CALCIUM-REQUIREMENT FOR A REVERSIBLE BINDING OF MEMBRANE PROTEINS TO RAT LIVER PLASMA MEMBRANES

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1. Introduction

Divalent cations are thought to be of importance for the structure and physiological functions of cell surfaces. For example, calcium has been shown to affect the adhesiveness, permeability and excitability of surface membranes [1–4]. Calcium is also involved in hormonal actions on the cell membrane [1]. Although little is known about the role of calcium in the structural organization of membranes, two structures known to be affected by calcium ions are the intercellular junctions known as desmosomes (maculae adherentes) and intermediate junctions (zonulae or fasciae adherentes). Removal of calcium causes disorganization of these junctions and separation of the cells held together by them [3,5–8]. It has also been reported [8] that cleavage of intermediate junctions by removal of calcium is accompanied by disorganization of the underlying actin-containing [3] microfilaments.

Although studies on the structure and function of plasma membranes require their isolation there is no method yet available for obtaining pure membrane preparations from eucaryotic cells. In addition to the contamination with intracellular membranes it is likely that different isolation procedures yield membrane preparations varying in chemical composition, structure and physical properties. That such variations are possible is shown in the present paper which demonstrates the occurrence of proteins that are reversibly bound to the plasma membrane. The binding is specifically mediated by calcium ions; removal of calcium by specific chelators results in the solubilization of these proteins.

2. Materials and methods

Plasma membranes were prepared from livers of male Sprague-Dawley rats weighing 200–250 g, essentially according to the method of Ray [9]. One modification was introduced. Before isopycnic centrifugation in the discontinuous sucrose gradient described by Ray the material was subjected to another discontinuous sucrose gradient of the following composition from the top to bottom of the tube: 4 ml of material suspended in homogenization medium (1 mM $NaHCO_3$–0.5 mM $CaCl_2$, pH 7.5), 9 ml of 37% (w/w) sucrose, 15 ml of 41% (w/w) sucrose. Centrifugation was carried out at 4°C for 2 h at 25 000 rev/min (rotor SW 25.1, Spinco Model L preparative ultracentrifuge). The sucrose solutions were buffered with 5 mM Tris–HCl, pH 7.4. The material from the interface between 37 and 41% sucrose was collected and subjected to flotation in the discontinuous sucrose gradient as described by Ray. The preparation of the membranes and subsequent experiments were carried out at 4°C. The plasma membranes were analyzed for activities of 5′-nucleotidase (marker for plasma membranes), glucose-6-phosphatase (marker for endoplasmic reticulum) and succinic-INT*-reductase (marker for mitochondrial inner membranes) as described by Morré [10]; cytochrome oxidase (marker for mitochondrial inner membranes) was determined according to Cooperstein and Lazarow [11] and

* INT, 2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium; EGTA, ethyleneglycol-bis (β-amino ethyl ether) *N,N′*-tetra-acetic acid.

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acid phosphatase (marker for lysosomes) was measured essentially as described by Trouet [12]. Inorganic phosphate was determined according to Fiske-Subbarow as described by Morré [10] or according to Bartlett [13].

The membranes were diluted, centrifuged for 30 min at 10 000 rev/min (Spinco rotor, SW 25.1), resuspended in Ca-medium (145 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM Tris–HCl, pH 7.4) and dialyzed 15–18 h against Ca-medium. The dialyzed membrane suspension was centrifuged for 30 min at 25 000 rev/min in a Spinco SW 36 rotor. The supernatant was denoted Ca-extract 1. The membrane pellet was resuspended and washed twice in Ca-medium, and was then suspended in EGTA*-medium (145 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 5 mM Tris–HCl, pH 7.4) and dialyzed against EGTA-medium. In some experiments a portion of the membranes was again suspended in Ca-medium instead of EGTA-medium and was dialyzed against Ca-medium. The membrane suspensions were centrifuged as described above and the supernatants were denoted EGTA-extract 1 and Ca-extract 2, respectively. The membrane pellet remaining after dialysis against EGTA-medium was again suspended in EGTA-medium, dialyzed against EGTA-medium and centrifuged. The resulting supernatant was denoted EGTA-extract 2.

To test the effect of Ca^{2+} on the binding of the solubilized proteins to the membranes the membrane suspension obtained after the first EGTA-dialysis was dialyzed against Ca-medium without first separating the EGTA-extract 1 from the membranes and was then centrifuged. The resulting supernatant was denoted Ca-extract 3. The remaining membrane pellet was suspended in EGTA-medium, dialyzed against EGTA-medium, and centrifuged. The resulting supernatant was denoted EGTA-extract 3.

All the extracts were dialyzed against distilled water and freeze-dried.

In another set of experiments a mitochondrial membrane fraction was used. This fraction is the material which banded in the interface between 45% and 48% sucrose in a regular fractionation according to Ray [9], and which has been shown to consist mainly of mitochondrial membranes with no contamination of plasma membranes [14]. These membranes were treated exactly as the plasma membranes and a Ca-extract 2 and EGTA-extract 1 were prepared.

Another control experiment was carried out by preparing a postmicrosomal supernatant of a liver homogenized in 1 mM $NaHCO_3$–0.5 mM $CaCl_2$, pH 7.5. The supernatant obtained after centrifugation at 41 000 × *g* for 30 min and then at 100 000 × *g* for 60 min was used. This supernatant was mixed in the cold with washed EGTA-extracted plasma membranes in 1 mM $NaHCO_3$–0.5 mM $CaCl_2$. The mixture was centrifuged and the membrane pellet was suspended in and dialyzed against Ca-medium. After two washes with Ca-medium the membranes were dialyzed against EGTA-medium. The resulting EGTA-extract was dialyzed against distilled water, lyophilized and analyzed on disc gel electrophoresis.

Perfusion of rat livers was carried out in the following way. Rats were anesthetized with ether. A catheter was inserted in the portal vein in situ, the lower caval vein was cut and perfusion was carried out with oxygenated buffers at temperatures of 37°C or 0–4°C (the same results were obtained at either temperature). The flow rate was kept at 50 ml/min by means of a peristaltic pump. The liver was gently excised as quickly as possible and placed on a nylon net. Perfusion was started with Ca-medium for 15 min to remove blood and was followed with Ca-medium or EGTA-medium for another 15–30 min. The perfusates were concentrated by ultrafiltration in an Amicon cell (membrane UM-10), dialyzed against distilled water and freeze-dried. When livers perfused in this way were used for preparation of membranes calcium was introduced in them again by perfusion for the last 5 min with Ca-medium.

Disc gel electrophoresis in sodium dodecyl sulphate (SDS) was carried out in 11% polyacrylamide gels according to Neville [15]. The freeze-dried extracts or perfusates were dissolved in 2% SDS–0.05 M Tris–H_2SO_4, pH 8.0 and β-mercaptoethanol was added to a final concentration of 10%. These mixtures were mixed with 1.5 volumes of a solution containing 10^{-5} M bromphenol blue, 3 mM dithiothreitol, 0.1% SDS and 4% sucrose dissolved in spacer gel buffer. Fifty or 100 μl (30–60 μg of protein) of the final mixture was applied to each gel. After electrophoresis, the gels were stained for 15 h in 0.01% Coomassie Brilliant Blue dissolved in methanol:acetic acid:water (5:1:5, v/v). Destaining was performed in methanol:acetic

acid:water (2:3:35, v/v). Bovine serum albumin, ovalbumin, IgG, cytochrome *c* and lathyritic rat skin collagen (the α_2 chain showed the same relationship between molecular weight and electrophoretic relative mobility as the other standard proteins) were run as standards of known molecular weights. The relative mobilities were used to estimate apparent molecular weights according to Neville [15].

3. Results and discussion

The marker enzyme analyses showed that the present modification of the membrane preparation resulted in plasma membranes of at least the same purity as membranes obtained by the original procedure described by Ray [9] (table 1). Electrophoretic analyses showed that a number of proteins could be solubilized by dialysis of the plasma membranes against Ca- or EGTA-media (fig.1). The proteins in the Ca-extract 1 probably represent non-specifically bound proteins described by Benedetti and Emmelot [7]. In the EGTA-extract 1 there appeared 4 components of interest. They are referred to as bands 1–4 (fig.1). Components 1, 3 and 4 all appeared as two very closely moving bands (doublets) on the gels. The molecular weights determined with disc gel electrophoresis were 67 000, 65 000, 45 000, 38 000, 37 000, 35 000

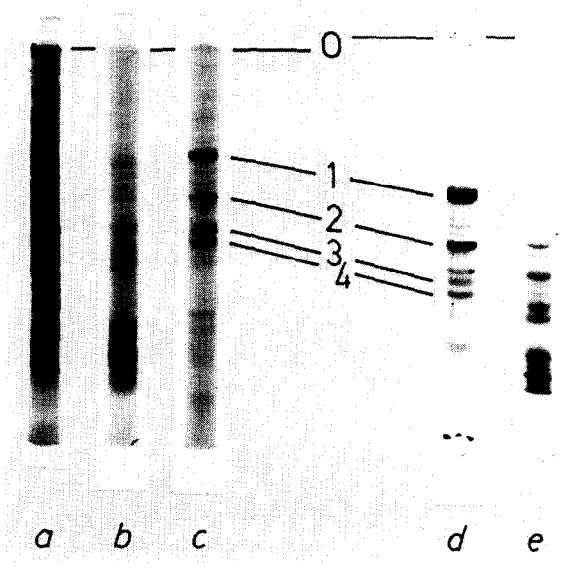

Fig.1. Disc gel electrophoresis of membrane extracts. a, b and c are extracts obtained from the same membrane preparation and d and e are obtained from another membrane preparation. The extracts in b and c were obtained from the same amount of membranes and equal volumes of the extracts were electrophoresed. The same is true for the extracts shown in d and e. The numerated bands are described in the text. 0, the origin, is the border between the spacer and separation gels. See text for further details. (a) Ca-extract 1, (b) Ca-extract 2, (c) EGTA-extract 1, (d) EGTA-extract 1, (e) Ca-extract 2.

Table 1
Relative specific activities of marker enzymes

Marker enzyme	Relative specific activity			
	Homogenate	Plasma membranes		
		Ray's procedure	Sedimentation	Flotation
5'-Nucleotidase	1.0	17.5	21.4	23.4
Glucose-6-phosphatase	1.0	0.85	0.63	0.60
Acid phosphatase	1.0	0	0.26	0.09
Cytochrome oxidase or succinic-INT-reductase	1.0	0.87	0.81	0.26

The enzymatic activities were measured in the homogenate and in plasma membranes prepared according to Ray [9] and according to the modified procedure. Membranes prepared with the latter procedure were analyzed both after the sedimentation and after the final flotation in the sucrose gradients as described in Materials and Methods. The relative specific activities were calculated by dividing the true specific activity with that of the homogenate, which thus will get the relative specific activity 1.0.

and 34 000 of components 1a, 1b, 2, 3a, 3b, 4a and 4b, respectively. Band 1, 3 and 4 only occurred in the EGTA-extract 1 but not in the Ca-extract 2. The proteins in band 1, 3 and 4 were reversibly bound again to the membranes when Ca-ions were introduced in the system as shown in fig.2. A control experiment where the EGTA-extract 1 in the absence of membranes was dialyzed against Ca-medium in the cold and centrifuged, showed that calcium did not merely precipitate the components 1, 3 or 4. Band 2 also occurred in the Ca-extract 2 and was not bound again to the membranes in the presence of Ca^{2+}. However, band 2 was always solubilized to a higher extent in the EGTA-medium than in the Ca-medium. One single extraction with EGTA-medium solubilized all of the components 1, 3 and 4, whereas component 2 continued to leak off the membranes with repeated treatments with Ca- or EGTA-medium (fig.2).

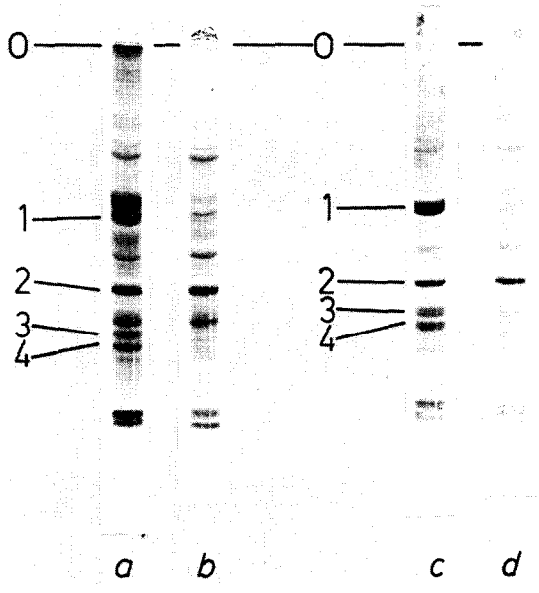

Fig.2. The Ca-dependent reversible binding of components 1, 3 and 4 to the membranes. After dialysis against EGTA-medium, giving EGTA-extract 1 the membranes were divided into two equal portions. One portion was treated so that Ca-extract 3 and EGTA-extract 3 were obtained. The other portion yielded the EGTA-extract 1 shown and was further treated so that EGTA-extract 2 was obtained. For further details see the text and the legend to fig.1. (a) EGTA-extract 1. The heavy band moving just behind band 1 is an irregularly occurring component (compare with fig.1), (b) Ca-extract 3, (c) EGTA-extract 3, (d) EGTA-extract 2.

None of the components 1–4 appeared in a Ca- or EGTA-perfusate from rat liver and membranes isolated from livers perfused with EGTA-medium still contained these components. Therefore, it seems likely that these proteins are localized on the cytoplasmic side of the membrane. They do not seem to be components common to all types of cellular membranes since the mitochondrial membranes definitely did not contain band 1 or 2 and probably not band 3 or 4. It thus seems reasonable to conclude that they are true components of the plasma membrane. However, there is a possibility that the proteins either are soluble cytoplasmic components or are present in some other subcellular compartment and during the homogenization of the liver in the presence of calcium become associated with the plasma membranes. At present this possibility cannot be ruled out; if must await the isolation of the proteins and subsequent analysis with immunofluorescence or immunoelectron microscopy to determine their subcellular localization in sections of liver tissues and of liver cells. However, the proteins are not present in a post-microsomal supernatant of a liver homogenate prepared in the presence of calcium. This was demonstrated by the fact that washed EGTA-treated membranes mixed with the supernatant under conditions identical to those for homogenization and subsequent membrane isolation, did not bind any proteins identified as band 1, 3 or 4. Thus, if the proteins 1, 3 and 4 occur as soluble cytoplasmic proteins in vivo all of them become bound to the plasma membranes during the homogenization and accordingly they must be present at rather low concentrations in the cytoplasm.

We have shown that rat liver plasma membranes isolated in the presence of calcium contain soluble proteins probably bound to the inside of the membranes. Some of these (band 1, 3 and 4) show a reversible binding to the membranes in the presence of calcium. One of them (band 2) is solubilized to a larger extent when calcium is removed but does not bind to the membranes again upon addition of calcium. Thus, membranes prepared in the presence or absence of calcium have significantly different

protein compositions. Possibly these proteins may be involved in the regulation of membrane functions mediated by calcium. In this context it is interesting to note that rat liver plasma membranes can stimulate formation of intercellular contacts between rat liver cells, but only if the membranes have been treated with EGTA-medium [16]. Membranes which have been in the presence of calcium continuously do not show this stimulatory effect. It is also interesting to note that band 2 has exactly the same electrophoretic mobility as the major protein in whole membranes solubilized with SDS and that its apparent molecular weight is identical to that of actin [17].

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