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Sodium-dependent and -independent Choline Uptake by Type II Epithelial Cells from Rat Lung

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Abstract. The uptake of ^{3}H-labeled choline by a suspension of isolated type II epithelial cells from rat lung has been studied in a Ringer medium. Uptake was linear for 4 min at both 0.1 μM and 5.0 μM medium choline; at 5 μM, only 10% of the label was recovered in a lipid fraction. Further experiments were conducted at the low concentration (0.1 μM), permitting characterization of the properties of high-affinity systems. Three fractions of choline uptake were detected: (i) a sodium-dependent system that was totally inhibited by hemicholinium-3 (HC-3); (ii) a sodium-independent uptake, when Na^+ was replaced by Li^+, K^+ or Mg^{2+}, inhibited by HC-3; (iii) a residual portion persisting in the absence of Na^+ and unaffected by HC-3. Choline uptake was sigmoidally related to the medium Na^+ concentration. Kinetic properties of the uptake of 0.1 μM ^{3}H-choline in the presence and absence of medium Na^+ were examined in two ways. (a) Inhibition by increasing concentrations of unlabeled choline (0.5–100 μM) was consistent with the presence of two Michaelis-Menten-type systems in the presence of Na^+; a Na^+-dependent portion (a mean of 0.52 of the total) had a K_m for choline of 1.5 μM while K_m in the absence of Na^+ (Li^+ substituting) was 18.6 μM. (b) Inhibition by HC-3 (0.3–300 μM) gave K_i values of 1.7 μM and 5.0 μM HC-3 for the Na^+-dependent and -independent fractions. The apparent K_m of the Na^+-dependent uptake is lower than that reported previously for lung-derived cells and is in the range of the K_m values reported for high-affinity, Na^+-dependent choline uptake by neuronal cells.

Key words: Na-dependent choline transport — Choline uptake kinetics — Alveolar epithelium — Inhibition of choline transport — Alveolar type II cells

Introduction

Lung surfactant is a complex material, elaborated and secreted by type II cells of the alveolar epithelium, which contains phosphatidylcholines among its characteristic components. These phospholipids are synthesized in the type II cells, using choline from the blood as substrate [16], but the mechanism(s) by which choline enters the cells are incompletely understood.

Three types of transport mechanism for choline have been recognized in vertebrate cells — a facilitated diffusion mechanism with apparent K_m 10 μM found in red cells [7] and two additional transport systems for which there is evidence of ion-coupling; these are distinguished by their affinity for choline and the inhibitor, hemicholinium-3 (HC-3). The high affinity system, K_m 0.5–3 μM choline, has K_i for HC-3 in the range 1–3 μM [24, 29], requires extracellular Na^+ and has been studied mainly in synaptosomes of cholinergic neurons and in the electric organ of certain eels [8, 23, 24, 27, 29]. The other type of mechanism has a lower affinity for choline, with reported K_m values in the range of 20–200 μM, is less clearly dependent on Na^+ and less effectively inhibited by HC-3, with K_i values in a similar range to the K_m [9, 12, 15, 21, 26, 28, 29]. This low-affinity system is more widely distributed, having been detected not only in cells which also contain the high affinity mechanism [e.g., 29] but also in a variety of other tissues [2, 7, 12, 14, 17, 21, 26].

There have been rather few studies of choline transport by alveolar epithelium, yet indications that it can be rate-limiting for the synthesis of phosphatidylcholines

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[30] make it an important process to understand. A considerable concentration gradient of choline can be established between cells and perfusate in perfused lungs [9, 11]. Early kinetic experiments of Fisher et al., [9], suggested that rat alveolar type II cells accumulated choline by a single, saturable mechanism with an apparent K_m of 18 μM. It was inhibited partially (50%) by HC-3 and by only 30–40% when mitochondrial ATP synthesis was inhibited. Although omission of medium Na^+ had no effect in these experiments with intact cells [9], further work using isolated vesicles of plasma membrane from rat type II cells showed an ''overshoot'' phenomenon for choline transport upon addition of Na^+ [10]. Work with the A549 cell line, originating from human lung epithelium, indicated an apparent K_m for choline of 10 μM with intact cells. Plasma membrane vesicles isolated from A549 cells showed an electrogenic, Na^+-induced overshoot while a Na^+-independent portion was sensitive to both electrical and proton gradients across the vesicular membrane [10]; later studies with these membrane vesicles determined K_m values of 4 and 40 μM for the Na^+-dependent and -independent transport respectively, when external choline was at 5 μM [15].

In the work described below, using a suspension of cells freshly isolated from rat lungs, we have confirmed the presence of parallel pathways for choline transport by type II cells and characterize the kinetics and HC-3-sensitivity of Na^+-dependent and -independent portions of choline uptake. By using a very low external choline concentration (0.1 μM), we have found that the transport characteristics of the Na^+-dependent mechanism of intact lung cells are similar to those reported for high-affinity choline uptake by cholinergic neurons. A preliminary account of some of this work has been presented [25].

Materials and Methods

Cell Isolation

Male albino rats (150–300 g) were anesthetized with pentobarbital (35 mg/kg i.p.). The isolation procedure followed that reported previously [3] with respect to lung lavage with Ca^{2+}- and Mg^{2+}-free medium, incubation with porcine elastase (type I, Sigma Chemical, St. Louis, MO) and filtration of the separated cells. At this point, the method was modified by separating the cells by centrifugation (30 min at 300 × *g;* room temperature) on a discontinuous density gradient of Percoll in the Ringer medium (*see below*) at d. 1.047 and 1.075. The type II epithelial cells were recovered from a band near the top of the Percoll d. 1.045; they were washed twice with, and finally taken up in, the Ringer medium. For experiments with modified Na^+ concentrations (*see below*), cells collected from the Percoll gradient were divided into batches, each of which was washed with one of the substituted Ringer solutions three times before being taken up in the same medium for preincubation.

The mean viability of the type II cells in 105 preparations, as determined by trypan blue exclusion, was 94.3 ± 0.9%, the yield of viable type II cells being $(29 \pm 1) \cdot 10^6$ cells per rat. The proportion of these cells in the suspension, estimated by fluorescence of phosphine R3 [13], was 90.3 ± 0.8%. Contaminating cells were: macrophages 3.6 ± 0.4, type I epithelial cells 2.4 ± 0.4 and ciliated cells 6.5 ± 0.5%.

Choline Transport

Incubation Media

The control Ringer medium used for the experiments and for some steps of the cell preparation (*see above*) contained (in mM): NaCl 136, KCl 5, $CaCl_2$ 1.8, $MgSO_4$ 1.3, Tris. HCl 10, glucose 10 and 1% (w/v) bovine serum albumin; the solution was at pH 7.4 and was gassed with O_2. Work with Na^+-free media tested five ways of replacing Na^+ in this control medium: NaCl was totally replaced by the chlorides of K^+, Li^+ or N-methyl-D-glucamine ($NMDG^+$); using Mg^{2+}, the usual NaCl and $MgSO_4$ were replaced by 100 mM $MgCl_2$ *plus* 36 mM $MgSO_4$; or the NaCl was replaced by 250 mM sucrose. To obtain intermediate concentrations of Na^+ at constant ionic strength, a suspension of cells taken up in control (i.e., 136 mM) Na^+ Ringer was mixed in appropriate proportions with cells taken up in Li^+ Ringer. The albumin used for Na^+-free media was previously dialyzed against the medium because of the presence of Na^+ in the purchased albumin. In some experiments, the suspension medium also contained a marker for intracellular water, either [C^3H_3] 3-O-methyl glucose or [$^{14}CH_3$]-antipyrine.

Incubation Procedures

General. The final pellets of the washed cells were taken up in a total of 2–4 ml of medium and transferred to plastic Erlenmeyer flasks (25 ml volume; gassed with O_2) for preincubation at 37°C in a shaking water bath for a minimum of 15 min. Portions of the preincubated suspensions were taken at intervals for the experimental incubation, all cells being used within 45 min of the start of preincubation. In most experiments, uptake of choline was initiated by transferring aliquots (0.2 ml) of the cell suspension to microcentrifuge tubes containing labeled choline in the quantity needed to give the final concentration required by the experiment (0.1–100 μM at constant total radioactivity). However, in experiments with HC-3, cells were further preincubated for 1 min in the microcentrifuge tubes in the presence of inhibitor, prior to initiation of choline uptake by the addition of labeled choline. Uptake was stopped at times indicated in Results by the addition of ice-cold stopping solution and immediate centrifugation in a microcentrifuge. The stopping solution consisted of the Ringer solution in which the cells had been incubated, with the addition of 100 μM HC-3 and 100 μM nonradioactive choline (the last was omitted when choline was assayed by HPLC). When 3-O-methylglucose was used as water marker, the stopping solution also contained phloretin (10 μM). In each experiment, 'zero-time' uptake was determined by adding 0.2-ml cell suspension to microcentrifuge tubes in ice, already containing stopping solution *plus* labeled choline. After centrifugation, a sample of the supernatant medium was taken for determination of the specific activity of medium choline (found to be $40–60 \cdot 10^3$ dpm/pmol in different experiments) and the remainder was removed by suction. The pellet was washed twice with the stopping solution. The effective removal of extracellular medium by the two washes was tested by determining the residual markers of intracellular water after a series of washes; both water markers reached a similar, constant level after 2 and 3 washes.

Kinetic Studies of Na^+ Dependence

A cell preparation from a single rat was used for each individual experiment, providing material for 22 incubation samples. The cells from

the gradient centrifugation were divided into two portions taken up respectively in the Na^+-medium or Li^+ medium (*see above*), then washed 3 times and preincubated for 15 min at 37°C in the same media. Two 'zero time' analyses (*see above*) were performed, one with preincubated cells from Na^+ medium and one from Li^+ medium (experiments of Fig. 4) or Li^+ medium plus HC-3 (300 μM; Fig. 5); there was no significant difference in the zero-time choline uptake of these two samples. Four cell samples provided duplicate control analyses in Na^+ or Li^+ medium without added test agent, i.e., unlabeled choline (Fig. 4) or HC-3 (Fig. 5). The remaining 12 samples gave duplicate comparisons of 3 concentrations of test agent in Na^+ or Li^+ medium which were varied for each experiment. Thirty three preparations of cells were used for the data of Fig. 4, and 13 for Fig. 5. The mean values of all experiments in each series were used for the kinetic analyses; therefore the interanimal variation inevitably limits the precision of the data and the quantitative conclusions drawn from them.

Analytical Procedures

Labeled choline, water markers and K^+ were extracted from the washed pellets with 0.1 N HNO_3 at room temperature overnight. Radioactivity was determined by β-emission liquid scintillation spectrometry and K^+ by emission flame photometry. In some cases, the cell pellets were extracted to separate aqueous and lipid-soluble derivatives [4]; incorporation of ^{3}H-choline into lipids was determined by counting radioactivity while the levels of choline and its conversion products in the aqueous phase were measured by HPLC [1]. The protein content of each tube was assayed by the Lowry method [18]. The final choline content of cell samples was calculated from the ^{3}H-content of the cells and the specific activity of the medium choline. Net uptake of choline was determined by subtraction of the zero-time contents (*see above*) from the total in each sample.

Kinetic Characterization of Pathways for Choline Influx

Using the equations described in Deves et al., [6] the data were fitted to either one or two Michaelis-Menten systems by an iterative, least-squares procedure. For analysis of the systems contributing to the influx of choline in sodium-containig media, the sodium-dependent contribution was determined directly in each batch of cells (*see* Results) and this fraction was then used as a fixed 'constant' in the kinetic analysis for that batch of cells (*see* Deves et al., [6]). Similarly, for the kinetic analysis of inhibition of choline influx by HC-3 in sodium-containing media, the fractional contribution of the sodium-dependent and -independent pathways was first determined (in the absence of HC-3) for that batch of cells; then, using this value as a fixed 'constant of proportionality, the two inhibitory constants for HC-3 in the presence of Na^+ were calculated. The value of lower affinity so computed was then directly compared with the value of the inhibition constant determined in the sodium-free medium.

Analysis of Results

The results in the text and tables are presented as mean ± SEM (number of observations). Significance of difference between sets of data was determined by 'Student's *t* test or by paired *t* test. Differences are considered significant when $P \leqslant 0.05$.

Results

Time Course Studies

Preliminary experiments using 5 μM choline [*cf* ref. 9] studied the uptake of this solute and the constancy of other cell parameters during experimental periods of 0.5–6 min (Table 1). Viability of the cells was determined immediately after completion of the cell preparation (i.e., before starting the preincubation), and again approximately 60 min later, after experimental incubation of all samples. There was no significant change of viability. The contents of K^+ and intracellular water were determined in each of the cell pellets from which choline was extracted and showed no significant change during the course of incubation.

The amount of labeled choline retained in the zero-time pellet after washing was substantial, giving an apparent concentration in the cell water of 40 μM (Table 1). However, since this was accumulated in less than 10 sec at 1°C in the presence of the stopping solution, it was presumably the result of binding to the cell exterior. Net uptake of choline, expressed per unit cell protein, was linear over the first 2 min (R 0.992) and continued during the entire 6-min period, finally attaining a content five times that of the zero-time samples. The apparent concentration of free ^{3}H-choline in the cell water increased throughout the 6 min, attaining a level thirty times that in the medium (Table 1). Comparisons at 5 μM choline showed no difference between uptake of ^{3}H- and ^{14}C-labeled choline (*not shown*).

Figure 1 shows the time course of choline uptake at both 0.1 and 5 μM medium choline, the latter with varying concentrations of the HC-3. Except at 200 μM HC-3, the uptake over 4 min did not deviate significantly from a straight line; this period was therefore adopted as the standard incubation time for subsequent experiments.

Metabolic Fate of Labeled Choline

The nature of the ^{3}H-choline recovered from the cell pellets after incubation at 37°C was investigated in two ways. First, separation into lipo- and hydrophilic fractions showed a small increase in lipophilic label in the first 30 sec, after which there was no further change (Fig. 2). By contrast, water-soluble label increased throughout the 4-min period of incubation studied and finally amounted to 90% of the total. Second, the aqueous choline fraction extracted from cells after 15- and 75-sec incubation with ^{3}H-choline was subjected to reversed phase HPLC; choline derivatives were measured both by counting the radioactivity in eluted fractions and by the response of the choline oxidase-based detector. Two peaks of radioactivity were detected. An early peak appeared after 2-min elution but produced no response in the choline detector; it represented phosphorylcholine. A second peak of ^{3}H occurred after 7 min elution and coincided both with the peak given by the HPLC detector and with the peak produced by a choline standard. The distribution of ^{3}H-labeled material in these two peaks changed during the course of incubation, with the phos-

Table 1. General characteristics and choline uptake of type II cells[a]

	Cells incubated with ^{3}H-choline for (min):				
	0	0.5	1.0	2.0	6.0
Trypan blue exclusion[b]	90.8 ± 1.6[c]				90.3 ± 2.1
(% type II cells)	(17)				(17)
K^+ content	403 ± 30	464 ± 64	458 ± 35	483 ± 38	500 ± 46
(μmol/g protein)	(30)	(33)	(29)	(28)	(12)
Water content	3.3 ± 0.3	3.0 ± 0.3	2.8 ± 0.2	2.9 ± 0.3	3.3 ± 0.2
(ml/g protein)	(31)	(33)	(32)	(28)	(12)
^{3}H-choline:					
Content of ^{3}H-choline[e]	118 ± 15	213 ± 30	254 ± 35	378 ± 56	591 ± 58
(nmol/g protein)	(32)	(34)	(33)	(30)	(12)
Apparent concentration of ^{3}H-choline[d]	[0]	43.0 ± 9.6	69.0 ± 20.0[f]	102.4 ± 29.4[f]	153.6 ± 20.0[g]
(μmol/l cell water)		(32)	(31)	(28)	(12)

[a] The cells were preincubated for 15 min at 37°C in the presence of ^{14}C-antipyrene as marker for intracellular water. Aliquots were then added in duplicate to ^{3}H-choline in microcentrifuge tubes to give a final concentration of 5 μM, and incubation continued for the periods indicated. Experiments were done with 17 different preparations; incubation was continued for 6 min only in the last 6 preparations.
[b] For cell counts with trypan blue, 1 sample before and 1 sample after incubation was taken from each preparation.
[c] Mean ± SEM (number of observations).
[d] The apparent concentration of ^{3}H-choline in the intracellular water was calculated after subtraction of the ^{3}H-choline in the zero time samples, on the assumption that the latter was predominantly present in an extracellular (possibly bound) form.
[e] Each value on this line differs significantly from every other, by paired two-tailed *t* test, $P = 0.013$–0.0002.
[f] Significantly different from value at $t = 0.5$ min, $P < 0.05$.
[g] Significantly different from value at $t = 0.5$ min, $P < 0.001$.

phorylcholine increasing at the expense of the choline. The mean values showed an increase of phosphorylcholine from 23.1 ± 4.5% of the total after 15 sec to 42.9 ± 5.9% after 75 sec. No peak corresponding to acetylcholine was evident.

Na^+ DEPENDENCE OF CHOLINE UPTAKE

Effect of Na^+ Substitution

For initial studies, cells were incubated with 0.1 μM choline in media in which Na^+ was totally replaced by other cations or sucrose (*see* Materials and Methods). With Li^+, K^+ or Mg^{2+} as substituting cations, net choline uptake was reduced by about 40%, showing the persisting activity of a sodium-independent system at this low concentration of choline (Table 2). In this series of experiments, the reduction of uptake in Li^+ as compared to Na^+ medium did not reach statistical significance; accordingly, a further series of studies was carried out, in which uptake in the Li^+ medium was significantly less than in Na^+ medium *viz.* 1.35 ± 0.20 *vs.* 2.00 ± 0.26 ($n = 15$ in each case; $P = 0.02$ by two-tailed, paired *t* test). Sucrose caused a substantial stimulation, a result contrary to the inhibition reported in A549 cells at 5-μM choline [15], but consistent with the stimulation of uptake of choline in mouse conceptus [26] and of the cationic amino acid, lysine, by the y^+L transport system in human red cells [5]. In the latter case, the effect was suggested to be due to the development of a negative surface potential in the presence of sucrose. Use of $NMDG^+$ gave variable results with the overall consequence being a marginal inhibition (Table 2).

Further experiments examined the effect of varying Na^+ concentration. Although all three inorganic cations gave similar results when totally replacing Na^+ (Table 2), reasons noted in the Discussion led to the choice of Li^+ as the most appropriate replacement for Na^+. Using this protocol, there was a sigmoid relationship between choline uptake and increasing concentrations of Na^+ (Fig. 3). These findings are similar to those reported by Wheeler for uptake of 0.5–1.0 μM choline by synaptosomes when Tris was the cation substituted for Na^+ [27].

The proportion of total choline uptake dependent on Na^+ varied somewhat between the various series of experiments. Thus, in Tables 2 (when Li^+ replaced Na^+) and 3 and Figs. 3, 4 and 5, the Na^+-dependent fraction in the absence of inhibitory substances (i.e., unlabeled choline or HC-3), was 0.47, 0.28, 0.66, 0.43 and 0.72, respectively, with a mean of 0.52. This appears to reflect biological variation of unknown cause between the different batches of animals used.

HC-3 Inhibition

Preliminary experiments with 5 μM ^{3}H-choline showed that 200 (*see* Fig. 1*b*) and 500 μM HC-3 gave a similar inhibition of uptake. A concentration of 300 μM HC-3

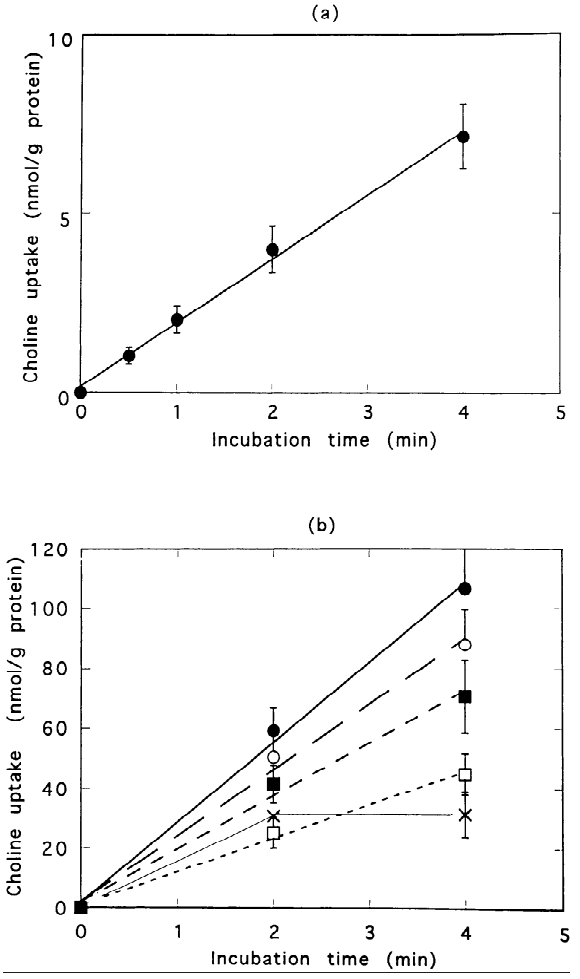

Fig. 1. Time course of uptake of labeled choline from medium containing (*a*) 0.1 μM choline and (*b*) 5 μM choline. Choline was added to the cells at $t = 0$ and samples were then taken at the times shown. In *b*, aliquots of the cells were preincubated for 1 min in the presence of the indicated concentrations of hemicholinium-3. Further details are given in Materials and Methods and Results. In *b*, points are the mean ± SEM of 14 observations, with the exception of those at 20 μM HC-3 which are the mean of 6; some error bars are omitted for clarity. Symbols representing HC-3 concentrations: ● None, ○ 20 μM, ■ 50 μM, □ 100 μM, ×200 μM. In *a*, points are the means of 15 observations.

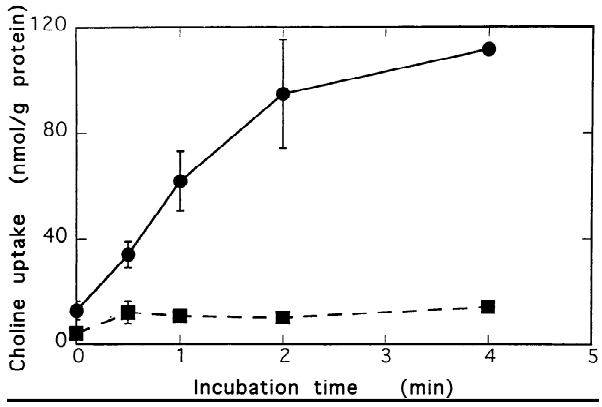

Fig. 2. Time course of the uptake of labeled choline into the aqueous and lipid-soluble materials extracted from type II epithelial cells. Uptake of 5 μM choline was initiated by addition of cells at $t = 0$ min. Samples were taken at the times noted. For further procedural details, *see* text. Points represent the mean ± SEM; $n = 2$ at 4 min, 10 at 0.5 and 2 min and 18 at 0 and 1 min. ● Aqueous phase; ■ Lipid phase.

was therefore chosen for a further study of its maximal inhibition of uptake of 0.1 μM choline in media with either 136 mM Na^+ or Li^+ as the major cation (Table 3). Substitution of Na^+ by Li^+ reduced uptake of 0.1 μM choline by 0.57 nmol/g protein · min^{-1} (i.e., 1.97 *minus* 1.40) and 80% of this Na^+-dependent uptake was inhibited by HC-3 (0.56 *minus* 0.11). In addition, HC-3 inhibited a considerable portion of the Na^+-independent uptake (1.40 *minus* 0.52). A residual uptake of 0.52 nmol/mg · min^{-1} was insensitive both to HC-3 and to removal of Na^+. These results suggest the presence of a minimum of three components contributing to uptake from this low concentration of choline, divided in this series of experiments as: (i) approximately 30% of the total, that requires Na^+ and is largely inhibited by 300 μM HC-3; (ii) a further fraction of 45% that is independent of Na^+ but sensitive to HC-3; (iii) 25–30% that neither requires Na^+ nor is inhibited by HC-3.

Kinetic Studies of Choline Uptake

Self-inhibition by Choline

Apparent K_m values for the uptake of 0.1 μM ^{3}H-choline were estimated from its inhibition by increasing concentrations of unlabeled choline. Paired incubations at each of the choline concentrations were carried out in Na^+ and Li^+ media. Analysis of the inhibition curve obtained with cells in the Na^+ medium (Fig. 4*a*) showed the presence of two kinetically separable components. The uptake rate was lower in Li^+ than in Na^+ medium, the difference being statistically significant ($P < 0.02$) at all concentrations except 2 and 100 μM. The Na^+-dependent uptake is given by difference and was calculated for each determination, rather than from the mean values of the curves in Na^+ and Li^+ media. At medium choline concentrations of 20–100 μM, the Na-dependent uptake was virtually unchanged and the uptake in Li^+ medium converged to the same level at 100 μM choline, suggesting that the residual uptake was a diffusional component. After subtraction of the latter, the data for the Na^+-dependent and Na^+-independent choline uptakes were individually fitted to a Michaelis-Menten relationship; the estimated K_m values were, respectively, 1.5 ± 0.3 and 18.6 ± 5.3 μM, with a proportionality constant of 0.42 for the relative contribution of the former to the saturable components. Reconstruction of a curve com-

Table 2. Effect of replacing Na^+ on choline uptake[a]

	Na^+ or replacement:					
	Na^+	$NMDG^+$	Li^+	K^+	Mg^{2+}	Sucrose
Choline uptake nmol/g protein · min^{-1}[b] (*n*)	1.37 ± 0.40 (11)	1.13 ± 0.27 (9)	0.72 ± 0.16 (11)	0.66[c] ± 0.16 (12)	0.75 ± 0.19 (6)	2.21[c] ± 0.49 (12)

[a] Cells collected from the Percoll gradient were divided into 4–6 batches, each of which was washed three times and preincubated for 15 min (37°C) in one of the modified Ringer media described in Materials and Methods. In each experiment, triplicate aliquots of each batch were then taken for assay of the 4-min uptake of ^{3}H-choline (0.1 μM) at 37°C. The solutions used to stop choline uptake and wash the Na-free cells after the transport assay were also modified by the same substitutions. [b]The values shown are derived from the net uptake of choline in 4 min, i.e., the cell content of ^{3}H after 4 min *minus* the ''zero-time'' content (*see* Materials and Methods); the latter, which was unaffected by the substitution of Na^+ amounted to a mean of 1.47 ± 0.22 (8) μmol choline/kg protein in these experiments. Results are given as mean ± SEM (number of observations). [c]Significantly different from value in Na^+ Ringer, $P \leq 0.05$.

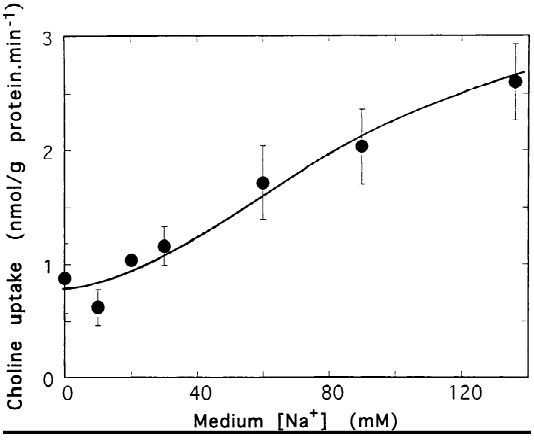

Fig. 3. Effect of Na^+ concentration on choline (0.1 μM) uptake. Cells were divided between Na^+ and Li^+ media in which they were washed three times and finally resuspended. The two suspensions were mixed in proportions giving the concentrations of Na^+ shown on the abscissa; corresponding Li+ concentrations were, 136 mM *minus* the Na^+ concentration. The mixtures were preincubated at 37°C in gassed vessels and aliquots were transferred to microcentrifuge tubes containing ^{3}H-choline for measurement of uptake during 4 min. For further details, *see* Materials and Methods. Points are the mean ± SEM, $n = 16$; values at 60, 90, and 136 mM Na^+ are significantly different from those at 0 and 10 mM Na^+, with $P < 0.02$, <0.01 and <0.001, respectively. The curve was drawn from computer analysis according to a sigmoid relationship.

Table 3. Effects of Na^+-free media and hemicholinium-3 on choline uptake[a]

HC-3 (μM)	Incubation medium:		
	Na^+	Li^+	Na^+ *minus* Li^+
Choline uptake (nmol/g protein · min^{-1})[b]			
0	1.97 ± 0.14 (17)	1.40 ± 0.14[c] (17)	0.56 ± 0.22
300	0.63 ± 0.06[d] (15)	0.52 ± 0.07[e] (16)	0.11 ± 0.15

[a] Procedures were as for Table 2, except that aliquots from the cell suspension preincubated at 37°C were preincubated for a further minute in microcentrifuge tubes with or without HC-3 (300 μM) before addition of ^{3}H-choline (0.1 μM). [b]The values are derived from the net uptake in 4 min, as described for Table 2. The ''zero-time'' uptake was unaffected by HC-3 or the absence of Na^+; for example, in the experiments of this table its mean value was 1.33 ± 0.20 (8) μmol/kg protein in Na^+ medium without HC-3 and 1.45 ± 0.20 (8) in Li^+ medium containing 300 μM HC-3. [c]$P \leq 0.05$ and [d]$P \leq 0.01$ for difference from Na^+ medium without HC-3. [e]$P \leq 0.05$ for difference from Li^+ medium without HC-3.

posed of choline entry through two systems having these characteristics appears adequate to describe the data obtained in Na^+-medium (Fig. 4*b*). The low K_m of the Na^+-dependent system, 1.5 μM, is within the range reported for the very-high-affinity choline uptake seen in cholinergic neurons [8, 23, 24, 27, 29].

These findings suggest that it would be difficult to determine accurately the very high affinity of the Na^+-dependent system at the higher choline concentrations used previously [15]. In fact, in self-inhibition experiments carried out at 2.25 μM labeled choline we detected two components, the estimated K_m of the higher affinity component (15.2 μM) being in keeping with earlier data [9, 15]. The lower affinity component of uptake at this concentration of choline was not further investigated but may, together with the sodium- and hemicholinium-independent portion of uptake at 0.1 μM choline, represent a diffusional component.

Inhibition by Hemicholinium-3

A similar analysis was performed on the inhibition by HC-3 of the uptake of 0.1 μM choline in Na^+ and Li^+ media; the net uptake in the Li^+ medium was significantly less than uptake in Na^+ medium ($P \leq 0.01$ to

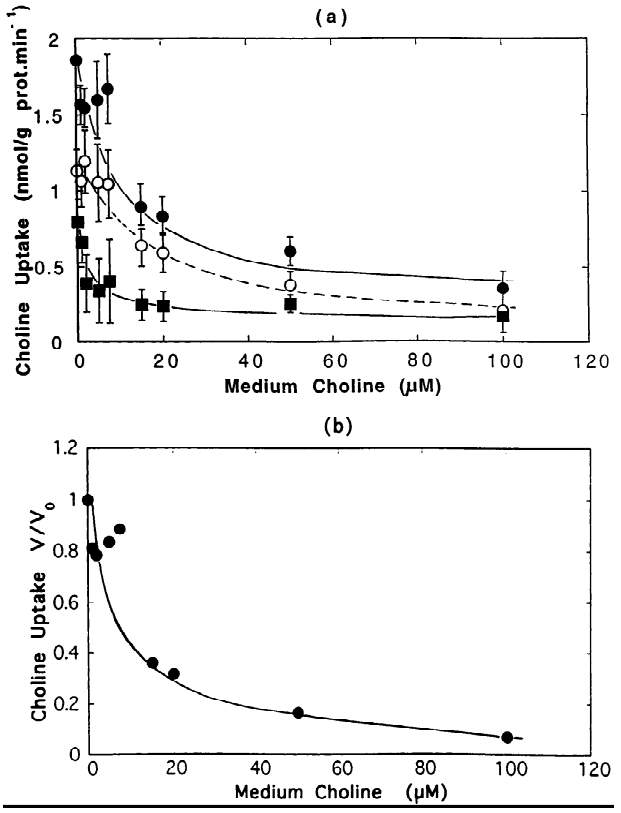

Fig. 4. Effect of increasing concentrations of unlabeled choline on the uptake rate of 0.1 μM ^{3}H-choline in the presence and absence of Na^+. Cells were washed, resuspended and preincubated in Na^+ or Li^+ medium, as for Fig. 3. Aliquots were transferred to microcentrifuge tubes containing ^{3}H-choline to give a final concentration of 0.1 μM and unlabeled choline to give the concentrations shown on the abscissa; uptake was determined after 4 min. (*a*) Net uptake of ^{3}H-choline in Na^+ Ringer (●), Li^+ Ringer (○) and the difference between them representing the Na^+-dependent uptake (■). Points are the mean ± SEM; n = 21–26, except at 0.1 μM (n = 68) and 100 μM (n = 16). Calculated K_m values are given in the text. (*b*) Reconstruction of the uptake in Na^+ Ringer by summation of the computer-derived curves of the Na^+-dependent and -independent (Li^+ Ringer) uptakes shown in *a*. The rates are normalized as V/V_o, where V_o is the uptake in the absence of unlabeled choline. The continuous line is the calculated curve; the points are the data from the curve in Na^+ Ringer shown in *a*.

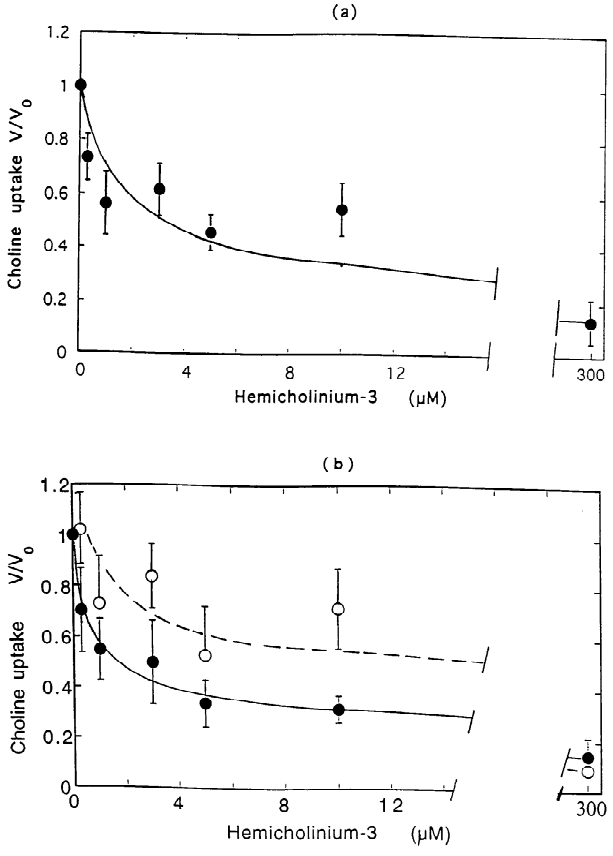

Fig. 5. Effect of increasing concentrations of hemicholinium-3 on uptake rate of choline (0.1 μM): (*a*) Rate of total uptake observed in Na^+ Ringer; the curve is drawn from the summation of the computer-derived curves for the Na^+-dependent and -independent fractions in part *b*. (*b*) Uptake in Li^+ Ringer (○) and the Na^+-dependent portion, i.e., the difference between Na^+ and Li^+ Ringers (●). Uptake rates are normalized as the ratio, V/V_o where V_o is the rate in the absence of HC-3. Methods as for Fig. 4 except that cells were preincubated for 1 min with the indicated concentrations of HC-3 before the addition of labeled choline; uptake was then stopped after 4 min uptake. Points are the mean ± SEM; n = 16–24 observations, except at 0 and 300 μM, where n = 30. Estimated K_i values are given in the text.

0.0001, by two-tailed, paired *t* test) at all concentrations of HC-3 except the highest (300 μM). In Fig. 5*b* the results are expressed as the ratios, V/V_o, for purposes of the kinetic analysis of the uptake (*see* Materials and Methods). Two components of the inhibition curve were detected in the Na^+ medium. The K_i for HC-3 of an Na^+-dependent portion of the uptake was estimated as 1.7 ± 0.4 mM, about six times less than that estimated for the Na^+-independent fraction (12.1 ± 4.2 μM); the constant for their relative contributions was 0.72. Reconstitution of the curve in Na^+ medium, calculated from the data obtained for the Na^+-dependent and -independent fractions, fitted the experimental points well (Fig. 5*a*).

Discussion

This work studied choline uptake by a freshly isolated suspension of intact type II epithelial cells from rat lung. Previous work on this subject used whole, perfused rat-lung, isolated type II cells in primary culture and whole cells or plasma-membrane vesicles of the lung epithelial cell line, A549 [9, 10, 15]. Using kinetic methods to study ion- and inhibitor-sensitivity (Figs. 4 and 5), our results confirm the existence of at least two types of

saturable choline transport mechanism in granular pneumocytes, a Na^+-dependent system with very high affinity for choline and a Na^+-independent mechanism of lower affinity. However, the degree of variability in the results from different cell preparations, to which we have drawn attention in Results, warrants some caution when considering the quantitative aspects of the results.

Technical Considerations

Much of the choline entering the cells was phosphorylated, but the amount incorporated into the lipid phase was small during the standard incubation period of 4 min. After the first 2 min at a medium concentration of 5 μM choline, the apparent concentration of ^{3}H-choline in the cell water was approximately 100 μM (Table 1); however, it can be calculated that the concentration of free ^{3}H-choline was approximately 50 μM, because at this time approximately 40% of the choline was present as phosphorylcholine and 10% as phospholipid. Thus, under the conditions of these observations, the concentration ratio, intracellular: extracellular, for free ^{3}H-choline was approximately 10:1. It should be noted that this level of intracellular choline can in principle be accounted for by equilibration according to a membrane potential of approximately −60 mV, which may be compared to the estimate of −40 mV for the membrane potential of a similar preparation of type II cells obtained by Peers et al. [20].

Justification for the use of Li^+ as a substituent for Na^+ is based on the following considerations: (i) it is a permeable, univalent cation unlikely to cause the osmotic and electrical effects expected for Mg^{2+} and K^+ respectively; (ii) it is accepted as a substitute for Na^+ by the Na/H exchanger, so limiting possible changes of cell pH that could affect choline uptake; (iii) it has been shown not to interact with a high affinity, Na^+-dependent 'choline transporter,' CHOT-1 [19, 22].

Some of the observed choline uptake must be attributed to the three types of cells contaminating the predominant (90%) type II cells in the suspension. Since these each contributed only 2–6% of the total cells, it seems unlikely that any of them contributed markedly to any of the three uptake systems we observed.

Comparison with Earlier Work

The total uptake of choline was shown to be reduced both by the absence of Na^+ from the medium and by the presence of HC-3, a known competitive inhibitor of choline uptake by other cell types, particularly (although not exclusively) of the Na^+-dependent type [15, 22, 24, 26, 28, 29]. This suggests the presence of at least two transport mechanisms, but some choline uptake persisted when HC-3 was present in Na^+-free medium, indicating the existence of a third pathway. The observations of Figs. 4 and 5 confirm this hypothesis and also provide direct evidence for the kinetic characterization of the first two pathways. The fact that the data for transport in the presence of Na^+ (Figs. 4*b* and 5*a*) are well described by the computed contribution of the Na^+-dependent and -independent routes provides evidence that this analysis is correct (Figs. 4*a* and 5*b*). The third pathway, characterized by its insensitivity to both Na^+ and HC-3, is discussed further below.

The results obtained from the kinetic experiments on the effects of Na^+ and HC-3 resemble the findings of others [10, 15] with lung-derived cells in suggesting that type II epithelial cells possess two saturable mechanisms for choline uptake. The apparent K_m for the higher affinity, Na^+-dependent uptake, estimated at 1.5 ± 0.3 μM, falls in the same range as that reported for the Na^+-dependent uptake by neuronal tissues [8, 23, 24, 27, 29]. A more precise estimate of the K_m of the lung system would require studies of a greater number of choline concentrations between 0.1 and 2 μM but the principal significance of our results is rather that they establish the K_m as being severalfold lower than previous estimates of the K_m for choline uptake by alveolar type II cells and significantly lower than the 4 μM reported for plasma-membrane vesicles of A549 cells [15]. As our results show, this difference is due to the previous use of a higher medium choline concentration. The dependence of choline transport on medium Na^+ concentration also resembles the high affinity neuronal system in showing a sigmoid relationship [27]. The questions of the stoichiometry and the nature of the relationship of Na^+ to choline transport raised by this finding require further study.

The inhibitory constant of the effect of HC-3 on Na-dependent choline uptake in the type II cells, estimated as 1.7 μM, was close to the highest values reported for neuronal tissue [29], although substantially higher than some other estimates, including that in A549 membrane vesicles [15, 24]. By contrast, our estimate of the K_i for the Na^+-independent fraction, approximately 12 μM, was rather lower than the values of 20–100 μM reported for other tissues [12, 15, 26, 28, 29]. Although the 'choline' transporter CHOT-1 was detected in lung tissue in very small amounts, its lack of sensitivity to HC-3 [9] makes it unlikely to have contributed to the choline uptake reported in the present work.

Cell Physiology of Pulmonary Choline Transport

Our findings and conclusions are summarized in Fig. 6. We propose that there are three routes available for choline entry into type II epithelial cells. Of highest affinity is the Na^+-dependent mechanism while an Na^+-independent system has a somewhat higher value for its K_m. It may be noted that the affinity of HC-3 as a com-

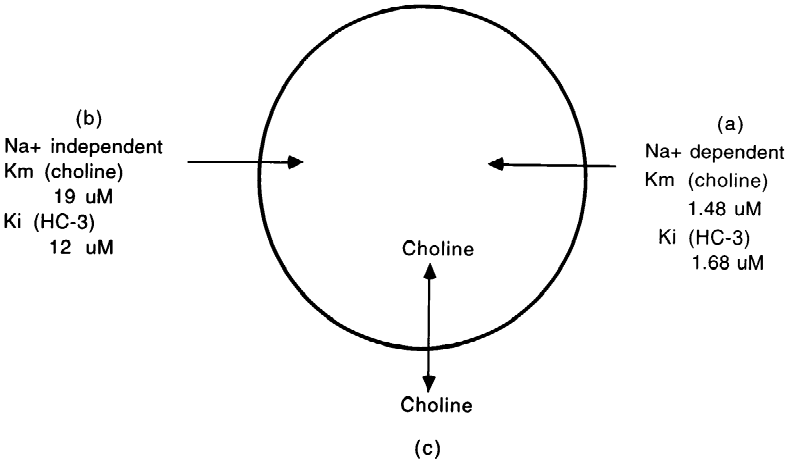

Fig. 6. Schematic representation of proposed pathways for choline uptake by type II cells of alveolar epithelium. (*a*) High affinity, Na^+-dependent uptake; (*b*) Lower affinity, Na^+-independent uptake; (*c*) Residual, presumed diffusive, system. Values for kinetic constants are derived from the results of Figs. 4 and 5.

petitive inhibitor is directly proportional to the affinity of choline as a substrate. Thirdly, our results provide some evidence of a third component, of very low affinity, which may be diffusional since it is Na^+-independent and insensitive to HC-3.

Physiologically, choline will play a central role in type II cell biology because of its requirement for the synthesis of phosphatidylcholine at rates adequate to sustain surfactant release. The observation that cells isolated from the intact, in vivo pulmonary epithelium have the classical ''neuronal'' high affinity, secondary active transport system for choline must therefore be of importance. Simple calculation shows that, assuming a membrane potential of −40 mV, and a coupling stoichiometry of 1:1 between choline and Na^+, a steady-state concentration ratio of intracellular to extracellular choline of 400 will be possible. Given that plasma choline is typically in the region of 20 μM, it seems likely that the Na^+-coupled transport system described in lung may be located at the apical surface; the Na^+-independent system (of lower affinity, but higher capacity) might then reasonably be looked for in the basolateral surface of the epithelial cells. Such a polarized distribution of transporters would permit scavenging of choline and its subsequent reincorporation into phosphatidylcholine.

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References

1. Bertrand, N., Bralet, J., Beley, A., 1990. Turnover rate of brain acetylcholine using HPLC separation of the transmitter. *J. Neurochem.* **55:**27–30
2. Bevan, C., Kinne, R.K.H. 1990. Choline transport in collecting duct cells isolated from rat renal inner medulla. *Pfluegers Arch.* **417:**324–328
3. Bland, R.D., Boyd, C.A.R. 1986. Cation transport in lung epithelial cells derived from fetal, new born, and adult rabbits. *J. Appl. Physiol.* **61:**507–515
4. Bligh, E.G., Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. *Canad. J. Biochem. Physiol.* **37:**911–917
5. Deves, R., Angelo, S. 1996. Changes in membrane and surface potential explain the opposite effects of low ionic strength on the two lysine transporters of human erythrocytes. *J. Biol. Chem.*, **271:**32034–32039
6. Deves, R., Chavez, P., Boyd, C.A.R. 1992. Identification of a new transport system (y^+L) in human erythrocytes that recognizes lysine and leucine with high affinity. *J. Physiol.* **454:**491–501
7. Deves R., Krupka, R.M. 1979. The binding and translocation steps in transport as related to substrate structure. A study of choline carrier of erythrocytes. *Biochim. Biophys. Acta* **557:**468–485
8. Ducis, I., Whittaker, V.P. 1985. High affinity, sodium-gradient-dependent transport of choline into vesiculated presynaptic plasma membrane fragments from the electric organ of *Torpedo marmorata* and reconstitution of the solubilized transporter into liposomes. *Biochim. Biophys. Acta* **815:**109–127
9. Fisher, A.B., Chander, A., Dodia, C., Reicherter, J., Kleinzeller, A. 1989. Choline transport by lung epithelium. *Amer. J. Respir. Cell Mol. Biol.* **1:**455–462
10. Fisher, A.B., Dodia, C., Chander, A., Kleinzeller, A. 1992. Transport of choline by plasma membrane vesicles from lung-derived epithelial cells. *Amer. J. Physiol.* **263:**C1250–C1257
11. Gardiner, J.E., Lee, H.S. 1982. Uptake of 1:^{14}C-choline by the guinea-pig lung in vitro: the effects of p-terphenyl hemicholinium-3. *Clin. Exp. Pharmacol. Physiol.* **9:**45–52
12. Grassl, S.M. 1994. Choline transport in human placental brush-border membrane vesicles. *Biochem. Biophys. Acta* **1194:**203–213
13. Kerr, J.S., Fisher, A.B., Kleinzeller, A. 1981. Transport of glucose analogues in rat lung. *Amer. J. Physiol.* **241:**E191–E195
14. Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M., Semenza, G. 1978. A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Their use in investigating some properties of D-glucose and choline transport systems. *Biochim. Biophys. Acta* **506:**136–154
15. Kleinzeller, A., Dodia, C., Chander, A., Fisher, A.B. 1994 Na^+-dependent and Na^+-independent systems of choline transport by plasma membrane vesicles of A549 cell line. *Am. J. Physiol.* **267:**C1279–C1287
16. Kuksis, A., Mookerjea, S. 1978. Choline. *Nutr. Rev.* **36:**201–207

17. Lee, R.D., Kwon, Y.-G., Morris, M.E. 1992. Hepatic transport of choline in basolateral liver plasma membrane vesicles. *FASEB J.* **8:***A*1485 (*Abstract)*
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193:**265–275
19. Mayser, W., Scloss, P., Betz, H. 1992. Primary structure and functional expression of a choline transporter expressed in the rat nervous system. *FEBS Lett.* **305:**31–36
20. Peers, C., Kemp, P.J., Boyd, C.A.R., Nye, P.C.G. 1990. Whole cell potassium currents in type II pneumocytes freshly isolated from rat lung: pharmacological evidence for two sub populations of cells. *Biochim. Biophys. Acta* **1052:**113–118
21. Porter, R.K., Scott, J.M., Brand, M.D. 1992. Choline transport into rat liver mitochondria. Characterization and kinetics of a specific transporter. *J. Biol. Chem.* **267:**14637–14646
22. Schloss, P., Mayser, W., Betz, H. 1994. The putative rat choline transporter CHO1 transports creatine and is highly expressed in neural and muscle-rich tissues. *Biochem. Biophys. Res. Comm.* **198:**637–645
23. Simon, J.R., Atweh, S., Kuhar, M.J. 1976. Sodium-dependent high affinity choline uptake: a regulatory step in the synthesis of acetylcholine. *J. Neurochem.* **26:**909–922
24. Sterling, G.H., Doukas, P.H., Ricciardi, F.J., Biedrzycka, D.W., O'Neill, J.J. 1986. Inhibition of high-affinity choline uptake and acetylcholine synthesis by quinuclidinyl and hemicholinium derivatives. *J. Neurochem.* **46:**1170–1175
25. Van Rossum, G.D.V., Boyd, C.A.R. 1995. Characteristics of choline uptake by lung type II epithelial cells. *FASEB J.* **9:**A860 (*Abstr.*)
26. Van Winkle, L.J., Campione, A.L., Mann, D.F., Wasserlauf, H.G. 1993. The cation receptor subsite of the choline transporter in preimplantation mouse conceptuses resembles a cation receptor subsite of several amino acid transporters. *Biochim. Biophys. Acta* **1146:**38–44
27. Wheeler, D.D. 1979. A model of high affinity choline transport in rat cortical synaptosomes. *J. Neurochem.* **32:**1197–1213
28. Wright, S.H., Wunz, T.M., Wunz, T.P. 1992. A choline transporter in renal brush-border membrane vesicles: energetics and structural specificity. *J. Membrane Biol.* **126:**51–65
29. Yamamura, H.I., Snyder, S.H. 1973. High affinity transport of choline into synaptosomes of rat brain. *J. Neurochem.* **21:**1355–1374
30. Yost, R.W., Chander, A., Dodia, C., Fisher, A.B. 1986. Synthesis of phosphatidylcholine by rat lung during choline deficiency. *J. Appl. Physiol.* **61:**2040–2044