DAILY RHYTHMIC CHANGES IN MG^{2+}-DEPENDENT ATPASE ACTIVITY IN HUMAN RED BLOOD CELL MEMBRANES IN VITRO

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SUMMARY

Outdated human red blood cells were kept in isotonic salt solution at 37°C. After membranes were prepared from samples taken every three hours the activity of the Mg-dependent ATPase was assayed. Significant daily changes in the enzyme activity (maximum at 8 h) indicate corresponding conformational changes in the membrane.

In the study of circadian rhythms mammalian erythrocytes offer several advantages. They are characterized by the absence of a cell nucleus, of mitochondria, and of protein synthesis, and can be kept in vitro without difficulty. If a circadian rhythm is observed in erythrocytes, as shown recently (1), its mechanism must be independent of the structures and functions mentioned above. This excludes some of the proposed models for the mechanism of the circadian clock (2,3), but allows further speculations on the relevance of membranes (4). Because several enzymes in red blood cells which appear to be reversibly bound to the membrane show circadian changes in their activities (1), it was considered possible that the binding and releasing process contributes to the mechanism of the basic oscillator. It was therefore of interest to study an enzyme such as the Mg-dependent ATPase, which is rather tightly bound to the membrane and supposed to show daily rhythmic changes in rat liver (5).

MATERIALS AND METHODS

Human blood bags with concentrated erythrocytes in ACD solution were obtained from the blood bank of the university clinics in Göttingen and used within 8 days after the expiration date. The bags contained blood of different donors. The red blood cells

were washed three times in a fivefold volume of isotonic NaCl, then suspended in the same volume of either NaCl or TKMS (64.5 mM tris(hydroxymethyl)aminomethane, 50 mM HCl, 25 mM KCl, 5mM $MgCl_2$, 250 mM sucrose) and incubated at 37°C. The incubation began at 11 h and was finished at 14 h on the next day. During this interval the erythrocytes were kept in the dark from 18 to 6 h. Every three hours a sample was taken and centrifuged for 5 min at 1000 g; the erythrocytes were then hemolysed by adding ten volumes of 10 mM tris-HCl/1 mM Na_2EDTA (pH 7.2) at room temperature - a procedure that avoids resealing of the membranes and retention of hemoglobin in the ghosts (6). A syringe was used for rapid mixing. After about 15 min the hemolysate was centrifuged 30 min at 16000 g. The remaining ghost pellet was resuspended and centrifuged again. This procedure was repeated until the ghosts showed a pink colour. The small red "button" at the bottom of the pellet was removed and the rest used to measure the Mg-ATPase activity. The assay was carried out at either 25 or 37°C with a system of auxiliary enzymes in a medium containing the following components: phosphoenolpyruvate (1.5 mM), $MgCl_2$ (4 mM), KCl (16 mM), NaCl (160 mM), tris-adenosine triphosphate (1.3 mM), reduced nicotineamideadeninedinucleotide (NADH) (0.4 mM), lactic dehydrogenase (EC 1.1.1.27) (10 U/ml), pyruvate kinase (EC 2.7.1.40) (1.3 U/ml), tris-HCl buffer (17 mM), ouabaine (g-strophantine) (0.8 mM). Ouabaine inhibits the (Na,K)-dependent ATPase. To a final volume of 1 ml 0.1 ml of the membrane suspension was added. Total protein concentration was determined according to Lowry et al. (7) using bovine serum albumin as a standard, hemoglobin concentration after the hemiglobincyanide method (8). The concentration of hemoglobin was then subtracted from the total protein content and the specific activity of the Mg-ATPase calculated with respect to the non-hemoglobin protein concentration.

RESULTS

The Mg-dependent ATPase in the membrane of human erythrocytes kept in vitro under light-dark conditions shows daily rhythmic changes in its activity with a maximum at 8 h. The difference between maximum and minimum is significant when applying a two-tailed t-test. The absolute values of the specific activity are different because of the different donors and different age of the blood bags; a rhythm, however, can be observed in all series even if the phase of the maximum may differ. The maximum value of the enzyme activity is found always between 2 and 8 h. The use of different incubation media had no influence on the rhythmicity. Changes in assay temperature markedly alter the activity level of the enzyme, indicating a striking temperature dependence of the ATPase.

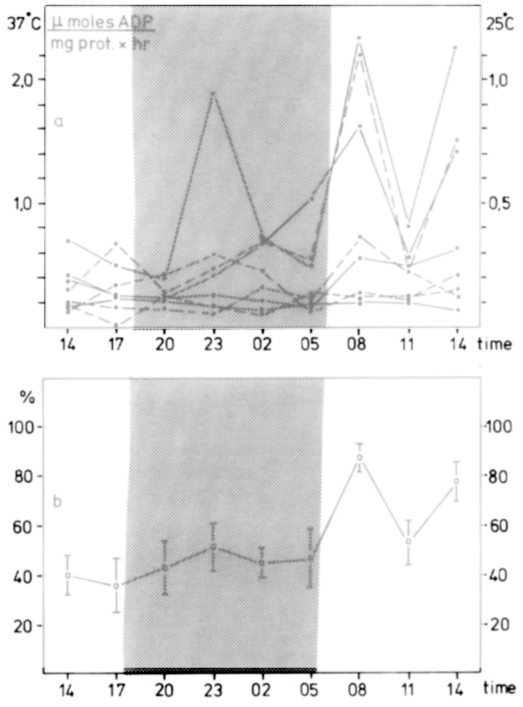

Figure 1. Daily rhythmic changes of the Mg-ATPase. (Shaded area indicates dark period.)

a Individual series of absolute activities, assayed at 25°C (——; right ordinate) or at 37°C (---; left ordinate).

b Mean values of relative activities, with standard error.

DISCUSSION

The results underline the importance of membranes for the oscillatory mechanism, although we have not yet analysed the Mg-dependent ATPase under constant conditions. The observed daily rhythmicity of this enzyme strongly indicates conformational changes of the membrane components that may cause the proposed reversible binding and release mechanism of other enzymes (1). It is possible that plasma lipoproteins in particular are involved in the control mechanism because they have been shown to affect Mg-ATPase activity (9).

As in the case of daily rhythmic changes of the isocitrate

dehydrogenase activity in isolated rat liver mitochondria which are controlled by changes of light and darkness (10) the influence of this factor on the Mg-dependent ATPase and on membrane conformation in general should be analysed.

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