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A PUTATIVE OPIOID-PEPTID PROCESSING ACTIVITY IN ENRICHED GOLGI FRACTION FROM RAT BRAIN

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A Golgi enriched fraction from rat brain was prepared. The preparation has no carboxypeptidase activity and is not contaminated with cytosol, mitochondria and lysosomes as judged by marker enzyme activities for these constituents. Associated with the Golgi membranes a putative opioid peptide processing activity was demonstrated, which acts on Dynorphin 1-13, α- and ß-Neoendorphin. The enzyme cleaves the bond between the paired basic residues, releasing Leucine-enkephalin-Arg^6. The activity has a pH-optimum around 9 and is inhibited by serine-protease inhibitors. Intracellular location and substrate specificity suggest that this endopeptidase activity may be involved in proenkephalin processing.

Peptide hormones are synthesized by limited proteolysis of the precursor proteins which are known as prohormones. Sequence analysis of these precursor proteins indicates that paired basic residues flank the biologically active peptides (1,2). It has therefore been proposed that paired basic residues serve as typical signals for processing the prohormones to the active hormones (3-5). The processing of the proenkephalin is thought to proceed by the sequential action of trypsin-like and carboxypeptidase B-like activities to yield the biological active peptides (6). Carboxypeptidase B-like activities which may be involved in the processing of proenkephalin and proopiomelanocortin or provasopressin have been demonstrated in various tissues (7-9). Trypsin-like activities have been found in secretory granules of islets of Langerhans that cleave proinsulin (10) and proopiomelanocortin (11). Recently, the purification and characterization of a pro-opiomelanocortin converting enzyme (12) and a putative prohormone processing protease (13) the former from pituitary and the latter from adrenal medulla, have been described. Since at least some steps of peptide processing from precursor proteins are thought to be located in the Golgi apparatus (14), we isolated an enriched

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Golgi fraction from rat brain in which we could demonstrate the presence of an endopeptidase cleaving at paired basic residues of opioid peptides. This enzyme is likely to be involved in the processing of enkephalins.

MATERIALS AND METHODS

Dynorphin 1-13, α-neoendorphin, ß-neoendorphin, phenylmethanesulfonylfluoride, D-Val-Leu-Lys-p-nitroanilid, L-lysin-p-nitroanilid, L-Arg-p-nitroanilid, leupeptin, phenanthroline, were purchased from SERVA, Heidelberg, FRG; antipain and leucine-hydroxamate were from SIGMA, München, FRG; Captopril was kindly donated by Fa. Heyden, München, FRG; leucinthiol was a gift from Dr. W.W.-C. Chan, monoamidin was kindly donated by Dr. O. Dann and thymosin $ß_4$ by Dr. E. Hannappel. All other reagents were of analytical grade.

Sprague-Dawley rats, 10-12 days old, kindly provided by the Institut für Toxikologie und Pharmakologie der Universität Erlangen-Nürnberg, were used in these experiments.

The preparation of the enriched Golgi fraction from rat brain was done according to the procedure described in (15) with minor modification. Only 10 or 20 rats were used for each experiment. Briefly, the animals were sacrified under carbondioxide anesthesia and the brains including cerebellum and medulla were removed and placed in ice-cold medium. The brains were sliced and treated with trypsin. After removal of the trypsin by repeated washings with soybean trypsin inhibitor and medium the tissue was passaged through a 100 mesh nylon screen and the neurons isolated by Ficoll density gradient centrifugation. The cells were homogenized by a motor-driven Teflon glass homogenizer. After removing the cell debris, the enriched Golgi fraction was isolated by sucrose density centrifugation as described in (15). Fraction F_1 was further analyzed.

The reaction mixture contained 10 µg of the various synthetic peptide substrates, 50 µl 0.2 M Tris-SO_4 buffer pH 7.8 (except otherwise indicated) and various amounts of enriched Golgi fraction F_1 in a final volume of 200 µl. Incubation conditions were chosen such that degradation of the peptide substrates at 22°C was linear with time and the amount of fraction tested. Sample blanks were included in each experiment. No spontaneous hydrolysis of the substrates occured during the incubation. The reaction was stopped by freezing the samples at -20°C. Products of enzymatic hydrolysis of synthetic peptides were separated as described in (16). Peptides and peptide fragments were separated on a Spherisorb RP-18 column (5 µ) 25 x 0.4 cm by application of a 30 min linear gradient of acetonitrile ranging from 15-30% in 0.1% H_3PO_4 adjusted to pH 2 with NaOH.

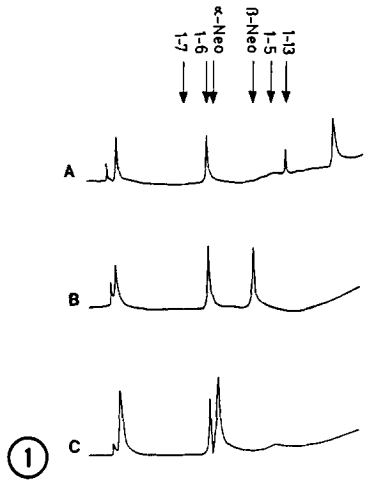
Emerging peaks were monitored at 206 nm. The respective peptide peaks were identified by cochromatography with authentic peptides and the degradation rates were calculated by monitoring the disappearence of substrate.

The fraction F_1 was characterized using activities known to be localized predominantly in specific organelles of rat tissues. For mitochondria NADH-cyt c reductase (17), for cytosol lactate-dehydrogenase (18), for lysosomes according to (19) with paranitrophenyl glucuronide or paranitrophenyl sulfat resp. and for Golgi apparatus UDP Gal : Glc Nac galactosyl transferase (20) was measured.

Protein was measured as described in (21).

RESULTS

The enriched Golgi fraction F_1 prepared from rat brain contained no lactate dehydrogenase, NADH-cyt c reductase and no glucuronidase and aryl-



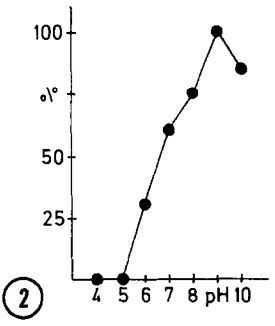


Fig 1: HPLC-profiles of the degradation of various opioid peptides by a opioid processing endopeptidase associated with the Golgi apparatus of rat brain.

Incubation was performed as described under Materials and Methods. A, Dynorphin 1-13 (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys); B, α-neoendorphin (Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys); C, ß-neoendorphin (Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro). The arrows above the panel indicate the elution position of the authentic substances. 1-5, (Tyr-Gly-Gly-Phe-Leu); 1-6, (Tyr-Gly-Gly-Phe-Leu-Arg); 1-7 (Tyr-Gly-Gly-Phe-Leu-Arg-Arg; 1-13, Dyn 1-13.

Fig 2: pH-profile of the degradation of ß-neoendorphin by the opioid-peptide processing endopeptidase associated with the Golgi apparatus of rat brain.

Incubations were performed in 50 mmol/l potassium phosphate containing 100 mmol/liter sodium chloride, adjusted to the various pH values by mixing KH_2PO_4 and K_2HPO_4 solutions. The points indicate relative hydrolysis.

sulfatase activity, indicating the complete absence of cytoplasm, mitochondria and lysosomes. However, UDP Gal : Glc Nac galactosyl transferase activity (250 nmol x mg^{-1} x h^{-1}) could be detected indicating the presence of Golgi membranes in the F_1 fraction.

Figure 1 shows the HPLC profiles of the degradation of α-, ß-neoendorphin and dynorphin 1-13 by the F_1 fraction. From all three peptides exclusively Dyn 1-6 is generated, which has been identified by cochromatography of the authentic substance. The pH profile of the F_1 activity of enriched Golgi fraction with ß-neoendorphin as substrate shows a maximum activity at pH 9 with no activity at pH 5 and below, indicating that no lysosomal protease is present in the preparation (Fig. 2). The results reported in Table 1 suggest the presence of an activity which cleaves the peptide bond

Table 1: Substrate specificity of the enriched Golgi fraction from rat brain with cleavage sites indicated by arrows

SUBSTRATE	STRUCTURE	RELATIVE ACTIVITY
α-neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg↓Lys-Tyr-Pro-Lys	0.35
β-neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg↓Lys-Tyr-Pro	0.5
Dyn 1-13	Tyr-Gly-Gly-Phe-Leu-Arg↓Arg-Ile-Arg-Pro-Lys-Leu-Lys	1
Dyn 1-9	Tyr-Gly-Gly-Phe-Leu-Arg↓Arg-Ile-Arg	0.28
Met-Enk-Arg-Gly-Leu	Tyr-Gly-Gly-Phe-Met-Arg↓Gly-Leu	0.45
	Not hydrolyzed by the preparation:	
Thymosin β_4	Ac-Ser-Asp-Lys-Pro-Asp-Met-Ala-Glu-Ile-Glu-Lys-Phe-Asp-Lys-Ser-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-Ser-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys. Glyn-Ala-Gly-Glu-Ser	—
Met-Enk	Tyr-Gly-Gly-Phe-Met	—
Leu-Enk	Tyr-Gly-Gly-Phe-Leu	—
Met-Enk-Arg	Tyr-Gly-Gly-Phe-Met-Arg	—
Met-Enk-Arg-Phe	Tyr-Gly-Gly-Phe-Met-Arg-Phe	—
Leu-Enk-Arg	Tyr-Gly-Gly-Phe-Leu-Arg	—
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	—
Lys-p-nitranilid		—
D-Val-Leu-Lys-p-nitranilid		—
Benzoyl-Arg-nitranilid		—

The peptides (20 µg) were incubated for 18-22 h with the Golgi preparation as described in Materials and Methods. The reaction mixtures were analyzed by reverse-phase HPLC. The liberated p-nitranilin was measured at 400 nm. Basic amino acids are underlined.

formed by arginine residue 6 in peptides having 8 to 13 amino acids. The degradation velocity was 0.7 µmol x mg^{-1} x h^{-1} with β-neoendorphin as substrate. Thymosin β_4 which contains a Lys-Lys sequence is not attacked. Shorter peptides like enkephalin or enkephalin containing peptides with

Table 2: Effect of various agents on opioid-peptide processing activity of rat brain

Agent	conc. in assay	type of protease inhibited	% activity of control
None			100
Phenyl-methan-sulfonyl-fluoride	0.1 mg/ml	Serine	66
Monoamidin	34 µg/ml	Serine	20
Antipain	0.1 mg/ml	Serine/-SH	32
Leupeptin	0.1 mg/ml	Serine/-SH	51
p-hydroxymercuri-benzoate	1 mM	-SH	95
$CuSO_4$	0.1 mM	-SH	85
$CoCl_2$	0.85 mM	-SH	76
Phenanthroline	1 mM	Metallo	97
EDTA	1 mM	Metallo	100
Captopril	1 mM	Metallo (ACE)	100
Leucine-hydroxamate	1 mM	Aminopeptidase	92
Leucinthiol	0.01 mM	Aminopeptidase	100
$ZnCl_2$	0.1 mM		100
$CaCl_2$	10 mM		100
$MgCl_2$	0.1 mM		95
ATP/$MgSO_4$	1 mM/0.5 mM		100

The activity was assayed with β-neoendorphin as substrate. ACE = angiotensin converting-enzyme.

C-terminal basic residues as well as bradykinin are not hydrolyzed by the preparation, indicating that there is no carboxypeptidase B-like or aminopeptidase activity in our F_1 preparation.

Of the protease inhibitors tested only those specific for serine-proteases had a marked effect (Table 2). The best inhibition was achieved with monoamidin, a known trypsin-inhibitor (22). The synthetic substrates Lys-p-nitranilid and benzoyl-Arg-nitranilid, frequently used to assay trypsin activity (23) are not attacked by the preparation, suggesting that the endopeptidase activity described here is not identical with trypsin.

DISCUSSION

In this paper we present evidence that there is a putative opioid peptide processing activity associated with Golgi membranes of rat brain. From the pH optimum and substrate specificity as well as the sensitivity towards inhibitors this endopeptidase activity can be attributed to a serine type protease distinct from trypsin. The enzyme cleaves enkephalin-containing peptides composed of at least 8 amino acids at paired basic residues, thus liberating hexapeptides with a C-terminal arginine. Several putative prohormone processing activities from bovine adrenal medulla, yeast, rat brain membranes and islets of Langerhans have been reported (10-13, 24-26). Among these activities there are serine type proteases with alkaline (13,25,26) or acid pH optima (12), thiol proteases with alkaline (27) or acid pH optima (10,12), which all act on paired or single basic residues. The activity reported here resembles the enzyme from yeast described by Mizuno et al. (26) since it (i) generates enkephalin hexapeptides with C-terminal basic amino acid and (ii) it acts on ß-neoendorphin as well.

On the other hand, the endopeptidase from the Golgi apparatus has a broader specificity than that from adrenal medulla which is specific for the Lys-Arg bond and does not cleave ß-neoendorphin (13). Another serine-typ enzyme from adrenal medulla was described by Lindberg et al. (25). Like these activities, the Golgi enzyme does not act on synthetic substra-

Table 3

Effect of various analogues on induced artificial propagation of sterlet

Hormone	Treatment	Apl.	Treated	Reacted /Ovulated/	%	Fertility rate of the ovulated eggs+ %
D-Phe^6-Gln^8-GnRH	5 x 30 µg/24 hours	ip.	10	10	100	80-90
D-Phe^6-Gln^8-GnRH-EA	5 x 30 µg/24 hours	ip.	10	10	100	80-90
Trp^7-Leu^8-GnRH	5 x 30 µg/24 hours	ip.	10	3	30	70-80
Trp^7-Gln^8-GnRH	5 x 30 µg/24 hours	ip.	10	1	10	60
Phe^7-Gln^8-GnRH	5 x 30 µg/24 hours	ip.	10	3	30	70-80
Phe^7-Leu^8-GnRH	5 x 30 µg/24 hours	ip.	10	0	0	0
D-Phe^6-Trp^7-Leu^8-GnRH	5 x 3o µg/24 hours	ip.	10	4	40	70-80
D-Phe^6-GnRH-EA	5 x 3o µg/24 hours	ip.	10	0	0	0
GnRH	5 x 30 µg/24 hours	ip.	10	0	0	0
Gln^8-GnRH	5 x 30 µg/24 hours	ip.	10	0	0	0

+ Fertility rate during in vitro fertilisation of the ovulated eggs

and FSH released into the medium during the incubation were measured by RIA. In this system the cGnRH, sGnRH and their DPhe^6 counterparts - except the extremely high dose - showed lower LH and FSH releasing activity than mGnRH, though the DPhe^6 substitution preserved it's ability ro rise the biological effect of the given peptide.

In the case of the in vivo experiments, we measured the effect of sGnRH, cGnRH and their analogs on the artificial propagation of fishes (Table 3). Surprisingly among the tested peptides the agonistic analogs of cGnRH were the most potent materials for the artificial propagation of fishes. In every cases, they had 100 % activity for inducing ovulation or spermiation; however the sGnRH and it's DPhe^6 analogs were less potent in stimulating reproductive functions. The untreated fish because they do not reproduce in captivity didn't show any changes. The DPhe^6 and the DPhe^6-ethylamide analogs of cGnRH were very potent in different dosages and type of treatment. With the aid of these analogs we were able to propagate artificially those fishes which couldn't be artificially propagated before, for example sterlet and the process was succesfully applied during and out of season too.

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