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HETEROGENEOUS DISTRIBUTION OF ENKEPHALIN-DEGRADING PEPTIDASES BETWEEN NEURONAL AND GLIAL CELLS

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Cultured neurones, astroblasts and astrocytes from murine brain have been screened with specific tests for the presence of peptidases capable of degrading enkephalin. Bestatin-sensitive aminopeptidases represent the major enkephalin-degrading activity in all cases. The dipeptidylaminopeptidasic activity is much higher in the neuronal than the glial cultures, whereas the opposite is true for the metallopeptidase called "enkephalinase". Only trace amounts of the dipeptidylcarboxypeptidase "angiotensin-converting enzyme" have been found. We conclude that bestatin-sensitive aminopeptidases on nerve cells are probable candidates for enkephalin-inactivating enzymes, whereas the "enkephalinase" on glial cells more likely serves a scavenger function.

Leu-enkephalin and Met-enkephalin (Tyr-Gly-Gly-Phe-$^{Leu}_{Met}$) are endogenous opiate peptides (1), which presumably act as neuromodulators in the central nervous system. It is generally assumed that the enkephalinergic signal is switched off by peptidases in the vicinity of the enkephalin-receptor. Several enzymes capable of hydrolyzing enkephalin in vitro have been partially purified from brain homogenates or crude membrane preparations [for review see (2)], but their localization and biological function in the central nervous system is not known so far. In the present study we have screened primary cultures of neurones, astroblasts and morphologically differentiated astrocytes from murine brain with specific tests for the presence of enkephalin-degrading peptidases.

MATERIALS & METHODS

Materials. [Tyr-^{3}H]Leu-enkephalin (50 Ci/mmol) and [Gly-^{14}C]Hippuryl-His-Leu (3 mCi/mmol) were purchased from New England Nuclear, [^{3}H]acetic anhydride (7 Ci/mmol) from Amersham. Tyrosine, Tyr-Gly, Tyr-Gly-Gly, Leu-enkephalin, N-succinyl-Ala-Ala-Phe-7-amido-4-methylcoumarin and Tyr-ß-naphthylamide were obtained from Bachem AG, Bubendorf, Switzerland, and Tyr-Gly-Gly-Phe from

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Cambridge Research Biochemicals Ltd., Cambridge, U.K. Bacitracin, puromycin and Leucine-aminopeptidase M (42 units/ml) were from Sigma Chemical Company. Captopril was a product from Squibb, Princeton, New Jersey, USA. Bestatin and phosphoramidon were generous gifts of Dr. Hamao Umezawa, Microbial Chemistry Foundation, Tokyo, Japan.

Cell culture. Neurones (3) and astroblast-rich cultures (4) from rat and mouse brains, respectively, were grown on plastic Petri dishes (diameter 50 mm) as described. Some of the astroblast-rich cultures were treated with 1 mM dibutyryl-cAMP for one week to induce differentiation (5) to astrocyte-like cells (in the following referred to as astrocytes). The protein content per dish was 0.49 mg for the neurones, 0.65 mg for the astroblasts and 0.29 mg for the astrocytes. At the start of the experiments, the cells were washed with 5 ml of HEPES-buffered medium and then maintained at 37°C in 2 ml of this medium, which consisted of 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 20 mM glucose and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid (HEPES) adjusted to pH 7.4 with NaOH. The osmolarity of the medium was 330 mOsmolxl^{-1}. As estimated from the percentage of lactate dehydrogenase in the 100,000xg supernatant of media which had been conditioned for 4 hours, the viability during the experiments was 95 % for the astrocytes and greater than 98% for the neurones and astroblasts.

Fragmentation analysis. [Tyr-^{3}H]Leu-enkephalin (10 µCi) was added to the different cultures and after two hours, aliquots of 20 µl were withdrawn from the media and spotted on silica gel plates. Tyr, Tyr-Gly, Tyr-Gly-Gly, Tyr-Gly-Gly-Phe and Leu-enkephalin were used as marker peptides. After development in $CHCl_3$-CH_3OH-CH_3COOH-H_2O (90:60:12:18 v/v/v/v), the plates were scanned for radioactivity using a Berthold Linear TLC Analyzer LB 2832, and the marker peptides were localized by spraying with ninhydrin.

Enzyme tests. All incubations were performed at 37°C. When inhibitors were used, the cultures were preincubated without the substrates for 15 min. At 0, 30, 60, 120 and 240 min after addition of the substrates, aliquots of the media were analyzed, and the enzymatic activities were calculated from the initial rates of product formation. In control experiments conditioned media were used. In the case of the dipeptidylaminopeptidases, total cell homogenates were also examined.

a) Aminopeptidases. [Tyr-^{3}H]Leu-enkephalin (10 µCi) was added to the different cultures (final concentration 100 nM). After given time intervals, aliquots of 20 µl were resolved by thin-layer chromatography as described for the fragmentation analysis. The plates were scanned for radioactivity and the percentage of [^{3}H]tyrosine formation was determined using the TLC Analyzer and a computer program.

b) Arylamidases. Tyr-ß-naphthylamide (final concentration 0.1 mM) was added to the cultures. At given times, aliquots of 75 µl were withdrawn and mixed with 75 µl of 200 mM glycin-NaOH buffer pH 10.5. The fluorescence of these samples was then determined in an Amicon fluorescence spectrophotometer (excitation at 340 nm, emission at 410 nm). The amount of ß-naphthylamine was calculated from a standard curve.

c) Dipeptidylaminopeptidases. The activity was determined as described for the aminopeptidases with the following modifications: the aminopeptidase inhibitor bestatin (100 µM) and the "enkephalinase" inhibitor phosphoramidon (1 µM) were present during the incubation, and the formation of [^{3}H]Tyr-Gly was determined.

d) "Enkephalinase". [Acetyl-^{3}H]N-acetyl-enkephalin was synthezised by acetylation of Leu-enkephalin with [^{3}H]acetic anhydride, and the product was purified by thin-layer chromatography on silica gel plates using the solvent system $CHCl_3$-CH_3OH-CH_3COOH (8:2:1 v/v/v), R_f = 0.42. [^{3}H]N-acetyl-enkephalin (10 µCi) were added to the cultures (final concentration 0.7 µM). At given times, aliquots of 20 µl were resolved by thin-layer chromatography as described for the purification of the substrate. N-acetylated tyrosine and peptides were used as marker substances and localized by spraying with Pauly's reagent. The plates were scanned for radioactivity and the percentage of [^{3}H]

N-acetyl-Tyr-Gly-Gly formation was determined using the TLC-Analyzer described above. R_f(N-acetyl-Tyr-Gly-Gly) = 0.18. In control experiments, 10 µM phosphoramidon was present in the cell cultures. The phosphoramidon-sensitive release of the tripeptide represents the activity of the "enkephalinase".
e) Thermolysin-like metallopeptidase. N-succinyl-Ala-Ala-Phe-7-amido-4-methylcoumarin (6) was added to the cultures (final concentration 0.1 mM). After given time intervals, aliquots of 75 µl were withdrawn, supplemented with 10 µM phosphoramidon and 2 milli-units of leucine-aminopeptidase M and incubated for 30 min. After addition of 75 µl of 200 mM glycine-NaOH buffer pH 10.5, the fluorescence (excitation at 383 nm, emission at 455 nm) was determined as described for the arylamidases. The amount of 7-amino-4-methylcoumarin was calculated from a standard curve. In control experiments, phosphoramidon was present in the cell cultures from the beginning. The phosphoramidon-sensitive activity represents the thermolysin-like metallopeptidase.
f) "Angiotensin-converting enzyme". The activity was determined as described using [Gly-^{14}C]hippuryl-His-Leu (7). In control incubations, 1 µM captopril was present in the cultures.
g) Lactate dehydrogenase. The activity was determined as described (8).

Protein determination. The cells of 10 culture dishes were scraped off with 10 ml of HEPES-buffered medium and homogenized by sonication. Protein was determined by a modification of the Lowry procedure (9).

RESULTS & DISCUSSION

N4TG1 neuroblastoma and human endothelial cells are known to hydrolyze enkephalin predominantly at the Tyr^1-Gly^2 peptide bond (10,11). Addition of [Tyr-^{3}H]Leu-enkephalin to primary cultures of neurones and astroblasts from rat and mouse brain, respectively, also leads to the formation of [^{3}H]Tyr as the major radioactive product (see Fig. 1a,b). In the neuronal culture, trace amounts of [^{3}H]Tyr-Gly and [^{3}H]Tyr-Gly-Gly are also present, whereas [^{3}H]Tyr-Gly could not be detected in the glial culture under these conditions. When the release of [^{3}H]Tyr is inhibited by bestatin, [^{3}H]Tyr-Gly is the main fragment in the neuronal culture and [^{3}H]Tyr-Gly-Gly in the glial culture (see Fig. 1c,d). Similar observations concerning the enkephalin-degrading activities in cultures of rat astroblasts have been reported recently (12). Differentiation of astroblasts upon treatment with dibutyryl-cAMP does not qualitatively change the fragmentation pattern (not shown). The different patterns of breakdown products produced by neuronal and glial cultures indicated a heterogeneous distribution of enkephalin-degrading peptidases between these cells and prompted us to determine the specific activities of the individual enzymes (Tab. 1)

As shown above, the liberation of N-terminal tyrosine is the major mode of enkephalin degradation by the two cell preparations, albeit their aminopepti-

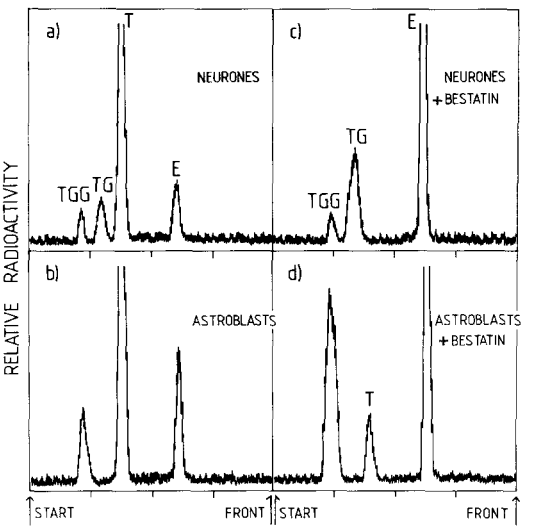

Fig. 1. Fragmentation of [Tyr-^{3}H]Leu-enkephalin by neurones and astroblasts.
[Tyr-^{3}H]Leu-enkephalin was added to cultures of neurones (a) and astroblasts (b). After two hours, aliquots of the media were analyzed by thin-layer chromatography as described under Methods. When bestatin (100 µM) was present during the incubation, the fragments shown in panels c and d were obtained. T, tyrosine; TG, Tyr-Gly; TGG, Tyr-Gly-Gly; E, enkephalin.

dasic activity differs quantitatively to some extent. The treatment of astroblasts with dibutyryl-cAMP seems to induce this activity threefold without affecting the other enzymes tested in this study. The Tyr-ß-naphthylamide hydrolyzing activity is present in comparable amounts in the three cultures.

Tab. 1. Specific activities of enkephalin degrading peptidases.

cell culture	specific activity ($\frac{\text{unit}}{\text{mg of protein}}$)					
	amino-peptidases	aryl-amidases	dipeptidyl-amino-peptidases	"enkephalinase"	thermolysin-like metallo-peptidase	"angiotensin-converting enzyme"
neurones	2.38	9.56	0.27	0.07	0.28	0.02
astroblasts	1.18	6.96	0.02	0.74	1.93	0.04
astrocytes	3.38	8.36	0.03	0.79	1.92	0.04

The enzyme activities were determined as described under Methods. In the radiochemical tests, one unit is defined as the formation of 1 % product per min.; in the fluorogenic tests, one unit is defined as the release of one nmol product per min. The values are the mean of duplicate determinations, which differed by less than 15 %. In control experiments with conditioned media it was found that soluble enzyme activities resulting from cell leakage can be neglected against the cell-bound activities except for the dipeptidylamino-peptidasic activity (neurones 0.2, astroblasts 0.01, astrocytes 0.03 units/ 2 ml of conditioned medium).

Tab. 2. Inhibition of the aminopeptidasic and arylamidasic activities.

cell culture	enzyme activities	inhibition (%)		
		bestatin	puromycin	bacitracin
neurones	aminopeptidases	100	86	89
	arylamidases	92	96	36
astroblasts	aminopeptidases	94	26	76
	arylamidases	82	80	22
astrocytes	aminopeptidases	96	67	72
	arylamidases	92	87	20

The enzyme activities were determined as described under Methods. The inhibitor concentration was always 100 µM.

Since the brain contains a variety of aminopeptidases and arylamidases (13), we have tried to differentiate between individual enzymes by use of aminopeptidase inhibitors. As shown in Tab. 2, significant differences are obvious, which indicate a heterogeneous cellular distribution of aminopeptidases and their non-identity to arylamidases.

The specific activity of the dipeptidylaminopeptidase (14), a hitherto poorly characterized enzyme, is much higher in the neuronal than the glial cultures. As estimated from the soluble dipeptidylaminopeptidasic activity in conditioned media (see legend to Tab. 1), the cell-bound activity is very low compared to the activity which leaks out of the cells during the incubation. This agrees with the observation that crude membrane preparations exhibit only trace amounts of this activity (12). On the other hand, total cell homogenates contain high dipeptidylaminopeptidasic activity, which also exhibits a heterogeneous cellular distribution: neurones 20.4, astroblasts 2.58, astrocytes 2.93 units/mg of protein. Considering these results it cannot be excluded that the cell-bound activity is due to an intracellular enzyme which adsorbs to the cells after leakage into the medium. Using histochemical methods, Gorenstein et al. demonstrated the presence of a dipeptidylaminopeptidase in neuronal populations of the brain (15). Since they employed the fluorogenic substrate Lys-Ala-ß-naphthylamide, which is also susceptible to hydrolysis by the lyso-

somal dipeptidylaminopeptidase II (16), it is not clear, whether this enzyme is identical to the enkephalin-degrading activity.

The "enkephalinase" hydrolyzes enkephalin at the Gly^3-Phe^4 peptide bond (17). The specific activity of this enzyme in the neuronal culture amounts to only one tenth of the specific activity in the glial cultures. Approximately the same difference has been found for the thermolysin-like metallopeptidase, which seems to be identical to the "enkephalinase" (18,19). Since the neuronal culture contains about 10 % non-neuronal cells, it is possible that the "enkephalinase" in this culture is produced by these cells.

The "angiotensin-converting enzyme", which also releases Tyr-Gly-Gly from enkephalin, is present only in trace amounts in the three cultures. This is confirmed by the fact that the hydrolysis of the Gly^3-Phe^4 peptide bond of N-acetyl-enkephalin can almost completely be inhibited (> 90 %) by the "enkephalinase" inhibitor phosphoramidon (19), whereas the "angiotensin-converting enzyme" inhibitor captopril (20) has little influence on this cleavage (< 10 %). It should be noticed that the "angiotensin-converting enzyme" may be produced by endothelial cells, which can be present in the primary cultures (21) and which are known to contain high amounts of this enzyme (22). No evidence for an enkephalin-degrading carboxypeptidase on the cells could be obtained.

These results demonstrate that certain plasma-membrane bound peptidases are not general membrane-constituent enzymes, but are heterogeneously distributed between different types of cells reflecting specialized functions. While neuronal peptidases may be involved in turning off peptidergic signals, glial peptidases more likely serve a scavenger function. Although the structural and functional integrity of cell interactions in the brain is not conserved in primary cultures of neurones and glial cells, the screening of these cultures with specific tests seems to be a useful approach to distinguish between these two functions and to select putative neuropeptide-inactivating enzymes. In the case of enkephalin, bestatin-sensitive aminopeptidases on nerve cells are probable candidates for such a role, whereas the "enkephalinase" may be involved in the hydrolysis of peptides outside the synaptic cleft.

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