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Rapid Report

Accelerated maximal velocity of the red blood cell Na^+/K^+ pump in hyperlipidemia is related to increase in 1-palmitoyl,2-arachidonoyl-plasmalogen phosphatidylethanolamine

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Among several phospholipid classes and molecular species of phosphatidylcholine and phosphatidylethanolamine (PE) analyzed, only the percentage of the molecular species 1-palmitoyl,2-arachidonoyl (16:0/20:4)-plasmalogen-(alkenylacyl)-PE showed positive relations to the maximal activity and to the dissociation constant of the red blood cell Na^+/K^+ pump for Na^+ in normo- and hyperlipidemic donors. A preferential interaction of this molecular species with the Na^+/K^+ pump is proposed.

More than 80% of K^+ uptake and Na^+ extrusiuon across the erythrocyte and many other cell membranes is accomplished by the Na^+/K^+ pump and the Na^+/K^+ cotransport system. The two transport systems have been shown to be sensitive to changes in the membrane lipid composition, e.g., to experimental variations in the cholesterol content [1,2] and to small alterations in the molecular species composition of phosphatidylcholine (PC) induced by the PC-specific transfer protein from bovine liver [3]. It was of interest, therefore, to investigate whether the kinetic properties of both transport systems were related to physiological or pathophysiological variations in lipid composition of the membrane in vivo.

A detailed analysis of red cell membrane phospholipid composition indicated that the percentages of certain phospholipid classes and of distinct molecular species of PC and phosphatidylethanolamine (PE) within the erythrocyte membrane exhibit considerable variations among normolipidemic and hyperlipidemic donors [4,5]. The activities of the Na^+/K^+ cotransport system and, to a lesser extent, that of the Na^+/K^+ pump are also known to show some interindividual variability among healthy donors. Accordingly, it was assessed whether the variability of the transport activities is somehow related to the membrane phospholipid composition in normolipidemic and hyperlipidemic individuals.

Seven normolipidemic donors (2 men, 5 women, mean age 39.9 ± 17.6 years, body mass index (BMI) 21.3 ± 1.1 kg/m^2, mean $\pm$S.D.) and 17 hyperlipidemic individuals were included in the study. Among the hyperlipidemic group there were five individuals with familial hypercholesterolemia (type IIa, 3 men, 2 women, mean age 43.5 ± 3.0, BMI 26.3 ± 3.0), referred to as HCH, nine with familial hypertriglyceridemia (type IV), one with familial dyslipoproteinemia (type III) and two with hyperchylomicronemia (type V). The latter 12 donors are referred to as individuals with elevated levels of triglyceride-rich lipoproteins (HTG) (8 men, 4 women, mean age 51.2 ± 8.2, BMI 26.6 ± 4.5).

Red cell membrane lipids were extracted by the method of Rose and Oklander [6]. Phospholipids (phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin, phosphatidylserine and phosphatidylinositol, phosphatidic acid, lyso-PC) were separated on Merck (Darmstadt) DC Fertigplatten (Kieselgel 60) using the solvent systems proposed by Broekhuyse and quantified by phosphate analysis.

The plasmalogen content of PE was determined after acid hydrolysis of the enol-ether bond in sn_1 of plasmalogen PE as described elsewhere [4]. The percentages of phospholipid classes are published in Ref. 4. Molecular species analysis of phospholipids (diacyl-

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PC and -PE, alkenylacyl-(plasmalogen)-PE) was performed as described in Ref. 5. Briefly, lipid extracts of the erythrocyte membrane were subjected to one-dimensional thin-layer chromatography and the ester bond in sn_3 of the phospholipid classes was cleaved by phospholipase C from *Chlostridium welchii* (PC) and *Bacillus cereus* (PE). The diradylglycerol species were derivatized using 3,5-dinitrobenzoylchloride and the diacyl-PC, diacyl-PE and alkenylacyl-PE derivatives were separated on HPTLC plates (Merck, Darmstadt). The dinitrobenzoyl-diradylglycerol derivatives of the phospholipid subclasses thus obtained were dissolved in acetonitrile/isopropanol (8:2 (v/v) and separated by HPLC with detection at 254 nm. Data for percentages of molecular species are given in Ref. 5.

The kinetics of the Na^+/K^+ pump were assessed by measurements of ouabain-sensitive Rb^+ uptake and Na^+ extrusion in Na^+ media as a function of both intracellular Na^+ (Na_i^+) and extracellular Rb^+ (Rb_o^+). The reversible increase of Na^+ and K^+ permeability in isotonic salicylate media containing varying proportions of Na^+ and K^+ (0°C, 3 h) was applied to modify the cell Na^+ content between 4–5 and 25–30 mmol/l at the expense of K^+ [7]. The kinetic properties of the Na^+/K^+- cotransport system were estimated by determining bumetanide-sensitive Rb^+ uptake.

For the determination of the kinetics of the Na^+/K^+ pump the flux experiment was initiated by adding 100-μl aliquots of the suspensions of preincubated cells of four different Na^+ contents to 1.75 ml of a 135 mM NaCl/10 mM choline chloride buffer (pH 7.4 at 37°C) containing 0.5 mM, 1 mM, 2 mM and 5 mM RbCl (replaced by choline chloride) without and with 0.1 mM ouabain, respectively. The kinetics of the Na^+/K^+-cotransport system were assessed in the cells with the highest and lowest cell Na^+ contents achieved after preincubation in the salicylate media by measuring Rb^+ uptake sensitive to inhibition by bumetanide (10 μM) in the same Na^+ buffer containing 0.1 mM ouabain and 1 mM, 2 mM, 5 mM and 10 mM RbCl, respectively (replaced by choline chloride). All washing and incubation solutions contained, in addition to the constituents mentioned, 5 mM glucose, 1 mM inorganic phosphate and 10 mM Mops, titrated to pH 7.4 at 37°C with Tris (300–305 mosmol/kg H_2O).

After 90 min of incubation at 37°C the tubes were transferred into an ice bath and centrifuged in the cold (30 s, 14000 × *g*). 40–60-μl aliquots of cells were washed three times by suspending in 2 ml ice-cold isotonic choline chloride and centrifugation in the cold (30 s, 14000 × *g*). The cells were hemolyzed in 1.4–1.6 ml 6% n-butanol in water (v/v) containing 0.1% CsCl (w/v). In the hemolysates, hemoglobin was determined, and Na^+, K^+ and Rb^+ concentrations were measured by atomic absorption (Perkin Elmer 420). All measurements were performed in duplicate with standard deviations of ±0.5% for hemoglobin, ±2.2% for Na^+, ±2.3% for K^+ and ±2.4% for Rb^+. Essentially similar results were obtained with incubation times of 60 min. 90 min of incubation were chosen to improve the accuracy of measurements of changes in red cell Na^+ content.

TABLE I

Kinetic parameters of the Na^+/K^+ pump and of the Na^+/K^+ cotransport system in normo- and hyperlipidemia

HTG, elevated levels of triacylglycerol-rich lipoproteins; HCH, hypercholesterolemia. Values given are mean ± S.D. Ranges in parentheses. The apparent V_{max} and K_s values of the Na^+/K^+ pump refer to saturating concentrations of the other cation. $*P < 0.05$; $**P < 0.025$ unpaired Student's *t*-test (hyperlipidemia vs. control) or analysis of variance followed by Duncan's test (other comparisons).

	Control ($n = 7$	Hyperlipidemia ($n = 17$)	HTG ($n = 12$)	HCH ($n = 5$)
Na^+/K^+ pump				
V_{max,Rb^+} (mmol/l per h)	4.78 ± 0.67 (3.66–5.88)	5.22 ± 0.76 (3.99–6.39)	5.30 ± 0.78 (3.99–6.39)	5.04 ± 0.77 (4.29–5.93)
K_{s,Rb_o^+} (mM)	0.68 ± 0.11 (0.56–0.84)	0.70 ± 0.13 (0.40–0.90)	0.68 ± 0.12 (0.40–0.86)	0.74 ± 0.15 (0.53–0.90)
V_{max,Na^+} (mmol/l per h)	5.78 ± 1.16 (4.27–7.44)	7.06 ± 1.07 ** (5.16–9.19)	7.10 ± 0.86 * (5.42–8.22)	6.96 ± 1.60 (5.16–9.19)
K_{s,Na_i^+} (mmol/l)	3.03 ± 0.60 (1.91–3.56)	3.32 ± 0.54 (2.42–4.19)	3.35 ± 0.56 (2.42–4.19)	3.25 ± 0.51 (2.86–4.11)
Na^+/K^+ cotransport				
V_{max,Rb^+} (mmol/l per h)	0.50 ± 0.17 (0.26–0.75)	0.61 ± 0.32 (0.17–1.27)	0.70 ± 0.32 (0.38–1.27)	0.39 ± 0.22 (0.17–0.70)
K_{s,Rb_o^+} (mM)	3.24 ± 0.25 (2.88–3.57)	3.39 ± 0.39 (2.84–4.59)	3.49 ± 0.41 (3.12–4.59)	3.16 ± 0.22 (2.84–3.41)

In the calculation of the apparent kinetic parameters of the Na^+/K^+ pump a modified Michaelis-Menten equation was used:

$$V = V_{max}/(1 + K_s/[S])^n \quad (1)$$

where V is the actual velocity of ouabain-sensitive transport at the respective substrate concentrations [S] of Na_i^+ or K_o^+ (Rb_o^+). V_{max} and K_s are the apparent maximum velocity and apparent dissociation constant, and n is the number of substrates that need to be bound (3 when transport is studied as a function of Na_i^+ and 2 when transport is evaluated as a function of Rb_o^+). Rearrangement of the above equation yields

$$V = V_{max}^{1/n} - K_s \cdot V^{1/n}/[S] \quad (2)$$

The left side of this equation was plotted against $V^{1/n}/[S]$ (modified Eadie plot) and V_{max} and K_s values were calculated from the intercept with the ordinate ($a = V_{max}^{1/n}$) and the slope ($b = -K_s$). The experimental data of ouabain-sensitive Rb^+ uptake and Na^+ extrusion were plotted against both red cell Na^+ and medium Rb^+. The r values of the modified Eadie plots ranged from 0.93 to 0.99 and from 0.98 to 0.99 for Na^+ and Rb^+ transport, respectively. Accordingly, the mean standard deviations of the intercepts and slopes of the Eadie plots were greater for ouabain-sensitive Na^+ extrusion ($4.0 \pm 1.4\%$ and $14.4 \pm 8.7\%$) than for ouabain-sensitive Rb^+ uptake ($1.5 \pm 1.2\%$ and $6.6 \pm 4.1\%$, respectively).

When estimating the kinetic properties of Na^+/K^+ cotransport the apparent V_{max} value of bumetanide-sensitive Rb^+ uptake (calculated by use of the Eadie plot) was found to be increased in the high Na^+ cells (0.55 ± 0.18) as compared to the V_{max} of 0.44 ± 0.16 mmol Rb^+ uptake/l per h) in low-Na^+ cells. Simultaneously, the apparent dissociation constant increased from 2.67 ± 0.21 in low-Na^+ to 3.58 ± 0.49 mM Rb_o^+ in high-Na^+ cells. The above pattern of kinetic constants of Na^+/K^+ cotransport was seen without exception in all experiments. In Table I, the mean values of the parameters determined at the two red cell Na^+ contents are evaluated.

The kinetic parameters of the Na^+/K^+ pump and the Na^+/K^+ cotransport system of 7 normolipidemic and 17 hyperlipidemic donors are given in Table I. The maximal Na^+ extrusion through the Na^+/K^+ pump was accelerated by about 20% in all hyperlipidemic patients, as well as in the group of donors with elevated levels of triacylglycerol-rich lipoproteins (HTG), a tendency towards an increase also being seen in the small group of hypercholesterolemic individuals (HCH). Also the maximal rate of Rb^+ uptake mediated by the Na^+/K^+ pump as well as the values for the apparent dissociation constant for intracellular Na^+ (K_{s,Na_i}) tended to be increased in hyperlipidemia. No differences among the groups were observed for the apparent affinity for extracellular Rb^+. Similarly, no differences in kinetic properties of Na^+/K^+ cotransport were seen between normo- and hyperlipidemia (Table I).

As can be deduced from the ranges given in Table I, the kinetic parameters of the two Na^+-transport systems varied by up to three-fold among the different donors studied. For the same donors considerable variations were observed in the percentages of red blood cell membrane phospholipid classes, plasmalogen-PE, certain molecular species of PC and PE and of the cholesterol to phospholipid ratio [4,5]. Thus, it was assessed whether there were relations between membrane lipid parameters and the kinetics of the Na^+-transport systems.

No significant relations were seen between the kinetics of the two transport systems and the percentages of phospholipid classes, the plasmalogen content of PE or the cholesterol contents. The kinetic properties of the Na^+/K^+-cotransport system were not significantly related to any of the 26 molecular species analyzed (9 in diacyl-PC, 9 in diacyl-PE and 8 in plasmalogen-PE [5]). In contrast, the maximal velocity of both Rb^+ uptake and Na^+ extrusion mediated by the Na^+/K^+-pump exhibited a significant positive relation to the species 1-palmitoyl,2-arachidonoyl-(16 : 0/20 : 4)-plasmalogen-PE. The data for Rb^+ uptake are shown in Fig. 1. The relation was evident both in the HTG as well as in the HCH group (see legend to Fig. 1). For the V_{max} of ouabain-sensitive Na^+ extrusion a similar correlation was obtained ($r = 0.60$, $2P < 0.01$). Also,

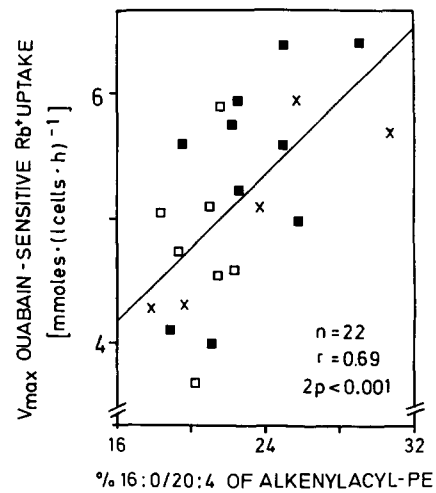

Fig. 1. Relation of the maximum velocity of ouabain-sensitive Rb^+ uptake (determined as a function of both extracellular Rb^+ and intracellular Na^+, see text) to the percentage of 16:0/20:4 in membrane plasmalogen PE. When the individuals with HTG and HCH were considered alone, the correlation coefficients were $r = 0.61$, $P < 0.05$ and $r = 0.88$, $P < 0.025$, respectively. ○, control; ●, HTG; ×, HCH.

the apparent affinity constant of the Na^+/K^+ pump for intracellular Na^+ showed a positive relation to the species 16:0/20:4 in plasmalogen-PE ($r = 0.54$, $2P < 0.01$). In multiple regression analysis including all phospholipid classes and molecular species analyzed (performed by using the SPSS/PC + program) only 16:0/20:4 in plasmalogen PE was significantly related to the maximal activity as well as to the apparent affinity of the Na^+/K^+ pump for intracellular Na^+. The apparent affinity constant of the Na^+/K^+ pump for extracellular Rb^+ was associated with 16:0/22:4 in plasmalogen PE ($r = -0.52$, $2P < 0.05$) and with 18:0/18:1 in diacyl PE ($r = -0.43$, $2P < 0.05$).

In hyperlipidemia, an increase in molecular species of erythrocyte membrane diacyl PC, diacyl- and alkenylacyl-PE with arachidonic acid in sn_2 has been noted previously [5]. The elevation of species with arachidonic acid was mainly restricted to the species containing palmitic acid at sn_1 (16:0/20:4), no differences being seen for the other quantitatively important species, namely 18:0/20:4. The results of the present study indicate that the (hitherto unknown) increase in maximal activity of the Na^+/K^+ pump in hyperlipidemia is most probably related to this rise in arachidonic acid in membrane phospholipids. More specifically, relations of the kinetic parameters of the Na^+/K^+ pump were observed to the species 16:0/20:4 in plasmalogen PE (Fig. 1 and text). No correlations were seen to molecular species with arachidonic acid in the same or other phospholipid subgroups. Furthermore, the specificity of these relations is reinforced by the lack of associations of the maximal velocity of the Na^+/K^+ pump to phospholipid classes or other molecular species of PC and PE.

On the basis of the results of the present study no conclusions can be drawn as to the causal nature of the results observed. However, a recent investigation indicates that more than 80% of the species 16:0/20:4 in plasmalogen-PE is present within the inner monolayer of the red blood cell membrane [8]: Thus, the putative preferential lipid–protein interaction of this plasmalogen species with membrane-embedded portions of the pump molecule could induce a conformational change of the protein, thereby hindering the access of intracellular Na^+ to its binding site.

An acceleration of Na^+/K^+ ATPase activity by PC molecules with arachidonic acid in sn_2 has already previously been noted in thymocytes [9]. In addition, in-vitro replacement of less than 10% of red blood cell membrane PC by 16:0/20:4-PC has also been shown to induce a slight increase in the Na^+/K^+ pump activity [3]. Accordingly, the phospholipid species containing a 16 C-atom chain in sn_1 and an arachidonic acid in sn_2 may provide an optimal molecular structure facilitating conformational changes of the pump protein, thereby increasing the maximal activity. In addition, the nature of the bond present in sn_1 of the phospholipid molecule could also play a role. This can probably be deduced from the observation that the kinetic parameters of the Na^+/K^+ pump were only related to 16:0/20:4 in plasmalogen PE and not to the corresponding species of diacyl PE. In this respect, it is worthwile to mention that a recent report has identified plasmalogen PE (together with anionic phospholipids) as a major component of lipids tightly bound to the sarcoplasmatic Ca^{2+} pump, e.g., after detergent treatment of the protein [10]. Since the genes for the different Ca^{2+} and Na^+/K^+ pumps are thought to have evolved from common ancestors, the two ATPases may share, apart from the well known activation of both proteins by anionic phospholipids [11], a preferential interaction with certain molecular species of plasmalogen PE.

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