J. Membrane Biol. 117, 153–161 (1990)

The Journal of
Membrane Biology

CCCP Activation of the Reconstituted NaK-Pump

Atsunobu Yoda and Shizuko Yoda
Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin 53706

Summary. In the NaK-ATPase proteoliposomes (PLs), the NaK-pump activity, Na^+ uptake, and ATP hydrolysis were apparently enhanced by carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) and other ionophores without ion gradients. These ionophore effects were not cation specific. Without ionophores, the PL's ATPase activity fell to its steady-state value within 3 sec at 15°C. This decrease in activity disappeared in the presence of CCCP. Since CCCP is believed to enhance proton mobility across the lipid bilayer and dissipate membrane potential (V_m), we postulated that a V_m build-up partially inhibits the PLs by changing the conformation of the NaK-pump, and that CCCP eliminated this partial inhibition. Since this activation required extracellular K^+ and high ATP concentration in the PLs, CCCP must affect the conversion between the phosphorylated forms of NaK-ATPase (EP); this step has been suggested by Goldschlegger et al. (1987) to be the voltage-sensitive step (*J. Physiol.* (*London*) **387:**331–355). Although cytoplasmic K^+ accelerated the change of ADP- and K^+-sensitive EP (E*P) to K^+-sensitive ADP-insensitive EP (E_2P), CCCP did not compete with cytoplasmic K^+ when cytoplasmic Na^+ was saturated. When the PLs were phosphorylated with 20 μM ATP and 20 μM palmitoyl CoA instead of with high concentration of ATP, CCCP increased the E*P content and decreased the ADP-sensitive K^+-insensitive EP (E_1P). The results described above suggest that CCCP affects the E_1P to E*P change in the $E_1P \rightarrow E^*P \rightarrow E_2P$ conversion and that this reaction step is inhibited by V_m.

Key Words NaK-pump · CCCP · membrane potential · NaK-ATPase proteoliposome · voltage-sensitive step · EP conversion · ionophore

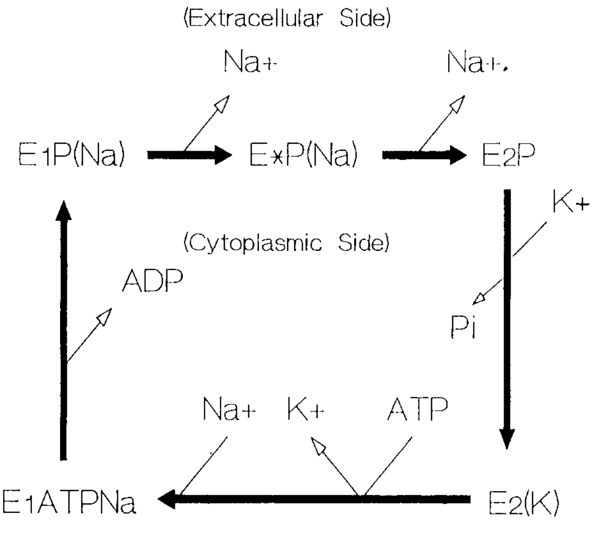
Introduction

The NaK-pump in animal cells uses the energy of ATP to maintain Na^+ and K^+ gradients and the membrane potential (V_m).[1] The details of the reaction mechanism of ATP-hydrolysis and the binding of Na^+ and K^+ to NaK-ATPase, the integral component of this pump, have been extensively studied. Investigations of the electrogenecity of the NaK-pump have been much rarer, however. But recent studies have clarified some aspects of this problem. After release of ATP from an inactive caged ATP[1] by a light flash, transient Na^+ fluxes were detected in the millisecond time range by Forbush [6]. These Na^+ fluxes were observed without K^+ and thought to be an early event in normal pump action. Using planar lipid bilayer-bound NaK-ATPase molecules, fast charge translocations were recorded after photochemical release of ATP from caged ATP [2, 3, 19]. These transient currents were also observed in the absence of K^+ and thought to be an electrical current equivalent to the above fast Na^+ fluxes. Since these transient Na^+-flux and charge movements occurred before the NaK-pump reaction reached its steady-state level, it is concluded that the Na^+-translocation step is electrogenic, and therefore the rate-limiting step does not precede the electrogenic step in the NaK-pump reaction. Since these electrochemical reactions are driven by ATP hydrolysis, it seems reasonable that the V_m influences the NaK-pump reaction [4, 5, 7, 16, 17].

Goldshlegger et al. [8] reported the activation of the NaK-pump by valinomycin (a K^+-ionophore) in the presence of a K^+ gradient. From this result they claimed that a decrease in V_m enhanced the Na-K exchange in the PLs by accelerating the E_1P to E_2P conversion.

Recently, E*P has been recognized as the intermediate in the change of E_1P to E_2P [18, 20, 26]. As described below, we found that both CCCP (a proton ionophore) and valinomycin apparently enhanced

[1] *Abbreviations used:* EP, phosphorylated form of NaK-ATPase; E_1P, ADP-sensitive K^+-insensitive EP; E*P, ADP- and K^+-sensitive EP; E_2P, K^+-sensitive ADP-insensitive EP; $E_2(K)$, K^+ occluded E_2 form of NaK-ATPase; Na^+_{cyt} and K^+_{cyt}, Na^+ and K^+ in the cytoplasmic (extravesicular) medium; Na^+_{ex} and K^+_{ex}, Na^+ and K^+ in the extracellular (intravesicular) medium; PL, reconsistituted NaK-ATPase proteoliposome; V_m, membrane potential; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; CHAPS, 3-[(3-cholamidopropyl) dimethyl ammonio]-1-propanesulfonate; CDTA, 1,1-diaminocyclohexanetetraacetic acid; caged ATP, adenosine-5'-triphosphate P^3-1-(2-nitrophenyl) ethyl ester; hemi-Na, hemi-sodium.



Scheme 1

the NaK-pump activity in the PLs even without salt or pH gradients.

In this study, we used CCCP instead of valinomycin in order to decouple ionophore effects from changes in ion-gradients during the NaK-pump reaction. Here, we present evidence that CCCP activation of the PL pump activity is due to the dissipation of V_m and effects the E_1P to E*P conversion.

Our working hypothesis in this study is represented in Scheme 1, which is modified from Post et al. (21).

Materials and Methods

Preparation of PLs from the Fragmental Electric Eel NaK-ATPase

1.2 mg of fragmental electric eel NaK-ATPase (specific activity 20 ± 3 μmol ADP/min/mg protein at 37°C) [24] was solubilized in 1 ml of 10 mM CHAPS solution containing 0.5 M NaCl, 0.5 mM EDTA, and 12.5 mM histidine-arginine buffer (pH 7.3) for 30 min at 15°C [25]. Egg phospholipid was prepared from eggs laid that day. After dehydrating the egg yolk with acetone (25 ml/yolk) twice, the insoluble yolk powder was treated with a chloroform-methanol mixture (2 : 1, vol/vol) (25 ml/yolk) for 2 hr at 5°C. After washing with one-fifth volume of 150 mM NaCl, the extract was dried. The residue was dissolved with chloroform (about 3 ml/yolk) and then precipitated with 10-fold volume of acetone. This precipitate was treated with Dowex 50 (Tris-form) to remove the potassium cognate as described previously [27] and kept at −80°C in the solid form.

In the present study, the liposomes were prepared with sonication from the homogeneous mixture of egg phospholipid described above,[2] phosphatidyl glycerol (Na salt) and cholesterol (9 : 1 : 3, wt/wt/wt). The reconstitution procedure was the same as that reported previously [25] except that 100 mM instead of 90 mM CHAPS was used to solubilize the liposomes. The final intravesicular medium contained 130 mM NaCl and 20 mM KCl. Each set of experiments was performed on the same batch of PLs.

Measurement of the Membrane Potential

The adsorption of potential sensitive dyes by liposomes from their media is dependent on the V_m of the liposomes. Apell and Bersch [1] reported that the fluorescence change of oxonol VI was a very useful indicator for V_m of the liposomes and PLs. However, the blank fluorescence was a major source of error in the measurements. In the same paper, they observed that the absorption spectrum of oxonol VI was shifted to longer wavelengths when the dye was adsorbed into the lipid bilayer. In the present study, this problem was overcome by measuring the absorption change at 620 nm with a double-beam spectrophotometer (a Hitachi type 220 double-beam spectrophotometer equipped with temperature control and a stirring device was used). The reaction mixture consisted of 130 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 0.1 mM ATP, 0.3 mM ouabain, 25 mM histidine buffer, 2 μM oxonol VI, and PL (0.4 ~ 0.7 mg lipid/ml). After preincubation of the reaction medium without $MgCl_2$ in both cuvettes, the reaction was started by adding $MgCl_2$ into the sample cuvette. The V_m was calibrated by the K-diffusion potential induced by valinomycin.

ATPase Assay

The ATPase activity of the PL was measured by the formation of ^{32}P-labeled inorganic phosphate from 0.2 mM [γ-^{32}P]ATP in the presence of NaCl, KCl, 0.3 mM ouabain, 5 mM $MgCl_2$, and PLs (about 1 mg lipid/ml) for 30 sec at 30°C as reported previously [23]. The total salt concentration in the reaction medium was kept at 150 mM with choline-HCl. The reaction was started by adding $MgCl_2$ and quenched by one-half volume of 24% $HClO_4$ containing 7% Na_2MoO_4. When the assay period was less than 10 sec, the reaction was quenched by one-half volume of only 24% $HClO_4$, and then 0.6 volume of 12% Na_2MoO_4 was added. The resultant ^{32}P-labeled phosphomolibdate was extracted with butyl acetate, and aliquots of the extract were counted. For the control, the $MgCl_2$ solution and quencher were added simultaneously.

Na^+ Uptake

The reaction conditions were similar to those for the ATPase activity except for using ^{22}Na-labeled NaCl and unlabeled ATP instead of unlabeled NaCl and [γ-^{32}P]ATP. The reaction was quenched by 60 mM $Tris_3$/CDTA after 30-sec incubation at 30°C. Compared to the ATPase assay, four times the amount of PL was used for the Na^+-uptake assay. For the control, the ATP was omitted. Other experimental details were the same as reported previously [25].

[2] It is possible to make PLs from commercially available egg phospholipids, but their maximum V_m were lower than those of the PLs from the egg phospholipid prepared as described above. The content of unsealed PLs was decreased to almost zero when the egg phospholipid described in the text was used.

Determination of EP Composition

The determination of the EP composition was performed at 15°C by the modified method as previously reported [25]. After 3 sec of the reaction of the PL (about 5 mg lipid/ml) in a 1-ml mixture of 70 mM choline-HCl, 30 mM NaCl, 50 mM KCl, 5 mM free $MgCl_2$, 20 μM [γ-^{32}P]ATP, 20 μM palmitoyl CoA, 25 mM histidine, and 1 mM EDTA (pH 7.4) with or without 1.0 μM CCCP, the phosphorylation was quenched and the dephosphorylation was started by the injection of 0.5 ml of 60 mM $Tris_3$/CDTA with or without 0.6 mM ADP. After 0.6–3.6 sec of dephosphorylation, the PLs were denatured with trichloroacetic acid. Other experimental details and calculation method for the EP composition were the same as reported previously. For the determination of EP level, the 60-mM $Tris_3$/CDTA solution and trichloroacetic acid were injected simultaneously after 3-sec phosphorylation as the same as for the EP composition at 0-sec dephosphorylation.

Reagents

ATP was obtained as a sodium salt from Pharmacia P-L Biochemicals and was changed to the Tris salt by passing through Dowex-50 (Tris-form) column. Palmitoyl CoA, ADP di-monocyclohexylammonium salt, and gramicidin were purchased from Sigma. Valinomycin, CCCP, and nigericin were obtained from CalBiochem. CHAPS and CDTA were purchased from Boehringer Mannheim and Aldrich, respectively. Hemi-sodium® was obtained from Eastman Kodak Company. Phosphatidyl glycerol Na-salt was obtained from Avanti Polar Lipids.

Results

Even though the salt composition and pH of the media on both sides of the PLs are the same, many ionophores increased the ATPase activity of the PLs to a large extent. Especially, valinomycin and CCCP were very potent activators (Fig. 1).[3] These ionophores did not change the ATPase activity of the fragmental NaK-ATPase. This ionophore activation of PLs required the presence of extracellular K^+ (K^+_{ex}). CCCP activated not only ATP hydrolysis of the PLs but also their Na^+-uptake without any significant change in the ratio between them (Table 1). The ATP hydrolysis of the PLs proceeds linearly for at least 30 sec despite the presence of CCCP or valinomycin (Fig. 2). These results from the time course experiments suggest that the activation of the NaK-pump by ionophores is not due to the depletion of any ligands in the intravesicular (extracellular) medium.[4]

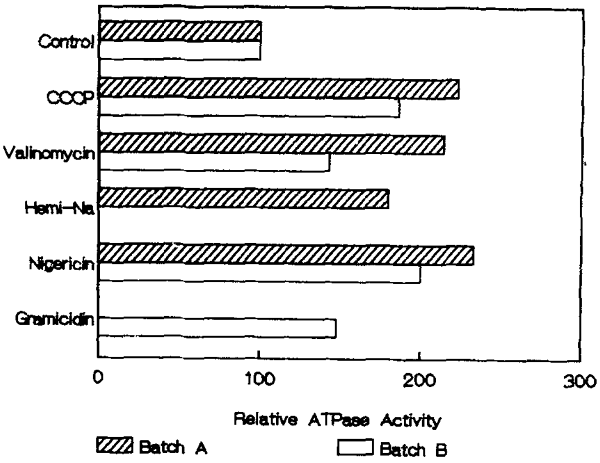


Fig. 1. Ionophore effect on ATP hydrolysis in the PLs. The reaction medium had the same salt composition as the intravesicular medium. The concentrations of the ionophores were 0.1 μM CCCP, 0.1 μM valinomycin, 2.0 μM hemi-Na, 2.0 μM nigericin, and 0.5 μM gramicidin. The activation was normalized by the control result (i.e., obtained without any ionophores). The liposomes for Batch A were prepared from the egg phospholipid prepared as described in the text, and those for Batch B were from the commercial source (Avanti Polar Lipid)

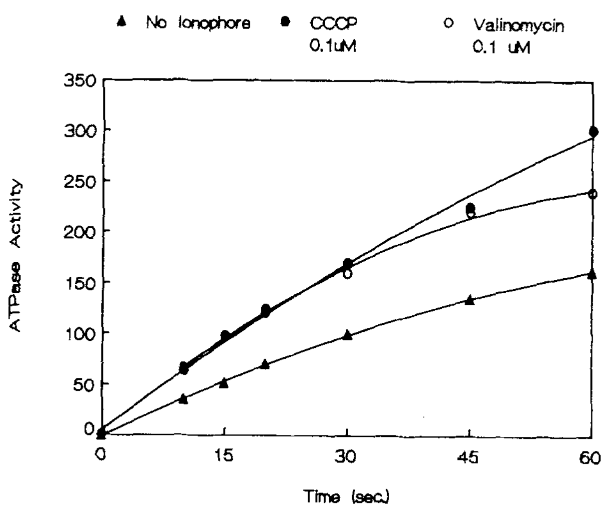


Fig. 2. Effects of valinomycin and CCCP on the time course of ATP hydrolysis by PLs. The reaction media with or without 0.1 μM ionophore are the same as in Fig. 1. The reaction was started by adding $MgCl_2$ and was terminated with injection of $HClO_4$-Na_2MoO_4 mixture. The experimental details are described in the text. The ATPase activity was normalized by the control result for 30-sec reaction

[3] The extent of this ionophore activation seems to vary with different lipid batches.

[4] The diameter of the PLs, which contain one molecule of NaK-pump per vesicle, is about 120 nm [23]. The intravesicular volume is therefore on the order of 10^{-13} μl. A single ion per vesicle corresponds to a concentration of 1.5 μM. If the turnover number of the PL is assumed to be 80 sec^{-1} at 30°C in the presence of an ionophore (equal to that of the fragmental NaK-ATPase), a 30-sec reaction of the NaK-pump lowers the intravesicular K^+ concentration from 20 to 13 mM. This reduction of K^+_{ex} has no serious effect on the NaK-pump activity, unlike the 60-sec reaction which causes a large decrease in the K^+_{ex} (to 5.5 mM), resulting in a decrease in the ATP hydrolysis (Fig. 2).

Table 1. CCCP effect on ATP-hydrolysis and Na^+-uptake of PLs

	ATP hydrolysis	Na^+ uptake	Na^+ uptake/ ATP hydrolysis
	(nmol/min/ml of the sample)		
−CCCP	515 ± 55	1630 ± 150	3.2 ± 0.6
+1.0 μM CCCP	1070 ± 100	3200 ± 200	3.0 ± 0.5

The assays were performed at 30°C for 30 sec. The extracellular medium contained 130 mM NaCl, 20 mM KCl, 12.5 mM histidine-arginine buffer, and 0.5 mM EDTA (pH 7.3). The cytoplasmic medium contained 30 mM NaCl, 50 mM KCl, 70 mM choline-HCl, 5 mM free $MgCl_2$, 0.1 mM ATP, 12.5 mM histidine-arginine buffer, and 0.5 mM EDTA (pH 7.3). Each value cited is the mean value and deviation of triplicate assays using the same sample.

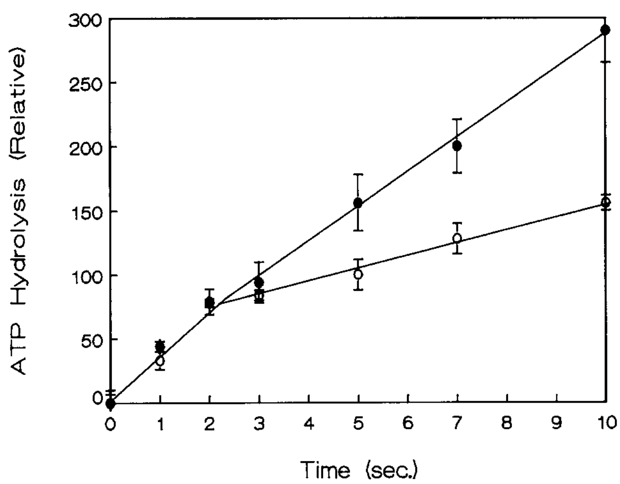


Fig. 3. Initial time course of the ATP hydrolysis of PLs. These experiments were performed at 15°C. The CCCP concentration was 1 μM. The reaction was terminated with 24% $HClO_4$, and then Na_2MoO_4 was added. The rest of the procedure was the same as in Fig. 2. In each set of experiments, the assay was triplicated at each time period, and the obtained ATPase activities from three sets were normalized by the control result for 5 sec without CCCP. The absence and presence of 1 μM CCCP in the media are shown by open and filled symbols, respectively

However, if we examined the initial stage of the PL ATP hydrolysis in detail, we saw that ATP was hydrolyzed monophasically in the presence of CCCP and other ionophores but biphasically in the absence of these ionophores. We observed these initial changes in the ATP hydrolysis more clearly in the lower temperature of 15°C (Fig. 3).

After this initial change, ATP-hydrolysis rates remained constant. These observations suggest that the accumulation of some factor in the initial stage changes the NaK-pump into its less active mode and that CCCP prevents this accumulation. As shown in Fig. 4, CCCP and other ionophores interfere with the buildup of V_m during the NaK-pump reaction. The ion mobility in the carrier-mediated transport system, which corresponds to the membrane conductance, depends on the type of carrier and membrane lipid component [15]. Therefore, the extent of V_m change and pump activation may vary with different ionophores and PL batches (*see* Figs. 1 and 4). In an experiment using phenol red containing PLs with low buffer concentrations, CCCP accelerated the alkalization of the extracellular medium during the NaK-pump reaction (Fig. 5). In the PLs, this pH change in the reaction medium should not enhance the NaK-pump reaction, because the NaK-ATPase activity is maximum around pH 7.3 and does not vary significantly between pH 7.0 and 7.7. Furthermore, an increase in buffer concentration from 12.5 to 50 mM did not significantly change the CCCP effect on the activation of the pump (from 65 ± 6% to 69 ± 5%). Thus, it is unlikely that the pH change causes enhancement of the NaK-pump activity. CCCP probably instead increases the conductance of the lipid bilayer by enhancing the proton mobility and thereby dissipating V_m. Compared with other ionophores, CCCP seems to be the most suitable

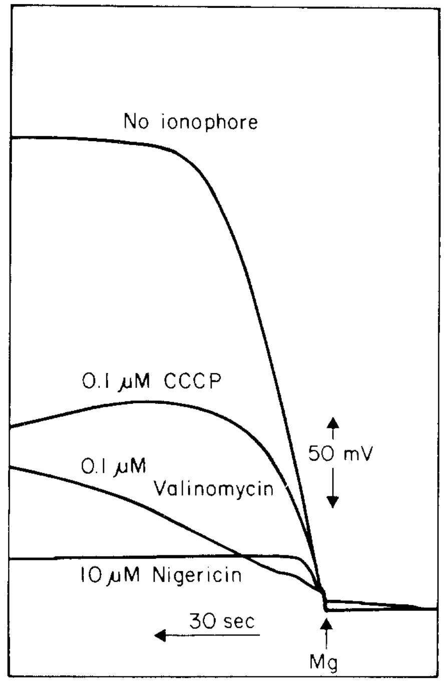


Fig. 4. Change in oxonol VI absorption with NaK-pump reaction. The reaction media and ionophore concentrations are the same as in Fig. 1. Both cuvettes contained 2 μM oxonol VI and PLs (0.5 mg lipid/ml). The reaction was started by adding $MgCl_2$ and the increase in V_m was measured by the increase in absorption at 620 nm. V_m was calibrated by the K^+-diffusion potential induced by valinomycin

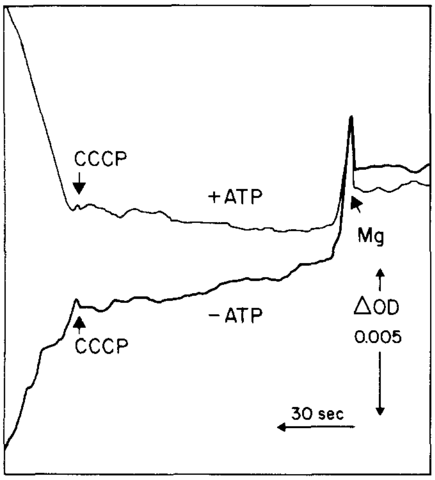


Fig. 5. Alkalization of the PLs by CCCP. The PLs used in this experiment contained phenol red (0.4 mg/ml) and 2.5 mM histidine buffer (pH 7.3). The alkalization of the intravesicular medium was followed by an increase in the absorption at 560 nm under the same reaction conditions as in Fig. 2 without oxonol VI. When the NaK-pump reaction occurred (+ATP), the addition of CCCP (0.1 μM in final concentration) to the sample cuvette indicated the alkalization of the intravesicular medium. When the pump reaction was absent (−ATP), the phenol red absorption decreased

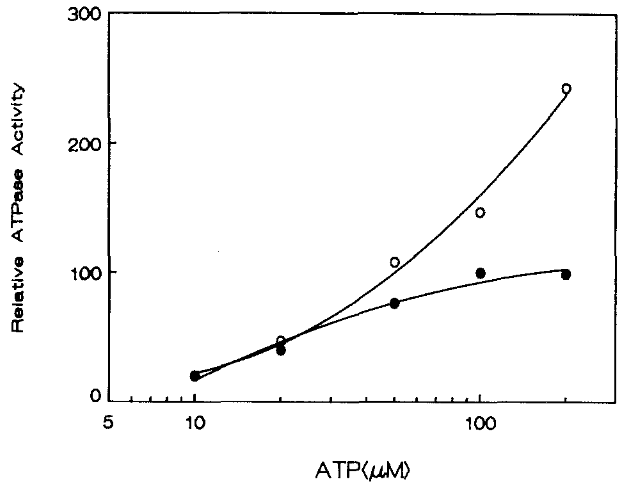


Fig. 6. CCCP effect on ATP hydrolysis of PL at different ATP concentrations. The CCCP concentration was 0.1 μM. The final concentrations of salts in the reaction medium (i.e., the cytoplasmic medium) were 30 mM NaCl, 50 mM KCl, and 70 mM choline-HCl. Other experimental procedures were the same as described in the text

reagent for studying ionophore effects on V_m, since it does not assist the alkali-ion transport directly.

The CCCP Effect Depends on the ATP Concentration

As shown in Fig. 6, the activation of ATP-hydrolysis in the PLs was observed only when the ATP concentration was above 20 μM. It is thought that at high concentrations ATP binds to the low affinity site of NaK-ATPase to accelerate the deocclusion of the K^+-occluded E_2-form [E_2(K)] [14, 21]. Under these conditions, the E_1P to E_2P conversion or the E_1P formation becomes at least one of the rate-limiting steps in the NaK-pump reaction [8, 13, 14, 21]. Therefore, the present result suggests that CCCP specifically accelerates the change of E_1P to E_2P or the phosphorylation of the enzyme.

Preincubation Effects on the ATP Hydrolysis of PLs

As described above, preincubating PLs in a cytoplasmic medium containing 130 mM Na^+, 20 mM K^+, 5 mM Mg^{2+}, and 0.2 mM ATP should increase the cytoplasmic negative potential. CCCP affected the pump activity of these charged PLs at 20 and 100 μM ATP, but did not at 10 μM ATP (Table 2, Expt. 1). Since this CCCP effect was not observed at 20 μM ATP in uncharged PLs, we decided to compare the effects of various preincubation treatments of the PLs at this ATP concentration. As shown in

Table 2. Effect of various preincubation treatments on the CCCP activation of PLs

	Additional ligands in preincubation	ATP concentration during assay (μM)	CCCP activation during assay (%)
Expt. 1	none	200	70 ± 10
	5 mM Mg^{2+}	10	7 ± 11
	5 mM Mg^{2+}	20	32 ± 8
	5 mM Mg^{2+}	100	86 ± 10
Expt. 2	none	100	60 ± 15
	none (no Mg^{2+})	20	0 ± 9
	5 mM Mg^{2+}	20	30 ± 10
	5 mM Mg^{2+}, plus 0.1 μM CCCP	20	−10 ± 5 and 5 ± 8

The PLs were preincubated at 30°C for 20 sec in a medium containing 130 mM Na^+, 20 mM K^+, 0.2 mM unlabeled ATP, 12.5 mM histidine buffer (pH 7.3) with the additional ligands listed. The assay was started by adding a ^{32}P-ATP solution with or without CCCP. Final concentrations of the assay medium were 130 mM Na^+, 20 mM K^+, 5 mM Mg^{2+}, 25 mM histidine buffer and ^{32}P-ATP as cited with or without 0.1 μM CCCP. After 30-sec incubation at 30°C, the liberated inorganic ^{32}P-phosphate was measured as described in the text. In this table, the CCCP activation is measured by the ratio between results in the presence and absence of 0.1 μM CCCP. In each set of experiments, triple assays were performed with or without CCCP. In Expt. 2, the deviation in ATPase activities in the presence of 20 μM ATP without CCCP was less than ±7%.

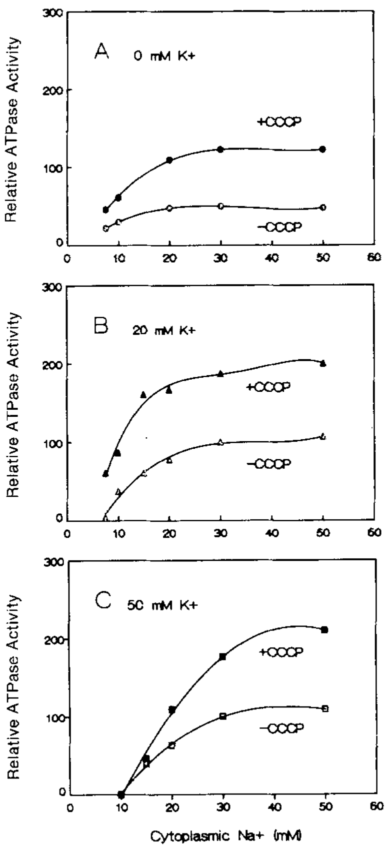


Fig. 7. CCCP effect on the ATPase activity of the PLs at different cytoplasmic Na^+ concentrations. The ATPase activity is normalized by the activity when the cytoplasmic salt concentrations were 30 mM Na^+, 50 mM K^+, and 70 mM choline-HCl without CCCP. The cytoplasmic K^+ concentrations are shown as: (*A*) 0 mM, (*B*) 20 mM, and (*C*) 50 mM. The absence and presence of 0.1 μM CCCP in the media are shown with open and filled symbols, respectively

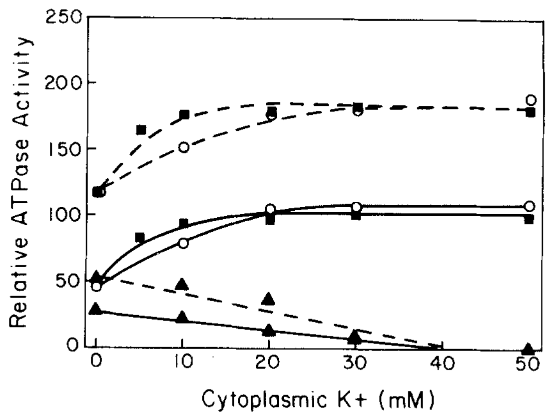


Fig. 8. CCCP effect on the ATPase activity of PLs at different cytoplasmic K^+ concentrations. The ATPase activity was normalized as in Fig. 6. The cytoplasmic Na^+ concentrations are shown as: ▲ 7.5 mM Na^+, ■ 30 mM Na^+, and ○ 50 mM Na^+. The absence and presence of 0.1 μM CCCP in the reaction media are shown with solid and dashed lines, respectively

Table 2, Expt. 2, the enhancement of ATPase activity by CCCP was observed only in the PLs which were pretreated with Na^+, K^+, Mg^{2+}, and 0.2 mM ATP (i.e., the same condition required to increase V_m). When other pretreatments (either the pump reaction did not occur or took place in the presence of CCCP) were used, the CCCP effect was not observed. These results suggest that a negative cytoplasmic potential is necessary for the enhancement of pump activity by CCCP.

Effects of Cytoplasmic Ions and CCCP on the ATP Hydrolysis of PL

As shown in Fig. 7*A–C*, Na^+_{cyt} controls the ATP hydrolysis of the NaK pump. When the concentration of Na^+_{cyt} was low, K^+_{cyt} competed with Na^+_{cyt} and increased the half-maximum concentration of Na^+_{cyt}. Even at such low levels of Na^+_{cyt}, CCCP activated the ATP hydrolysis, although this CCCP activation decreased as the K^+_{cyt} concentration increased (Fig. 8, ▲). On the other hand, when the Na^+_{cyt} concentration was saturated, both K^+_{cyt} and CCCP independently enhanced the ATPase activity of the PLs (Fig. 8, ○). Moreover, the CCCP enhancement was not affected by K^+_{cyt}. The addition of 0.1 μM CCCP also increased the ATPase activity in the PLs in the presence of saturated Na^+_{cyt} and K^+_{cyt} (30 mM each) to its maximum level; this level was also observed in the presence of 20 μM nigericin, 130 mM Na^+, and 20 mM K^+ on the cytoplasmic side.

The CCCP Effect on the EP Composition

It is well known that the EP level of fragmental NaK-ATPase decreases due to the formation of the K^+-occluded form and a rapid dephosphorylation when K^+ is present [21]. A similar K^+_{ex} effect is also observed in the PLs. Although this problem can be overcome in part by using high concentrations of ATP for phosphorylation, it makes the EP assay difficult because the specific radioactivity of ^{32}P-ATP decreases. Recently, Huang et al. [10–12] reported that acyl CoA's, e.g., palmitoyl CoA, accelerated the deocclusion of the K^+ from $E_2(K)$. In the present PLs, the addition of 20 μM palmitoyl CoA to 20 μM ATP activated the ATP-hydrolysis and increased the EP level (Table 3).

After the PLs containing 130 mM Na^+ and 20 mM K^+ inside were phosphorylated for 3 sec at 15°C in the presence of 20 μM palmitoyl CoA, 30 mM Na^+, 50 mM K^+ and 5 mM Mg^{2+} with 20 μM ATP,

Table 3. Effect of palmitoyl CoA on ATP hydrolysis and EP level of PLs

	ATP hydrolysis		EP level[a]
	No CCCP	+0.1 μM CCCP	No CCCP
No palmitoyl CoA	100 ± 5	118 ± 6	100 ± 14
20 μM palmitoyl CoA	122 ± 3	183 ± 5	198 ± 13

In both experiments, the ATP concentration was 20 μM, and the EP-level measurement was performed at 15°C for 3 sec. Other experimental conditions were the same as those for Table 1. The values cited are the percentage values against the value obtained without CCCP and palmitoyl CoA.

[a] The effect of 1 μM CCCP on the EP level was not significant.

Table 4. Effect of CCCP on EP composition

	Cytoplasmic ion concentration		CCCP	Dephosphorylation rate constant	Content of	
	NaCl, mM	KCl, mM	μM	sec^{-1}	E_1P, %	E^*P, %
Expt. 1	30	50	0	−0.30	100	0
			1.0	−0.33	71	29
Expt. 2	30	50	0	−0.27	100	0
			1.0	−0.28	80	20
Expt. 3	30	0	0	−0.25	93	8
			1.0	−0.27	78	22

The experimental details were described in the text and Fig. 9. The same sample was used in each set of experiments.

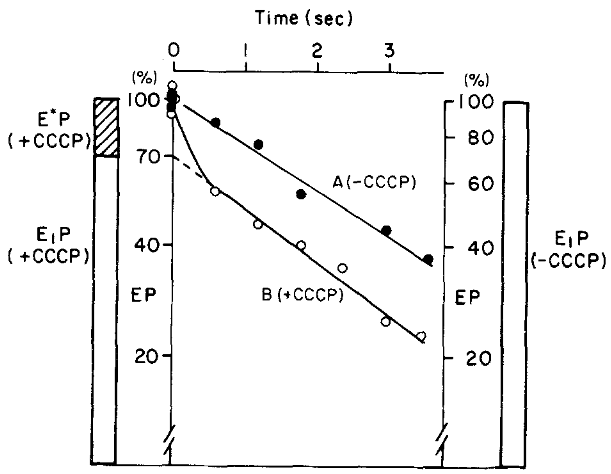


Fig. 9. CCCP effect on dephosphorylation of phosphorylated PL. The PLs containing 130 mM NaCl and 20 mM KCl as the intravesicular salts, were phosphorylated in 1 ml of a mixture containing 20 μM [γ-^{32}P]ATP, 20 μM palmitoyl CoA, 30 mM Na^+_{cyt}, 50 mM K^+_{cyt}, 70 mM choline-HCl, 5 mM free Mg^{2+}, 25 mM histidine, and 1 mM EDTA (pH 7.3) with or without 1.0 μM CCCP. After 3 sec of phosphorylation at 15°C, the dephosphorylation was started at 0 time, by injection of 0.5 ml of the quenching solution containing 60 mM $Tris_3$/CDTA, 20 mM NaCl, and 10 mM KCl. After 0.6–3.6 sec of dephosphorylation at 15°C, the PLs were denatured with trichloroacetic acid. The EP precipitate from an aliquot of the reaction mixture was collected on a nitrocellulose filter and counted after extensive washing with cold 0.5% trichloroacetic acid containing 1 mM P_i and 0.1 mM ATP. When 0.6 mM ADP was contained in the quenching solution, the EP values under both conditions were reduced to less than 5% within 0.6 sec (*data not shown*). Other experimental procedures are the same as those described in the text. The bars shown on both sides of the decay curves indicate each EP composition obtained from each dephosphorylation curve

the dephosphorylation was started by quenching the phosphorylation with 20 mM CDTA. The dephosphorylation curve was almost monophasic as shown in Fig. 9 (line *A*). The k_d of the dephosphorylation curve was -0.26 sec^{-1}, which is similar to that of E_1P (-0.30 sec^{-1}) [25]. If 0.2 mM (final) ADP was added with CDTA, more than 95% of the EP was dephosphorylated within 0.6 sec. These results suggest that the main component of the EP is E_1P. This E_1P value was calculated by extrapolating the dephosphorylation curve. When 1 μM CCCP was present during the phosphorylation of the PLs, the dephosphorylation curve was biphasic as shown in Fig. 9 (curve *B*). Since the resultant EPs are ADP sensitive, they could only be E_1P or E^*P; the E_1P content was 80 ~ 70% of the total, and the remainder was E^*P (Table 4). This change in the EP composition with the addition of CCCP suggests that CCCP accelerates the E_1P to E^*P change, increasing the E^*P content. Since CCCP activates the NaK-pump activity, it is unlikely that CCCP slows down the E^*P to E_2P conversion.

Discussion

The effect of V_m on the NaK-pump activity has been investigated by Läuger [17] and De Weer et al. [4, 5]. The electrical analog circuit of the cell which embedded the electrogenic NaK-pump in the cell membrane (=NaK-ATPase proteoliposome) is shown in Fig. 10. In this closed circuit, the following equation applies:

$$i = G_p(\mathrm{emf} - V_m) = G(V_m - E_m) + C\frac{dV_m}{dt}.$$

Since G varies with cations and anions mobilities, the relation between i and V_m is not easily determined, but it is clear that V_m affects i (= the pump activity).

The activation of the NaK-pump by the ionophores as shown in Fig. 1 implies the existence of

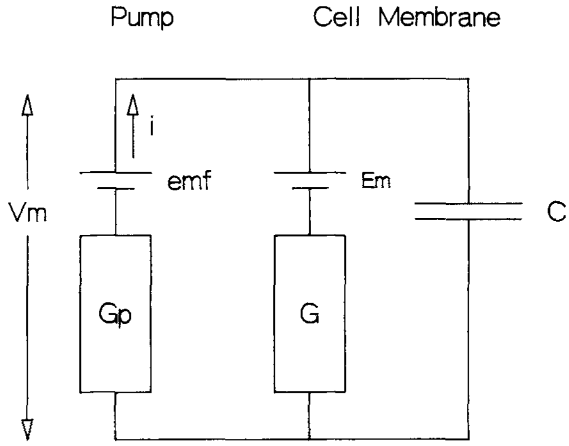


Fig. 10. Equivalent circuit for a cell which embedded the electrogenic NaK-pump in the cell membrane. *emf:* the electromotive force of the NaK-pump; G_p: the internal conductance of the pump; *Em:* the diffusion potential across the cell membrane which would exist in the absence of the pump; *G:* the conductance of the cell membrane; *i:* the pump current which corresponds to the pump activity; V_m: the membrane potential; *C:* membrane capacitance

an inhibitory mechanism in the PLs, even in the absence of alkali ion gradients across the lipid bilayer. There is no specificity among the ions transported by these ionophores (nigericin, valinomycin, hemi-sodium and CCCP). Since no ionophore activated the fragmental NaK-ATPase, this partial inhibition should be due to the barrier effect which is diminished by the ionophores. The lack of ion specificity among the ionophores implies that this partial inhibition in the PLs is due to the V_m across the lipid bilayer. In the case of CCCP, its activation of the pump reaction was apparently due not to the pH change since the pH used was the optimum one for the NaK-ATPase reaction, but instead to the enhancement of proton conductance in the lipid bilayer. The comparison of various preincubation conditions shown in Table 2 also supports the idea that CCCP eliminates the partial inhibition by dissipating V_m. In the absence of ionophores, the ATP hydrolysis in the PLs is biphasic. Thus, the PL ATP-hydrolysis rate decreased initially (within 3 sec at 15°C) before the ions inside vesicles (= extracellular ions) were depleted. After this initial change the rate of ATP-hydrolysis remained steady although the V_m build-up continued. In contrast, this initial rate change was not observed in the presence of CCCP. As discussed by Honig et al. [9], these results suggest that change of the intramembrane electric field by the V_m build-up causes the net displacement of bound charge on the transmembrane NaK-ATPase molecule, or the dipole reorientation, and that such changes may lead the conformational change of the enzyme. In the initial stage, the PL seems to build up enough V_m to cause such voltage-dependent conformation change on the NaK-pump. This charged NaK-pump is less active than the original neutral form.[5]

Goldshlegger et al. studied this V_m effect on the NaK-pump by imposing a diffusion potential with two ways; the rate measurement of active Na^+-K^+ exchange [8] and the fluorescence change of the fluorescein-labeled NaK-pump [22]. They concluded that an inside-negative potential accelerated the NaK-pump activity on inside-out pumps and that this effect occurs during the Na-transport due to the E_1P to E_2P conversion.

In the present study, the CCCP activation of the NaK-pump was not observed when the ATP concentration was less than 20 μM (Fig. 6). Since in the presence of these low ATP levels the rate-determining step of the NaK-pump is the change of $E_2(K)$ to E_1Na, the CCCP activation should be related to the change of E_1Na to $E_2(K)$ via phosphorylation (*see* Scheme 1).

When the extracellular K^+ (K^+_{ex}) was absent, the dephosphorylation of E_2P becomes the rate-limiting step of the NaK-pump [21]. Since CCCP did not activate the ATP hydrolysis in the PLs under these conditions, it is unlikely that CCCP activates the dephosphorylation of E_2P. Furthermore, CCCP did not change the half-maximum concentration of Na^+_{cyt} for the ATP hydrolysis in the PLs even in the presence of various concentrations of K^+_{cyt} (Fig. 7). The results of these two experiments indicate that the partial inhibition by V_m is due to suppression of the change of E_1P to E_2P, as Goldshlegger et al. [8] concluded from the imposed potential effect. Recently the ADP- and K-sensitive EP (E*P) has been shown to be the intermediate of the E_1P to E_2P change [18, 20, 26]. We showed that the change of E*P to E_2P was specifically accelerated by the cytoplasmic K^+ [25]. The K^+_{cyt} activated the NaK-pump when Na^+_{cyt} was saturated. As shown in Fig. 8, however, the CCCP activation of the NaK-pump was independent of K^+_{cyt} concentration when Na^+_{cyt} was saturated. Presumably, CCCP accelerates the E_1P to E*P change by dissipating V_m. The hypothesis of this acceleration mechanism is also consistent with the CCCP effect on the EP composition in the PLs when Na^+ and K^+ are present on both sides of

[5] From the data described in footnote 4, the capacitance of one PL is about 4×10^{-16} F. If the initial turnover of the PL is assumed to be 30 sec^{-1} at 15°C, as in the fragmental NaK-ATPase, the V_m build-up is about 15 mV/sec assuming no leak current. Therefore, the V_m required for the conformational change may be 30 to 50 mV. Since the V_m response to oxonol VI is slow, we were unable to detect this initial stage.

the lipid bilayer. After 3-sec phosphorylation with 20 μM ATP in the presence of palmitoyl CoA, the EP of the PLs saturated with Na^+ and K^+ on both sides was almost completely E_1P. The presence of 1 μM CCCP during the phosphorylation changed the EP composition to about 80% E_1P and 20% E*P. From this observation, we can conclude that the cytoplasmic negative potential changes the NaK-pump to the less active mode by suppressing the E_1P to E*P change. In vitro, the sodium and potassium channels permit ions to flow in and out of animal cells, thereby diminishing V_m. This V_m change enhances the NaK-pump activity in the cell, so that the ion gradient again increases as is also the case when CCCP is present.

This work was supported by National Institutes of Health Grant HL16549. A part of the preliminary results of this work was presented at the 33rd Annual Meeting of the Biophysical Society, Cincinnati, Ohio, February, 1989.

References

1. Apell, H.-J., Bersch, B. 1987. Oxonol VI as an optical indicator for membrane potentials in lipid vesicles. *Biochim. Biophys. Acta* **903:**480–494
2. Apell, H.-J., Borlinghaus, R., Läuger, P. 1987. Fast charge translocations associated with partial reactions of the Na,K-pump: II. Microscopic analysis of transient currents. *J. Membrane Biol.* **97:**179–191
3. Borlinghaus, R., Apell, H.-J., Läuger, P. 1987. Fast charge translocations associated with partial reactions of the Na,K-pump: I. Current and voltage transients after photochemical release of ATP. *J.Membrane Biol.* **97:**161–178
4. De Weer, P. 1984. Electrogenic pumps: Theoretical and practical considerations. *In:* Electrogenic Transport: Fundamental Principles and Physiological Implications. M.P. Blaustein and M. Lieberman, editors. pp. 1–15. Raven, New York
5. De Weer, P., Gadsby, D.C., Rakowski, R.F. 1988. Voltage dependence of the NaK-pump. *Annu. Rev. Physiol.* **50:**225–241
6. Forbush, B., III. 1984. Na^+ movement in a single turnover of the Na-pump. *Proc. Natl. Acad. Sci. USA* **81:**5310–5314
7. Glynn, I.M. 1984. The electrogenic sodium pump. *In:* Electrogenic Transport: Fundamental Principles and Physiological Implications. M.P. Blaustein and M. Lieberman, editors. pp. 33–48. Raven, New York
8. Goldshlegger, R., Karlish, S.J.D., Reppali, A., Stein, W.D. 1987. The effect of membrane potential on the mammalian sodium-potassium pump reconstituted into phospholipid vesicles. *J. Physiol. (London)* **387:**331–355
9. Honig, B.H., Hubbell, W.L., Flewelling, R.F. 1986. Electrostatic interactions in membranes and proteins. *Annu. Rev. Biophys. Biophys. Chem.* **15:**163–193
10. Huang, W.H., Kakar, S.S., Askari, A. 1986. Activation of (Na^+ + K^+)-ATPase by long-chain fatty acids and fatty acyl coenzymes A. *Biochem. Int.* **12:**521–528
11. Huang, W.H., Wang, Y., Askari, A. 1989. Mechanism of the control of (Na^+ + K^+)-ATPase by long-chain acyl coenzyme A. *J. Biol. Chem.* **264:**2605–2608
12. Huang, W.H., Xie, Z., Kakar, S.S., Askari, A. 1988. Control of the sodium pump by liponucleotides and unsaturated fatty acids: Side-dependent effects in red cells. *In:* The Na^+, K^+-pump. Part B. J.C. Skow, J.G. Norby, and A.B. Mansback, editors. pp. 401–407. Alan R. Liss, New York
13. Karlish, S.J.D. 1980. Characterization of conformational changes in (Na,K)ATPase labelled with fluorescein at the active site. *J. Bioenerg. Biomembr.* **12:**111–136
14. Karlish, S.J.D., Yates, D.W. 1978. Tryptophane fluorescence of (Na^+ and K^+)-ATPase as a tool for study of the enzyme mechanism. *Biochim. Biophys. Acta* **527:**115–130
15. Läuger, P. 1980. Kinetic properties of ion carriers and channels. *J. Membrane Biol.* **57:**163–178
16. Läuger, P. 1984. Thermodynamic and kinetic properties of electrogenic ion pumps. *Biochim. Biophys. Acta* **779:**307–341
17. Läuger, P. 1987. Dynamics of ion transport systems in membranes. *Physiol. Rev.* **67:**1296–1331
18. Lee, J.A., Fortes, P.A.G. 1985. Anthroylouabain binding to different phosphoenzyme forms of Na,K-ATPase. *In:* The Sodium Pump. I.M. Glynn and J.C. Ellory, editors. pp. 277–282. The Company of Biologists, Cambridge, U.K.
19. Nagel, G., Fendler, K., Grell, E., Bamberg, E. 1987. Na^+ currents generated by the purified (Na^+ + K^+)-ATPase on planar lipid bilayers. *Biochim. Biophys. Acta* **901:**239–249
20. Norby, J.G., Klodos, I., Christiansen, N.O. 1983. Kinetics of Na-ATPase activity by NaK-pump. *J. Gen. Physiol.* **82:**725–759
21. Post, R.L., Hegyvary, C., Kume, S. 1972. Activation by adenosine triphosphate in the phosphorylation kinetics of sodium and potassium ion transport adenosine triphosphatase. *J. Biol. Chem.* **247:**6530–6540
22. Rephaeli, A., Richards, D.E., Karlish, S.J.D. 1986. Electrical potential accelerates the E_1P(Na)-E_2P conformational transition of (Na,K)ATPase in reconstituted vesicles. *J. Biol. Chem.* **261:**12437–12440
23. Yoda, A., Clark, A.W., Yoda, S. 1984. Reconstitution of (Na^+ + K^+)-ATPase proteoliposomes having the same turnover rate as the membranous enzyme. *Biochim. Biophys. Acta* **778:**332–340
24. Yoda, A., Yoda, S. 1981. A new simple preparation method for NaK-ATPase-rich membrane fragments. *Anal. Biochem.* **110:**82–88
25. Yoda, A., Yoda, S. 1988. Cytoplasmic K^+ effects on phosphoenzyme of Na,K-ATPase proteoliposomes and on the Na^+-pump activity. *J. Biol. Chem.* **263:**10320–10325
26. Yoda, S., Yoda, A. 1986. ADP- and K^+-sensitive phosphorylated intermediate of Na,K-ATPase. *J. Biol. Chem.* **261:**1147–1152
27. Yoda, S., Yoda, A. 1987. Phosphorylated intermediates of Na,K-ATPase proteoliposomes controlled by bilayer cholesterol. *J. Biol. Chem.* **262:**103–109

Received 7 November 1989; revised 2 April 1990