Effect of Differentiation on the Leucine Enkephalin-Degrading Soluble Enzymes Released by the K562(S) Cell Line

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Leu-enkephalin hydrolysis kinetics were measured in the presence of soluble supernatants obtained from cultures of the K562(S) leukaemic cell line. Under these conditions, the substrate is degraded with formation of two distinct patterns of the hydrolysis by-products: in one pattern, similar amounts of Tyr and Tyr-Gly are formed; in the other, only Tyr-Gly can be measured. Kinetic data suggest that soluble proteolyses are released by these cells, and that either dipeptidylaminopeptidases alone, or both aminopeptidases and dipeptidylaminopeptidases are involved in substrate hydrolysis. This alternation of hydrolysis patterns appears consistent with existing data on the heterogeneity of K562 cells. In contrast with these results, chromatographic separation of the soluble enzymes indicates the release of all three classes of proteolyses known to hydrolyze enkephalins: aminopeptidases, dipeptidylaminopeptidases and dipeptidylcarboxypeptidases. In cells induced to differentiate by treatment with butyric acid, substrate hydrolysis is increased, and the pattern of the enzymes released is modified. In these cells, variations in both total proteolytic activity, and ratio between the three enzyme classes mentioned above are only minor, while the ratio between the different enzyme species within each class is greatly modified. Data obtained suggest that the expression of soluble enzymes is modified by differentiation. These data may also be interpreted as stressing the role of competition in controlling substrate hydrolysis by the multiple enzymes co-released by K562(S) cells.

KEY WORDS: Differentiation; leu-enkephalin, hydrolysis.

INTRODUCTION

As is the case with other plasma peptides, enkephalins (1,2) released into the bloodstream (3) are rapidly hydrolyzed (4) by enzymes belonging to three different compartments: those present in plasma as soluble molecules (e.g. 5), those embedded in the membrane of immunocompetent cells (e.g. 6,7) and those released by circulating cells, especially by immunocompetent cells (8). All these enzymes degrade the substrate at its two terminals; specifically, opioid peptides are hydrolyzed at the N-terminal region by aminopeptidases and dipeptidylaminopeptidases, at the C-terminal by dipeptidylcarboxypeptidases.

These enzymes—especially the membrane enzymes—are thought to hydrolyze plasma peptides in order to terminate their active life; they are also thought to be involved in the regulatory degradation of several neuropeptides and cytokines (9,10,11). Endopeptidase 24.11 hydrolyzes neurotensin (12), substance P (13) and enkephalins (14,13). Bradykinin, and the chemotactic peptide fMet-Leu-Phe are degraded by enkephalinase released by human neutrophils, so that a role of this enzyme in modulating chemotaxis has been suggested by...
Connelly et al. (9). Similarly, the involvement of enkephalinase in IL-1 degradation lead Pierart et al. (11) to hypothesize its role in the modulation of the immune response. Comparable roles have been suggested for soluble enzymes as well: Caughey et al. (15) evidenced the cleavage of substance P and vasoactive intestinal peptide by mastocyte-released enzymes, while the hydrolysis of kinetensin, leu-enkephalin and mast cell-active neurotensin by the enzymes released by human mast cells has been described by Goldstein et al. (16). These enzymes degrade neurotensin as well, and Cochrane et al. (17) evidenced increased hydrolysis of this peptide by stimulated as against resting mast cells.

Marini et al. (18), describing enkephalin hydrolysis by the soluble proteolyses released by immunocompetent cells, evidenced the involvement of all three classes of enzymes that degrade opioid peptides: aminopeptidases, dipeptidylaminopeptidases and dipeptidylcarboxypeptidases. In addition, in stimulated as against naive peripheral blood mononuclear cells (PBMC), leu-enkephalin hydrolysis was found to increase, and the ratio between the activity of the different enzyme classes was found to be altered (19,8). These modifications appear to be similar to those induced by the stimulation of several cell populations on the expression of membrane-bound enzymes, such as those reported above in the case of mast cells (17). Other cases described are modified expression of aminopeptidase N by ConA-stimulated T cells (20), that of several enzymes in leukocytes stimulated with C5a (21), the expression of aminopeptidase N by IL-4-stimulated immunocompetent cells (22), and of dipeptidyl peptidase IV and aminopeptidase N by human renal cells stimulated with IL-4 and IL-13 (23).

Above data only indicate that plasma peptides are in fact hydrolyzed by the above-mentioned proteolyses; the functional role of these enzymes in controlling the plasma peptide-mediated transmission of information is, however, corroborated by comportational data. According to Chipkin and Coffin (24), and to Saksida et al. (25), treatment with enkephalinase inhibitors induces antinociceptive effects that are suppressed by opioid antagonists. Similarly, Katoh et al. (26) evidenced that non-hydrolyzable enkephalin analogues—as well as proteolysis inhibitors—modify the behaviour of mice under stressful conditions, while naturally-occurring, hydrolyzable endorphins cannot modify these parameters.

Data reported pertain almost exclusively to membrane-bound enzymes. However, the role of the soluble proteolyses released by blood cells can be supposed to be similar, allowing for the differences in substrate accessibility caused by the reduced steric hindrance of soluble as compared to membrane enzymes. The possible relevance of these phenomena lead us to investigate the release of proteolytic enzymes in the K562(S) leukaemic subline (27). Upon stimulation with agents such as hemin or butyric acid (28), these cells differentiate, expressing both erythropoietic and granulopoietic markers (29). Cells of this line have therefore been used as a model to study the possible effects of differentiation on the expression of soluble enzymes capable to hydrolyze leu-enkephalin.

**EXPERIMENTAL PROCEDURE**

**Cell Culture.** Cells of the K562(s) subline were aliquoted and kept under liquid nitrogen. Soluble supernatants were obtained from freshly-cultured cells maintained 10 days in RPMI 1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% of foetal calf serum (Gibco), 100 units/ml penicillin and 0.1 mg/ml streptomycin.

Cell differentiation was induced additioning five hundred µl of butyric acid (30), 0.1 M in RPMI (1640 buffered with 8 X 10^{-3} M NaOH to 25 ml of cell suspension (2.0 X 10^{5} cells X ml^{-1}), for a final concentration of 2 mM. Cells were incubated 48 hours at 37°C, 5% CO_2. To evaluate differentiation, harvested cells were washed in PBS, resuspended at 1 X 10^{6} x ml^{-1} in the same buffer, and 25 µl of suspension were transferred to 96 wells round-bottom trays (Nunc). Concanavalin A (ConA) in phosphate buffered saline pH 7.2 (PBS) was added to cells to a final concentration ranging from 15 to 500 µg x ml^{-1}. Cells were incubated for 30 minutes at room temperature, and differentiation was estimated by the minimum ConA concentration capable of inducing agglutination (approximately 15 µl x ml^{-1} for non-differentiated cells, 250 µg x ml^{-1} for differentiated cells).

Soluble supernatants were prepared as follows: cells were resuspended in RPMI at a density of 2 X 10^{5} x ml^{-1}, transferred to 260 ml flasks (Nunc, Ry, Denmark), and cultured for 48 hours. Cells (1 X 10^{6}) were washed twice in PBS, resuspended at a concentration of 3.5 X 10^{6} x ml^{-1} and incubated 90 minutes at 37°C in a thermostat shaking bath. Incubation mixtures were transferred into ice, and centrifuged 10 minutes at 3,000 g. Pelletted cells were discarded, and supernatants were filtered through a 0.22 µm Millex filter (Millipore Co, Norwalk, CO, U.S.A.).

**Assay**

**Enzyme Assay.** Soluble supernatants obtained as described above were concentrated to 5 mg x ml^{-1} of protein on a Pellicon PCAC membrane (Millipore), dialyzed overnight at 4°C against 50 volumes of 5 mM N-(2-hydroxyethyl)-piperazine-N'-2 ethane sulfonic acid (HEPES), 10 mM NaCl, 2.5 X 10^{-3} M ZnCl_2 pH 7.2 and cleared by centrifugation (10 minutes at 12,000 g). Tritiated leu-enkephalin (5 µl corresponding to 1.7 X 10^{-12} mol) was added to 50 µl of each sample. Reactions were carried out at 37°C for the periods of time indicated in the Figures and stopped with 5 ul of acetic acid. Intact leu-enkephalin and its hydrolysis by-products were quantified by thin layer chromatography as described below. Protein concentration was estimated by its absorbance at 280 nm.

**Assay of Column Effluents.** Enzymes in the DEAE Fractogel effluent were measured as follows: samples were dialyzed overnight against 5 mM HEPES, 495 mM NaCl, 2.5 X 10^{-4} M ZnCl_2, pH 7.2. Tritiated leu-enkephalin (1.7 X 10^{-12} moles) was added to 25 µl of