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The role of calcium and cAMP in the mechanism of action of two melanocortins: αMSH and the $ACTH_{4-9}$ analogue Org 2766

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Abstract

Melanocortins accelerate functional recovery after nerve crush and enhance neurite outgrowth in vitro. To get more insight in the mechanism of action of melanocortins, we studied the effects of two neurotrophic peptides: alpha-melanocyte-stimulating hormone (αMSH) and an adrenocorticotropin$_{4-9}$ analogue Org 2766 on second messengers in cultures of spinal cord (SC), dorsal root ganglion (DRG) and Schwann cells. αMSH (10 μM) enhanced the forskolin-induced cAMP production in SC- (45%) and in DRG-cells (35%). Org 2766 (1 μM) induced an increase in cAMP only in SC-cells (39%). The peptides did not affect the cAMP levels in Schwann cells. Neither peptide evoked significant changes in the intracellular free calcium concentration ($[Ca^{2+}]_i$) in batch-measurements of all cell types, however, Ca^{2+}-imaging revealed an infrequent occurrence of large $[Ca^{2+}]_i$-elevations in individual SC-neurons. The results indicate that SC- and DRG-cells are targets for both peptides, while Schwann cells are not or exploit different pathways. We observed for αMSH that cAMP production always coincides with outgrowth stimulation, whereas for Org 2766 cAMP production and outgrowth stimulation appear not causally related. These differences in second messenger stimulation could be explained by receptor heterogeneity. We suggest that αMSH and Org 2766 act through different receptors, each with its own signalling pathways.

Keywords: αMSH; ACTH-analogue; Calcium; cAMP; Dorsal root ganglia; Spinal cord; Schwann cell

1. Introduction

Melanocortins, adrenocorticotropin-derived (ACTH-derived) peptides, are potential neurotrophic and neuroprotective agents with proven action on regenerating and developing neuronal tissue [3,20,37]. In vivo these peptides enhance sciatic nerve regeneration [5] and protect against the action of neurotoxic oncolytics, such as cisplatin [19] and taxol [22]. In primary neuronal cultures the peptides enhance neurite outgrowth [3], prolong cell survival [11] and protect against cisplatin-induced neurotoxicity [23].

A family of high affinity receptors specific for melanocortins has been characterized. Members of this family are probably involved in the neurotrophic action of melanocortins. Melanocortin-receptors (MC-R's) characterized until now all are adenylate cyclase-coupled receptors with 7 membrane-spanning domains [7,8,17,18,31]. It has been demonstrated that cyclic-AMP (cAMP) is involved in melanocortin-induced effects on cortical neurons [2,44], spinal cord neurons [25], astrocytes [45], rat brain slices [15] and adrenal cells [14]. Other second messengers may also play a role in the signal transduction cascade of melanocortins. In cultured adrenal cells treatment with ACTH and ACTH-derived peptides leads to a rise in the intracellular free calcium concentration ($[Ca^{2+}]_i$) [14,16,39] and αMSH increases $[Ca^{2+}]_i$ in mouse melanoma cells (B16F1) [29]. In adrenal zona fasciculata cells ACTH modulates the open time of dihydropyridin-sensitive L-type calcium channels, but others show that not the L- but the T-type channel is involved in ACTH-stimulated cortisol synthesis [13].

cAMP and calcium are both important regulators in the mechanism of neurite outgrowth [30]. An increase in cAMP, either caused by the adenylate cyclase activator forskolin or by treatment with cAMP-analogues

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results in an enhanced neurite-like outgrowth in mouse [27] and human neuroblastoma cells [32] and rat pheochromocytoma cells [26]. Neurite outgrowth is also strongly dependent on $[Ca^{2+}]_i$: the outgrowth of rat sensory neurons [1,12,28] and *Helisoma* neurons [30] is optimal in a narrow Ca^{2+}-window. A small increase in extracellular Ca^{2+} (3–6 mM) enhances neurite outgrowth [36], but a Ca^{2+} overload, induced by a massive depolarisation, leads to cell death [10]. The calcium channels involved in the regulation of neurite outgrowth are probably the L-type channels. Blockers of the L-type channel prevent neuronal damage during massive depolarisation [10] and reverse high potassium-induced neurite outgrowth inhibition [4]. Furthermore, the L-type calcium channels are clustered in the growth cone, allowing high local increases ('calcium hotspots') and hereby modulate growth cone motility [35]. Taken together, neurite elongation seems to be a complex interaction between several signal transduction pathways, in which cAMP and Ca^{2+} play important roles.

In order to study the mechanism of action of the neurotrophic effect of melanocortins we investigated the effect of αMSH and the $ACTH_{4-9}$ analogue Org 2766 (Met(O_2)-Glu-His-Phe-D-Lys-Phe) on cAMP production and $[Ca^{2+}]_i$ regulation in cultures of cells involved in peripheral nerve regeneration and neurite outgrowth: motor neurons (spinal cord), sensory neurons (dorsal root ganglia) and Schwann cells.

2. Materials and methods

2.1. Cell culture

2.1.1. Dissociated spinal cord cells (SC-cells)

Pregnant rats of an inbred Wistar strain (U:WU) were killed and the foetuses E15 were removed and put in 15 mM HEPES-buffered DMEM (pH 7.4; GIBCO Scotland). Spinal cords were dissected and the meninges with the dorsal root ganglia were removed. Spinal cords were dissociated to obtain a cell suspension as described before [42]. SC-cells were cultured in DMEM (GIBCO Scotland) supplemented with 5% (v/v) heat-inactivated horse serum (GIBCO Scotland).

2.1.2. Motor neurons

Motor neurons were isolated from E15 rat spinal cord according to Schnaar and Schaffner [34]. The cells were cultured in chemically defined medium (CDM), a modification of the N2 medium of Bottenstein and Sato [6]. CDM consisted of DMEM supplemented with 33.3 mM glucose (BDH England), 5 mg/l insulin, 100 mg/l bovine transferrin, 90 mg/l bovine serum albumin fraction V, 0.19 μM progesterone, 0.1 mM putrescine, 0.92 μM sodium selenite, 0.44 μM thyroxine, 0.51 μM triiodothyronine (all chemicals (except glucose) from Sigma, USA).

2.1.3. Dissociated dorsal root ganglion cells (DRG-cells)

Dorsal root ganglia were separated from the rat E15 spinal cord by careful dissection. The ganglia were dissociated in the same way as the spinal cords to get a single cell culture. DRG-cells were cultured in CDM supplemented with 1 μM 1-β-D-arabinofuranosylcytosine (ara-c) (Sigma, USA) and 3 ng/ml βNGF (Boehringer, Germany).

2.1.4. Primary Schwann cells

Schwann cells were obtained from sciatic nerves of 3–5 day old Wistar rats (U:WU) as described before [43]. The cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS; GIBCO Scotland) and 6 g/l (= 33.3 mM) glucose (DMEM-F10G6). After 24 h in culture the cells were treated with 10 μM ara-c for 72 h. Then the medium was replaced with DMEM-F10G6.

2.1.5. Schwann cell lines

The Schwann cell lines (U9204 passage 38 and U9208 passage 12, a gift of Dr. H.H.F.I. van Breugel) were prepared in our lab from primary Schwann cells. The cells were cultured in DMEM-F10G6.

All culture media contained 4 mM glutamine (GIBCO Scotland), 100 U/ml benzylpenicilline (Centrachemie, Netherlands), 100 μg/ml streptomycin sulphate (Pharmachemie, Netherlands). Cells were cultured in a humidified atmosphere under 6.5% of CO_2 in air at 37°C. Cells were cultured in poly-L-Lysin coated 24- or 96-well plates 135,000 or 20,000 cells/well for cAMP measurements or on glass coverslips for Ca^{2+} measurements.

2.2. cAMP measurements

After 48 h in culture the cells were rinsed with DMEM (10 min), subsequently replaced by DMEM with 1 mM IBMX (10 min; Sigma, USA). The cells were treated for 10 min with αMSH or Org 2766 (1 nM–100 μM) in the presence of 0.1 or 0.3 μM forskolin and 1 mM IBMX. The reaction was stopped and cAMP was extracted by replacing the incubation medium with ice-cold ethanol absolute. cAMP levels were measured with a RIA (NEN-DuPont).

2.3. Calcium measurements

$[Ca^{2+}]_i$ was monitored using fura-2. Cells grown on coverslips were incubated with 10 μM fura-2-AM (Sigma, USA) in DMEM for 30 min at 37°C. DMEM-fura-2AM was replaced with buffer (123 mM NaCl, 3 mM KCl, 20 mM $NaHCO_3$, 1.2 mM NAH_2PO_4, 10 mM D-glucose, 1.5 mM $MgSO_4$, 1.5 mM $CaCl_2$; pH 7.4; Merck, Germany) and incubated for an additional 15 min to allow complete hydrolysis of the dye. The loaded cells were used either for batch measurements in a spectrofluorometer (Perkin-Elmer LS50B) or for single cell Ca^{2+}-imaging.

2.3.1. Batch measurements

Cells cultured on round glass coverslips (14 mm Tamson) and loaded with fura-2-AM were mounted in a plastic cuvet containing 2 ml buffer (composition as above). The cuvet was mounted in a cuvet holder equipped with a thermostat (37°C) and a stirring device. The cuvet holder was constantly superfused with carbogen. The fura-2 signals were measured using the standard dual wavelength excitation (340 and 380 nm). Intracellular Ca^{2+} concentration was calculated according to Grynkiewicz [21]: $[Ca^{2+}] = K_d \cdot (R - R_{min}/R_{max} - R) \cdot \beta$; K_d(Fura) = 225 nM; $\beta = F_{380}$(EGTA)/F_{380}(ionomycin). The sample frequency was typically 0.53 Hz (a delay of 1.89 s between each ratio). The maximum 340/380 ratio (R_{max}) was obtained with 5 μM ionomycin (Sigma, USA) and the minimum 340/380 ratio (R_{min}) with 10 mM EGTA (Merck, Germany) at pH > 8.2.

2.3.2. Calcium imaging

Cells cultured on round glass coverslips (24 mm, No. 0, Chance Propper, England) were loaded with fura-2-AM and were mounted in a perfusion chamber (1 ml) on a 40×FLUOR objective lens of a

Nikon Diaphot inverted microscope with fluorescence illumination. The chamber was thermostatted at 37°C and constantly perfused with carbogen equilibrated buffer (composition as above). $[Ca^{2+}]_i$ was measured using the RMIRRA-system (Rudolf Magnus Institute, Utrecht University, The Netherlands), involving dual wavelength excitation (340 and 380 nm, 1.1 Hz) and on-line ratio calculation (0.56 Hz) of a 256×256 image (2×8 frames). Cells were isolated from background using total fluorescence and average ratios were calculated on line for each cell. The sample frequency was typically 0.33 Hz (for ratio images). Calibration as in the batch measurements.

2.3.3. Treatment

The cells were treated with the following agents: Org 2766 (Organon, The Netherlands), αMSH, bradykinin, substance P, serotonin, carbachol (Sigma, USA) and KCl (Merck, Germany). αMSH and Org 2766 were freshly dissolved in 10 mM HCl to get a 200 μM stock solution, buffer was added to get a 100 μM stock and dilutions were made in buffer. The other agents were stored as a stock solution at −80°C. Fura-2-AM stock (2 mM in DMSO) and ionomycin stock (1 mM in DMSO) were stored at −80°C until use. Tris-EGTA stock (1 M Tris–100 mM EGTA) was stored at −20°C.

3. Results

3.1. Cell specific action of melanocortins on cAMP production

All cell types were treated with melanocortins in combination with 0.1 μM or 0.3 μM forskolin. Others have shown that this combination results in a more consistent stimulatory effect of melanocortins [15,44]. The effect of αMSH and Org 2766 on the forskolin-induced increase in cAMP production depended on the cell type. Both peptides enhanced the adenylate cyclase activity in spinal cord cells (Fig. 1A), but only αMSH treatment led to an increase of the cAMP level in dorsal root ganglion cells (Fig. 1B). Schwann cells showed no stimulation of adenylate cyclase activity upon αMSH or Org 2766 treatment (Fig. 1C). The αMSH- or Org 2766-induced activation of adenylate cyclase in spinal cord cells resulted in a maximal effect of 145 ± 7% at 10 μM αMSH and of 138 ± 16% at 1 μM Org 2766. In dorsal root ganglion cells the αMSH treatment lead to a maximum stimulation of the cAMP production of 138 ± 3% at 1 and 10 μM of the peptide, whereas Org 2766 (1 nM–10 μM) had no effect.

3.2. Effect of melanocortins on $[Ca^{2+}]_i$

In spinal cord cells the resting $[Ca^{2+}]_i$ was 26 ± 4 nM ($n = 24$). Application of αMSH and Org 2766 over a wide concentration range (1 pM–1 μM) did not significantly alter the resting $[Ca^{2+}]_i$. Two typical experiments are shown in Fig. 2A and 2B. Depolarisation of these cells with 30 mM KCl evoked a 4.5-fold increase of $[Ca^{2+}]_i$ to 131 ± 10 nM ($n = 8$) (Fig. 2C). Preincubation with αMSH or Org 2766 (1 μM) did not modulate this K^+-evoked increase in $[Ca^{2+}]_i$ (Fig. 2C). Similar results were obtained in cultures from the dorsal root ganglion cells. In these cells the resting $[Ca^{2+}]_i$ was 45 ± 6 nM ($n = 16$) (Fig. 3A,B). These cells are known to react to the neuropeptide bradykinin by increasing the $[Ca^{2+}]_i$ through stimulation of IP_3 production [38]. This effect was confirmed in the current experiments, in which 1 μM bradykinin increased the $[Ca^{2+}]_i$ to 61 ± 6 nM. This response was not modulated by preincubation with αMSH or Org 2766 (1 pM–1 μM, Fig. 3C). In primary Schwann cells or the Schwann cell lines U9204 and U9208 no change in $[Ca^{2+}]_i$ was observed after application of 1 pM–1 μM of αMSH or Org 2766 (data not shown).

Although batch-wise measurements showed no increase in $[Ca^{2+}]_i$ upon treatment with αMSH or Org 2766, single cell Ca^{2+} measurements revealed that a few individual spinal cord cells responded to very low concentrations of both peptides (from 1 pM on). Fig. 4 shows a registration of the Ca^{2+} response in spinal

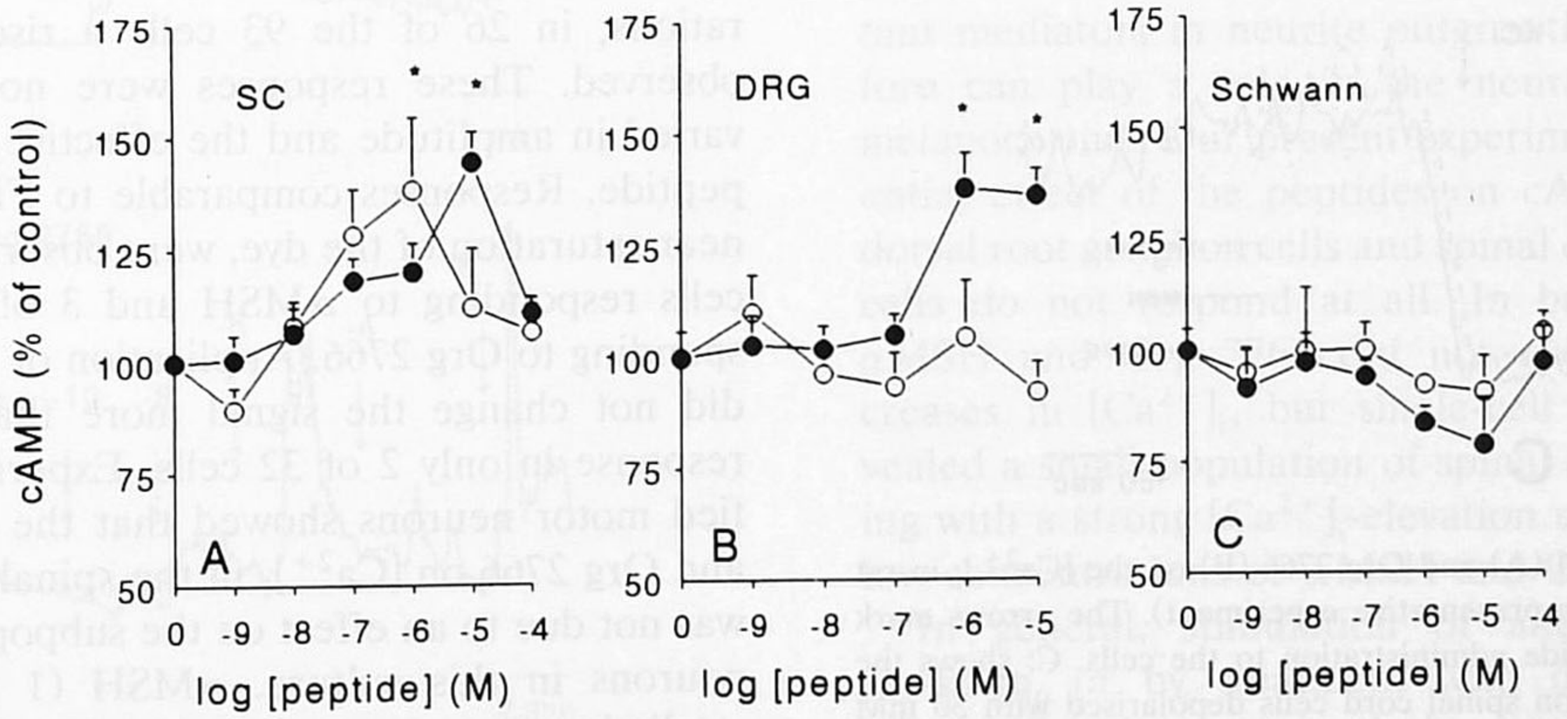

Fig. 1. The effect of αMSH (●) and Org 2766 (○) on the cAMP production in spinal cord (SC) cells (A), dorsal root ganglion (DRG) cells (B) and Schwann cells (C), in the presence of 0.1 μM (A,B) or 0.3 μM (C) forskolin. Data are expressed as mean ± S.E.M. of 9–12 observations obtained in 3–4 independent experiments (*$P < 0.05$, ANOVA followed by Bonferroni t-test).

cord cells after αMSH and Org 2766 treatment. After the initial increase the $[Ca^{2+}]_i$ returned to basal levels and the cells showed a second $[Ca^{2+}]_i$ elevation upon a second application of the peptide (higher concentration). The peptides induced a rapid increase of $[Ca^{2+}]_i$ and in some cells (top panel Fig. 4) the response was very strong, leading to a saturation of the dye (> 5 μM). After several applications the response was impaired. This impairment was not due to a general decline of the viability of the cell, since the cells still maintained a low steady-state $[Ca^{2+}]_i$ and responded to K^+ depolarisation and ionomycin. The peptide effects were scored in all cells with a low steady-state $[Ca^{2+}]_i$, indicating that these cells were viable. αMSH (1 pM–100 μM) was applied to a total of 146 spinal cord cells in 4 independent preparations. Upon application of αMSH we observed an increase in $[Ca^{2+}]_i$ in 29 cells. Org 2766 (1 pM–100 μM) was applied to 93 spinal cord cells distributed over 4 independent prepa-

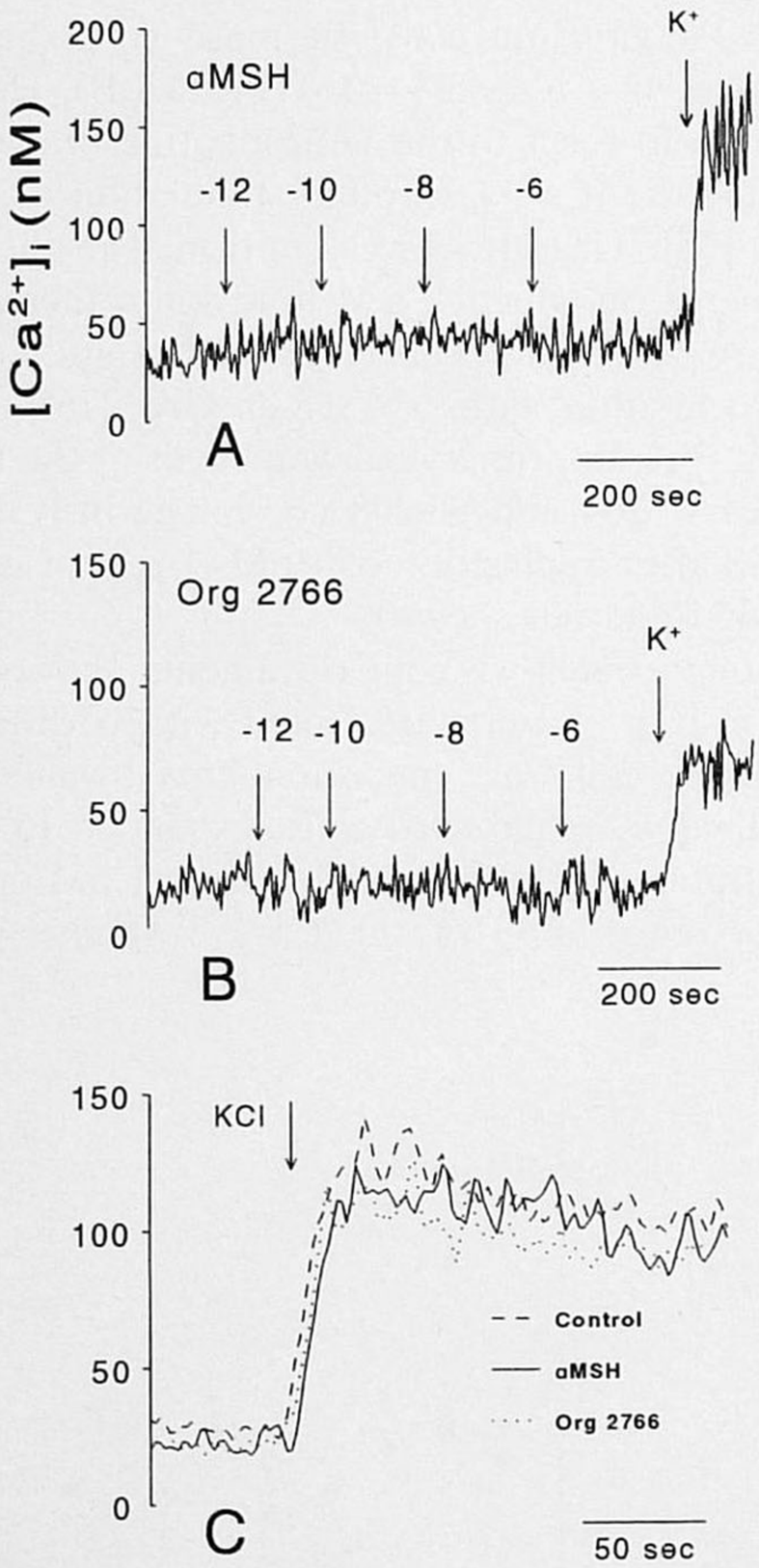

Fig. 2. Effect of αMSH (A) and Org 2766 (B) on the $[Ca^{2+}]_i$ in rat spinal cord cells (one representative experiment). The arrows mark the timepoints of peptide administration to the cells. C: shows the effect of the peptides on spinal cord cells depolarised with 30 mM KCl. Cells were treated with 1 μM αMSH or Org 2766, 5 min before KCl was administered. In C, data are represented as the mean of 9 (control), 5 (αMSH) and 7 (Org 2766) experiments.

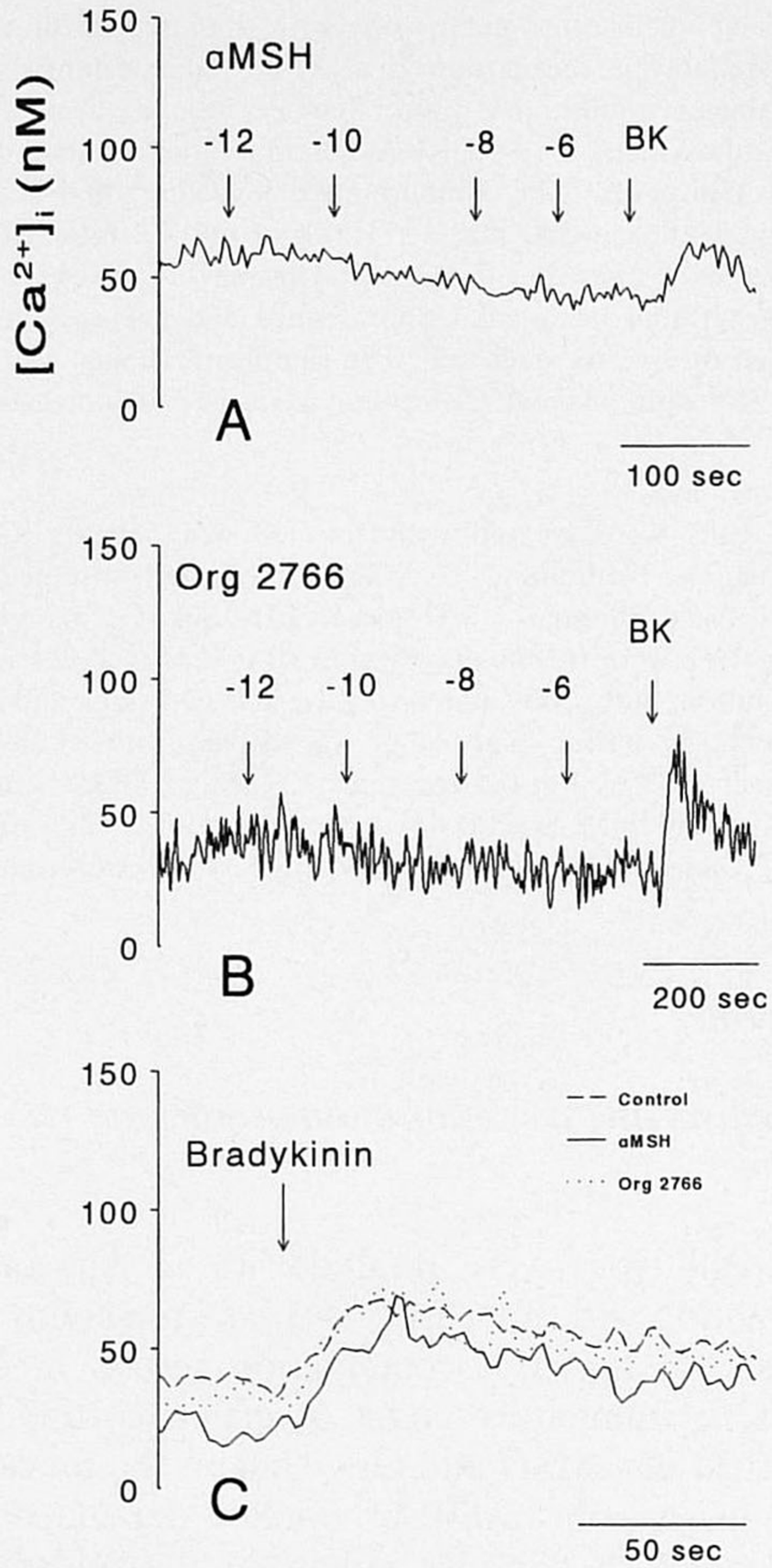

Fig. 3. Effect of αMSH (A) and Org 2766 (B) on the $[Ca^{2+}]_i$ in rat dorsal root ganglion cells (one representative experiment). The arrows mark the timepoints of peptide administration to the cells. C: shows the effect of the peptides on the bradykinin (1 μM)-induced rise in $[Ca^{2+}]_i$. Cells were treated with 1 μM αMSH or Org 2766, 5 min before bradykinin (BK) was administered. In C, data are presented as the mean of 10 (control), 4 (αMSH) and 3 (Org 2766) experiments.

rations; in 26 of the 93 cells a rise in $[Ca^{2+}]_i$ was observed. These responses were not consistent and varied in amplitude and the effective concentration of peptide. Responses comparable to Fig. 4, which were near saturation of the dye, were observed in 8 of the 29 cells responding to αMSH and 3 of the 26 cells responding to Org 2766. Application of the solvent alone did not change the signal more than 135%, with a response in only 2 of 32 cells. Experiments with purified motor neurons showed that the effect of αMSH and Org 2766 on $[Ca^{2+}]_i$ in the spinal cord cell culture was not due to an effect on the subpopulation of motor neurons in this culture. αMSH (1 pM–1 μM) was applied to 9 motor neurons (1 preparation); in none of the cells an increase in $[Ca^{2+}]_i$ was observed. Org 2766 (1 pM–1 μM) was applied to 20 motor neurons, 2 of

these cells showed an increase in $[Ca^{2+}]_i$ upon application of the peptide. Application of buffer, in which the peptides were dissolved, caused an increase in 3 of 22 cells. Although some motor neurons showed a rise in $[Ca^{2+}]_i$ upon application of Org 2766, these changes in $[Ca^{2+}]_i$ could not be distinguished from changes induced by application of the solvent. Fig. 5 shows a registration of changes in $[Ca^{2+}]_i$ of two representative motor neurons, which clearly respond to a local application of 6 mM KCl, but do not respond to the peptides.

In an earlier study we characterized the spinal cord cells, cultured under identical conditions, as neurons, because all cells showed neurofilament immunoreactivity with an antibody mainly directed against NF 200 kDa [42]. Furthermore the cells were glial fibrillary acidic protein (GFAP; a marker for astrocytes) and galactocerebroside (GalCer; a marker for oligodendrocytes) negative, indicating that there were no mature astrocytes or oligodendrocytes present in the culture [42]. In the present study we could not observe distinct morphological differences between the cells responding to αMSH or Org 2766 by a rise in $[Ca^{2+}]_i$ and the non-responsive cells in the same culture. Pharmacological characterization of these spinal cord neurons revealed that the cells responded with a rise in $[Ca^{2+}]_i$ upon application of substance P (14 of 91 cells), carbachol (3 of 80 cells), bradykinin (7 of 80 cells) and serotonin (2 of 21 cells). On the basis of these immunocytochemical, morphological and pharmacological characterizations, we were not able to distinguish a defined subpopulation of spinal cord neurons, that

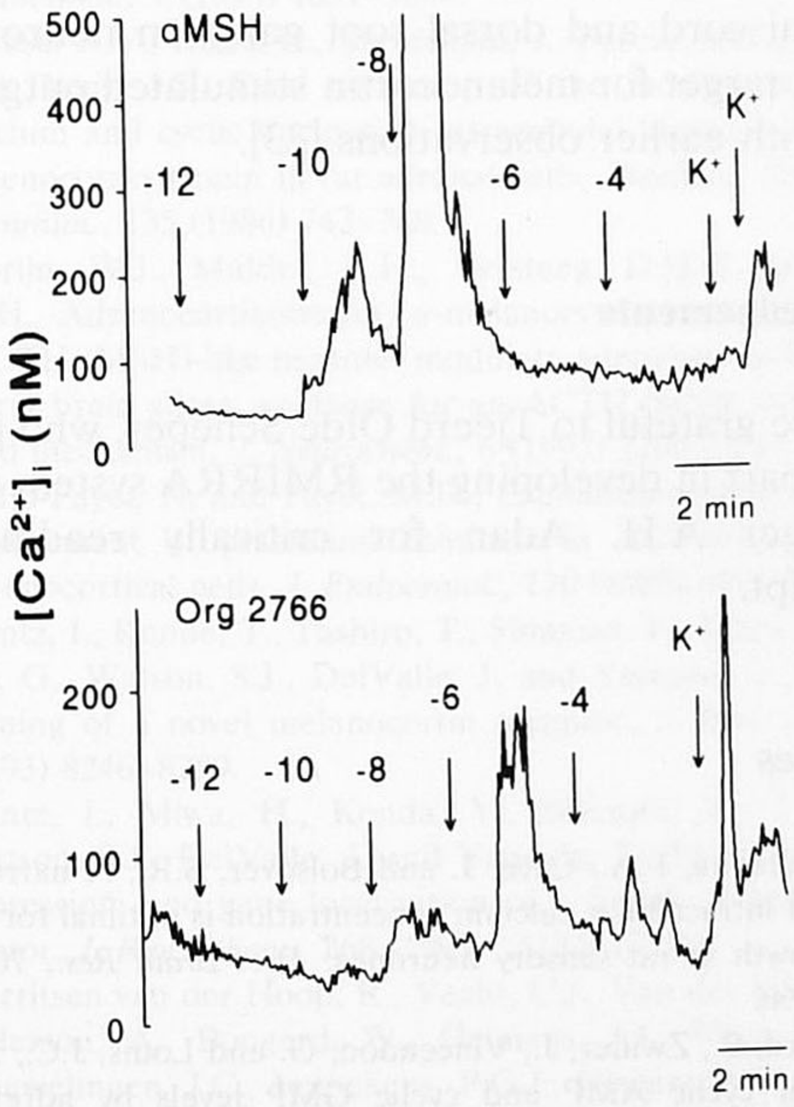

Fig. 4. This figure shows 2 single spinal cord neurons that responded to αMSH and Org 2766 treatment by an increase in $[Ca^{2+}]_i$. Arrows indicate timepoints of administration of the peptide.

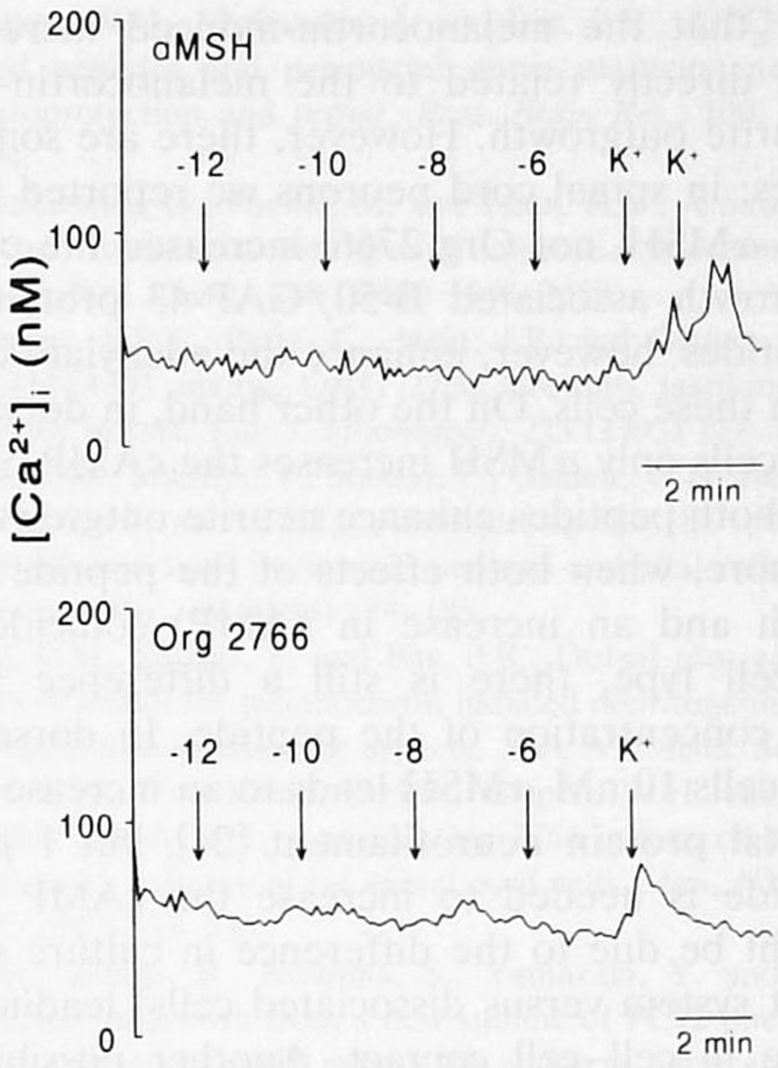

Fig. 5. Motor neurons did not respond specifically to application of αMSH or Org 2766. This figure shows a registration of changes in $[Ca^{2+}]_i$ in 2 representative motor neurons upon application of αMSH, Org 2766 or KCl. Only local application of 6 mM KCl resulted in a rise in $[Ca^{2+}]_i$ in both cells. Arrows indicate timepoints of administration of the peptide.

responded to application of αMSH or Org 2766 with a rise in $[Ca^{2+}]_i$.

4. Discussion

To gain insight in the mechanism of action of the neurotrophic properties of melanocortins, we monitored the effect of the melanocortins αMSH and the $ACTH_{4-9}$ analogue Org 2766 on the production of cAMP and the rise in intracellular Ca^{2+} in spinal cord cells, dorsal root ganglion cells and Schwann cells. Both second messengers, cAMP and Ca^{2+}, are important mediators in neurite outgrowth [1,30] and therefore can play a role in the neurotrophic action of melanocortins. Our present experiments show a differential effect of the peptides on cAMP production in dorsal root ganglion cells and spinal cord cells; Schwann cells do not respond at all. In batch-measurements αMSH and Org 2766 did not evoke detectable increases in $[Ca^{2+}]_i$, but single-cell measurements revealed a small population of spinal cord cells responding with a strong $[Ca^{2+}]_i$-elevation upon application of low concentrations of αMSH and Org 2766.

In general, stimulation of adenylate cyclase by forskolin, or by treatment with dibutyryl-cAMP increases neurite outgrowth in neuronal cells [27,32]. And as the cloned melanocortin receptors are all adenylate cyclase coupled [17,18,31], it is tempting to

speculate that the melanocortin-induced increase in cAMP is directly related to the melanocortin-stimulated neurite outgrowth. However, there are some discrepancies: in spinal cord neurons we reported earlier that only αMSH, not Org 2766, increased the content of the growth associated B-50/GAP-43 protein [42]. Both peptides, however, enhance the adenylate cyclase activity in these cells. On the other hand, in dorsal root ganglion cells only αMSH increases the cAMP production, but both peptides enhance neurite outgrowth [24]. Furthermore, when both effects of the peptide (more outgrowth and an increase in cAMP) coincide in a certain cell type, there is still a difference in the effective concentration of the peptide. In dorsal root ganglion cells 10 nM αMSH leads to an increase in the cytoskeletal protein neurofilament [24], but 1 μM of the peptide is needed to increase the cAMP levels. This might be due to the difference in culture system (an intact system versus dissociated cells) leading to a difference in cell–cell contact. Another possibility is that receptors, which are activated by other (related) ligands with high affinity, also bind αMSH when applied in a relatively high concentration (1 μM), leading to a stimulation of the cAMP production. Taken together, cAMP seems to play a role in the signal transduction system of melanocortins, but it is probably not the only mediator in and not exclusively responsible for the neurotrophic effect of the peptides.

Calcium is an important regulator of neurite outgrowth, however, the results of the batch-measurements of Ca^{2+} suggest that Ca^{2+} does not play a major role in the neurotrophic action of the melanocortins. The peptides also did not modulate the $[Ca^{2+}]_i$ regulation induced by other stimulations, such as K^+ depolarisation in spinal cord cells and bradykinin treatment in dorsal root ganglion cells. This suggests that the peptides do not modulate gating of Ca^{2+} channels in the plasma membrane, or release of Ca^{2+} from the endoplasmic reticulum. However, we found some neurons, only in the spinal cord cell cultures, which responded upon application of very low concentrations of αMSH or Org 2766 with a rise in $[Ca^{2+}]_i$. The subpopulation of Ca^{2+}-responsive cells is very small and not all of the responsive cells respond to the same concentration of peptide. This can explain why we could not observe a rise in $[Ca^{2+}]_i$ in the batch-experiments. The differences in response is probably due to the presence of several subpopulations of neurons in the spinal cord culture (e.g. motor neurons and interneurons). The cells that react are most probably not the motor neurons, because additional experiments showed that motor neuron cultures did not respond specifically to the peptides (Fig. 5).

All discrepancies described above can be due to the heterogeneity of the MC-receptor family. The various melanocortins have different affinities for the receptor subtypes [18,31,33] and it has been shown that Org 2766 has no binding affinity to the MC-3 receptor. Thus the differences in effects of αMSH and Org 2766 on $[Ca^{2+}]_i$ and cAMP might be due to the presence of several MC-receptor subtypes in the cell culture. Peptide treatment will result in a mixture of effects caused by different MC-receptor subtypes. Whether or not treatment with melanocortins will finally lead to enhancement of neurite outgrowth is probably dependent on the receptor types present in the cell culture.

From our experiments we can conclude that αMSH treatment in spinal cord- and dorsal root ganglion-cells is followed by a stimulation of adenylate cyclase. This increase in cAMP is probably involved in neurite outgrowth, as in both cell types αMSH treatment leads to an increase in neurite outgrowth [24,41]. In contrast, Org 2766 stimulates cAMP production in spinal cord cells, but has no effect on neurite outgrowth [42]. However, Org 2766 has no effect on cAMP in dorsal root ganglion cells, but does stimulate neurite outgrowth [24]. This indicates that other second messengers are necessary for Org 2766-stimulated neurite outgrowth. Our results suggests that αMSH and Org 2766 act via different receptors each with it's own signal transduction cascade. The neurotrophic response to melanocortins is probably a complex, pleiotropic interaction of several signal transduction systems, as observed for other growth factors as well (e.g. EGF [40]). Furthermore, as Schwann cells do not respond to αMSH or the analogue by an increase in cAMP or a rise in $[Ca^{2+}]_i$ these cells are probably not a target for melanocortins or mediate their effect through other second messenger pathways. This suggests that the spinal cord and dorsal root ganglion neurons are the main target for melanocortin stimulated outgrowth, in line with earlier observations [23].

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References

[1] Al-Mohanna, F.A., Cave, J. and Bolsover, S.R., A narrow window of intracellular calcium concentration is optimal for neurite outgrowth in rat sensory neurones, *Dev. Brain Res.*, 70 (1992) 287–290.

[2] Anglard, P., Zwiller, J., Vincendon, G. and Louis, J.C., Regulation of cyclic AMP and cyclic GMP levels by adrenocorticotropic hormone in cultured neurons, *Biochem. Biophys. Res. Commun.*, 133 (1985) 286–292.

[3] Bär, P.R., Schrama, L.H. and Gispen, W.H., Neurotrophic

effects of ACTH/MSH-like peptides in the peripheral nervous system. In D. De Wied (Ed.), *Neuropeptides: Basics and Perspectives*, Elsevier, Amsterdam, 1990, pp. 175–211.

[4] Bär, P.R., Veraart, C.P.W.M., Renkema, Hol, E.M. and Gispen, W.H., Nimodipine protects cultured spinal cord neurones from depolarization-induced inhibition of neurite outgrowth, *Cell Calcium*, 14 (1993) 293–299.

[5] Bijlsma, W.A., Jennekens, F.G.I., Schotman, P. and Gispen, W.H., Effects of corticotropin (ACTH) on recovery of sensorimotor function in the rat: structure-activity study, *Eur. J. Pharmacol.*, 76 (1981) 73–79.

[6] Bottenstein, J.F. and Sato, G., Growth of a neuroblastoma cell line in serum-free suplemented medium, *Proc. Natl. Acad. Sci. USA*, 76 (1979) 514–517.

[7] Chhajlani, V., Muceniece, R. and Wikberg, J.E.S., Molecular cloning of a novel human melanocortin receptor, *Biochem. Biophys. Res. Commun.*, 195 (1993) 866–873.

[8] Chhajlani, V. and Wikberg, J.E.S., Molecular cloning of the human melanocyte stimulating hormone receptor cDNA, *FEBS Lett.*, 309 (1992) 417–420.

[9] Coyne, M.D. and Pinkney, L., Adrenocorticotropin activates barium-conducting channels from bovine adrenocortical zona fasciculata cells in lipid bilayers, *Endocrinology*, 129 (1991) 263–269.

[10] Danks, A.M., Hammond, D.N., Wainer, B.H., Van Buskirk, R.G. and Isaacson, R.L., Cellular alterations produced by the experimental increase in intracellular calcium and the nature of protective effects from pretreatment with nimodipine, *Mol. Brain Res.*, 16 (1992) 168–172.

[11] Daval, J.L., Louis, J.C., Gerard, M.J. and Vincendon, G., Influence of ACTH on the growth of isolated neurons in culture, *Neurosci. Lett.*, 36 (1983) 299–304.

[12] Eichler, M.E., Dubinsky, J.M. and Rich, K.M., Relationship of intracellular calcium to dependence on nerve growth factor in dorsal root ganglion neurons in cell culture, *J. Neurochem.*, 58 (1992) 263–269.

[13] Enyeart, J.J., Mlinar, B. and Enyeart, J.A., T-type Ca^{2+} channels are required for adrenocorticotropin-stimulated cortisol production by bovine adrenal zona fasciculata cells, *J. Mol. Endocrinol.*, 7 (1993) 1031–1040.

[14] Farese, R.V., Rosic, N., Babischkin, J., Farese, M.G., Foster, R. and Davis, J.S., Dual activation of the inositol-triphosphate-calcium and cyclic nucleotide intracellular signaling systems by adrenocorticotropin in rat adrenal cells, *Biochem. Biophys. Res. Commun.*, 135 (1986) 742–748.

[15] Florijn, W.J., Mulder, A.H., Versteeg, D.H.G. and Gispen, W.H., Adrenocorticotropin/α-melanocyte-stimulating hormone (ACTH/MSH)-like peptides modulate adenylate cyclase activity in rat brain slices: evidence for an ACTH/MSH receptor coupled mechanism, *J. Neurochem.*, 6 (1993) 2204–2211.

[16] Gallo-Payet, N. and Payet, M.D., Excitation-secretion coupling: involvement of potassium channels in ACTH-stimulated rat adrenocortical cells, *J. Endocrinol.*, 120 (1989) 409–421.

[17] Gantz, I., Konda, T., Tashiro, T., Shimoto, Y., Miwa, H., Munzert, G., Watson, S.J., DelValle, J. and Yamada, T., Molecular cloning of a novel melanocortin receptor, *J. Biol. Chem.*, 268 (1993) 8246–8250.

[18] Gantz, I., Miwa, H., Konda, Y., Shimoto, Y., Tashiro, T., Watson, S.J., DelValle, J. and Yamada, T., Molecular cloning, expression, and gene localization of a fourth melanocortin receptor, *J. Biol. Chem.*, 268 (1993) 15174–15179.

[19] Gerritsen van der Hoop, R., Vecht, C.J., Van der Burg, M.E.L., Elderson, A., Boogerd, W., Heimans, J.J., Vries, E.P., Van Houwelingen, J.C., Jennekens, F.G.I., Gispen, W.H. and Neijt, J.P., Prevention of cisplatin neurotoxicity with and ACTH(4–9) analogue in patients with ovarian cancer, *N. Engl. J. Med.*, 322 (1990) 89–94.

[20] Gispen, W.H., Verhaagen, J. and Bär, P.R., ACTH/MSH-derived peptides and peripheral nerve plasticity: neuropathies, neuroprotection and repair, *Prog. Brain Res.*, 100, chapter 28, 1994.

[21] Grynkiewicz, G., Poenie, M. and Tsien, R.Y., A new generation of Ca^{2+} indicators with greatly improved fluoresence properties, *J. Biol. Chem.*, 260 (1985) 3440–3450.

[22] Hamers, F.P.T., Pette, C., Neijt, J.P. and Gispen, W.H., The ACTH-(4–9) analog, ORG 2766, prevents taxol-induced neuropathy in rats, *Eur. J. Pharmacol.*, 233 (1993) 177–178.

[23] Hol, E.M., Mandys, V., Sodaar, P., Gispen, W.H. and Bär, P.R., Protection by an $ACTH_{4-9}$-analogue against the toxic effects of cisplatin and taxol on sensory neurons and glial cells in vitro, *J. Neurosci. Res.*, 39 (1994) 178–185.

[24] Hol, E.M., Sodaar, P. and Bär, P.R., Dorsal root ganglia as an in vitro model for melanocortin induced neuritogenisis: pharmacological and mechanistic aspects, *Ann. NY. Acad. Sci.*, in press.

[25] Hol, E.M., Van Essen, E.H.R., Gispen, W.H. and Bär, P.R., αMSH and $ACTH_{4-9}$-analog Org 2766 induce cAMP increase in primary cultures of rat spinal cord cells, *Ann. NY Acad. Sci.*, 680 (1993) 533–535.

[26] Katoh-Semba, R., Kitajima, S., Yamazaki, Y. and Sano, M., Neuritic outgrowth from a new subline of PC12 pheochromocytoma cells: cyclic AMP mimics the action of nerve growth factor, *J. Neurosci. Res.*, 17 (1987) 36–44.

[27] Kumagai, C., Tohda, M., Isobe, M. and Nomura, Y., Involvement of growth-associated protein-43 with irreversible neurite outgrowth by dibutyryl cyclic AMP and phorbol ester in NG108–15 cells, *J. Neurochem.*, 59 (1992) 41–47.

[28] Lankford, K.L. and Letourneau, P.C., Evidence that calcium may control neurite outgrowth by regulating the stability of actin filaments, *J. Cell Biol.*, 109 (1989) 1229–1243.

[29] Mac Neil, S., Dobson, J., Bleehen, S.S. and Buffey, J., MSH increase intracellular calcium in melanoma cells, *Br. J. Dermatol.*, 123 (1990) 828.

[30] Mattson, M.P., Taylor-Hunter, A. and Kater, S.B., Neurite outgrowth in individual neurons of a neuronal population is differentially regulated by calcium and cyclic AMP, *J. Neurosci.*, 8 (1988) 1704–1711.

[31] Mountjoy, K.G., Robbins, L.S., Mortrud, M.T. and Cone, R.D., The cloning of a family of genes that encode the melanocortin receptors, *Science*, 257 (1992) 1248–1251.

[32] Nakagawa-Yagi, Y., Saito, Y., Takada, Y. and Nakamura, H., Suppresive effect of carbachol on forskolin-stimulated neurite outgrowth in human neuroblastoma NB-OK1 cells, *Biochem. Biophys. Res. Commun.*, 182 (1992) 45–54.

[33] Roselli-Rehfuss, L., Mountjoy, K.G., Robbins, L.S., Mortrud, M.T., Low, M.J., Tatro, J.B., Entwistle, M.L., Simerly, R.B. and Cone, R.D., Identification of a receptor for τ melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system, *Proc. Natl. Acad. Sci. USA*, 90 (1993) 8856–8860.

[34] Schnaar, R.L. and Schaffner, A.E., Separation of cell types from embryonic chicken and rat spinal cord: characterization of motoneuron-enriched fractions, *J. Neurosci.*, 1 (1981) 204–217.

[35] Silver, R.A., Lamb, A.G. and Bolsover, S.R., Calcium hotspots caused by L-channel clustering promote morphological changes in neuronal growth cones, *Nature*, 343 (1990) 751–754.

[36] Spoerri, P.E., Dozier, A.K. and Roisen, F.J., Calcium regulation differentiation: the role of calcium in GM1-mediated neuritogenesis, *Dev. Brain Res.*, 56 (1990) 177–188.

[37] Strand, F.L., Rose, K.J., Zuccarelli, L.A., Kume, J., Alves, S.E., Antonawich, F.J. and Garrett, L.Y., Neuropeptide hormones as neurotrophic factors, *Physiol. Rev.*, 71 (1991) 1017–1046.

[38] Thayer, S.A., Perney, T.M. and Miller, R.J., Regulation of calcium homeostasis in sensory neurons by bradykinin, *J. Neurosci.*, 8 (1988) 4089–4097.

[39] Tremblay, E., Payet, M.D. and Gallo-Payet, N., Effects of

ACTH and angiotensin II on cytosolic calcium in cultured adrenal glomerulosa cells. Role of cAMP production in the ACTH effect, *Cell Calcium*, 12 (1991) 655–673.
[40] Ullrich, A. and Schlessinger, J., Signal transduction by receptors with tyrosine kinase activity, *Cell*, 61 (1990) 203–212.
[41] Van der Neut, R., Bär, P.R., Sodaar, P. and Gispen, W.H., Trophic influences of αMSH and ACTH4–10 on neuronal outgrowth in vitro, *Peptides*, 9 (1988) 1015–1020.
[42] Van der Neut, R., Hol, E.M., Gispen, W.H. and Bär, P.R., Stimulation by melanocortins of neurite outgrowth from spinal and sensory neurons in vitro, *Peptides*, 13 (1992) 1109–1115.
[43] Van der Neut, R., Sodaar, P. and Bär, P.R., An enzyme-linked immunoadsorbent assay for laminin in primary Schwann cell cultures, *Neurosci. Res. Commun.*, 3 (1988) 119–125.
[44] Weiss, S., Sebben, M. and Bockaert, J., Corticotropin-peptide regulation of intracellular cyclic AMP production in cortical neurons in primary culture, *J. Neurochem.*, 45 (1985) 869–874.
[45] Zohar, M. and Salomon, Y., Melanocortins stimulate proliferation and induce morphological changes in cultured rat astrocytes by distinct transducing mechanisms, *Brain Res.*, 576 (1992) 49–58.