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Function and Regulation of Methionine^5-Enkephalin and Its Receptors in Murine Neuroblastoma Cells*

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A homogeneous class of enkephalin receptors found in murine neuroblastoma clone N1E-115 (Chang, K.-J., and Cuatrecasas, P. (1979) *J. Biol. Chem.* 254, 2610–2618) has been confirmed using a centrifugation assay employing cellular membranes. In intact N1E-115 cells, synthetic methionine^5-enkephalin inhibited prostaglandin E_1-induced intracellular cyclic AMP formation in a naloxone-sensitive manner. Upon demonstrating intracellular methionine^5-enkephalin immunocytochemically (Knodel, E., and Richelson, E. (1980) *Brain Res.* 197, 565–570), analyses of crude N1E-115 extract were made by radioimmunoassay or opiate receptor binding assay following fractionation by molecular sieve chromatography and high pressure liquid chromatography on a μ-Bondapak C_{18} column. Extracted methionine^5-enkephalin immunoreactive material behaved similarly to synthetic methionine^5-enkephalin in these analyses. Growth curve studies of the N1E-115 cells indicated that the quantity of methionine^5-enkephalin immunoreactive material synthesized per milligram of cellular protein and the maximum number of enkephalin receptor sites per milligram of membrane protein increased as the cells progressed from logarithmic to stationary phase, with no change in the apparent affinity of the enkephalin receptors for [^{3}H]methionine^5-enkephalin. These data suggest that adrenergic clone N1E-115 has functional methionine^5-enkephalin membrane receptors, that this clone synthesizes methionine^5-enkephalin, and that both the enkephalin receptor number and the content of stored methionine^5-enkephalin are regulated with respect to cell division.

After the demonstration of specific opiate binding sites in the brain (see review of Snyder and Childers, 1979), the enkephalin peptides were isolated from porcine brain, sequenced, and identified as the endogenous compounds which acted as agonists for the morphine receptor (Hughes *et al.*, 1975). Subsequently, a number of subclasses of opiate receptors in brain have been identified (see reviews of Chang *et al.*, 1980, and Hughes *et al.*, 1980), and a single class of enkephalin receptors has been found in cultured neuroblastoma clones (Chang and Cuatrecasas, 1979). The sequence of the opioid peptide methionine^5-enkephalin, Tyr-Gly-Gly-Phe-Met, while contained within the sequence of β-endorphin, has a sufficiently different distribution from the larger peptide throughout the nervous system to suggest that Met^5-enkephalin might be synthesized by other means than the breakdown of β-endorphin, which is synthesized primarily in the pituitary (see reviews of Adler, 1980 and Hughes *et al.*, 1980). A number of larger molecular weight precursors containing the sequence and the binding activity of Met^5-enkephalin have been found in the adrenal gland (see reviews of Lewis *et al.*, 1980, and Yang *et al.*, 1980), striatum (Lewis *et al.*, 1978), and hypothalamus (Huang *et al.*, 1979).

For a number of years, this laboratory has been investigating the function and control of the receptors present in the adrenergic clone, N1E-115, of murine neuroblastoma (Richelson, 1979), including histamine H_1 receptors which stimulate intracellular formation of cyclic GMP upon binding of agonist; muscarinic acetylcholine receptors which induce intracellular formation of cyclic GMP and inhibit intracellular formation of cyclic AMP upon activation; and prostaglandin E_1 receptors which stimulate formation of cyclic AMP in intact cells (Richelson, 1980) upon agonist binding. We confirm here the existence of enkephalin membrane receptors on N1E-115 cells and report the ability of Met^5-enkephalin to inhibit prostaglandin E_1 stimulation of intracellular cyclic AMP formation by this neuroblastoma clone. Evidence is presented that the N1E-115 clone synthesizes the opioid peptide Met^5-enkephalin, and that the amount of Met^5-enkephalin stored per milligram of cellular protein and the maximum number of [^{3}H]Met^5-enkephalin binding sites per milligram of membrane protein increase as the cells progress from logarithmic to stationary phase of cellular growth.

EXPERIMENTAL PROCEDURES

Cell Culture—Murine neuroblastoma cells (clone N1E-115) were cultured in Dulbecco-Vogt's modification of Eagle's medium (Grand Island Biological Company, Grand Island, NY) without antibiotics and supplemented with 10% (v/v) newborn calf serum (Grand Island Biological Company). Cells (passage number < 22) were grown in 20 ml of medium in 75-cm^2 Falcon flasks (250 ml; Becton, Dickinson and Company, Oxnard, CA) in a humidified atmosphere of 10% CO_2:90% air at 37 °C. Cells were routinely inoculated at a density of 1.0–1.5 × 10^6/flask and were fed daily beginning the 5th or 6th day after subculture by removal of 10 ml of growth medium and replacement with 10 ml of fresh medium. Cultures were free of mycoplasma by bacteriologic criteria.

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 (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). Cells to be used for Met^5-enkephalin extraction and for growth cycle studies were harvested with the D_1 solution containing no phenol red since this compound bound to Sep-Pak C_{18} cartridges. Small aliquots of the cellular suspension in the D_1 solution were routinely removed prior to

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[1] The abbreviations used are: Met^5-enkephalin, methionine^5-enkephalin; HPLC, high pressure liquid chromatography; Leu^5-enkephalin, leucine^5-enkephalin; PGE_1, prostaglandin E_1; [^{3}H]2-D-Ala-5-Met-enkephalinamide, [^{3}H]2-D-alanine-5-L-methionine-enkephalinamide.

centrifugation and used for enumerating cells (Coulter Electronics, Hialeah, FL) and assaying protein (method of Lowry *et al.*, 1951, with bovine serum albumin as the standard).

Met^5*-Enkephalin Receptor Binding Assay*—The cellular pellet was resuspended in 2–4 ml of 50 mM Tris, pH 7.4 at 0 °C, and homogenized with a Polytron PT45 homogenizer (Brinkmann) at setting 8 for 15 s. After dilution of the homogenate to 10–20 ml with the Tris buffer, a crude particulate fraction was prepared by centrifugation at 40,000 × *g* for 30 min in a Sorvall RC-5B refrigerated centrifuge (Du Pont, Newtown, CT). The pellet obtained was homogenized in Tris buffer and centrifuged as previously described. Tris buffer was added to the final pellet followed by homogenization to yield a 10% (w/v) suspension.

Opiate receptor binding was measured in a total volume of 1 ml of solution containing final concentrations of 50 mM Tris, pH 7.4 at 0 °C; 10 μM puromycin; and 0.5–10 nM [^{3}H]Met^5-enkephalin. Each reaction was run in duplicate in a polypropylene Microfuge tube of 1.5-ml capacity and was started by the addition of 100 μl of the 10% (w/v) homogenate. Nonspecific binding was determined by including 1 μM unlabeled Met^5-enkephalin. The incubation was done at 0 °C in the presence of puromycin to minimize enkephalin degradation by peptidases (Hazum *et al.*, 1979). The binding reaction was terminated routinely after 2 h by centrifugation at 8700 × *g* for 2.5 min in a precooled Beckman Microfuge B (Palo Alto, CA). After aspiration of the supernatant, the surface of each pellet was washed twice with 1 ml of Tris buffer, and the tip of each Microfuge tube was cut and placed in a scintillation vial. The pellet was incubated for at least 4 h in 7 ml of 3a70B complete counting cocktail (Research Products, Elk Grove Village, IL) for solubilization, followed by liquid scintillation counting in a Searle Isocap/300 counter (Des Plaines, IL).

Measurement of Relative Change in Cyclic AMP Production—Relative changes in prostaglandin E_1-stimulated cyclic AMP formation by intact murine neuroblastoma cells were measured by a method that involved prelabeling the intracellular stores of ATP in intact cells with [^{3}H]adenine and chromatographically isolating cyclic [^{3}H]AMP. Cells, 10–21 days after subculturing, were harvested as previously described and washed with a phosphate-buffered saline solution containing 110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 2.0 mM Na_2HPO_4, and 25 mM glucose (pH was adjusted to 7.35 with HCl, and osmolality was adjusted to 335–340 mosm with sucrose). Cells were resuspended in 2 ml of the phosphate-buffered saline solution, transferred to an Erlenmeyer flask (25 ml), and incubated with 20 μCi of [^{3}H]adenine (1 mCi/ml, 20 Ci/mmol) for 45 min at 37 °C in a shaker bath (GCA/Precision Scientific, Chicago, IL) at 40 rpm. The radioactive cell suspension was diluted with the phosphate-buffered saline solution to give ~250,000 cells/300-μl aliquot which was added to each well of a Linbro tissue culture multi-well plate (Cat. No. 76-033-05; Flow Laboratories, Hamden, CT). The cells were then incubated for 30 min at 37 °C with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (final concentration, 0.1 mM). When naloxone (final concentration, 50 μM) was used, cells were incubated an additional 15 min in the phosphate-buffered saline solution with or without (control) the antagonist. Next, cells were incubated with or without Met^5-enkephalin (final concentration as indicated in the figures) for 5 min prior to the incubation of cells with prostaglandin E_1 (concentration as indicated in the figures) for 10 min. The final assay volume after all additions was 500 μl. The reaction was stopped with 40 μl of 50% trichloroacetic acid and to each well was added 0.9 nCi of cyclic [^{14}C]AMP as an internal standard and 230 μl of 1.0 M Tris, pH 8.5 at 22 °C, to neutralize the trichloroacetic acid. The contents of each well were transferred with a Pasteur pipet to a chromaflex disposable column (Kontes, Vineland, NJ) containing Dowex 50-H^+ (0.8 × 4.0 cm; AG50W-X8, 100–200 mesh, H^+ form; Bio-Rad) equilibrated with 1 mM phosphate buffer, pH 7.0. Each well was rinsed with 0.5 ml of 5% trichloroacetic acid and 200 μl of 1.0 M Tris, which was also applied to the column. Each Dowex column was washed with 5.0 ml of 1 mM phosphate buffer, pH 7.0 (eluate discarded), and 6.0 ml of water, the eluate from which was allowed to drain directly onto a column of alumina (1 g; 0.8-cm diameter column; neutral chromatographic alumina, activity grade I, type WN-3; Sigma) equilibrated with 0.1 M imidazole-50 mM HCl, pH 7.0, buffer (imidazole-HCl buffer). The eluate from the alumina column was discarded, and the column was washed with 1 ml of imidazole-HCl buffer (eluate discarded) and then with 3.0 ml of imidazole-HCl buffer. Three ml of eluate was collected and was transferred to a scintillation vial, to which was added 7.0 ml of 3a70B complete counting cocktail, and the radioactivity was measured. All samples were corrected for the recovery of cyclic [^{14}C]AMP which was usually 60–70%.

Met^5*-Enkephalin Extraction From N1E-115 Cells*—Cells collected from 1–8 flasks were heated at 95 °C for 15 min in 5 ml of 0.1 N HCl, cooled on ice, and sonicated (Kontes, Vineland, NJ) for 30 s to ensure thorough disruption. After centrifugation at 16,300 × *g* for 20 min, a volume of the supernatant corresponding to the extract from 1–3 flasks of cells was applied to a C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA) which had been prewashed with 2–5 ml of absolute methanol and 5–12 ml of water. The column was then washed with 4 ml of 0.1 N 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 negative pressure on an Evapo-Mix (Buchler Instruments, Fort Lee, NJ). The residue, representing a crude cellular extract, was stored at −20 °C until use.

To estimate the recovery of Met^5-enkephalin-like material from the cells by the extractive procedure, 2–4 ng of synthetic Met^5-enkephalin was separately processed in parallel with the cellular samples, and the amount recovered measured by radioimmunoassay.

Met^5*-Enkephalin Radioimmunoassay*—Met^5-enkephalin immunoreactive material in cellular extracts was measured by a radioimmunoassay kit from Immuno Nuclear, Stillwater, MN (Cat. No. 1800). The amount of [^{125}I]Met^5-enkephalin bound to antibody was measured in the reaction precipitates (LKB 1275 mini gamma counter, Turku, Finland), and the quantity of unlabeled Met^5-enkephalin per sample (50–200 μl) was determined from a standard curve plotted on log-logit paper. The antibody in the radioimmunoassay was reported to have a cross-reactivity of 1.6, 0.002, and 0.002% with Leu^5-enkephalin, Substance P, and β-endorphin, respectively. Dried extracts were reconstituted before use in radioimmunoassay buffer for 30 min on ice, and centrifuged, if necessary, at 1000 × *g* for 5 min or 8700 × *g* for 1 min to remove undissolved material. Crude extract from an average of 50 × 10^6 N1E-115 cells (~2.5 confluent flasks; an average of 700 μg of extracted protein) contained 1 ng of Met^5-enkephalin immunoreactive material as measured by the radioimmunoassay.

High Pressure Liquid Chromatography—High pressure liquid chromatography was performed with a system composed of a model 100A solvent metering system (Beckman), 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 pre-column of the same material, and a Fisher Recordall Series 5000 Recorder (Pittsburgh, PA). The isocratic system used (21% *n*-propyl alcohol:0.057% acetic acid, pH 3.52) was a modification of the gradient system of Morris *et al.* (1980). The flow rate of the buffer was 1 ml/min and 1-min fractions were collected on an LKB 7000 Ultrorac (Bromma, Sweden).

For preparation of crude extract to be fractionated by HPLC prior to analysis in the opiate receptor binding assay and the radioimmunoassay, 26 flasks of N1E-115 cells in the stationary phase of growth were extracted as previously described, and the residue was resolubilized in the HPLC buffer and centrifuged at 8700 × *g* for 4.5 min. Aliquots of the supernatant corresponding to the extract from 1.1 flasks (11 μl; 88 μg of protein) were fractionated at 2500–2600 p.s.i. for 30 min. Twenty-one sequential runs were made, and all fractions having the same elution time were pooled. From the first 15 of each of these 30 pools, a volume corresponding to the chromatographed extract from two flasks of cells was retained for both the radioimmunoassay and the protein assay, and a volume corresponding to 9.5 flasks was retained for duplicate binding assays. The solvent from all samples was then removed at 37 °C on the Evapo-Mix or under N_2 and the residues stored at −20 °C until use. Of the remaining supernatant, a volume corresponding to the crude material from one flask of cells was retained for a protein assay, and duplicate volumes, each corresponding to the crude extract from one flask of cells, were chromatographed as above with the inclusion of 2.7 pmol of [^{3}H]Met^5-enkephalin and with separate fraction collection. The individual fractions from each of the duplicate runs were transferred to scintillation vials, mixed with 7 ml of Safety-Solve counting cocktail (Research Products), and the radioactivity measured in a Searle/Isocap 300 liquid scintillation counter.

Bio-Gel P-2 Column Chromatography—After resolubilization and centrifugation at 8700 × *g* for 1 min, crude extract (0.5 ml; 0.8–3 mg of protein) was fractionated on a Bio-Gel P-2 column (1.1 × 60 cm, Bio-Rad, 100–200 mesh) in 1 M acetic acid, pH 2.31, with 10-min

fractions (1.2 ml) collected on an LKB 7000 Ultrorac. Absorbance readings at 280 nm were made on alternate fractions using a Spectronic 710 spectrophotometer with a micro flow-through system (Bausch and Lomb, Rochester, NY). The remaining fractions were lyophilized (model 10-010; Virtis, Gardiner, NY) prior to analysis in the radioimmunoassay.

The void volume of the column was determined by chromatography of blue dextran (0.5 ml; 1 mg; a gift from Dr. Richard Hyslop, Mayo Foundation), and the elution position of Met⁵-enkephalin was assessed by chromatography of synthetic peptide (0.5 ml; 1–2 mg).

For rechromatography by HPLC of the peak of Met⁵-enkephalin immunoreactive material from Bio-Gel P-2 column chromatography, a crude extract was made, as previously described, from 28 flasks of cells. The extract was divided into two parts, and each half then resolubilized and centrifuged at 8700 × *g* for 1 min prior to fractionation (0.5 ml; 1.7–1.8 mg of protein) on the Bio-Gel P-2 column. Absorbance readings at 280 nm were made on alternate fractions. The remaining fractions were divided into two tubes, ⅓ of each fraction in one tube and ⅔ of each fraction in a second tube, and lyophilized. The tubes containing the ⅓ fractions were each resolubilized and assayed in the radioimmunoassay. Based on these analyses, two to five tubes (containing the ⅔ fractions) were chosen for each column from the peak of Met⁵-enkephalin immunoreactive material which had eluted at the position of synthetic Met⁵-enkephalin. The sample from each column was separately resolubilized in 22 μl of HPLC buffer and fractionated by the HPLC system described, in two injections (10 μl; ≤25 μg of protein) with pooling of fractions having the same elution times. The solvent from each set of fractions was then removed at 37 °C under N_2, and the residues were stored at 4 °C until analysis in the radioimmunoassay.

Growth Curve Experiments—Cells were kept in the logarithmic phase of growth for 3–7 weeks prior to the study by frequent subculturing (Richelson, 1973). On Day 0, 2 × 10^5 cells from a large pool were seeded into a number of flasks and cultured as previously described. On alternating days thereafter, flasks of cells were harvested for use in an extraction of Met⁵-enkephalin-like material or in a [³H]Met⁵-enkephalin binding assay. For Met⁵-enkephalin extraction, sufficient flasks were harvested for duplicate samples with 1.4–2.8 × 10^7 cells/sample. For a [³H]Met⁵-enkephalin binding assay, a total of 8.6–9.7 × 10^7 cells were harvested, and the 10% (w/v) membrane solution was prepared and used the same day.

Materials—Plasticware or silanized glassware were used for all experiments where synthetic Met⁵-enkephalin or Met⁵-enkephalin immunoreactive material was utilized. [³H]Met⁵-enkephalin was obtained from New England Nuclear and Amersham, and repurified before use by thin layer chromatography on cellulose or silica gel plates (Eastman, Rochester, NY) using the solvent system *n*-butyl alcohol:acetic acid:water (4:1:1) as suggested by New England Nuclear. Specific radioactivities ranged from 20–44 Ci/mmol. Due to the unavailability of [³H]Met⁵-enkephalin during the completion of one of the experiments reported here, [³H]2-D-alanine-5-L-methionine-enkephalinamide (specific activity, 32 Ci/mmol) was obtained from New England Nuclear and was repurified before use on silica gel plates in the above solvent system. [³H]Adenine and cyclic [¹⁴C]AMP were from Amersham. Nonradioactive synthetic Met⁵-enkephalin was supplied by Calbiochem and Boehringer-Mannheim. Nonradioactive Leu⁵-enkephalin was from Calbiochem.

Puromycin dihydrochloride, prostaglandin E_1, Sigmacote, and bovine serum albumin were supplied by Sigma. Morphine was obtained from N.I.D.A. (Rockville, MD), and naloxone was from Endo Laboratories, (Garden City, NY). 3-Isobutyl-1-methylxanthine was supplied by Aldrich. Methanol and *n*-propyl alcohol were obtained from Burdick and Jackson Labs (Muskegon, MI). All other reagents were analytical grade.

RESULTS

Met⁵-Enkephalin Binding Assay—Binding of [³H]Met⁵-enkephalin to membranes of murine neuroblastoma clone N1E-115 reached equilibrium after ~2 h at 0 °C (Fig. 1), with specific binding being at best ⅔ of total enkephalin binding (depending upon the lot and source of the radioligand). Binding was linear with respect to membrane protein over the range of 100–800 μg/ml of reaction mixture (data not shown). Sodium ions reduced by ⅓ to ½ the specific binding of [³H]Met⁵-enkephalin to membranes after a 2-h incubation at 0 °C (data not shown). In addition, preincubation of crude membrane fractions for 45 min at either 37 or 0 °C did not affect subsequent binding of [³H]Met⁵-enkephalin at 0 °C for 2 h (data not shown). However, freezing a membrane fraction for 9 days at −20 °C resulted in a 50% reduction of specific [³H]Met⁵-enkephalin binding.

[³H]Met⁵-enkephalin bound to cellular membranes in a saturable fashion (Fig. 2), and Scatchard analyses of these data were fitted with a straight line with average correlation and Hill coefficients of 0.8 and 1.4, respectively (*e.g.* Fig. 2, *inset*). With membranes from cells in the stationary phase of growth, the average equilibrium dissociation constant (K_D) for [³H]Met⁵-enkephalin was 2.4 nM, and the average maximum number of binding sites (B_{max}) was 55 fmol/mg of membrane protein (~10,000 binding sites/cell).

The [³H]Met⁵-enkephalin specific binding to N1E-115 membranes was progressively displaced by increasing concentrations of unlabeled Met⁵-enkephalin, naloxone, and morphine

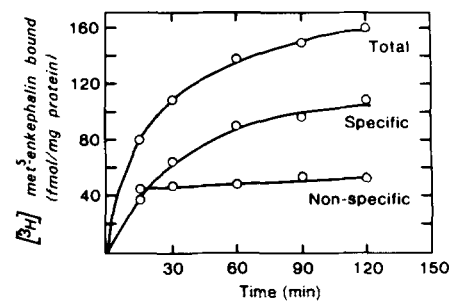

FIG. 1. **[³H]Met⁵-enkephalin binding to clone N1E-115 membranes as a function of time.** [³H]Met⁵-enkephalin was incubated at a final concentration of 3.9 nM with 530 μg of membrane protein from cells in the stationary phase of growth (passage number 12, 12 days after subculture) as described under "Experimental Procedures." Lengths of incubation at 0 °C were varied. Each point represents the average of triplicate samples. Nonspecific binding was measured in the presence of 1 μM unlabeled Met⁵-enkephalin. The data presented are from one of duplicate experiments.

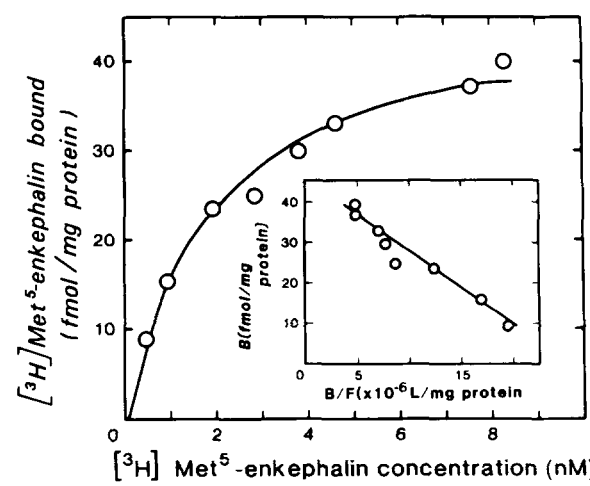

FIG. 2. **[³H]Met⁵-enkephalin binding to clone N1E-115 membranes as a function of radioligand concentration.** The assay was performed with increasing concentrations of [³H]Met⁵-enkephalin as described under "Experimental Procedures" with 600 μg of membrane protein from cells in the stationary phase of growth (passage number 16, 14 days after subculture). Each point, representing the specific binding at a given [³H]Met⁵-enkephalin concentration, was obtained by subtracting the average of duplicates for nonspecific binding at that concentration (measured in the presence of 1 μM unlabeled Met⁵-enkephalin) from the average of duplicates for total binding. The data presented are from one of seven Met⁵-enkephalin receptor binding assays. *Inset*, Scatchard plot of the given specific binding data done by the least squares method of linear regression analysis (correlation coefficient = 0.97).

(Fig. 3). The order of potency of these compounds at competing for [^{3}H]Met^5-enkephalin binding sites was Met^5-enkephalin > naloxone > morphine with calculated equilibrium dissociation constants for the receptor-inhibitor complex of 1.5, 13, and 130 nM, respectively.

Effects of Met^5-Enkephalin on Cyclic Nucleotide Synthesis—Met^5-enkephalin inhibited prostaglandin E_1-mediated cyclic [^{3}H]AMP formation by intact N1E-115 cells in a dose-dependent manner (Fig. 4). This effect of Met^5-enkephalin reached its maximum in the range of 0.05–0.10 μM. However, for reasons not understood, at concentrations of Met^5-enkephalin higher than 0.10 μM, the inhibitory effect of this opioid peptide on PGE_1-stimulated cyclic [^{3}H]AMP formation was less. When the experiment in Fig. 4 was repeated with Leu^5-enkephalin, an identical inhibitory curve was obtained (data not shown). Met^5-enkephalin or Leu^5-enkephalin alone had no effect on intracellular cyclic [^{3}H]AMP formation by these cells (data not shown).

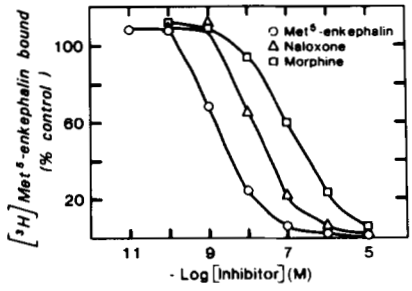

FIG. 3. **Inhibition of [^{3}H]Met^5-enkephalin binding to clone N1E-115 membranes.** [^{3}H]Met^5-enkephalin was incubated at a final concentration of 1.7 nM with 540 μg of membrane protein from cells in the stationary phase of growth (passage number 13, 12 days after subculture) in the presence of increasing concentrations of unlabeled inhibitors. Nonspecific binding was measured in the presence of 10 μM unlabeled Met^5-enkephalin, the duplicate values were averaged, and this value was subtracted from the average of the duplicates of total binding for each concentration of each inhibitor. The specific binding in the tubes containing inhibitors was then compared to that in control tubes lacking inhibitors. The IC_{50} value for each inhibitor was converted to an equilibrium dissociation constant for the receptor-inhibitor complex by the equation of Cheng and Prusoff (1973), using a K_D for [^{3}H]Met^5-enkephalin of 2.4 nM. These data are from one of duplicate experiments.

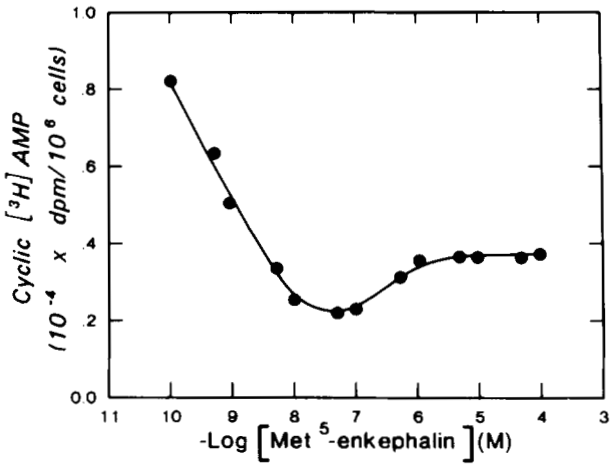

FIG. 4. **The effect of Met^5-enkephalin concentration on the production of prostaglandin E_1-stimulated cyclic [^{3}H]AMP in clone N1E-115.** Intact cells (passage number 17, 21 days after subculture) prelabeled with [^{3}H]adenine were incubated at 37 °C with, sequentially, 0.1 mM 3-isobutyl-1-methylxanthine for 30 min, increasing concentrations of Met^5-enkephalin for 5 min, and 1 μM prostaglandin E_1 for 10 min. The reaction was stopped and the cyclic [^{3}H]AMP was isolated as described under "Experimental Procedures." From each point (an average of duplicates) was subtracted the basal level of cyclic [^{3}H]AMP (an average of 1360 dpm/10^6 cells) in cells which had not been exposed to either prostaglandin E_1 or Met^5-enkephalin. Prostaglandin E_1-stimulation resulted in a 3.6-fold increase in intracellular cyclic [^{3}H]AMP formation above basal level in cells which had not been exposed to Met^5-enkephalin. These data are from one of three experiments.

The effect of 0.1 μM Met^5-enkephalin on the PGE_1 dose-response curve (Fig. 5) was to reduce the maximum response by ~50% and to slightly increase the EC_{50} of PGE_1. Prior incubation of cells with naloxone largely prevented the inhibition by Met^5-enkephalin of prostaglandin E_1-stimulated cyclic [^{3}H]AMP production. However, naloxone alone slightly reduced the maximal response to PGE_1 (Fig. 5).

Since activation of muscarinic receptors in N1E-115 cells causes an inhibition of PGE_1-mediated cyclic AMP formation and a stimulation of cyclic GMP formation (Matsuzawa and Nirenberg, 1975), the effect of Met^5-enkephalin on cyclic [^{3}H]GMP formation by these cells was tested with the use of the assay system previously described (Richelson *et al.*, 1978). Met^5-enkephalin at a final concentration of 0.1 μM in the absence of a phosphodiesterase inhibitor had no effect on cyclic [^{3}H]GMP synthesis by N1E-115 cells with a length of incubation up to 5 min.

Met^5-Enkephalin Immunoreactive Material Extracted from N1E-115 Cells—Diluted samples of the crude extract

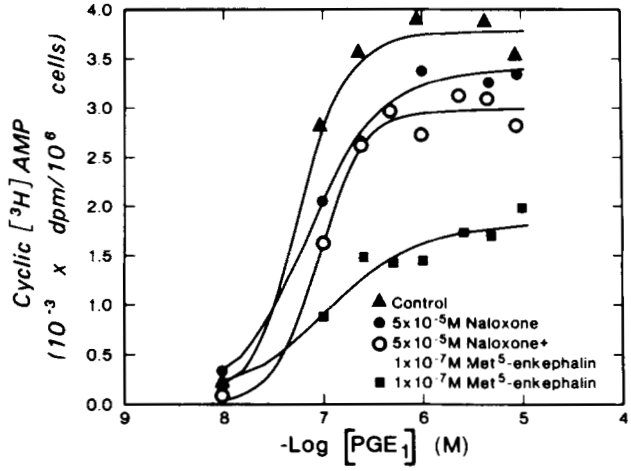

FIG. 5. **The effect of Met^5-enkephalin and naloxone on the dose-response curve of prostaglandin E_1-stimulation of cyclic [^{3}H]AMP formation in clone N1E-115.** Intact cells (passage number 17, 19 days after subculture) prelabeled with [^{3}H]adenine were incubated at 37 °C, sequentially, with 0.1 mM 3-isobutyl-1-methylxanthine for 30 min, with a phosphate-buffered saline solution (containing 110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 2.0 mM Na_2HPO_4, and 25 mM glucose with a pH of 7.35 and osmolality adjusted to 335–340 mosm with sucrose) or 50 μM naloxone for 15 min, with the phosphate-buffered saline solution or 0.1 μM Met^5-enkephalin for 5 min, and with increasing concentrations of prostaglandin E_1 for 10 min. The reaction was stopped and the cyclic [^{3}H]AMP was isolated as described under "Experimental Procedures." From each point (an average of duplicates) was subtracted the basal level of cyclic [^{3}H]AMP in cells which had been exposed to no agonists or antagonists (▲); to 50 μM naloxone (●); to 50 μM naloxone and 0.1 μM Met^5-enkephalin (○); and to 0.1 μM Met^5-enkephalin (■). The average basal level of cyclic [^{3}H]AMP for all four curves was 650 dpm/10^6 cells. Prostaglandin E_1-stimulation in the presence of no agonists or antagonists (▲) resulted in a maximum increase of 7-fold in the intracellular cyclic [^{3}H]AMP formation above basal level. The following values were obtained in paired *t* tests comparing two points on the maximum plateau of each experimental curve to two points on the maximum plateau of the control curve (▲): $p < 0.025$ (●), $p < 0.025$ (○), and $p < 0.0005$ (■). These data are from one of three experiments.

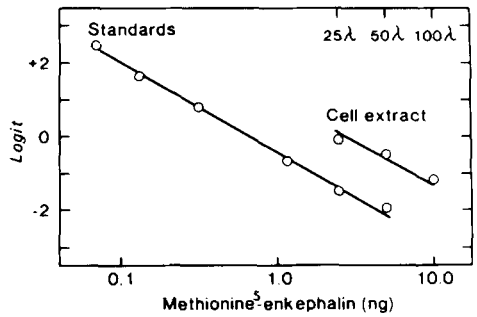

FIG. 6. **Met⁵-enkephalin immunoreactivity in clone N1E-115.** Crude extract was prepared from five flasks of cells (passage number 17, 9 days after subculture; see "Experimental Procedures"), and the residue was redissolved in 0.4 ml of radioimmunoassay buffer. Single aliquots of increasing volume from this solution were tested in the radioimmunoassay kit described in the text. These data are from one of three experiments.

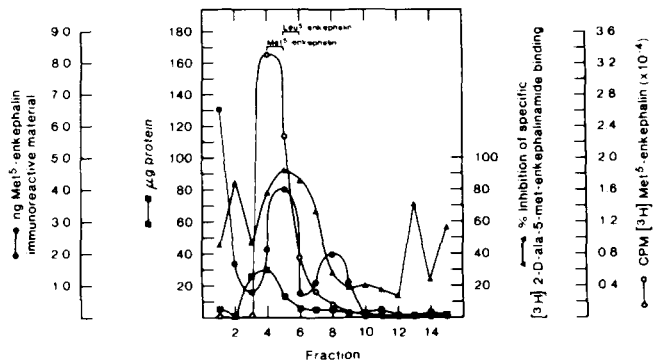

FIG. 7. **Elution profile from high pressure liquid chromatography of cellular extract from clone N1E-115.** The crude extract from 26 flasks of cells (passage number 12, 7 days after subculture) was resolubilized in the HPLC buffer and centrifuged at 8700 × *g* for 4.5 min. Twenty-one aliquots of the supernatant, each corresponding to extract from 1.1 flasks (11 μl; 88 μg of protein), were sequentially fractionated with a buffer flow rate of 1 ml/min, and 1-min fractions were collected. HPLC was done on a μ-Bondapak C_{18} column (0.39 × 30 cm) with an isocratic system of 21% *n*-propyl alcohol:0.057% acetic acid, pH 3.52, at 2500–2600 p.s.i. All fractions having the same elution time were pooled, and a volume corresponding to the chromatographed extract from two flasks of cells was retained for both the radioimmunoassay (●——●) and the protein assay (■——■), and a volume corresponding to 9.5 flasks was retained for duplicate binding assays (▲——▲). The solvent from all samples was removed at 37 °C on the Evapo-Mix or under N_2. Duplicate aliquots of the cellular supernatant each corresponding to the extract from one flask of cells were chromatographed as above with the inclusion of 2.7 pmol of [³H]Met⁵-enkephalin and with separate fraction collection. These fractions were transferred to scintillation vials, mixed with 7 ml of counting cocktail, and the radioactivity measured (○——○). The elution positions of synthetic Met⁵- and Leu⁵-enkephalin were assessed by chromatography of a standard mixture of 5 μg of each. The data shown are from one of duplicate experiments in which both the radioimmunoassay and protein assay data are the average of duplicates, the radioactivity data are from one of duplicate fractionations, and the binding data are calculated from the average specific binding of duplicates (see Fig. 2) from one of two assays (using 600 μg of membrane protein from cells with a passage number of 14, 13 days after subculture). (Due to the unavailability of [³H]Met⁵-enkephalin during the completion of this experiment, the two binding assays were performed with a final concentration of 3.5 nM of [³H]2-D-alanine-5-L-methionine-enkephalinamide.)

from N1E-115 cells yielded a dose-response curve parallel to the standard curve of the radioimmunoassay (Fig. 6). The routine recovery of Met⁵-enkephalin-like material by the described extractive procedure was 50–75%, as assessed by measurement of synthetic Met⁵-enkephalin processed in parallel. In addition, per cent recoveries within the same range were found in mixing experiments where Met⁵-enkephalin (either radioactively or nonradioactively labeled) was added directly to cells or to a cellular sonicate and its recovery quantitated at the end of the extraction procedure (data not shown). In the first case, analysis by radioimmunoassay indicated that the quantity of Met⁵-enkephalin-like material recovered from a cellular sample with added Met⁵-enkephalin was equal to the sum of the quantity of Met⁵-enkephalin immunoreactive material recovered from a parallel sample of cells alone and the quantity of synthetic Met⁵-enkephalin recovered from a parallel external standard alone. In the second case, a sonicate was spiked with [³H]Met⁵-enkephalin, and the radioligand recovered from the extraction was demonstrated to be nondegraded by thin layer chromatography.

However, the recovery of Met⁵-enkephalin-like material from fractionations of the crude extract, *e.g.* HPLC and Bio-Gel P-2 chromatography, was usually >100%, as measured by the radioimmunoassay. This might be explained by the presence of an interfering substance in the cellular extract which was partially or completely removed during fractionation, resulting in an apparent increase in the quantity of Met⁵-enkephalin immunoreactive material detected.

HPLC of Crude N1E-115 Extract—Microgram quantities of synthetic Met⁵-enkephalin and Leu⁵-enkephalin were resolved with elution times of 4.5 and 5.5 min, respectively, by high pressure liquid chromatography. When crude extract from 23 flasks of cells was fractionated by HPLC, by 21 sequential injections of the extract from 1.1 flasks, and the eluate was analyzed both by the radioimmunoassay and by the opioid receptor binding assay, the fractions having the greatest quantity of Met⁵-enkephalin immunoreactive material also demonstrated the greatest ability to inhibit [³H]2-D-Ala-5-Met-enkephalinamide binding to neuroblastoma membranes (Fig. 7). In addition, when identical aliquots of the crude extract were spiked with [³H]Met⁵-enkephalin and fractionated by HPLC, the peak of [³H]Met⁵-enkephalin radioactivity eluted at the same position as had the major peak of Met⁵-enkephalin immunoreactive material and binding activity. In addition, these three peaks coincided with the elution position of synthetic Met⁵-enkephalin. Several additional, more minor peaks of immunoreactivity and binding activity were eluted at positions before and after that of synthetic Met⁵-enkephalin; the substances present in these minor peaks are, as yet, undefined.

Bio-Gel P-2 Chromatography of Crude N1E-115 Extract—One peak of Met⁵-enkephalin immunoreactivity was routinely detected upon molecular sieve chromatography of the cellular extract (Fig. 8*a*). The elution volume of this peak (mean ± S.D. = 51 ± 6 ml; five determinations) corresponded to the elution volume of synthetic Met⁵-enkephalin (50 ± 1 ml; three determinations).

Rechromatography by HPLC of the peak of Met⁵-enkephalin immunoreactivity from the Bio-Gel P-2 column resulted in a peak of Met⁵-enkephalin immunoreactive material with an elution position identical to that of synthetic Met⁵-enkephalin in the same HPLC system (Fig. 8*b*).

Growth Curve Experiments—The stationary phase of cellular growth was reached at 8–10 days after subculturing, and the cellular number plateaued at 14–22 × 10^6 cells/flask (Fig. 9). The milligram protein/flask increased 8-fold between Days 2 and 13, while the milligram protein/10^6 cells decreased 10-fold during that period. The Met⁵-enkephalin immunoreactive material per milligram cellular protein increased to a range of 4- to 20-fold times the initial level during the course of the growth cycle; most of this increase occurred in the logarithmic phase of growth.

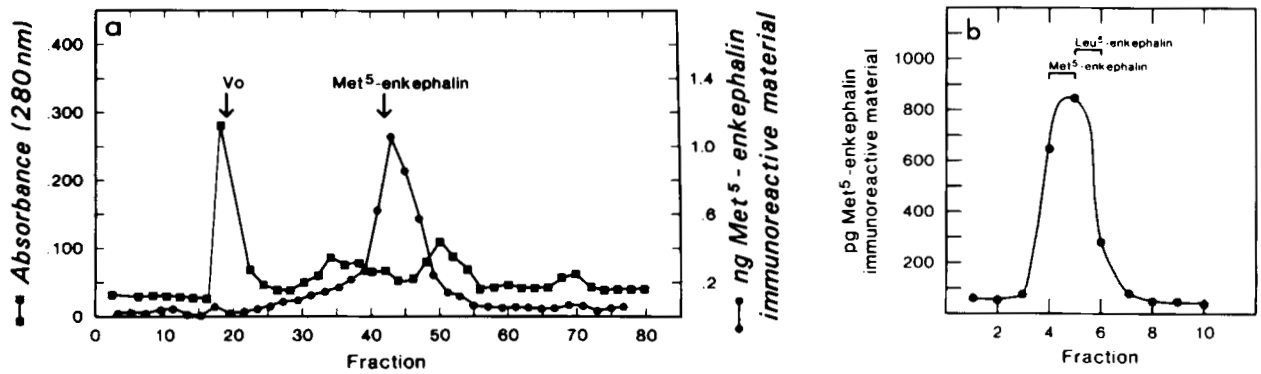

FIG. 8. **Elution profiles from Bio-Gel P-2 and high pressure liquid chromatography of cellular extract from clone N1E-115.** *a*, elution profile from Bio-Gel P-2 chromatography of cellular extract from clone N1E-115. The crude extract fractionated (0.8 mg of protein in 0.5 ml) was extracted as described under "Experimental Procedures" from 13 flasks of cells in the late logarithmic phase of growth (passage number 15, 5 days after subculture). After resolubilization and centrifugation at 8700 × *g* for 1 min, the cellular extract was chromatographed on a Bio-Gel P-2 column (1.1 × 60 cm) in 1 M acetic acid, pH 2.31, with 10-min fractions (1.2 ml) collected. Absorbance readings at 280 nm were made on alternate fractions, and the remaining fractions were lyophilized prior to analysis, in duplicate, in the Met^5-enkephalin radioimmunoassay (see text). The data shown are from one of six experiments. The elution position indicated for the void volume was determined by chromatography of blue dextran (1 mg in 0.5 ml) and was the average of two experiments. The elution position indicated for Met^5-enkephalin was assessed by chromatography of the synthetic peptide (1–2 mg in 0.5 ml) and was the average of three experiments. *b*, elution profile of Met^5-enkephalin-like material from Bio-Gel P-2 column rechromatographed by high pressure liquid chromatography. A crude extract was made from 28 flasks of N1E-115 cells (passage number 9, 7 days after subculture). The extract was divided into two parts, and each half was resolubilized, centrifuged at 8700 × *g* for 1 min, and fractionated on the Bio-Gel P-2 column described in Fig. 8*a* (0.5 ml; 1.7–1.8 mg of protein). Absorbance readings at 280 nm were made on alternate fractions, and the remaining fractions were divided into two tubes and lyophilized. One tube of each fraction (containing ⅓ of the original volume) was resolubilized and assayed in the radioimmunoassay. Based on these analyses, two to five tubes (containing ⅔ of the original volumes) were chosen for each column from the peak of Met^5-enkephalin immunoreactive material which had eluted at the position of synthetic Met^5-enkephalin. This sample from each column was resolubilized in 22 μl of HPLC buffer and fractionated in two injections by the HPLC system described in Fig. 7. The data presented are from one of these duplicate column samples. The fractions collected from the two injections (10 μl; 25 μg of protein) were pooled, and the solvent was removed at 37 °C under N_2. The residues were stored at 4 °C until analysis, in duplicate, in the radioimmunoassay.

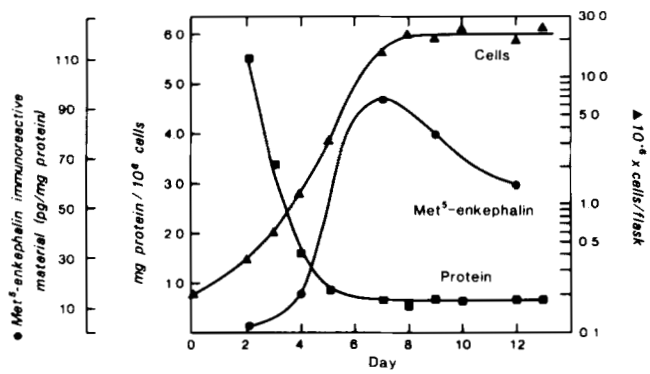

FIG. 9. **Growth curve of clone N1E-115 cells and its relationship to cellular protein and intracellular Met^5-enkephalin production.** On Day 0, 2 × 10^5 cells (passage number 20) which had been kept in the logarithmic phase of growth for 3–7 weeks were seeded into a number of flasks and cultured as described under "Experimental Procedures." For quantitation of stored Met^5-enkephalin immunoreactive material, duplicate samples of 1.4–2.8 × 10^7 cells were extracted and each analyzed in quadruplicate in the radioimmunoassay (see "Experimental Procedures") and the figures obtained corrected for recovery using recovery calculations for synthetic Met^5-enkephalin separately processed in parallel. Aliquots of the cellular suspension saved during harvesting were used for enumerating cells, in quadruplicate, and for assaying cellular protein, in triplicate, by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. These data are from one of duplicate experiments.

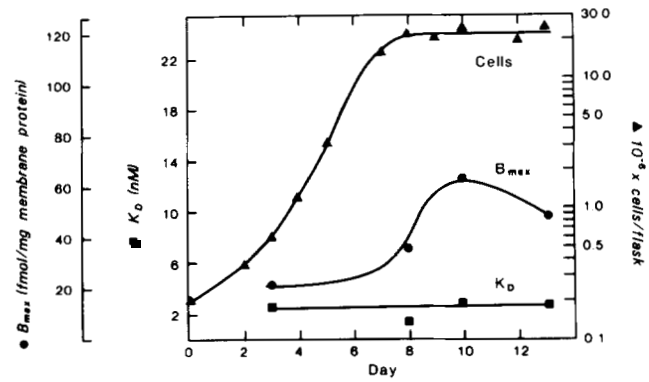

FIG. 10. **Growth curve of clone N1E-115 cells and its relationship to the maximum number of [^{3}H]Met^5-enkephalin receptor sites per milligram of membrane protein and the apparent equilibrium dissociation constant for [^{3}H]Met^5-enkephalin binding to neuroblastoma membranes.** On Day 0, 2 × 10^5 cells (passage number 20) which had been kept in the logarithmic phase of growth for 3–7 weeks were seeded into a number of flasks and cultured as described in the text. For a [^{3}H]Met^5-enkephalin binding assay, 8.6–9.7 × 10^7 cells were harvested, and a 10% (w/v) membrane solution was prepared and used the same day with 8–9 concentrations of radioligand as described under "Experimental Procedures." (see Fig. 2 for description of binding data manipulations). Scatchard analysis of the specific binding data from each assay by the least squares method of linear regression analysis provided the values for the K_D and B_{max}. Aliquots of the cellular suspension saved during harvesting were used for enumerating cells in quadruplicate. These data are from one of duplicate experiments.

The opioid receptor binding studies revealed no significant change in the value of the apparent equilibrium dissociation constant (K_D) for [^{3}H]Met^5-enkephalin during the course of the growth cycle, with an average value of 2.3 nM (Fig. 10). However, the maximum number of [^{3}H]Met^5-enkephalin receptor sites per milligram membrane protein (B_{max}) increased 2-fold as the cells progressed from logarithmic to stationary phase.

DISCUSSION

A homogeneous class of enkephalin receptors found in N1E-115 cells (Chang and Cuatrecasas, 1979) was confirmed by Scatchard analyses of data from the binding of [^{3}H]Met^5-enkephalin to cellular membranes. Ligand binding to the

receptors displayed saturability by the radioligand and linearity with tissue. Little endogenous opioid was found in the washed membrane fraction from the cells. Sodium ions reduced the specific binding of [^{3}H]Met^5-enkephalin to N1E-115 membranes in the same manner as they decrease the binding of opiate agonists to brain membranes (Pert and Snyder, 1974) and the binding of opioid peptides to brain membranes (Simantov *et al.*, 1978; Chang and Cuatrecasas, 1979) and to intact murine neuroblastoma clone N4TG1 cells (Miller *et al.*, 1978b). The order of potency of inhibitors for [^{3}H]Met^5-enkephalin binding to N1E-115 membranes, where Met^5-enkephalin was ~100 times more potent than morphine, was similar in rank to comparisons reported by Blume *et al.* (1977) using whole NG108-15 hybrid cells and by Chang *et al.* (1978) using intact murine neuroblastoma clone N4TG1 cells.

A comparison of the opioid receptors in N1E-115 cells to those in other neuroblastoma and neuroblastoma × glioma hybrid clones indicated that the K_D for [^{3}H]Met^5-enkephalin (2.4 nM) was similar to the values cited for enkephalin analogs (varying from 1–2 nM to 5 nM), although the number of opioid binding sites per cell was the lowest (others ranging from 18,000 to 410,000) (Blume *et al.*, 1977; Gerber *et al.*, 1978; and Chang *et al.*, 1978). Neuroblastoma cells have only the enkephalin type of opiate receptor with a low affinity for narcotics and a high affinity for enkephalins (Chang and Cuatrecasas, 1979). The large K_D for [^{3}H]dihydromorphine binding to NG108-15 cells (20–30 nM) found in the early study by Klee and Nirenberg (1974) is supportive of the above report, as is their finding of no narcotic receptors in neuroblastoma clone N1E-115 using the [^{3}H]dihydromorphine binding assay.

Rat brain membranes have two classes of sites for Met^5-enkephalin binding. Chang and Cuatrecasas (1979) reported a high affinity site with a K_D of 3.2 nM and a lower affinity site with a K_D of 6.2 nM. Simantov *et al.* (1978) found two equilibrium dissociation constants for Met^5-enkephalin binding with values of 1.8 and 5.8 nM. The Met^5-enkephalin receptor in N1E-115 cells, with a K_D of 2.4 nM, was more comparable to the higher affinity site in brain, *i.e.* the enkephalin type of opiate receptor.

The naloxone-sensitive inhibition of PGE_1-induced intracellular cAMP formation by Met^5-enkephalin in N1E-115 cells was similar to results found in other systems. After Collier and Roy (1974) reported that short-term incubation with morphine-like drugs inhibited the stimulation by prostaglandin E_1 and E_2 of cyclic AMP formation in homogenates of whole rat brain, the same inhibitory activity was found to be induced in intact or homogenized murine neuroblastoma and neuroblastoma × glioma clones by morphine-like compounds (Traber *et al.*, 1975; Sharma *et al.*, 1975; and Klee and Nirenberg, 1976) and opioid peptides (Klee and Nirenberg, 1976; and Wahlstrom *et al.*, 1977). In general, all of the above publications report naloxone-prevention of the opiate or opioid-induced inhibition of prostaglandin-stimulated cAMP formation.

This laboratory previously reported the presence of intracellular Met^5-enkephalin immunoreactivity in N1E-115 cells using indirect immunofluorescence (Knodel and Richelson, 1980). Based on this study, further investigations were made using a crude extract from these cells. Comparison of a dilution curve of the extract to the standard curve of the radioimmunoassay indicated that the Met^5-enkephalin-like material detected in the crude extract was immunologically similar to synthetic Met^5-enkephalin. The coincident elution during high pressure liquid chromatography of synthetic Met^5-enkephalin with the major peaks of Met^5-enkephalin immunoreactivity and inhibitory activity for [^{3}H]Met^5-enkephalin or [^{3}H]2-D-Ala-5-Met-enkephalinamide binding to neuroblastoma membranes indicated that the immunoreactive peptide in the crude extract had physical and receptor binding properties similar to those of Met^5-enkephalin. When cellular extract was fractionated by molecular sieve chromatography on a Bio-Gel P-2 column, the Met^5-enkephalin immunoreactive material had the same elution volume as did synthetic Met^5-enkephalin, indicating a similarity in apparent molecular weights. In addition, the Met^5-enkephalin-like material from the Bio-Gel P-2 column had the same elution position as did synthetic Met^5-enkephalin upon rechromatography by HPLC. Therefore, we believe that neuroblastoma clone N1E-115 synthesizes a peptide very similar to, if not identical with, Met^5-enkephalin. Further definitive structural analyses of this Met^5-enkephalin-like peptide are currently being done using mass spectrometry.

Glaser *et al.* (1980) have recently reported that an extract of neuroblastoma × glioma hybrid cells 108CC15 contains 0.057 ng/mg protein of immunoreactive Leu^5-enkephalin equivalents. Stationary cells of the N1E-115 clone contain 0.078 ng/mg cellular protein of Met^5-enkephalin immunoreactive material. The amount of Met^5-enkephalin measured by other authors in rat brain (assuming that 10% of the brain's weight is protein) range between 0.06 and 7.5 ng/mg of protein (Simantov and Snyder, 1976; Hughes *et al.*, 1977; Rossier *et al.*, 1977; Yang *et al.*, 1977; and Miller *et al.*, 1978a). Thus, N1E-115 cells have levels of Met^5-enkephalin that are comparable to the lowest estimates in whole brain and to the amount in neuroblastoma × glioma clone 108CC15.

We are currently studying the synthesis of Met^5-enkephalin by N1E-115 cells. While precursors for the opioid peptide were not routinely identified in the studies reported here, occasionally additional, presently unidentified peaks of Met^5-enkephalin immunoreactivity were seen during fractionation of the crude cellular extract (*e.g.* Fig. 7). We have no rigorous data on the ability of the radioimmunoassay to detect larger molecules containing Met^5-enkephalin or smaller, breakdown products of Met^5-enkephalin. Similarly, the opiate receptor binding assay occasionally detected other as yet unidentified fractions from HPLC analyses of crude extract which inhibited [^{3}H]Met^5-enkephalin or [^{3}H]2-D-Ala-5-Met-enkephalinamide binding to neuroblastoma membranes. However, precursors containing Met^5-enkephalin within larger molecular weight structures may not always be functionally active in the binding assay until they have been cleaved into their component parts by protease activity. The effect of protease inhibitor use during cellular extraction on the appearance of precursors and products is being studied.

Finally, several properties of neuroblastoma clone N1E-115 are known to be regulated with respect to cell division, including the activity of the enzyme tyrosine hydroxylase (Richelson, 1973) and the function of the muscarinic acetylcholine receptor (El-Fakahany and Richelson, 1980). Data presented here indicate that, in addition, the maximum number of [^{3}H]Met^5-enkephalin receptor sites per milligram of membrane protein and the quantity of stored Met^5-enkephalin immunoreactive material per milligram of cellular protein were regulated in a similar fashion. However, the apparent affinity of the enkephalin receptors for the [^{3}H]Met^5-enkephalin was not affected.

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