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KINETIC STUDIES ON THE INHIBITION OF $(Na^+ + K^+)$-ATPase BY DIKETOCORIOLIN B

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SUMMARY

Diketocoriolin B, a sesquiterpene antitumor antibiotic, inhibits particulate $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) of Yoshida sarcoma cells competitively, with respect to ATP, and uncompetitively with respect to Na^+ and K^+. The inhibition is reduced by the addition of phosphatidylserine.

Rat brain $(Na^+ + K^+)$-ATPase, which is solubilized by deoxycholate and requires phosphatidylserine for its activity, is also inhibited by diketocoriolin B competitively with respect to ATP and the inhibition was reversed by increasing the concentration of phosphatidylserine.

However, several differences are found between the solubilized and particulate systems: (a) 2 moles of diketocoriolin B interact with the former, while only one mole interacts with the latter, (b) K^+-dependent phosphatase activity of the former requires phospholipid and is sensitive to diketocoriolin B while the reverse is true with the latter.

Based on these kinetic studies, it is supposed that $(Na^+ + K^+)$-ATPase has two binding sites for phospholipid, one being essential for K^+-dependent phosphatase activity and when these two sites are filled with the appropriate phospholipids, ATP can bind to the enzyme.

INTRODUCTION

In a previous paper, it was reported that diketocoriolin B inhibited $(Na^+ + K^+)$-ATPase of cell membrane[1]. The mode of inhibition was specific and apparently different from that of other inhibitors, namely ouabain and oligomycin. To elucidate the mode of inhibition in detail, kinetic studies were performed using enzyme preparations from Yoshida sarcoma cells and rat brain.

Scheme I shows a generally accepted mechanism for the reaction of $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3)[2,3]. Reaction 3 is the K^+-dependent dephosphorylation reaction, which is inhibited by ouabain[4–7], while oligomycin inhibits

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$$E \xrightarrow[(1)]{ATP,\ Mg^{2+},\ Na^+} E\sim P \xrightarrow[(2)]{Mg^{2+}} E\text{-}P \xrightarrow[(3)]{K^+} E + P_i \qquad (I)$$

Reaction 2, the conformational change in the presence of Mg^{2+} (refs 8–10). As a result of binding studies by Shamoo *et al.*[11] and Jensen and Nørby[12], the involvement of the enzyme form "*E*–ATP" as an intermediate was revealed. Then, the reaction scheme of the $(Na^+ + K^+)$-ATPase reaction can be written as follows:

$$E \xrightarrow[(1)]{ATP,\ Mg^{2+}} E\cdot ATP \xrightarrow[(2)]{Na^+} E\sim P \xrightarrow[(3)]{Mg^{2+}} E\text{-}P \xrightarrow[(4)]{K^+} E + P_i \qquad (II)$$

The experiments in the present paper were intended to reveal the locus of action of diketocoriolin B in the sequential steps in Scheme II.

MATERIALS AND METHODS

Particulate $(Na^+ + K^+)$-ATPase was prepared as described previously[1]. When solubilized enzyme was needed, the particulate enzyme from rat brain was treated with deoxycholate according to the method of Tanaka *et al.*[13,14] and the precipitate obtained between 35 to 50% saturation of $(NH_4)_2SO_4$ was used after being dissolved in 10 mM Tris–HCl, pH 7.4. The enzyme solution remained active after storage for 5 days at −20 °C. To determine K^+-dependent phosphatase activity of the solubilized enzyme, the enzyme solution was passed through a column of Sephadex G-25 (coarse) which had been equilibrated with 10 mM Tris–HCl, pH 7.4. $(Na^+ + K^+)$-ATPase was assayed as described previously using 0.1-ml assay mixture. Other procedures were identical to those reported previously[1].

Sources of chemicals

ATP–Tris was prepared as described in a previous paper[1]. Phosphatidylserine and phosphatidylinositol from beef brain, phosphatidylcholine from egg yolk, phosphatidylethanolamine from *Azotobacter agilis*, and cardiolipin from beef heart were pure preparations kindly provided by the Department of Chemistry, National Institute of Health, Tokyo.

RESULTS

Studies with particulate enzyme preparation from Yoshida sarcoma cells

First, using a particulate enzyme preparation from Yoshida sarcoma cells, the effect of diketocoriolin B on the initial velocity of the $(Na^+ + K^+)$-ATPase reaction was determined at various concentrations of ATP. As shown in Fig. 1, diketocoriolin B is a competitive inhibitor with respect to ATP. As seen in the figure, the slope of the double reciprocal plot changes as a linear function of diketocoriolin B concentration and there is a linear competitive relationship between diketocoriolin B and ATP. Graphical extrapolation gave the apparent K_m for ATP and K_i for diketocoriolin B as $1.0 \cdot 10^{-3}$ M and $6.6 \cdot 10^{-6}$ M, respectively.

Next we determined the effect of diketocoriolin B on the initial velocity when the concentration of Na^+ was varied. The results are shown in Fig. 2a (Lineweaver–

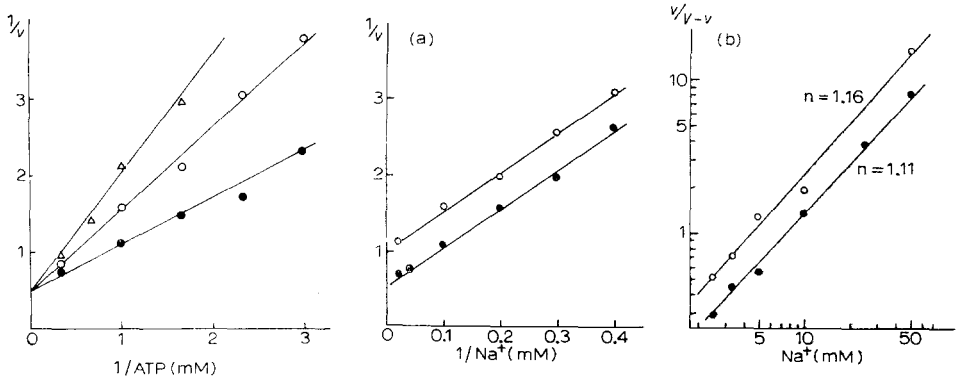


Fig. 1. Effect of diketocoriolin B on the substrate–velocity relationships of Yoshida sarcoma $(Na^+ + K^+)$-ATPase. The reaction mixture contained, in a total volume of 0.1 ml, 10 mM Tris–HCl (pH 7.4), 120 mM NaCl, 10 mM KCl, the enzyme preparation (20 μg protein), the indicated amount of ATP (Tris salt), $MgSO_4$ (the molar ratio of ATP/Mg^{2+} was fixed at 1/1.5), and either no diketocoriolin B (●), 2.5 μg/ml diketocoriolin B (○), or 5 μg/ml diketocoriolin B (△). It was incubated for 15 min at 37 °C and liberated P_i was determined. The rate of P_i liberation is expressed in relative units and is presented in the form of a Lineweaver–Burk plot.

Fig. 2. Effect of diketocoriolin B on the response of Yoshida sarcoma $(Na^+ + K^+)$-ATPase to Na^+. Incubations were carried out at 37 °C for 15 min in media containing 10 mM Tris–HCl (pH 7.4), 3 mM ATP (Tris salts), 4.5 mM $MgSO_4$, 10 mM KCl, the enzyme preparation (20 μg protein), and NaCl at the indicated concentrations in a total volume of 0.1 ml, with 5 μg/ml of diketocoriolin B (○), or without diketocoriolin B (●). The rate of P_i liberation is expressed in relative units and is presented in the form of a Lineweaver–Burk plot (a) and a Hill plot (b).

Burk plot) and in Fig. 2b (Hill plot). The Lineweaver–Burk plot shows a slightly increasing slope. The deviation from linearity is more critically demonstrated by the slopes, apparently larger than 1.0, in the Hill plot. However, the parallelism which is observed in the high concentration region of Na^+ (in Fig. 2a) suggests that diketocoriolin B is an uncompetitive inhibitor with respect to Na^+.

Interaction between diketocoriolin B and K^+ was examined by a similar experiment where the concentrations of ATP, Mg^{2+} and Na^+ were fixed, while that of K^+ was varied with the results shown in Fig. 3a (Lineweaver–Burk plot) and Fig. 3b (Hill plot). As in the case of Na^+, the parallelism which is observed in the high concentration region of K^+ (in Fig. 3a) suggests that diketocoriolin B uncompetitively inhibits the enzyme with respect to K^+.

The results described so far suggest that diketocoriolin B interacts with an enzyme form with which ATP reacts but not those forms with which Na^+ or K^+ react. This seems to be consistent with Scheme II in that diketocoriolin B reacts with E of the scheme, competitively with ATP. However, competition between diketocoriolin B and ATP for a common binding site on E is unlikely because of the dissimilarity of their structures and because of the ineffectiveness of diketocoriolin B on Mg^{2+}-ATPase, a closely similar enzyme also hydrolyzing ATP, of which the active site must be very similar to that of $(Na^+ + K^+)$-ATPase.

In consequence, another scheme, Scheme III, for the enzyme reaction can be proposed. The enzyme (E°) binds either to the inhibitor (I, which is diketocoriolin B)

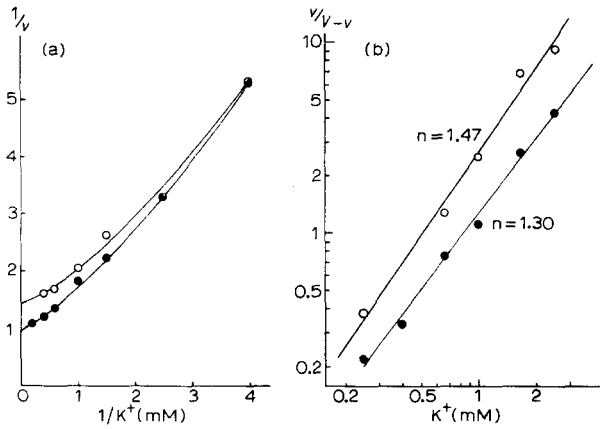
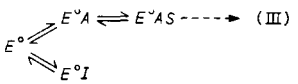


Fig. 3. Effect of diketocoriolin B on the response of Yoshida sarcoma $(Na^+ + K^+)$-ATPase to K^+. Incubation and analysis were carried as described in the legend to Fig. 2 except that the concentration of NaCl was fixed at 120 mM and the concentration of KCl was varied as indicated. The result is presented in the form of a Lineweaver–Burk plot (a) and a Hill plot (b). ●, control; ○, diketocoriolin B, 4 μg/ml.



or to a putative activator (A). The $E°A$ complex, if it is formed, can accept the substrate (S, which is ATP). It follows that $E°$ is an inactive form lacking the activator while $E°A$ is the native form of the enzyme. According to this scheme, the reaction velocity is expressed by the following equation, in which S, A and I are the concentrations of substrate, activator and inhibitor, respectively, while K_s, K_a and K_i are

$$v = \frac{V \cdot AS}{K_s K_a + K_s A + AS + \frac{K_s K_a}{K_i} I}$$

the dissociation constants of the respective complexes. This equation indicates that diketocoriolin B competes not only with the activator but also with ATP. Then, the nature of the activator must be clarified. $(Na^+ + K^+)$-ATPase is a lipoprotein which converts to the inactive form upon solubilization and therefore makes its purification difficult. Treatment with an organic solvent generally leads to inactivation[15]. Digestion with phospholipase A[16] or C[15–17] also markedly reduces its activity. On the other hand, after solubilization with deoxycholate, the activity of the enzyme preparation is restored by addition of phospholipid[13,14,18,19]. Based on this information, we thought that the proposed "activator" might be phospholipid and attempted to determine the effect of various phospholipids on inhibition of the enzyme by diketocoriolin B. As shown in Table I, phosphatidylserine appreciably reduced the diketocoriolin B inhibition. At increasing concentrations of phosphatidylserine, the inhibitory action of diketocoriolin B was significantly reduced, as shown in Table II, indicating competition between phosphatidylserine and diketocoriolin B. This supports the view that phosphatidylserine is the activator (A) which must first bind to $E°$, before ATP can be accepted.

TABLE I

REVERSAL OF THE DIKETOCORIOLIN B-INDUCED INHIBITION OF $(Na^+ + K^+)$-ATPase BY PHOSPHOLIPIDS

Incubation was carried out at 37 °C for 15 min with 10 mM Tris–HCl (pH 7.4), 3 mM ATP (Tris salt), 4.5 mM $MgSO_4$, 120 mM NaCl, 10 mM KCl, the particulate enzyme preparation of Yoshida sarcoma cells (20 μg protein), and phospholipid at a concentration of 100 μg/ml, in a total volume of 0.1 ml with or without 10 μg/ml of diketocoriolin B. Phospholipids were dispersed in 10 mM Tris–HCl (pH 7.4) by sonic vibration.

Additions	*P_i liberated (μmoles/mg protein per h)*		*Inhibition* (%)
	Diketocoriolin B (−)	*Diketocoriolin B* (+)	
None	5.09	0.98	80.0
Phosphatidylinositol	5.77	1.63	71.7
Phosphatidylethanolamine	5.24	1.15	78.1
Phosphatidylcholine	4.94	1.23	75.1
Cardiolipin	3.58	1.78	50.2
Phosphatidylserine	5.62	3.07	47.8

TABLE II

REVERSAL BY PHOSPHATIDYLSERINE OF THE DIKETOCORIOLIN B-INDUCED INHIBITION OF PARTICULATE $(Na^+ + K^+)$-ATPase OF YOSHIDA SARCOMA CELLS

Incubations were carried out as described in Table I with various concentrations of phosphatidylserine.

Diketocoriolin B (μg/ml)	*Phosphatidylserine* (μg/ml)	*P_i liberated* (*μmoles/mg protein per h*)	*Inhibition* (%)
0	0	7.45	
10	0	0.25	96.5
10	25	1.02	86.3
10	50	1.87	71.3
10	75	3.65	51.0

Studies with solubilized $(Na^+ + K^+)$-ATPase

The particulate enzyme preparation used in the preceding experiment inevitably contained phospholipid and, therefore, should be identified with $E^{\circ}A$ in Scheme III. For further study, we attempted to prepare enzyme dependent on exogenous phospholipid for activity. Solubilization of $(Na^+ + K^+)$-ATPase from Yoshida sarcoma cells with deoxycholate, Triton X-100, or sodium dodecyl sulfate was tried without success. There are several reports on successful solubilization of $(Na^+ + K^+)$-ATPase from various sources; from beef brain with lubrol[22], from guinea-pig kidney with lubrol and Triton X-100[20], from beef brain with deoxycholate[13,14,19], from rabbit kidney with deoxycholate[18], and from canine kidney with deoxycholate[21]. Generally, an enzyme solubilized with deoxycholate but not with lubrol requires phospholipid for

activity. Therefore, further experiments were conducted with an enzyme from rat brain solubilized with deoxycholate according to the method of Tanaka *et al.*[13,14], although the solubilizing process gave low recovery of the enzymatic activity and some modification of the enzyme characteristics. Restoration of the catalytic activity upon addition of various phospholipids to the solubilized enzyme was determined. As shown in Table III, marked activation was observed by phosphatidylserine while no or only slight activation was observed with other lipids. These results were consistent with the observations by Tanaka *et al.*[13,14] and by Towle and Copenhaver[18]. A mixture of phosphatidylserine and the solubilized enzyme at a protein/phosphatidylserine ratio of 1 showed a specific activity of 10 to 30 μmoles of ATP hydrolyzed/mg protein per h, while its $(Na^+ + K^+)$-ATPase/Mg^{2+}-ATPase ratio was 15. These characteristics were comparable to those of the particulate enzyme.

TABLE III

RECOVERY OF $(Na^+ + K^+)$-ATPase ACTIVITY BY THE ADDITION OF VARIOUS PHOSPHOLIPIDS

Incubations were carried out at 37 °C for 15 min in media containing 10 mM Tris–HCl (pH 7.4), 3 mM ATP (Tris salt), 4.5 mM $MgSO_4$, 120 mM NaCl, 10 mM KCl, and the solubilized enzyme preparation (10 μg protein) in a total volume of 0.1 ml, with or without phospholipid. Phospholipids were dispersed in 10 mM Tris–HCl (pH 7.4) by sonic vibration and added at a concentration of 100 μg/ml.

Phospholipid	*P_i liberated (μmoles/mg protein per h) in presence of*		*$(Na^+ + K^+)$-dependent activity*
	Mg^{2+}	*Mg^{2+}, Na^+, K^+*	
None	0.42	0.14	0
Phosphatidylinositol	0.14	1.90	1.76
Phosphatidylethanolamine	1.16	1.18	0.02
Phosphatidylcholine	0.50	1.28	0.78
Cardiolipin	0.78	0.38	0
Phosphatidylserine	0.72	12.0	11.3

Effect of diketocoriolin B on solubilized $(Na^+ + K^+)$-ATPase from rat brain

Inhibition by diketocoriolin B of the initial velocity of the enzyme reaction was determined at varied concentrations of ATP under conditions where the concentrations of phosphatidylserine, Na^+ and K^+ were fixed. The results are expressed as a Lineweaver–Burk plot in Fig. 4a which shows that diketocoriolin B inhibits the reaction in a competitive manner with ATP. In this respect, the mechanism of the inhibition was the same as that observed with the particulate enzyme. The figure also suggests that the degree of inhibition by diketocoriolin B is not a linear function of the amount of the inhibitor. To clarify this point, another experiment was conducted in which at fixed concentrations of ATP, the concentration of diketocoriolin B was varied. The results are expressed as a Dixon plot in Fig. 4b. The parabolic curves of the figure suggest that more than 2 moles of diketocoriolin B interact with the enzyme. To confirm this, the results shown in Fig. 4a were replotted according to the method of Johnson *et al.*[24,25], and are shown in Fig. 5a. The slope of the lines,

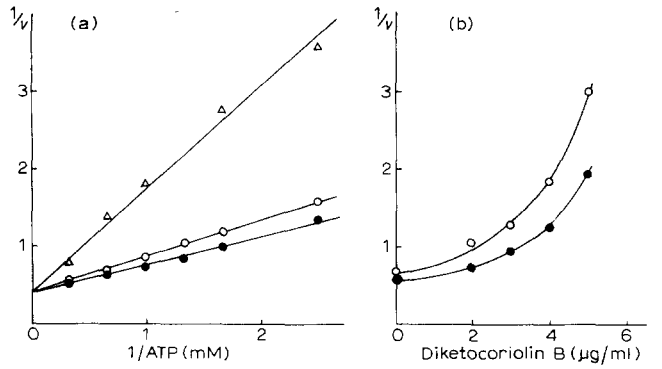


Fig. 4. Effect of diketocoriolin B on the substrate–velocity relationships of solubilized ($Na^+ + K^+$)-ATPase of rat brain. The experiment was conducted as in Fig. 1 except that the reaction mixture contained the solubilized ($Na^+ + K^+$)-ATPase of rat brain (12 μg protein) and phosphatidylserine (160 μg/ml). The results are expressed in the form of a Lineweaver–Burk plot (a): ●, control; ○, diketocoriolin B, 2 μg/ml; △, diketocoriolin B, 4 μg/ml. In panel (b), the effect of varying concentrations of diketocoriolin B was determined at either 1.5 mM ATP (○), or 3 mM ATP (●) and the data are presented in the form of a Dixon plot.

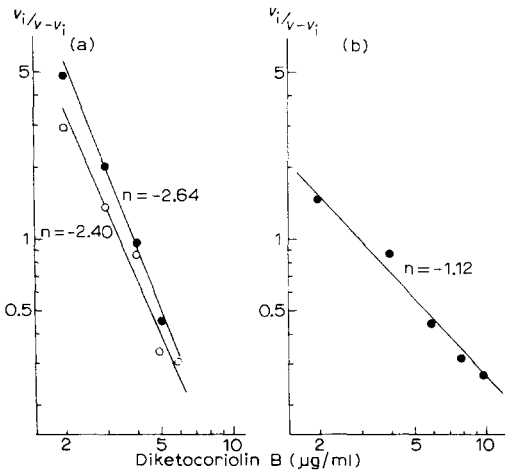


Fig. 5. Interaction between diketocoriolin B and rat brain ($Na^+ + K^+$)-ATPase. The results of Fig. 4b are used in a plot of log ($v_i/(v - v_i)$) as a function of the concentrations of diketocoriolin B on logarithmic coordinates, where "v" means the velocity without diketocoriolin B and "v_i" means that with the inhibitor at the indicated concentrations, according to the method of Johnson *et al.* and are shown in (a). A similar experiment was carried out with the particulate enzyme preparation from rat brain and the data are shown in (b).

about −2.5, supports the above mechanism. A similar experiment was conducted using a particulate enzyme from the same source, and resulted in a slope of −1.1 as shown in Fig. 5b. This agrees with observations on the particulate enzyme from Yoshida sarcoma cells (Fig. 1) and suggests that a single molecule of diketocoriolin B binds to a molecule of the particulate enzyme. To study the diketocoriolin B interaction with phosphatidylserine, initial velocities of the enzyme reaction were deter-

mined in the presence of various amounts of diketocoriolin B under conditions where the concentrations of ATP, Na^+ and K^+ were fixed while that of phosphatidylserine was varied. The results are expressed as a Lineweaver–Burk plot or Hill plot in Figs 6a and 6b, respectively. Fig. 6a indicates that the effect of diketocoriolin B is abolished by a large amount of phosphatidylserine. The finding is well explained if there are 2 binding sites for phosphatidylserine on an enzyme molecule and at each site phosphatidylserine competes with diketocoriolin B. In addition, it should be noticed that the activation of the enzyme by phosphatidylserine does not follow Michaelis–Menten kinetics as shown by the curved lines in Fig. 6a and by a slope of 1.5 to 2 in Fig. 6b. Although the interpretation of a Hill plot under the present conditions, where phosphatidylserine might exist as a micelle structure, is very difficult, the observed deviation from normal Michaelis–Menten kinetics might indicate some interaction between the sites for phosphatidylserine.

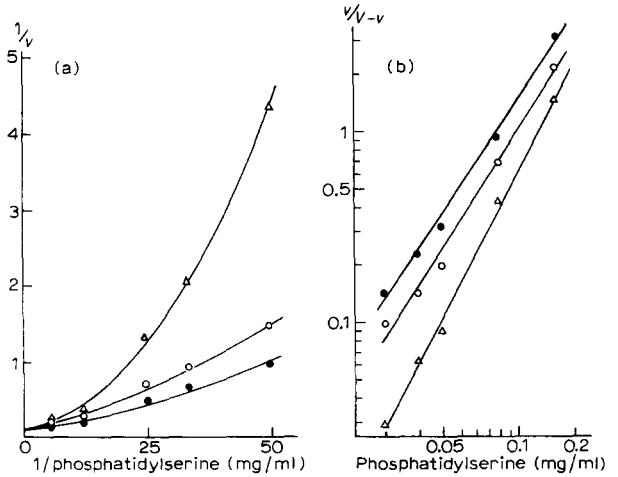


Fig. 6. Effect of diketocoriolin B on the reaction of the solubilized $(Na^+ + K^+)$-ATPase by phosphatidylserine. Incubations were carried out at 37 °C for 15 min in media containing 10 mM Tris–HCl (pH 7.4), 3 mM ATP (Tris salt), 4.5 mM $MgSO_4$, 120 mM NaCl, 10 mM KCl, the solubilized enzyme preparation (11 μg protein), phosphatidylserine at the indicated concentrations, and either no diketocoriolin B (●), 2 μg/ml diketocoriolin B (○), or 4 μg/ml diketocoriolin B (△) in a total volume of 0.1 ml. The rate of P_i liberation is expressed in relative units and is presented in the form of a Lineweaver–Burk plot (a) and a Hill plot (b).

We reported previously[1], using a particulate $(Na^+ + K^+)$-ATPase preparation from Yoshida sarcoma cells, that diketocoriolin B does not inhibit K^+-dependent phosphatase activity which is believed to represent the final step of reactions involved in $(Na^+ + K^+)$-ATPase[3,26–28]. We have now tested solubilized enzyme for sensitivity to diketocoriolin B. The enzyme preparation used for this purpose was the ammonium sulfate-precipitate fraction which had been freed from NH_4^+ by Sephadex G-25 column chromatography, since NH_4^+ substituted for K^+ in the reaction[26]. As is shown in Table IV, K^+-dependent phosphatase activity was observed only when phosphatidylserine was present in the system and interestingly, the activity was markedly inhibited by diketocoriolin B. In parallel with this experiment, a particulate

TABLE IV

EFFECT OF DIKETOCORIOLIN B ON K^+-DEPENDENT *p*-NITROPHENYL PHOSPHATASE ACTIVITY OF SOLUBILIZED ENZYME

Incubations were carried out at 37 °C for 15 min in media containing 50 mM Tris–HCl (pH 7.4), 2 mM *p*-nitrophenyl phosphate, 3 mM $MgCl_2$, 10 mM KCl, and the solubilized enzyme preparation (10 μg protein) in a total volume of 0.1 ml, with or without 100 μg/ml of phosphatidylserine. The reaction was terminated by adding 0.4 ml of 0.2 M Na_2CO_3. The absorbance of the resulting solution was read at 405 nm to determine the amount of liberated *p*-nitrophenol.

Diketocoriolin B (μg/ml)	*KCl*	*Phosphatidylserine*	*p-Nitrophenol* (*liberated μmoles/mg protein per h*)	
			Total activity	*K^+-dependent activity*
0	–	–	0.98	
5	–	–	1.04	
10	–	–	1.02	
0	+	–	0.94	0
5	+	–	0.78	0
10	+	–	0.82	0
0	–	+	2.12	
5	–	+	1.62	
10	–	+	1.40	
0	+	+	2.60	0.48
5	+	+	1.88	0.26
10	+	+	1.60	0.20

TABLE V

EFFECT OF DIKETOCORIOLIN B ON K^+-DEPENDENT *p*-NITROPHENYL PHOSPHATASE ACTIVITY OF THE PARTICULATE ENZYME

Incubation and analysis were performed as described in the legend to Table IV except that the particulate ($Na^+ + K^+$)-ATPase preparation of rat brain (33 μg protein) was used.

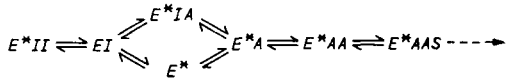
Diketocoriolin B (μg/ml)	*KCl*	*p-Nitrophenol* (*liberated μmoles/mg protein per h*)	
		Total activity	*K^+-dependent activity*
0	–	0.70	
10	–	0.78	
0	+	1.24	0.54
10	+	1.32	0.54

enzyme from the same source (rat brain) was tested. As shown in Table V, its K^+-dependent phosphatase activity was independent of exogenous phosphatidylserine and was completely insensitive to diketocoriolin B. There are contradictory reports

concerning the requirement for exogenous phosphatidylserine of the K^+-dependent phosphatase activity of solubilized $(Na^+ + K^+)$-ATPase. No requirement was reported by Towle[18] whereas the reverse was found by Tanaka *et al.*[14,23], both using deoxycholate-solubilized enzyme. Taniguchi *et al.*[16] reported that the enzyme preparation treated with phospholipase A or C was activated by exogenous phospholipid.

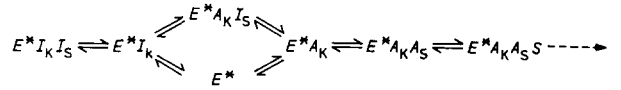
DISCUSSION

To explain all the data, Scheme III proposed above must be modified. Thus, we considered the Scheme IVa: E^* stands for the "naked" enzyme containing no "essential" phospholipid, and A stands for activator (phospholipid).



(IVa)

ATP, expressed as S, binds to the enzyme only after 2 sites for activator have been filled by phospholipid. Diketocoriolin B, expressed as I, competes with phospholipid at each binding site. However, this scheme is still imperfect because it cannot explain the following points: (1) 2 moles of diketocoriolin B interact with the solubilized enzyme while 1 mole of diketocoriolin B reacts with the particulate enzyme; (2) K^+-dependent phosphatase activity of the solubilized preparation required phospholipid and was sensitive to diketocoriolin B, while the reverse was true with the particulate enzyme. Accordingly, Scheme IVa was improved to Scheme IVb in which the 2 binding sites for phospholipids are discriminated from each other, that is, the presence



(IVb)

of the first phospholipid at site K is essential for K^+-dependent activity as well as for causing a conformational change in the enzyme molecule so that the second phospholipid may be accepted in Site S. Only after these events are completed, can ATP react with the enzyme–activator complex. In case of the particulate enzyme, diketocoriolin B competes with A only at Site S and accordingly does not inhibit K^+-dependent phosphatase. On the other hand, with the solubilized enzyme, diketocoriolin B can compete with A at both Site K and Site S leading to the inhibition of K^+-dependent phosphatase activity. In other words, E^*A_K and $E^*A_KA_S$ correspond to E° and $E^\circ A$ of Scheme III, respectively. $E^*A_KA_S$ also corresponds to E of Scheme I and II, which is the particulate enzyme itself.

There are several inhibitors of $(Na^+ + K^+)$-ATPase whose modes of action will be summarized below utilizing Scheme II. Ouabain and related cardiac glycosides interfere mostly with K^+-dependent Step 4 (refs 4–7). This step is also sensitive to the following: quindonium and related azasteroid[29,30]; hellebrigenin[31–33], a steroid of plant origin; erythrophleine[30] and related cytotoxic compounds, long-chain fatty acids[34]; and organic solvents like $CHCl_3$[35]. Some macrolide antibiotics such as oligomycin inhibit Mg^{2+}-dependent Step 3 (refs 8–10, 27). Sulfhydryl inhibitors such as *N*-ethylmaleimide are reported to inhibit Step 3 (refs 27, 36), however, their effects on the other steps are not negligible[37,38]. On the other hand, phlorizin and phloretin

modify the affinity of the enzyme to Na^+ resulting in blocking Step 2 (ref. 39). Ethacrynic acid and related diuretics inhibit Step 2, however, their effect on Step 4 is also reported[40–42]. It has been thought that chlorpromazine showing similar inhibition as the former two compounds works as a free radical[43]. Compared with these inhibitors, the mode of action of diketocoriolin B is very unique not only in view of its target step but also in view of its competition with phospholipid. Because of the latter point, diketocoriolin B is a unique enzyme inhibitor among enzyme inhibitors in general and furnishes a clue to the function of phospholipid in $(Na^+ + K^+)$-ATPase. There are several reports that membrane enzymes require phospholipid for their activities. Mg^{2+}-ATPase, K^+-dependent phosphatase and alkaline phosphatase all localized in the plasma membrane of rat liver cells, are inactivated by digestion with phospholipase C[15]. Widnell *et al.*[45] have reported that purified 5′-nucleotidase from rat liver contains sphingomyelin as the sole phospholipid component. Glucose-6-phosphatase in endoplasmic reticulum is inactivated by digestion with phospholipase C and reactivated upon addition of phosphatidylethanolamine[46]. NADH-cytochrome *c* reductase[47] and fatty acid desaturase[48] of the same organelle were reported to require phospholipid. β-Hydroxybutyl dehydrogenase[49], NADH-cytochrome *c* reductase[50] and Mg^{2+}-ATPase[51] in the mitochondrial membrane were reported to be dependent on phospholipid. And recently, it was reported that the hormone sensitivity of solubilized adenyl cyclase, another membrane enzyme, was restored by phosphatidylserine[52]. However, none of these studies elucidated the detailed function of phospholipid in any of these enzyme reactions.

Our scheme explaining the function of phospholipid in the $(Na^+ + K^+)$-ATPase reaction may be inconclusive because it is mostly based on evidence drawn from kinetic studies. Besides, the enzyme used was a partially purified one. For a complete scheme, additional direct demonstrations may be necessary. However, several recent works on the role of phospholipids in $(Na^+ + K^+)$-ATPase activity, which were performed from various viewpoints and employed methods different from each other, well support our scheme. Taniguchi and Tonomura[16] demonstrated that the formation and breakdown of the phosphorylated intermediate is inhibited by the removal of phospholipids. The specific requirement of phosphatidylserine for the formation of the intermediate was also indicated by Goldman and Albers[53]. Kimelberg and Papahadjopoulos[54] suggested that phospholipids can provide a cation-specific site, where head-group specificity of phospholipids is involved, while the fatty acid chains provide the appropriate environment in a rather non-specific manner.

During preparation of this paper, Palatini and Bruni[55] reported that the inhibition by oligomycin of mitochondrial ATPase and $(Na^+ + K^+)$-ATPase was reversed by some phospholipids. Using mitochondrial ATPase extracted from submitochondrial particle with cholate, Pitotti *et al.*[56] demonstrated that the acidic phospholipids and lysophosphatidylcholine stimulate the ATPase activity and that the activation deviates from normal Michaelis–Menten kinetics. More interestingly, they showed that the activation by phospholipids was competitively inhibited by oligomycin. Comparing these results with the data shown in the present report, it is suggested that a close similarity in the role of phospholipids for the activity of mitochondrial ATPase and $(Na^+ + K^+)$-ATPase exists. In this respect, it might be interesting to investigate the inhibition of mitochondrial ATPase by diketocoriolin B.

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