

Inhibition of Protein Kinase C, (Sodium plus Potassium)-activated Adenosine Triphosphatase, and Sodium Pump by Synthetic Phospholipid Analogues¹

Bin Zheng,² Kazuhiko Oishi,³ Mamoru Shoji, Hansjörg Eibl, Wolfgang E. Berdel, Joseph Hajdu, William R. Vogler, and J. F. Kuo

Departments of Pharmacology [B. Z., K. O., J. F. K.] and Medicine (Hematology/Oncology) [M. S., W. R. V.], Emory University School of Medicine, Atlanta, Georgia 30322; Max-Planck Institute for Biophysical Chemistry, Göttingen [H. E.], and Department of Medicine I (Hematology/Oncology), Technical University, Munich [W. E. B.], Federal Republic of Germany; and Department of Chemistry, California State University, Northridge, California 91330 [J. H.]

ABSTRACT

The effects and modes of action of certain antineoplastic phospholipid analogues (racemic 1-*O*-octadecyl-2-*O*-methyl glycerol-3-phosphocholine, BM 41.440, JH-1, CV-3988, and HePC) on (sodium plus potassium)-activated adenosine triphosphatase (Na,K-ATPase) and sodium pump activities were investigated. Inhibition of Na,K-ATPase in purified rat brain synaptosomal membranes by these lipids, in contrast to ouabain, was subject to membrane surface dilution and unaffected by whether the reaction was started with KCl, NaCl, or ATP. Kinetic analysis indicated that the analogues, again dissimilar to ouabain, were likely to interact directly or indirectly with sodium-binding sites of Na,K-ATPase located at the intracellular surface of the plasma membrane, a conclusion also supported by studies using the inside-out vesicles of human erythrocyte membranes. The studies also showed that ouabain (but not the lipids) increased the affinity constant of Na,K-ATPase for K⁺, whereas the lipids (but not ouabain) increased that for Na⁺. The lipids also inhibited ⁸⁶Rb uptake by intact human leukemia HL60 cells at potencies quite comparable to those seen for inhibition of purified protein kinase C or Na,K-ATPase. It is suggested that Na,K-ATPase (sodium pump) might represent a hitherto unrecognized site of action for the lipid analogues, and that the antineoplastic effects of the agents might be due to, in part, inhibition of both protein kinase C and Na,K-ATPase and perhaps other membrane-associated enzymes.

INTRODUCTION

Ether phospholipids and derivatives, as experimental anticancer agents, have become a focus of research in recent years (for reviews, see Refs. 1 and 2). For example, ET-18-OCH₃,⁴ the prototype of the alkyllysophospholipid class of agents (3), has been shown to inhibit growth (3, 4) and metastasis (5) of syngeneic murine tumors and the growth of some rat tumors (6) and human leukemic HL60 cells in culture (7, 8). Several modes or mechanisms of action have been postulated to account for its biological effects. These include enhancement of cytotoxic properties of macrophages (3-5), alterations of phospholipid metabolism (9), and inhibition of PKC (10). We reported previously that, besides ET-18-OCH₃ (10), the antineoplastic lipoidal amine CP-46,665-1 (11) and the thioether analogue of phospholipid BM 41.440 (12), as well as the clinically useful anticancer drugs Adriamycin (13) and tamoxifen (14), inhibited PKC at potencies comparable to those for *in vitro* inhibition of growth of cancer cells (see Refs. 1, 2, and 10 to 14 and papers

cited therein). These findings suggested that PKC might represent a potential site of action of the lipid agents and certain anticancer drugs.

Na,K-ATPase, like PKC, is a major membrane enzyme playing a pivotal role in cell function. Because Na,K-ATPase is also regulated by lipids (15), we suspected that certain lipid analogues shown to be PKC inhibitors would also inhibit Na,K-ATPase. We found in the present studies that ET-18-OCH₃, BM 41.440, JH-1, CV-3988, and HePC inhibited purified PKC and Na,K-ATPase as well as sodium pump in intact HL60 cells with comparable potencies.

MATERIALS AND METHODS

Materials. PS (bovine brain), ouabain, and histone H1 (type III-S) were purchased from Sigma Chemical Co. (St. Louis, MO); ET-18-OCH₃ was from Medmark Chemicals (Muenchen-Gruenwald, Federal Republic of Germany); BM 41.440 was from Boehringer Mannheim (Mannheim, Federal Republic of Germany); and CV-3988 was from Takeda Chemical Industry (Osaka, Japan). JH-1 (16) and HePC (17) were synthesized and provided by the corresponding authors.

Preparations of Na,K-ATPase and PKC. Na,K-ATPase was purified from rat cerebral cortex synaptosomes by extraction with sodium dodecyl sulfate and discontinuous sucrose density centrifugation, according to the procedure of Matsuda and Cooper (18) modified from the original method of Jorgensen (19). Mg²⁺-dependent ATPase activity in this enzyme preparation was less than 3% of the total ATPase activity. PKC was purified from pig brain extracts through the phenyl-Sepharose affinity step (20); the enzyme preparation was devoid of other contaminating protein kinase activities.

Assays for Enzymes. Na,K-ATPase activity was assayed as described (21). Briefly, the standard reaction mixture (0.5 ml) consisted of 3 mM [γ -³²P]ATP (containing about 2 to 8 × 10⁶ cpm), 4 mM MgCl₂, 20 mM KCl, 100 mM NaCl, 1 mM EDTA, 50 mM Tris/HCl (pH 7.4), and appropriate amounts of the enzyme (membrane) preparation. The reaction, started by the addition of either [γ -³²P]ATP, KCl, or NaCl and carried out for 15 min at 37°C, was terminated by the addition of 1 ml of ice-cold 5% (w/v) charcoal (Norite SG Extra; Baker) suspended in 0.1 M HCl, 1 mM NaH₂PO₄, and 1 mM Na₂P₂O₇, and the ³²P released was determined as described (22). Na-ATPase activity in the Na,K-ATPase preparations was assayed as described (23) except that the concentration of MgCl₂ was increased to 4 mM. Briefly, the standard reaction mixture (0.5 ml) consisted of 1 μM [γ -³²P]ATP (containing about 5 × 10⁶ cpm), 4 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, 50 mM Tris/HCl (pH 7.4), and appropriate amounts of the membrane preparation. The reaction was started with NPP, was carried out for 15 min at 37°C. PKC was assayed as described elsewhere (24). Briefly, the standard reaction mixtures (0.2 ml) contained 5 μmol of Tris/HCl (pH 7.5), 2 μmol of MgCl₂, 0.8 μg of protein of rat synaptosomal membrane (substituting 2 μg of PS), 40 μg of histone H1, either 0.8 μmol of ethyleneglycol bis(β-aminoethyl

Received 6/6/89; revised 11/21/89.

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¹ Supported, in part, by USPHS Research Grants CA-29850 (W. R. V.), CA-46750 (J. H.), and CA-36777, HL-15696, and NS-17608 (J. F. K.).

² On leave from Huasan Hospital, Shanghai Medical University, Shanghai, People's Republic of China.

³ Present address: Department of Molecular Pharmacology, Meiji College of Pharmacy, Setagaya-ku, Tokyo 154, Japan.

⁴ The abbreviations used are: ET-18-OCH₃, racemic 1-*O*-octadecyl-2-*O*-methyl glycerol-3-phosphocholine; PKC, protein kinase C; Na,K-ATPase, (sodium plus potassium)-activated adenosine triphosphatase; PS, phosphatidylserine; PC, phosphatidylcholine; lyso-PC, lysophosphatidylcholine; NPP, *p*-nitrophenyl phosphate; IC₅₀, concentration causing 50% inhibition; K_{0.5}, affinity constant.

ether)-*N,N,N',N'*-tetraacetic acid or 0.04 μmol of CaCl_2 , 1 nmol of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (containing about 1×10^6 cpm), and appropriate amounts of enzyme. The reaction, started with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, was carried out for 5 min at 30°C. The activities of all enzymes studied were linear as a function of incubation time or enzyme amount under the experimental conditions. All experiments concerning the effects of the agents on the enzymes as well as other experiments using membrane vesicles and HL60 cells (see below) were repeated 2 to 4 times to ascertain reproducibility of the findings reported herein.

Inside-out Membrane Vesicles of Erythrocytes. Vesicles were prepared from fresh human red blood cells according to the procedure of Blostein and Chu (25). The resulting vesicles were 80 to 85% inside-out as judged by the ratio of acetylcholinesterase activity in the presence or absence of Triton X-100 (26). The ATPase activity of the vesicles was assayed by the method of Carilli *et al.* (27). Briefly, vesicles were loaded by overnight equilibration at 0°C in loading medium (20 mM Tris/glycylglycine, pH 7.4, containing 1 mM MgCl_2 , 10 mM NaCl, and 5 mM KCl). The reaction mixture (0.1 ml) consisted of 10 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (containing about 5×10^6 cpm), and appropriate amounts of vesicles in the presence of 1 μM valinomycin, 20 mM Tris/glycylglycine (pH 7.4), and 0.5 μg of monensin (to dissipate ion gradients) and 100 μM ouabain (to inhibit Na,K-ATPase in unsealed vesicles).

^{86}Rb Uptake by HL60 Cells. Cultured human leukemic cells, from the mid-log growth phase, were used for ^{86}Rb uptake studies, essentially according to the procedures of Chopra and Gupta (28) described for HeLa cells and detailed in the legend to Fig. 8.

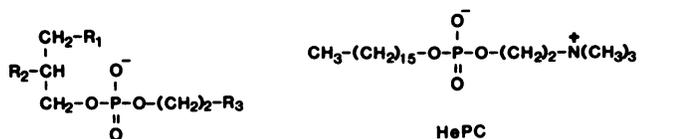
Treatment of Agents. Ouabain and all lipid analogues, dissolved in water, and strophanthidin, in dimethyl sulfoxide, were diluted to appropriate concentrations with 25 mM Tris/HCl (pH 7.4). Aliquots of these agents were added directly to incubation mixtures containing the enzyme or vesicle preparations or cells, as indicated in individual experiments.

Other Methods. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was prepared by the method of Post and Sen (29). Protein was determined according to the procedure of Lowry *et al.* (30), using bovine serum albumin as the standard.

RESULTS

The structures of representative synthetic phospholipid analogues used in the present studies are shown (Fig. 1). With the exception of HePC (hexadecyl phosphocholine), all other analogues (ET-18-OCH₃, BM 41.440, JH-1, and CV-3988) are derivatives of glycerol phospholipids.

Inhibition studies of PKC by the lipid analogues were carried out using a purified preparation of rat synaptosomal membranes (4 μg of protein/ml, or 5.5 μg of PS equivalents/ml) as the source of the phospholipid cofactor, instead of PS vesicles (4 to 10 $\mu\text{g}/\text{ml}$) under the standard assay conditions reported elsewhere (10–14, 24). The same concentration of membranes used in the above PKC studies was used as the source of purified Na,K-ATPase in most of the studies reported herein, in order



Compound	R ₁	R ₂	R ₃
ET-18-OCH ₃	-O-(CH ₂) ₁₇ -CH ₃	-O-CH ₃	$\text{N}^+(\text{CH}_3)_3$
BM 41.440	-S-(CH ₂) ₁₅ -CH ₃	-CH ₂ -O-CH ₃	$\text{N}^+(\text{CH}_3)_3$
JH-1	-O-(CH ₂) ₁₇ -CH ₃	-NH-CO-CH ₃	$\text{N}^+(\text{CH}_3)_3$
CV-3988	-O-CO-NH-(CH ₂) ₁₇ -CH ₃	-O-CH ₃	$\text{N}^+(\text{CH}_3)_3$

Fig. 1. Structures of some synthetic phospholipid analogues.

that the inhibition potencies of the analogues of both enzymes could be compared directly. The IC₅₀ values of the membrane-activated PKC for JH-1, CV-3988, ET-18-OCH₃, BM 41.440, and HePC were found to be 5, 5, 8, 9, and 9 μM , respectively (data not shown). The values for PS-activated PKC under the standard assay condition have been previously reported to be 10 μM for ET-18-OCH₃ (10) and 15 μM for BM 41.440 (12).

Because both PKC and Na,K-ATPase are membrane bound and require phospholipids for their activation, we examined if the lipid PKC inhibitors could also regulate Na,K-ATPase and sodium pump compared with ouabain, the classic inhibitor. Na,K-ATPase in purified rat brain membranes was inhibited by JH-1, CV-3988, and HePC, and this inhibition was attenuated by increasing amounts of the membrane preparation (Fig. 2, B to D). Thus the IC₅₀ values for JH-1, for example, were 9, 20, and 40 μM at membrane protein concentrations of 2, 4, and 8 $\mu\text{g}/\text{ml}$, respectively, consistent with the notion that the lipids are membrane interacting and subject to membrane surface dilution. The inhibitory potency of ouabain (non-lipid glycoside), in comparison, was unaffected by the membrane concentration, with an IC₅₀ of about 0.1 μM for all membrane concen-

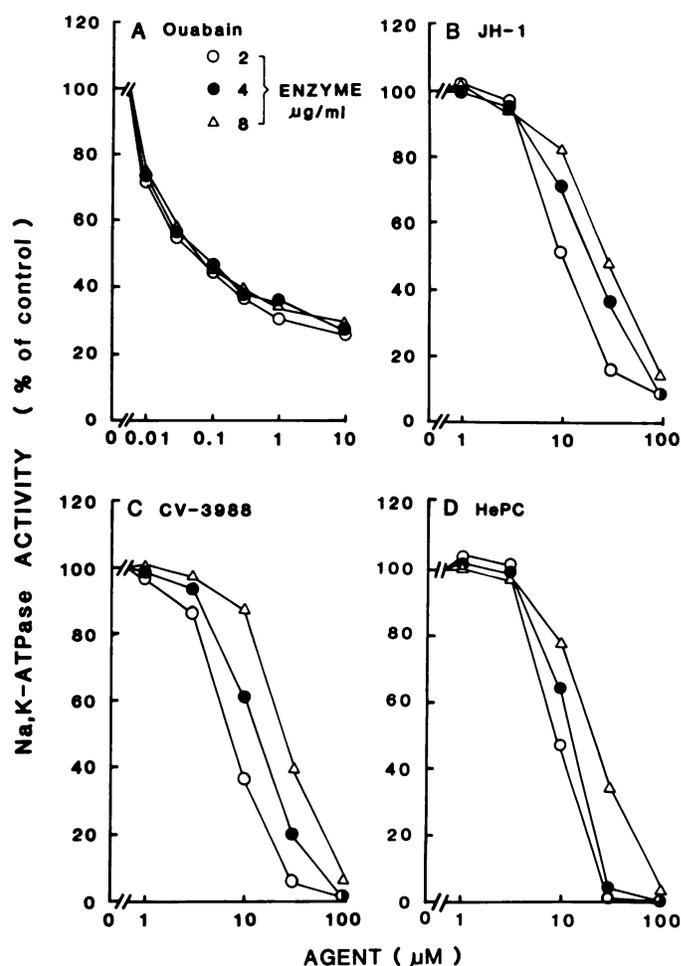


Fig. 2. Effects of ouabain, JH-1, CV-3988, and HePC on Na,K-ATPase in purified rat synaptosomal membranes as a function of the enzyme amount. The enzyme preparation (2, 4, or 8 μg of protein/ml) was preincubated for 30 min with various kinds and concentrations of the agents, as indicated. The reaction was started by the addition of KCl, and the incubation times were 30, 15, and 7.5 min, respectively, for the above-indicated enzyme amounts. The standard reaction mixture contained 100 mM NaCl, 20 mM KCl, 4 mM MgCl_2 , 1 mM EDTA, and 3 mM ATP. The activity values obtained in the absence of added agents (8.7 ± 0.4 μM of phosphate released/min/mg of protein) were taken as 100%. Points, mean of triplicate incubations with assay errors being less than 4%. See "Materials and Methods" for further details.

trations when the reaction was started with KCl (Fig. 2A). For the reason of simplicity and consistency, a fixed membrane concentration (4 μg of protein/ml) was used in subsequent experiments (Figs. 3 to 6).

In order to explore the mode of inhibition by the lipid metabolites on Na,K-ATPase, we examined their actions under various incubation conditions. Ouabain was more potent in inhibiting the enzyme when the reaction was started with KCl (IC_{50} of about 0.1 μM) than when NaCl or ATP (IC_{50} of about 1 μM for both) was used to initiate the reactions (data not shown). It has been reported previously that K^+ reduced the association and dissociation rate constants for the cardiac glycoside-ATPase interactions (31), suggesting that ouabain acted primarily on K^+ -binding sites located at the extracellular side of plasma membrane. JH-1, CV-3988, and HePC, in comparison, inhibited Na,K-ATPase with similar potencies whether the reaction was started with KCl, NaCl, or ATP (data not shown). We have observed similar phenomena for the other lipid analogues ET-18-OCH₃ and BM 41.440 (32). It appears that there is no strict structural requirement for Na,K-ATPase inhibition by lipid substances, consistent with the nonspecificity in the lipid requirement of Ca-ATPase or Na,K-ATPase activity (15).

The IC_{50} values for the agents of PKC and Na,K-ATPase from experiments shown above and of ^{86}Rb uptake by HL60 cells (see Fig. 8) are summarized (Table 1) for comparison.

The effects of the agents on the two partial reactions of Na,K-ATPase, *i.e.*, Na-ATPase and K-NPPase, were also examined (data not shown). The reactions were started with the addition of their respective substrates. Na-ATPase, stimulated by Na⁺ alone in the presence of low (micromolar) concentrations of ATP, is thought to represent the phosphorylation step (E1 stage) (33), whereas K-NPPase represents the dephosphorylation step (E2 stage) (34) of the Na,K-ATPase reaction. Ouabain inhibited Na-ATPase with a potency similar to its inhibition of Na,K-ATPase and an IC_{50} of about 2 μM . In comparison, ouabain was much less effective in inhibiting K-NPPase with an IC_{50} of about 100 μM ; this was due to a slow and incomplete binding of the glycoside to Na,K-ATPase in the absence of Na⁺ and in the presence of K^+ , conditions optimal for K-NPPase activity. All lipid analogues tested (JH-1, CV-3988, BM 41.440, ET-18-OCH₃, and HePC) had IC_{50} values of about 20 to 30 μM for the two partial reactions (data not shown), values comparable to those seen for Na,K-ATPase shown in Fig. 2 above.

Kinetic analysis of Na,K-ATPase inhibition by the analogues

Table 1 Summary of IC_{50} values for ouabain and lipid substances of PKC and Na,K-ATPase activities and ^{86}Rb uptake by HL60 cells

The values were taken from the present or previous studies. Synaptosomal membranes (4 μg of protein/ml or 5.5 μg of PS equivalents/ml) were used in the PKC assay as the source of phospholipid cofactor and in the Na,K-ATPase assay as the enzyme source, for the purpose of direct comparison.

Agent	IC_{50} (μM)		
	PKC	Na,K-ATPase	^{86}Rb uptake ^a
Ouabain	No effect	1	0.3
JH-1	5	15	25
CV-3988	5	15	25
HePC	8	15	40 ^b
ET-18-OCH ₃	9	16 ^c	9
BM 41.440	9	12 ^c	25
Lyso-PC ^d	4	15	90
Sphingosine ^d	10	16	4

^a Corrected for the ouabain-insensitive species, which accounted for 20% of total uptake activity.

^b Estimated from Fig. 8.

^c Taken from Ref. 32.

^d Taken from Ref. 39.

as a function of KCl concentration revealed that ouabain inhibited the enzyme competitively, whereas JH-1, CV-3988, and HePC noncompetitively, with respect to K^+ (Fig. 3). Skou (36) reported previously the same inhibitory kinetics for ouabain. It is worth noting that a mixed competitive and noncompetitive inhibition was also observed at high (micromolar) concentrations of ouabain (data not shown), suggesting that a heterotropic, allosteric interaction existed between the glycoside binding and K^+ activation (37). The degrees of cooperativity and $K_{0.5}$ for K^+ were analyzed using Hill plots (Fig. 4), based upon the data shown in Fig. 3 or similar experiments (data not shown). Similar degrees of cooperativity (Hill coefficients of about 1.2 to 1.9) for K^+ were observed in the absence or presence of any of the agents and, furthermore, only ouabain (but not JH-1, CV-3988, and HePC) increased the $K_{0.5}$ value (for example, from 1.1 to 4.3 μM at 0.4 μM ouabain). Kinetic analysis of inhibition by the agents as a function of NaCl concentration (Fig. 5) and Hill plots of the data (Fig. 6) are also presented. It was found that ouabain inhibited the enzyme noncompetitively, whereas the lipid analogues inhibited it competitively with respect to Na⁺. Hill plots indicated that, while none of the agents affected the degree of cooperativity (Hill coefficients of about 1.0 to 1.3), JH-1, CV-3988, and HePC (but not ouabain) increased the $K_{0.5}$ value for Na⁺ (for example, from 8.0 to 20.9 μM at 12 μM JH-1). These results suggested that the lipid analogues inhibited Na,K-ATPase by directly or indirectly interacting with the Na⁺-binding sites, whereas ouabain inhibition was likely in part due to its interactions with K^+ -binding sites.

Previous studies on the sodium pump indicated that there are multiple Na⁺-binding sites at both intra- and extracellular

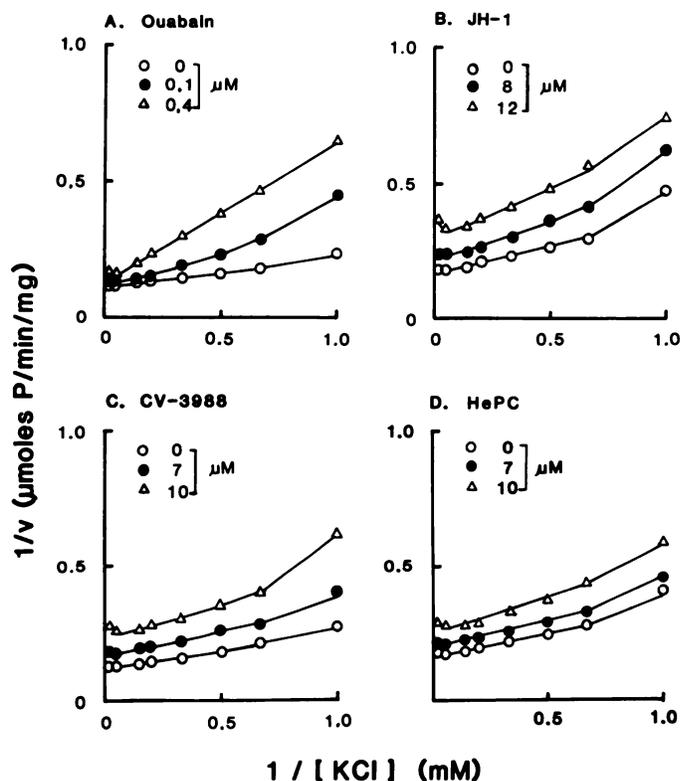


Fig. 3. Lineweaver-Burk plots showing inhibition by ouabain, JH-1, CV-3988, and HePC of K^+ -dependent activation of synaptosomal membrane Na,K-ATPase. The reaction, started with ATP, was carried out under the standard condition (containing 100 mM NaCl) except for the varying concentrations of KCl (1 to 50 mM) and the inhibitors, as indicated. The activity values presented were corrected for the basal activity (0.3 μM of phosphate/min/mg of protein) seen in the absence of added KCl. Points, mean of triplicate incubations with assay errors being less than 4%.

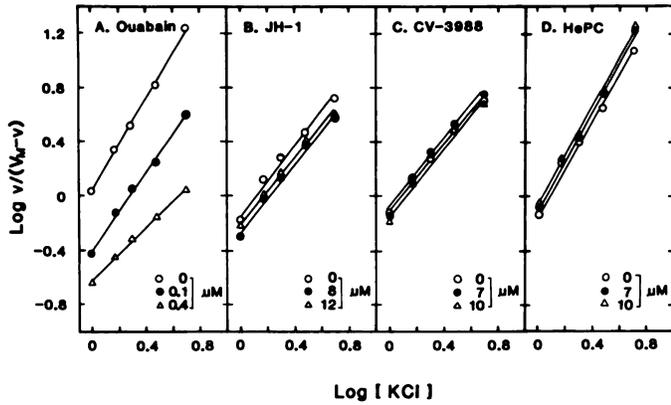


Fig. 4. Hill plots showing the effects of ouabain, JH-1, CV-3988, and HePC on K^+ -dependent activation of synaptosomal membrane Na,K-ATPase. The plots were based upon the data shown in Fig. 3 covering 1 to 50 mM KCl. v , observed reaction rate; V_m , maximal reaction rate (unit = μM of phosphate/min/mg of protein).

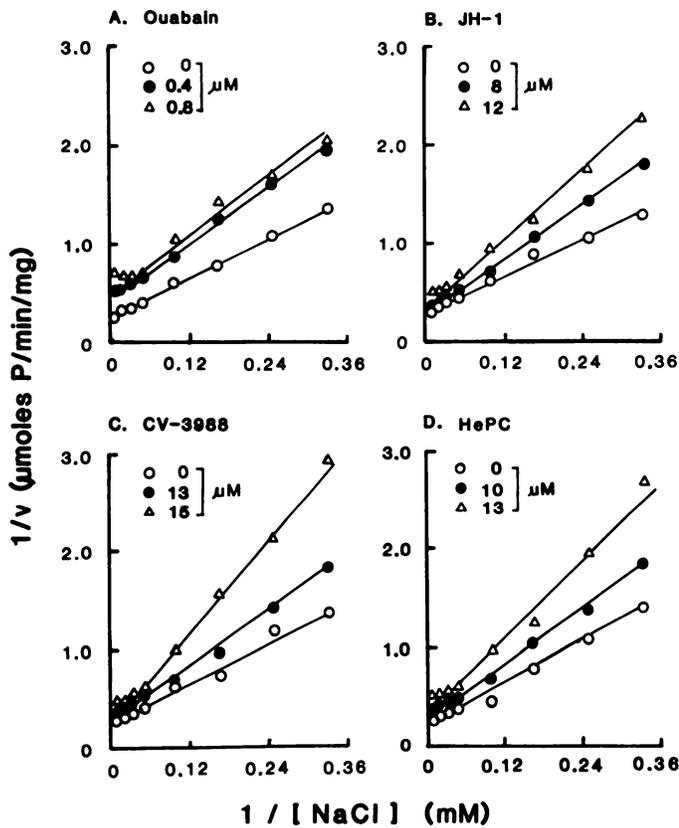


Fig. 5. Lineweaver-Burk plots showing inhibition by ouabain, JH-1, CV-3988, and HePC of Na^+ -dependent activation of synaptosomal membrane Na,K-ATPase. The reaction, started with ATP, was carried out under the standard condition (containing 20 mM KCl) except for varying concentrations of NaCl (3 to 100 mM) and the inhibitors, as indicated. The activity values presented were corrected for the basal activity ($3.5 \mu\text{M}$ of phosphate/min/mg of protein) seen in the absence of added NaCl. Points, mean of triplicate incubations with assay errors being less than 5%.

faces of the plasma membrane (38). Although the present kinetic studies, using the purified membrane fragments, suggested that the lipid analogues competed with Na^+ -binding sites on Na,K-ATPase, it is unclear where the lipids interacted. In order to further explore the problem, we used inside-out vesicles of erythrocyte membranes for the next series of experiments. In these experiments, Na,K-ATPase activity of the vesicles was defined as the ATPase activity that was resistant to ouabain (membrane-impermeable inhibitor) but was sensitive to stro-

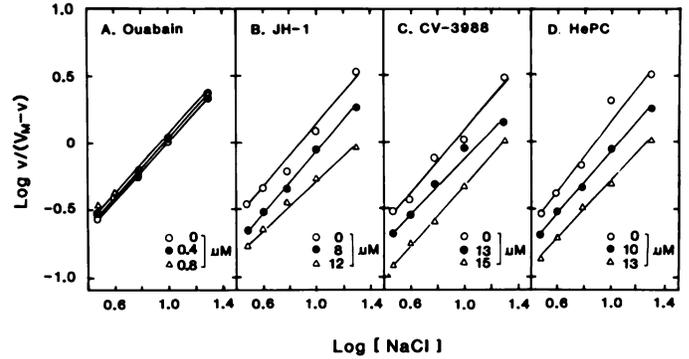


Fig. 6. Hill plots showing the effects of ouabain, JH-1, CV-3988, and HePC on Na^+ -dependent activation of Na,K-ATPase. The plots were based upon the data shown in Fig. 5 covering 3 to 100 mM NaCl.

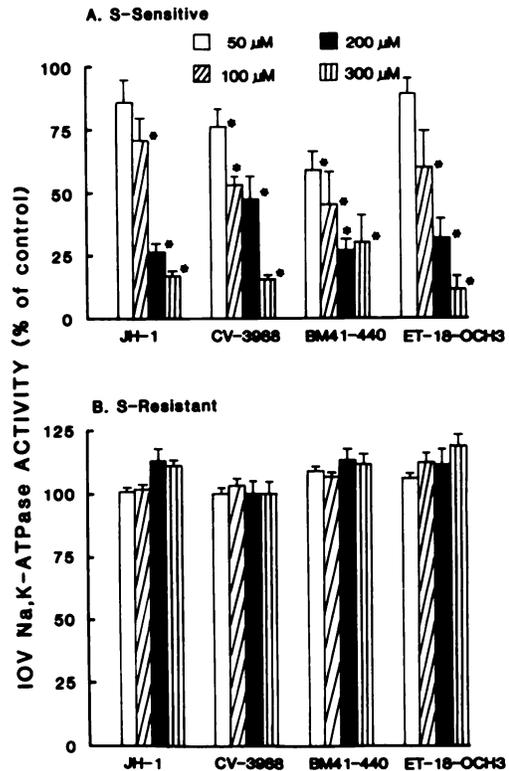


Fig. 7. Effects of JH-1, CV-3988, BM 41.440, and ET-18-OCH₃ on ATPase activities in inside-out vesicles (IOV) of human erythrocytes. The vesicles ($650 \mu\text{g}$ of protein/ml) were preincubated with or without 0.4 mM strophanthidin for 30 min, followed by further preincubation with or without the agents for an additional 15 min, as indicated. The reaction was started with ATP and carried out for 10 min at 37°C . The difference in ATPase activity seen in the absence (total activity) or presence (resistant activity) of strophanthidin was taken as the strophanthidin-sensitive Na,K-ATPase activity. Columns, mean of triplicate incubations, expressed as the percentage of the respective control values seen in the absence of the agents; bars, SE. The control values for strophanthidin-sensitive and -resistant ATPase activities were 0.73 and $1.60 \mu\text{M}$ of phosphate/min/mg of protein, respectively. See "Materials and Methods" for further details. S, strophanthidin.

phanthidin (membrane-permeable inhibitor) (27). JH-1, CV-3988, BM 41.440, and ET-18-OCH₃ showed a dose-dependent inhibition of strophanthidin-sensitive activity, with IC_{50} values estimated to be about 100 to 200 μM (Fig. 7). The requirement of high concentrations of the lipids to inhibit the vesicular Na,K-ATPase was probably due to surface dilution, because a high amount of membrane ($650 \mu\text{g}$ of protein/ml) was required for the assay. The strophanthidin-resistant activity, in comparison, was not inhibited by the lipid analogues (Fig. 7). Similar findings were also made for lyso-PC and sphingosine (39).

Finally, human leukemia HL60 cells were used to investigate

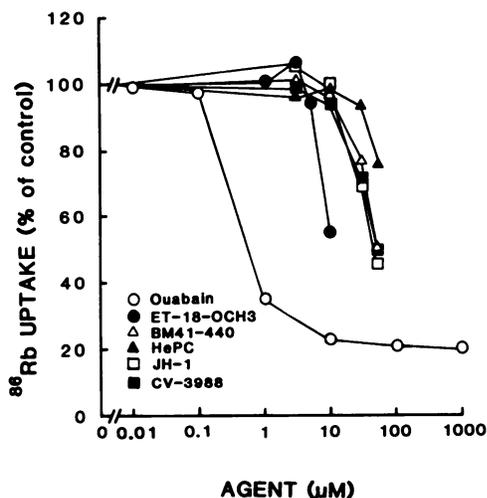


Fig. 8. Effects of ouabain and phospholipid analogues on ^{86}Rb uptake by HL60 cells. The cells ($7 \times 10^5/0.5$ ml/tube), in RPMI-1640 medium containing 10% fetal calf serum, were preincubated at 37°C for 3 h with ET-18-OCH₃ and 0.5 h with other analogues. The cells were not preincubated with ouabain. To each tube was then added ^{86}Rb (1 μCi), and the uptake was carried out for 0.5 h. The reaction was stopped by the addition of 1 ml of ice-cold washing buffer (25 mM Tris/HCl, pH 7.4, containing 145 mM choline chloride, 0.8 mM MgSO₄, and 1.8 mM CaCl₂), and the cells were washed 3 times with 1 ml of the same buffer. The cell pellets were finally dissolved in 0.25 ml of 0.5% deoxycholic acid in 0.1 N NaOH, and the ^{86}Rb taken up by the cells was determined using a gamma counter. Points, mean of triplicate determinations; the control activity value seen in the absence of added agents (151.3 ± 12.0 pmol of ^{86}Rb uptake/ 7×10^5 cells/h) was taken as 100%. The cell viability (as determined by trypan blue exclusion) in all treatments shown was $\geq 96\%$. Higher concentrations of the lipid agents caused extensive cell lysis and, therefore, could not be used for the studies.

the effects of the lipid analogues on the sodium pump in intact cells using ^{86}Rb uptake as an indicator of the pump activity. Ouabain at concentrations as high as 1000 μM inhibited the uptake by about 80%, indicating that only about 20% of the pump activity in HL60 cells was ouabain resistant. Preincubation of the cells for 0.5 or 3 h with varying concentrations of the lipid analogues resulted in a dose-dependent inhibition of the uptake (Fig. 8). Their IC₅₀ values, estimated to be 9 to 40 μM , are also summarized (Table 1). The IC₅₀ for ET-18-OCH₃ (3-h incubation) was 9 μM , whereas the values for others (0.5-h incubation) were 25 to 40 μM , which could be lower if the cells were pretreated with the agents for a longer time (e.g., 3 h). The notion was supported in part by our findings that the IC₅₀ for ET-18-OCH₃ was about 30 μM if the preincubation time was only 20 min.⁵

DISCUSSION

Negatively charged boundary phospholipids (such as PS), located at the inner membrane, have been suggested to act as endogenous activators of Na,K-ATPase or the sodium pump (15). Modification of the membrane lipid composition would therefore probably lead to changes in membrane properties and function. We observed in the present studies that several phospholipid analogues inhibited Na,K-ATPase of brain synaptosomal membranes, and inside-out vesicles from erythrocyte membranes, and ^{86}Rb uptake (sodium pump) into intact HL60 cells. We also observed that these lipid analogues, in contrast to ouabain, inhibited purified Na,K-ATPase competitively with respect to Na⁺ and noncompetitively with respect to K⁺, and that the inhibition was attenuated by increasing amounts of membranes. These findings suggest that the lipid analogues most likely interact with boundary phospholipid in the inner

membrane and, as a consequence, result in increased local fluidity and/or decreased interaction of acidic boundary phospholipids with Na,K-ATPase. Because ET-18-OCH₃ inhibited the phorbol ester-induced protein phosphorylation and differentiation of HL60 cells (40), coupled with the well-recognized fact that PS is the most effective phospholipid cofactor for PKC, it is likely that the *in vivo* actions of ET-18-OCH₃ and other lipid analogues might be mediated via similar membrane effects leading to PKC inhibition. Although the lipid analogues inhibited Na,K-ATPase in synaptosomal membranes and ^{86}Rb uptake into HL60 cells with comparable potencies, higher concentrations of the analogues were required to inhibit Na,K-ATPase in the inside-out vesicles of erythrocyte membranes. This discrepancy might have been due to the unnatural (*i.e.*, inside-out) state of the membranes of the blood cells, resulting in an exceedingly high membrane surface dilution for the lipid analogues.

Based upon studies on various compounds, Espan (41) has concluded that agents (positively charged, uncharged, or zwitterionic) that stabilize the bilayer phase of membranes are inhibitors, whereas agents (negatively charged, uncharged, or zwitterionic) that destabilize the bilayer phase by promoting its conversion to the hexagonal phase are activators of PKC. This generalization, although useful as a model, appears too simplistic. The criteria for modifications of physical and functional properties of the membrane as they are related to activation or inhibition of PKC (or Na,K-ATPase) appear more complex. This notion is supported, for example, by our recent studies that a distearoylphosphatidylcholine derivative that is methylated at position 8 of the fatty acyl group at position *sn*-2 of glycerol is a potent inhibitor, but the corresponding butylated analogue is a potent activator of PKC (42). The lipid analogues used in the present studies (see Fig. 1 for structures) and the distearoylphosphatidylcholine derivatives are all zwitterionic. It is of interest to know how the positive or negative charge, or both, of the agents could influence the membrane bilayer stability, leading to altered hydrophobic interactions between the enzyme protein and the membrane lipid, the events which are presumably essential for inhibition/activation of PKC or Na,K-ATPase.

We also observed in the present studies that the lipid analogues similarly inhibited purified PKC and Na,K-ATPase and ^{86}Rb uptake (sodium pump) in intact HL60 cells (for summary, see Table 1). It is conceivable, therefore, that the reported antineoplastic (1–8) and antidifferentiation effects (40) of these agents might be due to, in part, inhibition of both PKC and Na,K-ATPase, and that such a combination of inhibitions could produce a synergistic biological effect. However, PKC probably was not involved in the sodium pump activity, because phorbol esters, known to activate and down-regulate PKC in HL60 cells (12), had no effect on ^{86}Rb uptake by the cells (39).

It should be noted that HePC has a structure different from the glycerol phospholipid class of the agents such as ET-18-OCH₃ (see Fig. 1 for structures), representing a new class of antineoplastic agents (43–46). HePC was found to be as effective as glycerol lipids in inhibiting PKC, Na,K-ATPase, and ^{86}Rb uptake in the present studies. It is likely that HePC, lacking the glycerol backbone, has the minimal structural requirements essential for interacting with biomembranes, resulting in inhibition of certain membrane enzymes and process including selective tumor toxicity.

Lastly, we should probably attempt to understand the efficacy of the lipid agents in terms of their mol % with respect to membrane phospholipids. If one assumes that the average mo-

⁵ B. Zheng and J. F. Kuo, unpublished observations.

molecular weight of phospholipids is 800 and that biomembranes contain an equal amount of protein and phospholipids, then the amount of synaptosomal membrane protein (0.8 μg in a 0.2-ml incubation volume) used in the present studies would correspond to 0.8 μg , or 1.0 nmol, of total phospholipid. From the data given in Table 1, for example, the IC_{50} for ET-18-OCH₃ for PKC inhibition was 9 μM (in a 0.2-ml incubation volume), corresponding to 1.8 nmol of the agent. If one also assumes that 50% of the agent was actually incorporated into the membrane, then the molar ratio of the agent to total phospholipid in the membrane would be 0.9 to 1.0 = 0.9, or 90 mol %. This value appears to be high (or the ether lipid to be rather inactive), but a similar value (100 mol %) was also obtained in separate experiments for sphingosine (39), the putative endogenous PKC inhibitor (47). We have also noted that diacylglycerol (such as diolein), the acknowledged second messenger (48), half-maximally activated PKC at 2 μM under the same assay conditions (39), corresponding to 20 mol % for the activator. We reported previously that a half-maximal activation of PKC required 1 mol % of diolein in the mixed micelle assay system of Hannum *et al.* (47), which consisted of 0.3% Triton X-100 and 6 mol % of PS, corresponding to 17 mol % of diolein with respect to PS (49). Based upon the IC_{50} (16 μM) of Na,K-ATPase for ET-18-OCH₃ shown in Table 1 and the assumptions used in synaptosomal membranes, it was calculated that 160 mol % of the agent were required to inhibit the enzyme activity by 50%. Similarly, from the IC_{50} value (about 120 μM , in 0.1-ml incubation volume) for ET-18-OCH₃ of strophantidin-sensitive Na,K-ATPase in erythrocyte inside-out vesicles (65 μg of protein/0.1 ml) (Fig. 7), the value for the agent was calculated to be 7 mol %.

It was of interest to carry out similar calculations with the inhibition of ⁸⁶Rb uptake (sodium pump) in intact HL60 cells. We assumed that the number of cells (7×10^5) used in Fig. 8 would correspond to 5 mg of total cellular proteins or 1 mg (1250 nmol) of the membrane phospholipid. The IC_{50} of ET-18-OCH₃ was 9 μM (in 0.5-ml incubation volume) (Table 1). If only 10% of the agent (0.45 nmol) were actually taken up into cell membrane (90% remained bound to serum in the medium), then the ratio of the agent to total membrane phospholipid would be 0.45 to 1250 = 0.00036, or 0.036 mol %, a value clearly below the one that could cause cell lysis by lysophospholipids. Because the IC_{50} values for ET-18-OCH₃ of phorbol ester-stimulated endogenous protein phosphorylation and differentiation of HL60 cells (40) were similar to those seen for ⁸⁶Rb uptake (Fig. 8), it was concluded that the effects of the agent and its structural analogues on the intact cells were specific and were not due to a general phenomenon of membrane perturbation brought about by the lipids. The reasons for the requirement of about three orders of magnitude higher mol % of ET-18-OCH₃ to inhibit PKC or Na,K-ATPase using synaptosomal membrane fragments than to inhibit cellular activities in intact HL60 cells are unclear. It is plausible that the effects of the lipid agents on intact cells (such as cytotoxicity, inhibition of the sodium pump, and leukemia cell differentiation) are the combined results of their actions on the individual targets (such as PKC, Na,K-ATPase, and phospholipid-metabolizing enzymes), perhaps acting synergistically. The isolated membrane preparations (or PS vesicles), however, are still useful as a model in studies aiming to illustrate the modes or mechanisms of action of the lipid agents.

ACKNOWLEDGMENTS

We thank J. Marliese Casteel for her skillful preparation of the manuscript.

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