NEUROPEPTIDES

Neuropeptides (1987) 9, 25-32
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EVIDENCE FOR THE EXPRESSION OF PEPTIDES DERIVED FROM THREE OPIOID PRECURSORS IN NG 108CC15 HYBRID CELLS

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Abstract:

Five opioid peptides (immunoreactivity) derived from their respective opioid precursors were measured in neuroblastoma-glioma hybrid cells (NG 108CC15; pmol/g protein): heptapeptide (Tyr-Gly-Gly-Phe-Met-Arg-Phe), 13.0 +/- 2.6; α-neoendorphin, 6.6 +/- 0.8; dynorphin A, 4.4 +/- 1.5; dynorphin A 1-8, 1.3 +/- 0.29; ß-endorphin, 0.3 +/- 0.13. These peptides originate from preproenkephalin A (heptapeptide), prodynorphin (α-neonedorphin, dynorphin A, dynorphin A 1-8) and proopiomelanocortin (ß-endorphin). The data suggest the expression of all three known opioid precursors in a single hybrid cell line, permitting a simultaneous investigation of the processing of different opioid peptides under identical experimental conditions.

INTRODUCTION

NG 108CC15 cells have been used extensively as a model to study opioid mediated receptor responses <1>. More recently interest has focused on the opioid peptide content of these cells <2>.
Glaser et al. <3> were the first to obtain evidence for the presence of opioid peptides in NG 108CC15 cells, using a bioassay procedure. The demonstration of leu- and met--enkephalin in extracts of hybrid cells further confirmed this findings <13>. However, enkephalins may originate from different opioid peptide precursors: proopiomelanocortin, preproenkephalin A and prodynorphin (preproenekphalin B). Whether all these precursors are actually expressed in NG 108CC15 cells may be answered by investigating the presence of the individual opioid peptides derived from each.

The presence of two precursors has recently been reported. Studies demonstrating the presence of dynorphin A immunoreactivity <11> in NG 108CC15 cells suggested the presence of prodynorphin and the subsequent finding of ß-endorphin immunoreactivity <4> indicated the existence of the proopiomelanocortin precursor. The data communicated so far prompted investigations as to whether these hybrid cells are also equipped with the third known opioid precursor, proenkephalin A. The presence of three opioid precursors and their cleavage products in a single hybrid cell line would be compatible with the notion that a neuron may contain several neuroactive agents <20> and as such would provide the opportunity to study the regulation of all three opioid precursors under identical conditions.

METHODS

Hybrid cells (NG 108CC15) were raised as described by Hamprecht <1>. Briefly, confluent monolayers of cells were washed three times with cold phosphate buffered saline and harvested by shaking. The cells were pelleted at 1000 x g (10 min) and stored at -70°C.
For the extraction of opioid peptides each batch (5×10^7 cells) was treated with boiling HCl (0.1M, 0.4ml per 10^7 cells) for 10 min . After centrifugation (10 min, 10.000 x g) the supernatant was submitted to affinity chromatography (0.5 x 5.0 cm column). The affinity gel consisted of sepharose linked to an anti-ß-endorphin monoclonal antibody (3-E7) <4>. This 3-E7 antibody recognizes the amino acid sequence Tyr-Gly-Gly-Phe-Leu/Met, the common N-terminal sequence of all endogenous opioids, and, thus, common to the five opioid peptides here under investigation. The opioid-like material was eluted with 10 ml 2.5M acetic acid and subsequently lyophilized.
The content of the different opioid peptides in the samples was determined by radioimmunoassay (RIA), using highly specific antisera. Each peptide assay refers to a sample of 5×10^7 cells.
The immunoreactivity of the following peptides was measured (references for RIA protocol given in brackets): dynorphin A, dynorphin A 1-8 and α-neoendorphin <5>, ß-endorphin <6>, heptapeptide <7>. Additional assays were carried out for the octapeptide <7> and peptide F-amid <18>.
Respective crossreactivities of the used antisera to other opioids as measured by displacement curves were below 0.01%. An exception is dynorphin A antiserum which recognized dynorphin A 1-8 with a crossreactivity of 0.05%.

Gel chromatography:
Chromatography was carried out in acetic acid (1 M) on a G50 Sephadex (superfine) column (0.9 cm x 90 cm). Columns were calibrated with bovine serum albumin, α-neoendorphin,

dynorphin A, dynorphin A 1-8, ß-endorphin and heptapeptide. Fractions of 1ml were collected (flow rate 10 ml/h) and lyophilized. Recoveries of endogenous and synthetic peptides from the column were 90%.
High pressure liquid chromatography (HPLC):
The nature of the opioid peptide like immunoreactivity was further analysed by HPLC. Separations were carried out on a C18 (5 μm) column (Waters, Königsstein, Germany) using a gradient of 20% to 60% acetonitrile in 0.1% trifluoric acetic acid (flow rate 1.0 ml/min).
The peptide recovery for the analytical procedures was assessed by means of tritiated peptides as internal standards. All peptide concentrations reported are corrected for recovery.
Protein was determined according to the method of Spector <8>. Synthetic opioid peptides were purchased from Peninsula Laboratories (San Carlos, CA., USA), other chemicals from Merck (Darmstadt, Germany).

RESULTS

The concentrations of five opioid peptides measured in NG 108CC15 cells, harvested at the state of confluency, are given in table I.

Table 1. Opioid peptide content (immunoreactivity, pmol/g protein) in NG 108CC15 cells

peptide	precursor	content	n	re
heptapeptide	preproenkephalin A	13.0 ± 2.6	10	57
α-neoendorphin	preproenkephalin B	6.6 ± 0.8	20	51
dynorphin A	" "	4.4 ± 1.5	20	55
dynorphin A 1-8	" "	1.3 ± 0.29	10	65
ß-endorphin	proopiomelanocortin	0.3 ± 0.13	10	90

±: standard error of the mean; n= number of experiments; re= recovery in percent;

A direct comparison of peptide concentrations is permitted by the fact that they were obtained from cells raised and assayed under identical experimental conditions.
The highest concentration measured was the heptapeptide-like immunoreactivity. Table I shows it to be the most abundant of the five opioid peptids assayed: its concentration being

twice the amount of α-neoendorphin.
The heptapeptid-like immunoreactivity was verified by HPLC analysis of the affinity column purified eluates. Figure 1 demonstrates that 90% of the immunoreactivity eluted at an identical position as observed for the synthetic heptapeptide.
The concentration of α-neoendorphin appears to be slightly higher than that of dynorphin A. Further studies sought to determine the presence of peptides derived from pro-dynorphin, especially the occurence of dynorphin A 1-8 immunoreactivity, a typical candidate during the processing of dynorphin A <9>. The presence of this immunoreactivity was confirmed by chromatography. The concentration of dynorphin A 1-8 was found to be 30% of the amount for dynorphin A.
The data are in line with previous findings indicating a relatively low concentration of ß-endorphin in NG 108CC15 cells <4>.

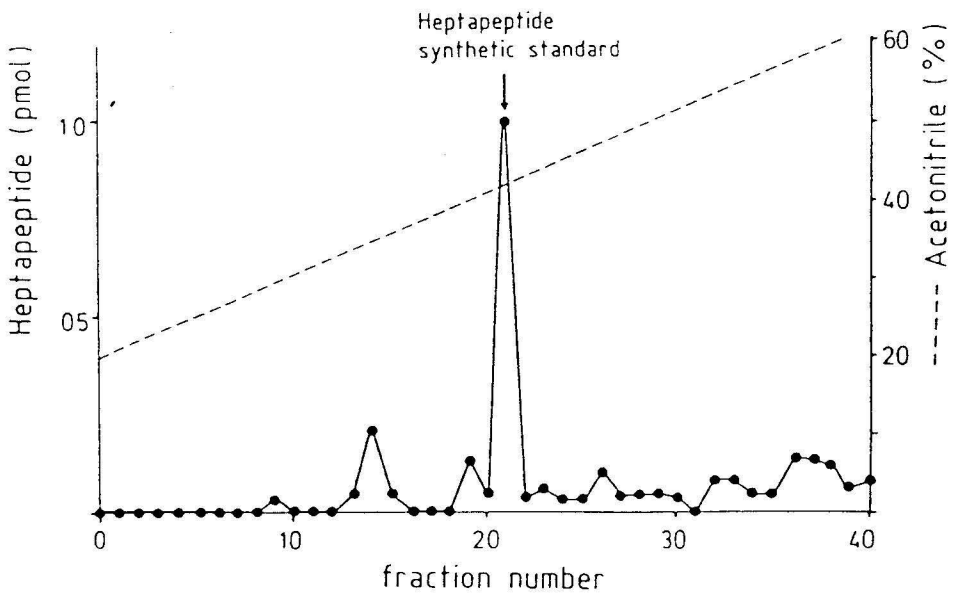

Figure 1: HPLC seperation of affinity purified material extracted from NG 108CC15 cells.

The affinity column eluates were also examined for other peptides which are derived solely from proenkephalin A.
A high specific antiserum (detection limit: 10 fmol/tube) failed to reveal significant amounts of the octapeptide (Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu). In addition, attempts were made to detect the proenkephalin A derived peptide F amid to see whether NG 108CC15 cells are able to amidate

opioids. A search in five batches showed no detectable amount (detection limit 100 fmol/tube). However the possibility of amidation of other opioid peptides can not be ruled out.

DISCUSSION

This study investigates the issue whether NG 108CC15 hybrid cells express the three known opioid peptide precursors. We report here the presence of immunoreactive opioid peptides known to originate from preproenkephalin A, prodynorphin and proopiomelanocortin. This finding strongly suggests the expression of this precursors. Moreover, this permits a comparison of peptides from three different opioid precursors in one homogenous cell type grown under identical conditions, in contrast to the situation in tissues <19> or even in primary cultures. Thus the NG 108CC15 cells represent an interesting example for the coexistence <20> of a neuronal transmitter (acetylcholin <21>) with opioid peptides.

Studies on the modulation of enkephalin content in hybrid cells have already been successfully conducted <15,17>. However, previously reported leu- and met-enkephalin in NG 108CC15 cells <12,13> could originate both from pro-dynorphin, from proenkephalin A or from proopiomelanocortin. In contrast heptapeptide as well as the octapeptide and peptide F-amid have as their only source proenkephalin A. The documentation of heptapeptide immunoreactivity thus strongly indicates the expression of the preproenkephalin A precursor.

Each peptide reported in our study is identified both at its N and C terminus. That is, the 3-E7 monoclonal antibody recognizes the N-terminal enkephalin sequence Tyr-Gly-Gly--Phe-Met/Leu common to all opioid peptides. The specific antisera employed here recognize the respective C-termini of the peptides.

It is interesting to note that differences in precursor processing and turnover apparently lead to a concentration of a product of the preproenkephalin A gene at least 40 times higher than that of ß-endorphin, the product of the proopiomelanocortin gene. Similar differences have been reported in specific tissues <16>.

The concentration of α-neoendorphin in NG 108CC15 cells is only slightly elevated in comparison to dynorphin A. It is concluded that their processing from prodynorphin is closely related, as has been suggested for rat and human tissue <5>. In support of further processing of dynorphin A in NG 108CC15 cells we report here the occurence of dynorphin A 1-8. This peptide occurs only as a third of the concentration of dynorphin A. An analogous ratio of processing can be found in posterior pituitary (0.5:1 <10>) . In brain (rat), however, dynorphin A 1-8 dominates dynorphin A <9>.

In principal the opioid content (pmol/g protein) of the different peptides expressed in NG 108CC15 cells is by a factor of about 15 lower than reported in rodent brain <5,14>.
The neuroblastoma glioma hybrid cells express a variety of other peptides <2>. In particular, vasoactive intestinal peptide (VIP) immunoreactivity is present in concentrations ranging from 0.94 to 4.14 pmol/g protein and co-release studies for opioids, acetylcholin and VIP were suggested for this hybrid cells <22>. The complete renin-angiotensin system has been identified in NG 108CC15 cells, angiotensin II content being 60 pmol/ g protein, renin 34 pg/mg protein and modulated in this cells <23>.

Our findings may contribute to the concept that NG 108CC15 cells are useful models to study the biochemistry of neuropeptides.

Acknoledgements

We thank Dr.T.Shippenberg for stylistic corrections of the text and Dr.T.Costa for helpful discussions.

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Received 17/9/86
Accepted 8/10/86