ and leukocytic extract on preformed E-P intermediates. 20 pmol of [γ-^{32}P]-ATP was reacted with ATPase for 30 s prior to zero time in a reaction mixture containing 20 mM NaCl and 3 mM $MgCl_2$. The reaction was continued for the specified time with the addition of distilled water (●), 1 mM KCl (○), leukocytic extract (■), or leukocytic extract and 1 mM KCl (□). 0.1 μmol unlabeled ATP and 0.1 μmol unlabeled P_i were added after termination of the reaction with 50% trichloroacetic acid.

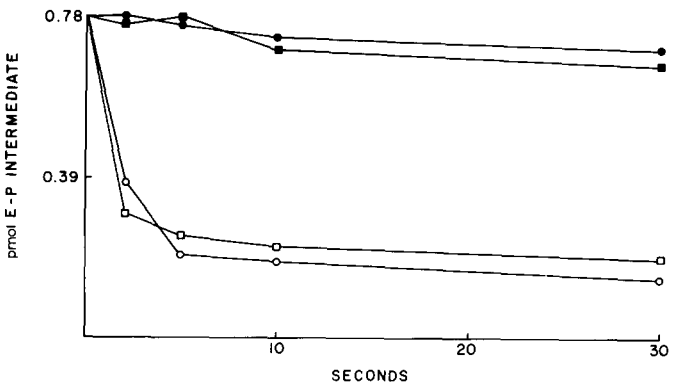

Fig. 3. Effect of 1 mM K^+ and protease-treated leukocytic extract on preformed E-P intermediates. A reaction mixture consisting of 4.5 ml of leukocytic extract and 0.1 ml of 0.1 M sodium borate buffer, pH 7.0, containing 5 mM $CaCl_2$ and 2.0 mg of protease, was incubated for 12 h at 37°C. The mixture was dialyzed against Tris · HCl buffer, pH 7.4, containing 0.1 mM EDTA. 30 pmol of [γ-^{32}P] ATP was reacted with ATPase for 30 s prior to zero time in a reaction mixture containing 20 mM NaCl and 3 mM $MgCl_2$. The reaction was continued for the specified time with the addition of distilled water (●), 1 mM KCl (○), protease-treated leukocytic extract (■), or protease-treated leukocytic extract and 1 mM KCl (□). 0.1 μmol unlabeled ATP and 0.1 μmol unlabeled P_i were added after termination of the reaction with 50% trichloroacetic acid.

of its effect on preformed E-P intermediates (Fig. 3). Similar results were obtained with leukocytic extract that had been treated with phenol or incubated at pH 1.1 for 16 h at 0°C.

Effect of (Na^+ + K^+)-ATPase inhibitors on activation by leukocytic extract

Ouabain (1 mM) did not prevent the effect of leukocytic extract on ATPase activity or on E-P intermediates although it inhibited both ATPase activity and formation of E-P intermediates. In addition, while ouabain decreased the effectiveness of K^+ in hydrolyzing preformed E-P intermediates, it had little effect on the action of leukocytic extract in preformed E-P intermediates. Similar results were obtained with dimethylsulfoxide, *N,N*-dimethylformamide, and hydroxylamine. Pretreatment of (Na^+ + K^+)-ATPase with iodoacetamide or *N*-ethylmaleimide decreased both enzymatic activity and E-P yield but did not prevent the effect of leukocytic extract on these parameters.

Effect of leukocytic extract on the K^+-phosphatase activity of (Na^+ + K^+)-ATP

Since the terminal hydrolytic step of the (Na^+ + K^+)-ATPase reaction is manifested as a K^+-dependent phosphatase activity, the effect of leukocytic extract on the *p*-nitrophenyl phosphatase and acetyl phosphatase activities of the system were investigated. The results (Fig. 4) demonstrated that both enzyme activities increased in a roughly linear manner with the concentration of leukocytic extract. In contrast, (Na^+ + K^+)-ATPase activity showed a linear stimulation only at low concentrations of leukocytic extract. Additionally, each enzyme differed in its sensitivity to activation. For example, the following activations were produced by 2 mg protein of one leukocytic extract preparation: ATPase, 38%; acetyl phosphatase, 268%; and *p*-nitrophenyl phosphatase, 152%. The higher protein concentration of the leukocytic extract used in this

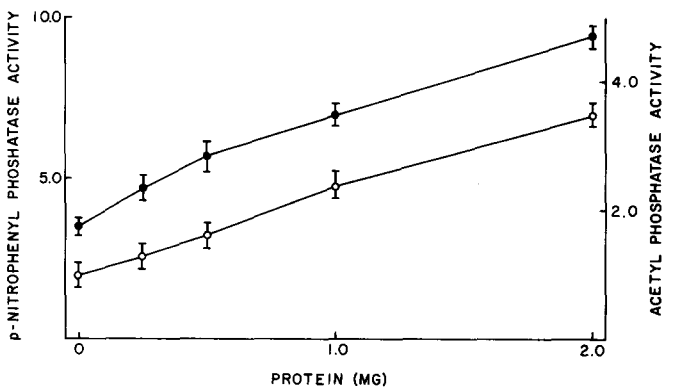

Fig. 4. Effect of leukocytic extract on *p*-nitrophenyl phosphatase and acetyl phosphatase activity. The results represent the mean ± S.D. of one experiment with triplicate assays for enzyme activity at each concentration of leukocytic extract. ●———●, *p*-nitrophenyl phosphatase; ○———○, acetyl phosphatase.

experiment compared to the experiment described in Fig. 1 is due to extra-leukocytic protein contamination of this extract. Leukocytic extract also stimulated the 5'-nucleotidase activity of whole brain microsomes. The activation of this enzyme was similar to that of $(Na^+ + K^+)$-ATPase.

Discussion

In this study the mode of action of an $(Na^+ + K^+)$-ATPase activator released by polymorphonuclear leukocytes was investigated by measurement of the ATPase E-^{32}P intermediates recovered on a Millipore filter. The sensitivity of E-P yield to various ions and ATPase inhibitors shows that the radioactivity trapped by the filter represents the phosphorylated intermediates of $(Na^+ + K^+)$-ATPase. Since leukocytic extract affected the steady state concentration of E-P intermediates and stimulated the K^+-phosphatase activity of $(Na^+ + K^+)$-ATPase, it appears that the point of action of the activator is the terminal hydrolytic step of the $(Na^+ + K^+)$-ATPase reaction sequence. The abolition of the effect after treatment of leukocytic extract with protease or phenol indicates that a proteinaeous substance, and not K^+ or K^+-like ions, was responsible for it.

Although the mechanism by which the activator stimulates $(Na^+ + K^+)$-ATPase is unknown, K^+ has been suggested to induce a change in the conformation of the enzyme, leading to a facilitation of the entrance of the acyl phosphate substrate [18] or water [19] into the hydrolytic site. Even though the failure of ouabain to inhibit the stimulatory effect of leukocytic extract suggests that K^+ and the ATPase activator act in a different manner, or at different points in the reaction sequence, both agents could act by inducing conformational changes. Recently Novogradsky [20] reported that concanavalin A (M_r = 26 000) a phytohemagglutinin, activated rat lymphocyte and brain microsomal $(Na^+ + K^+)$-ATPase and suggested that the activation may result from a membrane conformational change. The activation of 5'-nucleotidase by leukocytic extract suggests that any membrane conformational change induced by the activator may not be restricted to (Na^+K^+)-ATPase. The failure of iodoaceta-

mide- or *N*-ethylmaleimide treatment of (Na^+K^+)-ATPase to inhibit the activation suggests that enzyme sulfhydryl groups are not involved in the stimulatory mechanism.

The activation of microsomal K^+-phosphatase activity by leukocytic extract strongly suggests that the ($Na^+ + K^+$)-ATPase activator facilitates the dephosphorylation of the ($Na^+ + K^+$)-ATPase reaction. Although the relationship of K^+-phosphatase and ($Na^+ + K^+$)-ATPase is unclear, Robinson [18] has recently suggested that the K^+-phosphatase reaction reflects the terminal hydrolytic steps of the overall ($Na^+ + K^+$)-ATPase reaction but bypasses the initial Na^+-dependent formation of a phosphorylated intermediate. The difference in the sensitivity and activation kinetics of ($Na^+ + K^+$)-ATPase, acetyl phosphatase and *p*-nitrophenyl phosphatase extract may reflect certain dissimilarities in these enzyme activities which have been reported by other investigators [11,21].

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