Aldosterone-stimulated Sodium Uptake by Apical Membrane Vesicles from A6 Cells*

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The properties of the A6 amiloride-sensitive apical sodium transporter are similar to those of apical sodium transport systems found in the distal nephron and in frog skin and toad bladder (4-6). In these tissues, selective conservation of sodium is accomplished by the active transport of this ion from mucosa to serosa (7). This active mechanism is modulated by mineralocorticoids and antidiuretic hormone. The rate-limiting step in this process and the apparent primary site of action of these hormones are the apical sodium transport pathway (8-11). Sodium moves down its electrochemical gradient into the cells via this system and is subsequently actively pumped across the basal membrane into the serosal solution. The ability to induce the amiloride-sensitive apical sodium transporter in a cultured cell line under appropriate growth conditions makes the A6 cells a particularly valuable model system for studying the role of this transporter in salt and water homeostasis (2, 3). Further studies of this transport system using a new preparation enriched in apical membrane vesicles from A6 cells are presented here. These vesicles retain the amiloride-sensitive, aldosterone-stimulated sodium transport system and provide a much more direct means for studying it than can be attained with an intact cell preparation.

MATERIALS AND METHODS

Cell Culture—A6 cells were purchased from American Type Culture Collection (Rockville, MD) in the 68th plating. Stock cultures were carried as described previously (3), except that 5% rather than 10% fetal bovine serum was used in the growth medium. Lowering the serum concentration did not appear to alter the growth pattern of the cells or the development of transepithelial voltage. Vesicles were prepared from A6 cells grown in filter-bottomed cups. The cups were made from rings of acrylic tubing 1 cm high and 10.7 cm in diameter. A Millipore filter (HAWP) was attached to one end in the following way. The filters were soaked for 10 min in a solution of 30% ethanol in water (v/v) then removed and placed on a flat surface. The acrylic rings were dipped for 5 s in methylene chloride and firmly applied to the wet filters for 10 s. Three small polycarbonate plastic strips 0.25 mm thick were glued to the underside of each cup along its outer edge and the filter the bottom of a cup-like structure). In contrast, A6 cells grown on conventional plastic dishes showed no significant amiloride-sensitive component of apical sodium uptake (3). Since cells grown on plastic dishes have access to the culture medium from only their apical surface, whereas cells grown in filter-bottomed cups are bathed with medium from both above and below, this result provides good evidence that the amiloride-sensitive apical sodium entry pathway is only expressed when the cells are bathed with culture medium on both sides.

A6 is a continuous epithelial cell line derived from the kidney of the aquatic toad Xenopus laevis (1). Previous electrophysiological and radiotracer studies of intact A6 epithelia demonstrated the existence of amiloride-sensitive, aldosterone-stimulated transepithelial sodium transport in these cells (2). More recently, employing direct measurements of 22Na uptake into A6 monolayers (3), we have demonstrated that sodium transport across the apical surface of A6 epithelium is saturable ($K_m = 18$ mM, $V_{max} = 2.5$ nmol/min/cm²), competitively inhibited by amiloride ($K_i = 5 \times 10^{-8}$ M), and markedly stimulated by aldosterone (3-fold after 10-7 M aldosterone for 4 h). For these studies, A6 epithelia were grown in “filter-bottomed cups” (polycarbonate rings with Millipore filters glued to one end so that the ring forms the sides and the filter the bottom of a cup-like structure). In contrast, A6 cells grown on conventional plastic dishes showed no significant amiloride-sensitive component of apical sodium uptake (3).

Since cells grown on plastic dishes have access to the culture medium from only their apical surface, whereas cells grown in filter-bottomed cups are bathed with medium from both above and below, this result provides good evidence that the amiloride-sensitive apical sodium entry pathway is only expressed when the cells are bathed with culture medium on both sides.

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1 The abbreviation used is: 10 mM Tris/HEPES (N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid) buffered with Tris to pH 7.4.
A6 Membrane Vesicles

The cells were scraped into homogenization medium from the filters with a rubber policeman and diluted to a total volume of 30 ml. The scraped cells were left on ice for 10 min then homogenized at 4 °C in a VirTis model 23 homogenizer for 10 min at setting 4 and for 4 min at top speed. The homogenate was left on ice for 10 min and then centrifuged for 15 min at 5,000 × g. The supernatant was saved and centrifuged for 20 min at 43,000 × g. The resulting pellet was suspended in 3 ml of a buffer appropriate for the experiment and recentrifuged at 43,000 × g. This pellet was resuspended in the appropriate buffer, passed successively through a 22-, 25-, and a 30-gauge needle and stored on ice until use. This procedure typically yielded 2.0–5.5 mg of membrane protein. Aliquots of the homogenate and centrifugation were frozen in a dry ice/ethanol bath and stored above liquid nitrogen for the enzyme assays and occasionally for future transport experiments. We generally used fresh material for transport studies, but found no systematic difference in the transport properties of frozen vesicles.

Enzyme Assays—The purity of the vesicle preparation was monitored by measuring the activities of alkaline phosphatase and γ-glutamyltranspeptidase (apical membrane markers), glucose-6-phosphatase (an endoplasmic reticulum marker), and succinic dehydrogenase (a mitochondrial inner membrane marker). Na/K-ATPase activity was not measurable, owing to the presence of a high Mg-ATPase characteristic of other amphibian epithelial cells. Alkaline phosphatase, succinic dehydrogenase, and γ-glutamyltranspeptidase, and protein were measured as previously described (12). γ-Glutamyltranspeptidase was measured colorimetrically using Sigma kit 545.

Apical Cell Membrane Iodination—Covalent incorporation of 125I into the apical membrane of intact A6 cells growing in filter-bottomed cups was carried out using the New England Nuclear [125I]iodosulfanilic acid Labeling Kit (NEX-121) as follows. A filter-bottomed cup was washed 10 times with "A6 Ringer" (110 mM NaCl, 2.5 mM NaHCO₃, 3 mM KCl, 1 mM KH₂PO₄, 1 mM CaCl₂, 0.5 mM MgSO₄, 5 mM glucose) to remove adherent proteins. Disequilibrated [125I]iodosulfanilic acid prepared according to the instructions provided by New England Nuclear was diluted to 5 ml in A6 Ringer and added to the mucosal side of the cells (note that because the A6 cells formed a confluent monolayer the 125I-labeled reagent reached only the luminal surface of the cells). The filter-bottomed cup was then shaken gently at 4 °C for 5–5 h, after which the mucosal solution was removed by aspiration and the cup thoroughly washed six times in A6 Ringer containing 1% bovine albumin. The labeled A6 cells were then pooled with the cells from five untreated filter-bottomed cups and subjected to the vesicle preparation procedure described above.

125I Flux Measurements—Unless otherwise stated, flux measurements were performed under equilibrium exchange conditions. Filtration buffer (10 mM Tris/HEPES, 100 mM mannitol, 1 mM CaCl₂, and 1 mM NaCl) was passed successively through a 0.45-μm Millipore filter (HAWP, National Diagnostics) liquid scintillation fluid plus 1 μCi of [3H]mannitol/ml, and other additions as noted. A 50-μl aliquot of vesicles (approximately 1 mg of protein/ml) was placed in a glass test tube (12 × 75 mm) and at time 0 a 100-μl aliquot of incubation medium was added. After an appropriate time, the reaction was stopped by adding a 10-fold volume of ice-cold stop solution (A6 Ringer containing 10 mM mannitol and 10 mM amiloride). The vesicles were then immediately applied to a Millipore filter (HAWP) under light suction. The filter, which retained the vesicles, was then washed with a further 4.5 ml of cold stop solution, dissolved in 10 ml of Filtron X (National Diagnostics) liquid scintillation fluid plus 1 ml of water, and counted for radioactivity along with samples of the incubation medium and appropriate standards. The entire stopping and washing procedure took less than 30 s, during which the vesicles were in contact with the stop solution for less than 20 s. When the vesicles were left in the stop solution for an additional 30 to 45 s before filtration, no significant loss of 22Na or 3H was detectable.

All experimental points were carried out in at least triplicate at room temperature (25–28 °C). The errors quoted in the text and tables and the error bars shown in the figures are standard deviations. The simultaneously measured "uptake" of [3H]mannitol has been used to correct [22Na] fluxes for extrasaccular trapping by the membranes and filters. Sodium equilibrium exchange fluxes were calculated from 15- or 30-s points. In control experiments (not shown), we have verified that [22Na] uptake into the vesicles is linear with time over this interval to within the limits of accuracy of our measurements.

Chemicals—Aldosterone (chromatographic grade) was purchased from Calbiochem-Behring. Radiochemicals were from New England Nuclear. Amiloride was a gift from Merck Sharp and Dohme. The growth medium for cell culture was prepared by the National Institutes of Health Media Section. Fetal bovine serum was purchased from GIBCO.

RESULTS

Enzymatic Characterization of the Vesicle Preparation—The activities of alkaline phosphatase, γ-glutamyltranspeptidase, glucose-6-phosphatase, and succinate dehydrogenase in the initial homogenate and final vesicle preparation and the relative enzymatic enrichments and recoveries in the final vesicle fraction are shown in Table I. The membrane markers alkaline phosphatase and γ-glutamyltranspeptidase were enriched 9–10 times in the final vesicle fraction relative to the initial homogenate while contamination of the vesicles by endoplasmic reticulum and mitochondria as assessed by glucose-6-phosphatase and succinic dehydrogenase activity, respectively, was small.

We know of no histochemical evidence that alkaline phosphatase and γ-glutamyltranspeptidase activity are localized to the apical membrane of the A6 cells as they are in other epithelial cells. However, we have tested for alkaline phosphatase activity of the apical membrane of these cells as follows. A6 cells growing in filter-bottomed cups (2.5 cm in diameter) were exposed on both sides to a physiological salt solution at pH 8.1 (10 mM Na/HEPES, 0.5 mM MgCl₂, 4 mM KCl, 1 mM CaCl₂, 110 mM NaCl). The alkaline phosphatase substrate p-nitrophenyl phosphate was then added to either the apical or basal solution at a final concentration of 5.4 mM. The production of p-nitrophenyl in both solutions was subsequently monitored by measuring absorbance at 405 μM. With substrate on the apical side, p-nitrophenyl appeared in the apical solution at a rate of 26 nmol/h versus 7 nmol/h in the basal solution. On the other hand, when substrate was added to the basal side, p-nitrophenyl was produced at a rate of 5.4 nmol/h in the apical solution versus 1.6 nmol/h in the basal solution. These observations are consistent with a site of alkaline phosphatase activity in these cells localized to or closely associated with the apical membrane.

Fig. 1 illustrates the results obtained upon fractionation of cells whose apical membrane was labeled with [125I]iodosulfanilic acid. The average enrichment of 125I activity in the final vesicle fraction relative to the initial homogenate was 7.1 ± 0.88X (n = 3). As shown in the figure, the enrichment of 125I increased progressively at each stage of the vesicle preparation procedure. Also, although the absolute magnitude of 125I incorporation was different from preparation to preparation owing to varying times of exposure to [125I]iodosulfanilic acid (2–5 h), the relative enrichment of 125I activity in final pellet was similar in all experiments.

We conclude that our final vesicle preparation is enriched 7–10 times in apical membranes over the starting homogenate with little contamination by mitochondria and endoplasmic reticulum. At present, however, we cannot exclude the possibility that this preparation may be contaminated by basolateral membranes and/or other unassayed membrane components.

Sodium Fluxes—Fig. 2 shows the results of an experiment where 22Na uptake into the vesicle preparation after 5 min of incubation at 28 °C was measured as a function of extravesicular osmolarity. The osmolarity of the incubation medium was varied by changing its sucrose concentration. The results are plotted as uptake versus inverse osmolarity. The data fall on a straight line, indicating that sodium is being taken up into an osmotically active space. The non-zero intercept of this line on the vertical axis represents binding or trapping of
tracer Na and sufficient additional sucrose to give the extravesicular insensitive. In different experiments, the bound sodium osmolarities illustrated.

were prepared in Buffer AN with 100 mM mannitol replaced by 1 mM NaCl. We have not been able to determine the origin or significance of this variation; however, in all subsequent experiments presented in this paper, only the amiloride-sensitive component of sodium flux is considered. This component is taken to be the difference in flux observed in the presence and absence of $10^{-4}$ M amiloride. Amiloride was observed to have no effect on the accumulation of [$^{3}$H]mannitol (5 min) in the vesicle preparation, indicating that the action of the drug was specific to the sodium transport pathway.

An amiloride-sensitive component of sodium flux was found in most preparations, but its magnitude varied considerably from one preparation to another. Fig. 3 shows the relationship between the amiloride-sensitive flux observed in the vesicles and the transepithelial voltage generated by the tissue culture epithelium from which they were prepared. Generally speaking, low fluxes were associated with low potential differences between the amiloride-sensitive flux observed in the vesicles and transepithelial voltage, at least for cells with voltages $\geq 10$ mV. The full significance of the data shown in Fig. 3 remains to be clarified; however, in the latter stages of this study and in all the experiments presented here, only cells with transepithelial voltages greater than $10$ mV were included. These results argue against the likelihood of a direct relationship between amiloride-sensitive sodium flux in the vesicles and transepithelial voltage, at least for cells with voltages $>10$ mV. The full significance of the data shown in Fig. 3 remains to be clarified; however, in the latter stages of this study and in all the experiments presented here, only cells with transepithelial voltages greater than $10$ mV were included. These results argue against the likelihood of a direct relationship between amiloride-sensitive sodium flux in the vesicles and transepithelial voltage, at least for cells with voltages $>10$ mV.
used to prepare apical membrane vesicles. In these preparations, the amiloride-sensitive component of sodium flux was 76 ± 18% of the total sodium flux (equilibrium exchange flux of 1 mM NaCl). Similar stimulations were observed at 10 and 100 mM NaCl.

Fig. 4 illustrates the effect of amiloride on the equilibrium exchange rate of 1 mM sodium into the vesicles. Amiloride inhibition is dose-dependent and essentially complete at $10^{-4}$ M. Under the conditions of this experiment (1 mM NaCl), the $K_{0.5}$ of amiloride was approximately $7 \times 10^{-6}$ M. In the vesicle preparation used in Fig. 4, little, if any, amiloride-insensitive binding was seen.

Fig. 5 compares amiloride-sensitive sodium fluxes at 1 mM NaCl in vesicles prepared from cells treated with $10^{-7}$ M aldosterone for 4.5 h to fluxes in vesicles prepared from untreated cells from the same plating. A 2-fold stimulation in flux ($2.0 \pm 0.5, n = 7; p < 0.02$) is observed in the vesicles from treated cells. Similar stimulations were observed with vesicles from aldosterone-treated cells in flux studies carried out in 10 and 100 mM NaCl (data not shown).

The apical membrane origin of our A6 vesicle preparation is indicated by two independent observations. 1) The vesicles are enriched 9-10 times in alkaline phosphatase and $\gamma$-glutamyltranspeptidase, which generally are apical membrane markers. 2) When vesicles are prepared from A6 epithelia whose apical surface is covalently labeled with $[^{125}]$iodosulfanilic acid, a 7-fold enrichment in $[^{125}]$I activity is observed in the final vesicles preparation relative to the initial cell homogenate. Although mitochondrial and endoplasmic reticulum contamination of this preparation is low, we cannot, at present, exclude the possibility of contamination by other unassayed membrane fractions (e.g., basolateral membranes).

We would stress, however, that in this paper we concern ourselves only with the amiloride-sensitive component of sodium flux which has been established to be an apical membrane phenomenon (2, 3).

The sodium transport properties of the vesicles are in good agreement with our previous results on intact A6 epithelia. The $K_{0.5}$ value for amiloride inhibition reported here at 1 mM NaCl is $7 \times 10^{-8}$ M. The comparable value for intact cells can be calculated to be $5.3 \times 10^{-8}$ M from the data presented in reference (3). Also, the stimulation in sodium flux after aldosterone treatment was approximately 3-fold in the intact cells versus 2-fold in vesicles isolated from treated cells (Fig. 5). In both cases, the aldosterone-stimulated component of flux was completely amiloride-sensitive.

Chase and Al-Awquati (15) have recently published the results of sodium flux measurements on apical membrane vesicles prepared from the toad bladder epithelium. The purity of their final vesicle fraction was not reported; however, its sodium transport properties are markedly different from basolateral and mitochondrial membrane fractions obtained in the same preparation procedure. These authors find a $K_{0.5}$ for amiloride inhibition of sodium flux of $5 \times 10^{-8}$ M at 1 mM NaCl, a value in good agreement with the result reported here for A6 apical membrane vesicles. Studies in the toad bladder by Garty and Edelman (16) showed that the increase in apical sodium permeability elicited by aldosterone involved activation of sodium channels present in the apical membrane in nonconductive form. We demonstrate here that the activation of sodium channels by aldosterone can be maintained in the isolated A6 apical membrane vesicles.

The ability to isolate apical membrane vesicles containing the A6 amiloride-sensitive sodium transporter, coupled with the obvious advantages of working with a cultured cell line, provides an excellent system for studying the properties of this transporter and its control by aldosterone. This preparation is presently being used to investigate the electrical properties of the sodium transporter when it is incorporated into planar lipid bilayers (17).
REFERENCES

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