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Human placental syncytiotrophoblast expresses two pharmacologically distinguishable types of Na^+–H^+ exchangers, NHE-1 in the maternal-facing (brush border) membrane and NHE-2 in the fetal-facing (basal) membrane

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We investigated whether highly purified preparations of basal (fetal-facing) membrane isolated from normal term human placentas possess Na^+–H^+ exchanger activity. Uptake of Na^+ into basal membrane vesicles was stimulated many-fold by an outwardly directed H^+ gradient. This H^+-gradient-dependent uptake was inhibitable by amiloride and its analogues. Na^+ uptake in these vesicles did not occur via a Na^+ channel, as it was not influenced by changes in membrane potential and, in addition, was inhibited by benzamil only at high micromolar concentrations. The results indicate that the human placental basal membrane possesses Na^+–H^+ exchanger activity. We then studied whether this exchanger is similar to or distinct from the Na^+–H^+ exchanger described in brush border (maternal-facing) membrane preparations. For this purpose, we compared the pharmacological characteristics of the basal membrane Na^+–H^+ exchanger with those of the brush border membrane Na^+–H^+ exchanger. The basal membrane exchanger was about 20-fold less sensitive to inhibition by amiloride and about 70-fold less sensitive to inhibition by dimethylamiloride than was the brush border membrane exchanger. The exchanger activity in both membrane preparations was inhibitable by clonidine and cimetidine, but the inhibition patterns with these compounds were markedly different between basal and brush border membrane preparations. These data demonstrate that the basal membrane Na^+–H^+ exchanger is distinct from the brush border membrane Na^+–H^+ exchanger. The pharmacological profiles of these exchangers indicate that the human placental brush border membrane possesses the housekeeping or non-epithelial type Na^+–H^+ exchanger (NHE-1), whereas the basal membrane possesses the epithelial or apical type Na^+–H^+ exchanger (NHE-2).

INTRODUCTION

The syncytiotrophoblast of the human placenta is a polarized cell and its plasma membrane consists of two distinct regions, a brush border or microvillus membrane facing the maternal side and a basal membrane facing the fetal side. Methods are available to obtain membrane preparations from normal term human placentas which are highly enriched in either the brush border or the basal region. These membrane preparations have been used to investigate various transport mechanisms associated with these regions. Using this approach, we have previously shown that the brush border membrane possesses Na^+–H^+ exchanger activity [1]. This observation has subsequently been confirmed in other laboratories [2–4]. The Na^+–H^+ exchanger is a transport system which has been described in the plasma membrane of various cell types [5–10]. In recent years, however, evidence has been accumulating to indicate that there are two types of Na^+–H^+ exchangers [11–16]. The housekeeping type (NHE-1) is present in the plasma membrane of non-polarized cells such as fibroblasts, and the epithelial or apical type (NHE-2) is present in the brush border membrane of polarized epithelia such as intestinal and renal tubular cells. Even though non-polarized cells always appear to express only NHE-1, polarized epithelial cells may possess either NHE-2 only or both NHE-1 and NHE-2 [11–14]. These two types of Na^+–H^+ exchangers can be distinguished by their sensitivities to inhibition by amiloride and its analogues [11,12,14–16]. Recently we have demonstrated that the relative sensitivity to inhibition by clonidine and cimetidine can also be useful to differentiate between the two types [15,16]. Based on these pharmacological criteria, we have concluded that the Na^+–H^+ exchanger of the human placental brush border membrane is NHE-1 [15].

The purpose of the present investigation was to determine whether the basal membrane of the human placental syncytiotrophoblast possesses Na^+–H^+ exchanger activity, and, if it does, whether this exchanger is similar to or distinct from the Na^+–H^+ exchanger present in the brush border membrane. The results of the investigation lead to the conclusion that the human placental syncytiotrophoblast expresses both types of Na^+–H^+ exchanger, NHE-1 in the brush border membrane and NHE-2 in the basal membrane.

MATERIALS AND METHODS

Materials

Carrier-free $^{22}NaCl$ (radioactivity 800 mCi/ml), [*phenyl*-^{14}C]-tetraphenylphosphonium (TPP) bromide (specific radioactivity 19.2 mCi/mmol) and 1-[*propyl*-2,3-^3H]dihydroalprenolol (DHA) (specific radioactivity 53.1 Ci/mmol) were purchased from New England Nuclear–DuPont (Boston, MA, U.S.A.). Amiloride,

Abbreviations used: FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; DMA, dimethylamiloride; TMA, tetramethylammonium; TPP, tetraphenylphosphonium; DHA, dihydroalprenolol; NHE, Na^+–H^+ exchanger.

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clonidine and cimetidine were obtained from Sigma (St. Louis, MO, U.S.A.). Carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) was from Aldrich (Milwaukee, WI, U.S.A.). Benzamil and dimethylamiloride (DMA) were purchased from Research Biochemicals (Natick, MA, U.S.A.).

Isolation and characterization of brush border and basal membrane vesicles from normal term human placentas

Brush border membrane vesicles were prepared from normal term human placentas by an Mg^{2+} aggregation method [1,17]. The final membrane preparations were enriched in the brush border marker enzymes alkaline phosphatase and 5′-nucleotidase 20–25-fold compared with the homogenate of the washed placental tissue. (Na+K)-ATPase activity, a marker for basal membranes, was undetectable in these brush border membrane preparations. Basal membrane vesicles were prepared by a method originally developed in one of our laboratories [18] with minor modifications [19–21]. The purity of these preparations was assessed by determining the enrichment of DHA binding in these membranes relative to the tissue homogenate. Membrane preparations employed in the present investigation showed 48-fold enrichment in DHA binding. The cross-contamination with brush border membranes was low, since the enrichment in alkaline phosphatase activity was only 0.6-fold.

Following isolation, the membrane vesicles were preloaded with 300 mM-mannitol, buffered with either 20 mM-Hepes/Tris, pH 7.2, or 25 mM-Mes/Tris, pH 5.5. The protein concentration in the final preparations was adjusted to either 6 mg/ml (brush border membrane) or 8 mg/ml (basal membrane). The membrane vesicles were then frozen in small aliquots in liquid N_2 until use.

Uptake measurements

On the day of the experiment, the membrane vesicles were thawed at 37 °C and homogenized in a motor-driven Potter–Elvehjem homogenizer (10 strokes). During this step the membranes was kept on ice. Following homogenization, the membrane vesicles were kept at room temperature for 30 min and then used in uptake measurements. Sodium uptake measurements were made by a rapid filtration technique [22] using ^{22}Na tracer. Uptake of ^{22}Na was determined at a Na concentration of 0.5 mM either in the presence of a transmembrane pH gradient (pH_i 5.5, pH_o 7.2) or in its absence ($pH_i = pH_o = 7.2$). The uptake buffer was either 18 mM-Hepes/Tris, pH 7.5, or 20 mM-Hepes/Tris, pH 7.2, and contained 300 mM-mannitol or 150 mM-tetramethylammonium (TMA) chloride. After incubation for 30 s at room temperature, uptake was terminated by adding 3 ml of ice-cold stop buffer (5 mM-Hepes/Tris, 160 mM-KCl, pH 7.5) and the mixture was filtered. The uptake rate was approximately linear over this incubation period. The filter was washed with 3 × 5 ml of stop buffer and the radioactivity associated with the filter was counted. Sodium uptake which occurred via the Na^+–H^+ exchanger was calculated by subtracting the uptake measured in the presence of 2 mM-DMA from the total uptake measured in the absence of DMA.

Each experiment was done in replicates with two or three different membrane preparations. The results are presented as means ± S.E.M. Statistical significance was calculated by using Student's *t* test, and $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Evidence for the presence of a Na^+–H^+ exchanger in the basal membrane preparations

Uptake of Na in human placental basal membrane vesicle preparations was measured in the presence and absence of a transmembrane pH gradient. In the absence of a pH gradient ($pH_i = pH_o = 7.2$), uptake of Na at a concentration of 0.5 mM was 0.25 ± 0.01 nmol/30 s per mg of protein, of which 52% was DMA-sensitive. The presence of an outwardly directed H^+ gradient (pH_i 5.5, pH_o 7.2) stimulated Na uptake many-fold, most of which was DMA-sensitive. The value for Na uptake under these conditions was 0.94 ± 0.02 nmol/30 s per mg of protein in the absence of DMA and 0.12 ± 0.01 nmol/30 s per mg of protein in the presence of DMA. Thus approx. 90% of Na uptake which occurred in the presence of a pH gradient was DMA-sensitive. When the DMA-sensitive Na uptake alone was considered, it was found that the presence of an outwardly directed H^+ gradient stimulated this uptake by 6-fold.

The stimulation of the DMA-sensitive Na uptake by an outwardly directed H^+ gradient can occur by two mechanisms. First, Na entry into the vesicles may be stimulated by an inside-negative H^+-diffusion potential, and if the entry occurs via a Na^+ channel, the process is expected to be DMA-sensitive. Second, if the membrane preparations possess a Na^+–H^+ exchanger, Na uptake via the exchanger is expected to be stimulated by an outwardly directed H^+ gradient, because the gradient provides the driving force for the uptake. The exchanger-mediated Na uptake is also expected to be DMA-sensitive. In order to differentiate between these two mechanisms, we studied the influence of FCCP on the H^+-gradient-dependent, DMA-sensitive Na uptake (Fig. 1*a*). FCCP, being a H^+ ionophore, should facilitate H^+ diffusion across the membrane down its concentration gradient and enhance the generation of an inside-negative

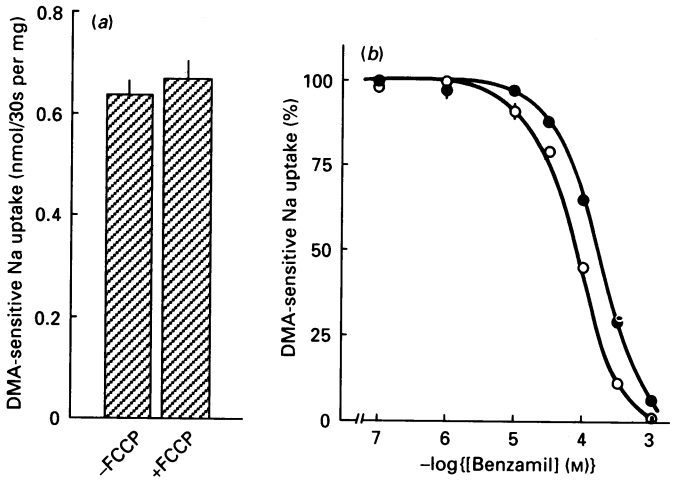

Fig. 1. Effect of inhibitors on H^+-gradient-dependent DMA-sensitive Na uptake

(*a*) Effect of FCCP-induced H^+-diffusion potential on the H^+ gradient-dependent, DMA-sensitive Na uptake. Uptake of Na (0.5 mM) was measured with a 30 s incubation in the presence of a H^+ gradient (pH_i 5.5, pH_o 7.2). In addition to the buffers, the intravesicular and extravesicular media contained 300 mM-mannitol. When present, the concentration of FCCP was 20 μM. Since the stock solution of FCCP was made in ethanol, an equal amount of ethanol alone was present during control uptake. The final concentration of ethanol was 0.66%. DMA-sensitive Na uptake was calculated by subtracting the uptake measured in the presence of 2 mM-DMA from the total uptake measured in the absence of DMA. (*b*) Concentration-dependent inhibition of H^+-gradient-dependent DMA-sensitive Na uptake by benzamil in placental basal (●) and brush border (○) membrane vesicles. Experimental conditions for Na uptake measurements were same as described above. Control uptake was 0.52 ± 0.02 nmol/30 s per mg of protein in basal membrane vesicles and 3.04 ± 0.06 nmol/30 s per mg of protein in brush border membrane vesicles. These values were taken as 100% for respective experiments.

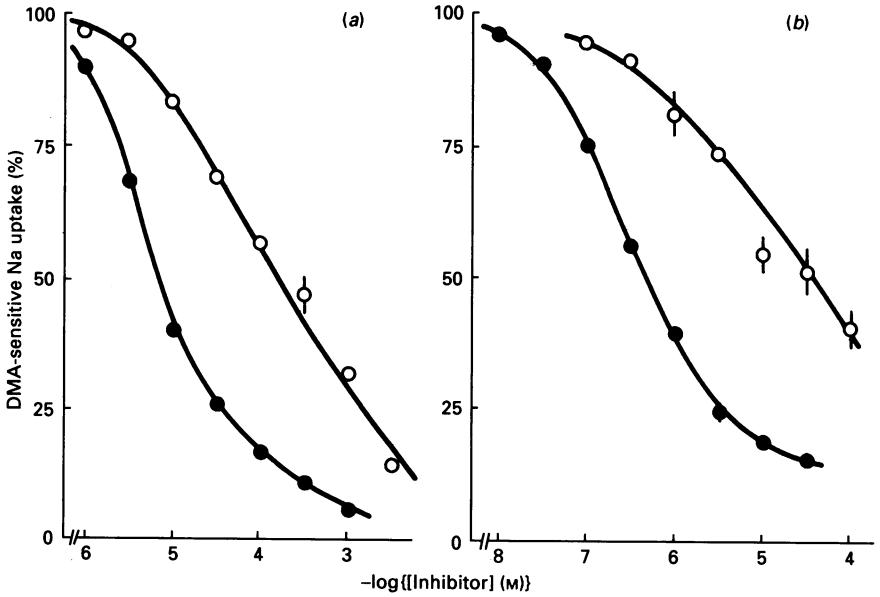

Fig. 2. Concentration-dependent inhibition of Na^+–H^+ exchanger activity by amiloride (*a*) and DMA (*b*) in placental basal (○) and brush border (●) membrane vesicles

The activity of the Na^+–H^+ exchanger was measured as the DMA-sensitive Na uptake, as described in the legend to Fig. 1. Control uptake was 0.57 ± 0.03 nmol/30 s per mg of protein in basal membrane vesicles and 3.77 ± 0.12 nmol/30 s per mg of protein in brush border membrane vesicles. These values were taken as 100% for the respective experiments.

H^+-diffusion potential. If the DMA-sensitive Na entry occurs via a Na^+ channel, it should be stimulated by FCCP. On the other hand, if the Na entry occurs via the electroneutral Na^+–H^+ exchanger [23], FCCP should not have an effect on the uptake. The results of the experiment showed that FCCP did not influence the DMA-sensitive Na uptake measured in the presence of an outwardly directed H^+ gradient. That FCCP did indeed produce an inside-negative membrane potential under these conditions was evident because the uptake of TPP^+ (a lipophilic cation whose uptake in influenced by membrane potentials) was significantly stimulated by FCCP under identical conditions ($39 \pm 4\%$; $P < 0.001$). These results suggest that Na uptake in these preparations was not mediated by a potential-sensitive uptake mechanism.

The participation of a Na^+ channel in the H^+-gradient-dependent DMA-sensitive Na uptake can also be investigated by studying the sensitivity of the uptake process to benzamil. This compound is known to inhibit the Na^+ channel at nanomolar concentrations, whereas concentrations which are many orders of magnitude greater than these are required to inhibit the Na^+–H^+ exchanger [24]. Therefore, we studied the concentration-dependent inhibition of the H^+-gradient-dependent DMA-sensitive Na uptake by benzamil in basal membrane preparations (Fig. 1*b*). The Na uptake was found to be inhibited by benzamil, but only at high micromolar concentrations (IC_{50} 180 μM). This suggests that the Na^+ channel was not responsible for the H^+-gradient-stimulated uptake of Na in these preparations. This suggestion is supported by the finding that similar concentrations of benzamil (IC_{50} 80 μM) were required to inhibit the H^+-gradient-dependent Na uptake in placental brush membrane vesicles, which are known to possess Na^+–H^+ exchanger activity.

Thus the characteristics of Na uptake in placental basal membrane preparations, which include stimulation by an outwardly directed H^+ gradient, inhibition by DMA, insensitivity to membrane potential changes and inhibition by relatively high concentrations of benzamil, indicate that the Na uptake in these membrane preparations occurs via a Na^+–H^+ exchanger.

Differentiation between the Na^+–H^+ exchanger in the basal membrane and the Na^+–H^+ exchanger in the brush border membrane

We then investigated whether the Na^+–H^+ exchanger of the placental basal membrane is similar to or distinct from the Na^+–H^+ exchanger of the placental brush border membrane. It has been shown in many laboratories that the two types of Na^+–H^+ exchangers (NHE-1 and NHE-2) which are known to exist in the plasma membrane of mammalian cells can be differentiated by their relative sensitivities to inhibition by amiloride and its analogues. This approach is successful in cultured cells [11,12,16] as well as in isolated membrane vesicles [14,15]. Therefore we compared the concentrations of amiloride and DMA required to inhibit Na^+–H^+ exchanger activity in basal and brush border membrane vesicles (Fig. 2). The IC_{50} values for amiloride to inhibit the brush border and basal membrane Na^+–H^+ exchangers were 7 μM and 140 μM respectively, showing that the brush border membrane Na^+–H^+ exchanger is about 20-fold more sensitive to inhibition by amiloride than the basal membrane Na^+–H^+ exchanger. This difference in susceptibility to inhibition was even greater when DMA was used as the inhibitor. The IC_{50} values for the brush border and basal membrane Na^+–H^+ exchangers were 0.45 μM and 31.6 μM respectively, showing a 70-fold difference between the exchangers. These results demonstrate that the Na^+–H^+ exchanger present in the placental basal membrane is distinct from the Na^+–H^+ exchanger of the placental brush border membrane. From what is known on the relative sensitivities of NHE-1 and NHE-2 to inhibition by amiloride and its analogues in other cell types, it appears that the human placental brush border membrane contains NHE-1 whereas the basal membrane contains NHE-2.

In addition to amiloride and its analogues, two other compounds, clonidine and cimetidine, have been shown to be inhibitors of the Na^+–H^+ exchanger in isolated membrane vesicles [25,26] and in cultured cells [16,27]. We have recently demonstrated that the relative sensitivity to inhibition by clonidine and cimetidine can provide an effective means of differentiation

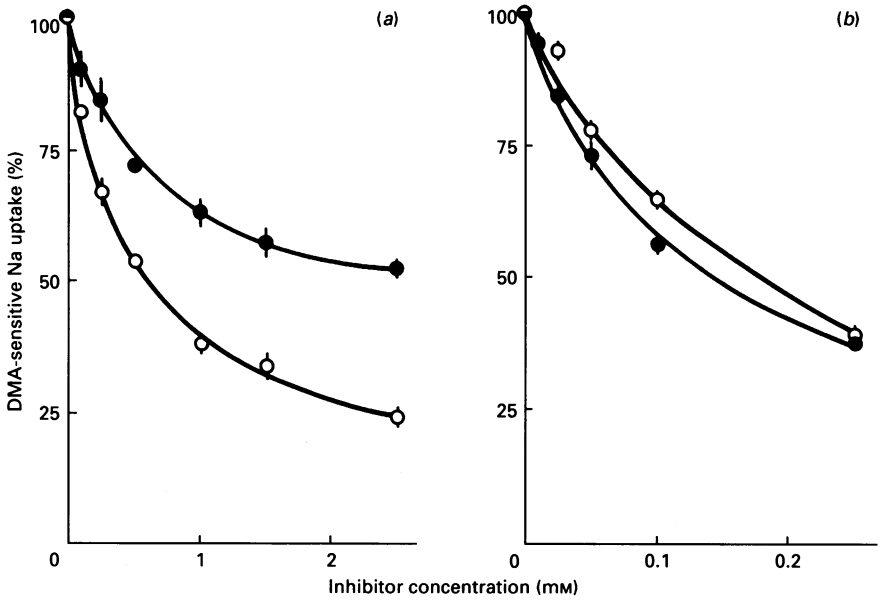

Fig. 3. Concentration-dependent inhibition of Na^+–H^+ exchanger activity by clonidine (○) and cimetidine (●) in placental basal (*a*) and brush border (*b*) membrane vesicles in the absence of Cl^-

The activity of the Na^+–H^+ exchanger was measured as the DMA-sensitive Na uptake, as described in the legend to Fig. 1. Control uptake was 0.63 ± 0.02 nmol/30 s per mg of protein in basal membrane vesicles and 3.84 ± 0.16 nmol/30 s per mg of protein in brush border membrane vesicles. These values were taken as 100% in respective experiments.

between the two types of Na^+–H^+ exchangers in isolated membrane vesicles [15] as well as cultured cells [16]. Therefore, in order to substantiate further that the placental brush border and basal membranes possess distinct types of Na^+–H^+ exchangers, we compared the effects of clonidine and cimetidine on Na^+–H^+ exchanger activity in placental brush border and basal membrane vesicles.

Fig. 3 shows the concentration-dependent inhibition of Na^+–H^+ exchanger activity by clonidine and cimetidine in placental brush border and basal membrane vesicles. In both membrane preparations, the activity was inhibitable by these compounds, but there was a marked difference in inhibitory potency. The basal membrane Na^+–H^+ exchanger was about 4-fold more sensitive to inhibition by clonidine than by cimetidine (IC_{50} for clonidine 0.6 mM; IC_{50} for cimetidine 2.5 mM) (Fig. 3*a*). These influences of clonidine and cimetidine on the basal membrane Na^+–H^+ exchanger were significantly different from their influences on the brush border membrane Na^+–H^+ exchanger (Fig. 3*b*). First, in contrast to the basal membrane exchanger where there is a marked difference in inhibition by clonidine and cimetidine, there was only a slight different in inhibition of the brush border membrane exchanger by these two substances (IC_{50} values for cimetidine and clonidine were 0.15 mM and 0.175 mM respectively). Second, both compounds inhibited the brush border membrane Na^+–H^+ exchanger more potently than the basal membrane Na^+–H^+ exchanger, the difference between the IC_{50} values for the inhibition of these exchangers being about 3.5-fold in the case of clonidine and about 17-fold in the case of cimetidine.

We have previously reported that the placental brush border membrane Na^+–H^+ exchanger is about 4-fold more sensitive to inhibition by cimetidine than by clonidine [15]. In the present study, however, the difference between the potencies of these compounds was less than 2-fold. The experimental conditions employed for the assay of Na^+–H^+ exchanger activity in these two studies were not identical. In the earlier study, we measured exchanger activity in the presence of Cl^-, whereas in the present study Cl^- was completely absent during the assay. It is known that the presence of Cl^- in the assay medium modifies the interaction of amiloride and other inhibitors with the Na^+–H^+ exchanger [28,29]. Since amiloride, clonidine and cimetidine all interact with the same site on the human placental brush border membrane Na^+–H^+ exchanger in a mutually exclusive manner [26,30], it is very likely that Cl^- in the assay medium exerts marked influences on the inhibitory characteristics of clonidine and cimetidine, as it does in the case of amiloride. Therefore we investigated the concentration-dependent inhibition of Na^+–H^+ exchanger activity in placental basal and brush border membrane preparations by clonidine and cimetidine in the presence of Cl^- (Fig. 4). Na^+–H^+ exchanger activity in the placental brush border membrane preparations was inhibitable by clonidine and cimetidine in the presence of Cl^- as it was in the absence of Cl^-, but the relative inhibitory potencies were found to be significantly different. The presence of Cl^- increased the difference between the IC_{50} values of the two inhibitors, cimetidine now being about 3-fold more potent than clonidine in inhibiting the placental brush border membrane exchanger (IC_{50} for cimetidine, 0.1 mM; IC_{50} for clonidine, 0.3 mM) (Fig. 4*b*). These values are comparable with the corresponding values obtained in the earlier study [15]. The presence of Cl^- also changed the inhibition characteristics of clonidine and cimetidine in the case of the Na^+–H^+ exchanger in placental basal membrane preparations (Fig. 4*a*). The difference between the IC_{50} values of clonidine and cimetidine observed in the absence of Cl^- was greatly decreased in the presence of Cl^- (IC_{50} values for clonidine and cimetidine were 0.33 mM and 0.45 mM respectively). However, the difference between the Na^+–H^+ exchangers of the placental basal and brush border membranes with respect to inhibition by clonidine and cimetidine is apparent even in the presence of Cl^-.

Relative activities of Na^+–H^+ exchangers in placental brush border and basal membrane preparations

The averaged results from 14–37 determinations using six different brush border and basal membrane preparations showed

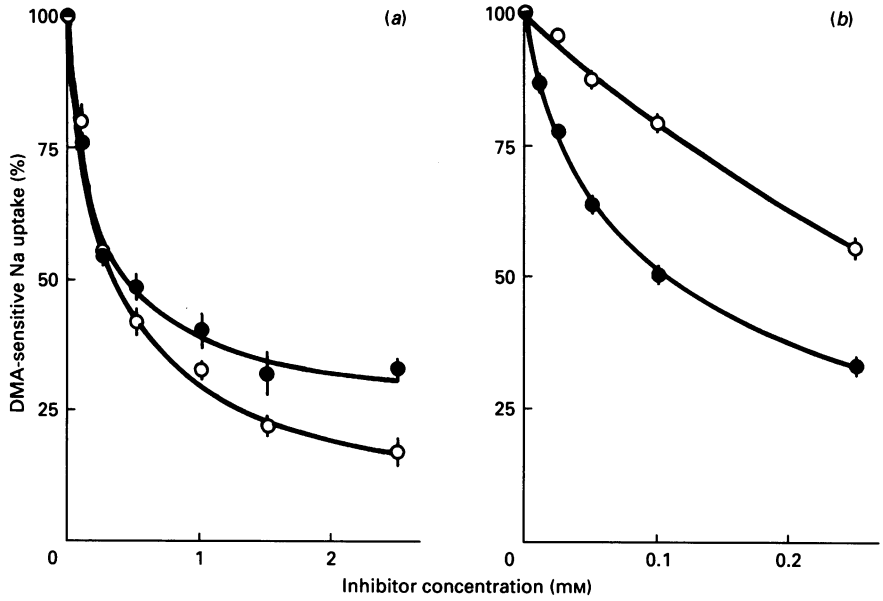

Fig. 4. Concentration-dependent inhibition of Na^+–H^+ exchanger activity by clonidine (○) and cimetidine (●) in placental basal (*a*) and brush border (*b*) membrane vesicles in the presence of Cl^-

The activity of the Na^+–H^+ exchanger was measured as the DMA-sensitive Na uptake as described in the legend to Fig. 1, except that the extravesicular medium contained 120 mM-TMA chloride instead of mannitol. Control uptake was 0.27 ± 0.01 nmol/30 s per mg of protein in basal membrane vesicles and 1.68 ± 0.04 nmol/30 s per mg of protein in brush border membrane vesicles. These values were taken as 100% in respective experiments.

that the activity of the Na^+–H^+ exchanger in the basal membrane was much lower than that in the brush border membrane. When both the extravesicular and the intravesicular media contained only the buffers and mannitol without any salt, the exchanger activity at a Na concentration of 0.5 mM was 3.68 ± 0.08 nmol/30 s per mg of protein in brush border membrane preparations. Under similar conditions, the exchanger activity in basal membrane preparations was only 0.64 ± 0.03 nmol/30 s per mg of protein. When the assay was done in the presence of TMA chloride in the extravesicular medium, it was found that Na^+–H^+ exchanger activity decreased significantly in both membrane preparations, but the ratio of the activities remained the same. Thus the activity of the Na^+–H^+ exchanger in basal membrane preparations is only one-sixth of the activity measured in brush border membrane preparations.

Uniqueness of the placental syncytiotrophoblast with respect to polarization of Na^+–H^+ exchanger activity

Intestinal and renal tubular cells as well as cultured cells of renal origin have been shown to express both NHE-1 and NHE-2 [11–14]. Since these cells are polarized, several investigators have studied the polarity of distribution of the two types of exchangers in these cells [11,12,14]. In all of these studies, only the expression of NHE-2 has been documented in apical membranes of these cells. NHE-1 has always been shown to be localized in the basolateral membrane. Even in the case of an opossum kidney cell line which expresses only NHE-2, it is the apical membrane that possesses the exchanger activity. Even though the human placental syncytiotrophoblast is a polarized cell and expresses both types of Na^+–H^+ exchangers as do the intestinal and renal tubular cells, the polarization of the exchanger activities in this cell is unique. Unlike the intestinal and renal tubular cells, which express NHE-1 in the basolateral membrane and NHE-2 in the apical membrane, the syncytiotrophoblast expresses NHE-1 in the apical membrane and NHE-2 in the basal membrane. The human placental syncytiotrophoblast and/or a cultured cell line of human placental origin which exhibits a similar polarization of the Na^+–H^+ exchanger activities will prove to be very valuable in future work aimed at determining the cellular mechanisms involved in selectively directing a particular type of Na^+–H^+ exchanger to either the apical membrane or the basal membrane.

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