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Sulfatide Role in the Sodium Pump

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Summary. Sodium efflux was studied in ^{22}Na-loaded red blood cells in the presence of arylsulfatase, an enzyme that specifically hydrolyzes sulfatide. Sodium efflux was inhibited in proportion to the amount of arylsulfatase present. Maximum inhibition was almost as high as the efflux obtained in medium with K^+ absent. At maximum inhibition 83.2% of the sulfatide content of the fragmented red blood cell membranes was hydrolyzed and ouabain-sensitive $(Na^+ + K^+)$-ATPase activity was inhibited by 100%. Sodium efflux, sulfatide content, and $(Na^+ + K^+)$-ATPase activity were unaffected with arylsulfatase in the presence of a high concentration of sulfatide. These results indicate that sulfatide plays a specific role in sodium and potassium ion transport. They also suggest that most sulfatide is localized externally in the red blood cell membrane.

Key words: Sulfatide, function, red cells, sodium transport

Analyses of the sulfatide content in rectal glands of spiny dogfish [10], bovine kidney medulla [9], salt glands of either duck or herring gull [11], and of human erythrocyte membranes [6] suggest that sulfatide may act as a lipid requirement for $(Na^+ + K^+)$-ATPase activity. In a previous paper [4] we showed that ouabain-sensitive ATPase activity in frog skin during larval development is fully inhibited. We also established a correlation between $(Na^+ + K^+)$-ATPase activity, sulfatide content, and sodium flux, indicating that sulfatide must be involved in the sodium pump mechanism. The sulfatide cofactor model [8] postulates affinity between sulfatide and potassium ions. As the affinity site for potassium on the external side of the membrane would be lacking when sulfatide is absent, total inhibition of potassium influx would be expected.

In the present paper we show that active sodium efflux in human red cells after sulfatide hydrolysis is reduced. This inhibition is of similar magnitude to that found in intact human red blood cells in the absence of external potassium.

Materials and Methods

Red blood cells isolated from heparinized fresh human blood, washed three times in isotonic saline solution, were loaded with ^{22}Na. The red cell loading, measurements of ^{22}Na loss, and determination of internal concentration of sodium ions of the loaded cells were carried out according to methods described previously [3].

^{22}Na-loaded red cells of final hematocrit of 1% were incubated for 10, 20, 40 and 60 min at 37 °C in Ringer's solution, potassium-free Ringer's solution, 100 mM potassium Ringer's solution, and Ringer's solution with ouabain (70 μM). The same procedure was repeated, adding arylsulfatase or microdispersed sulfatide in the medium. The Ringer's solution contained (in mM): 10, KCl; 145, NaCl; 1, $MgCl_2$; 1, $CaCl_2$; and 2.5, phosphate buffer (pH 7.4). In the potassium-free Ringer's solution or 100 mM potassium Ringer's solution, the NaCl concentration was adjusted to maintain the same ionic strength as that in normal Ringer's solution. Solid glucose was added to each incubation medium used at a final concentration of 11 mM. The arylsulfatase used [4] showed an enrichment of 1700-fold over the homogenate and a specific activity of 30 μmol of *p*-nitrocatechol sulfate hydrolyzed per min and per mg protein. It is necessary to point out that the analysis of mitochondrial phospholipids treated with 1.20 units of arylsulfatase by thin layer chromatography did not show any kind of lysocompound. Also, there is no protease contamination since rat kidney mitochondrial fraction crystalline bovine serum albumin fails to show protein breakdown after 60 min of incubation. Furthermore, mitochondrial enzymes like DPNH cytochrome *c* reductase and cytochrome *c* oxidase were not inhibited by the highest amount of arylsulfatase used in the assay.

Loss of ^{22}Na of red blood cells in Ringer's solution in the presence of 0.30, 0.60, and 1.20 units of the enzyme (a unit is defined as the amount of enzyme which hydrolyzes 1 μmol of *p*-nitrocatechol sulfate per min at 37 °C, pH 5.4) per ml of medium was measured. The highest amount of the enzyme used in the experiments was also added to the mediums containing ouabain or sulfatide micelles.

Sulfatides were obtained by extracting rat brain with 20 volumes of chloroform/methanol (2:1, vol/vol) at room temperature [14]. The residues were collected on a sintered glass, filtered, and

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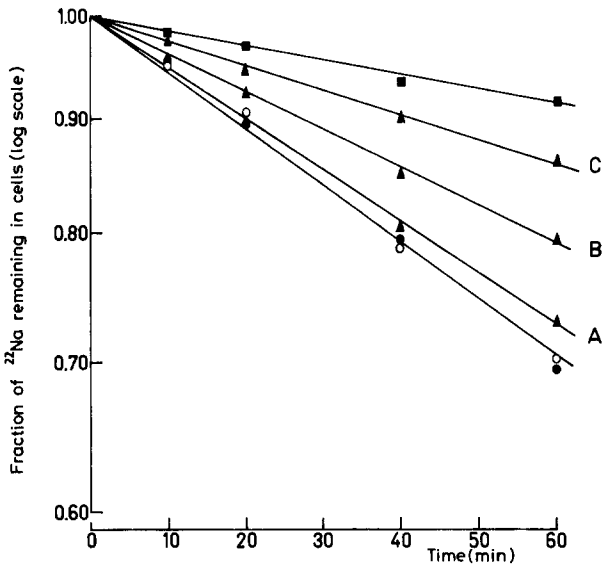

Fig. 1. The effect of arylsulfatase on the ^{22}Na loss from fresh human red blood cells. The cells were incubated for up to 1 hr at 37 °C. (●) Ringer's solution; (○) 100 mM K Ringer's solution; (■) Ringer's solution with ouabain (70 μM); (▲) Ringer's solution plus arylsulfatase: (*A*) 0.30 units/ml; (*B*) 0.60 units/ml; (*C*) 1.20 units/ml. The correlation coefficients ranged from 0.98 to 0.99

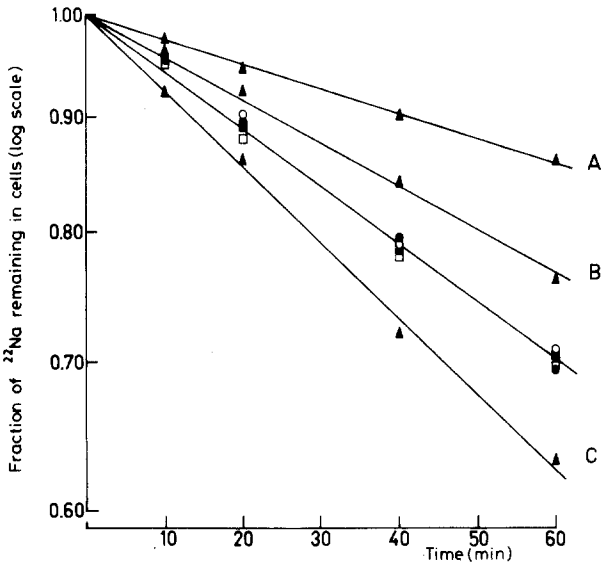

Fig. 2. The effect of sulfatide on the ^{22}Na loss from fresh human red blood cells. The cells were incubated for up to 1 hr at 37 °C. (●) Ringer's solution; Ringer's solution plus sulfatide: (■) 2.5 μg/ml; (□) 5.0 μg/ml; (○) 10.0 μg/ml; (▲) Ringer's solution-arylsulfatase 1.2 units/ml plus sulfatides: (*A*) 5.0 μg/ml; (*B*) 10.0 μg/ml; (*C*) 20.0 μg/ml

re-extracted with 10 vol chloroform/methanol. The two extracts were combined, evaporated to a small volume, and freed of nonlipid contaminants by chromatography on Sephadex G-25 [15].

The lipids, eluted with chloroform/methanol (19:1, vol/vol) saturated with water, were evaporated by means of a flash evaporator under reduced pressure to a moist residue and resuspended in a small volume of chloroform. Neutral lipid, cerebroside, and sulfatide were separated from the total lipid extract by silicic acid chromatography column (Unisil, Clarkson Chemical Co., Williamsport, Pa.), using as eluents chloroform, chloroform/acetone (1:1, vol/vol), and acetone, respectively [15]. The acetone-eluted sulfatide fraction was evaporated under nitrogen, and the moist residue was resuspended in a small volume of chloroform/methanol (2:1, vol/vol) and stored at −20 °C. The sulfatide thus obtained was microdispersed in aqueous medium by ultrasonic irradiation [1]. To clarify it, the microdispersion was spun down at 20,000 rpm in the N°40 Spinco rotor for 30 min. Sulfatide determinations were carried out as previously described [12], using bovine sulfatide (Applied Science Laboratories) as a standard.

$(Na^+ + K^+)$-ATPase activity at 37 °C was tested [4] in the fragmented membranes [5] of the loaded red cells incubated for 60 min in different mediums. To determine the sulfatide breakdown, sulfatide was extracted and determined in the loaded red cells incubated for 20, 40 and 60 min with 0.30 to 1.20 unit of arylsulfatase in Ringer's solution by the method mentioned above. The protein concentration was obtained using crystalline bovine serum albumin as a standard [13].

Radioactivity was measured in 1 ml of the supernatant dissolved in 10 ml of a Triton X-100-Toluene liquid scintillation mixture (333 ml Triton X-100, 666 ml Toluene, 4.0 g PPO and 0.05 g POPOP) in a Philips Spectrometer, P.W. 5403.

For each set of conditions the plots of ^{22}Na loss against time were extrapolated to zero time to estimate the extracellular ^{22}Na present at the beginning of the incubation period. The fractions of the initial intracellular radioactivity that remained inside the cells at each time were calculated and semilog plotted against time. Apparent rate constants were taken as the slope of the straight lines obtained. These values and the mean Na content values were used to calculate efflux.

Results

All figures show the fraction of ^{22}Na remaining in red cells, plotted as a function of time. The slopes of the curves give the apparent rate constants of the effluxes. Figure 1 represents the loss of ^{22}Na in Ringer's solution, 100 mM K Ringer's solution, ouabain, and in Ringer's solution with different amounts of arylsulfatase. This figure shows that as the amount of arylsulfatase increases, the slope of the ^{22}Na loss curve diminishes and approaches the slope of the curve for ^{22}Na loss in K-Free Ringer's solution (Fig. 3). The ^{22}Na loss curves for cells in Ringer's solution with microdispersed sulfatide with and without 1.20 units of arylsulfatase, respectively, are shown in Fig. 2. The different amounts of sulfatides used apparently do not change the slope of the curves. On the other hand, the action of arylsulfatase gradually diminishes when the sulfatide concentration increases in the medium. The slope of the ^{22}Na loss curve for 20.0 μg of sulfatide is higher than that obtained in Ringer's solution alone.

Figure 3 shows the curve in K-Free medium with 1.20 units of arylsulfatase, and the curves for a medium with 1.20 units of arylsulfatase in Ringer's solution or 100 mM K Ringer's solution, as well as the ^{22}Na loss curves in K-free medium with and without ouabain. It is evident that the slope of the ^{22}Na loss

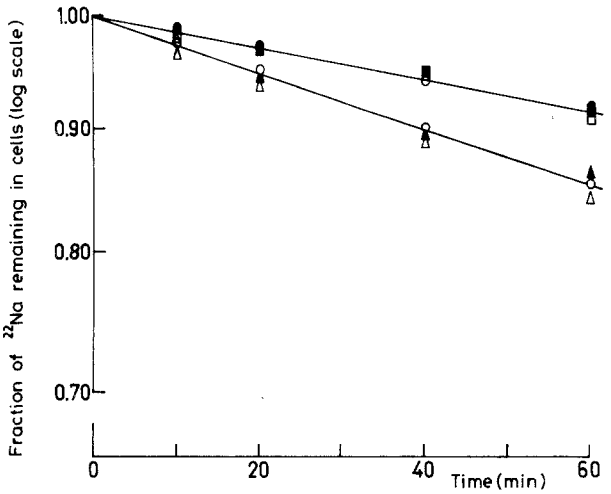

Fig. 3. The effect of 1.20 units/ml arylsulfatase on the ^{22}Na loss from fresh human red blood cells. The cells were incubated for up to 1 hr at 37 °C. (○) K-free Ringer's solution; (△) 100 mM K Ringer's solution plus 1.2 units arylsulfatase; K-free Ringer's solution with: (●) ouabain; (▲) 1.2 unit arylsulfatase; 1.2 units arylsulfatase plus ouabain in: (■) 10 mM K Ringer's solution; (□) 100 mM K Ringer's solution

curve with ouabain in Ringer's solution or 100 mM K Ringer's solution is unaffected by 1.20 units of arylsulfatase. Moreover, the slopes of the curves in the K-free medium with or without 1.20 units of arylsulfatase are very similar.

Effluxes calculated from the slopes of the ^{22}Na loss curves (figures) under the studied conditions are shown in Table 1. The highest amount of arylsulfatase in Ringer's solution caused a reduction of about 57% in the sodium efflux, which is close to the reduction observed in the K-free medium (52%). It may also be seen that with 1.20 units of arylsulfatase, the Na^+ effluxes from red cells in K-free Ringer's solution and Ringer's solution with or without ouabain were not significantly different. On the other hand, in normal Ringer's solution, 0.30 and 0.60 units of arylsulfatase reduce the sodium efflux to about 13 and 43%, respectively. Again, the highest amount of arylsulfatase added reduced the active sodium effluxes defined as the ouabain-sensitive sodium efflux to about 74%, while the reduction observed in the potassium-free medium is of the order of 72%. The sodium effluxes of red cells in Ringer's solution in the presence of sulfatide microdispersed with or without the addition of 1.20 units of arylsulfatase are shown in Table 2. The addition of 2.5 to 10.0 μg of sulfatide reduces the sodium efflux 1 to 4%, respectively. These amounts of sulfatide moreover produced 1.60 to 7.10% of hemolysis. Table 2 also shows that the inhibition induced by 1.20 units of arylsulfatase (Table 1) is diminished in the presence of sulfatide. Thus, 10 μg of sulfatide in the medium decreases the inhibition level of 61% of the sodium efflux caused by the arylsulfatase to about 20%, and with 20 μg of sulfatide the sodium efflux is 21% higher than the efflux in Ringer's solution alone, even with the enzyme present. It should be pointed out that when arylsulfatase is used in the medium in a concentration higher than 1.20 units, 36% of the cells were hemolyzed after 60 min after incubation. Under these conditions the efflux appears to diffuse freely.

Table 3 summarizes the sulfatide breakdown measured in the fragmented membrane isolated from the loaded red cells incubated for 20, 40 and 60 min in Ringer's solution with 1.20 units of arylsulfatase, respectively (i.e., from the same red cells from which the ^{22}Na loss curves were obtained), and the ($Na^+ + K^+$)-ATPase activity after 60 min incubation. The results show a typical enzymatic reduction. They also indicate that even the highest sulfatide breakdown level (83.2%) does not affect the ouabain-insensitive ATPase. On the other hand, the ouabain-sensitive ATPase is reduced by 31 and 51% with the use of

Table 1. Effect of arylsulfatase on sodium efflux in red blood cells

	Aryl-sulfatase (units/ml)	Ringer's solution	Inhibition (%)	Ouabain (70 μM)	Inhibition (%)	K-free medium	Inhibition (%)	100 mM K medium
Na efflux (mmol/liter cells/hr)	None	3.34		0.90	73.05	1.59	52.39	3.34
	0.30	2.91	12.87					
	0.60	2.20	34.13					
	1.20	1.45	56.59	0.85	74.55	1.53	54.19	1.61
Active Na efflux	None	2.44		0.00	100.00	0.69	71.72	2.44
(mmol/liter cells hr)	0.30	2.01	17.62					
	0.60	1.30	46.72					
	1.20	0.55	77.46	0.00	100.00	0.63	74.18	0.71

Na effluxes in red cells suspended in different media expressed as mmol/liter cells/hr. The values were calculated from the slopes of ^{22}Na loss curves (figures) and represent the mean of 4 experiments. The reproductibility of the estimated effluxes was better than ±0.03 mmol/liter cells/hr.

Table 2. Effect of sulfatide on sodium efflux in red blood cells

	Arylsulfatase (units/ml)	Sulfatide (μg/ml)	Ringer's solution	Inhibition (%)	Ouabain (70 μM)	Hemolysis range of values (%)
Na efflux	None	None	3.34	0.00	0.90	N.D.
(mmol/liter cells/hr)	None	2.5	3.31	0.90		0.00–1.60
	None	5.0	3.21	3.89		1.80–2.50
	1.20	5.0	1.31	60.78		N.D.
	None	10.0	3.22	3.59		5.40–7.10
	1.20	10.0	2.66	20.36		N.D.
	1.20	20.0	3.86	0.00		N.D.
Active Na efflux	None	None	2.44		100.00	
(mmol/liter cells/hr)	None	2.5	2.41	1.22		
	None	5.0	2.31	4.15		
	1.20	5.0	0.41	82.98		
	None	10.0	2.32	4.92		
	1.20	10.0	1.76	27.87		
	1.20	20.0	2.96	0.00		

Na effluxes of red cells in the presence of sulfatide microdispersion. Data are the mean values of 4 experiments expressed as mmol/liter cells/hr calculated from the sloped of ^{22}Na loss curves (figures).

N.D. = not detected.

Table 3. Effect of arylsulfatase on the loaded erythrocyte membranes

Arylsulfatase	Total ATPase	Ouabain-insensitive ATPase	% Sulfatide degradation after incubation (min)		
			20	40	60
None	1.39	0.94	N.D.	N.D.	N.D.
0.30 unit	1.26	0.95	21.5	40.1	58.9
0.60 unit	1.17	0.95	27.2	46.6	69.2
1.20 unit	0.93	0.93	32.6	57.1	83.2
1.20 unit plus 20 μg sulfatide	1.67	0.96	N.D.	N.D.	N.D.

The loaded red cells incubated for 20, 40 and 60 min at 37 °C in different mediums were fragmented. Half the final volume of 60 min was used to measure the ATPase activites. The other, 20 and 40 min, were extracted with chloroform/methanol (2:1, vol/vol). In the isolated glycolipid fraction, sulfatide amount was measured.

N.D. = not detected. Activities, expressed as μmol P_i/mg protein hr^{-1}. The sulfatide content of cell suspension was 7.75 μg/ml. Data are the average of 4 experiments.

0.30 and 0.60 units, respectively. Total inhibition of ouabain-sensitive ATPase is obtained with a 83.2% of sulfatide breakdown.

No breakdown of the structurally present sulfatide in the membrane was observed with 1.20 units of arylsulfatase in the incubation medium including 20 μg of sulfatide even after 60 min of incubation. Under these conditions ouabain-sensitive ATPase is increased by 53%. The sulfatide content of 1 ml cell suspension with a hematocrit of 1% was 7.75 μg (average of ten experiments). This value remains unchanged after 60 min incubation at 37 °C with or without 20 μg of sulfatide in Ringer's solution.

Discussion

Interest has been focused on the role of sulfatide in the sodium pump mechanism since red blood cell membranes have been shown to contain sulfatide, [6] and sulfatide hydrolysis known to inhibit ouabain-sensitive $(Na^+ + K^+)$-ATPase activity [4]. The present research compares normal sodium efflux with that obtained in the presence of different amounts of arylsulfatase.

The demonstration that membranes of loaded red cells treated with arylsulfatase show sulfatide breakdown, that cells are not hemolyzed when sodium efflux is reduced by about one third, and that the addition of sulfatide is able to protect the cells from enzyme action, seem to indicate that sulfatides are located externally in the red blood cell membrane and are involved in the sodium pump.

The breakdown of 83.2% of sulfatide that produced the highest reduction of the efflux seems to indicate that not all the sulfatide content functions in the sodium pump. The use of more than 1.20 units of enzyme hemolyses over 36% of the cells, and the ^{22}Na loss appears to occur by free diffusion. These results seem to confirm that sulfatide functions in maintaining the integrity of the membrane.

One-for-one sodium exchange and reduction in sodium efflux by about a third [7] has been shown

[3] in the absence of external potassium. Similar reduction is obtained in the presence of arylsulfatase.

In ATPase crude preparations treated with arylsulfatase, preliminary experiments show that the phosphorylation as well as the ouabain binding are blocked. On the other hand, it has been reported [2] that the ouabain binding site of the ATPase may involve a proteolipid component of the enzyme, but it is unknown if sulfatide is present in this component.

The cofactor site model [8] together with our sodium efflux findings in human red blood cells might suggest that sulfatides in the sodium pump are involved in specific K^+ binding or in the affinity site on the outside of the red cell membrane. Further potassium influx experiments are needed to distinguish between these two possibilities, since the evidence at present is too fragmentary for any clear choice.

Conclusions

The results reported in this work suggest the following conclusions:

a) Sulfatide is a lipid requirement for the sodium pump of the red blood cell.

b) The majority of the sulfatide content is located in the external monolayer of the red blood cell membrane.

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