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α-Melanocyte stimulating hormone promotes neurite outgrowth in chromaffin cells

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Chromaffin cells from adult bovine adrenal medulla were found to develop neurites when cocultured with pituitary intermediate lobe (IL) cells. In coculture 51.7% of the chromaffin cells extended neurites compared with 12% in control cultures (chromaffin cells alone). A soluble factor released by IL cells was apparently involved as medium conditioned by contact with IL cells also promoted neurite outgrowth. Moreover, the addition of αMSH, one of the pro-opiomelanocortin-derived peptides secreted by IL cells, alone reproduced this effect in a dose-dependent manner. The data provide evidence for a neurotrophic role of αMSH.

α-Melanocyte stimulating hormone; Neurite; Chromaffin cell; Neurotrophic factor; Neuronal plasticity

1. INTRODUCTION

α-Melanocyte stimulating hormone (αMSH) is a pro-opiomelanocortin-derived peptide produced mainly in the pituitary intermediate lobe (IL) [1], but also in the central [2,3] and peripheral [4] nervous systems. Though the peptide alters pigmentation in lower vertebrates, the role of αMSH in mammals is still unknown. Behavioural studies suggest that the peptide could influence neuronal function: in rodents αMSH modifies learning and avoidance responses [5] and such indirect evidence indicates that αMSH might enhance peripheral nerve regeneration [6]. All of this could be compatible with αMSH having a role in neuronal plasticity. Cultured chromaffin cells from bovine adrenal medulla provide a useful model to test αMSH neurotrophic properties because these cells can be induced to adopt a neurone-like phenotype ('transdifferentiation') under certain culture conditions [7–12]. In our system we put two cell groups into coculture: adult bovine chromaffin cells and porcine pars intermedia cells as a source of αMSH. We found that in serum-free conditions, the presence of IL cells or conditioned medium from such cells dramatically increased neurite outgrowth from the chromaffin cells. Moreover, *N*-acetylated αMSH and desacetylated αMSH, but not ACTH were shown to reproduce this neurotrophic effect.

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2. MATERIALS AND METHODS

2.1. *Preparation of cultures*

Briefly, porcine neurointermediate lobes were placed in a dissociation medium (0.2% dispase, 0.1% collagenase and 0.05% hyaluronidase) for 30 min. After rinsing the lobes were dissociated in 0.05% trypsin for 15 min, then resuspended in plating medium [13]. Chromaffin cells were isolated from bovine adrenal medulla by retrograde perfusion with collagenase and purified on a self-generating Percoll gradient [14].

Cells were plated at 2×10^5 cells/35 mm dish on

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collagen-coated glass coverslips. For cocultures, 1×10^5 of each cell type were plated together. Plating medium was 50% Dulbecco's modified Eagle's medium (DMEM). 50% Ham F12, pH 7.2, supplemented with 10% fetal calf serum, penicillin (1 μg/ml), streptomycin (1 μg/ml), kanamycin (0.1 mg/ml), glutamine (0.286 g/l), glucose (1.8 g/l) and sodium bicarbonate (2 g/l). After allowing 60 h for cell attachment, medium was changed to defined serum-free (N2) medium [15] with antibiotics as above. Thereafter, medium was changed every 3 days. Cytosine arabinoside (10^{-5} M) and fluorodeoxyuridine (10^{-5} M) were added from day 3. Cells were grown at 37°C under air/CO_2 (95%/5%).

2.2. *Production of conditioned medium (CM)*

Medium was 'conditioned' by contact with IL cells (4–8 days old, 10^5 cells/ml) for 24 h. This medium was harvested, centrifuged and diluted 1:1 with fresh defined serum-free medium.

2.3. *Immunofluorescence*

Cells were fixed in 4% paraformaldehyde in 0.12 M phosphate buffer (pH 7.0) and permeabilized in Triton X-100 and acetone [16]. Samples were preincubated in 5% sheep serum to reduce non-specific staining and then incubated in the primary antibody in Tris-buffered saline (TBS: 20 mM Tris, 150 mM NaCl, pH 7.4). Rabbit anti-αMSH antibody [17,18] was used to identify IL cells and rat anti-DBH [19] or rabbit anti-chromogranin A antibodies [20] were used as the chromaffin cell markers. Neurofilaments were marked using the mouse monoclonal NF 02.40 antibody [21]. Primary antibodies were used at a dilution of 1:1000, except anti-chromogranin A which was diluted 1:2500. Antibodies were visualized using fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG, tetramethylrhodamine isothiocyanate (TRITC)-conjugated sheep anti-mouse IgG (Sigma) or TRITC-conjugated goat anti-rat IgG (Nordic Immunologicals) at a 1:100 dilution in TBS.

2.4. *Neurite counts*

Cells were fixed and stained immunocytochemically for a chromaffin cell marker, either chromogranin A or DBH. Neurite outgrowth was scored by counting as positive, those cells having extensions twice as long as the cell body (>40 μm). For each dish, two independent counts of three transects, each including 100 cells, were carried out.

2.5. *Effects of POMC-derived peptides*

Peptides were dissolved in 0.1 M HCl, then diluted in defined medium to a concentration of 10^{-3} M. These stock solutions were sterilised by filtration through 0.22 μm filters. Concentrations were checked by radioimmunoassay [17], and appropriate dilutions in defined medium carried out.

Peptides were added to cultures from days 3 to 10. *N*-Acetylated αMSH was from Bachem, desacetylated αMSH from UCB and $ACTH_{1-24}$ from Ciba Geigy.

3. RESULTS AND DISCUSSION

Fig.1a–c shows a coculture marked with dopamine-β-hydroxylase (DBH) and αMSH antibodies to identify the chromaffin cells (fig.1b) and IL cells (fig.1c), respectively. The αMSH-producing cells were spherical and often closely associated with the chromaffin cells (fig.1a). Many of the DBH-positive cells put out numerous branched or unbranched neurite-like extensions of at least 40 μm in length, with some extensions attaining 1 mm. In chromaffin cells with neurite-like extensions, the intensity of the DBH staining was often diminished in the cell body and concentrated in the varicosities and tips of neurites suggesting that chromaffin granules are transported into the developing extensions (fig.1b). During the third week of coculture, neurofilament proteins were detected in chromaffin cell extensions (fig.1d,e) demonstrating that they are analogous to neuronal extensions. Chromaffin cells in coculture could be maintained up to 3 months, whereas control cultures did not survive more than 3 weeks. Indeed, in control cultures the majority of chromaffin cells remained spherical and only a few put out short extensions (fig.2a).

To determine if a diffusible factor from IL cells was involved in inducing neurite outgrowth, conditioned medium (CM) prepared from IL cultures (IL-CM) was used to culture chromaffin cells. Again, an increase in neurite outgrowth resulted, either in defined medium (fig.2b–d) or in medium supplemented with 5% fetal calf serum (not

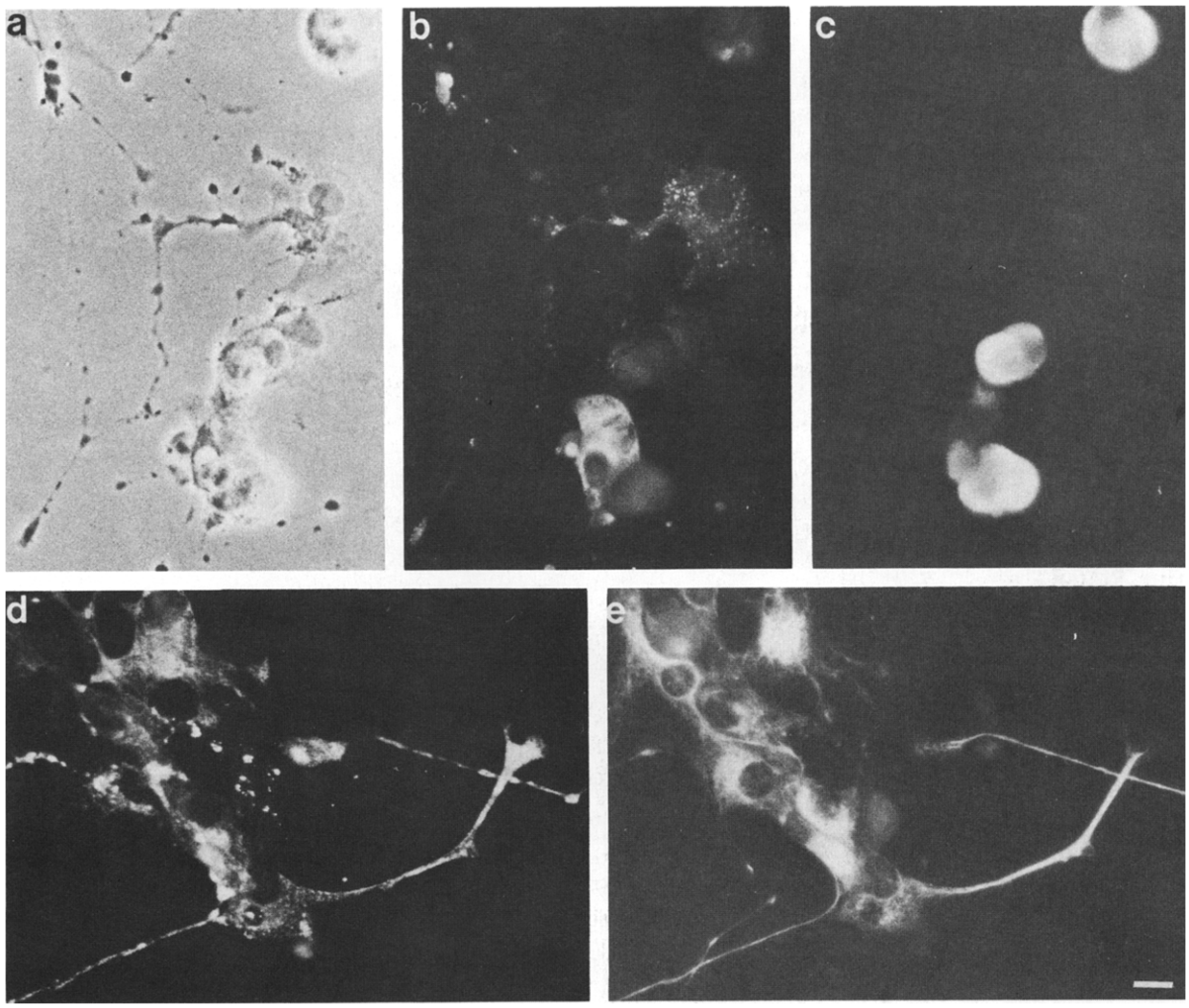

Fig.1. Chromaffin cells cocultured with IL cells (a–c) adopt neurone-like features (d,e). (a–c) Same field of 11-day-old coculture viewed in phase contrast (a) and after immunofluorescence staining, using anti-DBH for chromaffin cells (b) and anti-αMSH to identify IL cells (c). (d,e) Fluorescent images of chromaffin cells from an 18-day-old coculture stained with anti-DBH (d) and anti-neurofilament (e). Note colocalization of the markers in neurites. Bar, 15 μm.

shown). As in coculture, many chromaffin cells flattened and grew extensions, often branched and with varicosities (fig.2b). The extensions were immunopositive both for the chromaffin granule marker, chromogranin A (fig.2c), and for neurofilament proteins (fig.2d). Moreover, in contrast to controls, phosphorylated neurofilament proteins were detected in the transdifferentiated chromaffin cells in coculture and IL-CM (Grant, N. et al., submitted).

To quantify the relative effects of coculture conditions and IL-CM on neurite outgrowth, cells with neurites (>40 μm) were counted. As seen in fig.3a, the mean percentage of chromaffin cells developing neurites was higher in cocultures (51.7 ± 5.0%, mean ± SE) than in IL-CM (37.8 ± 1.8%) and both values were significantly different ($p < 0.001$) from controls (12.0 ± 1.1%). Since IL-CM mimics the effects observed in coculture, a soluble factor released by IL cells is probably involved in inducing neurite outgrowth.

One of the products of pro-opiomelanocortin (POMC) proteolysis secreted from the IL is αMSH, which is cleaved from ACTH. αMSH can

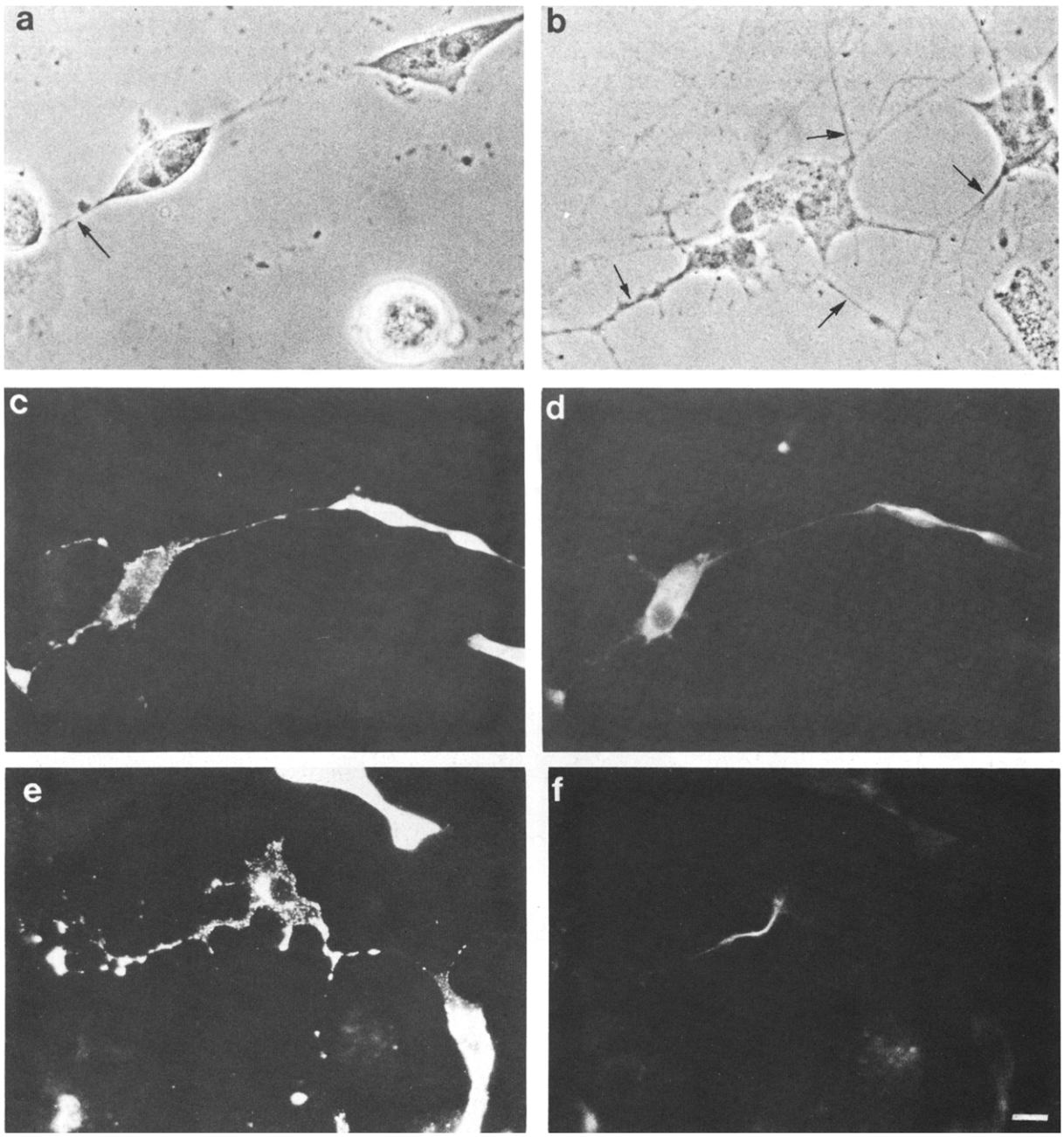

Fig.2. Comparison of cultures maintained in either control medium (a) or conditioned medium (b–d) at 21 days, or αMSH at 10 days (e,f). Arrows indicate short extensions in some control cells (a) and an extensive neurite network in cultures grown in conditioned medium (b). Colocalization of chromogranin A (c,e) and neurofilaments (d,f) in chromaffin cell extensions. Bar, 15 μm.

undergo N-terminal acetylation giving rise to the three forms found in IL extracts: *N*-acetylated (the form usually referred to as αMSH), *N,O*-diacetylated and desacetylated αMSH [17]. We initially measured the total αMSH levels in cocultures and IL-CM using a C-terminal-directed

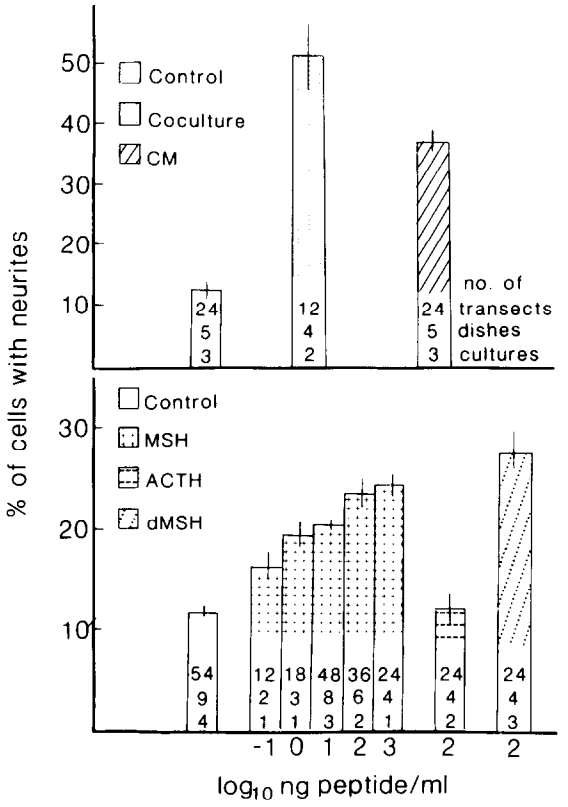

Fig.3. Neurite outgrowth from chromaffin cells is increased by coculture with IL cells, by IL conditioned medium and by αMSH. (Top) Percentage of cells with neurites in control, coculture and conditioned medium (CM). (Bottom) Percentage of cells with neurites in control, *N*-acetylated αMSH (MSH) at different concentrations, $ACTH_{1-24}$ and desacetylated αMSH (dMSH). All experimental groups except ACTH were significantly different from controls when analyzed using a Student's *t*-test, with n = number of transects counted.

antibody that recognises equally well the three forms of the peptide [17,18]. Medium taken from 1-week-old cocultures and IL-CM contained 81.2 ± 8.1 and 77.3 ± 7.8 ng/ml αMSH (mean ± SE, $n = 6$), respectively. Using an antibody (Patti) that recognises only acetylated forms of αMSH [22], we found that approx. 70% of the αMSH was acetylated, implying that the desacetylated form accounted for 30% of the total αMSH secreted. The media were also assayed for ACTH as neurotropic properties have been attributed to a fragment of the hormone $ACTH_{1-24}$ that includes the αMSH molecule [23]. Only low levels of ACTH were present (4.0 ± 1.5 ng/ml CM). We tested the effects of *N*-acetylated αMSH, desacetylated αMSH and ACTH on neurite outgrowth from bovine chromaffin cells, using each peptide at 100 ng/ml, approximately the concentration of αMSH found in IL-CM. *N*-Acetylated and desacetylated αMSH were equipotent in promoting neurite outgrowth (fig.2e,f); the mean percentage of chromaffin cells extending neurites being 23.9 ± 1.4% and 27.9 ± 1.7%, respectively, compared with 11.8 ± 0.5% for controls (fig.3). Interestingly, desacetylated αMSH is less effective than αMSH on the documented target for αMSH, melanocytes, which like chromaffin cells are derived from the neural crest. However, desacetylated αMSH is the major form present in the developing hypothalamus [24,25], and may play a neurotrophic role in the brain.

To characterize further the αMSH effect, a dose-response curve was carried out using *N*-acetylated αMSH, the principal form present in cocultures and CM. As seen in fig.3b, the effect of *N*-acetylated αMSH on neurite outgrowth was dose-dependent, and even the lowest dose tested (0.1 ng/ml; approx. 5×10^{-11} M) was significantly different from controls ($p < 0.01$). This concentration is in the range of αMSH concentrations reported for cerebro-spinal fluid [26]. ACTH alone did not affect neurite outgrowth in chromaffin cells (fig.3b).

The observation that coculture conditions and IL-CM are more effective than αMSH alone (fig.3) implies that other factors, perhaps POMC-derived molecules are involved. POMC processing gives rise to an array of peptides. Some of these neuropeptides and their derivatives have been shown to modify neuronal metabolism. Endorphins and related peptides modify turnover of a number of neurotransmitters [5] and even an endorphin fragment as short as two amino acids has been shown to alter cholinesterase activity in superior cervical ganglia [27].

The factors which favour transdifferentiation of adult bovine adrenal chromaffin cells are unclear. Unlike cells from rat adrenals, they respond poorly to nerve growth factor [9–12] and are unaffected by dibutyryl cyclic AMP [11]. However, they do respond positively to depolarizing agents [11] and to extracts of either bovine non-chromaffin

adrenal medulla cells or seminal vesicles [10]. The seminal vesicle is a source of POMC-derived peptides [24], so it is tempting to suggest that some of the neurite promoting effects of seminal vesicle extract might be due to such peptides.

Our results demonstrating enhanced neurite outgrowth from chromaffin cells suggest that *N*-acetylated αMSH and desacetylated αMSH must be added to the growing list of substances (protein growth factors, substrata, second messengers) which can influence neuronal plasticity. The present data coupled with the observation that αMSH immunoreactive neurons in the hypothalamus and thalamus project towards the hippocampus, forebrain and spinal cord [29], could implicate αMSH as a neurotrophic factor in the central nervous system.

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