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EFFECT OF PHOSPHOLIPASE A TREATMENT ON THE PARTIAL REACTIONS OF AND OUABAIN BINDING TO A PURIFIED SODIUM AND POTASSIUM ACTIVATED ADENOSINE TRIPHOSPHATASE

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Abstract—The effects of phospholipase A treatment on ouabain binding to and the partial reactions of a purified, mammalian NaK-ATPase (sodium and potassium activated adenosine triphosphatase) preparation were investigated. The treatment removed 70 per cent of the lipid phosphate without altering the relative quantities of the two protein subunits. Hydrolytic activity was reduced 70 per cent, whereas K^+-phosphatase and steady-state phosphorylation (minus K^+) were inhibited by 30 per cent. The phosphoenzyme formed was insensitive to both K^+ and ADP at 0°, but not at 22°. The number of [^{3}H]ouabain binding sites was not affected at 30°, but the rates of binding in the presence of Mg^{2+} plus inorganic phosphate and Mg^{2+}, ATP, sodium and potassium were reduced. At 0°, both the rates of binding and the apparent number of binding sites were markedly reduced.

It is now widely accepted that the membrane-bound NaK-ATPase‡ possesses an absolute requirement for lipids. Although several reports of the effects of disrupting this lipid–enzyme complex with detergents [1–6], organic solvents [7, 8], or phospholipases [9–13] can be found in the literature, much of the data are conflicting. Much of the variability in the observations can be ascribed to the different types of lipid depletion agents and the sources and types of NaK-ATPase preparations used, as well as to the methods of measuring enzymatic function.

This paper describes the effects of treating a purified mammalian NaK-ATPase with a purified phospholipase A fraction. ATP hydrolytic activity, phosphorylation, dephosphorylation, K^+-phosphatase activity, [^{3}H]ouabain binding and lipid content are reported.

EXPERIMENTAL PROCEDURES

Purification of NaK-ATPase. NaK-ATPase from frozen lamb kidney outer medulla was prepared essentially as described previously [14, 15] with the following modifications. Sodium iodide treatment of the microsomal fraction was carried out for 30 min. The sodium iodide-treated microsomes were adjusted to 0.1% sodium deoxycholate with 0.1 mg/mg of protein, stirred for 15 min, centrifuged for 1 hr at 100,000 *g*, suspended in the imidazole plus salts buffer (0.7 ml/mg of original sodium iodide microsomes), recentrifuged and resuspended in the same buffer to approximately 10 mg protein/ml. Sodium deoxycholate–sodium cholate (3:1) at 0.40% (w/v) and a detergent to protein ratio of 0.55 were used in the extraction of the deoxycholate-washed sodium iodide microsomes. The glycerol-precipitated enzyme fraction was extracted with 0.90 mg sodium deoxycholate–sodium cholate (3:1)/mg of protein. The resulting supernatant was adjusted to 25% saturation with a buffered ammonium sulfate solution, stirred for 3 min and centrifuged for 15 min at 75,000 *g*. The supernatant was decanted, diluted with approximately $\frac{1}{3}$ volume of 25 mM imidazole, pH 7.1, 1 mM Tris–EDTA, 20% glycerol and 10 mM 2-mercaptoethanol, and dialyzed overnight against 20 vol. of the same buffer minus 2-mercaptoethanol. The enzyme was then dialyzed for approximately 3 hr against 1 mM Tris–EDTA, pH 7.1, centrifuged at 100,000 *g* for 2 hr, resuspended in a small volume of 1 mM Tris–EDTA, and dialyzed for 3 days against 100 vol. of 1 mM Tris–EDTA, pH 7.1, with daily buffer changes. The dialyzed NaK-ATPase was precipitated at 100,000 *g* for 1 hr, resuspended in 1 mM Tris–EDTA to a protein concentration of 6–15 mg/ml, and could be stored at 3° for several weeks without any loss of NaK-ATPase activity.

Purification of phospholipase A. Phospholipase A (EC 3.1.1.4.) was partially purified from *Crotalus adamanteus* venom (Sigma Chemical Co.) by a modification of the procedure of Wells and Hanahan [16]. The lyophilized venom (500 mg) was suspended in 5 ml of 50 mM Tris–acetate, pH 8.0, 1 mM Tris–EDTA, 100 mM NaCl and centrifuged at 100,000 *g* for 1 hr. The supernatant fraction was applied to a Sephadex G-100 column (2.5 cm × 40 cm) that had been equilibrated in the same buffer. The fractions containing phospholipase A activity were pooled and

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dialyzed against 50 mM Tris-acetate, pH 8.0, and 1 mM Tris-EDTA. The dialyzed fraction was then added to a DEAE cellulose column (Whatman DE-52, 2.5 cm × 20 cm) in the same buffer and eluted with a 0 to 0.2 M NaCl gradient. The two peaks of phospholipase A activity were pooled, dialyzed against 25 mM imidazole, pH 7.3, containing 1 mM Tris-EDTA, concentrated, and stored at 3° in the same buffer containing 50% glycerol.

Phospholipase A treatment. NaK-ATPase (25 mg) and phospholipase A (5 mg) were preincubated for 3 min at 37° in 5 ml of 25 mM imidazole, pH 7.2, containing 1% fatty acid poor bovine serum albumin (FAP-BSA, Pentex). The reaction was initiated by adding $CaCl_2$ to a concentration of 5 mM and after 10 min was diluted with 3 vol. of cold 25 mM imidazole, pH 7.2, 1 mM Tris-EDTA and 1% FAP-BSA. The NaK-ATPase was pelleted by centrifuging at 100,000 *g* for 1 hr, washed twice with the imidazole-EDTA buffer plus FAP-BSA and twice with the same buffer minus FAP-BSA. The final pellet was resuspended in 25 mM imidazole, pH 7.2, and 1 mM Tris-EDTA to a protein concentration of 3–6 mg/ml. Two incubation controls, enzyme plus phospholipase A minus $CaCl_2$ and enzyme plus $CaCl_2$ with no phospholipase A, were utilized initially and found to yield identical results. Incubation with either $CaCl_2$ or phospholipase A alone had no measurable effect on the NaK-ATPase. Thereafter, the control enzyme was treated as described above, except that no phospholipase A was added to the incubation medium.

Measurement of NaK-ATPase activity. NaK-ATPase activity was measured at 37° by the spectrophotometric coupled-enzyme assay [17] in a medium containing 25 mM histidine, pH 7.2, 2.5 mM Na_2ATP, 5 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 1 mM Tris-EDTA, 0.4 mM NADH, 2 mM phosphoenolpyruvate, and pyruvate kinase/lactic acid dehydrogenase (Sigma).

Determination of K^+-phosphate activity. K^+-phosphatase activity was determined at 37° by the continuous spectrophotometric measurement at 410 nm of the amount of *p*-nitrophenol liberated from *p*-nitrophenylphosphate. The reaction mixture contained 30 mM imidazole, pH 7.8, 4 mM $MgCl_2$, 1 mM Tris-EDTA, 4 mM *p*-nitrophenylphosphate (Tris salt, Sigma), plus and minus 20 mM KCl.

Other measurements. Protein was determined by the method of Lowry *et al.* [18], with crystalline bovine serum albumin as standard. Lipids were extracted from the NaK-ATPase twice with 9 vol. of chloroform-methanol (2:1) at 25°. The extract was washed with 0.3 vol. of 0.9% NaCl and evaporated with nitrogen. Esterified fatty acids, lipid phosphate and cholesterol were determined as described by Entenman [19], Bartlett [20] and Zlatkis *et al.* [21] respectively. One-dimensional thin-layer chromatography of the chloroform-methanol extracted phospholipids was carried out by the method of Skipski *et al.* [22].

[^{3}H]ouabain binding. Ouabain binding was carried out at 30° essentially as described by Wallick and Schwartz [23]. Four different standard binding conditions were used: (a) (MgP_i) = 2.5 mM $MgCl_2$ and 2.5 mM inorganic phosphate; (b) (MgATP) = 2.5 mM $MgCl_2$ and 2.5 mM Tris-ATP; (c) (MgATPNa) = 2.5 mM $MgCl_2$, 2.5 mM Tris-ATP and 100 mM NaCl; and (d) (MgATPNaK) = 2.5 mM $MgCl_2$, 2.5 mM Tris-ATP, 100 mM NaCl and 1 mM KCl (30°) or 0.1 mM KCl (0°). In addition, each incubation medium contained 50 mM Tris-Cl, pH 7.4, and 2.5×10^{-6} M [^{3}H]ouabain (New England Nuclear, 100 mCi/m-mole). The binding reaction was initiated by adding 50–75 μg NaK-ATPase and stopped by adding unlabeled ouabain (final concentration 10^{-3} M), cooling and filtering through 0.22 μm Millipore filters. As previously described [23], the rate of binding k_{obs} was obtained by plotting ln $(A_e - A)/A_e - A_0)$ vs time, where A, A_0 and A_e are the amount of [^{3}H]ouabain bound to the enzyme at time = t, time = 0, and time = equilibrium respectively. With the conditions used, the rates of [^{3}H]ouabain binding exhibited first-order kinetics for both the native and the phospholipase-treated enzyme.

Phosphorylation. Steady-state phosphorylation of the NaK-ATPase was measured at 0° and 22° essentially as described by Post *et al.* [24]. The reaction mixture (0.5 ml) contained in 3 mM $MgCl_2$, 25 mM imidazole, pH 7.4, 20 mM NaCl and 40–75 μM [γ^{-32}P]ATP (New England Nuclear, 45 mCi/m-mole) and terminated after 5 or 10 sec by adding 3 ml of cold 10% trichloroacetic acid, 1.6 mM Na_2ATP and 1.5 mM H_3PO_4, followed by filtration through 0.45 μm Millipore filters and washing three times with 5 ml of the same solution.

To compare the approximate rates of dephosphorylation, the enzyme was first phosphorylated as described above and then a 100-fold excess of unlabeled ATP plus and minus 5 mM ADP or 0.01 to 100 mM KCl was added to the reaction mixture. The reaction was terminated 4 sec (22°) or 5 sec (0°) after this addition, as described above.

RESULTS AND DISCUSSION

Using the modified procedure described here for the purification of NaK-ATPase, approximately 30 mg of purified enzyme can be isolated from 100 g of lamb kidney medulla. This represents almost a 2-fold increase in the yield of protein and activity over that obtained with our previously reported procedure [14, 15]. Although this procedure requires 5–7 days to complete, we have found it to be particularly advantageous for the purification of large quantities (400–800 mg) of lamb kidney NaK-ATPase. The specific activity of the purified enzyme was between 900 and 1350 μmoles P_i/mg/hr, with less than 0.5 per cent of the activity ouabain insensitive. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate revealed the presence of only a Mr = 95,000 protein and a Mr = 40,000 glycoprotein. The enzyme preparation was vesicular and contained approximately 0.6 μmole phospholipid, primarily phosphatidylethanolamine and phosphatidylcholine and 0.16 μmole cholesterol/mg of protein.

Treatment of the NaK-ATPase at 37° for 10 min with purified phospholipase A reduced both the esterified fatty acid and the lipid phosphate content by 60–70 per cent, indicating that entire phospholipid molecules had been removed from the lipase-treated preparation (Table 1). Thin-layer chromatography of lipids extracted from both control and lipase-treated NaK-ATPase fractions revealed that virtually all of the phosphatidylethanolamine, phosphatidylserine

Table 1. Effects of phospholipase A treatment on NaK-ATPase*

	Control	Treated
Phospholipid (μmoles mg^{-1})	0.52	0.21
Esterified fatty acids (μmoles mg^{-1})	1.28	0.58
Cholesterol (μmoles mg^{-1})	0.16	0.13
NaK-ATPase (μmoles mg^{-1} hr^{-1})	1080	300
K^+-phosphatase (μmoles mg^{-1} hr^{-1})	220	160
Phosphoenzyme (pmoles mg^{-1})	1950	1370
[^{3}H]ouabain binding (pmoles mg^{-1})	2080	1950

* NaK-ATPase was digested for 10 min with phospholipase A and washed as described under Experimental Procedures. [^{3}H]ouabain binding values shown are the average of the equilibrium binding values determined at 30° with the four different ligand conditions [23].

and phosphatidylcholine had been removed. Only a trace of one lysophospholipid, lysophosphatidylethanolamine, was found to be associated with the lipase-treated enzyme. This is in contrast to the earlier report by Taniguchi and Tonomura [12], that phospholipase A digestion converted the phospholipids to lysophospholipids with only a slight decrease in the amount of lipid phosphate present. It appears that in our NaK-ATPase preparation cleavage of the β-fatty acid ester bond by phospholipase A reduces the hydrophobic interaction of the resulting lysophospholipid to such an extent that it is released from the lipoprotein complex. The cholesterol content of the NaK-ATPase was only slightly decreased after the phospholipase A digestion.

Control and phospholipase-treated NaK-ATPase fractions were subjected to SDS-polyacrylamide gel electrophoresis as described by Laemmli and by Lane [25, 26]. The protein profiles of the two enzyme fractions were identical and consisted of only two proteins. There was no detectable alteration in the molar ratio of the two proteins after digestion with phospholipase A. There was also no visible contamination of the lipase-treated enzyme fraction with either bovine serum albumin or phospholipase A.

Concomitant with the decrease in phospholipids, the NaK-ATPase activity was reduced by 60–80 per cent. The addition of aqueous emulsions of soybean lecithin (Sigma), phosphatidylserine (Nutritional Biochemicals), phosphatidylcholine (General Bio-chemicals), or a total lipid extract of the NaK-ATPase to the phospholipase-treated enzyme did not result in any significant restoration of NaK-ATPase activity.

K^+-phosphatase, which is thought to be a manifestation of the potassium-dependent dephosphorylation step in the reaction scheme of the NaK-ATPase, was reduced only about 30 per cent by phospholipase A digestion. The rate of potassium-dependent dephosphorylation was also measured directly by "chasing" the [^{32}P] phosphoenzyme with potassium (Fig. 1 and 2). When the "chase" experiment was conducted at 0°, the phosphoenzyme formed with the lipase-treated enzyme was observed to be very insensitive to added potassium. With the addition of 0.1 mM potassium to the phosphoenzymes, only 11.5 per cent of the control phosphoenzyme remained after 5 sec, com-

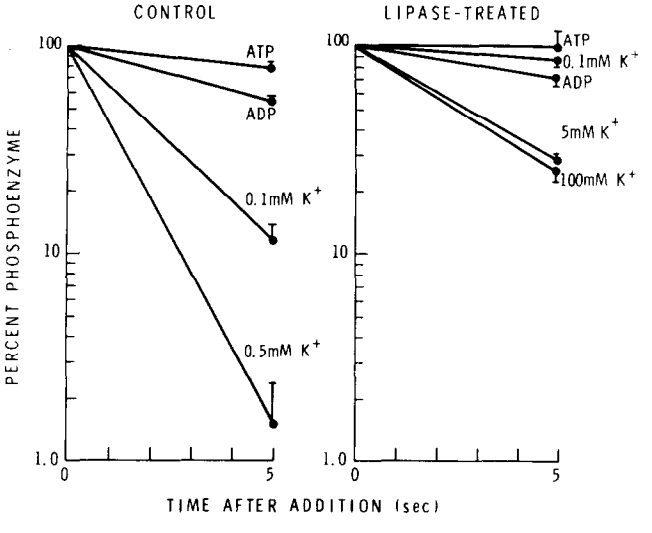

Fig. 1. Sensitivity of [^{32}P]phosphoenzyme to dephosphorylation at 0°. Control and lipase-treated NaK-ATPase were phosphorylated at 0° with [γ-^{32}P]ATP. At zero time (10 sec after initiation of phosphorylation with [γ-^{32}P]ATP), a 100-fold excess of unlabeled ATP plus and minus 5 mM ADP or the concentrations of KCl shown was added. The reaction was terminated 5 sec after this addition as described in Experimental Procedures. The points shown are means ± S. E. M. of two to six determinations.

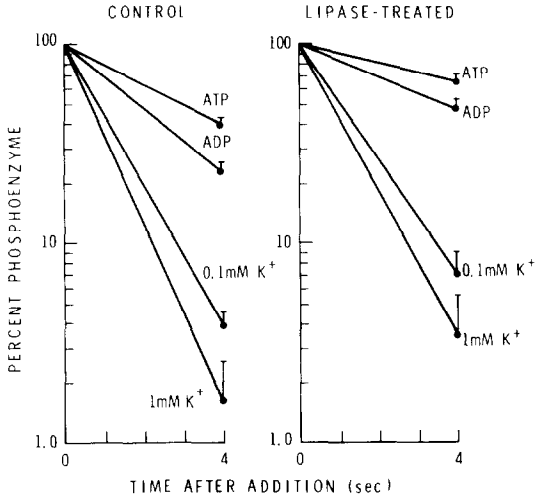

Fig. 2. Sensitivity of [^{32}P]phosphoenzyme to dephosphorylation at 22°. The reaction was carried out exactly as in Fig. 1, except that the temperature was 22° and the reaction was terminated 4 sec after the addition of excess unlabeled ATP. The points shown are means ± S. E. M. of two to six determinations.

pared with 86 per cent of the lipase-treated phosphoenzyme. When the "chase" concentration of potassium was increased to 100 mM, the control phosphoenzyme was completely dephosphorylated within the 5-sec measurement period, whereas 26 per cent of the lipase-treated phosphoenzyme remained. Neither the control nor the lipase-treated phosphoenzyme was very sensitive to ADP.

When the "chase" experiment was conducted at 22°, the lipase-treated phosphoenzyme was found to be much more sensitive to added potassium (Fig. 2). With 0.1 mM potassium, 3.8 per cent of the control phosphoenzyme and 7 per cent of the lipase-treated phosphoenzyme remained after 4 sec. Both phosphoenzymes were completely dephosphorylated within 4 sec in the presence of 5 mM potassium. Although the percentage of lipase-treated phosphoenzyme remaining after the 0.1 mM potassium "chase" was consistently higher than that of the control, the actual difference was so small that we cannot be certain whether this represents a decreased rate of dephosphorylation for the lipase-treated NaK-ATPase. It is interesting to note that a decrease in the rate of dephosphorylation would be consistent with the observed 30 per cent reduction in K^+-phosphatase activity of the lipase-treated enzyme.

In some respects, these results are similar to those reported by Taniguchi and Tonomura [12], where rates of phosphoenzyme decomposition were calculated from the ratio of hydrolytic activity to phosphoenzyme concentration at varying potassium concentrations. They estimated that the rate of phosphoenzyme decomposition at 37° with venom-treated microsomes was much less sensitive to increasing potassium than with untreated microsomes. We observed, however, that although the hydrolytic activity of our lipase-treated NaK-ATPase was reduced by 70 per cent at 37°, the rate of phosphoenzyme decomposition, as measured by the "chase" at 22°, appeared to be very similar to that of the control enzyme. Only at 0° was the difference in rates of potassium-dependent dephosphorylation large enough to be detected by the "chase" technique.

* E. T. Wallick, unpublished results.

Table 2. Steady-state phosphoenzyme levels for control and phospholipase A-treated enzymes at 0° and 22° *

KCl (mM)	Control (pmoles/mg)		Treated (pmoles/mg)	
	0°	22°	0°	22°
0	2130	1950	1440	1430
0.01	1830	1670	1200	1310
0.10	100	550	760	700
1.0	0	0	550	370
5.0	0	0	70	70
100.0	0	0	0	0

* Enzyme fractions were phosphorylated with [γ-^{32}P]-ATP as described under Experimental Procedures with varying concentrations of KCl included in the incubation medium. At 22° both enzymes were incubated for 5 sec and at 0° for 10 sec. A background level of 100 pmoles/mg, determined in the presence of 20 mM KCl, minus NaCl, was subtracted from all values.

The steady-state levels of phosphorylation for control and lipase-treated NaK-ATPase at various potassium concentrations were measured at both 0° and 22° (Table 2). In the absence of potassium, the level of control phosphoenzyme was consistently 30 per cent higher than that of the lipase-treated enzyme. In the presence of 0.1 to 5 mM potassium, the steady-state levels of the lipase-treated phosphoenzyme were always higher than those of the control enzyme at both temperatures.

Because the lipase-treated phosphoenzyme exhibited a marked difference in its sensitivity to the potassium "chase" at 0° and 22°, it was expected that there would also be a marked difference in the steady-state levels of lipase-treated phosphoenzyme at the two temperatures. However, as shown in Table 2, the steady-state levels of the lipase-treated phosphoenzyme were virtually the same at both temperatures. This suggests that for the lipase-treated enzyme, the effect of temperature on decomposition of the phosphoenzyme is very similar to the effect of temperature on the overall rate of formation of the phosphoenzyme. This is in contrast to the control enzyme where raising the temperature increases the rate of formation more than it does the rate of decomposition* (Table 2).

When measured at 30°, the total number of [^{3}H]-ouabain binding sites on the NaK-ATPase was not altered by phospholipase A treatment. For the four ligand conditions studied, both control and lipase-treated enzymes bound approximately 2000 pmoles/mg of protein. This is in contrast to the decreased amount of phosphoenzyme formed with the lipase-treated enzyme (Tables 1 and 2) and suggests that at least a portion of the lipase-treated NaK-ATPase can bind ouabain even though it cannot be phosphorylated.

As indicated in Table 3, the rate of [^{3}H]ouabain binding in the presence of MgATP was also

Table 3. Pseudo first-order rate constants for [^{3}H]ouabain binding to phospholipase A-treated and control NaK-ATPase at 30°*

Binding conditions	$k$sec^{-1} × 10^3)	
	Control	Treated
MgPi	62.1	32.3
MgATP	12.4	12.9
MgATPNa	35.2	27.3
MgATPNaK	16.8	9.94

* Phospholipase A treatment, binding conditions and determination of [^{3}H]ouabain binding rates are as described under Experimental Procedures, in the presence of 2.5 × 10^{-6} M [^{3}H]ouabain. The concentration of potassium used was 1 mM.

unchanged by phospholipase A digestion. With MgATPNa the rate of binding to the lipase-treated enzyme appeared to be slightly reduced and, in the presence of MgP_i and MgATPNaK, the rates of binding were reduced to approximately one-half those of the control enzyme. These observations are consistent with those reported previously by Taniguchi and Iida [13], following the digestion of an ox brain microsomal fraction with *Naja naja* venom, but inconsistent with their earlier work [27] in which no change in rates was noted. Also, Goldman and Albers [10] reported that *Naja naja* venom treatment of an *Electrophorus* NaK-ATPase fraction completely destroyed ouabain binding activity, as well as hydrolytic activity.

The decreased rate of ouabain binding to the lipase-treated enzyme in the presence of MgATPNaK suggested that this enzyme might have an altered sensitivity for potassium. To test this possibility, the dissociation constants for potassium and sodium with respect to ouabain binding were determined. Plots of $T_{1/2}$ for ouabain binding vs the reciprocals of sodium concentrations at various potassium concentrations were examined for both control and lipase-treated enzymes (Fig. 3). It was found that the data fit the following equation [28, 29]:

$$k_{obs} = \frac{k_{max}}{1 + \frac{K_a}{[Na^+]}\left(1 + \frac{[K^+]}{K_i}\right)} \quad (1)$$

where k_{obs} is the observed pseudo first-order rate constant, k_{max} is the maximum pseudo first-order rate constant (at infinite sodium) in the presence of

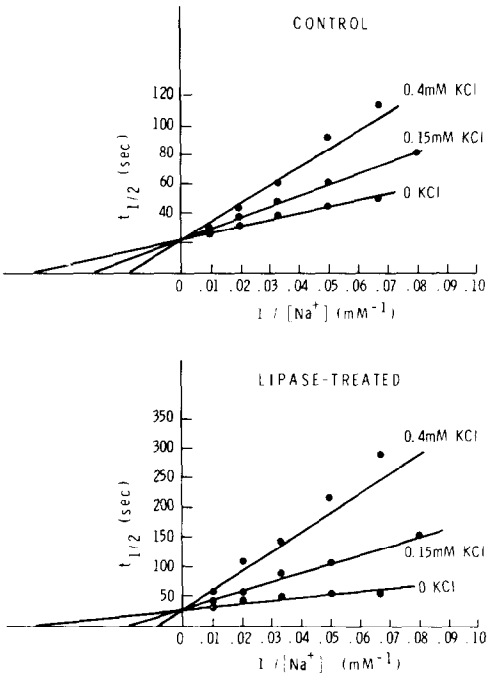

Fig. 3. Times for half-maximal [^{3}H]ouabain binding ($T_{1/2}$) vs reciprocal of NaCl concentration. [^{3}H]ouabain binding was carried out as described in Experimental Procedures in the presence of 2.5 mM $MgCl_2$, 2.5 mM Tris–ATP, 50 mM Tris–Cl, 2.5 × 10^{-6} M [^{3}H]ouabain and varying concentrations of NaCl and KCl at 30°. Since the reaction was first-order, $T_{1/2} = 0.693/k_{obs}$, where k_{obs} is the observed pseudo first-order rate constant as explained in Experimental Procedures. The lines drawn are those that would result from the values of k_{max}, K_a and K_i listed in Table 4 for Equation 1.

2.5 × 10^{-6} M ouabain, and K_a and K_i represent apparent dissociation constants for sodium and potassium respectively. The data were fit directly to the above equation using an iterative method [30, 31]. A good fit of the data to this equation was obtained, as shown in Fig. 3. The constants derived from these plots are shown in Table 4. As indicated, the affinity of the lipase-treated enzyme for potassium was approximately 2.5-fold greater than that of the control enzyme, whereas the affinity for sodium was unchanged.

[^{3}H]ouabain binding to control and lipase-treated NaK-ATPase was also measured at 0°. As with potassium-dependent dephosphorylation, we observed that

Table 4. Kinetic constants for the rate of [^{3}H]ouabain binding to control and phospholipase-treated NaK-ATPase at 30°*

Kinetic parameters	Control	Treated
k_{max}	0.0318 ± 0.0021 sec^{-1}	0.0248 ± 0.0015 sec^{-1}
$T_{1/2}$	21.8 sec	27.9 sec
K_a(Na$^+$)	19.7 ± 3.5 mM	19.8 ± 3.6 mM
K_i(K$^+$)	0.215 ± 0.038 mM	0.0826 ± 0.0124 mM

* The constants listed are those derived as explained in the text to give the best fit to Equation 1. The concentration of [^{3}H]ouabain was 2.5 × 10^{-6} M.

Table 5. [^{3}H]ouabain binding to phospholipase A-treated and control NaK-ATPase at 0 *

Binding conditions	Control		Treated	
	Equilibrium (pmoles/mg)	k(sec^{-1} × 10^3)	Equilibrium (pmoles/mg)	k(sec^{-1} × 10^3)
MgP_i	2423	1.32	1375	0.72
MgATPNa	2143	0.43	1434	0.25
MgATPNaK	1980	0.26	790	0.14

* Phospholipase A treatment, binding conditions and determination of [^{3}H]ouabain binding rates are as described under Experimental Procedures. In the presence of 2.5×10^{-6} M [^{3}H]ouabain. The concentration of potassium used was 0.1 mM. Equilibrium binding was routinely measured at 2, 4 and 6 hr for MgP_i, MgATPNa and MgATPNaK binding conditions respectively.

the effects of phospholipase A treatment on ouabain binding were much more pronounced at the lower temperature.

As illustrated in Table 5, the equilibrium levels of [^{3}H]ouabain binding to the lipase-treated enzyme were lower than those for the control NaK-ATPase. This apparent reduction in the number of binding sites on the lipase-treated enzyme was particularly evident in the presence of MgATPNaK. One explanation for this apparent reduction in binding sites is that the rates of binding to the lipase-treated enzyme are so slow at 0° that equilibrium was not attained within the incubation periods used (Table 5). This is supported by the observation that when aliquots of the incubation media containing lipase-treated NaK-ATPase were raised to 30° for 15 min at the end of the 0° incubation periods, the apparent levels of equilibrium binding were increased to 1870 pmoles/mg (MgP_i); 1840 pmoles/mg (MgATPNa); and 1612 pmoles/mg (MgATPNaK). If these values for equilibrium binding are used to calculate binding rates to the lipase-treated enzyme at 0°, the apparent pseudo first-order rate constants (k, sec^{-1} × 10^3) are reduced to 0.44 (MgP_i); 0.16 (MgATPNa); and 0.06 (MgATPNaK).

Attempts to attain these higher levels of "equilibrium" binding to the lipase-treated enzyme by extending the incubation at 0° to as much as 15 hr were not successful. After 15 hr at 0°, the observed values for [^{3}H]ouabain binding were essentially the same as those listed in Table 5. Furthermore, when aliquots of these incubation media were then raised to 30°, as described above, there was no increase in the amount of [^{3}H]ouabain bound. This suggests that the lipase-treated NaK-ATPase was denatured during the prolonged incubation at 0°, and that only those ouabain binding sites that were occupied by [^{3}H]ouabain prior to denaturation were expressed.

The inhibitory effects of phospholipase A treatment on both [^{3}H]ouabain binding and dephosphorylation are more pronounced at 0°, and in both cases the inhibition reflects an altered response to potassium. Dephosphorylation of the lipase-treated phosphoenzyme is less sensitive to potassium and this, in part, results in an increased steady-state level of phosphoenzyme. This phosphoenzyme is presumably an "E_2-P" form, since it is not sensitive to ADP [32]. In contrast, ouabain binding to the lipase-treated enzyme appears to be more sensitive to potassium.

One explanation for the contradictory effects of potassium on dephosphorylation and ouabain binding to the lipase-treated enzyme is that the potassium modulation of these two events is independent. Lindenmayer and Schwartz [28] concluded that potassium modulation of ouabain binding occurs through a potassium-activation site on the NaK-ATPase. The results presented here could suggest that potassium activation of dephosphorylation (turnover) and potassium modulation of ouabain binding are mediated through two independent potassium sites on the enzyme [33].

An alternative possibility is that the phospholipid depletion results in a decreased rate of phosphate release from the enzyme after potassium interaction. The decreased rate of phosphate release causes an increase in the amount of a $K \cdot E_2$-P form of the enzyme that does not bind ouabain. This $K \cdot E_2$-P form may be similar to the "potassium-complexed phosphoenzyme" of Post *et al.* [32], which was formed by phosphorylating the NaK-ATPase with P_i in the presence of excess potassium or rubidium.

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REFERENCES

1. R. Tanaka and L. G. Abood, *Archs Biochem. Biophys.* **108,** 47 (1964).
2. D. W. Towle and J. H. Copenhaver, Jr., *Biochim. biophys. Acta* **203,** 124 (1970).
3. P. Ottolenghi, *Biochem. J.* **151,** 61 (1975).
4. K. P. Wheeler, J. A. Walker and D. M. Barker, *Biochem. J.* **146,** 713 (1975).
5. K. P. Wheeler and J. A. Walker, *Biochem. J.* **146,** 723 (1975).
6. K. P. Wheeler, *Biochem. J.* **146,** 727 (1975).
7. J. Järnefelt, *Biochim. biophys. Acta* **266,** 91 (1972).
8. J. Noguchi and S. Freed, *Nature New Biol.* **230,** 148 (1971).
9. E. Erdmann and W. Schoner, *Biochim. biophys. Acta* **330,** 316 (1973).
10. S. S. Goldman and R. W. Albers, *J. biol. Chem.* **248,** 867 (1973).
11. B. Roelofsen and L. L. M. Van Deenen, *Eur. J. Biochem.* **40,** 245 (1973).
12. K. Taniguchi and Y. Tonomura, *J. Biochem., Tokyo* **69,** 543 (1971).
13. K. Taniguchi and S. Iida, *Molec. Pharmac.* **9,** 350 (1973).

14. L. K. Lane, J. H. Copenhaver, Jr., G. E. Lindenmayer and A. Schwartz, *J. biol. Chem.* **248,** 7197 (1973).
15. G. E. Lindenmayer, L. K. Lane and A. Schwartz, *Ann. N.Y. Acad. Sci.* **242,** 235 (1974).
16. M. A. Wells and D. J. Hanahan, *Meth. Enzym.* **14,** 178 (1969).
17. A. Schwartz, J. C. Allen and S. Harigaya, *J. Pharmac. exp. Ther.* **168,** 31 (1969).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
19. C. Entenman, *Meth. Enzym.* **3,** 317 (1957).
20. G. R. Bartlett, *J. biol. Chem.* **234,** 466 (1959).
21. A. Zlatkis, B. Zak and A. J. Boyle, *J. Lab. clin. Med.* **41,** 486 (1964).
22. V. P. Skipski, R. J. Peterson and M. Barclay, *Biochem. J.* **90,** 375 (1964).
23. E. T. Wallick and A. Schwartz, *J. biol. Chem.* **249,** 5141 (1974).
24. R. L. Post, S. Kume, T. Tobin, R. Orcutt and A. K. Sen, *J. gen. Physiol.* **54,** 306 (1969).
25. U. K. Laemmli, *Nature, Lond.* **227,** 680 (1970).
26. L. K. Lane, *Fedn Eur. Biochem. Soc. Lett.* **64,** 375 (1976).
27. K. Taniguchi and S. Iida, *Biochim. biophys. Acta* **233,** 831 (1971).
28. G. E. Lindenmayer and A. Schwartz, *J. biol. Chem.* **248,** 1291 (1973).
29. E. T. Wallick and A. Schwartz, *J. biol. Chem.* **249,** 5141 (1974).
30. G. N. Wilkinson, *Biochem. J.* **80,** 324 (1961).
31. W. W. Cleland, *Adv. Enzymol.* **24,** 1 (1967).
32. R. L. Post, G. Toda and F. N. Rogers, *J. biol. Chem.* **250,** 691 (1975).
33. G. E. Lindenmayer, A. Schwartz and H. K. Thompson, Jr., *J. Physiol., Lond.* **236,** 1 (1974).