Flow cytometry is a new method for the characterization of intestinal plasma membrane

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The highly differentiated plasma membrane of rabbit enterocytes constitutes an interesting model for membrane studies. Flow cytometry allows combined measurements of the size of membrane vesicles by light-scatter and fluorescence polarization at a single-particle level. The degree of fluorescence polarization of 1,6-diphenylhexa-1,3,5-triene was determined at 4, 18 and 37°C in the brush-border and basolateral plasma membranes. The fluorescence polarization was considerably higher in brush-border than in basolateral membranes. After incubation with dimethyl sulphoxide, the membrane fluidity decreased in both types of membranes. Moreover, a time-effect study of dimethyl sulphoxide showed changes in fluorescence polarization. Only in brush-border membrane a temporary fluid phase was observed. The different properties of the two membrane domains in relation to the lipid-protein dynamics of enterocytes are discussed.

Enterocytes, the predominant cell type lining the small intestine, are highly differentiated. These polarized cells have two membrane domains: the apical, differentiated in microvilli (brush-border) and in contact with the lumen, and the basolateral, devoid of microvilli. These two specialized membrane domains differ from each other not only by their ultrastructure but also in their protein and lipid composition (Kawai *et al.*, 1974; Christiansen & Carlsen, 1981; Kenny & Maroux, 1982).

The fluorescent probe 1,6-diphenylhexa-1,3,5-triene has been used previously to estimate membrane fluidity by measurements of the fluorescence polarization of the probe in the membrane bilayer (Cogan *et al.*, 1973; Lentz *et al.*, 1976; Schachter & Shinitzky, 1977; Shinitzky & Barenholz, 1978). Previous studies on the membrane fluidity of the enterocyte plasma membrane by using 1,6-diphenylhexa-1,3,5-triene have measured fluorescence polarization in membrane suspensions (Schachter & Shinitzky, 1977). However, determination of fluorescence polarization by flow cytometry has distinct advantages. Flow cytometry allows measurements at a single-particle level, keeping out the permanent problem of the depolarization owing to light-scattering. Moreover,

this technique permits efficient exclusion of disrupted particles and membrane debris, and, by calibrating the size of particles with latex beads, it is possible to calculate the average diameter of particles. These measurements were combined with fluorescence-polarization determination in order to estimate the degree of fluorescence polarization of intact membrane vesicles. Therefore we report on the use of an EPICS V cell sorter for studying membrane fluidity of brush-border and basolateral domains and the influence of temperature changes on membrane fluidity. Since dimethyl sulphoxide has been demonstrated to alter lipid-protein interactions, which are known to influence fluorescence-polarization values (Fourcade *et al.*, 1980; Mishal *et al.*, 1981), the effect of dimethyl sulphoxide on 1,6-diphenylhexa-1,3,5-triene polarization in both types of membranes was also studied.

Experimental

Membrane preparation

Membrane fractions of rabbit small intestine were prepared as described by Colas & Maroux (1980) and were then stored at 4°C in 10 mM-phosphate buffer (pH 7.4)/0.15 M-NaCl before flow-

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cytometric analysis. Protein concentration was measured by the Lowry *et al.* (1951) method.

Scatter pattern

The EPICS V cell sorter (Coulter Electronics, Hialeah, FL, USA), using an argon-ion laser model 171-17 (12 W), analysed vesicles in aqueous suspension as they passed through the laser beam. The laser excitation was 400 mW at 363 nm. The flow rate was adjusted to 1000 vesicles/s. The light scattered from particles was measured in the narrow angle of 0–9° with respect to the incident light. This permits the exclusion of disrupted particles by adequate gatings (Le Bouteiller *et al.*, 1983). The vesicle size was determined as described by David *et al.* (1981), with latex beads (Coultronics, Paris, France).

Fluorescence polarization

The stock solution of 1,6-diphenylhexa-1,3,5-triene (Sigma Chemical Co.) was prepared at 2 mM in tetrahydrofuran. Aqueous solutions of 1,6-diphenylhexa-1,3,5-triene were prepared as described by Schachter & Shinitsky (1977). Brush-border or basolateral membranes (2 mg of protein) were incubated for 30 min at 4, 18 or 37°C with 1,6-diphenylhexa-1,3,5-triene (final concn. 2 µM). Dimethyl sulphoxide (Prolabo, Paris, France) concentration during the different times of incubation with the membrane fractions (2 mg of protein) was 0.1% (v/v) in 10 mM-phosphate buffer (pH 7.4)/0.15 M-NaCl. This concentration, used also by Tapiero *et al.* (1980), did not affect cell growth. At 30 min before polarization measurement, 1,6-diphenylhexa-1,3,5-triene was added as described above. All solutions were equilibrated at the different temperatures for 15 min before use. Fluorescence polarization was measured with the cell sorter. Vesicles were analysed as they passed through the polarized beam of the laser (363 nm, set at 400 mW output) by simultaneous detection of apparent fluorescence intensity in planes parallel and perpendicular, respectively, to the polarization plane of the excitation light. The on-line computer calculated the fluorescence polarization P for each vesicle according to the following equation:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ were the fluorescence intensities recorded when fluorescent light passed through polarizers, oriented parallel and perpendicular, respectively, to the polarized excitation light. The EPICS V was calibrated with the aid of half-wave retardation plates [Orel Corp.].

Electron microscopy

Samples for thin sections were prepared as described by Gratecos *et al.* (1978).

Results and discussion

The light-scatter patterns of brush-border and basolateral vesicles are shown in Fig. 1. The binomial distribution of brush-border vesicles (Fig. 1a) corresponded to average diameters of 0.1 µm and 0.2 µm respectively. This is in agreement with electron-microscopic observations (Fig. 1c) of brush-border vesicles, corresponding to vesicles with diameters of 0.1 µm and 0.2 µm. The average diameter of basolateral vesicles was 0.2 µm by light-scatter measurement (Fig. 1b) and by microscopic observations (Fig. 1d).

In fluorescence-polarization studies, we employed 1,6-diphenylhexa-1,3,5-triene, generally used as a convenient probe for the lipid hydrocarbon chains (Andrich & Vanderkooi, 1976). Although criticized for the possible perturbations that probes might induce (Lakowicz *et al.*, 1979), 'membrane fluidity' is usually assessed by the fluorescence polarization of 1,6-diphenylhexa-1,3,5-triene. With microvillus and basolateral membrane fractions, we sought to overcome some of the limitations of the technique by using low amounts of 1,6-diphenylhexa-1,3,5-triene (membrane phospholipids/probe molar ratio of 200). As shown in Fig. 2, the fluorescence polarization (P) of brush-border vesicles was much higher than that of basolateral vesicles, whether measurements were done at 4, 18 or 37°C. This difference (P_1) was more pronounced at 4°C ($P_1 = 0.102$) than at 18°C ($P_1 = 0.063$) or 37°C ($P_1 = 0.052$). Therefore the flow-cytometry technique has allowed us to confirm results obtained by Hauser *et al.* (1982) by e.s.r. spectroscopy. We conclude that in rabbits membrane fluidity is lower in brush-border membranes than in basolateral membranes. Furthermore, the difference between P values measured at 4°C and at 37°C (P_2) was considerably higher in the brush-border ($P_2 = 0.073$) than in the basolateral fraction ($P_2 = 0.023$). Differences in the temperature-dependent shifts on the P values from high to low values became evident for brush-border compared with basolateral membranes. These results could be explained by the high activity of proetolytic enzymes in brush-border membranes (Kenny & Maroux, 1982), which, at higher temperature, could degrade membrane proteins, thus decreasing the high protein/lipid ratio in the bilayer.

Moreover, the scatter pattern of membrane vesicles did not change over a 3 h period at 4°C. However, the scatter pattern of brush-border vesicles started to deviate after 45 and 90 min at

Fig. 1. Scatter pattern of brush-border-membrane vesicles (a) and basolateral-membrane vesicles (b) analysed by cell sorter, and sections through the pellet of brush-border membrane (c) and basolateral membrane (d). The cell sorter analysed 5000 vesicles.

37°C and 18°C, respectively, indicating an accumulation of membrane debris. This effect was not seen with basolateral membranes.

To be able to compare the effects of dimethyl sulphoxide on brush-border and basolateral membranes, these experiments were done at 4°C. At the earliest time point (30 min), *P* values of brush-border vesicles were considerably higher in the presence of dimethyl sulphoxide, and the difference between *P* values measured in the absence and presence of dimethyl sulphoxide remained constant for up to 70 min. Thereafter, a sharp decrease in polarization was observed (0.320 at 84 min), followed by a slower rise to the initial value (Fig. 3a). An increase of *P* values was also observed with vesicles from basolateral mem-

branes incubated with dimethyl sulphoxide, but there was no temporary fluid state, as seen in brush-border vesicles (Fig. 3b). It has been reported that dimethyl sulphoxide raises the 'melting' temperature of acyl chains in artificial phospholipid membranes, indicating a stabilization of bilayers, probably related to a decrease in membrane fluidity (Lyman *et al.*, 1976). This could explain the dimethyl sulphoxide-induced decrease in fluidity observed for basolateral membranes and in brush-border fraction during earlier times. However, only in brush-border membranes did dimethyl sulphoxide induce a temporary fluid phase. Possibly this phase corresponds to a reorganization of lipids and proteins in the bilayer. For instance, dimethyl sulphoxide alters reversibly

Fig. 2. Fluorescence polarization at a single-particle level. Fluorescence polarization was measured with the cell sorter on brush-border (1) and basolateral (2) intact vesicles after incubation (30 min) with 2 μ M-1,6-diphenylhexa-1,3,5-triene at 4, 18 and 37°C. The cell sorter analysed 5000 vesicles. The values of fluorescence polarization (P) corresponded to the median value of histograms. At 4, 18 and 37°C P values for brush-border vesicles were 0.325, 0.273 and 0.252 respectively, and for basolateral vesicles were 0.223, 0.210 and 0.200 respectively.

the configuration of proteins (Rammner & Zaffaroni, 1967). Mishal *et al.* (1981) have shown that dynamic changes in the cell membrane occurred during dimethyl sulphoxide treatment that might be due to activation or inactivation of one or a group of enzymes by dimethyl sulphoxide. Capo *et al.* (1983) have shown that polarization of 1,6-diphenylhexa-1,3,5-triene was significantly decreased when the macrophage-like cell line P388 D1 was induced to differentiate by dimethyl sulphoxide, and they correlated this observation with changes in lipid composition. In our case,

Fig. 3. Effect of dimethyl sulphoxide on fluorescence polarization (P) at different times detected with the cell sorter for brush-border fraction (a) and basolateral fraction (b).

Brush-border or basolateral vesicles were incubated with 0.1% dimethyl sulphoxide (●) or without dimethyl sulphoxide (■).

further experiments would be needed to show whether dimethyl sulphoxide acts on lipid dynamics or on protein-lipid interactions. Our present studies have been done on isolated membranes, and it would be interesting to study the effect of dimethyl sulphoxide on the differentiation of isolated crypt cells. Indeed, dimethyl sulphoxide has been previously reported to induce differentiation of erythroleukaemic, promyelocytic and neuroblastoma cells (Friend *et al.*, 1971; Kimhi *et al.*, 1976; Collins *et al.*, 1978).

Flow cytometry could be a very useful technique for membrane and intact-cell studies. Moreover, the analysis on a single-particle level permits the sorting of these particles in order to test their differences in protein and lipid composition and their enzymic activities. Such results could be correlated with the different models of membrane bilayers in order to gain better knowledge of the structure and the function of enterocytes.

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