

## FACTORS FROM GLIAL CELLS REGULATE CHOLINE ACETYLTRANSFERASE AND TYROSINE HYDROXYLASE ACTIVITIES IN A HYBRID-HYBRID CELL LINE

Rolf HEUMANN, Mucella ÖCALAN and Bernd HAMPRECHT

*Max-Planck-Institut für Biochemie, 8033 Martinsried, FRG*

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### 1. Introduction

The existence of factors inducing cholinergic properties in non-committed cells derived from the neural crest has been inferred or demonstrated in studies of three embryonic systems [1–5]. One of them consists of cultured superior cervical ganglion cells of newborn rats. Normally these cells become adrenergic. However, in the presence of medium that had previously been exposed to certain non-neuronal cells (conditioned medium) they turn cholinergic [5].

Here we present an *in vitro* system of a permanent hybrid-hybrid cell line which allows convenient and reliable studies of the induction of cholinergic properties by factors released from glial-type and heart cells. The hybrid-hybrid cells, generated by fusion of the adrenergic mouse-neuroblastoma cells N115-BU-8 [6] with the cholinergic mouse-neuroblastoma × rat glioma hybrid cells 108CC15 [7], contain choline acetyltransferase (EC 2.3.1.6) and acetylcholine as cholinergic, tyrosine hydroxylase (EC 1.14.16.2) and noradrenaline as adrenergic properties [8].

### 2. Materials and methods

#### 2.1. Cell lines

Clonal hybrid-hybrid cells (NH15-CA2) were generated as in [6,8]. C6-4-2 cells are polyploid cells derived from C6 glioma [9] by sequential fusion of appropriate mutants [6]. The neuroblastoma × glioma hybrid cells 108CC15 [7], the 5-bromodeoxyuridine resistant mouse fibroblasts B82 [10] and the HTC rat

hepatoma cells [11] have been described. As described [7] the cell lines were grown in Dulbecco's modified Eagle's medium (henceforth called medium DME) supplemented with 10% fetal calf serum (henceforth called serum), 0.1 mM hypoxanthine (H), 1  $\mu$ M aminopterin (A) and 16  $\mu$ M thymidine (T), except B82 for which HAT was substituted by 0.1 mM 5-bromodeoxyuridine.

#### 2.2. Primary cultures

Heart primary cultures were prepared from newborn mice by treating the minced tissues 4–5 times at 37°C for 5 min with a mixture of 0.025% (w/v) trypsin and 0.25% (w/v) collagenase in Puck's medium D1 containing 200 U/ml penicillin and 200  $\mu$ g/ml streptomycin and adjusted to 320 mOsmol by the addition of glucose and sucrose. The dissociated cells were collected in serum. They were cultivated in medium DME + serum + 20 U/ml penicillin + 20  $\mu$ g/ml streptomycin (growth medium). Primary brain cell cultures consisting mainly of astroblasts were prepared as in [12].

#### 2.3. Preparation of conditioned media

Three days after cells ( $6 \times 10^6$  C6-4-2,  $2 \times 10^6$  B82,  $\sim 3 \times 10^6$  C6 or HTC) were seeded into culture flasks (Nunc, 175 cm<sup>2</sup>) the growth medium was renewed. The next day cells were washed with  $\sim 100$  ml medium DME and exposed to  $\sim 180$  ml medium DME containing 1.1 mM dibutyryl cyclic AMP with or without hypoxanthine, aminopterin and thymidine, depending on the cell line. Two to three days later the conditioned medium was harvested and centrifuged (10 min, 1300 × g). If the conditioned medium

was prepared in roller tubes,  $4 \times 10^7$  cells of the cell lines were inoculated and grown for 6–8 days. Then the cells were washed with ~300 ml medium DME before they were allowed to condition for 1 day 400–450 ml medium DME containing 1.1 mM dibutyryl cyclic AMP. Heart cells were seeded at  $1-3 \times 10^6$  viable cells/flask (175 cm<sup>2</sup>, Greiner, Nürtingen), brain cells at  $3 \times 10^6$  cells/50 mm diam. plate (Greiner). After incubation in growth medium for 1–2 weeks, these cultures were washed with medium DME (100 and 5 ml, respectively) and exposed to 35 or 6 ml, respectively, of medium DME + 1.1 mM dibutyryl cyclic AMP (including 10% serum in the one case of expt. 3, table 2). The duration of conditioning was 2–3 days for heart cells and 7 days for brain cells.

#### 2.4. Exposure of hybrid–hybrid cells to conditioned medium

This was either in 50 mm diam. plates (fig. 1–3 and table 2) or in Linbro wells (table 1) in which  $7-10 \times 10^4$  or  $4 \times 10^4$  cells, respectively, were seeded in medium DME + serum + hypoxanthine + aminopterin + thymidine. One day later the medium was replaced by conditioned medium to which had been added either 10% serum or 10% of a 10-fold concentrated solution in medium DME of the 5 special ingredients (see table 1) of medium N2 [13]. In all cases the medium contained 1.0 mM final conc. dibutyryl cyclic AMP. Unless stated otherwise, the conditioned N2-type medium was used. The conditioned medium was renewed (daily) only in the plates. After treatment with conditioned medium for 4 days (fig. 1–3) or 2 days (table 1), respectively, the cells were harvested from the plates or Linbro wells and assayed for choline acetyltransferase and tyrosine hydroxylase specific activities [8,14]. All data are the means of values obtained with duplicate incubations.

### 3. Results

After 4 days of exposure of hybrid–hybrid cells to chemically defined N2 medium [13] conditioned by C6-4-2 cells the specific activity of choline acetyltransferase has increased 6-fold (fig. 1A, curve c). In contrast, it remains unchanged if unconditioned medium (fig. 1A, curve a) or medium exposed to B82

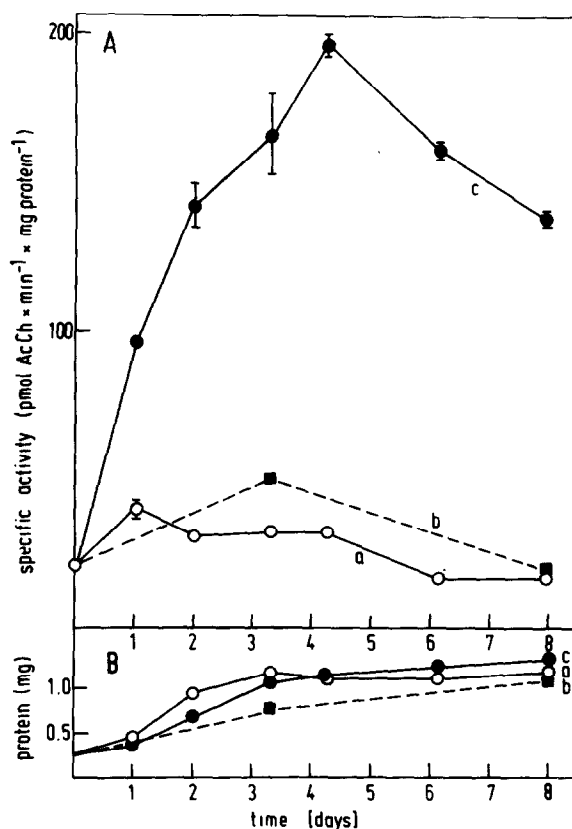


Fig. 1. (A). Specific activity of choline acetyltransferase after various times of exposure to unconditioned medium (curve a) or to medium conditioned by C6-4-2 passage no. 17 (curve c) or B82 passage no. 80 (curve b). (B) Protein per plate of hybrid–hybrid cells (passage no. 10). The designation of the curves in (B) corresponds to that in (A). Viability and cell number at day 3 in conditioned and unconditioned medium 95–98% and  $3.8 \pm 0.5 \times 10^5$ , respectively.

fibroblasts (fig. 1A, curve b), to hepatoma cells or to the hybrid–hybrid cells (see legend of table 2) is used. These differences in specific activity are not due to differences in protein content per plate (fig. 1B).

With repeated subculturing, the hybrid–hybrid cells do not lose their responsiveness to medium conditioned by the polyploid glioma line. During at least 34 passages the factor of relative stimulation remains constant at  $6.2$  ( $SD \pm 0.6$ ).

The question arose whether one of the 5 characteristic ingredients of medium N2 [13] was especially essential for the action of conditioned medium.

Table 1  
Relative elevation of choline acetyltransferase specific activity by C6-4-2 conditioned medium

Supplement omitted	Relative stimulation (%)
None	100
Transferrin	83
Insulin	76
Selenite	68
Putrescine	90
Progesterone	79

In each incubation only 1 of the 5 supplements of medium N2 [13] was omitted. 100% stimulation corresponds to a 3.9-fold elevation above the control level

Table 1 demonstrates that omission of any one of them causes a partial loss of response to conditioned medium, the maximal loss of 33.3% of the response being found if no selenite is added (table 1). A reduced stimulating influence of conditioned medium on choline acetyltransferase specific activity is

observed if the special ingredients of the chemically defined medium N2 (table 1) are substituted by serum (table 2). In medium N2, serum markedly raises the activities of both choline acetyltransferase and tyrosine hydroxylase. At saturating concentrations of serum (2–6%), dialyzed C6-4-2-conditioned medium (see below) still increases choline acetyltransferase activity (data not shown). These observations indicate independent actions of serum and dialyzed conditioned medium.

Not only tumor-derived cell lines release choline acetyltransferase stimulating activity into the culture medium but also primary cultures of heart and brain cells (table 2).

The magnitude of the response of the hybrid-hybrid cells ( $6.0\text{-fold} \pm 0.6\text{ SD}$  in 4 independent sets of experiments) renders the method sufficiently sensitive to discriminate between a dialyzable and a non-dialyzable fraction of the stimulating activity (fig. 2, curves a,c). The dose-response curve for the latter reaches a plateau (fig. 2, curve a). Interestingly,

Table 2  
Elevation of specific choline acetyltransferase activity by various sources of conditioned medium

Cells used for conditioning the medium	Exp. no.	Duration of treatment (days)	Fold stimulation in the presence of:	
			Medium N2	Serum
Unconditioned control	1–7	variable	1.0	1.0
Cell lines				
C6-4-2	1	4	–	1.9
(rat glioma)	2	5	–	3.1
	7	4	–	2.3
	8	4	6.3	1.6
	4	4	6.1	–
	4	3	5.2	–
	4	2	4.6	–
C6 (rat glioma)	2	5	–	2.9
Mouse primary cultures				
Heart	3	8	–	1.9
	5	3	2.6	–
Brain	6	2	2.4	–

The fold stimulation is the mean of data obtained from duplicate plates. Negative controls: After 4 days of exposure to media conditioned by the cell lines B82, HTC or NH15-CA2 the fold stimulation in the presence of serum ranged close to unity: 0.85, 0.86 (both expt. 1) and 0.94 (expt. 7), respectively

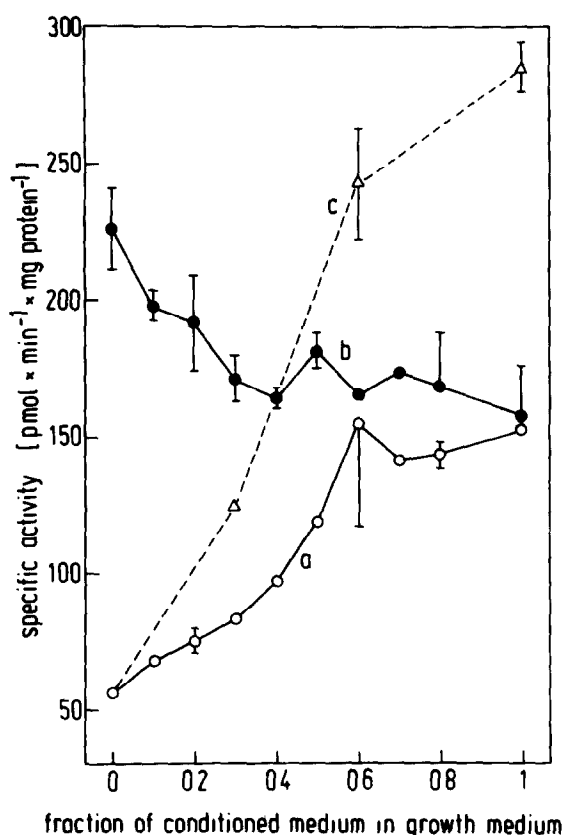


Fig.2. Specific activities of choline acetyltransferase (curve a) and tyrosine hydroxylase (curve b) after exposure of cells to various concentrations of dialyzed medium conditioned by C6-4-2 cells. Curve c: choline acetyltransferase specific activity after exposure of cells to undialyzed conditioned medium. 450 ml of conditioned medium (42  $\mu$ g protein/ml) were sterily dialyzed against 25 l medium DME with one medium change after 7 h. Protein, cell number and viability of hybrid-hybrid cells (passage no. 13) remained constant at  $1.17 \pm 0.08$  mg/plate,  $4.05 \pm 0.6 \times 10^6$  cells/plate and 97–99%, respectively, irrespective of the presence or absence of conditioned medium. Concentration of conditioned medium protein at half-maximal stimulation of choline acetyltransferase specific activity: 17.6  $\mu$ g/ml.

curve b (fig.2) indicates a decrease of TH specific activity due to conditioned medium.

The stimulating activity produced by C6-4-2 cells is heat-labile. At 60°C the activity is half-maximally lost after 60 min (fig.3). Boiling for 5 min completely inactivates the non-dialyzable fraction of the stimulating activity (data not shown).

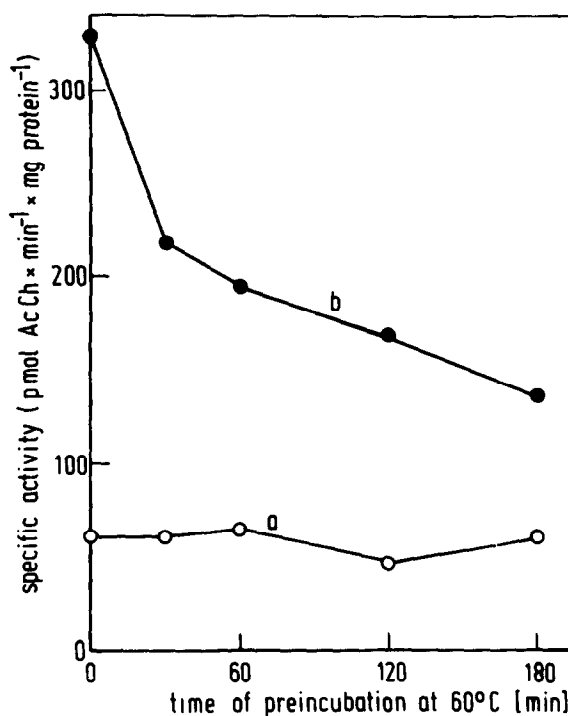


Fig.3. Influence of the time of heating at 60°C on the activity of medium conditioned by C6-4-2 cells (curve b). The unconditioned medium controls remain constant (curve a). Average protein of hybrid-hybrid cells (passage no. 10)  $0.67 \pm 0.08$  mg/plate.

#### 4. Discussion

As demonstrated here the simultaneously cholinergic and adrenergic hybrid-hybrid cells strongly increase their specific activity of choline acetyltransferase on prolonged exposure to chemically defined medium conditioned by a polyploid glioma cell line. The height of this response, its high reproducibility, its stability during repeated subcultivations and the easiness of handling such a permanent cell line make the hybrid-hybrid cells a highly appropriate system for studying factors involved in intercellular communication between neurons and cells they can be associated with, e.g., glial and heart cells.

On dialysis half of the choline acetyltransferase enhancing activity is lost from C6-4-2 conditioned medium. This indicates that part of the activity is associated with a non-dialyzable factor, probably of high molecular weight. The partial loss of activity

during dialysis may be due to either a second factor of low molecular weight or the uptake into the conditioned medium of an inhibitory component present in the dialysis medium DME.

The maximal effect by conditioned chemically defined medium N2 is observed after ~4 days. This period has to be preceded by a period of growth in medium containing serum. If the cells are cultured in unconditioned medium N2 for 5 days they lose their responsiveness to conditioned medium completely (data not shown). This phenomenon points to material in serum that is required to bring the hybrid-hybrid cells into a responsive state.

The possibility that the stimulating activity in conditioned medium selects cells of high specific activity of CAT is unlikely. Conditioning of the growth medium does not change the content of cellular protein in the culture (cf. fig.1 and legend of fig.2).

Increasing concentrations of conditioned medium, restored to its normal content of low molecular weight ingredients by dialysis against medium DME, cause an increasing response until a plateau is reached. The dose-response curve (fig.2) points to a saturable cellular receptor for the non-dialyzable component of conditioned medium. The sensitivity to heat indicates that the non-dialyzable component could be a protein.

In vivo the primordial neural crest cells are regulated by factors from the surrounding tissue to become cholinergic or adrenergic [1,2]. In addition, in the rat gut, cells derived from the neural crest transiently become adrenergic before turning cholinergic [3,4,15]. Medium conditioned by blood vessels or by heart cells induces cholinergic properties in cultured sympathetic neurons. Primary cultures of whole brain are rather ineffective [16]. Skeletal muscle cells in culture produce a factor that induces choline acetyltransferase activity in cultured neurons from spinal cord [17]. It would be important to know if the factors involved in these phenomena were similar to or identical with the factors in the conditioned media inducing choline acetyltransferase activity in the hybrid-hybrid cells.

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