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αMSH but not Org 2766 induces expression of c-*fos* in cultured rat spinal cord cells

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α-MELANOCYTE stimulating hormone (αMSH) and Org 2766 (an adrenocorticotrophic hormone (ACTH) MSH_{4-9} analogue) stimulate sprouting after nerve damage *in vivo*. In cultures of rat spinal cord cells only αMSH enhances neurite outgrowth. In order to analyse the mode of action of αMSH and to get a clue why its action differs from that of Org 2766, we studied whether expression of c-*fos* was induced in spinal cord cells by treatment with αMSH or Org 2766. We found a rapid and transient induction of both c-*fos* mRNA and protein after treatment with 100 μM αMSH. Treatment with Org 2766 (100 pM–100 μM) had no effect on c-*fos* expression. These results suggest that c-*fos* is involved in the signal transduction system of αMSH stimulated neuronal outgrowth *in vitro*.

Key words: Melanocortins; αMSH; Org 2766; Spinal cord cells; c-*fos*; Neurite outgrowth; Regeneration; Plasticity; Neuropeptides

Introduction

α-Melanocyte stimulating hormone (αMSH) (acetylated $ACTH_{1-13}$-NH_2) and adrenocorticotrophic hormone (ACTH) fragments and analogues such as Org 2766 (an ACTH/MSH_{4-9} analogue (Met(O_2)-Glu-His-Phe-D-Lys-Phe)) enhance nerve regeneration in rats after damage to the sciatic nerve.[1,2] αMSH also has a trophic effect on neurones in culture: in organotypic cultures of rat spinal cord slices αMSH (0.1–10 nM) stimulates neuronal outgrowth by 25–40%.[3] In cultures of dissociated spinal cord cells, αMSH treatment (10 nM–100 μM) increases dose-dependently the amount of the growth associated protein B50/GAP43 and the cytoskeletal protein neurofilament (NF).[4] For both proteins a maximum increase was reached with 100 μM αMSH (B50/GAP43: 36%; NF: 64%). Org 2766 has a beneficial effect on nerve repair in rats,[5–7] but in rat spinal cord cultures this peptide has no effect on the expression of B50/GAP43.[4] How αMSH and Org 2766 exert their action in the nervous system is not known, but available data strongly suggest a receptor-mediated mechanism of action. Recently ACTH/MSH receptors, present on melanoma and adrenal cells, have been cloned[8,9] and the presence of a different ACTH/MSH receptor subtype in neuronal tissue is suggested.[10]

ACTH, administered to rats, results in an enhanced production of the transcription factor c-*fos* in the adrenal gland.[11,12] This suggests that c-*fos* is involved in the nuclear regulation of the expression of late response genes after stimulation of the ACTH receptor. In PC12 cells c-*fos* expression is induced by growth factors such as nerve growth factor (NGF)[13] and epidermal growth factor (EGF).[14] A possible function in the nervous system for immediate early genes like c-*fos* is to mediate the long-term response of neurones to external stimuli. Thus, they may function as mediators in neuronal differentiation and plasticity.[15] Similarly, the putative ACTH/MSH receptor on neuronal cells may be coupled to c-*fos*, which may act as an intermediate in the signal transduction pathway of the neurotrophic peptides αMSH and Org 2766. To test this hypothesis we examined the effect of αMSH and Org 2766 on the expression of c-*fos* in dissociated spinal cord cells. This culture system was chosen because only αMSH has a trophic effect on these neurones, although both peptides enhance nerve repair *in vivo*.

Materials and Methods

Cell culture: Pregnant rats of an inbred Wistar strain (U:WU) were killed and the foetuses (E15) were removed and put in Dulbecco's modification of Eagle's medium (DMEM) (15 mM HEPES, pH 7.4, Flow Laboratories). Spinal cords from the foetuses were dissected and dissociated as described earlier.[3] The cells (124 000 per cm^2) were cultured in poly-L-lysine-coated 8-well Lab-Tek® Chamber Slides™ (Bayer) or in plastic petri-dishes (Nunc), at 37°C in a humidified atmosphere under 5% CO_2 in air. The culture medium consisted of DMEM supplemented with 5% v/v heat-inactivated horse serum (Gibco), 4 mM glutamine, 100 μg ml^{-1} streptomycin and 100 U ml^{-1} penicillin. Vero cells (American Tissue Culture catalogue-CCL81) were cultured in DMEM containing 10% foetal calf serum, 4 mM glutamine, 100 μg ml^{-1} streptomycin and 100 U ml^{-1} penicillin. Schwann cells (passage 20–25) were cultured in the same medium with 6 g l^{-1} glucose.

In situ hybridization: For *in situ* hybridization spinal cord cells were fixed at 15, 30, 45, 60 and 90 min after

treatment (other cells only at 30 min) with 2% paraformaldehyde (PFA) in 0.1 M Na_2HPO_4/NaH_2PO_4 (pH 7.4) for 45 min, followed by methanol for 30 min. An 1.0 kb *Eco*RI cDNA fragment of human c-*fos*[16] was inserted in both the sense and antisense orientation in pBluescript®II KS (Stratagene). Human c-*fos* DNA shows 89% homology to rat c-*fos* DNA. Anti-sense and sense RNA probes were obtained by *in vitro* transcription of the *Hind*III (Boehringer) linearized template with T7 RNA polymerase (Promega) in presence of ^{35}S-labelled αUTP (Amersham). The probe was hydrolysed by 0.2 M carbonate buffer pH 10.2 to obtain fragments of about 150 nucleotides. Before hybridization the cells were rinsed with phosphate-buffered saline (PBS pH 7.4) and 2 × SSC (double strength standard sodium citrate, 0.3 M NaCl, 0.03 M tri-sodium-citrate-2-hydrate, pH 7.0). Subsequently proteins were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl. Cells were rinsed with 2 × SSC before aldehyde glycination (0.1 M Tris-HCl, 0.1 M glycine, pH 8.0). The cells were dehydrated with ethanol, hybridization mix was added (1.4×10^6 cpm ^{35}S-labelled probe/slide) and the cells were hybridized for 5 h at 62°C. After hybridization the cells were rinsed twice with 2 × SSC/50% formamide at 50°C followed by 0.1 × SSC/20 mM β-mercapto-ethanol at 62°C. The cells were dehydrated with graded ethanol, put in xylol for 20 min and finally in 100% ethanol. They were left to dry and then exposed to Kodak NTB-2 liquid film emulsion for 10 days. After developing the film in D19 (Kodak), cells were counterstained with haematoxylin and embedded in Depex®. The results were quantified by counting 350–400 cells per treatment and calculating the percentage of responsive cells.

Immunocytochemistry: For detection of the c-*fos* protein spinal cord cells were fixed with 4% PFA for 15 min at 30, 60, 90, 120 and 240 min after treatment. The cells were rinsed four times with Tris-buffered saline (TBS, 0.05 M Tris, 0.9% NaCl, pH 7.4) and incubated overnight at 4°C with a rabbit antiserum against a synthetic c-*fos* peptide sequence (c-*fos* ab-2, Oncogene Sciences), diluted 1:200–500 in 0.05 M Tris with 0.9% NaCl, 1% BSA and 0.5% Triton X-100 (pH 7.4). The cells were rinsed four times with TBS and incubated in biotinylated goat–anti-rabbit antiserum (Vector) for 1 h at room temperature, followed by incubation with avidin–biotin complex (Vector). The immunoreaction was visualized by incubation with 0.02% 3′,3′-diaminobenzidine, 0.02% nickel ammonium sulphate, 0.03% H_2O_2 in 0.05 M Tris (pH 7.4). The cells were embedded in Depex® (glass slides) or in Aquamount (Petri dishes).

Treatment: After 48 h the culture medium was replaced by DMEM without serum or growth factors, and after 65 h in culture cells were treated as follows. To validate the *in situ* hybridization and immunocytochemical staining, they were incubated with 200 ng ml^{-1} phorbol 12-myristate 13-acetate (PMA; Sigma) or DMEM supplemented with 5% horse serum (DMEM-H5), PMA[14] and serum[17] are known to induce a c-*fos* response. Peptide treatment consisted of 100 pM–100 μM αMSH (Sigma) or Org 2766 (gift from Organon Int). Stock solutions of PMA (1 mM in DMSO) and αMSH or Org 2766 (1 mM in 10 mM HCl) were diluted in buffered culture medium without serum to obtain a 10× concentrated solution, which was added to the cultures.

Results

Spinal cord cells did not express c-*fos* mRNA at any of the time points tested after treatment with vehicle (diluted HCl or DMSO) (Fig. 1a). Treatment with

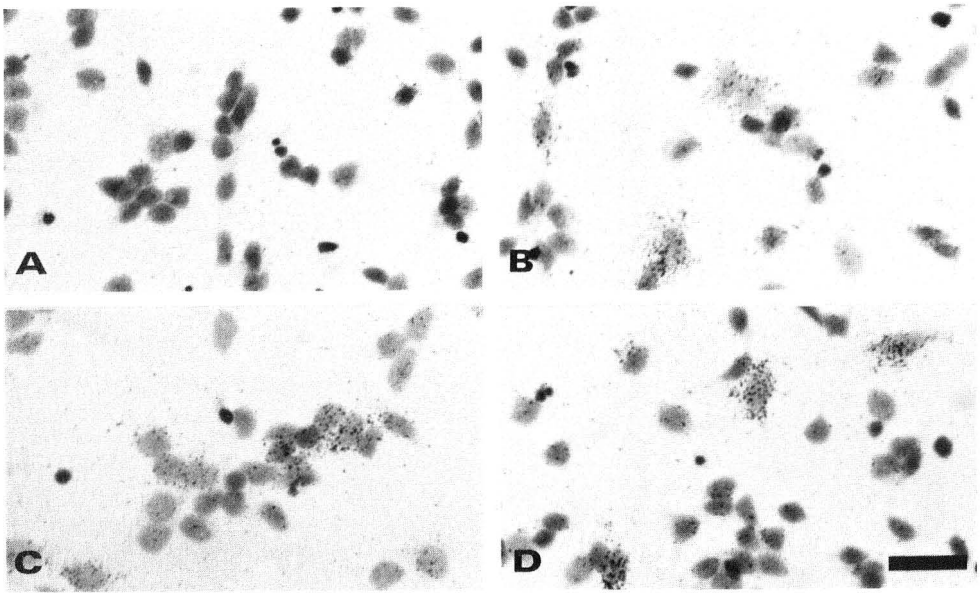

FIG. 1. Induction of c-*fos* mRNA in dissociated spinal cord cells treated for 30 min with (**A**) vehicle, (**B**) DMEM + 5% horse serum, (**C**) 200 ng ml^{-1} PMA and (**D**) 100 μM αMSH. Scale bar = 30 μM.

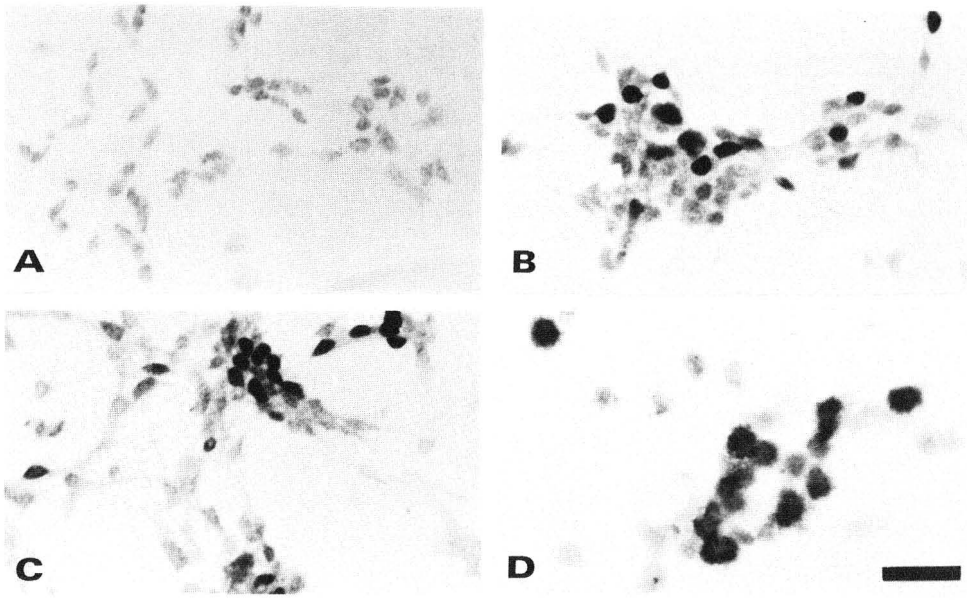

FIG. 2. C-*fos* immunoreactivity in spinal cord cells treated for 90 min with (**A**) vehicle, (**B**) DMEM + 5% horse serum, (**C**) 200 ng ml^{-1} PMA and (**D**) 100 μM αMSH. Scale bar = 30 μM.

DMEM-H5 (Fig. 1b) and 200 ng ml^{-1} PMA (Fig. 1c) resulted in a clear induction of c-*fos* mRNA within 30 min, with a maximal c-*fos* signal intensity occurring between 30 and 45 min after addition of serum or PMA to the culture medium. Expression had returned to control level at 90 min after treatment. Treatment with 100 μM αMSH induced expression of c-*fos* mRNA with a maximum between 30 and 45 min (Fig. 1d). Other concentrations of αMSH (100 pM–10 μM) had no effect on the c-*fos* expression and Org 2766 had no effect over the range tested (100 nM–100 μM). To check if the cells did not only express c-*fos* mRNA but also translate it into protein, we again treated the cells with serum, PMA and 100 μM αMSH and monitored c-*fos* protein. No c-*fos* was detected when cells were treated with vehicle (Fig. 2a) but treatment with DMEM-H5 (Fig. 2b), 200 ng ml^{-1} PMA (Fig. 2c) and 100 μM αMSH (Fig. 2d) resulted in a clear increase of the c-*fos* protein production. A maximum was observed between 60 and 120 min after treatment, and after 4 h no more c-*fos* was present. These results were observed in five independent experiments. Two representative experiments are summarized in Fig. 3.

In order to study the cell specificity of the c-*fos* response on αMSH treatment, we performed control experiments on a kidney cell line (Vero cells) and on a Schwann cell line (Table 1). Vero and Schwann cells responded to serum treatment with a marked increase in c-*fos* mRNA within 30 min. They also responded to PMA treatment, but to a lesser extent. Schwann cells reacted to αMSH (10–100 μM) treatment with an increase in c-*fos* positive cells, whereas αMSH had no effect on the c-*fos* expression in Vero cells.

Discussion

To gain insight in the mode of action of two melanocortins,[3,4] we treated dissociated spinal cord cells with αMSH and Org 2766 in concentrations ranging from 100 pM to 100 μM and followed induction of the nuclear transcription factor, c-*fos*. Our results demonstrate that c-*fos* expression is induced rapidly (within 15 min) and transiently (after 90 min no mRNA could be detected) by αMSH at a concentration of 100 μM. Characterization of this culture has shown that no astrocytes and oligodendrocytes are present, and only a small number of fibroblasts (< 1%).[4] As these easily recognizable cells did not respond to αMSH-treatment by an increased c-*fos* expression, the data indicate that under these conditions only neurones respond. DMEM-H5 did induce c-*fos* expression in fibroblasts, which is in agreement with the induction of c-*fos* by serum in the fibroblast cell-line NIH 3T3.[17]

The concentration of αMSH that maximally induced c-*fos* production in this study is the same as the one that stimulated outgrowth in the same cells as shown earlier.[4] Lower concentrations of αMSH (100 pM–1

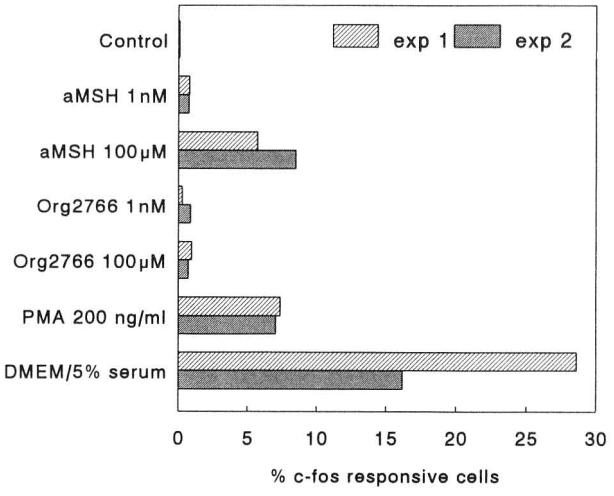

FIG. 3. Induction of c-*fos* mRNA in dissociated spinal cord cells in two independent experiments. Each bar represents the percentage of c-*fos* positive cells of in total 350–400 cells counted per treatment.

Table 1. The effect of different treatments on c-*fos* expression in cultured cells

	Spinal cord cells	Schwann cells	Vero cells
Vehicle	–	–	–
PMA	+	+	+
H5	++	++	++
αMSH	+	+	–
Org 2766	–	–	–

The effect of phorbol ester (PMA), DMEM + 5% horse serum (H5), αMSH and Org 2766 on c-*fos* mRNA expression in three cell types. All cells were treated for 30 min with a range of peptide concentrations (100 pM–100 μM), or a positive control (5% serum or 200 ng ml^{-1} PMA). C-*fos* mRNA was detected with an *in situ* hybridization. '–' < 1% of the cells show c-*fos* induction; '+' 5–10%, and '++' > 15% of the cells are c-*fos* positive.

μM) did not increase the NF levels,[4] and did not induce c-*fos* expression (this study). Furthermore, Org 2766 has neither effect on outgrowth (production of B50/GAP43) nor on the c-*fos* expression (this study) in spinal cord cells.[4] These parallel observations strongly suggest that c-*fos* expression is part of the pathway that is initiated by αMSH treatment and that Org 2766 triggers a different pathway. Direct proof that c-*fos* expression is a necessary step in the outgrowth response must however still be obtained.

C-*fos* may regulate the expression of proteins that are involved in neurite outgrowth. A candidate for such a protein may be the growth associated protein B50/GAP43, which is increased after 100 μM αMSH treatment[4] and which contains a consensus sequence for an AP-1 site[18] in its promoter sequence. Such a site is crucial to the action of c-*fos* as a transcriptional activator. Another candidate might be the gene for the cytoskeletal protein NF: the human NF-M gene has an AP-1 site in its promoter region,[19] suggesting that c-*fos* is an important factor in the regulation of the NF protein. The increased c-*fos* expression[13] and production of NF-L and NF-M mRNA[20] after NGF treatment in PC12 cells support the suggestion of a regulatory role of c-*fos* in NF production. In our cells the production of c-*fos* protein is enhanced by αMSH treatment, which may be compared to the induction of c-*fos* expression by growth factors, such as NGF and EGF.[13,14]

In the Schwann cells, αMSH (10 μM–100 μM) enhances the expression of c-*fos* mRNA (Table 1). This finding suggests that a similar type of ACTH/MSH receptor (which does not interact with Org 2766) is present on spinal cord cells and Schwann cells. The finding that αMSH even in the relatively high concentration of 100 μM had no effect in Vero cells, indicates that changes in pH or membrane fluidity possibly caused by a high concentration of peptide do not induce c-*fos* expression. Thus, the αMSH effect in spinal cord neurones and Schwann cell line seems to be specific for neuronal cells.

Expression of c-*fos* is under control of several regulatory elements.[21,22] cAMP and Ca^{2+} regulate the c-*fos* expression via the cAMP/calcium responsive element. The peripheral receptor for ACTH/MSH is linked to adenylate cyclase[8] and there are indications that cAMP is important for the action of melanocortins in the nervous system.[23,24] Thus, cAMP may be the link between the αMSH message and the c-*fos* response observed.

Conclusion

The differential effects of Org 2766 and αMSH on neurite outgrowth observed *in vitro* are reflected in the c-*fos* response of spinal cord cells to these peptides. Another transcription factor may be active as a mediator of the Org 2766 effect *in vitro*, but from this study we conclude that c-*fos* is involved in the mechanism of action of the neurotrophic peptide αMSH.

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