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AMILORIDE-SENSITIVE SODIUM UPTAKE INTO HUMAN PLACENTAL BRUSH BORDER MEMBRANE VESICLES

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Abstract—Sodium transport into human placental brush border membrane vesicles was examined in the presence of an outwardly directed sodium gradient leading to the formation of an intravesicular negative charge. ^{22}Na entered the vesicles in a time dependent fashion. The activation energy of the uptake process was calculated and was found to be 11.2 kcal/mol, similar to the value of ionic diffusion in free solution. Amiloride inhibited Na uptake in a concentration dependent fashion with an IC_{50} value of 3.08 μM. Neither ouabain nor bumetanide had an effect on Na uptake at concentrations up to 100 or 1000 μM, respectively. The system presented here indicates Na transport via channels without involvement of the Na–K-ATPase or the Na–K–Cl cotransporter. The system may be useful in investigating Na transport defects in cystic fibrosis.

Key words: sodium transport; human placental brush border membrane vesicles; amiloride; cystic fibrosis

The brush border of the syncytiotrophoblast layer of the placenta is important in the regulation of solute transport between the mother and the foetus [1]. BBMV† from placenta have been used to study some of these transport phenomena [2–10]. Isolated BBMV have advantages over whole cell or organ preparations since the possibility of interference in apical transport processes by regulatory and compensatory mechanisms associated with baso-lateral membranes or other subcellular organelles is eliminated. However, important elements such as enzymes required for optimal transport processes may be lost or inactivated during vesicle preparation procedures. The study of ion transport across placental membrane vesicles provides information pertaining to placental transport processes in particular and epithelial ion transport in general.

The study of sodium transport in epithelia may be important in relation to the disease CF since sodium transport, along with chloride transport, is thought to be defective in some tissues in CF [11–14]. Since the placenta has the same genotype as the foetus and is known to express the CF gene, the gene responsible for the production of the CFTR [15], placental BBMV may be good models in the study of possible Na transport defects in CF. A preliminary report of this work has been published in abstract form [16].

MATERIALS AND METHODS

Chemicals. $^{22}NaCl$ (0.2 mCi/mL) was purchased from Amersham (U.K.). Dowex cation exchange resin (50-X8-100) was obtained from the Sigma Chemical Co. (Poole, U.K.). Dulbecco's modified Eagle medium (DMEM) was from Flow Laboratories (Irvine, U.K.). Bumetanide was a gift from Leo Laboratories (Dublin, Ireland). Amiloride was a gift from Merck, Sharp and Dohme (Hoddesdon, U.K.). All other chemicals were of the highest grade commercially available.

Preparation of BBMV. BBMV were prepared from human term placenta using the method of Ganapathy *et al.* [10]. Placentae were collected from a local maternity hospital and were transported back to the laboratory within 30–60 min of delivery in DMEM maintained at 4°. All of the subsequent steps of the vesicle isolation procedure were carried out at 4°. Maternal facing tissue (140 g) was removed from the placenta and washed three times in buffer containing 300 mM mannitol, 10 mM Tris–HEPES (pH 7.0). Tissue was then chopped very finely and agitated for 1 hr using a magnetic stirring bar. Tissue was then filtered through gauze and the homogenate was centrifuged at 1000 *g* for 10 min to remove blood and cellular debris. The resulting supernatant was centrifuged at 10,000 *g* for 15 min and the supernatant resulting from this step was ultracentrifuged at 86,000 *g* for 35 min. The resulting pellets were collected in 50 mL of the preparation buffer and homogenized using a Dounce Homogenizer. This suspension was made up to 10 mM with respect to $MgCl_2$, agitated for 10 min at 4° and allowed to stand for a further 10 min. The supernatant was centrifuged for 15 min at 3000 *g* to pellet the non-brush border membanes which had been precipitated by Mg. The resulting supernatant was ultracentrifuged at 86,000 *g* for 35 min to harvest the BBMV. BBMV were resuspended in the appropriate buffer and were snap frozen and stored in liquid nitrogen prior to use. For sodium transport studies, the BBMV were resuspended in medium containing 130 mM sodium gluconate and 10 mM imidazole-acetate (pH 7.0).

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† Abbreviations: BBMV, brush border membrane vesicles; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator.

The membrane vesicles were found to be enriched in the brush border marker enzymes alkaline phosphatase and in γ-glutamyl transpeptidase by factors of 25.3 and 53.4, respectively, and were found to be relatively homogenous in shape and orientation by transmission and scanning microscopy and in size by flow cytometry [17].

Preparation of Dowex columns. Dowex 50W (50-X8-100) was converted from the acidic form to the Tris form by extensive washing with distilled and deionized water followed by titration with 1 M Tris base and further washing [18]. Before use, each column was washed with 2 mL ice-cold sucrose buffer.

Sodium transport assay. Sodium uptake into the BBMV was measured in the presence of an outwardly directed sodium gradient leading to an interior negative membrane potential according to a modification of the method of Garty *et al.* [19]. We modified the assay in that we preincubated our vesicles in sodium gluconate medium instead of the sodium chloride medium used in the work reported by Garty *et al.* [19]. In order to study the time course of Na uptake, 500 μL of BBMV in 130 mM Na gluconate, 10 mM imidazole acetate (pH 7.0) were added to a Pasteur pipette plugged with Dacron wool which was packed with Dowex 50-X8-100 cation exchange resin (Tris form) in order to remove extravesicular Na and, in doing so, generated an outwardly directed Na gradient leading to the efflux of intravesicular Na and the formation of a membane potential (negative inside). The BBMV were eluted with 2 × 750 μL additions of 250 mM sucrose and the eluted membrane vesicles were collected in 2 mL sucrose buffer. At this point, 10 μL of ^{22}NaCl (2 μCi) was added to the uptake system. At desired time points, aliquots of 250 μL were removed from the uptake reaction and were added in duplicate to further Dowex columns in order to remove extravesicular ^{22}Na, and the vesicles were eluted with 2 mL of 250 mM sucrose, 10 mM imidazole-acetate (pH 7.0). Samples were counted in an LKB γ Counter. The cpm values were converted to dpm and expressed as pmol ^{22}Na/mg protein. Protein was measured using the method of Lowry *et al.* [20].

Calculation of activation energy. The activation energy of the ^{22}Na uptake into placental BBMV was calculated by measuring uptake over 2 min at four different temperatures. The temperatures which were chosen were 4°, 12°, 25° and 37°. The following procedure was adhered to for all temperatures: 500 μL of BBMV were preincubated for 10 min at the appropriate temperature, were passed through a Dowex cation exchange column and were eluted with 2 × 750 μL of sucrose buffer which had been maintained at the appropriate temperature. The eluted BBMV were incubated for 2 min. The uptake reaction was initiated by adding 750 μL of BBMV to 750 μL of pre-equilibrated sucrose buffer which contained 3.75 μL of ^{22}Na (0.75 μCi). The uptake reaction was allowed to proceed for 2 min after which time 3 × 250 μL samples were added to Dowex columns to remove extravesicular ^{22}Na. The BBMV were eluted with 2 × 1 mL of sucrose buffer. The data were plotted as temperature (in Kelvin) versus $\log_{10}$ pmol ^{22}Na/mg protein. The slope of the line was calculated and was used to solve the Arrehenius equation for E_A, the activation energy of the process.

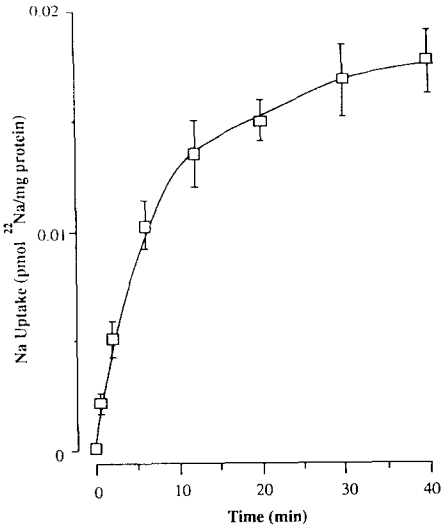

Fig. 1. Time course of ^{22}Na accumulation into human placental BBMV following the imposition of an inside negative membrane potential. ^{22}Na values are expressed as pmol ^{22}Na/mg protein. Data represent the means ± SEM of four independent experiments performed in duplicate.

Effects of amiloride, ouabain and bumetanide. Samples of BBMV (100 μL) were passed through Dowex cation exchange columns and were eluted with two 400 μL washes of sucrose buffer to set up an inside negative membrane potential. Drugs were added at 1 in 100 dilutions to give the required final concentrations. Control tubes contained vehicle alone. Amiloride and ouabain were added as aqueous solutions; bumetanide was dissolved in DMSO. ^{22}Na uptake in the presence of increasing concentrations of drug was measured over 2 min. Uptake was stopped by passing the vesicles through further Dowex cation exchange columns to remove extravesicular ^{22}Na. The data was plotted as % of control against log concentration of drug.

RESULTS

Time course of ^{22}Na *uptake*

Uptake of ^{22}Na into placental BBMV was measured as a function of time following the imposition of an inside negative membrane potential (Fig. 1). Results are expressed as pmol ^{22}Na/mg protein. Uptake was linear with time up to approximately 6 min and tended towards equilibrium by 30–40 min. In further experiments, uptake was measured over 2 min as this was chosen as an appropriate point on the linear portion of the graph.

Activation energy

The activation energy of ^{22}Na uptake was estimated by measuring ^{22}Na uptake into BBMV at different temperatures, plotting the data as described in Materials and Methods, and solving the Arrhenius

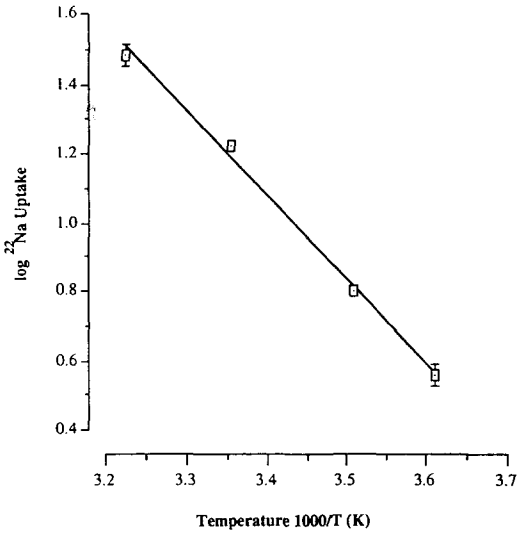

Fig. 2. Effect of temperature on ^{22}Na uptake into placental BBMV. Uptake was measured over 2 min following the imposition of an inside negative membrane potential. Uptake was measured at 4°, 12°, 25° and 37°. The activation energy for the process was calculated by solving the Arrhenius equation and was found to be 11.2 ± 0.6 kcal/mol. Results are expressed as 1000/temperature (in degrees Kelvin) versus $\log_{10}$ of the ^{22}Na uptake values. Data represent the means ± SEM of five independent experiments performed in triplicate.

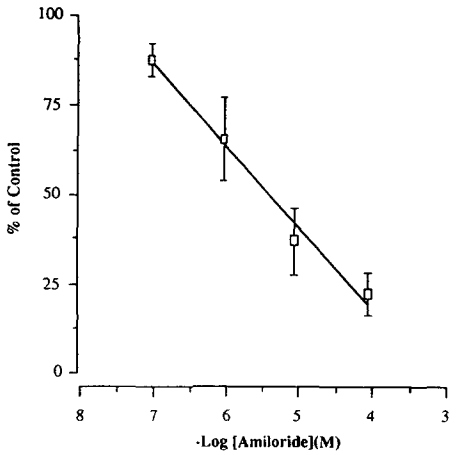

Fig. 3. Effect of increasing concentrations of amiloride (0–100 μM) on ^{22}Na uptake into placental BBMV. ^{22}Na uptake was measured over 2 min. Results are expressed as % reduction from control values. The IC_{50} for ^{22}Na uptake inhibition was found to be 3.1 ± 0.7 μM (means ± SEM of three independent experiments performed in duplicate).

equation for E_A, the activation energy (Fig. 2). We calculated this to be 11.2 ± 0.6 kcal/mol (N = 5), a value which is similar to the accepted value for ionic diffusion in aqueous solution and is also similar to activation energy values reported for chloride ion conductance in other systems [7, 21, 22].

Effects of drugs

The effects of amiloride, ouabain and bumetanide on ^{22}Na uptake were examined. Of the three pharmacological agents used, only amiloride, an established sodium channel inhibitor, had a significant effect, causing concentration-dependent inhibition of uptake (Fig. 3). The IC_{50} was calculated and was found to be 3.08 μM. In contrast, neither ouabain nor bumetanide had any significant effect on ^{22}Na uptake (Fig. 4A, B). In the case of bumetanide, at higher concentrations (100 and 1000 μM) there tended to be a certain amount of inhibition; however, this was found to be not statistically significant (Fig. 4B).

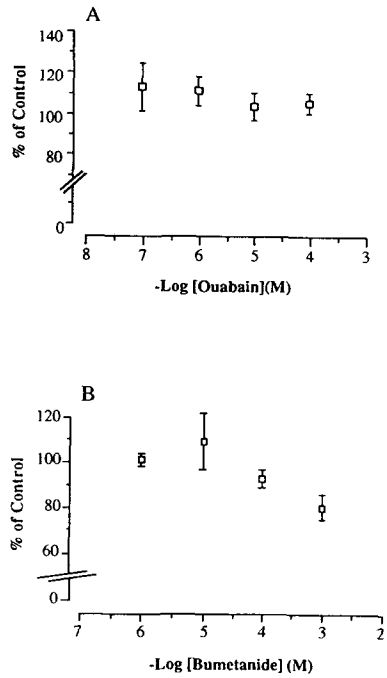

Fig. 4. Effect of increasing concentrations of (A) ouabain and (B) bumetanide on ^{22}Na uptake into placental BBMV. ^{22}Na uptake was measured over 2 min. There was no significant effect on ^{22}Na uptake observed. Data represent the means ± SEM of three independent experiments performed in duplicate.

DISCUSSION

In this study we have used human placental BBMV to study conductive Na uptake using a modification of the method of Garty *et al.* [19]. The modification was that BBMV were preloaded with sodium gluconate medium instead of sodium chloride medium. This alteration was made for the following reason: since the assay exploits the different membrane permeabilities of ions, we needed an anion which was much less permeable than sodium. If the anion had membrane permeability properties equal to or greater than those of sodium, then the removal of extravesicular Na and the resulting

outward Na gradient would dissipate very quickly, resulting in the loss of the membrane potential required for isotope uptake. We have previously shown that BBMV from human placenta contain a chloride conductance [17] and, for this reason, the impermeant anion gluconate was chosen as the counter ion for sodium since the membrane permeability of gluconate is much less than that of Na and Cl. In this way, we examined Na uptake into vesicles which contained a Na conductance and, in agreement with Garty *et al.* [19], the point of maximum isotope uptake corresponded to around 1% of the total added radioactivity. The assay system was set up so that the pH was equal inside and outside the BBMV thus minimizing the possible occurrence of H^+ gradients during the course of the experiments. Several groups [4–6] have provided evidence to show that, under different experimental conditions, Na ions enter placental BBMV via a Na–H exchanger. Post and Dawson [23] showed that in the basolateral membrane of turtle colon, there existed a Na–H exchanger which could, under some circumstances, exhibit Na conductance. In the light of these findings it is possible that under the experimental conditions employed in this paper, the placental BBMV Na–H antiporter is operating in conductive mode.

Na entered the BBMV in a time dependent fashion, reaching a maximum between 30 and 40 min. The activation energy of this uptake process was calculated and its value of 11.2 kcal/mol was similar to the value for ionic diffusion in free solution. This was taken as evidence that the Na uptake being examined was a channel mediated rather than a carrier mediated process.

We examined the effect of amiloride (0–100 μM) on Na transport of placental BBMV and we have shown that this drug inhibited uptake in a concentration dependent fashion. The IC_{50} value of 3.1 μM for amiloride is close to values found by other workers for Na–H exchange [4, 5]. However, Garty *et al.* [19], using similar conditions to those used in the present study, found similar sensitivities for amiloride-sensitive Na-specific channels in toad bladder membrane vesicles as those reported in this present study. In our present studies, at maximum amiloride concentration (100 μM) Na uptake was reduced to approximately 15% of total. The residual Na uptake under these conditions may represent binding of Na to the BBMV. It has been shown [24] that in airway epithelial cultures, the amiloride-sensitive equivalent short circuit current of CF cultures is two to three times larger than normal, i.e. amiloride produces a greater effect in CF epithelia. Jorissen *et al.* [25] reported the presence of an amiloride-insensitive cation channel in cultures of human nasal epithelia and found no difference in the activity of this channel in CF epithelia over controls. Therefore, the amiloride-sensitive channel which we have observed in human placental BBMV may be affected in CF. Indeed, some clinical studies on amiloride treatment by aerosol for CF patients have indicated some promising beneficial effects [26].

We also investigated the effects of ouabain and of bumetanide on placental Na channel activity. Ouabain, at concentrations up to 100 μM, did not significantly affect Na uptake. This drug affects the Na–K–ATPase system which has been shown previously to be located on the placental basal membrane [1]. The lack of effect on Na conductance in our placental BBMV suggests that these vesicles were relatively pure and were not significantly contaminated by basal membranes. Bumetanide did not affect Na transport in placental BBMV. Bumetanide, along with other loop-blocking diuretics such as furosemide and piretanide, selectively inhibits the Na–K–2Cl exchanger. The relative lack of effect of bumetanide on apical sodium channel activity shown in this paper is in accordance with the accepted selectivity of these drugs, and the lack of significant effect at concentrations up to 1000 μM rules out any involvement of the Na–K–2Cl cotransporter in the sodium uptake system.

In conclusion, we have described a Na uptake process in placental BBMV which is driven by a membrane potential (negative inside). The calculated activation energy suggests that the process is conductive transport and is blocked concentration dependently by amiloride with an IC_{50} of 3.08 μM. Neither ouabain nor bumetanide had a significant effect on Na uptake at concentrations up to 100 μM. Since Na transport in epithelia is known to be defective in CF [24], the study of Na transport in placental BBMV from normal and CF individuals may provide information on the mechanisms of abnormal epithelial Na channel function in CF.

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