Inhibition by Lead Ion of *Electrophorus* Electroplax (Na\(^+\) + K\(^+\))-Adenosine Triphosphatase and K\(^+\)-p-Nitrophenylphosphatase*

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Inorganic lead ion in micromolar concentrations inhibits *Electrophorus* electroplax microsomal (Na\(^+\) + K\(^+\))-adenosine triphosphatase ((Na\(^+\) + K\(^+\))-ATPase) and K\(^+\)-p-nitrophenylphosphatase (NPPase). Under the same conditions, the same concentrations of PbCl\(_2\) that inhibit ATPase activity also stimulate the phosphorylation of electroplax microsomes in the absence of added Na\(^+\). Enzyme activity is protected from inhibition by increasing concentrations of microsomes, ATP, and other metal ion chelators. The kinetics follow the pattern of a reversible noncompetitive inhibitor. No kinetic evidence is elicited for interactions of Pb\(^{2+}\) with Na\(^+\), K\(^+\), Mg\(^{2+}\), ATP, or p-nitrophenylphosphate. Na\(^+\)-ATPase, in the absence of K\(^+\), and (Na\(^+\) + K\(^+\))-NPPase activity at low [K\(^+\)] are also inhibited. ATP inhibition of NPPase is not reversed by Pb\(^{2+}\). The calculated concentrations of free Pb\(^{2+}\) that produce 50% inhibition are different for ATPase and NPPase activities. Pb\(^{2+}\) may act at a single independent binding site to produce both stimulation of the kinase and inhibition of the phosphatase activities.

Inorganic lead ion has been found to stimulate phosphorylation by [γ-\(^32\)P]ATP of electroplax (1) and rat brain (2) microsomal (Na\(^+\) + K\(^+\))-activated adenosine triphosphatase in the absence of added sodium ion. This effect of lead ion is surprising because Na\(^+\) exerts specific effects in the regulation of enzyme phosphorylation that are related to its unique role in the physiologic function of sodium and potassium transport and since Na\(^+\) appears to be an absolute requirement for phosphorylation under physiologic conditions (3). However, the molecular mechanism involved in Na\(^+\) regulation of enzyme phosphorylation is not understood, and lead ion may be a useful probe in studies of the enzyme's reactions. This paper describes the characteristics of Pb\(^{2+}\) inhibition of the phosphorylation functions of electroplax enzyme. It is found that Pb\(^{2+}\) inhibits (Na\(^+\) + K\(^+\))-ATPase, Na\(^+\)-ATPase, K\(^+\)-NPPase, and (Na\(^+\) + K\(^+\))-NPPase activities noncompetitively in concentrations similar to those that abolish the Na\(^+\) requirement for phosphorylation of electroplax microsomes. It is suggested that all the effects may arise from an action of Pb\(^{2+}\) at a single independent Pb\(^{2+}\) binding site on the enzyme.

**METHODS AND MATERIALS**

**RESULTS**

**Effects of Pb\(^{2+}\) on (Na\(^+\) + K\(^+\))-ATPase**— PbCl\(_2\) produces very little or no inhibition of Mg\(^{2+}\)-ATPase activity while strongly inhibiting (Na\(^+\) + K\(^+\))-ATPase activity. The value for total [PbCl\(_2\)] is 4 μM, and the calculated free [Pb\(^{2+}\)] is 0.5 μM for this experiment in the presence of 3 mM MgCl\(_2\) and 3 mM ATP (Fig. 1). In earlier experiments, under the same conditions but with dilute enzyme suspended in imidazole added last to reaction mixtures, the value for total [PbCl\(_2\)] was 10 to 15 μM. The reason for the difference is not known. A clear dependence on time of exposure to lead could not be demonstrated. Effects on the microsomes of prior dilution in imidazole are also of possible pertinence.

The inhibition due to PbCl\(_2\) can be prevented by the inclusion of various chelators or thiol in the incubation medium (Fig. 1). In this experiment, the enzyme was added last to the reaction mixture. Under these conditions, the order of potency for reduction of inhibition appears to be EDTA > RAL = DL-penicillamine > EGTA = 1,3-dithiothreitol. In separate experiments performed as in Fig. 2, 0.1 mM L-cysteine reduced the inhibition due to 10 μM PbCl\(_2\) from 86% to 41% while concentrations up to 1 mM of glutathione, L-cystine, DL-α-lipoic acid (oxidized), or coenzyme A produced little or no alteration in the inhibition (data not shown). Table I shows that BAL and EDTA are also able to reverse inhibition when added to enzyme suspensions containing PbCl\(_2\) after sufficient time for Pb\(^{2+}\) to inhibit the enzyme. It is not known whether the small differences in reversal related to temperature in these experiments are significant.

We wished to compare the inhibition of hydrolysis with the stimulation of phosphorylation. Since the phosphorylation experiments employ 50 times more microsomal protein than do

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\* The abbreviations used are: (Na\(^+\) + K\(^+\))-ATPase, (Na\(^+\) + K\(^+\))-activated adenosine triphosphatase; K\(^+\)-NPPase, K\(^+\)-activated p-nitrophenylphosphatase; EGTA, ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid; BAL, 2,3-dimercaptopropanol.

\* Methods and Materials and References are presented as a miniprint supplement immediately following this paper. (Figs. 1 through 11 and Tables I through III are found on pp. 5204-5205.) They are also available as 17 pages of full size photocopies from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20014. Request JBC Document Number 76M-1366. Cite the authors, and include a check or money order for $2.55 per set of photocopies.
the hydrolysis assays and the dissociation constant for lead enzyme appears to be less than $10^{-4}$ M, it was necessary to study the effects of protein concentration on Pb$^{2+}$ inhibition. In addition, such experiments can provide information about the reversibility of inhibition when low concentrations of inhibitor are used (9). Fig. 3A shows that the rate of ATP hydrolysis is not a linear function of the enzyme concentration but curves upward exhibiting an activation effect. Since the addition of BAL tends to straighten out the curve (Fig. 3A), it is suggested that the lower specific activities obtained with diluted microsomes are due to enzyme denaturation, and that this denaturation is decreased by BAL. The denaturation is probably related to sulfhydryl oxidation. If it were due to the presence of inhibitors in the microsomes, one would expect the specific activity to fall with increasing microsomal concentration. Unfortunately, BAL or other protective agents cannot be used in experiments designed to study Pb$^{2+}$ inhibition. Fig. 3A shows that the percentage of Pb$^{2+}$ inhibition tends to decrease, but not stoichiometrically, at the higher microsomal concentrations. The inset in Fig. 3A shows, however, that the activity curves at low enzyme concentrations with and without PbCl$_2$ are readily extrapolated to the origin, indicating reversible inhibition (9). The decrease in Pb$^{2+}$ inhibition at higher microsomal concentrations may be due to binding of Pb$^{2+}$ at nenzymatic low affinity sites or effects of dilution on the accessibility of enzyme sites to lead ion (or both). Fig. 3B shows the same departures from linearity for hydrolysis of p-nitrophenylphosphate. These results indicate that the kinetics of lead actions cannot be compared at widely divergent concentrations of microsomes.

PbCl$_2$ inhibition was studied under approximately the same conditions employed for measurements of steady state levels of phosphorylation, i.e. 0.1 mg of microsomal protein and 1-min incubations at 2°C. Under these conditions, the values for total [PbCl$_2$], are 15 and 21 μM at 1 and 3 mM ATP, respectively (Fig. 4). The value for total [PbCl$_2$] at 15 to 20 μM (Fig. 5). Although the prior exposure of microsomes to PbCl$_2$ does not appear to alter this value significantly, it seems to potentiate inhibition of phosphorylation by excessive concentrations of PbCl$_2$ (Fig. 5).

Hill plots for the activation of ATPase by Na$^+$ and K$^+$ (not shown) demonstrated no significant effect of PbCl$_2$ on the values for n or the [S]$_{50}$ for either Na$^+$ or K$^+$ whether the enzyme was first exposed to 4 μM total PbCl$_2$ as described under "Methods and Materials" and added last to complete media containing 15 μM total PbCl$_2$. Approximately 50% inhibition was obtained in both sets of experiments. The reason for the lower apparent affinity for PbCl$_2$ in the latter method is not understood. The important point is that PbCl$_2$ produced only a decrease in the maximal observed velocity and had no effect on the activation parameters. This indicates that the extent or rate of development of inhibition is not dependent on Na$^+$ or K$^+$ concentrations.

A separate experiment was performed with a 4-fold greater concentration of enzyme to test the inhibition of Na$^+$-stimulated ATPase in the absence of K$^+$. In the presence of 40 mM NaCl, 0.2 μM of protein/μl, no added KCl, but other conditions as in Fig. 1. 10 μM total PbCl$_2$ produced 70% inhibition of the Na$^+$-dependent increment in activity.

Mg$^{2+}$ activation is shown in Fig. 6. The presence of 20 μM total PbCl$_2$ does not significantly change the value for total [MgCl$_2$]$_{50}$ (0.2 to 0.25 mM in the presence of 3 mM ATP). The extent of Pb$^{2+}$ inhibition is markedly potentiated by concentrations of MgCl$_2$ higher than 0.4 mM. This observation excludes the possibility that lead inhibition is due to competition with Mg$^{2+}$ either for an enzyme site or for binding to ATP$^{4-}$. The potentiating effect of MgCl$_2$ on Pb$^{2+}$ inhibition probably could be due to displacement of Pb$^{2+}$ from PbATP$^{2-}$.

Fig. 7 shows that the inhibition due to PbCl$_2$ is reduced by increasing ATP concentrations at a constant level of MgCl$_2$. Since the concentration of MgCl$_2$ in this experiment is 750 times that of PbCl$_2$, the concentrations of MgATP$^{2-}$ and Mg$^{2+}$ at given levels of added ATP are not significantly changed by the presence or absence of PbCl$_2$. The effect of ATP on the extent of inhibition at given concentrations of total PbCl$_2$ could be explained by its influence on the concentration of free lead ion.

In order to test the possibility of interactions between Pb$^{2+}$ and PbATP$^{2-}$, Mg$^{2+}$, MgATP$^{2-}$, or ATP$^{4-}$, a series of PbCl$_2$ concentration curves at three different levels of MgCl$_2$ and ATP were performed (Table II). In these experiments, the enzyme was added to the reaction medium last. Under these three sets of conditions, the concentrations of free and complexed magnesium ions and ATP vary 20-fold. Although the observed values for total [PbCl$_2$]$_{50}$ under the three conditions also vary through the same range, from 3.5 μM to 68 μM, the corresponding calculated values for free [Pb$^{2+}$]$_{50}$ are all within a 2-fold range of 1.5 to 3.3 μM. Therefore, the apparent affinity for Pb$^{2+}$ as measured by inhibition of ATP$^{4-}$ hydrolysis is not significantly altered by ATP$^{4-}$, Mg$^{2+}$, MgATP$^{2-}$, or PbATP$^{2-}$.

The observed effects of ATP and MgCl$_2$ on the extent of inhibition at given concentrations of PbCl$_2$ can be explained simply by their influences on the concentration of free lead ion.

**Effects of Pb$^{2+}$ on NPPase Activity** - K$^+$-stimulated NPPase activity is believed to represent the phosphatase function in the overall enzyme cycle (8). Fig. 8 shows that the value for total [PbCl$_2$]$_{50}$ as measured by inhibition of K$^+$-NPPase activity is 1.3 μM. In this experiment, the residual activity with 20 μM PbCl$_2$ corresponds to that proportion of activity seen in the absence of added KCl. The inhibition of the non-K$^+$-dependent portion was found to vary in different experiments from 0% to 30% under conditions that maximally inhibited the K$^+$-dependent portion. Although information concerning dissociation constants for complexes of p-nitrophenylphosphate with Mg$^{2+}$ or Pb$^{2+}$ is lacking, these constants are expected to be much greater than those for the metal ion complexes with ATP. Therefore, the observed value of 1.3 μM for total [PbCl$_2$]$_{50}$ as measured by K$^+$-NPPase inhibition may be close to the free [Pb$^{2+}$] under these conditions.

The K$_m$ for p-nitrophenylphosphate is 1.87 mM without PbCl$_2$ and 1.96 mM with PbCl$_2$ (Fig. 9). The V$_{max}$ on the other hand, is reduced from 10.07 to 0.035 μmol·mg$^{-1}$·min$^{-1}$ by 1.5 μM total PbCl$_2$ in this experiment. A Hill plot for the K$^+$-activation of NPPase showed no effect of 1.5 mM PbCl$_2$ on the values for n or [S]$_{50}$ (data not shown).

The activation by Mg$^{2+}$ is shown in Fig. 10. There appears to...
be no change in the total [MgCl_2]_5 produced by 1.5 μM total PbCl_2 (0.5 mM) but in contrast to results with ATPase, there is no potentiation of inhibition by high concentrations of Mg^{2+}. The failure of MgCl_2 to potentiate the inhibition due to PbCl_2 is consistent with the assumption of insignificant metal ion complex formation by p-nitrophenylphosphate.

Thus, there is no kinetic evidence for an interaction of Pb^{2+} with the substrate site or activation sites for Mg^{2+} or K^+.

However, there appears to be evidence for separate regulatory sites for Na^+ and K^+ in addition to a catalytic K^+ site as measured by NPPase activity of electroplax microsomes in media containing dimethylsulfoxide (11). Fig. 11A shows that this observation also holds for the electroplax enzyme in aqueous suspension. The stimulation of NPPase by [K^+] less than 1 mM is potentiated by Na^+; this effect exhibits a plateau from 0.25 mM to 1 mM K^+; between 1 mM and 6 mM K^+ the same concentration of Na^+ becomes inhibitory. Since two qualitatively different K^+ sites can be distinguished by their differing responses to Na^+, it is of obvious interest to test their responses to Pb^{2+}. Fig. 11B shows that the (Na^+ + K^+)-dependent NPPase activity found at 1 mM K^+ is also inhibited by 3 μM total PbCl_2 just as is the K^+-NPPase at higher [K^+].

Experiments employing enzyme prepared from other tissues have shown a synergistic stimulation of NPPase by Na^+ plus ATP at low [K^+] (12-15). In addition, it has been shown that the simultaneous presence of Na^+ plus ATP at high [K^+] reverses the inhibition produced by either substance alone under this condition (cf. Ref. 16 for electroplax). Since Pb^{2+} appears to mimic an action of Na^+ in that Pb^{2+} stimulates enzyme phosphorylation, it is conceivable that Pb^{2+} might also, in the presence of ATP, mimic the action of Na^+ in inhibition. In a separate experiment, we determined that the total [ATP]_5 for inhibition of K^+-NPPase is 60 μM in the presence of 5 mM MgCl_2, 5 mM p-NPP, and 20 mM KCl (data not shown). The data in Table III indicate that the addition of 15 μM total PbCl_2 is equally inhibitory with or without 60 μM ATP at 1 mM or 20 mM KCl. It can also be seen that the inhibition due to ATP is not reversed by Pb^{2+}. Thus, Pb^{2+} does not appear to mimic this potentiating effect of Na^+ on K^+-NPPase with or without ATP present.

**DISCUSSION**

Lead ion at concentrations near 10^-6 M is a reversible inhibitor of electroplax microsomal (Na^+ + K^+)-ATPase and K^+-NPPase activities. Little or no inhibition of nonspecific ATPase is found. Metal ion chelators, such as EDTA, EGTA, penicillamine, thiols, and ATP readily prevent the inhibition. BAL and EDTA were also shown to reverse the inhibition. Effects of MgCl_2 and ATP on inhibition appear related to the concentration of ATP^2- available to form PbATP^2-. The fact that ATP in concentrations employed for enzyme assays reverses inhibition by chelating Pb^{2+} makes it impossible to accurately determine the stability of lead enzyme by measuring inhibition. Treatment of enzyme with Na^+, K^+, Mg^{2+}, and ATP prior to Pb^{2+} does not alter the extent of subsequent inhibition. The extent and rate of development of inhibition are not influenced by the concentrations of Na^+ or K^+ present throughout the exposure of enzyme to Pb^{2+}. The nature of the liganding group on the enzyme responsible for binding Pb^{2+} is not known. Candidates include sulfhydryl (17, 18), carboxylate (18, 19), and imidazole groups (20).

It is of technical interest that Pb^{2+} inhibition is reduced at higher protein concentrations indicating probable binding to low affinity nonenzymic sites which tend to buffer the free [Pb^{2+}]. This makes it difficult to compare inhibition kinetics from experiments employing widely different protein concentrations or different tissues and types of enzyme preparations.

The only observed kinetic effect of Pb^{2+} is to reduce the V_{max} for hydrolysis of both ATP and p-nitrophenylphosphate under all conditions investigated. No evidence for an effect of Pb^{2+} on the apparent affinities for Na^+, K^+, Mg^{2+}, MgATP^2-, or p-nitrophenylphosphate, or vice versa, could be obtained, as measured by inhibition of hydrolysis. The inhibition follows the pattern of a reversible, noncompetitive inhibitor which produces an EI complex incapable of catalyzing hydrolysis no matter what substrates or physiologic ligands are present. The calculated concentrations of free Pb^{2+} required for 50% inhibition of hydrolysis of both ATP and NPP are in the same range. Two classes of K^+ sites detected by their stimulation of NPPase are distinguished as described by earlier studies (11); the low affinity site is inhibited by Na^+ and the high affinity site is potentiated by Na^+. The same concentrations of Pb^{2+} that inhibit (Na^+ + K^+)-ATPase and Na^+-ATPase also inhibit K^+-NPPase and (Na^+ + K^+)-NPPase. The simplest assumption is that Pb^{2+} binding to a single independent site inhibits a reaction of the enzyme necessary for all these activities.

In a previous communication we have shown that PbCl_2 abolishes the physiologic requirement of Na^+ for microsomal enzyme phosphorylation by ATP (1). The Pb^{2+}-dependent phosphoprotein in the absence of Na^+ was shown to be similar to the usual Na^+-dependent product in pH stability, molecular size in sodium dodecyl sulfate, specificity for ATP, and ouabain sensitivity, but insensitive to Na^+. Yet, there is no kinetic evidence obtained with electroplax enzyme for Na^+-Pb^{2+} interactions, and it cannot be presumed that Pb^{2+} binds to a Na^+ site. It is shown in this paper that the value for total [PbCl_2]_5 for inhibition of the ATPase activity is similar to that found for stimulation of phosphorylation when obtained under the same conditions. It is possible that effects on both the kinase and phosphatase enzymes may ensue from a single action of Pb^{2+} at an independent site to produce or stabilize an enzyme conformation that can be phosphorylated but that cannot catalyze hydrolysis of the enzyme phosphate or of p-nitrophenylphosphate. A previously discussed enzyme kinetic model has dealt with evidence consistent with the assumption of reversibly alternating conformations distinguishable by Na^+-sensitive kinase or K^+-sensitive phosphatase activities (5, 21, 22).

In terms of this model, the effect of Pb^{2+} may be represented as follows.

\[ E + Pb^{2+} \rightleftharpoons Pb^{2+}E, \]

\[ Mg^{2+} + Pb^{2+}E + ATP \rightleftharpoons Pb^{2+}E + P + ADP \]

\[ Pb^{2+}E + ATP \rightleftharpoons Pb^{2+}E + P + ADP \]

\[ E \] represents any species of enzyme and Pb^{2+}E represents a modified form of E_1 (5) that can be phosphorylated in the absence of Na^+ but that cannot undergo conformational change to E_2 which would be necessary for hydrolytic activity. The failure of Pb^{2+} to reverse ATP inhibition of NPPase and the absence of measurable Pb^{2+}-stimulated hydrolytic activity are consistent with this assumption. Pb^{2+}E_P would appear to provide a protected hydrophobic environment for the phosphate. It is not yet clear whether Reaction II is reversible, and no implication is given regarding the energy level of the phosphate bond in Pb^{2+}E_P. Residual reactivity of the lead enzyme with physiologic ligands in studies of phosphorylation and transphosphorylation is the subject of a future report.

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Pb**+ Inhibition of Na**+ + K**+-ATPase and K**+-NPPase

**Fig. 1.** Time-course of inhibition of Na**+ + K**+-ATPase by Pb**+ ions. The reaction mixture was incubated at 37°C for 10 min. The reaction was started by addition of ATP and Mg**2+. The enzyme was preincubated with various concentrations of Pb**+ for 10 min before the addition of ATP and Mg**2+. The reaction was stopped by addition of trichloroacetic acid. The reaction mixture was then centrifuged, and the optical density of the supernatant was measured at 340 nm. The results are expressed as a percentage of the control. The control value was 100%. The curves represent the mean ± SEM of three experiments. (A) Without Pb**+ ions, (B) with 5 mM Pb**+ ions, (C) with 10 mM Pb**+ ions, (D) with 15 mM Pb**+ ions, (E) with 20 mM Pb**+ ions, (F) with 25 mM Pb**+ ions, (G) with 30 mM Pb**+ ions, (H) with 35 mM Pb**+ ions, (I) with 40 mM Pb**+ ions, (J) with 45 mM Pb**+ ions, (K) with 50 mM Pb**+ ions, (L) with 55 mM Pb**+ ions, (M) with 60 mM Pb**+ ions.
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