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Control of the sodium-proton antiporter in human placental microvillous membranes by transport substrates

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The microvillous membrane of the human placental syncytiotrophoblast contains an amiloride-inhibitable, electroneutral, Na^+/H^+ antiporter. The kinetic characteristics of this antiporter have been investigated to determine its response to alterations in intracellular and extracellular H^+ and Na^+ concentrations. Antiporter activity was measured using a pH-sensitive fluorescent probe entrapped in placental microvillous vesicles. We report here on the kinetic characterization of the antiporter, a transporter which displays simple, saturable kinetics for the external site but complex kinetics at the internal site. Measurement of the external Na^+ and H^+ dependences demonstrated that Na^+ and H^+ compete for binding to a single external binding site which displays saturation kinetics. The external K_m determined for Na^+ was 8.2 ± 4.0 mM, while the external pK was 7.29 ± 0.02. The V_{max} calculated from these experiments was 0.57 ± 0.10 nequiv./s per mg membrane protein. By contrast, the internal dependences for both Na^+ and H^+ showed significant deviations from simple linear kinetics. Decreasing internal pH to 6.0 stimulated Na^+/H^+ exchange to a greater degree than predicted for a single-site saturable binding model, in a manner which suggested allosteric activation. At the other extreme, Na^+/H^+ exchange ceased above an internal pH of 7.1, despite the existence of an inwardly-directed Na^+ gradient. Increasing intracellular Na^+ caused inhibition of Na^+/H^+ exchange but the intracellular Na^+ dependence showed that the effect is due to a mechanism more complex than simple, competitive inhibition between Na^+ and H^+. These results show that the microvillous Na^+/H^+ antiporter is insensitive to changes in extracellular Na^+ and H^+ concentrations in the physiological range. Changes in intracellular Na^+ and H^+ however are likely to cause marked changes in antiporter activity. These characteristics suggest that cellular Na^+ and H^+ concentrations are tightly controlled in the placental syncytiotrophoblast and that the Na^+/H^+ antiporter may play a significant role in their regulation.

Introduction

Carrier-mediated, transmembrane Na^+/H^+ exchange has been observed in most mammalian tissues, where it is involved in diverse cellular functions. These include the regulation of intracellular pH (and thus the indirect regulation of pH dependent metabolic processes), control of intracellular Na^+ concentrations and the regulation of cellular volume through effects on cell water content [1–3]. In epithelial tissues, the Na^+/H^+ exchange transporter or antiporter regulates transepithelial transport of Na^+ and of the solutes to which Na^+ transport is commonly coupled (e.g., chloride, amino acids, glucose and phosphate). More recently, another role has been identified for the Na^+/H^+ antiporter in the control of cell growth and differentiation [4,5]. Stimulation of Na^+/H^+ exchange by the antiporter appears to be an intermediate step in the genomic and proliferative responses to a variety of agents, especially growth factors [6,7].

Because Na^+ and H^+ fluxes are crucial components of the cellular ionic environment, the antiporter is involved in ionic homeostasis. The Na^+/H^+ antiporter may affect (or be affected by) the operation of other cellular ion transporters which carry Na^+ or H^+, or by transporters which influence the intracellular concentrations of Na^+ and H^+. Thus operation of the antiporter

Abbreviations: NMG, *N*-methylglucamine; 6CF, 6-carboxyfluorescein; MVM, microvillous membrane.

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may be coupled to cellular ionic movement via transporters such as the Na^+/K^+- and H^+-ATPases, Cl^-/HCO_3^- exchanger and $Na^+/K^+/2Cl^-$ cotransporter.

The placenta serves as the fetal lung, kidney and intestine, and so placental Na^+ and H^+ transport is of major importance for fetal growth and development. In addition to the fetal requirement for accumulation of Na^+, the transport of several essential nutrients (e.g., phosphate, amino acids) is coupled to the transmembrane sodium gradient across the plasma membrane of the placental syncytiotrophoblast [8,9]. Additionally, extrusion of placental and fetally-derived H^+ is an important component in maintaining placental and fetal acid-base balance.

The placental syncytiotrophoblast contains an amiloride-inhibitable, pH-dependent Na^+ transporter, located on the maternal-facing or microvillous membrane surface. Balkovetz et al. [10] and Chipperfield et al. [11] identified this transporter in term human placenta using $^{22}Na^+$ as a probe, but its functional characteristics are unexplored. Although the role of the Na^+/H^+ transporter in this tissue has not been defined, it is probable that it is important for the regulation of placental intracellular pH and cellular volume. In addition to its cellular functions, it is well situated to control the transplacental transport of Na^+, H^+ and substances which are coupled to the movement of these ions. To determine the role of the transporter under physiological conditions, knowledge of its kinetic characteristics is necessary. Given the possible role of the antiporter in cellular ionic homeostasis, it is also important to understand the effect of alterations in intracellular and extracellular Na^+ and H^+ concentrations on the exchange process. We report here on experiments designed to determine the kinetic parameters for the placental Na^+/H^+ antiporter and to investigate the dependence of exchange activity on Na^+ and H^+, the transport substrates.

Methods and Materials

Microvillous membrane vesicle preparation

Microvillous membranes were prepared from normal, term (38–41 week gestation) placentae [12]. Briefly, after removal of the chorionic plate and a decidual layer, the tissue was washed with 0.9% NaCl and homogenized in 250 mM sucrose, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 10 mM Tris-Hepes (pH 7.0). The homogenate was centrifuged at $10000 \times g$ for 15 min, the supernatant retained and the pellet was rehomogenized and recentrifuged. The combined supernatants were centrifuged at $27500 \times g$ for 30 min and the pellet was resuspended in the homogenization buffer. $MgCl_2$ was added to the suspension (12 mM final concentration) to aggregate non-microvillous membranes and the suspension was stirred on ice. The mixture was centrifuged at $2500 \times g$ for 15 min to remove the Mg^{2+}-precipitated membranes and the supernatant was centrifuged at $47500 \times g$ for 30 min to produce a microvillous membrane (MVM) fraction. The MVM fraction was washed and resuspended in 250 mM sucrose, 10 mM Tris-Hepes (pH 7.0), frozen in liquid N_2 and stored at −70 °C. Membranes prepared in this manner are enriched > 20-fold in the microvillous marker, alkaline phosphatase and were essentially free from contamination by syncytiotrophoblast basal membranes, mitochondria, microsomes and lysosomes as measured by marker enzyme analysis [12]. Contamination by nonsyncytial plasma membranes was minimal, as determined by immunoblotting [12]. The MVM vesicle orientation was previously determined to be approx. 90% right-side out [12].

Fluorophore loading and measurement of H^+ flux

MVM were equilibrated overnight at 4 °C in buffer solutions chosen to set the intravesicular composition, including the internal Na^+ concentration (Na_i^+) and internal pH (pH_i). The loading buffer also contained 0.2 mM 6-carboxyfluorescein (6CF), a pH-sensitive fluorophore [13]. MVM were washed three times in > 20 volumes of the same loading buffer minus fluorophore then resuspended by repeated passage through a 23-gauge needle. The loss of 6CF from MVM prepared in this manner was less than 5% per hour when the vesicles were stored at 4 °C. Solutions chosen to set the extravesicular Na^+ concentration (Na_0^+) and pH (pH_0) were formulated to give ionic and osmotic strengths identical to the intravesicular solutions using NMG and gluconate to make the balance of the buffers. Buffers contained 50 mM K-gluconate for the purposes of clamping the membrane potential to zero (in the presence of valinomycin), except for those experiments performed to investigate the electrogenicity of Na^+/H^+ transport. Solutions were buffered with either 50 mM Mes or 50 mM Hepes, and experiments were carried out at room temperature. Immediately prior to assay, valinomycin was added to the MVM from ethanolic stock to give a final concentration of 25 μg/mg membrane protein. The final ethanol concentration was less than 1% and did not affect H^+ flux measurements.

Experiments were performed by diluting MVM (approx. 50 μg membrane protein) into 2 ml of medium stirred continuously in a thermostatted (23 °C) acrylic cuvette. The time course of 6CF fluorescence was measured using an integration time of 0.5 s over 120 s. After 90 s, monensin was added from ethanolic stock (final concentration 100 μg/mg membrane protein), permitting rapid equilibration of intravesicular and extravesicular Na^+ and H^+ and providing a reference level of 6CF fluorescence. Fluorescence was excited at 490 nm and the emission was measured using a 515 nm cut-on

filter (Melles-Griot, Irvine, CA) in a SLM 8000C spectrofluorimeter (SLM Instruments, Urbana, IL) interfaced to a System 1800A AT-compatible computer (Rose Hill Systems, Scotts Valley, CA).

H^+ fluxes (J_H; nequiv./s per mg membrane protein) were calculated from the time course of 6CF fluorescence, the MVM buffer capacity, and the 6CF fluorescence vs. pH calibration curve as described previously [13,14]. The buffer capacity of the MVM, B(pH), was measured as described previously [14] by titration of MVM in deionized, distilled water containing 0.05% Triton X-100. B(pH) in the pH range 5.0–7.5 was approximated by the equation, $B(\text{pH}) = 185(\text{pH}) - 782$ (nequiv./pH unit per mg membrane protein), similar to the previously measured buffer capacity. The fluorescence response of 6CF to pH was determined previously [13] and the calibration curve is described by the equation,

$$f(\text{pH}) = 1.8(\text{pH})^3 - 1.04(\text{pH})^2 + 0.18(\text{pH}) - 0.0095$$

Fluorescence time courses were normalized to the final value of fluorescence, measured after the equilibration of intravesicular and extravesicular Na^+ and H^+ concentrations following addition of monensin. The H^+ flux was calculated from

$$J_H = B(\text{pH})[f(t)/f(0)]f(\text{pH}_i)R/f'(\text{pH}_i)$$

where B(pH) is the intravesicular buffer capacity in nequiv./pH unit per mg protein, $f(\text{pH}_i)$ is the initial fluorescence value determined from the calibration curve, $f'(\text{pH}_i)$ is the derivative of $f(\text{pH}_i)$ with respect to pH evaluated at pH_i, $f(t)/f(0)$ is the normalized initial slope of the 6CF fluorescence time course and R is the fraction of 6CF present in the intravesicular space (determined as described previously [13]). Curve fitting was carried out using a generalized non-linear Newton's method. Flux rates were compared by Student's t-test. All measurements were made at least in triplicate and all experiments were performed using at least three different placental preparations. Data are presented as the means ± standard deviation.

Materials

Carboxyfluorescein was obtained from Molecular Probes Inc. (Eugene, OR) and other chemicals were purchased from Sigma Chemcial Company (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ).

Results

Exchange inhibition

Amiloride inhibition is an important tool in the study of the Na^+/H^+ antiporter, distinguishing uncoupled H^+ or Na^+ fluxes from the coupled activity of the antiporter. Amiloride inhibition of Na^+-stimulated H^+ flux was measured by addition of MVM containing 50 mM NMG-gluconate ($\text{pH}_i = 6.0$) to external solutions containing 50 mM Na-gluconate ($\text{pH}_0 = 7.4$) in the presence of varying concentrations of amiloride (Fig. 1). Control H^+ efflux was measured in the absence of external Na^+, after addition of MVM to an external solution containing 50 mM NMG-gluconate ($\text{pH}_0 = 7.4$). The apparent K_i determined from these experiments was 47 ± 14 μM ($n = 3$). Harmaline and quinidine, both previously reported as inhibitors of Na^+/H^+ exchange, were also effective in inhibiting the placental microvillous antiporter. Exchange activity in the presence of 0.2 mM harmaline, quinidine and amiloride was 72 ± 13%, 22 ± 14% and 11 ± 8%, respectively, significantly less than control activity (0.64 ± 0.12 nequiv./s per mg); $P < 0.05$; $n = 3$).

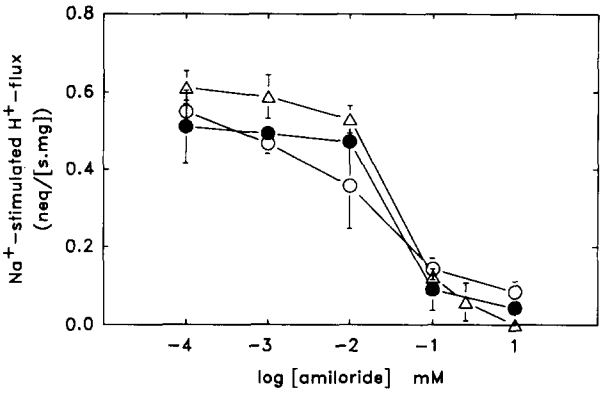


Fig. 1. Amiloride inhibition of Na^+/H^+ exchange. Effect of amiloride on Na^+-stimulated H^+-efflux (nequiv./s per mg) produced when microvillous vesicles (≈ 50 mg membrane protein/50 μl; $\text{pH}_i = 6.0$) containing 0.1 mM 6CF and valinomycin were added to an external solution (1.95 ml) containing 50 mM Na-gluconate ($\text{pH}_0 = 7.4$). Control rate measured after addition of MVM to 50 mM NMG-gluconate ($\text{pH}_0 = 7.4$). Each point represents mean ± standard deviation for triplicate measurements.

Voltage dependence of exchange

The Na^+/H^+ antiporter characteristically displays electroneutral transport [15]. The electrogenneicity of placental Na^+-stimulated H^+-flux was assessed by measuring exchange activity in the presence and absence of a transmembrane potential. MVM loaded with 50 mM K-gluconate, 50 mM NMG-gluconate ($\text{pH}_i = 6.0$) were added to external solutions at pH 7.4 containing 10 mM K-gluconate 40 mM NMG-gluconate (voltage) or 50 mM K-gluconate (no voltage), plus 50 mM Na-gluconate. In the presence of valinomycin, the membrane potential was clamped to 0 mV with an external K^+ concentration of 50 mM, and to 30 mV (internal negative) with an external K^+ concentration of 10 mM. The Na^+-stimulated H^+-flux measured in the presence of a 30 mV membrane potential (0.47 ± 0.09 nequiv./s per mg); $n = 3$) was not different from the flux measured in

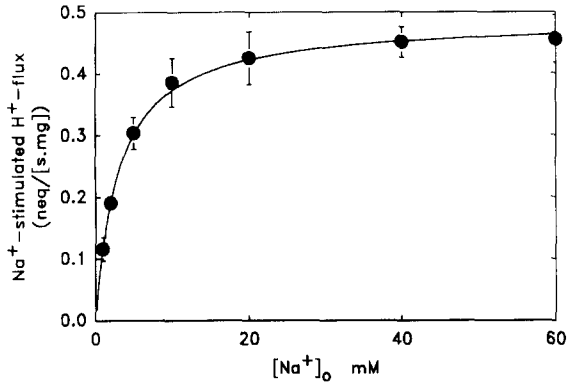


Fig. 2. External Na^+ dependence of Na^+/H^+ exchange. Representative example of Na^+-stimulated H^+-efflux measured over a range of external Na^+ concentrations. MVM containing 50 mM NMG-gluconate ($pH_i = 6.0$) were added to external solutions at pH 7.4 containing a range of Na-gluconate concentrations (balance NMG-gluconate). Other conditions as for Fig. 1. Curve fitted to saturable model.

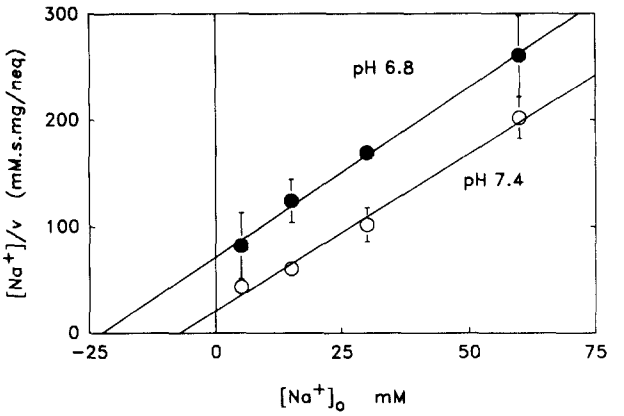


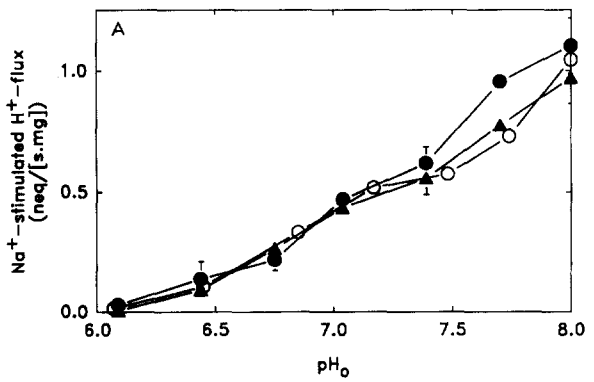
Fig. 3. External Na^+ dependence of Na^+/H^+ activity at pH 6.8 and 7.4. Typical example of the external Na^+ dependence of Na^+-stimulated H^+-efflux measured at external pH of 6.8 and 7.4. MVM containing 50 mM NMG-gluconate ($pH_i = 6.0$) were added to external solutions containing Na-gluconate with a pH of 6.8 or 7.4. Other conditions as for Fig. 1. Data graphed as a Hanes-Woolf plot and curves fitted by linear regression.

the absence of a potential (0.46 ± 0.07 nequiv./s per mg; $n = 3$). Na^+-stimulated H^+-flux was not altered by a membrane potential, demonstrating the electroneutrality of Na^+/H^+ exchange.

Dependence of H^+-efflux on external Na^+

To determine the degree to which H^+-efflux depends on external Na^+ concentration (Na_0^+), MVM containing 50 mM NMG-gluconate ($pH_i = 6.0$) were added to solutions containing a range of Na-gluconate concentrations at pH 7.4. The H^+-flux stimulated by Na^+ was best fitted to a single-site, saturable model (Fig. 2), with a V_{max} of 0.57 ± 0.10 nequiv./s per mg and a K_m of 8.2 ± 4.0 mM ($n = 6$). Similar parameters were found when Li^+ was substituted for Na^+; the V_{max} was 0.71 ± 0.07 nequiv./s per mg and the K_m was 4.8 ± 0.7 mM ($n = 3$). Substitution of Rb^+ or Cs^+ for Na^+ were without effect on H^+-efflux (data not shown).

To study the interaction between Na^+ and H^+ at the external transport site(s), the external Na^+ dependence of H^+-efflux was measured at two different values for the external pH ($pH_0 = 6.8$ and $pH_0 = 7.4$). The presence of competition between Na^+ and H^+ for binding to a single external transport site should result in an increased K_m for Na_0^+ when external pH is decreased (increased $[H^+]_0$). The Na^+-stimulated H^+-fluxes determined from a typical experiment are shown as a Hanes-Woolf plot ($[Na^+]_0/v$ vs. $[Na^+]_0$) in Fig. 3. The V_{max} values calculated from these plots were 0.36 ± 0.05 nequiv./s per mg ($pH_0 = 7.4$) and 0.41 ± 0.06 nequiv./s per mg ($pH_0 = 6.8$) for three placental preparations, values which did not differ significantly. However, the K_m value calculated at pH 6.8 (27.2 ± 4.2 mM) was significantly higher ($P < 0.05$) than that obtained at pH 7.4 (10.0 ± 3.3 mM). These results, in addition to confirming the adherence of the transport mechanism to single-site, saturable kinetics, demonstrate the competi-



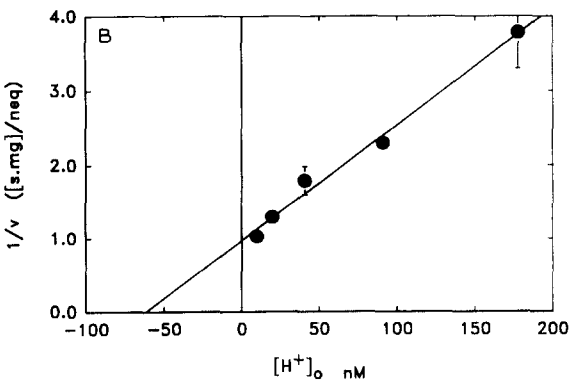


Fig. 4. (A) Effect of external pH on Na^+/H^+ exchange activity. Change in Na^+-stimulated H^+-efflux with alterations in external pH for three separate placental preparations. MVM containing 50 mM NMG-gluconate were added to external solutions containing 50 mM Na-gluconate, at a range of external pH values between 6.0 and 7.4. Other conditions as for Fig. 1. (B) Linearized plot of the external pH dependence. Transformation of the data for one of the curves in Fig. 4A into a Dixon plot. Curve fitted by linear regression.

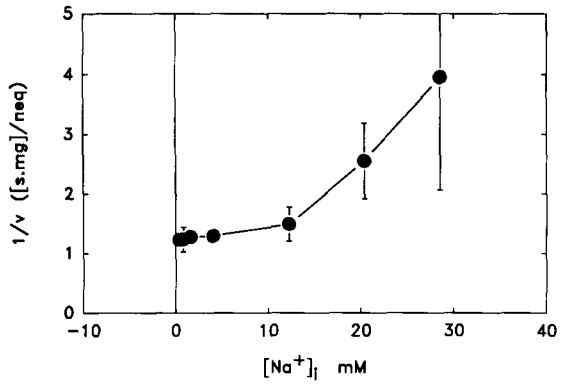


Fig. 5. Reciprocal plot of internal Na^+ dependence. Dixon plot of the inhibition of Na^+-stimulated H^+-efflux by Na_i^+; one example representative of three separate experiments. MVM equilibrated with varying concentrations of Na-gluconate (balance NMG-gluconate, pH 5.8) were added to an external solution containing 50 mM Na-gluconate (pH_0 7.2). Other conditions as for Fig. 1.

tive interaction between Na^+ and H^+ at the external transport binding site.

In the light of the observed external Na^+ dependence, decreasing external pH (increasing $[H^+]_0$) should inhibit Na^+-stimulated H^+-efflux by increasing the competition between H^+ and Na^+ for the external site. The effects of external pH on Na^+-stimulated H^+-efflux were measured by addition of MVM containing 50 mM NMG-gluconate ($pH_i = 6.0$) to solutions containing 50 mM Na-gluconate, adjusted to pH values between 6.0 and 8.0 (Fig. 4A). The results show a reduction in the flux to zero as pH_0 was decreased to 6.0. Competitive inhibition between H^+ and Na^+ should appear linear when graphed as a Dixon plot ($1/v$ vs. [inhibitor]). This transformation of the experimental data is shown in Fig. 4B. The linearity of the plot demonstrates that transport conforms to single-site, saturable binding. A K_i value for H^+ was determined from the point on the abscissa where a horizontal line crossing the ordinate at $1/V_{max}$ intersects the experimental curve [16]. Using the V_{max} value obtained above (0.57 ± 0.10 nequiv./s per mg), the K_i determined was 51 ± 2 nM ($n = 3$), equivalent to a pK value for the external H^+ binding site of 7.29 ± 0.02. Intersection of the $1/V_{max}$ line with the experimental curve above the abscissa indicates that the inhibition of Na^+-stimulated H^+-efflux by external H^+ is consistent with competitive inhibition [16].

Dependence on internal Na^+

The effect of internal Na^+ (Na_i^+) on Na^+/H^+ exchange activity was determined by measuring the inhibition of Na^+-stimulated H^+-efflux by increasing $[Na^+]_i$. MVM equilibrated with a range of $[Na^+]_i$ (0–50 mM) at pH 5.8 were added to solutions containing 50 mM Na-gluconate at an external pH of 7.2. The Dixon plot of this data is non-linear (Fig. 5), showing that the inhibition of Na^+-stimulated H^+-efflux by Na_i^+ does not follow simple linear inhibition kinetics. These experiments cannot distinguish between the possible causes of non-linear kinetics, which include partial or mixed inhibition and cooperative interaction [16].

Internal pH

The internal pH dependence (pH_i) of Na^+/H^+ exchange was determined by addition of MVM containing 50 mM NMG-gluconate (pH_i 6.0 to 7.4) to solutions containing 50 mM Na-gluconate ($pH_0 = 7.4$). The results showed an increase in the Na^+-stimulated H^+-efflux with decreasing pH_i (Fig. 6A). Extrapolation of this data showed that the activity of the antiporter decreased to zero at a pH_i of 7.1 ± 0.1. When replotted in double-reciprocal form (Fig. 6B), there was upward deviation from linearity, indicative of positive cooperativity. The intercept on the ordinate ($1/V_{max}$) resulted in a value for V_{max} of 0.48 ± 0.17 nequiv./s per mg ($n = 3$), similar to the value obtained from the Na_0^+-dependence data. The reciprocal plots were linearized by plotting $1/v$ against $1/[H^+]^2$, suggesting two internal binding sites for H^+ and providing estimates of V_{max}

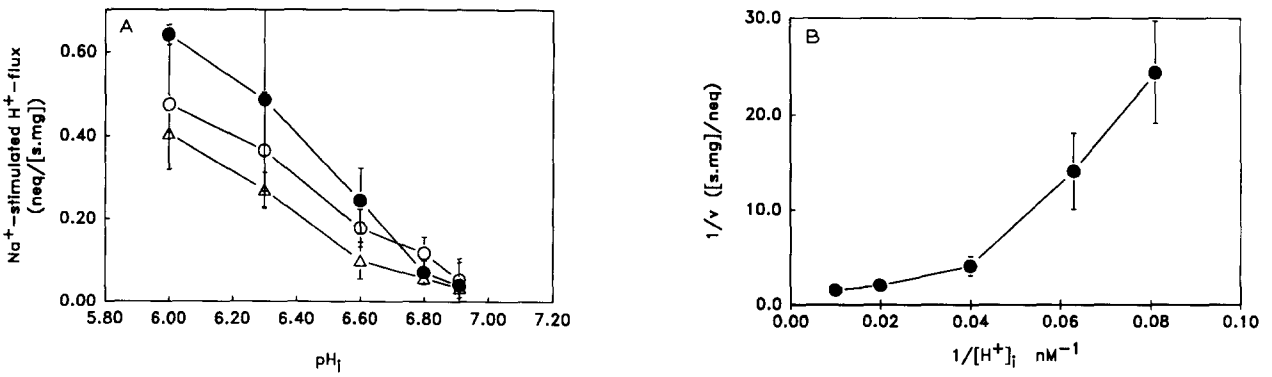


Fig. 6. (A) Internal pH dependence of Na^+/H^+ exchange. Effect of changes in internal pH on the rate of Na^+-stimulated H^+-efflux. H^+ fluxes were measured after addition of MVM containing 50 mM NMG-gluconate (pH 6.0 to 7.4) to an external solution containing 50 mM Na-gluconate (pH 7.4). Other conditions as for Fig. 1. (B) Lineweaver-Burk plot of internal pH dependence. Double-reciprocal plot of one of the internal pH dependence curves shown in Fig. 6A.

and K'_H, a constant which comprises the association constant and interaction factors. The V_{max} obtained from this transformation is 0.59 ± 0.21 nequiv./s per mg ($n = 3$), the same as that obtained from the external Na^+ dependence data. K'_H derived from this plot was 83 ± 66 nM, equivalent to a pK of 7.1 ± 0.3 for the internal site. The slope of a Hill plot ($v/(V_{max} - v)$ vs. $[H^+]$) provides a measure of the number of interacting substrate sites on the transporter. The slopes from a Hill plot of this data gave a value for n_{app} of 2.5 ± 0.4 for three separate placental preparations, supporting the suggestion of allosteric interaction.

Discussion

Our study of the kinetics of Na^+/H^+ exchange indicates the presence of active, carrier-mediated transport in the microvillous membrane and confirms information in previous reports, in which amiloride and its analogs were shown to inhibit placental Na^+/H^+ exchange [10,11]. The inhibition constant determined here for the placental Na^+/H^+ antiporter is similar in magnitude to those obtained in a variety of other tissues [17–19]. The pattern of inhibition determined for amiloride, quinidine and harmaline was also comparable to that measured in other systems [18,20]. Kulanthaivel et al. have suggested that, based on inhibition patterns, the placental microvillous membrane antiporter has characteristics similar to the renal basolateral Na^+/H^+ antiporter, a plasma membrane exchanger, rather than the renal brush border exchanger [21]. In basal membranes isolated from placental syncytiotrophoblast using the same preparative procedure [12], Na^+/H^+ exchange activity was not observed (data not shown).

Kinetics of the external site

The dependence of Na^+/H^+ exchange on Na_0^+ was fitted to a model for a single, saturable transport site, yielding a K_m of 8.2 ± 4.0 mM and a V_{max} of 0.57 ± 0.10 nequiv./s per mg. The K_m and V_{max} values are similar to values for the placental antiporter obtained by Balkovetz et al. (7.8 mM, 0.65 nequiv./s per mg [10]) and the K_m is similar to that obtained by Chipperfield et al. [9.6 mM [11]), both obtained by measuring the transport of $^{22}Na^+$.

The lack of a voltage effect on Na^+-stimulated H^+-efflux demonstrates that exchange does not involve net transport of charge (electroneutral process), similar to that noted for renal microvillous vesicles, ileal brush border vesicles and fibroblasts [4,22,23].

Electroneutrality is consistent with a stoichiometry of 1 : 1 or greater, however, the external Na^+ dependence of H^+-efflux conforms to single-site, saturable kinetics, and therefore a single external binding site for Na^+ and H^+ is probable. The inhibitory effect of decreasing pH_0 is consistent with saturation kinetics, implying a single external binding site for H_0^+. The competition between Na^+ and H^+ for a single external site was confirmed by the comparison between the external Na^+ dependence at $pH_0 = 7.4$ and $pH_0 = 6.8$, in which the V_{max} remained constant despite a large increase in the K_m for Na_0^+. Thus the external kinetics of the antiporter conform to those of a simple, saturable transporter, lacking allosteric interactions.

Kinetics of the internal binding site

The dependence of Na^+-stimulated H^+-flux on internal H^+ and Na^+ concentrations, by contrast with their external dependencies, deviated significantly from simple saturation kinetics. At higher internal H^+ concentrations, where saturation of the transport site by H^+ and a maximal transport rate would be expected (zero order kinetics), placental Na^+/H^+ exchange activity continued to increase. In view of the data demonstrating electroneutral transport via a single external site, these findings are consistent with an internal (allosteric) modifier site separate from the transport site which can bind H^+ and activate transport. These results suggest an allosteric site comparable to those observed in vesicles and cells from other tissues [3,4,6,19].

The internal Na^+ dependence also shows marked variation from simple, saturation kinetics. In this case, at higher internal Na^+ concentrations, exchange activity was inhibited to a degree greater than that predicted for simple competition between H_i^+ and Na_i^+. Unlike the effects of internal H^+, however, non-linear Dixon or Hill plots cannot be taken as evidence of allosteric interaction, since a variety of partial and mixed inhibition schemes will generate non-linear plots [16]; the only conclusion that can be drawn from this data is that the effects of internal Na^+ are more complex than simple linear inhibition.

As the data reported here indicates, Na^+/H^+ exchange activity is halted at a pH_i of approx. 7.1, despite the presence of an inwardly directed gradient of Na^+. In vivo this would result in an inactive antiporter in all but the most extreme of conditions. However, there is strong evidence to suggest that in many tissues the pH at which exchange ceases (the 'set point') can be altered by factors which stimulate protein kinase C [24]. The stimulatory effects of these extracellular factors (including platelet-derived growth factor, epidermal growth factor, interferon and vasopressin) on Na^+/H^+ appear to occur via phosphorylation of the antiporter, modifying the internal H^+ binding site such that the set point is increased from 7.0 to 7.4. Recently, data has been obtained suggesting that protein kinase C-mediated phosphorylation of the antiporter in placental microvillous membranes also can increase Na^+/H^+ exchange activity [25].

It should be noted that the vesicles obtained using the preparative procedure described here [12] comprise both right-side out (88%) and inside out (12%) vesicles. It is possible therefore that the sidedness of the activating and inhibitory effects observed in the kinetic results may be underestimated because of the small population of inside-out vesicles present in the experimental samples, however, this will not affect the conclusions derived from these results.

Effect of changes in Na^+ and H^+ on Na^+/H^+ antiporter activity and ionic homeostasis

Na^+ is actively transported out of the syncytiotrophoblast by the basal membrane Na^+/K^+-ATPase, creating an inwardly directed gradient across the syncytiotrophoblast plasma membranes. The intracellular Na^+ concentration in the human syncytiotrophoblast has not been measured but the gradient is probably, like most animal cells, 5–10-fold. In the rabbit placenta for example, the intracellular and extracellular Na^+ concentrations are 25 mm and 145 mM [26]. In the interior of most cells the H^+ concentration is higher than or equal to that of extracellular fluid. These conditions strongly favour the extrusion of intracellular H^+ in exchange for extracellular Na^+ in vivo. The passive distribution of H^+ determined by cellular membrane potentials (-20 to -40 mV, internal negative [27]) would predict a lower pH_i than that observed in vivo and lower than that compatible with normal metabolic function. Thus the antiporter may function in part to perform the necessary uphill extrusion of H_i^+ and maintain pH_i.

The physiologic level of extracellular sodium greatly exceeds the K_m for Na_0^+ and the antiporter will therefore operate under conditions of near saturation at the external site. In this situation, changes in Na_0^+ and H_0^+ within the physiologic range will have little effect on the operation of the antiporter. As a consequence of the non-linear kinetics of the internal transport site however, relatively small changes in Na_i^+ and H_i^+ may have significant regulatory effects. The lack of both a reliable value for syncytial Na_i^+ and knowledge of the inhibitory mechanism by which Na_i^+ acts make prediction of the effects of changes in Na_i^+ difficult. Examination of Fig. 5 suggests that below an internal Na^+ concentration of 10–15 mM there is simple competition between Na_i^+ and H_i^+. A reduction in Na_i^+ will therefore stimulate transport activity as a result of a reduction in the rate of Na_0^+/Na_i^+ exchange. Previous studies examining the effects of Na_i^+ have also shown stimulation of exchange activity by reductions in Na_i^+ [3,20,28]. These studies however looked only at the depletion of Na_i^+ from normal intracellular levels and did not measure the effects of increased Na_i^+. The non-linear inhibitory effects of Na_i^+ on Na^+-stimulated H^+-efflux occur above 10 mM, where increased Na_i^+ appears to produce greater inhibition than that predicted by simple competitive behaviour. The antiporter may therefore act to maintain intracellular Na^+ by self-inhibition of Na^+ influx. In the placental context, this function may be important for maintenance of the transmembrane Na^+ gradient, the driving force behind the transfer of many essential fetal nutrients.

Non-linear kinetics were observed for the pH_i dependence of exchange activity. With evidence of a single external site and electroneutral exchange, this data strongly suggests the presence of a second, separate internal H^+ binding site which modulates the activity of the antiporter. The fact that the curvilinear reciprocal plot was linearized by plotting $1/v$ against $1/[H_i^+]^2$ suggests the presence of two internal binding sites, presumably the transport site and the regulatory site [16]. This mode of regulation provides an important method for the fine control of H^+ extrusion in response to increased intracellular acid loading. This is of particular importance in the placenta since transplacental passage is the only route for excretion of fetally produced H^+. The placental syncytiotrophoblast will be involved in the transport of H^+ generated by both fetus and placenta, a load which will increase markedly under conditions of metabolic acidosis, a problem common to a number of pathophysiological states in pregnancy [29]. In addition to regulation of intracellular pH, the antiporter is likely be involved in the maintenance of the fetal acid-base balance, thus a mechanism to activate proton extrusion under acidotic conditions would be of considerable value to the fetus.

Role of the antiporter in NaCl transport and volume control

We have previously demonstrated the existence of a Cl^-/HCO_3^- exchanger on the microvillous membrane which, under in vivo conditions, will exchange extracellular Cl^- for intracellular HCO_3^- [30]. Operation of this transporter in combination with the Na^+/H^+ antiporter will cause uptake of NaCl into the placental syncytiotrophoblast by the 'dual countertransport' mechanism. Intracellular Na^+ will be pumped out of the cell into the fetal extracellular space by the Na^+/K^+-ATPase mounted on the basal (fetal-facing) membrane of the placental epithelium. In the presence of a suitable exit step for Cl^- across the basal membrane, these mechanisms provide a pathway for the maternofetal transfer of NaCl. The V_{max} for Na^+/H^+ exchange calculated here (0.57 ± 0.10 nequiv./s per mg) is comparable to that determined for the Cl^-/HCO_3^- transporter (0.54 nmol/s per mg [27]).

In a number of cells, Na^+/H^+ exchange is activated as a response to cellular shrinkage [9,23]. The cellular property which enables volume control by the Na^+/H^+ antiporter is the high degree of buffering by proteins and fixed sites within the cell. Although Na^+/H^+ ex-

change is isosmotic, the internal H^+ lost in exchange for Na^+ is replaced from intracellular buffer sites, resulting in the net uptake of one osmole of Na^+. Seen in isolation, activation of the antiporter will therefore be accompanied by water influx. There are however a number of other transport mechanisms, like the Cl^-/HCO_3^- transporter which, operating in concert with the antiporter, may also produce osmolar movement. Prediction of the cellular responses to osmotic challenge and the role of the Na^+/H^+ antiporter will require greater knowledge of the mechanisms and coupling of syncytiotrophoblast transporters.

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