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Ethanolamine base exchange enzymatic activities in spontaneous transformed glial cell lines

Effect of dibutyryl cyclic AMP treatment

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Cultured astrocytes derived from neonatal rats (normal cells) displayed maximal ethanolamine base exchange enzymatic activity (EBEE) when cultures reached confluency and cells almost ceased to divide. At this stage, ethanolamine phosphotransferase (EPT) and choline base exchange enzyme (CBEE) activities reached a plateau. In spontaneously transformed glial cells, no differential activity variation either between EPT and CBEE, or between EPT and EBEE was observed. The EBEE activity was mainly localized in the microsomal fraction and was completely absent from plasma membranes. Dibutyryl cyclic AMP (db-cAMP) treatment of the transformed cells reversed the pattern of these activities to that of normal cells. Moreover, treatment of the transformed cells with medium conditioned by normal astroblasts markedly increased EBEE activity. This study demonstrates that (i) variation of EBEE activity during cell growth differs in normal and in transformed cultured glial cells. (ii) EBEE activity may be modulated via both db-cAMP and normal cell conditioned medium. Our findings suggest a possible implication of EBEE in the maturation and contact inhibition of cell growth.

Ethanolamine base exchange enzyme; Primary culture; Transformation; Subcellular fractionation; Dibutyryl cyclic AMP; (Astrocyte)

1. INTRODUCTION

Phospholipids play a major role in the modulation of the biophysical properties of the membrane, in addition to their involvement in membrane functions such as synaptic transmission, receptor-ligand affinity and enzyme activities [1–5]. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the major phospholipids of eukaryote cells [6]. They are synthesized by two different pathways: (i) via the Kennedy process, or (ii) via methylation of ethanolamine phospholipids and exchange of polar head groups (base exchange).

Base exchange reactions have been studied specifically in nerve tissue. These reactions are not energy dependent [7,8], but require calcium [7]. Calmodulin may modulate the activity of the enzymes catalysing the base exchange reaction [9]. Kanfer [10] suggested that coupling may occur between methylation of phospholipids and base exchange. Indeed, monomethylethanolamine and dimethylethanolamine can be incorporated into the respective phospholipids by the base exchange reaction [11]. Subsequently, these phospholipids are methylated by phospholipid methyltransferase to produce PC [11].

Recently, we have shown [12] that in cultured astrocytes the ethanolamine base exchange enzyme (EBEE) specific activity reached a maximum on the 19th day, whereas ethanolamine phosphotransferase (EPT) reached a plateau after 15 days of culture. Similarly, Saito et al. [13] have observed that EBEE activity varies with the age of rat brain and reaches a maximum between 22 and 27 days of age.

Since the maximum activity of EBEE occurred on day 19 when cells in the culture reached confluence and ceased to divide, we investigated the activity of some exchange and transferase enzymes

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involved in the synthesis of PC and PE in spontaneously transformed glial cells. In the present study, we examined the activities of EPT, EBEE and choline base exchange enzyme (CBEE) in spontaneously transformed cells as a function of the age of the culture. The localization of the EBEE activity and the effect of dibutyryl cyclic AMP (db-cAMP) on this activity are also reported.

2. MATERIALS AND METHODS

2.1. *Primary cultures of astrocytes*

Astrocyte primary cultures derived from neonatal rat brain hemispheres were obtained according to Sensenbrenner et al. [14]. The cells were seeded at a density of 4×10^6 cells on 80 mm diameter culture dishes (Falcon 3003, USA). Culture medium (Dulbecco's modified Eagles, DMEM, Gibco, Grand Island, NY) was supplied with 10% fetal calf serum (FCS, Gibco). Cells were grown in a 95% air/5% CO_2 humid atmosphere at 37°C and medium was changed at 5 day intervals.

2.2. *Spontaneously transformed cell lines*

Spontaneously transformed astrocyte cell lines were derived from the primary cultures by repetitive subculturing as reported previously [15]. To each subculture a passage number was attributed and passages (P) 51, 65, 81 and 92 were obtained by trypsinization of 7 days in culture, in vitro, transformed cell cultures (P 50, 64, 80 and 91) with 0.05% trypsin and 0.04% tyrode KCl [15]. The harvested cells were seeded at a density of 400×10^3 cells per petri dish (Falcon 3001; 35 mm in diameter). After 1 day in culture (DIC) the growth medium was discarded, the cells washed twice with DMEM and fed with either culture medium or culture medium supplemented with dibutyryl cAMP (Sigma) at a final concentration of 1 mM and 10% FCS. Cell viability of both cell types was tested by Trypan blue dye exclusion and cells counted as previously described [16].

2.3. *Subcellular fractionation*

Nuclei and cell debris ($900 \times g$ fraction; P_1), P_2 ($7000 \times g$ fraction), P_3 ($17000 \times g$ fraction) and P_4 ($104000 \times g$ fraction) were isolated according to Mersel et al. [12]. Ultrastructural studies and investigation of mitochondrial and microsomal enzymatic markers indicated that P_2 (heavy) and P_3 (light) subcellular fractions were enriched in mitochondria whereas P_4 was enriched in microsomes.

2.4. *Enzymatic assays*

CDP-ethanolamine:1,2-diacylglycerol EPT and EBEE or CBEE activities were assayed essentially according to Freysz et al. [17] and Saito et al. [13] with some modifications described previously [12]. The specific activities of radioactive [1,2-^{14}C]-cytidine diphosphate (ICN, Irvine, USA), [*methyl*-^{14}C]choline chloride (Amersham, England) and [2-^{14}C]ethanolamine hydrochloride (Amersham, England) were 97 mCi, 58 mCi and 49 mCi/mmol, respectively. The final concentrations of these compounds in the enzymatic assay were 0.16, 2.3 and 2.5 mM, respectively, and the reaction volume was 400 μl.

2.5. *Lipid and protein determination*

The lipids were extracted according to Folch et al. [18]. Phospholipids, including radioactive enzymatic products, were detected and quantified as described previously [12]. Phosphorus and protein were determined according to Rouser et al. [19] and Lowry et al. [20] respectively.

3. RESULTS

Primary cultures of neonatal rat astrocytes and the transformed cell lines attained confluence on the 16th DIC and 2nd DIC, respectively. Addition of db-cAMP to the culture medium did not affect the phospholipid pattern but the growth rate was 30% decreased. The distribution of the major phospholipids was similar in both cell types (choline phospholipid, 42%; ethanolamine phospholipid, 30%; serine and inositol phospholipid, 15%; sphingomyelin, 13%) and remained constant during cell growth. Thus the phospholipid profile seemed to be similar in normal and transformed cells.

Cells were assayed for EPT, CBEE and EBEE activities as a function of the age of the culture (fig.1A). For normal cells, the specific activity of EPT increased 3.2-fold from the 12th DIC to the 16th DIC and remained constant thereafter (400 pmol·mg protein^{-1}·min^{-1}). The specific ac-

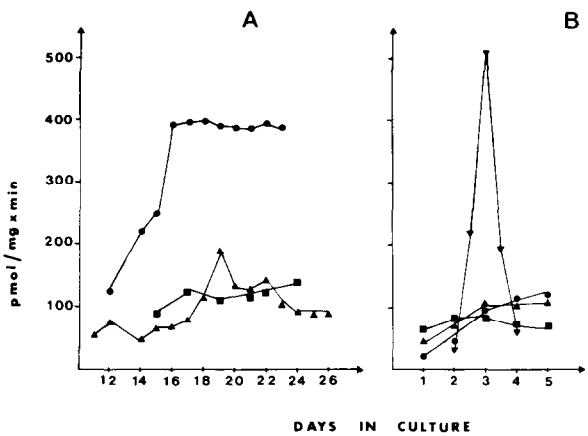

Fig.1. Enzymatic specific activities of ethanolamine phosphotransferase (●—●), choline base exchange (■—■) and ethanolamine base exchange (▲—▲) as a function of culture age. (A) Primary astrocyte cultures. (B) Spontaneous transformed glial cell lines. (▼—▼) Treatment with dibutyryl cyclic AMP. The results are the mean values of 8 experiments. The standard deviation for EPT, CBEE and EBEE specific activities was ± 5%, ± 17% and ± 10%, respectively.

tivity of EBEE was constant until the 16th DIC, increased 3.8-fold up to the 19th DIC (190 pmol·mg protein^{-1}·min^{-1}) and decreased gradually to reach the basal level, close to that of DIC 12–16, on the 24th DIC (75 pmol·mg protein^{-1}·min^{-1}). In contrast, the specific activity of CBEE was constant between the 15th and 24th DIC (100 pmol·mg protein^{-1}·min^{-1}). However, in the transformed cells (fig.1B), EPT, CBEE and EBEE specific activities (pmol·mg protein^{-1}·min^{-1}) reached a plateau on the 2nd DIC (100 for EPT and EBEE and 80 for CBEE). After 24 h treatment with db-cAMP, EBEE specific activity increased 5-fold between the 2nd and 4th DIC; thus, as for the normal cells, the peak of EBEE activity was observed at the time cells attained confluence (fig.1B). By contrast, db-cAMP did not enhance the specific activities of CBEE and EPT.

These data demonstrate that: (i) the differential variation of EPT, CBEE and EBEE specific activities observed in the primary cell cultures was not found in the spontaneous transformed cells. (ii) db-cAMP treatment restored the pattern seen in the primary cell cultures. The effect was not the result of the decreased growth rate by 30%, since the EBEE specific activity should increase by 1.3-fold instead of the observed 5-fold increase. Incubation of the cellular homogenate in the assay buffer containing 1 mM db-cAMP did not raise EBEE activity. Incubation of 2nd DIC transformed cells with conditioned medium from the 19th DIC normal cells (culture medium was changed on the 16th DIC and removed on the 19th DIC) for 24 h, increased EBEE specific activity 3.5-fold. This enzymatic activity was not present in the conditioned medium itself.

Our findings indicate that either the 19th DIC primary cultures or db-cAMP-treated cell lines release a factor, or promote a mechanism, which modulated the EBEE activity of the transformed cell lines. As for primary cultures [12], EBEE activity could not be detected in plasma membrane isolated from the spontaneously transformed cultured cells. Subcellular fractionation indicated that EBEE activity was essentially found in the microsomal fraction (P_4) (table 1) and this activity was increased as a function of age: 2-, 2.3- and 6-fold on the 2nd, 3rd and 4th DIC, respectively. Concomitantly, the percent activity recovered in P_4 was 37%, 50% and 60%, respectively.

Table 1

Effect of dibutyryl cAMP on the specific activity of ethanolamine base exchange[a] in different cellular fractions isolated from spontaneously transformed glial cell lines

	Days in culture					
	2		3		4	
Cellular fractions	C	T	C	T	C	T
Cells[b]	70 ± 3.7 (100)	35 ± 3 (100)	104 ± 7.0 (100)	514 ± 13.0 (100)	104 ± 7.0 (100)	67 ± 6.0 (100)
P_1	90 ± 7.8 (28)	ND	63 ± 6.0 (20)	ND	40 ± 3.6 (6)	ND
P_2	78 ± 7.0 (17)	76 ± 7.0 (1)	48 ± 4.0 (11)	2445 ± 31.0 (53)	47 ± 4.0 (13)	70 ± 6.0 (1)
P_3	70 ± 6.0 (18)	70 ± 6.0 (1)	100 ± 5.0 (11)	2100 ± 40.0 (39)	105 ± 5.0 (21)	81 ± 7.2 (1)
P_4	142 ± 7.1 (37)	989 ± 20.0 (98)	238 ± 11.0 (50)	78 ± 7.0 (8)	630 ± 18.0 (60)	613 ± 18.0 (98)

[a] Results are expressed in pmol/min per mg protein

[b] Cells, total cellular homogenate; C, control; T, dibutyryl cAMP-treated cells; P_1, 900 × *g* fraction; P_2, 7000 × *g* fraction; P_3, 17000 × *g* fraction; P_4, 104000 × g fraction

The results in brackets express the percentage of ethanolamine base exchange activity found in the cellular subfractions as compared to the total cellular homogenate. The results are the mean values of 4 experiments (passage no.51, 65, 81 and 92) each performed in duplicates. ND, not detected

When cultured cell lines were treated with db-cAMP, EBEE specific activity in P_4 was highly enriched (table 1) (28- and 10-fold for the 2nd DIC and 4th DIC, respectively) and almost all the activity (98%) was recovered in this subcellular fraction.

However, after 48 h of treatment (3rd DIC), the EBEE activity was mainly recovered in P_2 and P_3 fractions (53% and 39%, respectively) with 4.7 and 4.1 enrichment. The microsomal fraction was not enriched in EBEE specific activity and the recovery was only 8% (table 1).

These results clearly demonstrate that (i) EBEE activity was mainly localized in the microsomal subcellular fraction; the enrichment and recovery increased either as a function of time in culture or via db-cAMP treatment (2 DIC and 4 DIC). (ii) Enrichment and recovery found in P_2 and P_3 after 48 h of db-cAMP treatment paralleled the peak of specific activity observed in total cellular homogenate on the 3rd DIC (fig.1B).

4. DISCUSSION

In a previous report, we showed that EBEE activity was maximal when astrocyte primary cultures reached confluence and ceased to divide (19th DIC), and the activity, which was principally located in the microsomal fraction on the 16th and 24th DIC, was also essentially found in P_2 and P_3 subcellular fractions at this particular stage. Ultrastructural studies indicated that on the 19th DIC mitochondria were seen in the proximity of RER and that P_2 was contaminated with these organelles. Therefore we concluded that mitochondrial-associated RER structures containing EBEE activity sedimented with the heavy mitochondrial fraction. However, since the light mitochondrial fraction was not contaminated by RER, the increase of EBEE specific activity in P_3 seemed to be related to an activation of the enzyme.

In order to examine the role of these enzymatic activities in differentiation and transformation, we have investigated an additional base exchange enzyme (CBEE) activity and compared the variation of EBEE, CBEE and EPT activities between normal and spontaneous transformed cells during cellular growth. The transient maximal activity of EBEE on day 19 was exclusively observed in the normal culture system, indicating the possible involvement of this enzyme in cell differentiation, especially at the stage where cells attain confluence.

It is well known that db-cAMP inhibits the division of some tumor cell types [21] in which cAMP metabolism is altered [21]. Treatment of cell lines with this compound also restores certain morphological and biochemical properties found in the normal cell type [22,23]. Moreover, addition of db-cAMP to astrocyte primary cultures induces morphological and biochemical differentiation changes [24,25]. More information could be obtained by studying the effect of db-cAMP on the variation and localization of EBEE activity during the development of the cell lines. Indeed (i) db-cAMP restored the pattern observed in the case of the normal cells, i.e. a peak of EBEE activity, mostly contained in P_2 and P_3 subcellular fractions; (ii) the 19th DIC primary culture conditioned medium markedly enhanced EBEE activity of the cell lines. It is noteworthy that the concentration of db-cAMP we used was similar to that utilized by other workers who investigated the effect of this compound on 'in vitro cellular models' [26–28], and in particular cultured glial cells [14,29].

This observation supports the view that the modulation of EBEE activity occurs due to a 'factor' either released by the normal cells or induced by db-cAMP and suggests a correlation between EBEE activity and cellular differentiation. In conclusion our findings suggest that EBEE activity is probably involved in differentiation and/or in 'contact inhibition' of cell growth in our biological system. Previous data which showed subtle changes at the level of both cell surface PE and protein components [16,30] support such a contention.

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