Biochimica et Biophysica Acta, 727 (1983) 39–44
Elsevier Biomedical Press

BBA 71479

CHOLESTEROL MODULATION OF ($Na^+ + K^+$)-ATPase ATP HYDROLYZING ACTIVITY IN THE HUMAN ERYTHROCYTE

PHILIP L. YEAGLE

Department of Biochemistry, SUNY/Buffalo School of Medicine, Buffalo, NY 14214 (U.S.A.)

(Received May 6th, 1982)

Key words: ($Na^+ + K^+$)-ATPase; Cholesterol; Erythrocyte membrane; Vesicle; Lipid-protein interaction

The cholesterol content of human erythrocyte membranes has been modified by incubation of intact cells with sonicated egg phosphatidylcholine/cholesterol vesicles and with egg phosphatidylcholine vesicles. ($Na^+ + K^+$)-ATPase ATP hydrolyzing activity was measured as a function of membrane cholesterol content. High membrane cholesterol inhibits the ATPase activity of the enzyme and low membrane cholesterol activates that enzyme activity. The most likely mechanism of inhibition is suggested to comprise direct cholesterol-protein interactions which lead to a low activity conformation. Ouabain binding studies show that the inhibition is not due to a loss of enzyme from the membrane.

Cholesterol can influence a number of human erythrocyte properties. One of the most notable is an extensive folding of the plasma membrane due to increased membrane cholesterol levels [1]. Spur cell anemia can develop due to increased plasma cholesterol levels resulting from liver cirrhosis that leads to increased cholesterol/phospholipid ratios in the human erythrocyte membrane [2]. Recently, some transport functions of the membrane have been shown to be differently affected by varying the cholesterol content of the human erythrocyte membrane [3].

($Na^+ + K^+$)-ATPase hydrolyzing activity from other sources has been shown in recombinant membrane systems to be inhibited by cholesterol [4]. In the human erythrocyte membrane, conflicting views of the effects of cholesterol on the ($Na^+ + K^+$)-ATPase transport function have been advanced [1,5–7]. The purpose of this study was to look at the effect of membrane cholesterol on ($Na^+ + K^+$)-ATPase hydrolyzing activity. Since 20–50% of the total production of cellular ATP is used by the ($Na^+ + K^+$)-ATPase [8], any effects of membrane cholesterol on ATP utilization by the ($Na^+ + K^+$)-ATPase might have important consequences for cellular metabolism. In this study, membrane cholesterol levels are shown to modulate the ATP hydrolyzing activity of the ($Na^+ + K^+$)-ATPase in the human erythrocyte membrane, with high levels inhibiting the enzyme and low levels activating the enzyme relative to normal, unmodified erythrocytes.

Materials and Methods

Acrylamide, *N,N'*-methylenebisacrylamide, sodium dodecyl sulfate and other electrophoresis supplies were purchased in electrophoresis grade from Bio-Rad Laboratories. Triton X-100 was obtained from Calbiochem. Pyruvate kinase, lactate dehydrogenase, histidine, ouabain, NADH, ATP and ADP were obtained from Sigma. Cholesterol was obtained from Eastman and exhibited a single spot when analyzed on thin-layer chromatography in petroleum ether/diethyl ether/glacial acetic acid (90 : 10 : 1, v/v) visualized by acid charring. Egg phosphatidylcholine was supplied by Avanti Biochemicals and produced a single spot with two-di-

mensional thin-layer chromatography: (a) chloroform/methanol/ammonia (65 : 25 : 5) and (b) chloroform/acetone/methanol/acetic acid/water (6 : 8 : 2 : 2 : 1). The plates were developed by sulfuric acid charring. ^{3}H-labeled ouabain was obtained from New England Nuclear at a specific activity of 14.0 Ci/mmol.

Fresh blood was drawn after an overnight fast from normal human donors with no known recent medication history and between 22 and 35 years of age. No clinically obese individuals were among the group. Blood was drawn into a 1% EDTA solution in normal saline and used immediately. Erythrocytes and plasma were separated by centrifugation in an SS-34 rotor in an RC5-B superspeed centrifuge at $20000 \times g$ for 15 min at 5°C. The buffy coat was aspirated away.

Erythrocytes were washed with 10 vol. of the incubation buffer by recentrifugation as described above. The incubation buffer comprised 150 mM NaCl/5 mM KCl/5.5 mM Na_2HPO_4/0.8 mM NaH_2PO_4/0.5 mM $CaCl_2$/10 mM glucose (pH 7.4).

The following procedure was used to add cholesterol to the erythrocytes. A 1 : 1 weight ratio of egg phosphatidylcholine and cholesterol was dissolved in chloroform, dried under a stream of nitrogen and then under vacuum. A buffer with 150 mM NaCl/10 mM Tris (pH 8) was added and the lipid was suspended at 8 mg/ml by repeated pipetting with a Pasteur pipette at 23°C. This suspension was sonicated in an ice bath for three 5-min periods and centrifuged at 40000 rpm in a 50 rotor in a Beckman L5-50B ultracentrifuge at 5°C. The upper 85% of the supernatant was collected. Then 6 ml of the vesicles were added to 2 ml of the washed cells and 12 ml of the incubation buffer were added. The cells were incubated at 37°C for 18 h, under N_2, in a shaking water bath.

The following procedure was used to lower the cholesterol content of the erythrocytes. Lyophilized egg phosphatidylcholine was suspended in the incubation buffer at 3 mg/ml by repeated pipetting with a Pasteur pipette at 23°C. The suspension was sonicated and centrifuged as described above. To 2 ml washed cells were added 6 ml of the sonicated vesicles and 12 ml of the incubation buffer. The cells were incubated at 37°C for 18 h under N_2 in a shaking water bath. Normal cells were incubated in the same buffer, but without vesicles, at 37°C for 18 h under N_2 in a shaking water bath.

After the incubations described above, the cells were washed by centrifugation, as before, three times with the incubation buffer. Then, using the procedure of Dodge et al. [9], white or slightly pink ghosts were made. All buffers were purged with nitrogen before use to reduce oxidation. The ghosts were frozen and the $(Na^+ + K^+)$-ATPase assays were performed another day.

Analysis of ghosts

The ghosts were analyzed for $(Na^+ + K^+)$-ATPase activity as described below. They were analyzed for phosphate content by the method of Barlett [10], for protein according to Lowry et al. [11] using 3% sodium dodecyl sulfate, and for cholesterol by the enzymatic method of Allain et al. [12] in Triton X-100. The phospholipid content was examined with thin-layer chromatography of a chloroform/methanol (2 : 1) extract of the aqueous membrane preparation (4 : 1, organic/aqueous). Two-dimensional thin-layer chromatography was employed as described as described above on silica gel G plates. The plates were first developed with ninhydrin to locate phosphatidylethanolamine and phosphatidylserine. The plates were then charred after spraying with 50% sulfuric acid, to locate the sphingomyelin and the phosphatidylcholine. To determine the relative amount of phospholipid in each spot, the spots were scraped from the plate and a phosphate analysis was performed. A clear area of the plate was scraped to serve as a blank. Finally, the proteins of the membrane were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis as described below. All assays including electrophoresis, were performed in triplicate.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed in tube gels in a water-jacketed Buchler electrophoresis apparatus maintained at 15°C with a Fisher refrigerated water bath. A discontinuous system was employed using a 3% stacking gel at pH 8.8 on top of a 6% running gel at pH 6.8. Gels were stained with Coomassie blue and scanned on a Gilford gel

scanner at 550 nm. Photocopies of the gel scans were obtained, and the peaks cut and weighed to determine the relative amount of protein. All analyses were performed in triplicate.

$(Na^+ + K^+)$-ATPase assays

A coupled enzyme assay was used to measure $(Na^+ + K^+)$-ATPase activity at 37°C. The activity was measured in the assay buffer, with and without 0.1 mM ouabain. The assay buffer comprised 100 mM triethanolamine (pH 7.2), 5 mM $MgSO_4$, 100 mM NaCl, 25 mM KCl, 5 mM ATP, 0.15 mM phospho*enol*pyruvate, 0.2 mM NADH, 10 IU pyruvate kinase/ml and 20 IU lactate dehydrogenase/ml. The course of the reaction was monitored at 340 nm continuously and was linear for at least 0.5 h. In the buffer with ouabain, the KCl was omitted and NaCl substituted. Four different concentrations of enzyme (80, 120, 160 and 200 μg erythrocyte ghost protein per 1 ml assay buffer) were used in each assay, and the results were averaged. The activity with ouabain was subtracted from the activity without ouabain to obtain the $(Na^+ + K^+)$-ATPase activity. Known amounts (from solutions made from dry weight) of ADP were used to calibrate the system.

Ouabain binding

Binding of ^{3}H-labeled ouabain to normal cells and to cholesterol-enriched cells were measured. The cells were washed by centrifugation, as described above, three times with 140 mM choline chloride. Subsequently, the cells were washed into 150 mM NaCl/30 mM Hepes (pH 7.4). Packed cells were diluted 1 : 10 into this buffer. ^{3}H-labeled ouabain was added (2 ng) to 200-μl aliquots of the cells. To determine nonspecific binding, 100 μM ouabain was added in addition to the ^{3}H-labeled ouabain. The cells were then incubated at 37°C for 60 min. Following the incubation the cells were washed twice with 1 ml 140 mM choline chloride. To the final pellet were added 200 μl 5% trichloroacetic acid. The precipitate was spun down and the clear supernatant added to Liquiscint scintillation fluid and counted. Cells with cholesterol removed were not studied due to their fragility. Cell counts were obtained with a hemocytometer and a phase star microscope. Titrations with unlabeled or labeled ouabain showed that the sites were saturated under these conditions. All determinations were done in triplicate and the results averaged.

Results

The results of modifying the cholesterol content of human erythrocytes using the procedure described above can be seen in the abscissa of Fig. 2.

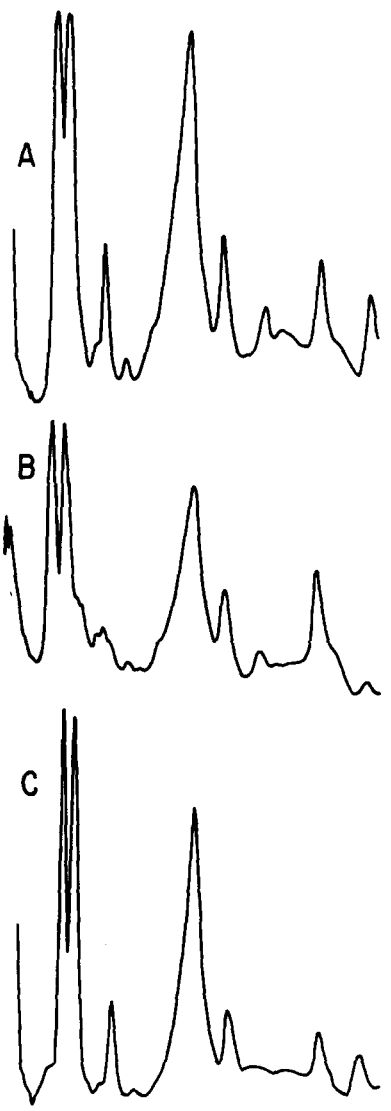

Fig. 1. Scans of sodium dodecyl sulfate polyacrylamide gel electrophoresis of human erythrocyte membrane protein performed as described in the text. (A) cholesterol-enriched membranes; (B) cholesterol-depleted membranes; (C) unmodified membranes.

TABLE I

PHOSPHOLIPID COMPOSITION OF ERYTHROCYTES (MOL%)

System	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-serine	Sphingo-myelin
Normal	30±5	30±3	19±3	26±1
Cholesterol-depleted	35±6	23±6	12±6	30±6
Cholesterol-enriched	32±3	29±6	16±4	31±1

The cholesterol/phospholipid ratio is considerably altered due to this procedure, which is essentially the same as that reported elsewhere [13]. While activity measurements proved consistent in all cases, the membranes with a lower cholesterol content were more fragile and frequently partially lysed before the ghosts were made.

The lack of other modifications to the membrane was demonstrated in two ways. Fig. 1 shows sodium dodecyl sulfate polyacrylamide gel electrophoresis results for each of the preparations from a typical experiment. No apparent change in membrane protein content is noted. Table I lists the phospholipid composition of the normal and cholesterol modified membranes, determined as described in Materials and Methods. No large changes in phospholipid composition are noted. These results indicate that in most cases no net flux of vesicle phosphatidylcholine into the red cell membrane occurred during the period in which a net flux of cholesterol either into or out of the cell occurred. Therefore, the only significant change in either lipid or protein content of these membranes is a change in the cholesterol content. (In a few experiments an increase of phosphatidylcholine content did occur in the cholesterol depleted membranes, but it did not appear to cause a variation in the activity results.)

This study focuses on the effects of such changes in cholesterol content on the rate of consumption of ATP by the enzymes of the erythrocyte plasma membrane. In particular, attention is centered on the $(Na^+ + K^+)$-ATPase.

$(Na^+ + K^+)$-ATPase activity

The activity of the $(Na^+ + K^+)$-ATPase was measured in the membranes with modified cholesterol content as described in Materials and Methods. Activities could then be expressed per mg erythrocyte ghost protein. However, this method for normalizing the activity did not prove to be satisfactory. Some hemoglobin appeared to bind to the membranes of cells depleted of cholesterol during the process of making ghosts, which produced a small, artificial increase in the protein content. Therefore, another method was needed. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of 40 μg erythrocyte ghost membrane protein from each preparation was performed as described in Materials and Methods. The gels of all the samples were made and stained at the same time with the same material to permit direct comparison. Three gels were run on each sample and the results were averaged. The intensity of band 3 was measured and this served as a means of comparing the amount of membrane material in each of the samples which had been assayed for $(Na^+ + K^+)$-ATPase activity. No change in the relative amounts of band 3, spectrin (bands 1 and 2), and actin were observed, indicating no change in the band 3 'concentration' in the

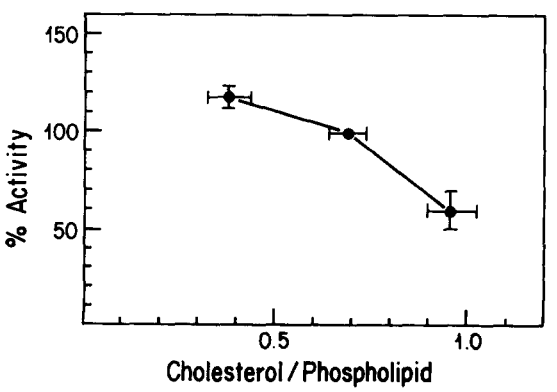

Fig. 2. $(Na^+ + K^+)$-ATPase ATP hydrolyzing activity in human erythrocyte ghosts as a function of cholesterol/phospholipid ratio in the membranes. The activity is presented as a percentage of the normal, as described in the text. The data represent the average of the data from six different individuals.

erythrocytes. This is further substantiated by the observation of no significant change in the phospholipid/protein ratio as determined by phosphate assay and assay by the method of Lowry et al. [11], between normal and cholesterol-enriched preparations. After adjusting the activity for the amount of membrane material determined as just described, the activities were recalculated as a percentage of the activity observed in the unmodified membranes. A summary of these data, obtained from six different individuals, is presented in Fig. 2. The activity in the cholesterol-enriched erythrocytes was significantly different from that in the cholesterol-depleted erythrocytes ($\alpha < 0.01$, two-tailed Student's t-test). The $(Na^+ + K^+)$-ATPase activity in a typical experiment without cholesterol modification was found to be $6.7 \cdot 10^{-3}$ μmol P_i/min per mg ghosts protein. This was normalized by total protein content and not by the procedure, described above, used to normalize the data in Fig. 2.

The normalization procedure using band 3 provides an accurate and simple means to compare activities provided that the number of $(Na^+ + K^+)$-ATPase enzyme molecules per cell does not change during the cholesterol modification experiments. Therefore, the total number of ouabain-binding sites per cell was determined for the normal cells and for the cells enriched in cholesterol. When the cells were depleted of cholesterol, they became too fragile to perform the ouabain-binding experiments. The results were calculated in terms of pmol ouabain/10^9 cells (normal = 0.19 ± 0.02; cholesterol-enriched = 0.20 ± 0.02). No significant difference in the amount of ouabain bound between the unmodified cells and the cholesterol-enriched cells was observed at saturation of the sites. This result is not surprising, because the $(Na^+ + K^+)$-ATPase is an integral membrane protein which requires detergent for its removal from the membrane, as is the case for band 3. Therefore, the activity changes seen in Fig. 2 are most likely due to specific activity changes and not to changes in the number of $(Na^+ + K^+)$-ATPase enzyme molecules in the membranes.

Discussion

These data show that high membrane cholesterol in the human erythrocyte inhibits and low membrane cholesterol stimulates the rate of hydrolysis of ATP by the $(Na^+ + K^+)$-ATPase. These data from the enzyme in its native membrane closely resemble the results for a $(Na^+ + K^+)$-ATPase from a different plasma membrane, reconstituted into a defined lipid medium [4]. Thus, the effect of high membrane cholesterol on plasma membrane $(Na^+ + K^+)$-ATPase may be systemic rather than peculiar to one tissue. This proposal needs to be tested experimentally.

At least three different mechanisms can be suggested for the inhibition of $(Na^+ + K^+)$-ATPase activity. One is that the freedom of movement of molecules in the membrane is restricted generally by the addition of cholesterol to a phospholipid bilayer. Such an effect is seen in permeability measurements of phospholipid bilayers containing cholesterol [14]. This general restriction, if felt by the protein, would reduce the conformational flexibility of the membrane-bound enzyme. If such flexibility was required for ATPase activity, then the hydrolysis of ATP by the enzyme would be inhibited by the addition of cholesterol to the membrane. This explanation is consistent with the data presented here, and has been suggested by others [4,15].

A second possibility would be to invoke a direct interaction between cholesterol and the $(Na^+ + K^+)$-ATPase which would modulate the activity. Direct interaction between cholesterol and proteins has been suggested for two other human erythrocyte membrane proteins, glycophorin (unpublished data) and band 3 [17].

A third mechanism that is possible is a change in the lateral distribution of membrane lipids induced by changes in membrane cholesterol content, which then modulates enzyme activity.

Several pieces of information are available which assist in distinguishing between these possibilities. It has been shown that a decrease in erythrocyte membrane cholesterol content causes an increase in the dissociation constant for internal Na^+ and an increase in the Na^+ flux [7]. No change in affinity for external cations was noted [7].

The changes in affinity for internal Na^+ could be mimicked by the addition of cationic and neutral amphiphiles. These resulted in a decrease in membrane order, as does removal of cholesterol, and an increase in internal Na^+ affinity [5]. Thus,

the first mechanism suggested above is useful for interpreting changes in internal Na^+ affinity [5]. External cation affinity does not change and is, therefore, insensitive to a change in membrane order [5].

A different mechanism must be invoked to explain changes in ATPase activity and Na^+ flux. High pressure, which increases membrane order, stimulates the ATPase activity [16] while cholesterol, which also increases membrane order, inhibits ATPase activity. Therefore, a simple change in membrane order cannot explain both effects. Na^+ flux is stimulated by a reduction in cholesterol [7], while cationic and neutral amphiphiles do not stimulate to the same extent when present at concentrations sufficient to cause changes in membrane order comparable to that resulting from cholesterol depletion [5].

The second mechanism above, however, is useful for interpreting these latter data. A direct cholesterol-($Na^+ + K^+$)-ATPase interaction could modulate ATPase activity by converting the enzyme to a low-activity form, independent of any changes in membrane order. This would provide a mechanism for inhibition by cholesterol and allow for a different mechanism, perhaps a pressure-induced protein conformation change, to explain the pressure effects on ATPase activity. A direct cholesterol-protein interaction has also been suggested to explain cholesterol effects on Na^+ flux [5].

The third possibility mentioned above is also a possible contribution, but adequate methods do not yet exist to investigate fully what role, if any, it may have.

Regardless of the mechanism, the inhibition of the ($Na^+ + K^+$)-ATPase by membrane cholesterol has fascinating implications for cellular metabolism. Since 20–50% of total cellular ATP produced in erythrocytes is used by the plasma membrane ($Na^+ + K^+$)-ATPase [8], any change in that usage would be important. Thus, cholesterol inhibition of the ($Na^+ + K^+$)-ATPase would lead to a reduced ATP demand, an effect which would feed back into glycolysis.

Several studies have shown that increased serum cholesterol levels can lead to increases in plasma membrane cholesterol in erythrocytes [18], platelets [2], macrophages [19], liver cells [20] and lymphoblasts [15]. Thus, increased serum cholesterol could cause a systemic decrease in plasma membrane ($Na^+ + K^+$)-ATPase usage of ATP. A possible metabolic effect of such a change is suggested by the correlation of a reduced number of ATPase sites per cell with clinical obesity in humans [21]. These suggestions will be explored with further experimentation.

Acknowledgments

I thank M. Lund for excellent technical work and Dr. G. Willsky for help in the ouabain-binding experiments. The work was supported by a grant from the National Institutes of Health (HL23853).

References

1 Cooper, R.A., Arner, E.C., Wiley, J.S. and Shattil, S.J. (1975) J. Clin. Invest. 55, 115–121
2 Cooper, R.A. (1969) J. Clin. Invest. 48, 1820–1827
3 Grunze, M., Forst, B. and Deuticke, R. (1980) Biochim. Biophys. Acta 600, 860–869
4 Kimelberg, H.K. and Papahadjopoulos, D. (1974) J. Biol. Chem. 249, 1071–1080
5 Giraud, F., Claret, M., Bruckdorfer, K.R. and Chailley, B. (1981) Biochim. Biophys. Acta 647, 249–258
6 Owen, J.S. and McIntyre, N. (1978) Biochim. Biophys. Acta 510, 168–176
7 Giraud, F., Claret, M. and Garay, R. (1976) Nature 264, 646–648
8 Whittam, R. and Blond, D.M. (1965) Biochem. J. 92, 147–158
9 Dodge, J.T., Mitchell, C.D. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–129
10 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–472
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–271
12 Allain, C.C., Poon, L.S., Chen, C.S.G., Richmond, W. and Fu, P.C. (1974) Clin. Chem. 20, 470–477
13 Lange, Y., Cutler, H.B. and Steck, T.L. (1980) J. Biol. Chem. 255, 9–31–9336
14 De Gier, J., Mandersloot, J.G. and Van Deenen, L.L.M. (1968) Biochim. Biophys. Acta 150, 666–672
15 Cooper, R.A. (1974) J. Supramol. Struct. 8, 413–430
16 Goldinger, J.M., Kang, B.S., Choo, Y.E., Paganelli, C.V. and Hong, S.K. (1980) J. Appl. Physiol. 49, 224–231
17 Klappaif, E. and Schubert, D. (1977) FEBS Lett. 80, 423–425
18 Cooper, R.A., Diloy-Pway, N., Lands, P. and Greenberg, M.S. (1977) Clin. Invest. 51, 3182–3190
19 Dianzani, M.U., Torrielli, M.V., Canuto, R.A., Garcia, R. and Feo, F. (1976) J. Path. 118, 193–199
20 Grandison, A.S. and Green, C. (1976) Biochem. Soc. Trans. 4, 645–646
21 De Luise, M., Blochbeon, G.L. and Flier, J.S. (1980) N. Engl. J. Med. 303, 1017–1027