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# DEGRADATION OF DYNORPHIN-(1-13) AND DYNORPHIN-(1-17) BY THE NEUROBLASTOMA CELL MEMBRANE. EVIDENCE FOR THE INVOLVEMENT OF A CYSTEINE PROTEASE

Mitsuo Satoh, Hideyoshi Yokosawa, and Shin-ichi Ishii

Department of Biochemistry, Faculty of Pharmaceutical Sciences,
Hokkaido University, Sapporo 060, Japan

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SUMMARY: The membrane of mouse neuroblastoma N-18 cells degraded dynorphin-(1-13), dynorphin-(1-17), and Leu-enkephalin. The degradation of the former two peptides was inhibited strongly by N-ethylmaleimide, moderately by diisopropylphosphorofluoridate and phosphoramidon, and slightly by bestatin. When Leu-enkephalin was the substrate, however, the effects of phosphoramidon and bestatin were marked and those of N-ethylmaleimide and diisopropylphosphorofluoridate were negligibly small. Captopril did not affect the degradation of the two dynorphins and Leu-enkephalin, but inhibited the further cleavage of N-terminal fragments generated from dynorphin-(1-13) by the N-ethylmaleimide-sensitive protease. Thus, a cysteine protease and, probably, a serine protease are responsible to the initial fragmentation of the dynorphins. 

Since the pioneering work of Hughes et al., (1) on the isolation of endogenous opioid peptides, Met- and Leu-enkephalins, a variety of enkephalin-containing peptides have been isolated and classified into three groups on the basis of their precursor structures (2,3). Among them, a group of Leu-enkephalin-containing peptides including dynorphin-(1-17) (4,5), dynorphin-(1-8) (6,7), α- and β-neo-endorphins (8,9), and rimorphin (10) have been isolated and are found to be derived from a common precursor, prodynorphin (proenkephalin B). These Leu-enkephalin-containing peptides are thought to function as neurotransmitters and/or neuromodulators rather than as precursors for Leu-enkephalin, because these peptides are more potent than Leu-enkephalin (11,12) and bind to an opiate receptor distinct from the Leu-enkephalin-binding receptor (13,14).

It is at present believed that the physiological activity of opioid peptides is terminated through enzymatic degradation. Met- and Leu-

Abbreviations: DFP, diisopropylphosphorofluoridate; HPLC, high-performance liquid chromatography.

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enkephalins are degraded by the action of two peptidases, a phosphoramidon-sensitive neutral endopeptidase (enkephalinase, endopeptidase-24.11, EC 3.4.24.11) and a bestatin-sensitive aminopeptidase (15). Furthermore, β-neo-endorphin and dynorphin-(1-8) have been reported to be degraded by angiotensin-converting enzyme (peptidyldipeptide hydrolase, EC 3.4.15.1) and the enkephalin-degrading aminopeptidase (16,17). However, an inactivating mechanism for Leu-enkephalin-containing peptides composed of 12 or more amino acid residues has not been well elucidated except for one report (18) that dynorphin-(1-13) was degraded by membrane-bound enzymes from rat brain.

In this study, we used a homogenous cell line, the neuroblastoma N-18 cell (19), in tissue culture, as a possible model for understanding the inactivation system of opioid peptides composed 12 or more amino acid residues in neurons, and found that two distinct novel proteases, a cysteine protease and a serine protease, were responsible to the initial cleavages of dynorphin-(1-13) and dynorphin-(1-17).

## MATERIALS AND METHODS

Leu-enkephalin, dynorphin-(1-13), dynorphin-(1-17), E-64, and phosphoramidon were purchased from the Peptide Institute, Osaka, Japan. Captopril and bestatin were kindly provided by Dr. A. Awaya of Mitsui Pharmaceuticals Inc., Tokyo, and Dr. W. Tanaka of Nippon Kayaku Co., Tokyo, respectively.

An established neuroblastoma cell line N-18 derived from mouse neuroblastoma C-1300 (19) was cultured and collected as described previously (20). The detached cells were washed with 10 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl, suspended in a 10-fold volume of 10% (w/w) sucrose, and homogenized with a Teflon homogenizer (1,200 rpm, 10 strokes). The homogenate was centrifuged at 800 x g for 20 min and then at 9,000 x g for 20 min. The plasma membrane was prepared from the second pellet by using discontinuous sucrose density gradient centrifugation according to the method of Jones and Matus (21). The membrane preparation was suspended in 5 mM Tris-HCl (pH 8.1) and stored at -80°C.

Proteins were determined by the method of Bradford (22) using bovine serum albumin as a standard.

The degradation of Leu-enkephalin, dynorphin-(1-13), and dynorphin-(1-17) by the membrane preparation was allowed to proceed at 37°C in a reaction mixture containing the following components in a final volume of 0.05 ml: 75 mM Tris-HCl (pH 8.1), 150 mM NaCl, 5mM $CaCl_2$, 0.06 mM peptide, and 3 μg of membrane preparation.

Degradation of the substrates was followed by HPLC on a reversed-phase column (4.6 x 250 mm) of Nucleosil $5C_{18}$ (Marchery, Nagel, and Co.), monitored at 210 nm using a Toyo Soda UV-8 model II spectrophotometer. Isocratic elution was carried out with 33%, 35%, and 35% acetonitrile in 0.1% trifluoroacetic acid for dynorphin-(1-13), dynorphin-(1-17), and Leu-enkephalin, respectively. Alternatively, the cleavage products were separated by elution from the same column, which had been preequilibrated with 1% acetonitrile in 0.1% trifluoroacetic acid, with a 32-min linear gradient of 1-65% acetonitrile in 0.1% trifluoroacetic acid. The

chromatographic equipment consisted of a 110B solvent delivery module, a 340 organizer, and a 420 controller (Beckman Instruments, Inc.).

The cleavage products separated by HPLC were lyophilized and hydrolyzed with 6 M HCl containing 1% (v/v) 2-mercaptoethanol at 110°C for 24 hr, and then their amino acid compositions were determined on a Hitachi 835 amino acid analyzer.

## RESULTS AND DISCUSSION

Degradation products of Leu-enkephalin, dynorphin-(1-13), and dynorphin-(1-17) by the membrane preparation of neuroblastoma N-18 cells were initially analyzed by HPLC under conditions of isocratic elution. The chromatographic peak area of each of the starting materials decreased almost linearly as a function of time, so long as more than 50% of the materials remained. At the concentrations of 0.06 mM, Leu-enkephalin, dynorphin-(1-13), and dynorphin-(1-17) were found to be degraded at rates of approximately 80, 50, and 30 pmol/hr/μg membrane protein, respectively. The results are consistent with the fact that dynorphin-(1-13) and dynorphin-(1-17) have longer duration of action than the small dynorphins; dynorphin-(1-7), dynorphin-(1-8), and dynorphin-(1-9) (14).

The effects of various inhibitors on the degradation of the large dynorphins and Leu-enkephalin by the membrane preparation are shown in Table I. The degradation of Leu-enkephalin was inhibited by phosphoramidon and slightly by bestatin, whereas that of dynorphin-(1-13) and dynorphin-(1-17)

TABLE I
Effects of inhibitors on the degradation* of Leu-enkephalin and the large dynorphins by neuroblastoma N-18 cell membrane

| Inhibitor | Conc. (mM) | Inhibition (%) | | |
|---|---|---|---|---|
| | | Leu-enkephalin | Dynorphin-(1-13) | Dynorphin-(1-17) |
| N-Ethylmaleimide | 1.0 | 5 | 72 | 81 |
| | 0.1 | 0 | 59 | 64 |
| | 0.01 | 1 | 33 | 27 |
| DFP | 1.0 | 0 | 15 | 27 |
| | 0.1 | 3 | 4 | 7 |
| | 0.01 | 1 | 3 | 2 |
| Phosphoramidon | 0.1 | 42 | 14 | 15 |
| | 0.01 | 17 | 2 | 1 |
| | 0.001 | 3 | 0 | 0 |
| Bestatin | 1.0 | 15 | 3 | 4 |
| | 0.1 | 11 | 2 | 3 |
| | 0.01 | 9 | 1 | 3 |
| Captopril | 0.1 | 0 | 0 | 1 |
| E-64 | 0.2 | 0 | 0 | 0 |

*The extents of degradation were measured by the reductions in chromatographic peak areas of Leu-enkephalin, dynorphin-(1-13), and dynorphin-(1-17) during incubation for 8, 15, and 30 hr, respectively.

was inhibited by N-ethylmaleimide and DFP. p-Chloromercuribenzoate and p-aminobenzamidine at the concentration of 1.0 mM also inhibited the degradation of dynorphin-(1-13) and dynorphin-(1-17) (75 and 81% inhibition with the former inhibitor, and 11 and 21% with the latter, respectively). Phosphoramidon and bestatin exhibited less inhibitory effects on the degradation of the two dynorphins than on that of Leu-enkephalin. Captopril and E-64 showed no inhibitory effects with all three peptides. These results suggest that an N-ethylmaleimide-sensitive protease and a DFP-sensitive protease are involved in the degradation of dynorphin-(1-13) and dynorphin-(1-17), but not of Leu-enkephalin. Furthermore, lack of susceptibility to E-64 indicates that the N-ethylmaleimide-sensitive protease is distinct from cathepsin B of lysosomal origin.

The proteolytic cleavage products from dynorphin-(1-13) were then fractionated by HPLC with a linear gradient of acetonitrile concentration. Chromatograms of the products obtained under four different incubation conditions are shown in Fig. 1. Ten peaks (a-j) were detectable in the absence of inhibitors (see Fig. 1(b)). The peptide material in peak i was identical to the substrate, dynorphin-(1-13). On the other hand, the products in the presence of captopril gave 12 peaks (as shown in Fig.1 (c)), five of which (A-E) appeared at the positions different from any of the peaks detected on Fig. 1 (b). However, the extent of degradation of dynorphin-(1-13) itself was not affected with captopril as described in Table I. N-Ethylmaleimide suppressed the appearance of most of the fragment peaks except peak h (Fig. 1(d)). Amino acid compositions of the fragments separated by HPLC are shown in Table II. The results allowed their assignment. As shown in Table II, several fragments originated from the C-terminal portion of substrate, together with Tyr-Gly-Gly (peak a), tyrosine (peak b), and phenylalanine (peak d), accumulated in the absence of captopril, while those from the N-terminal portion were additionally found in the presence of captopril. The results suggest that putative membrane-bound endopeptidases first split dynorphin-(1-13) around the

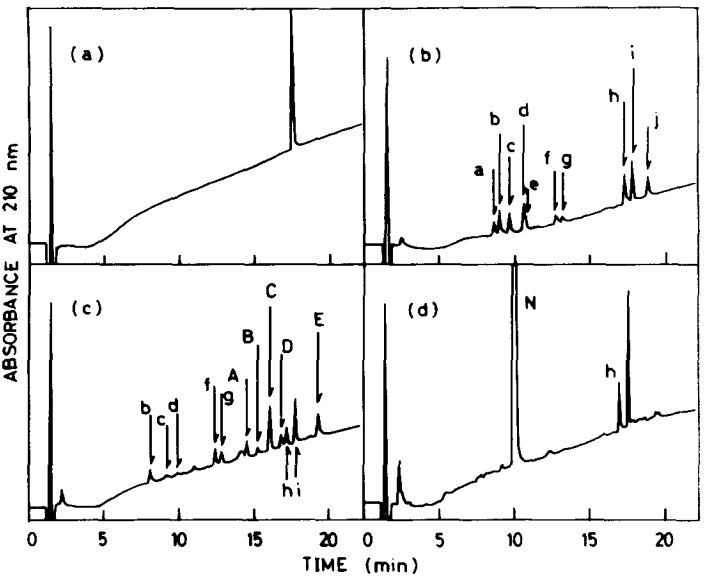


Fig. 1. Degradation of dynorphin-(1-13) by neuroblastoma cell membrane. The reaction mixture was incubated at 37°C for 0 (a) and 24 hr (b) in the absence of inhibitor, for 24 hr (c) in the presence of 0.1 mM captopril, and for 24 hr (d) in the presence of 1.0 mM N-ethylmaleimide. At an indicated time, 5 µl-aliquots were analyzed by HPLC using a Nucleosil $5C_{18}$ column (4.6 x 250 mm) which had been preequilibrated with 1% acetonitrile in 0.1% trifluoroacetic acid. Elution was carried out with a 32-min linear gradient of 1-65% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. The absorbance at 210 nm was monitored. Peak N is that formed with N-ethylmaleimide.

TABLE II

Amino acid compositions of the peptides isolated from a 24-hr reaction product of dynorphin-(1-13)[a] with neuroblastoma N-18 cell membrane

| Peak | Amino acid (Mol %) | | | | | | | | Fragment identified | Yield (%)[b] | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Tyr | Gly | Phe | Leu | Arg | Ile | Pro | Lys | | (-) | (+)[c] |
| a | 26 | 74 | | | | | | | (1-3) | 5 | 0 |
| b | 100 | | | | | | | | (1) (= Tyr) | 17 | 16 |
| c | | | | | 26 | 23 | 26 | 26 | (8-11) | 8 | 2 |
| d | | | 100 | | | | | | (4) (= Phe) | 17 | 4 |
| e | | 18 | | | 35 | 21 | 9 | 18 | (7-11) | 2 | 0 |
| f | | | | 21 | 19 | 17 | 9 | 34 | (8-13) | 5 | 11 |
| g | | | | 15 | 36 | 14 | 7 | 28 | (7-13) | 4 | 7 |
| h | 5 | 27 | 13 | 14 | 19 | 6 | 8 | 7 | (1-11)+(2-5)[d] | 6+5 | 2+0 |
| i | 5 | 16 | 7 | 15 | 24 | 7 | 8 | 15 | Complete | | |
| j | 14 | 45 | 20 | 20 | | | | | (1-5) | 9 | 0 |
| A | | 32 | 16 | 18 | 32 | | | 2 | (2-7) | | 10 |
| B | | 42 | 19 | 19 | 19 | | | | (2-6) | | 4 |
| C | 13 | 29 | 14 | 14 | 29 | | | | (1-7) | | 13 |
| D | 10 | 35 | 16 | 18 | 21 | | | | (1-6) | | 4 |
| E | 6 | 19 | 9 | 17 | 12 | 8 | 11 | 17 | (1-8)+(10-13)[e] | | 7+7 |

[a] The sequence, Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys.
[b] Determined on the basis of the amount of dynorphin-(1-13) degraded.
[c] (-), Without any inhibitor; (+), with 0.1 mM captopril.
[d] The two standard peptides, fragments (1-11) and (2-5), were eluted at the position of peak h. The amino acid composition shown in this line is for the material in this peak without any inhibitor.
[e] Tentatively assigned.

$Arg^6$-$Arg^7$ bond, followed by the dipeptidyl carboxypeptidase action of angiotensin-converting enzyme on the generated N-terminal fragments. The involvement of angiotensin-converting enzyme is supported by the previous reports that β-neo-endorphin (16) and dynorphin-(1-8) (17) were degraded by this enzyme. The material in peak h of Fig. 1 (d) was unambiguously identified as fragment (1-11) on the basis of its composition (Tyr, Gly, Phe, Leu, Arg, Ile, Pro, Lys in a molar ratio of 7 : 20 : 9 : 10 : 26 : 9 : 9 : 9). The fact that this is the almost sole fragment detectable in the cleavage product obtained in the presence of N-ethylmaleimide indicates the involvement of, at least, an N-ethylmaleimide-sensitive protease in the initial cleavage around the $Arg^6$-$Arg^7$ bond. The formation of fragment (1-11) in the presence of N-ethylmaleimide may be due to the action of a DFP-sensitive protease and/or angiotensin-converting enzyme. Detection of this fragment in the presence of N-ethylmaleimide and captopril (date not shown) supports the involvement of DFP-sensitive protease.

It has been shown (23) that the removal of the N-terminal tyrosine residue from enkephalin results in loss of its opiate activity. The detection of free tyrosine as peak b in the presence or absence of captopril may imply the action of an aminopeptidase in the membrane on the N-terminal fragments generated by the N-ethylmaleimide-sensitive endopeptidase. As the membrane-bound enkephalin-degrading aminopeptidase has been reported to be inactive on dynorphin-(1-13) (24), the initial cleavage of dynorphin-(1-13) by the N-ethylmaleimide-sensitive endopeptidase is considered to be necessary for the action of the membrane-bound aminopeptidase.

Lesile and Goldstein (18) have reported the generation of dynorphin-(1-12) from dynorphin-(1-13) with rat brain membranes. However, this could not be confirmed with our membrane preparation under the more physiological conditions (i.e. 0.15 M NaCl) used in this study.

In conclusion, the action of the N-ethylmaleimide-sensitive cysteine protease described in this communication will have physiologically important consequences for dynorphin; first, an increased susceptibility to the attack

of membrane-bound aminopeptidase and angiotensin-converting enzyme; second, a change in receptor specificity; finally, a drastic decrease in opiate activity. The cysteine protease seems to share these roles partly with a DFP-sensitive protease in the membrane. To elucidate the function of these proteases, attempts to purify them from the neuroblastoma cell and from rat brain are now in progress.

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