USE OF A CONCANAVALIN A POLYMER TO ISOLATE RIGHT SIDE-OUT VESICLES OF PURIFIED PLASMA MEMBRANES FROM EUKARIOTIC CELLS

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SUMMARY : We have purified two plasma membrane populations using a Concanavalin A polymer. It was assumed that vesicles retained by the polymer were right side-out, whereas vesicles not retained were inside-out. 5'-nucleotidase and ($Na^+ + K^+$) stimulated Mg^{++} ATPase activities were at least two fold higher in inside-out than in right side-out vesicles, though recovered total activity was about 80 % for both enzymes together. Moreover, Concanavalin A modified 5'-nucleotidase activity of right side-out vesicles according to the dose used.

In order to study the mechanism by which a surface membrane effector could stimulate or inhibit a given cell function, we needed to isolate right side-out purified plasma membrane vesicles.

Plasma membranes are asymetrical and glycoproteins are mainly exposed on their external surface (1 - 3) and when vesiculated they can expose carbohydrates either on the outside surface (right side-out vesicles) or onn the inside surface (inside-out vesicles). Lectins are (glyco) proteins with specific binding sites for carbohydrates (4) : for instance, Concanavalin A (Con A) is known to bind specifically α D-glucose and α D-mannose and their derivatives (5).

Thus we thought to use Con A polymer to isolate right side-out vesicles which could be ultimately removed by α D-methylmannopyranoside.

MATERIEL and METHODS.

Cells.

We have isolated several cell lines in tissue culture from the murine plasmocytoma MOPC 173 (6) ; one of which, MF_2S^+, has been adapted back to the mouse and grows as ascites. Ascites cells are harvested a week after injection and washed twice in saline buffer. Contaminating red blood cells are eliminated by hypotonic shock (15 sec.) followed by two washes in the buffer.

Plasma membranes purification.

The technique used was already described by L. Lelièvre (7) and

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was adapted to these cells by slight modifications. 40-50 % of the initial 5'-nucleotidase total activity was recovered in the plasma membranes, and this enzymatic activity is 10 to 18 fold purified with respect to the specific activity in the lysate.

Polymerization of Con A.

The polymer is prepared according to the technique of Avrameas and Ternynck (8). In brief, one part of Con A (Calbiochem) is copolymerized with four parts (w:w) of Bovine Serum Albumin (BSA, Sigma) in 0.2 M acetic acid-Na acetate buffer, pH 5.0, using glutaraldehyde (Fisher Scientific Co.) at 10 mg/100 mg of protein. After about one hour, the gel is washed five times in a 40 mM imidazol-HCl, 5 mM $MgCl_2$, 1 mM magnesium titriplex, buffer, pH 7.4. For use, the final concentration is 1.5 mg of Con A per ml.

Determination of enzymatic activities.

Enzymatic assays were performed with about 60 µg of protein per ml of incubation medium. 5'-nucleotidase (E.C.3.1.3.5.) activity was measured by the method of Emmelot and Bos (9) -(Na^+ + K^+)- stimulated Mg^{++} - ATPase (E.C. 3.6.1.3) was measured by a slight modification of the method of Bakkeren and Bonting (10). Protein concentrations were determined by the method of Lowry et al. (11). Phosphorus present in the reaction medium was determined by a modification of the Marsh technique (12).

Determination of sialic acids.

The total amount of sialic acids was estimated by incubating 100 µg of plasma membrane (expressed in protein equivalent) with 1 ml of 0.1 N H_2SO_4, 80° C, 1 hour, and measurements are made according to Warren's technique (13). Specific measurements of the exposed sialic acids was made by adding Clostridium Perfringens neuraminidase (E.C. 3.2.1.18. Sigma - 50 µg per mg of membrane protein).

RESULTS.

Separation of two plasma membrane populations.

1 mg of plasma membrane was mixed with 10 ml of Con A polymer and gently stirred with a magnetic barrel for 1 hour. The suspension is centrifuged 10 mn at 2,000 g and the pellet is washed twice in the imidazol buffer, supernatants being pooled.

To remove sticking membranes from the polymer, the pellet is suspended in a 0.2 M α D-methylmannopyranoside (αMM - Calbiochem) imidazol buffer and kept for one hour under stirring. Centrifugations and washes are performed as above.

Supernatants obtained before and after treatment by α MM are then centrifuged 40 mn at 40,000, leading to two pellets : A vesicles (not sticking

Material	Protein (mg)	Sialic acids: Total content (nmole/mg of protein)	Sialic acids: released by neuraminidase (nmole/mg of protein)	Specific activities (µmoles Pi/h/mg of protein): $(Na^+ + K^+)Mg^{2+}$ ATPase	Specific activities (µmoles Pi/h/mg of protein): 5'-nucleotidase
Purified membranes	1	390	610	6.3	15
A vesicles	0.4	400	430	9	21
B vesicles	0.4	375	840	3.6	8.5

Table : Properties of three different subpopulations of MOPC 173 plasma membranes.

to the Con A polymer) and B vesicles (sticking to the Con A polymer). All procedures were performed at 4° C.

Protein and sialic acids measurements (see table).

80 % of the utilized plasma membrane proteins were recovered in equal amounts in pellets A and B. This separation is not due to unspecific adsorption on the polymer since a second treatment of the A vesicles by the polymer did not lead to any loss of plasma membranes by fixation.

The total amount of sialic acids estimated by acid hydrolysis was found to be 400 nmoles per mg of protein in A vesicles and 375 nmoles per mg of protein in B. Release by neuraminidase was twice higher in B than in A vesicles i. e. 840 nmoles/mg of protein in B and 430 nmoles/mg of protein in A. This discrepancy could be due to a loss during the acid hydrolysis ; however the interesting fact is that the same amount of sialic acids is found in A and B vesicles, whereas, by enzymatic degradation occuring on the exposed sites, much more sialic acids are found in the B than in the A vesicles. Perhaps therefore we can assume that the relative large amount of free sialic acids found after neuraminidase treatment of the A particles could be due to a partial permeability of the vesicles to the enzyme.

Enzymatic activities.

Preliminary experiments (done with L. Lelièvre) have shown that cells just before lysis have three fold lower $(Na^+ + K^+)$ stimulated Mg^{++} ATPase and 5'-nucleotidase activities than the lysate from the same cells. Thus we could assume the hydrolytic sites of these enzymes being located on the cytoplasmic surface of the cell membrane.

From three different plasma membrane isolations, eight separations of vesicles populations were performed : the specific activities (expressed in

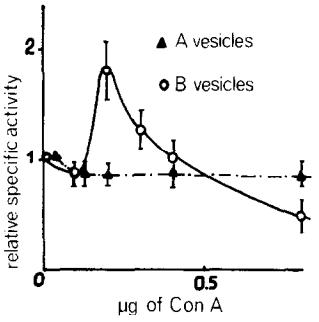

Figure 1 : Modulation of the 5'-nucleotidase activity by adding various amounts of Con A to A (▲) or B (O) vesicles. (Kinetics are linear up to the end of the test : 30 mn).

µmoles Pi/h/mg of protein) of 5'-nucleotidase and (Na^+ + K^+) stimulated Mg^{++} ATPase were respectively (as a mean of the results) 21 and 9 for the A vesicles, and 8.5 and 3.6 for the B population, i. e. the enzymatic activities were at least two fold higher in A than in B vesicles (table 1).

In all instances, total activities recovered in the two vesicles populations represented about 80 % of the original activities in the purified membranes.

Modulation of the 5'-nucleotidase activity by Con A.

To 40 µg of plasma membrane proteins increasing amounts of Con A from 0.1 to 1 µg were added ; the mixtures were incubated for 30 mn at 37° C to reach the equilibrium binding (14). The results are shown in figure 1 : the lectin has no effect what so ever on the A vesicles, whereas the B vesicles have a biphasic reaction : from 0.1 to 0.3 µg of Con A, the 5'-nucleotidase activity increased from 100 % to 160-200 %, while from 0.5 to 1 µg of Con A the activity was decreased to 40 % of the original activity.

DISCUSSION.

Two plasma membrane populations have been separated by á Con A polymer from a purified plasma membrane preparation obtained from the murine plasmocytoma MOPC 173, substrain MF_2S. It was assumed that membrane vesicles which were not retained by Con A were inside-out (A fraction) whereas membrane vesicles retained by Con A and ultimately removed by α MM were right side-out (B fraction). This assumption is sustained by the following results :

- The A vesicles have no mannose nor glucose exposed to the outside whereas B vesicles have such carbohydrates, though both populations have carbohydrates (as estimated by sialic acids).

- Treatment by neuraminidase led to twice the amounts of free sialic acids from the B vesicles as compared to the A vesicles, whereas the total amount of sialic acids was about the same in both populations.

- Having shown that 5'-nucleotidase activity is much higher in the lysate immediately after lysis than in the cell suspension, we could consider this activity due to the cytoplasmic side of the plasma membrane.
Moreover the activity is also higher in the A (inside-out) vesicles than in the ones of B ; the same results were observed for the ($Na^+ + K^+$) stimulated Mg^{++} ATPase activity which is known to hydrolyse ATP at the internal face of the plasma membrane of red blood cells (15, 16) and of all other cells studied (17).

It has been shown that the binding of various effectors on the cell surface can enhance membrane enzymatic activities (18 - 21) or inhibit such activities (21). Here we have demonstrated that Con A did not modify the 5'-nucleotidase activity on the A vesicles. By contrast, increasing amounts of Con A mixed with B vesicles modified the 5'-nucleotidase through a biphasic curve : low doses of Con A (0.1 to 0.3 µg) stimulated until 200 % the original activity of 40 µg of membrane, whereas relatively large doses (0.5 to 1 µg) lowered the activity to 40 %. These effects must be reversable by α D-methyl-mannose since B vesicles being removed from the polymer by this carbohydrate have still a 5'-nucleotidase activity associated with them.

We try to investigate the mechanism by which the lectin is able to stimulate or inhibit the 5'-nucleotidase activity : we cannot rule out a direct action of Con A on 5'-nucleotidase since it seems that this enzyme is a glycoprotein (22). Such studies could shed some light on the mechanism by which any effector stimulate or inhibit a given cell activity, depending upon the dose of the effector used.

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