J. Membrane Biol. 133, 99–106 (1993)

The Journal of
Membrane
Biology

Molecular Species of Membrane Phospholipids Containing Arachidonic Acid and Linoleic Acid Contribute to the Interindividual Variability of Red Blood Cell Na^+-Li^+ Countertransport: In Vivo and In Vitro Evidence

Bernd Engelmann*, Jochen Duhm, Ulrike M. Schönthier, Sabine Streich, Jos A.F. Op den Kamp†, and Ben Roelofsen†

Physiology Institute, University of Munich, D-8000 Munich 2, Germany, and †Centre for Biomembranes and Lipid Enzymology, University of Utrecht, The Netherlands

Summary. Previous studies indicate a particular sensitivity of red blood cell Na^+-Li^+ countertransport activity to small variations in the fatty acid composition of membrane phospholipids. To assess whether the interindividual variability of Na^+-Li^+ countertransport is related to differences in the species pattern of erythrocyte phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in vivo, the molecular species composition of PC and PE as well as the kinetics of Na^+-Li^+ countertransport were analyzed in parallel in normo- and hyperlipidemic donors. Both in diacyl-PC and in diacyl-PE the species 16:0/20:4 and 16:0/18:2 were, respectively, positively and negatively related to the apparent maximal velocity of Na^+-Li^+ countertransport. The sum of all species with 20:4 at sn_2 of diacyl-PE exhibited a strong positive ($r = 0.82$, $2p < 0.001$), and those containing 18:2 a negative correlation ($r = -0.63$, $2p < 0.01$) to the transport activity. Essentially similar connections were observed between these species and the apparent affinity of the transport system for intracellular Na^+. To evaluate whether the associations between molecular species of membrane phospholipids and Na^+-Li^+ countertransport activity were indicative of a causal relationship, the species 16:0/20:4-PC and 16:0/18:2-PC were selectively introduced into the erythrocyte membrane by means of the PC-specific transfer protein. Replacement of 11% of native PC by 16:0/18:2-PC inhibited the transport rate by about 25%. Exchange of 6 and 9% of PC with 16:0/20:4-PC, in contrast, accelerated the transport rate by 30 and 60%, respectively. The accordance between the in vivo relations and the results of the in vitro modification strongly suggests that elevations and reductions in the arachidonic acid and linoleic acid content of membrane PC and PE contribute to the interindividual variability of red blood cell Na^+-Li^+ countertransport activity and its acceleration in hyperlipidemias.

Key Words red blood cells · Na^+-Li^+ countertransport · diacyl-phosphatidylcholine · diacyl-phosphatidylethanolamine · alkenylacyl-phosphatidylethanolamine · phosphatidylcholine-specific transfer-protein

Introduction

The activity of red blood cell Na^+-Li^+ countertransport is elevated in patients with essential hypertension [3], hyperlipidemias [5] and diabetic nephropathy [15]. Recent studies indicate that a high maximal velocity of countertransport may be associated with insulin resistance [6], a condition common to part of the patients with each of the above named cardiovascular risk factors [21]. A rise in Na^+-Li^+ countertransport has been proposed as a marker for those at high risk for cardiorenal complications associated with essential hypertension [19, 29].

The reasons for the pathological elevation and interindividual variability of Na^+-Li^+ countertransport in healthy individuals are not known. The maximal velocity of Na^+-Li^+ countertransport exhibits a phenotypically stable genetic determination [12, 26]. This could be interpreted to indicate that the increased activity observed in patients with cardiovascular risk factors is due to the presence of a higher number of transport molecules within the red blood cell membrane. Studies of Levy and Livne suggested, however, that the acceleration in Na^+-Li^+ countertransport may be caused by changes in the lipid composition of the erythrocyte membrane [14].

Two recent investigations indicate that Na^+-Li^+ countertransport is particularly sensitive to changes in the fatty acid composition of phosphatidylcholine (PC), the quantitatively dominant phospholipid of the human red blood cell membrane. Small variations in the molecular species[1] composition of PC

* *Correspondence address:* Physiologisches Institut, Universität München, Pettenkoferstr. 12, D-8000 München 2, Germany.

[1] Phospholipid molecules with defined fatty acid composition at the C_1- and C_2 atom (at sn_1 and sn_2) of the glycerol backbone.

were found to cause considerable alterations in Na^+-Li^+ countertransport activity [8, 9]. Thus, in principle, limited changes in the fatty acid composition could contribute to the acceleration of Na^+-Li^+ countertransport in red blood cells of patients with cardiovascular risk factors.

To assess whether the activity of the transport system was related to individual molecular species of erythrocyte phospholipids in vivo, the present study analyzed in parallel the molecular species composition of membrane PC and phosphatidylethanolamine (PE) as well as kinetic properties of Na^+-Li^+ countertransport (apparent maximal velocity and cation binding affinities). In erythrocytes from normolipidemic (control) and hyperlipidemic donors, positive and negative relations of the V_{max} values of Na^+-Li^+ countertransport were observed to molecular species of PC and PE containing, respectively, arachidonic acid (20:4) and linoleic acid (18:2) at sn_2. These results prompted us to investigate whether a selective replacement of the endogenous PC with species containing 20:4 or 18:2 at sn_2 would affect the transport system in a similar way as suggested by the above-mentioned relations.

Materials and Methods

Subjects

Red blood cells of 7 normolipidemic and 16 hyperlipidemic individuals were analyzed. Among the hyperlipidemic group there were 9 individuals with familial hypertriglyceridemia (phenotype IV), one with hyperchylomicronemia (phenotype V) and one with familial dyslipoproteinemia (phenotype III), these 11 donors being referred to as patients with elevated levels of triglyceride-rich lipoproteins; five patients had hypercholesterolemia (all with familial hypercholesterolemia (phenotype IIa)). As compared to normolipidemic donors, hyperlipidemic patients had higher levels of the following plasma lipoprotein parameters (mean values ± SD of normolipidemic (values given first in the parentheses) and hyperlipidemic donors (values given second in the parentheses)) (mmol/liter ± SD): total cholesterol (4.71 ± 0.66 *vs.* 7.49 ± 1.40), total triglycerides (0.75 ± 0.27 *vs.* 6.38 ± 6.23), LDL cholesterol (2.76 ± 0.54 *vs.* 3.72 ± 1.56), VLDL cholesterol (0.33 ± 0.16 *vs.* 2.63 ± 1.95) and lower levels for HDL cholesterol (1.53 ± 0.21 *vs.* 0.99 ± 0.31), all differences being statistically significant with $2p < 0.05$ or less.

There were no indications for further complicating diseases such as diabetes mellitus or thyroid dysfunctions. None of the women was pregnant or taking contraceptives. All individuals were normotensive with diastolic blood pressure below 90 mmHg and systolic blood pressure below 140 mmHg. Seven patients were on medical treatment at the time of the study. Blood was drawn from an antecubital vein of fasting donors between 8:00 and 9:00 a.m. and anticoagulated by heparin.

Molecular Species Analysis of Red Blood Cell PC and PE

The method used was essentially the same as the one described by Takamura et al. [25]. The lipids from erythrocytes, washed four times with an isotonic $MgCl_2$-sucrose buffer, were extracted according to Rose and Oklander [22]. PC and PE of 0.5–1 ml of packed erythrocytes were separated by one-dimensional thin layer chromatography (tlc) with the solvent $CHCl_3/CH_3OH/NH_3/H_2O$ (90:54:5.5:5.5, v:v) and extracted from the silica according to Arvidson [1]. The phospholipids were dispersed by sonication either in 2 ml of 50 mM tris-(hydroxymethyl)-aminomethane, 30 mM boric acid and 5 mM $CaCl_2$ (pH 7.4; PC) or in 2 ml of 30 mM K_2HPO_4, 30 mM boric acid (pH 7.0; PE), containing, in addition, 200 IU of phospholipase C (PLC, from *Chlostridium welchii* in the case of PC, from *Bacillus cereus* in the case of PE). Diethylether (4 ml) was added and the mixtures were incubated under argon for 8–12 hr at 37°C. One-dimensional tlc with diethylether/hexane (3:2) was employed to check for the completeness of the PLC-mediated formation of diradylglycerol.

For the derivatization of diradyglycerol, 25 mg of 3,5-dinitrobenzoylchloride and 1 mg of dry pyridine were added to the lipids which had been previously dried under vacuum. The mixture was heated for 10 min at 65°C. After immersion into an ice bath for 15 sec, 1 ml of ice-cold H_2O, 2 ml of ice-cold 0.1 M HCl and 2 ml of hexane were added. The water phase was reextracted two times with 2 ml of hexane, the combined hexane phases evaporated under N_2, and the sediment redissolved in a small aliquot of hexane. The sample was applied to high performance tlc plates (HPTLC, Merck Darmstadt, FRG) and developed in hexane/diethylether (7:3) in order to separate the different phospholipid subclasses (diacyl-PC, alkenylacyl- and diacyl-PE). The R_f values for the subclasses were 0.23 (diacyl) and 0.33 (alkenylacyl). The spots corresponding to the subclasses were extracted with diethylether. After evaporation of the ether phase the sediment was dissolved in 100 μl of acetonitrile/iso-propanol (8:2) for injection into the HPLC.

The separation of the dinitrobenzoyldiradylglycerol derivatives was performed by HPLC using a Knauer liquid chromatograph (HPLC pump 64, Knauer, Berlin) with detection at 254 nm by the use of a variable wavelength UV detector (Gynkotek, Germering, FRG). A Lichrosorb ODS column (10 μm, 4.6 × 250 mm, Gynkotek) served to separate the single molecular species at room temperature with the solvent acetonitrile/iso-propanol (8:2). The flow rate was 1 ml/min.

Identification of the molecular species was achieved by fatty acid analysis of the peaks collected and by comparison with retention time values published by Takamura et al. [25]. For determination of fatty acids, the DNBC-derivatives were hydrolyzed with 14% borontrifluoride in methanol [16]. The fatty acid methyl esters and dimethylacetals thus formed were analyzed by gas chromatography.

The percentages of individual molecular species of two samples obtained from the same donor at the same day treated independently from the start of the lipid extraction were found to agree within <7% for molecular species with percentages of >5% of total species composition.

In Vitro Modification of the Molecular Species Composition of PC

A limited replacement of endogenous erythrocyte PC by the molecular species 1-palmitoyl, 2-linoleoyl-PC and 1-palmitoyl, 2-

arachidonoyl-PC (16:0/18:2-PC and 16:0/20:4-PC, both obtained from Sigma, Deisenhofen, FRG) was performed essentially as described in ref. [8]. Briefly, donor vesicles containing the respective molecular species of PC, cholesterol, phosphatidic acid (45:45:10, mol:mol) and, in addition, ^{3}H-labeled cholesterol ether (as nonexchangeable marker) were incubated for 30–120 min with washed erythrocytes in the presence of the phosphatidylcholine-specific transfer protein (PC-Tp, isolated from bovine liver, *see ref.* [8]). The incorporation of the species was assessed by gas chromatographic analysis of the fatty acid composition of PC. Replacement of endogenous PC by the newly introduced species was calculated according to [27].

Determination of the Maximal Velocity of Na^+-Li^+ Countertransport

Na^+-Li^+ countertransport was assessed as phloretin-sensitive Li^+ uptake [7, 23]. Preincubation for three hours with four salicylate media containing different proportions of Na^+ and K^+ at 0°C (hematocrit 4–6%) [28] was used to modify the red blood cell Na^+ content between 4 and 26 mmol/liter at the expense of K^+. After two washings with isotonic NaCl and three further washings with choline chloride (room temperature), the cells were incubated for 90 min at 37°C (hematocrit about 5%) in isotonic choline chloride media containing 1, 2, 5, and 10 mM LiCl (replaced by choline), 0.1 mM ouabain, and either 0.25 mM phloretin (dissolved in ethanol) or ethanol alone. All media contained, in addition, 5 mM glucose, 1 mM inorganic phosphate, and 10 mM morpholinopropane sulfonic acid, titrated to pH 7.40 at 37°C with tris-(hydroxymethyl)-aminomethane (Tris-MOPS, 300–305 mosmol $(kg\ H_2O)^{-1}$).

Aliquots of fresh, preincubated and incubated cells (40–110 μl) were washed three times with a 20-fold excess of ice-cold 150 mM choline chloride. The cells were hemolyzed by adding 1.2–1.6 ml 6% *n*-butanol in water (v:v) containing 0.1% cesium chloride (w:v). In the hemolysates, hemoglobin was determined in duplicate (cyanmethemoglobin method) and Li^+, Na^+ and K^+ concentrations were measured in duplicate or triplicate using an atomic absorption spectrophotometer (Perkin Elmer 420). The standard deviations in duplicate measurements were 0.8% for hemoglobin, 1.9% for Li^+, 2.2% for Na^+ and 2.3% for K^+. The red blood cell cation contents and transport rates given refer to 5.2 mmol hemoglobin tetramer (335.4 g), the mean hemoglobin content of 1 liter of human erythrocytes. Comparable results were obtained when the incubation time of Li^+ uptake was 60 min.

The apparent kinetic parameters of Na^+-Li^+ countertransport (V_{max} and K_m values for intracellular Na^+ and extracellular Li^+) were calculated by means of Eadie-Hofstee plots with phloretin-sensitive Li^+ uptake as a function of either the four cellular Na^+ contents or of the four extracellular Li^+ concentrations. Most of the Eadie-Hofstee plots exhibited correlation coefficients with absolute values of $r > 0.98$. Exclusion criterion in the kinetic evaluation was an absolute r value <0.93. For estimation of kinetic parameters pertaining to saturating concentrations of both intracellular Na^+ and extracellular Li^+, the four values of maximal velocity obtained from plots of the experimental data as a function of cell Na^+ (medium Li^+) were plotted as a function of medium Li^+ (cell Na^+). The V_{max} values given in Results represent the means of the two V_{max} values thus obtained. The corresponding affinities for extracellular Li^+ and intracellular Na^+ ranged (±SD) between 4.03 ± 0.86 – 9.11 ± 0.61 mM Li^+ and between 8.78 ± 1.75 – 26.6 ± 2.69 mmol Na^+/liter cells among the different donors, respectively.

Determination of the Activity of Na^+-Li^+ Countertransport at 2 mM Extracellular Li^+

The method used was essentially the same as described in [8]. Briefly, erythrocytes which had been preincubated either with vesicles alone or, additionally, with the PC-Tp (*see above*), were washed three times with 150 mM choline chloride and thereafter incubated for 60 min at 37°C in media containing (mM) 75 $MgCl_2$, 90 sucrose, 10 Tris-MOPS, 5 glucose, 2 LiCl, 0.2 ouabain and either 0.25 phloretin (dissolved in ethanol) or ethanol alone (pH 7.4). The cellular L^+ and Na^+ concentrations were measured in butanol hemolysates and normalized to 5.2 mmol hemoglobin tetramer as described above. The data of phloretin-sensitive Li^+ uptake given in Results were corrected for a constant red blood cell Na^+ content of 10 mmol/liter by assuming simple Michaelis-Menten kinetics and an apparent K_m value of 10 mM cellular Na^+.

Results

To assess whether the maximal velocity of Na^+-Li^+ countertransport was related to individual molecular species of erythrocyte membrane phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in vivo, in normolipidemic and hyperlipidemic donors PC and PE were isolated from red blood cell lipids and analyzed for their molecular species composition by HPLC (*see* Materials and Methods). The separation of different molecular species from diacyl-PC and two subgroups of PE (diacyl- and alkenylacyl-PE) is illustrated in Fig. 1. In diacyl-PC the quantitatively predominant species contain linoleic acid (18:2) and oleic acid (18:1) at sn_2. Arachidonic acid (20:4) is present in high amounts at the sn_2 position of diacyl-PE and constitutes the major fatty acid at sn_2 of alkenylacyl-PE.

The maximal velocity of Na^+-Li^+ countertransport was positively related to the species 16:0/20:4 in diacyl-PC ($r = 0.53$, $2p < 0.05$), diacyl-PE ($r = 0.55$, $2p < 0.01$) and in alkenylacyl-PE ($r = 0.49$, $2p < 0.05$). A negative connection was observed with respect to the species 16:0/18:2 in both diacyl-PC ($r = -0.48$, $2p < 0.05$) and diacyl-PE ($r = -0.46$, $2p < 0.05$). The V_{max} value of the transport system was also positively associated with other species of diacyl-PE containing 20:4 (18:0/20:4: $r = 0.52$, $2p < 0.05$; 18:1/20:4: $r = 0.52$, $2p < 0.05$). In addition, a positive relation was observed to the species 18:0/20:4 ($r = 0.53$, $2p < 0.05$) in alkenylacyl-PE.

The maximal velocity of Na^+-Li^+ countertransport showed a positive correlation to the apparent affinity of the transport system for intracellular Na^+ ($K_mNa_i^+$; $r = 0.69$, $2p < 0.01$) while the relation to the affinity for extracellular Li^+ was not significant. The values for $K_mNa_i^+$ were positively related to

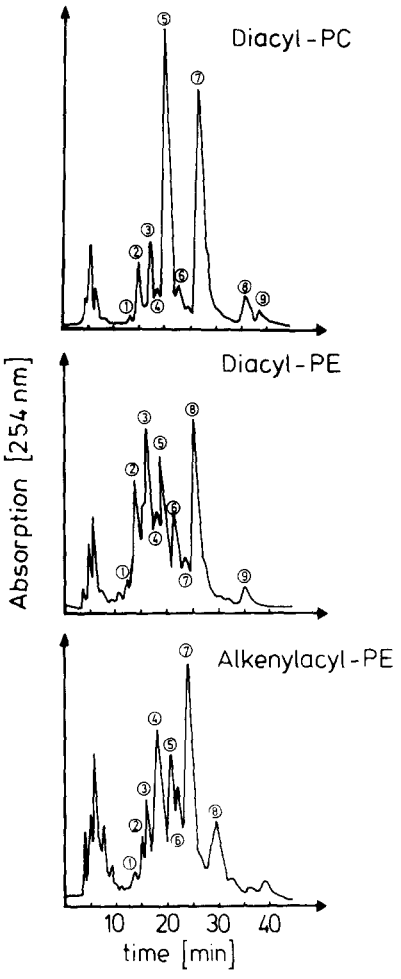

Fig. 1. Separation of molecular species of diacyl-phosphatidylcholine (PC), diacyl-phosphatidylethanolamine (PE) and alkenylacyl-PE by HPLC. Representative example from a donor with hypercholesterolemia. By means of gaschromatographic analysis of the peaks and comparison with retention times published by Takamura et al. [25] the following molecular species could be identified (the number before and after the slash indicate the fatty acids or fatty aldehydes at sn_1 and sn_2 of the glycerol backbone, respectively; percentages of total species in parentheses): **Diacyl-PC:** (*1*) 16:0/20:5 (0.6%). (*2*) 16:0/22:6 (5.5%). (*3*) 16:0/20:4 (8.5%). (*4*) 18:1/18:2 (4.2%). (*5*) 16:0/18:2 (35.5%). (*6*) 18:0/20:4 (4.8%). (*7*) 16:0/18:1 + 18:0/18:2 (33.7%). (*8*) 18:0/18:1 (4.6%). (*9*) 16:0/18:0 (2.5%). **Diacyl-PE:** (*1*) 16:0/22:6 (3.3%). (*2*) 18:1/20:4 (12.1%). (*3*) 16:0/20:4 (15.6%). (*4*) 18:1/18:2 (12.5%). (*5*) 16:0/18:2 (15.4%). (*6*) 18:0/20:4 (13.4%). (*7*) 18:1/18:1 (7.0%). (*8*) 16:0/18:1 (19.2%). (*9*) 18:0/18:1 (3.7%). **Alkenylacyl-PE:** (*1*) 16:0/20:5 (1.8%). (*2*) 18:1/22:6 (3.7%). (*3*) 16:0/22:6 + 18:1/20:4 (9.4%). (*4*) 16:0/20:4 (17.9%). (*5*) 18:0/22:6 (16.5%). (*6*) 16:0/22:4 (10.8%). (*7*) 18:0/20:4 (26.9%). (*8*) 18:0/22:4 (13.1%).

16:0/20:4 ($r = 0.47$, $2p < 0.05$) and to 18:0/20:4 in diacyl-PE ($r = 0.68$, $2p < 0.01$). A negative association was observed to 16:0/18:2 in diacyl-PE ($r = -0.54$, $2p < 0.05$).

When Na^+-Li^+ countertransport activity was assessed as phloretin-sensitive Li^+ uptake in media with 2 mM Li^+ in cells with Na^+ contents close to the in vivo values, the connections to molecular species of PC and PE were essentially similar as for the V_{max} values of the transport system (*not shown*). In all donors investigated there was a strong positive relation between the maximal velocity and the transport rate of Na^+-Li^+ countertransport determined at 2 mM Li_o^+ ($r = 0.79$, $2p < 0.001$).

The percentages of other molecular species (apart from those mentioned above) did not exhibit significant relations to the activity of Na^+-Li^+ countertransport. Notably, there were no significant connections between the percentages of species of diacyl-PC and diacyl-PE and the V_{max} values of two other Na^+ transport systems (Na^+-K^+ cotransport and Na^+-K^+ pump, *data not shown*).

A strong positive relation of the V_{max} values of Na^+-Li^+ countertransport was found to the sum of those molecular species of diacyl-PE with 20:4 at sn_2 (Fig. 2, upper panel), while a negative relation was observed to species with 18:2 at sn_2 of the same phospholipid subgroup (Fig. 2, lower panel). The correlations in Fig. 2 were also significant for the hyperlipidemic group alone (*see legend to* Fig. 2). The sum of species of diacyl-PE with arachidonic acid and linoleic acid, respectively, exhibited also positive and negative relations to the apparent K_m Na_i^+ values of Na^+-Li^+ countertransport ($r = 0.73$, $2p < 0.001$; $r = -0.54$, $2p < 0.01$). In addition, there were positive relations of the sum of those species of diacyl-PE containing 18:0 (stearic acid) at sn_1 to the apparent $K_m Na_i^+$ value ($r = 0.68$, $2p < 0.001$) as well as to the maximal velocity of Na^+-Li^+ countertransport ($r = 0.49$, $2p < 0.05$).

To assess whether the positive and negative relations of Na^+-Li^+ countertransport to molecular species of phospholipids containing arachidonate and linoleate were indicative of a causal relationship, part of the endogenous PC in the membrane of intact erythrocytes from normolipidemic donors was replaced by either 16:0/18:2-PC or 16:0/20:4-PC. Red cells were incubated for different time intervals with vesicles containing the respective species in the presence of the PC-specific transfer protein which mediates a strict 1:1 exchange of PC molecules located in the outer monolayer of the erythrocyte membrane with those present within the vesicles.

Replacement of 5% of endogenous red blood cell PC by 16:0/18:2-PC did not affect the transport rate of Na^+-Li^+ countertransport (Fig. 3, left panel). Higher incorporation levels reduced Na^+-Li^+ countertransport, the transport activity being decreased by about 25% when 11% of native PC was exchanged. Enrichment with 16:0/20:4-PC acceler-

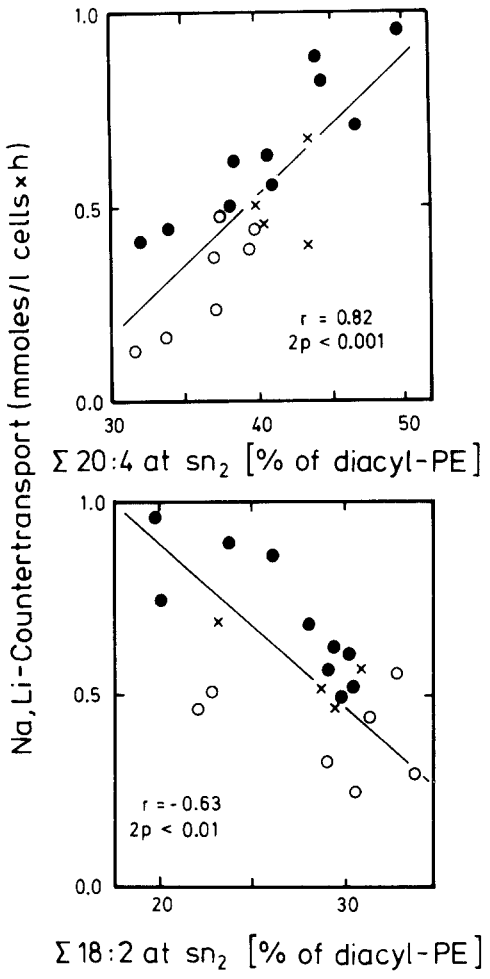

Fig. 2. Relations between the sum of percentages of molecular species with arachidonic acid (20 : 4) and linoleic acid (18 : 2) at sn_2 of diacyl-PE and the maximal velocity of Na^+-Li^+ countertransport in normolipidemic and hyperlipidemic donors. The molecular species containing 20 : 4 were: 16 : 0/20 : 4, 18 : 0/20 : 4 and 18 : 1/20 : 4, those with 18 : 2: 16 : 0/18 : 2 and 18 : 1/18 : 2. (○) Normolipidemia; (●) Patients with elevated levels of triglyceride-rich lipoproteins; (×) patients with hypercholesterolemia. When the relations were analyzed for the group of patients with elevated levels of triglyceride-rich lipoproteins alone, the correlation coefficients were: $r = 0.90$, $2p < 0.01$ (sum of species with 20 : 4) and $r = -0.82$, $2p < 0.01$ (species with 18 : 2).

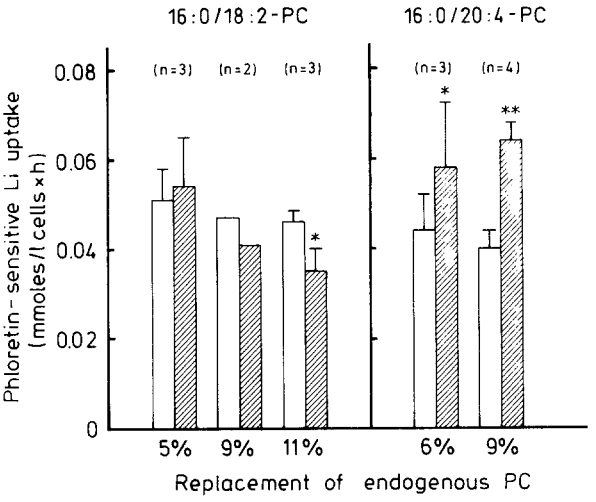

Fig. 3. Effect of replacement of native red blood cell PC by, respectively, 16 : 0/18 : 2-PC and 16 : 0/20 : 4-PC on the transport rate of Na^+-Li^+ countertransport (determined as phloretin-sensitive Li^+ uptake from media containing 2 mM Li^+, *see* Materials and Methods) in normolipidemic donors. Open bars: Erythrocytes treated with vesicles containing the respective molecular species (absence of the PC-specific transfer protein (PC-Tp), control). Hatched bars: Red blood cells incubated additionally with the PC-Tp. Replacement of 5–11% of native PC by 16 : 0/18 : 2-PC did not affect phloretin-resistant Li^+ uptake (control cells: 0.049 ± 0.014, modified cells: 0.048 ± 0.014 mmol/liter cells × hr, $n = 8$, ± SD). Exchange of 6 and 9% of endogenous PC by 16 : 0/20 : 4-PC slightly stimulated phloretin-resistant Li^+ uptake (control cells: 0.042 ± 0.008, modified cells: 0.046 ± 0.008 mmol/liter cells × hr, $n = 7$), the difference, however, not being significant. The enhancement of phloretin-resistant Li^+ uptake was small (+10%) compared to the 30–60% elevation of phloretin-sensitive Li^+ uptake indicating that the incorporation of 16 : 0/20 : 4-PC did not grossly perturb the determination of Na^+-Li^+ countertransport activity. Mean values ± SD $^*P < 0.05$, $^{**}P < 0.005$ (*vs.* control (without PC-Tp), Student's paired *t*-test, n = number of different donors).

ated the transport rate by 32 and 60% at replacement values of 6 and 9%, respectively (Fig. 3, right panel). Accordingly, in vitro incorporation of the two molecular species exerted an effect on the rate of Na^+-Li^+ countertransport corresponding to the relations observed in vivo.

Discussion

Previous studies indicated a particular sensitivity of the activity of the Na^+-Li^+ countertransport system to membrane lipids, in particular towards defined molecular species of phosphatidylcholine (PC) [8, 9]. To analyze whether the interindividual variability of the transport activity was in fact related to distinct molecular species of red cell membrane phospholipids in vivo, in the present study a detailed analysis of the molecular species composition of erythrocyte PC and phosphatidylethanolamine (PE) was performed in normo- and hyperlipidemic individuals, together with the determination of the kinetics of Na^+-Li^+ countertransport. PC and PE are the quantitatively most prominent phospholipids of the erythrocyte membrane comprising about 60% of total red blood cell phospholipids.

Molecular species from diacyl-PC (comprising approximately 95% of total PC in human erythrocytes [17]) and two subgroups of PE, diacyl- and alkenylacyl-PE, which constitute up to 95% of total

PE in red blood cells [17] were separated by HPLC. Previous analyses of the species composition of erythrocyte PC and PE subgroups using different derivatization procedures yielded comparable results with respect to both the nature and the percentages of the species analyzed [13, 17].

Positive correlations were observed in vivo between the percentages of the species 16 : 0/20 : 4 in each of the three phospholipid subgroups and the maximal activity of Na^+-Li^+ countertransport (*see* Results). Negative associations were found to 16 : 0/18 : 2 in diacyl-PC and diacyl-PE. The sum of species with 20 : 4 at sn_2 of diacyl-PE and of those with 18 : 2 at the sn_2 position of this phospholipid exhibited, respectively, strong positive and negative associations to the V_{max} values and binding affinities for intracellular Na^+ of the transport system (Fig. 2 and Results). The maximal activities of the Na^+-K^+ cotransport and the Na^+-K^+ pump were not related to any of the species of diacyl-PC and -PE. Taken together, these results indicate specific positive and negative associations between the interindividually differing activity of Na^+-Li^+ countertransport and the relative amounts of molecular species of PC and PE containing 20 : 4 and 18 : 2, respectively.

In addition, strong positive relations of the maximal velocity of Na^+-Li^+ countertransport to percentages of certain phospholipid classes were observed, i.e., to PC and the PC/sphingomyelin ratio [10]. Multiple regression analysis revealed that the association between the sum of species of diacyl-PE with arachidonic acid at sn_2 and Na^+-Li^+ countertransport activity was independent of the connection of the transport system to phospholipid classes.

To assess whether the changes in arachidonic acid and linoleic acid content of membrane phospholipids were causally related to the interindividual activity of Na^+-Li^+-countertransport, the molecular species composition of red blood cell membrane PC was modified in vitro. By means of the PC-specific transfer protein (PC-Tp) the species 16 : 0/20 : 4-PC and 16 : 0/18 : 2-PC were selectively incorporated into the erythrocyte membrane. Since, apart from the modification of the species composition of PC, no other lipid components of the membrane are altered by this procedure, potential effects on the transport rate can be specifically attributed to the replacement of native species by the newly introduced species. To avoid prolonged incubation of the erythrocytes preincubated with PC-Tp and vesicles, Na^+-Li^+-countertransport was directly assessed as phloretin-sensitive Li^+ uptake in erythrocytes with Na^+ contents close to the in vivo values (at 2 mM extracellular Li^+, *see* Materials and Methods). The values thus obtained were highly significantly related to the maximum velocities determined in the kinetic assay (*see* Results).

Replacement of 6 and 9% of endogenous PC by 16 : 0/20 : 4-PC accelerated the transport rate of Na^+-Li^+ countertransport by up to 60% (Fig. 3). Thus, a minute modification of less than 2% of total erythrocyte membrane lipids (assuming that total phospholipids make up approximately 50% of total membrane lipids and that PC constitutes about 30% of total phospholipids) led to a considerable increase in transport activity.

When 11% of endogenous PC was exchanged with 16 : 0/18 : 2-PC, the transport rate of Na^+-Li^+ countertransport was reduced by about 25% (Fig. 3). Again, a small experimental change in the species composition of PC considerably influenced the transport activity. It should be noted in this context that replacement of 11% of endogenous PC by 16 : 0/18 : 2-PC will increase the relative amount of this species in the erythrocyte membrane from about 30% (*see* legend to Fig. 1 and ref. [17]) to approximately 38% since part of the PC transferred from the cell membrane to the vesicles contains also 16 : 0/18 : 2-PC. Together with the positive and negative correlations between molecular species containing 20 : 4 and 18 : 2 and the maximal activity of Na^+-Li^+ countertransport observed in vivo (Fig. 2), these in vitro results argue for a causal relationship between the variations in molecular species composition of membrane PC and PE and the individual activity of Na^+-Li^+ countertransport in the normo- and hyperlipidemic donors studied.

Alterations in membrane fluidity are unlikely to account for the stimulatory and inhibitory effects of 16 : 0/20 : 4 and 16 : 0/18 : 2 on the activity of Na^+-Li^+ countertransport, since the transition temperatures of 18 : 2 and 20 : 4 are nearly identical [24]. Data from previous studies [8, 9], together with the present results, indicate that both a certain length and a minimal number of double bonds of the fatty acids in sn_2 position are required for activation of the transport. Only fatty acids at sn_2 with more than two double bonds and longer than 18 C atoms considerably accelerate the transport rate (20 : 4 and 22 : 6); shorter fatty acids are less effective or inhibitory (16 : 0, 18 : 1 and 18 : 2) ([8, 9] and this study). These results could indicate that a certain bilayer thickness is required for optimal activation of Na^+-Li^+ countertransport.

The activity of Na^+-Li^+ countertransport is elevated under several pathological conditions, all being well established cardiovascular risk factors (essential hypertension [3], hyperlipidemia [4, 5], diabetes mellitus [15]). Interestingly, an elevation of arachidonic acid, together with a reduction in lino-

leic acid in total membrane lipids, has already been observed in erythrocytes [20] and platelets [18] of patients with essential hypertension. Accordingly, these changes in the fatty acid composition could in part account for the acceleration of Na^+-Li^+ countertransport in essential hypertension.

Recent evidence indicates that the percentages of molecular species with arachidonic acid at sn_2 of PC and PE are increased in the red blood cell membrane in hyperlipidemias [11]. Plasma VLDL and total cholesterol levels correlated positively with red blood cell membrane phospholipid species containing arachidonic acid. Accordingly, the well-known genetic control of the individual activity of Na^+-Li^+ countertransport could ultimately be determined by the level of plasma lipoproteins which are known to be in part genetically regulated, e.g., by means of changes in membrane phospholipid species.

An association between an increase in Na^+-Li^+ countertransport activity and insulin resistance has been suggested [6]. It is well known that insulin resistance coupled with hyperinsulinemia is present in subgroups of patients suffering from essential hypertension, hyperlipidemia and diabetes mellitus [21]. An increased insulin concentration in plasma could lead to a rise in arachidonic acid content in membrane phospholipids, since the hormone—at least in diabetic animals—stimulates the formation of arachidonic acid from linoleic acid [2]. This would explain the association of a high percentage of arachidonic acid with a low content in linoleic acid in hyperlipidemia [11] as well as in essential hypertension [18, 20]. Thus, in view of the results of the present study, the removal of the inhibitory effect exerted by molecular species with linoleic acid at sn_2, as well as the activating influence of arachidonic acid, should accelerate synergistically Na^+-Li^+ countertransport activity under the above-mentioned pathological conditions.

In conclusion, the results of the present study indicate that an increase and a reduction of molecular species of membrane phospholipids containing arachidonic acid and of linoleic acid, respectively, contribute to the interindividual variability of red blood cell Na^+-Li^+ countertransport, its acceleration in hyperlipidemias and, possibly, also in essential hypertension.

The authors wish to thank Dr. W.O. Richter (II. Medizinische Klinik, Klinikum Großhadern, Universität München) for selection of the patients and Dr. T. Brosche (Universität Erlangen-Nürnberg) for gaschromatographic analyses. This study was supported in part by a grant of the Wilhelm-Sander-Stiftung to B.E.

References

1. Arvidson, G.A.E. 1968. Structural and metabolic heterogeneity of rat liver glycerophosphatides. *Eur. J. Biochem.* **4:**478–486
2. Brenner, R.R. 1990. Endocrine control of fatty acid desaturation. *Biochem. Soc. Trans.* **18:**773–775
3. Canessa, M., Adragna, N., Solomon, H.S., Connolly, T.M., Tosteson, D.C. 1980. Increased sodium-lithium countertransport in red cells of patients with essential hypertension. *N. Engl. J. Med.* **302:**772–776
4. Carr, S.J. Thomas, T.H., Laker, M.F., Wilkinson, R. 1990. Elevated sodium-lithium countertransport: a familial marker of hyperlipidaemia and hypertension? *J. Hypertension* **8:**139–146
5. Corrocher, R., Steinmayr, M., Ruzzenente, O., Brugnara, C., Bertinato, L., Mazzi, M., Furri, C., Bonfanti, F., de Sandre, G. 1985. Elevation of red cell sodium-lithium countertransport in hyperlipidemias. *Life Sci.* **36:**649–655
6. Doria, A., Fioretto, P., Avogaro, A., Carraro, A., Morocutti, A., Trevisan, R., Frigato, F., Crepaldi, G., Viberti, G.C. 1991. Insulin resistance is associated with high sodium lithium countertransport in essential hypertension. *Am. J. Physiol.* **261:**E684–E691
7. Duhm, J., Becker, B.F. 1977. Studies on the lithium transport across the red cell membrane. IV. Interindividual variations in the Na^+-dependent Li^+ countertransport system of human erythrocytes. *Pfluegers Arch.* **370:**211–219
8. Engelmann, B., Op den Kamp, J.A.F., Roelofsen, B. 1990. Replacement of molecular species of phosphatidylcholine: influence on erythrocyte Na transport. *Am. J. Physiol.* **258:**C682–C691
9. Engelmann, B., Duhm, J. 1991. Effect of cholesterol and dipalmitoyl phosphatidylcholine enrichment on the kinetics of Na^+-Li^+ exchange of human erythrocytes. *J. Membrane Biol.* **122:**231–238
10. Engelmann, B., Duhm, J., Schönthier, U.M., Streich, S. 1993. Relations of sodium-lithium countertransport kinetics to plasma and red cell membrane phospholipids in hyperlipidemia. *Atherosclerosis (in press)*
11. Engelmann, B., Schönthier, U.M., Richter, W.O., Duhm, J. 1992. Changes of membrane phospholipid composition of human erythrocytes in hyperlipidemias. II. Increases in distinct molecular species of phosphatidylethanolamine and phosphatidylcholine containing arachidonic acid. *Biochim. Biophys. Acta* **1165:**38–44
12. Hasstedt, S.J., Wu, L.L., Ash, K.O., Kuida, H., Williams, R.R. 1988. Hypertension and sodium-lithium countertransport in Utah pedigrees: Evidence for major locus inheritance. *Am. J. Hum. Genet.* **43:**14–22
13. Hullin, F., Bossant, M.-J., Salem, N., Jr. 1991. Aminophospholipid molecular species asymmetry in human erythrocyte plasma membrane. *Biochim. Biophys. Acta* **1061:**15–25
14. Levy, R., Livne, A. 1984. The erythrocyte membrane in essential hypertension. Characterization of the temperature dependence of lithium efflux. *Biochim. Biophys. Acta* **769:**41–48
15. Mangili, R., Bending, J.J., Scott, G., Li, L.K., Gupta, A., Viberti, G.C. 1988. Increased sodium-lithium countertransport in red cells of patients with insulin-dependent diabetes and nephropathy. *N. Engl. J. Med.* **318:**146–150
16. Morrison, W.R., Smith, I.M. 1964. Preparation of fatty acid methylesters and dimethylacetals from lipids with boron-trifluoride methanol. *J. Lip. Res.* **5:**600–608

17. Myher, J.J., Kuksis, A., Pind, S. 1989. Molecular species of glycerophospholipids and sphingomyelins of human erythrocytes: Improved method of analysis. *Lipids* **24:**396–407
18. Naftilan, A.J., Dzau, V.J., Loscalzo, J. 1986. Preliminary observations on abnormalities of membrane structure and function in essential hypertension. *Hypertension* **8(suppl. II):**174–179
19. Nosadini, R., Semplicini, A., Fioretto, P., Lusiani, L., Trevisan, R., Donadon, V., Zanette, G., Nicolosi, G.L., Dall'Aglio, V., Zanuttini, D., Viberti, G.C. 1990. Sodium-lithium countertransport and cardio-renal abnormalities in essential hypertension. *Hypertension* **18:**191–198
20. Ollerenshaw, J.D., Heagerty, A.M., Bing, R.F., Swales, J.D. 1987. Abnormalities of erythrocyte membrane fatty acid composition in human essential hypertension. *J. Hum. Hypertension* **1:**9–12
21. Reaven, G.M. 1988. The Banting lecture 1988: role of insulin resistance in human disease. *Diabetes* **37:**1595–1607
22. Rose, H.G., Oklander, M.J. 1965. Improved procedure for the extraction of lipids from human erythrocytes. *J. Lip. Res.* **65:**428–431
23. Sarkadi, B., Alifimoff, J.K., Gunn, R.B., Tosteson, D.C. 1978. Kinetics and stoichiometry of Na-dependent Li transport in human red blood cells. *J. Gen. Physiol.* **72:**249–265
24. Stubbs, C.D., Smith, A.D. 1984. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochim. Biophys. Acta* **779:**89–137
25. Takamura, H., Narita, H., Park, H.J., Tanaka, K., Matsuura, T., Kito, M. 1987. Differential hydrolysis of phospholipid molecular species during activation of human platelets with thrombin and collagen. *J. Biol. Chem.* **262:**2262–2269
26. Turner, S.T., Weidman, W.H., Michels, V.V., Reed, T.J., Ormson, C.L., Fuller, T., Sing, C.F. 1990. Distribution of sodium-lithium countertransport and blood pressure in Caucasians five to eighty-nine years of age. *Hypertension* **13:**378–391
27. Van Meer, G., Op den Kamp, J.A.F. 1982. Transbilayer movement of various phosphatidylcholine species in intact human erythrocytes. *J. Cell. Biochem.* **19:**193–204
28. Wieth, J.O. 1970. Paradoxical temperature dependence of sodium and potassium fluxes in human red cells. *J. Physiol.* **207:**563–580
29. Yap, L., Arrazola, A., Soria, F., Diez, J. 1989. Is there increased cardiovascular risk in essential hypertensive patients with abnormal kinetics of red blood cell sodium-lithium countertransport? *J. Hypertension* **7:**667–673

Received 2 September 1992; revised 7 December 1992