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Met-Enkephalyl-Arg^6-Phe^7 immunoreactivity in a human neuroblastoma cell line: effect of dibutyryl 3′:5′-cyclic AMP and reserpine

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Summary

The carboxy terminal part of the proenkephalin A sequence is the 31 amino acid peptide B, which has as its final seven amino acids the sequence of the opioid peptide Met-enkephalyl-Arg^6-Phe^7. Using a radioimmunoassay which recognises both these peptides we have investigated the relative amounts of peptide B and Met-enkephalyl-Arg^6-Phe^7 in a human neuroblastoma cell line. We show that these cells contain peptide B-like immunoreactivity but not its heptapeptide fragment. This may be due to lack of proteolytic activity cleaving Met-enkephalyl-Arg^6-Phe^7 from its precursor, peptide B. On treatment with dibutyryl cyclic AMP the level of immunoreactivity approximately doubles, due to increased amounts of peptide B-like immunoreactivity. Treatment with reserpine, which increases conversion of peptide B to the heptapeptide in bovine chromaffin cells in culture does not stimulate the accumulation of Met-enkephalyl-Arg^6-Phe^7 in the human neuroblastoma cells. The results are discussed with respect to peptide processing.

enkephalin; opioid peptide; proteolysis; cyclic AMP; neuroblastoma; reserpine

Introduction

Based on the characterisation of the larger fragments of the proenkephalin A sequence found in bovine adrenal medulla, it may be proposed that one of the primary

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cleavage points for proenkephalin A is at the double basic residues immediately following the sole Leu-enkephalin sequence; this removes the large C-terminal fragment peptide B [1]. The carboxy-terminal sequence of this peptide is the active opioid heptapeptide Met-enkephalyl-Arg^6-Phe^7, which is itself preceded by a double basic residue in both the bovine and human sequences [2–4]. Using a radioimmunoassay to the heptapeptide which cross-reacts with peptide B, we have previously shown that, while in the bovine adrenal medulla the molar amounts of peptide B far exceed those of the heptapeptide, in the bovine brain (striatum) the heptapeptide accumulates while peptide B does not [5]. In contrast, in extracts of the human post-mortem adrenal more immunoreactivity is found in the form of the heptapeptide than in the form of peptide B [6], commensurate with the increased processing of other opioid peptides in these extracts [7].

Bovine adrenal chromaffin cells in primary culture contain large quantities of proenkephalin A products. Eiden and co-workers [8,9] have shown that culture of these cells in the presence of the cyclic AMP analogue dibutyryl cyclic AMP, or in conditions elevating endogenous cyclic AMP levels, results in increased levels of proenkephalin products as a result of increased proenkephalin A mRNA levels. However, culture in the presence of reserpine, a drug which depletes vesicular stores of catecholamine, results in the increased processing of the larger proenkephalin products to the smaller [9], in part at least, as a result of changes in proteolytic enzyme activity [10]. Consistent with these observations we have recently shown using bovine chromaffin cells that overall Met-enkephalyl-Arg^6-Phe^7 immunoreactivity is raised by treatment with dibutyryl cyclic AMP and forskolin, that the presence of reserpine increases the amount of heptapeptide relative to peptide B and furthermore, that these changes are reflected in the more functional index of stimulated release [11].

In looking for continuous cell lines which synthesise the proenkephalin A products, we have found a human neuroblastoma cell line with measurable amounts of Met-enkephalyl-Arg^6-Phe^7 immunoreactivity. Here we present a partial characterisation of this immunoreactivity and describe the response to elevated cyclic AMP and reserpine.

Methods

Cell culture

The cells used in these experiments were the human neuroblastoma cell line SK-N-MC, kindly provided by Dr. C.R. Snell (MRC Neuroendocrinology Unit, Newcastle, U.K.), used at passage numbers 42–44. Cells were grown in 9-cm Nunc tissue culture dishes in 10% fetal calf serum, in a 1:1 mixture of DMEM and Hams' F12, with gentamycin. For experimental work cells were plated into 24-well Linbro multiwell plates (Flow Laboratories, Irvine, Scotland) and used just before becoming confluent. Alternatively, they were grown on 9-cm dishes for subsequent chromatography of the extracts. Where appropriate, 3-isobutyl-1-methylxanthine (IBMX, 50 μM), prostaglandin E_1 (PGE_1, 10 μM), dibutyryl cyclic AMP (1 mM), and reserpine (5 μM) were added to the culture medium. Treatment with drugs was for 4 days prior

to extraction of the cells. The culture dish was cooled on ice and extracted into cold 0.1 N HCl by scraping cells free, sonication (on ice for about 10 s) and centrifugation (10000–12000 × *g* for 10 min) to separate the protein, which was estimated by the method of Lowry et al. [12]. Previous studies with various tissues and with bovine adrenals in cell culture using this extraction have indicated a recovery of >90%.

Tissue culture supplies, except where indicated, were from GIBCO, Paisley, Scotland, and drugs were from Sigma.

Radioimmunoassay

The antiserum and assay procedure for the Met-enkephalyl-Arg^6-Phe^7 assay was essentially as described previously [6], except that the assay buffer was changed to 20 mM sodium phosphate, with 8.8 g/l NaCl, 100 mg/l BSA, 1 g/l gelatin, 0.2 g/l Triton X-100, at a pH of 7.4. The antiserum was used at a final concentration of 1:30000, with an assay volume of 200 µl containing about 10000 cpm of chloramine T synthesised ^{125}I-labelled Met-enkephalyl-Arg^6-Phe^7. Separation of bound from free was by a second antibody procedure using immobilised goat anti-rabbit antiserum (Saccell, Wellcome). This assay cross-reacts on an equimolar basis with both Met-enkephalyl-Arg^6-Phe^7 and with peptide B (peptides were from Peninsula). This is consistent with the previously presented data [6], indicating that the antiserum recognises the free carboxyl terminus of the heptapeptide.

The assay for total opioid-like immunoreactivity was as described earlier [7]; it is dependent upon alpha-amino acetylation of the sample, followed by radioimmunoassay with an antiserum which recognises the acetylated enkephalins with or without carboxy terminal extensions. All endogenous opioid peptides have either Leu- or Met-enkephalin as their amino terminus, and are therefore recognised by this assay.

Chromatography

Gel exclusion chromatography was with Sephadex G50 fine equilibrated and run in 40% acetic acid. Column dimensions were either 120 × 1.4 cm or 55 × 0.9 cm. Reverse-phase high-pressure liquid chromatography (HPLC) used a Hypersil 5 ODS column (0.5 × 25 cm) from HPLC Technology (Macclesfield, U.K.), eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid; the gradient was 6–30% (5 min), 30–60% (50 min). Recovery of Met-enkephalyl-Arg^6-Phe^7 through chromatography steps was higher than 80%.

Results

Levels of Met-enkephalyl-Arg^6-Phe^7 immunoreactivity in untreated cultures of SK-N-MC cells varied from about 0.4 to about 1.75 pmol/mg protein (compare Tables I and II). The results of characterisation of this immunoreactivity with gel exclusion chromatography and reverse-phase HPLC is shown in Fig. 1. When the sample was run on a Sephadex G50 column in 40% acetic acid the immunoreactivity was found in a single peak, co-eluting with the bovine peptide B marker. No peak of immunoreactivity was seen in the position of Met-enkephalyl-Arg^6-Phe^7. Pooling

TABLE I

Effect of IBMX + PGE_1 treatment on opioid peptide-related immunoreactivity

	pmol/mg protein	
	Control	IBMX + PGE_1
Met-Enkephalyl-Arg^6-Phe^7 immunoreactivity	1.76 ± 0.13	3.58 ± 0.44*
Total opioid peptide-like immunoreactivity	0.80 ± 0.04	1.54 ± 0.03*

* Significantly different from controls, $P < 0.01$ (Student's 't' test).

of the peak eluting on the G50 with bovine peptide B, and separation by HPLC, revealed a single peak of immunoreactivity, eluting significantly in advance of the bovine peptide B marker. This is consistent with the material being human peptide B, since the two species differ in sequence in a manner which would predict that the human peptide would elute before the bovine peptide (see Discussion). No human peptide B was available as a marker.

In initial experiments IBMX/PGE_1 treatment was used to investigate the responsivity of cells to manipulation of intracellular cyclic AMP. Preliminary investigations showed that these cells respond with an increased cyclic AMP level (in untreated cells levels of cyclic AMP were below detectable limits in our assay conditions, so an estimation of increase relative to control was not produced). The response of the cells is seen in Table I to be an approximately 2-fold increase in Met-enkephalyl-Arg^6-Phe^7 immunoreactivity. Additionally, Table I shows the response of an assay measuring total opioid peptide immunoreactivity; this also approximately doubled in response to IBMX/PGE_1 treatment. If the response to IBMX/PGE_1 treatment is a response to increased cyclic AMP levels, then it should be possible to mimic this with dibutyryl cyclic AMP. This is shown in Table II. Again the level of immunoreactivity

TABLE II

Effect of dibutyryl cyclic AMP and reserpine on Met-enkephalyl-Arg^6-Phe^7 immunoreactivity

	Met-enkephalyl-Arg^6-Phe^7 immunoreactivity (pmol/mg protein)
Control	0.40 ± 0.03
Dibutyryl cyclic AMP	0.81 ± 0.07*
Reserpine	0.36 ± 0.02
Dibutyryl cyclic AMP + reserpine	0.51 ± 0.02**

* Significantly different from control value, $P < 0.01$, ** $P < 0.05$. Value for dibutyryl cyclic AMP-treated and dibutyryl cyclic AMP + reserpine-treated were significantly different, $P < 0.01$ (Student's 't' test).

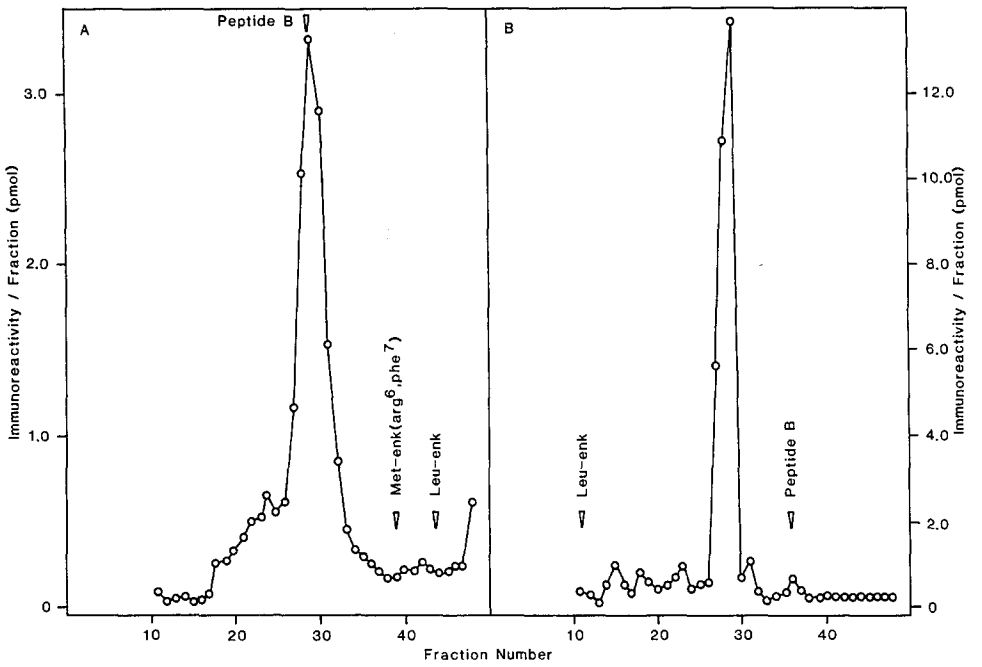

Fig. 1. Met-enkephalyl-Arg^6-Phe^7 immunoreactivity in fractions from the chromatography of extracts of SK-N-MC cells (A) on a Sephadex G50 column in 40% acetic acid (dimensions 120 × 1.4 cm). Elution of marker peptides, as indicated, was based on the elution of ^{125}I-peptide B, ^{125}I-labelled Met-enkephalyl-Arg^6-Phe^7 and [^{3}H]Leu-enkephalin. (B) Material from fractions 28, 29 and 30 in (A) were pooled and eluted on reverse-phase HPLC with an acetonitrile gradient (6–30% in 5 min, 30–60% in 50 min). Aliquots of fractions were assayed for Met-enkephalyl-Arg^6-Phe^7 immunoreactivity.

in the Met-enkephalyl-Arg^6-Phe^7 assay approximately doubles. When treated with reserpine there is no significant change in immunoreactivity; the non-significant reduction seen in Table II was seen over several experiments. It is interesting to note that when cells were being treated with dibutyryl cyclic AMP, the addition of reserpine also resulted in a small decrease in immunoreactivity which sometimes reached significance, as in Table II.

In order to see whether any of these treatments change the processing of the peptide B-like immunoreactivity revealed in Fig. 1, short Sephadex G50 columns were used to provide a low-resolution characterisation sufficient to distinguish between peptide B and the heptapeptide. Fig. 2 shows that following treatment with dibutyryl cyclic AMP the increase in activity is caused by an increase in the peptide B-like material; no immunoreactivity in the position of the heptapeptide appears. Fig. 3 shows that reserpine, alone or in combination with cyclic AMP, also failed to modify the position of the immunoreactivity on gel exclusion chromatography.

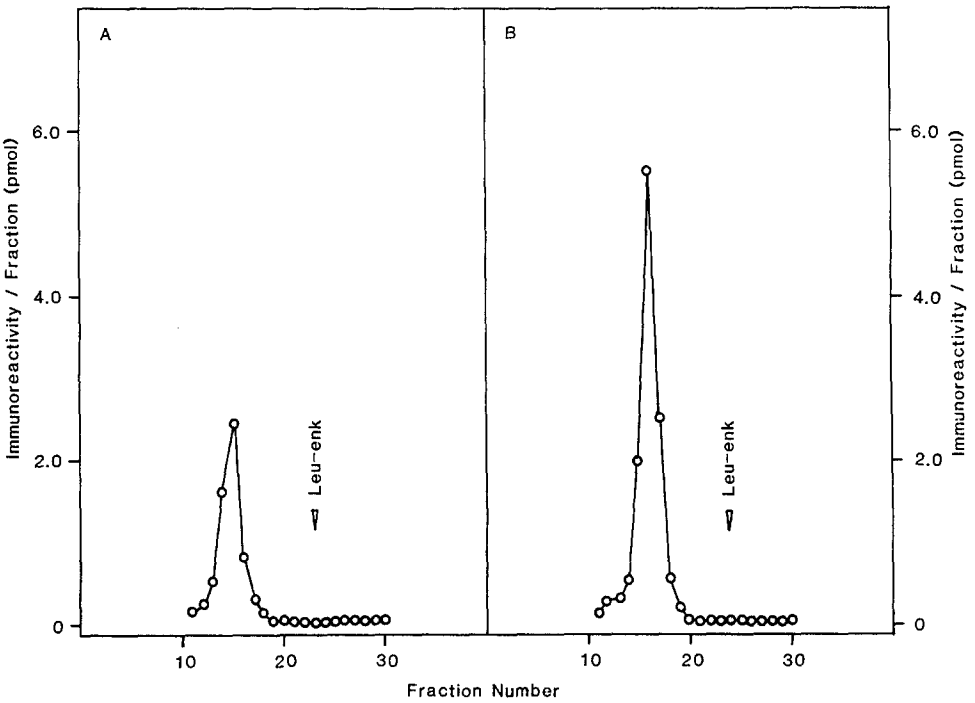

Fig. 2. Met-enkephalyl-Arg^6-Phe^7 immunoreactivity from the chromatography of extracts of SK-N-MC cells on Sephadex G50 columns (55 × 0.9 cm) run in 40% acetic acid. (A) Control cells; (B) cells after 4 days with 1 mM dibutyryl cyclic AMP added to the medium.

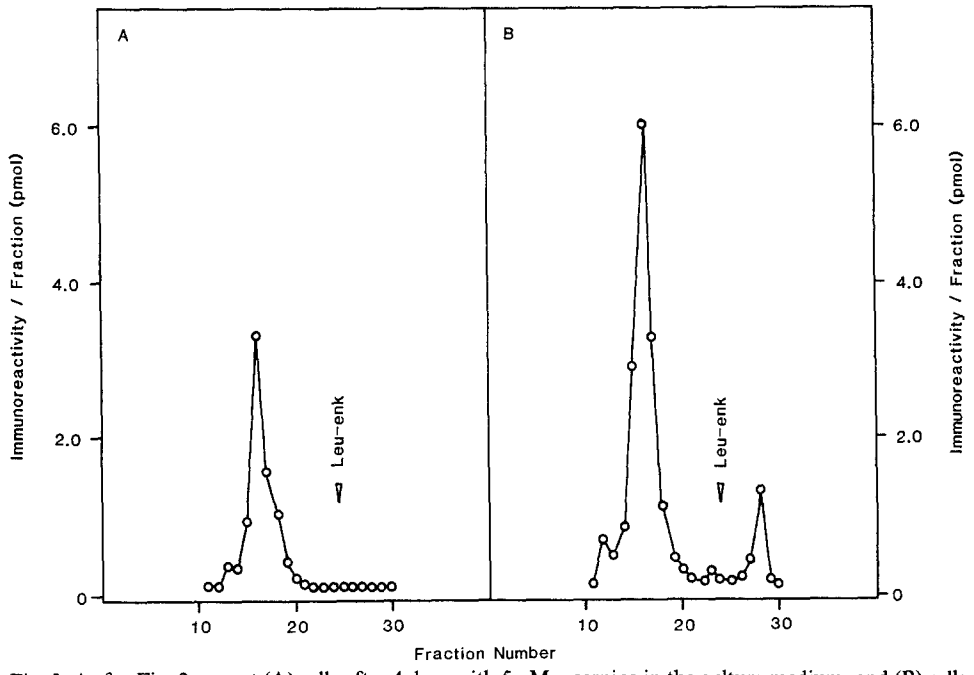

Fig. 3. As for Fig. 2, except (A) cells after 4 days with 5 μM reserpine in the culture medium, and (B) cells after 4 days with 5 μM reserpine and 1 mM dibutyryl cyclic AMP in the medium.

The principal type of cell culture used in the study of proenkephalin A products has been the primary culture of bovine adrenal chromaffin cells; this is in part due to the high levels of peptides sustained in the cells [13]. However, there have been some published studies on the levels of opioid peptides in continuous cell lines [e.g. 14–16]. Analysis of these cell lines is hampered by the relatively low levels of peptides; it can be shown however, that they respond in a similar way to conditions of elevated cyclic AMP, i.e. an elevation of the level of proenkephalin A products [15,16].

In looking for cell lines of use in investigating these peptides, we have been particularly interested in cells of human origin. We found sufficient immunoreactivity in the SK-N-MC cells to both Met-enkephalyl-Arg^6-Phe^7 and the total opioid immunoassay to enable further investigation. The Met-enkephalyl-Arg^6-Phe^7 immunoreactivity provides an attractively simple approach to the proenkephalin A products, since the sequence occurs only once, at the carboxy terminus of the precursor, and upon processing only two forms of immunoreactivity, the 31 amino acid fragment peptide B, and the heptapeptide product, are formed. It seems likely that the peptide B is normally removed from the precursor first and this then serves as the precursor for the heptapeptide, since the precursor fragment lacking only the carboxy terminal heptapeptide has never been reported, whereas the fragment lacking only peptide B has been reported [17]. This is central to the interpretation of results presented here, since the unexpected finding that, on a gel filtration analysis the Met-enkephalyl-Arg^6-Phe^7 immunoreactivity behaves like peptide B, with no heptapeptide-like immunoreactivity, may indicate that the enzyme responsible is absent or inactive. One alternative interpretation is that the heptapeptide is degraded or lost to the culture medium; the latter is unlikely, since we failed to find immunoreactivity released from these cells, either basic or stimulated with high potassium (results not shown). A further alternative explanation, that the heptapeptide is broken down during the extraction procedure, is made unlikely by the stability of Met-enkephalyl-Arg^6-Phe^7 (both endogenous and exogenous) in numerous studies using the same extraction methods with adrenal and brain tissue.

The HPLC analysis of the gel filtration peak reveals immunoreactivity which is less hydrophobic than the bovine adrenal peptide B marker. The latter is an accurate marker for the human peptide on gel exclusion, but the difference in sequence between bovine and human peptide B [3,4] is, judged by published hydrophobicity data [18], sufficient to account for the different behaviour on a reverse-phase column. This data does not present an unequivocal identification of the peptide as peptide B, but is consistent with this interpretation.

The observed elevation of immunoreactivities with treatment with IBMX/PGE$_1$ or with dibutyryl cyclic AMP may be due to increased transcription of the proenkephalin A gene, an interpretation encouraged by the observation of increased proenkephalin A mRNA seen with similar treatment of bovine chromaffin cells [9]. The Sephadex G50 analyses show the increase to be due to accumulation of peptide B-like immunoreactivity; in other words if the lack of heptapeptide-like material is due to failure to convert the precursor peptide B, then increasing the concentration of this precursor still does not result in formation of the heptapeptide product. This may be due to a lack of proteolytic activity.

The action of reserpine on these cells is complicated by the observation, in analyses not reported here, that the cells do not contain detectable levels of catecholamines. In addition to its action in depleting catecholamines, reserpine has been reported to increase peptide levels [19] by increased translation of messenger and by increased proteolytic processing [9]. We have shown [11] that reserpine treatment of bovine chromaffin cells results in the increased accumulation of Met-enkephalyl-Arg^6-Phe^7 at the expense of levels of peptide B-like material. We wished here to see if we could stimulate the cells to produce the heptapeptide from peptide B by reserpine treatment. The observation that reserpine failed to cause the accumulation of measurable levels of Met-enkephalyl-Arg^6-Phe^7, even under conditions of elevated peptide levels caused by dibutyryl cyclic AMP, is again consistent with a lack of proteolytic activity. The tendency of reserpine to cause a small fall in peptide levels, sometimes reaching statistical significance, seems inconsistent with the suggestion that this treatment leads to increased translation of proenkephalin A mRNA.

One interpretation of these findings is that we have a human neuroblastoma cell line which is lacking in proteolytic activity able to convert peptide B to the heptapeptide Met-enkephalyl-Arg^6-Phe^7. If this is the case then this indicates a specific deficit, since cleavage at the double basic residue giving rise to peptide B remains. An alternative explanation is that the peptide B-like material seen here is to some degree different from the normal peptide B sequence, and cannot therefore serve as a substrate for the proteolytic enzyme.

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