Inhibition of Na,K-ATPase and Sodium Pump by Protein Kinase C Regulators Sphingosine, Lysophosphatidylcholine, and Oleic Acid*

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The effects and modes of action of certain lipid second messengers and protein kinase C regulators, such as sphingosine, lysophosphatidylcholine (lyso-PC), and oleic acid, on Na,K-ATPase and sodium pump were examined. Inhibition of purified rat brain synaptosome Na,K-ATPase by these lipid metabolites, unlike that by ouabain, was subject to membrane dilution (i.e. inhibition being counteracted by increasing amounts of membrane lipids). Kinetic analysis, using the purified enzyme, indicated that sphingosine and lyso-PC were likely to interact, directly or indirectly, with Na+-binding sites of Na,K-ATPase located at the intracellular face of plasma membranes, a conclusion also supported by studies on Na,K-ATPase and 22Na uptake using the inside-out vesicles of human erythrocyte membranes. The studies also showed that ouabain (but not sphingosine and lyso-PC) increased the affinity constant (K_o.5) for K+, whereas sphingosine and lyso-PC (but not ouabain) increased K_o.5 for Na+. Sphingosine and lyso-PC inhibited 86Rb uptake by intact human leukemia HL-60 cells at potencies comparable to those for inhibitions of purified Na,K-ATPase and protein kinase C. It is suggested that Na,K-ATPase (sodium pump) might represent an additional target system, besides protein kinase C, for sphingosine and possibly other lipid second messengers.

Na,K-ATPase, an integral component of the sodium pump, is critically involved in the maintenance of cellular integrity (1) and regulation of cellular activities and functions, such as contractility (2), growth (3, 4), and differentiation (5). Numerous reports have documented an importance of lipids in Na,K-ATPase activity, as evidenced by its inactivation by treatments with detergents or phospholipases (6) and restoration of its activity upon repurification (7). Ouabain, a therapeutic useful cardiac glycoside, is a well-known inhibitor of Na,K-ATPase (1). Certain endogenous "ouabain-like" factors that inhibit Na,K-ATPase and the sodium pump have been shown to elevate in the plasma and urine of patients with essential hypertension (8) and chronic renal insufficiency (9). Acute and chronic sodium loading and expanded extracellular volume in the human and experimental animals have also been shown to increase levels of the inhibitory factors.

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EXPERIMENTAL PROCEDURES

Materials—D-Sphingosine, lyso-PC (oleoyl), NPP (Tris salt), ATP (vanadium-free), ouabain, strophantidin, nonenins, PS (bovine brain), histone H1, and BSA (fatty acid-free) were purchased from Sigma; oleic acid was from Aldrich; 32Na was from Du Pont New England Nuclear; 86Rb was from Amersham Radiochemicals.

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. Inhibition of purified Na,K-ATPase activity by these lipid second messengers. Inhibition of purified Na,K-ATPase activity by these lipid second messengers. Inhibition of purified Na,K-ATPase activity by these lipid second messengers. Inhibition of purified Na,K-ATPase activity by these lipid second messengers. Inhibition of purified Na,K-ATPase activity by these lipid second messengers.
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1 mM EDTA, 50 mM Tris/HCl (pH 7.4), and appropriate amounts of the membrane preparation. The reaction was started with [γ-32P]ATP and carried out for 15 s at 37 °C; the 32P released was determined as described above for Na,K-ATPase. The activity of K-NPPase was assayed by measuring the absorbance at 410 nm of p-nitrophenol released as described (23). Briefly, the standard reaction mixture (0.5 ml) consisted of 3 mM NPP, 4 mM MgCl2, 20 mM KC1, 1 mM EDTA, 50 mM Tris/HCl (pH 7.4), and appropriate amounts of the membrane preparation. The reaction started with NPP, was carried out for 15 min at 37 °C. PKC was assayed as described elsewhere (19). Briefly, the standard reaction mixtures (0.2 ml) contained 0 mM of Tris/HCl (pH 7.5), 2 mM of MgCl2, 2 mM of PS, 40 μg of histone H1, either 0.5 mM of EGTA or 0.04 mM of CaCl2, 1 mM of [γ-32P]ATP (containing about 1 × 106 cpm), and appropriate amounts of the enzyme. For the reaction, started with [γ-32P]ATP, was carried out for 5 min at 30 °C. The activities of all enzymes studied were linear as a function of incubation time and enzyme amount under the experimental conditions. All experiments for the effects of the agents on the enzymes as well as other experiments using membrane vesicles and HL-60 cells (see below) were repeated 2-4 times to ascertain reproducibility of the findings reported herein.

Inside-out Membrane Vesicles and 32Na Uptake—The vesicles were prepared from fresh human red blood cells according to the procedure of Blose and Chu (26). The resulting vesicles were 86-85% inside-out as judged by the ratio of acetylcholinesterase activity in the presence or absence of Triton X-100 (27). The ATPase activity of the vesicles was assayed by the method of Carilli et al. (26). Briefly, the vesicles were loaded by overnight equilibration at 0 °C in loading medium (20 mM Tris/glycyglycine (pH 7.4), containing 1 mM MgCl2, 10 mM NaCl, and 5 mM KCl). The reaction mixture (0.1 ml) consisted of 10 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM [γ-32P]ATP (containing about 5 × 106 cpm), and appropriate amounts of the vesicle in the presence of 1 mM valinomycin, 20 mM Tris/glycyglycine (pH 7.4), and 0.5 μg of monensin (to dissipate ion gradients) and 100 μM ouabain (to inhibit Na,K-ATPase in the unsealed vesicles). Measurement of 32Na uptake by the inside-out vesicles was carried out by the method of Blose and Chu (26). Briefly, the vesicles were incubated in loading medium overnight at 0 °C. Aliquots (10 μl containing 75 μg of protein) were then incubated in 0.1 ml of loading medium with 1 mM ATP and 32NaCl (1 μCi) for 10 min at 37 °C and the reaction was terminated by the addition of 2 ml of the ice-cold loading medium. 32Na uptake by the vesicles was determined by filtration over Millipore filter membranes (HAWP 02500, 0.45-μm filter size).

32Rb Uptake by HL-60 Cells—The cultured human leukemia cells, from the mid-log growth phase, were used for 32Rb uptake studies, essentially according to the procedures that Chopra and Gupta (29) described for HeLa cells and detailed in the legends to Figs. 8 and 9 of this paper.

Treatments of Agents—All agents except ouabain and strophedrin were dissolved in a mixture (1:1, v/v) of methanol: chloroform. Appropriate aliquots of the solutions were dried under the stream of N2 gas followed by sonication in appropriate volumes of 25 mM Tris/HCl (pH 7.4) for 1 min at room temperature. Ouabain, dissolved in water and strophedrin, in dimethyl sulfoxide, were diluted to appropriate concentrations with 25 mM Tris/HCl (pH 7.4). Aliquots of the agents were added directly to the incubation mixtures containing the enzyme, vesicle preparations, or cells. In 32Rb uptake experiments, sphingosine was added to HL-60 cells as the sphingosine-BSA complex (1:1 molar ratio) prepared by the method of Merrill et al. (30) to the purpose of reducing cytotoxicity of sphingosine (31). Other Methods—[γ-32P]ATP was prepared by the method of Post and Sen (32). Protein was determined according to the procedure of Lowry et al. (33), using BSA as standard.

RESULTS

In the present studies, we examined whether and how certain lipid PKC modulators could regulate Na,K-ATPase and sodium pump compared with ouabain, the classic inhibitor. Na,K-ATPase in the purified rat brain synaptosomes was found to be inhibited by sphingosine, lyso-PC, and oleic acid and the inhibition was attenuated by increasing amounts of the membrane preparation (Fig. 1, B-D). Thus the IC50 values for sphingosine, for example, were 10, 37, and 110 μM at membrane protein concentrations of 2, 8, and 32 μg/ml, respectively, consistent with the notion that the lipids are membrane interacting and subject to membrane surface dilution. The inhibition 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 concentrations (Fig. 1A). For the reason of simplicity and consistency, a fixed membrane concentration (4 μg of protein/ml) was used in subsequent experiments (Figs. 2-7).

It seemed desirable that some phospholipids or membranes were present in the assay systems for both PKC and Na,K-ATPase, so that the inhibitory potencies for the lipid agents can be directly compared. When the brain membrane (4 μg of protein/ml) was used as the source of phospholipid cofactor instead of PS, as in the standard conditions, for PKC activation, sphingosine and lyso-PC inhibited PKC with IC50 values of 10 and 4 μM, respectively (Fig. 2). The corresponding IC50 values for the two agents of Na,K-ATPase activity in the same concentration of the brain membrane were 16 and 15 μM, respectively (Fig. 2).

In order to explore the mode of inhibition by the lipid metabolites on Na,K-ATPase, we examined their actions under various reaction conditions. Ouabain was more potent in inhibiting the enzyme when the reaction was started with KCl than that with NaCl or ATP (data not shown), consistent with the report (34) that ouabain acted primarily on K+ binding sites located at the extracellular side of plasma mem-
bran. Sphingosine, lyso-PC, and oleic acid, in comparison, inhibited Na,K-ATPase with similar potencies whether the reaction was started with KCl, NaCl, or ATP (data not shown).

The effects of the agents on the two partial reactions of Na,K-ATPase, i.e., Na-ATPase and K-NPPase, were also examined (Fig. 3). 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) (35), whereas K-NPPase, the dephosphorylation step (E2 stage) (36) of Na,K-ATPase reaction. Ouabain inhibited Na-ATPase at a potency similar to its inhibition of Na,K-ATPase shown earlier in Fig. 1A (Fig. 3A), both reactions started with ATP. In comparison, ouabain was much less effective in inhibiting K-NPPase (Fig. 3B), as reported previously by Akera et al. (37). Sphingosine slightly activated Na-ATPase at concentrations around 10 μM followed by inhibition of the activity at higher concentrations (Fig. 3A). Sphingosine, in comparison, inhibited K-NPPase in a simple dose-dependent manner (Fig. 3B). Lyso-PC and oleic acid similarly inhibited both Na-ATPase and K-NPPase (Fig. 3) as they did Na,K-ATPase.

Kinetic analysis of Na,K-ATPase inhibition by the agents as a function of KCl concentration revealed that ouabain inhibited the enzyme competitively, whereas sphingosine and lyso-PC noncompetitively, with respect to K⁺ (Fig. 4). Skou et al. (38) 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 as previously reported by others (37, 39). The degree of cooperativity and Kₐ₅ for K⁺ were analyzed using Hill plots (Fig. 5), based upon the data shown in Fig. 4 or similar experiments (data not shown). The same degree of cooperativity for K⁺ was observed in the absence or presence of the agents and, furthermore, only ouabain (but not sphingosine and lyso-PC) increased the K₀₅ value. Kinetic analysis of inhibition by the agents as a function of NaCl concentration (Fig. 6) and Hill plots of the data (Fig. 7) were also presented. It was found that ouabain inhibi-
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FIG. 4. Lineweaver-Burk plots showing inhibition by ouabain, sphingosine, and lyso-PC of K+-dependent activation of rat brain 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 (0.6-50 mM) and ouabain (A), sphingosine (B), and lyso-PC (C), as indicated. The activity values presented were corrected for the basal activity (0.2 μmol of P/min/mg of protein) seen in the absence of added KCl. The data shown are the means of triplicate incubations with assay errors being less than 4%.

FIG. 5. Hill plots showing the effects of ouabain, sphingosine, and lyso-PC on K+-dependent activation of Na,K-ATPase. The plots were based upon the data shown in Fig. 4 covering 0.6-5 mM KCl. v, observed reaction rate; Vₘ, maximal reaction rate (unit = micromole of P/min/mg of protein).

FIG. 6. Lineweaver-Burk plots showing inhibition by ouabain, sphingosine, and lyso-PC of Na+-dependent activation of rat brain membrane Na,K-ATPase. The reaction, started with ATP, was carried out under the standard condition (containing 20 mM NaCl) except for varying concentrations of NaCl (3-200 mM) and ouabain (A), sphingosine (B), and lyso-PC (C), as indicated. The activity values presented were corrected for the basal activity (3.3 μmol of P/min/mg of protein) seen in the absence of added NaCl. The data presented were the means of triplicate incubations with assay errors being less than 5%.

FIG. 7. Hill plots showing the effects of ouabain, sphingosine, and lyso-PC on Na+-dependent activation of Na,K-ATPase. The plots were based upon the data shown in Fig. 6 covering 3-20 mM NaCl.

...ited the enzyme noncompetitively, whereas sphingosine inhibited it competitively with respect to Na+. The inhibition by lyso-PC, in comparison, appeared more complex, showing competitive kinetics at 15 μM but a mixed-type kinetics at 19 μM, suggesting a positive heterotropic allosteric interaction (39) between lyso-PC binding and Na+ activation. Hill plots indicated that, while none of the agents affecting the degree of cooperativity, sphingosine and lyso-PC (but not ouabain) increased the K₀.₅ value for Na+. The results suggested that sphingosine and lyso-PC 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 sodium pump indicated that there are multiple Na+-binding sites at both intra- and extracellular faces of the plasma membrane (40). Although the present kinetic studies, using the purified membrane fragments, suggested that sphingosine and lyso-PC competed with Na+-binding sites on Na,K-ATPase, it is unclear where the lipids interact. In order to explore further the problem, we employed inside-out vesicles of erythrocyte membranes for the next series of experiments. Na,K-ATPase activity of the vesicles is defined as the ATPase activity that is resistant to ouabain (membrane-impermeable inhibitor) but is sensitive to strophanthidin (membrane-permeable inhibitor) (28). It was found that sphingosine (1100 μM) and lyso-PC (360 μM) inhibited strophanthidin-sensitive activity 34 and 47%, respectively, and the inhibition was already maximal at 1-min preincubation of the vesicles with the agents, indicating that the action of the lipids with the outer membranes of the inside-out vesicles was rapid (data not shown). The requirement of high concentrations of the lipids to inhibit the vesicular Na,K-ATPase was likely due to a surface dilution because a high amount of membrane (750 μg of protein/ml) was required for the assay. The strophanthidin-resistant activity, in comparison, was not inhibited by the metabolites. It was further noted that the strophanthidin-sensitive ²²Na uptake into the inside-out vesicles was also inhibited by sphingosine and lyso-PC at concentrations comparable to their inhibition of Na,K-ATPase of the vesicles, and that the strophanthidin-resistant uptake was not inhibited (data not shown). Ouabain was without effect on both species of ²²Na uptake. The notion that the inhibitions of Na,K-ATPase activity and ²²Na uptake in the vesicles were not due to possible general membrane perturbation caused by the lipids was supported by the findings that acetylcholine esterase activity, located at the intravesicular face of the...
inside-out vesicles (i.e. originally at the extracellular side of intact erythrocytes), was not detected in the vesicles treated with the lipid metabolites (data not shown).

Finally, human leukemia HL-60 cells were employed to investigate the effects of the lipid metabolites on sodium pump in intact cells as indicated by \(^{86}\)Rb uptake. Ouabain at 1 or 0.01 mM (data not shown) inhibited the uptake about 90%, indicating that only about 10% of the pump activity in HL-60 cells was ouabain-resistant. Preincubation of the cells for one-half h with varying concentrations of lyso-PC in serum-containing medium resulted in a dose-dependent inhibition of the uptake; for example, a 71% inhibition was noted at 200 \(\mu\)M lyso-PC without causing cell membrane damage (Fig. 8A). Higher concentrations of lyso-PC caused extensive cell membrane damage (data not shown). A longer preincubation (23 h) with lyso-PC caused little or no inhibition, presumably due to its active metabolism by the cells. Preincubation of HL-60 cells with oleic acid for one-half h (Fig. 8A), but not 23 h (data not shown), resulted in a slight stimulation of \(^{86}\)Rb uptake. Lyso-PC was much more effective in inhibiting the sodium pump activity when the cells were incubated in the serum-free medium (Fig. 8B) compared to the inhibition seen in the presence of serum shown above (Fig. 8A). Thus a 60% inhibition was noted at 30 \(\mu\)M lyso-PC and this occurred without preincubating the cells with the lipid. Oleic acid, however, was without effect (Fig. 8B).

When HL-60 cells were incubated in the serum-free medium, sphingosine (as a BSA complex) inhibited \(^{86}\)Rb uptake 38 and 60% at 15 and 20 \(\mu\)M, respectively, without pretreating the cells with sphingosine (Fig. 8B). Sphingosine, when added as a free form, was extremely lytic especially in the serum-free medium, making it impossible to study the uptake by the cells under these conditions.

**DISCUSSION**

It has been suggested that negatively charged boundary phospholipids (such as phosphatidylserine and phosphatic acid), located at the inner membrane, act as endogenous activators of Na,K-ATPase or sodium pump (41). Modifications of the membrane lipid compositions or structures would therefore likely lead to changes in membrane fluidity and function. In this respect, it is of interest that cholesterol depletion or exposure of neutral or cationic amphiphiles (such as chlorpromazine, imipramine, and hexyl alcohol) caused a decreased affinity of the internal sites of the membrane for Na\(^{+}\), without affecting the affinity of the external membrane sites for K\(^{+}\) in human erythrocytes (42). It was well recognized that cholesterol interacted with erythrocyte phospholipids, causing a decreased fluidity of the lipid matrix (43-45). We observed in the present studies that sphingosine and lyso-PC inhibited Na,K-ATPase of brain synaptosome fragments and inside-out vesicles of erythrocyte membranes, \(^{22}\)Na uptake into inside-out vesicles, and \(^{86}\)Rb uptake into intact HL-60 cells. We also observed that sphingosine and lyso PC 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 membrane (Fig. 1, B-D) or liposomes (PC:cholesterol = 10:7.5) (data not shown). These findings, when taken together, suggested that the lipid metabolites were likely to interact with boundary phospholipids in the inner membrane and, as a consequence, caused an increased local fluidity and/or decreased interactions of acidic boundary phospholipids with Na,K-ATPase. The mode of action of oleic acid appeared complex and site of its interactions could not be accurately determined. Although oleic acid inhibited Na,K-ATPase in synaptosome fragments as did others, it had variable effects on strophanthin-sensitive and -resistant ATPase in inside-out vesicles of erythrocyte membrane (data not shown) and slightly stimulated \(^{86}\)Rb uptake into HL-60 cells (Fig. 8). It has been
reported that inhibition of Na,K-ATPase by unsaturated fatty acids was reversed by increasing concentrations of K⁺ (46), that fatty acids stimulated Ca²⁺-ATPase even in the calmodulin-deficient membrane (47), and that long-chain acyl-coenzyme A derivatives activated Na,K-ATPase only at suboptimal (micromolar), but not at the optimal concentrations (millimolar) of ATP with complex mechanisms, including increased rate of K⁺ deocclusion at the intracellular side of the membrane and blockade of the extracellular "allosteric" Na⁺ sites (48).

Several lipid metabolites have been shown or suggested to function as intracellular mediators, including unsaturated fatty acids (18), lyso-PC (19), diacylglycerol (15), and sphingosine and related substances (16). Biological significance of these lipid metabolites in transmembrane signal transduction is based largely upon their abilities to positively or negatively regulate PKC. In the present studies, we presented evidence to suggest that Na,K-ATPase or sodium pump might be an additional site or target with which certain lipid second messengers could potentially interact to alter cellular activities. We reported that ET-1%OCH₃ and BM 41.440, alkyl ether inhibitors (49, 50), were also equally potent inhibitors of Na,K-ATPase. It appears that the target systems for certain lipid second messengers might involve inhibition of both PKC and Na,K-ATPase. It appears that the target systems for certain lipid second messengers and pharmacological agents might expand to include, in addition to PKC, other membrane-associated enzymes such as Na,K-ATPase. The biological actions of sphingosine might also include a direct inhibition of calmodulin-dependent enzymes such as myosin light chain kinase (55), which, in comparison, was not inhibited by BM 41.440 and related ether lipids (50). It should be noted that PKC probably was not involved in sodium pump activity, because tumor-promoting phorbol ester, known to activate and down-regulate PKC in HL-60 cells (50), had no effect on Rb uptake by the cells.²

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