Peptidases involved in the degradation of neurotensin by human brain synaptic membranes

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The functioning of neuropeptides as neurotransmitters or neuromodulators requires a mechanism for their inactivation and this is thought to involve membrane-bound synaptic peptidases (Turner, 1986). Several rat synaptosomal peptidases have been shown to be involved in the degradation of neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Pro-Tyr-Ile-Leu-OH) in vitro, the main points of hydrolysis being Arg9-Arg10, Pro10-Tyr11 and Tyr11-Ile12 (McDermott et al., 1983; Checler et al., 1983, 1986). This communication describes attempts to identify the peptidases involved in neurotensin inactivation in human brain.

Synaptosomes were prepared from human post-mortem brain (12 g of frontal cortex; post-mortem delay 15 h) by the method of Dodd et al. (1981). Crude synaptic membranes were prepared by lysis of the synaptosomes for 1 h at 4°C in 5 mM-Tris/HCl (pH 8) and pelleted by centrifugation (160 000 g, 15 min). The membranes were washed twice with 150 mM-NaCl in 10 mM-Tris/HCl (pH 7.4) and twice with 10 mM-Tris/HCl (pH 7.4) at 4°C. Neurotensin (40 μg) was incubated with membranes (1.2 mg of protein/ml) in 10 mM-Tris/HCl (pH 8) and pelleted by centrifugation (12 g of frontal cortex; post-mortem delay 15 h) by the method of Dodd et al. (1981). Crude synaptic membranes were prepared by lysis of the synaptosomes for 1 h at 4°C in 5 mM-Tris/HCl (pH 8) and pelleted by centrifugation (160 000 g, 15 min). The membranes were washed twice with 150 mM NaCl in 10 mM-Tris/HCl (pH 7.4) and twice with 10 mM-Tris/HCl (pH 7.4) at 4°C. Neurotensin (40 μg) was incubated with membranes (1.2 mg of protein/ml) in 10 mM-Tris/HCl (pH 7.4) at 37°C for 15–180 min. The reaction mixture was then acidified with an equal volume of 1% trifluoroacetic acid (TFA) and the degradation products were analysed by reversed-phase h.p.l.c. (Nova-Pak C18 cartridge; 2.1–49% acetonitrile gradient over 20 min containing 1 μM-TFA; 1 μl/min; u.v. monitoring at 214 nm). Products were identified by comparing their elution times with those of standards (McDermott et al., 1982, 1983). After 2 h incubation in the absence of inhibitors, the following were the major peptidase degradation products and their molar ratios: neurotensin [1–8], 100; neurotensin [1–10], 77.8; neurotensin [1–11], 16.1. The lack of corresponding C-terminal neurotensin fragments is explained by their further rapid degradation to amino acids including tyrosine which was identified in the h.p.l.c. profile. The partial inhibition of neurotensin degradation by carboxypeptidase A-Ala-Ala-Phe-p-aminobenzoate (CPE-AAF-pAB; an inhibitor of endopeptidase 24.15; Chu & Orlowski, 1984) was mainly due to prevention of neurotensin [1–8] formation (Table 1), consistent with the inhibition of a membrane-bound variant of endopeptidase 24.15 (EC 3.4.24.15) which cleaves the Arg9-Arg10 bond. Thiorphan (Roques et al., 1980), an inhibitor of endopeptidase 24.11 (EC 3.4.24.11; cleaving at Pro10-Tyr11 and Tyr11-Ile12), had only a small overall inhibitory effect; this was accompanied by the almost complete inhibition of neurotensin [1–11] formation but by an increase in neurotensin [1–10] formation. Therefore a second enzyme (in addition to endopeptidase 24.11) capable of hydrolysing the Pro10-Tyr11 bond must be present on human synaptic membranes as previously reported for rat membranes (Checler et al., 1986). Captopril, an inhibitor of angiotensin-converting enzyme (Ondetti et al., 1977), had no overall effect on neurotensin degradation, but appeared to inhibit conversion of neurotensin [1–10] to neurotensin [1–8] (Checler et al., 1983). Dynorphin [1–13] was the most effective inhibitor; the pattern of product formation was consistent with inhibition of the endopeptidase 24.15-like enzyme (McDermott et al., 1986) and the Pro10-Tyr11-cleaving peptidase, while leaving endopeptidase 24.11 unaffected.

Table 1. Effect of inhibitors on the degradation of neurotensin by human synaptic membranes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition (%)</th>
<th>Formation of neurotensin fragments (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1–8</td>
<td>1–10</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>50 μM-CPE</td>
<td>55</td>
<td>34</td>
</tr>
<tr>
<td>10 μM-Thiorphan</td>
<td>15</td>
<td>64</td>
</tr>
<tr>
<td>1 μM-Captopril</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td>6 μM-Dynorphin (1–13)</td>
<td>84</td>
<td>6</td>
</tr>
<tr>
<td>50 μM-CPE + 10 μM-thiorphan</td>
<td>78</td>
<td>16</td>
</tr>
</tbody>
</table>

Neurotensin was incubated with human synaptic membranes in the presence of the appropriate inhibitor. Extent of degradation and degradation products were analysed by reversed-phase h.p.l.c.

In summary, human synaptic membranes appear to have three main neurotensin-degrading enzymes, all metalloendopeptidases as reported for rat synaptic membranes. It is not yet clear which is responsible for controlling neurotensin levels in vivo.

Abbreviations used: pGlu, pyroglutamic acid; TFA, trifluoroacetic acid; CPE-AAF-pAB, carboxypeptidase A-Ala-Ala-Phe-p-aminobenzoate.

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High M, multicatalytic peptidases in human and rat brain

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Cytosolic high-molecular-mass (> 500 kDa) proteases have been implicated in the extralysosomal degradation of proteins and they appear to be involved in the degradation of ubiquitin-protein conjugates in at least some cell types. In this study, two high-Mₙ cytosolic proteases have been isolated and partially characterized from both post-mortem human and rat brain. These soluble enzymes all appear to have similar Mₙ values but exhibit different elution characteristics on ion-exchange chromatography.

Human temporal cortex (25 g, 24 h post-mortem delay) was homogenized in 5 vol. of 0.05M-Tris/HCl pH 7.34 buffer containing 1 mM-EDTA and 1 mM-dithiothreitol, the homogenate was centrifuged (27000 g for 15 min at 4°C) and the supernatant applied to a DE52 anion-exchange column (28 cm x 2.5 cm). A linear 200 ml Tris/HCl gradient (0.05-0.4 M) was applied, the fractions collected (3 ml) and assayed for enzyme activity. This was done by incubating an aliquot (15 μl) with each of two fluorogenic substrates Z-Gly-Gly-Arg-NMec (R) and glutaryl-Gly-Gly-Phe-NMec (F, 125 nM, pH 7.34, 1 h at 37°C total volume 300 μl). After adding ethanol (600 μl), the fluorescence of the liberated NMeC was measured (excitation 380 nm; emission 440 nm). Two peaks of activity (I and II) were identified (Fig. 1a) which were applied to a high-performance ion-exchange column (TSK-DEAE 5PW, 7.5 mm x 75 mm) eluting with a 30 min linear gradient of Tris/HCl (pH 7.34; 0.05-0.4 M). Flow rate was 1 ml/min and 1 min fractions were collected. Peak I eluted at 0.27 M- and peak II at 0.3 M-Tris/HCl. However, subsequent chromatography of these fractions on a high-performance gel exclusion column (TSK G4000SW, 7.5 mm x 600 mm) indicated that both peptidase fractions had a molecular mass of 630 kDa (Fig. 1b). Rat brain (38 g) was extracted and subjected to anion-exchange chromatography as above. In addition to peaks I and II, an additional peptidase peak was identified which only hydrolysed substrate R. Peaks I and II from rat brain were subjected to size exclusion chromatography and were found to have a molecular mass of 690 kDa.

Both human enzymes (peaks I and II) hydrolysed substrates R and F at pH 7.4; their activity against substrate R increased around 2.5-fold at pH 10. No increase in activity was observed in the presence of 2 mM-ATP. The effect of various inhibitors on the activity of the major enzyme (peak I) against substrates R (at pH 10) and F (at pH 7.4) was as follows (relative activity in parentheses, control = 100):

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>R (pH 10)</th>
<th>F (pH 7.4)</th>
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<tbody>
<tr>
<td>1 mM-I,10-phenanthrol</td>
<td>87</td>
<td>97</td>
</tr>
<tr>
<td>1 mM-phenylmethylsulphonyl fluoride</td>
<td>97</td>
<td>92</td>
</tr>
<tr>
<td>0.5 mM-leupeptin</td>
<td>94</td>
<td>94</td>
</tr>
<tr>
<td>0.5 mM-EDTA</td>
<td>94</td>
<td>94</td>
</tr>
<tr>
<td>1 mM-phenylarsine oxide</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>0.5 mM-p-coumaric acid</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>0.5 mM-hydroxylamine</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>0.5 mM-mercuric chloride</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>0.5 mM-chloroacetate</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>0.5 mM-trypsin</td>
<td>92</td>
<td>92</td>
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</table>

1 mM-EDTA completely inhibited both human and rat brain enzyme activity.

Abbreviation used: NMec, methylcoumarylamide.

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