Relative sensitivity to inhibition by cimetidine and clonidine differentiates between the two types of Na⁺–H⁺ exchangers in cultured cells

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Available evidence indicates that there are two types of Na⁺–H⁺ exchangers, type A (housekeeping type) and type B (epithelial or apical type), in mammalian cells. We have recently reported, using isolated membrane vesicles, that these two types can be differentiated by their relative sensitivities to inhibition by clonidine and cimetidine [Kulanthavel, Leibach, Mahesh, Cragoe & Ganapathy (1990) *J. Biol. Chem.* **264**, 1249–1252]. The present study was undertaken to determine whether this approach is also effective in cultured cells. The JAR human placental choriocarcinoma cell line and the opossum kidney (OK) cell line, when grown as confluent monolayer cultures on an impermeable plastic support, express Na⁺–H⁺ exchanger activity which is measurable by determining Na⁺ uptake into the cells from the culture medium. The JAR cell Na⁺–H⁺ exchanger was found to be about 100 times more sensitive to inhibition by dimethylamiloride than the OK cell Na⁺–H⁺ exchanger. Inhibition studies with clonidine and cimetidine were able to differentiate between these two exchangers very clearly. Cimetidine was 18 times more potent than clonidine in inhibiting the JAR cell Na⁺–H⁺ exchanger. In contrast, clonidine was at least 8 times more potent than cimetidine in inhibiting the Na⁺–H⁺ exchanger of the OK cell. The results show that the JAR cell expresses the type A Na⁺–H⁺ exchanger, whereas the OK cell expresses the type B Na⁺–H⁺ exchanger. This approach also proved to be very effective in correctly identifying the type of Na⁺–H⁺ exchanger in a third cell line (HeLa). It is concluded that the relative susceptibility to inhibition by clonidine and cimetidine offers an easy and efficient means of differentiating between the two types of Na⁺–H⁺ exchangers in cultured cells.

INTRODUCTION

A transport mechanism catalysing the transmembrane exchange of Na⁺ and H⁺ was first described in the brush border membrane of intestinal and renal tubular cells [1]. Since then, the presence of the Na⁺–H⁺ exchanger has been demonstrated in the plasma membrane of almost every cell type. This ubiquitous transport system participates in many important cellular functions, such as regulation of intracellular pH, transcellular movement of acid and base equivalents, cell growth and proliferation, and regulation of cell volume [2–7]. The activity of this exchanger is under regulation by various hormones, growth factors, neurotransmitters, chemotactic peptides, and several other biologically active agents [6,7].

It has become increasingly evident in the last couple of years that there are in fact two types of Na⁺–H⁺ exchangers. These are generally referred to as the epithelial (brush border, apical) type and the housekeeping type [8,9]. The Na⁺–H⁺ exchanger present in the brush border membrane of polarized epithelia such as the intestinal and renal tubular cells is classified as the epithelial type, whereas the Na⁺–H⁺ exchanger present in the plasma membrane of non-polarized cells such as fibroblasts is classified as the housekeeping type. This nomenclature is simple and convenient, but suffers from some inconsistencies, as evident from recent investigations. Epithelial cells do not always exclusively express the epithelial type Na⁺–H⁺ exchanger. There are instances where they possess the housekeeping type only, or both the epithelial type and the housekeeping type [8–11]. In addition, we have recently reported that the Na⁺–H⁺ exchanger in the brush border membrane of the human placental syncytiotrophoblast, a

polarized epithelial cell, belongs to the housekeeping type rather than the epithelial type [12]. Therefore, for the sake of clarity, we will use in the present paper the terms ‘type A’ and ‘type B’ to denote the housekeeping type and the epithelial type Na⁺–H⁺ exchangers respectively. The two types of exchangers differ markedly in their physiological functions as well as in their regulation by protein kinases [6,7,9,13–19]. They also appear to be under separate genetic control [8,20]. In addition, there is evidence for a difference in their molecular sizes [21–27]. These observations raise the possibility that the two types of Na⁺–H⁺ exchangers may indeed be two distinct, yet related, proteins.

An increasing number of reports describe the use of cultured cell lines to investigate the function and regulation of the Na⁺–H⁺ exchanger. In many cases, specific cell lines are used as models to study the role of the Na⁺–H⁺ exchanger in specific tissues [e.g. HT-29 and Caco-2 cell lines for the small intestine, LLC-PK₁ and opossum kidney (OK) cell lines for the proximal tubule, and the MDCK cell line for the collecting duct]. However, owing to the marked differences in function and regulation between the two types of Na⁺–H⁺ exchangers, the results from the cell line studies cannot be extrapolated to the tissue unless it is documented that a particular cell line expresses the same Na⁺–H⁺ exchanger(s) as does the normal tissue for which the cell line serves as the model. The differential sensitivities of the exchangers to inhibition by amiloride and its analogues can be a useful test to identify the type of the exchanger in a cultured cell [8,9,11,28]. However, since the IC₅₀ values (concns. giving 50 % of maximum inhibition) determined for a particular type of Na⁺–H⁺ exchanger vary significantly among different cell lines [8,11,29], this approach alone may not be adequate for this purpose. Additional tests are

Abbreviations used: DMA, dimethylamiloride; OK, opossum kidney; IC₅₀, concn. giving 50 % of maximum inhibition.

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therefore required to determine unambiguously the exchanger type in cultured cells. In a recent study with isolated membrane vesicles [12], we observed that the relative inhibition by clonidine and cimetidine can be employed to distinguish between the type A and type B $\text{Na}^+\text{-H}^+$ exchangers. The purpose of the present investigation was to determine the usefulness and efficacy of this approach in distinguishing between the two types of exchangers in cultured cells.

MATERIALS AND METHODS

Materials

Carrier-free $^{22}\text{NaCl}$ (specific radioactivity 800 mCi/ml) was purchased from DuPont-New England Nuclear (Boston, MA, U.S.A.). The JAR human placental choriocarcinoma cell line was obtained from the American Type Culture Collection, Rockville, MD, U.S.A. Opossum kidney cells (OK cell line) were obtained from Dr. D. G. Warnock, University of Alabama at Birmingham, AL, U.S.A. HeLa cells (HeLa Ohio) were originally obtained from Flow Laboratories (McLean, VA, U.S.A.). Penicillin/streptomycin, Fungizone (amphotericin B), fetal bovine serum, fetal calf serum and calf serum were purchased from Flow Laboratories. Culture media were either from Flow Laboratories or from GIBCO Laboratories (Grand Island, NY, U.S.A.). Clonidine, cimetidine and ouabain were from Sigma (St. Louis, MO, U.S.A.). Dimethylamiloride (DMA) was obtained from Research Biochemicals, Inc. (Natick, MA, U.S.A.). All other chemicals were of analytical grade.

Cell culture

The cell lines were cultured in 75 cm² Corning culture flasks and maintained at 37 °C in a CO₂ incubator. The culture medium varied depending upon the type of cell line (RPMI-1640 medium supplemented with 10 % fetal bovine serum for the JAR placental choriocarcinoma cell line, modified minimum essential medium supplemented with 10 % heat-inactivated fetal calf serum for the OK cell line, and Eagle's minimum essential medium supplemented with 10 % calf serum for the HeLa cell line). All media contained 50 units of penicillin/streptomycin/ml and 0.125 µg of Fungizone/ml. In all cases, confluent cultures were treated with phosphate-buffered saline (8.1 mM-Na₂HPO₄, 1.4 mM-KH₂PO₄, 137 mM-NaCl, 2.7 mM-KCl, pH 7.2) containing 0.1 % trypsin and 0.25 mM-EDTA, and subcultures were started from the released cells. For the uptake studies, cultures were started by seeding 35 mm disposable Falcon Petri dishes with approx. 1.5×10^6 cells/dish and allowed to grow to confluency. JAR cells received 2 ml of fresh culture medium 24 h after the subculture and were used the next day. The OK cell subcultures received 2 ml of fresh culture medium every other day and were used for uptake measurements 5–7 days after starting the subculture. HeLa cells were used for uptake measurements 1–2 days after starting the subcultures.

Assay of $\text{Na}^+\text{-H}^+$ exchanger activity

The activity of the $\text{Na}^+\text{-H}^+$ exchanger in confluent monolayer cultures were measured by determining ^{22}Na uptake into the cells for 2 min at room temperature (22–23 °C). The uptake medium contained 20 mM-Hepes/Tris (pH 7.5), 140 mM-choline chloride, 1 mM-NaCl, 5.4 mM-KCl, 1.8 mM-CaCl₂, 0.8 mM-MgSO₄, 5 mM-glucose, 0.5 mM-ouabain and traces of ^{22}Na . The assays were done with and without an NH₄Cl prepulse. For measurement of exchanger activity without the prepulse, the monolayer cultures were washed once with the wash buffer, whose composition was the same as that of the uptake medium but without ouabain and NaCl (labelled as well as unlabelled), prior to the initiation of ^{22}Na uptake. For measurement of exchanger activity with the

NH₄Cl prepulse, the monolayer cultures were incubated for 15 min at room temperature with the wash buffer containing 25 mM-NH₄Cl (the concentration of choline chloride was decreased to 115 mM to maintain osmolarity). Following this preincubation, the cells were washed twice with NH₄Cl-free wash buffer prior to the initiation of ^{22}Na uptake. Uptake was terminated by aspirating the uptake medium from the dishes and washing the monolayers with 4 × 1.5 ml of wash buffer. After washing, 1 ml of 0.3 M-NaOH was added to each dish and allowed to stand for 1 h, at which time the contents of the dish were transferred to a scintillation vial and radioactivity measured by liquid scintillation spectrometry. DMA-sensitive ^{22}Na uptake was determined by subtracting the uptake measured in the presence of 1 mM-DMA from the total uptake measured in the absence of DMA.

Protein determination

Protein was assayed for each experiment using cultures grown concurrently and under conditions identical to those used for uptake measurements. After aspirating the culture medium, deionized water (1 ml) was added to each dish. The dishes were frozen and thawed twice, after which the dish contents were suspended to form a homogenate using a 1 ml syringe and a 25 gauge needle. The protein concentration in the homogenate was determined by the method of Lowry *et al.* [30] using BSA as the standard.

Statistics

Each experimental point was determined with duplicate or triplicate dishes and each experiment was repeated between two and five times. The results are expressed as means ± S.E.M. of these replicate values.

RESULTS

$\text{Na}^+\text{-H}^+$ exchanger activity in the JAR human placental choriocarcinoma cell line

We measured the 2 min uptake of Na^+ in confluent monolayer cultures of the JAR human placental choriocarcinoma cell line at a Na^+ concentration of 1 mM (Table 1). In control cells (i.e. cells which were prepulsed with choline chloride), Na^+ uptake was 1.61 ± 0.13 nmol/2 min per mg of protein, of which 65 % was DMA-sensitive. On the other hand, in cells which were prepulsed with NH₄Cl, Na^+ uptake was 7.17 ± 0.29 nmol/2 min per mg of protein. DMA-sensitive Na^+ uptake represented about 90 % of this total uptake. Thus pretreatment of the cells with NH₄Cl instead of choline chloride resulted in 6-fold stimulation of DMA-sensitive Na^+ uptake. The NH₄Cl prepulse technique is widely used in cultured cells to acidify the intracellular pH [6]. Since such a manoeuvre stimulated the uptake of Na^+ , almost all of which was inhibitable by DMA, this can be taken as strong evidence for the presence of a $\text{Na}^+\text{-H}^+$ exchanger in JAR human placental choriocarcinoma cells.

Relative potencies of cimetidine and clonidine in inhibiting $\text{Na}^+\text{-H}^+$ exchanger activity in JAR cells

Amiloride and its analogues are the most widely used inhibitors of the $\text{Na}^+\text{-H}^+$ exchanger [31]. Recent studies with intact cells and isolated membrane vesicles have identified additional inhibitors of the $\text{Na}^+\text{-H}^+$ exchanger, which include cimetidine [32], loperamide [33], β -adrenergic receptor antagonists [34], muscarinic acetylcholine receptor antagonists [22] and α -adrenergic receptor agonists [29,35]. Some of these inhibitors have been found to be useful in the identification of the $\text{Na}^+\text{-H}^+$ exchanger protein in the renal brush border membrane (type B) [22,24]. We have recently provided evidence for the usefulness of two inhibitors, clonidine and cimetidine, in differentiating be-

Table 1. Na⁺-H⁺ exchanger activity in the JAR human placental choriocarcinoma cell line

Confluent monolayer cultures of JAR cells were incubated for 15 min with a Na⁺-free buffer containing either choline chloride (composition: 25 mM-Hepes/Tris, 140 mM-choline chloride, 5.4 mM-KCl, 1.8 mM-CaCl₂, 0.8 mM-MgSO₄, 5 mM-glucose, pH 7.5) or NH₄Cl (composition as above, except containing 115 mM-choline chloride and 25 mM-NH₄Cl). Following the incubation, the monolayers were washed twice with the choline chloride buffer. Uptake was initiated by adding to the cells 1 ml of the choline chloride buffer containing 1 mM-NaCl and traces of ²²Na. Uptake measurements were made with a 2 min incubation in the absence and the presence of 1 mM-DMA. Uptake of Na⁺ which occurred via the Na⁺-H⁺ exchanger was calculated by subtracting uptake measured in the presence of DMA from total uptake measured in the absence of DMA. The values represent means ± S.E.M. (n = 8).

Uptake conditions	Na ⁺ uptake (nmol/2 min per mg of protein)	
	Without NH ₄ Cl prepulse	With NH ₄ Cl prepulse
Control	1.61 ± 0.13	7.17 ± 0.29
DMA	0.57 ± 0.03	0.76 ± 0.05
DMA-sensitive	1.04 ± 0.13	6.41 ± 0.29

tween type A and type B Na⁺-H⁺ exchangers in isolated membrane vesicles [12]. The type A Na⁺-H⁺ exchanger is about 4-fold more sensitive to inhibition by cimetidine than by clonidine, whereas the order of potency is reversed for the type B exchanger, for which clonidine is about 4-fold more potent than cimetidine as an inhibitor of the exchanger. In order to see whether this approach can be used to identify the type of the Na⁺-H⁺ exchanger present in JAR placental choriocarcinoma cells, we investigated the effects of clonidine and cimetidine on Na⁺-H⁺ exchanger activity in confluent monolayer cultures of these cells. Exchanger activity was measured as DMA-sensitive Na⁺ uptake (2 min incubation, 1 mM-Na⁺) after the cells were prepulsed with NH₄Cl. Fig. 1 shows the effects of increasing concentrations of these two compounds on exchanger activity. Both compounds inhibited DMA-sensitive Na⁺ uptake in JAR cells. The approximate IC₅₀ values for cimetidine and clonidine were 15 and 270 μM respectively. Thus cimetidine was 18-fold more potent than clonidine in inhibiting the Na⁺-H⁺ exchanger in JAR cells, suggesting that this Na⁺-H⁺ exchanger is similar to that in the human placental brush border membrane.

Na⁺-H⁺ exchanger of the OK cell line and its inhibition by cimetidine and clonidine

From the relative inhibitory potencies of cimetidine and clonidine, it appeared that the Na⁺-H⁺ exchanger of the JAR placental cell line measured in the present study is a type A exchanger. However, since the usefulness of this approach to distinguish between the two types of Na⁺-H⁺ exchangers has been demonstrated only in isolated membrane vesicles, it was necessary to prove the efficacy of the approach in intact cells in order to strengthen the above conclusion regarding the identity of the JAR cell line Na⁺-H⁺ exchanger. Therefore we chose the OK cell line as a model for determining the relative potencies of cimetidine and clonidine in inhibiting the type B Na⁺-H⁺ exchanger in an intact cell system. The OK cell line expresses a Na⁺-H⁺ exchanger [36,37] which is inhibitable by protein kinase C [38]. Since protein kinase C activates the type A but inhibits the type B exchanger, the OK cell line Na⁺-H⁺ exchanger thus appears to be of type B.

Fig. 1. Inhibition of Na⁺-H⁺ exchanger activity by clonidine and cimetidine in the JAR human placental choriocarcinoma cell line

Confluent monolayer cultures of JAR cells were prepulsed with 25 mM-NH₄Cl, following which the activity of the Na⁺-H⁺ exchanger (DMA-sensitive Na⁺ uptake) was determined by measuring a 2 min uptake of ²²Na (concentration of Na⁺ was 1 mM) in the absence and presence of 1 mM-DMA. Clonidine (●) and cimetidine (○) were added to the uptake buffer at the indicated concentrations (clonidine concentration range 0.05–1 mM; cimetidine concentration range 0.01–0.2 mM). Control activity measured in the absence of clonidine and cimetidine was 8.2 ± 0.2 nmol/2 min per mg of protein (100%). The values represent means ± S.E.M. (n = 4).

Table 2. Na⁺-H⁺ exchanger activity in the OK cell line

Experimental procedures were as described in the legend to Table 1. The values represent means ± S.E.M. (n = 6).

Uptake conditions	Na ⁺ uptake (nmol/2 min per mg of protein)	
	Without NH ₄ Cl prepulse	With NH ₄ Cl prepulse
Control	1.80 ± 0.06	6.84 ± 0.65
DMA	0.70 ± 0.02	0.29 ± 0.04
DMA-sensitive	1.11 ± 0.06	6.54 ± 0.65

We measured the uptake of Na⁺ in confluent monolayer cultures of OK cells with and without an NH₄Cl prepulse (Table 2). In control cells, Na⁺ uptake was 1.80 ± 0.06 nmol/2 min per mg of protein, of which 62% was inhibited by DMA. Na⁺ uptake increased to 6.84 ± 0.65 nmol/2 min per mg of protein in cells pretreated with NH₄Cl, and approx. 90% of this uptake was sensitive to inhibition by DMA. Thus an NH₄Cl prepulse stimulated DMA-inhibitable Na⁺ uptake by about 6-fold. These results provide evidence for the presence of a Na⁺-H⁺ exchanger in the OK cell line, confirming earlier observations by many investigators.

Having established that Na⁺-H⁺ exchanger activity can be measured in the OK cell line under the same experimental conditions employed for the JAR placental cell line, we investigated the effects of cimetidine and clonidine on OK cell line Na⁺-H⁺ exchanger activity. As in the JAR cell line, exchanger

Fig. 2. Inhibition of $\text{Na}^+\text{-H}^+$ exchanger activity by clonidine and cimetidine in the OK cell line

Experimental procedures were as described in the legend to Fig. 1, except that the concentrations of the inhibitors used were different: the concentration range for both clonidine (●) and cimetidine (○) was 0.1–2.5 mM. Control activity of the $\text{Na}^+\text{-H}^+$ exchanger in the absence of clonidine and cimetidine was 7.2 ± 0.6 nmol/2 min per mg of protein (100%). The values represent means $\pm$ S.E.M. ($n = 4$).

Fig. 3. Concentration-dependent inhibition of $\text{Na}^+\text{-H}^+$ exchanger activity by DMA in JAR and OK cell lines

Confluent monolayer cultures of JAR cells (●) and OK cells (○) were prepulsed with 25 mM- NH_4Cl , following which a 2 min uptake of Na^+ (Na^+ concentration 1 mM) was determined. DMA was added to the uptake buffer at indicated concentrations (range: JAR cells, 10^{-10} – 10^{-3} M; OK cells, 10^{-9} – 10^{-3} M). Control uptake (100%) was 6.0 ± 0.2 nmol/2 min per mg of protein in JAR cells and 7.8 ± 0.7 nmol/2 min per mg of protein in OK cells. The values represent means $\pm$ S.E.M. ($n = 5$). IC_{50} values: JAR cells, 56 nM; OK cells, 6.3 μM .

activity was measured as DMA-inhibitable Na^+ uptake (2 min incubation, 1 mM- Na^+) after the cells were prepulsed with NH_4Cl . Fig. 2 shows that the $\text{Na}^+\text{-H}^+$ exchanger present in the OK cell line is also sensitive to inhibition by clonidine and cimetidine. The IC_{50} values calculated from the dose-response curves of these inhibitors were 300 and > 2500 μM respectively. Therefore

clonidine was at least 8 times more potent than cimetidine in inhibiting the $\text{Na}^+\text{-H}^+$ exchanger in this cell line. This is in contrast to the effects of these compounds on the JAR cell line $\text{Na}^+\text{-H}^+$ exchanger, in which cimetidine was a more potent inhibitor than clonidine.

It can be concluded from these results that the relative sensitivity to inhibition by cimetidine and clonidine clearly differentiates between the $\text{Na}^+\text{-H}^+$ exchangers expressed in the JAR and OK cell lines. In addition, the data show that the JAR placental cell line possesses the type A $\text{Na}^+\text{-H}^+$ exchanger, whereas the OK cell line possesses the type B $\text{Na}^+\text{-H}^+$ exchanger.

Relative inhibition of the $\text{Na}^+\text{-H}^+$ exchangers in the JAR and OK cell lines by DMA

Many studies have demonstrated differences in the sensitivities of the two types of $\text{Na}^+\text{-H}^+$ exchangers to inhibition by amiloride or its analogues. These studies have been done with either intact cells or isolated membrane vesicles. In LLC-PK₁/PKE 20 cells, the basolateral membrane $\text{Na}^+\text{-H}^+$ exchanger (type A) is many times more sensitive to inhibition by ethylisopropylamiloride than is the apical membrane $\text{Na}^+\text{-H}^+$ exchanger (type B) [8,9]. Similar results have been obtained in MDCK cells [11]. In isolated membrane vesicles, using human placental brush border membranes as the source of the type A $\text{Na}^+\text{-H}^+$ exchanger and rabbit renal brush border membranes as the source of the type B $\text{Na}^+\text{-H}^+$ exchanger, we have shown that the type A exchanger is more sensitive to inhibition than type B, not only by ethylisopropylamiloride but also by other amiloride analogues (t-butyl amiloride, hexamethylene amiloride and DMA) and by amiloride, the parent compound [12]. A recent study using brush border and basolateral membrane vesicles isolated from the villous cells of the rabbit ileum has demonstrated that the basolateral membrane $\text{Na}^+\text{-H}^+$ exchanger is about 10 times more sensitive to inhibition by amiloride than is the brush border membrane $\text{Na}^+\text{-H}^+$ exchanger, suggesting that the two exchangers may be type A and type B respectively [39]. Therefore, in order to strengthen our conclusion drawn from the inhibition studies involving cimetidine and clonidine that the JAR cell line $\text{Na}^+\text{-H}^+$ exchanger is type A and the OK cell line $\text{Na}^+\text{-H}^+$ exchanger is type B, we assessed the sensitivities of these exchangers to inhibition by DMA. In these experiments the activity of the $\text{Na}^+\text{-H}^+$ exchanger was measured in the cells after an NH_4Cl prepulse and the concentration-dependent inhibition of the activity by DMA was determined (Fig. 3). The JAR cell line $\text{Na}^+\text{-H}^+$ exchanger was about 100 times more sensitive to inhibition by this amiloride analogue than was the OK cell line $\text{Na}^+\text{-H}^+$ exchanger. The IC_{50} values for these exchangers were 56 nM and 6.3 μM respectively. The difference in the sensitivities of these two types of $\text{Na}^+\text{-H}^+$ exchangers to inhibition by DMA is similar to what has been observed with other amiloride analogues. These data strongly support our conclusion regarding the identities of the exchangers in the JAR and OK cell lines.

Inhibition of the $\text{Na}^+\text{-H}^+$ exchanger by DMA, cimetidine and clonidine in the HeLa cell line

The general applicability of the approach involving cimetidine and clonidine to identifying the type of $\text{Na}^+\text{-H}^+$ exchanger in cultured cells was tested on HeLa cells. The HeLa cell line is a non-polarized epithelial cell which is known to express $\text{Na}^+\text{-H}^+$ exchanger activity [40,41]. Since the activity of this exchanger is stimulated by phorbol esters [40], it is probably type A. However, to confirm this, we studied the effect of DMA on the activity of the $\text{Na}^+\text{-H}^+$ exchanger in this cell line. Exchanger activity could be measured in confluent monolayer cell cultures under the conditions described for the JAR and OK cell lines. DMA-sensitive Na^+ uptake was stimulated in the HeLa cell line by an

Fig. 4. Inhibition of the Na⁺-H⁺ exchanger activity by DMA, clonidine and cimetidine in the HeLa cell line

Confluent monolayer cultures of HeLa cells were prepulsed with 25 mM-NH₄Cl, following which a 2 min uptake of Na⁺ (Na⁺ concentration 1 mM) was determined. (a) Inhibition by DMA (concentration range 10⁻¹⁰-10⁻³ M; control uptake 2.2 ± 0.1 nmol/2 min per mg of protein; IC₅₀ 30 nM). (b) Inhibition by clonidine (●) and cimetidine (○). The concentration range was 0.025-1 mM for clonidine and 5-200 μM for cimetidine. Only the DMA-sensitive Na⁺ uptake was used in calculations. This uptake, when measured in the absence of clonidine and cimetidine, was 1.5 ± 0.1 nmol/2 min per mg of protein (100%). The values represent means ± S.E.M. (n = 4).

NH₄Cl prepulse, as in the other two cell lines. Fig. 4(a) shows the effect of increasing concentrations of DMA on the activity of the HeLa cell line Na⁺-H⁺ exchanger after the cells were pretreated with NH₄Cl. DMA was found to be a very potent inhibitor of the exchanger, with an IC₅₀ value of 30 nM. This high sensitivity to DMA inhibition indicates that the Na⁺-H⁺ exchanger expressed in this cell line does indeed belong to type A.

Fig. 4(b) shows that cimetidine was more potent than clonidine in inhibiting the HeLa cell line Na⁺-H⁺ exchanger. The IC₅₀ values for cimetidine and clonidine were 18 and 150 μM respectively. The greater potency of cimetidine compared with clonidine in inhibiting exchanger activity thus strongly supports the conclusion that the HeLa cell line Na⁺-H⁺ exchanger is of type A.

DISCUSSION

Our previous studies have shown that clonidine, an α₂-adrenergic receptor agonist [35], and cimetidine, a H₂-histamine receptor antagonist [32], inhibit the activity of the Na⁺-H⁺ exchanger in human placental brush border membrane vesicles. These inhibitors interact with the Na⁺-binding site of the exchanger in a mutually exclusive manner [42]. This is the same site involved in the interaction with amiloride [35,42]. The inhibition caused by these compounds is competitive with respect to Na⁺ if the experiments are done in the presence of Cl⁻. Recently we reported that the relative inhibition by clonidine and cimetidine can be used to differentiate the Na⁺-H⁺ exchanger of the human placental brush border membrane (type A) from that of the rabbit renal brush border membrane (type B) [12]. The present study shows that this approach can be used as an efficient means to distinguish the two types of Na⁺-H⁺ exchangers in cultured cells also. We demonstrate that in cultured cells, as in isolated membrane vesicles, the type A Na⁺-H⁺ exchanger is many-fold more sensitive to inhibition by cimetidine than by clonidine and, in contrast, the type B Na⁺-H⁺ exchanger is

many-fold more sensitive to inhibition by clonidine than by cimetidine.

The inhibition caused by clonidine and cimetidine is mediated not through their receptors but through direct interaction with the Na⁺-H⁺ exchanger, as the inhibition is demonstrable even in isolated membrane vesicles where the participation of receptor-coupled second messenger systems is very unlikely. In addition, the IC₅₀ values are many orders of magnitude greater than what is expected for the action of these compounds through their receptors. This conclusion is also supported by the fact that there are no specific high-affinity binding sites for clonidine in the brush border membranes of the human placenta and the rabbit kidney, suggesting the absence of the α₂-adrenergic receptor in these membranes (D. F. Balkovetz, F. H. Leibach & V. Ganapathy, unpublished work). Even in cultured cells, clonidine and cimetidine inhibit the Na⁺-H⁺ exchanger by direct interaction rather than through receptors. The relatively high concentrations of these compounds required for the observed inhibition in cultured cells make the involvement of receptors in the mechanism of this inhibition very unlikely. There is in fact strong supporting experimental evidence for the direct interaction of many imidazole and guanidinium compounds, which include clonidine and cimetidine, with Na⁺-H⁺ exchangers in cultured cells [29,43]. Moreover, a recent study [44] on the Na⁺-H⁺ exchanger of OK cells, one of the cell lines used in our present investigation, has demonstrated that stimulation of the α₂-adrenergic receptor with adrenaline has no influence on the exchanger unless the cells had been previously exposed to parathyroid hormone to suppress exchanger activity. Even under these conditions, the α₂-adrenergic receptor agonist leads to the restoration rather than inhibition of the exchanger activity. The available data thus strongly support our conclusion that clonidine and cimetidine directly interact with the substrate-binding site of both types of Na⁺-H⁺ exchangers.

The observation that there is a marked difference in the relative sensitivities of the type A and type B Na⁺-H⁺ exchangers to inhibition by clonidine and cimetidine suggests that there may be a difference in the nature of the substrate-binding sites of these two exchangers. This suggestion is also supported by the different sensitivities of the two types of Na⁺-H⁺ exchangers to inhibition by amiloride and its analogues. We and other investigators have reported that the type A exchanger is more sensitive to inhibition by amiloride than is the type B exchanger, and that the difference between the sensitivities of the exchangers becomes even greater with amiloride analogues that possess hydrophobic substitutions. It is therefore likely that the substrate-binding site of the type A Na⁺-H⁺ exchanger is relatively more hydrophobic than that of the type B exchanger. This would allow a better interaction of the type A exchanger with the hydrophobic amiloride analogues. Whereas the type A Na⁺-H⁺ exchanger has been cloned and its amino acid sequence is known [26], there is no information available as yet on the primary structure of the type B exchanger. Therefore the above suggestion regarding the possible difference in the hydrophobicity of the substrate-binding sites of the two Na⁺-H⁺ exchangers has to remain speculative until conclusive evidence can be obtained by comparing the amino acid sequences of the two exchangers.

In the present study the activity of the Na⁺-H⁺ exchanger was determined using cells grown as confluent monolayers on an impermeable plastic support and by measuring Na⁺ uptake into the cells from a medium added to the culture dish. Under these experimental conditions, only the upwardly facing surface of the cell is readily accessible to the assay medium. Therefore, even though polarized epithelial cells can express Na⁺-H⁺ exchanger activity in apical and/or basolateral membranes, the present study is concerned only with the Na⁺-H⁺ exchangers expressed in

the apical membranes of the two polarized epithelial cells used (JAR and OK). The polarity of distribution of Na⁺-H⁺ exchanger activity in the OK cell line has already been determined [45]. In this cell line the exchanger is exclusively localized in the apical membrane, and activity is thus measurable by the technique employed in our current study. The basolateral membrane does not possess any Na⁺-H⁺ exchanger activity. The Na⁺-H⁺ exchanger activity of the JAR cell line has not been previously investigated. Our study demonstrates for the first time that the apical membrane of this cell line expresses a Na⁺-H⁺ exchanger. The experimental technique employed here does not, however, allow us to determine whether or not the basolateral membrane of this cell line possesses Na⁺-H⁺ exchanger activity.

There are some interesting aspects with respect to the Na⁺-H⁺ exchanger of the JAR cell apical membrane. The present investigation demonstrates that this exchanger is of type A, in contrast with the Na⁺-H⁺ exchanger of the OK cell apical membrane which is of type B. Previously, only the expression of the type B Na⁺-H⁺ exchanger had been documented in apical membranes of confluent cultures of polarized epithelial cells; hence this is the first report of the presence of the type A Na⁺-H⁺ exchanger in the apical membrane of a polarized cell. Another point of interest is the similarity between the Na⁺-H⁺ exchangers of the JAR cell apical membrane and the syncytiotrophoblast brush border membrane, i.e. both belong to type A. Thus the JAR cell line, which originates from a human placental choriocarcinoma, represents a very useful model for investigating the function and regulation of the Na⁺-H⁺ exchanger present in the brush border membrane of the normal human placental syncytiotrophoblast. A note of caution is required, however, in that the present study was carried out with cells grown on an impermeable plastic support. Under these culture conditions the cells may not be fully polarized. Therefore, in order to demonstrate unequivocally that the type A Na⁺-H⁺ exchanger measured in the present study is indeed a component of the apical membrane of a fully polarized JAR cell, additional experiments are needed with JAR cells grown on a permeable filter support, where cells are truly polarized.

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