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Effects of Pb^{++} and other Divalent Cations on Ouabain Binding to *E. electricus* Electroplax ($Na^+ + K^+$)-Adenosinetriphosphatase

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SUMMARY

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$PbCl_2$ reduces the level of [^{3}H]ouabain binding to ($Na^+ + K^+$)-adenosinetriphosphatase in *E. electricus* electroplax microsomal preparations in the presence of Mg^{++} and ATP with or without Na^+. The inhibition is competitive with ATP, and Na^+ is cooperative with Pb^{++} in reducing the affinity for ATP at a site involved in ouabain binding. The ATP site involved in ouabain binding is not the substrate site since no such cooperative interactions among Pb^{++}, Na^+, and ATP could be observed with regard to phosphorylation or inhibition of hydrolysis. ADP also stimulates ouabain binding, which is inhibited competitively by Pb^{++}. Cd^{++}, Cu^{++}, Hg^{++}, and Zn^{++} also inhibit ouabain binding in the presence of Mg^{++}, ATP, with or without 50 mM Na^+ but only the inhibition by Cd^{++} is potentiated by 50 mM Na^+. $BaCl_2$, $FeCl_2$, $CaCl_2$, $MnCl_2$, $NiCl_2$, $CoCl_2$, and $SrCl_2$ at concentrations of 60 μM did not inhibit drug binding.

INTRODUCTION

The rate of ouabain binding to ($Na^+ + K^+$)-ATPase from various tissues can be accelerated in two different pathways of enzyme reactions: (1) Na^+-stimulated, Mg^{++} plus nucleotide-dependent and (2) Na^+-inhibited, Mg^{++} plus P_i-dependent (1–4). These and related studies (5, 6) indicate complicated effects of ligands on multiple conformational properties or restraints of the enzyme. In any attempt to assign effects on the enzyme to specific ligand sites, those effects that are obligatorily dependent on the production of phosphoenzyme need to be identified. There has been some controversy as to the role of ATP in ouabain binding (5).

Recent studies have shown that Pb^{++}, while an inhibitor of ATP hydrolysis (7, 8), stimulates phosphorylation by ATP of the catalytic polypeptide of *E. electricus* electroplax (7, 9) and of rat brain (10) ($Na^+ + K^+$)-ATPase in the absence of Na^+. The Na^+- and Pb^{++}-dependent [^{32}P]peptides in proteolytic digests of labeled electroplax enzyme are electrophoretically identical (11). The present study investigates whether Pb^{++} stimulates ouabain binding. The results describe inhibition by Pb^{++} and other divalent cations of ouabain binding and lead to the conclusion that ouabain binding does not depend on enzyme phosphorylation.

METHODS AND MATERIALS

Tritiated ouabain was a product of New England Nuclear Co., Boston, and Tris nucleotides were obtained from Sigma Chemical Co., St. Louis. Microsomal preparations of ($Na^+ + K^+$)-ATPase were prepared from

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electroplax of *E. electricus* as described (9). Binding of [^{3}H]ouabain to microsomes was assayed by the method of filtration as reported earlier (4). Microsomes were routinely exposed at twice the final concentration of $PbCl_2$ for 10 min at 2° prior to addition of reaction mixtures. In the usual assay, [^{3}H]ouabain bound to microsomes was measured after incubation of 40 μg microsomal protein for 15 min at 23° in 40 μl in media containing, in final concentrations, 75 mM imidazole (pH 7.4), 0.01 mM [^{3}H]ouabain (0.5 Ci/mmol), 2.5 mM $MgCl_2$, 1.25 mM ATP, and other additions, including NaCl, as noted. Where other divalent cations were tested, microsomes were first exposed to the indicated chloride salt in twice the final concentration for 10 min at 2°.

RESULTS AND DISCUSSION

Table 1 shows that the Pb^{++} inhibition of ouabain binding depends to some extent on the length of time the microsomes are first exposed to Pb^{++}; this relationship is observed for up to 10 min at 2°. The addition of Na^+ to the reaction mixture increases the per cent inhibition produced by Pb^{++} (Table 1). In other experiments, the order of exposure of microsomes to Mg^{++}, ATP, and Pb^{++} did not alter the subsequent inhibition of ouabain binding. The presence of Na^+, however, in the Pb^{++} exposure medium containing Mg^{++} and ATP increased the inhibition effect of Pb^{++} when ouabain was subsequently added. In some experiments this potentiating effect of Na^+ was about 20% greater when Na^+ was added to microsomes after the Pb^{++} as compared to the effect of Na^+ added at the same time as the Pb^{++}. In all following experiments, microsomes were first exposed to Pb^{++} for 10 min at 2° prior to addition of reaction mixtures.

As expected, incubation for 15 min at 23° under the conditions studied in Table 1 produces maximum or saturation levels of ouabain binding even in the absence of Na^+, (4). In order to determine whether the effect of Pb^{++} is on the rate or on the saturation level of binding, incubations with ouabain were performed as indicated in Table 1 but the intervals were varied. Microsomes were first exposed to Pb^{++} for 10 min at 2° prior to addition of reaction mixtures. Levels of ouabain binding with or without Na^+ were found to vary less than 10% during incubations for 15, 30, and 60 min while the inhibition due to Pb^{++} was 94, 94, and 90% in the presence of Na^+, and 40, 50, and 49% in the absence of Na^+. The effect of Pb^{++} is, therefore, on the level of binding. Rates of Pb^{++} dissociation and association must be very much faster than the rate of ouabain binding.

Figure 1 shows the inhibition produced by chloride salts of Cd^{++}, Hg^{++}, Cu^{++}, and Pb^{++} in the absence of Na^+. The $[I]_{0.5}$ values are 15 to 20 μM for Cd^{++}, Hg^{++} and Cu^{++}, and 28 μM for Pb^{++}. In other experiments ouabain binding measured as in Figure 1 was not inhibited by 60 μM $BaCl_2$,

TABLE 1

Effects of Pb^{++} exposure time and Na^+ on inhibition of [^{3}H]ouabain binding

Microsomes, 40 μg protein, were exposed to 55 μM $PbCl_2$ at 2° for the indicated times and then diluted with an equal volume of reaction mixture containing 0.01 mM ouabain, 3 mM $MgCl_2$, 1 mM ATP plus or minus 125 mM NaCl in final concentrations. Other conditions were as in METHODS

Pre-exposure time	$PbCl_2$	No Na^+		125 mM Na^+	
		[^{3}H]bound	inhibition	[^{3}H]bound	inhibition
min		*pmoles·mg^{-1}*	%	*pmoles·mg^{-1}*	%
2	−	762		777	
2	+	599	21	252	78
5	−	738		747	
5	+	498	33	159	79
10	−	752		847	
10	+	422	44	122	86
20	−	786		844	
20	+	433	45	154	82

$FeCl_2$, $CaCl_2$, $MnCl_2$, $NiCl_2$, $CoCl_2$, or $SrCl_2$. $ZnCl_2$, 60 μM, produced 30% inhibition. Zn^{++}, undoubtedly, is a more potent inhibitor than indicated in these results since imidazole, which binds Zn^{++}, was used in this study.

In earlier studies, increasing the concentration of microsomes in assay mixtures was found to reduce the apparent potency of Pb^{++}, presumably because of Pb^{++} binding to nonenzyme sites (9). Therefore, the $[PbCl_2]_{0.5}$ of 28 μM for inhibition of ouabain binding (performed with 40 μg microsomal protein) represents an affinity for Pb^{++} that may be 1.5 to 2 times lower than that represented by the value of 20 μM (9) obtained for stimulation of phosphorylation or inhibition of hydrolysis (performed with 100 μg microsomal protein). Although the potencies of Pb^{++} as measured by effects of ouabain binding and phosphorylation are close, the data do not allow the two effects to be assigned to the same Pb^{++} binding site.

Table 2 shows the effect of Na^+ on the inhibition produced by the metal ions. Only the inhibition produced by Pb^{++} and Cd^{++} is potentiated by Na^+. We could not explain the grouping, as measured by their effects on ouabain binding, of all the metal ions used in this study by the order of their affinities for sulfide or other possible enzyme functional groups (12) or for chelators (13) or from their effects on phosphorylation. Of these divalent ions, only Pb^{++} stimulates phosphorylation (10).

Figure 2 shows that Na^+ is cooperative with Pb^{++} in producing inhibition of ouabain binding. The Pb^{++} response curve is sigmoidal and is shifted by Na^+ toward lower concentrations of Pb^{++}. Values for $[PbCl_2]_{0.5}$ are 23 and 36 μM with and without 125 mM NaCl, respectively, under the conditions of this experiment.

Decreasing the concentration of $MgCl_2$ in

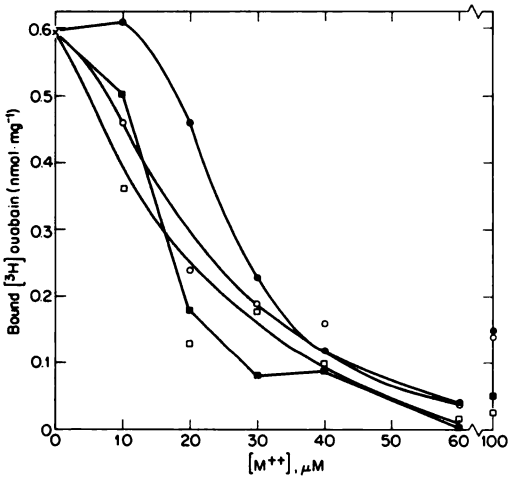

FIG. 1. *Divalent cation inhibition of ouabain binding*

The experiment was performed as described in Methods except that reaction mixtures contained 3 mM $MgCl_2$, 1 mM ATP, and no NaCl. ●——●, $PbCl_2$; ○——○, $CuCl_2$; ■, $HgCl_2$; □, $CdCl_2$.

TABLE 2

M^{++} inhibition of ouabain binding: interactions with Na^+

Chloride salts were used. Reaction mixtures contained 3 mM $MgCl_2$, 1 mM ATP plus or minus 50 mM NaCl in final concentrations. Other conditions were as in METHODS except that incubation with ouabain was for 2 min at 23°. Differences due to Na^+ in the presence of Cu^{++} and Hg^{++} are not significant. Control values for ouabain binding were 0.31 and 0.49 μmol·mg^{-1} without and with Na^+, respectively. These are amounts of binding obtained in 2 min and are not saturation levels. Thus the effect of Na^+ on the rate of binding is discernible in the control data.

M^{++} added		No Na^+	50 mM Na^+
	μM	% inhibition	
Pb^{++}	28	34	99
Cd^{++}	15	46	96
Cu^{++}	15	60	65
Hg^{++}	15	43	37
Zn^{++}	125	75	50

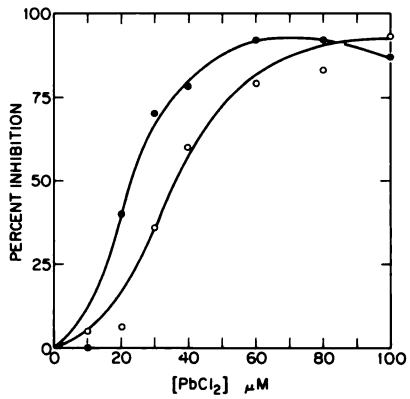

FIG. 2. *Effect of Na^+ on Pb^{++} inhibition of ouabain binding*

Conditions were as described in METHODS. ○——○, no Na^+; ●——●, 125 mM NaCl.

the reaction mixtures reduces the percent of inhibition due to $PbCl_2$. In the presence of 1 mM ATP, 27.5 μM $PbCl_2$, and no NaCl, inhibition of ouabain binding measured as in METHODS, was 58, 32, 16, and 0% in the presence of 3, 2, 1, and 0.5 mM $MgCl_2$, respectively. This effect of reducing [Mg^{++}] can be accounted for by the increasing formation of $PbATP^-$, although an additional competitive effect of Mg^{++} cannot be excluded.

The cooperative effects of Pb^{++} and Na^+ on the apparent affinity for ATP are shown in Figures 3 and 4. The $[ATP]_{0.5}$ values for ouabain binding are less than 0.05 mM under these conditions in the presence or absence of Na^+, provided that Pb^{++} is excluded. The inclusion of 27.5 μM $PbCl_2$ increases the $[ATP]_{0.5}$ to about 0.1 mM in the absence of Na^+ (Fig. 3) and to about 0.5 mM in the presence of Na^+ (Fig. 4). Of course, a portion of the ATP reversal of Pb^{++} inhibition is related to chelation of Pb^{++} but this could not account for the almost complete reversal when 1 mM ATP is added in the presence of 3 mM $MgCl_2$ (Fig. 3) or the difference due to Na^+. Pb^{++} also increases the $[ADP]_{0.5}$ value as measured by ouabain binding in the absence of Na^+ from about 0.05 to about 0.5 mM (Fig. 5). The ADP stimulation of ouabain binding could not be accounted for by presumed conversion to ATP since the adenylate kinase activity in these microsomal preparations as detected by the formation of [^{14}C]AMP from [^{14}C]-

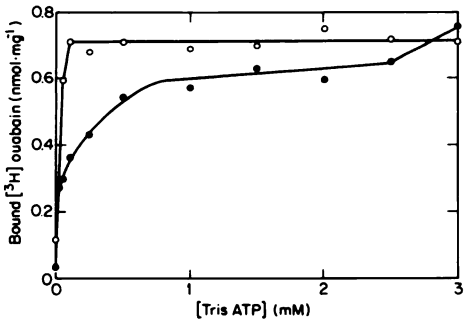

FIG. 3. *Pb^{++} inhibition of ATP-dependent ouabain binding in the absence of Na^+*

The experiment was performed as described in METHODS except that $MgCl_2$ was 3 mM and various ATP concentrations were used without added Na^+. ○—○, no $PbCl_2$; ●—●, 27.5 μM $PbCl_2$.

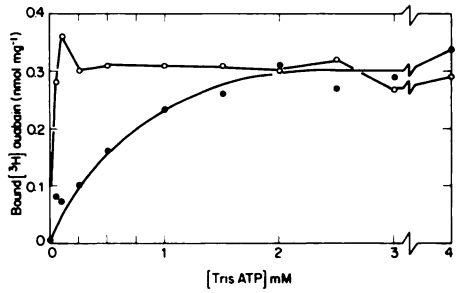

FIG. 4. *Pb^{++} inhibition of ATP-dependent ouabain binding in the presence of Na^+*

The conditions were as described in METHODS, except that various ATP concentrations were used in the presence of 125 mM NaCl. ○—○, no $PbCl_2$; ●—●, 27.5 μM $PbCl_2$.

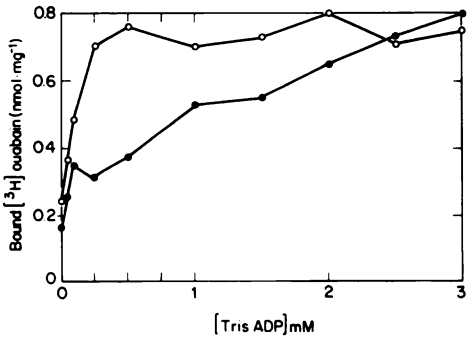

FIG. 5. *Pb^{++} inhibition of ADP-dependent ouabain binding in the absence of Na^+*

The experiment was performed as in Figure 3. ○—○, no $PbCl_2$; ●—●, 27.5 μM $PbCl_2$.

ADP is less than needed to convert 2% of the ADP to ATP under the present conditions (11). Experiments with Na^+ and ADP were not performed for further comparison. The parsimonious assumption that ATP and ADP act at the same site, while not obligatory, is consistent with the fact that both nucleotides support Na^+-stimulated ouabain binding (4). This could be tested with additional experiments. This assumption suggests that the cooperativity between Na^+ and Pb^{++} would be stronger with ADP than with ATP because of the lower affinity for ADP.

Prior studies have shown that $PbCl_2$ in concentrations less than 60 μM stimulates and produces no inhibition of phosphorylation, whether or not Na^+ is present (7, 9). Moreover, Na^+ does not potentiate Pb^{++} inhibition of hydrolysis (9) and there is no

evidence for interaction between Pb^{++} and ATP as measured by hydrolytic activity (9). Therefore, the nucleotide site interactive with Na^+ and Pb^{++} and involved in ouabain binding is not the substrate site involved in phosphorylation or hydrolysis.

The strongest evidence that phosphorylation is necessary for ouabain binding is the absent or inhibitory effects of nonphosphorylating ATP analogues on ouabain binding (14, 15). Differences in metal ion chelation (16) or in conformation could explain these results with ATP analogues (17). On the other hand, other studies showing that ADP supports Na^+-stimulated ouabain binding almost as well as ATP suggest that enzyme phosphorylation is not critical for Na^+-stimulated ouabain binding (4). The present findings add further support to this conclusion. An alternative explanation could be that Na^+ and nucleotides produce conformational effects on the enzyme apart from any consequent to enzyme phosphorylation. The nucleotide site, *in situ,* is on the cytoplasmic surface while the ouabain site is on the external surface of the cell (18, 19). These presumed locations, together with the data provided here, suggest that these nucleotide-produced conformational effects are transmitted through the cell membrane. While these effects are important for ouabain binding, it is not known whether the identical effects are important in regulation of cation translocation. ATP may have activating influence, as indicated in studies measuring hydrolytic activity (20).

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