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INTERACTION OF CLONIDINE WITH HUMAN PLACENTAL Na^+-H^+ EXCHANGER

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Abstract—The effect of clonidine, an α_2-adrenergic receptor agonist, on the Na^+-H^+ exchanger in human placental brush-border membrane vesicles was examined. The exchanger was inhibited by clonidine. The inhibition was freely reversible, and the apparent inhibition constant for the process was 250 μM. The nature of inhibition was found to be competitive with respect to Na^+. The Dixon plot ($1/v$ versus clonidine concentration) was linear ($r^2 = 0.998$), indicating the interaction of the drug with a single site on the exchanger protein. Similar kinetic analyses with amiloride, a potassium-sparing diuretic, and cimetidine, a histamine type II receptor antagonist, revealed that these drugs also inhibited the Na^+-H^+ exchanger by interacting with a single site on the protein. The presence of clonidine increased the intercepts without affecting the slopes of the $1/v$ versus amiloride concentration and the $1/v$ versus cimetidine concentration plots. These results demonstrate that all three drugs, amiloride, cimetidine and clonidine, interact with the human placental Na^+-H^+ exchanger at a single site in a mutually exclusive manner, and the site of interaction is identical with the Na^+-binding site on the external surface of the exchanger protein.

A plasma membrane system which catalyzes the exchange of extracellular Na^+ for intracellular H^+ has been identified in many cell types [1]. There has been a surge of interest in this transport system in recent years because activation of this system appears to be involved in the initiation of cell growth and proliferation in response to many mitogenic growth factors [2, 3]. In polarized epithelial cells of mammalian origin such as the absorptive cells of small intestine and renal proximal tubule, the Na^+-H^+ exchanger is restricted to the brush-border membrane [4–7]. We have demonstrated recently that the brush-border membrane isolated from normal human term placenta also possesses a highly active Na^+-H^+ exchanger (manuscript submitted for publication).

The Na^+-H^+ exchanger is inhibited by many drugs such as amiloride [8], harmaline [9], and quinidine [10, 11]. Amiloride is a reversible, competitive inhibitor of the exchanger and it competes with Na^+ for the same binding site on the protein [12]. Quinidine, on the other hand, interacts with the Na^+-binding site as well as with at least one additional site that is not shared by Na^+ and amiloride [11]. Since judicial use of inhibitors could provide an effective means of understanding the mechanisms involved in the Na^+-H^+ exchange process, we screened various other drugs for their abilities to inhibit the Na^+-H^+ exchanger. We have demonstrated recently that cimetidine, a histamine type II receptor antagonist, is a potent inhibitor of Na^+-H^+ exchanger in human placental and rabbit renal brush-border membranes (manuscript submitted for publication). The characteristics of cimetidine inhibition of the exchanger are similar to those of amiloride inhibition. The present paper reports the inhibition of the human placental Na^+-H^+ exchanger by clonidine, an α_2-adrenergic receptor agonist, and also describes the nature of interaction of the drug with the exchanger.

METHODS AND MATERIALS

Preparation of placental brush-border membrane vesicles. Human placentas were obtained from normal, uncomplicated term pregnancies within 30 min of delivery and processed immediately. Brush-border membrane vesicles were prepared by a previously described procedure [13], with a few modifications. After removing the decidua from the maternal surface, the villous tissue was separated from the chorionic plate, minced thoroughly with a sharp scalpel, and collected in a beaker. The tissue was washed three times with 300 ml of ice-cold buffer (10 mM Hepes†/Tris, pH 7.0) containing 300 mM mannitol to remove blood. The washed tissue was agitated in 400 ml of the same buffer at 4° on a magnetic stirrer. This procedure resulted in separation of brush-border membranes from the syncytiotrophoblast layer. The placental slurry was filtered through four layers of cotton gauze and the filtrate was centrifuged at 8,000 *g* for 15 min. The supernatant fraction was again centrifuged at 60,000 *g* for 30 min. The pellets were suspended in 50 ml of the same buffer using a Dounce glass homo-

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† Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; and Mes, 2(*N*-morpholino) ethanesulfonic acid.

genizer with ten strokes. 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 let stand for 10 min at 4°. The suspension was then centrifuged at 3,000 *g* for 15 min to remove the Mg^{2+}-aggregated, non-brush-border membranes. The supernatant fraction containing brush-border membranes was centrifuged at 60,000 *g* for 30 min. The membrane pellets were suspended in preloading buffer and centrifuged again at 60,000 *g* for 30 min. This washing procedure was repeated one more time, and the final pellets were suspended in a small volume of the preloading buffer by passing through a 25-gauge needle. In most experiments, the preloading buffer was 25 mM Mes/5 mM Tris, pH 5.5, containing 150 mM KCl. In some experiments, 18 mM Hepes/12 mM Tris, pH 7.5, containing 150 mM KCl was used as the preloading buffer. Protein concentration in the final preparation was adjusted to 6 mg/ml. The membrane suspension was then distributed in small aliquots in plastic tubes and stored in liquid nitrogen until use. Alkaline phosphatase and 5′-nucleotidase, marker enzymes for human placental brush-border membrane, were enriched in these preparations 19.1 ± 2.2- and 18.5 ± 2.8-fold, respectively, compared to the homogenate of the starting placental tissue.

Uptake studies. Uptake of radiolabeled Na^+ in membrane vesicles was determined at room temperature (20–22°) by a rapid filtration technique [14] using Millipore filters (pore size, 0.65 μm). The specific conditions for each uptake experiment are given in the respective figure legends. In general, uptake was initiated by mixing 40 μl of membrane suspension with 160 μl of uptake buffer containing labeled Na^+. The composition of the uptake buffer in most experiments was: 18 mM Hepes, 12 mM Tris, 150 mM KCl, 0.625 mM NaCl, pH 7.5. The uptake was terminated by the addition of 3 ml of ice-cold stop buffer (18 mM Hepes/12 mM Tris, pH 7.5, containing 150 mM KCl) and the mixture was filtered. The filter was washed three times with 5 ml of the stop buffer and then transferred to a counting vial. The radioactivity associated with the filter was determined by liquid scintillation spectrometry.

Materials. Cimetidine and clonidine were purchased from Sigma. Amiloride was a gift from Prof. K. Green, Department of Physiology, Medical College of Georgia. All other chemicals were of the highest purity available. Carrier-free $^{22}NaCl$ (radioactivity, 200 μCi/ml) was obtained from the Radiochemical Center, Amersham.

RESULTS

Effect of clonidine on Na^+ uptake. Uptake of Na^+ in human placental brush-border membrane vesicles was studied in the presence and absence of an outward-directed H^+ gradient. Figure 1 shows that the initial rates of Na^+ uptake were many times greater in the presence of a H^+ gradient ($[pH]_i = 5.5$; $[pH]_o = 7.2$) compared to the uptake rates in the absence of a H^+ gradient ($[pH]_i = [pH]_o = 7.5$). The time course of Na^+ uptake exhibited the overshoot phenomenon, but this transient accumulation of Na^+ within the vesicles above the equilibrium concentration was observed only when the H^+ gradient was present across the membrane. In the absence of a H^+ gradient, uptake of Na^+ was slow and gradually reached the equilibrium value without exhibiting the overshoot. These results indicate that Na^+ uptake in these vesicles is energized by an outward-directed H^+ gradient and thus provide evidence for the existence of Na^+-H^+ exchanger in these membranes. Figure 1 also shows that the H^+ gradient-dependent Na^+ uptake was inhibited drastically by clonidine and the active accumulation of Na^+ within the vesicles was abolished. However, the equilibrium uptake of Na^+ remained the same even in the presence of the inhibitor, indicating that the intravesicular volume was not affected by clonidine.

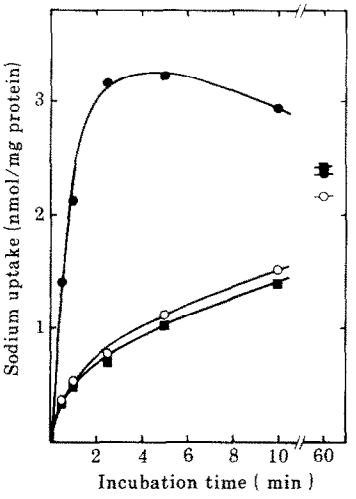

Fig. 1. Effect of clonidine on proton gradient-dependent Na^+ uptake. Membrane vesicles were suspended in either 25 mM Mes/5 mM Tris buffer, pH 5.5, containing 150 mM KCl or 18 mM Hepes/12 mM Tris buffer, pH 7.5, containing 150 mM KCl. Uptake of Na^+ (0.5 mM) was measured by incubating 40 μl of membrane suspension (0.24 mg protein) with 160 μl of uptake buffer (18 mM Hepes/12 mM Tris, 150 mM KCl, pH 7.5) containing labeled Na^+. Final concentration of clonidine was 2 mM. Key: (■—■) $[pH]_i = [pH]_o = 7.5$; (●—●) $[pH]_i = 5.5$; $[pH]_o = 7.2$; and (○—○) $[pH]_i = 5.5$; $[pH]_o = 7.2$ plus clonidine.

The effect of increasing concentrations of clonidine on the H^+ gradient-dependent Na^+ uptake was then studied. In this experiment, the membrane vesicles were preloaded with pH 5.5 buffer and uptake (1 min) was determined at pH 7.2. Final concentration of Na^+ in the incubation medium was 0.5 mM, and clonidine concentration was varied from 0 to 5 mM. Na^+ uptake in the absence of the inhibitor was 2.95 ± 0.13 nmoles/mg protein. Uptake of Na^+ decreased with increasing concentrations of clonidine (Fig. 2). The contribution of the Na^+-H^+ exchanger to the total uptake of Na^+ in these vesicles was calculated by determining the uptake of Na^+ in the presence of 2 mM amiloride and subtracting this amiloride-insensitive uptake from the total uptake. The Na^+-H^+ exchanger was found to be responsible for 90% of Na^+ uptake in these vesicles. The inhibition of the amiloride-sensitive Na^+ uptake by clonidine was then calcu-

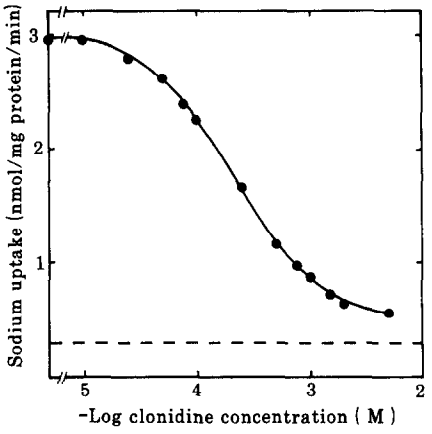

Fig. 2. Dose–response of clonidine inhibition. Uptake of Na^+ (0.5 mM) was measured for 1 min in the presence of a proton gradient ($[pH]_i = 5.5$; $[pH]_o = 7.2$). Concentration of clonidine was varied from 0.01 to 5 mM. The dashed line indicates Na^+ uptake in the presence of 2 mM amiloride.

lated, and the results are given in Fig. 3 as a Dixon plot ($1/v$ vs $[I]$). The relationship was linear ($r^2 = 0.998$), indicating that clonidine interacts with the Na^+-H^+ exchanger at a single site. The K_i for the inhibition of amiloride-sensitive Na^+ uptake by clonidine was approximately 250 μM.

Reversibility of inhibition. The reversibility of clonidine inhibition of the Na^+-H^+ exchanger was then determined. The membrane vesicles were preincubated at pH 7.3 with 0, 100 μM and 1 mM clonidine at room temperature for 30 sec in the absence of Na^+. These vesicles were then diluted 1 in 10 in uptake buffer containing labeled Na^+ and different concentrations of clonidine. Amiloride-sensitive Na^+ uptake was measured for 1 min, and the results are given in Fig. 4. When the concentration of clonidine during preincubation as well as during the uptake measurement was zero, the uptake of Na^+ was

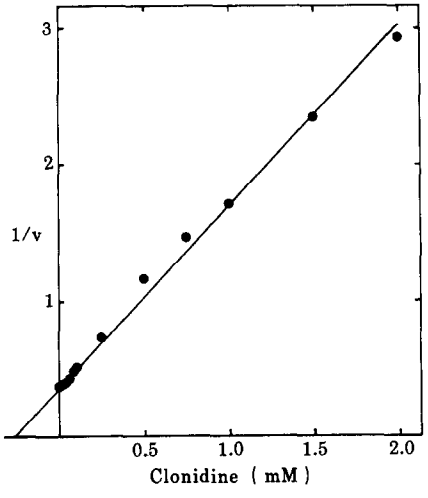

Fig. 3. Dixon plot ($1/v$ vs $[I]$) of clonidine inhibition. The same data in Fig. 2 are used here after subtracting the amiloride-insensitive Na^+ uptake from the uptake values obtained in the absence of amiloride.

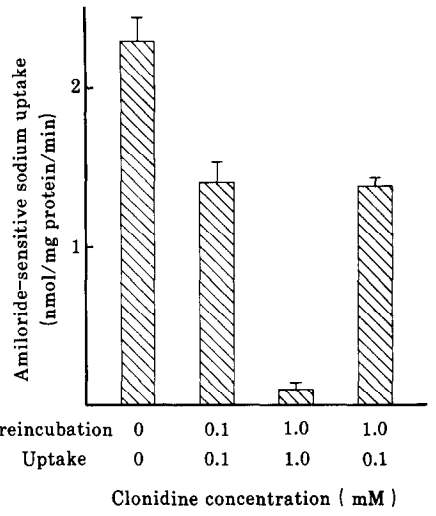

Fig. 4. Reversibility of clonidine inhibition. Membrane vesicles preloaded with 25 mM Mes/5 mM Tris, pH 5.5, and 150 mM KCl were used. Ten microlitres of membrane suspension (0.14 mg protein) was preincubated for 30 sec with either 90 μl of uptake buffer (18 mM Hepes/12 mM Tris, pH 7.5, with 150 mM KCl), 90 μl of 0.11 mM clonidine or 90 μl of 1.11 mM clonidine (the solutions of clonidine were made in the uptake buffer). The pH of this preincubation mixture was 7.3. After preincubation, 1 min Na^+ uptake (0.5 mM) was measured at pH 7.3 by incubating these vesicles with either 900 μl of the uptake buffer or 900 μl of the uptake buffer containing 0.1 mM or 1 mM clonidine. Labeled Na^+ was added to the uptake buffer. Results are given as the mean ± S.D. from three determinations.

2.28 ± 0.14 nmoles/mg protein. The uptake was reduced to 1.40 ± 0.13 and 0.10 ± 0.04 nmoles/mg protein, respectively, when the clonidine concentration during preincubation and uptake was 100 μM and 1 mM. But, when the vesicles were preincubated with 1 mM clonidine and the uptake was measured with only 100 μM clonidine, Na^+ uptake was 1.37 ± 0.04 nmoles/mg protein. If the inhibition of the exchanger by clonidine were irreversible, uptake of Na^+ in the vesicles preincubated with 1 mM clonidine should have been 0.10 ± 0.04 nmole/mg protein irrespective of clonidine concentration during uptake measurement. Instead, the inhibition of Na^+ uptake by clonidine was not influenced by the clonidine concentration during preincubation, but was dependent on clonidine concentration during uptake. Thus, when clonidine was diluted during uptake measurement, the inhibition decreased accordingly. These results demonstrate that the inhibition of the Na^+-H^+ exchanger by clonidine is freely reversible.

Nature of inhibition. The effect of clonidine on kinetic constants (affinity constant, K_t and maximal velocity, V_{max}) for Na^+ uptake via the Na^+-H^+ exchanger was then studied. Uptake of Na^+ was measured in the presence and absence of 2 mM amiloride, and only the amiloride-sensitive Na^+ uptake was used to determine the kinetic constants. The effect of Na^+ concentration on the amiloride-sensitive Na^+ uptake in the presence and absence of 150 μM clonidine is described in Fig. 5 in which the

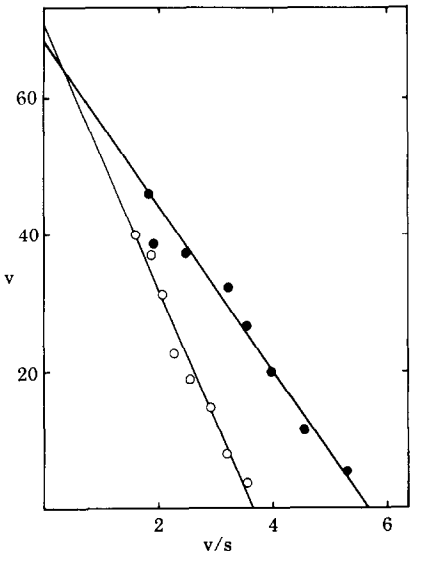

Fig. 5. Kinetics of clonidine inhibition of amiloride-sensitive Na^+ uptake. Uptake of Na^+ was measured for 1 min in the presence of a proton gradient ($[pH]_i = 5.5$; $[pH]_o = 7.2$) over a Na^+ concentration range 1–25 mM. Amiloride-sensitive Na^+ uptake was calculated by subtracting the Na^+ uptake in the presence of 2 mM amiloride from the Na^+ uptake values obtained in the absence of amiloride. Key: (v) nmoles/mg protein/min; (s) Na^+ concentration (mM); (●—●) control; and (○—○) 150 μM clonidine.

results are presented as Eadie–Hofstee plots (v vs v/s). In the absence of clonidine, Na^+ uptake was saturable with respect to Na^+ and the apparent K_t for the process was 11.0 ± 0.6 mM and V_{max} was 64.1 ± 2.4 nmoles/mg protein/min. In the presence of clonidine, Na^+ uptake was inhibited, and yet obeyed Michaelis–Menten saturation kinetics. However, the apparent K_t for the uptake process was increased 2-fold (19.2 ± 0.5 mM), but there was no significant change in the maximal velocity (70.1 ± 1.5 nmoles/mg protein/min). These data demonstrate that clonidine is a competitive inhibitor of the Na^+-H^+ exchanger and that the inhibitor and Na^+ compete for the same binding site on the exchanger protein.

Interaction of clonidine and amiloride with the Na^+-H^+ *exchanger.* Amiloride has been shown to interact with the Na^+-binding site of the Na^+-H^+ exchanger in renal brush-border membranes [12]. Since our present study shows that clonidine also competes with Na^+ for the same site, we designed experiments to determine if amiloride and clonidine are mutually exclusive inhibitors. In these experiments, uptake of Na^+ (0.5 mM) was measured as a function of amiloride concentration (0–10 μM) in the presence and absence of 200 μM clonidine. Na^+ uptake via the Na^+-H^+ exchanger was calculated by subtracting from these uptake values the uptake of Na^+ measured in the presence of 2 mM amiloride. The results are given in Fig. 6 as Dixon plots ($1/v$ vs $[I]$). The plots were linear ($r^2 > 0.99$) showing that amiloride interacts with a single site on the exchanger protein. The presence of clonidine caused a 2.2-fold increase in the negative x-intercept of the Dixon plot, but did not change the slope of the plot significantly. These results indicate that amiloride and clonidine interact with the same site and are mutually exclusive inhibitors of the exchanger.

Interaction of clonidine and cimetidine with the Na^+-H^+ *exchanger.* We have demonstrated recently that cimetidine is a potent inhibitor of human placental Na^+-H^+ exchanger and that cimetidine and Na^+ compete for the same site on the exchanger protein (manuscript submitted for publication). Therefore, we tested whether cimetidine and clonidine are also mutually exclusive inhibitors of the exchanger. In these experiments, the amiloride-sensitive Na^+ uptake was measured as a function of cimetidine concentration (0–75 μM) in the presence

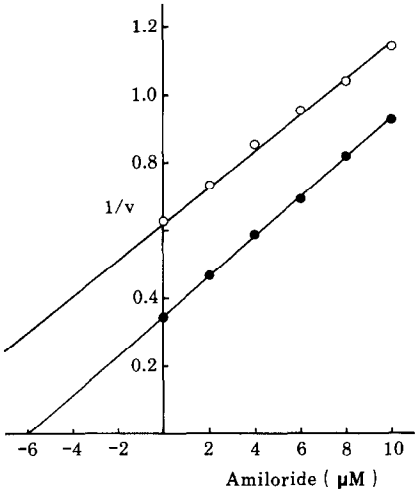

Fig. 6 Effect of clonidine on kinetics of amiloride inhibition. Uptake of Na^+ (0.5 mM) was measured for 1 min in the presence of a proton gradient ($[pH]_i = 5.5$; $[pH]_o = 7.2$). Amiloride concentration was varied from 0 to 10 μM, and clonidine concentration, when present, was 200 μM. Only the amiloride-sensitive Na^+ uptake values were used in this plot. Key: (●—●) control; and (○—○) clonidine.

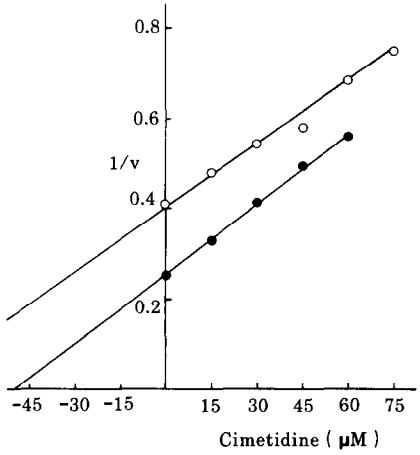

Fig. 7. Effect of clonidine on kinetics of cimetidine inhibition. Uptake of Na^+ (0.5 mM) was measured for 1 min in the presence of a proton gradient ($[pH]_i = 5.5$; $[pH_o =$ 7.2). Cimetidine concentration was varied from 0 to 75 μM, and clonidine concentration, when present, was 150 μM. Only the amiloride-sensitive Na^+ uptake values were used in this plot. Key: (●—●) control; and (○—○) clonidine.

and absence of 150 μM clonidine. The data are presented in Fig. 7 as Dixon plots ($1/v$ vs $[I]$). The linearity of the plots ($r^2 > 0.99$) indicates that, like amiloride, cimetidine also interacts with a single site on the Na^+-H^+ exchanger. The effect of clonidine on the Dixon plot was to cause a 1.9-fold increase in the negative x-intercept, without significantly changing the slope of the line. Thus, the results in Figs. 6 and 7 suggest that all three inhibitors, amiloride, cimetidine and clonidine, interact with a single site on the exchanger protein in a mutually exclusive manner and that the site of interaction in all three cases is identical with the Na^+-binding site.

DISCUSSION

The data presented in this paper demonstrate that clonidine, a widely used α_2-adrenergic receptor agonist, is an inhibitor of the human placental Na^+-H^+ exchanger. The inhibitory effect was freely reversible and the inhibition was competitive with respect to Na^+. Thus, the inhibition of the exchanger by clonidine is very similar to the inhibition by other drugs, amiloride and cimetidine. The K_i for the inhibition process by clonidine however, was high (K_i = 250 μM) compared to inhibition by amiloride (K_i = 6 μM) and cimetidine (K_i = 40 μM). Kinetic analysis of the inhibition of the exchanger by amiloride, cimetidine and clonidine indicates that a single binding site on the exchanger protein was responsible for interaction with all three drugs. The site of interaction with the drugs was identical with the external Na^+-binding site. It has been demonstrated with the renal brush-border Na^+-H^+ exchanger that Na^+, Li^+, H^+ and NH_4^+ are all substrates for transport by this system and that they compete for the same external binding site on the exchanger protein [5, 15]. These results collectively indicate that the inhibitors, amiloride, cimetidine and clonidine, and the substrates, Na^+, Li^+, H^+, and NH_4^+, all compete for binding to a single site on the external surface of the exchanger protein in a mutually exclusive manner.

It may be argued that clonidine, being a weak base, could collapse the H^+ gradient across the membrane and thereby inhibit the H^+ gradient-driven Na^+ uptake rather than directly interacting with the Na^+-binding site of the exchanger. However, the data from the reversibility experiment show that this argument is untenable. The membrane vesicles were preincubated with 0.1 mM or 1 mM clonidine in the presence of a H^+ gradient ($[pH]_i = 5.5$; $[pH]_o = 7.3$). After 30 sec of preincubation, Na^+ uptake was measured at pH 7.3, clonidine concentration during uptake being 0.1 mM. If clonidine were to dissipate the H^+ gradient, one would expect the Na^+ uptake in the vesicles that were preincubated with 1 mM clonidine to be less than the Na^+ uptake in the vesicles that were preincubated with 0.1 mM clonidine. But, the data in Fig. 4 show that Na^+ uptake was identical in both cases. Therefore, the inhibition of the H^+ gradient-driven Na^+ uptake by clonidine is not due to the dissipation of the driving force.

Apart from its role in the regulation of intracellular pH, the Na^+-H^+ exchanger in the brush-border membrane of kidney, intestine and placenta is involved in the transport of Na^+ and consequently in the osmotically-coupled water movement across the membrane. Genetic impairment of this exchange system in human small intestine leads to secretory diarrhea resulting in excessive loss of Na^+ and water in the feces [16]. It is therefore clear that the Na^+-H^+ exchanger plays a prominent role in electrolyte and fluid transfer across the brush-border membrane of the absorptive cells of intestine and most likely also in kidney and placenta. Clonidine is currently being evaluated as a potential antidiarrheal drug. This drug has been shown to inhibit secretory diarrhea in a patient with bronchogenic adenocarcinoma [17], to prevent opiate withdrawal symptoms including diarrhea [18], and to control diabetic diarrhea [19]. Even though the effects of clonidine on the intestinal Na^+-H^+ exchanger have not been examined, it is very likely that it is also inhibited by this drug. Therefore, the intestinal Na^+-H^+ exchanger could not be the locus for the antidiarrheal action of clonidine because the inhibition of the exchanger is expected to worsen the diarrhea rather than to control it. Moreover, it is doubtful if the luminal concentration of clonidine will ever be high enough to cause significant inhibition of the exchanger under physiological conditions due to the high inhibition constant of the drug and due to high concentrations of Na^+ in the intestinal fluid. For the same reasons, it is not clear at present whether therapeutic levels of this drug would affect the physiological function of the human placenta. However, we believe that the data from the present study showing that clonidine, cimetidine and amiloride interact with the same site on the Na^+-H^+ exchanger could be potentially useful in understanding the chemical nature of the binding site as well as the overall mechanism of the exchange process.

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