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AN IMPROVED METHOD FOR ISOLATION OF BASOLATERAL MEMBRANES FROM RAT KIDNEY

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Summary

We have described a method using differential centrifugation and free-flow electrophoresis for the isolation of basolateral membranes from rat renal cortical tissue. Purity of the membrane preparation was assessed by determination of enzymic constituents and substantiated by observation of transport characteristics for D- and L-glucose, L-proline and L-glutamine. Specific activity of $(Na^+ + K^+)$-ATPase in the pooled electrophoretic fractions was 15.4 times that in the cortical homogenate, and the relative purification of $(Na^+ + K^+)$-ATPase compared to alkaline phosphatase was 25-fold. L-Glucose showed no Na^+-gradient dependence and was used as a measure of diffusion. Uptake of D-glucose as well as L-proline followed the same time course in the presence or absence of an Na^+ gradient. Only with L-glutamine was uptake stimulated by the presence of an Na^+ gradient. Our data indicate we have sucessfully isolated basolateral membranes which are free of brush border contamination in superior yield and with greater facility than previously reported.

Introduction

A decade ago, Fitzpatrick et al. [1] demonstrated that plasma membranes from renal proximal tubule cells could be isolated by differential centrifugation in relatively large quantities. This membrane preparation consisted of

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about 70% isolated basolateral (antiluminal) membranes and 30% brush border (luminal) membranes, as assessed by use of marker enzymes. Further centrifugation in an attempt to purify the basolateral membranes has been only minimally successful. To date, the most successful purification of renal cortical antiluminal membranes has been through the use of a free-flow electrophoresis which allows the separation and isolation of both basolateral and brush border membranes [2]. The major advantage of the latter method is the parallel isolation from a single tissue sample of two different membrane fractions representing the different poles of a single cell. The major disadvantage is that the quantitative recovery of minimally contaminated basolateral membranes by this method is relatively low. We have utilized the method of Fitzpatrick et al. [1] for the initial preparation of basolateral membranes in combination with free-flow electrophoresis in order to improve the yield and purity of a basolateral membrane preparation and have assessed the transport characteristics of vesicles made from these membranes. These results form the basis of this paper.

Materials and Methods

Membrane preparation. Renal cortical plasma membranes were isolated by differential centrifugation using a modification of the method described by Fitzpatrick et al. [1]. Adult male Sprague-Dawley rats fed ad libitum on Purina rat chow and water were killed by decapitation, the kidneys removed, decapsulated and placed in saline on ice. Kidney cortical slices were obtained with a Stadie-Riggs microtome and were homogenized (1 : 3, w/v) in buffer I (0.25 M sucrose in 0.01 M triethanolamine hydrochloride titrated to pH 7.4 with NaOH) using eight strokes in a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle (0.01 inch clearance) at 300 rev./min. All operations thereafter were performed at 4°C. The homogenate was centrifuged at $1475 \times g$ for 10 min. The resultant supernatant was discarded and the pellet was resuspended in 2 M sucrose (1 ml per g starting tissue) and homogenized with three strokes of a motor-driven Potter-Elvehjem tissue grinder at 1000 rev./min. This suspension was centrifuged for 10 min at $13\,300 \times g$. The supernatant was diluted to isotonicity with the addition of 7 vols. of distilled water, homogenized with three strokes of the motor-driven Potter-Elvehjem tissue grinder, and centrifuged at $33\,000 \times g$ for 15 min. After centrifugation, half of the supernatant was discarded and the fluffy, upper pellet was resuspended in the remaining supernatant. This was homogenized with three strokes of the motor-driven Potter-Elvehjem tissue grinder and centrifuged at $33\,000 \times g$ for 15 min. The upper, fluffy part of the pellet was resuspended in buffer I (1 ml per g starting tissue), homogenized with three strokes of the motor-driven Potter-Elvehjem tissue grinder, and again centrifuged at $33\,000 \times g$ for 15 min. Following the last centrifugation, the upper part of the pellet was resuspended in electrophoresis running buffer (0.25 M sucrose in 8.5 mM triethanolamine titrated with acetic acid to pH 7.4) to a protein concentration of 3.5—5.5 mg/ml and injected into a Bender and Hobein VaP5 free-flow electrophoresis apparatus (Munich, F.R.G.) through the port above fraction 70 at a rate of 6 ml/h. During the free-flow run, the voltage was

maintained at 875 V and the temperature was maintained at 4.5°C, conditions similar to those previously reported [2,3]. Most free-flow electrophoresis machines are designed to maintain a constant current and allow both voltage and resistance to vary. In the method described here, the current was adjusted manually throughout the run in order to maintain a constant voltage. As a result, no evidence of any shift in the path which the membranes traversed in the separation chamber was observed with extended running time. This allows an unlimited total volume of material to be separated. In our standard runs, an average of 7 ml of membrane suspension were injected. Running buffer flowed through the separation chamber at a rate of 160 ml/h. The electrode buffer consisted of 85 mM triethanolamine titrated to pH 7.4 with acetic acid. After separation, the samples were assayed for appropriate membrane enzyme markers and protein concentration. Those fractions containing (Na^+ + K^+)-ATPase activity, the marker enzyme for basolateral membranes [2], were pooled and collected by centrifugation at 33 000 × *g* for 15 min. Basolateral membranes were resuspended in buffer II (100 mM mannitol plus 2 mM Hepes adjusted to pH 7.5 by the addition of Tris) to a final protein concentration of 0.2—0.4 mg/ml as determined by using the method of Lowry et al. [4].

Enzyme determinations. All materials used were of the highest quality available. Enzyme levels were determined on aliquots of the initial cortical homogenate and from pooled basolateral membrane fractions after electrophoresis. These determinations were performed according to standard procedures: alkaline phosphatase (E.C. 3.1.3.1) [5], acid phosphatase (E.C. 3.1.3.2) [5], succinic dehydrogenase (E.C. 1.3.99.1) [6], the γ-glutamyltransferase (E.C. 2.3.2.2) component activities of γ-glutamyl transpeptidase and γ-glutamyl hydrolase [7] and (Na^+ + K^+)-ATPase (E.C. 3.6.1.3) [8]. Glucose-6-phosphatase (E.C. 3.1.3.9) activity was measured in an assay modified from that of Hubscher and West [9]. 0.1 ml of 0.2 M glucose 6-phosphate, 0.5 ml of 0.05 M maleate buffer containing 4 mM EDTA, pH 6.0, and 0.1 ml of membrane suspension were incubated for 15 min at 37°C. The reaction was stopped by adding 1.0 ml of 20% trichloroacetic acid to precipitate protein. After centrifugation, 1.0 ml of the supernatant was used for determination of inorganic phosphate as previously described [8].

Determination of metabolic activity. Basolateral membrane vesicles were incubated for 20 min with 0.02 mM [^{14}C]glutamine at 22°C. Trichloroacetic acid, at 4°C, was then added to the incubation mixture to a final concentration of 10%. The mixture was allowed to rest on ice for 10 min after which it was centrifuged at 31 000 × *g* for 15 min. The supernatant was spotted on Whatman No. 2 paper and descending chromatography performed with a solvent mixture of butanol/acetic acid/water (4 : 1 : 2). After the chromatographic run, the air-dried paper was cut in 0.5 cm strips and counted in a liquid scintillation counter.

Transport measurements into basolateral membrane vesicles. The measurement of amino acid uptake by membrane vesicles using Millipore filtration on HAWP filters (0.45 μm) was performed using the technique described by McNamara et al. [10]. All uptake experiments were performed at 22°C. Trapped and diffused space was measured using L-[^{3}H]glucose as previously described [11].

Materials. Radioactive compounds were purchased from New England Nuclear Corp.: [U-^{14}C]proline (261 mCi/mM), [U-^{14}C]glutamine (226 mCi/mM), D-[U-^{14}C]glucose (4 mCi/mM), and L-[U-^{3}H]glucose (17.46 Ci/mM).

Results

Luminal and antiluminal marker enzyme determinations were made on sequential free-flow electrophoresis fractions and the results are shown in Fig. 1A. The luminal marker enzyme activity measured was alkaline phosphatase and the antiluminal marker enzyme activity was ($Na^+ + K^+$)-ATPase. The two populations of cellular membranes migrated at different rates due to membrane surface charge and size, and were separated into two major regions. Fractions 2—26 contained primarily basolateral membranes and fractions 26—42 contained brush border membranes. The profile of protein concentration in the fractions is shown in Fig. 1B. Protein concentrations of free-flow electrophoresis fractions 2—34 were greater than 0.25 mg/ml and did not fall below this value until after fraction 34. In order to determine whether or not any other cellular membranes migrated or were associated with the basolateral membranes, free-flow electrophoresis fractions 2—21 were pooled and specific activities of six marker enzymes were determined in these pooled basolateral membrane fractions as well as in the initial homog-

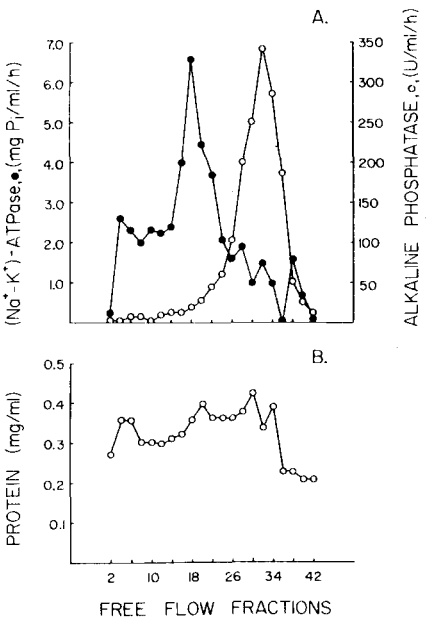

Fig. 1. Separation of isolated renal cell membranes by free-flow electrophoresis. Following differential centrifugation, isolated renal cellular membranes were separated using a VaP5 free-flow electrophoresis apparatus as described in the text. The enzymatic activities of both ($Na^+ + K^+$)-ATPase (●———●) and alkaline phosphatase (○———○) were determined in the fractions and are shown in A. The profile of protein concentrations is shown in B for the same fractions. This figure represents the profiles of one free-flow electrophoretic run with duplicate determinations for each point shown. Three separate preparations showed profiles similar to the ones presented here.

TABLE I

SPECIFIC ACTIVITIES OF ENZYMES IN ORIGINAL HOMOGENATE AND BASOLATERAL PREPARATION FROM ADULT RAT KIDNEYS

Basolateral membranes were isolated and enzyme assays performed as described in Materials and Methods. Units for the specific activity of the enzyme measured are indicated in parentheses under the enzyme name. Values shown are the mean ± S.E. from *n* experiments performed in triplicate. BL/H is the ratio of activity in basolateral membranes to activity in homogenate.

Enzymes	Cellular marker	Basolateral	*n*	Homogenate	*N*	BL/H Ratio
$(Na^+ + K^+)$-ATPase (nmol P_i liberated/mg per h)	Basolateral membrane	108.44 ± 16.42	7	7.06 ± 4.08	7	15.36
Mg^{2+}-ATPase (nmol P_i liberated/mg per h)		46.12 ± 7.18	6	14.12 ± 4.11	6	3.27
Alkaline phosphatase (U/mg per h)	Brush border membrane	195.53 ± 14.94	3	338.93 ± 17.72	4	0.58
Acid phosphatase (U/mg per h)	Lysosomes	67.55 ± 5.93	7	127 ± 14.38	7	0.53
Glucose-6-phosphatase (mmol P_i liberated/mg per h)	Microsomes	7.08 ± 2.08	4	10.86 ± 2.01	4	0.65
Succinate dehydrogenase (A_{490}/mg per h)	Mitochondria	0.78 ± 0.22	5	9.73 ± 2.94	5	0.09

enates. The results are shown in Table I. The specific activities of $(Na^+ + K^+)$-ATPase, the basolateral marker, and Mg^{2+}-ATPase, a generalized membrane marker, were the only enzyme activities which were enriched in the pooled fraction compared to the initial homogenate. The relative specific activity for $(Na^+ + K^+)$-ATPase of 15 indicates that the pooled fraction consisted primarily of basolateral membranes. The relative specific activities for the four other subcellular marker enzymes measured in the pooled fractions were less than unity when compared to the original homogenate, indicating a low degree of contamination of the pooled fraction by other cellular components. Quantitative recovery of these various marker enzymes from the initial homogenate were determined to be 21% for $(Na^+ + K^+)$-ATPase, 4.5% for Mg^{2+}-ATPase and less than 0.9% for the other four enzymes, listed in Table I, in the pooled basolateral membrane. In addition to the marker enzymes shown in Table I, the γ-glutamyltransferase component activities, γ-glutamyl transpeptidase and γ-glutamyl hydrolase, were assayed and showed relative specific activities of 0.1 compared to the original homogenate. Chromatographic analysis of incubation mixtures of basolateral membrane vesicles and 0.02 mM [^{14}C]glutamine showed no conversion to glutamic acid after 20 min at 22°C. Since hydrolysis of glutamine has been shown to be a property of isolated brush border membrane vesicles [11], the absence of this activity is indicative of little brush border membrane contamination associated with the isolated basolateral membrane.

The pooling of free-flow fractions 2—21 yielded approx. 5 mg of basolateral membrane protein per 10 g of rat renal cortex. This yield is obtained after a 90 min electrophoretic separation (total preparation time from initial homogenate to isolated basolateral membranes was 3.5 h). The pooled basolateral fractions contained a mixture of open sheets and sphere-like vesicles (50 : 50) when placed in buffer II as determined by the degree of oubain-sensitivity

of (Na^+ + K^+)-ATPase following the method of Kinne [12]. About 50% of the membrane vesicles were oriented right-side-out and 50% of the membrane vesicles were oriented inside-out as determined by ATPase activity after deoxycholate treatment [13]. L-Glucose was used to measure intravesicular volume which was determined to be 2 μl per mg of protein for membranes suspended in buffer II. This buffer was 100 mosM with respect to mannitol. Intravesicular size was decreased by increasing the medium osmolarity with sucrose as shown in Fig. 2, where the uptake of L-glucose by basolateral membranes was inversely proportional to medium osmolarity. By extrapolating the line in Fig. 2 to an infinitely high osmolarity where the vesicles could contain an infinitely small intravesicular space, no uptake of this isomer of glucose could be detected as the line intersected the ordinate at zero. Thus, no binding component for L-glucose appeared to exist in the membrane.

Since carrier systems for sugar and amino acid transport are characterized by their stereospecificity, double-labelled experiments using the D- and L-stereoisomers of glucose enabled the simultaneous measurements of L-glucose diffusion and D-glucose uptake, which is carrier-mediated in isolated renal brush border membrane vesicles [3]. Uptake of D-glucose in excess of L-glucose was determined and is presented in Fig. 3A. The uptake in excess of diffusion by basolateral membranes in the presence of an inward Na^+ gradient showed no statistical difference compared to uptake when Na^+ was equilibrated across

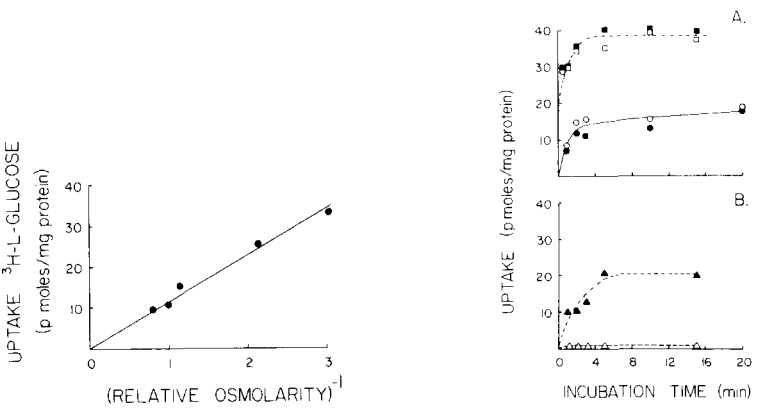

Fig. 2. Effect on medium osmolarity on the uptake of L-glucose by basolateral membranes. Basolateral membranes were allowed to incubate for 30 min in medium containing buffer II and NaCl plus various concentrations of sucrose in order to change the osmolarity of the incubation medium. L-[^{14}C]-Glucose was then added and incubated with the vesicles for an additional 24 min, after which the mixture was filtered and radioactivity determined as described in the text. Results shown are the mean of at least 11 determinations at each relative osmolarity. Relative osmolarity is defined as 300 mosM = 1 = isomolar and 100 mosM = 0.1.

Fig. 3. Time course of uptake of D-glucose, L-proline, and L-glutamine. The time course of 0.05 mM D-[^{14}C]glucose (●,○) and 0.02 mM L-[^{14}C]proline (■,□) uptake (A) and 0.02 mM L-[^{14}C]glutamine (▲,△) uptake (B) by isolated rat renal basolateral membrane vesicles was measured under either Na^+-gradient conditions (solid symbols) or sodium equilibrated conditions (open symbols) by rapid filtration as described in the text. The uptake levels represent uptake in excess of diffusion as assessed by the simultaneous determination of L-[^{3}H]glucose space. Each point represents the mean of at least eight determinations. No significant difference was observed between values obtained under Na^+-gradient and equilibrated conditions in A. In B, the differences in the curves drawn are significant with the standard errors less than ±5% of the mean.

the membrane. Thus, D-glucose uptake by these membranes appears to be independent of the Na^+ gradient or non-electrogenic in nature. Similarly, uptake of L-proline appeared to be independent of an Na^+ gradient when L-proline replaced D-glucose in such double-labelled experiments as are depicted in Fig. 3A.

In contrast to the observations with D-glucose and L-proline, the time course of glutamine uptake by basolateral membrane vesicles exhibited a marked stimulation under an Na^+-gradient (extravesicular → intravesicular) as compared to uptake when Na^+ was equilibrated across the membrane. Fig. 2B shows the uptake of glutamine in excess of diffusion as measured with L-glucose. Under Na^+-equilibrated conditions, little or no uptake in excess of diffusion occurred. However, when an inward Na^+ gradient was imposed, glutamine uptake exceeded both the level of diffusion as measured with L-glucose and the level of glutamine uptake when Na^+ was equilibrated across the membrane. Thus, glutamine uptake by basolateral membrane vesicles appears to be stimulated by an inward NaCl gradient in the time course studied.

Discussion

We have described a method for isolation of purified basolateral membranes from rat renal cortex by differential centrifugation and free-flow electrophoresis which provides more highly purified membranes in larger yield more rapidly than those obtained by previous methods [2]. The integration of two previously described methods takes advantage of the positive attributes of both and should permit a greater facility in the assessment of transport and other characteristics of basolateral membranes from renal cortex. In our method, 5 mg of basolateral membranes can be isolated from 10 g of cortex by a single electrophoretic run. These basolateral membranes show an enrichment of $(Na^+ + K^+)$-stimulated ATPase of 15.4 compared to the cortical homogenate and a relative specific activity of $(Na^+ + K^+)$-ATPase to alkaline phosphatase of more than 25, yet they show no alkaline or acid phosphatase enrichment compared to the homogenate. In contrast, a single electrophoretic run using the method of Heidrich et al. [2] results in 60% of our yield of basolateral membranes as measured by protein determination. However, their single-run preparation has a significant degree of lysosomal and brush border marker enzymes associated with it which have been removed during our isolation procedure. Indeed, Heidrich et al. [2] required a second electrophoretic run of their combined basolateral membrane fractions to obtain a maximum enrichment of $(Na^+ + K^+)$-stimulated ATPase of 15.8 compared to the cortical homogenate and a relative specific activity of $(Na^+ + K^+)$-ATPase to alkaline phosphatase of only 9.4. These relative enrichment factors apply to a single fraction in the second run. Examination of the protein profile of our fractions obtained on a single electrophoretic run reveals about equal amounts of protein in the brush border and basolateral fractions as opposed to the single-run profile shown by Heidrich et al. [2] where the pooled basolateral fractions contain less than 30% of the protein.

Since the VaP5 free-flow electrophoresis apparatus, buffer and running conditions we are using are similar to those described by Heidrich et al. [2],

the observed differences in yield and purity of a single run must be due to inherent differences in the material injected in the VaP5 apparatus. We utilized a crude plasma membrane fraction isolated by using a modification of the method by Fitzpatrick et al. [1] which included a greater percentage of basolateral membranes as the starting material for our electrophoretic separation. Heidrich et al. [2] utilized a crude brush border preparation as starting material. By starting with the larger membrane fragments, we eliminated a great deal of the brush border contamination in our final preparation. Presumably, the larger membrane fragments remaining after high shearing forces are used contain proportionately lower percentages of microvilli than whole cell membranes, since such shearing forces tend to result in the release of single microvilli [14]. This could explain the much lower alkaline phosphatase contamination observed in our preparation. In essence, Heidrich et al. [2] demonstrated electrophoretic separation of a predominantly brush border fraction and removed basolateral contamination; we have used electrophoresis to isolate basolateral membranes by removing brush border contaminants, thus resulting in a larger yield.

In order to carry out transport experiments, the largest yield of membranes with the highest purity from combined fractions is the aim of the separation procedure. The method we have employed to obtain basolateral membranes from a single free-flow electrophoretic run results in a spectrum of fractions, Nos. 2—21, all of which appear to contain basolateral membrane protein which is relatively uncontaminated by brush border or subcellular organelle markers. By pooling these fractions, we realized a generous yield of purified membranes with a relative enrichment for $(Na^+ + K^+)$-ATPase compared to alkaline phosphatase of 25.

In light of this improved yield and purification, we have carried out transport studies with D-glucose, L-proline and D-glutamine and demonstrated that our basolateral membrane vesicles, which had intravesicular volumes and osmotic activities similar to those previously reported [12], have transport characteristics which differ from those in brush border membranes. In the uptake of both D-glucose and L-proline by basolateral membranes, no 'overshoot' phenomenon in the presence of an Na^+ gradient was observed. This is in contrast to the observation of Slack et al. [15] but does not differ from their conclusion that there is no Na^+-stimulated overshoot of glucose and proline in purified basolateral membranes. They attributed their observed overshoot in the presence of an Na^+ gradient to contamination of their basolateral membranes by brush border membranes. Our transport studies with more highly purified basolateral membranes confirm this.

Rapid hydrolysis of glutamine to glutamic acid did not occur during the uptake of glutamine by basolateral membranes. Such hydrolysis occurs in the presence of brush border membranes which contain glutaminase (γ-glutamyl transpeptidase) [11]. This again indicates negligible contamination of our preparation by brush border membranes. There was no glutamine uptake in excess of diffusion by basolateral membranes under conditions where Na^+ was equilibrated across the membrane, unlike the finding in brush border membranes [11]. There was, however, uptake of glutamine in excess of diffusion by our basolateral membranes under Na^+-gradient conditions, which

has also been observed in brush border membrane vesicles [11]. We feel, however, that the increased uptake of glutamine under Na^+-gradient conditions is an inherent characteristic of the basolateral membrane since no brush border contamination of our preparation can be demonstrated and agrees with the observation by others [16,17].

Acknowledgement

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