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Choline transport in human placental brush-border membrane vesicles

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Abstract

Pathways for transport of choline by human placental epithelia were investigated using brush border membrane vesicles isolated by divalent cation precipitation. The presence of choline transport mechanisms mediating Na^+-choline cotransport, choline/H^+ exchange and facilitated diffusion were assessed from [^{3}H]choline tracer flux measurements. The rate and magnitude of intravesicular choline accumulation was unaffected by the imposition of an inwardly directed Na^+ gradient suggesting an absence of a mechanism mediating brush border membrane Na^+-choline cotransport. The imposition of inside-acid or inside-alkaline pH gradients was observed to have no significant effect on choline uptake suggesting choline is not a substrate for placental epithelial organic cation/H^+ exchange. Conditions favoring the development of an inside-negative K^+ diffusion potential was observed to induce a concentrative accumulation of choline to levels exceeding equilibrium suggesting the presence of a conductive uptake pathway for choline in placental brush border membrane. Evidence to suggest conductive choline uptake resulted from a mediated transport process includes a demonstration of the counterflow phenomena, the concentration-dependent inhibition by hemicholinium-3 ($I_{C50} \cong 100\ \mu M$) and the saturable rate of conductive choline uptake ($K_m \cong 300\ \mu M$, $V_{max} \cong 30$ nmol/mg per min). Substrate specificity studies of the mechanism mediating conductive choline uptake suggest the interaction of choline with the transport protein occurs at a minimum of two sites: a site of negativity with the positively charged nitrogen group and a site of hydrogen bonding to the primary alcohol. Several commonly prescribed pharmaceuticals known to cross the placental barrier including imipramine, verapamil, propranolol, quinine, flurazepam, amiloride and ritodrin were observed to inhibit conductive choline uptake suggesting an interaction with the mechanism mediating conductive choline transport. Conductive choline uptake was unaffected by the presence of the basic amino acids lysine, arginine and histidine; the neurotransmitters serotonin, dopamine and histamine and the vitamins thiamine and carnitine which suggests the mechanism mediating conductive choline transport is not a pathway for placental uptake of these compounds.

Key words: Choline; Placental; Transport; Vesicle

1. Introduction

Normal growth and development of the human fetus is critically dependent upon an adequate delivery of maternal blood-borne nutrients across the placental epithelia. At the cellular level net maternal to fetal transfer of metabolites arises from the polarized distribution of membrane proteins mediating active and passive transport at the apical and basal membrane of syncytiotrophoblast cells. Typical of epithelial cells and well-suited to the purposes of transcellular transport, the apical or maternal surface of syncytiotrophoblast cells is amplified morphologically in the form of a brush-border. Similar to renal and intestinal epithelia the isolation as membrane vesicles of this morphologically specialized membrane has greatly facilitated the study of placental epithelial transport by the identification and characterization of transport pathways present at the maternal side of the syncytiotrophoblast [1]. In contrast, and do primarily to the only recently described methodologies for basal membrane vesicle isolation, the presence and nature of transport pathways at the fetal-facing membrane of the syncytiotrophoblast remain largely unknown [2,3]. The importance of mem-

Abbreviations: TMA, tetramethylammonium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholine)ethanesulfonic acid; HC-3, hemicholinium-3.

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brane vesicle studies to elucidating, in detail, the precise molecular mechanisms of placental epithelial transport pathways is underscored by the limited information available from studies using alternative experimental models of this epithelia. Toward the further definition of placental epithelial transport function we have conducted membrane vesicle studies designed to identify and characterize molecular mechanisms of organic and inorganic anion transport at the maternal-facing membrane of the syncytiotrophoblast [4–7]. More recently we have extended our study of placental epithelial transport function to include an investigation of the possible presence of an apical membrane transport pathway for the quaternary amine choline. Choline is an essential nutrient utilized by the developing fetus in three major biochemical pathways: (1) the synthesis of phosphatidyl choline and sphingomyelin which are major phospholipid constituents of cell membranes; (2) the synthesis of the neurotransmitter acetylcholine; (3) the conversion to betaine which in turn contributes methyl groups towards the synthesis of methionine [8]. Furthermore, recent evidence suggests de novo synthesis of choline by either the fetus or placenta as unlikely sources of fetal choline and that the fetal metabolic requirement for choline is met almost exclusively by transplacental transport from the maternal circulation [9]. Presently, little is known of the pathways for choline transport across the apical or maternal-facing membrane of placental epithelia. Accordingly, we have investigated the possible presence of three choline transport mechanisms in human placental brush-border membrane vesicles: Na^+-choline cotransport, H^+/choline exchange and mediated conductive choline transport. This report describes evidence suggesting the presence of a mediated conductive mechanism as the pathway for placental brush-border membrane choline uptake from the maternal circulation. The mechanism mediating conductive choline uptake is further characterized with regard to interaction with both endogenous and exogenous substrates.

2. Materials and Methods

2.1. Membrane preparations

Brush-border membrane vesicles were isolated from human term placenta by divalent cation aggregation and differential centrifugation as previously described [4,5]. Briefly, the villous tissue of placenta obtained within 15 min of elective caesarean section was quickly dissected and minced into small (~ 1 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 approx. 30 min using a motor-driven spatula. The tissue suspension was filtered through cotton gauze, and phenylmethylsulfonylfluoride was added to a final concentration 0.2 mM. The filtrate was centrifuged at 8100 rpm for 15 min using an SS-34 rotor (Sorvall). The low-speed pellet was discarded, and the supernatant was centrifuged at 19000 rpm for 40 min. The high-speed pellet was gently resuspended, and $MgCl_2$ added to a final concentration of 12 mM. After incubating for 10 min the membrane suspension was centrifuged at 5000 rpm for 15 min to pellet the Mg^{2+}-induced membrane aggregates. The low-speed supernatant was centrifuged at 19000 rpm for 40 min, and the resulting pellet (brush border membrane vesicles) 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 vesicle fraction was typically enriched 25.4 ± 1.3 (S.E., $n = 7$)-fold in alkaline phosphatase activity [10] compared to homogenates of villous tissue. Typical membrane marker enzyme enrichments for the basal membrane (Na^+/K^+-ATPase), 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 [11–13]. Protein was determined by a sodium dodecylsulfate-Lowry assay using bovine serum albumin as the standard [14].

2.2. Isotopic flux measurements

Frozen (−70°C) aliquots of membrane vesicles were thawed at room temperature and isosmotic solutions of appropriate ionic composition were added to obtain the desired intravesicular solution described for each experiment in the figure and table legends. The membrane suspension was incubated for 120 min at room temperature to attain transmembrane equilibration of the added media. The extravesicular media were prepared similarly, the final composition for each experiment is given in the figure and table legends. Intravesicular [^{3}H]choline content was assayed at least in triplicate at 37°C by a rapid filtration technique previously described [15]. Briefly, a small aliquot of media (40 μl or 95 μl) containing radiolabelled substrate was placed at the bottom of a glass test tube followed by positioning a 10 μl, or 5 μl aliquot of membrane suspension (100–200 μg) on the test tube wall immediately above the aliquot containing radiolabelled substrate. Vesicle uptake of radiolabelled substrate was initiated by rapidly mixing the two aliquots using a vortex and after a predetermined time interval the uptake reaction was quenched by rapid dilution with isosmotic potassium gluconate, 20 mM Hepes/K (pH 7.5) 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 quench buffer. The process of quenching, filtration and washing occurred routinely

within a 15s 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 by the nonspecific retention of isotope by the filters. While absolute choline uptake values expressed per mg protein varied from membrane preparation to membrane preparation, relative changes in choline uptake resulting from the conditions set forth in each experiment were highly reproducible. Where appropriate, statistical significance has been determined using unpaired t-test for two means where $P < 0.05$ is taken as a statistically significant value.

2.3. Materials

Valinomycin, choline, taurine, TMA, phosphocholine, L-carnitine, guanidine, acetazolamide, verapamil, propranolol, quinine, amiloride, ritodrine, procainamide, and terbutaline were purchased from Sigma. Ethanolamine, *N*,*N*-dimethylethanolamine, trimethylamine, dimethylamine, methylamine, γ-butyrobetaine, *S*-(−)-(3-chloro-2-hydroxypropyl)trimethylammonium, D,L-carnitinamide, 2-hydroxyethyltriethylammonium, butyrylcholine, benzoylcholine, hemicholinium-3, 1-(2-hydroxyethyl)quinolinium, were purchased from Aldrich. Acetylcholine, acetyl-L-carnitine, phenytoin, imipramine, flurazepam, lidocaine, disopyramide and glutethimide were purchased from Research Biochemicals. Trimethylphenylammonium and betaine were purchased from Kodak. Thiocholine was purchased from K and K Laboratories. Carbamylcholine was purchased from Mann Research Laboratories. Thalidomide was a gift from Dr. G. Vogelsang, Johns Hopkins Oncology Center, Baltimore, MD. Methacholine and homocholine were gifts from Dr. O.M. Brown, Department of Pharmacology, SUNY Health Science Center, Syracuse, New York. [^{3}H]Choline was obtained from New England Nuclear. Valinomycin was solubilized in 95% ethanol and added to the membrane suspension in a 1:100 dilution. Equivalent volumes of ethanol were added to control aliquotes of membrane. All solutions were prepared with distilled-deionized water and filtered through 0.22 μm Millipore Filters.

3. Results

Membrane transport pathways for choline at the apical or maternal-facing side of human placental epithelial cells was first investigated by testing for the possible presence of Na^+-choline cotransport. The presence of a brush-border membrane Na^+-choline cotransport mechanism was assessed by determining the ability of an imposed, inwardly directed Na^+ concentration gradient to serve as a driving force for intravesicular choline accumulation. As shown in Table 1 the uptake of choline in the presence of an inwardly directed Na^+ gradient was essentially indistinguishable from choline uptake measured in the absence of a cation gradient where $K_o^+ = K_i^+$. Furthermore, no significant difference in choline uptake was observed when measured in the presence of a Na^+gradient or gradients of Li^+ and *N*-methyl-D-glucamine. The inability of an inwardly directed Na^+ gradient to increase intravesicular choline accumulation beyond the levels observed in the absence of cation gradient or in the presence of substitute cation gradients suggests placental brush-border membrane choline uptake is not coupled to Na^+ via a Na^+-choline cotransport mechanism.

Table 1
Effect of cation concentration gradients on choline influx (values in pmol/mg)

$K_o^+ = K_i^+$	$Na_o^+ > Na_i^+$	$Li_o^+ > Li_i^+$	*N*-methyl-D-glucamine$_o$ > *N*-methyl-D-glucamine$_i$
72±5	78±8	80±6	92±9

Brush border membrane vesicles were pre-equilibrated with 125 mM KCl, 50 mM mannitol, 20 mM Hepes/K (pH 7.5). The 15 s uptake of choline (25 μM) occurred from extravesicular solutions containing 100 mM (Na, Li, K, *N*-methyl-D-glucamine) Cl, 25 mM KCl, 50 mM mannitol, 20 mM Hepes/K (pH 7.5). The mean±S.E. choline uptake was obtained from three separate membrane preparations.

The identity of the placental brush-border membrane choline transporter was investigated further by testing for the presence of a mechanism mediating proton for choline exchange. The possible presence of a brush-border membrane H^+/choline exchange mechanism was assessed by determining the ability of an imposed proton gradient to serve as a driving force for transmembrane influx and efflux of choline. As shown in Table 2, compared to the absence of a pH gradient at pH 6 or pH 7.5 an inside-acid pH gradient resulted in a small but insignificant increase in choline uptake. Similarly, no significant difference was observed when comparing choline uptake measured in the presence of an inside-alkaline pH gradient to ei-

Table 2
Effect of proton concentration gradients on choline influx (values given in pmol/mg)

pH_o 6/ pH_i 6	pH_o 7.5/ pH_i 6	pH_o 7.5/ pH_i 7.5	pH_o 6/ pH_i 7.5
67±5	83±5	71±5	63±10

Brush-border membrane vesicles were pre-equilibrated with 125 mM KCl, 52 mM Mes, 42 mM Hepes/K (pH 6) or 125 mM KCl, 52 mM mannitol, 42 mM Hepes/K (pH 7.5). The 15s uptake of choline (25 μM) occurred from extravesicular solutions containing 125 mM KCl and where pH_o 6 = pH_i 6, 52 mM Mes, 42 mM Hepes/K; pH_o 7.5/pH_i 6, 47 mM mannitol, 5 mM Mes, 42 mM Hepes/K; pH_o 7.5/pH_i 7.5, −52 mM mannitol, 42 mM Hepes/K; pH_o 6/pH_i 7.5, 52 mM mannitol, 38 mM Mes, 4 mM Hepes/K. The mean±S.E. choline uptake was obtained from measurements using three different membrane preparations.

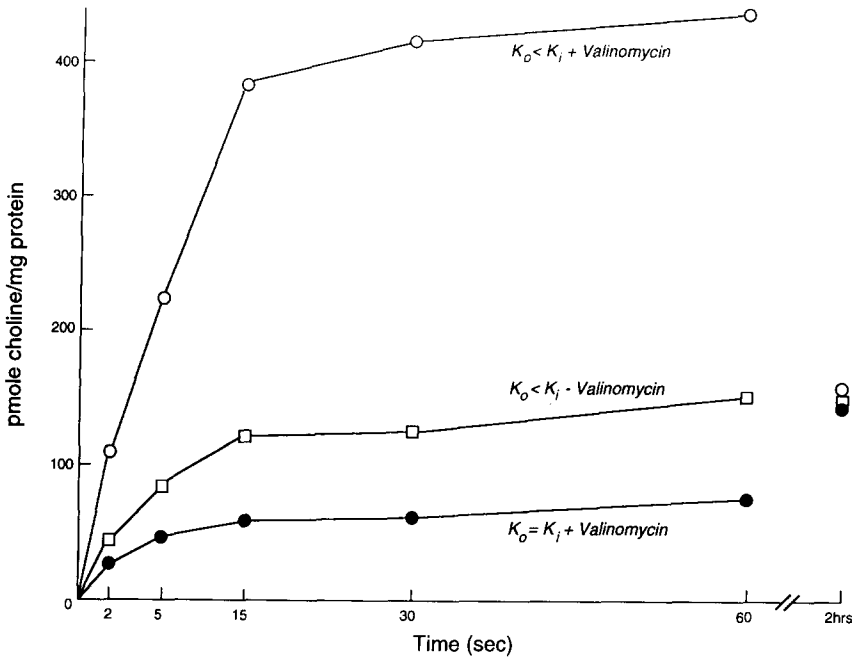

Fig. 1. Effect of membrane potential on choline influx. Brush border membrane vesicles were pre-equilibrated with 150 mM potassium gluconate, 20 mM Hepes/K (pH 7.5). Uptake of choline (25 μM) occurred from extravesicular solutions containing $K_o = K_i$-150 mM potassium gluconate, 20 mM Hepes/K (pH 7.5) or $K_o < K_i$-142.5 mM *N*-methyl-D-glucamine gluconate, 7.5 mM potassium gluconate, 20 mM Hepes/K (pH 7.5). Where indicated membranes were incubated with valinomycin (0.25 mg/ml) or an equivalent volume of ethanol for a minimum of 30 min. A representative experiment of three independent observations each performed with a different membrane preparation is shown.

ther the absence of a pH gradient or an inside-acid pH gradient. While consistent with the operation of a H^+/choline exchange mechanism the level of choline uptake resulting from imposed inside-acid and inside-alkaline pH gradients was not sufficiently increased or decreased respectively to indicate the presence of a direct chemical coupling of H^+ to choline transport. Perhaps more consistent with the observed levels of pH gradient-induced choline uptake is the possible indirect coupling of choline uptake via a conductive pathway to inside negative and positive proton gradient-induced voltage differences. The later possibility is supported

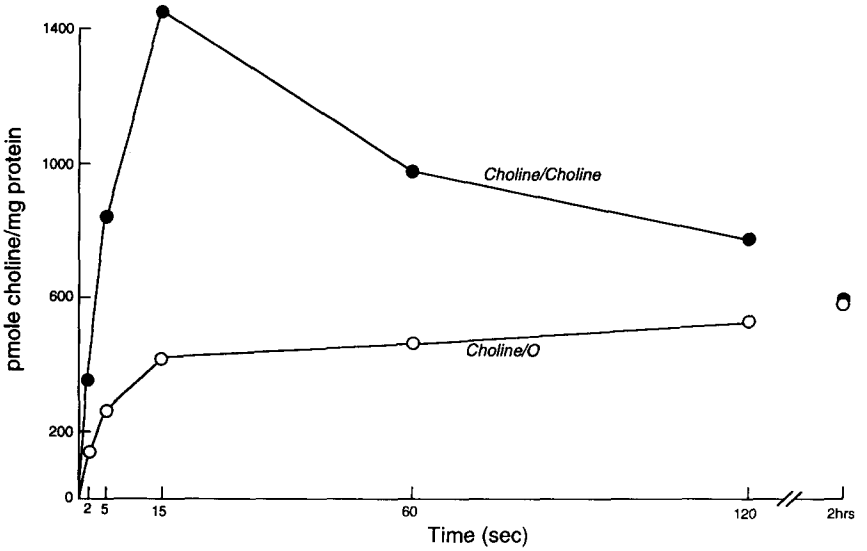

Fig. 2. Effect of intravesicular choline on choline influx. Brush border membrane vesicles were pre-equilibrated with 125 mM KCl, 50 mM mannitol, 20 mM Hepes/K (pH 7.5) and 5 mM choline Cl (choline/choline) or 5 mM KCl (choline/o). Uptake of choline (250 μM) occurred from an extravesicular solution containing 125 mM KCl, 50 mM mannitol, 20 mM Hepes/K (pH 7.5). Membrane vesicles were preincubated with valinomycin as described in the legend to Fig. 1. A representative experiment of three independent observations is illustrated.

by a description of the intrinsic proton permeabilities, mediated or otherwise, of placental brush-border membrane vesicles [16].

To the extent that a conductive pathway for choline is present in placental brush-border membrane then an inside-negative, valinomycin-induced, K^+ diffusion potential should serve as a driving force for concentrative accumulation of intravesicular choline. As shown in Fig. 1 in the presumed absence of a transmembrane voltage difference where intra- and extravesicular K^+ was equal choline uptake by valinomycin pretreated membranes was low and slowly approached an equilibrium value measured at 2 h. The imposition of an outwardly directed K^+ gradient resulted in a marked stimulation of choline uptake by membranes pretreated with and without valinomycin but only exceeded equilibrium in membranes made sufficiently K^+ permeable by addition of valinomycin. This observation suggests an inside-negative voltage difference may serve as a driving force for the concentrative accumulation of intravesicular choline via a conductive uptake pathway. The observed coupling of choline uptake to an inside-negative voltage difference may have occurred as a result of a mediated choline transport process or via some form of 'leak' pathway possibly introduced during membrane vesicle preparation. An attempt was made to distinguish between these two possibilities by testing the effect of trans choline on the rate and magnitude of radiolabeled choline uptake. Membrane vesicles were preloaded with or without 5 mM choline and diluted 20-fold into an extravesicular solution containing, in both instances, 250 μM radiolabeled choline. As shown in Fig. 2, compared to those vesicles initially devoid of intravesicular choline both the rate and magnitude of radiolabelled choline uptake was markedly stimulated into vesicles where a large outward choline concentration gradient was initially imposed. The observed transtimulation of radiolabeled choline uptake by intravesicular choline demonstrates the property of counterflow, a membrane transport phenomena associated with the presence of a carrier mediated transport process [17,18]. Importantly, this observation further suggests conductive choline uptake occurs by a mediated transport process and is not an artifact of membrane vesicle preparation.

The properties of brush-border membrane conductive choline transport were next assessed with regard to its choline transport inhibitor sensitivity as a second criteria used to evaluate the presence or absence of mediated choline transport. The concentration-dependent inhibition of conductive choline uptake by hemicholinium-3, a well-known inhibitor of choline transport in a variety of cell types [19] is shown in Fig. 3. In keeping with the properties expected for a mediated transport process conductive choline uptake was progressively reduced in the presence of increasing concentrations of hemicholinium-3 ($IC_{50} \cong 100$ μM).

A further characterization of the mechanism mediating conductive choline transport included a determination of its kinetic properties as estimated from initial

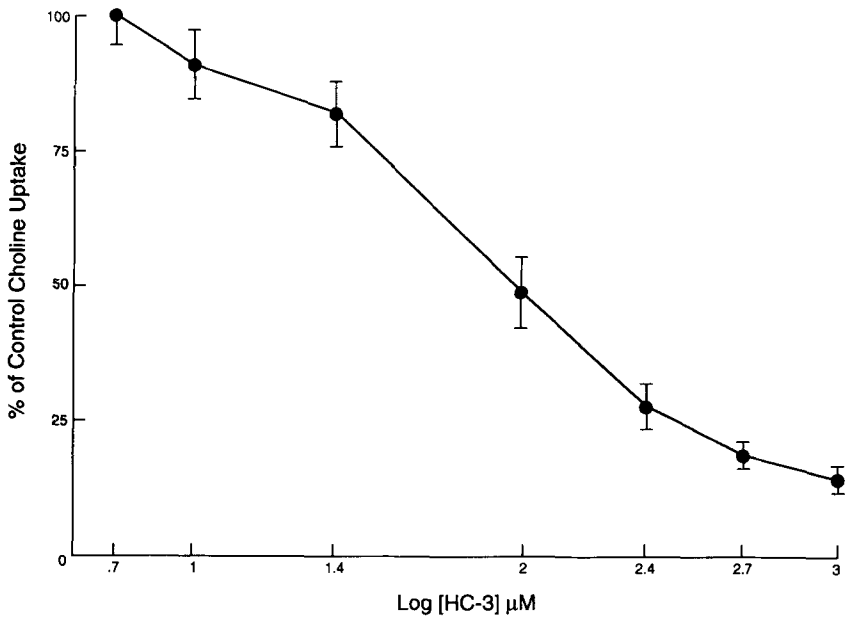

Fig. 3. Effect of hemicholinium-3 on conductive choline influx. Brush border membrane vesicles were pre-equilibrated with 150 mM potassium gluconate, 20 mM Hepes/K (pH 7.5). The 2 s uptake of choline (25 μM) was measured from extravesicular solutions containing 142.5 mM *N*-methyl-D-glucamine gluconate, 7.5 mM potassium gluconate, 20 mM Hepes/K (pH 7.5) and HC-3 concentrations of 5 μM, 25 μM, 100 μM, 250 μM, 500 μM and 1000 μM. Membrane vesicles were preincubated with valinomycin as described in the legend to Fig. 1. The mean ± S.E. of four experiments each performed with a different membrane vesicle preparation is shown. Control choline uptake was 100 ± 16 pmol/mg.

rates of choline uptake. The data shown in Fig. 4 illustrates the relationship between the initial rate of choline uptake and the extravesicular choline concentration. Again, consistent with the presence of mediated transport, conductive choline uptake demonstrates the property of substrate saturability as the initial rate of choline uptake is observed to approach a constant value at approx. 2 mM choline. A Hanes-Woolf replot of the data also shown in Fig. 4, is linear which suggests that choline interacts with the transporter at a single saturable site with a K_m value of approx. 300 μM and which maximally transports choline at an approximate rate of 30 nmol/min per mg of protein.

The requirements for recognition of transportable or interactive substrates by the transport protein mediating conductive choline influx were investigated by testing the ability of various chemical analogues of choline to block occupancy and, therefore, uptake of radiolabeled choline. Choline analogues with chemical modifications of the primary alcohol side chain indicated the possible importance of hydrogen bonding to a substrate binding site via the terminal hydroxyl group. Thus, as shown in Table 3, the presence of amine analogues closely resembling choline but lacking a terminal hydroxyl group such as thiocholine, phosphocholine, betaine, γ-butyrobetaine, L-carnitine and *S*-(−)-(3-chloro-2-hydroxypropyl)trimethylammonium did not significantly reduce the level of conductive choline uptake compared to control. Interestingly, a second set of amine analogues also lacking a terminal hydroxyl group including acetylcholine, methacholine, benzylcholine, butyrylcholine and carbamylcholine was observed to decrease conductive choline uptake by approx. 20–40% which indicates a limited interaction with the transport protein and may reflect subtle differences among these compounds to engage a substrate binding site by hydrogen bonding. Also notable was the reduced level of inhibition measured in the presence of homocholine compared to the level measured in the presence choline which suggests the distance between the positively charged amine and the terminal hydroxyl group is important for permitting optimal orientation to their respective sites of interaction within the transport protein.

An additional determinant for interaction of choline with the transport protein was the number and size of alkyl side chains bonded to the positively charged nitrogen group. As shown in Table 3 the amine analogues ethanolamine and *N*,*N*-dimethylamine, which closely resemble choline except for fewer methyl carbon-nitrogen bonds, reduced conductive choline uptake to levels lower than observed in the presence of choline suggesting a greater affinity of the amine binding site for a less-alkylated nitrogen group. This characterization of the choline transporter amine binding site is further suggested by the observed inability of (2-hydroxyethyl)triethylammonium, a choline analogue with increased nitrogen group alkylation, to reduce conductive choline uptake.

The substrate specificity of the placental choline transport pathway was studied further with regard to possible interaction with therapeutically relevant, charged and uncharged heterocyclic amines. As shown

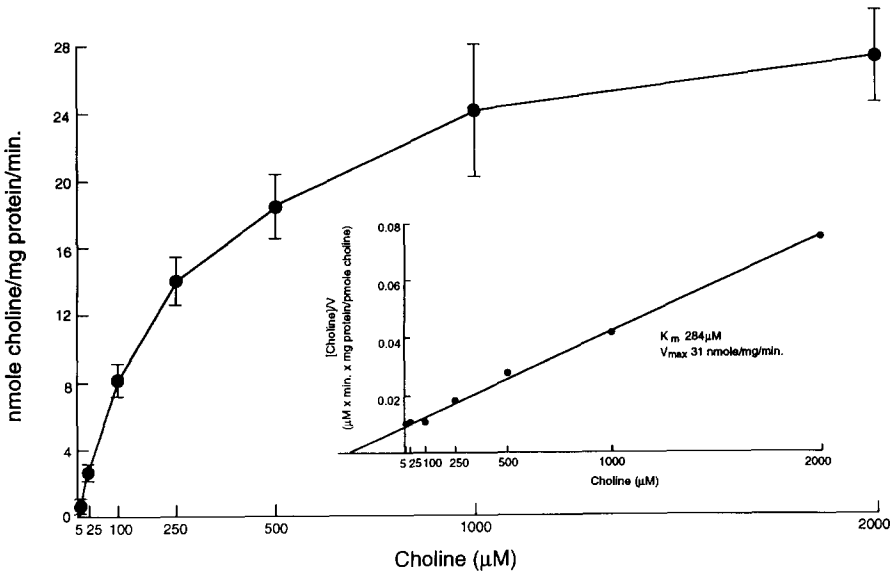

Fig. 4. Kinetics of conductive choline influx. Brush border membrane vesicles were pre-equilibrated with 150 mM potassium gluconate, 20 mM Hepes/K (pH 7.5). Choline uptake (2 s) was measured from extravesicular solutions containing 142.5 mM *N*-methyl-D-glucamine gluconate, 7.5 mM potassium gluconate, 20 mM Hepes/K (pH 7.5) and the choline concentrations shown. Mem-brane vesicles were preincubated with valinomycin as described in the legend to Fig. 1. The mean ± S.E. of four experiments each performed with a different membrane preparation is illustrated.

at the top of Table 4, conductive choline uptake was essentially unaffected by the presence of compounds such as phenytoin, acetazolamide, thalidomide and glutethimide which possess no net positive charge. In contrast, a marked inhibition of conductive choline uptake was noted when measured in the presence of compounds which do possess positively charged amino groups including imipramine, verapamil, propranolol, quinine, amiloride, flurazepam and ritodrine. However, a small subset of positively charged drugs including procainamide, lidocaine, disopyramide and terbutaline which may be further distinguished by the extent of alkylation on or close the nitrogen group, was observed to have either no effect or to reduce conductive choline minimally. Finally, evidence obtained from similar experiments (data not shown) further suggests other biologically relevant amines such as the neurtransmitters dopamine, histamine and serotonin; the vitamin thiamine and the basic amino acids lysine, arginine and histidine are not substrates for the conductive choline transport mechanism present in placental brush border membrane.

Table 3
Substrate specificity of conductive choline influx

Substrate	Choline Uptake /Control (%)
Methylamine	105 ± 8
Dimethylamine	108 ± 7
Trimethylamine	96 ± 4
Tetramethylammonium	93 ± 12
Trimethylphenylammonium	55 ± 7
Ethanolamine	11 ± 2
Taurine	98 ± 4
N,N-Dimethylethanolamine	6 ± 1
Choline	35 ± 4
Homocholine	63 ± 6
Thiocholine	101 ± 8
Phosphocholine	101 ± 1
Acetylcholine	68 ± 7
Methacholine	65 ± 2
Butyrylcholine	83 ± 6
Benzylcholine	75 ± 7
Carbamylcholine	56 ± 6
Betaine	106 ± 5
γ-Butyrobetaine	103 ± 8
L-Carnitine	117 ± 9
Acetyl-L-carnitine	120 ± 2
DL-Carnitinamide	105 ± 6
S-(−)-(3-Chloro-2-hydroxyl propyl) trimethylammonium	100 ± 1
(2-Hydroxyethyl)triethylammonium	108 ± 4
1-(2-Hydroxyethyl)quinolinium	78 ± 3
Guanidine	112 ± 5

Brush-border membrane vesicles were pre-equilibrated with 150 mM potassium gluconate, 20 mM Hepes/K (pH 7.5). The 2 s uptake of choline (25 μM) was measured from extravesicular solutions containing 142.5 mM *N*-methyl-D-glucamine gluconate, 75. mM gluconate, 20 mM Hepes/K (pH 7.5) and 500 μM of the compounds listed below. The data are shown as the percent of choline uptake measured in the absence of test compounds (129 ± 15 pmol/mg protein). The mean ± S.E. of seven experiments, each performed with a different membrane preparation is shown.

Table 4
Drug interaction of conductive choline influx

Substrate	Choline Uptake /Control (%)
Phenytoin	102 ± 7
Acetazolamide	101 ± 7
Thalidomide	97 ± 5
Glutethimide	82 ± 3
Imipramine	10 ± 2
Verapamil	26 ± 1
Propranolol	30 ± 2
Quinine	33 ± 3
Amiloride	44 ± 3
Flurazepam	43 ± 3
Ritodrine	47 ± 1
Procainamide	95 ± 4
Lidocaine	85 ± 8
Disopyramide	75 ± 7
Terbutaline	92 ± 6

Pre-equilibration of brush-border membrane vesicles and measurement of choline influx is described in the legend to Table 3. The data are shown as the percent of choline uptake measured in the absence of the test compounds (134 ± 25 pmol/mg protein). The mean ± S.E. of four experiments, each performed with a different membrane preparation is shown.

4. Discussion

The present study was conducted as an effort toward the identification and characterization of membrane transport pathways mediating placental epithelial uptake of choline from the maternal circulation. An interest in defining the functional properties of the mechanism(s) mediating placental choline uptake at the maternal-facing brush border membrane and efflux at the fetal-facing basal membrane arises from an apparent inability of the fetus or the placenta to undertake de novo synthesis of choline and, therefore, the fetal requirement for this essential nutrient must be met by transplacental transfer. The possible presence of three choline transport mechanisms mediating Na^+-choline cotransport, H^+/choline exchange and conductive choline uptake was assessed from studies of radiolabelled choline uptake by isolated placental brush border membrane vesicles. Among the three choline transport mechanisms examined sufficient evidence was obtained to suggest the presence of a mediated conductive uptake pathway in the placental brush border membrane.

The uptake of choline from the synaptic cleft of cholinergic neurons occurs largely as a result of a Na^+-choline cotransport mechanism with high affinity for choline and present in the presynaptic membrane [19]. Given this precedent for the existence of a mecha-

nism coupling Na^+ and choline transport in nerve tissue we first investigated pathways for placental brush border membrane choline transport by testing for the possible presence of a Na^+-choline cotransporter. To the extent that a Na^+-choline cotransport mechanism was present in placental brush border membranes then the imposition of an inwardly directed Na^+ concentration gradient should serve to drive intravesicular choline accumulation to levels significantly beyond those observed in the absence of a Na^+ gradient or in the presence of substitute cation gradients. The data shown in Table 1 indicate the imposition of an inwardly directed Na^+ gradient has no effect on the magnitude of brush border membrane choline uptake and, therefore, suggests the absence of a Na^+-choline cotransport mechanism coupling Na^+ and choline influx in placental brush border membrane. The absence of a placental brush border membrane Na^+-choline cotransport mechanism suggested by the results of these studies performed using membrane vesicle preparation is consistent with previous observations made from studies of choline transport in human term placental fragments [20,21] and the perfused guinea-pig placenta [22]. Where the possible coupling of Na^+ to choline transport has been investigated in other non-neuronal tissues such as the renal proximal tubule [23] or the red blood cell [24] no evidence for Na^+-choline cotransport was obtained.

Placental brush border membrane pathways mediating choline transport was next investigated by testing for the possible presence of a mechanism mediating the coupled exchange of choline for protons. Previous membrane vesicle studies of organic cation transport across the apical membrane of renal proximal tubule cells have demonstrated the presence of an organic cation/proton exchange mechanism for which choline is a substrate [25]. Furthermore, the presence of a placental epithelial organic cation/proton exchange mechanism has been described from membrane vesicle studies using guanidine as a model organic cation [26]. Accordingly, we have assessed the possible presence of an organic cation/proton exchange mechanism as a pathway for placental epithelial choline uptake by determining the ability of imposed proton gradients to serve as a driving force for intravesicular choline accumulation. The data shown in Table 2 indicate no significant effect of inwardly or outwardly directed proton gradients on choline uptake compared to the absence of a proton gradient at pH 6 or pH 7.5. The observed inability of an imposed proton gradient to stimulate influx or efflux of choline further indicates a lack of direct chemical coupling between protons and choline and thus suggests the absence of a transport mechanism mediating choline for proton exchange. This finding is also consistent with the observed inability of choline to compete with guanidine for occupancy of interactive sites within the placental brush border membrane organic cation/proton exchanger [26]. Although no significant effect of an imposed pH gradient on choline uptake was observed, our attention was drawn to the small difference in choline uptake when measured in the presence of an inward compared to an outward proton gradient. In an attempt to minimize the rate of pH gradient dissipation the effect of inward and outward directed pH gradients on choline uptake was determined in the absence maneuvers designed to minimize proton gradient-induced diffusion potentials. This suggests the small differences in choline uptake noted in the presence of inward and outward pH gradients may have resulted from the generation of inside positive and negative membrane potentials and the possible presence of a conductive uptake pathway for choline. The precedent for mediated conductive choline transport has been established from studies of renal inner medulla collecting duct cells [27], renal proximal tubules [28] and renal cortical brush border [23] and basolateral membrane vesicles [29]. Accordingly, placental brush border membrane transport pathways for choline were further investigated by assessing the possible presence of a mechanism mediating conductive choline uptake. To the extent that a conductive pathway for choline is present in placental brush border membrane vesicles then a valinomycin-induced, inside-negative K^+ diffusion potential would be expected to serve as a driving force for the concentrative accumulation of choline. Compared to the level of choline uptake by valinomycin pretreated membranes in the presumed absence of a transmembrane voltage difference where $K_o^+ = K_i^+$, a marked stimulation of choline uptake, achieving levels well above chemical equilibrium, was observed in valinomycin pretreated membranes when measured in the presence of an outward K^+ gradient where the development of an inside-negative voltage difference would be expected. Although these results clearly demonstrate the presence of a placental brush border membrane conductive uptake pathway for choline, the question arises as to whether choline uptake occurred via mediated transport or some form of nonmediated 'leak' pathway. We sought to distinguish between these two possibilities by assessing the properties of conductive choline uptake with regard to inhibitor sensitivity, substrate saturability and the ability to demonstrate the phenomena of counterflow. Whereas conductive choline uptake by a nonmediated 'leak' pathway would be expected to be inhibitor-insensitive, the presence of a mediated conductive mechanism should demonstrate the property of inhibitor sensitivity. The observed concentration-dependent inhibition of placental brush border membrane conductive choline uptake by hemicholinium-3 ($IC_{50} \cong 100\ \mu M$), a well-known inhibitor of choline transport in many different tissues, suggests the pres-

ence of a mediated conductive uptake mechanism for choline in this epithelia [19]. Interestingly, the HC-3 inhibitor sensitivity of conductive choline uptake determined here in placental brush border membrane is remarkably similar to the HC-3 inhibition of choline uptake observed in human term placenta fragments [30], the in vivo perfused guinea pig [22] and renal brush border membrane vesicles [24].

The property of substrate saturability was assessed as a second criteria by which to judge the presence of a mediated conductive choline uptake pathway in placental brush border membrane. Consistent with the presence of a mediated transport mechanism, the estimated initial rate of conductive choline uptake was observed to approach a maximum at a choline concentration of approx. 2 mM. A kinetic replot of these data occurs as a straight line suggesting the mechanism mediating placental brush border membrane conductive choline transport interacts with choline at a single saturable site with a K_m value of approx. 300 μM and a maximal transport rate of approx. 30 nmol/mg per min. The K_m value of the conductive uptake mechanism for choline determined from the present investigation using membrane vesicles compares closely to the K_m value previously described from a kinetic analysis of choline uptake by human term placental fragments [20].

The phenomena of counterflow is a third criteria by which to assess the possible presence of a mediated pathway for conductive choline uptake in placental brush border membranes. Counterflow results from the trans-stimulation of radiolabeled substrate accumulation in the pre-sence of a large outward gradient of unlabeled substrate and may be explained mechanistically in theoretical terms by assuming the presence of a 'mobile carrier' which moves across the membrane more rapidly in the loaded than unloaded state [17,18]. The stimulation of radiolabeled choline uptake measured in the presence compared to the absence of an outward gradient of unlabelled choline show in Fig. 2 demonstrates counterflow, a membrane transport phenomena consistent with the presence but not the absence of mediated choline transport. A similar trans-stimulation of radiolabelled choline uptake by gradients of unlabeled choline has also been reported in recent studies of electrogenic choline uptake by renal brush border membrane vesicles [23]. Thus, the evidence obtained from counterflow experiments, when considered together with the observed inhibitor sensitivity and substrate saturability of conductive choline uptake, strongly suggests the presence of a mediated conductive mechanism for choline in placental brush border membranes.

An attempt was made to define the chemical determinants of substrates which permit interaction with the substrate binding sites within the mechanism mediating conductive choline transport. Consistent with the presence of a substrate binding site capable of hydrogen bonding, chemical analogues of choline lacking a terminal hydroxyl group were either without effect or competed poorly with choline for occupancy within the transporter. A similar finding was made from studies of placental choline transport using the dually perfused placenta of guinea-pig in which the presence of betaine was observed to have no effect on placental choline uptake [22]. Interestingly, substrate specificity studies of electrogenic choline uptake in renal brush border membrane vesicles also demonstrated a requirement for the primary alcohol which may suggest both the renal and placental choline transporter possess a similar site for hydrogen bonding to substrate [23]. As suggested by Wright et al. [23] the site of hydrogen bonding may be the carbonyl oxygen of an amide associated with arginine, glutamine or a peptide bond. Substrate specificity studies of placental conductive choline uptake also revealed the degree of nitrogen group alkylation as a second chemical determinant important for recognition of transportable substrates. Whereas ethyl for methyl group substitution at all three alkyl bonding sites on the nitrogen was prohibitive for interaction with the choline transporter, decreased methylation of the nitrogen group resulted in an apparent increased affinity of the transporter for substrate. The mechanism mediating choline transport in guinea-pig placenta would appear to share a similar specificity with regard to nitrogen group alkylation as both ethanolamine and dimethylethanolamine were observed to decrease choline uptake in this species as well [22]. Similar to the placental choline transporter, the degree of nitrogen group alkylation was also determined to be important for substrate interaction with the mechanism mediating electrogenic choline transport in renal brush border membranes [23]. Both the renal and placental choline transporter demonstrated a similar pattern of sensitivity to inhibition by chemical analogues of choline differing in nitrogen group alkylation with the exception that ethanolamine inhibited choline transport in placenta but not in kidney. Thus, in addition to possessing a substrate binding site capable of hydrogen bonding to the primary alcohol of choline, the choline transport protein would also appear to interact with substrate at a second site which identifies positive charge and is sensitive to the degree of alkylation about the positive charge. Furthermore, the decreased ability of homocholine to compete with choline for occupancy within the transporter also suggests the molecular distance separating the positively charged nitrogen group from the primary alcohol as a third determinant limiting either access to or interaction with the two substrate binding sites. Having identified a number of chemical determinants important for substrate interaction with the choline transport pro-

tein, we sought to further characterize the choline transporter with regard to interaction with several exogenous and endogenous compounds. Among the exogenous compounds examined were a number of drugs commonly prescribed during pregnancy which inhibited conductive choline transport including: the tricyclic antidepressant, imipramine; the antihypertensive, verapamil; the β-adrenergic receptor blocker, propranolol; the antimalarial agent, quinine; a benzodiazopene, flurazepam, prescribed for insomnia; the diuretic, amiloride and the β_2-receptor agonist, ritodrine which is given to prevent preterm labor. These in vitro observations of drug interaction with the mechanism mediating placental choline uptake suggests not only possible effects of these compounds on maternal-to-fetal delivery of choline but also the choline transport mechanism as a possible pathway for maternal-to-fetal drug transfer. Interestingly, neither thiamine nor carnitine, two vitamins essential for normal fetal development, were observed to inhibit placental brush border membrane choline uptake which is in contrast to the finding obtained for thiamine from studies of perfused guinea-pig placenta [22] and suggests alternate transport pathways for these vitamins in human placenta. The observed inability of the basic amino acids lysine, arginine and histidine to reduce placental brush border choline uptake suggests the mechanism mediating conductive choline transport is not the pathway for placental basic amino acid absorption from the maternal circulation. Finally, the neurotransmitters dopamine, serotonin and histamine were also observed to be without effect on placental brush border membrane choline uptake which further distinguishes the mechanism mediating conductive choline uptake from those placental brush border transport pathways mediating Na and Cl dependent catecholamine transport [30] and organic cation/proton exchange [26].

The evidence obtained from the present investigation of mechanisms mediating placental epithelial choline uptake at the maternal-facing, brush border membrane strongly support the presence of a mediated conductive pathway for choline absorption from the maternal circulation. Thus, to the extent that the choline transport mechanism identified is the only pathway for placental choline uptake then placental choline accumulation from the maternal circulation would appear to be passive and limited by the electrochemical potential for choline across the brush border membrane. The presence of a mediated conductive pathway for choline in placental brush border membrane is remarkably consistent with observations made from the single previous study of choline accumulation by human term placental fragments [20]. Choline uptake by human placental fragments was observed to be increased as a result of substituting sucrose but not Li^+ for Na^+ in the extracellular buffer. The stimulation of choline uptake noted in the presence of extracellular sucrose but not Li^+ suggests the increased accumulation of choline was due to membrane hyperpolarization secondary to a net efflux of positive charge from cells bathed in sucrose but not Li^+. Similarly, choline accumulation by placental fragments was sequentially reduced by stepwise increments in extracellular K^+ which suggests choline uptake is inhibited by membrane potential depolarization. Finally, the observed inhibition of choline uptake by placental fragments incubated with metabolic poisons or ouabain is also consistent with a dependence of choline accumulation on membrane potential.

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