ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 174, 744–746 (1976)

COMMUNICATIONS

Lead Ion Activates Phosphorylation of Electroplax Na^+- and K^+- Dependent Adenosine Triphosphatase [(NaK)-ATPase] in the Absence of Sodium Ion

$PbCl_2$ in micromolar concentrations stimulates phosphorylation of electroplax microsomal protein in the absence of Na^+. Other divalent cations showed little or no such effect. The (Mg^{2+} + Pb^{2+})- and (Mg^{2+} + Na^+)-dependent membrane-bound protein kinase activities in electroplax particulate preparations exhibit properties in common, including their acid stability, ouabain sensitivity, ATP specificity, and molecular size. It is concluded that the (Mg^{2+} + Pb^{2+})-dependent phosphoprotein is part of the Na^+-, K^+-dependent adenosine triphosphatase [(NaK)ATPase]. The Pb^{2+}-dependent product, in contrast to the Na^+-dependent one, is insensitive to K^+ and the hydrolysis of ATP is thus inhibited.

We report here that micromolar concentrations of $PbCl_2$, in the absence of Na^+, stimulate the phosphorylation of endogenous protein in *E. electricus* electroplax microsomes enriched in (NaK)-ATPase activity.[1] The resulting phosphoprotein is insensitive to K^+ but has a number of other properties in common with the Na^+-stimulated phosphoenzyme believed to be the catalytic unit of (NaK)-ATPase. Previously, it has been believed that phosphorylation of this enzyme by ATP has an absolute requirement for Na^+ (1). Concentrations of lead that stimulate phosphorylation are known to inhibit (NaK)-activated ATP hydrolysis (2). It is possible that the Pb^{2+}-stimulated phosphorylation is related to the mechanism of Pb^{2+} inhibition of (NaK)-ATPase. The purpose of this report is to demonstrate the similarity of the Pb^{2+}- and Na^+-dependent phosphoproteins.

Electroplax microsomes were prepared as described (3) except that the homogenizing and washing solution consisted of 5 mM Tris–HCl (pH 7.4) and 0.1 mM Tris–EDTA. Final pellets were resuspended in water. The formation of acid-stable, non-exchangeable phosphoprotein was measured as follows. Incubations were performed at 2°C for 45 s and were initiated by the addition of microsomes, 100 μg of protein, to media containing 75 mM imidazole–HCl (pH 7.4), 1 mM Tris–[γ-^{32}P]ATP (10^4 cpm/nmol), 3 mM $MgCl_2$, and either 100 mM NaCl (Na^+ medium) or 80 μM $PbCl_2$ (Pb^{2+} medium) in final volumes of 50 μl. Reactions were terminated by the addition of 50 μl of cold 10% trichloroacetic acid (TCA) and the remaining steps were carried out as described earlier (4). It was found that Pb^{2+} in the concentrations used did not alter values for the blanks prepared by denaturing the protein prior to addition of the labeled ATP. In Pb^{2+}-medium, the level of phosphorylation was highest at 15 s of incubation and diminished from that value by 9% at 60 s, 18% at 120 s, and 26% at 180 s.

Figure 1 shows that in the presence of Na^+ and Mg^{2+}, the addition of $PbCl_2$ does not alter the level of ^{32}P incorporation into phosphoprotein, except at the highest $PbCl_2$ concentration. In the absence of Na^+, however, $PbCl_2$ increases the level of phosphoprotein almost to the extent produced by Na^+. Since Pb^{2+} is about 250 times more potent than Na^+ (4), the possibility of Na^+ contamination is excluded as a cause of phosphorylation in Pb^{2+} medium. In addition, the levels of ^{32}P incorporation are not additive in the presence of saturating concentrations of both Na^+ and Pb^{2+}. This eliminates the possibility of simultaneous phosphorylations of separate sites and indicates that either the identical site is involved or that phosphorylation of one site is paralleled by inhibition at another site.

We proceeded to compare the Na^+- and Pb^{2+}-stimulated reactions. Ouabain is a specific inhibitor of (NaK)ATPase and inhibits (Mg^{2+} + Na^+)-dependent phosphorylation of the enzyme by ATP (3). We pretreated microsomes in solutions of 75 mM imidazole–HCl (pH 7.4) and 3 mM $MgCl_2$ with and without 0.5 mM ouabain at 2°C for 1 h. The microsomes were then phosphorylated as described above except that 0.1 mM ouabain was included in the media for the ouabain-treated samples. Phosphoprotein formation in control microsomes was 0.951 and 0.761 nmol·mg^{-1} in Na^+- and Pb^{2+}-media, respectively, while in ouabain-treated samples, the corresponding values were 0.078 and 0.074 nmol·mg^{-1}. Thus, both the (Mg^{2+} + Pb^{2+})- and (Mg^{2+} + Na^+)-dependent reactions are inhibited by ouabain.

[1] Abbreviations used: (Na, K)-ATPase, Na^+- and K^+-dependent adenosine triphosphatase; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

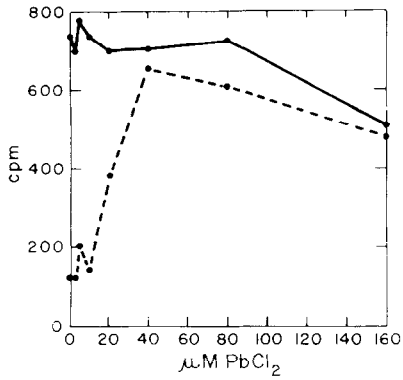

FIG. 1. Pb^{2+}-stimulated phosphorylation of electroplax microsomes. Incubations were performed as described in the text. The ordinate shows net counts per minute after subtraction of values for blanks. (●——●), 100 mM NaCl present; (●- - -●), no NaCl added.

Other divalent cations were tested under similar conditions, and we found that 0.1 mM $FeCl_2$ or $BaCl_2$ produced ouabain-sensitive phosphoprotein increments that were 7 and 17%, respectively, of that produced by 0.1 mM $PbCl_2$. Total increments of less than 9% were produced by 0.1 mM chloride salts of Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Sr^{2+}, and Hg^{2+} as compared to Pb^{2+}.

The Pb^{2+}-dependent phosphorylation, approximately as the Na^+-stimulated reaction (5), was found to be partially dependent on added Mg^{2+}. Values obtained with no added cation and in the presence of 3 mM $MgCl_2$ only, 80 μM $PbCl_2$ only, and both combined were 0.016, 0.125, 0.205, and 0.564 nmol of ^{32}P·(mg of protein)$^{-1}$, respectively. Thus it appears that either $MgATP^{2-}$ or $PbATP^{2-}$ can act as substrate and that both ions together are synergistic.

It is well known that K^+ decreases the levels of (Mg^{2+} + Na^+)-dependent phosphorylation of (NaK)-ATPase (1). This K^+ effect may ensue from inhibition of enzyme phosphorylation as well as from activation of dephosphorylation (6). It was found that the levels of (Mg^{2+} + Pb^{2+})-dependent phosphorylation were not significantly altered by the presence of 50 mM KCl in the media from the start of incubations for periods from 15 to 120 s. At 180 s of incubation, the level of phosphorylation was 19% higher in the presence of K^+. In contrast, the presence of 50 mM KCl in Na^+ media reduced the level of phosphorylation by an average of 55% at each interval, as expected (4). In a separate experiment, 50 mM KCl or an equivalent volume of water was added at the end of 45 s of incubation under phosphorylating conditions, and 15 s later TCA was added. The final ^{32}P incorporation into protein in Na^+ media was 0.779 and 0.393 nmol·mg^{-1} without and with K^+, respectively. On the other hand, in the Pb^{2+} media, ^{32}P incorporation into protein was 0.648 and 0.632 nmol·mg^{-1}, respectively. Thus, in contrast to effects on the Na^+ product, K^+ does not discharge the (Mg^{2+} + Pb^{2+})-dependent phosphoprotein once it is formed and K^+ does not inhibit its formation under these conditions. It is not yet known whether the insensitivity to K^+ is due entirely to the presence of Pb^{2+} or, possibly, also to the absence of Na^+. In addition, the effect of Mg^{2+} on the K^+ sensitivity of this reaction is not yet known.

The stabilities of the (Mg^{2+} + Na^+)- and (Mg^{2+} + Pb^{2+})-dependent phosphoproteins were found to be similar. The TCA-precipitated pellets obtained in both media were resuspended in the indicated buffers and incubated at 40°C. At various intervals, portions of the suspensions were again tranferred to ice-cold TCA and the acid-soluble supernatant fractions were obtained by sedimentation. Both phosphoproteins released 53% of their ^{32}P into the acid-soluble fraction at 60 min in 50 mM glycine-HCl (pH 2) and 57 to 60% of their ^{32}P at 15 min in 75 mM imidazole-HCl (pH 7.4).

Nucleotide affinity as measured by Na^+-stimulated phosphorylation is highest for ATP (5). In Pb^{2+} media containing 0.1 mM [γ-^{32}P]ATP, the addition of 1 mM UTP, CTP, or GTP (Tris salts) produced no inhibition. Instead, there was 31, 42, and 43% stimulation of [^{32}P]phosphate incorporation, respectively, as compared to the controls. In the same experiment, 1 mM ADP produced 16% inhibition and 1 mM adenosine or 5'-AMP produced no significant effect. Tris-P_i, 1 mM, formed a visible lead phosphate precipitate and reduced the protein phosphorylation by 69% of the control value. Experiments employing 1 mM Tris-$^{32}P_i$ and 1 mM Tris-[^{14}C]ATP in Pb^{2+} media failed to demonstrate significant label incorporation under the same conditions in which labeling by [γ-^{32}P]ATP was found. Thus, the (Mg^{2+} + Pb^{2+})-dependent phosphorylation is specific for ATP and involves the transfer of the terminal phosphate of ATP to the protein.

Finally, we wished to compare molecular sizes of the Pb^{2+}- and Na^+-dependent phosphoproteins. The TCA-washed phosphoproteins obtained in Pb^{2+} and Na^+ media were dissolved in sodium dodecyl sulfate (7) and subjected to polyacrylamide-gel electrophoresis (SDS-PAGE) as described (8). Figure 2 shows that only one labeled protein band is observed in each case and that these bands have the same mobility. About 85% of the acid-stable phosphate dissociates during solubilization and electrophoresis, in each case running ahead of the dye marker. Companion gels stained for protein with Coomassie blue demonstrate patterns similar to those already published for the electroplax microsomes (7) and do not exhibit any protein bands below the dye marker. The ^{32}P-labeled protein band corresponds in molecular size to the 98,000 molecular weight protein shown to undergo Na^+-stimulated phosphorylation

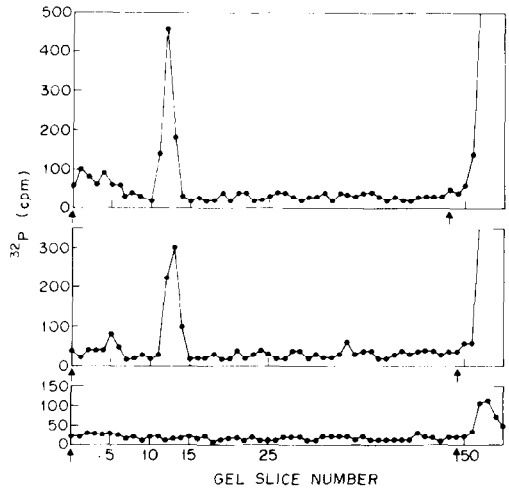

FIG. 2. Polyacrylamide-gel electrophoresis of electroplax microsomal phosphoproteins. Microsomes, 100 μg of protein, were incubated for 45 s at 2°C with 0.1 mM [γ-^{32}P]ATP (2 × 10^5 cpm/nmol), 1 mM $MgCl_2$, 75 mM imidazole (pH 7.4), and either 80 μM $PbCl_2$, 100 mM NaCl, or 50 mM KCl. Proteins were precipitated and washed as described in the text and, after storage in liquid N_2 overnight, were resuspended in cold water and again sedimented. The pellet was finally solubilized in 1% sodium dodecyl sulfate, 1% β-mercaptoethanol, and 25 mM Tris-PO_4 (pH 6.8) at 23°C for 30 min. Samples were counted directly in scintillation solvent and portions containing 50 μg of protein were applied to SDS gels for electrophoresis. After electrophoresis, the gels were sliced and the slices averaging 1.1 mm each were incubated in 30% H_2O_2 at 45°C overnight prior to scintillation counting. The entire stacking gel was counted in one piece. *Upper graph:* Pb^{2+} medium; *middle graph:* Na^+ medium; *lower graph:* K^+ medium. Left arrow indicates counts per minute in stacking gels; right arrow indicates position of dye marker at the end of electrophoresis. R_f values for the first peaks are 0.250 and 0.255, respectively, for the Pb^{2+} and Na^+ media. From our standard curve, an R_f of 0.255 corresponds to a mass of 98,000. Total phosphate incorporation in the SDS-solubilized pellets: Pb^{2+} medium, 0.596 nmol of $^{32}P \cdot mg^{-1}$; Na^+ medium, 0.636 nmol of $^{32}P \cdot mg^{-1}$; K^+ medium, 0.052 nmol of $^{32}P \cdot mg^{-1}$.

in other (NaK)-ATPase preparations (1).

These results raise a number of questions regarding the mechanism of (NaK)-ATPase as well as the possible biochemical effects of Pb^{2+} in tissue.

Our working hypothesis is that Pb^{2+} through binding to enzyme ligands abolishes the requirement for Na^+-induced conformational changes in the enzyme otherwise necessary for phosphorylation of the catalytic site of (NaK)-ATPase and, in the presence of ATP, also inhibits an enzyme conformational transition necessary for K^+-activated hydrolysis of the phosphoenzyme. The second effect would account for the observed inhibition by lead ion of ATP hydrolysis (2). These two effects might ensue from Pb^{2+} binding to either a single site or multiple sites. The interactions with Na^+ and K^+ are under investigation currently. The mechanisms involved in Na^+–enzyme interactions leading to transfer and conservation of bond energy from ATP are not understood. The effects of lead ion might be a useful clue for physicochemical approaches to this question.

In addition, it is noted that although other divalent cations are known to be involved in phosphoryl transfers (9), this is the first known report of Pb^{2+}-stimulated kinase activity in any system. The implications for both the biochemistry and toxicity of inorganic lead in tissues remain to be explored.

ACKNOWLEDGMENT

Supported by National Science Foundation Grant No. PCM75-05979.

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Received October 23, 1975