Biochemical Pharmacology, Vol. 28, pp. 405–409.

0006-2952/79/0201-0405 $02.00/0

CHOLINE AND DIETHYLCHOLINE TRANSPORT INTO A CHOLINERGIC CLONE OF NEUROBLASTOMA CELLS*

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(*Received* 7 *April* 1978; *accepted* 20 *June* 1978)

Abstract—The transport of choline and diethylcholine has been investigated in a cholinergic clone ($S20F_3$) of mouse neuroblastoma cells. Choline transport was linear for the first 20 min of incubation and was temperature dependent at low concentrations ($< 1 \times 10^{-5}$ M). Diethylcholine transport was linear for the first 10 min of incubation and was also temperature dependent at low concentrations. High affinity ($K_m < 1 \times 10^{-6}$ M) and low affinity ($K_m > 1 \times 10^{-5}$ M) components of transport were found for both compounds. The transport system had a greater apparent affinity (lower K_m) for choline than for diethylcholine (3-fold), but maximal transport velocities were about equal. Each compound competitively inhibited the other's high affinity transport. Hemicholinium (1×10^{-5} M) slightly inhibited high affinity choline transport but triethylcholine (1×10^{-6}–1×10^{-4} M) did not. Choline transport was also found to be dependent on the pH and pCO_2 of the medium.

(2-Hydroxyethyl) methyldiethylammonium (diethylcholine; DEC) has been shown to block cholinergic transmission at a variety of nicotinic and muscarinic sites [1, 2]. A good deal of indirect evidence exists that the mechanism of blockade is through the *in vivo* synthesis of a false cholinergic transmitter from DEC [1–5]. However, the possibility that DEC blocks cholinergic transmission via a hemicholinium-like action cannot be ruled out.

Cholinergic neurons have a unique high affinity choline transport system ($K_t < 1 \times 10^{-5}$ M) that is linked to the synthesis of acetylcholine such that choline transport is the rate-limiting step in the synthesis [6–8]. Certain cholinergic clones of cultured mouse neuroblastoma cells have also been shown to possess this high affinity transport system [9, 10] although some laboratories have not been able to repeat these finds [11, 12].

We present here our findings on the existence of a high affinity choline transport system in the $S20F_3$ clone of neuroblastoma cells. We have also studied the interaction of choline and DEC transport into these cells in order to distinguish between the two probable mechanisms of cholinergic blockage by DEC.

* This work was supported in part by research grants CH-81 awarded by the American Cancer Society and CA-17584 awarded by the National Cancer Institute, NIH.

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MATERIALS AND METHODS

Materials. Diethylaminoethanol, iodomethane, triethylcholine iodide, hemicholinium bromide (HC-3), and choline chloride were obtained from commercial sources. Tritiated choline chloride (8.4 Ci/m-mole, uniformly labeled) and tritiated iodomethane (3.15 Ci/m-mole) were purchased from Amersham Corp., Arlington Heights, IL, and New England Nuclear Corp., Boston, MA, respectively. Diethylcholine was synthesized in this laboratory by the method of Holton and Ing [13].

Two buffers were used in the transport experiments. The phosphate buffered saline (PBS) had the following composition: 138 mM NaCl, 2.7 mM KCl, 5.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$. The bicarbonate-phosphate buffered saline (BPBS) consisted of all the ingredients of the PBS except that the molarity was reduced by 13.1 per cent and that 20 mM of $NaHCO_3$ was added. The BPBS was saturated with 5% CO_2–95% air before it was used. Both buffers had a pH of 7.3–7.4.

Tritiated diethylcholine iodide. The synthesis of tritium labeled DEC was essentially the same as that of unlabeled DEC. Diethylaminoethanol and [^{3}H]-iodomethane (31.7 μmoles of each) were combined at room temperature in 0.5 ml of ethyl ether. The precipitated product was purified three times with ethyl ether. After drying with N_2, the product was dissolved in 1.0 ml ethanol, transferred to a screw-capped vial and dried in a high vacuum. The product was weighed and then stored at $-20°$ as a 100 μM solution in ethanol. The specific activity of the product was 3.1 Ci/m-mole based on the starting material.

Neuroblastoma cells. The cholinergic $S20F_3$ clone of mouse neuroblastoma cells was used throughout

this study. It contained high activity of acetyl CoA: choline-*O*-acetyltransferase (EC 2.3.1.5; ChAc) and was a gift from Dr. Marshall Nirenberg at The National Heart and Lung Institute/National Institutes of Health. Cells were subcultured for experiments from confluent 250 ml Falcon flasks containing a stock culture that received fresh medium every other day and was subcultured within 2 days of confluency. Experimental cultures were grown in 35 mm diameter Falcon or Costar tissue culture dishes. Each dish received 1×10^5 cells and 2 ml of culture medium. The medium was changed every other day and cells were used for an experiment on day 5 after passage or 18 to 24 hr after the last change of medium.

Cells were grown in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, NY—GIBCO No. 196G) supplemented with fetal calf serum (10%), L-glutamine (1%), penicillin (50 units/ml), and streptomycin (50 μg/ml). Cell incubation conditions were maintained at 37° with no light in an atmosphere of 95% air, 5% CO_2, and 100% humidity.

Transport kinetics. Each confluent plate of cells was washed once with 1.0 ml of buffer (BPBS at 37°, saturated with 5% CO_2–95% air) and then incubated for an appropriate time period with 1.0 ml of the same buffer. Appropriate tritium-labeled compounds and transport-blocking drugs were added for incubation and afterwards each plate was washed four times with ice-cold PBS within 45 sec. The last wash was completely aspirated and the plate was allowed to dry. The dry cells were dissolved in 0.5 ml formic acid (< 20 min). A 0.2-ml aliquot of dissolved cells was transferred to 10 ml of scintillation solution (100 g naphthalene and 7 g of 2,5-diphenyloxazol in 1 liter dioxane) and counted in a Beckman scintillation counter. A 10- or 20-μl aliquot of dissolved cells was used to determine the protein content.

Control plates received no tritium-labeled compound and were used to determine the scintillation blank and the radioactivity quenching. Scintillation counts per min were converted into pmoles of substrate and the results were expressed as either pmoles/mg of protein or pmoles/min/mg of protein. A least squares fit of a line to a Lineweaver–Burk plot was used to calculate K_m and V_{max}. The standard errors of K_m and V_{max} were found by the method of Bull [14].

Protein. Protein was determined by the method of Bradford [15]. The dye concentrate was purchased from Bio-Rad Laboratories, Rockville Center, NY. Bovine serum albumen was used to construct a standard curve.

RESULTS

Preliminary experiments on choline transport in $S20F_3$ neuroblastoma cells were carried out with a standard procedure and resulted in erratic and non-reproducible data. To determine if sudden changes in the pH and pCO_2 of the growth media and incubation buffer were responsible for this problem, V_{max} and K_m for choline transport were measured under two different incubation conditions. In the first method, cells and culture media were allowed to equilibrate with humid, 37° air (a medium pH change from 7.3 to 7.7) and then incubated with PBS (pH 7.35 with air) and [^{3}H]choline. In the second method, cells were not equilibrated with air (no change of medium pH) and were incubated with BPBS (PBS plus bicarbonate and CO_2; pH 7.3–7.4 with 5% CO_2–95% air). Incubation with PBS resulted in an absence of a high affinity component of transport and a reduction in low affinity transport compared to incubation with BPBS (Table 1). Incubation with PBS is a standard method used by others in transport kinetics experiments but in this laboratory the abrupt changes in pH and pCO_2 had a significant effect on choline transport. Therefore, all subsequent experiments were done with the second incubation method. The membrane transport of choline in $S20F_3$ cells was found to be linear for the initial 20 min of incubation and that of DEC was linear for the initial 10 min of incubation (Fig. 1). The transport of choline was found to have both high and low affinity components as was the transport of DEC (Fig. 2, Table 2). The high and low apparent transport constants (K_m values) were lower for choline than those for DEC. The maximal high affinity transport (V_{max}) of choline was about the same as that of DEC but the maximal low affinity transport of DEC was slightly higher than that of choline (Table 2).

The transport of choline and DEC was also measured at 0° in the high affinity transport range to determine if the transport was energy dependent. The rate of choline transport at 0° was only 3.2 per cent of that at 37° at concentrations ranging from 1.2×10^{-7} M to 1.2×10^{-6} M. The transport rate of DEC at 0° was 4.0 per cent of the normal transport at a concentration range of 1.0×10^{-7} M to 5.4×10^{-6} M (Fig. 3).

Several cholinergic blocking agents were tested for

Table 1. Effect of incubation buffer and atmosphere on choline transport*

	PBS with air	BPBS with 5% CO_2–95% air
V_{max} low affinity†	92.0 ± 10.0‡	155.0 ± 1.5
K_m low affinity	2.0 ± 0.2 × 10^{-5} M	9.9 ± 1.0 × 10^{-6} M
V_{max} high affinity		6.1 ± 0.8
K_m high affinity		2.7 ± 0.3 × 10^{-7} M
Choline range	1.2 × 10^{-7}–2.3 × 10^{-4} M	1.0 × 10^{-8}–4.8 × 10^{-5} M

† Cells, culture media, and buffers were equilibrated with the appropriate atmosphree before media were exchanged for buffer plus [^{3}H]choline.

‡ V_{max}, pmoles/min/mg of protein.

* All values were taken from best fit lines generated form at least 16 points at four substrate concentrations.

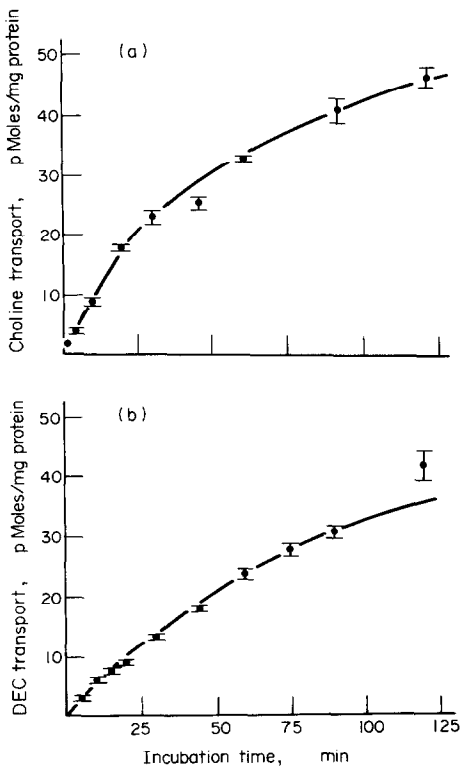

Fig. 1. [^{3}H]choline (A) and [^{3}H]DEC (B) transport as a function of time in S20F$_3$ cells. Bars represent standard errors of the mean for $n = 4$. The concentration of choline was 1.2×10^{-7} M and that for DEC was 1×10^{-7} M.

their ability to inhibit the high affinity transport of choline. Hemicholinium caused a slight competitive inhibition at 1×10^{-5} M (Fig. 4). Lower concentrations of HC-3 had little or no effect. Triethylcholine had little effect on the transport at doses up to 1×10^{-4} M. Diethylcholine had no effect on the transport at concentrations ranging from 1×10^{-8} to 1×10^{-6} M. However, there was a competitive inhibition of choline transport by DEC at 1×10^{-5} M and a mixed type of inhibition at 1×10^{-4} M (Fig. 5).

The effect of choline on the high affinity DEC transport was tested in order to determine if the same carrier system was responsible for both choline and DEC transport. Choline competitively inhibited DEC transport at 1×10^{-5} M but had no effect at 1×10^{-6} M or lower concentrations (Fig. 6).

DISCUSSION

The main purpose of these experiments was to provide additional evidence to elucidate the mechanism of cholinergic transmission blockade produced by DEC. It was found that DEC competitively inhibited high affinity choline transport and was itself transported by an energy-dependent system. Diethylcholine transport had both high and low affinity components and the high affinity component was competitively inhibited by choline. This strongly suggests that DEC is a competitive substrate for transport into neuroblastoma by the choline high affinity carrier. The same results were obtained for two other choline analogs, monoethylcholine and pyrrolcholine, in crude rat forebrain synaptosomes [8]. Coupling this information with the fact that DEC is readily acetylated by choline acetyltransferase, it can be concluded that DEC probably blocks cholinergic transmission primarily through a false transmission and not simply via the inhibition of choline transport. This is in agreement with other workers [16–19] who have demonstrated the existence of a whole class of false cholinergic transmitters. However, the final proof of false transmission, that is the calcium-dependent release of acetyl-DEC, has not been done.

Another important result of these experiments was the finding of high and low affinity transport systems for choline in neuroblastoma cells which is in agreement with Massarelli *et al.* [9] but opposed to the findings of two other laboratories [11, 12]. In one of these studies [11], the investigators may not have used low enough choline concentrations to detect the high

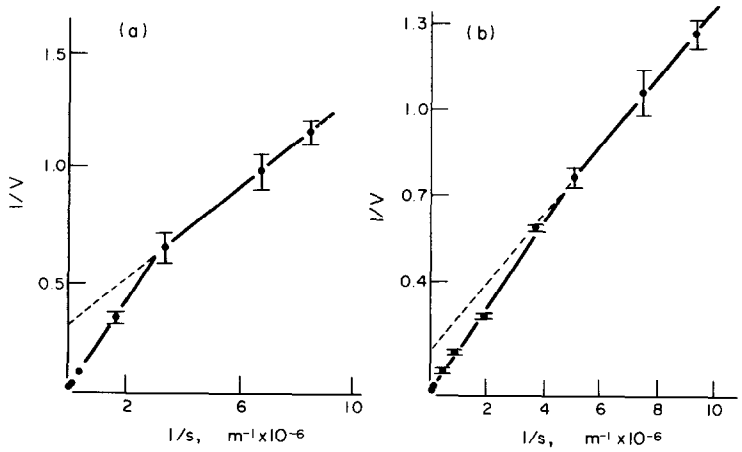

Fig. 2. Lineweaver–Burk plot of [^{3}H]choline (A) and [^{3}H]DEC (B) transport kinetics in S20F$_3$ cells of a substrate range from 1×10^{-7} M to 1×10^{-5} M. Bars represent standard errors of the mean of four experiments. Ordinate units are pmoles^{-1} · min · mg of protein. Cells were incubated with labeled compound for 10 min. The line is a least squares fit.

Table 2. Michaelis-Menten constants for transport of choline and DEC in $S20F_3$ cells*

	Choline†	DEC
V_{max} low‡	155.0 ± 1.5	233.0 ± 21.8
K_m low	9.9 ± 1.0 × 10^{-6} M	3.6 ± 0.3 × 10^{-5} M
V_{max} high†	6.1 ± 0.8	7.0 ± 1.3
K_m high	2.7 ± 0.3 × 10^{-7} M	9.0 ± 1.4 × 10^{-7} M
Substrate range	1.0 × 10^{-8}–4.8 × 10^{-5} M	2.1 × 10^{-8}–8.6 × 10^{-5} M

* All values were taken from best fit lines generated from at least 16 points at four substrate concentrations.

† Transferred from Table 1 for comparison.

‡ V_{max}, pmoles/min/mg of protein.

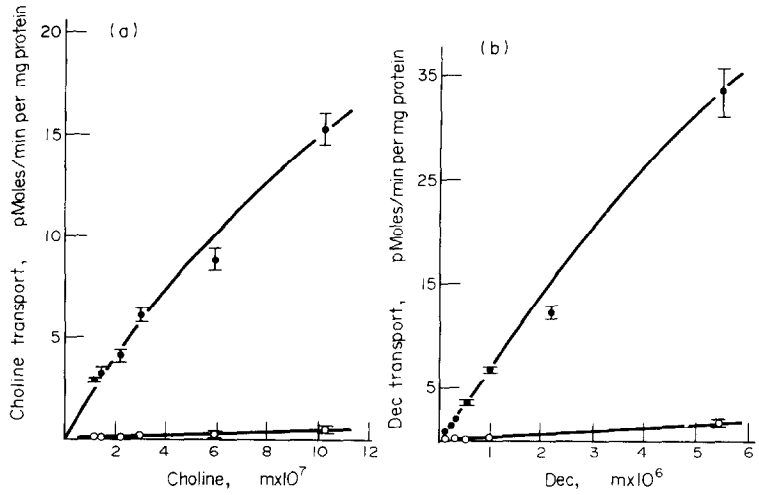

Fig. 3. [^{3}H]choline (A) and [^{3}H]DEC (B) transport as a function of temperature and concentration. Bars represent the standard errors of the mean of four experiments. Key: (●) 37° incubation; and (○) 0° incubation. Cells were incubated with labelled compound for 10 min.

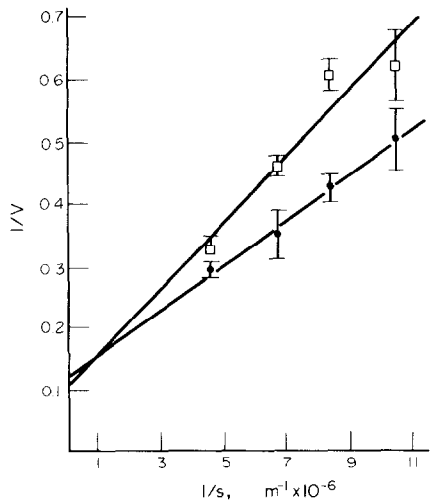

Fig. 4. Lineweaver–Burk plots of choline transport inhibition by heimicholinium. Key: (●) choline only; and (□) choline with 10^{-5} M HC-3. See legend fo Fig. 2 for other information.

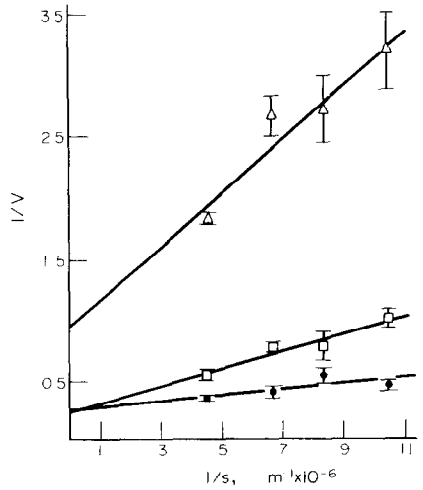

Fig. 5. Lineweaver–Burk plots of choline transport inhibition by DEC. Key: (●) choline only; (□) choline with 10^{-5} M DEC; and (△) choline with 10^{-4} M DEC. See legend of Fig. 2 for other information.

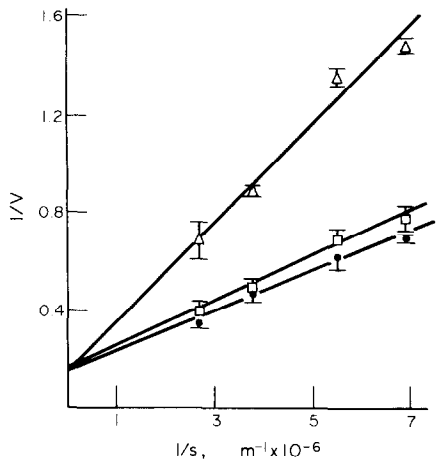

Fig. 6. Lineweaver–Burk plots of DEC transport inhibition by choline. Key: (●) DEC only; (□) DEC with 10^{-6} M choline; and (△) DEC with 10^{-5} M choline. See legends of Fig. 2 for other information.

affinity component but the discrepancy may just be the result of different incubation media and clonal cell lines.

Previously, this high affinity transport system had been shown to be sodium dependent [9, 20] and we have now shown that it is also dependent on pH and pCO_2. Our experiments, however, do not distinguish between the effects of changes in pH or pCO_2 alone.

One of the important features of most peripheral and central high affinity choline transport systems is its complete inhibition by low concentrations of HC-3 ($< 1 \times 10^{-5}$ M) [8, 21]. As has been shown here and by others [22], the high affinity choline transport in neuroblastoma cells was relatively insensitive to HC-3 as was transport in guinea pig cortical synaptosomes [23], embryonic chick nerve cell cultures [10], and rat cortical slices [24]. However, low concentrations of HC-3 inhibited the increase in choline transport caused by potassium depolarization of cortical slices [24]. In our study, HC-3 significantly inhibited the choline transport at 1×10^{-5} M but the magnitude of inhibition was in the range of HC-3 inhibition of low affinity choline transport ($K_i > 3 \times 10^{-5}$ M). This differential effect of HC-3 suggests that there are both HC-3 sensitive and insensitive types of high affinity choline transport systems and that the sensitivity may be partially governed by the state of membrane polarization.

One additional result of these experiments was an inability of triethylcholine to block choline transport. The lack of effect of triethylcholine is not surprising inasmuch as it is less potent than DEC in inhibiting choline transport [8] and requires millimolar concentrations to inhibit the neuromuscular junction *in vitro*.

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