Biochimica et Biophysica Acta, 642 (1981) 96—105

BBA 79153

CARDIOLIPINS ARE 'IN VITRO' INHIBITORS OF RAT BRAIN ($Na^+ + K^+$)-DEPENDENT ATPases

A PROBABLE MECHANISM OF ACTION *

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(Received August 11th, 1980)

Key words: Cardiolipin; ($Na^+ + K^+$)-ATPase inhibitor; Diphosphatidylglycerol; Δ^9-Tetrahydrocannabinol; (Rat brain)

Summary

Cardiolipins were found to potentiate the 'in vitro' inhibitory activity of (—)-Δ^9-tetrahydrocannabinol on ($Na^+ + K^+$)-dependent rat brain ATPases. The compounds were found to be powerful inhibitors by themselves. At optimal concentrations of cations (Na^+, K^+, Mg^{2+}), the compounds were found to be noncompetitive inhibitors of ATP ($K_i = 3.5 \cdot 10^{-6}$ M) and 'uncompetitive' inhibitors of Na^+. From gas-liquid chromatographic analysis of the cardiolipin preparations it can be inferred that their effectiveness as inhibitors is related to the linoleic acid contents. The preliminary data presented here suggest that cardiolipins inhibit the Na^+-dependent phosphorylation step in the hydrolysis of ATP.

Based on the observations reported in this work, a hypothesis is presented suggesting that there may be a functional or evolutionary explanation for the paucity of cardiolipins in cell plasma membranes.

Introduction

Probably the most thoroughly studied of the membrane-bound enzymes is the ($Na^+ + K^+$)-ATPase. The enzyme preparation requires phospholipids for

* Part of this work was presented at the 64th Annual Meeting of the Federation of American Societies for Experimental Biology, Anaheim, CA, April 13—18, 1980.

Abbreviations: K^+ neutral phosphatase, potassium-stimulated nitrophenyl phosphohydrolase (EC 3.1.3.1); (—)-Δ^9-tetrahydrocannabinol, also known as Δ^1THC.

activity and their removal leads to inactivation. Exposure of the enzyme to detergents and phospholipases causes an inactivation which is reversed by addition of phospholipids. These findings have been interpreted as suggestive of a role of phospholipids in the active structure of the $(Na^+ + K^+)$-ATPase [1—4]. It has been suggested that under physiological conditions the lipid responsible for maintaining the activity of the $(Na^+ + K^+)$-ATPase may be phosphatidylserine [5]. Several reports [6—9] attest to the role of phospholipids in restoring enzymatic activity to $(Na^+ + K^+)$-ATPase preparations in which enzyme inhibition by deoxycholate treatment was essentially complete. Phospholipids are not the only lipids found to restore enzymatic activity to such preparations; gangliosides [10] and sulfatides [11] have been found to restore activity as well. Our own studies have demonstrated that phosphatidylethanolamine restores enzymatic activity to rat brain $(Na^+ + K^+)$-ATPases previously inactivated by the addition of cannabinoids and specifically by (—)-Δ^9-tetrahydrocannabinol [12].

Diphosphatidylglycerols or cardiolipins are highly amphipathic phospholipid molecules found in the single cellular membrane of bacteria as well as in the inner membrane of mitochondria, where they make up over 10% of the total lipids [13]. These membranes are not characterized by having $(Na^+ + K^+)$-ATPases as one of their integral proteins. On the other hand, cardiolipins have not been reported among the phospholipids known to restore enzymatic activity to microsomal $(Na^+ + K^+)$-ATPases. Our observation that cardiolipins potentiated the inhibitory activity of (—)-Δ^9-tetrahydrocannabinol on rat brain microsomal $(Na^+ + K^+)$-ATPase preparations led us to study their effects on the kinetics of these ATPases in more detail.

Materials and Methods

Trizma hydrochloride, Trizma base, ouabain octahydrate, ATP (disodium salt), *p*-nitrophenyl phosphate, phosphatidylethanolamine and linoleic acid (99% pure) were purchased from Sigma. Stock ATP solutions neutralized to pH 7.2 with 1 M Trizma base were kept frozen in small aliquots until used. Ouabain was stored under refrigeration as a 10 mM solution in water until used.

Three commercial preparations of cardiolipins purchased from different sources were used without further purification (Sigma Lot No. 104C-0016, 9.3 mg/ml; Sigma Lot No. 57C-0018, 4.5 mg/ml; U.S. Biochemical Control No. 10818, 5.9 mg/ml).

The (—)-Δ^9-tetrahydrocannabinol used in this work was chromatographically pure both by thin-layer (TLC) and high-pressure liquid chromatography (C^8 Bondapack column, CH_3CN/H_2O (90 : 10, v/v) used as eluant). All other reagents were either U.S.P. or C.P. grade.

Precoated silica gel plates from Kontes-Quantum were used for TLC. Plates were developed in $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v), hexane/diethyl ether/acetic acid (90 : 15 : 1, v/v) or $CHCl_3/CH_3OH$/28% NH_4OH (65 : 25 : 4, v/v) and visualized by exposure to iodine vapor and by staining with phosphomolybdate.

Preparation of $(Na^+ + K^+)$-ATPase from adult rat brain. Adult Sprague-Dawley rats weighing 200—250 g were killed by decapitation and their brains

were removed. The cerebellum was discarded and the enzyme was prepared by a simple modification of the procedure described by Ahmed and Judah [14] which consisted of using sucrose instead of mannitol to prepare the solutions. The specific activity of these preparations was 1500—1800 nmol P_i/mg protein per min and was 75—80% sensitive to 1 mM ouabain. Protein was measured by the modified Folin's method of Lowry et al. [15]. Enzyme activity was measured as P_i liberated using Sigma equine ATP as substrate. No attempt was made to remove the vanadate found by other investigators [16,17] to be present in these substrate preparations and to be strongly inhibitory to ATPases. Such inhibition was not apparent in this work. Incubations were performed at 37°C at a pH of 7.02. The total volume of the incubation mixture was 1 ml. The procedure for the assays has been described elsewhere [18]. In the assays where ouabain, (—)-Δ^9-tetrahydrocannabinol, cardiolipins or linoleic acid were used as inhibitors, the blanks contained the corresponding amounts of inhibitor in their respective solvents. As a rule, the assay mixture contained 10—40 μg protein, 1 μg (—)-Δ^9-tetrahydrocannabinol, 2.5—5 μg cardiolipins or linoleic acid and from 1 to 10 μmol ATP. ($Na^+ + K^+$)-ATPase was determined as the difference in activities between total ATPase and ouabain-insensitive ATPase. The latter was measured omitting K^+ from the medium in the presence of 1 mM ouabain.

Assay for K^+ neutral phosphatase. The phosphatase was assayed essentially as described by Nagai et al. [19] using *p*-nitrophenyl phosphate as substrate. The total protein in the assays varied between 80 and 120 μg. Its activity was calculated as the difference between activity measured in the presence of Mg^{2+} plus K^+ (5 and 10 mM) and the activity detected with Mg^{2+} alone. The reaction was started by adding the substrate to a final concentration of 5 mM, allowed to proceed for 15 min at 37°C and was stopped by adding 2 ml of 0.05 M NaOH. The mixtures were centrifuged and the *p*-nitrophenol liberated was read at 510 nm. Control assays were run in the presence of appropriate concentrations of solvents used as vehicle for the inhibitors ((—)-Δ^9-tetrahydrocannabinol, cardiolipins, ouabain). Blanks were included to correct for absorption due to the presence of inhibitors.

Analysis of the fatty acid composition of commercial preparations of cardiolipins was obtained by gas-liquid chromatography of the fatty acid methyl esters. Transmethylation was performed using 15% (v/v) CF_3CO_2H in CH_3OH (1 ml/mg or less of sample) at 60°C for 90 min. Excess reagent was removed by evaporation with a stream of N_2 at room temperature, and the residue was dissolved in an appropriate quantity of hexane (e.g., 50 μl) for analysis. This simple technique was found to give the same results as the more conventional 10—15% HCl in CH_3OH followed by extraction and neutralization of excess acid. No difference was noted in the CF_3CO_2H procedure when an aqueous $NaHCO_3$ washing step was included. The more convenient procedure given above was therefore employed as the standard method.

Gas-liquid chromatography was performed on a Perkin-Elmer Sigma 1 chromatograph, using a 180 × 0.2 cm glass column containing 10% silar 10CP.

A multilevel program was employed to analyze conveniently the straight-chain fatty acid methyl esters from C6 to C24. The column was held at 110°C for 3 min; then raised at 9.50°C/min to 175°C; held for 5 min at 175°C; and

finally raised at 10°C/min to 220°C, and kept at 220°C for 10 min. Data were collected for 28 min. Authentic samples (Supelco and Applied Science Laboratories) of all fatty acid methyl esters were used for identification of the peaks.

Results

Table I shows the effects of various substances known to be inhibitors of $(Na^+ + K^+)$-dependent ATPases on the total activity of a rat brain microsomal preparation of ATPase. It is apparent that while phosphatidylethanolamine reverses the inhibition caused by (—)-Δ^9-tetrahydrocannabinol, cardiolipins potentiate this inhibition. Phosphatidylethanolamine has no appreciable effect on the inhibition caused by ouabain while cardiolipins slightly increase the inhibition.

Fig. 1 illustrates the residual enzymatic activity obtained when ATPases were incubated in the presence of varying amounts of cardiolipins. Concentrations of 3 μM (5 μg/ml) in one specific preparation (preparation I, Sigma Lot No. 104C-0016) were enough to inhibit about 80% of the total ATPase activity. This inhibitory activity was variable, depending on the preparation used. Preparations I and II were found to be free of impurities by TLC using two different solvent systems: $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v) and $CHCl_3/CH_3OH/28\%$ NH_4OH (65 : 25 : 4, v/v). Preparation III showed an impurity (about 12%) assumed to be a lysocardiolipin from its chromatographic behavior.

To find out whether these differences in inhibitory activity were in any way related to differences in fatty acid composition, we performed gas-liquid chromatographic analysis. The results are shown in Table II. What is clearly apparent from these results is that the higher the content of linoleic acid (C18:2), in the cardiolipin preparation, the higher is its inhibitory activity.

The possible link between C18:2 contents and ATPase inhibitory activity was studied as follows. Concentrations of C18:2 resembling those present in the incubation mixture, where cardiolipins concentrations were enough to produce a

TABLE I

POTENTIATION OR REVERSAL OF INHIBITORY ACTIVITY ON RAT BRAIN ATPases BY PHOSPHOLIPIDS

10 μg of protein were present in the incubation mixture. The same amounts of (—)-Δ^9-tetrahydrocannabinol (Δ^9 THC) and ouabain were used in all cases. Cardiolipin: sample I; Sigma Lot No. 104 C-0016. The values obtained for residual activity are the average of two experiments (three determinations per experiment).

Inhibitor added	Residual activity (nmol P_i/mg protein per min)	% inhibition
None	1640 ± 40	
Δ^9THC ($3 \cdot 10^{-9}$ mol)	840 ± 35	48.8 ± 2.2
Δ^9THC + phosphatidylethanolamine ($7 \cdot 10^{-8}$ mol)	1030 ± 54	37.2 ± 3.3
Δ^9THC + cardiolipin ($3 \cdot 10^{-9}$ mol)	350 ± 30	78.6 ± 1.9
Ouabain (10^{-7} mol)	426 ± 25	74.0 ± 1.5
Ouabain + cardiolipin ($3 \cdot 10^{-9}$ mol)	222 ± 20	86.5 ± 1.2
Ouabain + cardiolipin ($1.5 \cdot 10^{-9}$ mol)	300 ± 15	81.7 ± 1.1
Ouabain + phosphatidylethanolamine ($7 \cdot 10^{-8}$ mol)	330 ± 22	79.9 ± 1.3

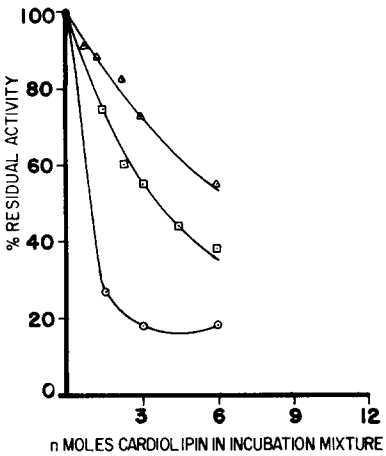

Fig. 1. Total ATPase activity as a function of concentration of cardiolipins. ⊙———⊙, preparation I; △———△, preparation II; ⊡———⊡, preparation III. Enzymatic activity was determined as described in the text. Each point traced is the average of two experiments; three determinations per experiment. The same holds true for points traced in Figs. 2—4.

60—80% inhibition of total ATPase activity, were added to the incubation mixture. For amounts of C18:2 varying from 12 to 16 nmol per ml of incubation mixture the percent inhibition was from 52 to 58%, in good agreement with values found for the inhibitory effects in the presence of 6 nmol of cardiolipin from preparations I and III (see Fig. 1).

Once the inhibitory properties of cardiolipins on these enzyme preparations were substantiated, kinetic studies on enzyme activity were performed in an attempt to determine the type of inhibition exerted by this particular inhibitor.

Throughout these kinetic studies the assumption was made that cardiolipins inhibit ($Na^+ + K^+$)-ATPases selectively and that the maximum inhibition attribut-

TABLE II

FATTY ACID COMPOSITION OF CARDIOLIPIN PREPARATIONS

The values of % composition are the average of four independent determinations. Preparation I, Sigma Lot No. 104C-0016; preparation II, Sigma Lot 57C-0018; preparation III, U.S. Biochemicals Control No. 10818.

Fatty acid	% composition		
	Preparation I	Preparation II	Preparation III
Lower than C_{14}	12.6	7.0	6.2
C14:1	3.6	7.3	0.1
C16:0	2.9	9.1	2.3
C16:1	1.1	2.1	9.4
C16:2	1.9	13.1	0.5
C18:0	3.3	9.1	1.4
C18:1	6.6	7.7	7.6
C18:2	68.0	17.2	58.7
C18:3	0	0.6	5.2
Higher than C18:3	—	28.8	8.6

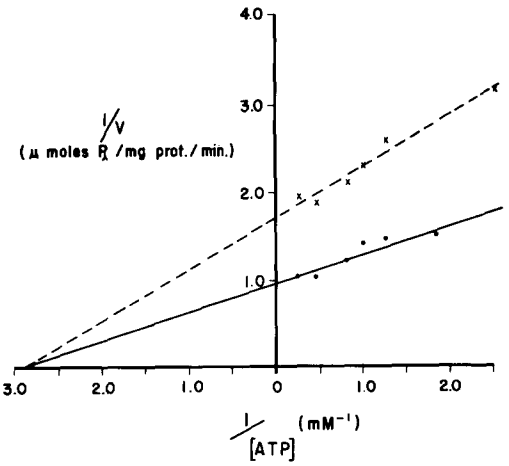

Fig. 2. Double-reciprocal plot using variable concentrations of ATP as substrate under optimal concentrations of cations. ●———●, uninhibited reaction; X———X, in the presense of $1.5 \cdot 10^{-9}$ mol of cardiolipin (preparation I). Enzymatic activity was determined as described in the text.

able to them was 80% of the total ATPase activity.

Experiments conducted in the presence of optimal concentrations of cations with ATP as substrate yielded the data shown as a double-reciprocal plot in Fig. 2. Under the experimental conditions used, cardiolipins acted as a non-competitive inhibitor of ATP. The K_i value under these experimental conditions was $3.5 \cdot 10^{-6}$ M. Experiments performed using optimal concentrations of Mg^{2+} and K^+ with varying concentrations of Na^+ and with optimal concentrations of Mg^{2+} and Na^+ using varying concentrations of K^+ are shown in Figs. 3 and 4 respectively. In Fig. 3, the data as plotted are suggestive of the so called uncompetitive inhibition. When the variable cation is K^+ (Fig. 4), the nature of the inhibition is different, suggesting a mixed inhibition.

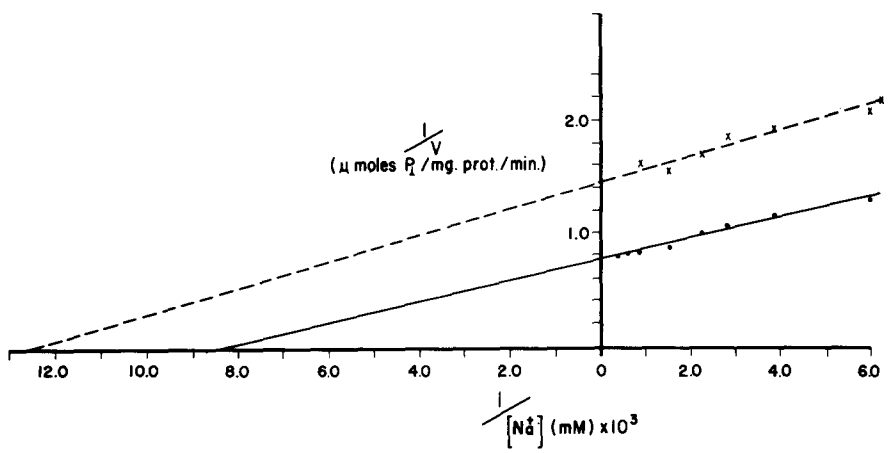

Fig. 3. Double-reciprocal plot using variable concentrations of Na^+, optimum concentrations of Mg^{2+} and K^+ and saturating concentrations of ATP. ●———●, uninhibited reaction; X- - - - -X, in the presence of $1.5 \cdot 10^{-9}$ mol of cardiolipins (preparation I). Enzymatic activity was determined as described in the text.

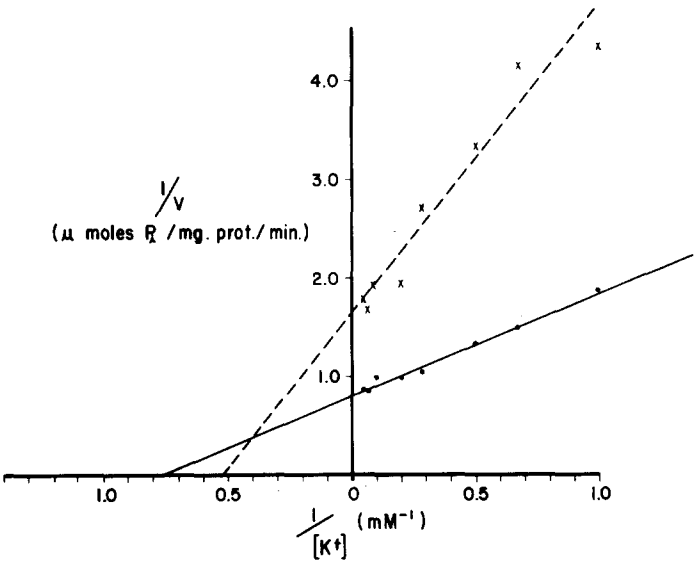

Fig. 4. Double-reciprocal plot using variable concentrations of K^+, optimum concentrations of Mg^{2+} and K^+ and saturating concentrations of ATP. •———•, uninhibited reaction; X- - - - -X, in the presence of $1.5 \cdot 10^{-9}$ mol of cardiolipins (preparation I). Enzymatic activity was determined as described in the text.

Table III shows a comparison between the effects of ouabain and cardiolipins on the K^+ neutral phosphatase activity of the preparations. Concentrations of ouabain which caused an 80% inhibition of total ATPase activity caused a much lower inhibition of the K^+ neutral phosphatase (about 25%). A similar degree of inhibition (about 33%) was observed when cardiolipins (preparation I) were used as inhibitors. These concentrations of cardiolipins were enough to cause about 80% inhibition of total ATPase activity.

Discussion

The results presented in Table I and Fig. 1 clearly show that cardiolipins are in vitro inhibitors of the ATPase preparations used in this work and the inhibi-

TABLE III

A COMPARISON OF THE INHIBITORY EFFECTS OF OUABAIN AND CARDIOLIPINS ON THE K^+ NEUTRAL PHOSPHATASE OF RAT BRAIN

p-Nitrophenyl phosphate was used as substrate. Total protein present in incubation mixture, 80 μg. The values shown for the activity are the average of two experiments (three determinations per experiment).

Inhibitor	Activity (nmol P_i/mg protein per min)	% inhibition
None	872 ± 60	—
Ouabain ($5 \cdot 10^{-8}$ mol)	650 ± 44	25.3 ± 5.1
Ouabain (10^{-7} mol)	642 ± 43	26.4 ± 5.0
Cardiolipin sample I ($1.5 \cdot 10^{-8}$ mol)	578 ± 46	33.7 ± 5.3
Cardiolipin sample I ($3.0 \cdot 10^{-8}$ mol)	588 ± 45	32.6 ± 5.6

tory activity is particularly selective for the ($Na^+ + K^+$)-ATPase. These particular phospholipids are not known to be constituents of the cellular membranes of brain cells. The results shown are unexpected in the light of information available as to the effects of other phospholipids on ATPase preparations from various sources, including rat brain [1—9]. A recent report by Ernster et al. [20] mentions that previously inhibited mitochondrial ATPase can be reactivated by a dialyzable factor claimed to be a cardiolipin. This result although at first instance may seem to be contrary to our finding is not necessarily so. Mitochondrial ATPases are not ($Na^+ + K^+$)-dependent.

The data presented in Table II can be explanatory of the results shown in Fig. 1. The differences in degree of inhibition could be explained by differences in composition of the various cardiolipids used. It is apparent that the most striking differences in composition can be ascribed to differences in the linoleic acid (C18:2) contents of the preparations. It is to be noted that while preparations I and III contained similar amounts of C18:2 (and their inhibitory activities were similar), preparation II contained much smaller amounts of C18:2 than preparations I and III and likewise, the inhibitory activity was lower. The experiments using linoleic acid lend support to the hypothesis that inhibition can be ascribed to the hydrocarbon chain, since the results have shown that the acid itself is highly inhibitory. The results suggest that the conformation assumed by the hydrocarbon chain determines, to a large extent, the inhibitory action. More detailed experiments on this aspect are in progress in our laboratory.

The kinetic data are presented as Lineweaver-Burk plots in Figs. 2—4. The same ratio of inhibitor to enzyme was used in all the experiments. Since ATP, Na^+ and K^+ can be considered substrates of the enzyme, the figures represent the results of experiments in which the corresponding substrates were used at variable concentrations. The kinetic data presented in Fig. 2, by themselves, do not allow a conclusion as to whether combination of the enzyme with the inhibitor occurs prior to or after the enzyme combines with the substrate; i.e., either of these situations is possible:

$$E + I \rightarrow EI + S \rightarrow EIS$$

$$E + S \rightarrow ES + I \rightarrow ESI$$

where E, I, EI, S, ES and EIS are enzyme, inhibitor, enzyme-inhibitor complex, substrate, enzyme-substrate complex, and enzyme-inhibitor-substrate complex, respectively. Both schemes lead to the same results. The data obtained when using saturating concentrations of ATP and varying concentrations of cations give a different picture. When Na^+ is used in varying concentrations, the inhibition is of the type named uncompetitive. In this kind of inhibition, the inhibitor combines only with the enzyme-substrate complex [21]. This kind of inhibition is not unusual for enzymes such as ATPases, having two or more substrates [22]. A postulated scheme for the hydrolysis of ATP suggests an activating effect of Na^+ on the enzyme prior to phosphorylation [23], i.e.:

$$E_2 + Na^+ \rightarrow E_1Na^+ + ATP \rightarrow E_1Na^+P + ADP$$

where E_2 and E_1 are different conformational states of the enzyme. Since in

our measurements we are determining hydrolysis of ATP, it can be proposed, taking these data in conjunction with those obtained in Fig. 2 and discussed above, that the enzyme-substrate complex to which the inhibitor binds is E_1Na^+, thus inhibiting the next step which would be:

$$E_1Na^+ + ATP \rightarrow E_1Na^+P + ADP$$

A possible reaction would be $E_1Na^+ + I \rightarrow E_1Na^+I$, where E_1Na^+ and E_1Na^+I are the enzyme-substrate and enzyme-substrate-inhibitor complex, respectively.

The mixed inhibition which is apparent from Fig. 4 is to be expected from the results obtained in Figs. 2 and 3 and discussed above.

A possibility that looked very appealing was that cardiolipins may be acting as chelating agents at the pH used for the enzyme assays. These substances are negatively charged and may bind Mg^{2+}, an indispensable cation for enzyme activity. This possibility was ruled out when we found (results not shown) that increasing the concentration of Mg^{2+} 3-fold did not diminish the inhibitory effects of cardiolipins on enzyme preparations.

In assaying for K^+ neutral phosphatase activity (Table III) the intension was to find out where in the sequence of reactions leading to the hydrolysis of ATP do the cardiolipins exert their action. An impressive body of data available supports the hypothesis that hydrolysis of ATP occurs via phosphorylation of the enzyme followed by a dephosphorylation. Thus, in the second step of ATP hydrolysis the enzyme acts as a phosphatase. $(Na^+ + K^+)$-ATPase preparations usually show an ouabain-inhibitable, K^+-dependent phosphatase activity [24, 25]. Whether the two activities belong to the same or different enzymes has not been definitely established [19,26]. From the point of view of kinetics, the similarity is useful because by the use of phosphate esters other than ATP as substrate, valuable information can be obtained as to the mechanism of inhibition. The kinetics of inhibition of these reactions have been used to speculate on the mechanism of action of ATPase inhibitors [18]. The data presented in Table III, show a moderate (33%) inhibition by cardiolipins on the activity of the K^+ neutral phosphatase. Under the assumption that the action of the phosphatase is identical to the dephosphorylation reaction of the ATPase, the conclusion should be that cardiolipins inhibit the Na^+-dependent phosphorylation step in the hydrolysis of ATP. However, the data in Table III also show that the K^+ neutral phosphatase was very moderately sensitive to ouabain. These data do not help to conclude whether the ATPase and phosphatase activities reside on the same enzyme.

A point that merits discussion is that while plasma membranes rich in $(Na^+ + K^+)$-ATPases are phospholipid rich, cardiolipins are not found among those phospholipids. On the other hand, in bacterial and mitochondrial membranes, which are rich in cardiolipins, the ATPases are essentially Mg^{2+} activated and thus ouabain insensitive. We have tested ouabain-insensitive (Mg^{2+}-dependent) ATPases from guinea-pig ileum, and in these preparations cardiolipins do not appear to exert significant inhibitory activity (Toro-Goyco, E., unpublished observations). All these findings lead us to suggest that there could be a functional or evolutionary explanation for the absence of cardiolipins from plasma cell membranes; their presence there would be inhibitory to the sodium pump and consequently an obstacle to ion transport. $(Na^+ +$

K^+)-ATPase is not the only enzyme inhibited by cardiolipins. Bovine liver glutamate dehydrogenase has been found to be inhibited by cardiolipins [27]. This enzyme, which exists in mitochondria, is not found imbedded in the mitochondrial membranes but in the matrix.

Acknowledgments

We thank Messrs. J. Mussenden and G. García de la Noceda for the art work, and Mrs. María M. Rivera and Esther Torres for typing the manuscript.

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