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Properties of Plasma Membranes from Granulation Tissue with Reference to Extracellular Matrix

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Treatment with neuraminidase decreased the activity of Na^+,K^+-activated Mg^{2+}-adenosine triphosphatase in plasma membranes isolated from experimental granulation tissue but not that of 5′-nucleotidase or leucine-β-naphthylamidase. A temporary lowering of the pH of the plasma membrane suspension to 2 – 3 inactivated all three enzymes, which remained inactive after the pH had been readjusted to 7.4. Addition of dextran preparations to the membrane suspension decreased the activity of adenosine triphosphatase. Ethanol (0.4 %) had a similar effect. These marker enzymes of plasma membranes were not affected by additions of hyaluronate, chondroitin sulfate, protein polysaccharide or soluble collagen. Serotonin stimulated the adenosine triphosphatase activity slightly.

About 10 – 20 % of the protein in the plasma membrane preparation was extracted with EDTA. This "fuzzy coat" fraction yielded a distinct gel-electrophoretic protein pattern. Hyaluronidase was not helpful in cleaving this surface layer from the plasma membranes.

The main purpose of the present work was to explore the effects of extracellular components on the isolated plasma membranes of the granulation tissue. The activities of the marker enzymes were considered as the most sensitive parameters of the membrane functions. The extracellular matrix should be important in any modification of the activities of the connective-tissue cells, especially when the composition or the properties of the intercellular space have been altered, *e.g.*, in rheumatoid disease or after an injury. Since the fibroblast is the main source of the extracellular matrix, the relations of collagen and acidic mucopolysaccharides to the plasma membranes are of special interest. The accessibility of the receptors for hormones and for the "inflammation mediators" regulates the activation of the connective tissue to the granuloma formation and fibrosis. The chemical anatomy of the cell membrane with its "fuzzy coat" [1,2] is relevant for the social functions of the cell such as adhesion, contact inhibition, charge, transport and intercellular communication.[3]

MATERIAL AND METHODS

Plasma membrane preparations and their treatment. The plasma membranes were prepared without the use of enzymes or heavy metals from experimental granulation tissue as described in detail elsewhere.[4] Mature granulation tissue was pressed through a sieve plate, the homogenization completed with a Dounce homogenizer and the bulk of the heavy plasma membranes isolated by an isopycnic centrifugation in discontinuous sucrose gradient where the main fraction accumulated to the interphase of 28/38 %, w/v, sucrose. Both fresh and frozen (at −70 °C) membranes were used, though during storage some of the enzymic activities were lost. The concentration of the membranes in the suspension did not affect the specific enzymic activities calculated per protein. The activity of Na^+,K^+-activated Mg^{2+}-adenosine triphosphatase was about 15 – 20 % higher in 40 mM Tris-HCl buffer than in Krebs-Ringer medium buffered with 20 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid; Sigma, St. Louis, Mo., USA).

Interaction of the plasma membranes with various substances. The membrane suspension (about 1 mg protein/ml), either in Tris-HCl buffer or Krebs-Ringer-Hepes medium, was preincubated with several concentrations of the additive for 30 – 60 min at 37 °C (Table 1). Then the substrate solutions of the marker enzymes were added with the appropriate cofactors for

the determination of enzymic activities (see below).

In the experiments with neuraminidase from *Vibrio comma* (Behringwerke, Marburg, Germany; ORKD No. T 1070S and 572S) the membranes were treated for various periods and with various concentrations of the enzyme (Fig. 1). Collagenase and hyaluronidase were commercial preparations, not homogeneous in polyacrylamide-gel electrophoresis.

Dextran preparations were obtained from Pharmacia, Uppsala, Sweden. Bradykinin (B-1377), ganglioside (Type III: G-2375), lysolecithin (L-6626), spermine (S-2876) and spermidine (S-2501) were from Sigma, serotonin (No. 85030) from Fluka AG (Buchs, Switzerland) and histamine from E. Merck AG (Darmstadt, Germany). The other preparations were made in our laboratory. Prostaglandins were a gift from The Upjohn Company through Mr. O. Oinonen, Helsinki, Finland.

Separation of the "fuzzy coat". The plasma membranes were treated with 0.01 M EDTA in 0.05 M Tris-HCl buffer, pH 6.8, for 5 h at +4° C(the control samples without EDTA) and centrifuged at 100 000 *g* for 45–60 min. In some experiments the membrane suspension was subjected to mechanical shear in Couette's rotation viscometer at +4 °C. The gap between the cylinder and the jacket was 0.75 mm, the diameter 1.8 cm, and the speed 150 rpm. The control samples were kept in the same condition without shear. Both the pelleted materials and the supernatants were analyzed for proteins and the enzyme activities. In the supernatants the values were so small that they are not considered reliable.

Analytical. The marker enzymes determined were 5′-nucleotidase (EC 3.1.3.5),[5] Na^+,K^+-activated Mg^{2+}-adenosine triphosphatase (EC 3.6.1.3),[6] and leucine-β-naphthylamidase (EC 3.4.11.1).[7] Also the following components were analyzed: liberated inorganic phosphate,[8] protein,[9] cholesterol,[10] and *N*-acetylneuraminic acid.[11] The polyacrylamide-gel electrophoresis has been described in detail earlier[4] and the protein staining was performed according to Lenard.[12]

RESULTS

Effects of extracellular constituents and pertinent enzymes. Connective-tissue components had no clear effect on the marker enzymes of

Table 1. Effects of various additions on the activities of the characteristic enzymes of plasma membranes.[a]

(0) No effect, (+) activation, (−) inhibition

Addition and the largest final concentration	5′-Nucleotidase	Na^+,K^+ activated ATPase	Leucine-β-naphthylamidase
Hyaluronate, 1 %	(−)	(−)	0
Chondroitin sulfate-4, 4 %	0	0	0
Proteoglycan, 4 %	0	0	0
Heparin, 0,1 %	0	0	0
Dextran T10 (Fig. 3)	0	–	0
Dextran T200 (Fig. 3)	0	–	0
Dextran sulfate (Fig. 3)	(−)	–	0
Soluble collagen, 2 %	0	0	0
Serum albumin, 2 %	0	0	0
Immunoglobulin, crude, 0.5 %	0	0	0
Lipoproteins (HDL, LDL, VLDL,) 100 μg protein/ml	0	0	0
Ganglioside, crude, 0.1 mg/ml	–	0	0
Lysolecithin, 0.1 mg/ml	0	–	0
Hyaluronidase*	0	0	0
Collagenase*	0	(−)	(−)
Neuraminidase* (Fig. 1)	0	–	0
Spermine, spermidine, 10 mM	0	0	0
Bradykinin,* 0.1 mM	0	0	0
Prostaglandins E_1, E_2, $F_{2}\alpha$, 10 μg/ml	0	0	0
Serotonin,* 10^{-4} M	0	(+)	0
Histamine,* 10^{-2} M	0	0	0
Ethanol, 0.4 %	0	–	0

[a] All the experiments were carried out in Tris-HCl buffer, except those marked * performed in Krebs-Ringer medium buffered with Hepes. Serotonin and histamine were studied with both solvents.

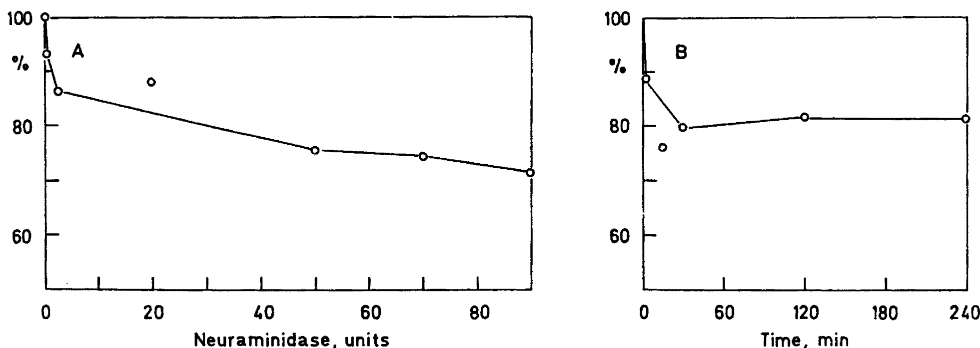

Fig. 1. Effect of a pretreatment of the plasma membranes with neuraminidase on the activity of Na^+,K^+-activated Mg^{2+}-adenosine triphosphatase. A. Effect of various amounts of the enzyme (duration 15 min); B. Effect of the duration of the treatment (neuraminidase 10 U/sample). The solvent was Krebs-Ringer-Hepes medium.

the plasma membrane preparations (Table 1), presumably because the membranes contained some acidic mucopolysaccharides and collagen already. Only hyaluronate (0.5 – 2 %) may have had some inhibitory effect, at least more than chondroitin sulfate, both on Na^+,K^+-activated adenosine triphosphatase and on 5′-nucleotidase although it is difficult to assess the effect of hyaluronate quantitatively. The membranes stained with Alcian blue, which was partially removed with 0.2 M $MgCl_2$.

Experiments were made therefore with pertinent hydrolytic enzymes. The effects of hyaluronidase and collagenase were slight and variable, and the presence of impurities in the enzyme preparations cannot be ruled out. Neuraminidase had a clear effect on Na^+,K^+-activated adenosine triphosphatase, but only 20 – 25 % of the total activity could be inhibited (Fig. 1).

Prompted by an occasional observation that even a short exposure to low pH decreased the subsequent enzymic activities of plasma membranes at neutral environment, a systematic experiment was carried out (Fig. 2). All the marker enzymes proved sensitive although at different pH.

Rheumatoid synovial fluid, human serum, and immunoglobulins were inert with respect to the enzymic activities of the plasma membranes.

Effects of artificial macromolecules. High-molecular-weight dextran and dextran sulfate depressed the activity of Na^+,K^+-activated

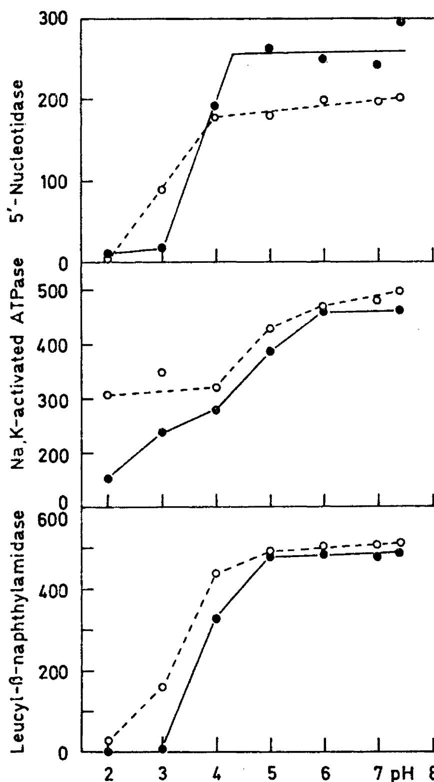

Fig. 2. Effect of the temporary lowering of pH on the subsequent activity of 5′-nucleotidase, Na^+,K^+-activated Mg^{2+}-adenosine triphosphatase, and leucyl-β-naphthylamidase of plasma membranes. The duration at the indicated pH (●) 30 min or (○) 5 min. The solvent was Krebs-Ringer-Hepes medium.

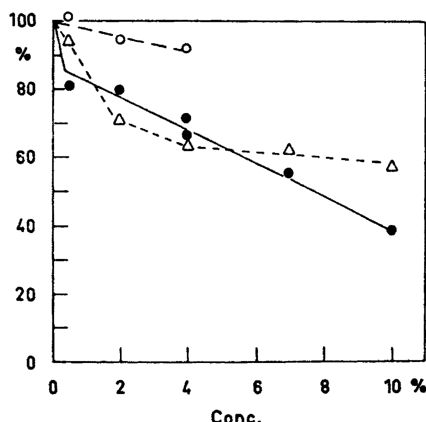

Fig. 3. Effect of dextran preparations on the activity of Na^+,K^+-activated Mg^{2+}-adenosine triphosphatase of plasma membranes. (△) Dextran sulfate, (○) dextran T10 (mol wt. 10 000) and (●) dextran T2000 (mol wt. 2 000 000). The solvent was Tris-HCl buffer.

Mg^{2+}-adenosine triphosphatase (Fig. 3). The effect was not due to an increase of colloid osmotic pressure because low-molecular-weight dextran was less effective. With dextran sulfate the inhibitory effect levels off at low concentration but with dextran it is more concentration-dependent.

Low-molecular-weight substances. The polyamines which one would expect to combine with the acidic groups in the membrane did not have any effect.

The effect of ethanol in "physiological" concentrations, a decrease by 13 – 27 % in the activity of Na^+,K^+-activated Mg^{2+}-adenosine triphosphatase, has not been described earlier on isolated membranes but on slices and microsomal preparations.[13] The effect of serotonin was investigated very thoroughly because serotonin is alleged to stimulate fibroblast functions at low concentrations (*cf.* Ref. 14). In these preparations and with the present parameters the stimulating effect of serotonin at $10^{-5} - 10^{-4}$ M is very small (about + 10 %) if any.

5′-Nucleotidase is sensitive to gangliosides and Na^+,K^+-activated Mg^{2+}-adenosine triphosphatase to lysolecithin (0.1 mg/ml). Several detergents in the concentrations of 1 mg/ml activate the insoluble 5′-nucleotidase of these

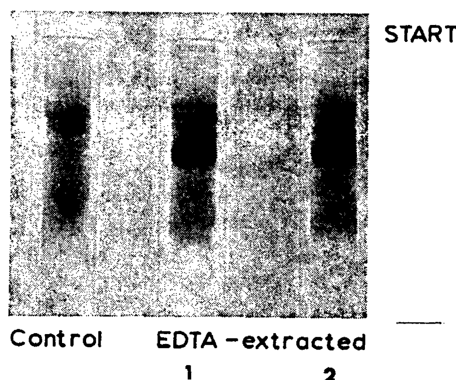

Fig. 4. Effect of the extraction with EDTA with (2) and without (1) mechanical shear on the gel-electrophoretic distribution of proteins in supernatant material detached from plasma membrane preparations. The samples were obtained from the experiment No. 1, Table 2.

Table 2. Effects of EDTA and the treatment with mechanical shear on the plasma membranes.

Conditions of treatment	Protein mg/ml	Enzyme activities, μmol/(h/mg protein) 5′-Nucleotidase	Na+,K+-act. ATPase	Leucine-β-naphthylamidase
Exp. 1 (duration 5 h)				
Control	2.15	3.9	26.2	2.6
Extraction with EDTA	2.00	2.7	30.9	2.8
Extraction with EDTA at shear	1.90	2.1	29.6	2.9
Exp. 2 (duration 2 h)				
Control	0.36	7.5	28.0	5.5
Extraction at shear	0.32	5.8	28.9	4.9

The solvent was 0.05 M Tris-HCl, pH 6.9 and the temperature +4 °C. The data refer to the pelleted material.

plasma membranes (T. Vihersaari, personal communication). The inactivating effect of EDTA may have bearing on the finding that 5′-nucleotidase is located on the cell surface (Table 2).

"Fuzzy coat". The membranes were treated with hyaluronidase, extracted with EDTA and other solutions, and subjected to increased mechanical shear in a rotation viscometer. Hyaluronidase did not detach a significant amount of protein from the membranes, affect the enzymic activities or change the gel-electrophoretic distribution of membrane proteins. Instead, EDTA liberated to the supernatant proteins having a specific gel-electrophoretic pattern (Fig. 4). The membranes lost to the solution mainly 5′-nucleotidase. The mechanical shear liberated additional protein and further inactivated 5′-nucleotidase (Table 2). No cholesterol or *N*-acetylneuraminic acid was released.

DISCUSSION

The preparation of granulation-tissue plasma membranes is not sensitive to substances that are intimately associated with the cell membranes in the tissues or to enzymes that degrade the components of the extracellular matrix. Either the characteristic marker enzymes are not in this case the proper probes of the membrane function or their sensitivity is lost during the preparation of the membranes. Other enzymes, *e.g.*, adenylate cyclase, may be more sensitive, or the membrane may respond only *in situ*, as part of a living cell.

The effect of dextran preparations may depend on exclusion phenomena [15] which interfere with the formation of a complex between Na^+,K^+-activated Mg^{2+}-adenosine triphosphatase and its substrate. It has been observed that dextran increases the zeta-potential on the surface of erythrocytes.[16] It remains to be investigated whether the activity of adenosine triphosphatase is in any way related to the double layer. The effect of neuraminidase is in agreement with the fact that Na^+,K^+-activated Mg^{2+}-adenosine triphosphatase is a sialoprotein.[17] Ethanol presumably disturbs the relationships between adenosine triphosphatase protein and the stimulating phospholipid in the membrane, or interferes with the local structure of water.

The irreversible inactivation at low pH is similar for 5′-nucleotidase and leucine-β-naphthylamidase. The dissociation point of dicarboxylic amino acids [18] and hexuronic acids is at pH 3 – 4 and the secondary structures of macromolecules may be lost and inactive complexes formed between adjacent substances. The mechanism for the inactivation of adenosine triphosphatase may be different. The curves in Fig. 2 do not resemble those for the pH dependence of the activities of the enzymes.

The "fuzzy coat" [19] of cells has attracted considerable attention because it is suggested to be different in transformed cells.[20] Its preparation from the plasma membranes originating from solid tissue, obtained without destroying the membranes or covalent bonds, has not been described earlier. This material contains a characteristic protein pattern as seen in Fig. 4. The EDTA-liberated acidic mucopolysaccharides as well as glycoproteins from erythrocyte membranes are heterogenous, markedly different from those shown in Fig. 4.[21] A growth-enhancing protein has been obtained from cell surface of cultured fibroblasts with urea-EDTA.[22]

As the next step in this study we suggest a more sensitive and specific analysis of the composition of the EDTA-liberated fraction. This plasma membrane has been prepared without enzymes and heavy metals and represents the native state as closely as possible. The "fuzzy coat" fraction is neither a part of the plasma membrane proper nor does it belong to the matrix. It conceivably contains the substances just leaving the cell as well as material influencing the social functions of the cells.

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