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# Influence of δ-Opioid Receptors on Production of Labeled Methionine$^5$-Enkephalin in Murine Neuroblastoma Cells

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**Abstract:** Synthesis of methionine$^5$-enkephalin by intact cells of murine neuroblastoma clone N1E-115 has been demonstrated both immunocytochemically and biochemically. In addition, N1E-115 cells possess homogeneous enkephalin (δ) receptors which inhibit prostaglandin $E_1$-induced intracellular cyclic AMP formation. An assay was developed for measuring de novo synthesis of methionine$^5$-enkephalin by pulsing cells in culture with radioactive methionine and isolating this pentapeptide to radiochemical purity by a procedure that included immunoaffinity chromatography specific for oxidized methionine$^5$-enkephalin. This assay indicated that production of radiolabeled-methionine$^5$-enkephalin was increased upon lengthy exposure of intact N1E-115 cells in the late logarithmic phase of growth to a nonproteolyzable analog of methionine$^5$-enkephalin. This increase in synthesis of intracellular methionine$^5$-enkephalin relative to control cells was prevented by prior incubation of the clone with naloxone, indicating that the response was mediated by the δ receptor. **Key Words:** δ Receptors—Methionine$^5$-enkephalin—Neuroblastoma—Opioid. **Gilbert J. A. and Richelson E.** Influence of δ-opioid receptors on production of labeled methionine$^5$-enkephalin in murine neuroblastoma cells. *J. Neurochem.* **44**, 922–928 (1985).

The synthesis and regulation of the endogenous opioid methionine$^5$-enkephalin (Met$^5$-enkephalin, Tyr-Gly-Gly-Phe-Met) have been subjects of interest since the discovery of this peptide and leucine$^5$-enkephalin (Leu$^5$-enkephalin) by Hughes et al. (1975). Although the precursory origin of both of these opioids is now well accepted (for review, see Rossier, 1982), much has yet to be learned about regulation of the synthesis and metabolism of Met$^5$-enkephalin and Leu$^5$-enkephalin.

*In vitro* synthesis of Met$^5$-enkephalin has been demonstrated by pulse-labeling experiments with radioactive amino acids in chromaffin cells from bovine adrenal medulla (Rossier et al., 1980; Tan and Yu, 1980; Wilson et al., 1980), myenteric plexus-longitudinal muscle preparation from guinea pig (Sosa et al., 1977), slices of the corpus striatum from guinea pig (McKnight et al., 1979), whole brain slices from guinea pig (Jones and Marchbanks, 1983), human placenta (Tan and Yu, 1981), and cell cultures of murine spinal cord and fetal brain (Neale et al., 1980). Where applied, the synthesis of this pentapeptide by the cells/tissue in question has been further investigated in terms of release, proteolysis, and production under the influence of certain exogenous compounds.

The synthesis of Met$^5$-enkephalin by intact cells of murine neuroblastoma clone N1E-115 has been demonstrated both immunocytochemically (Knodel and Richelson, 1980) and biochemically (Gilbert et al., 1982). To study the regulation of the pentapeptide's production by this adrenergic clone, an assay was developed for marking the synthesized Met$^5$-enkephalin by means of a radioactive precursor and isolating the labeled peptide to radiochemical homogeneity. By use of this technique, it was discovered that the intracellular synthesis of Met$^5$-enkephalin changes following lengthy exposure of the cells to an agonist of the enkephalin (δ) receptors on the cellular surface.

## MATERIALS AND METHODS

### Preparation of antigen

The procedure used for the preparation of antigen was

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*Abbreviations used:* DAMA, 2-D-Alanine-5-L-methionine-enkephalinamide; dBcAMP, $N^6$,$O^{2'}$-Dibutyryl adenosine 3′:5′-cyclic monophosphate; Leu$^5$-enkephalin, Leucine$^5$-enkephalin; Met$^5$-enkephalin, Methionine$^5$-enkephalin; Met$^5$-*O*-enkephalin, Oxidized methionine$^5$-enkephalin; $PGE_1$, Prostaglandin $E_1$.

a modification of that of Clement-Jones et al. (1980). Met$^5$-enkephalin (0.5 mg/0.5 ml of 10 m*M* HCl) was oxidized by the addition of chloramine T (1.5 mg/1.5 ml of 0.05 *M* sodium phosphate, pH 7.4) for 30 s. The reaction was terminated by the addition of 15 μl of 2-mercaptoethanol. After 10-fold dilution with 0.1 *M* HCl, the oxidized Met$^5$-enkephalin was isolated by chromatography on a Sep-Pak $C_{18}$ cartridge (Waters Associates, Milford, MA) which had been prewashed with 5 ml of absolute methanol, 12 ml of water, and 5 ml of 0.10 *M* HCl. Following application, the cartridge was rinsed in a stepwise fashion with 4 ml of 0.01 *M* HCl which was sequentially made 10%, 20%, 25%, 28%, 30%, 40%, and 50% in methanol. Oxidized Met$^5$-enkephalin (Met$^5$-*O*-enkephalin) was eluted by the 20% and 25% methanol rinses, which were then pooled and dried under negative pressure on an Evapo-Mix (Buchler Instruments, Fort Lee, NJ).

The residue was solubilized in 0.25 ml of 0.05 *M* sodium phosphate (pH 7.4) and combined with bovine thyroglobulin (2.5 mg/1.0 ml in 0.05 *M* sodium phosphate, pH 7.4). This solution was made 0.1% in glutaraldehyde (vol/vol) and was incubated for 30 min at room temperature with gentle stirring. This conjugate was dialyzed versus three changes of 200 volumes of water for 24 h at 4°C, and the dialysate was lyophilized. The lyophilizate was resolubilized in 1 *M* $CH_3COOH$ and chromatographed on a Bio-Gel P-2 column, as described, for complete separation of the conjugate from the unbound Met$^5$-*O*-enkephalin. The chromatographed conjugate was lyophilized, and an aliquot was assayed for protein (method of Lowry et al., 1951, with bovine serum albumin as the standard) before use as an antigen.

## Preparation of antibody

The procedure used for the production of antibody was a modification of that of Clement-Jones et al. (1980). Two New Zealand white rabbits were injected intradermally at numerous sites with 1 mg of antigen emulsified in complete Freund's adjuvant (Difco, Detroit, MI) by the double-hubbed needle method described by Herbert (1978). The rabbits were injected for 2 months on a triweekly schedule and thereafter on a monthly or bimonthly basis.

The two-stage assay used for detecting antibody in rabbit serum and for assessing specificity of the antisera was similar to the radioimmunoassay kit for Met$^5$-enkephalin from Immuno Nuclear (Stillwater, MN). Prior to use, however, the $^{125}$I-labeled Met$^5$-enkephalin and all competing unknowns were oxidized with hydrogen peroxide, as described by Clement-Jones et al. (1980).

In the first stage, 0.0–0.2 ml of 0.1 *M* sodium phosphate, 0.1% bovine serum albumin, 0.01% Merthiolate (pH 6.4) buffer; 0.1 ml of a serial dilution of serum; 0.0–0.2 ml of competing peptide; and 0.1 ml of [$^{125}$I]Met$^5$-*O*-enkephalin (≈50 pg/ml) were incubated at 4°C for 16–24 h. Bound [$^{125}$I]Met$^5$-*O*-enkephalin was separated from free label by the addition of 0.1 ml of 5.0 mg/ml bovine serum albumin in assay buffer, and 0.5 ml of saturated ammonium sulfate. The tubes were incubated at 4°C for 15 min, and centrifuged at 2000 × *g* in a Sorvall RC-5B refrigerated centrifuge (DuPont, Newtown, CT) for 10 min. Radioactivity in the pellets was measured in a LKB 1275 mini gamma counter (Turku, Finland).

## Preparation of immunoaffinity column

A crude preparation of the IgG fraction of immunized rabbit serum was made using the procedure of Livingston (1974). The serum was made 50% saturated with ammonium sulfate by the addition of solid ammonium sulfate accompanied by mixing. After incubation on ice for 15 min, the solution was centrifuged at 5000 × *g* for 10 min. The pellet was dissolved in a volume of 10 m*M* potassium phosphate (pH 6.8) equal to the original volume of serum, and the 50% ammonium sulfate precipitation and centrifugation were repeated.

The pellet was then dissolved in a volume of 0.1 *M* sodium bicarbonate (pH 8.0) equal to the original volume of serum, and dialyzed at 4°C versus three changes of 100 volumes of 0.1 *M* sodium bicarbonate (pH 8.0). The dialysate was diluted with the same buffer to a concentration of ~3 mg/ml.

The crude IgG obtained was covalently attached to Sepharose by a modification of the procedure of Wilchek et al. (1971). Prior to activation, Sepharose 4B was washed under gentle vacuum in a scintered glass funnel with a volume of water 40 times that of the packed Sepharose. The washed Sepharose, 10 g wet weight, was suspended in 30 ml of water, and was stirred continuously during the addition of 1 g of solid cyanogen bromide. The pH was quickly raised to 11 by the addition of 5 *M* NaOH and was maintained at pH 10.8–11.2 for 8 min by the slow addition of 3 *M* NaOH. The reaction was terminated by filtration and washing of the activated resin with 40 volumes of cold water. The resin was then added to the IgG solution in a ratio of gel weight/antibody weight of 60:1. This suspension was gently stirred at 4°C for 16 h.

The suspension was then centrifuged for 1 min at 200 × *g*, and the pellet was resuspended in 2 volumes of 0.05 *M* Tris (pH 7.4) and incubated at 4°C for several days to ensure the masking of all activated groups on the resin that were not attached to antibody. A spectrophotometric reading of the supernatant of the incubation solution indicated that ~90% of the crude antibody had been bound to the Sepharose by this procedure.

The affinity resin was poured into 12 individual polypropylene columns with attached one-way stopcocks (Kontes, Vineland, NJ) giving final immunosorbent volumes of 1 ml each. The columns were then equilibrated in 0.1 *M* sodium phosphate (pH 6.4) and stored at 4°C. Chromatography of radioactively labeled, synthetic Met$^5$-*O*-enkephalin on the affinity resin thus prepared by the elution method described as follows, demonstrated a percentage recovery of 70%.

## Cell culture

Murine neuroblastoma cells (clone N1E-115) were cultured in Dulbecco-Vogt's modification of Eagle's medium (DMEM, Grand Island Biological Company, Grand Island, NY) without antibiotics and supplemented with 10% (vol/vol) newborn calf serum (Grand Island Biological Company). Cells (passage number <22) were grown in 20 ml of medium in 75 cm$^2$ Corning flasks (Corning Glass Works, Corning, NY) in a humidified atmosphere of 10% $CO_2$/90% air at 37°C. Cells were routinely inoculated at a density of 0.5–1.0 × 10$^6$/flask and were fed daily beginning the fifth or sixth day after subculture by removal of 10 ml of growth medium and replacement with 10 ml of fresh medium.

Cells were harvested by aspiration of culture medium, incubation of the cellular monolayer for 10 min at 37°C in 10 ml of modified Puck's $D_1$ solution without antibiotics and without phenol red (Honegger and Richelson, 1976), disruption of the layer by agitation of the flask, and collection of the cells by centrifugation at 300 × *g* for 1–2 min at 4°C in a CRU-5000 centrifuge (Damon/IEC Division, Needham Heights, MA). A small aliquot of the cellular suspension in the $D_1$ solution was routinely removed prior to centrifugation and used for enumerating cells (Coulter Electronics, Hialeah, FL).

### Labeling of N1E-115 cells with radioactive methionine

Each assay included triplicate flasks for each control and experimental point, each of the three flasks in a set being treated identically. Growth medium was aspirated from a flask of cells close to confluence and was replaced with 10 ml of fresh DMEM supplemented with 10% newborn calf serum. To this medium was added the appropriate concentration of the compound under study (or an equal volume of buffer to a control flask) and the flask was incubated at 37°C in a humidified atmosphere of 10% $CO_2$/90% air for varying lengths of time, depending on the experiment. To the flask was then added 10 μCi of either [$^3$H]- or [$^{35}$S]methionine (with specific activities of 74 Ci/mmol and 1055–1237 Ci/mmol, respectively), and the flask incubated for 2 h. After extraction of each flask by the described procedure, an average was made of the values obtained for each set of three flasks.

### Met$^5$-enkephalin extraction from N1E-115 cells

The isolation procedure employed was a modification of that published for isolation of Met$^5$-enkephalin (Gilbert et al., 1982). Cells from an individual flask were harvested after labeling, heated at 95°C for 15 min in 5 ml of 0.1 *M* HCl, cooled on ice, and sonicated (Kontes) for 30 s to ensure thorough disruption. All of the following steps were done at 4°C unless otherwise indicated. After centrifugation at 16,300 × *g* for 20 min in a Sorvall RC-5B, the volume of the supernatant was measured and made 10% in trichloroacetic acid by the slow addition of the appropriate amount of 50% trichloroacetic acid (wt/vol, in water). After incubation for 20 min, the solution was centrifuged at 44,500 × *g* for 20 min, and the supernatant was applied at room temperature and a flow rate of 1 ml/min to a Sep-Pak $C_{18}$ cartridge which had been prewashed with 5 ml of absolute methanol followed by 12 ml of water. The column was then washed with 4 ml of 0.1 *M* HCl and 4 ml of water and the eluates discarded. Met$^5$-enkephalin-like material was eluted with 4 ml of absolute methanol which was subsequently removed by evaporation under a gentle stream of nitrogen at 50°C.

The residue, representing a crude cellular extract, was oxidized by the procedure of Clement-Jones et al. (1980) employing a 10-fold higher concentration of hydrogen peroxide. After removal of solvent by evaporation under a gentle stream of nitrogen at 50°C, the residue was dissolved in 0.5 ml of 0.1 *M* sodium phosphate (pH 6.4) and applied to an immunoaffinity column of rabbit antibody to Met$^5$-*O*-enkephalin attached to Sepharose 4B. The column was then rinsed with 3 ml of 0.1 *M* sodium phosphate (pH 6.4) with a flow rate of ~1 ml/10–20 min, and the eluate was discarded. The bound, radioactively labeled Met$^5$-*O*-enkephalin was removed by application to the column of 9 ml of 3 *M* sodium thiocyanate in 0.1 *M* sodium phosphate (pH 6.4) with a flow rate of ~1 ml/10–15 min. The affinity column was rinsed with 8 ml of 0.1 *M* sodium phosphate (pH 6.4) after use.

Sodium thiocyanate was removed from the sample by chromatography on a Sep-Pak $C_{18}$ cartridge as described earlier. The residue remaining after evaporation of the methanol rinse was solubilized in 0.5 ml of 1 *M* $CH_3COOH$, and the sample was transferred in duplicate halves to plastic scintillation vials (Research Products International, Mount Prospect, IL) and mixed with 7 ml Safety-Solve counting cocktail (Research Products International), and the radioactivity was measured in an Isocap/300 liquid scintillation counter (Searle, Des Plaines, IL). The results of an extraction were expressed as average dpm/$10^6$ cells upon correcting the cpm obtained for the efficiency of the Searle for measuring $^3$H or $^{35}$S in unknowns of this nature (~40% and ~80%, respectively) using the external standard ratio method.

### High pressure liquid chromatography

HPLC was performed with a system composed of a model 100A solvent metering system (Beckman, Berkeley, CA), a model 210 sample injection valve (Beckman), a model 160 absorbance detector with a fixed wavelength of 254 nm (Beckman), a μ-Bondapak $C_{18}$ column (0.39 × 30 cm; Cat. No. 27324; Waters) with a precolumn of Bondapak $C_{18}$/Corasil (Waters), and a Fisher Recordall Series 5000 Recorder (Pittsburgh, PA). The isocratic system used (55% methanol/0.057% acetic acid, pH 4.00) was a modification of the system of Humbert et al. (1979). The flow rate of the buffer was 1 ml/min and 1-min fractions were collected on a LKB 7000 Ultrorac (Bromma, Sweden). Part of the fractions from each run were transferred to scintillation vials, mixed with 7 ml of Safety-Solve counting cocktail, and the radioactivity measured.

### Bio-Gel P-2 column chromatography

Samples to be chromatographed (≤2 mg) were dissolved in 0.5 ml of 1 *M* acetic acid (pH 2.3) made 5% in glycerol and fractionated on a Bio-Gel P-2 column (1.1 × 60 cm, Bio-Rad, 100–200 mesh) in the same buffer with 10-min fractions (1.2 ml) collected on a LKB 7000 Ultrorac. Absorbance readings at 280 nm were made on fractions using a Spectronic 710 spectrophotometer with a micro flow-thru system (Bausch and Lomb, Rochester, NY). Part of the fractions obtained were measured for radioactivity as previously described.

### Materials

Plasticware or silanized glassware were used for all experiments. L-[$^{35}$S]-Methionine and [$^{125}$I]Met$^5$-enkephalin were from New England Nuclear (Boston, MA). L-[$^3$H]-Methionine was purchased from Amersham (Arlington Heights, IL). [$^3$H]Met$^5$-enkephalin from Amersham was repurified before use by TLC on silica gel plates (MCB, Cincinnati, OH) using the solvent system *n*-butyl alcohol/acetic acid/water (4:1:1) as suggested by New England Nuclear. Met$^5$-enkephalin, 2-D-alanine-5-L-methionine-enkephalinamide (DAMA), and carboxypeptidase B were obtained from Boehringer-Mannheim (Indianapolis, IN). Prostaglandin $E_1$ ($PGE_1$), Sigmacote, hydrogen peroxide, 2-mercaptoethanol, bovine serum albumin, chloramine T, thyroglobulin, puromycin, and cycloheximide were sup-

plied by Sigma (St. Louis, MO). Trypsin-TPCK was purchased from Worthington (Freehold, NJ). Methanol was obtained from Burdick and Jackson Labs (Muskegon, MI). Cyanogen bromide was supplied by Eastman Kodak (Rochester, NY). Sepharose 4B was from Pharmacia (Piscataway, NJ). All other reagents were analytical grade.

## RESULTS

### Titer and specificity of antiserum

As detected by the assay described, both of the immunized rabbits developed antibodies to the Met[5]-*O*-enkephalin hapten with the maximum titer achieved being 1:1000. The antiserum employed for the immunoaffinity columns had a titer of 1:250. When analyzed for their ability to compete with [$^{125}$I]Met[5]-*O*-enkephalin for this rabbit antiserum, Met[5]-*O*-enkephalin and oxidized Gly-Gly-Phe-Met were comparable in increasing concentrations from 100 pg to 50 ng/tube, while Leu[5]-enkephalin subjected to the hydrogen peroxide treatment demonstrated no consistently increasing displacement in the same range.

### Pulse-labeling of N1E-115 cells

A time course of recovery of newly synthesized Met[5]-*O*-enkephalin indicated that while some radioactive peptide could be isolated from the N1E-115 cells after 1 h of incubation with labeled precursor, the minimum pulse time required for recovery of a consistent and significant level of radioactive product was 2 h (Fig. 1). Under the conditions of labeling employed, the incorporation of radioactivity into total cellular protein was linear both with respect to pulse time and micro-Curies of labeled amino acid employed/flask.

### Identification of isolate

The isolate from the labeling and purification procedure, which included an immunoaffinity chromatography step specific for Met[5]-*O*-enkephalin, was analyzed by molecular sieve chromatography on a Bio-Gel P-2 column (Fig. 2a). The $^{35}$S peak from this column was rechromatographed by reverse-phase chromatography on an HPLC (Fig. 2b). In both cases, the isolate containing the [$^{35}$S]methionine label eluted in a position identical to that of synthetic, Met[5]-*O*-enkephalin.

As verification of the proteinaceous nature of the isolated product, compounds known to inhibit protein synthesis at the RNA level reduced the formation of labeled Met[5]-*O*-enkephalin. The amount of isolate obtained was reduced by preincubation of cells with cycloheximide or puromycin prior to incubation with radioactive methionine (see Table 1).

Furthermore, as would be expected of the pentapeptide Met[5]-enkephalin, treatment of the labeled isolate with carboxypeptidase B and trypsin had no effect. The elution position from HPLC of the peptide prior and subsequent to protease treatment, was identical to that of synthetic, Met[5]-*O*-enkephalin.

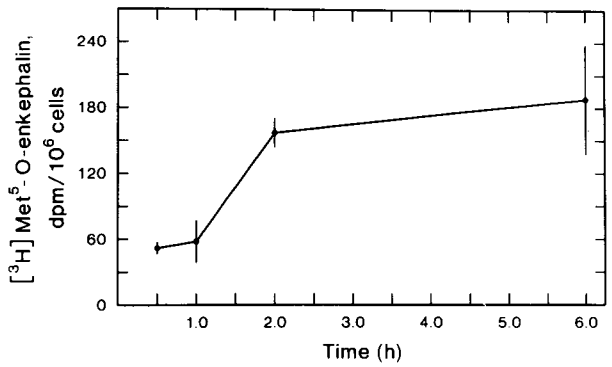


**FIG. 1.** The effect of length of pulsing with radiolabeled methionine on the intracellular production of labeled Met[5]-*O*-enkephalin in N1E-115 cells. Individual flasks of control cells in the late logarithmic phase of growth (passage number 10; 3 days after subculture; 0.98–1.91 × $10^6$ cells/flask) were pulsed with [$^3$H]methionine in 10 ml of fresh DMEM supplemented with 10% newborn calf serum for the indicated lengths of time. The Met[5]-*O*-enkephalin was then extracted and the radioactivity was measured as described under Materials and Methods. Each point was the average of triplicates, with the bar indicating the SEM. Significance difference from the 30 min value using *t* statistic for two means was p = 0.4, 0.001, and 0.025, respectively, for the 60 min, 2 h, and 6 h values. These data were from one of two similar experiments.

### Labeled-Met[5]-*O*-enkephalin isolation procedure

For each experiment, triplicates of each time/concentration point in an experiment were done, each of the triplicates employing a separate flask of cells. By adding a quantity of synthetic [$^3$H]Met[5]-enkephalin within a range of 8–1600 pg to broken cells prior to sonication and measuring the amount recovered from the total extraction procedure, the routine recovery of isolated Met[5]-*O*-enkephalin was ascertained to be 10%.

### Production of labeled Met[5]-*O*-enkephalin by N1E-115 cells

Supporting the earlier demonstration that the quantity of intracellular Met[5]-enkephalin is regulated in clone N1E-115 with respect to cellular division (Gilbert et al., 1982), the isolation of significant levels of labeled Met[5]-*O*-enkephalin with this assay was dependent upon the use of cells that were close to confluence. Control cells that had been at maximal population for more than 7 days produced 26 ± 1 dpm/$10^6$ cells (mean ± SE, n = 3) of isolated [$^3$H]- or [$^{35}$S]Met[5]-*O*-enkephalin after a preincubation of 1–2 h and were unresponsive to treatments which, in late logarithmic cells, proved to modify the quantities of labeled Met[5]-*O*-enkephalin subsequently produced. Control cells in late logarithmic phase produced an average of 159 ± 55 dpm/$10^6$ cells after 0.08–1.0 h of incubation prior to labeling (n = 22), 115 ± 31 dpm/$10^6$ cells follow-

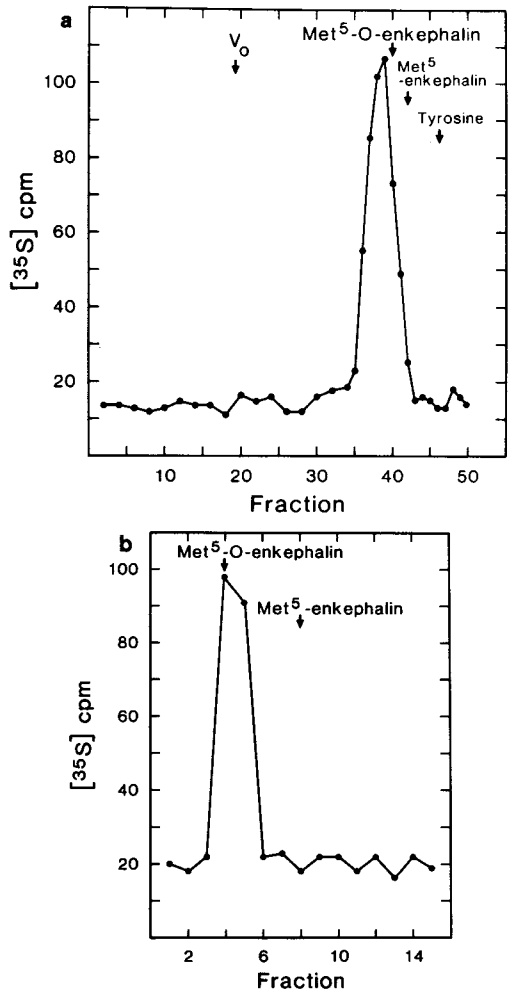


**FIG. 2.** Elution profiles from Bio-Gel P-2 and HPLC of radioactive isolate from clone N1E-115. **a:** Elution profile from Bio-Gel P-2 chromatography of radioactive isolate from clone N1E-115. The purified isolate fractionated was labeled and extracted as described under Materials and Methods from five flasks of cells in the late logarithmic phase of growth (passage number 15, 7 days after subculture). After resolubilization of ~5% of the product from the second $C_{18}$ chromatography in 0.5 ml of 1 *M* acetic acid, the isolate was chromatographed on a Bio-Gel P-2 column (1.1 × 60 cm) in 1 *M* acetic acid (pH 2.3) with 10-min fractions (1.2 ml) collected. One-twelfth of the indicated fractions was measured for radioactivity. The data shown were from one of three similar experiments. The elution position indicated for Met$^5$-*O*-enkephalin was assessed by chromatography of synthetic peptide (0.1–0.5 mg in 0.5 ml) which had been oxidized by the procedure described, and represents the average of two experiments. **b:** Elution profile of Met$^5$-*O*-enkephalin-like material from Bio-Gel P-2 column rechromatographed by HPLC. The remainder of the peak tubes of radioactivity from the Bio-Gel P-2 chromatography described in (a) were combined, lyophilized, and subjected to HPLC as described under Materials and Methods. One-minute (1 ml) fractions were collected, and one-tenth of each fraction was measured for radioactivity. The data shown were from one of three similar experiments.

**TABLE 1.** *Conditions affecting synthesis of intracellular Met$^5$-O-enkephalin*

| Prelabeling condition | dpm/flask | dpm/$10^6$ cells | Percent control |
|---|---|---|---|
| Control, for 30 min | 244 ± 21 | 101 ± 7 | 100 |
| $10^{-5}$ *M* Cycloheximide, for 30 min | 221 ± 53 | 68 ± 9[a] | 67 |
| Control, for 30 min | 804 ± 264 | 293 ± 87 | 100 |
| $10^{-4}$ *M* Puromycin, for 30 min | 288 ± 31 | 86 ± 27[a] | 29 |
| Control, for 4 h 15 min | 632 ± 114 | 204 ± 69 | 100 |
| Control, for 15 min + $10^{-7}$ *M* DAMA, for 4 h | 1180 ± 110 | 353 ± 18[a] | 173 |
| $5 \times 10^{-5}$ *M* Naloxone, for 15 min + $10^{-7}$ *M* DAMA, for 4 h | 870 ± 187 | 230 ± 62[b] | 113 |

Values are the average of triplicates ± SE; number of experiments = 2; 2.21–5.68 × $10^6$ cells/flask in late logarithmic phase of growth.

[a] Significant difference, $p < 0.05$ from matching control using one-tailed *t* test for two means.

[b] Significant difference, $p > 0.05$ from matching control using one-tailed *t* test for two means.

ing 1.25–2.0 h of preincubation (n = 8, p = 0.4), and 113 ± 48 dpm/$10^6$ cells after 4–6 h (n = 3, p = 0.4).

### Influence on production of intracellular Met$^5$-*O*-enkephalin by δ receptors

The production of radioactive Met$^5$-*O*-enkephalin was changed in intact N1E-115 cells after a lengthy exposure of the clone to a nonproteolyzable analog of Met$^5$-enkephalin. Cells exposed to $10^{-7}$ *M* DAMA for less than 4 h demonstrated no significant alteration in the amount of labeled Met$^5$-*O*-enkephalin synthesized. However, cells incubated with the analog for a greater length of time exhibited an increase in the quantity of radioactive Met$^5$-*O*-enkephalin produced (see Table 2). As seen in Table 1, the effect of stimulating N1E-115 cells with DAMA for 4 h was prevented by preincubating for 15 min with $5 \times 10^{-5}$ *M* naloxone.

## DISCUSSION

The adrenergic neuroblastoma clone N1E-115, possessing both homogeneous δ receptors and the ability to synthesize Met$^5$-enkephalin, was employed to ascertain the relationship between δ-receptor function and the intracellular production of this pentapeptide. The assay described here was developed to measure de novo synthesis of Met$^5$-enkephalin in cultured cells by isolating labeled Met$^5$-*O*-enkephalin subsequent to pulsing with radiolabeled methionine. It was discovered that incubation

TABLE 2. *Effect of length of incubation with 0.1 μM DAMA on intracellular production of Met$^5$-O-enkephalin*

| Prelabeling condition | dpm/flask | dpm/$10^6$ cells | Percent control |
|---|---|---|---|
| Control, for 15 min | 270 ± 29 | 86 ± 13 | 100 |
| 0.1 μ*M* DAMA, for 15 min | 254 ± 11 | 91 ± 17[a] | 106 |
| 0.1 μ*M* DAMA, for 30 min | 279 ± 31 | 69 ± 17[a] | 80 |
| 0.1 μ*M* DAMA, for 1 h | 169 ± 15 | 58 ± 10[a] | 67 |
| Control, for 15 min | 114 ± 20 | 28 ± 10 | 100 |
| 0.1 μ*M* DAMA, for 2 h | 263 ± 71 | 74 ± 24[a] | 264 |
| 0.1 μ*M* DAMA, for 4 h | 365 ± 55 | 65 ± 8[b] | 232 |
| 0.1 μ*M* DAMA, for 5.5 h | 293 ± 46 | 73 ± 14[b] | 261 |

Values are the average of triplicates ±SE; number of experiments = 2; 1.82–7.29 × $10^6$ cells/flask in late logarithmic phase of growth; during extraction, samples were serially fractionated on Sep-Pak $C_{18}$, affinity, and second Sep-Pak $C_{18}$ columns in groups of 12, which included three sets of experimental triplicates and one set of controls.

[a] Significant difference, $p > 0.05$ from matching control using one-tailed *t* test for two means.

[b] Significant difference, $p < 0.05$ from matching control using one-tailed *t* test for two means.

of the clone in the late logarithmic phase of growth with DAMA for 4 h or longer resulted in an increase in the formation of intracellular Met$^5$-enkephalin to levels greater than those of unexposed cells. This change in peptide production was prevented by prior incubation with naloxone, indicating that this effect was mediated by the δ receptor.

A physiological correlate for the naloxone-reversible increase in synthesis of Met$^5$-enkephalin following chronic exposure of the clone to this analog is not readily discernible. One possibility may be the role Met$^5$-enkephalin is believed to play as an endogenous analgesic that is released under stressful or painful conditions (for review, see Hughes, 1983). In situations calling for the use of such an endogenous opiate, a mechanism ensuring increased production even in the presence of greater than normal quantities would appear to be advantageous for replenishment of opioid as it is used and/or proteolytically degraded.

A mechanism by which enhanced Met$^5$-enkephalin production is effected by the δ receptor might be the interaction of the δ receptor with adenylate cyclase. As is well established, the δ receptor exercises dual regulation of adenylate cyclase in that acute exposure to agonist results in inhibition of receptor-stimulated production of cyclic AMP, whereas chronic incubation produces a state of tolerance and dependence (for review, see Gilbert and Richelson, 1983). At this point the receptor-stimulated adenylate cyclase activity has adjusted to normal levels in the continuing presence of the δ receptor agonist. Specifically, Met$^5$-enkephalin is reported to produce tolerance in 108CC15 (NG108-15) neuroblastoma × glioma hybrid cells within 1–3 h at a concentration of 1 μ*M* (Brandt et al., 1976) and 12 or more h at 10 μ*M* (Lampert et al., 1976). In the latter study, an increase in basal activity of adenylate cyclase was demonstrated upon development of tolerance to the peptide in addition to enhancement of $PGE_1$-stimulated adenylate cyclase activity.

The production of Met$^5$-enkephalin is controlled in N1E-115 cells with respect to cellular division (Gilbert et al., 1982). The same is true of the synthesis of opioid peptides in NG108-15 neuroblastoma × glioma hybrids (Glaser et al., 1982). Recently, Braas et al. (1983) have demonstrated that incubation of NG108-15 cells in the logarithmic phase of growth for 1–5 days with the nonhydrolyzable analogs of cyclic AMP, 8-bromo-adenosine 3′,5′-cyclic monophosphate and $N^6$,$O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate (dBcAMP), results in morphological differentiation of the cells as well as increasing production of Leu$^5$- and Met$^5$-enkephalin, as detected by immunocytochemical staining. Furthermore, similar results were found when the NG108-15 clone was stimulated to produce intracellular cyclic AMP by incubation with $PGE_1$ and 3-isobutyl-1-methylxanthine for the same length of time. Treatment of a number of neuronal clones with dBcAMP, including N1E-115 cells, gave comparable results to those in NG108-15 hybrids. Thus, increased levels of cyclic AMP can play a direct role in the regulation of Met$^5$-enkephalin synthesis in cultured cells in the logarithmic phase of growth.

Therefore, it seems reasonable to speculate that the increased formation of labeled Met$^5$-*O*-enkephalin seen in these N1E-115 cells following a 4–6 h incubation with DAMA was due to the chronic exposure of δ receptor to agonist resulting in enhanced production of intracellular cyclic AMP (i.e., tolerance) which then directly stimulated the synthesis of Met$^5$-enkephalin. Preliminary experiments to test this hypothesis have demonstrated that incubation of N1E-115 cells with $PGE_1$ for periods of 2–6 h has resulted in a statistically significant increase in production of radiolabeled Met$^5$-*O*-enkephalin relative to control cells.

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