Volume 213, number 2, 406–410 FEB 04537 March 1987

Neurotrophic effect of naturally occurring long-chain fatty alcohols on cultured CNS neurons

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Received 5 January 1987; revised version received 23 January 1987

A long-chain fatty alcohol,*n*-hexacosanol, that we have isolated from the Far-Eastern traditional medicinal plant, *Hygrophila erecta*, Hochr., is shown to promote the maturation of central neurons. Added at 500 nM to fetal rat brain neurons in culture, it increased both neurite outgrowth by a factor of 4–6 and the number of collaterals, especially in multipolar neurons. The biochemical differentiation of cultured neurons was also strikingly enhanced by this compound: it increased the protein content and almost doubled the activities of two neuron-specific enzymes, phosphate-activated glutaminase and neuron-specific enolase, by 92 and 78%, respectively. Extensive studies with several synthetic long-chain fatty alcohols showed that the neurotrophic activity was maximal for *n*-hexacosanol. It is suggested that some long-chain fatty alcohols with an appropriate length of hydrocarbon chain might play an important role in central neuron development.

Long-chain fatty alcohol; Growth factor; (Central neuron, Medicinal plant)

1. INTRODUCTION

The isolation of neurotrophic hormones or factors that promote growth or differentiation of central neurons is a major problem in developmental biology. While NGF has been studied extensively with peripheral neurons [1–4], few neurotrophic factors have been shown to be active on the central part of the nervous system. These are mainly hormones or compounds present in brain extracts or in culture-conditioned media [5]. We have thus looked for neurotrophic factors in plants which have been used for healing wounds, screening the crude extracts and monitoring the isolation of active components by their effect on fetal rat brain neurons [6]. We used a similar approach with other animal cell cultures to isolate other types of biologically active substances. This method takes advantage of the sensitivity and reliability of in vitro cell cultures, compared with in vivo tests on animals [7]. In the present report, we describe the potent activity of simple long-chain fatty alcohols, isolated from a tropical plant, *Hygrophila erecta* Hochr. (Acanthaceae), reported to be used in Vietnam and India to cure wounds [8]. The effect, detected by morphological changes and substantiated by a variety of biochemical assays, is maximal for the C_{26}-alcohol, *n*-hexacosanol. This finding is particularly interesting as it has recently been shown that developing rat sciatic nerves as well as developing rat brain biosynthesize long-chain alcohols [9,10].

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2. MATERIALS AND METHODS

The neuronal cultures, obtained from the cerebral hemispheres of 13-day-old rat embryos, were maintained as monolayer cultures in 35 mm

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00145793/87/$3.50

culture dishes. They were grown on polylysine-coated dishes and cultured with 3 ml of a chemically defined medium prepared with Eagle-Dulbecco medium supplemented with 5 μg/ml insulin, 100 μg/ml transferrin, 2×10^{-8} M progesterone, 10^{-12} M estradiol, 3×10^{-8} M selenium, 10^{-4} M putrescine and 15 mM KCl. The cells were seeded at 5×10^5 cells per dish and the medium was not changed during the 4 days of culture. The composition of the cell population was assessed by an immunohistochemical demonstration of tetanus toxin binding (96% of neurons) and by autoradiography after L-[^{3}H]glutamate uptake (67% positive cells) [6].

In typical experiments, 8 μl of plant extract or of synthetic compounds in ethanol were added on the first day of culture. In control dishes, 8 μl of ethanol were added in parallel (0.26% final concentration or 46 mM). It should be noted that controls without ethanol were also performed, but biochemical measurements as well as microscopic observation did not show any significant difference from controls with 8 μl ethanol. It has also been shown that ethanol had no effect on enolase activity in neuronal cultures below 100 mM [11].

After 4 days of culture, the cells were scraped off and sonicated in 0.05 M imidazole-HCl buffer, pH 7.2, containing 0.5 mM EDTA, as described [6]. The incubation mixture for the phosphate-activated glutaminase (PAG) assay contained 0.15 M potassium phosphate, 40 mM glutamine, 50 mM Tris-HCl, 0.1 mM dithiothreitol, 0.2 mM EDTA, 2 mM NAD, 2.5 mM ADP, 80 mM hydrazine and 100 μg glutamate dehydrogenase (Boehringer). The reaction was started at 37°C by adding 100 μl of cell homogenate. The absorbance versus a blank prepared by omitting glutamine from the incubation mixture was determined kinetically at 340 nm in 8 min. The initial velocity of the reaction was calculated using an Uvikon 810 spectrometer (Kontron).

Separation and quantification of neuron-specific and non-neuronal enolases were measured by a modification of the method described in [12]. Briefly, the separation was achieved by ion-exchange chromatography on DEAE-cellulose columns: non-neuronal enolase was eluted with a buffer containing 40 mM KCl; the hybrid of non-neuronal and neuron-specific enolase eluted with a buffer containing 130 mM KCl; and neuron-specific enolase (NSE) was eluted by a 240 mM KCl buffer. Initial velocity at 23°C was measured kinetically at 240 nm in the presence of 1 mM 2-phosphoglycerate with an Uvikon 810 spectrometer (Kontron).

The plant material was collected from the glass houses of the University Paul Sabatier (Toulouse, France) and from the Phytotron of CNRS (Gif-sur-Yvette, France). It was identified to be authentic material by the Service de Botanique of the University Paul Sabatier. The dry plant material was extracted by methanol and the extract subjected to counter-current distribution between hexane and aqueous methanol. The biologically active hexane extract was extensively chromatographed over silica-gel columns to obtain a biologically active fraction which was shown to be homogeneous on TLC plates. The plant components or the synthetic compounds were added once at the beginning of the cell culture and no culture medium change was performed until the end of the experiment (4 days). ^{1}H and ^{13}C NMR, and GC-MS analysis have shown that the active fraction was constituted by saturated straight-chain fatty primary alcohols from 24 to 30 carbon atoms. This fraction of compounds was difficult to separate into individual fatty alcohols and they were correlated chemically and biologically with the synthetic commercially available compounds which were obtained from Fluka (Buchs, Switzerland).

3. RESULTS AND DISCUSSION

3.1. *Morphological studies*

The addition of the active fraction isolated from *H. erecta* had a striking effect on the morphological differentiation of cerebral neurons. After 4 days in culture with 190 ng/ml of the active fraction, the neurons extended very long neurites that formed a dense network by contacting each other (fig.1): these fibers also showed many collaterals, as well as spines. Three main types of neurons could be recognized: bipolar neurons with a spindle-shaped cell body; pyramidal neurons with a triangular cell body; and stellate-shaped multipolar neurons. In most cells, one of the fibers was much longer and showed many more collaterals than the others: occasionally recurrent branches towards the cell body or another neurite

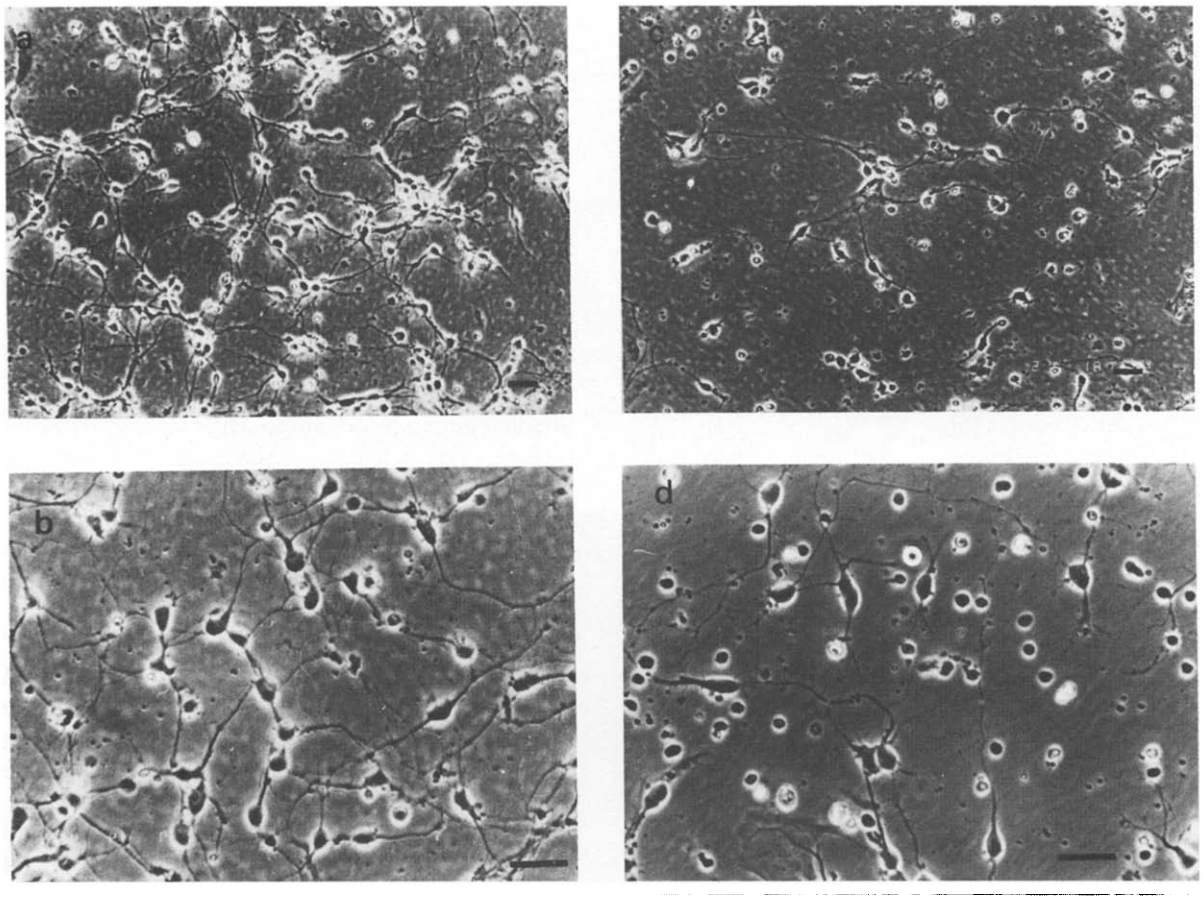

Fig.1. Phase contrast microscopy of 4-day-old neuronal cultures after treatment with the active fraction obtained from *H. erecta* (a and b). The cultures were obtained from cerebral hemispheres of 13-day-old rat embryos as described [6]. They were grown on polylysine-coated dishes and cultured with 3 ml of a chemically defined medium. 8 μl of the active fraction in ethanol were added once at the beginning of the culture (a and b): in control cultures 8 μl of ethanol were added at the same time (c and d). Each bar represents 20 μm. Note the great number of cells and the extent of neurite formation in treated cultures compared to controls: many collaterals arising from the primary processes can be seen at the higher magnification (b).

could also be seen. However, in control cultures, the fiber length, as well as the number of collaterals per cell were much smaller (table 1). The same types of neurons could be seen, but we rarely observed one fiber much longer than the others, or recurrent collaterals.

The morphometric analysis of the effect of the active fraction was carried out using a locally developed Fortran program. It showed that the cell perimeter was not significantly different from the control (except for multipolar neurons), but the length of fiber and the number of collaterals were much increased in treated cultures (table 1). The differences were particularly striking for pyramidal and multipolar neurons, the fiber length was 4–6-times greater than in controls and the number of collaterals was 7–11-times greater. Thus pyramidal and multipolar neurons could synthesize more than 0.5 mm total fiber length per cell during 4 days in culture with the active fraction, while control neurons synthesized only 0.1 mm total fiber length per cell.

It should be noted that a similar extent of neurite elongation was observed with 500 nM pure hexacosanol, but no computer analysis was performed with this synthetic substance.

Table 1
Morphometric analysis of the effect of the active fraction from *Hygrophila erecta*

	Control			Active fraction		
	Bipolar (15)	Pyramidal (10)	Multipolar (10)	Bipolar (14)	Pyramidal (7)	Multipolar (14)
Cell perimeter	35.5 ± 3.0	35.3 ± 6.4	28.2 ± 0.5	35.5 ± 3.0	35.9 ± 2.2	39.5 ± 8.5
Fiber length	199.4 ± 45.8	140.8 ± 32.4	89.3 ± 20.5	334.6 ± 4.6	540.5 ± 124.3	566.2 ± 130.2
Collaterals	5.0 ± 1.0	2.0 ± 0.4	1.0 ± 0.15	7.0 ± 1.4	14.0 ± 2.5	11.0 ± 2.2

The morphometric analysis was performed on 4-day-old cultures after 96 h treatment with the active fraction of *H. erecta*. An IBM 3080 computer, a Tektronix 4957 graphic tablet and a specially designed Fortran program were used. The initial seeding concentration was 10^5 cells per dish. According to the number of primary processes arising from the soma, neurons were divided into bipolar neurons (two processes), pyramidal neurons (three processes) and multipolar neurons (more than three processes): the number of unipolar neurons was not sufficient to perform a statistical analysis. Cell perimeter and total fiber length per cell are expressed in μm. The number of collaterals is the mean value per cell. The number of cells analysed for each cell type is indicated between brackets. The means ± standard deviation were compared to the controls using the Student's *t*-test. The differences in fiber length and the number of collaterals between treated cells and controls were significant with $P < 0.001$. The cell perimeters were not significantly different from the control except for multipolar cells ($P < 0.001$)

3.2. *Biochemical analysis*

Two enzyme activities were measured in cultures treated during 4 days with 190 ng/ml of the active fraction, namely neurospecific enolase which has been shown to be a specific marker of neuronal maturation [13] and phosphate-activated glutaminase which is mainly involved in the metabolism of the neurotransmitter glutamate [14]. It should be noted that a majority of the neurons present in our cultures have been shown to be glutamatergic [6]. Neuron-specific enolase was increased by 78% and phosphate-activated glutaminase increased by 92% over controls (fig.1). In the same culture, the protein content was increased by 49% over the controls. It should be noted that the enhancement of enzyme activities was already observed after 24 h in culture and reached a plateau after 48 h. The control values for glutaminase activity are in good agreement with those reported in various cerebellar or cerebral cultures [15,16]. It should also be emphasized that both glutaminase and neurospecific enolase have been shown to increase with the morphological and functional maturation of neurons [13,16–18].

Studies with synthetic long-chain *n*-alcohols showed that *n*-hexacosanol (26 carbon atoms) was the most potent in changing the defined neuronal biochemical markers: at a concentration of 500 nM, it induced an increase of phosphate-activated glutaminase by 94% and of neuron-specific enolase by 59% over the controls (fig.2), total protein content increased by 33% in the same experiment. Other long-chain fatty *n*-alcohols containing 16, 20, 22, 24 or 30 carbon atoms did not show any significant increase of these biological activities at the designed concentration. Moreover, saturated fatty acids were also tested and were approx. 10-times less potent than the corresponding alcohols.

Thus, it seems that the increase of biochemical activities is specifically related to a certain chain length (26 carbon atoms) of *n*-alcohols and to the presence of the alcoholic function. These results also show that the neurotrophic effect is not closely related to the lipophilic properties of *n*-alcohols, but may be specific to *n*-hexacosanol.

The relatively high concentrations used in this study also show that this compound has no toxic effects, in contrast with other fatty compounds like short fatty acids. This property enabled us to use *n*-hexacosanol at an optimal concentration (500 nM), which was able to induce very significant quantitative effects on the activity of some neurospecific enzymes as well as on the outgrowth of neurites (increased by more than 400% compared to control).

3.3. *Conclusion*

It is surprising that such a deceptively simple long-chain *n*-alcohol might display such high and varied activities generally associated with protein

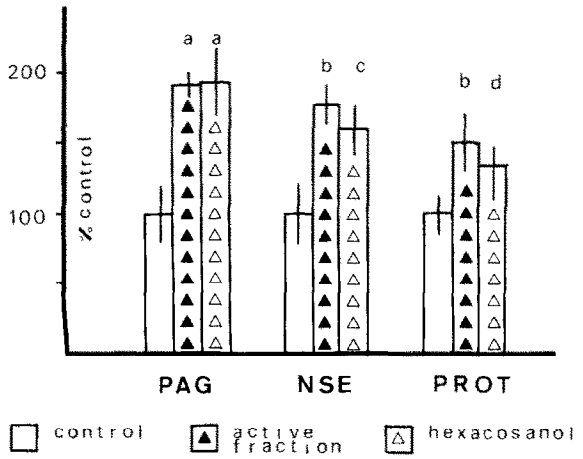

Fig.2. Effect of the active fraction and of hexacosanol on 4-day-old neuronal cultures. On the first day of culture, the active fraction or hexacosanol was added to the culture dish containing 3 ml of a chemically defined medium [6] at a final concentration of 500 nM. The activities of phosphate-activated glutaminase (PAG) were measured after 4 days of culture. The results are the mean ± SD of 5 determinations and are expressed as percentage of the controls (0.639 ± 0.128 nmol/min per dish). Activities of neuron-specific enolase (NSE) are the mean ± SD of 3 determinations and are expressed as percentage of the control (3.98 ± 0.93 nmol/min per dish). The amount of protein (PROT) was determined according to Lowry et al. [20] and is expressed as mean ± SD of 5 determinations. Control value is 52 ± 8 μg/dish. For each parameter, the first column represents the control value, the second is the value after treatment with the active fraction (black triangles) and the third is the value after hexacosanol addition (white triangles). The means ± SD were compared by Student's t-test and found to be significantly different from the controls with: $P < 0.001$ for a; $P < 0.01$ for b; $P < 0.02$ for c; $P < 0.05$ for d.

growth factors. However it was reported that another simple long-chain fatty alcohol containing 30 carbon atoms, the triacontanol, is a growth promotor in plant kingdom [19] and also that long-chain fatty alcohols are synthesized in large amounts mainly from corresponding fatty acids in the developing sciatic nerves [9,10].

It is clear that n-hexacosanol may display the effects of a trophic factor on rat central nervous system neurons. While this result poses a question concerning the physiological significance of these substances and their regulation, it remains to be seen whether this finding is sufficiently general to be of therapeutic use.

ACKNOWLEDGEMENTS

We thank Professor G. Ourisson, J. Mark and G. Kaufmann for their comments and suggestions. Miss N. Tholey is gratefully thanked for the elaboration of the image analysis program.

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