proteolytic activity in the membrane lysates, spectrins and protein 3 very quickly undergo degradation, and in this case an increase in the number of spots is observed on the two-way gels. The possibility cannot be ruled out that the large number of polypeptides found by the workers cited above on two-dimensional gels, with the simultaneous absence of high-molecular-weight components, can be attributed to uncontrolled proteolysis. In fact, the number of proteins composing the human erythrocyte membrane is not so great.

The result of the present investigation was thus to obtain two-way gels of human erythrocyte membrane proteins by electrophoresis showing all the previously classified proteins and about 50 minor components. The preliminary protein map obtained can serve as the basis for further filling in of detail in the study of the protein composition of erythrocyte membranes and the compiling of a more complete catalog. The map which we obtained may perhaps also serve as a high-resolution tool in the search for the primary biochemical defect in corresponding hereditary diseases.

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CHANGES IN K^+, Na^+, and H^+ PERMEABILITY OF VESICLES FROM ISOLATED APICAL EPITHELIOCYTE MEMBRANES OF THE RABBIT SMALL INTESTINE DURING Ca-ACTIVATED LIPOLYSIS

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KEY WORDS: membrane permeability, epitheliocytes, lipolysis.

The system of membrane phospholipid metabolism in epitheliocytes of the mammalian small intestine contains Ca^{++}-activated phospholipases A_2 and A_1 (or lysophospholipase) [3]. No detailed information could be found on phospholipase compartmentalization in the literature. It can be postulated that these enzymes may participate in the regulation of epitheliocyte membrane permeability in the small intestine, at least under pathological conditions accompanies by de-energization of the cells, a raised Ca^{++} level in the cytosol, and impossibility of phospholipid resynthesis [1, 8].

With the above facts in mind it was interesting to study permeability of apical membranes of small intestinal epitheliocytes located on the boundary between the external and internal media of the body, and this was the aim of the present investigation.

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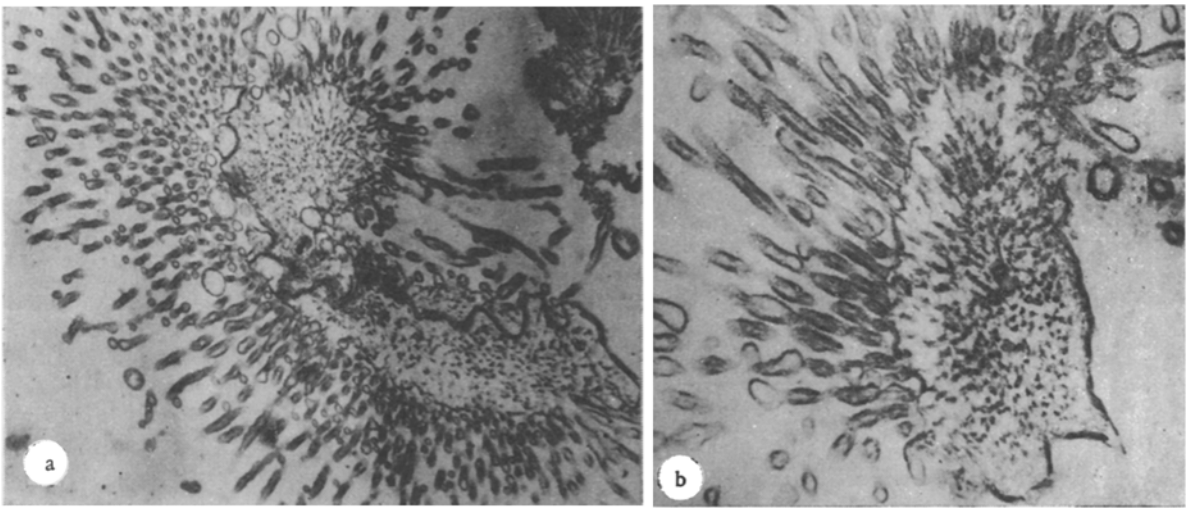

Fig. 1. Fraction of apical membranes of enterocytes of rabbit small intestine: a) 14,000 ×; b) 26,000 ×.

EXPERIMENTAL METHOD

Epitheliocyte apical membrane vesicles (EAMV) were isolated from the rabbit small intestine by the method in [10] in the writers' modification. Scrapings of the small intestinal mucosa of noninbred male rabbits deprived of food for 24 h, and weighing under 2 kg, were obtained in the cold and homogenized in medium containing 0.25 M sucrose, 1 mM EDTA, 0.5 mM dithiothreitol, and 2 mM HEPES, pH 7.4. The homogenate was centrifuged at 2000g (10 min), the residue removed from the supernatant was homogenized and centrifuged at 60g (10 min), and the residue thus obtained was removed. This operation was repeated twice. To obtain improved purification from mitochondria, the fraction was exposed for 2 min with $CaCl_2$ (1 mM), inorganic phosphate (10 mM), and sodium succinate (10 mM) at room temperature, then diluted in 4-5 volumes of ice-cold isolation medium, and centrifuged at 2000g (10 min); after repeated centrifugation at 2000g for 10 min the residue was collected. By treatment in this way the mitochondria were subjected to osmotic shock, as a result of active calcium accumulation. Further purification was carried out by centrifugation in a stepwise sucrose density gradient (45.7-50%), on which the previously purified fraction was layered, and centrifuged for 2 h at 100,000g. The fraction on the boundary between the sucrose layers contained 20-35 times greater alkaline phosphatase activity (determined with the aid of kits from Sigma, USA) than initially, and very low succinate dehydrogenase activity (determined by the method in [6]). Examination of the fraction thus obtained by transmission electron microscopy revealed mainly open fragments of brush border, i.e., apical plasmalemma of epitheliocytes with typical microvilli and a terminal network measuring up to 6-10 μ (Fig. 1). To obtain closed membrane vesicles, free from the terminal network [9], the fraction thus obtained was treated with ultrasound on the UZDN-1 apparatus (44 kHz, 50 mA) for 15 sec, three times, $MgCl_2$ (0.1 mM) was added, and the sample was centrifuged at 2000g for 10 min; vesicles were sedimented from the resulting supernatant at 4000g (10 min).

Changes in the volume of the EAMV were recorded by measuring trnsmittance of the suspension at a wavelength of 600 nm on a modified FÉK-56 photoelectric colorimeter.

Changes in permeability of the EAMV were studied by the osmotic compression—swelling method after addition of potassium or sodium acetate in the presence of carriers [2]: the K^+ carrier valinomycin, the H^+ carrier (CCCP), or the H^+/Na^+ exchanger gramicidin D (GCD).

Lipids were extracted [5] and re-extracted with diethyl ether, and the extracts were pooled and evaporated in vacuo.

Free fatty acids (FFA) were estimated with the aid of rhodamine 6G [7]. Thin-layer chromatography (TLC) was carried out on silica-gel 60-254 plates (Metschek, West Germany) in a solvent system of chloroform—methanol—water—heptane (65:25:4:9), and then in a system of ethanol—diethyl ether—water—acetone (5:80:5:10), and developed in vapor from crystalline iodine.

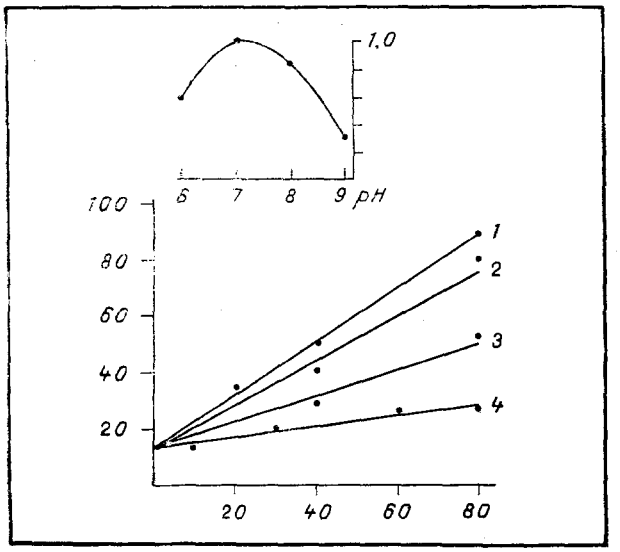

Fig. 2

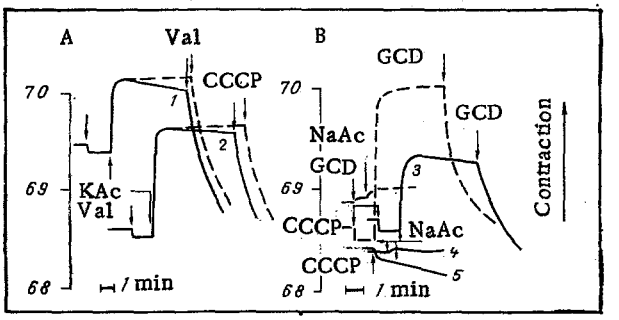

Fig. 3

Fig. 2. Dependence of rate of FFA release (in nmoles/mg protein) during Ca^{++}-activated lipolysis in preparations of apical membranes of rabbit enterocytes on pH. Abscissa, time (in min). pH values: 1) 7.0, 2) 8.0, 3) 6.0, 4) 9.0. Incubation medium: 110 mM KCl, 5 mM HEPES (at 6.0 and 7.0), or Tris (at pH 8.0 and 9.0). Results of 4-8 measurements given; standard error at each point does not exceed 10% of arithmetic mean.

Fig. 3. Changes in transmittance of suspension of apical membrane vesicles in response to introduction of potassium (A) and sodium (B) acetate after incubation of vesicles in presence of 1 mM $CaCl_2$. Vertical axis — transmittance (in %). Incubation medium: KCl (A) or NaCl (B) 10 mM, HEPES 1 mM, $CaCl_2$ 1 mM, EAMV 50 μg protein/ml, pH 7.4. N-ethylmaleimide (0.5 mM) was added to block specific carriers [11]. Arrows indicate addition of: potassium (KAc) or sodium (NaAc) acetate, 50 mM of each, valinomycin (Val) 5 μM, CCCP 5 μM, GCD 2 μM. Broken line indicates experiments with original EAMV, 1, 2) experiments with EAMV, preincubated for 1 h in KCl medium; 3) the same, in NaCl medium; 4, 5) the same as in (3) but preincubation time of EAMV was 90 min.

EXPERIMENTAL RESULTS

On incubation of the sonicated (but not purified from the terminal network) preparations of enterocyte brush border in the presence of 1 mM $CaCl_2$ (37°C) accumulation of FFA was observed with pH-optimum in a neutral medium. EDTA almost completely inhibited this lipolysis (Fig. 2). According to the results of TLC, no significant increase in the level of lysophospholipids could be detected, evidence of hydrolysis of phospholipids in the first and second positions, i.e., of activation of phospholipases A_1 and A_2.

The original FFA level in EAMV preparations purified from the terminal network was 40-50 nmoles/mg protein, or 12-15 moles % relative to phospholipids, rising to 40-50 moles % after incubation for 1 h.

Addition of potassium acetate to the suspension of original EAMV, in the absence of ionophores, or even after addition of CCCP or valinomycin (inducers of permeability for H^+ and K^+, respectively) beforehand, led to an increase in transmittance, evidence of shrinking of the vesicles (the broken line in Fig. 3A), without subsequent swelling due to the absence of any marked permeability of the vesicles for both K^+ and H^+. If an inadequate type of permeability is induced by introduction of the second ionophore, the EAMV quickly swell.

Preincubation of the vesicles for 30 min with Ca^{++}, during which FFA accumulated to the amount of 25-30 moles % relative to phospholipids, was not accompanied by changes in the osmotic behavior of the EAMV. Preincubation of the EAMV with Ca^{++} for 1 h had the result that after contraction of the vesicles in response to introduction of potassium acetate, they began to swell in the presence of both valinomycin and CCCP, although the rate of swelling was low compared with maximal (observed after addition of the second ionophore) (Fig. 3A: 1, 2). This is evidence of the appearance of marked permeability of the EAMV for both H^+ and K^+. Os-

motic responses of the EAMV in medium with sodium acetate were rather stronger (Fig. 3B). On addition of sodium acetate to the suspension of original vesicles, preceded by CCCP, just as in the first case contraction of the vesicles was observed without their subsequent swelling, which could later be abolished by the addition of GCD, whereas addition of sodium acetate, preceded by GCD, did not cause contraction of the vesicles, as was to be expected. If, however, the EAMV were preincubated with Ca^{++} for 1 h, addition of sodium acetate after CCCP was accompanied by an increase in transmittance of the suspension by a smaller amount than in the control, followed by a decrease of transmittance at an appreciable rate, evidence of permeability of the vesicles for Na^{+} (Fig. 3B: 3). After incubation of the EAMV with Ca^{++} for 1.5 h, no marked osmotic responses could be found to addition of sodium acetate (Fig. 3B: 4, 5), just as to addition of potassium acetate, evidence of a sharp increase in peremability of the EAMV for cations.

Significant changes in permeability of the EAMV were thus observed during deep (more than 50 moles %) hydrolysis of phospholipids. This was evidently related to two circumstances: the fact that the plasmalemma of epitheliocytes is rich in cholesterol, and the fact that no significant quantities of lysophospholipids, which are more effective detergents than FFA [3, 4], are formed during hydrolysis of phospholipids. An example of this, which stands out clearly, is the sharp increase in permeability of the inner mitochondrial membrane (containing trace quantities of cholesterol) during hydrolysis of as little as 10 moles % phospholipids by endogenous phospholipase A_2, leading to the formation of FFA and lysophospholipids [8]. Meanwhile phospholipase activity in the brush border of rabbit epitheliocytes is sufficiently high and may participate in the development of cell necrosis. The intensity of the changes of permeability under these circumstances will actually depend on the absolute and relative levels of hydrolysis products of phospholipids (FFA and lysophospholipids) which, in turn, will depend on the ratio between the levels of phospholipases A_2 and A_1 (lysophospholipase).

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