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SEPARATION OF BASOLATERAL PLASMA MEMBRANES FROM SMOOTH ENDOPLASMIC RETICULUM OF THE RAT ENTEROCYTE BY ZONAL ELECTROPHORESIS ON DENSITY GRADIENTS

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Summary

Basolateral plasma membranes of rat small intestinal epithelium were purified by density gradient centrifugation followed by zonal electrophoresis on density gradients. Crude basolateral membranes were obtained by centrifugation in which the marker enzyme, $(Na^+ + K^+)$-ATPase, was enriched 10-fold with respect to the initial homogenate. The major contaminant was a membrane fraction derived from smooth endoplasmic reticulum, rich in NADPH-cytochrome *c* reductase activity. The crude basolateral membrane preparation could be resolved into the two major components by subjecting it to zonal electrophoresis on density gradients. The result was that $(Na^+ + K^+)$-ATPase was purified 22-fold with respect to the initial homogenate. Purification with respect to mitochondria and brush border membranes was 35- and 42-fold, respectively. Resolution of $(Na^+ + K^+)$-ATPase from NADPH-cytochrome *c* reductase by electrophoresis was best with membrane material from adult rats between 180 and 250 g. No resolution between the two marker enzymes occurred with material from young rats of 125 to 140 g. These results demonstrate that zonal electrophoresis on density gradients, a simple and inexpensive technique, has a similar potential to free-flow electrophoresis.

Abbreviation: SDS, sodium dodecyl sulfate.

Introduction

Epithelial cells which perform transepithelial transport of solutes and water are polarized structures with two distinct plasma membranes. Isolation of brush border membranes from small intestine and kidney cortex can easily be performed and, as a consequence, there is extensive literature on transport studies in isolated brush border membrane vesicles [1—4]. Isolation of the basolateral membrane of these cells has presented more problems and relatively few studies have been performed with basolateral membrane vesicles [5—7].

Starting from homogenates of rat kidney cortex, density gradient centrifugation followed by free-flow electrophoresis is needed to separate basolateral from brush border membranes and other cellular organelles [5,8]. Preparative procedures for the isolation of basolateral membranes from rat small intestine have been published [9,10]. Recently it was shown that these basolateral fragments, which appeared homogeneous during density gradient centrifugation, were heavily contaminated with smooth endoplasmic reticulum [11]. Using free-flow electrophoresis, Mircheff et al. [11] were able to separate basolateral membranes from endoplasmic reticulum fragments. Free-flow electrophoresis has also been used successfully in separating apical membranes of parietal cells of hog gastric mucosa [12] in isolating synaptosomes [13], erythrocyte membrane ghosts [14] and lysosomes [15]. However, the use of free-flow electrophoresis has so far been restricted to a few laboratories and the high cost of the apparatus may be the factor restricting a more general application. Recently Walters and Bont [16] developed an electrophoretic method which is expected to have a similar potential to free-flow electrophoresis but its cost is negligible. We set out to purify basolateral fragments of rat small intestine by zonal electrophoresis on density gradients in order to compare the results with those obtained by Mircheff et al. [11] who used free-flow electrophoresis. The results of both electrophoretic methods were in good agreement and zonal electrophoresis on density gradients can therefore be considered as an attractive alternative to free-flow electrophoresis.

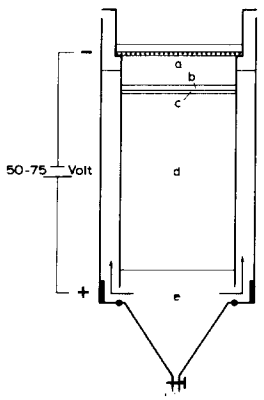
Methods and Materials

Male wistar rats of 120 to 300 g have been used, but in a single experiment the weights of the rats were equal to within 20 g. Rats were killed and the isolated intestine was rinsed as described before [9]. The mucosa from the entire length of the small intestines of four rats was scraped off and homogenized in 25 ml of 5 mM EDTA (pH 7.4) in a loose-fitting Dounce apparatus (ten strokes). After dilution to 250 ml with isolation buffer (5% sorbitol, 12.5 mM NaCl, 0.5 mM EDTA, 5 mM imidazole-histidine, pH 7.4), the homogenate was centrifuged at 25 000 × *g* for 15 min (average speed) in a 410 type rotor (Damon/IEC centrifuge type B60). The pellet, P_o, was taken up in 60 ml of isolation buffer and resuspended in a Dounce apparatus (60 strokes). The suspension was brought to 40% sorbitol by adding 1.4 vols. of 65% sorbitol and distributed to centrifuge tubes. An overlay of 3 ml of 15% sorbitol was applied and the tubes were centrifuged for 2 h at 100 000 × *g* in a swing-out rotor (type 402, Damon/IEC, maximal speed). The white band between the 15 and 40%

layers was collected (crude basolateral membranes), diluted with isolation buffer and centrifuged at 240 000 × *g* for 15 min (rotor type 460, maximal speed). The pellet was resuspended in 15 ml of solution which contained 7% sorbitol, 10 mM Tris-acetate, pH 7.4, and 0.1 mM EDTA. This suspension was homogenized with a motor-driven glass-Teflon potter (40 strokes) and layered on the gradient in the electrophoresis vessel.

Zonal electrophoresis on density gradients

The electrophoresis apparatus used has been described in detail previously [16]. In Scheme I a schematic drawing is given of the apparatus after applying



a : upper electrode: 0.2 M TRIS-acetate pH 7.4 : 300 ml
b : overlay: 6% Sorbitol, 10 mM TRIS-acetate : 100 ml
c : sample in 7% Sorbitol : 15 ml
10 mM TRIS-acetate pH 7.4
0.1 mM EDTA
d : linear gradient 10 to 20% Sorbitol : 1000 ml
10 mM TRIS-acetate pH 7.4
0.1 mM EDTA
e : 30 % Sorbitol, 0.4 M Bicine pH 8.0 : 600 ml

Scheme I. Schematic drawing of the electrophoresis apparatus after loading of electrode buffers, gradient, sample and overlay. The inner cylinder has a diameter of 8 cm. The protein content of sample c varied between 20 and 50 mg among different experiments. Bicine, *N,N*-bis(2-hydroxyethyl)glycine (Merck, Darmstadt).

the gradient, sample and overlay. To apply the sample smoothly as a thin film without disturbing the gradient, a sieve was used as described by Walters and Bont [16]. Electrophoresis is carried out at 4°C. After electrophoresis, the inner cylinder is lowered and pressed down on an 0-ring at the bottom of the outer cylinder. Then, the gradient was fractionated in samples of 20 ml. Absorbance at 230 nm was used to detect the membranes. All of the membranes were present in a total volume of about 300 ml. The fractions were centrifuged at 240 000 × *g* for 20 min, the pellets were taken up in 1 ml isolation buffer and their enzymic content was analyzed.

SDS-acrylamide gel electrophoresis

Peak fractions of basolateral membranes (50 μg of protein) and smooth endoplasmic reticulum (50 μg of protein) obtained by zonal electrophoresis (Fig. 1) were loaded on 10% acrylamide/0.2% SDS slab gels (thickness 1 mm) with a 3% acrylamide stacking gel and run in the discontinuous buffer system described by Laemmli [17]. The gel was stained in a solution containing 0.2% Coomassie brilliant blue R-250 in 50% methanol/3% acetic acid for 45 min, destained in 10% methanol/5% acetic acid and dried on BioRad gel-slab dryer. Molecular weight determinations were carried out according to the method of Weber and Osborn [18] using skeletal muscle myosin (200 000 daltons), phos-

phorylase *a* (94 000 daltons), bovine serum albumin (68 000 daltons), catalase (58 000 daltons), ovalbumin (43 000 daltons) and chymotrypsinogen (27 000 daltons) as markers.

Enzyme assays

The following enzymes were assayed according to published procedures: $(Na^+ + K^+)$-ATPase, a basolateral membrane marker [19]; sucrase or invertase, a brush border marker [20]; succinic dehydrogenase [21]; NADPH-cytochrome *c* reductase [22]. Protein was determined by means of a Coomassie Blue binding assay (Bio-Rad).

Reagents

NADPH was obtained from Boehringer GmbH (Mannheim). Glucose oxidase, peroxidase, dianisidine (3,3-dimethoxybenzidine), cytochrome *c*, ATP and *p*-iodonitrotetrazolium were obtained from Sigma (St. Louis, MO). All other chemicals were obtained from Merck (Darmstadt).

Results

The two centrifugation steps prior to electrophoresis resulted in a good separation of basolateral membranes from soluble protein, mitochondria and brush borders as shown in Table I. The major contaminant of the crude basolateral membrane fraction is a membrane population most likely derived from smooth endoplasmic reticulum, as indicated by the NADPH-dependent cytochrome *c* reductase activity. Table I shows that $(Na^+ + K^+)$-ATPase is enriched up to 10-fold and that it is also purified 14- and 21-fold with respect to mitochondria and brush borders, respectively. Mircheff and Wright [19] have shown previously that basolateral membranes and part of the endoplasmic reticulum co-purify during equilibrium centrifugation. We subjected the crude basolateral membrane fraction directly to zonal electrophoresis. The results are shown in Fig. 1 $(Na^+ + K^+)$-ATPase and alkaline phosphatase co-purified in a rather broad band. The peak of NADPH-cytochrome *c* reductase was always relatively sharp and ahead of the $(Na^+ + K^+)$-ATPase peak. The protein distribution among the fractions never showed discrete peaks. The small mitochondrial contamination always peaked between the two major markers. The small amount of brush border membranes was recovered from the cathodic end of the protein band,

TABLE I

COMPOSITION OF THE CRUDE BASOLATERAL MEMBRANE (BLM) FRACTION WITH RESPECT TO THE INITIAL HOMOGENATE ($S_O + P_O$)

Results are expressed as percentages of the total activity estimated as the sum of the contents of supernatant (S_O) and pellet (P_O). Values presented are means ± S.E.; number of observations in parentheses.

Crude BLM	$(Na^+ + K^+)$-ATPase	Succinic dehydrogenase	Sucrase	NADPH-cytochrome *c* reductase	Protein	
Yield in % of ($S_O + P_O$)	25.1 ± 3.1 (11)	1.8 ± 0.4 (7)	1.2 ± 0.3 (5)	9.8 ± 1.8 (9)	2.6 ± 0.3 (8)	%

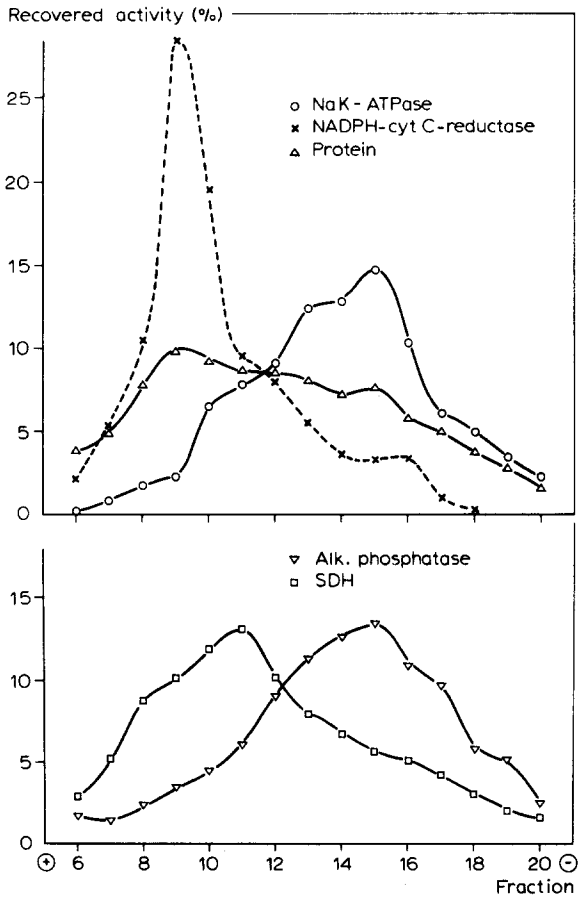


Fig. 1. Marker enzyme distribution after electrophoresis of the crude basolateral membrane fraction (Table I). Electrophoresis was performed at 2.0 V/cm, 34 mA for 8 h in a cold room (4°C). Enzyme activities are expressed as percentage of total recovery in the pellets of electrophoresis fractions numbered 6—20. The rats in this experiment weighed 180—200 g. Note than the succinic dehydrogenase (SDH) content in the crude basolateral fraction is only 1.8% of the initial homogenate (Table I).

behind the $(Na^+ + K^+)$-ATPase peak. The mitochondrial contamination of the basolateral membrane peak fraction 15 was approx. 0.1% of the initial succinic dehydrogenase activity while the sucrase activity in fraction 15 was approx. 0.2% of the initial activity. This means that $(Na^+ + K^+)$-ATPase was purified 35- and 42-fold with respect to succinic dehydrogenase and sucrase, which is comparable to the results obtained with free-flow electrophoresis by Mircheff et al. [11]. Quantitative data from six electrophoresis experiments on the purification of basolateral membrane fragments are shown in Table II. After the two purification steps, centrifugation and electrophoresis, $(Na^+ + K^+)$-ATPase was enriched 22-fold. The total recovery of protein and $(Na^+ + K^+)$-ATPase after pelleting the electrophoresis fractions was about 60% of the applied quantity. When $(Na^+ + K^+)$-ATPase assays were performed directly in the electrophoresis fractions, the recovery of $(Na^+ + K^+)$-ATPase amounted to 100%. This indicates that the electrophoresis step did not inactivate $(Na^+ + K^+)$-ATPase but that

TABLE II

SPECIFIC ACTIVITIES OF $(Na^+ + K^+)$-ATPase AFTER DENSITY GRADIENT CENTRIFUGATION AND SUBSEQUENT ELECTROPHORESIS

$(Na^+ + K^+)$-ATPase activity is expressed in μmol P_i/h per mg protein and represents mean values ± S.E.; number of experiments in parentheses. S_O, supernatant; P_O, pellet.

	$S_O + P_O$	Crude basolateral membranes	Peak fraction of electrophoresis (20 ml)
$(Na^+ + K^+)$-ATPase specific activity	1.61 ± 0.14 (9)	15.9 ± 1.5 (11)	35.2 ± 2.0 (6)
Yield (%)	100	25.1	4.0

membrane material was not totally recovered during harvesting by centrifugation.

With a smaller electrophoresis apparatus (diameter of inner cylinder 4 cm) we could raise the electric field strength to 5 V/cm without an increase in temperature during electrophoresis. This small apparatus was used for purification of basolateral membranes from duodenal epithelium and a separation between NADPH-cytochrome *c* reductase and $(Na^+ + K^+)$-ATPase could be realized within 3 h at 5 V/cm and 24 mA.

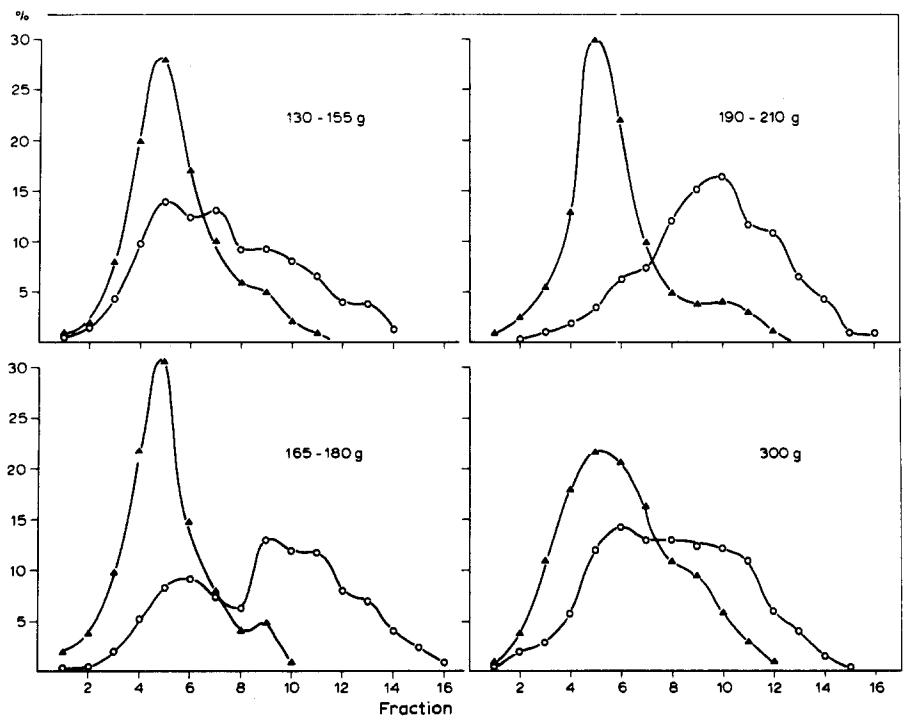


Fig. 2. Age effect of the rats used on the distribution of basolateral membranes and smooth endoplasmic reticulum markers after electrophoresis. The experiments have been performed twice for the age group given. Marker activities are given as percentage of total recovery after electrophoresis. ○———○, $(Na^+ + K^+)$-ATPase; ▲———▲, NADPH-cytochrome *c* reductase. Enzyme activities are expressed as percentage of total recovery in the pellets of electrophoresis fractions.

In the course of the experiments we became aware of an effect of the rats' age on the resolution of the two marker enzymes. With young rats (120—135 g) we were unable to resolve ($Na^+ + K^+$)-ATPase from NADPH cytochrome *c* reductase. In Fig. 2 the results of varying the age of rats are summarized. The best electrophoretic separation between basolateral membranes and endoplasmic reticulum is obtained with the intestine from rats between 180 and 250 g. With increasing age, the resolution appeared to decrease again.

Fig. 3 shows the protein composition of the smooth endoplasmic reticulum and basolateral membrane peak fractions obtained by zonal electrophoresis as analyzed on SDS-acrylamide gels. Each staining pattern consists of a least 15 different protein bands with molecular weights between about 25 000 and 200 000. No major protein bands of lower molecular weight were detected on small-pore gels (15—20% acrylamide, results not shown). Most illustratively for the difference between both membrane fractions is the 57 000 dalton protein

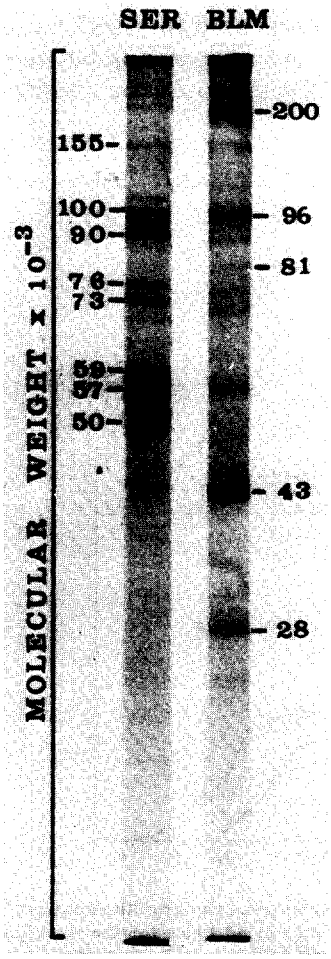


Fig. 3. Protein-staining patterns of single peak fractions of basolateral membranes (BLM) and smooth endoplasmic reticulum (SER).

heavily stained in the smooth endoplasmic reticulum pattern but nearly absent in the basolateral membrane, as well as the 43 000 and 200 000 dalton bands that are most pronounced in the plasma membranes but only weakly visible in the endoplasmic reticulum staining pattern. On acrylamide gels of various composition (experiments not shown), the latter two proteins comigrate exactly with actin and myosin, respectively, suggesting a specific and tight association of basolateral membranes with cytoskeleton elements of the enterocyte.

Discussion

In this study we have used a novel electrophoretic method to purify basolateral plasma membranes from rat small intestinal cells. By a combination of centrifugation techniques and zonal electrophoresis on density gradients, $(Na^+ + K^+)$-ATPase-containing membranes could be purified 20- to 25-fold. The overall yield of basolateral membranes was about 15% of the initial quantity. These results are comparable with those obtained by Mircheff et al. [11], who used a combination of zonal centrifugation and free-flow electrophoresis to purify basolateral membranes from rat duodenum. Their yield was higher but this is explained by the use of a zonal centrifugation step prior to electrophoresis. Our results show clearly that zonal electrophoresis on a density gradient has the same potential as free-flow electrophoresis. The advantages of zonal electrophoresis on density gradients are the low costs and the simple equipment. An advantage of free-flow electrophoresis is the relatively short time it takes to separate membrane fractions. However, with a smaller electrophoresis vessel we were able to increase the field strength up to 5 V/cm without an increase in temperature and under these conditions the electrophoresis could be finished within 3 h. With free-flow electrophoresis the sample size is in theory unlimited, since loading of the sample is continuous. In practice, the problem of sample size can be overcome with zonal electrophoresis simply by increasing the diameter of the electrophoresis vessel, so more protein can be layered as a thin starting layer on top of the gradient. The method described here should also be of value in separating brush border from basolateral membranes of rat kidney cells, since it is known that these plasma membranes can be separated only on the basis of the difference in their surface charge [8].

In contrast with the complete resolution of a mixture of three proteins into three sharp bands as described by Walters and Bont [16], we never found a complete resolution of the membrane fractions and observed relatively broad bands. The reason for this difference must be that the membrane fractions are not homogeneous, either in origin or in vesicle size. In our study, whole small intestine was used and there may be considerable variation in membrane properties along the intestinal tract. An inhomogeneity in membrane vesicle size of fragmented biomembranes has been demonstrated recently by Bont et al. [23] in studies with an analytical centrifuge. Also, the membrane material in our study did not have a uniform vesicle size (data not shown). Although these factors influence the resolution of membrane mixtures, our results demonstrate that zonal electrophoresis on density gradients is a valuable, attractive, low-cost technique for purification of plasma membranes.

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