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Binding of ethylenediamine to phosphatidylserine is inhibitory to Na^+/K^+-ATPase

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Covalent linkage of ethylenediamine with the Na^+/K^+-ATPase complex from rabbit kidney outer medulla by the use of the water-soluble carbodiimide, *N*-ethyl,*N'*-(3-dimethylaminopropyl)carbodiimide, resulted in a 73% reaction with phosphatidylserine and only 27% with carboxylic groups in the proteic component of the enzyme. Condensation products from the reaction between phosphatidylserine and ethylenediamine, *N*-(*O*-phosphatidylseryl)ethylenediamine, *N*,*N'*-bis(*O*-phosphatidylseryl)ethylenediamine and its intermediary product *O*-phosphatidyl-[*N*,*N'*-bis(seryl)]ethylenediamine, were synthesised. Symmetrically substituted ethylenediamine was the most likely condensation product of ethylenediamine with endogenous phosphatidylserine. The synthesised lipids were incorporated in proteoliposomes containing Na^+/K^+-ATPase and only the addition of the phospholipid phosphatidylcholine. The ratio of phospholipid to protein was 52 (w/w). These proteoliposomes were perforated by the addition of 0.5% cholate and both the Na^+-dependent phosphorylation level and its dependence on Na^+, Mg^{2+} and ATP were measured. Phosphatidylcholine alone increased the half-maximal activation concentration for Na^+ ($[Na^+]_{0.5}$) from 0.2 to 1–2 mM, for Mg^{2+} from 0.1 to 0.8 μM and for ATP from 0.02 to 0.3 μM. The K_i for K^+ (in the absence of Na^+) was unaffected: 12.8 μM vs. 12.5 μM in the non-reconstituted system. Replacing 10 mol% of phosphatidylcholine by phosphatidylethanolamine or phosphatidylserine had no significant effect on $[Na^+]_{0.5}$: 1.1 and 0.7 mM, respectively. Replacing 5 mol% phosphatidylcholine by the bis(phosphatidylseryl) substituent of ethylenediamine further increased $[Na^+]_{0.5}$ to 13.7 mM, while half-maximal activation concentrations for Mg^{2+} and ATP were unaltered. The mono-phosphatidylseryl derivatives of ethylenediamine, each 5 mol%, also increased $[Na^+]_{0.5}$, but to a lesser extent (3.2–3.8 mM). In addition to their competitive effects, the phosphatidylseryl-substituted ethylenediamine compounds exerted a slowly-increasing non-competitive inhibition, not only in phosphorylation, but also in overall ATPase activity, which was reduced, although not abolished, by exogenous protein (bovine serum albumin). A detergent-like action in the usual sense is unlikely since liposomes containing these lipids remained intact. These studies prove that phospholipids are not only required for optimal activity of this transport enzyme, but in excess or in compositions deviating from the normal, may also be inhibitory.

Introduction

Na^+/K^+-ATPase, the enzyme responsible for active Na^+ and K^+ transport over the plasma membrane, is not only embedded in the phospholipid bilayer, but is also dependent on phospholipids for its action [1]. Although the polar headgroup specificity would be limited [1], counterarguments have been proposed in favour of the interaction with the negatively-charged phospholipids PS and PI as being essential for activation [2]. In addition, size and unsaturation of acyl side

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Abbreviations: Eda, ethylenediamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; SM, sphingomyelin; PS-Eda(Ser) (compound VIa), *O*-phosphatidyl-[*N*,*N'*-bis(seryl)]ethylenediamine; PS_2Eda (compound VIIa), *N*,*N'*-bis(*O*-phosphatidylseryl)ethylenediamine; PS-Eda (compound VIIIa), *N*-(*O*-phosphatidylseryl)ethylenediamine; EP, phosphorylation level (steady-state); EDC, *N*-ethyl,*N'*-(3-dimethylaminopropyl)carbodiimide ; CMC, 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide metho-*p*-toluenesulfonate; EPIS, *N*-ethyl-5-phenylisoxazolium-3'-sulfonate; TPS, 2,4,6-triisopropylbenzenesulfonyl chloride; Z, benzyloxycarbonyl; Trt, (trityl), triphenylmethyl; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; HOBt, hydroxybenzotriazole; CDTA, *trans*-1,2-diaminocyclohexane tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TDM, 4,4'-tetramethyldiaminodiphenylmethane; Mo or Mo-blue, Molybdenum blue reagent; n_H, Hill coefficient; *K*, Nernst partition quotient; r_{max}, element of maximal concentration in counter-current distribution.

chains are of importance in relation to the balance between membrane order and fluidity required for enzyme activity [3–5].

Recently [6,7], we discovered that ethylenediamine (Eda) is an inhibitory probe for Na^+ and K^+ activation sites (or access to these sites). Only the double-protonated Eda was a strong inhibitor (I_{50} = 0.45 mM in Na^+-activated phosphorylation), indicating that during its inhibition, it occupies paired, negatively-charged cation activation sites, which are 3–4 Å apart [7]. Likely candidates for such activation sites are carbodiimide-sensitive carboxylic acid groups [8,9]. On the assumption that these carbodiimide-reactive carboxyls include or overlap the Eda binding sites, we have analysed cross-linking between Eda and the Na^+/K^+-ATPase proteolipid complex using the water-soluble *N*-ethyl,*N'*-(3-dimethylaminopropyl)carbodiimide (EDC). Strikingly, the majority (around 73%) of radioactively-labeled ethylenediamine appeared in the lipid moiety. More specifically, label was associated with PS, despite a predominance of carboxyl group pairs in the protein as compared to PS-twins in the lipid (40 vs. 27.8 mol/mol Na^+/K^+-ATPase [1,10]).

In order to establish the involvement of the phospholipid as an inhibitory site of action of ethylenediamine, we synthesised the mono- and bis(phosphatidylseryl) derivatives of ethylenediamine, PS-Eda (compound VIIIa) and PS_2Eda (compound VIIa). The effects on phosphorylation and overall activity of Na^+/K^+-ATPase in PC liposomes vehicles were investigated, in addition to effects of the intermediate synthetic product of PS_2Eda, PS-Eda(Ser) (compound VIa). In agreement with the concept of paired occupancy of carboxyl groups by Eda, the bis(phosphatidylseryl) derivative VIIa displayed the strongest inhibition toward Na^+ activation of the Na^+/K^+-ATPase.

Materials and Methods

Enzyme preparation and overall hydrolytic activity

Na^+/K^+-ATPase was prepared and purified from rabbit kidney outer medulla according to the isopycnic zonal centrifugation method of Jørgensen [11], following solubilisation of contaminating protein by dodecylsulfate treatment. Removal of ATP, used to stabilise the enzyme during isolation, by hydrolysis, washing and storage of the enzyme was carried out according to Schoot et al. [12], except for the composition of the storage medium, which contained 50 mM imidazole-HCl (pH 7.0), 10% (w/v) sucrose and no CDTA.

Protein was determined by the method of Lowry et al. [13], following trichloroacetic acid precipitation and using bovine serum albumin as a standard [11]. Specific activity of the enzyme preparations, as determined under standard conditions (pH 7.4, 37°C [12]), ranged between 1000 and 1500 μmol/mg protein per h. Overall activity of proteoliposomes was assayed in a medium (0.3 ml) of identical composition: 100 mM Na^+, 10 mM K^+, 5 mM Mg^{2+}, 5 mM ATP, 30 mM imidazole-HCl (pH 7.4), 0.1 mM EDTA and 14.5 μg enzyme protein/ml. [γ-^{32}P]ATP was added to the ATP resulting in a specific radioactivity in the order of 0.15 Ci/mol. Initiating by the addition of 15 μl 100 mM ATP to 285 μl of the assay medium, the reaction was traced for 15 min at 37°C. At intervals of 5 min, 2 ml 10% (w/v) of analytical grade charcoal in 5% (w/v) trichloroacetic acid (stopping solution) was added. Blanks without enzyme served as controls, and blanks where stopping solution was added prior to the ATP served as zero-time controls. The suspension was centrifuged at 0°C for 10 min at 2000 × *g*, and 0.5 ml aliquots of the supernatant fluid were taken for determination of ^{32}P via liquid scintillation counting; 5-μl aliquots of ATP served as standards.

Phosphorylation

The method of phosphorylation of the enzyme at 23°C and pH 7.0 was in principle based on our previous method [7]: 20 μl [γ-^{32}P]ATP (0.15 mM, 240–2425 cpm/pmol) were added to 130 μl of a reaction medium containing proteoliposomes or enzyme (15 μg on protein basis) in triethanolamine-HCl (50 mM, pH 7.0), Na^+ (concentrations as specified), $MgCl_2$ (0.1 mM) and cholate (0.5%). All concentrations indicate final concentrations, except for ATP, which became 20 μM upon dilution. The proteoliposomes were preincubated in the reaction medium with cholate for 3 min at 23°C, prior to addition of ATP, in order to maximise the number of phosphorylation sites accessible to the substrate, while keeping detergent inactivation (about 10%) to a minimum. Phosphorylation was quenched 5 s after initiating the reaction by the addition of 5 ml 5% (w/v) trichloroacetic acid, containing 0.1 M phosphoric acid. The sediment was washed three times on 25 mm diameter, 1.2 μm pore size membrane filters (Schleicher and Schuell, Dassel, Germany) with the quenching solution and counted for ^{32}P in a liquid-scintillation analyser after addition of 4 ml Opti-Fluor (Packard Instruments, Groningen, The Netherlands). Data were corrected for blanks in which the quenching medium was added prior to ATP. Aliquots (2 μl) of the ATP solution served as standards. Phosphorylation for 10 or 15 s yielded levels equivalent to those at 5 s, proving a steady-state had been reached. In view of a developing inhibition exerted by some of the phospholipids (see results), the time lag between the start of reconstitution and phosphorylation was kept constant (2.5–3.5 h, fixed time per experiment).

Preparation of the (proteo)liposomes

Liposomes were prepared according to the procedure of Rabon et al. [14]. Per ml of eventual liposomal

suspension the protocol was as follows: a solution of phosphatidylcholine in chloroform, equivalent to 39 mg (49.6 μmol) PC, plus 2 mg (5.2 μmol) cholesterol was evaporated in a 15 ml round-bottom glass tube under a stream of N_2, while swirling on a Vortex mixer. The lipid was dissolved again in ether (1 ml) and treated twice in the same way. The film was then dissolved again in 1 ml diethyl ether, and 1 ml buffer medium (50 mM triethanolamine-HCl, pH 7.0) was added. The ether was evaporated, while stirring, by a controlled stream of N_2, using a bubble counter (three bubbles per s). The resulting liposomal suspension was sonicated for 30 min at maximal output (Branson Sonifier B-12) in a sonicator ice-cooled water bath (Heat Systems Ultrasonics). The liposomes could be stored at 4°C for at least a week [15]. A maximum of 10 mol% of PC was replaced by another phospholipid, as specified in the results. The liposomal volume (percentage of the total suspension) was determined according to Oku et al. [16]. To this end liposomes were prepared in the presence of 0.02 mM calcein; 5 μl was added to 1 ml 50 mM triethanolamine (pH 7.0) and the fluorescence (exitation 490 nm, emission 520 nm, slit width 10 nm) was recorded. Fluorescence of the extravesicular calcein was quenched by the addition of 5 μl 10 mM $CoCl_2$ and intravesicular fluorescence was quenched by opening of the liposomes by addition of 50 μl 5% cholate. The ratio of fluorescence decrease (corrected for dilution) before and after addition of detergent yielded the relative intravesicular volume.

Proteoliposomes were prepared (in 15 ml round-bottom glass tubes) by treating enzyme protein (6.3–6.4 mg/ml) with triethanolamine cholate solution (5% in cholate initially, 0.91% (w/v) finally) for 1 min at room temperature. Liposomes were then added (1.33 ml = 52 mg PC per mg protein), mixed and frozen in liquid N_2. Thawing was carried out at room temperature. This freeze-thawing was repeated twice more and the obtained proteoliposomes were sonicated for 6 min, as indicated above for liposomes.

The cholate used in our studies (preparation of proteoliposomes and opening of the vesicles) was purified by decolourisation of a hot ethanolic solution of cholic acid with a few spoonfuls of analytical grade charcoal [17]. After filtration, the solvent was removed by rotatory evaporation and the residue recrystallised from dilute acetic acid. The crystals were dried under vacuum over NaOH. A 5% solution in H_2O was made by the addition of triethanolamine to a concentration of 210 mM, giving rise to a pH of 7.3. Residual [Na^+] and [K^+] were shown to be 12.3 μM and 4.4 μM, respectively. It was observed that the imidazole salt is poorly soluble, and although the Tris salt is soluble, Tris cannot be used, since it is inhibitory to phosphorylation [18].

Covalent coupling of ethylenediamine to Na^+/K^+-ATPase and individual (phospho)lipids

Coupling of [^{14}C]ethylenediamine (up to 150 μM, 22 Ci/mol) to the enzyme (0.5 mg/ml) was accomplished by the addition of 0.2 vol. of an aqueous solution of *N*-ethyl,*N'*-(3-dimethylaminopropyl)carbodiimide-HCl (EDC, 5 mM) to 0.8 vol. of a buffered enzyme suspension, containing 62.5 mM triethanolamine-HCl (pH 7.0). The covalent coupling reaction was run overnight at 22°C in stoppered glass tubes (about 12 ml), and under N_2 to prevent carbamate formation from ethylenediamine and CO_2. Duplicate aliquots of 40 μl were mixed with 4 ml 5% (w/v) trichloroacetic acid to reduce the coupling of ethylenediamine to proteolipid (where formation of trichloroacetic anhydride would become predominant). After standing for 1 h at room temperature, the samples were filtered and washed three times on the filter with 5% trichloroacetic acid. Contents of the filters were then extracted for 30 min with 1 ml 10% (w/v) sodium dodecylsulfate and counted for ^{14}C, following mixing with 10 ml Insta-Gel (Packard Instrument B.V., Groningen, The Netherlands). Blanks, serving as correction for aspecific ethylenediamine binding, were prepared by the use of non-radioactive Eda (50 mM, pH 7.0) in addition to the radioactive chemical. These counts are related to protein + lipid-associated ethylenediamine coupling. Aliquots (5 μl) of [^{14}C]ethylenediamine served as standards.

To the remainder of the coupling medium, 4 ml 5% trichloroacetic acid was also added, but instead of filtration, 3-fold centrifugation and resuspension was applied. After the ultimate centrifugation step, the lipid was extracted with chloroform-methanol as outlined below. Coupling agents, other than EDC (CMC, 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide metho-*p*-toluenesulfonate [19]; EPIS, *N*-ethyl-5-phenylisoxazolium-3'-sulfonate [20,21]) were inferior to EDC: 90 and 98% lower coupling within 1 h, respectively. Concentrations of EDC higher than 2 mM yielded suboptimal incorporation of ethylenediamine, possibly because of side reactions: formation of intra- and intermolecular cross-links [22]. 1 mM EDC was, therefore, applied, yielding optimal coupling to protein.

Coupling of [^{14}C]ethylenediamine to individual (phospho)lipids followed the same procedure as that applied to the Na^+/K^+-ATPase preparation, but under swirling (Vortex, model K-500–4). In order to approach the natural conditions amounts of specific lipids (2 mg/ml $CHCl_3/CH_3OH$ (2:1, v/v)), as calculated from the literature [1,23] and corresponding to 0.25 mg Na^+/K^+-ATPase protein, were as follows: SM (20 μl), PC (40 μl), PI (10 μl), PS (15 μl), PE (30 μl), PA (arbitrarily 25 μl), cholesterol (30 μl); (0.5 mg/ml):

palmitic acid (10 μl), stearic acid (5 μl) and oleic acid (5 μl) in a total volume of 0.5 ml. Addition of trichloroacetic acid at the end of incubation was omitted, but the lipid was extracted directly (see below procedure).

Lipid extraction

Lipid extraction followed the procedure of Folch et al. [24], using 3-ml portions of $CHCl_3/CH_3OH$ (2:1, v/v, 1st extraction) and $CHCl_3/CH_3OH/H_2O$ (2:1:0.12, v/v, 2nd and 3rd extraction). H_2O (0.2–0.3 ml) was added to bring about phase separation. Extraction was carried out by stirring (Vortex, model K-500–4) for 30, 60 and again 60 min, respectively, in stoppered glass tubes under N_2. Phase separation was accelerated by centrifugation, the lower phases were combined and made up to 10 ml by addition of $CHCl_3/CH_3OH/H_2O$. The combined lower phases were extracted with 2.2 ml 0.1 M KCl (once) and 2.2 ml 0.1 M KCl, saturated with $CHCl_3/CH_3OH/H_2O$ (twice). The KCl-extracted lower layer was evaporated to dryness in a hot water bath under a stream of N_2 and the residue was taken up in a few hundred microliters toluene-ethanol (4:1, v/v). Aliquots of this solution were used for thin-layer chromatography [25], determination of lipid phosphorus [26], or lipid-associated ethylenediamine binding following the coupling with [^{14}C]ethylenediamine by EDC.

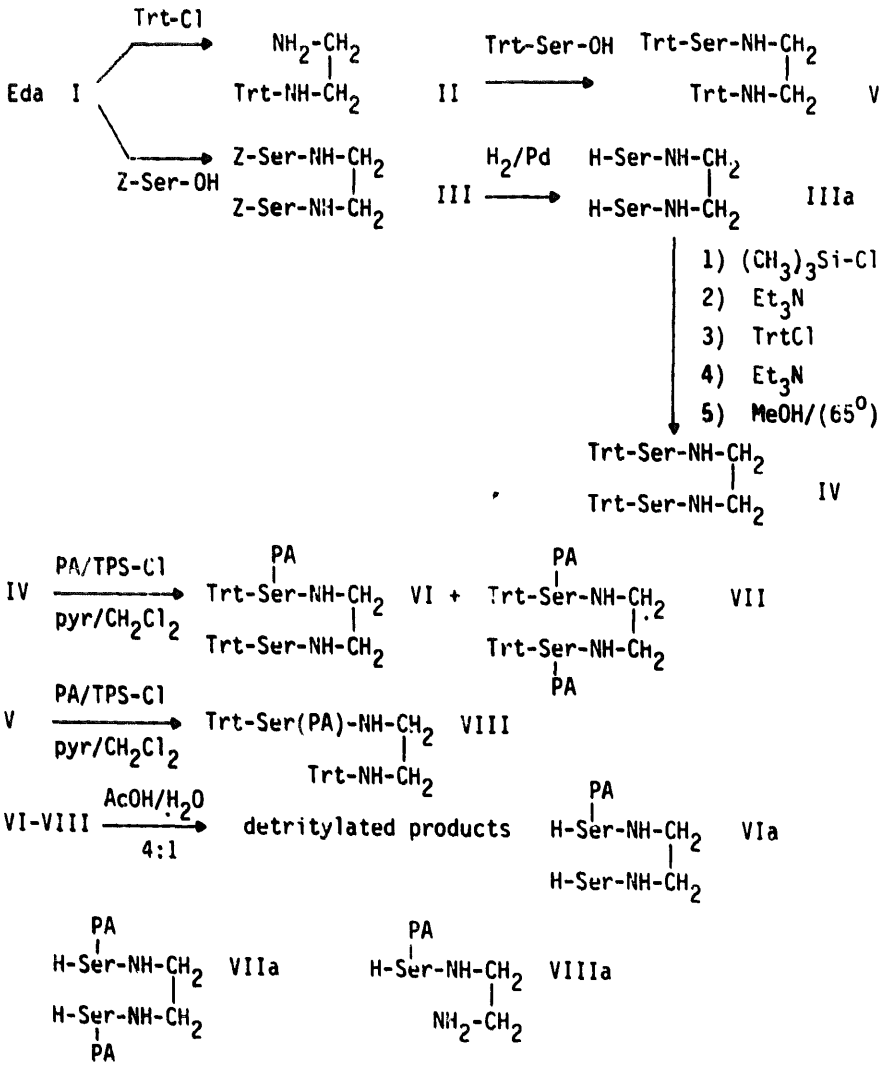

Fig. 1. Synthetic chart for the preparation of the phosphatidylserylethylenediamine derivatives (compounds VIa–VIIIa). Nomenclature and abbreviations of the intermediary and final products: I, ethylenediamine (Eda); II, *N*-tritylethylenediamine (Trt-Eda); III, *N,N'*-bis(*N*-benzyloxycarbonylseryl)ethylenediamine ((Z-Ser)$_2$Eda); IIIa, *N,N'*-bis(seryl)ethylenediamine [(H-Ser)$_2$Eda]; IV, *N,N'*-bis(*N*-tritylseryl)ethylenediamine ((Trt-Ser)$_2$Eda); V, *N*-(*N*-tritylseryl)-*N'*-tritylethylenediamine (Trt-Ser-Eda(Trt)); VI, *O*-phosphatidyl-[*N,N'*-bis(*N*-tritylseryl) ethylenediamine] (Trt-Ser(PA)-Eda(Trt-Ser)); VIa, *O*-phosphatidyl-[*N,N'*-bis(seryl)]ethylenediamine (Ser(PA)-Eda(Ser) or PS-Eda(Ser)); VII, *N,N'*-bis(*N*-trityl-*O*-phosphatidylseryl)ethylenediamine ([Trt-Ser(PA)]$_2$Eda); VIIa, *N,N'*-bis(*O*-phosphatidylseryl)ethylenediamine ([Ser(PA)]$_2$ Eda or PS$_2$Eda); VIII, *N*-(*N*-trityl-*O*-phosphatidylseryl)-*N'*-tritylethylenediamine (Trt-Ser(PA)-Eda(Trt)); VIIIa, *N*-(*O*-phosphatidylseryl)ethylenediamine (Ser(PA)-Eda or PS-Eda).

Thin-layer (phospho)lipid chromatography

Aliquots, containing 5–10 μg of the various lipids were applied on 10 × 10 cm silica gel plates, having been preheated (150°C, 30 min) to remove moisture and pre-eluted with $CHCl_3/CH_3OH$ (2:1, v/v) to remove any lipophilic contamination. The spots were dried under N_2 and chromatographed with $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (40 : 10 : 10 : 1, v/v) until the solvent front reached the top of the plate. The plate was then dried under N_2 and rechromatographed in the same direction up to 1.2 cm from the top, using a solvent, containing the same components but in the proportions 120:46:19:3 (v/v) [27]. The plate was air-dried and subjected to I_2 vapour. The spots were encircled with pencil and destained by evaporation overnight. In the case of the covalent coupling of [^{14}C]ethylenediamine, the thin layer plate was autoradiographed for 5 days and the radioactive spots scraped off and counted for ^{14}C.

Synthesis of mono- and bis(phosphatidylseryl)ethylenediamine (compounds VIIIa and VIIa)

These compounds were synthesized by condensation of phosphatidic acid to the N-protected headgroup components (V and IV) by means of the sterically-hindered coupling agent 2,4,6-triisopropylbenzenesulfonyl chloride (TPS [28,29]), and subsequent removal of the trityl groups by hydrolysis in acetic acid/water [30].

The reaction scheme, including the synthesis of IV and V, is outlined in Fig. 1.

$(TrtSer)_2$Eda (IV) was synthesized by coupling of benzyloxycarbonyl serine (Z-Ser) to Eda (I), reductive removal of the Z-groups, and tritylation of $(H\text{-}Ser)_2$Eda (IIIa), rather than by direct acylation with Trt-Ser, because of an allegedly poor coupling rate of tritylamino acids [31]. This route could not be applied in the synthesis of the N-tritylated monoseryl-Eda intermediate (V) because of poor solubility of the free base (*N*-SerEda) in the tritylation medium. In the latter case direct coupling of Trt-Ser to Trt-Eda [32] was carried out (Fig. 1).

All steps were checked by thin layer chromatography on silica gel with fluorescence indicator, elemental (CHN) analysis, ^{1}H-NMR (90 or 400 MHz) and mass spectroscopy or analysis of the constituent residues following hydrolysis. Phosphate was assessed according to Fiske and SubbaRow [33] and the modification of Broekhuyse [34], using $H_2SO_4/HClO_4$ (5:1, v/v) as destruction medium. Ethylenediamine was monitored as fluorescamine derivative [35], using HPLC (Lichrosorb RP-18 250 × 4 mm, pressure 65–66 bar, 22°C, eluent 0.1 M sodium phosphate (pH 7.5)/CH_3OH (3:2, v/v), flow 0.5 ml per min) in combination with spectrofluorometry (excitation 390 nm, emission 480 nm; retention time: serine 7.6 min, ethylenediamine 12.6 min). Fatty acid residues were assayed using BCl_3-catalysed methanolysis for 30 min at 100°C [36] in combination with GLC (fused silica-Chrompack WCOT CP-Sil 88–50 m/0.22 mm, 90–225°C: 16 C°/min; retention times fatty acids, min: 16:0, 10.5; 18:1, 12.3; 21:0, 14.2).

Detection of spots on TLC was carried out by the following means:

1. UV fluorescence quenching (illumination at 254 nm) by aromatic residues.
2. Spraying with ninhydrin (0.06% (w/v) in butanol/CH_3COOH (100:4, v/v)), followed by heating for 10 min at 80°C on -NH_2 or Trt-NH-.
3. Spraying with 4,4'-tetramethyldiaminodiphenylmethane (TDM) reagent ([37], solution E: 0.3% (w/v) ninhydrin in butanol/CH_3COOH (100:3, v/v)), after dipping in a Cl_2 atmosphere (chlorination tank) for 10 s and removal of excess Cl_2 by air. Primary, secondary and tertiary amines, phenols and carboxylic acid salts all stain. Spraying could be applied after ninhydrin staining.
4. Spraying with H_2SO_4/CH_3OH (1:1, v/v) on trityl compounds, including $TrtOCH_3$.
5. Spraying with Molybdenum blue reagent [38] on phosphate esters. The reagent could be applied after ninhydrin. Coincidence of ninhydrin- and molybdenum-positive material proves the coupling of phosphatidic acid and the phospholipid headgroup (see below).

Synthesis of the intermediary products

Z-Ser was synthesized according to Wünsch [39], Trt-Cl according to Vogel [40] and TrtSer according to Barlos et al. [32] (Procedure C). The latter product melted at 143–145°C. The R_f value in SiO_2-TLC (isopropyl-OH/25% NH_3/H_2O (7:1:2, v/v) as eluent) was 0.64, the spot being positive with UV and ninhydrin, with a trace contaminant at $R_f = 0.4$, which was only ninhydrin-positive. The R_f value of the major product in $CHCl_3/CH_3OH$ (9:1, v/v) was 0.21–0.26.

Trt-Eda (II). Ethylenediamine (50 mmol) in 25 ml dry, methanol-free $CHCl_3$ was treated at room temperature with 0.5 equivalent TrtCl, dissolved in 25 ml $CHCl_3$ (dropwise addition under stirring). A white solid, presumably ethylenediamine-HCl, precipitated and was removed by filtration. Following evaporation of the solvent, the residue solidified and was dissolved in 10 ml $CHCl_3/CH_3OH$ = 19:1, filtered through a 8.5 × 5 cm SiO_2 60 H column and eluted with the same solvent at a rate of 1 ml/min (46 5-ml fractions, 11 of 10 ml). Fractions 39–57 containing only the monotrityl compound (R_f on SiO_2-TLC with $CHCl_3/CH_3OH$ = 9:1 as solvent 0.14, Trt_2Eda 0.84, spots UV- and ninhydrin-positive) were combined, yielding a yellow glass (32% yield), which crystallised upon standing (m.p. 67–68°C). CHN-analysis (%): C (83.09), H (7.32),

N (9.27); calculated C (83.40), H (7.33), N (9.26). ^{1}HNMR (90 MHz, $CDCl_3$): phenyl (δ 7.35 ppm, m) 15 H; α-CH_2 (δ 2.85 ppm, t) 2 H; β-CH_2 (δ 2.23 ppm, t) 2 H.

(Z-Ser)$_2$Eda (III). 5 g Z-Ser (20.9 mmol) + 0.82 equivalent hydroxybenzotriazole · H_2O (HOBt · H_2O) + 0.41 equivalent ethylenediamine were dissolved in 20 ml tetrahydrofuran, and dimethylformamide was added to keep all components in solution. Then 1.03 equivalents DCC were added as a solid, the mixture being stirred under Ar for 72 h. Excess DCC was converted to DCU by the addition of about 1 ml CH_3COOH and further standing (1 h). After filtration, the solvent was removed under reduced pressure and the residue extracted with ice-cold tetrahydrofuran to remove HOBt and residual DCU (R_f values 0.46 and 0.74, respectively, on SiO_2-TLC, $CHCl_3/CH_3OH/CH_3COOH$ (95:20:3, v/v) as solvent, both spots being TDM-positive, while only the one with the lowest R_f value was UV-positive). The remaining solid was recrystallised from methanol, yielding pure III in 54% yield (R_f 0.49 on SiO_2-TLC with $CHCl_3/CH_3OH/CH_3COOH$ (95:20:3, v/v), spot weakly UV-positive, strongly TDM-positive). The compound decomposed at about 200°C, turning yellow and melting completely at 225°C. CHN-analysis (%): C (57.35), H (5.99), N (11.08); calculated C (57.36), H (6.02), N (11.15). ^{1}H-NMR (90 MHz, CD_3OD): phenyl (δ 7.3 ppm, s) 10 H; benzyl-CH_2 (δ 5.1 ppm, s) 4 H; α-seryl-CH (δ 4.11 ppm, t) 2 H; β-seryl-CH_2 (δ 3.7 ppm, d) 4 H. $[\alpha]_D^{23°} = +2.6°$ ($c = 1$, dimethylformamide).

(H-Ser)$_2$Eda (IIIa). Compound III (3 mmol, 1.5 g) in a 1% (w/v) solution in methanol was subjected to hydrogenolysis using 10% Pd on carbon as the catalyst (total H_2 consumption 140 ml). The solution was filtered over Celite and the solvent was removed by evaporation, leaving a white crystalline mass in 100% yield. In contrast to the starting material, it did not travel on SiO_2-TLC in $CHCl_3/CH_3OH/CH_3COOH$ (only one TDM- and ninhydrin-positive spot was observed at the origin). IIIa was not analysed further but directly transferred as a whole to the next tritylation step.

(Trt-Ser)$_2$Eda (IV). Compound IIIa was N-tritylated in much the same way as indicated for serine [32]. Following protection of all functional groups with trimethylsilyl functions, selective N-protection was performed with trityl chloride and triethylamine in boiling chloroform. Some care had to be given to the complete removal of the protecting trimethylsilyl groups on oxygen. The final product required heating under reflux for about 4 h in methanol/$(C_2H_5)_3N$ (0.1 equivalent) to convert all ninhydrin-positive (transient) spots in TLC to one spot: R_f 0.3 ($CHCl_3$/MeOH (24:1, v/v)).

After evaporation of the solvent, the residue was purified by chromatography using a SiO_2 column (4.5 × 5 cm) and $CHCl_3/CH_3OH$ (24:1, v/v) as the solvent (5 ml) and the eluent (5-ml fractions). In total, 35 fractions were collected, changing to $CHCl_3/CH_3OH$ (9:1, v/v) as eluent at fraction 26. TLC was carried out with $CHCl_3/CH_3OH$ (24:1, v/v). TrtOCH_3 (R_f 0.77, UV-positive, ninhydrin-negative) preceded the product (fractions 11–24). The product (R_f 0.26, UV- and ninhydrin-positive) was eluted in the fractions 15 to 32. Weakly UV- and ninhydrin-positive material (R_f 0.11) was present in fractions 28 and on. Fractions 15–32 were combined and crystallised upon evaporation.

The residue was dissolved in 5 ml $CHCl_3$ and the solution was added dropwise under stirring to 30 ml hexane, cooled to the temperature of solid CO_2/acetone (−77°C). A faintly yellow solid (R_f 0.33) precipitated, leaving TrtOCH_3 (R_f 0.77) in solution. Yield 58%; the product liquefied above 150°C with no sharp melting point. CHN-analysis (%): C (75.84), H (6.47), N (7.40); calculated C (76.86), H (6.45), N (7.79). ^{1}H-NMR (90 MHz, $CDCl_3$): amido (δ 7.7 ppm, s) 2 H; phenyl (δ 7.3 ppm, m) 30 H; overlapping peaks of α-seryl-CH, Eda-CH_2, α-seryl-NH and β-seryl-CH_2 (δ 2.7–3.8 ppm) 12 H. Major mass spectrum peaks (fast atomic bombardment) 115 (McLafferty transformed segment minus N), 243 (trityl) and 719 (total molecule). $[\alpha]_D^{23°} = -47.4°$ ($c = 1$, $CHCl_3/CH_3OH$ (9:1, v/v)).

Trt-Ser-Eda(Trt) (V). Trt-Eda (7.68 mmol) was dissolved with 1 equivalent Trt-Ser in 50 ml dimethylformamide together with 1.1 equivalents hydroxybenzotriazole and 1.05 equivalents DCC (added in 20 ml dimethylformamide). The reaction mixture was left under Ar at room temperature overnight. Precipitated DCU (which started to form after 1 h) was removed by filtration and the clear supernatant added dropwise under stirring to 400 ml saturated $NaHCO_3$. The resultant white precipitate was dissolved in diethyl ether. The solution was filtered and the ether layer was washed with saturated NaCl and finally dried over Na_2SO_4. Following filtration and evaporation of the solvent, the residue was purified by counter-current distribution since various attempts at purification by recrystallisation were unsuccessful. The two-phase solvent system (MeOH/EtOAc/hexane/water (4:2:6:1, v/v) for apolar compounds) of Montaque et al. [41] was used. After 80 transfers, $K = 0.6$ and $r_{max} = 30$ in this system. Yield 71%, m.p. 101–104°C. The compound was chromatographically pure ($R_f = 0.68$ in $CHCl_3$/MeOH (9:1, v/v)). CHN-analysis (%): C (80.74), H (6.79), N (6.35); calculated C (81.74), H (6.54), N (6.65). ^{1}H-NMR (400 MHz, $CDCl_3$): amido (δ 7.7 ppm, s) 1 H; phenyl (δ 7.35 ppm, m) 30 H; β-seryl-CH_2 (δ 3.6 ppm, t) 2 H; α-seryl + β-Eda-NH (δ 3.3 ppm, m) 2 H; α-Eda-CH_2 (δ 3.0 ppm, t) 2 H; α-seryl-CH (δ 2.7 ppm, d) 1 H; β-Eda-CH_2 (δ 2.3 ppm, t) 2 H. $[\alpha]_D^{23°} = -72.7°$ ($c = 1$, $CHCl_3/CH_3OH = 9:1$, v/v).

CHN-analysis and ^{1}H-NMR demonstrated the presence of EtOAc (0.3 mol/mol) as solvating ester.

Coupling of phosphatidic acid (PA) and V or IV

To a 1–2% (5–20 ml) solution of PA in a mixture of dry, methanol-free CH_2Cl_2 and pyridine (1:1, v/v), 1–1.1 equivalents compound V and three equivalents TPS were added. The reaction mixture was kept for 48 h at room temperature under Ar. Excess TPS was hydrolysed by the addition of 2.25 ml tetrahydrofuran/H_2O (8:1, v/v) per ml of reaction medium, leaving the medium under Ar overnight. The organic phase was washed 4-fold with a saturated $NaHCO_3$ solution containing NaCl to give a clear phase separation. The upper phase contained the UV-, Mo-blue- and ninhydrin-positive product (R_f around 0.5 on SiO_2-TLC in $CHCl_3$/CH_3OH (9:1, v/v)), which solidified on evaporation of the solvent. Residual pyridine was azeotropically removed by coevaporation with toluene (three times). The major part of unreacted PA was removed by column chromatography (10 × 2 cm, SiO_2 60 in 5% CH_3OH in $CHCl_3$ (v/v)). The UV + ninhydrin + Mo-positive material was combined, yielding 340 mg of solid upon evaporation of solvent, starting from 210 mg of PA.

The esterification of PA with the two seryl hydroxyls of IV was carried out in a two-step procedure in order to avoid excessive formation of pyrophosphatidic acid, the first coupling being performed as described for V. Another equivalent of PA was added to the reaction medium and again three equivalents of TPS were administered. The medium was incubated for 48 h under Ar. Excess TPS was hydrolysed in H_2O (10%, v/v), which caused no phase separation. The reaction proceeded overnight in an atmosphere of Ar. Subsequently, 2 vols. of CH_2Cl_2 were added and the organic phase was treated as before. The resultant crude product was used in the next step, postponing the removal of PA (and pyro-PA) by column chromatography until after the detritylation stage (next section).

Detritylation of the protected compounds

The coupling products were homogenised in excess 80% CH_3COOH and kept at 30–35°C for about 1 h. Compound VII required addition of ~6% (v/v) of $CHCl_3$ + a few drops of CH_3OH to obtain complete solution. The solvent was removed by evaporation and residual acetic acid was swept out by azeotropic distillation with cyclohexane.

Compounds VIa–VIIIa were purified from detritylation products of IV and V by extraction of a solution of the residue in $CHCl_3$/MeOH (10:1, v/v) with solutions of $KHSO_4$ (pH 2), satd. $NaHCO_3$, H_2O and satd. NaCl. If greater amounts of PA and its anhydride were present, as occurs in the preparation of VIa + VIIa, the crude compounds were additionally subjected to chromatography over DE52 (acetate form) using $CHCl_3$/MeOH systems of increasing MeOH concentrations (10–50%); 10–30% MeOH elutes VIa + VIIa, whereas 50% MeOH elutes the PA and pyro-PA. Amounts of 247 mg (75%) of VIa and VIIa and 255 mg (77%) of VIIIa were obtained. The yield of VIa and VIIa is based on the presence of a mass ratio of 35:60 (cf. next purification step).

Purification of VIIIa and VIIa

Crude material from the detritylation reaction was purified by counter-current distribution, using an apparatus with elements of 5 ml lower phase. The solvent system was CCl_4/MeOH/H_2O (62:35:3.15, v/v) [42]. After 240 transfers, pure VIIIa was found in the elements No. 165–195, $K = 3.0$; total yield 46.3 mg or 13.9%. The pure compound (VIIIa) had $R_f = 0.65$ on TLC in the system $CHCl_3$/MeOH/17% NH_3 (41:41:18, v/v).

Compound VIIa contained VIa and some other impurities, and required 840 transfers for purification. $K = 0.17$, $r_{max} = 120$ ($R_f = 0.11$ on TLC in $CHCl_3$/MeOH/17%NH_3 (20:5:1, v/v)). The yield was 60 mg (19.7%) with respect to the starting material (PA).

Polar by-products, with a higher mobility in this system than VIIa, like VIa ($K = 5$, $r_{max} = 100$ after 120 transfers, $R_f = 0.06$ on TLC) and another (unknown) impurity with $K = 1.4$ ($r_{max} = 87$, $n = 320$; $R_f = 0.11$) were obtained in yields of 35 (9.4%) and 15.9 mg, respectively. All synthesised phospholipids contained the constituent fatty acids (palmitic and oleic acid) and phosphate in equimolar quantities, but the Eda/phosphate ratio in VIIa was half that of the others.

Commercial sources

All phospholipids were obtained from Avanti Polar Lipids, Alabaster, AL, USA. Cholesterol was obtained from Sigma, St. Louis, MO, USA.

Free fatty acids, including C*21*, used to standardise the GLC determinations, were obtained from Supelco, Inc., Bellefonte, PA, USA. Coupling reagents were obtained either from Sigma: *N*-ethyl,*N'*-(3-dimethylaminopropyl)carbodiimide-HCl, 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide metho-*p*-toluenesulfonate and *N*-ethyl-5-phenylisoxazolium-3'-sulfonate, or from Janssen, Beerse, Belgium: 2,4,6-triisopropylbenzenesulfonyl chloride. Non-radioactive ATP (diNa-salt) was obtained from Boehringer-Mannheim, Germany; non-radioactive ethylenediamine from Merck, Darmstadt, Germany; radioactive chemicals, i.e. [γ-^{32}P]ATP (code PB 170) and [^{14}C]ethylenediamine · 2HCl from Amersham International, Little Chalfont, Amersham, UK; L-serine from Ajinomoto, Tokyo, Japan; cholic acid was from Matheson Coleman & Bell, East Rutherford, NJ, USA; calcein from Aldrich,

Steinheim, Germany. Bovine serum albumin (Fraction V) was obtained from Sigma, St. Louis, MO, USA and freed from fatty acids according to Chen [43], centrifuging for another 30 min at 250000 × *g* and replacing the neutralisation by dialysis for 64 h vs. 2 liters of physiological saline (changing each 24 h), followed by 6 h vs. aqua bidest and lyophilisation. BCl_3 (10% (w/v) in methanol) was obtained from Alltech/Applied Science, Deerfield, IL, USA. Column materials: SiO_2 60 (grain size 63–200 μm) and SiO_2 60 H from Merck, Darmstadt, Germany; DE 52 from Whatman Biochemicals, Maidstone, UK, and silica gel 60 F254 thin-layer chromatography plates (glass, 0.25 mm thick) from Merck, Darmstadt. All further chemicals were of analytical grade.

Results

Linking ethylenediamine to Na^+/K^+-ATPase

Following covalent coupling of 25–150 μM radioactive ethylenediamine to Na^+/K^+-ATPase by means of the carbodiimide EDC, 69–79% (average 73 ± 2%) of the tracer could be extracted with chloroform/methanol (Fig. 2), indicating that most of the Eda was coupled to lipids. Extrapolation to infinite concentration yielded maximal coupling values of Eda to protein and to lipid of about 1:1. The Scatchard plot of coupling to protein is virtually horizontal, indicating aspecific binding under the present conditions, although it remains possible that the plot will curve downward at higher Eda concentrations (positive cooperativity). The extent to which the 27% non-lipid-extractable radioactivity is due to cross-linking by Eda of lipid to protein and to cross-linking of neighbouring carboxyl groups within the same protein was not investigated. Following polyacrylamide gel electrophoresis of the holoenzyme, however, subdivision of radioactive Eda between the α and β protein subunit was analysed, and yielded a 7:3 molar ratio.

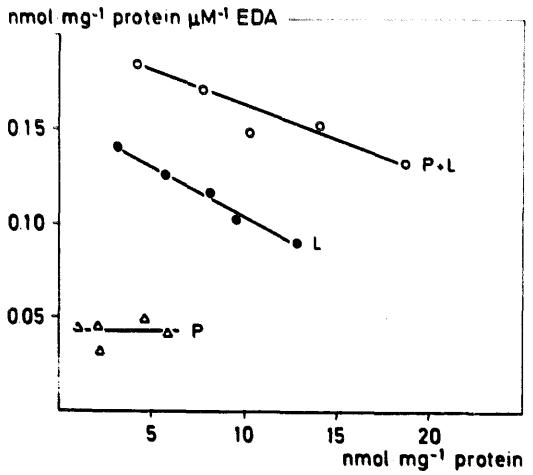

Fig. 2. Scatchard plots of ethylenediamine binding to the protein and lipid moiety of Na^+/K^+-ATPase. The coupling of ethylenediamine (Eda, 25–150 μM) to the enzyme took place during overnight (16 h) incubation in the presence of 1 mM EDC. Binding to the total complex of protein (P) plus lipid (L), lipid alone (L, following its extraction from the protein, but expressed per mg protein) and protein alone (P, determined by deduction) are presented. The initial ethylenediamine concentrations are corrected for the binding to P+L.

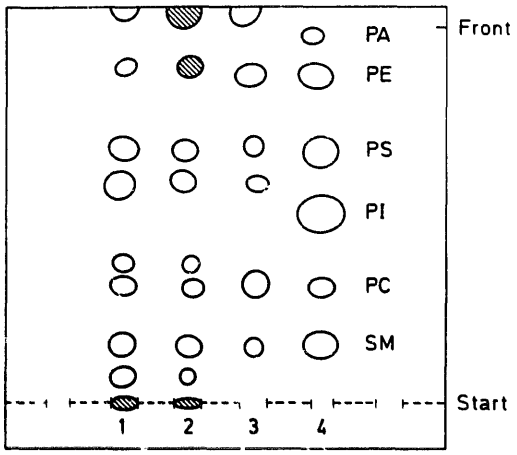

Fig. 3. Thin-layer chromatogram of the Na^+/K^+-ATPase lipid extracts. Lipid extracts were prepared, following coupling of [^{14}C]ethylenediamine (100 μM) to the enzyme by 1 mM EDC during overnight (16 h) incubation in the presence of 50 mM non-radioactive ethylenediamine (blank, lane 1), absence of non-radioactive ethylenediamine (lane 2), and absence of any coupling (no ethylenediamine, no EDC, lane 3). Lane 4 contains a set of standard phospholipids: SM, PC, PI, PS, PE, and PA. Shown are I_2-stained spots which, when hatched, contain radioactivity. Top spots represent free fatty acids.

Specificity of ethylenediamine coupling within the lipid moiety

Lipid components within the chloroform-methanol extract of ethylenediamine labelled Na^+/K^+-ATPase were separated via TLC (Fig. 3). About 90% of the label ran equidistantly with PE, whereas about 10% coincided with the free fatty acid top spot. Only 1% of the radioactivity remained at the origin, possibly due to a contamination by protein or ethylenediamine itself. The free fatty acids may have been generated by hydrolysis of the phospholipids during overnight coupling since they were absent in the standard phospholipid mixture (lane 4).

Identification of the ethylenediamine-lipid coupling products

A number of lipid components, such as SM, PC, PI, PS, PE, PA, cholesterol, palmitic (16:0) acid, stearic (18:0) acid and oleic (18:1) acid, were tested in the Eda coupling assay. Only PS yielded a coupling product that comigrated with PE as well as with the coupling product of Eda with Na^+/K^+-ATPase lipids (Fig. 4). In addition, the thin-layer top spots indicate the occurrence of coupling between free fatty acids and the radioactive Eda, the product of which is located in that

position. The coupling of Eda with pure PS and PS in Na^+/K^+-ATPase is about commensurate (6.8% in the Na^+/K^+-ATPase complex vs. 8.8% for the pure lipid, and double these values on double substitution) under identical coupling conditions at 100 μM Eda.

Comparison of the ethylenediamine-lipid products with the synthetic products

Since phosphatidylserine predominantly condensed with the added ethylenediamine, we synthesised the single- (VIIIa, PS-Eda) and double- (VIIa, PS_2Eda) substituted ethylenediamine. Compound VIa (PS-Eda(Ser)) is the unprotected intermediary product in the synthesis of VII (Fig. 1). Only VIIa resembled the coupling product of Eda and the natural lipids of Na^+/K^+-ATPase in so far as mobility in TLC was concerned (lane 2, Fig. 5). The slightly lower mobility than that of compound VIIa from endogenous PS (which comigrates with PE, Fig. 3) may be due to the difference in fatty acid composition; the 'artificial' product contained equimolar amounts of fatty acids 16:0 and 18:1, whereas the natural product contained 27% each of 18:0 and 18:1, in addition to 9–16 mol% of 16:0, 18:2 and 20:4 (average chain length 18 instead of 17 and unsaturation index 113 instead of 50 [23]).

Spots of the compounds VIIIa and VIa (lane 1 and 3, respectively, Fig. 5) were definitively non-coincident with the coupling product of Eda with the lipids of natural origin in Na^+/K^+-ATPase. VIIIa comigrated with PC, whereas the slightly more polar VIa showed a slight lag. Despite their non-identity with the coupling product of Eda and the natural phospholipids (being more like VIIa), we compared their effects on the activity of Na^+/K^+-ATPase, since they offer an opportunity to investigate a charge difference. VIIIa and VIa are positively-charged, whereas VIIa is neutral under assay conditions.

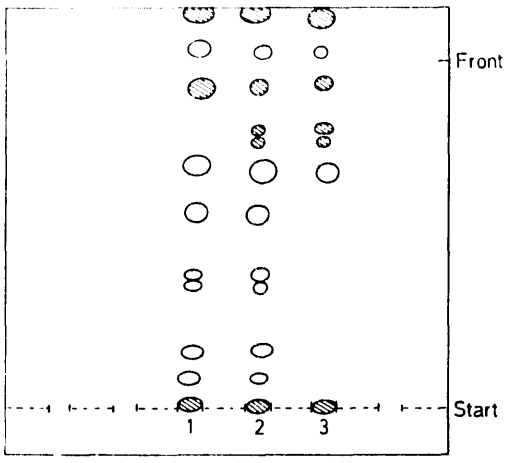

Fig. 4. Comparison of ethylenediamine-lipid coupling products from Na^+/K^+-ATPase and pure bovine brain PS by thin-layer chromatography. Coupling of [^{14}C]ethylenediamine, 75 μM, to Na^+/K^+-ATPase (lane 1), 100 μM to PS (lane 3) and lipid extraction occurred as in Fig. 3, lane 2. Lane 2 in this figure is an average mixture of material from Na^+/K^+-ATPase and pure PS: 13.5 and 2.7 μg of phospholipid, respectively; lane 1: 27 μg, lane 3: 5.4 μg. Standard phospholipids were omitted from this chromatogram, but the reader is referred to Fig. 3. Hatching indicates radioactivity, not necessarily I_2-staining as for the open spots.

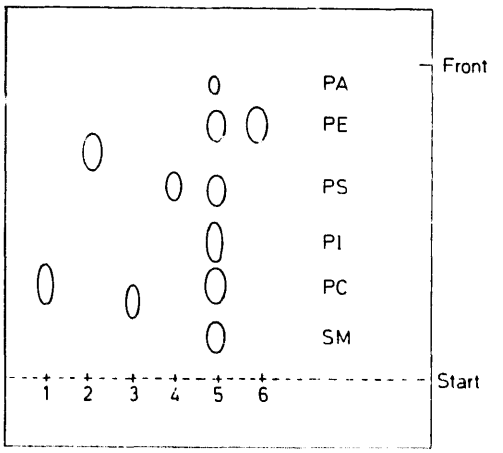

Fig. 5. Comparison of synthetic monophosphatidylseryl- (VIIIa) and bis(phosphatidylseryl)ethylenediamine (VIIa) with the natural phospholipids. A thin-layer chromatogram of VIIIa (lane 1), VIIa (lane 2), VIa (lane 3) and another, unknown, side product of the VIIa synthesis (lane 4) is shown. Roman numeral labelling is given in Fig. 1. Lane 5, standard phospholipids, lane 6, pure PE (for meaning of abbreviations, see the abbreviations footnote). The synthetic products (2 μl of 1% (w/v) solutions in $CHCl_3/CH_3OH$ (9:1, v/v)) and the PE and PS spots all stain with ninhydrin and Mo-blue reagent. The remaining spots only stain with Mo-blue.

Phospholipid effects on activation of Na^+/K^+-ATPase

Phosphorylation in PC proteoliposomes. Phosphatidylcholine (PC) was used as carrier and standard phospholipid for the formation of lipid bilayers [44]. The effects of moderate replacement of PC (up to 10 mol%) by other phospholipids on the activity of Na^+/K^+-ATPase was investigated. In order to avoid variations in the ratio of closed and leaky vesicles, all were opened by preincubation with 0.5% cholate with little detergent inactivation (see Phosphorylation under Methods).

The phospholipids contain counter-cations, contributing essential quantities of Na^+ (up to about 0.5 mM) and K^+ (up to about 25 μM) to the phosphorylation medium. For this reason, buffer-activated phosphorylation [45] could not be studied with the reconstituted system and we thus confined ourselves to Na^+-activated phosphorylation. To enable the interpretation of inhibitory lipid effects in terms of inhibition by counter-cation K^+ or by the lipid itself, the type and extent of K^+ inhibition in proteoliposome phosphorylation was determined. With increasing K^+ concentrations, the $[Na^+]_{0.5}$ increased exponentially

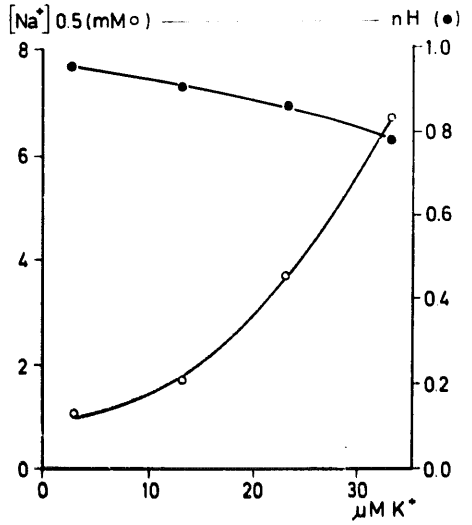

Fig. 6. Effect of K^+ on Na^+-activated phosphorylation in permeabilised phosphatidylcholine-containing proteoliposomes. The $[Na^+]_{0.5}$ values (left ordinate, ○) and Hill coefficients (n_H, right ordinate, ●) have been determined from Eadie-Scatchard and Hill plots at $[Na^+] = 0.5$–20 mM. Maximal phosphorylation capacity under each condition is 1.8 nmol/mg protein.

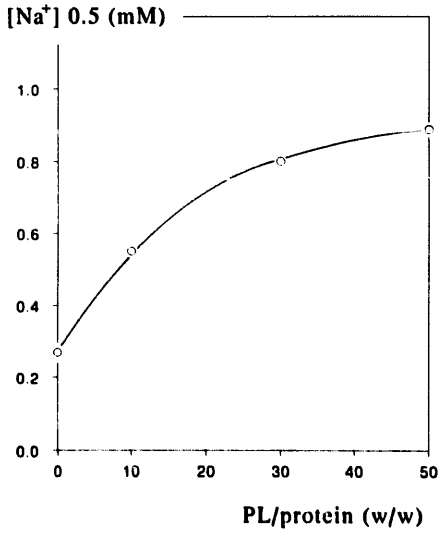

Fig. 7. Decrease of Na^+-affinity in the phosphorylation by $CHCl_3/CH_3OH$-extracted phospholipids from rabbit kidney outer medulla (microsomal fraction). Shown is the weight ratio of liposomal phospholipid (PL) to enzyme protein. No cholesterol was added in the preparation of liposomes because of microsomal lipids already containing 33% cholesterol [23]. The phospholipid weight has been calculated on the basis of an average molecular weight of 750 [23]. $[Na^+]$ and $[K^+]$ in the phosphorylation at the maximally applied PL/protein ratio of 50 was 0.73 mM and 7.2 μM, respectively. The $[Na^+]_{0.5}$ determination took these figures into account.

(Fig. 6) from an extrapolated value of 0.9 mM in the absence of K^+, to 6.6 mM at 33.3 μM (including residual) K^+. The maximal phosphorylation capacity (at infinite $[Na^+]$) was unaffected (competitive inhibition). Plotting $[Na^+]_{0.5}$ vs. $[K^+]^2$ yielded a straight line (not shown), indicating that two K^+ ions cooperate in the inhibition. This is in agreement with the concept of cooperation in the occlusion of K^+ ions [46]. In addition, they appeared to introduce negative cooperativity between Na^+ activation sites (a decrease of the Hill-coefficient, Fig. 6). The $[Na^+]_{0.5}$ value of 1 mM (average 1.4 ± 0.3 mM from four experiments, Table I) in the absence of K^+ is 5-fold higher than the $[Na^+]_{0.5}$ value for non-reconstituted enzyme (0.2 mM, Ref. 7). Fig. 7 shows that the increased $[Na^+]_{0.5}$ is not due in particular to the alien PC (from egg). Phospholipids,

TABLE I

Effects of phospholipids on kinetic parameters of Na^+-activated phosphorylation

The parameters were determined via Eadie-Scatchard and Hill plots. For the determination of the $[Na^+]_{0.5}$ and n_{H,Na^+}, Na^+ concentrations of 0.1–3.0 mM (free enzyme, lines 1+2), 0.5–20 mM (proteoliposomes, lines 3–6) or 2–100 mM (proteoliposomes with PS-substituted Eda, lines 7–9) were used at 0.1 mM Mg^{2+} and 20 μM ATP. For the determination of $[Mg^{2+}]_{0.5}$, Mg^{2+} concentrations of 5–100 μM at 100 mM Na^+ and 20 μM ATP were used, and for the determination of $[ATP]_{0.5}$, ATP concentrations of 1–10 μM at 0.1 mM Mg^{2+} and 100 mM Na^+ were used. The Mg^{2+} concentration was corrected for binding to ATP [45] and the ATP concentration for consumption during phosphorylation. Symbols (VIa–VIIIa) for the synthetic products are from Fig. 1.

Phospholipid	mol%	$[Na^+]_{0.5}$ (mM)	n_{H,Na^+}	$[Mg^{2+}]_{0.5}$ (μM)	$[ATP]_{0.5}$ (μM)	EP_{max} (nmol/mg protein)
Endogenous	–	0.2	1.8	0.1 [a]	0.02 [a]	2.65
+cholate (0.5%)	–	0.3	1.3	–	–	2.65
PC (lipid/protein, 52 w/w)	100	1–2	1–1.2	0.8	0.3	1.90
PC+PE	90+10	1.1	1.2	–	–	1.98
PC+PS	90+10	0.7	1.4	–	–	1.84
PC+PA	90+10	7.8	0.6	–	–	1.90
PC+VIa	95+5	3.2	1.0	–	–	0.68
PC+VIIa	95+5	13.7	1.0	1.1	0.5	0.80
PC+VIIIa	95+5	3.8	1.0	–	–	0.96

[a] From Ref. 7.

extracted from the microsomal fraction of rabbit kidney outer medulla (the membranes containing the native enzyme) induced a similar reduction in Na^+ affinity. The effect of detergent (0.5% cholate) on the $[Na^+]_{0.5}$ of native enzyme was only moderate (becoming 0.3 mM, and EP_{max} remaining unchanged), although the Hill coefficient was reduced from 1.8 to 1.3. This indicates that lipids counteract Na^+ activation of phosphorylation. Replacing 10 mol% of PC in the reconstitution by PE ($[Na^+]_{0.5} = 1.1$ mM, $n_H = 1.2$), PS ($[Na^+]_{0.5} = 0.7$ mM, $n_H = 1.4$) or PA ($[Na^+]_{0.5} = 7.8$ mM, $n_H = 0.6$) did not change either the maximal phosphorylation capacity, the affinity for Na^+, or the Hill-coefficient (Table I), except for PA, which may have been due in part to contaminating K^+ (20 μM).

The effect of lipids on the affinity for K^+ in the phosphorylation reaction was minimal. The K_i value of 12.8 μM, derived from Fig. 6 is very similar to the value of 12.5 μM in buffer-activated phosphorylation (no Na^+: Fig. 9 of Ref. 45) of the non-reconstituted system. Other affinities of the PC proteoliposomes are (Table I): $[Mg^{2+}]_{0.5} = 0.8$ vs. 0.1 μM for the non-reconstituted system, and $[ATP]_{0.5} = 0.3$ vs. 0.02 μM for the non-reconstituted system. Hence, the additional lipids also reduced the affinity for Mg^{2+} and ATP about 10-fold.

Effects of PS-substituted ethylenediamine. Compound VIIa (PS_2Eda), being most similar to the coupling product of ethylenediamine and the endogenous phospholipids of Na^+/K^+-ATPase (cf. Figs. 4 and 5) was studied more extensively.

Addition of increasing amounts of VIIa to PC proteoliposomes decreased the EP level measured at 2 mM Na^+ to 3% of the original level (Fig. 8). In addition, at a Na^+ concentration of 100 mM, the EP

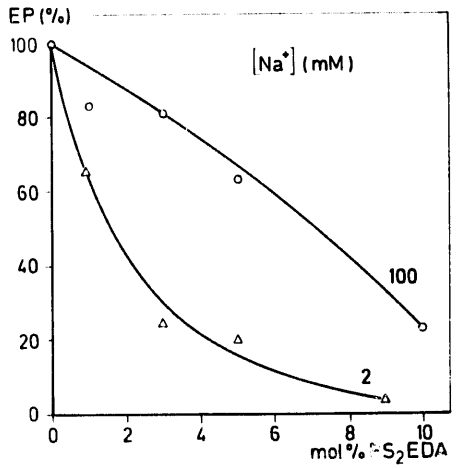

Fig. 8. Dose-dependent inhibition of Na^+-activated phosphorylation by bis(phosphatidylseryl)ethylenediamine (VIIa, PS_2Eda) in phosphatidylcholine-constituted proteoliposomes. $[Na^+]$ in the phosphorylation media was 2 (△) and 100 mM (○). 100% is 1.5 nmol EP per mg protein (○) or 1.3 nmol EP per mg protein (△).

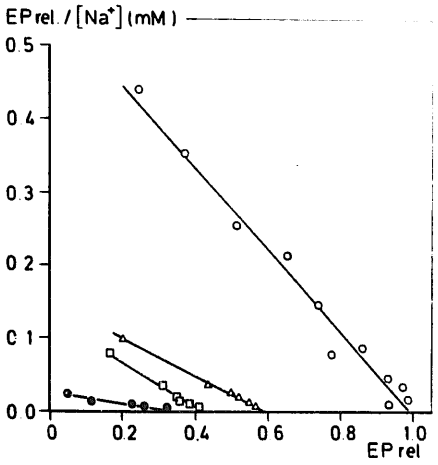

Fig. 9. Eadie-Scatchard plots of Na^+-activated phosphorylation in proteoliposomes, containing PC alone (○), PC+5% VIIIa (△), PC+5% VIa (□) and PC+5% VIIa (●). Phosphorylation levels ($EP_{rel.}$) are expressed as fractions of the control maximum (○). The control plot is the mean of two experiments with VIIa and VIa + VIIIa, repectively. $[Na^+]_{0.5}$ values obtained from regression analysis are: 1.8 mM (control), 3.2 mM (PC+VIa), 3.9 mM (PC+VIIIa) and 14 mM (PC+VIIa). Roman numeral labels are given in Fig. 1.

level decreased, although to a lesser extent than at 2 mM Na^+. Scatchard analysis at 5% VIIa (Fig. 9) shows that $[Na^+]_{0.5}$ increased from 1.8 mM to 14 mM. With both VIIIa (PS-Eda) and VIa (PS-Eda(Ser)) (each 5%), the $[Na^+]_{0.5}$ also increased, but only by a factor 2 (Fig. 9, Table I). In addition, the maximal phosphorylation capacity decreased by 43–66%.

The extent of competitive inhibition was not due to contaminating K^+ introduced by VIa–VIIIa. The mono-substituents of Eda hardly affected K^+ contribution to the phosphorylation medium, which was in the range of 7–9 μM. The di-substituent VIIa contributed maximally 1.2 μM K^+ per mol%, which could, therefore, have yielded an increase in $[Na^+]_{0.5}$ at 5 mol% of 50–100% (Fig. 6) instead of the 600% recorded. The $K_{0.5}$ values for Mg^{2+} and ATP, rising from 0.8 to 1.1 μM and 0.3 to 0.5 μM, respectively, were virtually unaffected.

Attempts were made to reveal a few additional characteristics of the non-competitive fraction of inhibition, such as time course of the inhibition, the effect of detergent (cholate) and effect of additional protein (bovine serum albumin, complexing, e.g., inhibitory fatty acid, generated by hydrolysis). Fig. 10 shows that the non-competitive fraction of the inhibition exerted by PS-substituted Eda increased with time. While PC proteoliposomes displayed a stable phosphorylation capacity over a period of more than 3 h from mixing enzyme and liposomes, inclusion of 5 mol% VIIa caused a first order non-competitive decrease ($k = 0.25$ h^{-1}). In view of this time course, phosphorylations commenced 2.5–3.5 h (fixed time per experiment) after

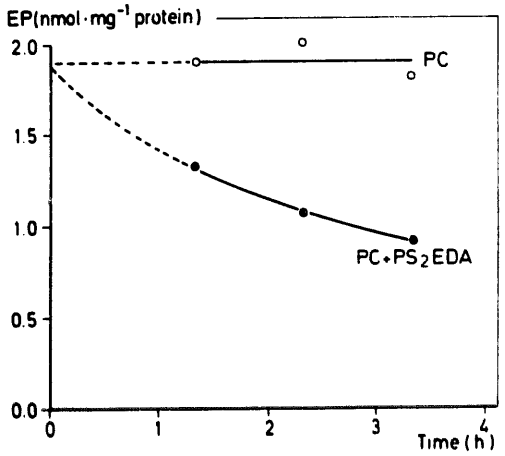

Fig. 10. Time-course of inhibition of Na^+-activated phosphorylation in PC-proteoliposomes by bis(phosphatidylseryl)ethylenediamine (VIIa, PS_2Eda). Upper line: control (○), lower curve (●) proteoliposomes, containing PC and 5 mol% PS_2Eda. Zero-time is the moment of mixing enzyme and liposomes; [Na^+] = 100 mM.

mixing enzyme and liposomes with an expected non-competitive inhibition between 45 and 59% (Fig. 10).

It is essential to discriminate between a genuine direct inhibition by VIIa and an indirect one, e.g., VIIa destabilising the enzyme or making it more prone to inactivation by cholate. The effect of increasing cholate on phosphorylation of VIIa containing PC proteoliposomes was, therefore, investigated and compared to PC proteoliposomes in the absence of VIIa (Fig. 11). The latter type of proteoliposomes underwent essentially more (48%) stimulation upon increasing cholate concentration to 0.5% than the VIIa-containing proteoliposomes (14%, lower graph of Fig. 11). This finding leads to the positive conclusion that cholate in the concentrations shown, was not inhibitory and that stimulation in the 3 min solubilisation period prevailed (upper curve in Fig. 11). The lower curve of Fig. 11 may raise the suspicion that either VIIa prevented cholate from opening the phospholipid bilayer or that VIIa itself had a detergent-like effect and prevented liposome closure. The liposomal volume as a percentage of the total suspension was, thus, determined according to the Co^{2+} calcein fluorescence quenching method of Oku et al. [16]. The liposomal volume of PC liposomes, containing 5 mol% VIIa was 25.1 ± 2.9% ($n = 4$) and, thus, was not significantly different from liposomes lacking VIIa: 23.7 ± 1.9% ($n = 3$). Each type of liposome totally lost its calcein fluorescence by Co^{2+} complexation upon addition of cholate (final concentration 0.5%) within 3 min (opening up of the liposomes). Hence, PS_2Eda had no detergent-like effect on liposomes nor did it prevent the detergent (cholate) from opening the vesicles.

Finally, the stabilising effect of addition of exogenous protein (fatty acid free bovine serum albumin, 15 mg/ml) during liposome preparation did not abolish the inhibition of VIIa (5 mol%)-containing proteoliposomes. The albumin enhanced the phosphorylation

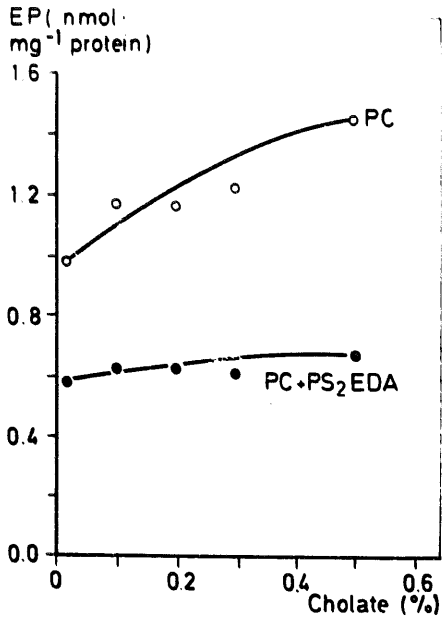

Fig. 11. Effect of cholate on the Na^+-activated phosphorylation in PC-proteoliposomes with and without PS_2Eda. Upper curve: control containing PC as sole exogenous phospholipid; lower curve: proteoliposomes, containing in addition 5 mol% bis(phosphatidylseryl)-ethylenediamine (VIIa, PS_2Eda). [Na^+] during phosphorylation was 100 mM.

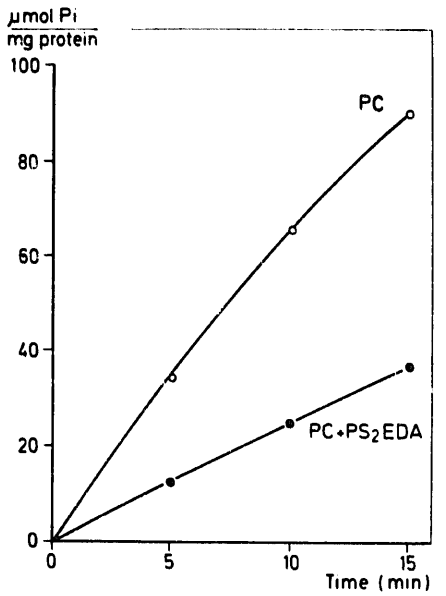

Fig. 12. Inhibition of overall Na /K^+-ATPase activity by bis(phosphatidylseryl)ethylenediamine (VIIa, PS_2Eda). Upper curve: control, containing PC as sole exogenous phospholipid; lower curve: proteoliposomes, containing in addition 5 mol% bis(phosphatidylseryl)ethylenediamine (PS_2Eda). The time course of P_i release (from ATP) is shown.

level under standard conditions (saturating ligands) of the control system (PC proteoliposomes) by 15–18%, and decreased the inhibition by VIIa from 62 to 34% (each 3.5 h from the start of making the proteoliposomes). It is, therefore, unlikely that free fatty acids were the cause of the non-competitive component of inhibition exerted by VIIa (Discussion).

Overall Na^+/K^+-ATPase activity in proteoliposomes

Since phosphorylation, as studied above, is not rate-limiting to overall Na^+/K^+-ATPase activity [47], it was interesting to know the effect of inhibitory VIIa on the overall activity. For instance, if the non-competitive effect of VIIa on phosphorylation is due to inactivation (i.e., cancelled enzyme molecules) or due to a hampered deocclusion of K^+ (a step prior to phosphorylation and rate-limiting [47]), then the appearance of a reduced phosphorylation level should be reflected in the overall activity. The latter appeared to be the case (Fig. 12). Despite a reduced control activity, displayed by PC proteoliposomes, which was about half that of the free enzyme (since cholate could not be used due to considerable inhibition at 37°C), the inhibition (64%) exerted by VIIa (5 mol%) was evident and of the same order of magnitude as that of the phosphorylation (Fig. 10).

Discussion

Phospholipids and conformational transitions

The present data clearly indicate that lipids have a profound influence on activation of Na^+/K^+-ATPase. Hitherto, it was established that fluidity of the membrane governs conformational transitions in the protein ($E_2 \rightarrow E_1$, $E_1P \rightarrow E_2P$), involved in cation transport through the lipid barrier with E_1 (E_2P) having the larger entropical freedom [48,49]. Lipid effects on cation activation might be interpreted in view of this simplifying principle of membrane fluidity, since E_1 has the higher affinity for the intracellularly-activating substrate and cations (ATP, Mg^{2+}, Na^+), and E_2 the higher affinity for the extracellularly-activating cation (K^+ [50]). A shift of the equilibrium to E_2 by phospholipids, represented by PS, as opposed to a shift to E_1 by low-affinity ATP binding, has been demonstrated by low-angle laser light scattering photometry [51]. These shifts were accompanied by association of the $\alpha\beta$-subunit protomer or $(\alpha\beta)_2$-dimer to higher aggregates and vice versa.

A shift to E_2 could explain the observed increased half-maximally-activating substrate and cation concentrations. Similar data can be found in the literature. For instance, the Na^+-ATPase-driven uncoupled Na^+ efflux from proteoliposomes (phospholipid/protein = 20:1, w/w) displays a K_m for Na^+ (after correction for inhibition by Mg^{2+} [45]) of 2 mM [52], as compared to 0.4 mM for the Na^+-ATPase activity without added phospholipid [53]. The increase in the K_m for ATP in the Na^+-Na^+ exchange (at about 100 mM Na^+), due to reconstitution, was only moderate (from 0.2 to 0.3 μM [54]), but the increase in the K_m for Na^+ was 4-fold (from 1.7 to 6.6 mM [55]). In agreement with our data on K^+, the affinity (K_m = 0.12 mM) for this cation in the Na^+-K^+ exchange at the outer face of the membrane was not altered by the addition of phospholipid (PC [56]). The inhibitory effect (half-maximal at about 10 mg phospholipid/mg protein) was also displayed by 'natural' phospholipids, extracted from rabbit kidney outer medulla microsomes (Fig. 7). Hence, the effect is not due, in this case, to a composition of the phospholipid deviating from the natural, but rather to a general conformational inhibition at above optimal level, such as that which may occur in the $E_2 \rightarrow E_1$ transition.

It is interesting that inhibition is related to ligands (Na^+, Mg^{2+}, ATP) that activate at the inner face of the membrane, from which the greater part of the enzyme protrudes [57]. This part may, therefore, undergo relatively more interaction with the added phospholipids than those sites allowing access to extracellular K^+.

Phospholipids and charge

Another reason for reduction in the affinity for activating ligands via screening of electrostatic forces exerted by negatively-charged phospholipids, has also been described [58]. In previous studies in our laboratory [6,7] on stimulatory and inhibitory effects of amino compounds in the phosphorylation reaction, we detected the strong inhibitory action of ethylenediamine, and established that the I_{50} for Eda is much less than that for propylamine with CH_3- substituted for the second -NH_2. Eda with one protonated amino group was less inhibitory than the dual-protonated form. Hence, we concluded that during inhibition Eda spans neighbouring negative charges involved in cation activation (carboxyl groups), which are 3–4 Å apart. Consequently, part of the inhibition caused by Eda may be due to such charge screening.

Binding of Eda to Na^+/K^+-ATPase was ouabain-insensitive [7], unlike Na^+ and K^+ binding [46], suggesting that the diamine binds to the lipid moiety for which ouabain displays no affinity. Nor do the K_i values for binding of Na^+ and K^+ (17–20 mM [7]) in their competition with Eda indicate binding to the protein in its E_1Na and E_2K conformation, respectively. This suggests that Eda indirectly inhibits cation activation by binding to the lipid moiety, and more specifically to the carboxyl groups of PS.

The main purpose of the organic synthetic work was to establish whether or not the product formed during cross-linkage between membrane lipids and Eda resulted in a mono- or di-substitution product with PS. Indeed, formation of a di-substituted product was es-

tablished, and this product, in agreement with the dual carboxyl occupation theory for Eda, inhibited the activation by Na^+ more strongly than the mono-substitution products. That the latter were not formed during cross-linking of Eda and the membrane lipids could mean that membrane PS is present in clusters near active sites, although a more simple explanation is that coupling was given ample time (16 h) to form dimers. Although we have not confirmed whether or not PS-Eda is formed as an intermediary during cross-linking, the major conclusion from this work is that the strongest inhibition in terms of apparent K_m for Na^+ is exerted by PS_2Eda. This may occur as a result of its stronger anchorage in the membrane, and/or the lipophilic areas of the enzyme.

PS_2Eda did not increase the inhibition of activation by Mg^{2+} and ATP over that exerted by PC alone. This in contrast to the effect of free Eda on the native enzyme with increases of $[Mg^{2+}]_{0.5}$ and $[ATP]_{0.5}$ from 0.1 to 13 μM and 0.02 to 0.04 μM, respectively [7]. In explanation, it may be proposed that free Eda interacts with additional sites on the protein besides those which are affected by PS_2Eda. An argument that PC had already seriously compromised the Mg^{2+} and ATP affinities so as to prevent further inhibition by PS_2Eda is unlikely since, firstly, PS_2Eda can further increase $[Na^+]_{0.5}$ and, secondly, $[Mg^{2+}]_{0.5}$ and $[ATP]_{0.5}$ have not reached their limits, and may also be increased, e.g., by free Eda (see above) and by K^+ [59], respectively. For the latter reason, as concluded before [7], activations by Mg^{2+} and ATP are at sites, probably within the large cytoplasmic loop between the 4th and 5th transmembrane segment [57], other than the Na^+-activation sites, which may be located in the 7th transmembrane domain [60].

Non-competitive inhibition

The natural phospholipids (PC, PE, PS and PA) only exerted a competitive effect on Na^+-activation, whereas the synthetic ones (PS-Eda, PS-Eda(Ser) and PS_2Eda) displayed an additional non-competitive effect, which could be reduced by exogenous protein (bovine serum albumin). Our data cannot discriminate between inactivation and partial non-competitive inhibition. Protection by exogenous protein suggests stabilisation against inactivation, rather than partial non-competitive inhibition. A feasible cause would be the presence of fatty acids in the synthetic phospholipids, exerting a detergent-like effect. Fatty acids could neither be detected in these preparations by TLC nor generated from them by the enzyme preparation, which is devoid of phospholipase A activity (unpublished experiments). In addition, inhibition by fatty acids, being virtually limited to the unsaturated species [61], does not occur whilst the concentration of Na^+/K^+-ATPase is ≥ 50 μg/ml and the concentration of oleic acid $\leq$ 100 μM [61]. This is related to the extremely high fatty acid binding capacity of the enzyme preparation itself: 662 nmol/mg protein or 263 mol/mol (60 mol/mol of which are contained by the enzyme already [23] as compared to 6 mol of high-affinity fatty acid binding sites per mol of bovine serum albumin with a preference of 1.7–4.0 for 16:0 over 18:1 [62]). It would imply that under the present conditions (0.1 mg Na^+/K^+-ATPase, 2 mg bovine serum albumin (mol. wt. 66500) per ml), a concentration of 150 μM oleic acid (derived from 331 μM VIIa) or 20% hydrolysis would be tolerated by the system. For the mono PS-substituted VIa and VIIIa the percentage hydrolysis could be twice as high. It seems impossible that such a high fatty acid concentration could escape detection on TLC and would probably have caused lysis of the liposomes, which did not occur. The non-competitive inhibition caused by PS-substituted ethylenediamine derivatives is, therefore, verified and is probably due to distortion of the enzyme, to rate limitation of one of the partial reactions involved, or a combination of the two.

That inhibition by PS_2Eda, in contrast to that by Eda^{2+} [7], is partially non-competitive may be due to the covalent binding of Eda to PS. In the latter case Na^+ is obviously unable to substitute for Eda and so unable to protect against the inactivation. Their common competitive inhibition of Na^+ activation underlines their functional similarity. Apparently, by contact with the enzyme, in hydrogen bonding or salt bridge formation, the serum albumin protects against the strains of inactivation. Poor activation of VIIa-containing proteoliposomes by detergent (Fig. 11) must mean that the active sites, hidden inside the liposomes, which are perforated by the detergent, are inhibitory sites (screened by covering with the lipid or inhibited conformationally by contact with the embedding lipid).

Conclusions

In conclusion, phospholipids are a prerequisite for the activity of Na^+/K^+-ATPase and at the same time inhibitory above optimal levels occurring in the native membrane or in a composition deviating from the natural as occurs upon administration of Eda-linked PS. Our data extend the functional role of phospholipids to beyond that of a simple matrix of fluidity, merely involved in allowing conformational changes, like $E_2 \rightarrow E_1$ and $E_1P \rightarrow E_2P$, to take place, which carry out cation transport. This action may depend on the fatty acyl side chains and their degree of unsaturation. In addition, our data on the inhibition by Eda-linked PS point to a second role of phospholipids, related to the polar headgroup and involved in cation activation. It is possible that blocking negatively-charged phospholipids (e.g., by Eda) in the neighbourhood of the protein may cause inhibition, for instance of occlusion of [63] and activation by [7] cations.

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