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Hydrolysis of rat melanin-concentrating hormone by endopeptidase 24.11 (neutral endopeptidase)

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Melanin-concentrating hormone (MCH) is a cyclic peptide which behaves as an antagonist of the pituitary melanotropic hormone α-melanocyte-stimulating hormone in fishes. Cloning of the rat MCH cDNA precursor recently revealed the presence of an additional putative peptide named NEI. The present work examined the susceptibility of these novel peptides to hydrolysis by various purified exo- and endo-peptidases including endopeptidases 24.11 (NEP), 24.15, 24.16, angiotensin-converting enzyme, leucine aminopeptidase and carboxypeptidase A. NEP attacked MCH at three sites of the molecule with an apparent affinity of about 12 μM and a $k_{cat.}$ of 4 min^{-1}. The first site of cleavage was at Cys-7–Met-8, i.e. within the peptide loop formed by the internal disulphide bridge. NEP could therefore be considered as an MCH-inactivating peptidase since the degradation products generated are probably devoid of biological activity. In contrast, NEI neither inhibited the degradation of the NEP chromogenic substrate glutaryl-Phe-Ala-Phe-*p*-aminobenzoate nor was susceptible to proteolysis by NEP. Unlike NEP, angiotensin-converting enzyme, endopeptidase 24.15 and endopeptidase 24.16 appeared totally unable to cleave MCH, whereas the peptide was readily degraded by aminopeptidase M and carboxypeptidase A.

INTRODUCTION

Melanin-concentrating hormone (MCH) is a cyclic peptide which has been shown to participate in the control of melanophores in teleost fishes (Kawauchi *et al.*, 1983; Baker, 1988; Eberle, 1988). In such species, salmon MCH was shown to behave as an antagonist of the pituitary melanotropic hormone α-melanocyte-stimulating hormone (Kawauchi *et al.*, 1983; Gilham & Baker, 1984; Wilkes *et al.*, 1984*b*) since it was reported to stimulate aggregation of melanosomes within melanophores both *in vitro* and *in vivo*. Interestingly, high concentrations of this peptide induced an inverse action leading to melanin dispersion in other species such as frogs and lizards (Wilkes *et al.*, 1984*a*; Baker, 1988). Besides its influence on pigment cells, salmon MCH was also reported to strongly inhibit stress-induced or corticotropin-releasing factor-stimulated α-melanocyte-stimulating hormone and adrenocorticotropin secretions in teleosts (Baker *et al.*, 1986), whereas it seemed to elicit a similar although weaker activity on the rat pituitary (Baker *et al.*, 1985). This pharmacological profile could probably be ascribed to mediation via specific receptors, suggesting the presence of MCH-like peptides in the central nervous system of other classes of vertebrates including mammals.

Immunological approaches have demonstrated the occurrence of putative MCH-like peptides in the brain of various classes of vertebrates (Baker, 1988; Eberle, 1988). The staining of perikarya appeared to be predominantly associated with the dorsolateral zone of rat and human hypothalamus (Skofitsch *et al.*, 1985; Bresson *et al.*, 1987) with widespread projections throughout the central nervous system. The peptide probably responsible for the label obtained with the anti-(salmon MCH) antibodies has been purified from rat hypothalamus and characterized as a 19-amino-acid peptide which differs from the salmon congener by substitution of four residues together with a two-amino-acid extension at the *N*-terminus (Vaughan *et al.*, 1989). Nahon *et al.* (1989*a*) reported on the cloning and sequencing of MCH cDNA isolated from a rat hypothalamic library. Interestingly, the primary structure of the precursor protein not only confirmed the sequence of purified rat hypothalamic MCH but also revealed the presence of two putative additional peptides, named NGE and NEI according to Tatemoto's nomenclature (Tatemoto & Mutt, 1981), which apparently do not correspond to MCH-related peptides. However, it has been shown that MCH, NGE and NEI are co-expressed in the dorsolateral hypothalamus (Nahon *et al.*, 1989*a*,*b*). It is therefore tempting to speculate on a possible concomitant participation of these neuropeptides in a complex behavioural or physiological function. The possibility that such a role could be masked by efficient and rapid proteolytic inactivation has prompted us to examine the susceptibility of rat MCH and NEI to various purified peptidases that were previously shown to participate in the metabolism of several neuropeptides belonging to the opiates, tachykinins and other peptide families.

MATERIALS AND METHODS

Rat MCH and NEI were kindly provided by Dr. C. Hoeger and Dr. J. Rivier (The Clayton Foundation for Peptide Biology, The Salk Institute for Biological Studies, La Jolla, CA, U.S.A.). Carboxypeptidase A (di-isopropyl phosphorofluoridate-treated), angiotensin-converting enzyme and leucine aminopeptidase (microsomal, type IV) were from Sigma. glutaryl-Phe-Ala-Phe-*p*-aminobenzoate (Glut-F-A-F-pAB) was from Novabiochem.

Endopeptidase 24.15 and endopeptidase 24.16 were purified as previously described (Checler *et al.*, 1986; Barelli *et al.*, 1991). Endopeptidase 24.11 (neutral endopeptidase, NEP) was kindly provided by Dr. P. Crine and Dr. G. Boileau (Département de Biochimie, Université de Montréal, Montréal, Quebec, Canada). H.p.l.c. analysis of the kinetics of Glut-F-A-F-pAB hydrolysis by purified NEP indicated that a single cleavage was observed after 5 min which led to the formation of two degradation products (not shown) eluted with the retention times of Glut-F-A and F-pAB which corresponded to the fragments reported to be generated by NEP (Pozsgay *et al.*, 1986). These two products concomitantly increased in a time-dependent manner and were

Abbreviations used: MCH, melanin-concentrating hormone; NEP, endopeptidase 24.11; Glut-F-A-F-pAB, glutaryl-Phe-Ala-Phe-*p*-aminobenzoate.

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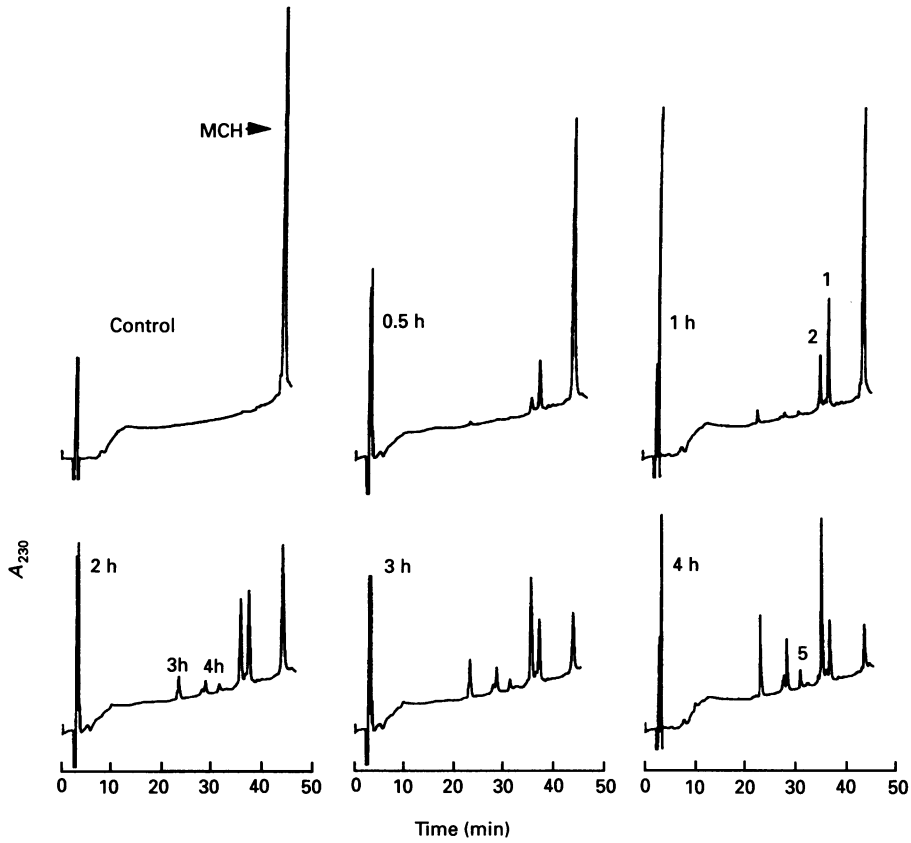


Fig. 1. Kinetic analysis of MCH degradation by NEP

MCH (2 nmol, 20 μM) was incubated for the indicated times at 37 °C in the absence (control) or presence of 0.9 μg of NEP in a final volume of 100 μl of 20 mM-Tris/HCl, pH 7.5. Incubations were stopped and incubation mixtures analysed by h.p.l.c. as described in the Materials and methods section. Degradation products are numbered according to their order of appearance.

recovered in maximal amounts after 60 min. The NEP inhibitor, phosphoramidon, elicited a dose-dependent and complete inhibition of Glut-F-A-F-pAB hydrolysis with an IC_{50} of 50 nM (not shown) in agreement with the K_i value previously reported (Mumford *et al.*, 1981). The fact that the two degradation products remained unchanged between 60 and 120 min although Glut-F-A-F-pAB appeared to be completely degraded indicated that the two fragments recovered did not undergo secondary processing, therefore suggesting that purified NEP was not contaminated by additional peptidases.

Peptide incubation and h.p.l.c. analysis

Peptides (2–5 nmol) were incubated with several peptidases for various times at 37 °C in a final volume of 100 μl of 20 mM-Tris/HCl, pH 7.5, containing 0.02% (w/v) BSA. Inhibition studies were performed by prior incubation of saturating concentrations of various specific inhibitors. Incubations were terminated by acidification (10 μl of 1 M-HCl), and the incubation mixtures centrifuged (10000 *g*, 10 min). Supernatants were submitted to reverse-phase column chromatography and analysed by h.p.l.c. with the trifluoroacetic/triethylamine system previously described (Barelli *et al.*, 1988).

Preparation and sequencing of MCH-degradation products

Rat MCH (7 nmol) was incubated overnight with 0.9 μg of NEP. Degradation products were separated by h.p.l.c. as described above. Sequencing was performed on an Applied Biosystems 477A protein–peptide sequencing system equipped with an on-line phenylthiohydantoin analyser, model 120A.

RESULTS

Rat MCH was readily degraded by NEP (Fig. 1). Proteolytic activity was fully blocked by 1 μM-phosphoramidon (Fig. 2*a* and *b*). A complete dose–inhibition curve allowed the determination of an IC_{50} of about 0.1 μM and therefore the derivation of a K_i value of about 35 nM (Fig. 2*c*) in agreement with that deduced from experiments performed with the synthetic chromogenic substrate of NEP (see the Materials and methods section).

The kinetics of MCH degradation by NEP is illustrated in Fig. 1. Five major fragments could be recovered which were amenable to sequencing. The first two degradation products (1 and 2) were derived from proteolytic attack within the peptide loop formed by the internal disulphide bridge of MCH (Fig. 3). Products 3, 4 and 5 appeared later (Fig. 1), the two former fragments (i.e. 3 and 4) probably being generated by cleavage at the *C*-terminus of MCH or product 2 (Fig. 3). It is noteworthy that, although formation of degradation products 3, 4 and 5 continued to increase with time, formation of fragment 1 began to diminish after 3 h of incubation, indicating that it was susceptible to further processing into a secondary product, which could be fragment 2, that was still being augmented at this incubation time (Fig. 1). It should be noted that after 24 h, products 1 and 2 were virtually undetectable whereas the other fragments were recovered in maximal amounts (not shown). This suggested that these three peptides are the final degradation products of MCH, in agreement with the fact that apposition of their sequences leads to a reconstituted intact MCH (see Fig. 3). In contrast, NEI remained virtually intact after a long incubation with NEP (not

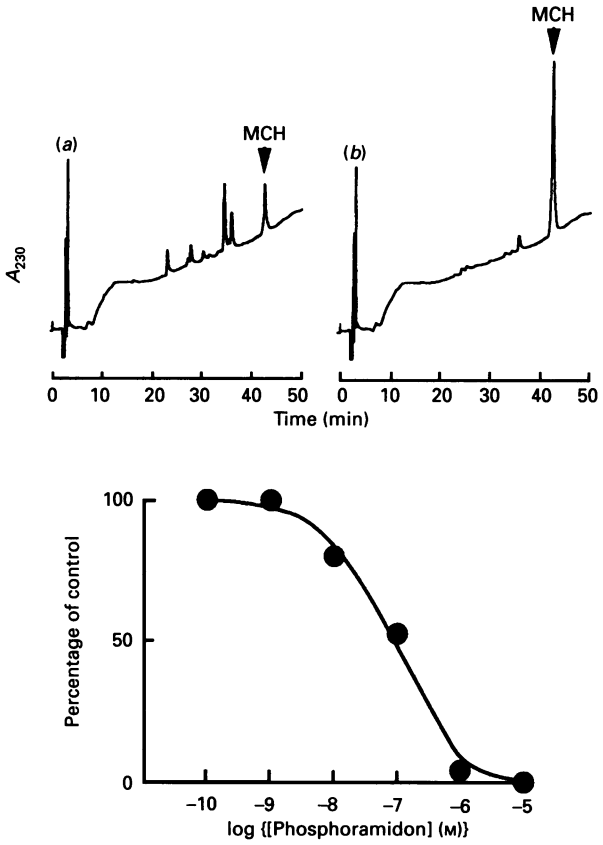
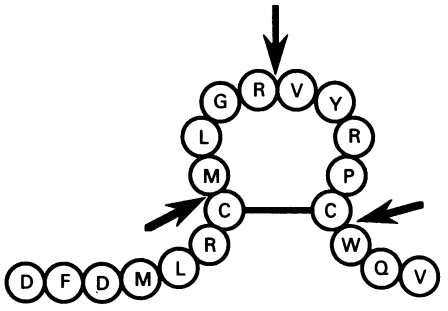


Fig. 2. Effect of phosphoramidon on MCH hydrolysis by NEP

MCH (2 nmol, 20 μM) was incubated for 2 h with 0.9 μg of NEP at 37 °C in the absence (*a*) or presence (*b*) of 1 μM-phosphoramidon. Samples were acidified and analysed by h.p.l.c. as described in the Materials and methods section. A complete inhibition curve was obtained in the above conditions by varying the concentrations of phosphoramidon (*c*). Values are expressed as the percentage of MCH degradation obtained in the absence of phosphoramidon (taken as 100).



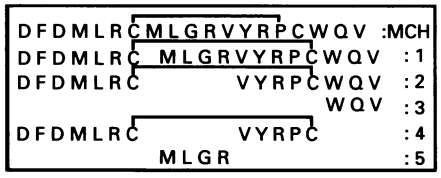


Fig. 3. Model of MCH degradation by NEP

Arrows indicate sites of cleavage of MCH generated by NEP. The degradation products (numbered as in Fig. 1) were recovered and sequenced as described in the Materials and methods section.

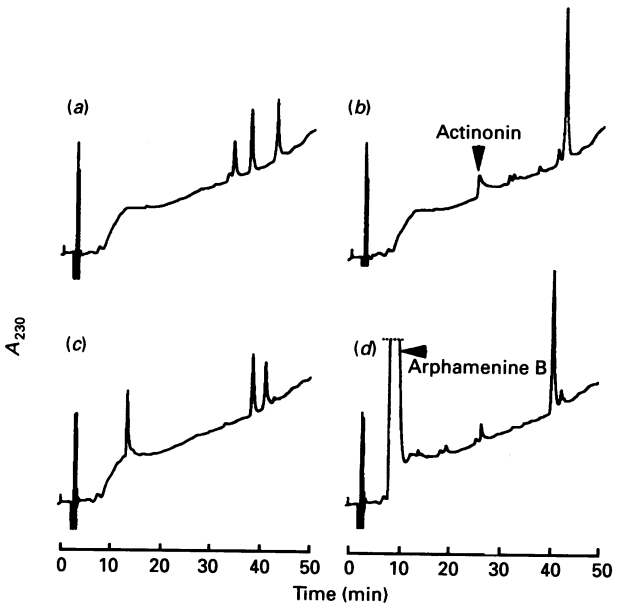


Fig. 4. MCH hydrolysis by aminopeptidase M and carboxypeptidase A

MCH (2 nmol, 20 μM) was incubated for 30–60 min at 37 °C in a final volume of 100 μl of 20 mM-Tris/HCl, pH 7.5, containing 0.02 % BSA, in the presence of 2.5 μg of aminopeptidase M (*a*) or 8 μg of carboxypeptidase A (di-isopropyl phosphorofluoridate-treated) (*c*). Inhibition experiments were performed by prior incubation with 0.1 mM-actinonin (*b*) or 1 mM-arphamenine B (*d*) for aminopeptidase M and carboxypeptidase A respectively. Samples were acidified and analysed by h.p.l.c. as described in the Materials and methods section.

shown). Interestingly, MCH totally resisted proteolysis by other peptidases such as angiotensin-converting enzyme, endopeptidase 24.15 and endopeptidase 24.16 (not shown) but appeared to be susceptible to aminopeptidase M and carboxypeptidase A, as expected from its free *N*- and *C*-termini (Fig. 4*a* and *c*). Preincubation of aminopeptidase M and carboxypeptidase A with 0.1 mM-actinonin and 1 mM-arphamenine B (Fig. 4*b* and *d*) respectively led to full protection of MCH against hydrolysis, therefore confirming that proteolysis was indeed due to these two peptidases and not to contaminating peptidases present in the commercial enzyme preparations.

H.p.l.c. analysis showed that hydrolysis of the NEP chromogenic substrate Glut-F-A-F-pAB was potently inhibited by MCH but not by NEI (Fig. 5). Fig. 6 shows that Glut-F-A-F-pAB hydrolysis by NEP was dose-dependently inhibited by MCH with IC_{50} of about 25 μM, whereas a concentration of 100 μM-NEI appeared totally unable to compete for Glut-F-A-F-pAB hydrolysis by NEP (not shown). From the above statements and according to the simple relationship:

$$IC_{50} = K_i\left(1+\frac{S}{K_m}\right) \quad \text{(Cheng \& Prusoff, 1973)}$$

it was possible to deduce a K_i value of about 12 μM which corresponded to the mean apparent affinity (K_m) of MCH for NEP. Experiments carried out at a concentration of 100 μM-MCH (i.e. in conditions of maximal velocity) allowed determination of a $k_{cat.}$ value of 4 min^{-1} (not shown). The same batch of NEP gave a $k_{cat.}$ value of about 500 min^{-1} for [methionine-5]enkephalin.

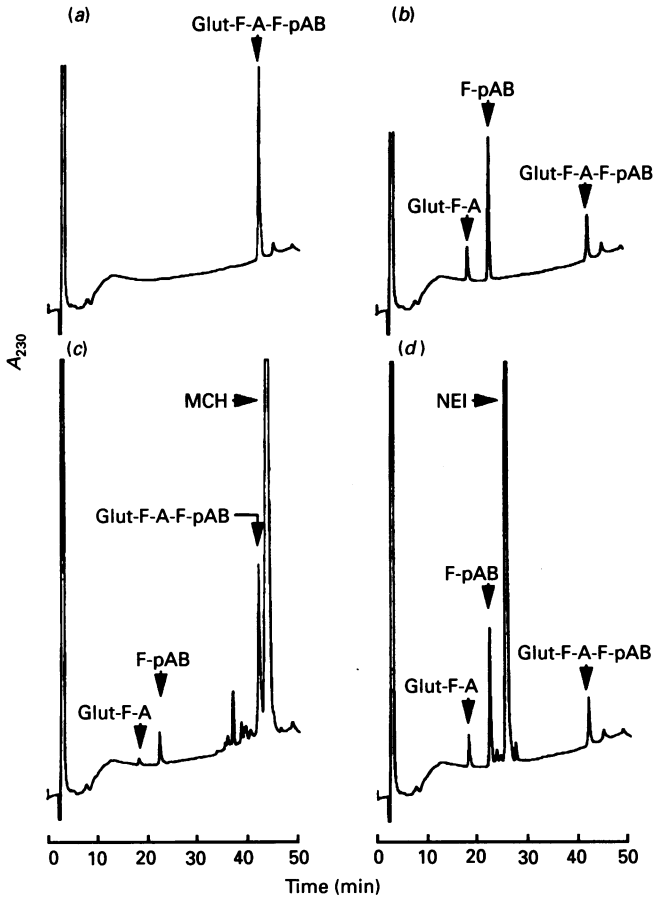


Fig. 5. Degradation of Glut-F-A-F-pAB by NEP: effect of MCH and NEI

Glut-F-A-F-pAB (5 nmol, 50 μM) was incubated for 30 min at 37 °C with 0.5 μg of NEP in the absence (*b*) or presence of MCH (*c*) or NEI (*d*) (15 nmol, 150 μM) in a final volume of 100 μl of 20 mM-Tris/HCl, pH 7.5, containing 0.02 % BSA. Incubations were stopped and mixtures analysed by h.p.l.c. as described in the Materials and methods section. The sequence of NEI is as follows: Glu-Ile-Gly-Asp-Glu-Glu-Asn-Ser-Ala-Lys-Phe-Pro-Ile-NH_2.

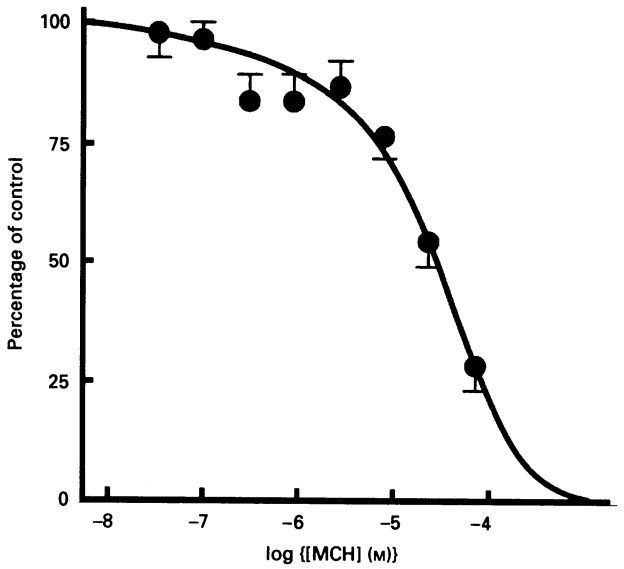


Fig. 6. Inhibition of Glut-F-A-F-pAB hydrolysis by MCH

Glut-F-A-F-pAB (0.5 mM) was incubated for 45 min at 37 °C with 0.09 μg of NEP in a final volume of 100 μl of 20 mM-Tris/HCl, pH 7.5, containing 0.02 % BSA. Incubations were stopped by addition of 1 μM-phosphoramidon; then 5 μg of leucine aminopeptidase was added to release the chromophore. Spectrophotometric analysis was performed as described in the Materials and methods section. Values are expressed as the percentage of control obtained in the absence of MCH and represent means ± S.E.M. of four independent determinations.

DISCUSSION

The present paper establishes that NEP displays the ability to hydrolyse MCH. The K_m value (12 μM) shows MCH to be one of the natural peptides that exhibits the highest affinity for NEP *in vitro*. However, it is clear that NEP has a rather low $k_{cat.}$ value when compared with that for [methionine-5]enkephalin (see the Results section). It is noteworthy that the $k_{cat.}$ value determined in the present study with this opioid (500 min^{-1}) is in agreement with previous values reported by others [21.2 min^{-1} (Almenoff *et al.*, 1981); 138 min^{-1} (Malfroy & Schwartz, 1982*b*); 304 min^{-1} (Malfroy & Schwartz, 1984); 1268 min^{-1} (Malfroy & Schwartz, 1982*a*)]. Altogether, this indicates that our batch of NEP compares well with NEP previously purified from rat kidney or bovine pituitary.

The first two sites of cleavage were inside the peptide loop formed by the intramolecular disulphide bridge. A secondary process consisted of the release of the *C*-terminal tripeptide. The susceptibility of MCH to proteolysis by NEP corroborated previous studies showing that several disulphide-bridge-containing peptides, such as somatostatin, natriuretic peptides and endothelins, are readily broken down by NEP (Vanneste *et al.*, 1988; Sakurada *et al.*, 1990; Vijayaraghavan *et al.*, 1990*b*). As demonstrated in MCH, these peptides were also shown to be primarily attacked within the peptide loop. Furthermore, human α-atrial natriuretic factor and endothelin 1 also undergo an additional attack leading to the removal of the tripeptide from their *C*-terminus (Vanneste *et al.*, 1988; Vijayaraghavan *et al.*, 1990*a*). This is in agreement with previous studies indicating that NEP preferentially releases di- or tri-peptides from free *C*-termini (Malfroy & Schwartz, 1982*a*; Hersh & Morihara, 1986). This is probably due to Arg-102 of NEP interacting with a free carboxylic moiety (Bateman *et al.*, 1989; Beaumont *et al.*, 1991). Interestingly, the ability of NEP to hydrolyse MCH is not shared by other endopeptidases. Indeed endopeptidase 24.15, endopeptidase 24.16 and angiotensin-converting enzyme were totally unable to degrade MCH.

The question arises as to whether NEP can be considered an MCH-inactivating peptidase, i.e. an enzyme producing degradation products that are totally devoid of biological activity. Very little is known about the structure–function of rat MCH. The only available data are from studies on salmon MCH (Baker *et al.*, 1990). Structure–activity studies performed with a number of cyclic and linear fragments and analogues of salmon MCH indicated that the cyclic portion, MCH (5–14), deleted from its *N*- and *C*-terminal sequences only exhibited 0.1 % potency when tested using a fish melanophore assay (Lebl *et al.*, 1988; Baker *et al.*, 1990). On the other hand, the exocyclic parts of the molecule, MCH(1–4, 15–17), displayed minimal activity (Baker *et al.*, 1990). This seems to indicate that salmon MCH biological activity is probably borne by both endo- and exo-cyclic sequences. Since there existed a 90 % homology between the loop structure of salmon and rat MCH and the *C*-terminus is also highly conserved (Baker *et al.*, 1990), one could speculate that the rat MCH sequence responsible for biological activity is probably similar to those of salmon MCH. In this context, the sites of proteolysis of rat MCH by NEP allowed us to consider NEP an

MCH-inactivating peptidase. This is not the case for NEI which remained intact after long incubations with NEP.

Studies concerned with the search for a physiological role for MCH in mammalian brain should consider the possibility of MCH encountering NEP during peptide diffusion to its receptors, particularly after intracerebroventricular injection. Since NEP has been shown to be particularly abundant in choroid tissues and meninges (Zajac *et al.*, 1987; Bourne *et al.*, 1989), such studies should therefore be performed in the presence of the NEP inhibitor phosphoramidon, in order to protect MCH from NEP proteolytic activity.

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