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RESEARCH PAPER

Novel Choline Transport Characteristics in Caco-2 Cells

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ABSTRACT

Choline transport is characterized by sodium-dependent high-affinity, sodium-independent low-affinity, and sodium-independent blood–brain barrier transport mechanisms. Each defined mechanism has specific characteristics with regard to affinity for choline, transport capacity, and inhibition by hemicholinium. The purpose of this study is to determine the characteristics of choline transport across Caco-2 monolayers. ***Methods.*** *Choline transport across Caco-2 cell monolayers was determined in both the apical to basal direction and the opposite direction. Further, the determination of calcium dependence and specific inhibitors was made. Determination of the apparent permeability of choline was calculated by established methods.* ***Results.*** *The apical to basal Caco-2 permeability coefficient is* $11.11 \pm 0.33 \times 10^{-6}$ *cm/sec with 21.3% of the choline associating with the cells. Meanwhile the basal to apical value is approximately 50% less (*$5.55 \pm 0.14 \times 10^{-6}$ *cm/sec), suggesting an active apical to basal transport mechanism. Choline transport in this system was inhibited by nifedipine (82%), verapamil (80%), EGTA (36%), and cyclosporin (15%).* ***Conclusions.*** *Choline transport across Caco-2 cells is demonstrated to be active and both pH- and* Ca^{2+}*-dependent. Furthermore, choline transport across Caco-2 monolayers has unique characteristics when compared to traditional choline transport models.*

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INTRODUCTION

Choline is a precursor for both phosphatidylcholine, an essential phospholipid in eukaryotic cell membranes, and the neurotransmitter acetylcholine.[1] While humans can synthesize choline *de novo*, dietary choline is considered essential for prenatal memory development[2] and liver function.[3,4] However, choline is a charged molecule at physiological pH, and is thus limited in its absorption by a local intestinal transport system.

Choline transport across enterocytes has not been consistently described. Kuczler et al.[5] defined [^{14}C]-choline uptake across the mucosal border of guinea-pig jejunum as a saturable, non-sodium-dependent, carrier-mediated process at low mucosal choline concentrations and at high concentrations (greater than 4 mM) operating by passive diffusion. However, Hegazy and Schwenk[6] defined choline transport across isolated intestinal epithelial cells of guinea pigs as saturable and Na^+-dependent. Furthermore, the authors defined the choline transport with an apparent K_m value of 119 μM and V_{max} of 208 pmol/(mg protein/min).

Recently, Caco-2 cell lines have been used to characterize intestinal nutrient and drug transport.[7–10] Caco-2 cells are derived from a human colon carcinoma and display in vivo enterocyte-like characteristics when differentiated. They show microvilli and tight junctions, express di/tri-peptide and amino acid carriers, bile acid transporters, vitamin and hexose transporters, have brush-border hydrolases, and contain an Na^+/H^+ antiport.[11] Of interest for the present study, choline transport across Caco-2 cells has been demonstrated to be sodium-independent with a K_m of ~39 μM and a V_{max} of 1.4 nmol/mg/10 min. Furthermore, choline transport was inhibited by hemicholinium-3, and the cationic drugs etilefrin, atropine, atenolol, and clonidine.[12,13]

The demonstrated characteristics of choline uptake across Caco-2 cells are not consistent with the traditional high- or low-affinity choline transport models. High-affinity choline transporters are found in erythrocytes, fibroblasts and photoreceptor cells, which require exogenous choline for phospholipid synthesis.[14] High-affinity transport has also been characterized in cholinergic nerve terminals linked to the synthesis of acetylcholine.[15] Transport of choline via this transporter is carrier-mediated, saturable, and dependent on sodium.[16] Low-affinity choline transporters are found in cell bodies and provide choline for phosphatidylcholine synthesis. Transport of choline in the low-affinity system is linearly dependent on choline concentration and may operate by passive diffusion.[17]

The Caco-2 cells are not the only cell type to show novel choline transport characteristics. For example, choline transport across the blood–brain barrier is sodium-independent, carrier-mediated, and saturable with an affinity between the two traditional systems.[18] This suggests choline transport is unique, based on tissue location and need of exogenous choline. Therefore, the purpose of this study was to further characterize essential choline transport across Caco-2 cells. Furthermore, this characterization may have importance in predicting the interaction or transport of cationic drugs from the intestine to plasma.[12]

MATERIALS AND METHODS

Materials

The following materials were purchased: [^{3}H]-mannitol (728 MBq/μmol) from Dupont NEN (Regensdorf, Switzerland); [^{3}H]-choline chloride (3150 MBq/μmol) from Amersham International (Buckinghamshire, UK); Hanks buffered salt solution (HBSS) and phosphate buffered saline (PBS) from GibcoBRL (Paisley, Scotland); Dulbecco's modified Eagles medium (DMEM) with 4.5 g/L glucose, penicillin G, streptomycin sulfate and HEPES from Sigma Chemicals (St. Louis, MO). Falcon 12-mm polyethylene-teraphthalate (PET) filter inserts were purchased from Becton Dickinson GmbH (Heidelberg, Germany). The epithelial volt-ohm meter (EVOM) and Endohm-12 chamber were supplied by World Precision Instruments (Sarasota, FL). Verapamil was obtained from Fluka Chemicals (Buchs, Switzerland). Probenecid was purchased from ICN Biomedicals GmbH (Eschwege, Germany). Cyclosporin-A was prepared to its highest purity from Novartis. All other chemicals were from Sigma Chemicals (St. Louis, MO).

Caco-2 Monolayers

The human colon carcinoma cell line (Caco-2) used in our laboratory originated from University Hospital, Utrecht, Netherlands, and was cultured in a similar fashion to that done by Artursson[8] and

Hidalgo et al.[19] Briefly, the cells were maintained in 25-cm^2 flasks containing DMEM supplemented with 10% fetal calf serum, 1% non-essential amino acids, penicillin (161 U/mL), and 100 μg/mL streptomycin. They were incubated at 37°C in a Heraeus Cytoperm incubator set to 95% humidity and 5% CO_2. Before reaching confluency, at 6 days post-culturing, the cells were removed by trypsinization (0.25% trypsin and 0.02% EDTA in PBS). Cells were resuspended in culture medium and seeded onto 0.9-cm^2 Falcon HD PET filter inserts (0.45 μm pore size) in 12-well plates at 65,000 cells/cm^2. The DMEM was replaced every 2–3 days. The cells reached confluence between 5 and 7 days after seeding on filters, but were allowed to develop for 21–24 days before transport studies were initiated. Caco-2 cells were used between passages 75 and 85.

Monolayer Transport

All transport experiments were conducted at 37°C, for 2 hr in transport medium [HBSS (pH 7.4) containing 25 mM D-glucose and 25 mM HEPES buffer], except for one study that examined the effect of a pH gradient on choline transport. For this one study, the apical medium only was reduced to pH 6.0 using 25 mM MES buffer instead of 25 mM HEPES. Before initiation of the study, Caco-2 cell monolayers on Falcon inserts were switched to clean 12-well plates and had culture medium replaced with transport medium. The monolayers were allowed to equilibrate at 37°C for 60 min. Each Falcon insert was measured for trans-epithelial electrical resistance (TEER) at this time using an EVOM volt-ohm meter and an end-ohm-12 chamber. Transport studies in the apical to basolateral direction (Ap to Bas) had 1.5 mL of transport medium placed in the basolateral chamber. Initiation of the study began when 0.5 mL of transport medium supplemented with the test compound was placed in the apical chamber. In transport studies in the basolateral to apical direction (Bas to Ap), 0.5 mL of transport medium was added first to the apical chamber followed by 1.5 mL of transport medium supplemented with [^{3}H]-choline and the inhibitor applied to the basolateral chamber. Two hundred microliters were removed from the basolateral chamber (acceptor side) in Ap to Bas direction studies, while 75 μL was removed from the apical chamber in Bas to Ap studies at 10, 20, 30, 45, 60, 90 and 120 min. Volumes taken were replaced with fresh transport medium pre-warmed to 37°C. At each of these times, just prior to sampling, the 12-well plates were gently swirled. After 120 min, samples were also taken from the donor side and TEER measurements were repeated to confirm that cellular integrity was maintained throughout the study. If TEER of monolayers minus that of blank inserts fell below 250 Ω cm^2, cells were considered leaky and results from these monolayers were not used for subsequent analysis. Usually, TEER of our Caco-2 cells ranged from 350 to 600 Ω cm^2. Monolayers and wells were washed twice in excess PBS before the cells were trypsinized in 0.25% trypsin and 0.02% EDTA for 10 min at 37°C. Cells were scraped from the filters and the filters removed from the inserts.

Cells, filters, and all samples collected during the 120-min study had Lumasafe liquid scintillation cocktail (Lumac LSC, Groningen, Netherlands) added and were counted in a Beckman Tri-carb β counter to determine the amount of radiolabeled [^{3}H]-choline transported into and through the cell monolayers.

In studies involving the addition of the inhibitors such as verapamil (100 μM), CsA (10 μM), probenecid (1.5 mM), nifedipine (100 μM), and EGTA (200 μM), the compounds were present in both apical and basolateral chambers at the concentrations described. Transport media supplemented with individual inhibitors were only added at the same time as test compounds so that no pre-incubation with either compound occurred. Otherwise these studies were identical to the normal Ap to Bas or Bas to Ap direction studies, except that the fresh media also contained the individual inhibitors. All of these experiments were conducted with a pH 7.4 transport buffer present on both sides of the membrane.

CALCULATIONS

Apparent permeability through Caco-2 cells was calculated as described previously:[20]

$$P_{app}\ (\text{cm/sec}) = \frac{\Delta Q}{\Delta t 60 A C_0} \quad (1)$$

where $\Delta Q/\Delta t$ is the permeability rate (μg/min), C_0 is the initial concentration in the donor chamber (μg/mL), and A is the surface area of the membrane (cm^2).

All compounds used in our study were radiolabeled, which allowed measurement of the radioactivity associated with both the cells and filters. We could then correct for these factors in the determination of permeability. Therefore, a modification of Artursson's[7] equation was created, as shown here in expanded format to first determine corrected clearance volumes [Eq. (2a)], followed by the effective permeability (P_{eff}) determined from the slope of the regression line of the clearance volumes vs. time [Eq. (2b)]:

$$\text{Cl.vol} = A_a/(\{C_{d_0} \times V_d - [A_a + (C_{a'} \times V_s)] - (A_c + A_f) \times n/n_{fin}\}/V_d) \quad (2a)$$

where:

Cl.vol = clearance volume (mL)
n = time (min)
A_a = amount in acceptor compartment at time n (dpm)
C_{d_0} = concentration in donor compartment at time 0 (dpm/mL)
V_d = volume of donor compartment (mL)
$C_{a'}$ = concentration in acceptor compartment at the previous time point (dpm/mL)
V_s = sample volume of previous time point (mL)
$A_c + A_f$ = amount of compound associated with cells and filters, respectively, after cells are removed from the filters by trypsin treatment at the end of the study (dpm)
n_{fin} = final time point (min)

$$P_{eff} = J_{ss}/A \quad (2b)$$

where J_{ss} is the rate of clearance which equates to $\text{Cl.vol}/n$ (mL/sec), A is the area of the monolayer (cm^2), and P_{eff} is the effective permeability (cm/sec).

Statistical Analysis

Transport of Caco-2 [^{3}H]-choline and inhibition of transport was evaluated by one-way analysis of variance (ANOVA) followed by a Bonferoni's multiple comparison test. Errors are reported as standard error of the mean unless otherwise indicated. Differences were considered statistically significant at $p < 0.05$ (GraphPad Prism version 2.01 for Windows, GraphPad Software, San Diego, CA).

RESULTS

Choline P_{eff} values determined in this study were calculated as described above. Figure 1 shows potential active transport of choline across Caco-2 cells working in the apical to basolateral (0.01 μM choline Ap to Bas P_{eff} $11.11 \pm 0.33 \times 0^{-6}$ cm/sec) direction. Transport of choline in this direction is approximately twice that in the basolateral to apical direction (0.01 μM choline Bas to Ap P_{eff} $5.55 \pm 0.14 \times 10^{-6}$ cm/sec). In both experiments approximately 20% of the choline was associated with the cell, possibly representing cellular choline uptake.

In determining if choline transport was dependent on calcium, the calcium antagonists verapamil and nifedipine were included in the transport media. Figure 2 shows a statistically significant reduction of choline transport in the apical to basolateral direction by approximately 73% and 81%, respectively. A small, but significant, reduction in basolateral to apical choline flux was also seen under the same conditions.

Figure 3 demonstrates the effects of efflux protein inhibitors on choline transport across Caco-2 cells. The inhibitors cyclosporin-A and probenecid were added during transport at concentrations of 10 μM and 1 mM, respectively. Cyclosporin-A had a minor reduction (~14.5%) in apical to basolateral transport of choline and no effect in the opposite direction. Probenecid produced similar results with a 24% reduction in the apical to basolateral direction and no effect in the opposite direction. The small reduction of choline transport may be accounted for by cyclosporin-A having some calcium antagonistic activity.

To confirm choline transport across Caco-2 cells is calcium-dependent, EGTA was added at a concentration of 200 μM. Figure 4 demonstrates a significant decrease of choline transport in the apical to basolateral direction but also an equalization of bi-directional transport. To ensure EGTA was not increasing the pore size of the Caco-2 cells, and subsequent increased paracellular transport, TEER measurements were monitored and remained (500–600 Ω cm^2) unaffected throughout the 2-hr study.

Determination of pH influence on choline transport across Caco-2 membranes was accomplished by changing the pH on the apical side of the membrane to 6.0. The media was composed as previously described except 25 mM MES buffered

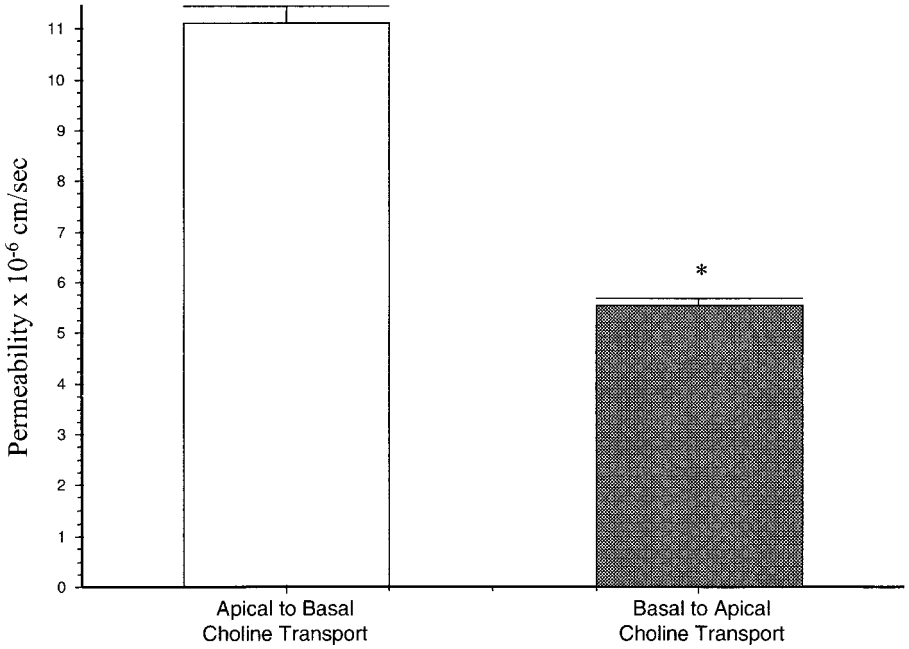

Figure 1. A comparison of PEFF for apical to basal and basal to apical [^{3}H]-choline (mL/sec/g). An asterisk (*) indicates a significant difference ($p < 0.05$). Data are mean ± SEM ($n = 3$).

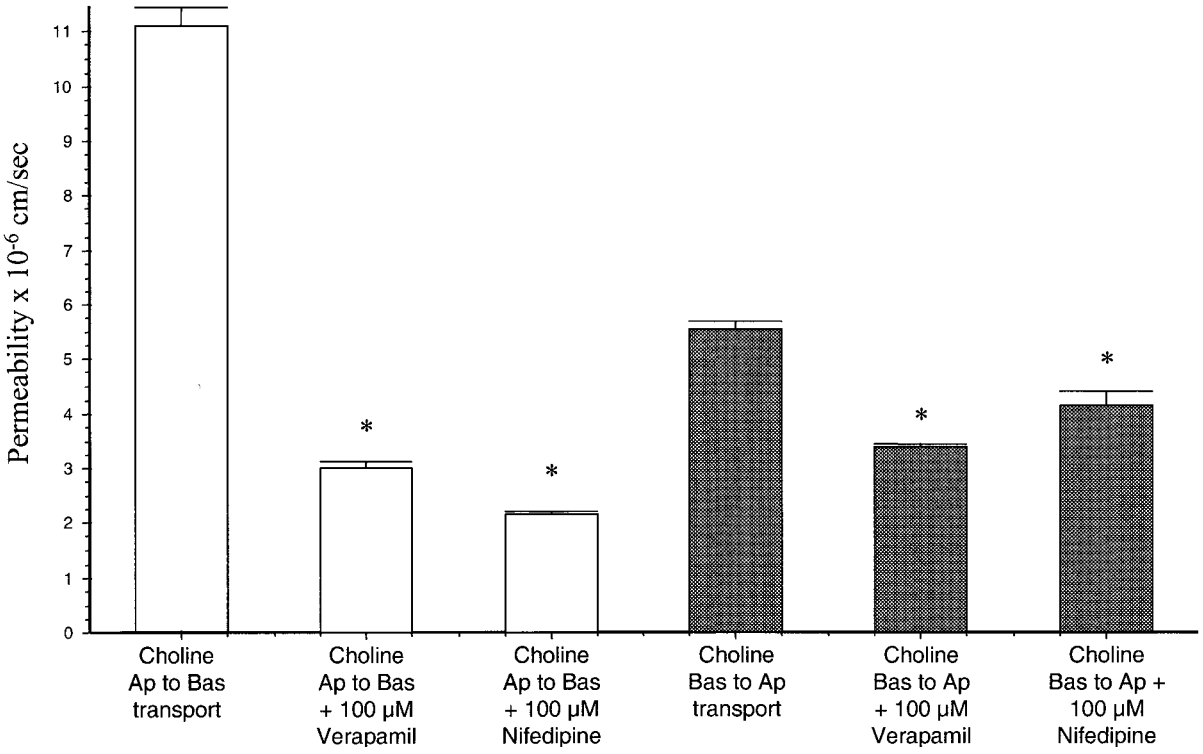

Figure 2. Effects of 100 µM concentrations of verapamil and nifedipine on Caco-2 [^{3}H]-choline (mL/sec/g) PEFF using 0.01 µM choline concentrations. Data are mean ± SEM ($n = 3$). *Significant difference ($p < 0.05$).

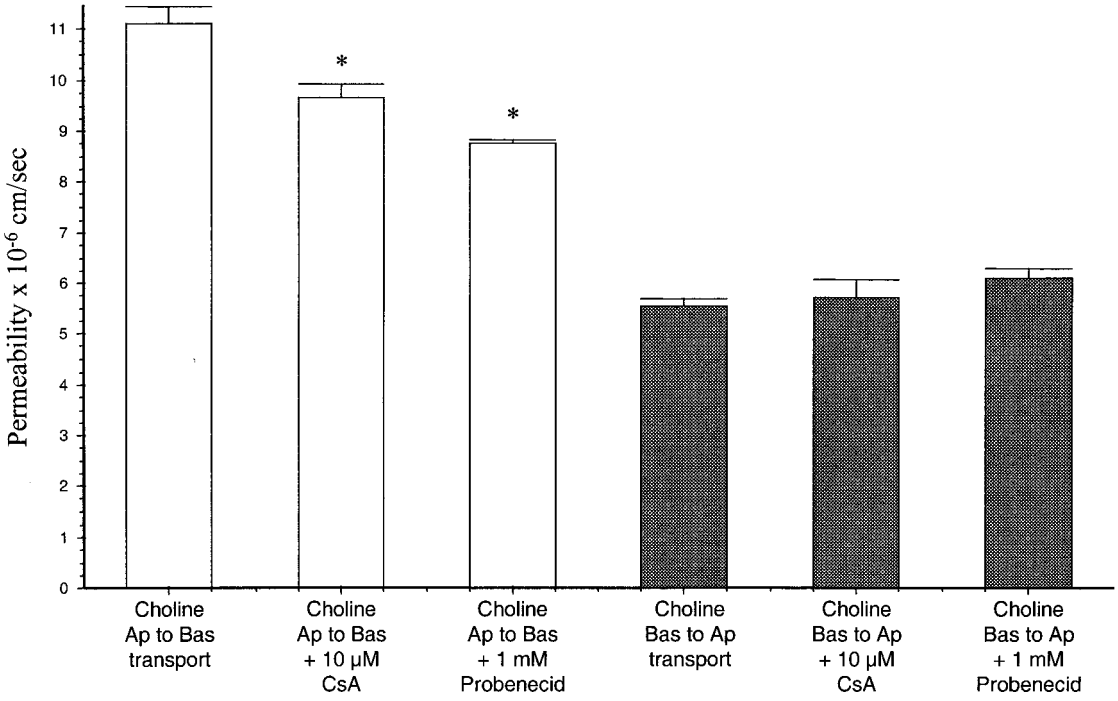

Figure 3. Effects of P-gp inhibitors cyclosporin and probenecid on Caco-2 [^{3}H]-choline (mL/sec/g) PEFF using 0.01 µM choline concentrations. Data are mean ± SEM ($n = 3$). *Significant difference ($p < 0.05$).

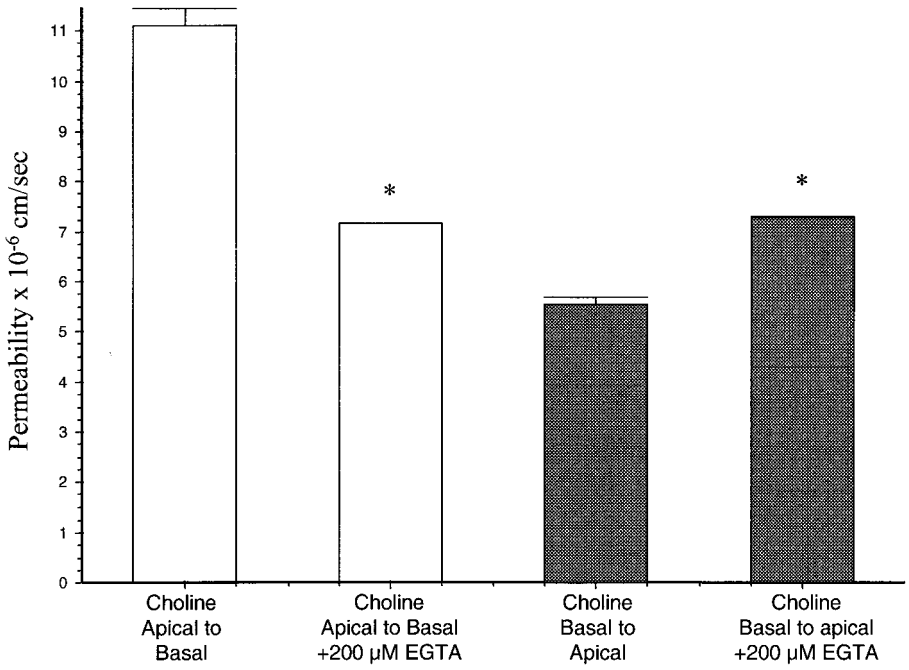

Figure 4. Effects of the calcium chelator EGTA on Caco-2 [^{3}H]-choline (mL/sec/g) PEFF using 0.01 µM choline concentrations. Data are mean ± SEM ($n = 3$). *Significant difference ($p < 0.05$). For points where no error bars are seen they are within the plotted data point.

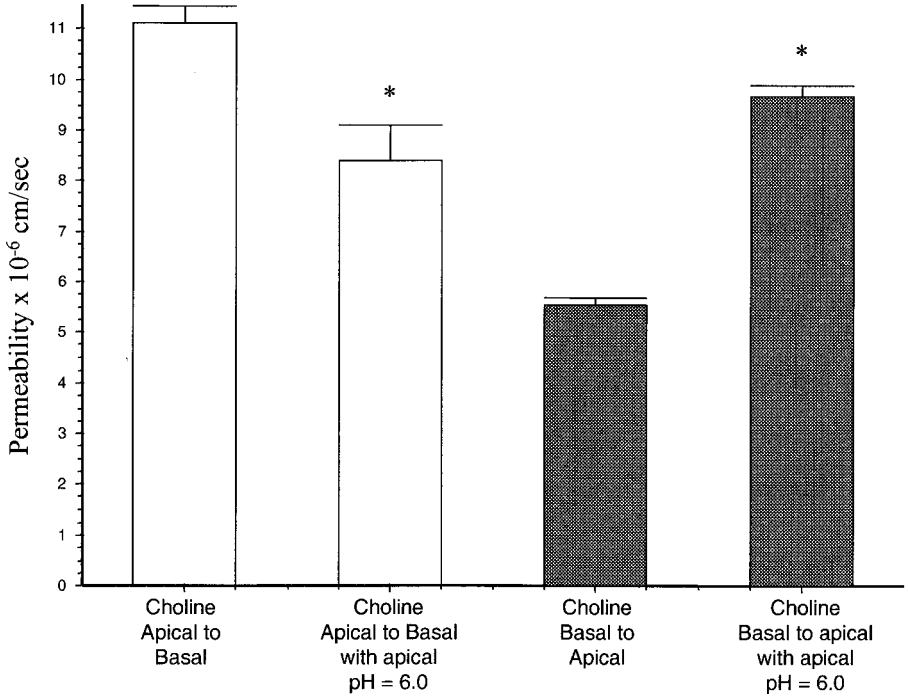

Figure 5. Effects of using 25 mM MES buffered HBSS at pH 6.0 on the apical side of the membrane (maintaining pH 7.4 on the basolateral side) on Caco-2 [^{3}H]-choline (mL/sec/g) PEFF using 0.01 μM choline concentrations. Data are mean ± SEM ($n = 3$). *Significant difference ($p < 0.05$).

HBSS was used. Figure 5 illustrates a 25% reduction in choline transport in the apical to basolateral direction, however, in the basolateral to apical direction an increase in choline transport was observed (5.5 to $9.67 \pm 0.23 \times 10^{-6}$ cm/sec).

DISCUSSION

The results of the studies presented herein demonstrate an active choline transport mechanism in Caco-2 cells that is dependent on both extracellular calcium and pH. This paper extends previous work evaluating the pharmacokinetics of Caco-2 choline transport.[12,13]

The Caco-2 choline transporter has been shown to have a K_m of ~39 μM and a V_{max} of ~1.4 nmol/min/g.[13] Similarly, the experiments herein demonstrate active choline transport at a concentration of 10 nM with apical to basolateral flux exceeding the opposite direction flux by approximately twofold (Figure 1). This is consistent with active choline transport in rat enterocytes.[21,22]

High-affinity ($K_m \sim 2$–20 μM) choline transport is considered to be sodium-dependent. This high-affinity, sodium-dependent choline transporter has been demonstrated in cholinergic nerve terminals.[15] However, recently it has been demonstrated that in-vivo choline transport at the blood–brain barrier is sodium-independent with an intermediate affinity. Furthermore, this transporter interacts with the monovalent cations: Cs^+, K^+, and Li^+,[18] and the divalent metals: Mn^{2+} and Cd^{2+}.[23] Similarly, choline transport across Caco-2 cells has an intermediate affinity for choline, is sodium-independent, and does not interact with Li^+.[13]

To further characterize Caco-2 choline transport and its dependency or interaction with monovalent or divalent cations, various pharmacological inhibitors were added to both the apical and basal compartment in different procedures. Figure 2 demonstrates marked apical to basal choline transport reduction when the calcium antagonists and weak P-gp substrates, verapamil and nifedipine, were added to the apical chamber. Furthermore, a significant, but not as prominent, reduction of basal

to apical choline transport was also seen. The results of these two experiments would suggest that transport of choline across Caco-2 cells may be calcium-dependent or mediated by P-gp efflux.

To exclude the possibility that protein-mediated efflux is not influencing choline transport, two experiments were conducted. First, cyclosporin-A (CsA), a stronger inhibitor of P-gp than verapamil,[24] was incorporated into the transport media. Figure 3 demonstrates a small, but significant, reduction in choline transport only in the apical to basal flux. However, CsA does have some Ca^{2+} antagonistic activity, possibly causing this small reduction. Further evidence that choline efflux is not present is shown in Fig. 3 with administration of probenecid. Probenecid is an inhibitor of multispecific organic anion transport and is also used to inhibit multidrug resistance-associated protein.[25] Probenecid addition again resulted in a small, but significant, decrease of choline apical flux and an insignificant increase in basal flux. Experiments with the efflux protein inhibitors suggest that a minute portion of choline transport may be mediated by efflux proteins, but may still be associated with Ca^{2+} antagonistic activity.

To confirm Caco-2 choline transport calcium-dependency, experiments with a calcium chelator were completed. Figure 4 illustrates 200 μM of EGTA significantly decreased apical to basal transport of choline. However, of substantial interest, EGTA increased basal to apical choline transport. Thus, when comparing bi-directional choline flux, after the addition of EGTA, choline transport was essentially equalized. This equalization suggests that once the usable media calcium was removed from the extracellular environment, choline transport occurs by passive permeability. Furthermore, the passive permeability demonstrated was not a result of increased paracellular junction opening as the TEER size was unaffected after 2 hr, remaining approximately 500–600 $\Omega\,cm^2$.

In determining if pH alters choline transport across Caco-2 cells, the transport medium was altered to achieve pH 6.0 on the apical side and pH 7.4 on the basolateral side. Apical choline permeability decreased approximately 30% and basal flux increased 76% (Fig 5). This suggests that apical and basal flux is dependent on pH, similar to the hydrogen antiport transport mechanism for the cationic metal cadmium in brush-border membrane vesicles.[26]

In summary, active Caco-2 choline transport can be characterized as calcium- and pH-dependent. Furthermore, this model is unique when compared to the traditional high- and low-affinity choline transport models. While the Caco-2 choline transporter may not represent a unique protein, it may demonstrate novel characteristics from residing in the intestinal environment. Further work should be carried out to determine if an antiport is conclusively involved, and to better understand the role of this transporter in drug absorption.

ABBREVIATIONS

TEER = Trans-epithelial electrical resistance
Ap = Apical
Bas = Basolateral
Cl.vol = Clearance volume (mL)
n = Time (min)
A_a = Amount in acceptor compartment at time n (dpm)
C_{d_0} = Concentration in donor compartment at time 0 (dpm/mL)
V_d = Volume of donor compartment (mL)
$C_{a'}$ = Concentration in acceptor compartment at the previous time point (dpm/mL)
V_s = Sample volume of previous time point (mL)
$A_c + A_f$ = Amount of compound associated with cells and filters respectively after cells are removed from the filters by trypsin treatment at the end of the study (dpm)
n_{fin} = Final time point (min)

REFERENCES

1. Klein, J.; Gonzales, R.; Köppen, A.; Löffelholz, K. Free Choline and Choline Metabolites in the Rat Brain and Body Fluids: Sensitive Determination and Implications for Choline Supply to the Brain. Neurochem. Int. **1993**, *22*, 293–300.
2. Zeisel, S.H. Choline: Needed for Normal Development of Memory. J. Am. Coll. Nutr. **2000**, *19* (5), 528s–531s.
3. Zeisel, S.H.; DaCosta, K.A.; Franklin, P.D.; Alexander, E.A.; Lamont, J.T.; Sheard, N.F.; Beiser, A. Choline, an Essential Nutrient for Humans. FASEB J. **1991**, *5* (7), 2093–2098.
4. Hensley, K.; Kotake, Y.; Sang, H.; Pye, Q.N.; Wallis, G.L.; Kolker, L.M.; Tabatbaie, T.; Stewart, C.A.; Konishi, Y.; Nakae, D.; Floyd, R.A. Dietary Choline Restriction Causes Complex 1 Dysfunction and

Increased H_2O_2 Generation in Liver Mitochondria. Carcinogenesis **2000**, *21* (5), 983–989.
5. Kuczler, F.J.; Nahrwold, D.L.; Rose, R.C. Choline Influx Across the Brush Border of Guinea Pig Jejunum. Biochim. Biophys. Acta **1977**, *465* (1), 131–137.
6. Hegazy, E.; Schwenk, M. Choline Uptake by Isolated Enterocytes of Guinea Pig. J. Nutr. **1984**, *114* (12), 2217–2220.
7. Artursson, P.; Karlsson, J. Correlation Between Oral Drug Absorption in Humans and Apparent Drug Permeability Coefficients in Human Intestinal Epithelial (Caco-2) Cells. Biochem. Biophys. Res. Comm. **1991**, *175* (3), 880–885.
8. Artursson, P. Epithelial Transport of Drugs in Cell Culture. I: A Model for Studying the Passive Diffusion of Drugs Over Intestinal Absorptive (Caco-2) Cells. J. Pharm. Sci. **1990**, *79* (6), 476–482.
9. Delie, F.; Rubas, W. A Human Colonic Cell Line Sharing Similarities with Enterocytes as a Model to Examine Oral Absorption: Advantages and Limitations of the Caco-2 Model. Crit. Rev. Ther. Drug Carr. Syst. **1997**, *14*, 221–286.
10. Pinto, M.; Robine-Leon, S.; Appay, M.; Kedinger, M.; Triadou, N.; Dussaulx, E.; Lacroix, B.; Simon-Assmann, P.M.; Haffen, K.; Fogh, J.; Zweibaum, A. Enterocyte-like Differentiation and Polarization of the Human Colon Carcinoma Cell Line Caco-2 in Culture. Biol. Cell **1983**, *47*, 323–330.
11. Makhey, V.D.; Guo, A.; Norris, D.A.; Hu, P.; Yan, J.; Sinko, P.J. Characteristics of the Regional Intestinal Kinetics of Drug Efflux in Rat and Human Intestine and in Caco-2 Cells. Pharm. Res. **1998**, *15* (8), 1160–1167.
12. Möbus, K.; Brandsch, M.; Neubert, R. Transport of Cationic Drugs by a Choline Transport System in Caco-2 Cells. Arch. Pharm. Pharm. Med. Chem. **2000**, *333*, 22.
13. Möbus, K.; Brandsch, M.; Neubert, R. High-Affinity Uptake of Choline in Caco-2 Cell Monolayers. In Proceedings of 3rd World Meeting APV/APGI, Berlin, April 3–6, 2000; 351–352.
14. Martin, K. Concentrative Accumulation of Choline by Human Erythrocytes. J. Gen. Physiol. **1968**, *51* (4), 497–516.
15. Tuček, S. Problems in the Organization and Control of Acetylcholine Synthesis in Brain Neurons. Progr. Biophys. Mol. Biol. **1984**, *44* (1), 1–46.
16. Yamamura, H.I.; Snyder, S.H. High Affinity Transport of Choline into Synaptosomes of Rat Brain. J. Neurochem. **1973**, *21* (6), 1355–1374.
17. Choi, R.L.; Freeman, J.J.; Jenden, D.J. Kinetics of Plasma Choline in Relation to Turnover of Brain Choline and Formation of Acetylcholine. J. Neurochem. **1975**, *24* (4), 735–741.
18. Allen, D.D.; Smith, Q.R. Characterization of the Blood–Brain Barrier Choline Transporter Using the In-Situ Rat Brain Perfusion Technique. J. Neurochem. **2001**, *76* (4), 1032–1041.
19. Hidalgo, I.J.; Raub, T.J.; Borchardt, R.T. Characterization of the Human Colon Carcinoma Cell Line (Caco-2) as a Model System for Intestinal Epithelial Permeability. Gastroenterology. **1989**, *96* (3), 736–749.
20. Crowe, A.P.; Lemaire, M. In Vitro and In Situ Absorption of SDZ_RAD Using a Human Intestinal Cell Line (Caco-2) and Single Pass Perfusion Model in Rats: Comparison with Rapamycin. J. Pharm. Sci. **1998**, *15* (11), 1666–1672.
21. Saitoh, H.; Kobayashi, M.; Sugawara, M.; Iseki, K.; Miyazaki, K. Carrier-Mediated Transport System for Choline and Its Related Quaternary Ammonium Compounds on Rat Intestinal Brush Border Membrane. Biochim. Biophys. Acta **1992**, *1112*, 153–160.
22. Tsubaki, H.; Komai, T. Intestinal Absorption of Choline in Rats. J. Pharmacobiodyn. **1987**, *10*, 571–579.
23. Lockman, P.R.; Roder, K.E.; Allen, D.D. Inhibition of the Rat Blood–Brain Barrier Choline Transporter by Manganese Chloride. J. Neurochem. **2001**, *79* (3), 588–594.
24. Romsicki, Y.; Sharom, F.J. Phospholipid Flippase Activity of the Reconstituted P-Glycoprotein Multidrug Transporter. Biochemistry **2001**, *40* (23), 6937–6947.
25. Evers, R.; Zaman, G.J.; Van Deemter, L.; Jansen, H.; Calafat, J.; Oomen, L.C.; Oude-Elferink, R.P.; Borst, P.; Schinkel, A.H. Basolateral Localization and Export Activity of the Human Multidrug Resistance-Associated Protein in Polarized Pig Kidney Cells. J. Clin. Invest. **1996**, *97* (5), 1211–1218.
26. Endo, T.; Kimura, O.; Sakata, M. Secretory Transport of Cadmium Through Intestinal Brush Border Membrane Via H(+)-Antiport. Toxicology **2000**, *150* (1–3), 129–136.