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DIFFERENCE IN PUTRESCINE TRANSPORT IN UNDIFFERENTIATED VERSUS DIFFERENTIATED MOUSE NB-15 NEUROBLASTOMA CELLS

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SUMMARY: The transport of [^{14}C]putrescine into control and cAMP-induced differentiating mouse NB-15 neuroblastoma cells was studied and compared. Results indicated a steady and significant decrease in the rate of putrescine transport of the cAMP-treated culture when compared to that of the control over a five-day culture period. The decrease in the rate of putrescine transport of the differentiating neuroblastoma cells may be attributable to an increase in the apparent K_m value of the transport system. The V_{max} values for the control and cAMP-tested cultures (day 4 cultures) were identical (5.3 nmol/mg protein/hr) while the apparent K_m values were different, being 2.75μM and 28.5μM for the control and cAMP-treated cultures respectively. The decrease of putrescine transport in the differentiating cells was specific, concomitant measurements of aminoisobutyrate transport revealed little difference between the control and cAMP-treated culture. The inhibition of putrescine transport may represent one of the earlier changes of membrane function associated with cell differentiation.

Mouse neuroblastoma cells in culture can be induced to differentiate by cyclic AMP and agents which increase intracellular cAMP content (1,2). Differentiated neuroblastoma cells are characterized by long neurite outgrowth (>50μM), increased amounts of cAMP-binding protein and neurotransmitter metabolizing enzymes (1-4).

Our previous studies of the biochemical processes involved in the differentiation of mouse neuroblastoma cells have demonstrated differences in the regulation of ornithine decarboxylase activity (ODC, EC.4.1.1.17) in the differentiated and undifferentiated mouse neuroblastoma cells (5). ODC is the rate-limiting enzyme for the biosynthesis of polyamines (6). Polyamines are ubiquitous organic cations in prokaryotic and eukaryotic cells

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(7,8). Their multiple functions and physiological roles in various biochemical processes are currently being investigated in many laboratories (7-11).

Polyamines also exist in body fluid (9-12). Studies have shown that extracellular polyamines can be transported intracellularly and can regulate the activities of the polyamine-synthesizing enzymes (13). That the transport of polyamines may be important in the regulation of growth is suggested by the following studies. Pohjanpelto (14) reported that putrescine transport is greatly increased in human fibroblasts which are initiated to proliferate. Lajtha and Sershen (15) have shown that the transport system of diamines (putrescine and cadaverine) is different from the transport systems of amino acids in mouse brain slices. Kano and Oka (16) have demonstrated that the active transport system for polyamines in mouse mammary explants can be stimulated by insulin and prolactin.

In the present study, the transport rates of putrescine into the non-differentiating and differentiating neuroblastoma cells in tissue culture were measured and compared over a five-day culture period. We observed a significant decrease of putrescine transport associated with the differentiation of neuroblastoma cells.

MATERIALS AND METHODS

Chemicals. [1,4-^{14}C]Putrescine dihydrochloride (96.4mCi/mmol) was purchased from Amersham, Arlington, Ill. α-[methyl-^{3}H]aminoisobutyric acid was obtained from New England Nuclear, Boston, Mass. Putrescine·2HCl, spermidine·3HCl, spermine·4HCl, cadaverine, aminoisobutyric acid (AIB), dibutyryl cyclicAMP, (Bt_2cAMP) and phenylmethylsulfonyl fluoride (PMSF) were from the Sigma Chem. Co. St. Louis, MO. 3-isobutyl-1-methyl xanthine (IBMX) was from Aldrich Chem. Co., Milwaukee, WI. Dulbecco's modified Eagle medium, fetal calf serum were from Gibco, Grand Island, N.Y. All other chemicals were of standard reagent grade.

Cell culture and differentiation. Mouse NB-15 neuroblastoma cells (a gift of Dr. W. Gibson, Rutgers Univ.) were grown as monolayer cultures in Dulbecco's modified Eagle medium (with 4500 mg glucose per liter, without sodium pyruvate), supplemented with 10% fetal calf serum (normal growth medium). The growth medium also contained 50 U/ml penicillin and 50μg/ml streptomycin. The cell culture was maintained in a Forma water-jacketed CO_2 incubator (95% air, 5% CO_2) at 37°C. The differentiation of neuroblastoma cells was initiated by adding 1mM Bt_2cAMP and 0.5mM IBMX to the cell culture 15 hrs after plating (the seeding density was approximately $1x10^4$ cells/cm^2). Neurite outgrowth was detectable within 2 hrs after adding the drugs, while increases in acetyl-

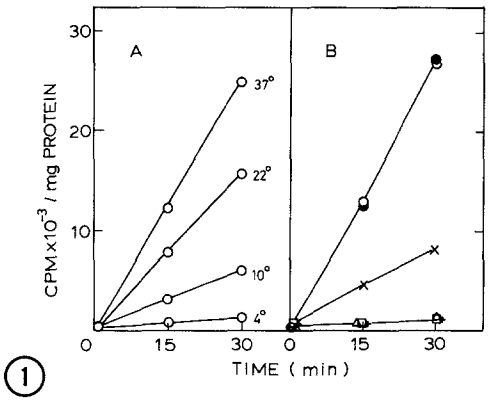
cholinesterase and cAMP-binding protein(s) were clearly demonstrable two days after adding Bt_2cAMP (data not shown). Cells grown in the normal growth medium will be designated as NB cells denoting non-differentiating neuroblastoma cells whereas cells grown in the differentiation medium (i.e. normal growth medium with 1mM Bt_2cAMP and 0.5mM IBMX) will be designated as ND cells denoting differentiating neuroblastoma cells.

Transport of radioactive substances. For studies of the transport rate of putrescine into cells as a function of time in tissue culture, time zero (t=0) refers to the time point when Bt_2cAMP and IBMX were added to the cell culture. At various times thereafter, duplicate cultures of NB and ND cells were removed from the incubator and quickly washed and reincubated in fresh Dulbecco's medium prewarmed to 37°C. [^{14}C]Putrescine and [^{3}H]AIB were added to a final concentration of 0.5μM and 0.1μM respectively. The uptake of radioactivity over a 20 min incubation period was taken as an index of the rate of transport. After incubation, cells were washed three times with ice-cold Hank's salt solution containing 5mM each of unlabeled putrescine and AIB. The washed cells were then scraped with a rubber policeman into 20mM Tris-HCl (pH 7.2) containing 1mM PMSF. The cells were broken by sonication and aliquots were solubilized in aquasol for radioactivity counting using a Beckman LS 7000 Liquid Scintillation Counter. Protein concentration was determined by Lowry's method (17). For the determination of K_m and V_{max} of the putrescine transport system, NB and ND cells at their early stationary phase of growth (t=~80hr) were used.

RESULTS AND DISCUSSION

Putrescine transport in mouse neuroblastoma cells appeared to be an active transport process as shown by its temperature dependency and its sensitivity toward uncouplers such as dinitrophenol. Spermidine, spermine and cadaverine inhibited putrescine transport suggesting that they may compete for a common transport system (Fig. 1). These results are consistent with findings obtained in other mammalian systems (14-16).

The putrescine transport systems of NB and ND cells were studied by comparing their initial rates of putrescine transport at various times over a five-day culture period (Fig. 2A). A marked difference in the transport pattern between NB and ND cells was observed. The rate of putrescine transport in NB cells was high during the entire log phase of growth (t= 0~80hr) and started to decline when cells became confluent. This decline was most likely due to cell density inhibition as shown by many workers in other cell systems (18,19). The putrescine transport of ND cells showed a 27% decrease when compared to that of the control 6 hrs after the addition of Bt_2cAMP and IBMX, and remained significantly lower than that of the control



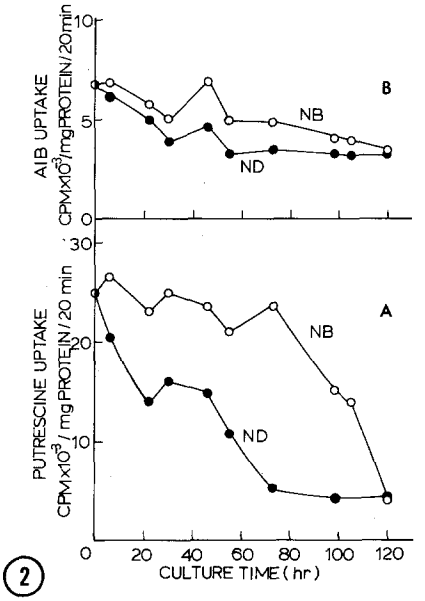


Fig. 1. Effects of temperature (A) and drugs (B) on the transport of putrescine into NB-15 neuroblastoma cells.
(A) NB-15 cells were pre-incubated at various temperatures for 20 min. The transport experiment was then initiated by adding [^{14}C]putrescine to the cell cultures as described in "Materials and Methods".
(B) Various drugs were added 10 min before the addition of [^{14}C] putrescine to the cultures. The putrescine uptake was measured as described under "Materials and Methods". Control, (-○-); 1mM Bt_2cAMP (-●-); 5μM spermidine (-□-); 5μM spermine (-△-); 5μM cadaverine (+); 1mM dinitrophenol (X).

Fig. 2. Initial rate of transport of [^{14}C]putrescine (A) and [^{3}H] aminoisobutyrate (B) into NB and ND cells as a function of time in tissue culture. NB-15 cells were seeded at 1x10^{4}cells/cm^2 in 60mm tissue culture dishes. Fifteen hours after seeding, cell cultures were treated with (-●-) or without (-○-) 1mM Bt_2cAMP plus 0.5mM IBMX. The control culture was designated as NB cells and the cAMP-treated culture was designated as ND cells. Time zero refers to the time point when Bt_2cAMP and IBMX were added to the cell culture. The initial rate of putrescine transport was determined at the indicated times as described under "Material and Methods." Each point represents the average of 3 separate experiments. Standard error is within the range of 10% for each point.

over the entire five-day culture period. The inhibition of putrescine transport in ND cells was apparently not due to a direct effect of cAMP on the transport system but rather related to some biochemical events triggered by cAMP which in turn led to alteration of the transport system. This interpretation is supported by the fact that the addition of 1mM Bt_2cAMP to the experimental

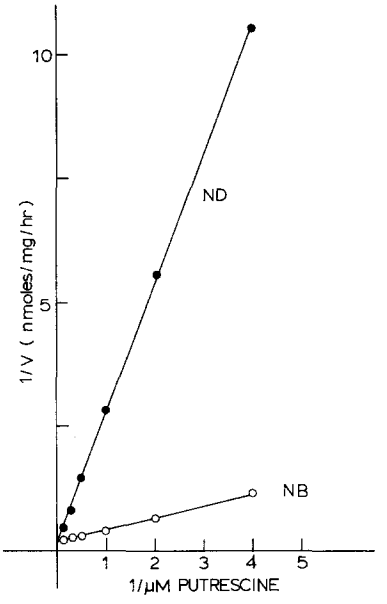


Fig. 3 Apparent K_m value and V_{max} for putrescine transport by NB (-○-) and ND (-●-) cells. Both NB and ND cells at t=~80hr were exposed to various concentrations of unlabeled putrescine with constant amounts of [^{14}C]putrescine (0.1μCi/ml) for 30min. The radioactivity in cells was determined as described in "Material and Methods". Each point represents the mean of duplicate measurements.

medium during the transport measurement had no effect on putrescine transport (Fig. 1B). Similar observation has been made by Pohjanpelto (14) using human skin fibroblasts.

To study the specificity of the effects of cAMP, the transport of AIB in the NB and ND cells was determined and compared with the transport of putrescine under identical conditions. Control experiments indicated that AIB did not affect putrescine transport suggesting separate transport systems for these two compounds. Results in Fig. 2B showed that only a slight inhibition of AIB transport occurred in ND cells when compared to NB cells.

In order to determine the basis of the decreased putrescine transport of ND cells, the K_m and V_{max} values of the transport system of ND cells was studied and compared to that of NB cells. Results are shown in Fig. 3. The V_{max} and K_m of putrescine transport of NB cells were 5.3 nmol/mg protein/hr and 2.75μM respectively. These values are similar to those obtained in

human fibroblasts (14). The V_{max} of putrescine transport for ND cells was identical to that obtained for NB cells while the K_m of putrescine transport for ND cells, being 28.5μM, was 10 times greater than that of NB cells. Thus the increase of K_m in ND cells probably accounted for the decrease of putrescine transport observed in these cells.

Differentiation of mouse neuroblastoma cells in vitro constitutes a good system to study the biochemistry of tumor reversion and differentiation process. The molecular mechanism of cAMP-induced differentiation of neuroblastoma cells is of general interest because cAMP is known to be a potent inducer of differentiation in many other cell types (20-22).

Since polyamines are ubiquitous and are involved in growth regulation, the alteration of polyamine metabolism may be of importance in cell differentiation. Indeed, several studies have shown that specific changes of ODC activity occurred during the differentiation of chondrocytes (22), bone marrow cells (23) and muscle cells (24). We have recently found that the differentiation of mouse neuroblastoma cells was accompanied by a marked decrease of ODC activity and spermidine content (Chen et al., manuscript submitted). The present finding that a specific change in the putrescine transport system occurred in ND cells, namely the K_m of the transport system, supports the notion that polyamine metabolism may be involved in the differentiation. In addition, the inhibition of putrescine transport in ND cells may represent one of the earlier membrane changes associated with the differentiation of neuroblastoma cells.

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