Characterization of an ATP-driven H⁺ pump in human placental brush-border membrane vesicles

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The presence of an ATP-driven H⁺ pump as measured by H⁺ uptake upon addition of ATP was not demonstrable in human placental brush-border membrane vesicles when used in their native form, owing to their right-side-out orientation. However, the presence of the H⁺ pump in these membranes became evident when the membrane vesicles were transiently exposed to 1 % cholate, with subsequent removal of the detergent to re-form the vesicles. Apparently, cholate pretreatment reoriented the H⁺ pump from an inward-facing configuration to outward-facing. Consequently, H⁺ uptake in response to externally added ATP was easily demonstrable in these cholate-pretreated vesicles by using the ΔpH indicator Acridine Orange. In addition, bafilomycin A₁-sensitive ATPase activity was measurable in cholate-pretreated vesicles, but not in native intact vesicles, indicating reorientation of the H⁺ pump. The reoriented H⁺ pump was electrogenic because H⁺ uptake was stimulated by an inside-negative anion-diffusion potential or when the vesicles were voltage-clamped. ATP supported H⁺ uptake with an apparent K_m of 260 μM. ITP and GTP supported the pump activity partially, whereas CTP and UTP did not. Mg²⁺ and Mn²⁺ were the most preferred bivalent cations. Co²⁺ and Zn²⁺ showed partial activity, whereas Ca²⁺ and Ba²⁺ showed little or no activity. The pump was inhibited by nanomolar concentrations of bafilomycin A₁ and micromolar concentrations of *N*-ethylmaleimide, *p*-chloromercuribenzenesulphonate, *NN*-dicyclohexylcarbodi-imide and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, but was relatively insensitive to oligomycin, vanadate and NaN₃. The inhibition by *N*-ethylmaleimide was protectable by ATP. It is concluded that human placental brush-border membranes possess an ATP-driven H⁺ pump and that, on the basis of its characteristics, it belongs to the class of vacuolar (V-type) H⁺ pumps.

INTRODUCTION

Human placenta performs various functions for the developing fetus, which in adults are handled by a multitude of organs such as intestine, kidney, lung and liver. Placental transfer is the only mechanism by which essential nutrients enter the fetal circulation. Similarly, catabolic end products are eliminated from the fetal circulation via the placenta. The cell layer that is primarily responsible for this transport function is the syncytiotrophoblast. This syncytium is polarized, similar to the transporting epithelial cells of the intestine and kidney, exhibiting an asymmetry introduced by the different transport capabilities of the brush-border (maternal-facing) and basal (fetal-facing) membranes [1]. The polarity of the syncytiotrophoblast dictates the vectorial movement of solutes and ions across the placenta.

Transfer of H⁺/OH⁻ across the brush-border and basal membranes plays a critical role in acid/base homeostasis of the fetus. These two poles of the syncytiotrophoblast plasma membrane can be purified with little cross-contamination, and the resulting membrane vesicles can be conveniently used to characterize various transport systems inherent in these membranes. For many years, our laboratory has been investigating H⁺-coupled transport phenomena in brush-border membrane vesicles isolated from term placentas with an aim to delineate the various mechanisms available for H⁺ movement across this membrane. These investigations have so far led to the identification of three transport systems in these membrane vesicles which are coupled to H⁺, namely Na⁺-H⁺ exchanger [2,3], organic-cation-H⁺ antiporter [4] and lactate-H⁺ symporter [5]. Studies from other laboratories [6,7] have demonstrated that the

placental brush-border membrane possesses a SO₄²⁻ transport system which is independent of Na⁺. It has been suggested that the operational mechanism for this transport system may be either SO₄²⁻/OH⁻ exchange or SO₄²⁻/H⁺ symport [8]. In addition to these co-transport systems available for H⁺, Cabrini *et al.* [9] have described a H⁺-conductance pathway in placental brush-border membranes which is stilbene-sensitive and modulated by various anions.

A totally different mechanism for transmembrane H⁺ movement has been described in the plasma membrane of certain cell types. This mechanism is responsible for primary active H⁺ secretion and is mediated by a H⁺-translocating ATPase (H⁺ pump). Such an ATP-driven H⁺ pump has been demonstrated in the luminal membrane of renal proximal tubule [10,11], collecting duct [10,12] and urinary bladder [13–16]. We have reported recently that the brush-border membranes isolated from human term placenta also possess an ATP-driven H⁺ pump and that the catalytic activity of this H⁺ pump is inhibited when the membranes are treated with tyrosyl-group-specific reagents [17]. The present study was undertaken to determine the general characteristics of the placental H⁺ pump. The significance of a better understanding of the nature of this H⁺ pump is underscored by the findings that in many cells a plasma-membrane-associated H⁺ pump plays a role in receptor-mediated transport pathways (receptor-mediated endocytosis and receptor-coupled transmembrane transport) [18,19] and that these transport pathways are responsible in the placental syncytiotrophoblast for the transport of a number of important substances across the placenta (e.g. immunoglobulins, cholesterol, folate and Fe²⁺) [1]. Therefore delineation of the characteristics of the placental H⁺

Abbreviations used: DCCD, *NN*-dicyclohexylcarbodi-imide; NEM, *N*-ethylmaleimide; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; pCMBS, *p*-chloromercuribenzenesulphonate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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pump is not only relevant to the mechanisms of the handling of H^+ by the placenta, but may also prove to be useful to understand the coupling of the H^+ pump with other transport pathways.

MATERIALS AND METHODS

Materials

Nucleotides, sodium cholate, *N*-ethylmaleimide (NEM), oligomycin, *NN*-dicyclohexylcarbodi-imide (DCCD), vanadate (Na_3VO_4), *p*-chloromercuribenzenesulphonate (pCMBS), NaN_3 , 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), Acridine Orange and Sephadex G-50 (bead size 20–80 μm) were all purchased from Sigma, St. Louis, MO, USA. Polystyrene disposable cuvettes were obtained from Sarstedt, Newton, NC, U.S.A. 5-[1,2- ^3H]Hydroxytryptamine (serotonin) binoxalate (sp. radioactivity 23.4 Ci/mmol) was purchased from DuPont–New England Nuclear, Boston, MA, U.S.A. All chemicals were of analytical grade.

Preparation of human placental brush-border membrane vesicles

Brush-border membrane vesicles were prepared from human term placentas as previously described [2,17,20] by a Mg^{2+} -aggregation method. Only normal placentas which were obtained within 2 h of delivery were used. All the steps involved in the isolation procedure were carried out at 4 °C. Briefly, the villous tissue was collected after removing decidua and chorionic plate. The tissue was cut into small pieces and washed with an iso-osmotic buffer (10 mM-Hepes/Tris, pH 7.0, containing 300 mM-mannitol) to remove blood. The washed tissue fragments were then agitated in 300 ml of the same buffer for 30 min on a magnetic stirrer. This procedure resulted in the release of brush-border membranes from syncytiotrophoblast. The slurry was then filtered through three layers of cheesecloth and the resulting filtrate was centrifuged at 8000 *g* for 15 min. The supernatant containing brush-border membranes was again centrifuged at 73000 *g* for 30 min. The pellets representing crude brush-border membranes were suspended in 50 ml of buffer by using 10 strokes with a Dounce homogenizer (loose-fitting pestle). A stock solution of 1 M- $MgCl_2$ was added to the homogenate to give a final concentration of 12 mM- $MgCl_2$. The mixture was stirred for 1 min and left for 10 min. It was then centrifuged at 3000 *g* for 15 min to remove Mg^{2+} -aggregated non-brush-border membranes. The supernatant containing brush-border membranes was centrifuged at 73000 *g* for 30 min. The pellets were suspended in 5 mM-Hepes/Tris buffer (pH 7.4) containing 150 mM-KCl and centrifuged again. The resulting pellets were finally suspended in a small volume of the above buffer. Protein concentration of the suspension was adjusted to 10 mg/ml, and it was stored in liquid N_2 in small portions. The purity of the brush-border membranes was occasionally checked by determining the activity of alkaline phosphatase, a brush-border marker enzyme [21]. The enrichment of the activity in membrane preparations (compared with the activity in the original homogenate of the washed placental tissue) normally varied within a range of 20–30-fold, with an average value of 23 ± 3 ($n = 4$).

Detergent treatment and reorientation of the H^+ pump

Treatment of placental brush-border membranes with cholate was performed as previously described [17,22]. On the day of the experiment, the frozen brush-border membrane vesicles were thawed at 37 °C, cooled down again on ice and diluted with 5 mM-Hepes/Tris buffer (pH 7.4) containing 150 mM-KCl to a protein concentration of 3.7 mg/ml. A stock solution of 10% sodium cholate in water was added to the membrane suspension dropwise, with frequent mixing of the detergent–membrane mixture.

The final concentration of the detergent was 1% and that of protein was 3.3 mg/ml. After keeping the mixture on ice for 3 min, it was passed through a Sephadex G-50 column in order to remove the detergent. The column was equilibrated with 5 mM-Hepes/Tris buffer (pH 7.4) containing 150 mM-KCl before application of the sample. For a 5 mg protein sample, the size of the column was normally 1 cm $\times$ 15 cm. The turbid fractions containing the re-formed vesicles were collected and used for measurement of the H^+ -pump activity. The protein concentration in these fractions was always within the range of 1.5–2.5 mg/ml.

Determination of ATP-driven H^+ pump

The ATP-driven H^+ pump was assayed by measuring the uptake of H^+ into the vesicles upon addition of ATP to the external medium. H^+ uptake was determined by monitoring intravesicular acidification resulting from H^+ influx, and this was done by measuring the absorbance decrease of the ΔpH indicator Acridine Orange as described by Sabolic *et al.* [23]. Briefly, cholate-treated membrane vesicles (150–250 μg of protein) were added to a cuvette containing 1 ml of 50 mM-Hepes/Tris, 150 mM-KCl, 5 mM- $MgCl_2$ and 6 μM Acridine Orange, pH 7.4. When the absorbance of the dye as measured by dual-wavelength spectrophotometry (492–540 nm) with an Aminco DW-2C dual-wavelength spectrophotometer had stabilized, 20 μl of a 100 mM-ATP solution, whose pH had been adjusted to 7.4 with Tris, was added. This resulted in a final ATP concentration of 2 mM in the cuvette. After addition of ATP, the decrease in absorbance was recorded. When inhibitors were used, controls received the same amount of solvent (water for vanadate, NaN_3 and pCMBS, or ethanol for valinomycin, DCCD, NEM, NBD-Cl and oligomycin). Initial rates of the H^+ -pump activity were calculated from the slopes of plots of the absorbance decrease of Acridine Orange against time.

Measurement of 5-hydroxytryptamine uptake

Uptake of 5-hydroxytryptamine into membrane vesicles was measured by a rapid-filtration technique described previously [24]. Millipore filters (DAWP type; 0.65 μm pore size) were used in these experiments. Uptake was measured in the presence of an inwardly directed transmembrane NaCl gradient (initial zero-trans conditions).

Statistics

The activity of the H^+ pump was determined in duplicate or triplicate in each membrane preparation. Each experiment was repeated with 2–3 different membrane preparations and the results are given as means $\pm$ S.E.M. The H^+ -pump activity, expressed as $\Delta A/\text{min}$ per mg, varied between different membrane preparations. The range was 0.0138–0.0695, with an average value of 0.0300 ± 0.0026 (8 membrane preparations). Owing to the variability in the activities of the H^+ pump in different membrane preparations, the results of certain experiments are given in terms of percentage activity rather than absolute activity. When original tracings are given, each is a representative of the experiment done in replicates. 5-Hydroxytryptamine uptake measurements were made in triplicate with two different membrane preparations. The variability among the triplicate values was less than $\pm 5\%$ of the mean value. Statistical analysis was done by Student's *t* test, and $P < 0.05$ was considered significant.

RESULTS

Reorientation of the ATP-driven H^+ pump

The ATP-driven H^+ pump in human placental brush-border membrane vesicles was measured by monitoring ATP-dependent

Fig. 1. ATP-driven H^+ uptake in native and cholate-pretreated placental brush-border membrane vesicles

Native membrane vesicles (intact, 250 μ g of protein) or cholate-pretreated membrane vesicles (150 μ g of protein) were suspended in 1 ml of 50 mM-Hepes/Tris buffer (pH 7.4) containing 150 mM-KCl, 5 mM-MgCl₂, 6 μ M Acridine Orange as Δ pH indicator, and no (-val) or 10 μ M-valinomycin (+val) to clamp the membrane potential difference. Measurement of H^+ uptake was initiated by the addition of ATP (final concn. 2 mM). When indicated, nigericin (10 μ M) was added to dissipate the ATP-generated transmembrane pH gradient. The curves redrawn from the original show the time-dependent decrease in the Acridine Orange absorbance and are from a representative experiment.

intravesicular acidification (i.e. H^+ influx) with Acridine Orange, an indicator of transmembrane pH gradient. The absorbance of Acridine Orange measured in a dual-wavelength spectrophotometer as $A_{492-540}$ decreases when an inside-acidic pH gradient develops and increases when an inside-alkaline pH gradient develops across the vesicular membrane. As shown in Fig. 1, addition of ATP to intact placental brush-border membrane vesicles failed to cause any significant change in the absorbance of Acridine Orange. It is known that the brush-border membrane vesicles prepared from human placentas exist predominantly (> 90%) in the form of right-side-out vesicles [25]. Therefore, if these membranes contain an ATP-driven H^+ pump, the ATP-binding site, which is located on the cytoplasmic side of the pump, will be on the inner side of the vesicular membrane and thus will be inaccessible to the externally added ATP in the native right-side-out vesicles. However, when these membrane vesicles were transiently exposed to cholate, with subsequent removal of the detergent to re-form the vesicles, ATP-dependent intravesicular acidification could be easily demonstrated. The absorbance of Acridine Orange decreased with time in response to the externally added ATP in cholate-pretreated membrane vesicles. This change in the absorbance was not due to a better access of ATP to the internal ATP-binding site of the H^+ pump in cholate-treated vesicles than in control vesicles, because if this were to be the case, H^+ will be pumped out of the intravesicular medium, which will generate an inside-alkaline pH gradient across the membrane and thus result in an increase, and not a decrease, in the Acridine Orange absorbance. The conclusion that addition of ATP did develop an inside-acidic pH gradient across the membrane in these vesicles is supported by the observation that nigericin, which effectively collapses transmembrane pH gradients in the presence of K^+ , caused an immediate increase in the absorbance. Apparently, transient exposure of the native membrane vesicles to cholate reorients the ATP-driven H^+ pump from the inside of the membrane to the outside. This reorientation renders the ATP-binding site accessible to the externally added ATP, and the pump catalyses the ATP-driven H^+ transport from outside to inside of these cholate-pretreated vesicles.

Fig. 2. Calibration of the Acridine Orange signal in cholate-pretreated brush-border membrane vesicles

Cholate-pretreated vesicles (70 μ g of protein/assay) were loaded with 100 mM-KCl/5 mM-Hepes/Tris, pH 7.0, and suspended in 1 ml of buffer containing 2.5, 5, 7, 10 or 20 mM-KCl, 5 mM-Hepes/Tris, pH 7.0, and 6 μ M Acridine Orange. The total Cl^- concentration was kept at 100 mM by addition of appropriate amounts of tetramethylammonium chloride. At zero time, 10 μ M-nigericin was added. The Δ pH indicated at the curves in the left panel was calculated by assuming that $[K^+]_i/[K^+]_o = [H^+]_i/[H^+]_o$ through K^+/H^+ exchange via nigericin. The right panel is a replot of maximal absorbance change as a function of calculated Δ pH.

Fig. 2 describes the relationship between the Acridine Orange absorbance change and the transmembrane pH gradient. In this experiment, the changes in the Acridine Orange signal were measured in cholate-pretreated brush-border membrane vesicles in response to experimentally induced transmembrane pH gradients of varying magnitude. The pH gradients were generated by using nigericin in the presence of outwardly directed K^+ gradients of different magnitude. Nigericin catalyses K^+/H^+ exchange, and therefore the Δ pH across the membrane at different K^+ gradients can be calculated, assuming that $[K^+]_i/[K^+]_o$ equals $[H^+]_i/[H^+]_o$ in the presence of nigericin. The results show that the change in the Acridine Orange absorbance (ΔA) increased when the magnitude of the transmembrane pH gradient increased. The relationship between the two parameters was, however, not linear. Nonetheless, the experiment demonstrates that the changes in the Acridine Orange absorbance in response to the addition of ATPMg²⁺ in cholate-pretreated membrane vesicles were indeed due to the generation of an inside-acidic transmembrane pH gradient by the activity of the H^+ pump.

In order to demonstrate the ATP-dependent intravesicular acidification in cholate-treated membrane vesicles by a procedure other than the measurement of Acridine Orange absorbance, we studied the effects of ATPMg²⁺ on 5-hydroxytryptamine uptake in these vesicles. Uptake of 5-hydroxytryptamine in human placental brush-border membrane vesicles occurs via a NaCl-gradient-dependent transport mechanism, and this uptake is stimulated by an outwardly directed H^+ gradient ($pH_i < pH_o$) [24]. The results of the experiment, given in Table 1, show that preincubation of the cholate-pretreated membrane vesicles with ATPMg²⁺ stimulated subsequent uptake of 5-hydroxytryptamine.

Table 1. ATPMg²⁺-dependent stimulation of 5-hydroxytryptamine uptake

Cholate-pretreated brush-border membrane vesicles were preloaded in 1 mM-Hepes/Tris buffer (pH 7.5) containing 350 mM-mannitol. These vesicles were preincubated for 5 min at room temperature (22 °C) with preincubation buffer (25 mM-Hepes/Tris/300 mM-mannitol, pH 7.5) with or without Mg²⁺, ATP and ATP plus Mg²⁺. Uptake of 5-hydroxytryptamine was initiated by addition of uptake buffer (25 mM-Hepes/Tris/150 mM-NaCl, pH 7.5) containing radio-labelled 5-hydroxytryptamine. Uptake was measured for 15 s. When present during preincubation, the concentrations of Mg²⁺, ATP and FCCP were 5 mM, 5 mM and 20 μ M respectively. The concentration of 5-hydroxytryptamine during uptake measurement was 50 nM. The data represent means $\pm$ S.E.M. from six determinations done with two membrane preparations.

Addition	5-Hydroxytryptamine uptake	
	(pmol/15 s per mg of protein)	(%)
None	17.2 $\pm$ 0.1	100
Mg ²⁺	16.7 $\pm$ 0.2	97
ATP	14.4 $\pm$ 0.1	84 (100)
ATPMg ²⁺	23.0 $\pm$ 0.4	134 (160)
ATPMg ²⁺ plus FCCP	17.8 $\pm$ 0.3	105 (124)

Fig. 3. Effects of anions and valinomycin on ATP-driven H⁺ uptake in cholate-pretreated membrane vesicles

Cholate pretreatment and the subsequent detergent removal on a Sephadex G-50 column were done in the presence of 5 mM-Hepes/Tris buffer (pH 7.4), containing 150 mM-potassium gluconate, resulting in the preloading of the cholate-treated vesicles with the above buffer. These vesicles (150 μ g of protein) were suspended in 1 ml of 50 mM-Hepes/Tris buffer (pH 7.4), containing 125 mM-potassium gluconate, 5 mM-MgSO₄, 6 μ M Acridine Orange and 25 mM of the indicated anions as potassium salts. H⁺ uptake was initiated by addition of 2 mM-ATP. The experiment was done both in the absence (curves a) and in the presence (curves b) of 10 μ M-valinomycin. The curves, redrawn from the original, show the time-dependent decrease in the Acridine Orange absorbance and are from a representative experiment.

This stimulation was significantly decreased when the preincubation was done in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine (FCCP), a protonophore. These data suggest that preincubation of the vesicles with ATPMg²⁺ (but not with Mg²⁺ or ATP alone) causes intravesicular acidification.

Taken collectively, these results demonstrate that the human placental brush-border membrane vesicles possess an ATP-driven H⁺ pump and that the activity of the pump can be measured by monitoring ATP-dependent intravesicular acidification after a transient exposure of the membrane vesicles to cholate. Subsequent experiments on the characterization of the placental brush-border membrane H⁺ pump were therefore performed with cholate-pretreated membrane vesicles.

Electrogenic nature of the reoriented H⁺ pump

Fig. 1 also describes an experiment in which the effect of valinomycin on the activity of the reoriented H⁺ pump was studied. The rate of decrease in the absorbance of Acridine Orange upon addition of ATP significantly increased in the presence of valinomycin. K⁺ was present both inside and outside of the vesicles during this experiment, and hence addition of valinomycin is expected to short-circuit the membrane under the experimental conditions. Since abolishing the transmembrane potential difference by this manoeuvre increased the ATP-driven intravesicular acidification, the data can be taken as supporting evidence for the electrogenic nature of the H⁺ pump.

We have also investigated the electrogenicity of the H⁺ pump with additional experiments in which the influence of various anions with different membrane permeabilities on the activity of the reoriented H⁺ pump was studied (Fig. 3). In the absence of valinomycin (curves a), the initial rate and magnitude of the decrease in the absorbance depended on the anion in the medium. The activity of the H⁺ pump as measured by the absorbance changes was smallest for sulphate and gluconate, comparatively larger in the presence of nitrate and chloride, and largest in the presence of iodide and thiocyanate. It is evident that increasing permeability of anions increased the rate and magnitude of ATP-driven H⁺ influx, indicating that the reoriented pump is electrogenic and requires movement of anions into the vesicles for charge compensation. Addition of valinomycin increased the activity of the pump in the presence of all of the above-mentioned anions (curves b). Since valinomycin, a K⁺ ionophore, brings about charge compensation very effectively by increasing the movement of K⁺ across the membrane, the activity of the pump was comparable in the presence of all of these anions. As expected, a comparison of the activities in the presence and absence of valinomycin reveals that the ionophore-induced stimulation of the ATP-driven H⁺ uptake was greatest in the presence of weakly permeant anions such as gluconate and sulphate, whereas highly permeant anions such as thiocyanate were capable of charge compensation even without valinomycin. Accordingly, addition of valinomycin had the smallest effect on the H⁺ uptake in the presence of these anions.

Nucleotide specificity

We investigated the nucleotide specificity of the reoriented H⁺ pump by measuring the ability of various nucleotides to stimulate H⁺ uptake in cholate-pretreated membrane vesicles. At a concentration of 2 mM, the activity of the H⁺ pump was the highest in the presence of ATP (results not shown). GTP and ITP were able to support H⁺ uptake to some extent (26% and 20% respectively, compared with ATP). On the contrary, UTP and CTP were totally inactive.

Bivalent-cation specificity

We then investigated the bivalent-cation specificity of the reoriented H⁺ pump by studying the influence of various bivalent cations on the ATP-dependent H⁺ uptake in cholate-pretreated membrane vesicles. The activity of the H⁺ pump was maximal in the presence of Mg²⁺ (results not shown). At equal concentration

Fig. 4. Influence of ATP concentration on the initial rate of H⁺ uptake in cholate-pretreated membrane vesicles

Cholate-pretreated membrane vesicles (195–225 μg of protein) were suspended in 1 ml of 50 mM-Hepes/Tris buffer (pH 7.4), containing 150 mM-KCl, 5 mM-MgCl₂, 10 μM valinomycin and 6 μM Acridine Orange. H⁺ uptake was initiated by addition of various concentrations of ATP (range 0.077–1 mM). The initial rates of H⁺ uptake were calculated from slopes of the original curves. The data represent means ± S.E.M. from four determinations done with two membrane preparations. Inset shows Lineweaver-Burk plot of the same data.

Fig. 5. Inhibition of ATP-driven H⁺ uptake by NEM in cholate-pretreated membrane vesicles

Cholate-pretreated membrane vesicles (100–250 μg of protein) were incubated for 15 min at room temperature (22 °C) with various concentrations of NEM (range 0.5–7.5 μM) in 50 mM-Hepes/Tris buffer (pH 7.4), containing 150 mM-KCl, 5 mM-MgCl₂, 10 μM-valinomycin and 6 μM Acridine Orange, before initiation of H⁺ uptake by addition of 2 mM-ATP. Initial rates of H⁺ uptake were calculated from slopes of the original curves. For each membrane preparation, the rates of H⁺ uptake in the presence of NEM were determined as percentages of control uptake rate in the absence of NEM. The data represent means ± S.E.M. from four determinations done with two membrane preparations.

Table 2. Sensitivity of the reoriented H⁺ pump to inhibitors

Cholate-pretreated brush-border membrane vesicles (230 μg/assay) were suspended in 1 ml of 50 mM-Hepes/Tris buffer (pH 7.4), containing 150 mM-KCl, 5 mM-MgCl₂, 10 μM-valinomycin and 6 μM Acridine Orange. Inhibitors were added either 30 min (DCCD) or 15 min (NEM, NBD-Cl, pCMBS) before initiation of the assay. Vanadate, NaN₃ and oligomycin were added shortly before the initiation of the assay. The assay was started by addition of ATP (final concn. 2 mM). The data represent means ± S.E.M. from four determinations done with two membrane preparations.

Inhibitor	Concn.	H ⁺ uptake	
		(ΔA/min per mg)	(%)
None		0.0391 ± 0.0011	100
Vanadate	1 mM	0.0326 ± 0.0020	83
NaN ₃	2 mM	0.0329 ± 0.0012	84
Oligomycin	10 μg/ml	0.0331 ± 0.0017	85
NEM	10 μM	0	0
NBD-Cl	10 μM	0	0
pCMBS	10 μM	0	0
DCCD	20 μM	0.0242 ± 0.0013	62
	200 μM	0	0

(5 mM), Mn²⁺ supported 91% of the activity observed with Mg²⁺. Co²⁺ and Zn²⁺ showed much lesser activity (34% and 10% respectively, compared with Mg²⁺), and Ca²⁺ and Ba²⁺ showed little or no activity.

Influence of ATP concentration on the activity of the reoriented H⁺ pump

The effect of ATP concentration on the activity of the H⁺ pump was studied in cholate-pretreated membrane vesicles. The pump activity was measured by determining the initial rates of decrease in the absorbance of Acridine Orange in the presence of

increasing concentration of ATP (range 0.077–1 mM). As shown in Fig. 4, the activity was a hyperbolic function of ATP concentration. The Lineweaver-Burk plot (1/[ATP] versus 1/activity) was linear (*r* = 0.986), indicating a single saturable mechanism, and the apparent dissociation constant (*K_m*) for ATP was 0.26 mM (Fig. 4, inset). However, it has to be mentioned here that this *K_m* value is only an approximate estimate, because the kinetic analysis was done not by measuring the H⁺-pump activity directly (i.e. H⁺ transport), but by simply measuring the change in the Acridine Orange absorbance, a parameter not linearly related to the magnitude of the ΔpH across the membrane.

Sensitivity of the reoriented H⁺ pump to various inhibitors

There are three major families of H⁺-translocating ATPases, namely P-ATPases (E₁–E₂ type), F-ATPases (F₀–F₁ type) and V-ATPases (vacuolar type) (for a review, see [26]). These three types of H⁺-ATPases exhibit different sensitivities to various inhibitors, and this offers an efficient probe to distinguish them from each other. In order to find out which class of these H⁺-translocating ATPases the human placental brush-border membrane H⁺ pump belongs to, we investigated the effect of a number of inhibitors on ATP-driven H⁺ uptake in cholate-pretreated membrane vesicles. The results of these experiments are given in Table 2. NaN₃ and oligomycin at concentrations greater than those needed for complete inhibition of F-ATPases decreased H⁺ uptake only by about 15%. Vanadate, an effective inhibitor of P-ATPases, including K⁺/H⁺-ATPase, caused only a slight inhibition (17%) of H⁺ uptake in these vesicles. DCCD is an inhibitor of all three types of H⁺-ATPases, but the potency, which varies for each type, is in the following order: F-ATPases > V-ATPases > P-ATPases. It was found in the present study that DCCD is a potent inhibitor of H⁺ uptake in cholate-pretreated placental brush-border membrane vesicles. NBD-Cl, to which the relative sensitivity of the three types is in the order

Fig. 6. Protection of the ATP-driven H^+ pump from NEM-induced inhibition by ATP

Cholate-pretreated membrane vesicles (800 μ g of protein) were incubated for 15 min at room temperature (22 °C) with (curves b and d) or without (curves a and c) 40 μ M-NEM in the presence (curves c and d) or absence (curves a and b) of 5 mM-ATP. The incubation buffer was 5 mM-Hepes/Tris (pH 7.4), containing 150 mM-KCl and 5 mM $MgCl_2$. After the incubation, the vesicles were passed through a Sephadex G-50 column to remove ATP and NEM that had not reacted and then used for H^+ -uptake measurements. The vesicles (80 μ g of protein) were suspended in 1 ml of 50 mM-Hepes/Tris buffer (pH 7.4) containing 150 mM-KCl, 5 mM- $MgCl_2$, 10 μ M-valinomycin and 6 μ M Acridine Orange, and H^+ uptake was initiated by addition of 2 mM-ATP. The curves, redrawn from the original, show the time-dependent decrease in the Acridine Orange absorbance and are from a representative experiment. The values given in parentheses for curves (b) and (d) represent H^+ -uptake activity as a percentage of their corresponding control activity (curves a and c respectively).

of V-ATPases > F-ATPases > P-ATPases, also inhibited the placental H^+ pump very effectively. The thiol-group reagents NEM and pCMBS are considered as specific inhibitors of V-ATPases at low concentrations. In the present study, these two reagents completely blocked H^+ uptake at a concentration of 10 μ M. These inhibition studies suggest that the H^+ pump of the cholate-pretreated placental brush-border membrane vesicles belongs to the category of V-ATPases.

Since NEM is one of the specific inhibitors of V-ATPases [26], we investigated the effect of NEM on the activity of the placental H^+ pump. Fig. 5 describes the dose-dependent inhibition of the ATP-driven H^+ uptake by NEM in cholate-pretreated membrane vesicles. The IC_{50} value for this inhibition was found to be approx. 2 μ M.

Protection of the reoriented H^+ pump by ATP from NEM-induced inhibition

The V-type H^+ -ATPases from several sources have been shown to be protected by ATP from the inhibition caused by NEM, an alkylating agent for thiol groups [26]. This suggests that the NEM-reactive thiol groups are located at or near the ATP-binding site of the V-type H^+ -ATPases. Since our studies on the sensitivity of the placental brush-border membrane H^+ pump to various inhibitors including NEM indicated that this H^+ pump belongs to the class of V-type H^+ -ATPases, we investigated the ability of ATP to protect the pump from the NEM-induced inhibition. In order to provide proper controls, cholate-pretreated membrane vesicles were incubated in four ways: (a) in the absence of ATP and NEM, (b) in the presence of NEM alone, (c) in the presence of ATP alone, and (d) in the presence of ATP plus

Fig. 7. Inhibition of ATP-driven H^+ uptake by bafilomycin A_1

Cholate-pretreated vesicles (200 μ g of protein/assay) loaded with 150 mM-KCl/5 mM-Hepes/Tris, pH 7.0, were added to cuvettes containing 1 ml of 150 mM-KCl/5 mM- $MgCl_2$ /50 mM-Hepes/Tris, pH 7.0, plus 6 μ M Acridine Orange, 10 μ M-valinomycin and bafilomycin A_1 at concentrations indicated. The measurement was started by addition of 2 mM-ATP. Curves shown are redrawn traces from a representative experiment.

NEM. After incubation, the vesicles were passed through a Sephadex G-50 column to remove ATP and NEM that had not reacted, and then used for measurement of ATP-driven H^+ uptake (Fig. 6). In the absence of ATP, NEM caused complete inhibition of the H^+ pump (curve b versus curve a). However, when the vesicles were incubated with NEM in the presence of ATP, the pump was almost totally protected from this inhibition. The activity of the H^+ pump in vesicles incubated with ATP plus NEM (curve d) was about 80% of that in the corresponding control vesicles, which were incubated with ATP alone (curve c). These results strongly suggest that, similar to the V-type H^+ -ATPases from other sources, the H^+ pump of the placental brush-border membrane also possesses NEM-reactive thiol group(s) at or near its ATP-binding site.

Inhibition of the placental H^+ pump by bafilomycin A_1

Bowman *et al.* [27] reported that bafilomycin A_1 , a macrolide antibiotic, is a specific and potent inhibitor of vacuolar type H^+ -ATPases. Therefore we investigated the sensitivity of the human placental H^+ pump to this inhibitor (Fig. 7). It was found that bafilomycin A_1 inhibited the H^+ -pump activity at nanomolar concentrations.

Effects of bafilomycin A_1 on ATPase activity in placental brush-border membrane vesicles

Because H^+ transport into the vesicles in response to the externally added ATP could be demonstrated only in cholate-pretreated membrane vesicles, but not in native membrane vesicles, it appeared that cholate treatment resulted in the reorientation of the H^+ pump, exposing the ATP-binding site of the pump to the exterior. If this were the case, it should be possible to demonstrate bafilomycin-sensitive ATPase (i.e. H^+ -ATPase) activity in cholate-pretreated membrane vesicles, but

not in native vesicles. Therefore we determined ATPase activity by the liberation of phosphate in native and cholate-pretreated membrane vesicles in the presence and absence of bafilomycin A_1 . In native membrane vesicles, the ATPase activity in the absence of bafilomycin was $0.359 \pm 0.057 \mu\text{mol/min per mg}$, which was not different from the activity measured in the presence of bafilomycin ($0.368 \pm 0.010 \mu\text{mol/min per mg}$). Thus bafilomycin-sensitive ATPase activity was not detectable in native membrane vesicles. In contrast, the ATPase activity in cholate-pretreated membrane vesicles was $0.189 \pm 0.003 \mu\text{mol/min per mg}$ in the absence of bafilomycin. This activity was significantly decreased when the assay was done in the presence of bafilomycin ($0.168 \pm 0.014 \mu\text{mol/min per mg}$; $P < 0.05$). However, the bafilomycin-sensitive ATPase activity was very small in these membrane vesicles and represented only 6% of the activity reported in pig renal brush-border membrane vesicles [22] (in the latter case, the H^+ -ATPase activity was measured as the NEM-sensitive ATPase after cholate treatment). Nonetheless, the appearance of a measurable bafilomycin-sensitive ATPase activity in placental brush-border membrane vesicles after cholate treatment suggests that the exposure of the vesicles to the detergent reoriented the H^+ pump. The ATPase activity measured in the absence of bafilomycin in cholate-treated vesicles was less than the ATPase activity measured under similar conditions in control vesicles. This was most likely due to inactivation of the ecto-ATPases, which are unrelated to the H^+ pump, by the detergent.

DISCUSSION

The present paper describes the general characteristics of the H^+ pump in brush-border membrane vesicles isolated from human term placentas. Since these vesicles are oriented predominantly right-side-out, with the ATP-splitting site of the H^+ pump facing the vesicle interior, H^+ uptake into the vesicles in response to the externally added ATP, as detected by the decrease in absorbance of Acridine Orange, could not be demonstrated in native vesicles. However, when these vesicles were transiently exposed to cholate, the vesicles which were reformed upon removal of the detergent were found to be capable of transporting H^+ from outside to inside when ATP was added to the external medium. Apparently, cholate treatment resulted in the reorientation of a part or all of the H^+ pumps with their ATP-binding site now facing the external side of the vesicles. This conclusion is supported by the observation that the H^+ -ATPase activity which is inhibitable by bafilomycin A_1 is measurable only in cholate-pretreated, but not in native, membrane vesicles. The reorientation of the H^+ pump by this manoeuvre thus offers an easy and convenient way to characterize the pump. A similar technique has been used to investigate the properties of the H^+ pump present in renal brush-border membrane vesicles [22].

The characteristics of the human placental H^+ pump as determined with cholate-pretreated membrane vesicles can be summarized as follows. The ATP-driven H^+ pump is electrogenic, resulting in the transfer of positive charge, and is inhibitable by nanomolar concentrations of bafilomycin A_1 . Because of its electrogenic nature, the activity of the pump is stimulated by voltage-clamping of the vesicles or by addition of permeant anions such as Cl^- , I^- or SCN^- to the assay medium, whose movement across the membrane compensates for the pump-mediated transfer of positive charge. The pump requires ATP for its activity, indicating coupling of ATP hydrolysis to H^+ transport. The apparent dissociation constant for ATP is $260 \mu\text{M}$. GTP and ITP support the pump activity to some extent, but CTP and UTP are totally inactive. Studies on bivalent-cation

specificity reveal that Mg^{2+} or Mn^{2+} is required for the activity of the H^+ pump. Co^{2+} and Zn^{2+} can substitute to a small but significant extent, whereas Ba^{2+} and Ca^{2+} cannot. Potent inhibitors of the pump include ADP, NEM, NBD-Cl, pCMBS and DCCD. NaN_3 , oligomycin and vanadate have little inhibitory effect on the pump. The inhibition of the pump by NEM is protectable by ATP, suggesting the location of the NEM-reactive thiol group(s) at or near the ATP-binding site of the pump.

There are three types (P, F and V) of H^+ -ATPases (H^+ pumps), which can be distinguished from each other by their sensitivities to various inhibitors (for a review see [26]). The inhibition studies with the human placental H^+ pump strongly indicate that this pump belongs to the class of V-type H^+ -ATPases. This type of H^+ pump has been described in various intracellular compartments such as clathrin-coated vesicles, endosomes, lysosomes, Golgi-derived vesicles and chromaffin granules. Since purified placental brush-border membrane vesicles were employed in the present study, it is unlikely that the H^+ pump detected in these membranes is due to contamination of above-mentioned intracellular organelles. Moreover, if these organelles are present in the brush-border membrane preparation, the ATP-binding site of the H^+ pump of these organelles should be accessible to externally added ATP [28–30]. The fact that the H^+ pump activity was not measurable in our study using native membrane vesicles indicates the absence of intracellular organelles containing vacuolar H^+ pumps, and supports the conclusion that the pump is localized in the brush-border membrane. We therefore consider that the characteristics described in this paper are those of the human placental brush-border membrane H^+ pump, assuming that cholate pretreatment has not altered the characteristics.

Even though the properties of various H^+ pumps belonging to the category of the V-type are generally comparable, some subtle differences do exist among them. For example, the H^+ pumps of Golgi and clathrin-coated vesicles have been shown to be highly specific for ATP [28,29], whereas the H^+ pumps of lysosomes, endosomes as well as various secretory granules are relatively non-specific in their nucleotide requirement [28,30–32]. The present study shows that GTP can support the placental H^+ pump to a considerable extent. Although the H^+ -ATPase of the kidney medulla accepts ITP nearly as effectively as it does ATP as a substrate [12], the ability of ITP to support the placental H^+ -pump activity is only one-fifth of that of ATP. It appears that the placental H^+ pump is not completely specific for ATP. In fact, with regard to the nucleotide specificity of H^+ pumps, a study by Moriyama & Nelson [33] has concluded that all V-type H^+ -ATPases may catalyse GTP (and ITP)-dependent H^+ transport, and that the previous works which described an absolute specificity for ATP for H^+ pumps of Golgi and clathrin-coated vesicles might have overlooked the GTP (and ITP)-dependent activity owing to the particular experimental conditions employed in those studies.

The ATP-driven H^+ pump of the placental brush-border membrane is electrogenic in nature, as indicated by the greater activity of the pump in the presence of permeant anions than of impermeant anions. This characteristic is common to all V-type H^+ pumps. The physiological permeant anion is Cl^- , and its involvement in the activation of ATP-driven H^+ uptake has been studied in great detail (for a review see [26]). This requirement for Cl^- is due to the dissipation of the membrane potential generated by ATP-driven H^+ transport by means of parallel movement of Cl^- . However, this Cl^- movement is not catalysed by the H^+ pump. The Cl^- conductance present in the H^+ -pump-containing membranes is largely responsible for this process [34]. A Cl^- transporter necessary for the maintenance of the H^+ pump

activity in clathrin-coated vesicles has recently been characterized [35]. In renal endosomes, which also contain a H^+ pump, a Cl^- channel has been demonstrated by single-channel recording [36]. There is ample evidence that human placental brush-border membranes contain Cl^- conductance [36–39]. This Cl^- -transport mechanism may be responsible for the maintenance of the H^+ -pump activity *in vivo*.

In summary, the present study provides evidence for the existence of an ATP-driven H^+ pump in brush-border membrane vesicles isolated from human placentas. Under conditions *in vivo*, the direction of the pump-catalysed H^+ transport will be from syncytiotrophoblast to maternal blood. This H^+ pump, together with the Na^+-H^+ exchanger recently characterized in the placental brush-border membrane [2,3], could participate in the elimination of H^+ from the fetus. Metabolically derived CO_2 may enter the syncytiotrophoblast from the fetal circulation via the basal membrane and then be converted into H^+ and HCO_3^- by placental carbonic anhydrase [40]. The generated H^+ may leave the cell via the brush-border membrane catalysed by the H^+ pump as well as by the Na^+-H^+ exchanger. These two H^+ -transporting mechanisms may also play a role in the regulation of intracellular pH of the syncytiotrophoblast, because both these transport systems have been shown to participate in pH regulation in other cell types (for a review see [41]). In addition, the H^+ pump may play an obligatory role in receptor-coupled transport pathways which mediate the transport of many important substances such as immunoglobulins, cholesterol, folate and Fe^{2+} across the placenta.

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