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Proton Transport in Human Placental Microvillous Membrane Vesicles

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= ABSTRACT =

The change of the acridine orange absorbance was used to monitor the formation and/or dissipation of a pH gradient in microvillous membrane vesicles (MVV) isolated from human term placenta. Under Na^+ efflux conditions, an acidification of the intravesicular space occured and it was completely inhibited by 0.1 mM amiloride. Under K^+ efflux conditions, an acidification of the intravesicular space occured and it was potentiated by valinomycin or FCCP. An inwardly directed chloride gradient also induced a minor intravesicular acidification, but it was not observed in voltage-clampled MVV. The initial rate of the dissipation of a pH gradient was accelerated by pulse injections of Na^+ in a saturable manner and Li^+ could replace Na^+. The kinetic parameter of Na^+ in placental Na^+/H^+ exchange was similar to that of renal Na^+/H^+ exchange. Amiloride was a inhibitor of directly coupled Na^+/H^+ exchange and its IC_{50} in placental MVV was about 14-fold higher than that in renal brush border membrane. These results indicate that Na^+/H^+ exchanger exists in human placental MVV and that its kinetic characteristics is similar to that of renal Na^+/H^+ exchanger but its pharmacological characteristics is different. In placental MVV K^+, H^+, and, relatively minor chloride conductances are present. The magnitude of Cl^-/OH^- exchange, even though it exists, seems to be smaller than that of Na^+/H^+ exchange.

Key Words: Proton transport, Microvillous membrane vesicles, Human placenta.

INTRODUCTION

The human placental epithelium performs a critical function in fetal development by mediating the transfer of metabolites between maternal and fetal circulations. At the cellular level, the syncytiotrophoblast serves as the functional unit of the placenta by expressing a polarized distribution of transport process at its apical and basal membranes. The morphological specialization of the apical or maternal side of the syncytiotrophoblast as brush border is certainly well-suited to the purpose of transcellular transport (Shennan & Boyd, 1987). Similar to renal and intestinal epithelia the isolation as membrane vesicles of this morphologically specialized membrane has greatly facilitated the study of placental epithelial transport by the identification and characterization of transport pathways at the apical side of the syncytiotrophoblast. Furthermore, the availability of isolated membrane vesicle preparations may prove to be especially significant for investigations of placental transport function because, unlike renal or intestinal epithelia, only a limited amount of information may be obtained from the

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alternative experimental models currently at hand. Since Smith et al. (1974) reported at first the successful isolation of microvillous membrane vesicles (MVV) from human placenta, extensive studies about mechanisms of organic solute and anion transport have been made in MVV (Shennan & Boyd, 1987).

Passive and ion-coupled proton permeabilties in biological membranes are important for regulation of cell pH and for the net transepithelial transport of ions and proton equivalents. In biological membranes, passive proton transport in HCO_3^- -free media occurs primarily by electrogenic passive diffusion and electroneutral Na^+/H^+ and Cl^-/OH^- countertransport (Reenstra et al, 1981; Verkman & Ives, 1986). Although it has been reported that Na^+/H^+ exchange is present in placental microvillous membrane (Balkovetz et al, 1986; Ganapathy et al, 1987), the relative contribution of Na^+/H^+ exchange, proton conductance and Cl^-/OH^- exchange in proton transport in placental microvillous membrane has not been known.

Acridines have been used to examine pH gradient in several different vesicular systems (Aickin & Thomas, 1977; Cala, 1980; Johson et al, 1976; Thomas, 1977; Thevenod et al, 1989). These methods follow absorbance intensity and record the change in extravesicular probe concentration that results from uptake of the probe into the intravesicular space. A distinct advantage of these optical methods is that they provide a direct "on-line" measurement of the pH gradient. Therefore, the present study used the acridine orange (AO) absorbance change to characterize the pathway of proton transport in MVV from human full term placenta.

METHODS

Membrane preparations

MVV from normal term placenta were isolated by the method described by Balkovetz et al. (1986). Placenta was obtained within 15 min of delivery by elective Caesarean section and chilled on ice. All subsequent steps of the procedure were carried out on ice or in referigerated centrifuges. The villous tissue was quickly dissected from the chorionic plate and minced into small fragments. The tissue fragments were rinsed three times in 300 mM mannitol, 10 mM HEPES-Tris (pH 7.4) and gently stirred for approximately 30 min using a teflon spatula. The tissue suspension was filtered through cotton gauze. The filterate was centrifuged 10,000g for 10 min using a SS-34 rotor (Sorvall). The low-speed pellet was discarded and the supernatant was centrifuged at 48,000g for 30 min. The high-speed pellet was gently resuspended and $MgCl_2$ was added to a final concentration of 10 mM. After treating for 15min the membrane suspension was centrifuged at 3,000g for 15 min to pellet the Mg^{2+}-induced aggregates. The low-speed supernant was centrifuged at 100,000g for 30 min and the resulting pellet (MVV) was resuspended and washed twice in buffers designated for each experiment. The vesicles were preloaded with a buffer of desired ionic composition by an incubation for 2 h at room temperature. The composition of the buffer is given in the legend to the figure. The final protein concentration was measured and adjusted to be 10 mg/ml placental MVV preparations.

Brush border membrane vesicles (BBMV) and synaptosomes were prepared using a Mg^{2+} precipitation method from rabbit renal cortex (Aronson, 1978) and using the method of Hajos (1975) from rabbit cerebral cortex, respectively. The vesicles were preloaded with 100 mM mannitol, 100 mM KCl, 20 mM Mes-Tris (pH 6.0 at 25° C). The final protein concentration was adjusted to be 10 mg/ml for each membrane preparations.

Measurements of pH gradient and membrane potential

Proton uptake experiments were performed at 25°C on a Hewlett-Packard diode-array spectrophotometer (HP8452A) in the dual wa-

velength mode, using acridine orange (Burhan et al, 1982). The absorbance was monitored at 494 nm using 546 nm as the reference wavelength. The cuvette was filled with 2 ml buffer containing 6 μM acridine orange. The composition of the buffer is given in the legend to the figure. The ionophores used in this study were added from ethanol stock solutions. The experiment was started by an injection of 10 μl of membrane suspension to obtain a final protein concentration of 100 μg/2 ml.

The measurement of potential change in MVV was performed at 25°C on a Hewlett-Packard diode-array spectrophotometer (HP8452A) in the dual wavelength mode, using 3,3'-diethyloxadicarbocyanine iodide (DiO-C_2-(5)) (Kragh-Hansen et al, 1982). The absorbance was monitored at 574 nm using 604 nm as the reference wavelength. The detailed experimental procedure was given in the legend to the figure 2.

Transport studies

The uptake of [^{14}C]succinate was measured by a rapid filtration technique similar to that described by Berner & Kinne (1976). Briefly, the reaction was initiated by adding membrane vesicles to buffer (a 1:10 dilution of membrane vesicle suspension) containing radioactive substrate at 25° C. The composition of the incubation medium was indicated in the figure legend. At specified times, 100 μl samples were taken and quickly filtered under vaccum through Millipore filters (HAWP; 0. 45 μm pore size). Filters were washed with 5 ml of ice-cold stop solution, containing 100 mM mannitol, 100 mM NaCl and 10 mM HEPES-Tris, pH 7.5. The filters were dissolved in methoxyethanol counted by liquid-scintillation counter (Packard 300C).

Measurements of protein and marker enzymes

Protein was determined by the method of Bradford (1976), with gamma-globulin as a standard. Na-K-ATPase activity was measured by the method of Jϕrgensen and Skou (1972) and alkaline phosphatase activity by the method of Linhardt and Walter (1963).

Data analysis

Results are expressed as means±S.E.M.. Statistical comparisions were made with student's t-test. P values greater than 0.05 were considered to be nonsignificant.

Materials

^{14}C-succinate was obtained from Du Pont-New England Nuclear (Boston, MA), DiO-C_2-(5) from Eastman Kodak (Rochester, NY), and acridine orange, amiloride, FCCP*, HEPES, MES and valinomycin from Sigma Chemical (St. Louis, MO). All other chemicals were of at least reagent grade. Tetramethylammonium (TMA) gluconate was made by titrating solutions of TMA hydroxide with gluconic acid.

*FCCP, carbonyl cyanide p-trifuoromethoxyphenyl hydrazone

RESULTS

Characterization of MVV preparation

The purity of the prepararred MVV was measured by comparing the enzyme activities in the homogenate and the final vesicle preparation. Alkaline phosphatase, an enzyme known to be present in the microvillous membrane, was enriched more than 18-fold in all preparations. The activity of Na^+-K^+-ATPase was slightly enriched in these preparations (Table 1). These data are similar to those observed by others (Karl & Fisher, 1990; Glazier et al, 1988). As they explained, the modest increase in Na^+-K^+-ATPase activity may be due to the fact that the placenta consists of structures which are mostly devoid of Na^+-K^+-ATPase. Therefore, the whole tissue homogenate is relatively low in activity, making detection difficult, and the slightest contamination during purification could result in apparent enrichment.

In order to test functional integrity of our MVV preparations, we measured the uptake

Table 1. Specific activities of marker enzymes in the placental microvillous membrane and in the homogenate

Enzyme	Homogenate	Microvillous membrane	Enrichment factor
Alkaline phsophatase (nmole p-nitrophenol/mg/min)	0.38±0.05	6.92±1.31	18.21
Na^+-K^+-ATPase (nmole Pi/mg/min)	3.28±1.33	12.29±1.40	3.74

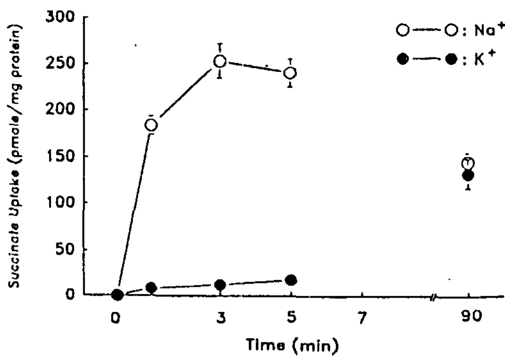

Fig. 1. Uptake of succinate in the presence of a Na^+ gradient or a K^- gradient. The membrane vesicles were preloaded with 300 mM mannitol, 10 mM Hepes-Tris, pH 7.5. Uptake of succinate was measured during incubation of vesicles in a medium containing 10 μM succinate, 10 mM Hepes-Tris (pH 7.5), 100 mM mannitol and either 100 mM NaCl (○) or 100 mM KCl (●). Data were means±S.E.M. of duplicate measurements from 3 different preparations.

of succinate, which has been known to be transferred by Na^+-cotransport mechanism in MVV (Ganapathy et al, 1988; Ogin & Grassl, 1989). Time-dependent uptake of succinate was shown in Fig. 1. The presence of an inwardly directed Na^+ gradient stimulated the transport of succinate relative to that with a potassium gradient. The uptake was rapid at the beginning, representing an "overshoot" phenomenon which peaked at 3min, and gradually approached equilibrium by 90min, the last time point measured. These results indicate that Na^+ cotransport system for succinate is present in our MVV.

Proton transport pathway in MVV

In order to study pathways for proton transport in placental MVV we changed the ion gradients imposed (Na^+,K^+, Cl^- or H^+) and observed effects of cation specific ionophores. Vesicles preloaded with a buffer containing 100mM KCl were injected into a buffer containing TMA-Cl instead of KCl (Fig. 2). In the absence of ionophore (curve b) an intravesicular acidification was observed compared to the condition where vesicles were diluted with a buffer containing KCl (curve a). The absorbance change induced by the K^+ gradient was increased by the addition of FCCP (curve c) or valinomycine (curve d), suggesting that the intrinsic proton and potassium conductances are considerable. In MVV the simultaneous presence of potassium selective ionophore valinomycin and of the protonophore FCCP further increased an intravesicular acidification as indicated by the large and fast decline of the acridine orange absorbance (curve e). As the preset transmembrane ion gradients collaped with time, the pH gradient generated was transient. The pattern of the absorbance change was very similar to that of valinomycin-induced hyperpolarization signal which was recorded by a potential sensitive dye in the same experimental condition (Fig. 2, inset).

Vesicles preloaded with a buffer containing 100mM NaCl were injected into a buffer containing TMA-Cl, instead of NaCl (Fig. 3). In MVV a transient intravesicular acidification was observed as indicated by the transient de-

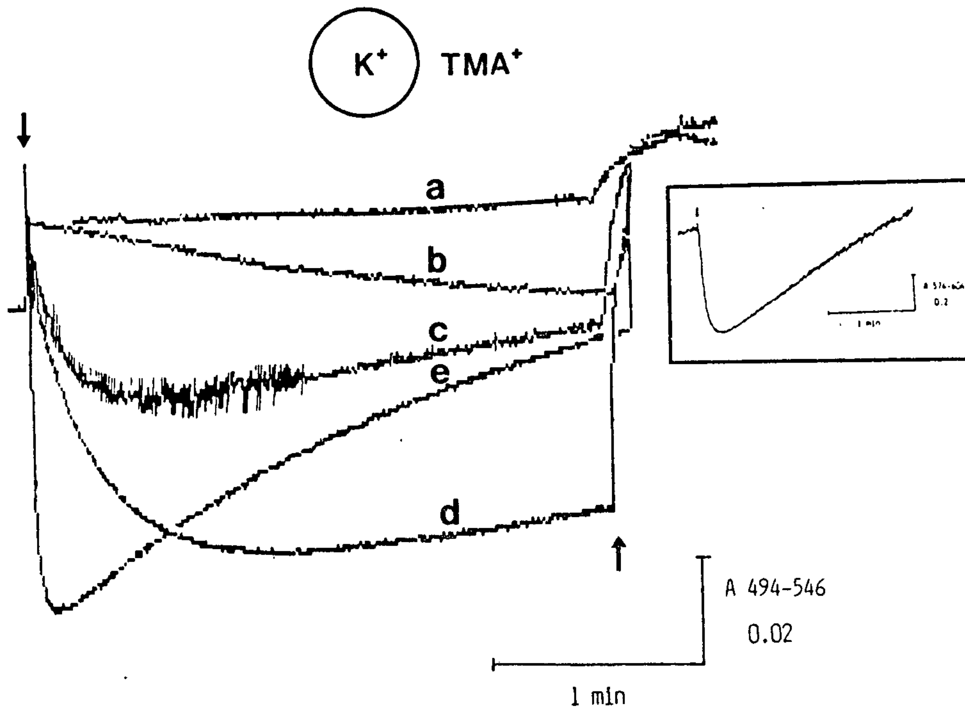

Fig. 2. *K^+-gradient dependent formation of pH gradient in placental MVV Vesicles were preloaded in 100mM mannitol, 100 mM KCl, 10 mM Hepes adjusted with Tris to pH 7.5. 10 μl (100 μg) of placental MVV were injected into 2 ml of a buffer containing 100 mM TMA chloride, 100 mM mannitol and 10 mM Hepes-Tris, pH 7.5, 6 μM acridine orange and 0.5% ethanol (↓). Curve b: absence of ionophores; curve c: 5 μM valinomycin; curve d: 10 μM FCCP; curve e: 5 μM valinomycin and 10 μM FCCP. In curve a vesicles were injected into a buffer containing 100 mM KCl instead of TMA Cl. 5 μl of 1% Triton X-100 was added to the cuvette (↑). Inset: The typical recording of potential change in placental MVV. Vesicles were preloaded in 100 mM mannitol, 100 mM KCl, 20 mM Hepes-Tris, pH 7.5. 50 μl(500 μg protein) of placental MVV were injected to 2 ml buffer containing 100 mM TMA Chloride, 100 mM mannitol, 20 mM Hepes-Tris, pH 7.5, 15 μM D_iO-C_2-(5). After stabilization of absorbance, 5 μM valinomycin was injected to induce hyperpolarization of MVV (↓).*

crease of the acridine orange absorbance (curve a). Sodium efflux related influx of protons can be attributed to at least two different events: a) conductive coupling of sodium efflux and proton influx; b) direct coupling via a common transport pathway (Na^+/H^+ exchange). To distinguish between these possibilities we tested the effects of amiloride and FCCP. The intravesicular acidification was decreased by the addition of FCCP (curve b) and completely abolished by 0.1mM amiloride (curve c), which indicated that Na^+/H^+ exchange is responsible for the intravesicular acification induced by a outwardly directed Na^+ gradient. In the presence of amiloride, an increase of the proton conductivity by FCCP did not induce in-travesicular acidification compared to the condition in the absence of FCCP (curve d), suggesting that the intrinsic sodium conductance in MVV is very low.

When an inwardly directed Cl^- gradient was imposed, acidification of the vesicle interior occured as shown in Fig. 4. If the decrease in

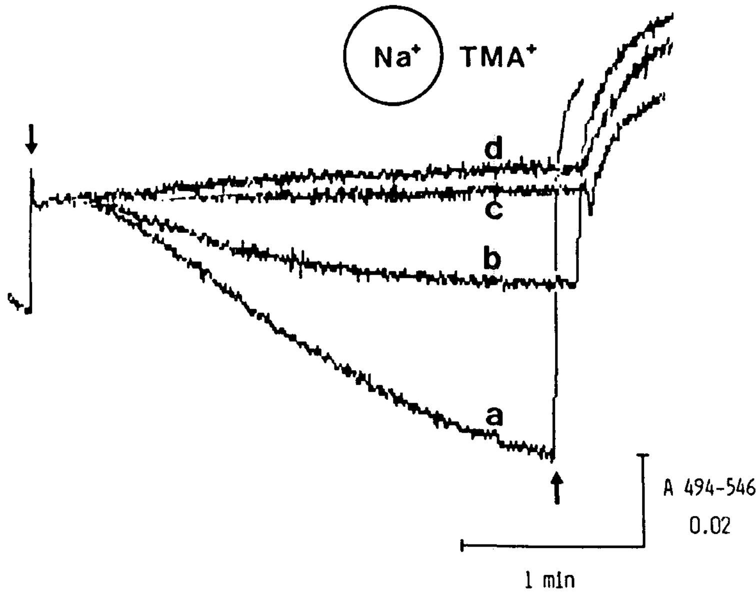

Fig. 3. *Na^{+}-gradient dependent formation of pH gradient in placental MVV. Vesicles were preloaded in 100 mM mannitol, 100 mM NaCl and 10 mM Hepes adjusted with Tris to pH 7.5. 10 μl (100 μg) placental MVV were injected into 2 ml of a buffer containing 100 mM TMA chloride, 100 mM mannitol, 10 mM Hepes-Tris, pH 7.5, 6 μM acridine orange and 0.5% ethanol (↓). Curve a: absence of ionophores; curve b: 10 μM FCCP; curve c: 0.1 mM amiloride; curve d: 10 μM FCCP and 0.1 mM amiloride. 5 μl of 1% Triton X-100 was added to the cuvette (↑).*

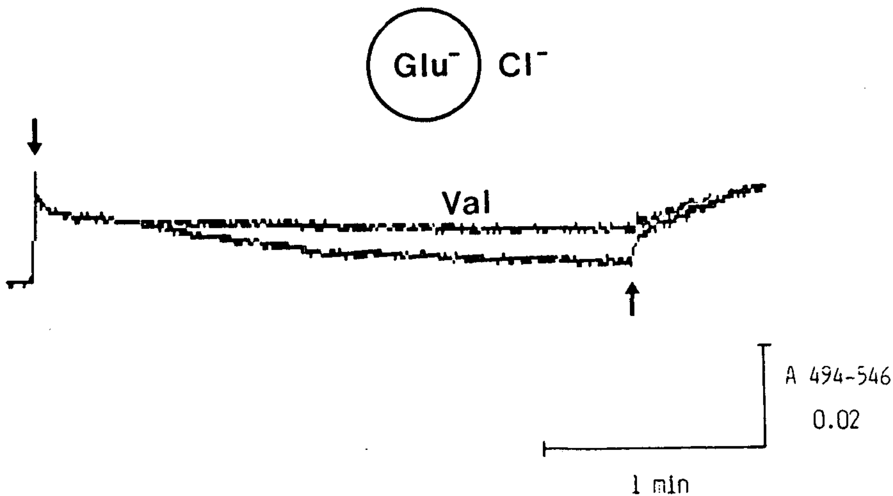

Fig. 4. *Effect of chloride gradient on the formation of a pH gradient in placental MVV. Vesicles were preloaded with 100 mM TMA gluconate, 50 mM K^{+}-gluconate and 10 mM Hepes adjusted with Tris to pH 7.5. 10 μl(100 μg) placental MVV were injected into 2 ml of a buffer containing 100 mM TMA chloride, 50 mM KCl, 10 mM Hepes-Tris, pH 7.5, 6 μM acridine orange and 0.5% ethanol (↓). 5 μl of 1% Triton X-100 was added to the cuvette (↑).*

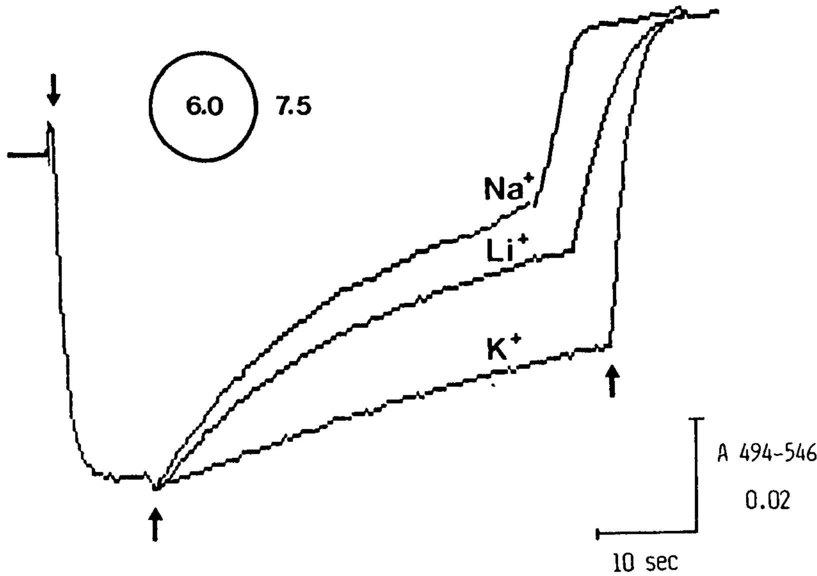

Fig. 5. Effect of sodium on the dissipation of a preset pH gradient in placental MVV. Vesicles were preloaded in 100 mM mannitol, 100 mM KCl, 10 mM Mes adjusted with Tris to pH 6.0. 10 μl (100 μg protein) of placental MVV were injected into 2 ml buffer containing Hepes-Tris (pH 7.5), instead of Mes-Tris, 6 μM acridine orange (↓). After about 5 s concentrated salt solution (adjusted to pH 7.5) were injected (↑). Salt injection increased the salt concentration in the cuvette by 25 mM NaCl, 25 mM LiCl or 25 mM KCl.

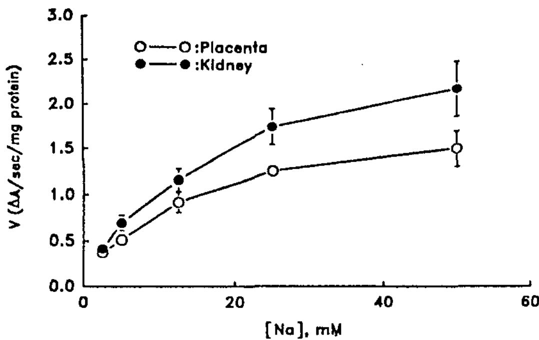

Fig. 6. Effect of external Na^+ on initial rate of Na^+/H^+ exchange in placental MVV and kindney cortical BBMV. Experimental condition was as same as described in Fig. 5. The concentration of Na^+ was varied from 2.5 mM to 50 mM and the total concentration of injected salt was maintained at 50 mM, replacing Na^+ with TMA^+. Initial rates were measured during the 2-s period after addition of Na^+ to the external buffer. Data were means ± S.E.M. of duplicate measurements from 3 different preparations.

absorbance of acridine orange by inwardly directed Cl^- gradient result from Cl^-/OH^- exchange which is an electroneutral process, it would occur in voltage clamped vesicles. However, it was not observed in voltage clamped vesicles ($K_{in}=K_{out}$ with valinomycin), suggesting that an intravesicular negative potential generated by inwardly directed Cl^- gradient induces intravesicular acidification.

Characteristics of placental Na^+/H^+ exchanger

The activity of the Na^+/H^+ exchange system can be studied by proton efflux experiments using the absorbance of AO. Proton efflux experiments were performed by diluting vesicles, preloaded with 100mM KCl at pH 6. 0, with external buffer containing 100mM KCl at pH 7.5; steady-state decrease of AO absorbance developed during the next 5s. NaCl was then added and the rate of AO absorbance recovery was measured (Fig. 5). Increase in AO ab-sorbance recovery was ob-

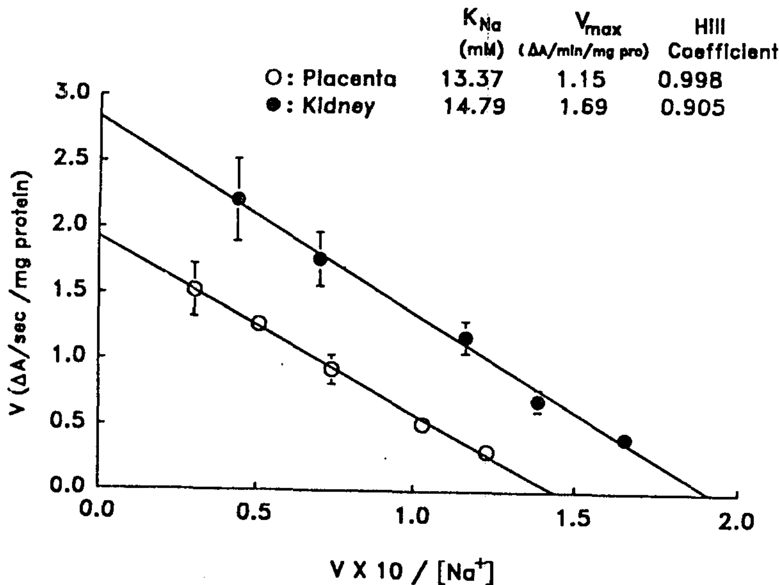

Fig. 7. Eadie-Hofstee plot of the data in Fig. 6: Initial rate (v) versus initial rate divided by external Na^+ *concentration (v/S).*

served by the addition of Na^+. Li^+, which is one of the substrates for Na^+/H^+ exchanger (Mahnensmith & Aronson, 1985), also increased absorbance recovery, although its magnitude was smaller than that induced by Na^+. In contrast, only minor change in AO absorbance recovery was observed after addition of K^+.

To compare kinetic characteristics of Na^+/H^+ exchange in placental microvillous membrane and renal brush border membrane, initial rates were calculated from the tangents to the absorbance recovery curves for the first two seconds after NaCl addition (Fig. 6). In both preparations the initial rate of AO absorbance recovery was increased and saturated as the external concentration of NaCl was increased. The initial rate (v) for the Na^+/H^+ exchanger was plotted against initial rate divided by external Na^+ concentration (v/S) in Fig. 7. The Na^+ concentrations required for the half-maximal rate of AO absorbance recovery (K_{Na}) in placental MVV and renal BBMV were 13.37 ± 1.12 and 14.79 ± 1.34 mM, respectively, and the maximal rates (V_{max}) were 1.15 ± 0.10 and 1.69 ± 0.18 ($A_{494-546}$/min/mg protein), respectively. Hill coefficients calculated form the data in Fig. 7 were 0.998 for placental MVV and 0.905 for renal BBMV. These values were nearly one, indicating an apparent lack of cooperativity of the antiporter.

We have compared the sensitivities of the placental, renal and synaptosomal Na^+/H^+ exchangers to amiloride. The experiments were conducted under identical conditions, using vesicles from human placenta, rabbit kidney cortex and rabbit cerebral cortex. In each case, the Na^+/H^+ exchanger activity was quantified by measuring the initial rate of AO absorbance recovery in the presence of an outwardly directed proton gradient ($pH_i = 6.0$; $pH_o = 7.5$). The results were given in Fig. 8. The Na^+/H^+ exchanger of each preparation was inhibited by amiloride in a dose-dependent manner. The Na^+/H^+ exchanger of placental brush-border membrane exhibited greater sensitivity to amiloride than that in the Na^+/H^+

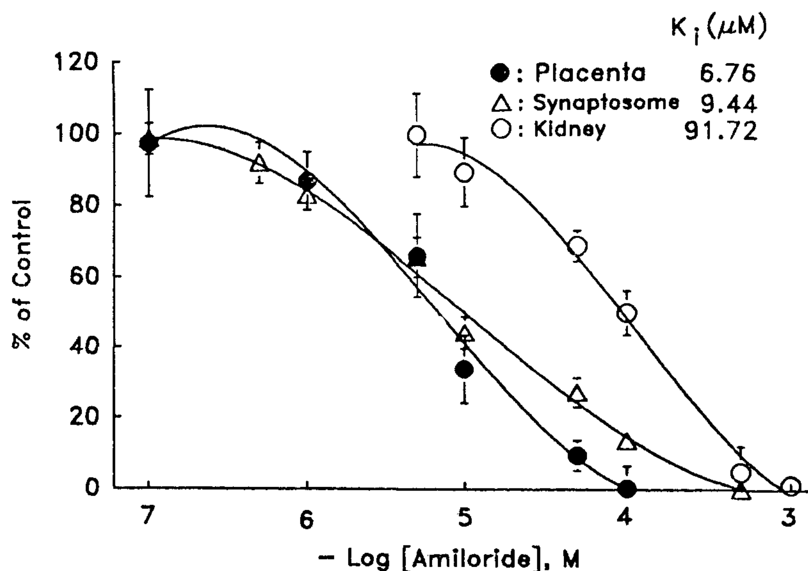

Fig. 8. Dose-response of amiloride inhibition in placental MVV, kidney BBMV and synaptosome. The absorbance change by the addition of 25 mM Na^+ was measured in the presence of a proton gradient ($[pH]_i=6.0$; $[pH]_o=7.5$) and various concentrations of amiloride. Data were means±S.E.M. of duplicate measurements from 3 different preparations and were plotted as % of control value.

exchanger of the renal brush-border membrane. The inhibition constants for amiloride (K_i) were given in Fig. 8. The kidney/placenta ratio of the K_i value was 13.6 and the synaptosome/placenta ratio was1.4.

DISCUSSION

The present study was designed to identify hydrogen transport pathways in MVV from human full term placenta. The experimental data indicate that Na^+/H^+ exchange and proton conductance are major pathways for proton transport across placental microvillous membrane. In this experiment, we could not observe absorbance change by Cl^-/OH^- exchange. However, Illsley et al. (1988) have observed Cl^-/OH^- exchange in microvillous membrane vesicles using SPQ (6-methoxy-N [3-sulfopropyl] qui-nolinium), a chloride-sensitive dye. Such a discrepancy between the experimental results using pH-sensitive and chloride-sensitive dyes was also observed in renal BBMV (Cassano et al, 1984; Seifter et al, 1984; Chen et al, 1988; Shiuan & Weinstein, 1984). This could be explained by the difference in passive permeabilities to Cl^- and proton in the membrane. It is not possible to demonstrate Cl^-/OH^- exchange from measurements of intravesicular pH by optical probes such as acridine orange and 6-carboxyfluorescein, when the magnitude of Cl^-/OH^- exchange is much less than that of the passive proton flux. In contrast, it is possible to measure lower rates of Cl^-/OH^- exchange when Cl^- rather than pH is the measured quantity because of the lower rate of intrinsic Cl^- flux.

The data from this experiment indicate that the conductance pathways for K^+ and Cl^- are present in placental MVV and that the permeability of MVV to Na^+ is very low compared to those of K^+ or H^+. It is not clear whether the conductance pathways for ions observed in this experiment are also present

in microvillous membrane of intact syncytiotrophoblast cells, since it has been reported that ionic permeability of membrane can be changed during preparation of membrane vesicles (Sabolic & Burckhardt, 1984; Glazier et al, 1988). Since, despite this problem, the present data of ionic conductive pathways are compatible to those of other studies using radioisotope (Chipperfield et al, 1986; Christine & Shennan, 1987) or fluorescent dyes (Illsley et al, 1988), the K^+, H^+ and Cl^- conductances observed in this experiment should not be considered as artifacts which are only observed in our MVV preparations.

Since the original demonstration in 1976 of the presence of the Na^+/H^+ exchanger in brush-border membranes isolated from small intestine and renal proximal tubule (Murer et al, 1976) the Na^+/H^+ exchanger has been identified in cells as widely differing in structure and function as fibroblasts, neuronal and glial cells, lymphocytes, neutrophils, platelets, myocytes, and epithelial cells from renal tubule and intestine (Mahnensmith & Aronson, 1985). The data from this experiment indicate that, as previously observed (Balkovetz et al, 1986; Ganapathy et al, 1987), Na^+/H^+ exchange is present in placental microvillous membrane and that the Na^+/H^+ exchanger in placenta is kinetically similar to that in kidney but has pharmacological characteristics differnt from kidney. Recently, Haggerty et al. (1988) published evidence indicating that two types of Na^+/H^+ exchangers with distinct pharmacological properties exist in cultered kidney cells (LLC-PK_1 and Madin-Darby canine kidney), one in the brush-border membrane and the other in the basolateral membrane. These two exchangers, which appear to be under separate genetic control, can be distinguished by their sensitivities to inhibition by amiloride or its analogs. The brush-border membrane Na^+/H^+ exchanger is less sensitive to the inhibitors than the basolateral exchanger. A similar observation has been made more recently in intestinal epithelial cells (Knickelbein et al, 1988). Interestingly, the exchanger found in the plasma membrane of other nonpolarized cells also shows high sensitivity to amiloride similarly to that of the the basolateral exchanger of the cultured renal cells. It is, therefore, apparent that the Na^+/H^+ exchanger of the human placental brush border membrane is more similar to the exchanger found in the plasma membrane of other nonpolarized cells than to the exchanger in the renal brush-border membrane.

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