Membrane transport pathways for transplacental transfer of CO₂/HCO₃⁻ were investigated by assessing the possible presence of a Cl⁻/HCO₃⁻ exchange mechanism in the maternal-facing membrane of human placental epithelial cells. Cl⁻/HCO₃⁻ exchange was tested for in preparations of purified brush border membrane vesicles by ³⁶Cl⁻ tracer flux measurements and determinations of acidine orange fluorescence changes. Under 10% CO₂/90% N₂ the imposition of an outwardly directed HCO₃⁻ concentration gradient (pH₆, 6/pH, 7.5) stimulated Cl⁻ uptake to levels approximately 2-fold greater than observed at equilibrium. Maneuvers designed to offset the development of ion gradient-induced diffusion potentials (valinomycin, K⁺ = K₀) significantly reduced HCO₃⁻ gradient-driven Cl⁻ uptake but concentrative accumulation of Cl⁻ persisted. Early time point determinations performed in the presumed absence of membrane potential suggests the reduced level of HCO₃⁻ gradient-driven Cl⁻ uptake resulted from a more rapid dissipation of the HCO₃⁻ concentration gradient. Concentrative accumulation of Cl⁻ was not observed in the presence of a pH gradient alone under 100% N₂, suggesting a preference of HCO₃⁻ over OH⁻ as a substrate for transport. As monitored by acidine orange fluorescence the Cl⁻ gradient-dependent collapse of an imposed pH gradient (pH₆, 8.5/pH, 6) was accelerated in the presence of CO₂/HCO₃⁻ when compared with its absence, indicating coupling of HCO₃⁻ influx to Cl⁻ efflux. Increasing concentrations of the anion exchange inhibitor 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid were observed to cause a stepwise reduction in HCO₃⁻ gradient-driven Cl⁻ uptake (150 - 25 μM) further suggesting the presence of a Cl⁻/HCO₃⁻ exchange mechanism. The results of this study provide evidence for a 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid-sensitive Cl⁻/HCO₃⁻ exchange mechanism in the maternal-facing membrane of human placental epithelial cells. The identification of an ion-coupled HCO₃⁻ transport pathway in placental epithelia may suggest functional roles in mediating transplacental transfer of CO₂ as well as maintenance of fetal acid/base balance.

The human placenta performs an important function in normal fetal development by serving as an interface for net transfer of carbon dioxide from fetal to maternal blood supplies. In this capacity the placenta may also function to maintain normal fetal acid/base status when confronted with changes in maternal blood pH or PCO₂ levels. Both of these processes require transfer of CO₂ as a gas or related species (H₂CO₃, HCO₃⁻), across the fetal-facing and/or maternal-facing membrane of syncytiotrophoblast cells. In its simplest form CO₂ transport across the syncytiotrophoblast may occur entirely as the highly permeable gaseous species moving down its fetal-maternal concentration gradient. Alternatively, gaseous CO₂ may enter the syncytiotrophoblast cell across its basal membrane where HCO₃⁻ is formed for export by mediated transfer across the maternal-facing brush border membrane. Intracellular conversion of CO₂ to HCO₃⁻ may be facilitated by base equivalents generated from a Na⁺/H⁺ exchange mechanism present in the brush border membrane (1). Although as yet unknown in humans, placental carbonic anhydrase activity has been measured in all species examined (rat, hamster, guinea pig, sheep, pig), which may further suggest the formation of HCO₃⁻ from CO₂ (2). Investigations of Cl⁻ transport pathways in human placental brush border membranes indicate the presence of a DIDS-sensitive Cl⁻ uptake and a DIDS-insensitive voltage-dependent Cl⁻ uptake which suggests the existence of both an electroneutral anion exchanger and a Cl⁻-coductive pathway (3, 4). These observations indicate the possible presence of a syncytiotrophoblast anion exchange mechanism at the maternal-facing membrane that may couple the favorable transmembrane Cl⁻ gradient to the efflux of intracellular HCO₃⁻. The presence of a Cl⁻-coductive pathway may serve to recycle Cl⁻ back across the brush border membrane thus preventing an increase in intracellular Cl⁻.

In an effort to determine a possible role for ion-coupled HCO₃⁻ transport pathways in transplacental CO₂ transfer and/or placental maintenance of fetal acid/base balance, the presence of a Cl⁻/HCO₃⁻ exchange mechanism was assessed using preparations of purified brush border membrane vesicles. Evidence supporting the existence of a Cl⁻/HCO₃⁻ exchange mechanism was obtained from ³⁶Cl⁻ tracer uptake studies and acidine orange fluorescence measurements.

EXPERIMENTAL PROCEDURES

Membrane Preparations—Brush border membrane vesicles were isolated from human term placenta by divalent cation aggregation and differential centrifugation as described previously (5). Briefly, the villous tissue of placenta obtained within 15 min of elective cesarean section was quickly dissected and minced into small (~ cm) fragments at 4 °C. The tissue fragments were rinsed three times in 300 mM mannitol, 10 mM HEPES/TMA, pH 7, and gently stirred for approximately 30 min using a Teflon spatula. The tissue suspension was filtered through cotton gauze, and phenylmethylsulfonyl fluoride was added to a final concentration of 0.2 mM. The filtrate

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1The abbreviations used are: DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TMA, N-trimethylammonium; MES, 4-morpholineethanesulfonic acid; TAPS, N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid.
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was centrifuged at 8,100 rpm for 15 min using an SS-34 rotor (Sorvall). The low speed pellet was discarded and the supernatant was centrifuged at 19,000 rpm for 40 min. The high speed pellet was gently resuspended and MgCl₂ was added to a final concentration of 12 mM. After incubating for 10 min the membrane suspension was centrifuged at 5,000 rpm for 15 min to pellet the Mg²⁺-induced aggregates. The low speed supernatant was centrifuged at 19,000 rpm for 40 min and the resulting pellet (brush border membrane vesicles) was resuspended and washed twice in buffers designated for each experiment. Membranes were stored frozen (−70 °C) and used within 2 weeks of preparation. The isolated membrane vesicles were enriched 25.4 ± 1.3 (S.E., n = 7)-fold in alkaline phosphatase activity (6) compared with homogenates of villous tissue. Membrane marker enzyme enrichments for the basal membrane (N₅₄/K₆₆ATPase), mitochondria (succinic dehydrogenase), and endoplasmic reticulum (NADH dehydrogenase) were 0.68 ± 0.05 (S.E., n = 7), 0.43 ± 0.02 (S.E., n = 7), and 0.34 ± 0.03 (S.E., n = 7), respectively (7-9). Protein was determined by a sodium dodecyl sulfate-Lowry assay using bovine serum albumin as the standard (10).

Isotopic Flux Measurements—Frozn (−70 °C) aliquots of membrane vesicles were thawed at room temperature and ionic solutions of appropriate ionic composition were added to obtain the desired intravesicular solution described for each experiment in the figure legends. The membrane suspension was incubated for 90 min at room temperature to attain transmembrane equilibration of the added media. During the pre-equilibration period the membranes were gassed continuously with humidified 100% N₂ or 90% N₂, 10% CO₂. The extravesicular media were prepared similarly, and the final composition for each experiment is given in the figure legends. Intravesicular ³²Cl content was assayed in triplicate at 37 °C in the continued presence of either 100% N₂ or 90% N₂, 10% CO₂ by a rapid filtration technique previously described (11). A metronome was used to determine Cl⁻ uptake values at 4 s or less (12). The uptake reaction was quenched by the rapid addition of 210 mM potassium gluconate, pH 5.0, at 4 °C. The diluted membrane suspension was passed through a 0.65-μm Millipore filter (DAWP) and washed with an additional 9 ml of the quench buffer. The process of quenching, filtration, and washing occurred routinely within a 15-sec period. The filters were dissolved in 3 ml of Ready-Solv HP (Beckman) and counted by scintillation spectroscopy. The timed uptake values obtained were corrected for the nonspecific retention of intravesicular fluorescence. While absolute Cl⁻ uptake values expressed per mg of membrane protein varied from membrane preparation to membrane preparation, relative changes resulting from experimental manipulations were highly reproducible.

Fluorescence Determinations of ΔpH—Changes in intravesicular pH in response to an imposed pH gradient (pH, 8.5/pH, 6) and the experimental conditions described were monitored by an acridine orange fluorescence spectrofluorimeter (13). Fluorescence was measured at excitation and emission wavelengths of 496 and 530 nm, respectively, with a bandpass of 3.6 nm. Equal aliquots of thawed membranes washed into either 165 mM KCl, 10 mM MES/TMA, pH 7.5, were kept at 4 °C. The diluted membrane suspension was passed through a 0.65-μm Millipore filter (DAWP) and washed with an additional 9 ml of the quench buffer. The process of quenching, filtration, and washing occurred routinely within a 15-sec period. The filters were dissolved in 3 ml of Ready-Solv HP (Beckman) and counted by scintillation spectroscopy.

RESULTS AND DISCUSSION

HCO₃⁻ Gradient-driven Cl⁻ Influx—The presence of a Cl⁻/HCO₃⁻ exchange mechanism in human placental brush border membrane vesicles would be suggested by the ability of a HCO₃⁻ concentration gradient to serve as a driving force for intravesicular concentrative Cl⁻ accumulation. The time course of intravesicular Cl⁻ accumulation (Cl⁻ = 4.15 mM) is illustrated.
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Fig. 3. Early time point determinations of HCO₃ gradient-driven Cl⁻ influx. Brush border membrane vesicles were pre-equilibrated as described in the legend to Fig. 1. Uptake of ³⁶Cl (4.15 mM) occurred from extravesicular solutions described in the legend to Fig. 1. Where indicated (+ VAL) membrane vesicles were preincubated with valinomycin (VAL) (0.17 mg/ml) for a minimum of 30 min. A representative experiment of three independent observations is illustrated.

Fig. 4. OH⁻ gradient-driven Cl⁻ influx. Brush border membrane vesicles were pre-equilibrated under 10% CO₂/90% N₂ as described in the legend to Fig. 1 for (pH, 6/pHi, 7.5 + CO₂/HCO₃) or under 100% N₂ with (pH, 6/pHi, 7.5 – CO₂/HCO₃) 110 mM TMA gluconate, 57.3 mM potassium gluconate, 52 mM mannitol, 45.3 mM HEPES, 23 mM TMA (OH⁻). Uptake of ³⁶Cl (4.15 mM) occurred from extravesicular solutions under 10% CO₂/90% N₂ as described in the legend to Fig. 1 for (pH, 6/pHi, 7.5 + CO₂/HCO₃) and (pH, 6/pHi, 7.5 + CO₂/HCO₃) containing DIDS at the concentrations shown. Membranes were preincubated with valinomycin (0.17 mg/ml) for a minimum of 30 min. The data are illustrated as the mean ± S.E. of three experiments, each performed using a different membrane preparation.

flux coupling consistent with the operation of a Cl/HCO₃ exchange mechanism. However, as the HCO₃ gradient-induced stimulation of Cl⁻ uptake may have resulted from indirect electrical coupling to an ion gradient (H⁺, OH⁻, HCO₃⁻)-induced inside positive diffusion potential, the nature of HCO₃ coupling to Cl⁻ uptake was examined by determining the effect of maneuvers designed to minimize membrane potential development. To the extent that Cl⁻ uptake was electrostatically coupled to ion gradient (H⁺, OH⁻, HCO₃⁻)-induced diffusion potentials, a reduced level of Cl⁻ uptake

in Fig. 1 as a function of an imposed transmembrane pH and HCO₃⁻ gradient. In the absence of an imposed HCO₃⁻ concentration gradient (pH, 6/pH, 6) Cl⁻ uptake was low and slowly approached an equilibrium value at 1.5 h. The imposition of an outwardly directed HCO₃⁻ gradient (pH, 6/pH, 7.5) resulted in a marked stimulation of Cl⁻ uptake accumulating to levels approximately 2-fold greater than observed at equilibrium. The concentrative accumulation of Cl⁻ noted in the presence of an outwardly directed HCO₃⁻ gradient suggests an anion
would be expected in the presence of charge compensating movements of K\(^+\) across valinomycin-treated membranes. Although, as shown in Fig. 2, the stimulation of Cl\(^-\) uptake measured in the presence of an outwardly directed HCO\(_3\) gradient was reduced in membranes incubated with valinomycin, the concentrative accumulation of Cl\(^-\) persisted. While these results may indicate the presence of a Cl\(^-\)-conductive pathway also identified in previous studies of placental brush border membranes (3, 4), Cl\(^-\) uptake was observed to exceed equilibrium levels when measured in the presumed absence of membrane potential differences. This finding suggests a direct chemical coupling of HCO\(_3\) efflux to Cl\(^-\) influx consistent with the existence of a Cl/HCO\(_3\) exchange mechanism.

The effect of short-circuiting membrane potential development to reduce HCO\(_3\) gradient-driven Cl\(^-\) uptake may have also occurred in part as a result of promoting a more rapid dissipation of the imposed HCO\(_3\) concentration gradient. To test this possibility measurements of HCO\(_3\) gradient-driven Cl\(^-\) uptake were performed at early time points in vesicles treated with and without valinomycin. As shown in Fig. 3 Cl\(^-\) uptake was essentially identical in the presence and absence of valinomycin at 1 s but thereafter was progressively reduced in short-circuited membranes. These data suggest that after only 1 s the imposed HCO\(_3\) gradient had not dissipated to levels where differences in Cl\(^-\) uptake may be distinguished between membranes treated with and without valinomycin. The similarity of 1-s Cl\(^-\) uptake values in the presence and absence of valinomycin would not be expected if the effect of blunting membrane potential development was entirely due to inhibition of conductive Cl\(^-\) uptake. We submit this observation as further evidence for the presence of Cl/HCO\(_3\) exchange mechanism by suggesting the decreased Cl\(^-\) uptake measured in short-circuited membrane vesicles resulted at least partly from a more rapid decay of the driving force for exchange.

In an effort to distinguish between HCO\(_3\) and OH\(^-\) as the preferred driving force for exchange with Cl\(^-\), measurements of Cl\(^-\) uptake by valinomycin-pertreated membranes were performed in the presence of the same pH gradient (pH, 6/pH, 7.5) gassed with and without CO\(_2\). As illustrated in Fig. 4 the imposition of an inside alkaline pH gradient in the nominal absence of CO\(_2\) induced only a small increase in Cl\(^-\) uptake compared with control values determined in the absence of a pH gradient (pH, 6/pH, 6). Notably, a concentrative accumulation of Cl\(^-\) was only observed in the presence of a pH gradient and CO\(_2\)/HCO\(_3\) which strongly suggests HCO\(_3\) as the preferred anion for exchange with Cl\(^-\).

**Fluorescence Studies of Cl\(^-\)/Gradient-driven HCO\(_3\) Influx**—The presence of a placental brush border membrane Cl/HCO\(_3\) exchange mechanism was investigated further by examining the reciprocal role relationship between Cl\(^-\) and HCO\(_3\) as the driving force for anion exchange. Not only should gradients of HCO\(_3\) drive Cl\(^-\) uptake as previously shown but a Cl\(^-\)-gradient-dependent accumulation of intravesicular HCO\(_3\) should also be observed to indicate the presence of Cl/HCO\(_3\) exchange. Using the fluorescent pH probe acridine orange, the rate of intravesicular alkalinization in response to an imposed pH gradient (pH, 8.5/pH, 6) was determined in the absence and presence of CO\(_2\)/HCO\(_3\). To the extent that a Cl/HCO\(_3\) exchange mechanism was operative in these membrane vesicles then the Cl\(^-\) gradient-dependent rate of intravesicular alkalinization would be greater in the presence than absence of CO\(_2\)/HCO\(_3\). The marked stimulation of Cl\(^-\)-gradient-dependent alkalinization conferred by the presence of CO\(_2\)/HCO\(_3\) as shown in Fig. 5 suggests Cl\(^-\) efflux is coupled to HCO\(_3\) influx consistent with the presence of a Cl/HCO\(_3\) exchanger.

**Effect of DIDS on HCO\(_3\) Gradient-driven Cl\(^-\) Influx**—Finally, the concentration-dependent inhibition of HCO\(_3\) gradient-driven Cl\(^-\) uptake by the Cl/HCO\(_3\) exchange inhibitor DIDS was assessed to verify further its existence in placental brush border membrane (14). Shown in Fig. 6 are 15-s Cl\(^-\) uptake values measured in the presence of an outwardly directed HCO\(_3\) gradient and extravesicular DIDS concentrations ranging from 10 to 250 μM. As would be expected for the presence of a Cl/HCO\(_3\) exchange mechanism HCO\(_3\) gradient-driven Cl\(^-\) uptake was decreased as a function of inhibitor concentration (I₅₀ ~ 25 μM).

In conclusion, the existence of a DIDS-sensitive, Cl/HCO\(_3\) exchange mechanism has been demonstrated in brush border membrane vesicles isolated from the maternal surface of human placental epithelial cells. The presence of this ion-coupled membrane transport pathway for HCO\(_3\) suggests a possible role in mediating transplacental transfer of CO\(_2\) from fetus to mother. The identified HCO\(_3\) transport pathway may be postulated to serve an additional function in maintaining fetal acid/base balance when the placenta is exposed to changes in maternal blood pH and pCO\(_2\) levels.

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