The metabolism of neuropeptides

Both phosphoramidon-sensitive and Captopril-sensitive metallopeptidases are present in the electric organ of *Torpedo marmorata*

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A membrane fraction from the electric organ of *Torpedo marmorata* hydrolyses the Gly³–Phe⁴ bond of [D-Ala²,Leu⁵]enkephalin as well as the Gly–His bond of benzoyl-Gly-His-Leu. The hydrolysis of benzoyl-Gly-His-Leu is completely inhibitable by Captopril ($I_{50} = 19$ nM), consistent with peptidyl dipeptidase activity, but enkephalin hydrolysis is inhibited to a maximum of only 70%. The residual activity hydrolysing enkephalin is inhibited by phosphoramidon ($I_{50} = 15$ nM) and therefore resembles endopeptidase-24.11, a mammalian plasma-membrane enzyme implicated in the metabolism of neuropeptides. Both enkephalin-hydrolysing activities in *Torpedo* electric organ are inhibited by 1,10-phenanthroline, like their mammalian counterparts. The peptidases may function in the hydrolysis of endogenous peptides or in neurotransmitter exocytosis in the electric organ.

Endopeptidase-24.11 (EC 3.4.24.11) and peptidyl dipeptidase (angiotensin-converting enzyme, EC 3.4.15.1) are two well-characterized mammalian plasma-membrane metalloenzymes involved in the metabolism of biologically active peptides (Matsas *et al.*, 1983; Relton *et al.*, 1983; Saffer, 1976; Erdös *et al.*, 1978). Both enzymes can hydrolyse the Gly³–Phe⁴ bond of enkephalin, are present in synaptic membranes and have been implicated in the physiological inactivation of this opioid peptide (Schwartz, 1983; Benuck & Marks, 1979). The two enzymes can be distinguished by, among other criteria, their differential sensitivity to inhibitors. Endopeptidase-24.11 is competitively inhibited by phosphoramidon ($I_{50} = 8$ nM; Fulcher & Kenny, 1983), whereas peptidyl dipeptidase is uniquely sensitive to Captopril ($I_{50} = 23$ nM; Cushman *et al.*, 1977). Opiates have been shown to modify cholinergic transmission (Bixby & Spitzer, 1983), and an enzyme resembling mammalian peptidyl dipeptidase has been identified in *Torpedo californica* on the basis of its sensitivity to Captopril (Altstein *et al.*, 1984). In the present paper, we demonstrate that a mem-

brane preparation from the electric organ of *Torpedo marmorata* hydrolyses the Gly³–Phe⁴ bond of [D-Ala²,Leu⁵]enkephalin and that this is attributable to two separate enzyme activities. One of these activities also hydrolyses the artificial substrate Bz-Gly-His-Leu and is inhibited by Captopril, and therefore bears a close similarity to mammalian peptidyl dipeptidase. The other activity, comprising approx. 30% of the total, has characteristics similar to those of endopeptidase-24.11, an enzyme previously identified only in mammalian tissues and particularly enriched in kidney microvilli (for review see Kenny & Maroux, 1982). The role of the phosphoramidon-sensitive metallopeptidase in the electric organ may be in the metabolism of endogenous peptides or in neurotransmitter exocytosis (Baxter *et al.*, 1983).

Experimental

Materials

[D-Ala²,Leu⁵]Enkephalin was supplied by Cambridge Research Biochemicals (Harston, Cambridge, U.K.). Bz-Gly-His-Leu and Bz-glycine (hippurate) were from Sigma Chemical Co. Captopril (SQ14225; D-3-mercapto-2-methylpropanoyl-L-proline) was a gift from the Squibb Institute for Medical Research, Princeton, NJ, U.S.A. Other

Abbreviations used: Bz, benzoyl; h.p.l.c., high-pressure liquid chromatography.

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materials were from sources previously noted (Fulcher *et al.*, 1982; Matsas *et al.*, 1983).

Preparation of a membrane fraction

Torpedo electric organ was obtained from specimens of *T. marmorata* supplied by the Station Biologique d'Arcachon, France, and maintained in sea-water aquaria at 12–15°C for periods up to 16 weeks. Electric-organ tissue was removed from fish anaesthetized by chilling in ice, homogenized in 5 vol. of 10mM-Tris/HCl buffer, pH 7.4, in a Braun Multiprac homogenizer, and centrifuged at 100000g for 45 min. The membrane pellet was washed and re-centrifuged once with 10mM-Tris/HCl buffer, pH 7.4, and then with the same buffer containing 0.5M-NaCl. After centrifugation, the membrane pellet was resuspended in 10mM-Tris/HCl buffer, pH 7.4, at a protein concentration of 5 mg/ml and stored frozen at –70°C until required. Subcellular fractionation of electric-organ tissue was carried out as described previously (Dowdall *et al.*, 1982).

Assay of [D-Ala²,Leu⁵]enkephalin hydrolysis

[D-Ala²,Leu⁵]Enkephalin (0.5 mM) was incubated at 37°C for 1 h with *Torpedo* membranes (50 µg) in 0.1M-Tris/HCl buffer, pH 7.4, containing 0.1mM-bestatin in a total volume of 100 µl as described previously (Matsas *et al.*, 1983). Incubation of subcellular fractions was carried out in an iso-osmotic buffer system consisting of 300mM-NaCl/100mM-Tris/HCl buffer, pH 7.4. The reaction was stopped by the addition of 30 µl of 30% (v/v) acetic acid, and the supernatant obtained after centrifugation was analysed by h.p.l.c.

Assay of BzGly-His-Leu hydrolysis

The hydrolysis of Bz-Gly-His-Leu was also measured by an h.p.l.c. method. Bz-Gly-His-Leu (5mM) was incubated in 0.1M-Tris/HCl buffer, pH 8.3, containing 0.1mM-bestatin in a total volume of 100 µl. The reaction was stopped with 30 µl of 30% (v/v) acetic acid, and the products (hippurate and His-Leu) were resolved and quantified by h.p.l.c.

Peptide separation by h.p.l.c.

Reaction conditions for hydrolysis of [D-Ala²,Leu⁵]enkephalin or Bz-Gly-His-Leu were established such that less than 15% of the particular substrate was hydrolysed, and the reaction rates under these conditions were linear with respect to time of incubation and protein concentration. Peptide reaction products were resolved by h.p.l.c. as described previously (Matsas *et al.*, 1983). Quantification was performed by using the Waters 730 Data Module. Retention times were: [D-Ala²,Leu⁵]enkephalin, 16.6 min; Tyr-D-Ala-Gly,

5.34 min; Phe-Leu, 12.5 min; Bz-Gly-His-Leu, 14.6 min; Bz-Gly, 10.2 min; His-Leu, 3.8 min; bestatin, 15.2 min.

Results and discussion

Hydrolysis of [D-Ala²,Leu⁵]enkephalin by *Torpedo* membranes

The specific activity of [D-Ala²,Leu⁵]enkephalin hydrolysis (3.7 nmol/min per mg of protein) was comparable with that previously observed in pig caudate synaptic membranes (Matsas *et al.*, 1983). Significant hydrolysis of the enkephalin analogue occurred only at the Gly³–Phe⁴ bond, producing the products Tyr-D-Ala-Gly and Phe-Leu (Fig. 1). In the absence of bestatin from the incubation, the dipeptide Phe-Leu was further hydrolysed by an aminopeptidase present in the membrane preparation. Mammalian endopeptidase-24.11 and peptidyl dipeptidases are both Zn²⁺-containing en-

Fig. 1. Hydrolysis of [D-Ala²,Leu⁵]enkephalin by *Torpedo* membranes

The peptide products of [D-Ala²,Leu⁵]enkephalin hydrolysis were resolved by h.p.l.c. (see the Experimental section). The resolved peaks correspond to (1) acetic acid, (2) Tyr-D-Ala-Gly, (3) Phe-Leu, (4) bestatin and (5) [D-Ala²,Leu⁵]enkephalin.

zymes, sensitive to chelating agents. More than 95% inhibition of enkephalin hydrolysis by *Torpedo* membranes was achieved in the presence of 1 mM-1,10-phenanthroline. Titration of activity with 1,10-phenanthroline indicated the involvement of enzymes with an I_{50} value of approx. 100 μ M towards this chelating agent (Fig. 2a). When the *Torpedo* electric organ was subjected to subcellular fractionation (Dowdall *et al.*, 1982), the activity hydrolysing [D-Ala²,Leu⁵]enkephalin was detectable only in membrane fractions and was enriched 4.1-fold in the synaptosomal preparation relative to the original homogenate. Treatment with 0.1% Triton X-100 demonstrated that less than 12% of the synaptosomal peptidase activity was present in an occluded form.

Hydrolysis of Bz-Gly-His-Leu by *Torpedo* membranes

Although mammalian peptidyl dipeptidase hydrolyses the Gly³-Phe⁴ bond of enkephalin (Benuck & Marks, 1979), the classical substrate for assay of this enzyme is Bz-Gly-His-Leu (Saffer, 1976). The *Torpedo* membranes efficiently hydrolysed this substrate, releasing the dipeptide His-Leu, and the activity was completely inhibitable by Captopril (Fig. 2b) with an I_{50} value of approx. 19 nM.

Inhibition of [D-Ala²,Leu⁵]enkephalin hydrolysis

When hydrolysis of [D-Ala²,Leu⁵]enkephalin by *Torpedo* membranes was examined, a maximum

inhibition of only 70% was achieved with Captopril (Fig. 2c), although a similar concentration caused half-maximal inhibition. These observations suggested that the *Torpedo* membranes contained at least two metallopeptidases. The principal activity, which hydrolysed both [D-Ala²,Leu⁵]enkephalin and Bz-Gly-His-Leu, was sensitive to Captopril and therefore resembled mammalian peptidyl dipeptidase.

The possibility that the second activity, which hydrolysed [D-Ala²,Leu⁵]enkephalin but not Bz-Gly-His-Leu, may resemble mammalian endopeptidase-24.11 was explored by using the inhibitor phosphoramidon (Kenny, 1977; Matsas *et al.*, 1983; Relton *et al.*, 1983). A combination of Captopril (1 μ M) and phosphoramidon (1 μ M) completely inhibited the hydrolysis of [D-Ala²,Leu⁵]enkephalin by *Torpedo* membranes. When inhibition by phosphoramidon alone was examined, a complex titration curve was obtained (Fig. 3a), reflecting the fact that both endopeptidase-24.11 and peptidyl dipeptidase are sensitive to this inhibitor, although with markedly different sensitivities (endopeptidase-24.11, I_{50} = 8 nM; peptidyl dipeptidase, I_{50} > 100 μ M for the mammalian kidney enzymes; R. Matsas, A. J. Kenny & A. J. Turner, unpublished work). Since endopeptidase-24.11 is not inhibited by micromolar concentrations of Captopril (Fulcher & Kenny, 1983; Relton *et al.*, 1983), it proved possible to examine selectively the effect of phosphoramidon under conditions where the peptidyl dipeptidase is

Fig. 2. Effects of 1,10-phenanthroline and Captopril on hydrolysis of [D-Ala²,Leu⁵]enkephalin and Bz-Gly-His-Leu by *Torpedo* membranes

(a) Inhibition of [D-Ala²,Leu⁵]enkephalin hydrolysis by 1,10-phenanthroline; (b) inhibition of Bz-Gly-His-Leu hydrolysis by Captopril; (c) inhibition of [D-Ala²,Leu⁵]enkephalin hydrolysis by Captopril. In the last case, the maximum inhibition obtained was 71%. The inclusion of 1 μ M-phosphoramidon in addition to Captopril resulted in complete inhibition of [D-Ala²,Leu⁵]enkephalin hydrolysis. The hydrolysis of Bz-Gly-His-Leu by *Torpedo* membranes was inhibited by 95% by 1 mM-1,10-phenanthroline (results not shown).

Fig. 3. Effect of phosphoramidon on $[\text{D-Ala}^2, \text{Leu}^5]\text{enkephalin}$ hydrolysis in (a) the absence and (b) the presence of $10 \mu\text{M}$ -Captopril

In the latter case, phosphoramidon appears to inhibit a single enzyme activity with an I_{50} value of approx. 15 nM .

completely inhibited (Fig. 3b). In this case, a sharp titration curve was obtained for phosphoramidon inhibition ($I_{50} = 15 \text{ nM}$), indicating the additional presence in the *Torpedo* membranes of an enzyme activity with similar characteristics to mammalian endopeptidase-24.11 (see, e.g., Kenny & Maroux, 1982). The relative concentrations of the two enzymes differ, though, from those in pig caudate synaptosomes, where the endopeptidase, rather than peptidyl dipeptidase, is the major activity hydrolysing $[\text{D-Ala}^2, \text{Leu}^5]\text{enkephalin}$ (Matsas *et al.*, 1983).

Conclusions

Membrane preparations from the electric organ of *T. marmorata* contain enkephalin-hydrolysing activities resembling two peptidases previously believed to be of restricted distribution among mammals. Peptidyl dipeptidase occurs in a number of mammalian tissues, but its major location is considered to be lung (Cushman & Cheung, 1971), where it inactivates bradykinin and also catalyses the hydrolysis of angiotensin I to yield the vasoactive peptide angiotensin II. Since a similar activity has now been detected in a specialized tissue, the electric organ of *Torpedo*, its role in the metabolism of biologically active peptides clearly needs to be re-assessed. Endopeptidase-24.11 was, until a few years ago, thought to be located

exclusively in kidney microvilli, which is reflected in its official enzyme classification as 'kidney brush border neutral proteinase' (Nomenclature Committee of the International Union of Biochemistry, 1979). It is now also known to be located in membrane fractions from various mammalian tissues, including pituitary and brain (Orlowski & Wilk, 1981; Matsas *et al.*, 1983). This phosphoramidon-sensitive endopeptidase has been implicated in the physiological inactivation of enkephalins and substance P (Schwartz, 1983; Matsas *et al.*, 1983) and, at the mammalian neuromuscular junction, in neurotransmitter exocytosis (Baxter *et al.*, 1983). The functions of the *Torpedo* endopeptidase, and how closely it resembles its mammalian counterpart, must await its purification and further characterization. *Torpedo* tissue may prove useful for exploring the physiological functions of both the Captopril-sensitive and phosphoramidon-sensitive metallopeptidases.

Enkephalins and other neuropeptides are widely distributed throughout the animal kingdom (Kilpatrick *et al.*, 1983; Kreiger, 1983), and, in the mammalian nervous system, can co-exist with other neurotransmitters (Osborne, 1983). The possibility that endogenous peptides may modulate acetylcholine action, or be co-localized with acetylcholine in the *Torpedo* electric organ, a tissue thought to be exclusively of cholinergic innervation, deserves consideration.

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