RADIOIMMUNOASSAY OF METHIONINE ENKEPHALIN
AND INTERFERENCE BY BRAIN FACTOR OF
IMMUNOREACTIVITY AND OPIATE RECEPTOR
BINDING ACTIVITY

Masakatsu TAKAHASHI, Hiroshi KANETO, Eiko UENO,
Joe WATANABE, Masao KOIDA, Hiroshi OGAWA*
and Haruaki YAJIMA*

Department of Pharmacology, Faculty of Pharmaceutical Sciences,
Nagasaki University, Nagasaki 852 and *Faculty of Pharmaceutical Sciences,
Kyoto University, Sakyo-ku, Kyoto 606, Japan

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Abstract—We developed a radioimmunoassay of methionine-enkephalin (met-enk)
which was found to be highly specific to the peptide and 1 pmole was the detection
limit. Interference by the following peptides was practically negligible; leucine-
enkephalin, LAP- or CNBr-treated met-enk, α- and β-endorphins. Morphine and
its congeners were totally inert. Measurement of met-enk in rat brain P2 fraction
revealed besides met-enk, the presence of the factor which cross-reacted with the
antisera and also appeared to bind with the brain opiate receptor. Though the
nature of the factor has not yet been characterized, it is suggested that for qualitative
assessment of the tissue level of met-enk, a method to selectively inactivate either
the peptide or the factor should be developed first.

It is now firmly established that opiate receptors exist for endogenous ligands and
that these ligands may serve as neurotransmitters or hormones. Several opiate-like peptides
have been isolated (1-4) and structurally elucidated as being relative to the pituitary hormone,
β-lipotropin (5). Though receptor binding is the common property shared by all these
peptides, they apparently differ in tissue distribution patterns (6, 7) and in pharmacological
properties (4, 8-12), suggesting that one peptide may play different roles. To elucidate
roles of these peptides, one research step is to individually follow the function-related turnover
of the peptides, and here development of a specific assay method for each is required.

We succeeded in developing a radioimmunoassay specific for one of these peptides,
methionine-enkephalin (met-enk). Three types of antisera directed to the peptide were
prepared and two were found to provide radioimmunoassay with satisfactory specificity and
sensitivity. When the assay method was applied for estimation of met-enk content of a
brain subcellular fraction, it was found that the tissue contained, besides met-enk, the factor
which cross-reacted with the antisera and also appeared to inhibit binding of met-enk to the
brain opiate receptor. Though the nature of the factor has yet to be characterized, its
presence in brain tissue necessitated correction of the value estimated by radioimmunoassay.

MATERIALS AND METHODS

N-(m-Maleimidebenzoyloxy)-succinimide (MBS) (13) was kindly provided by Dr. T.
Kitagawa of Nagasaki University. Leucine-enkephalin and met-enk were synthesized as described previously (14). Synthetic α- and β-endorphins were purchased from Peninsula Lab., [3H]-acetic anhydride (5.6 Ci/mmole) from The Radiochemical Center., [tyrosyl-3,5-3H]-met-enk (22 Ci/mmole) from New England Nuclear and leucine aminopeptidase (LAP) from Boeringer Mannheim. [Acetyl-3H]-N-acetyl-met-enk was prepared by reaction of met-enk with [3H]-acetic anhydride in absolute pyridine and purified by thin layer chromatography. Throughout this experiment, the protein was estimated according to the method of Lowry et al. using bovine serum albumin (BSA) as the standard (15).

Preparation of immunogen: Met-enk was a) first linked to p-diazophenylacetic acid and the derivative to BSA using water soluble carbodiimide (CDI), b) directly conjugated to BSA with CDI, or c) first derivated with MBS and then coupled to dithiothreitol reduced BSA (13). In each case, coupling was followed by extensive dialysis. Amino acid analysis (16) of the dialyzed conjugate estimated the binding ratio to BSA on the molar basis, 10 by a), 11 by b) and 10 by c), respectively.

Immunization: Two mg of the immunogen emulsified in complete Freund's adjuvant was injected into several sites of a rabbit including back and foot pads. Booster doses of 1 mg were given at 2 week intervals and at least 3 times. Rabbits were bled 10 days after the last booster injection.

Radioimmunoassay: The reaction mixture in 0.40 ml of phosphate buffered saline contained diluted antiserum, 20 nmoles of bacitracin, normal rabbit serum γ-globulin (1.0 mg as protein) and [3H]-met-enk (2.3 pmoles, 4.4 × 10⁴ cpm) or [3H]-N-acetyl-met-enk (15 pmoles, 3.7 × 10⁴ cpm). After incubation at 4°C for 3 hr, the mixture was half saturated with ammonium sulfate and left to stand at 4°C for 1 hr. The formed precipitate was separated by centrifugation and dissolved in Aquasol (5 ml) for counting.

Radio receptor assay: The assay was carried out as described by Terenius (17). The crude synaptosomal fraction (P₂) was prepared from male Wistar rats weighing 150-200 g according to the method of Whittaker et al. (18). The reaction mixture contained 2.0 pmoles of [3H]-met-enk (2.0 × 10⁴ cpm), 20 nmoles of bacitracin and the P₂ membrane fraction (0.60 mg as protein) in 0.50 ml of 0.10 M Tris-HCl buffer (pH 7.4). The mixture was incubated at 25°C for 20 min and the reaction terminated by centrifugation. For counting, the pellet was digested using Protosol (1 ml).

Extraction, fractionation and assay of met-enk in the P₂ fraction: The P₂ fraction described above was extracted 3 times with 10 ml each of 1 N acetic acid at 4°C. The supernatants were combined and heated in boiling water for 15 min. After being delipidized with ether, the solution was lyophilized. The residue was taken up in 2 ml of 1 N acetic acid and applied on a Biogel P-2 column (1.6 × 35 cm) buffered with 1 N acetic acid. The column was eluted with 1 N acetic acid at a flow rate of 24 ml/hr and 2 ml of fraction was collected per tube. Absorbency at 280 nm and reactivities with fluorescamine and AgCl were monitored. Six series of fractions (refer to Fig. 2) were separately combined and lyophilized. The lyophilizate was dissolved in 2 ml of water and after adjustment of the pH to 7.0 with 1 N ammonium hydroxide, the peptide content was estimated by radioim-
Treatment of met-enk with CNBr and LAP: CNBr treatment was carried out by allowing 2 μmoles of met-enk to react with 50 mg of CNBr in 0.20 ml of 0.1 N HCl at 25°C for 2 hr. The reaction was terminated by lyophilization. For preparation of LAP-digested met-enk, 2.0 μmoles of met-enk containing [3H]-met-enk was incubated with 1 U (10 ng) of the enzyme in 1.5 ml of 60 mM Tris-HCl (pH 7.4) containing 2.5 μmoles of MgCl₂ at 37°C for 60 min. Under this condition, 99% of tyrosine was released. The same incubation condition was applied for treatment of tissue extract which was assayed to contain less than 0.10 μmole of the peptide. Absence of inhibitory material of LAP in the extract was confirmed by release of [3H]-label from [3H]-met-enk added to the extract.

RESULTS

Development of radioimmunoassay of met-enk

The antiserum prepared by method a) was highly specific for met-enk (Fig. 1a). With this assay system, less than 1 pmole of the peptide could be detected. Cross-reactivity with α- and β-endorphins, leucine-enkephalin, CNBr- or LAP-treated met-enk was practically negligible. The serum was thus used for estimation of met-enk in brain extract described below.

A comparable sensitivity and a higher selectivity were attained with the antiserum to CDI-coupled met-enk (Fig. 1b). With this serum, 1 pmole of met-enk was detectable. To be noted, is that the serum did not interact with leucine-enkephalin.

On the other hand, conjugation of met-enk to BSA using the MBS method directed the specificity to N-acetyl-met-enk rather than met-enk itself (Fig. 1c). The cross-reaction with endorphins was considerably high. The serum was not suitable for assay of met-enk.

Estimation of met-enk in extract of rat brain P₂ fraction

When assayed before gel-filtration, the extract of rat brain P₂ fraction contained 0.60 nmole of met-enk per brain in radioimmunoassay. The elution pattern of met-enk-like activity from a Biogel P-2 column is shown in Fig. 2. The immunoactivity was present in all but F fraction with a peak in fraction C. As to the receptor binding activity, a similar elution profile was obtained but this activity was considerably higher in fraction B to E than the immunoactivity. The recovery rate of the immunoactivity was estimated to be between 90 to 100% in three independent experiments.

Attempting to differentiate met-enk from the other factors including leucine-enkephalin, we observed the manner in which LAP digestion changed the respective activity of each fraction. Incubation with LAP reduced both activities of fractions B to E, but even after digestion, which would almost completely degrade met-enk, as evidenced by release of [3H]-tyrosine from [3H]-met-enk, the activities were never abolished.

DISCUSSION

The radio receptor assay of the opiates first described by Pert and Snyder (20) has proved to be a powerful tool for characterization of the opiate receptor in various tissues and also
FIG. 1. Comparison of the specificity to met-enk of three types of antisera. a) the antisera directed to met-enk coupled to p-diazo phenylacetic acid, b) the antisera to met-enk directly conjugated with BSA and c) the antisera prepared by MBS method. In a) and b), the affinities of various peptides were compared as % inhibition of binding of [3H]-met-enk to the sera and in c) as that of binding of [3H]-N-acetyl-met-enk, respectively. The peptides are indicated by (▲) for met-enk, (▼) α-endorphin, (▼) β-endorphin, (×) leucine- enkephalin and (○) N-acetyl-met-enk, respectively.

FIG. 2. Elution pattern of a met-enk-like substance in a P2 extract on Biogel P-2 column. An extract from eight brains was applied on the column. The type a) antibody in Fig. 2 was employed for radioimmunoassay. Preliminary calibration of the column determined that β-endorphin was to be eluted between tubes 14 and 22 and met-enk 28 and 37, respectively.
for identification of the endogenous opioids. This method is however not convenient for assay of an individual peptide in tissues because it measures the common property of the endogenous peptide and preliminary separation into each individual peptide is included in the assay procedure. In contrast, the specificity of the radioimmunoassay may be directed to any such peptide under study. Several reports describing preparation of antisera to enkephalins have appeared (21-25), but only the serum developed by Watson et al. (25), met-enk, seems to be highly specific.

The antisera elicited in experiments toward two types of antigens were satisfactorily selective for qualitative measurement of met-enk and such is an efficient tool for researching the enzyme activity which generates met-enk from $\beta$-endorphin (Aono et al. in preparation), especially since endorphins are substantially inert to the sera. In addition, the lack of recognition of the opiates by any of the antisera suggests the potential utility of the radioimmunoassay in the study on the function-related change of met-enk level during development of tolerance to and physical dependence on the opiates.

While testing such utility, it was noticed however that the acid extract of the rat brain P₂ fraction contained unknown factor(s) which interacted with the antisera and rather strongly with the opiate receptor. The factor was not separable from met-enk by gel-filtration thus making it difficult to directly measure met-enk content in spite of a sharp specificity of the used antisera. LAP-digestion which was able to degrade met-enk and leu-enk, failed to abolish both activities. The effect of CNBr-treatment on the activities of each fraction was tested but due to the ambiguity of the obtained results (i.e. the treatment generally tended to decrease the activities but in some cases rather enhanced), the method was tentatively abandoned. Thus, it may be concluded that the brain P₂ fraction contains acid-heat extractable materials which interfere with the radioimmunoassay and which also exhibit opiate receptor binding activity. For accurate assay of met-enk level in this fraction, a method to selectively inactivate either the peptide or the materials has to be developed.

The presence of endogenous factors different from those seen in the endorphin family, have been reported by three research groups (26-30). We are now attempting to determine whether the factor found in the P₂ fraction corresponds to any of those reported or is totally different.

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