CHOLINE STIMULATES THE SYNTHESIS AND ACCUMULATION OF ACETATE IN A CHOLINERGIC NEUROBLASTOMA CLONE

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SUMMARY : The NS 20 mouse neuroblastoma clone was shown to synthesize acetylcholine from labelled glucose or acetate as precursor of the acetyl moiety of acetylcholine ; in both cases, the synthesis was stimulated by the presence of exogenous choline. In addition, we report that acetate is accumulated in the NS 20 clone by a mechanism that is highly temperature-dependent and is stimulated by the presence of externally added choline.

INTRODUCTION

To date, few details are available concerning the biosynthesis of acetylcholine (ACh) in the cholinergic mouse neuroblastoma clones. Some of the clones have been analyzed for the transport of choline (1,2), but were found to be devoid of the specific uptake system characteristic of cholinergic nerve terminals (3,4). Therefore it has been questioned to what extent the presence of choline acetyltransferase (ChAc, EC 2.3.1.6) is a sufficient marker of a cholinergic phenotype and whether the ChAc-containing cells could serve as a model for the study of ACh synthesis.

In this paper, we describe the synthesis of ACh in the NS 20 cholinergic clone using both glucose and acetate as precursors of the acetyl moiety of ACh. In addition, we have examined the accumulation of acetate into the cells as a function of the choline concentration

MATERIALS AND METHODS

Cell Culture The clones NS 20 and NIE 115 were a gift of Dr M.Nirenberg. The cells were grown in Dulbecco's modified Eagle's medium (Gibco H-21) containing 5% Gibco fetal calf serum and antibiotics (penicillin, streptomycin and fungizone at 100 U, 50 µg and 0.05 µg/ml respectively) in bacteriological Petri dishes (Planet) without stirring in a water-saturated atmosphere of 5% CO_2/95% air. Under these conditions cells grew in suspension forming small aggregates easily dissociable. Cells were 90 ± 5% viable as judged by trypan blue dye exclusion. Suspension culture conditions were chosen for the following reasons : a) no significant differences were found in ChAc and ACh levels between suspension and monolayer cultures in comparable phases of growth cycle, and b) collecting and sampling of cells were faster, more efficient and less deleterious to cells.

Abbreviations: ACh, acetylcholine ; ChAc, choline acetyltransferase

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Unlabelled ACh was measured by the radio-enzymic assay described by GUYENET et al. (5) using both thin-layer chromatography and choline kinase to further remove the residual choline. All determinations presented in this paper were done in triplicate ; the values presented are the average of these measurements.

Synthesis of (^{3}H) or (^{14}C)ACh in the NS 20 clone from (^{3}H)glucose and (^{14}C) acetate. In all experiments, the suspension cultures were washed and resuspended in medium containing in mM : NaCl, 130 ; KCl, 5.6 ; $NaHCO_3$, 16.2 ; NaH_2PO_4, 1.2 ; $MgCl_2$, 1.2 ; $CaCl_2$, 2.2 and eserine salicylate 0.049. Aliquots containing 4 X 10^5 NS 20 cells in 0.2 ml (240 µl protein) were preincubated for 3 min at 37°C in 2 ml plastic tubes with varying concentrations of choline. The samples were gassed under O_2/CO_2 (95:5) by means of a two-needle attachment in the cover of the tube. Labelled glucose or acetate (20 µl) was added and incubated for an additional 15 min at 37°C. The reaction was terminated by the addition of 300 µl of 8.5% Cl_3AcH containing (^{14}C-Ac)ACh (5nCi) or (^{3}H-Ac)ACh (20 nCi) as a recovery standard. The labelled ACh that was synthesized over the 15 min period was first extracted and then isolated by thin-layer chromatography as described by LEFRESNE et al.(6).

Accumulation of acetate into the neuroblastoma clones in one minute. The suspension cultures were washed and resuspended in the medium described above except that 5 mM glucose was also added. Aliquots of 0.2 ml (2 X 10^5 cells which corresponds to 120 µg and 230 µg protein for the NS 20 and NIE 115 respectively) were preincubated for 5 min at 37°C under O_2/CO_2 (95:5). The reaction was started by the addition of 20 µl of (^{14}C)acetate. The samples were incubated for 1 min at 37°C or at 0°C and the reaction terminated by the addition of 750µl of cold incubation medium. The samples were centrifuged (3 min, 170g) and subsequently analyzed for their radioactivity by the method of GUYENET et al.(7). The net accumulation is expressed in nmol of acetate taken up per minute per mg of protein ; the values were obtained by subtracting the accumulation observed at 0°C (which corresponds to the amount penetrating by passive diffusion) from the accumulation measured at 37°C. A one minute incubation period was chosen since this was the shortest time interval within which these uptake experiments could be completed. As an initial velocity was measured, an incubation period as short as possible was used.

Percentage conversion of (^{14}C) acetate into (^{14}C)ACh. The NS 20 cells (10^6/200 µl) were prepared as described in the previous section. The reaction was started by the addition of 20 µl of (1-^{14}C)acetate (5.9 µCi) containing various concentrations of sodium acetate in the presence or absence of 1 mM choline. The samples were incubated for 3 min at 37°C or at 0°C and the reaction terminated by the addition of 750 µl of cold incubation medium. The samples were centrifuged (3 min, 170 g) at 4°C and the cellular pellet was analyzed for (i)(^{14}C)ACh by the procedure described previously and (ii) for the total radioactivity contained within the cells. The "percentage conversion" corresponds to the amount of (^{14}C)ACh accumulated in the cells expressed in percent of the total ^{14}C radioactivity found in the cellular pellet.

RESULTS AND DISCUSSION

ACh synthesis using glucose and acetate as precursors

The NS 20 in suspension culture was found to contain high levels of endogenous ACh (2 nmol/mg protein). Therefore some mechanism for the production of ACh had to be present within the cells. We examined the effectiveness of both glucose and acetate as precursors of the acetyl moiety of ACh.

Fig. 1a shows that (^{3}H)glucose was an effective precursor for the synthesis of (^{3}H)ACh at a rate which was increased

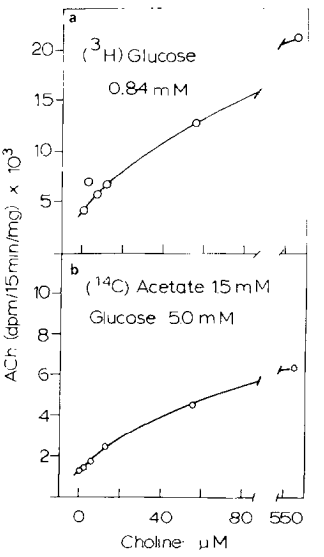

Figure 1. Synthesis of (^{3}H) or (^{14}C)ACh in NS 20 using (^{3}H)glucose or (^{14}C)acetate in the presence of increasing concentrations of choline. (a) NS 20 incubated with 0.084 mM (6-^{3}H)glucose (93 µCi/ml). (b) NS 20 incubated with 5.0 mM glucose plus 1.5 mM (1-^{14}C)acetate (33.8 µCi/ml).

by the addition of choline. This last result agrees with the prominant role played by extracellular choline in the synthesis of ACh in cholinergic ganglion (8) and brain cholinergic nerve terminals (7).

Acetate was also found to act as a precursor of the acetyl moiety of ACh in the cholinergic clone. This result is in contrast to that observed with mammalian brain cholinergic neurons which cannot use acetate as a precursor (7,9-13). Fig.1b illustrates the formation of (^{14}C)ACh over 15 min in the NS 20 clone incubated in the presence of (^{14}C)acetate. The rate of labelled ACh synthesis was also stimulated by the presence of exogenous choline.

In the experiments with acetate, unlabelled glucose was added to the incubation medium to allow the intermediary metabolism of the cells to function under physiological conditions. Even in the presence of this high concentration of unlabelled glucose (5 mM), corresponding to the physiological level in the blood, (^{14}C)acetate was still incorporated into (^{14}C)ACh. This

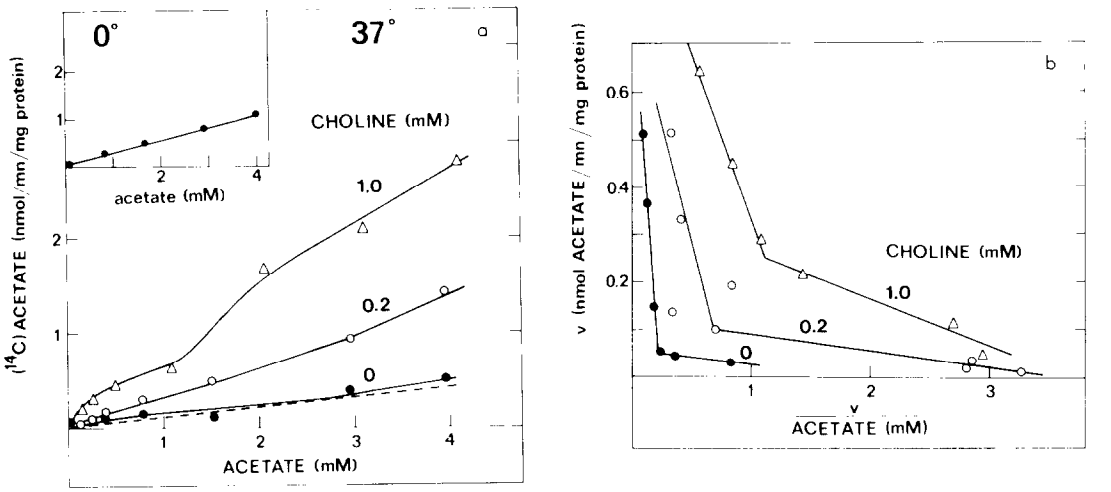

Figure 2. Velocity of the accumulation of (^{14}C)acetate in the cholinergic NS 20 clone. (a) Effect of increasing concentrations of choline. The reaction was started by addition of 20 µl of (2-^{14}C)acetate (0.001 µCi up to an acetate concentration of 0.04 mM and 1.0 µCi up to an acetate concentration of 4 mM) and choline to give the final concentration indicated on the Figure. The dashed line shows the accumulation of acetate into the NIE 115 clone in the presence of 1 mM choline. INSET: Initial accumulation of (2-^{14}C)acetate at 0°C in the NS 20 clone. The results are the same in the presence or absence of 1 mM choline. (b) Eadie-Hofsee plot of the accumulation of (^{14}C)acetate into the NS 20 in the presence of varying concentrations of choline. With acetate concentrations up to 1.0 mM, the Km values for acetate accumulation were calculated from the Eadie-Hofsee plot to be 36, 36 and 100 µM at 0, 0.2 and 1.0 mM choline; the corresponding Vmax values were 0.06, 0.134 and 0.37 nmol/min/mg protein. With acetate concentrations beyond the millimolar range, the Km values were 3, 1 and 0.7 mM in the presence of choline concentrations of 0, 0.2 and 1.0 mM respectively. The corresponding Vmax values in this case were 0.75, 0.8 and 1.1 nmol/min/mg protein.

result favors the hypothesis that acetate deriving from the hydrolysis of the released ACh can be physiologically used in the synthesis of the neurotransmitter and is preferentially used for the synthesis of ACh.

Therefore it would appear that the neuroblastoma is more closely related to other systems which can use acetate as a precursor molecule (i.e. the rat and lobster neuromuscular juntion (14,15), the corneal epithelium (16), the Torpedo electric organ (17,18) and the cat superior cervical ganglion (TUCEK & COLLIER, personal communication)) rather than to the brain cholinergic neurons.

Normal neuronal tissue cannot synthesize choline and it must be provided from an external source. This may alsc be true for the cholinergic neuroblastoma. Indeed with no choline added to the incubation medium, there is only a samll amount of ACh synthesized from glucose or acetate. This residual activity may be attributed to (i) a small intracellular pool of choline which remains despite the "washing" procedures in a medium free of choline or (ii) to the hydrolysis of phospholipids during the course of the incubation.

Accumulation of acetate in the NS 20 and NIE 115

Since acetate was found to be a good precursor for ACh, (^{14}C)acetate initial accumulation (one minute) was examined in the NS 20 and in the catecholaminergic clone (NIE 115). In the absence of choline the rate of (^{14}C)acetate accumulation was low and of the same order of magnitude in the two clones. When 0.2 - 1 mM choline was added to the incubation medium, no change in the kinetics of (^{14}C)acetate uptake could be observed in the NIE 115 clone, while a marked stimulation of the initial accumulation of (^{14}C)acetate occurred in the cholinergic NS 20 clone (Fig.2a). Indeed at a concentration of choline (1 mM), the one-minute initial accumulation of (^{14}C)acetate in the NS 20 clone was approximately 20 times higher (Fig. 2a) than that found in the catecholaminergic clone (NIE 115). Kinetic analysis of the NS 20 data using an Eadie-Hoftsee plot (19) gave results compatible with the presence of two saturable acetate uptake systems (Fig. 2b). The Km of the acetate uptake mechanism which operates at low as well as high acetate concentrations can be modified by the presence of choline. This suggests that there is probably a continuous modification of the acetate uptake as a function of the choline concentrations.

It should be pointed out that while (^{14}C)acetate incorporation into (^{14}C)ACh was found consistently, the stimulation by choline of the initial accumulation of (^{14}C)acetate into the cholinergic cells tended to vary among experiments. As opposed to the effect of choline on the (^{14}C)acetate accumulation, the addition of acetate (1 mM) to the incubation medium did not modify the choline transport in the cholinergic nor in the catecholaminergic cells (data not shown).

Therefore choline appears to play a regulatory role in

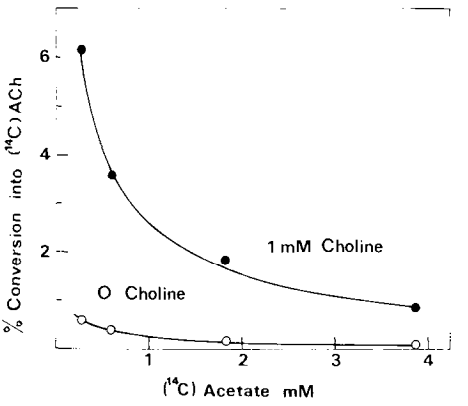

Figure 3. Percentage conversion of (^{14}C)acetate into (^{14}C)ACh in the presence and absence of 1 mM choline.

the accumulation of acetate in the NS 20 neuroblastoma. It will be of interest to determine whether choline can regulate the uptake of acetate in other neuronal systems which utilize acetate in the synthesis of ACh.

Percentage conversion of acetate into ACh

Since acetate was found to be accumulated by a "high" and "low" affinity process, we determined the percentage conversion of acetate into ACh over a short time period (3 min) in a concentration range which corresponds to these two affinity systems.

In the absence of any added choline, the percent conversion varied from 0.6% to 0.08% when the (^{14}C)acetate concentration was increased from 0.3 mM to 4 mM ; under the same conditions but in the presence of 1 mM choline, this percent conversion varied from 6% to 0.09% (fig. 3). Therefore, at low concentrations of acetate, that is in the "high affinity" range, there is a greater percentage conversion of acetate into ACh. This is analogous to the "high affinity" choline uptake system in cholinergic nerve terminals (3,4); at low choline concentrations, the percentage conversion of choline into ACh can be as high as 80%. In contrast, LANKS et al.(2) have shown that only 0.5% of the total choline is converted into ACh in the "high affinity" range in the NS 20 neuroblastoma. It thus remains to be determined whether the neuroblastoma are defective in this

property or whether the cells have not attained a state of differentiation wherein these properties are expressed.

The percentage conversion of acetate into ACh was increased in the presence of 1 mM choline (Fig. 3). Therefore choline exerts a critical function in (i) the initial accumulation of ^{14}C-acetate in the NS 20 neuroblastoma (Fig. 2)(ii) in the synthesis of ACh from acetate over a 15 min period (Fig. 1) and (iii) in the percentage conversion of acetate into ACh over the range of the "high" and "low" affinity uptake systems (Fig. 3).

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