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# Neutral Endopeptidase 24.11: Structure, Inhibition, and Experimental and Clinical Pharmacology

BERNARD P. ROQUES, FLORENCE NOBLE, VALÉRIE DAUGÉ, MARIE-CLAUDE FOURNIÉ-ZALUSKI, AND ANN BEAUMONT

*Pharmacochimie Moléculaire et Structurale (Unité 266 Institut National de la Santé et de la Recherche Medicale - Unité de Recherche Associée D 1500 Centre National de la Recherche Scientifique, Faculté de Pharmacie, 4 Avenue de l'Observatoire 75270, Paris Cédex 06, France*

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## I. Introduction

Considerable interest in membrane-bound peptidases emerged at the end of the 1970s following the discovery that inhibition of ACE* (peptididyl-dipeptidase, E.C. 3.4.15.1), the enzyme implicated in the formation of angiotensin II from angiotensin I, produced antihypertensive effects (Ondetti et al., 1977) and 3 years later that inhibition of another membrane-bound Zn metallopeptidase, involved in the inactivation of the opioid peptide enkephalins in the brain, induced analgesic responses (Roques et al., 1980). Given its enkephalin-metabolizing activity, this latter enzyme was designated "enkephalinase" (Malfroy et al., 1978b) and some time later was shown to be identical with NEP (E.C. 3.4.24.11), an already well-characterized Zn metallopeptidase known to be present in the brush border cells of the proximal tubules of the kidney (Kerr and Kenny, 1974a,b).

As studies of metabolizing enzymes progressed, it became apparent that, as was the case for their peptide substrates, these peptidases were widely distributed, and the concept of "one peptidase: one substrate" was not to be realized (Schwartz, 1983; Kenny, 1986). For instance, NEP was recently found to participate in clearing the circulating ANP from plasma (Koehn et al., 1987; Stephenson and Kenny, 1987a; Sonnenberg et al., 1988; Olins et al., 1989). Moreover, NEP was also shown to be one of the many proteins used in immunology and leukemia research as lymphocyte markers and was designated CALLA (CD10) (Greaves et al., 1975; Letarte et al., 1988).

It now appears that, both in nervous and peripheral tissue, peptides are degraded extracellularly by what is probably a limited number of enzymes with relatively broad specificities. Most of these extracellular peptide-degrading enzymes are ectoenzymes, i.e., integral membrane proteins that have their active sites facing the extracellular space, and many are Zn metallopeptidases (Turner et al., 1987; Maroux, 1987). Despite their relatively broad specificities, a certain in vivo specificity seems to be achieved, governed by both the distribution of a given peptidase and that of its potential substrates.

Thus, in the brain and the spinal cord, the enkephalins, like morphine and other classical opiates, interact with different binding sites such as the recently cloned and sequenced δ-opioid receptor (Kieffer et al., 1992; Evans et al., 1992) to produce a multiplicity of pharmacological responses, such as analgesia and euphoria. Increasing the levels of endogenous opioid peptides by inhibiting their inactivating enzymes was, therefore, proposed as a means of eliminating or minimizing the serious drawbacks (respiratory depression, constipation, tolerance, physical, and psychic dependence) induced by chronically administered opiates (Roques et al., 1980). These side effects are thought to be caused by an overstimulation of opioid receptors in all brain areas in which the enkephalins might be either tonically or phasically released. A similar approach is now being followed to potentiate the diuretic, natriuretic, and vasorelaxant effects of circulating ANP with the aim of obtaining new antihypertensive agents (reviewed by Roques and Beaumont, 1990).

The literature concerning these subjects is already voluminous and includes recent reviews of the development of inhibitors for various Zn metallopeptidases (Ro-

** Abbreviations: ACE, angiotensin-converting enzyme; NEP, neutral endopeptidase; ANP, atrial natriuretic peptide; CALLA, common acute lymphoblastic leukemia antigen; APN, aminopeptidase N; DAP, dipeptidylaminopeptidase; SP, substance P; i.c.v., intracerebroventricular; CCK, cholecystokinin; FTI, N-([fluoresceinyl]-N′-[1-(6-(3-mercapto-2-benzyl-1-oxopropyl)-amino-1-hexyl]-thiocarbamide; TLN, thermolysin; CNS, central nervous system; VTA, ventral tegmental area; CGRP, calcitonin gene-related peptide; ANF, atrial natriuretic factor; SHR, spontaneously hypertensive rat; ALL, acute lymphoblastic leukemia; HACBO-Gly, N-[(2RS)-4-(hydroxyamino)-1,4-dioxo-2-(phenylmethyl)butyl]-glycine; DAMGO, Try-D.Ala-Gly-(Me)Phe-Gly.ol; DPDPE, Tyr-D.Pen-Gly-Phe-D.Pen; DSTBULET, Tyr-D.Ser(O-tertiobutyl)-Gly-Phe-Leu-Thr; BUBU, Tyr-D.Ser(O-tertiobutyl)Gly-Phe-Leu-Thr(O-tertiobutyl); DTLET, Tyr-D.Thr-Gly-Phe-Leu-Thr; BDNL, Boc-Tyr($SO_3H$)Nle-Gly-Trp-Nle-Asp-Phe$NH_2$; RB 101, N-[(*R*,*S*)-2-benzyl-3[(*S*)(2-amino-4-methylthio)butyldithio]-1-oxopropyl]-L-phenylalanine; [$^{125}$I]RB 104, 2-{(3-[$^{125}$I]iodo-4-hydroxy)-phenylmethyl}-4-N-[3-hydroxyamino-3-oxo-1-(phenyl methyl)propyl]amino-4-oxobutanoic acid; BC 264, Boc-Tyr($SO_3H$)-gNle-mGly-Trp-(NMe)Nle-Asp-Phe$NH_2$; RB 38A, N-[3(*R*)hydroxyaminocarbonyl)-2-benzyl-1-oxopropyl]-L-phenylalanine.

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ques et al., 1982a,b; Ondetti and Cushman, 1984; Roques, 1985; Roques and Fournié-Zaluski, 1986; Chipkin, 1986; Thorsett and Wyvratt, 1987; Rich, 1990) and their pharmacological effects (Roques and Fournié-Zaluski, 1985, 1986; Schwartz et al., 1985; Roques, 1988a, 1991; Dickenson, 1986; Needelman et al., 1989; Le Bien and McCormack, 1989; Noble et al., 1992d). The main results reported in these papers will be recalled when necessary, but the present review is mainly devoted to the molecular biology and structural characterization of NEP and the potential use of these results for designing either selective or mixed inhibitors of NEP and APN, another Zn metalloenzyme involved in enkephalin metabolism, or NEP and ACE. We have also looked at the distribution of NEP and discussed its possible role in various tissues. Finally, we have tried to provide an extensive survey of the in vivo actions of the peptidase inhibitors and to show how these molecules can be used (*a*) to achieve a better understanding of the physiological functions of peptidergic systems and (*b*) in therapeutics.

## II. Neutral Endopeptidase 24.11: A Pharmacologically Relevant Multisubstrate-metabolizing Enzyme

The observation that enkephalins were rapidly degraded by brain tissue homogenates suggested that peptidases could be involved in the physiological inactivation of these endogenous opioid peptides. This accounted for the weak and transient analgesia obtained only with high doses (0.1 mg) of i.c.v. administered Met-enkephalin in mice (Belluzi et al., 1976) and the higher potency of enkephalin analogues protected from peptidase inactivation by modifications such as replacement of $Gly^2$ by an amino acid of the dextro configuration (Pert et al., 1976), amidation, or reduction of the free carboxyl group, N-methylation of the $Gly^3$-$Phe^4$ bond, or replacement of $Phe^4$ by its D-isomer (Fournié-Zaluski et al., 1979). Because the enkephalins are unable to enter cells, the detection of the metabolites Tyr, Tyr-Gly-Gly, and Tyr-Gly following i.c.v. administration of Leu-enkephalin suggested that the peptide was degraded by aminopeptidase, DAP, and peptidyl-dipeptidase activities located at the cell surface and acting, therefore, as ectoenzymes (Hambrook et al., 1976; Dupont et al., 1977; Craves et al., 1978).

The existence of a relatively low affinity-binding site ($K_D$ approximately 90 nM) for [$^3$H]Leu-enkephalin in rat striatal membranes different from the receptor-binding site (Malfroy et al., 1978a) was subsequently attributed to the existence of a Tyr-Gly-Gly-releasing enzyme designated enkephalinase (Malfroy et al., 1978b), and its presence in brain was confirmed by Sullivan et al. (1978). In addition, it was shown that the tripeptide was not produced by the sequential action of a carboxypeptidase (Guyon et al., 1979). The $K_m$ of the enkephalins for enkephalinase was subsequently shown to be higher (approximately 20 μM) (Vogel and Altstein, 1979; Fournié-Zaluski et al., 1979) than previously estimated (approximately 90 nM). The physiological relevance of enkephalinase in enkephalin metabolism was firmly established by the naloxone-reversible antinociceptive properties elicited by the synthetic enkephalinase inhibitor, thiorphan (Roques et al., 1980). However, the reported increase in enzyme levels after chronic morphine treatment (Malfroy et al., 1978b) was not confirmed (Barchas et al., 1981), particularly when enzyme levels were measured using quantitative autoradiography (Delay-Goyet et al., 1989a).

ACE was also found to cleave the $Gly^3$-$Phe^4$ bond of the enkephalins (Erdös et al., 1978); however, although they were initially suggested to be identical (Swerts et al., 1979a; Benuck and Marks, 1979), enkephalinase and ACE were shown to be two distinct enzymes (Swerts et al., 1979b; Buckett, 1979). The enkephalins were found to have a low affinity for ACE ($K_m$ approximately $10^{-3}$ M), thereby excluding a major role for this enzyme in the in vivo metabolism of these peptides. However, the extended opioid peptide, Met-enkephalin-$Arg^6$-$Phe^7$, could be transformed to the less potent peptide Met-enkephalin by ACE (Benuck et al., 1982). This might explain the often reported mood elevation in patients treated with ACE inhibitors. Interestingly, probably for conformational reasons, β-endorphin, as well as dynorphin 1–13 and 1–17, appear to be resistant to NEP, ACE, and, to a lesser extent, APN (Turner et al., 1987). The coidentity of enkephalinase and NEP was clearly established when the two enzymes were found to be immunologically indistinguishable (Relton et al., 1983; Matsas et al., 1983).

Because of their rather homogeneous distribution apparently unrelated to that of enkephalins or opioid receptors, a physiological role for aminopeptidases as enkephalin-metabolizing enzymes was initially questioned (Schwartz et al., 1981). Nevertheless, two aminopeptidases, differing in their sensitivity to the natural inhibitors puromycin and bestatin, were purified (Hersh, 1981; Hui, et al., 1983); both were capable of cutting the $Tyr^1$-$Gly^2$ bond of enkephalins. Bestatin, but not puromycin, was shown to potentiate the analgesic effect of i.c.v. injected Met-enkephalin (Carenzi et al., 1981), suggesting that the puromycin-sensitive aminopeptidase was not the relevant enzyme. The common identity of the membrane-bound bestatin-sensitive aminopeptidase and APN was finally established by titration with an antibody directed toward the kidney enzyme (Matsas et al., 1985; Gros et al., 1985). The preferential involvement of APN in the physiological inactivation of enkephalins was demonstrated by the increased analgesic potency of inhibitors more selective for APN than bestatin, such as kelatorphan (Waksman et al., 1985a).

In addition to these two enzymes, a membrane-bound DAP was identified in rat brain (Gorenstein and Snyder, 1979) and purified from pig kidney (Chérot et al., 1986a).

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TABLE 1
*Selected mixed enzyme inhibitors of NEP/APN or NEP/ACE*

Mixed inhibitors NEP/APN or NEP/ACE

$S_1$, $Zn^{2+}$, $S'_1$, $S'_2$; (-) (+)

| Structure | Compound | $K_I$ (nM) NEP | $K_I$ (nM) APN |
|---|---|---|---|
| $^-O$-HN-C(=O)-$CH_2$-CH($CH_2$Φ)-CONH-CH($CH_3$)-$COO^-$ | *Kélatorphan* | 1.8 | 380 |
| $^-O$-HN-C(=O)-$CH_2$-CH($CH_2$Φ)-CONH-CH($CH_2$Φ)-$COO^-$ | *RB 38A* | 0.9 | 120 |
| $^-O$-HN-C(=O)-$CH_2$-CH($CH_2$Φ)-NHCO-$CH_2$-CH($CH_3$)-$COO^-$ | *JFH 19* | 0.2 | 2.000 |
| $^-O$-HN-C(=O)-$CH_2$-CH($CH_2$Φ)-CONH-CH—CH-$COO^-$ (ring) | *XJ 9* | 3.8 | 74 |
| $^+H_3N$-CH(($CH_2$)$_2$-$SCH_3$)-$CH_2$-$S^-$ | *PC 18* | >10.000 | 8 |
| $S^-$-$CH_2$-CH($CH_2$Φ)-CONH-CH($CH_2$Φ)-$COO^-$ | *ST43* | 1.5 | >10.000 |
| | | **NEP** | **ACE** |
| $S^-$-$CH_2$-CH($CH_2$Φ)-CONH-CH($CH_2$-CH($CH_3$)-$CH_3$)-$COO^-$ | *ES34* | 4.5 | 55 |
| $S^-$-$CH_2$-CH($CH_2$Φ)-CONH-CH($OCH_2$Φ)-$COO^-$ | *ES 37* | 5.2 | 14 |
| $CH_3$-CH($CH_3$)-CH($S^-$)-CONH-CH(CH($CH_3$)-$CH_2$-$CH_3$)-CONH-CH($CH_2$Φ-OH)-$COO^-$ | *PC 57* | 1.4 | 0.2 |

However, it has been demonstrated using specific inhibitors that this enzyme is not critically involved in enkephalin metabolism (Chérot et al., 1986b).

The role of NEP and APN in enkephalin degradation has now been amply confirmed by the use of numerous inhibitors, either specific for one enzyme or able to inhibit both (see tables 1 and 2 and section IX). A clear participation of NEP in the in vivo inactivation of other neuropeptides is still not well established. However, as discussed in the following section, NEP could participate in the interruption of the physiological actions of SP in the rat substantia nigra (Mauborgne et al., 1987a) and neurotensin in structures as yet not well defined (Checler et al., 1983; Coquerel et al., 1986). The involvement of NEP in the physiological inactivation of other neuropeptides will have to be confirmed by the use of specific antagonists of neuropeptides capable of reversing both the pharmacological response induced by the endogenous effector and that induced by an NEP inhibitor. As discussed at the end of this review, these requirements are fulfilled for ANP; this peptide is, therefore, the second peptide messenger whose action is clearly regulated by NEP. This does not exclude the participation of the peptidase in other peptidergic pathways which remain to be characterized.

## III. Neutral Endopeptidase 24.11 Purification and Assay of Neutral Endopeptidase 24.11 Activity

NEP has been purified from various tissues, such as kidney (Kerr and Kenny, 1974b), pituitary (Almenoff et al., 1981), brain (Relton et al., 1983), and intestine (Fulcher et al., 1983), using various detergents or lipophilic solvents to solubilize this membrane-bound protein. Immunoaffinity chromatography with polyclonal

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(Fulcher et al., 1983) and, especially, monoclonal antibodies (Aubry et al., 1987) has greatly facilitated the rapid preparation of large quantities of pure enzyme for functional and structural studies.

Numerous sensitive assays have been developed that are based on the wide substrate selectivity of NEP. Current methods include measuring the tripeptide fragments [$^3$H]Tyr-Gly-Gly or [$^3$H]Tyr-D-Ala-Gly formed by cleavage of the Gly-Phe bond of [$^3$H]Leu-enkephalin or [$^3$H]D-Ala$^2$-Leu-enkephalin, respectively (Vogel and Altstein, 1977; Llorens et al., 1982). [$^3$H]Leu-enkephalin is also currently used to study APN activity. Internally quenched fluorogenic substrates such as the NEP selective, commercially available, dansyl-D-Ala-Gly-Phe($pNO_2$)-Gly allows continuous recording of NEP activity (Florentin et al., 1984).

NEP activity can also be detected using a two-step reaction catalyzed sequentially by NEP and APN. The substrate benzyl-Gly-Arg-Arg-Leu-2NA is cleaved by NEP at the Arg-Leu bond, leading, after addition of APN, to the formation of 2-naphtylamine by removal of the N-terminal amino acid, Leu. 2-Naphtylamine can then be quantified by fluorescence after diazotation (Almenoff and Orlowski, 1984). Owing to the common identity of NEP and CALLA and to the clinical interest in characterizing and quantifying this protein on the surface of various cells, new methods have been recently proposed. A highly sensitive enzyme-linked immunosorbent assay method for NEP applicable to material of porcine and human origin has been developed (Howell et al., 1991). The limits of using immunological methods to quantify NEP are their species specificity and their generally lower sensitivity compared to enzymatic methods (Beaumont et al., 1989; Milhiet, 1992). Thus, numerous lymphoid cells, defined as CALLA negative using immunological detection by flow cytometry, were shown to have low but significant levels of NEP activity at the surface when [$^3$H]D-Ala$^2$-Leu-enkephalin was used as substrate. However, due to the low specificity of NEP, enzymatic methods require control with selective inhibitors. For this reason, highly sensitive methods based on the use of highly selective fluorescent (Milhiet et al., 1992b) or radioiodinated (Fournié-Zaluski et al., 1992c) NEP inhibitors have been recently developed. The major advantage of these compounds over monoclonal antibodies is that they recognize NEP independently of the species and the degree of glycosylation of the enzyme. A recent study has shown, for example, that, of 20 human monoclonal antibodies tested, only four cross-reacted with a rat cell line (Hélène et al., 1992).

The fluorescent thiorphan derivative ($IC_{50}$ = 10 nM on NEP) has been used to detect NEP on the cell surface, to follow its intracellular traffic using photomicroscopy (Milhiet et al., 1993), and to quantify the presence of the enzyme on various human or rodent cells by flow cytometry (Milhiet et al., 1992b). However, the most interesting method of directly characterizing the presence of NEP, whatever the origin and the cellular or subcellular localization of the metallopeptidase, is based on the fact that NEP preserves some of its enzymatic activity after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Sullivan and Johnson, 1989), a property probably due to the high content of disulfide bonds in the peptidase. Thus, the highly potent ($K_D$ = 0.03 nM) and specific inhibitor [$^{125}$I]RB104 can be used to visualize and quantify as little as 2 ng of the enzyme in crude tissue extracts after gel electrophoresis (Fournié-Zaluski et al., 1992c; Soleilhac et al., 1992).

## IV. Substrate Specificity and Mechanism of Action of Neutral Endopeptidase 24.11

NEP is a glycosylated Zn metallopeptidase inhibited by nonspecific metal-chelating reagents, such as $\beta$ mercaptoethanol or ethylenediaminetetraacetic acid (Kerr and Kenny, 1974a,b). The Zn metallopeptidases form a large group of enzymes which include, in addition to NEP, APN, carboxypeptidases A, B, and E, ACE, collagenases, and the bacterial endopeptidase TLN (reviewed by Vallee and Auld, 1990).

As shown from the crystallographic analyses of carboxypeptidase A (Lipscomb, 1980) and TLN (Kester and Matthews, 1977a), all of the Zn metalloproteases have similarities in their active sites and in their respective mechanisms of action (Kester and Matthews, 1977b; reviewed by Matthews, 1988). Schematically, the hydrolysis of a peptide bond by these enzymes involves (*a*) the coordination of the oxygen of the scissile bond to the Zn atom; (*b*) a glutamate-promoted nucleophilic attack by a water molecule on the carbonyl carbon polarized by the Zn ion; and (*c*) the protonation of the nitrogen of the peptide bond to be cleaved, leading to a breakdown of the weakened linkage between the tetrahedral carbon and the protonated nitrogen atoms, with subsequent release of the two peptide fragments, one of them corresponding to a single amino acid in the case of exopeptidases (aminopeptidases or carboxypeptidases). Crystallographic studies of TLN complexed with carboxyl or hydroxamate inhibitors have suggested that hydrolysis occurs through the formation of a pentacoordinate complex of the metal, including the oxygen of the scissile bond and the three Zn coordinating amino acids of the peptidase, without displacement of the water molecule initially bound to the Zn atom (Hangauer et al., 1984; Matthews, 1988).

The specificity of the Zn metallopeptidases is essentially ensured by Van der Waals and ionic interactions between their $S_2$, $S_1$, $S_1'$ and $S_2'$ subsites and the lateral chains of the corresponding $P_2$, $P_1$, $P_1'$ and $P_2'$ moieties of the substrate. Specificity is also determined by several well-positioned hydrogen bonds between donor and acceptor groups of the bound molecule and the polar residues of the peptidases, such as Asn$^{112}$ and Arg$^{203}$ in TLN.

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The structure of NEP is discussed in greater detail in the following section, but for the purposes of this section it is important to note that the enzyme preferentially cleaves peptides on the amino side of hydrophobic residues (Phe, Leu, Met), suggesting the occurrence of a lipophilic $S_1'$ subsite similar to that found in TLN (Kerr and Kenny, 1974b). A more detailed comparison of these enzymes has shown that the presence of a Phe in the $P_2$ position optimizes the specificity and the turnover rate constant of NEP, provided that the $S_1$ subsite is occupied by a Gly or Ala residue, a restriction that does not occur in TLN (Hersh and Morihara, 1986; Pozsgay et al., 1986). However, when we consider the design of better adapted inhibitors, the results of enzymatic studies suggesting the presence of putative well-defined subsites in metallopeptidases must be used cautiously. The rate of cleavage of substrates is critically dependent on the dissociation rate of the two metabolites formed, which is a function of their solvatation and the energy-consuming transconformational processes associated with their release from the enzyme's active site. Most of these parameters have no influence on the binding affinity of inhibitors which is dominated by ionic and Van der Waals interactions. Inhibitors (or antagonists in the case of receptors) are, therefore, better adapted to scrutinize the presence and the nature of putative subsites in proteins.

NEP has a broad selectivity and can cleave various short linear or cyclic peptides such as endothelin (Fagny et al., 1991) or the ANP (Stephenson and Kenny, 1987a), as well as polypeptides of intermediate or long length, such as insulin-B chain (approximately 3000 daltons) (Kerr and Kenny, 1974b) and interleukin-$\alpha_1$ (17,000 daltons) (Pierart et al., 1988), although it sometimes acts more efficiently as a dipeptidylcarboxypeptidase than as a true endopeptidase. The $K_m$ of Leu-enkephalin-amide, for example, is about 20 times higher than that of Leu-enkephalin. This finding was interpreted as being due to a favorable ionic interaction between the free COOH-terminal carboxyl group of Leu-enkephalin and a well-positioned, positively charged amino acid in the active site (Fournié-Zaluski et al., 1979; 1981a), leading NEP to be considered as more closely structurally related to a carboxypeptidase than to an endopeptidase (Malfroy and Schwartz, 1982, 1985; Hersh and Morihara, 1986). However, the enzyme also behaves as a very efficient endopeptidase, cleaving various linear peptides in vitro, such as SP and neurokinins (Matsas et al., 1983, 1984b; Stephenson and Kenny, 1987b), gastrin and $CCK_8$ (Matsas et al., 1984a; Durieux et al., 1985; Durieux et al., 1986; Zuzel et al., 1985), neurotensin (Checler et al., 1983), and Met-enkephalin-Arg-Phe. The chemotactic peptide formyl-Met-Leu-Phe was also shown to be hydrolyzed by NEP. The colocalization of NEP immunoreactivity with senile plaques in brain of patients suffering from Alzheimer disease warrants an investigation of a possible role of NEP in $\beta$-amyloid protein degradation (Sato et al., 1991). We have recently shown that in vitro NEP cleaves the $\beta$-amyloid (fragment 1–40) at several sites (unpublished results).

The active site of NEP, especially the catalytic subsite, has been shown to be large (Fournié-Zaluski et al., 1983), accounting for the ability of the enzyme to cut the Cys-Phe bond of ANP, i.e., at a site within the ring formed by the two half-cystines present in this peptide, SLRRSSCFGGRMDRIGAQSGLGCNSFRY (Stephenson and Kenny, 1987a). It is important to notice that TLN cleaves the enkephalins and ANP at the same bonds as does NEP (E. Lucas Soroca and B. P. Roques, unpublished results), emphasizing again the close correspondence in the active sites of both enzymes.

In addition to the enkephalins and Met-enkephalin-Arg-Phe, NEP shows little activity toward other opioid peptides. All of these peptides are characterized by the N-terminal sequence of Met or Leu-enkephalin, but the efficiency of NEP in cleaving their Gly[3]-Phe[4] bonds is exquisitely sensitive to the length of the amino acid sequence added at the COOH terminus (Turner et al., 1987). Thus, dynorphin 1–9, dynorphin 1–13, $\alpha$- and $\beta$-neoendorphin, and $\beta$-endorphin are poor substrates, suggesting a conformationally related hindered access of the enzyme-sensitive bonds to the active site. This is an important observation because it indicates that the "opioid" pharmacological effects induced by NEP inhibitors, and probably by mixed inhibitors acting on both NEP and APN, are mainly due to the protection of the two endogenous enkephalins and perhaps partially to that of the extended heptapeptide, Met-enkephalin-Arg-Phe.

Like NEP, with which it is often found colocalized, the membrane-bound APN also has a broad specificity (McDonald and Barrett, 1986), although hydrophobic residues, preferentially aromatic, in the $NH_2$-terminal position are more rapidly removed. In addition, the $S_1'$ and $S_2'$ subsites of APN also seem to prefer hydrophobic residues (Hernandez et al., 1988; Xie et al., 1989a,b).

The possible involvement of NEP in the degradation of a peptide in a crude tissue preparation contaminated by other peptidases is usually demonstrated by using either thiorphan or phosphoramidon as selective inhibitors. Nevertheless, the in vitro degradation of a peptide by an enzyme, even using a tissue in which both molecules are localized, does not necessarily mean that the peptidase is responsible for its metabolism in vivo. Thiorphan, for example, has little effect on the degradation of rat striatal $CCK_8$ (Butcher et al., 1989) or spinal SP (Yaksh and Chipkin, 1989; Mauborgne et al., 1991) in spite of the presence of NEP in these tissues. However, in other brain regions, such as the substantia nigra, SP might well be degraded by NEP (Mauborgne et al., 1987a). Owing to the wide distribution of NEP in peripheral tissues, it is probable that other putative physiological substrates are yet to be characterized.

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## V. Design of Selective Neutral Endopeptidase 24.11 and Aminopeptidase N Inhibitors

Several recent reviews have been devoted to the synthesis of NEP and aminopeptidase inhibitors (Roques and Fournié-Zaluski, 1986; Thorsett and Wyvratt, 1987; Rich, 1990) and to the differences in the active sites of NEP and ACE (Roques, 1985). Given the large similarities in the active sites of Zn metallopeptidases (reviewed by Matthews, 1988), the rational design of potent and selective NEP inhibitors and mixed inhibitors of NEP and APN was based on the synthesis of molecules that contain a strong metal-coordinating group and that are able to satisfy all of the possible energetically favorable interactions with at least one of the subsites surrounding the catalytic site. Moreover, for the exopeptidases, the presence of a positively charged group on the $P_1$ residue or a negatively charged group on the $P_2'$ residue can be used to increase the selectivity of inhibitors for aminopeptidases or peptidyl-dipeptidases, respectively (Roques and Fournié-Zaluski, 1986; Roques, 1988b). Because ACE is also able to cleave the enkephalins at their $Gly^3$-$Phe^4$ bond, showing that its active site has some similarities with that of NEP, a comparison of the inhibitory potencies of some inhibitors will be given for both enzymes to illustrate how this problem has been overcome or how this information is now used to design mixed NEP/ACE inhibitors. Only the most representative compounds in each series of inhibitors, characterized by the nature of the Zn coordinating group, are reported.

### *A. Development of Selective Inhibitors of Neutral Endopeptidase 24.11*

*1. Thiol inhibitors.* Extensive studies of enkephalin analogues and dipeptides have shown that the specificity of NEP is essentially ensured by the $S_1'$ subsite, which interacts preferentially with aromatic or large hydrophobic moieties. The $S_2'$ subsite has a poor specificity, although in contrast to ACE, but like TLN, a proline in this position leads to poor inhibitors (Fournié-Zaluski et al., 1979, 1981a; Llorens et al., 1980). It was also observed that N-methylation of the $Phe^4$-$Met^5$ amide bond in enkephalin analogues or in various dipeptides decreased their affinities for NEP but not for ACE (Fournié-Zaluski et al., 1979, 1981a). This observation is in agreement with the good affinity for ACE of inhibitors bearing an N-methylated amino acid or a proline in the $P_2'$ position. This property of ACE was advantageously taken into account in the synthesis of captopril [(*S*)-2-methyl-3-mercaptopropionyl]-L-proline (Ondetti et al., 1977).

The above observations were used to design the first described synthetic potent NEP inhibitor, thiorphan (HS-$CH_2$-CH($CH_2\phi$)-CONH-$CH_2$-COOH, N[(*R,S*)(3-mercapto-2-benzyl-propanoyl)]-glycine ($K_I$ = 4 nM) (table 2) (Roques et al., 1980). Thiorphan is only about 50-fold more potent in inhibiting NEP than ACE. However, its two enantiomers (*R* and *S*) have the same inhibitory potency toward NEP but not for ACE (*S*-isomer = 140 nM; *R*-isomer = 860 nM), indicating large differences in the stereochemical requirements for optimal interactions in the active sites of the two enzymes. The similar affinities of *R*- and *S*-thiorphan for NEP contrast with the 100-fold better inhibitory potency of L-Phe-Ala ($IC_{50}$ approximately 1 μM) as compared with D-Phe-Ala ($IC_{50}$ approximately 100 μM), demonstrating the energetically greater importance of the binding of the coordinating group (thiol) with the Zn atom than the stereochemically dependent Van der Waals interactions governing subsite recognition (Bouboutou et al., 1984; Fournié-Zaluski et al., 1985). These findings illustrate that caution must be taken when directly extrapolating the results of inhibition induced by di- or tripeptides to the design of potent and selective inhibitors. For example, Phe-Asp is among the weakest NEP dipeptide inhibitors (Llorens et al., 1980), but when an Asp residue was introduced in place of Gly in thiorphan, the inhibitor thus obtained kept the strong inhibitory potency of its parent compound (Beaumont et al., 1992).

To try to increase NEP selectivity, various structural modifications of the $P_1'$ and/or $P_2'$ moieties of thiorphan were made (Roques et al., 1982a; Roques, 1985; Fournié-Zaluski et al., 1981a, 1982, 1984b; Gordon et al., 1983; Thorsett and Wyvratt, 1987). However, this generally resulted in the synthesis of numerous highly potent mixed inhibitors of NEP and ACE (table 1), such as ES37 (NEP: $K_I$ = 5.2 nM; ACE: $K_I$ = 12 nM) or ES34 (NEP: $K_I$ = 4.5 nM; ACE: $K_I$ = 55 nM) (Fournié-Zaluski et al., 1984b), subsequently designated SQ 28,133 (Seymour et al., 1991c). A clear increased selectivity for NEP was observed by replacing the benzyl group of thiorphan with a cyclohexyl residue, but this was also associated with a decreased affinity (NEP: $IC_{50}$ = 31 nM; ACE: $IC_{50}$ > 10,000 nM). Modifications of the COOH-terminal carboxyl group of thiorphan, such as esterification or amidification, led to a modest decrease in inhibitory potency for NEP, which is in agreement with the endopeptidase nature of this enzyme, but to a larger loss in ACE inhibition, which underlies the critical role played by the COOH-terminal carboxyl group and by its spatial orientation in ACE substrate recognition (Fournié-Zaluski et al., 1984b). In agreement with the flexibility of the side chain of the arginine residue ($Arg^{102}$) located at the surface of NEP and at the edge of the $S_1'$ subsite (Beaumont et al., 1992), an increased selectivity was obtained by replacing the glycine moiety of thiorphan with longer amino-alkyl carboxylic acids, such as amino heptanoic acid in SQ 29,072 [HS-$CH_2$-CH($CH_2\phi$)-CONH($CH_2$)$_6$-COOH] (Seymour et al., 1989b) or by a heterocyclic hydrazide as in RU 44004 (*R,S*)HS-$CH_2$-CH($CH_2\phi$)-CONH-$NC_4H_8O$ (table 2).

The first described thiol inhibitors capable of interacting with the $S_1$, $S_1'$, $S_2'$ domain of NEP, such as IGM22, HS-CH($CH_2$CH($CH_3$)$_2$)-CH($CH_2\phi$)-CONH-CH($CH_3$)-

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TABLE 2
*Relatively selective NEP inhibitors*

ENDOPEPTIDASE-24.11 ACTIVE SITE

| Inhibitor | $K_I$ (nM) NEP | $K_I$ (nM) ACE |
|---|---|---|
| Thiorphan | S 4<br>R 4 | S 140<br>R 860 |
| Rétrothiorphan | S 210<br>R 2.3 | > 10.000 |
| Acetorphan (R,S)<br>Sinorphan (S)<br>Retorphan (R)<br>(prodrugs) | 10 000 | N.D. |
| SQ 29 072 | 26 | > 10.000 |
| SCH 39 370 | 11 | > 10.000 |
| IGM 22 | 4 | > 10.000 |
| HACBO-Gly | 1.7 | > 10.000 |
| RB 104 | 0.05 | > 10.000 |
| RU 44004 | 200 | > 10.000 |
| UK 69578 | 28 | > 10.000 |

COOH (Fournié-Zaluski et al., 1992b; Gomez-Monterrey et al., 1993), have $IC_{50}$ values in the same range as thiorphan, an observation in agreement with the absence of a true hydrophobic $S_1$ subsite in the peptidase (table 2). Replacement of the carboxyl group of thiorphan and its derivatives by the acidic -$SO_3H$ moiety did not significantly increase their inhibitory potencies but improved the pharmacokinetic properties of these inhibitors (Mimura et al., 1992).

If we take into account that modifying the $P_1'$ and $P_2'$ residues cannot ensure a complete differentiation of NEP and ACE inhibition, modification of the amide $P'_1$-$P'_2$ bond of the inhibitors was considered the most promising discriminating factor. This was confirmed by retroinversion of the amide bond of thiorphan, because the resulting retrothiorphan HS-$CH_2CH(CH_2\phi)$-NHCO-$CH_2$-COOH is almost as potent as thiorphan, having an $IC_{50}$ of 6 nM, but displays a drastic loss of potency for ACE ($IC_{50}$ > 10,000 nM) (Roques et al., 1983). This successful approach was based on the assumption that the retroinversion of the natural amide bond of thiorphan would allow the respective groups of (*R*)-retrothiorphan to maintain similar interactions, including hydrogen bonding, with NEP but not with ACE, given the stringent stereochemical requirements of the $S_1'$-$S_2'$ subsites of the latter enzyme. As hypothesized, (*R*)retrothiorphan ($K_I$ = 2.3 nM) is a better NEP inhibitor than its *S*-isomer ($K_I$ = 210 nM).

Because inhibition experiments using separate *R*- and *S*-isomers of thiorphan and retrothiorphan (Fournié-Zaluski et al., 1986) indicated that the specificity of NEP and TLN were very similar, computer studies and crystallographic determination of the three-dimensional structures of *S*-thiorphan and *R*-retrothiorphan bound

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to TLN were undertaken. These studies showed that, as anticipated, the carbonyl oxygen and amide hydrogen display very similar hydrogen bonding despite the inversion of the -CONH- linkage. The good agreement between the computer-modeling results (Benchetrit et al., 1987) and crystallographic analysis (Roderick et al., 1989) suggest that docking experiments, using a reconstituted active site of NEP, may soon be feasible. The topological concept of retroinversion isomers (Goodman and Chorev, 1979), extended for the first time to enzyme inhibitors in retrothiorphan, has since been successfully extended to other NEP inhibitors. These inhibitors have the added advantage of an enhanced resistance to enzymatic degradation.

Another class of mercapto inhibitors, N-mercaptoacetyldipeptides, has been reported by two different groups (Altstein et al., 1982, 1983; Van Amsterdam et al., 1987). These compounds were more potent than might be expected, given the size of the extended chain bearing the thiol group. This can be explained if the mercapto acetyl function is assumed to act as a bidentate ligand for the Zn ion.

The thiol inhibitors, including thiorphan, are relatively hydrophilic and have limited ability to penetrate the gastrointestinal and blood-brain barriers. An improvement in the bioavailability of thiorphan has been obtained by protecting its thiol and carboxyl hydrophilic groups (Roques et al., 1980, 1982a,b; Fournié-Zaluski et al., 1981a,b). The resulting prodrugs, such as acetorphan [(*R,S*)$CH_3COS$-$CH_2$-CH($CH_2\phi$)-CONH-$CH_2$-$CO_2CH_2\phi$] or its *S*-isomer, sinorphan, are rapidly transformed to thiorphan by esterases in the brain and blood. This prodrug approach is currently used with other thiol-containing NEP inhibitors.

*2. Carboxyl inhibitors.* Introduction of a carboxyl group at the $NH_2$ terminus of the dipeptide Phe-Leu resulted in a molecule with an $IC_{50}$ of 0.1 $\mu$M. Unlike ACE inhibitors, however, the affinities of these compounds for NEP were not modulated by the relative positions of the carboxyl group and the phenyl moiety, which supports the idea of a larger catalytic site in NEP (Fournié-Zaluski et al., 1982, 1983). Consequently, some of these molecules are relatively potent, mixed inhibitors of NEP and ACE. Based on the high inhibitory potency for ACE exhibited by enalapril, a tripeptide analogue that interacts with the $S_1$, $S_1'$, and $S_2'$ subsites of the enzyme (reviewed by Thorsett and Wyvratt, 1987), carboxyl-derived inhibitors of NEP and various N-carboxyl alkyl dipeptides bearing an hydrophobic chain on the carboxyalkyl group were synthesized (Mumford et al., 1982; Chipkin, 1986; Northridge et al., 1989; Haslanger et al., 1989). The significant increase in potency obtained with these compounds and the influence of the chirality of the side chains suggested the existence of a $S_1$ subsite that could exhibit some selectivity for NEP recognition. However, nonspecific Van der Waals interactions and bidentate coordination of the carboxyl group are more probable explanations (Roques et al., 1982a,b; Elliot et al., 1985).

Three carboxyl NEP inhibitors have so far been extensively studied, SCH 32,615, SCH 39,370, and UK 69,578 (table 2). In the latter compound, a cyclopentyl group and a *p*-aminocyclohexane carboxyl moiety were introduced in the $P_1'$ and $P_2'$ positions, respectively, to improve the selectivity for NEP (Fournié-Zaluski et al., 1984b). Prodrugs of these molecules can be easily obtained by esterifying the carboxyl function with lipophilic alcohols.

*3. Bidentate inhibitors.* A detailed analysis of NEP inhibition by $NH_2$-terminal hydroxamates was performed by Bouboutou et al. (1984), using four series of novel dipeptides analogues. This study showed that (*a*) hydroxamates are more efficient than N-formyl-N-hydroxyamino derivatives, a result identical with that obtained for TLN (Nishino and Powers, 1978); (*b*) the insertion of a methylene spacer between the Zn-chelating group and the benzyl-bearing carbon increases the inhibitory potency of the molecules; and (*c*) all of the inhibitors have poor affinities for ACE ($IC_{50}$ > 10,000 nM). Among these derivatives, HACBO-Gly, [HO-NHCO-$CH_2$-CH($CH_2\phi$)-CONH-$CH_2$-COOH] ($K_D$ = 0.4 nM) was developed as a tritiated probe, e.g., [$^3$H]HACBO-Gly, for binding studies and in vitro or in vivo visualization of NEP by autoradiography (Waksman et al., 1984; Salès et al., 1991).

The optimization of the interactions of hydroxamate inhibitors belonging to the HACBO-Gly series in the active site of NEP (Fournié-Zaluski et al., 1985; Xie et al., 1989a,b) has shown that the absolute configuration of the $P_1'$ residue, as well as the size and the hydrophobicity of the $P_2'$ residue, does not greatly influence enzyme recognition. However, these two parameters have played an important role in the design of selective or mixed inhibitors of NEP, APN, and DAP; this will be discussed later. The retroinversion of the amide bond in dipeptide hydroxamates only led to very efficient NEP inhibitors with compounds containing a methylene spacer between the hydroxamate group and the carbon bearing the $P_1'$ benzyl side chain (Fournié-Zaluski et al., 1989). The first of this series (retro-HACBO-Gly) is a highly potent and selective NEP inhibitor with an $IC_{50}$ of 0.5 nM. An increase in NEP affinity was also obtained by monosubstitution of the $P_2'$ residue. Thus, the inhibitor, (*S,S*)HONH-CO-$CH_2$-CH($CH_2\phi$)-NHCO-$CH_2$-CH($CH_3$)-COOH (JFH19), is more than 100- and 2000-fold more selective for NEP ($IC_{50}$ approximately 0.15 nM) than for APN and DAP, respectively (Fournié-Zaluski et al., 1989).

The replacement of Gly in retro-HACBO-Gly by a highly hydrophobic aromatic moiety in RB104 led to a large increase in NEP affinity (table 2). [$^{125}$I]RB104 is the most potent NEP inhibitor described so far ($K_D$ = 0.03 nM), a property that has been used to directly

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visualize NEP in crude membrane fractions after gel electrophoresis (Fournié-Zaluski et al., 1992c).

*4. Phosphorus-containing inhibitors.* Another interesting series of inhibitors are the phosphorus-containing dipeptides, among which is the natural competitive inhibitor of NEP, phosphoramidon, produced by *Streptomyces tanashiensis*; this compound was initially described as a TLN inhibitor (Umezawa, 1972). Various potent phosphorylated inhibitors of NEP have been described (Altstein et al., 1982; Garcia-Lopez et al., 1985), and more recently, phosphoramidate derivatives have also been reported (Elliot et al., 1985). However, these compounds, although potentially able to interact with the $S_1$, $S_1'$, and $S_2'$ subsites of NEP, are less efficient than the phosphorylated dipeptide. Nevertheless, this series of potential inhibitors deserves further investigation.

### *B. Aminopeptidase N and Dipeptidyl Peptidase Inhibitors*

Various natural aminopeptidase inhibitors have been isolated from actinomycetes. These include puromycin, which is nonselective and weakly active, and the more potent peptide analogues bestatin, $H_2N$-$CH(CH_2\phi)$-$CH(OH)CO$-Leu (Umezawa et al., 1985), amastatin, $H_2N$-$CH$-$[CH_2CH(CH_3)_2]$-$CH(OH)CO$-Val-Val-Asp, and derivatives (Rich et al., 1984). However, these molecules have little selectivity for APN.

Simple molecules that recognize only the $S_1$ subsite and interact with the Zn atom, such as substituted aminoethanethiols, were found to be highly potent APN inhibitors (Chan, 1983; Pickering et al., 1985; Fournié-Zaluski et al., 1992a). Recently, $\alpha$-thiolbestatin analogues (Ocain and Rich, 1988) and sulfur-containing modified di- or tripeptides (Gordon et al., 1988) have been synthesized. Several of these compounds exhibit high inhibitory potencies on various aminopeptidases, but their $IC_{50}$ values for APN are low (>10 $\mu$M). To improve the bioavailability of phenylalanine-thiol ($IC_{50}$ approximately 20 nM), a hydrophobic carbamate group was introduced on the thiol function, leading to an aminopeptidase inhibitor designated carbaphethiol. This compound is reported to elicit antinociceptive activity after i.v. administration in mice (Gros et al., 1988).

Inhibitors of DAP were designed by taking into account the requirement for a charged amino group in the $P_2$ position and the hydrophobicity of the extended $S_1$, $S_1'$, and $S_2'$ active site. The most potent DAP inhibitor, Tyr-Phe-NHOH ($K_I$ approximately 10 nM), is also highly selective with $IC_{50}$ values > 10,000 nM for NEP, APN, and ACE (Chérot et al., 1986b).

## VI. Development of Mixed Inhibitors of Neutral Endopeptidase 24.11 and Aminopeptidase N and of Neutral Endopeptidase 24.11 and Angiotensin-converting Enzyme

Because the enkephalins are degraded by more than one enzyme, i.e., NEP, APN, and possibly DAP, numerous mixed inhibitors of these enzymes have been designed. This was achieved using the hydroxamate group as a Zn-chelating moiety by hypothesizing that the strength of its coordination to the metal should counterbalance a less than perfect fit of the inhibitor side chains to the active sites of the three metallopeptidases (Bouboutou et al., 1984; Fournié-Zaluski et al., 1985). Accordingly, kelatorphan [(*R,S*)(HONHCO-$CH_2$-$CH(CH_2\phi)$-CONH-$CH(CH_3)$-COOH] strongly inhibits NEP ($IC_{50}$ = 1.8 nM), APN ($IC_{50}$ = 380 nM), and DAP ($IC_{50}$ = 0.9 nM), whereas its *S,S*-stereoisomer is a highly potent and selective NEP inhibitor with $IC_{50}$ values of 1.8 nM for NEP, 29,000 nM for APN, and 100 nM for DAP (table 1).

A large number of analogues have been synthesized using this new concept, and all have a pseudodipeptide structure. RB38A [(*R,S*)HONH-CO-$CH_2$-$CH(CH_2\phi)$-CONH-$CH(CH_2\phi)$-COOH] is as active as kelatorphan on DAP and NEP but is a more potent inhibitor of APN ($IC_{50}$ = 120 nM) (Schmidt et al., 1991). In contrast, the *S,S*-stereoisomer RB38B is a selective and very efficient NEP inhibitor ($IC_{50}$ = 5.6 nM). It was interesting to observe that a hydrophobic, large residue in the $P'_2$ position significantly increased APN recognition without affecting NEP and that retroinversion of the amide bond also led to highly efficient mixed inhibitors (Fournié-Zaluski et al., 1985; Hernandez et al., 1988).

At the present time, kelatorphan and retrokelatorphan-related bidentates are the only mixed inhibitors with nanomolar affinities for both NEP and APN. However, their high water solubility, although favorable for binding studies and inhibiting enzymes easily accessible from the circulation, prevents them from crossing the blood-brain barrier. Efforts to improve their bioavailability have met with little success. Another strategy was, therefore, used, which consisted of linking highly potent thiol-containing APN and NEP inhibitors by a disulfide bond. In addition to the easy modulation of their hydrophobicity, one of the main advantages of these mixed inhibitor prodrugs is the stability of the disulfide bond in plasma, contrasting with its relatively rapid breakdown in brain (Fournié-Zaluski et al., 1992b). Among the various compound synthesized, RB101 [$H_2N$-$CH(CH_2$-$CH_2$-S-$CH_3)$-$CH_2$-S-S-$CH_2$-$CH(CH_2\phi)$-CONH-$CH(CH_2\phi)$-$COOCH_2\phi$] has been shown to be very active in antinociceptive tests after i.v or s.c. administration at low doses (Noble et al., 1992a).

Relatively few mixed NEP/ACE inhibitors have been synthesized so far, but this number will probably grow in the near future, and attention must be paid to the introduction of pharmacokinetically favorable cyclic moieties in the structure of these compounds, thereby taking advantage of the now well-established structural differences in the active site of NEP and ACE (Roques et al., 1983; Fournié-Zaluski et al., 1984b; Roques, 1985). Based on these findings, we have recently synthesized

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three series of new mixed NEP/ACE inhibitors (B. P. Roques and M.-C. Fournié-Zaluski, French patent 91.09306) that exhibit nanomolar affinity and are capable of blocking almost completely the two peptidases after oral administration in mice ($ED_{50}$ = 0.5 to 5 mg/kg).

## VII. Molecular Biology of Neutral Endopeptidase 24.11

### A. Complementary DNA Cloning and Sequencing

The primary structure of rabbit NEP was elucidated by cloning and sequencing the DNA complementary to the mRNA coding for the kidney enzyme (Devault et al., 1987). The metallopeptidase consists of 749 amino acids with five N-glycosylation sites accounting for the $M_r$ 94,000 evaluated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein also contains 12 cysteine residues which are likely to be involved in disulfide bridges (Tam et al., 1985). NEP has a short $NH_2$-terminal cytoplasmic domain (27 amino acids), followed by a 23-residue hydrophobic domain, which anchors the protein in the plasma membrane, and a large extracellular domain that contains the active site (fig. 1). These observations are consistent with previous suggestions concerning the topological organization of ectoenzymes such as APN (Maroux, 1987).

Although the sequence of NEP shows only a weak homology with those of other Zn metallopeptidases, some of the most important amino acids in the active site of TLN appear to have been conserved (Benchetrit et al., 1988). Several of these residues are included in a consensus sequence VxxHExxH, which has been found in numerous other Zn endopeptidases, such as human collagenase, human ACE (Soubrier et al., 1988), and the exopeptidase APN (Jongeneel et al., 1989; Hélène et al., 1991), but not in the Zn carboxypeptidases so far sequenced (Erdös and Skidgel, 1989; Vallee and Auld, 1990). The two histidines of this sequence ($His^{583}$ and $His^{587}$ in NEP) are Zn-coordinating ligands and the glutamate ($Glu^{584}$ in NEP) plays a role in catalysis by polarizing a water molecule. An additional short homologous sequence was also found, which in TLN contains

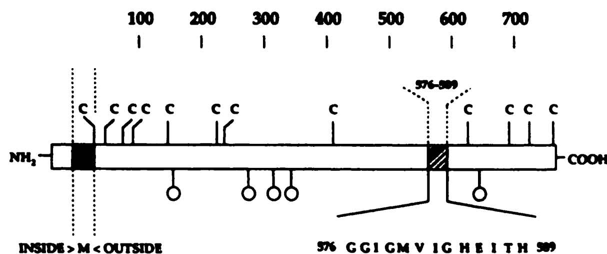


FIG. 1. Schematic model of the primary structure of NEP. The intracellular domain (residues 1 to 27) is followed by a membrane-spanning domain (M, residues 28 to 50) with the rest of the enzyme being in the extracellular space. C, 12 cysteine residues; "lollypops," putative glycosylation sites; hatched region, consensus sequence with residues at the active site of TLN.

an essential histidine residue thought to be involved in stabilizing the transition state of the enzyme-substrate complex ($His^{637}$ in NEP). This observation was in agreement with previous reports of the similarities between NEP and TLN (Roques et al., 1983; Fournié-Zaluski et al., 1984b), including the presence of a critical His residue at the active site of NEP (Beaumont and Roques, 1986) and in contrast to a Tyr residue in carboxypeptidases (Lipscomb, 1980). However, subsequent site-directed mutagenesis of $His^{637}$ showed that it has little, if any, role in NEP activity (Devault et al., 1988a), and $His^{711}$ has since been proposed as an essential active site residue from affinity labeling and site-directed mutagenesis studies (Bateman et al., 1990; Kim et al., 1992a). As previously proposed in the case of TLN (Hangauer et al., 1984), $His^{711}$ could be involved in transition state stabilization through hydrogen bonding between the $His^{711}$-NH group and the oxygen of the amide bond to be cleaved. The role of an additional tyrosine residue in stabilizing the polarized substrate remains to be firmly established.

Several other putative active site residues, which could correspond to those located in the active site of TLN, were also proposed (Benchetrit et al., 1988) by using the recently developed technique of hydrophobic cluster analysis, which predicted that, despite their very low sequence homologies, the enzymes might have a very similar active site arrangement.

Interestingly, the exopeptidase APN also possesses the consensus sequence VxxHExxH (Olsen et al., 1988), showing that, as suggested (Fournié-Zaluski et al., 1985; Fournié-Zaluski, 1988), the active site structure and mechanism of action of APN may be closer to a Zn endopeptidase than to that of classical exopeptidases. This has been confirmed both by identifying some of the critical amino acids (His, Arg) present in the active site by use of selective chemical reagents (Hélène et al., 1991) and the proposed model of inhibitor binding confirmed by crystallographic analysis of bestatin complexed to leucine-aminopeptidase, another Zn-containing enzyme (Burley et al., 1992).

Endopeptidase 24.11 from rat kidney and brain (Malfroy et al., 1987) and human placental NEP (Malfroy et al., 1988) show a high degree of homology (>90%) with the rabbit enzyme, with the only important difference being the number of possible glycosylation sites; there are five in rabbit and six in human and rat NEP (Malfroy et al., 1988). These differences in glycosylation have no effect on the enzymatic activity, but they could explain the inconsistencies in the cross-reactivity of antibodies used to localize NEP from different species (Fulcher et al., 1983; Lecavallier et al., 1989). Differences in glycosylation could also affect antibody recognition in tissues of the same species, because human NEP from the fibroblasts and kidney has been found to have a molecular weight of approximately 90 kDa, whereas the molecular

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weight of neutrophil NEP was found to be 110 kDa (Braun et al., 1984). The recombinant rabbit kidney enzyme has a molecular weight of 87 and 94 kDa when expressed in insect or in Cos cells, respectively (Fossiez et al., 1992; Devault et al., 1987).

### *B. Site-directed Mutagenesis*

The role of several of the putative active site residues of NEP has been tested by site-directed mutagenesis using recombinant enzymes expressed at the cell surface of COS-1 cells. Replacing $Glu^{584}$ by Val abolished the degradation of [$^3$H]Leu-enkephalin by the enzyme, and similar results were obtained with an $Asp^{584}$ mutant, showing that shortening the side chain of $Glu^{584}$ by a single methylene group is sufficient to suppress the polarization of the water molecule involved in the catalytic process (Devault et al., 1988b). Interestingly, the mutations were unable to change the affinity of the inhibitor [$^3$H]HACBO-Gly, suggesting that $Glu^{584}$ is not involved in the binding of this Zn-directed bidentate-containing inhibitor, a finding that can be used to design compounds capable of profiting from this potential additional interaction.

Several groups had suggested the presence of a substrate-binding Arg residue, using the reagents butanedione and phenylglyoxal (Malfroy and Schwartz, 1982; Beaumont and Roques, 1986; Bateman et al., 1989). Site-directed mutagenesis has also shown the presence in the NEP active site of two arginine residues: $Arg^{102}$, initially identified by its labeling by [$^{14}$C]phenylglyoxal (Bateman et al., 1989), and $Arg^{747}$ (Beaumont et al., 1991), which was first proposed to be in the active site by the hydrophobic cluster analysis and to correspond to $Arg^{203}$ in TLN (Benchetrit et al., 1988). Site-directed mutagenesis of the two indicates that they could both play a role in substrate binding, $Arg^{102}$ interacting with the free carboxyl group of the $P_2'$ residue of some substrates and $Arg^{747}$ with the carbonyl amide group of the $P_1'$ residue. The proposed localization of these two arginines could help to explain why NEP has both endopeptidase and dipeptidylcarboxypeptidase activities. By mutating $Arg^{102}$ to Glu it has proved possible to invert the specificity of the enzyme by charge polarity reversal, thereby opening the way to the design of recombinant enzymes with appropriate properties, such as for the enantiomeric separation of unnatural amino acids (Beaumont et al., 1992). This result, confirmed by the replacement of $Arg^{102}$ by several other amino acids (Kim et al., 1992b), also indicates that $Arg^{102}$ is located at the edge of the active site, because previous studies have shown that charge polarity reversal does not seem to work with a residue located in the interior of an active site (Hwang and Warshel, 1988).

The third Zn ligand was identified by site-directed mutagenesis as $Glu^{646}$, and replacement of this residue by Val led to a complete loss of enzyme activity and [$^3$H]HACBO-Gly-binding affinity (Le Moual et al., 1991). Interestingly, $Asp^{646}$-NEP exhibited similar $K_m$ and $K_I$ values for the substrate [$^3$H]D-Ala-Leu-enkephalin and the inhibitor [$^3$H]HACBO-Gly, respectively, but had a reduced rate of hydrolysis and a lower affinity for the Zn. These results have been interpreted by a small distortion in the geometry of the Zn-binding residues, provoking a small change in the spatial position of $Glu^{584}$ which is linked to one of these residues, $His^{583}$. This could result in a decreased nucleophilicity of the water molecule bound to both the Zn atom and $Glu^{584}$ residue (Le Moual et al., 1991) (fig. 2).

Point mutation experiments have also confirmed that, as suggested (Benchetrit et al., 1988), $Asn^{542}$ and $Ala^{543}$ of NEP are the corresponding residues to $Asn^{112}$ and $Ala^{113}$ in TLN (H. Le Moual, B. P. Roques, P. Crine, and G. Boileau, to be published).

Crystallographic studies have shown the close correspondence between TLN and other Zn metallopeptidases with regard to the location and function of amino acids crucially involved in binding and substrate hydrolysis (Matthews, 1988; Roderick et al., 1989). Results of these studies are in agreement with the often underlined conservation of active site geometries in the superfamily of Zn metallopeptidases (Vallee and Auld, 1990). The knowledge of the position of the disulfide bonds and of the most important amino acids involved in substrate and inhibitor binding, including those belonging to the

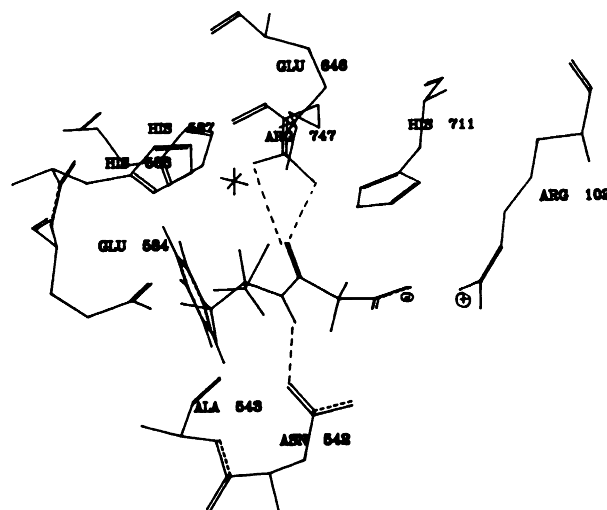


FIG. 2. A schematic representation of the binding of the NEP inhibitor, retrothiorphan (table 2), to the active site of the enzyme, with the sulfhydryl group of the inhibitor pointing toward the Zn atom. Site-directed mutagenesis studies have confirmed the proposed roles of $Glu^{584}$ in catalysis (Devault et al., 1988b), $His^{583}$, $His^{587}$, and $Glu^{646}$ in Zn binding (Devault et al., 1988a; Le Moual et al., 1991), $Arg^{747}$ (Beaumont et al., 1991) and $Arg^{102}$ (Bateman et al., 1989; Beaumont et al., 1991, 1992) in ligand binding, and $His^{711}$ which may be involved in transition state binding (Bateman et al., 1990). $Asn^{542}$ and $Ala^{543}$ are probably involved in substrate binding (Benchetrit et al., 1988). All of these residues except $Arg^{102}$ have their homologues in the active site of TLN (Matthews, 1988), and the binding mode of retrothiorphan is taken from data obtained by cocrystallization of the inhibitor with this enzyme (Roderick et al., 1989).

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relatively specific $P_1'$ subsite of NEP, which may be determined using affinity labeling (Beaumont et al., 1987), could permit the tertiary structure of the active site of NEP to be modeled through the use of computer calculations coupled to graphics analysis. This approach represents an attractive challenge to develop more potent and selective "tailor-made" inhibitors (Benchetrit et al., 1987). However, even with all the information required, care must be taken because, in spite of a remarkably similar active site topology, Zn metallopeptidases could have their respective polypeptide chains following completely different courses, as shown for TLN and carboxypeptidase A.

Crystallization of NEP appears to be hampered by its glycosylation and the presence of a highly hydrophobic membrane-spanning sequence, causing aggregation in the absence of detergents. To try to overcome the latter problem, the secretion of a soluble form of the recombinant NEP from COS-1 cells was induced by fusion of the cDNA encoding the signal peptide of a secreted protein (proopiomelanocortin) to the cDNA sequence of the complete ectodomain of NEP (Lemay et al., 1989). The recombinant soluble NEP, which has since been expressed in large quantities using insect cells transfected by a genetically modified baculovirus (Fossiez et al., 1992), is fully active and, therefore, could be useful as a first step in obtaining an enzyme that will readily crystallize. Attempts to obtain an even shorter, soluble form of NEP were unsuccessful, very likely because the modifications removed sequences containing one or more cysteine residues that are probably involved in disulfide bridges (Waksman et al., 1993).

### *C. Human Neutral Endopeptidase 24.11 (Common Acute Lymphoblastic Leukemia Antigen) Gene*

The human NEP/CALLA gene spans more than 80 kilobases and contains 24 miniexons with (*a*) exon 3 encoding the initiation site, the cytoplasmic, and transmembrane domains; (*b*) exon 19 encoding the consensus sequence VxxHExxH associated with Zn binding and substrate catalysis; and (*c*) exon 24 encoding the terminal 32 amino acids of the protein and containing the entire 3′-untranslated region (D'Adamio et al., 1989). Analysis of clones differing from one another in the 5′-untranslated region suggests that NEP gene transcription could be controlled by alternative splicing of a common or distinct pre-mRNAs, leading to differentially controlled gene expression in a tissue-specific and/or developmentally regulated manner (D'Adamio et al., 1989).

This could account for the considerable changes in the expression of CALLA/NEP at the surface of lymphoid cells in various physiopathological conditions (Le Bien and McCormack, 1989). A similar mechanism of regulatory control has been proposed to explain the large differences in the rate of synthesis between fetal and adult ANP in intestinal microvili (Danielsen et al., 1987, and references cited therein). NEP expressed on cultured synovial fibroblasts is negatively regulated by phorbol diesters (Werb and Clark, 1989), although it remains to be determined whether this regulation occurs at the 5′-untranslated region of the gene. Interestingly, autoradiographic studies with the NEP inhibitor [$^3$H]HACBO-Gly have shown a large increase in the peptidase concentration in the spinal cord during an early stage of human development (Salès et al., 1989) and, even more intriguing, a transient hyperexpression of NEP in heart, intestine, and bone marrow in rat fetus (Dutriez et al., 1992). The NEP gene is located on human chromosome 3 which also encodes somatostatin, transferrin, and its receptor, enzymes such as acetylcholinesterase and β-galactosidase, and the oncogenes c-*raf-1*, c-*erb-A*, and c-*erb-B*. Deletions and translocations of this chromosome are frequent in small cell lung and renal cell carcinomas and malignant cutaneous T-cell lymphoma (Tran-Paterson et al., 1989). Inasmuch as NEP is expressed in these tissues, it will be important to study the NEP gene in both normal and tumor cells to reveal possible alterations associated with malignancy.

Because the gene for human APN has been located on chromosome 15q,13q-ter and that of another ectoenzyme, γ-glutamyl transpeptidase, is on chromosome 22, it appears that the genes of these ectoenzymes are not under a common regulatory DNA control (Kruse et al., 1988).

A short mRNA encoding a protein of 255 amino acids and derived from the NEP gene by alternative splicing has been characterized in brain tissue (Llorens-Cortes et al., 1990). Although the putative protein would contain the consensus sequence of Zn metallopeptidases, some important amino acids present in the active site of NEP are lacking (Arg[102], Asn[542], and Ala[543]). Whether this protein is indeed expressed and, if so, whether it is located at the cell membrane and has enzymatic activity remains to be determined. It is perhaps worth noting that, when a domain close to that which would be lacking from this enzyme was deleted from recombinant NEP, no enzyme was detected at the surface of the COS-1 cells (G. Waksman et al., 1993).

A cDNA clone isolated from a human lung library was also shown to encode a shorter form of NEP characterized by the deletion of exon 16 (corresponding to the 27-amino acid sequence Ala[465] to Glu[491]). The recombinant form of this new NEP, probably produced by alternative splicing, was found at the surface of COS-7 cells, but deletion of the 27-amino acid segment reduces enzymatic activity by a factor of at least 150 (Iijima et al., 1992). Two new shorter transcripts (1.8 and 0.8 kDa) have been detected in both B- and T-cells by northern blot analysis, using probes corresponding to the $NH_2$-terminal and COOH-terminal coding sequence of CALLA (Mari et al., 1992).

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## VIII. Localization of Neutral Endopeptidase 24.11

NEP can hydrolyze a variety of regulatory peptides in vitro. Therefore, it was essential to know its precise localization and that of its possible substrates and their receptors to determine its in vivo specificity. Several groups have studied the distribution of NEP in the CNS and peripheral organs.

### A. Central Nervous System

The first precise localization of NEP in the CNS was obtained by quantitative autoradiography using the tritiated inhibitor [$^3$H]HACBO-Gly, which selectively interacts with the peptidase with a high affinity ($K_D = 0.5$ nM) (Waksman et al., 1984, 1985b, 1986a). The same approach was independently pursued for the visualization of brain ACE using the potent and selective inhibitor, [$^3$H]captopril (Srtittmatter et al., 1984).

[$^3$H]HACBO-Gly-binding sites were found to be discretely distributed in rat brain, with the highest concentrations in the choroid plexus, substantia nigra, caudate putamen, globus pallidus, olfactory tubercle, nucleus accumbens, and the substantia gelatinosa of the spinal cord (fig. 3). Moderate binding levels were found in the amygdala, the interpeduncular nucleus, the molecular layer of the cerebellum, the periaqueductal gray matter, and the hippocampus. An intense accumulation of silver grains, intermingled with streaks of nonlabeled zones corresponding to white matter tracts, was observed in the region connecting the substantia nigra and the caudate putamen and which was particularly dense in the entopeduncular nucleus. A similar pattern has been reported in a nigrostriatal pathway for ACE, visualized with [$^3$H] captopril (Strittmatter et al., 1984).

A good correspondence was found between the distribution of the enzyme and opioid receptors (Waksman et al, 1986a, and references cited therein). Thus, in the caudate putamen, [$^3$H]HACBO-Gly binding overlapped both patchy $\mu$-sites and diffusely labeled $\delta$-receptors and paralleled that of $\mu$- and $\delta$-opioid receptors in the pyramidal cells of the CA1 and CA3 regions of the hippocampus. High concentrations of both NEP and $\mu$-receptors were found in the periaqueductal gray matter and the substantia gelatinosa of the spinal cord, areas that are implicated in pain perception and analgesia (table 3). Low but measurable levels of $\delta$-opioid-binding sites were found in the substantia gelatinosa, a finding that is in agreement with a selective involvement of this opioid receptor type in spinal analgesia (Dickenson et al., 1986, 1987a). High resolution autoradiography, using emulsion-coated coverslips (Zajac et al., 1988), showed that in the medial portion of lamina V and in the motoneuronal area only $\mu$-binding sites occurred, whereas NEP was sparsely distributed in the intermediolateral column, most likely in Schwann cells as suggested by Matsas et al. (1986), who studied the pig peripheral nervous system. In the cerebellum, high levels of NEP, contrasting with the sparse distribution of both enkephalins and opioid receptors, were found, probably presynapticaly, located on $\gamma$-aminobutyric acid terminals. In human brain, a high density of NEP overlapping that of $\mu$- and $\delta$-opioid receptors was found in the caudate putamen, globus pallidus, and substantia nigra. Lower levels were found in the periaqueductal gray and cortical layers (Roques, 1989).

Similar results were obtained in the pig CNS using a polyclonal antibody and immunoperoxidase staining (Matsas et al., 1986) and in rat brain using a $^{125}$I-iodinated monoclonal antibody (Pollard et al., 1989). In both of these studies, the distribution of NEP was compared with that of Leu-enkephalin and SP, confirming that SP could serve as substrate for the peptidase in some regions, especially the substantia nigra (Mauborgne et al., 1987a). NEP immunoreactivity has also been found in the pig (Barnes and Kenny, 1988) and rat (Pollard et al., 1989) adenohypophysis. The peptide(s) processed in this region is as yet unknown. In the median eminence, the relative distribution of dense enkephalin and NEP immunoreactivities indicates that the endogenous opioid peptides might reach the hypothalamohypophyseal microcirculation to induce growth hormone secretion from the pituitary. The localization of NEP in some regions has also been investigated by a fluorescent histochemical method, leading to results largely in agreement with those obtained using the previously discussed methods (Back and Gorenstein, 1986, 1989).

*1. Distribution and ontogenesis of neutral endopeptidase 24.11 in spinal cord and meninges of humans and rats.* In adult human spinal cord, specific [$^3$H]HACBO-Gly binding was found in the substantia gelatinosa, with a similar distribution to that already described for the rat (Zajac et al., 1987a,b). In the fetus, the enzyme and $\mu$- and $\delta$-opioid receptors appear at an early stage of development (14 weeks), mainly localized in the superficial layer of the dorsal horn (Salès et al., 1989). This period, 2 weeks before the appearance of the enkephalins, corresponds to the development of diverse types of synapses (Rizui et al., 1986), suggesting that NEP and the receptors could serve to modulate developmental activity, such as axonal growth (Hammer, 1985). NEP concentrations in the substantia gelatinosa decrease in the neonate, which could be related to the final correct formation of axonic connections. In agreement with this, opioid agonists and antagonists have brain growth-retarding and -stimulating effects, respectively (Akoev et al., 1989). Receptor occupancy by the endogenous neuropeptides could, therefore, participate in transient synaptic circuitry and in the final establishment of neural connections. These results could have important repercussions in understanding the formation of nociceptive pathways and of disturbances of infants born to opiate-addicted mothers. Moreover, the presence in human fetal spinal cord of the

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structural and biochemical elements involved in enkephalinergic transmission, including regulation of enkephalin diffusion by meningeal NEP, as discussed below, could offer new possibilities for treating pain in infants (Salès et al., 1989, and references cited therein).

High levels of NEP have been found in the rat and pig choroid plexus, using [$^3$H]HACBO-Gly (Waksman et al., 1986a; Salès et al., 1991) and a polyclonal antibody (Kenny et al., 1987), respectively. The enzyme is uniquely located on the brush border of the apical surface, whereas ACE and APN appear to be much more abundant on the endothelial lining of microvessels (Kenny et al., 1987). NEP may thus play a role in regulating neuropeptide concentrations in the CSF. The enzyme has been detected in the meninges of rat and human spinal cord with [$^3$H]HACBO-Gly (Zajac et al., 1987a) and in the brain and spinal cord of pig by immunohistochemistry (Matsas et al., 1986). In addition, the rat and human meninges were shown to contain high concentrations of ACE and aminopeptidase activity (Zajac et al., 1987a). The role of these enzymes in the meninges could be to maintain the homeostatic concentration of neuropeptides in the CNS. In the dura mater, they could be localized in a structure comparable to the arachnoid villus of the brain, which resorbs cerebrospinal fluid back into the blood stream, and in the pia matter they could be part of a similar system for the fluid entering into the CNS. A specific [$^3$H]HACBO-Gly binding was found in the fibroblastic, but not in the meningothelial, component of human meningiomas which originate from arachnoid cells (Mailleux et al., 1990). NEP inhibitors could, therefore, be useful for the classification of the histological phenotypes of the meningiomas, for clinical diagnosis of small meningiomas using position emission tomography scanning and for the treatment of surgically inaccessible meningiomas.

*2. Lesion studies and cellular localization of neutral endopeptidase 24.11 in brain tissues.* The levels of NEP

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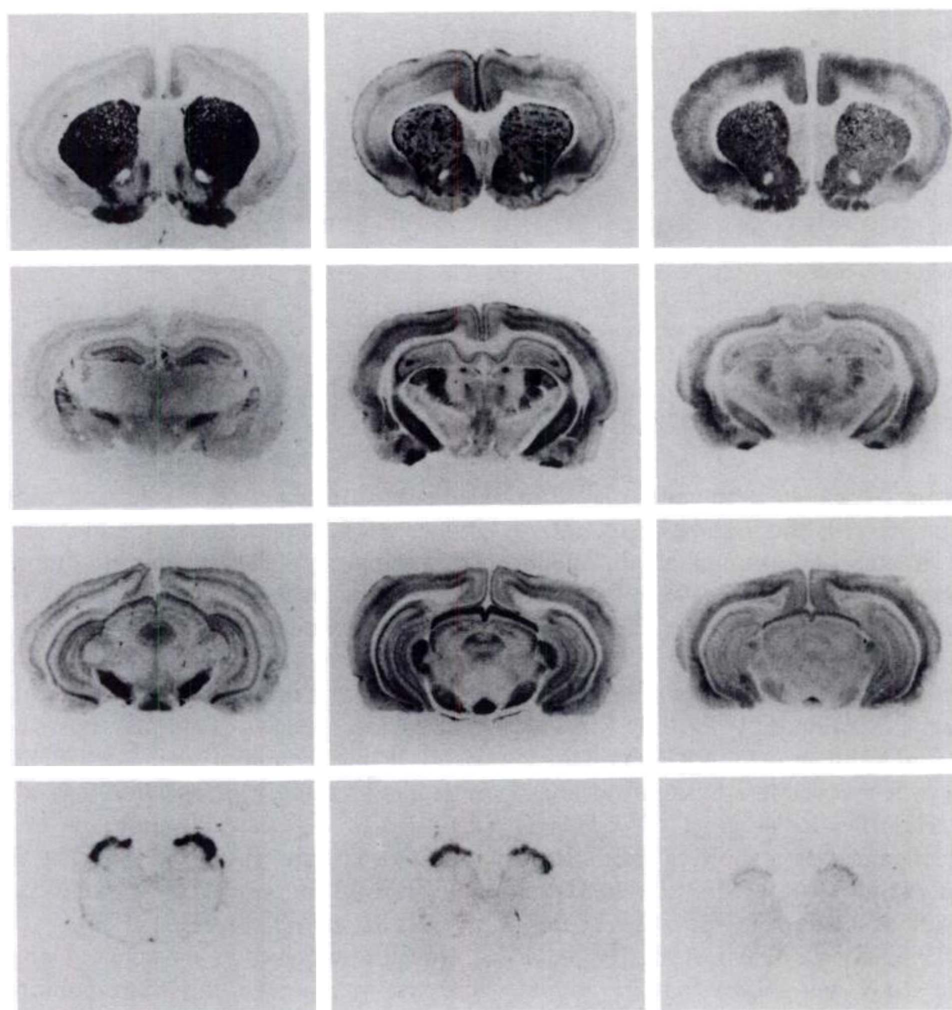

FIG. 3. Autoradiograms showing the relationship between the distribution of NEP (left) and the $\mu$-opioid (center) and $\delta$-opioid (right) receptor subtypes at different levels of the rat brain. Tissue sections were incubated with 3 nM [$^3$H]HACBO-Gly (left), 4 nM [$^3$H]DAMGO (center), and 4 nM [$^3$H]DTLET (right) and exposed to LKB Ultrofilm for 2 to 3 months. From top to bottom, caudate-putamen, thalamus-hypothalamus, substantia nigral, and spinal cord. Optimal photographic representation of individual autoradiograms appears in this figure. For a quantitative comparison of the levels of NEP and of $\mu$- and $\delta$-opioid-binding sites, see table 3.

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TABLE 3
*Comparative distribution of NEP and μ- and δ-opioid receptors and enkephalins in rat brain**

| | NEP | μ | δ | Enkephalins |
|---|---|---|---|---|
| **Telencephalon** | | | | |
| Olfactory tubercle | ++++ | ++ | +++ | +++ |
| Cortex layers I and IV | + | +++ | + | ++ |
| Cortex layers II and V | + | ++ | ++ | ++ |
| Cortex layers VI | 0(+) | ++ | ++ | + |
| Nucleus accumbens | +++ | +++++ | +++ | +++ |
| Caudate putamen | +++ | +++++ | +++ | +++ |
| Globus pallidus | +++ | ++ | + | +++ |
| Hippocampus CA1 | + | +++ | + | + |
| Hippocampus CA3 | + | +++ | + | + |
| Dentate gypus | +++ | +++ | + | + |
| Lateral septum | + | ++ | + | + |
| Posteromedial cortical amygdaloid nucleus | ++ | +++++ | ++ | 0 |
| **Diencephalon** | | | | |
| Medial habenula | ++ | +++++ | ++ | + |
| Mediodorsal thalamic nucleus | + | ++++ | ++ | + |
| Ventroposterior thalamic nucleus | + | +++ | + | ++ |
| Entopedoncular nucleus | +++ | +++ | + | 0 |
| Ventromedial hypothalamic nucleus | + | +++ | + | +++ |
| Arcuate hypothalamic nucleus | + | + | 0 | +++ |
| Mammilary bodies | ++ | ++ | + | + |
| Choroid plexus | +++++ | 0 | 0 | ND |
| **Mesencephalon** | | | | |
| Superior colliculus | | | | |
| Superficial gray layer | ++ | ++++ | ++ | 0(+) |
| Intermediate gray layer | + | ++ | + | 0(+) |
| Inferior colliculus | + | ++++ | ++ | ++ |
| Medial geniculate nucleus | + | ++++ | ++ | ++ |
| Interpedoncular gray matter | +++ | ++++ | ++ | ++ |
| Substantia nigra | ++++ | ++++ | ++ | ++ |
| Periacqueductal gray matter | ++ | ++++ | + | ++ |
| **Cerebellum** | | | | |
| Granulayer layer | 0(+) | 0 | 0 | + |
| Molecular layer | ++ | 0 | 0 | ++ |
| Spinal cord: substantia glatinosa | +++ | +++ | + | ++ |

* Densities range from very high (+++++) to null (0); ND, not determined.

and μ- and δ-receptors have also been measured after different lesions of the rat CNS and in some degenerative nervous diseases in humans (Waksman et al., 1986b, 1987; Delay-Goyet et al., 1987; Roques, 1989). Injection of kainic acid in the caudate putamen, which first destroys neuronal perikarya with a subsequent degeneration of nerve terminals, induced a time-dependent reduction (52% after 3 weeks) in [$^3$H]HACBO-Gly binding, suggesting a preferential localization of NEP on intrinsic neostriatal neurons with a possible additional localization on glial cells (Horsthemke et al., 1983). This is consistent with histochemical data showing that a large number of neostriatal perikarya have NEP activity (Back and Gorenstein, 1989) or have been found to express mRNA by in situ hybridization (Wilcox et al., 1989). An electron microscopic study has shown that in the rat some of these NEP-containing neurons correspond to medium spiny striatofugal neurons (Marcel et al., 1990).

It is interesting to notice that most of the striatal enkephalinergic neurons also express the dopamine $D_2$ receptor mRNA (Le Moine et al., 1991). Lesions of the caudate putamen with kainic acid were also shown to induce after 3 weeks a distant and large decrease in NEP (approximately 60%) and in μ and δ (approximately 40%) binding sites in the globus pallidus and substantia nigra, indicating that in both regions a large percentage of NEP and opioid receptors are presynaptically localized on axon terminals originating from striatal neurons (Waksman et al., 1986b). This has been confirmed by an electron microscopic immunocytochemical study showing that in these regions NEP was almost completely localized to the plasma membrane of axons and axon terminals (Barnes et al., 1988) and by the low density of NEP mRNA found in the rat globus pallidus by in situ hybridization (Wilcox et al., 1989).

It still remains to be determined whether the striatal enkephalinergic neurons corresponding to the major opioidergic pathway connecting the neostriatum to the pallidum (Del Fiacco et al., 1982) also express NEP and what type of striatal neurons express μ- and δ-opioid receptors in the globus pallidus. In addition to the striatopallidal projections, distinct striatal neurons that send

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their axons to the substantia nigra and contain classical transmitters such as GABA, or neuropeptides such as SP and/or dynorphin, are also associated with NEP (Waksman et al., 1986a) and ACE (Strittmatter et al., 1984) as shown by the large loss of both peptidases following kainic acid lesions of the striatum. Furthermore, the substantia nigra contains very high levels of SP and moderate levels of enkephalins. Therefore, it is possible that the signal conveyed by these peptides is presynaptically regulated by both peptidases in this region (Mauborgne et al., 1987a).

Lesions of the dopamine nigrostriatal or mesolimbic pathways by injection of 6-hydroxydopamine into the substantia nigra or the VTA (Waksman et al., 1986b) do not induce any significant change of NEP labeling by [$^3$H]HACBO-Gly in the caudate putamen, globus pallidus, and substantia nigra of rats, whereas a decrease in NEP activity was initially reported following similar lesions (Malfroy et al., 1979). However, this treatment was shown to induce a reduction in both $\mu$- and $\delta$-opioid-binding sites by about 25% in the caudate putamen and the nucleus accumbens and by 40% in the globus pallidus (Waksman et al., 1986b, 1987; Pollard et al., 1977a,b), suggesting the association of opioid receptors with dopamine terminals of neurons originating from the substantia nigra or the VTA. However, in another study no significant change was observed in the nucleus accumbens (Dilts and Kalivas, 1989). Although unlikely, the opioid receptor down-regulation could be related to a transynaptic degeneration subsequent to the loss of accumbens dopaminergic innervation (Unterwald et al., 1989) or to the well-known enhancement in preproenkephalin mRNA and Met-enkephalin levels and in enkephalin release following blockade of dopaminergic transmission. Terminals containing Leu-enkephalin immunoreactivity have been shown to be associated in the VTA with the cell bodies of both dopaminergic and nondopaminergic neurons (Sesack and Pickel, 1992) which is consistent with studies hypothesizing that endogenous enkephalins could produce a part of their effects through dopamine-dependent and dopamine-independent pathways (Kalivas et al., 1983; Daugé et al., 1992).

Prefrontocortical ablation produced no changes in NEP and opioid receptors in the caudate putamen (Waksman et al., 1987), suggesting that cortical neurons histochemically reactive for NEP activity (Back and Gorenstein, 1989) and expressing NEP mRNA (Wilcox et al., 1989) do not project to the neostriatum. Because lesioning the dopamine cells projecting to the neostriatum did not produce significant changes in NEP, the possible localization of the peptidase on striatal afferences remains unknown. However, it can be noticed that the axon terminals where NEP was found to be localized correspond mainly, if not exclusively, to local arborizations of intrastriatal neurons (Marcel et al., 1990).

A large depletion in NEP and in both $\mu$- and $\delta$-opioid receptors, but not in neurotensin receptors, has been observed in the caudate putamen, the globus pallidus, and the substantia nigra in patients with Huntington's disease (Roques, 1989), which is characterized by a severe loss of neurons. Contrastingly, in those with Parkinson's disease, no changes in enzyme or opioid receptor levels were observed in several forebrain regions (Delay-Goyet et al., 1987), whereas the neurotensin-binding site decreased by 50% in the nigrostriatal neurons (J. M. Zajac, W. Rosténe, and B. P. Roques, unpublished results). ACE levels were also reported to be modified in Huntington's and Parkinson's diseases (Arregui et al., 1978).

The nociceptive neurons of the dorsal horn are characterized by a dense enkephalin immunoreactivity associated with opioid receptors and NEP (Waksman et al., 1986a; Zajac et al., 1989), particularly in laminae I, II, and V, as well as around the central canal. Unilateral dorsal root rhizotomy produced a 60% decrease in $\mu$- and $\delta$-opioid receptors, whereas NEP levels were unaltered (Zajac et al., 1989). This indicates that, unlike NEP, most of the $\mu$- and $\delta$-opioid-binding sites are presynaptically located on afferent fibers. However, the 40% of binding sites remaining probably reflect the existence of a significant population of postsynaptic receptors, in agreement with the observation that numerous spinothalamic neurons receive direct enkephalinergic inputs (Ruda, 1982).

It seems that the enkephalins and NEP are therefore present in interneurons strategically positioned to release the opioid peptides, not only presynaptically at the terminal afferent C-fibers in the substantia gelatinosa but also near the dendrites or cell bodies of the spinothalamic neurons in more internal layers of the rat spinal cord (Besse et al., 1990) (fig. 4). In agreement with this, iontophoretic studies have demonstrated an inhibition of nociceptive dorsal horn neurons by stable enkephalin analogues and by kelatorphan (Morton et al., 1987). In support of this finding the expression of c-Fos evoked by various nociceptive stimuli was inhibited in a naloxone-reversible manner not only by morphine but also by kelatorphan i.v. injected in rats (Tölle et al., 1992; J. M. Besson, C. Abbadie, and B. P. Roques, unpublished results). The enkephalin action could be both postsynaptic, by hyperpolarization of the target neurons via an increased potassium conductance (Yoshimura and North, 1983), and presynaptic, by regulation of the release of other messengers, especially peptides such as SP and CGRP, from afferent terminals as discussed later.

This plausible neuronal organization could account for the independent control of nociception resulting from selective stimulation of spinal $\mu$- and $\delta$-receptors, the latter being selectively recruited by the endogenous enkephalins to give analgesia (Dickenson et al., 1987b; Sullivan et al., 1989).

*3. Cellular and subcellular distribution of neutral en-*

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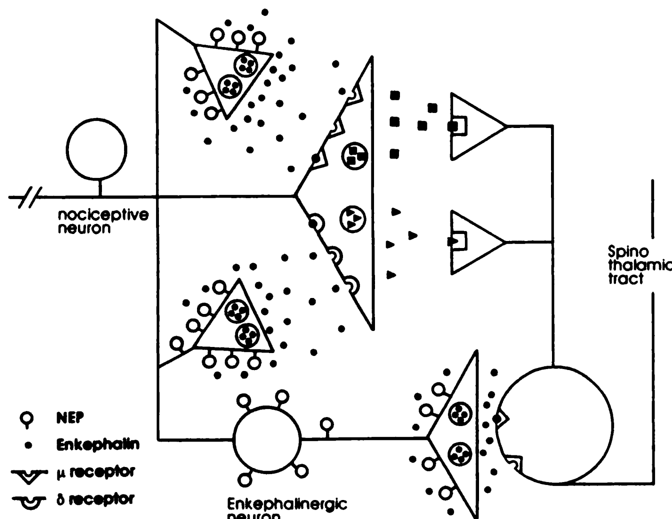


FIG. 4. Schematic representation of the spinal localization of NEP in a model of "neuropeptide extended synaptic area." The enkephalins, released from the spinal interneurons, interact presynaptically with opioid receptors located on the afferent fiber terminals to modulate peptide secretion (SP, CGRP). The concentration of the peptides at the receptor level is dependent on both their passive dilution in the extended synaptic volume and the efficiency of the inactivation processes ensured by peptidases, such as NEP, mainly located on the enkephalin neurons. The fast enkephalin-induced responses observed in electrophysiological experiments could correspond to the release of the opioid peptide into a classical synaptic area at the cell body level of spinothalamic neurons.

*dopeptidase 24.11 in the central nervous system.* Lesion studies generally support a predominantly neuronal localization of NEP, although the enzyme could be present on oligodendrocytes surrounding the fibers of the striatopallidal and striatonigral pathways (Waksman et al., 1986b; Marcel et al., 1990) and on Schwann cells in the peripheral nervous system (Matsas et al., 1986; Kioussi and Matsas, 1991; Kioussi et al., 1992). An electron microscopic localization of immunoreactive NEP in the rat neostriatum has shown that the peptidase is mainly associated with plasma membranes of neurons and to a lesser extent with oligodendrocytes and astrocytes (Marcel et al., 1990). Immunoreactive NEP was not preferentially concentrated at the level of specific membrane interfaces, such as the synapse, but rather almost uniformly distributed on the surface of neuronal perikarya and dendrites. The observation that the enzyme is not strictly synaptosomal agrees with previous reports showing that NEP is distributed all along the nigrostriatal pathway (Waksman et al., 1986a). The relatively large concentration of NEP found on striatal glial cells contrasts with its distribution in the pig globus pallidus (Barnes et al., 1988) where the enzyme was found to be almost exclusively located on synaptic and axonal neuronal membranes in possible agreement with a suggested presynaptic localization of the peptidase on a striatopallidal pathway (Waksman et al., 1986a,b).

At variance with ACE, no intracellular immunoreactive NEP was observed in the striatum. Owing to the problems (diffusion, reduced accessibility of antigenic site, epitope selectivity, etc.) encountered with the use of immunoreactive material, it is important to reassess the cellular and subcellular distribution of NEP, for instance, by electron microscopic analysis of [$^{3}$H]RB104 binding, which has been shown to be unchanged after treatment of various tissues by glutaraldehyde (N. Salès and B. P. Roques, unpublished results).

Electron microscopy studies have also shown that the enkephalins are distributed in large vesicles scattered all along the axon and are sparsely localized at the synapse (Coulter, 1988). Furthermore, electron microscopic studies, using the selective $\mu$ ([$^{125}$I]FK 33,825) and $\delta$ ([$^{125}$I] azido-DTLET) agonists (Hamel and Beaudet, 1987; Pasquini et al., 1988, 1992) have shown that only a small proportion (approximately 20%) of $\mu$- and $\delta$-receptors are localized at the synapse, suggesting that endogenous opioid peptides primarily act nonjunctionally on the plasma membranes of dendrites, axons, and, probably, neuronal perikarya. This reinforces the hypothesis that interruption of enkephalinergic transmission could be ensured by a gradual decrease of endogenous peptide concentrations through inactivation by NEP, the enzyme being probably located at the various neuronal sites of peptide release (Waksman et al., 1986a).

Electron microscopic immunoperoxidase staining of the substantia nigra of pig brain with an antibody against NEP showed that the peptidase is present in the plasma membranes of axons, axon boutons, and some dendrites. Both pre- and postsynaptic membranes and occasionally dendritic membranes were stained, but no peroxidase reaction was observed in the cytoplasm (Barnes et al., 1992). The localization of NEP in the substantia nigra confirms its probable role in the inactivation of SP and enkephalins in this region. The lack of intracellular immunolocalization of NEP observed in the globus pallidus of pig (Barnes et al., 1988) and rat (Marcel et al., 1990) contrasts with the presence of the peptidase in synaptic vesicles and the cytoskeleton of rat nucleus tractus solitarius neurons (Lasher et al., 1990). These apparent discrepancies are probably due (*a*) to differences in the antibodies used and (*b*) to the existence of various cellular forms of NEP that might be recognized by one antibody but not by another. NEP was also detected by immunochemistry using a monoclonal antibody against NEP purified from rat kidney in the nucleus tractus solitarius of the rat, particularly in the commissural nucleus of the nucleus tractus solitarius which receives the cardiac afferents of the vagus nerve, in fibers bordering the area postrema, and in quantities in the area postrema itself (Lasher et al., 1990).

In immunoreactive presynaptic terminals, the staining was associated with synaptic vesicles and with both the pre- and postsynaptic plasma membrane. Most of these presynaptic terminals formed axo-dentritic synapses, typical of vagal afferent terminals. This neuroanatomical

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localization suggests that the enzyme could be involved in the cleavage of SP released from the vagal afferent. The metabolites formed, SP 1–7 and SP 7–11, could be involved in blood pressure regulation, the former by depressor and bradycardiac effects, the latter producing conversely a long-lived pressor effect through activation of postsynaptic receptors (Hall et al., 1987, 1989a,b). It would be interesting to know whether other peptides such as CCK are metabolized by the peptidase in this region.

*4. In situ hybridization of neutral endopeptidase 24.11 messenger RNA in the central nervous system.* There have been few studies of the distribution of NEP mRNA. Wilcox et al. (1989) found high densities in the rat brain striatum, whereas none was detected in the globus pallidus and substantia nigra, in agreement with the probable presynaptic localization of pallidal and nigral NEP on terminals issuing from striatal neurons (Waksman et al., 1986a). Using a more efficient mRNA probe, Gaudoux et al., (1993) recently found NEP mRNA in brain structures, such as the red nucleus, in which the enzyme was initially reported to be not expressed. It would be interesting to determine the brain structures to which these NEP-containing neurons project, possibly through retrograde transport of an inhibitor such as [$^{125}$I]RB104. Moreover, the modulation of the multiexon NEP gene expression (Llorens-Cortes et al., 1990; Mari et al., 1992) could play a role in the ontogeny of numerous organs (Dutriez et al., 1992) and in the course of differentiation of lymphoid progenitor cells (reviewed by Le Bien and McCormack, 1989). The substrate candidates for these assumed autocrine or paracrine NEP-regulated functions remain unknown.

### *B. Localization of Neutral Endopeptidase 24.11 in Peripheral Tissues*

The presence of NEP in peripheral organs has been investigated using an enzymatic assay (Llorens and Schwartz, 1981), and more precisely, by immunological methods in pigs (Gee et al., 1983, 1985; Bowes and Kenny, 1986; Matsas et al., 1986) and rats (Ronco et al., 1988), and by autoradiographic visualization of [$^{3}$H] HACBO-Gly labeling in vitro or in vivo in adult rats or during ontogenesis (fig. 5) (Salès et al., 1991; Dutriez et al., 1992). The use of such an inhibitor for autoradiography has advantages over the previous methods by which quantitation could be hampered by differences in enzymatic activity in different homogenates or by differences in the tertiary structure and epitope presentation of the enzyme in various tissues, as shown in the case of ACE (Jackson et al., 1986; Strittmatter and Snyder, 1987). In contrast, binding of an inhibitor directed toward the active site of the metallopeptidase offers a quick, simple, and reproducible method for visualization and quantitation of NEP in its intact membrane-anchored form. It offers the additional advantage that the accessibility of the enzyme to circulating inhibitors also can be studied. [$^{125}$I]RB104 (Fournié-Zaluski et al., 1992c) and the radioiodinated derivative of SCH 39370, [$^{125}$I]SCH 47896 (Kanazawa et al., 1992), can also be used to determine the distribution of NEP after only some days of exposure.

NEP is particularly abundant in membranes of the brush border epithelial cells of intestine and kidney, the lymph nodes, and placenta; it is found at lower concentrations in lung, testis, prostate, fibroblasts, neutrophils, chondrocytes in articular cartilage, exocrine glands, and various epithelial and endocrine cells (reviewed by Kenny et al., 1987, and Salès et al., 1991).

The distribution of NEP in a great variety of tissues suggests multiple physiological functions, as discussed later in the pharmacological sections, for the renal tubules, the vasculature epithelium, the alveolar cells of the lung, or the intestinal mucosa. The presence of NEP in lymph nodes and bone marrow suggests that the peptidase could have a critical role in degrading as yet unknown immunoregulatory peptides. As already discussed, NEP was recently shown to be identical with CALLA expressed transiently at the surface of lymphohematopoietic cells (reviewed by Le Bien and McCormack, 1989).

Elucidating the functions of ectoenzymes such as NEP, ACE, and APN on circulating lymphoid cells, including macrophages, is an exciting area of research aimed at understanding the complex interrelationships between neuroendocrinological and immunological systems. NEP might also participate in chemotactic responses, phagocytosis, inflammatory processes, and peripheral analgesia. The tripeptide formyl-Met-Leu-Phe, one of the major chemotactic peptides produced by bacteria, has been shown to be cleaved by NEP present on the surface of the polymorphonuclear leukocyte neutrophils. When activated, these cells destroy bacterial cells and host tissue and, hence, contribute to the inflammatory response (Connelly et al., 1985). NEP could also play a regulatory role in controlling inflammatory processes triggered by other in vitro substrates of the enzyme, such as SP, bradykinin, angiotensin II etc., which are released by the injured tissue (Dubner and Ruda, 1992).

In rats, the ectoenzyme has been found in high concentrations in the tissue belonging to reproductive organs (Kenny et al., 1987; Ronco et al., 1988; Sales et al., 1991), which also express the proenkephalin gene (Kilpatrick and Rosenthal, 1986), but the role of the enzyme and its putative peptide substrates (vasoactive intestinal peptide, neuropeptide Y, enkephalins) remain to be determined.

NEP seems to be involved in the degradation of peripheral enkephalins released from myenteric nervous plexi, where opioid receptors have also been detected. The main action of the enkephalins in this region could be to control presynaptically the release of classical

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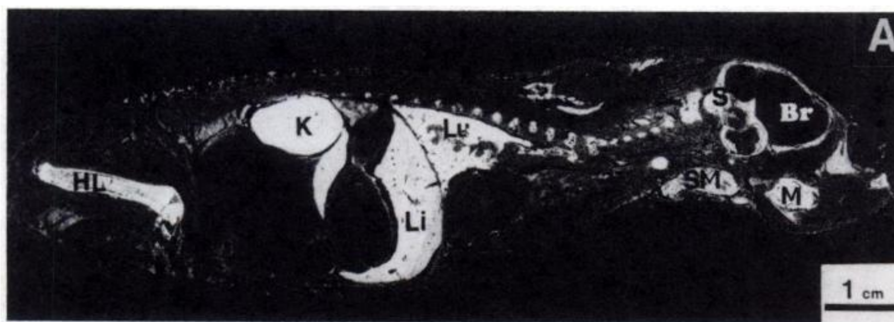


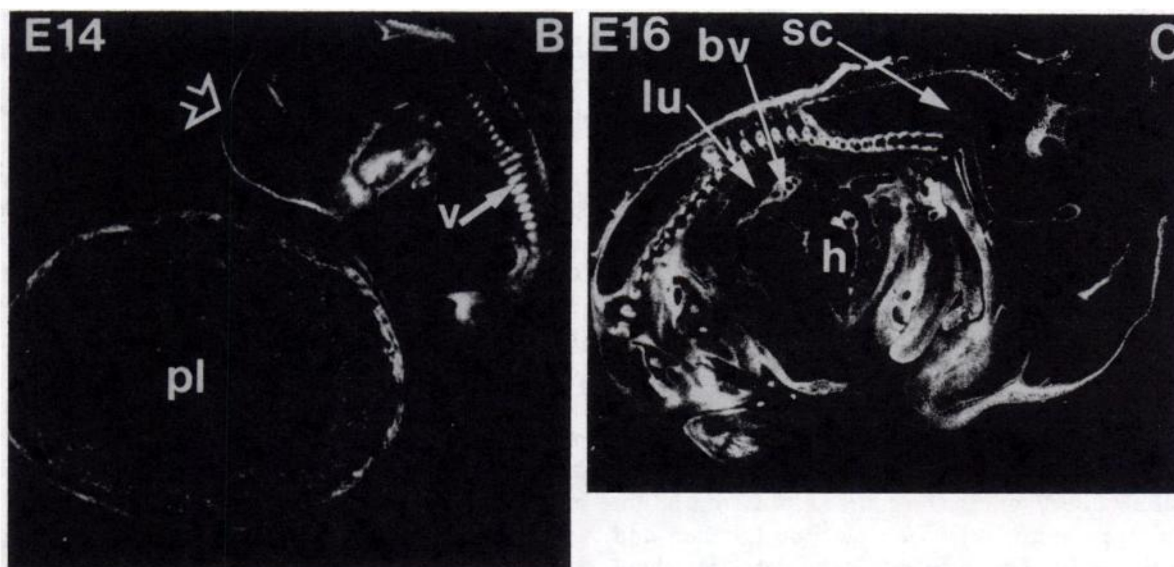


FIG. 5. A, Ex vivo autoradiogram, generated from a sagittal section of a rat given 60 mCi, i.v., [$^3$H]HACBO-Gly 10 min prior to killing. Br, brain; K, kidney; HL, Hind limb; Li, Liver; Lu, Lung; M, mandibula; Sm, submaxillary gland. The brain is not labeled because [$^3$H]HACBO-Gly does not cross the blood-brain barrier. Nonspecific binding (not shown) determined by i.v. administration of 10 mg HACBO-Gly was negligible except in the kidney, which is a major route of elimination of the inhibitor. B, In vitro labeling of a sagittal section of rat embryo by 3 nM [$^3$H] HACBO-Gly at gestational day 14. Open arrow, head; pl, placenta; v, vertebrae. C, In vitro labeling of a sagittal-near medial section of rat embryo by 3 nM [$^3$H]HACBO-Gly at gestational day 16. The head is on the right side. bv, blood vessels; h, heart; lu, lung; sc, spinal cord.

neurotransmitters such as acetylcholine, noradrenaline, or dopamine (North and Egan, 1983). This peripheral action of enkephalins probably accounts for the naloxone-reversible antidiarrheal effects of opiates such as loperamide or acetorphan, as discussed in the pharmacological section.

The distribution of NEP in peripheral organs has also been investigated after an i.v. injection of [$^3$H]HACBO-Gly (Salès et al., 1991). The difference between this and the previous methods is that only the peptidase that is easily accessible from the circulation is observed. For instance, no binding occurred in the brain because the radiolabeled probe is unable to cross the blood-brain barrier. As expected, a very dense labeling of [$^3$H] HACBO-Gly was observed in the kidney, including the glomeruli, lymph nodes, the lung, the salivary glands, and fat deposits in the neck region, but the most interesting result was the unexpectedly high binding found in the bones including the skull, vertebrae, and mandibula and in the bone marrow and joints, where it could be involved in the reorganization of the extracellular matrix during embryonic osteogenesis (Mikuni-Takagari and Cheng, 1987) (fig. 5A). Finally, it is important to note that several tissues containing important concentrations of NEP (testes, prostate, eye, gut brush border) are inaccessible to the i.v. injected inhibitor due to the presence of functional barriers.

### *C. Pre- and Postnatal Ontogeny of Neutral Endopeptidase 24.11 in Central and Peripheral Tissues*

A comparison of NEP localization with the appearance of morphologically differentiated structures and with the onset of physiological functions was carried out using [$^3$H]HACBO-Gly (fig. 5, B and C) to try to shed some light on the physiological role of the peptidase and on the pharmacology and possible side effects resulting from

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the therapeutic use of its inhibitors (Dutriez et al., 1992, and references cited therein).

NEP expression takes place prenatally in mesoblastic and ectoblastic structures. In the prenatal and developing CNS of the rat, the olfactory bulbs are the only structures strongly and permanently labeled before and, to a lesser extent, after birth, suggesting the possibility of an NEP-regulated role for peptides in olfaction at birth. Surprisingly, it was only at the end of the second postnatal week that NEP was demonstrated in the nigrostriatal tracts, and its appearance seems, in this case, to be more closely related to that of $\delta$- than $\mu$-opioid receptors (Kent et al., 1982; Spain et al., 1985). Other NEP-rich brain structures in the adult rat, such as the neocortex and the cerebellum (Waksman et al., 1986a), were not labeled during the first postnatal month, demonstrating a late or very progressive appearance of the endopeptidase unrelated to the development and differentiation of these brain areas.

The only brain region where NEP is transiently expressed is the mesencephalic flexure at approximately the time when nigral neurons extend processes toward their striatal targets (Dutriez et al., 1992). On the other hand, NEP expression is strongly enhanced during the development of sensory organs such as the eyes or the vibrissae. Therefore, it would be important to study the expression of putative NEP substrate(s) during the same periods. The presence of SP (Tervo et al., 1982) in the adult retina and ocular nerve endings is well documented, although other peptides such as vasoactive intestinal peptide, neuropeptide Y, CGRP, and enkephalins have also been found in eye tissues (Stone et al., 1987). In peripheral non-nervous system organs, such as the kidney, the lungs, and the salivary glands, the appearance and the tissue localization of NEP closely parallel the morphological differentiation of the structures, suggesting a progressive involvement of the enzyme in adult physiological functions. In the placenta, NEP is more abundant in maternal structures at early stages and in fetal structures at late stages of gestation. Therefore, it is possible that NEP fulfills a protective role against foreign peptides, first for the mother and then for the fetus (Dutriez et al., 1992). In addition, the heavy labeling of the terminal portion of the intestine may be related to the inactivation of peptides in the amniotic fluid.

There is also considerable evidence implicating several putative NEP substrates such as gastrin, neuropeptide Y, CGRP, and CCK in the control of gastrointestinal epithelial cell proliferation (Goodlad and Wright, 1987). Interestingly, the CGRP-like peptide bombesin accelerates the growth of the entire gastrointestinal tract in suckling rats (Lehy et al., 1986). The rather weak and stable labeling of bone marrow could be mainly due to CALLA-positive lymphoid progenitor cells in the course of their differentiation (Le Bien and McCormack, 1989) and/or to neutrophils.

In the heart and major blood vessels, in the genital tubercle, and in the cervical region, including the nuchal ligament and the brown fat pads, the presence of ANP or of ANP receptors has been shown in the adult, but often in more limited amounts, suggesting the involvement of NEP in developmental events (Vollmar et al., 1990). In several tissues where NEP has been localized, such as the testis, adipocytes, interscapular brown fat pads, and bone, ANP receptors are physiologically coupled to cyclic GMP. Neuropeptide Y, a 36-amino acid tyrosine-rich peptide also cleaved in vitro by NEP, has been found to be localized with the metallopeptidase in the brown fat, where the peptide could exert antilipolytic effects (Valet et al., 1990). The distribution of NEP closely parallels that of ANP during heart development, both being present in the ventricle at early stages but in the atrial region only later. Likewise, it is interesting to observe that, contrasting with the rather high concentration of NEP in heart and aorta in the fetus, the enzyme is barely detectable in blood vessels in the adult rat (Tamburini et al., 1989; Soleilhac et al., 1992). It would be interesting to know whether this hyperexpression of NEP in the cardiovascular system is related to a concomitant increase in released ANP, which could be transiently involved in trophic processes.

### D. Neutral Endopeptidase 24.11 in Physiological Fluids

A soluble form of NEP has been found in various human physiological fluids such as plasma, cerebrospinal fluid, amniotic fluid, and seminal plasma (Spillantini et al., 1990), and its concentration seems to increase during inflammatory processes in the synovial fluid (Appelboom et al., 1991), in sarcoidosis, or in adult respiratory distress syndrome (Johnson et al., 1985). Moreover, NEP was reported to increase in the serum of patients with end-stage renal failure (Deschodt-Lanckman et al., 1989). The pathological relevance of this increase in soluble NEP is unknown. Moreover, whether the peptidase corresponds to a secreted or mature form without the transmembranous anchoring fragment, or is released by a shedding process, remains to be established. However, secretion of metalloendoproteinase by synoviocytes, chondrocytes, and osteoblasts (or osteoclasts) plays an important role in the destruction of the extracellular matrix in rheumatoid arthritis. It could be interesting to investigate a potential therapeutic action of NEP inhibitors on membrane-bound and/or soluble NEP found at high levels in joints (Salès et al., 1991).

## IX. Modulation of Neutral Endopeptidase 24.11 Expression and Possible Role as Regulator of Protein Turnover at the Cell Surface

NEP is synthesized as a nonglycosylated precursor of apparent molecular weight of 85 kDa which is subsequently N-glycosylated in the endoplasmic reticulum before a final activation step in the Golgi, resulting in a 93-kDa protein. The biosynthetic process takes about 90

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min and seems to be identical in various cells (Stewart and Kenny, 1984; Lorkowski et al., 1987; Lemay et al., 1990). However, two glycosylation variants of NEP have been characterized by anion exchange chromatography (Vida and Hersh, 1992). They differ by their content in N- and O-linked carbohydrates and possibly have differences in their polypeptide backbones.

Little is known about the possible modulation of NEP expression. However, a decrease in NEP activity has been reported after treatment of cells by phorbol esters (Erdös et al., 1989; Werb and Clark, 1989). In contrast, formyl-Met-Leu-Phe, the calcium ionophore A23187, and the tumor necrosis factor led to an increase in membrane-bound NEP on neutrophils (Shipp et al., 1991a). An increase in NEP activity also was found after glucocorticoid treatment of transformed human tracheal epithelial cells (Borson and Gruenert, 1991). Curiously, the various factors that regulate the expression of NEP seem to control the expression of secreted Zn metalloendoproteinases such as stromelysin and collagenase in an inverse manner. Thus, phorbol esters were shown to increase the levels of mRNAs encoding the latter enzymes, whereas glucocorticoids and tumor necrosis factor block their mRNA transcription (Lennarz and Strittmatter, 1991, and references cited therein). The levels of NEP expressed in human endometrial stroma cells in monolayer culture were shown to increase in response to progesterone treatment (Casey et al., 1991). Therefore, it was hypothesized that NEP in the endometrium may occupy a pivotal position in the endometrial blood flow and myometrial contractions induced by endothelin-1 (Dickinson et al., 1991). A decrease in progesterone levels at the end of an ovarian cycle may be associated with a decline in NEP, facilitating the endothelin-1-evoked vasospasm of spiral arterioles that precedes menstruation. Conversely, the increase in NEP activity that occurs at the mid-luteal phase could serve to maintain a low level of the vasoconstrictor peptide endothelin-1, thus facilitating blastocyst implantation. Recently, NEP levels were shown to increase during the growth of leukemic cells in culture; this phenomenon may be related to the augmentation of cell contact (Milhiet et al., 1992a).

When incubated with leukemia cells (Reh-6), the potent inhibitor HACBO-Gly produced a slight (−15%) but significant and selective modulation of membrane-bound NEP expression; the latter effect could be due to an internalization of the complexed enzyme with a slow compensatory synthesis of the peptidase (Milhiet et al., 1992a). Another study (Jochen and Berhanu, 1987) has shown that phosphoramidon increases the intracellular accumulation of insulin in adipocytes, suggesting a role for NEP in receptor-induced internalization of the hormone. The growth of small cell carcinomas of the lung is regulated by an autocrine loop whereby tumor cells secrete bombesin, express bombesin receptors, and respond to bombesin stimulation by increased proliferation. Low levels of NEP are present at the surface of small cell carcinomas, and the enzyme is able to cleave bombesin, whose production seems to be increased in cigarette smokers. The bombesin-dependent growth of small cell carcinomas in culture is potentiated by phosphoramidon (Shipp et al., 1991b).

Few studies have been devoted to the cellular traffic of NEP. However, the transfection of MDCK epithelial cells with recombinant rabbit kidney NEP has provided an interesting experimental model for studying this process (Jalal et al., 1991). A significant internalization of NEP located at the surface of MDCK cells has been demonstrated by videomicroscopy using the fluorescent inhibitor FTI (section II). The disappearence of NEP from the surface was associated with a fluorescent honeycomb pattern, suggesting that the inhibitor was located on the inner leaflet of the plasma membrane. The coincubation of FTI with a monoclonal antibody, which does not inhibit FTI binding, produced a large increase in intracellular fluorescence. All of these results suggest an endosomal/lysomal internalization pathway for the complex NEP-inhibitor or monoclonal antibody-NEP-inhibitor which could be used both for studies of intracellular components of traffic and to improve the vectorization of drugs inside NEP-bearing cells (Milhiet et al., 1993). Membrane fusion events seem to require proteolytic cleavage of membrane proteins (Lucy, 1984) which may, for example, be important for the fusion and endocytosis of influenza virus into mast cells (Bosch et al., 1981). We have recently shown in preliminary studies that the expression of NEP, as well as CD4, at the surface of the lymphoblastic cells CEM are decreased by infection with the human immunodeficiency virus 1 retrovirus (Milhiet (1992)). This suggests that NEP/CD10 can be associated with the CD4-controlled intracellular penetration of the retrovirus. Furthermore, a possible role of NEP in cells that can be infected in the absence of CD4 remains to be explored. In line with this, APN was recently shown to be a receptor for the enteropathogenic coronavirus, transmissible gastroenteritis virus (Delmas et al., 1992), and for the human coronavirus 229E (Yeager et al., 1992).

It is also interesting to observe that NEP is expressed at the surface of many lymphoblastic cells, including the macrophages, which are known to act as a virus reservoir and are probably capable of entering the brain. More generally, it is possible that the permanent reorganization of the plasma membranes for exocytosis of neurotransmitter-filled vesicules or endocytosis of regulatory peptides, bacteria, and viruses requires the action of a metallopeptidase, possibly NEP, as occurs in the case of adrenal chromaffin cells and mast cells (Mundy and Strittmater, 1985, and references cited therein). Thus, both soluble and particulate metalloendoproteinases, the latter inhibited by phosphoramidon, have been shown to play a critical role in exocytoses phenomena, such as catecholamine secretion in chromaffin cells (Farach et

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al., 1987) or the acrosome reaction that occurs in sperm prior to egg fertilization (Strouss et al., 1988). Nevertheless, although NEP was found at the surface of these specialized cells, its direct implication in exocytosis remains to be established. Inhibitors of NEP and/or antibodies could be useful probes for testing its potential role in these processes.

Recently, cultured T-lymphocytes (Jurkat T-cells) were shown to express membrane-bound NEP, and the production of interleukin-2 by activated Jurkat cells was reduced in a dose-dependent manner by selective NEP inhibitors (retrothiorphan, phosphoramidon, thiorphan). This suggests that NEP on human T-cells could be involved in the production of lymphokines possibly through the processing of an activating factor at the surface of the lymphocyte (Mari et al., 1992).

## X. Pharmacological Studies

The initial report of the naloxone-reversible antinociceptive responses induced by i.c.v. injection of thiorphan (Roques et al., 1980) prompted numerous pharmacological studies in which the role of the endogenous opioid peptides in the CNS and the periphery was characterized (reviewed by Dickenson, 1986; Roques and Fournié-Zaluski, 1985, 1986; Schwartz et al., 1985; Roques, 1991; Roques and Beaumont, 1990). These investigations were based on the reasonable assumption that inhibitors of enkephalin degradation produce their physiological effects by increasing the extracellular levels of endogenous opioid peptides released from enkephalin-containing neurons, either tonically or following stimuli-evoked depolarization (phasic release). The effects of the inhibitors will depend, therefore, on (*a*) the magnitude and duration of the enkephalin release evoked by a particular stimulus, which probably varies in the different enkephalinergic pathways (Besson and Chaouch, 1987); (*b*) the efficiency of the inhibition (selective inhibition of NEP or APN or inhibition of both); (*c*) the efficiency of clearance mechanisms (Mulder et al., 1989); and (*d*) because in vitro the enkephalins have a 5- to 10-fold higher affinity for $\delta$- than for $\mu$-binding sites, one must recognize that the relative occupancy of $\mu$- and $\delta$-receptors will, therefore, be modulated both by the levels of circulating opioid peptides and by the local concentrations of opioid receptors.

These basic considerations could explain why in many pharmacological assays the administration of the rather $\mu$-selective antagonist naloxone does not produce a measurable effect on the response induced by the enkephalins. Thus, naloxone fails to affect pain thresholds in normal volunteers (El-Sobky et al., 1976) but lowers the thresholds of patients with naturally high pain thresholds (Buchsbaum et al., 1977). Furthermore, the beneficial effects of acupuncture and electrical nerve stimulation for the reduction of pain in humans is completely (Mayer et al., 1977) or partially reversed by naloxone (Chapman and Benedetti, 1977; Sjölund and Eriksson, 1979). The variable clinical outcomes observed probably result from differential recruitment of opiate and nonopiate inhibitory systems (reviewed by Mayer and Watkins, 1984). This emphasizes the importance of studying the inhibitors after local administration and of comparing the responses observed with those obtained with selective $\mu$- and $\delta$-agonists in the presence or absence of $\mu$- or $\delta$-selective antagonists. In addition, systemic administration of peptidase inhibitors is likely to produce an initial homogeneous distribution of the drug in various brain regions, giving a global representation of the effects induced by blockade of endogenous enkephalin metabolism and allowing the advantages and limits of the therapeutic benefits that could come from the clinical use of these inhibitors to be defined. As shown by the comparative studies of the distribution of NEP and $\mu$- and $\delta$-opioid receptors, the complementary targets of the enkephalinergic systems are located in brain areas not only involved in the control of pain but also in regions accounting for the multiple effects of morphine, especially those related to euphoria and reward, respiratory depression, and physical dependence (Waksman et al., 1986a).

In this section, we have reviewed the pharmacological responses induced by administration of selective or mixed inhibitors of NEP and APN and their relationships with the stimulation of $\mu$-opioid, $\delta$-opioid, or both opioid receptors. Moreover, the effects of inhibiting peripheral ANP have been surveyed. Recent clinical studies of these inhibitors as novel analgesics, antidiarrheal agents, and antihypertensives are discussed.

### *A. In Vitro and in Vivo Studies of Neuropeptide Degradation by Neutral Endopeptidase 24.11 and Aminopeptidase N*

The protection of exogenous or endogenous NEP-sensitive peptides can be studied using slices of various tissues. This preparation is physiologically more relevant than homogenates in which the anatomical organization of the tissue is destroyed. Moreover, the endogenous peptides can be released from tissue slices by depolarization and the metabolites measured in the superfusion medium (Patey et al., 1981; Waksman et al., 1985a; Bourgoin et al., 1986). Under these conditions, an enhanced recovery of a given neuropeptide, resulting from addition of a selective peptidase inhibitor, strongly supports the involvement of the peptidase in the metabolism of the peptide. This has been well illustrated with the mixed NEP/APN inhibitor kelatorphan (table 1), which almost completely inhibited [$^3$H]Met-enkephalin degradation by rat brain or spinal cord slices (Waksman et al., 1985a; Bourgoin et al., 1986). In these experiments, the NEP inhibitor thiorphan reduced the formation of [$^3$H]Tyr-Gly-Gly but enhanced [$^3$H]Tyr levels, whereas the opposite effect was observed with the APN inhibitor bestatin, showing that a blockade of both enzymes is

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required to obtain an important increase in the extracellular levels of endogenous enkephalins. The greater efficiency of kelatorphan, as compared to the combination of thiorphan and bestatin, is due both to its better selectivity for APN than bestatin (Waksman et al., 1985a) and to kinetically dependent favorable conditions for dual inhibition because a single compound instead of two interacts with peptidases expected to be topologically sequestered (Roques and Fournié-Zaluski, 1986).

The release of endogenous messengers from neurons can also be measured by using the radioligand displacement assay proposed by Chavkin and collaborators (Wagner et al., 1990). This approach combines the advantages of the brain slice preparation with the receptor selectivity of radioligands, thus constituting a sensitive measure of transmitter release under physiologically relevant conditions, i.e., after focal electrical stimulation of the preparation. It has been possible, using this technique, to demonstrate that, when protected from degrading enzymes by kelatorphan or a mixture of peptidase inhibitors, the endogenous enkephalins released by stimulation of the hippocampal perforant pathway interact with $\mu$-opioid receptors. The levels of norepinephrine released in the guinea pig hippocampus from axon terminals of neurons whose perikarya are located in the locus coeruleus have been estimated using the displacement of [$^3$H]propanolol-specific binding (Simmons et al., 1992). The secretion of norepinephrine was reduced in a naloxone-reversible manner by exogenous administration of a $\mu$-agonist or by endogenous opioid peptides concomitantly released with the catecholamine by tissue stimulation. This presynaptic activation of $\mu$-receptors probably increases calcium conductance (Williams et al., 1988), thus blocking calcium-activated fusion of norepinephrine-containing vesicles (Simmons et al., 1992). Electrophysiological studies have shown that adrenergic terminals form excitatory synaptic connections on GABAergic interneurons in the CA3 region of the hippocampus (Caudle et al., 1991). From these results it appears that the endogenous opioid peptides participate in the regulation of information processed by the noradrenergic system in the hippocampus, i.e., selective attention and arousal. On the other hand, focal stimulation of the mosey fibers releases endogenous dynorphin that bind, $\kappa_1$-receptors in the guinea pig hippocampus (Wagner et al., 1991).

The dorsal zone of the spinal cord, which is enriched in peptidases such as NEP and in $\mu$- and $\delta$-opioid receptors, the latter being mainly presynaptically located on afferent fibers (Besse et al., 1990), is an ideal region for exploring the role of the peptidase on peptidergic neurons. Various nociceptive stimuli (thermal, chemical, mechanical) have been shown to enhance Met-enkephalin levels in the spinal cord (Yaksh and Elde, 1981; Cesselin et al, 1982; Le Bars et al., 1987; Cesselin et al., 1989; Bourgoin et al., 1990). Kelatorphan almost completely prevented the spinal degradation of exogenous [$^3$H]Met-enkephalin in superfusions of halothane-anesthetized rats (Bourgoin et al., 1986). In contrast, in the same in vivo model, thiorphan or bestatin alone were inactive and, when coadministered, were only half as active as kelatorphan. Moreover, when the spontaneous outflow of endogenous Met-enkephalin was measured, there was a 2-fold better recovery in the presence of kelatorphan and a 5-fold enhancement during noxious stimulation (muzzle pinching) with no apparent change in the release process itself. This latter result shows that protection of extracellularly released enkephalins has no significant effect on the secretion of the opioid peptides, indicating that mixed inhibitors can be used to investigate the existence of tonically or phasically active enkephalinergic pathways (Dickenson et al., 1986; Williams et al., 1987; Tölle et al., 1992; Roques, 1991).

Recently, the NEP inhibitors, SCH 32,615 (20 to 100 mg/kg, i.v.), SCH 34,826 (30 to 100 mg/kg, p.o.), and thiorphan (10 to 30 mg/kg, p.o.), have been shown to cause a dose-dependent increase in both the resting (Yaksh et al., 1991; Yaksh and Chipkin, 1989) and the $K^+$-evoked (Yaksh and Chipkin, 1989) levels of enkephalins in rat spinal perfusates, reaching up to 10-fold the control values. These results are somewhat surprising given that thiorphan has a poor penetration of the blood-brain barrier and that even a mixture of acetorphan and carbaphethiol, two inhibitors with improved bioavailability that do enter the CNS, was not found to give a similar effect (Llorens-Cortes et al., 1989).

Tissue slices and spinal cord superfusion have also been used to investigate peptidase selectivity, showing, for example, that endogenous SP seems to be partially degraded by both NEP and APN in slices of substantia nigra (Mauborgne et al., 1987a). Although kelatorphan seemed to be less efficient than thiorphan in protecting the released peptide, this was shown to be due to enhanced enkephalin levels stimulating $\delta$-opioid receptors with a consequent reduction in SP release. In agreement with this finding, $\delta$-selective agonists also decreased SP release in the spinal cord (Mauborgne et al., 1987b), and it would be interesting to test the effect of $\mu$-agonists because the $\delta$-antagonist ICI 154,129 alone leads to an increase in the $K^+$-evoked release of SP.

In rat spinal cord slices, $K^+$- or veratridine-induced SP release was selectively decreased by opioid $\delta$-agonists and enhanced by a $\mu$-agonist (Mauborgne et al., 1987b). These opposing effects probably occurred in capsaicin-sensitive fibers whose terminals are located in the substantia gelatinosa, because no opioid agonist control was observed on the $K^+$-evoked release of SP remaining after destruction of neonatal C-afferent fibers with capsaicin (Pohl et al., 1989). These data show that the negative influence of opioids on the release of hyperalgesic substances, such as SP, in the spinal cord results essentially from the stimulation of spinal $\delta$-receptors. This is sup-

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ported by pharmacological studies showing that δ-agonists are efficient analgesics in the spinal cord and that the antinociceptive responses induced by kelatorphan at the spinal level are selectively blocked by δ-antagonists and are independent of μ-induced effects (Dickenson et al., 1988).

In in vivo experiments, the δ-antagonist naltrindole enhanced the outflow of SP like material (+170%), an observation supporting a tonic inhibitory control due to the stimulation of δ-receptors by endogenous opioids (Collin et al., 1991). Interestingly, at variance with findings in the substantia nigra, SP appears to be inactivated in the spinal cord by a peptidase activity different from NEP (Mauborgne et al., 1991). This bacitracin-sensitive enzyme could correspond to an SP-degrading activity previously shown to be present in human supraspinal fluid (Nyberg et al., 1984). Accordingly, in the primate, it has been shown that the NEP inhibitor SCH 32615 after i.v. administration can enter the CNS and enhance extracellular levels of enkephalin (9-fold), encrypted enkephalins (11-fold), and SP (2-fold) in cerebrospinal fluid, as compared to vehicle injected into animals. Plasma peptide levels of enkephalin were not altered by the inhibitor (Yaksh et al., 1991).

In slices of rat substantia nigra (Benoliel et al., 1992), neither thiorphan nor bestatin affected the $K^+$-evoked release of $CCK_8$-like immunoreactivity, a finding in agreement with degradation studies that suggested that a thiol or a serine $CCK_5$-releasing protease (McDermott et al., 1983; Rose et al., 1988), but not NEP, could be involved in the main physiologically relevant metabolic pathway of $CCK_8$ (Durieux et al., 1986). In apparent contradiction, a combination of these inhibitors, or kelatorphan, significantly enhanced $CCK_8$ overflow (Benoliel et al., 1992). Again, this was shown to be due to elevated levels of endogenous enkephalins in the extracellular fluid, which, in turn, activate δ-opioid receptors with a consequent stimulation of $CCK_8$ release. μ-Opioid receptor agonists, on the other hand, reduced $CCK_8$ overflow. These in vitro results have been recently confirmed by i.c.v. or i.v. injections of kelatorphan or RB101, respectively, which increased $CCK_8$ release through stimulation of the opioid δ-receptor by the protected enkephalins (Ruiz-Gayo et al., 1992b).

The expected increase in brain enkephalin levels following inhibition of their degradation has proved difficult to quantify, because levels of Met-enkephalin in the extracellular medium have been reported as ranging from 1% to 20% of the total after administration of selective or mixed peptidase inhibitors (Cesselin et al., 1981, 1982; Waksman et al., 1985a; Llorens-Cortes et al., 1986). Nevertheless, a direct demonstration of the increase in "synaptic" levels of enkephalins following inhibitor administration was obtained by in vivo binding experiments performed under the conditions commonly used for pharmacological studies (Meucci et al., 1989; Ruiz-Gayo et al., 1992a). Thus, after i.c.v. injection into mouse brain, Met-enkephalin at a high concentration (7.5 nmol) inhibited about 40% of the binding of the μ-agonist [$^3$H] DAMGO but not that of the δ-ligand [$^3$H]DTLET. Partial protection of Met-enkephalin degradation by coadministration with thiorphan slightly improved the inhibition of [$^3$H]DAMGO binding, whereas the mixed inhibitor RB38A completely blocked [$^3$H]DAMGO binding and inhibited 80% of [$^3$H]DTLET binding. However, the most important result was the ability of RB38A alone, but not thiorphan or bestatin, to inhibit [$^3$H]DAMGO binding in a dose-dependent manner with a maximum inhibition (60%) occurring at a dose (150 μg) at which mixed inhibitors have been shown to completely inhibit in vivo enkephalin catabolism (Bourgoin et al., 1986), demonstrating that the increase in tonically released endogenous enkephalins is too low to saturate μ-opioid receptors. These results have been confirmed by the displacement of [$^3$H]diprenorphine from opioid-binding sites following i.v. administration of RB101 at doses at which this mixed inhibitor was found to be pharmacologically active (Ruiz-Gayo et al., 1992a). Interestingly, the displacement of [$^3$H]diprenorphine binding was increased in stressed mice, which is in agreement with the well-known enhanced release of endogenous enkephalins elicited by stressful stimuli (Akil et al., 1976a).

Another approach has been to use radioimmunoassay to measure the turnover rate of Tyr-Gly-Gly, a process expected to reflect the activity of enkephalinergic neurons, because this metabolite is formed from Met- and Leu-enkephalin and from Met$^5$-enkephalin-Arg$^6$-Phe$^7$ (Llorens-Cortes et al., 1985). Thiorphan or bestatin modified the level of Tyr-Gly-Gly formed in mouse striatum slices, and an apparent rapid turnover rate of 18 ± 2 pmol/mg/h was calculated. Given the complex mechanisms leading to the final active peptide from preproenkephalin (protein synthesis including posttranslational processing, maturation, axonal transport), the rapid turnover of Tyr-Gly-Gly associated with the high percentage (approximately 20%) of extrasynaptosomal Met-enkephalin suggests a large rapidly mobilizable intracellular pool of enkephalins.

This method has also been used to measure changes in enkephalin release induced by noxious stimuli in the superfused spinal cord (Llorens-Cortes et al., 1989), yielding results generally similar to those obtained by measuring Met-enkephalin levels (Bourgoin et al., 1986). However, the interpretation of the results obtained in in vivo experiments is complicated by differences in the release of opioid peptides that contribute to Tyr-Gly-Gly formation (Llorens-Cortes et al., 1989). Thus, the basal levels of Tyr-Gly-Gly, found to be about 10-fold higher than those of Met-enkephalin in the spinal cord fluid, were not altered after systemic administration of either acetorphan or the combination of acetorphan and carbaphethiol. In the presence of inhibitors, muzzle pinch-

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ing tended to further elevate extracellular Met-enkephalin levels, although the levels reached remained lower than those observed in the spinal cord superfused by kelatorphan (Bourgoin et al., 1986). Nevertheless, in microdialysis experiments, the measurement of Tyr-Gly-Gly levels could be an interesting method of evaluating the in vivo functioning of enkephalinergic neurons (Houdi and Van Loon, 1990).

### *B. Limited Antinociceptive Effects of Selective Neutral Endopeptidase 24.11 or Aminopeptidase N Inhibitors*

The ability of NEP and/or APN inhibitors to potentiate the analgesic effects of exogenous enkephalins or enkephalin analogues is well documented. A variety of inhibitors have been tested, such as thiorphan or bestatin alone or in association (Roques et al., 1980; Carenzi et al., 1981; Zhang et al., 1982; Chipkin et al., 1982a,b,c; Vaught and Chipkin, 1982; Carenzi et al., 1983a,b; Chaillet et al., 1983b; Davis et al., 1983; Hachisu et al., 1985), retrothiorphan (Roques et al., 1983) and phosphoryl (Altstein et al., 1982), or carboxyl-containing inhibitors (Fournié-Zaluski et al., 1981a, 1982, 1983; Murthy et al., 1984; Chipkin et al., 1988). Different analgesic tests have been used, such as the hot plate test in mice (Roques et al., 1980; Hachisu et al., 1985), the tail withdrawal test in mice (Roques et al., 1980), the tail flick test in rats (Chipkin et al., 1982b; Hachisu et al., 1985), and the writhing test in mice (Hachisu et al., 1985); different routes of administration have also been used (reviews in Roques and Fournié-Zaluski, 1986; Chipkin, 1986). The antinociceptive responses observed in these studies were antagonized by prior administration of naloxone. In a typical study (Fournié-Zaluski et al., 1984a), bestatin strongly potentiated the analgesic responses in the mice hot plate test of Tyr-Gly-Gly-MePhe-Met-ol, a peptide hydrolyzed by aminopeptidases at the Tyr-Gly bond, whereas thiorphan was unable to increase the noxious threshold, because the Gly-MePhe bond is protected from NEP action. The reverse occurred with Tyr-D-Ala-Gly-Phe-Met, the activity of which was potentiated by thiorphan but remained unchanged after coadministration of bestatin, due to the D-Ala$^2$ residue which blocks aminopeptidase action. With Met-enkephalin, inhibition of both NEP and aminopeptidases, especially APN (Waksman et al., 1985a; Giros et al., 1986), is required to obtain an antinociceptive response.

The most interesting properties of the inhibitors are their intrinsic opioidergic actions. This was first recognized when i.c.v. injected thiorphan (50 $\mu$g) was shown to increase the noxious threshold of mice in the hot plate test (table 4); this effect was blocked by prior (table 4) administration of naloxone (Roques et al., 1980). In this test the antagonist exhibited pronociceptive properties. The photoaffinity label azidothiorphan (Fournié-Zaluski et al., 1981b), which has a high affinity for NEP ($K_I$ = 0.75 nM), was found to be as potent as thiorphan in the vocalization and hot plate jump tests after i.c.v. injection in mice, although its longer lasting responses suggested an irreversible in vivo binding (Beaumont et al., 1987). The more lipophilic prodrug of thiorphan, acetorphan (also designated ES52 or GN52), injected i.v. in mice strongly inhibited brain NEP and produced long-lasting potentiation of antinociceptive effects elicited by i.c.v. administered D-Ala$^2$-Met-enkephalin (Lecomte et al., 1986). Acetorphan has also been reported to be active after i.v. administration in the writhing and the hot plate tests in mice, although it was unable to modify the nociceptive activities of dorsal horn neurons in the anesthetized rat (Villanueva et al., 1985). However, because of the rather mild thermal nociceptive stimulus used in this study, the levels of enkephalin released and their degradation by APN would result in a receptor occupancy too low to give a pharmacological response. This probably also explains why acetorphan does not affect flexion reflexes or pain sensation in humans (Willer et al., 1986). Likewise, in a double-blind study of 84 patients requiring myelography, i.v. infusion of 150 mg of thiorphan over 30 min produced a reduction in the postmyelographic side effects (headache, nausea, and vomiting) but did not reduce the lumbar puncture pain (Floras et al., 1983).

Unexpectedly, thiorphan has been reported to produce a weak but significant analgesia in the tail flick test in mice, albeit after administration of a very high dose (300 mg/kg, i.p.). Under these conditions the concentration of thiorphan in the brain was calculated to be about 1000 times higher than the $IC_{50}$ of the inhibitor, and when the brain tissue from treated animals was incubated with [$^3$H]Met-enkephalin, no [$^3$H]Tyr-Gly-Gly formation was observed, but, strikingly, a large amount of [$^3$H]Tyr-Gly, only 2-fold lower than that of [$^3$H]Tyr, was found (Hachisu et al., 1985). Owing to the very high dose of thiorphan used, this could result from the interaction of the inhibitors not only with NEP but also with various aminopeptidases.

Phosphoramidon administered i.c.v. to rats, via an implanted cannula, produced a strong but not dose-dependent reduction in the vocalization threshold (Rupreht et al., 1983). The potent NEP inhibitor, phosphoryl-Phe-Leu, produced naloxone-reversible antinociceptive responses in the hot plate test in mice (Altstein et al., 1982). The N-carboxyalkyldipeptide, SCH 34826, which is about 10-fold less potent than thiorphan as an NEP inhibitor, was found to be active at 100 mg/kg, p.o., in the mouse low-temperature hot plate test, the mouse acetic acid-induced writhing test, and the rat yeast-paw test (Chipkin et al., 1988). In vivo it is deesterified to SCH 32615, the active constituent, which induces antinociceptive responses in the hot plate and tail flick test in rats after microinjection into the periaqueductal gray, the ventral medulla, and the amygdala (Al-Rodhan et al., 1990), as well as after i.v. administration in the acetic acid-induced writhing test in mice, rat yeast inflamed-

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TABLE 4
*Supraspinal antinociceptive effects of selective or mixed inhibitors*

| Compounds | Administration | Analgesic tests | Effects | Receptors involved | References |
|---|---|---|---|---|---|
| **NEP inhibitors** | | | | | |
| Thiorphan | i.c.v. | Hot plate (mice) | + | | Roques et al., 1980 |
| Acetorphan | i.v. | Hot plate (mice) | + | | Lecomte et al., 1986 |
| | | Writhing (mice) | ++ | | |
| Phosphoramidon | i.c.v. | Paw pressure (rats) | + | | Rupreht et al., 1983 |
| SCH 32826 | p.o. | Writhing (mice) | ++ | | Chipkin et al., 1988 |
| | | Hot plate (mice) | + | | |
| | | Inflamed paw pressure (rats) | + | | |
| SCH 32615 | PAG | Hot plate (rats) | ++ | | Al-Rodhan et al., 1990 |
| | | Tail flick (rats) | ++ | | |
| | i.v. | Writhing (mice) | ++ | | Chipkin and Coffin, 1991 |
| **APN inhibitors** | | | | | |
| Bestatin | i.c.v. | Hot plate (mice) | + | | Chaillet et al., 1983b |
| | | Writhing (mice) | ++ | | |
| Carbaphethiol | i.v. | Hot plate (mice) | + | | Gros et al., 1988 |
| **NEP + APN inhibitors:** | | | | | |
| Bestatin + thiorphan | i.c.v. | Hot plate (mice) | +++ | μ | Chaillet et al., 1984 |
| **Mixed inhibitors** | | | | | |
| Kelatorphan | i.c.v. | Hot plate (mice) | +++ | | Schmidt et al., 1991 |
| | | Writhing (mice) | +++ | | |
| | | Tail flick (mice) | + | | |
| RB38A | i.c.v. | Hot plate (mice) | ++++ | | Schmidt et al., 1991 |
| | | Writhing (mice) | ++++ | | |
| | | Tail-flick (mice) | +++ | | |
| | | Tail-flick (rats) | ++ | | |
| | | Tail electrical stimulation | | | |
| | | Motor response | ++ | | |
| | | Vocalization | + | | |
| | | Vocalization postdischarge | +++ | | |
| | | Paw pressure | ++++ | | |
| RB101 | i.v. | Hot plate (mice) | ++++ | μ | Noble et al., 1992a |
| | | Writhing (mice) | ++++ | μ | |
| | | Tail flick (rats) | ++ | μ/δ | |
| | | Tail electrical stimulation | | | |
| | | Motor response | ++ | μ/δ | |
| | | Vocalization | +++ | μ | |
| | | Vocalization postdischarge | ++++ | μ | |
| | i.p. | Hot plate (mice) | ++++ | | |

paw test, and hot water bath tail flick test in squirrel monkeys (Chipkin and Coffin, 1991) (table 4).

Stress-induced analgesia, which produces a brief naloxone-reversible analgesia probably due to an increased release of enkephalins in regions involved in pain control, is sensitive to inhibitors (Akil et al., 1976b; reviewed by Mayer and Watkins, 1984). Thus, thiorphan potentiates foot-shock stress-induced analgesia in rats (Chipkin et al., 1982c), immobilization stress-induced analgesia in mice (Greenberg and O'Keefe, 1982), warm water swim stress-induced analgesia in mice (O'Connor and Chipkin, 1984), and transcranial electrostimulation analgesia (Malin et al., 1989) measured by the tail flick test. Likewise, the antinociceptive properties of NEP inhibitors are modulated by stressful REM (rapid eye movement) sleep deprivation (Ukponmwan et al., 1986).

The analgesic effect of i.c.v. administered Met-enkephalin-Arg-Phe in mice or rats is strongly potentiated by ACE inhibitors, such as captopril or HOE 498 (2-[N-[(*S*)-1-carboxy-3-phenylpropyl]-L-alanyl-(1S,3S,5S)-2-azabicyclo[3-3.0]octane-3-dicarboxylic acid), but only weakly by thiorphan; this suggests that the peptide could be metabolized by ACE in addition to bestatin-sensitive aminopeptidases (Mellstrom et al., 1986, 1987; Norman et al., 1985). The physiological relevance of this degradation pathway remains to be determined, because Met$^5$-enkephalin-Arg-Phe derives from the preproenkephalin precursor and is, therefore, probably coreleased with Met- and Leu-enkephalin. However, because NEP and ACE are colocalized in several brain regions, participation of both peptidases in the metabolism of the various preproenkephalin derived peptides cannot be disregarded. Interestingly, HOE 498 had analgesic activity on the hot plate test after i.c.v. injection in mice at a dose only 5-fold higher than that of thiorphan (Norman et al., 1985). Curiously, in the same test, although very efficient in potentiating the effects of Met-enkephalin-Arg$^6$-Phe$^7$, captopril was about 10-fold less potent than

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HOE 498. Furthermore, mood elevating properties have been observed in depressed patients treated with ACE inhibitors (Cohen and Kurz, 1982). The higher affinity of Met-enkephalin-Arg[6]-Phe[7] for opioid receptors as compared to Met-enkephalin might compensate for its lower tissue concentration and could explain the antinociceptive properties of ACE inhibitors. However, no firm conclusions can be drawn until the effects of opioid antagonists are known.

The aminopeptidase inhibitor bestatin has been reported to reduce the nociceptive threshold in the hot plate, writhing, and tail electrostimulation tests in mice (Chaillet et al., 1983b; Costentin et al., 1986) after i.c.v. administration, although other investigators have failed to find any intrinsic antinociceptive properties (Carenzi et al., 1981, 1983a; Zhang et al., 1982). Bestatin derivatives potentiate the Met[5]-enkephalin induced analgesia (Matsuoko et al., 1988). Carbaphethiol (Gros et al., 1988), a prodrug of the APN inhibitor phenylalaninethiol (Pickering et al., 1985), was found to be weakly active in the hot plate test after i.v. injection in mice (table 4).

No significant antinociceptive responses were observed in a large series of APN inhibitors derived from $\alpha$-amino acids and bearing a free thiol group. However, as disulfide forms, they strongly potentiate the weak but nonsignificant analgesic responses induced by acetorphan (Fournié-Zaluski et al., 1992a). Taken together these results emphasize the necessity of completely inhibiting NEP and APN to obtain a significant morphine-like analgesia (Roques and Fournié-Zaluski, 1986).

### *C. Enhanced analgesic Responses Induced by Mixed Inhibitors of Enkephalin-degrading Enzymes*

As expected, coadministration of NEP and APN inhibitors produces stronger analgesic responses than those achieved by inhibiting only one enzyme. This was shown by studies using bestatin and thiorphan, carbaphethiol, and acetorphan or NEP and APN disulfide inhibitors in the hot plate (jumping), vocalization during electrical stimulation of the tail, and writhing tests (Carenzi et al., 1983b; Chaillet et al., 1983b; Zhang et al., 1982; Fournié-Zaluski et al., 1984a; Gros et al., 1988; Hachisu et al., 1987; Noble et al., 1992a; Fournié-Zaluski et al., 1992b). However, thiorphan plus bestatin had no significant action in the tail flick, tail withdrawal, and hot plate (paw licking) tests in either mouse or rat (Chaillet et al., 1983b; Chipkin et al., 1982b; Chipkin, 1986). Because naloxone alone has no pronociceptive effects in these tests, it has been proposed that the ability of the inhibitors to induce analgesia is restricted to tests in which naloxone has intrinsic activity (Costentin et al., 1986).

Mixed inhibitors are particularly effective. For example, kelatorphan was shown to decrease the dose of Met-enkephalin required to obtain 50% analgesia ($ED_{50}$) by a factor 50,000 (Fournié-Zaluski et al., 1984a). Under these conditions, the $ED_{50}$ of Met-enkephalin was not very different from that of DAMGO, a peptidase-resistant enkephalin analogue. These findings are in agreement with the similar in vitro affinities of both compounds for the opioid receptors.

Complete inhibition of enkephalin metabolism by i.c.v. kelatorphan, RB38A, or i.v. RB101, induced naloxone-antagonized antinociceptive responses in all the various assays commonly used to select analgesics (Fournié-Zaluski et al., 1984a; Xie et al., 1989a,b; Schmidt et al., 1991; Noble et al., 1990) (table 4). Endogenous enkephalins completely protected from metabolizing enzymes by mixed inhibitors after central or peripheral administration are, therefore, able to elicit pain suppressive effects not only in tests in which naloxone produces pronociceptive effects (Schwartz et al., 1985; Costentin et al., 1986) but more generally in morphine-sensitive assays (Noble et al., 1992a). However, even at very high concentrations (150 $\mu$g, i.c.v.), at which they have been shown to completely inhibit enkephalin metabolism (Waksman et al., 1985a; Bourgoin et al., 1986), mixed inhibitors such as RB38A were unable to produce the maximum analgesic effect induced by morphine, except in the hot plate and the writhing tests (Schmidt et al., 1991). In agreement with the previously discussed in vivo binding experiments (Meucci et al., 1989; Ruiz-Gayo et al., 1992a), these results indicate that the local increase in enkephalin concentration is still too low to saturate opioid-binding sites. This would eliminate, or at least minimize, receptor overstimulation, which is thought to be responsible for the major side effects of morphine and its analogues. Another mixed inhibitor, mercaptoacetyl-Phe-Phe (phelorphan), induced a large increase in extracellular striatal Met-enkephalin and a maximum antinociceptive response in the mouse hot plate test after i.c.v. administration (Van Amsterdam and Llorens-Cortes, 1988).

The similar analgesic responses obtained with morphine and endogenous enkephalins in several assays (Roques and Fournié-Zaluski, 1986; Roques, 1991) suggest that a smaller fraction of the total population of functional receptors is probably required with the opioid peptides because of their higher intrinsic efficacy (Porreca et al., 1990b; Noble et al., 1992b). Unfortunately, because the bidentate-containing inhibitors are unable to cross the blood-brain barrier, the effects resulting from acute or chronic complete inhibition of enkephalin-degrading enzymes could not be investigated following administration of the inhibitors by a clinically relevant route. Therefore, as previously discussed, new lipophilic thiol-containing APN and NEP inhibitors linked by their mercapto groups, such as RB101, N-[(*R,S*)-2-benzyl-3-[(*S*)(2-amino-4-methylthio)butyldithio]-1-oxopropyl]-L-phenylalanine benzyl ester, have been synthesized (Fournié-Zaluski et al., 1992b).

RB101 is the first systemically active prodrug gener-

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ating, through a biologically dependent cleavage of the disulfide bond, the potent APN [(*S*)2-amino-1-mercapto-4-methylthiobutane ($IC_{50}$ = 11 nM)] and NEP [N-[(*R*,*S*)-2-mercapto-methyl-1-oxo-3-phenylpropyl]-L-phenylalanine ($IC_{50}$ = 2 nM)], inhibitors. RB101 easily crosses the blood-brain barrier, as shown by the complete inhibition of cerebral NEP following i.v. injection in mice (Noble et al., 1992a). The prodrug induces strong, dose-dependent antinociceptive responses in mice after i.v., i.p., or s.c. administration in the hot plate ($ED_{50}$ = 9 mg/kg. i.v) (fig. 6A) and phenylbenzoquinone-induced writhing ($ED_{50}$ = 3.25 mg/kg, i.v) tests. RB101 is also active in the tail flick and tail-electric stimulation tests in rats. In contrast, as disulfide forms, the above selective APN or NEP inhibitors are inactive after i.v. administration and, even when coadministered, are 3-fold less potent than RB101 alone (table 4).

In all of the tests used, the pain-alleviating effect of RB101 was suppressed by naloxone but, except for the tail flick and the motor response to tail-electric stimulation, not by the $\delta$-selective antagonist naltrindole. The maximum analgesic effects of RB101 occur 10 to 15 min after i.v. injection and are still significant 30 to 40 min later. In the hot plate test, RB101 was only 3.5-fold less active than i.v. administered morphine ($ED_{50}$ = 1.6 mg/kg). As discussed later, RB101 is the first compound that permits the analgesia produced by elevating the extracellular levels of enkephalins to be critically compared with that produced by morphine.

In the presence of increasing concentrations of naloxone, i.v. injection of the mixed inhibitor RB101 or the highly $\mu$-selective agonist DAMGO resulted in similar $pA_2$ values (Noble et al., 1992a). In addition, thiorphan and acetorphan were both shown to induce analgesia in DBA/2J mice but not in C57BL/6J mice, a strain characterized by a genetic insensitivity to the preferential $\mu$-agonist morphine (Michael-Titus et al., 1989). All of these results support a preferential involvement of $\mu$-receptors in supraspinal analgesia, at least regarding thermal nociceptive stimuli (Gacel et al., 1981; Chang et al., 1982; Chaillet et al., 1984; Fang et al., 1986; Daugé et al., 1987; Shook et al., 1987; Baamonde et al., 1991; Noble et al., 1992a); however, a possible modulation of the analgesic effects through $\mu$-/$\delta$-receptor coupling cannot be completely eliminated as a possibility (Heyman et al., 1989; Porreca et al., 1990a).

### *D. Inhibitor-induced Spinal Antinociception*

The physiological relevance of the neuroanatomical organization of the spinal enkephalinergic system to control nociceptive messages (see section VII) is strongly supported by electrophysiological and pharmacological experiments in which kelatorphan was used to reveal the role of endogenous enkephalins. In anesthetized spinal cats, the selective inhibition of nociceptive responses by Met-enkephalin, administered in the substantia gelatinosa, was markedly potentiated by coadministration of kelatorphan, an effect blocked by electrophoretically administered naloxone (Morton et al., 1987). Neurons of the substantia gelatinosa were also inhibited by kelatorphan administration; this effect was naloxone reversible. In contrast, kelatorphan, administered near the cell bodies of laminae IV and V neurons neither altered evoked responses nor potentiated Met-enkephalin inhibition of these cells. When injected in the substantia gelatinosa, however, the inhibitor reduced the nociceptive responses of some laminae IV and V neurons, an effect blocked by electrophoretically applied naloxone, suggesting that some dorsal horn neurons are tonically inhibited by an action of opioid peptides in the substantia gelatinosa (Morton et al., 1987).

Several studies have used the expression of immediate early genes as markers for neuronal activity in an attempt to differentiate the pain modulatory effects of exogenously administered opioids from tonically released endogenous opioid peptides. The majority of c-*FOS*-like immunoreactive neurons, which are concentrated in neuronal area laminae I and II of the lumbar dorsal horn, receive inputs from enkephalin-immunoreactive axonal varicosities (Pretel and Piekut, 1991). Tölle et al. (1992)

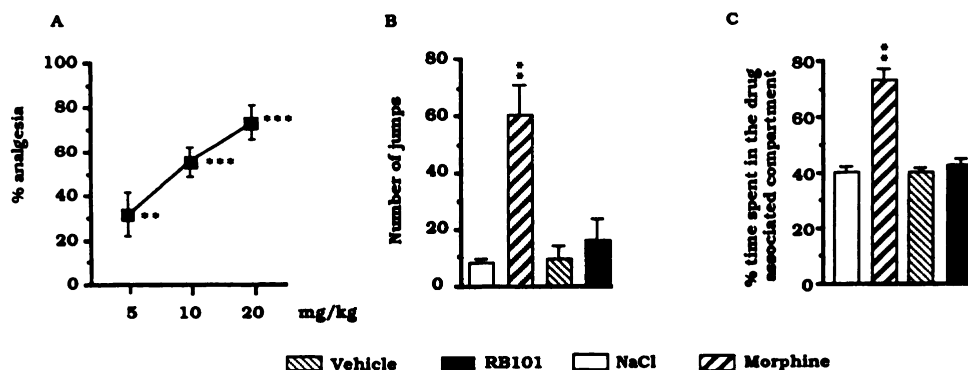


FIG. 6. Analgesic effects of a mixed NEP and APN inhibitor (RB101) in mice and lack of physical and psychic dependence after chronic treatment. A, Antinociceptive effect of RB101 administered i.v. in the hot plate jump test. B, Comparison of the withdrawal symptoms induced by naloxone after chronic treatment with morphine (6 mg/kg, i.p.) and RB101 (160 mg/kg, i.p.), injected twice a day for 5 days. C, Comparison of the dependence effects induced by morphine (6 mg/kg, i.p.) and RB101 (160 mg/kg, i.p.), injected every other day for 8 days.

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observed that, when administered before heat stimulation, both morphine and, to a lesser extent, kelatorphan reduced the induction of immediate early genes (c-*FOS*, *NGF1-a*, and c-*JUN*) in the superficial dorsal horn and the deep dorsal horn of rats. Naloxone, in contrast, increased the level of expression of these genes.

Consequently, enhancing the local concentration of opioid peptides in the spinal cord by inhibiting their catabolism mimics the effects of exogenously applied opiates on immediate early gene expression. Nevertheless, the effects observed after kelatorphan administration indicate that the local increases in enkephalin levels are too low to saturate opioid-binding sites. This limited opioid receptor occupation by the endogenous ligands is in agreement with in vivo binding studies (Meucci et al., 1989; Ruiz-Gayo et al., 1992a). On the other hand, the decrease of immediate early gene expression by kelatorphan and the increase by naloxone supports the existence of a tonically active opioidergic gating system in the dorsal horn. Accordingly, thiorphan and SCH 32615 displayed strong naloxone-reversible antinociceptive responses in the hot plate, paw pressure, and tail flick tests in rats after intrathecal drug administration (Yaksh and Harty, 1982; Oshita et al., 1990).

The role of endogenous opioid peptides and their receptors has been examined on the C- and $A_\beta$-evoked responses of convergent dorsal horn neurons in the halothane-anesthetized intact rat (reviewed by Dickenson, 1991). When an acute noxious stimulus was induced by transcutaneous electrical stimulation, the direct spinal application of bestatin, thiorphan, or kelatorphan reduced noxious C-fiber-evoked activity in a dose-dependent manner. Kelatorphan was considerably more effective than thiorphan or bestatin alone and significantly more efficient than the combination of both inhibitors (Dickenson et al., 1987b). The shape of the dose-response curve to kelatorphan paralleled that of selective δ-agonists, such as DPDPE or DSTBULET, but not that of μ-agonists, such as DAMGO. This suggests a preferential involvement of δ-opioid receptors in the enkephalin-mediated control of nociceptive messages conveyed by afferent C-fibers (table 5). This hypothesis was confirmed using the selective δ-opioid antagonist ICI 174,864 which reversed the antinociceptive properties of kelatorphan that had been locally infused onto the spinal cord (Dickenson et al., 1986). In addition, the effects of kelatorphan were additive with those of the selective μ-agonist DAMGO but not with those of the selective δ-agonist DSTBULET (Dickenson et al., 1986, 1988).

These studies confirm that endogenous and exogenous δ-opioid receptor agonists act on a common binding site to produce spinal antinociception. Accordingly, no antinociceptive cross-tolerance between morphine and δ-agonists, such as DSTBULET and BUBU, was observed after systemic administration in normal and arthritic rats (Desmeules et al., 1993; Kalso et al., 1992, 1993).

Kelatorphan and DSTBULET have also been tested on the response of convergent dorsal horn neurons to a more prolonged chemical noxious stimulus elicited by an s.c. injection of 5% formalin. At a dose of 50 μg, both kelatorphan and DSTBULET completely abolished C-fiber-evoked responses, and these effects were selectively antagonized by ICI 174,864 (Sullivan et al., 1989) (table 5). The mechanism of action of the δ-induced reduction in nociceptive responses is unknown but could involve presynaptic inhibition of SP release (Mauborgne et al., 1987b). At low doses, neither DSTBULET nor kelatorphan, unlike μ-agonists, induced excitatory effects on spinal neurons (Sullivan et al., 1989). The facilitations occurring with μ-agonists could be related to their potency in enhancing the release of either SP (Mauborgne et al., 1987b) or excitatory amino acids or to the presynaptic autoinhibition of Met-enkephalin release (Ueda et al., 1986; Kayser and Guilbaud, 1985; Ghelardini et al., 1989); the latter effect would imply that the putative autoreceptor might be of the μ-opioid receptor subtype. However, other authors have shown that the paradoxical analgesia produced by low doses of the opiate antagonist naloxone (presumably resulting from antagonism at opioid presynaptic autoreceptors) is mediated by interaction at a site with characteristics of the δ-opioid receptor (Taiwo et al., 1989; Kamei et al., 1992). Whatever the mechanism implicated, the opioid antagonist-induced antinociceptive responses are very weak compared to the effects of enzyme inhibitors, showing that enzy-

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TABLE 5
*Spinal antinociceptive effects of selective and mixed inhibitors**

| Compounds | % Inhibition C-fiber-evoked activity | Receptors involved | References |
| --- | --- | --- | --- |
| Transcutaneous electrical stimulation | | | |
| Bestatin | 17 | | Dickenson et al., 1986, 1988 |
| Thiorphan | 25 | | |
| Kelatorphan | 50 | δ | |
| Formalin test: | | | |
| Kelatorphan | 100 | δ | Sullivan et al., 1989 |

* Administration was by intrathecal injection.

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matic metabolism can be considered as the main process involved in clearing enkephalins from their site of action.

Given that there are pharmacologically discernable $\mu$- and $\delta$-receptor populations in the spinal cord that independently modulate noxious transmission, mixed inhibitors such as kelatorphan and/or selective $\delta$-agonists may be of clinical interest in patients insensitive or tolerant to morphine; these drugs also may be useful as a means of avoiding or minimizing unwanted side effects mediated by stimulation of the $\mu$-receptor. This novel approach to analgesia has provided preliminary promising clinical results after intrathecal administration of either kelatorphan (J. Meynadier, M. C. Fournié-Faluski, and B. P. Roques, unpublished results) in morphine-tolerant patients or bestatin and thiorphan in normal patients (Meynadier et al., 1988).

### *E. Analgesic Effects of Selective and Mixed Inhibitors in Chronic Pain*

The Freund's adjuvant-induced arthritic rat is a widely used model of chronic pain that is characterized by an increased sensitivity to $\mu$-agonists (Neil et al., 1986). Intracellular opioid peptide levels are markedly enhanced in the spinal cord of these animals, but this is associated with a large reduction (50% to 60%) in extracellular opioid levels. This latter effect was not due to changes in spontaneous outflow or in the rate of degradation of the peptides, because the same relative reduction persisted when kelatorphan was added to the superfusion medium (Bourgoin et al., 1988). Noxious stimuli produced a significant enhancement of Met-enkephalin release that was of the same order in arthritic and in control rats, suggesting that it is the basal and not the tonic activity of spinal enkephalinergic neurons that is reduced in arthritic rats (Bourgoin et al., 1988).

The ventrobasal complex of rat brain, which contains neurons activated by various noxious mechanical, thermal, or visceral stimuli, is sensitive to both morphine and aspirin in arthritic rats. The activity of these neurons is depressed by 56% by 5 to 10 mg/kg, i.v., ES52 (acetorphan), and the antinociceptive responses induced by mechanical and thermal stimuli are reversed by naloxone (Kayser et al., 1984). Nonnoxious messages were also depressed and, in addition, acetorphan produced a large expansion of the receptive field of nonnoxious ventrobasal neurons, emphasizing the complexity of pain control by endogenous enkephalins and raising the question of their possible involvement in peripheral analgesia (Stein et al., 1989).

Kelatorphan, at doses as low as 2.5 mg/kg, i.v., and at which acetorphan was ineffective, produced potent naloxone-reversible antinociceptive responses in normal rats that were comparable to those induced by 1 mg/kg, i.v., morphine. At higher doses (5, 10, and 15 mg/kg, i.v.), however, the effects of kelatorphan were no more pronounced than those of acetorphan (Kayser et al., 1989). Unlike acetorphan, kelatorphan was much more effective in raising the vocalization threshold in arthritic (244%) than in normal (144%) rats, even at 5 mg/kg, i.v.

Autoradiographic studies have shown that, at both supraspinal and spinal levels, the enhanced efficiency of endogenous enkephalins in the arthritic rat is not directly related to changes in the levels of NEP or $\mu$- and $\delta$-opioid-binding sites (Delay-Goyet et al., 1989b). Because the penetration of kelatorphan into the brain is very poor, its efficiency in reducing pain in arthritic rats after peripheral administration could be due to an increased permeability of the blood-brain barrier. Another simpler explanation could be that kelatorphan acts at the periphery to modify the strength of noxious stimuli at the primary level of nociceptors by increasing the concentrations of peripherally released endogenous opioid peptides. Indeed, several studies have shown that opioid agonists modulate responses to noxious pressure through a peripheral opioid receptor-specific mechanism (Stein et al., 1988, 1989). Moreover, on a model of unilateral inflammatory "pain" (intraplantar injection of Freund's complete adjuvant), the stress of a forced cold water swim induced a greater elevation of paw pressure threshold in inflamed than in noninflamed paws (Stein et al., 1990). This antinociceptive response was significantly potentiated and prolonged in rats that received an intraplantar injection of thiorphan plus bestatin, showing that this effect seems to be mediated, at least in part, by endogenous opioid peptides. Evidence for a peripheral site of action of enkephalin-like peptides in this model was provided by the antagonism of the action of the inhibitors by s.c. quaternary naltrexone, administered at doses shown to act exclusively at a peripheral level (Parsons and Herz, 1990). As expected, slightly higher effects were observed in the same model following systemic administration of RB38A or RB101 (R. Maldonado, O. Valverde, and B. P. Roques, unpublished results).

Inflammatory processes in the skin seem to be associated with intensive local activation of the lymphoid systems. The macrophages and B- and T-cells recruited overexpress proopiomelanocortin and proenkephalin mRNAs, possibly induced by a paracrine mechanism ensured by the secreted lymphokines. Carboxypeptidase E activity seems also to be increased, and numerous cells infiltrating the inflamed subcutaneous tissue are stained intensively by $\beta$-endorphin and Met-enkephalin antibodies (Przewlocki et al., 1992).

On the other hand, the flare reaction is associated with an increase in the release of SP and CGRP from sensory nerve terminals, the latter peptide producing extravasation. All of these results show that peptides producing hyperalgesia (SP, CGRP) or analgesia (enkephalins) are present in inflamed tissue. Under these conditions, opioid receptors could be also hyperexpressed in nociceptors, and because NEP is present on lymphoid cells

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(Beaumont et al., 1989), a part of the effect of opiates or peptidase inhibitors could occur at the primary site of pain processing. A part of the antinociceptive effects of selective or mixed NEP inhibitors could, therefore, be due to the protection of the endogenous opioid peptides from degrading enzymes at the level of peripheral nociceptors (Kayser et al., 1989), which may have clinical value in inflammatory disorders. Even though acetorphan administered p.o. (200 mg) in humans slightly potentiated the increases in flare areas and wheal volumes induced by SP intradermally administered to volunteers (Nichol et al., 1992), the nociceptive stimulus triggered by endogenous SP could be overcome by a larger increase in pain-alleviating peptides such as the enkephalins.

### F. Interactions between the Cholecystokinin and Enkephalin Systems in the Control of Pain

Anatomical studies have shown that the distribution of both CCK and CCK receptors parallels that of endogenous opioids and opioid receptors in the pain-processing regions of both the brain and the spinal cord (Gall et al., 1987; Pohl et al., 1990). This overlapping distribution has focused investigations on the role of CCK in nociception. Electrophysiological and behavioral studies suggest that CCK acts as an endogenous antagonist of opioid-induced antinociception (reviewed by Baber et al., 1989).

The supraspinal interactions between endogenous enkephalins and CCK have been investigated using the hot plate test in mice. Activation of CCK-A receptors, by i.c.v. administration of the $CCK_8$ analogue BDNL, potentiated the analgesic effects of RB101 and DAMGO (i.v.), whereas activation of CCK-B receptors, by i.c.v. injection of the selective CCK-B agonist BC 264, reduced them (Noble et al., 1993b). Taken together these results suggest the occurrence of a regulatory mechanism between the CCK and enkephalin systems in the control of pain. Schematically, the potentiation of the effects of DAMGO or RB101 by BDNL could be related to both an increase in released enkephalins associated with CCK-A receptor activation, in agreement with a previous study using $CCK_8$ and a cocktail of peptidase inhibitors (Hill et al., 1987), and/or to a direct improvement in the efficacy of transduction processes occurring at the $\mu$-sites, which might be allosterically evoked by CCK-A site occupation (Magnuson et al., 1990). Moreover, CCK-B receptor activation could, in turn, negatively modulate the opioidergic system, in agreement with the blockade of these binding sites by L-365,260 which significantly increased the antinociceptive responses induced by RB101 (Maldonado et al., 1993) or morphine in nonacclimated rats exposed to a novel environment (Lavigne et al., 1992).

If stimulation of CCK sites modulates the functioning of the opioidergic system, this could in turn regulate the release of CCK peptides. Thus, the binding of enkephalins protected by RB101 or of BUBU to $\delta$-sites was shown to increase the synaptic concentration of CCK (Ruiz-Gayo et al., 1992b), which could counterbalance the hyperalgesic effects of BC 264 through stimulation of CCK-A sites and subsequent enhancement of enkephalin release and/or $\mu$-CCK-A receptor interactions. This hypothesis is supported by the BUBU-induced reversion of the hyperalgesic effect of BC 264 in the hot plate test. On the other hand, activation of $\mu$-receptors was reported to exert an inhibitory influence on the supraspinal release of CCK (Benoliel et al., 1991). Accordingly, this results in a potentiation of the $\mu$-related response evoked by DAMGO or RB101 (after nociceptive stimulus) by reducing the negative influence that results from the binding of CCK to B sites (fig. 7).

### G. Tolerance, Dependence, and Side Effects of Selective and Mixed Inhibitors of Neutral Endopeptidase 24.11 and Aminopeptidase N

Few studies have been carried out to assess the possible tolerance and dependence effects resulting from chronic administration of inhibitors of enkephalin-degrading enzymes. Some physical dependence has been observed after naloxone administration to rats chronically treated with i.c.v. thiorphan (Bean and Vaught, 1984), whereas no sign of a withdrawal syndrome was seen in mice challenged with naloxone after chronic i.v. administration of acetorphan (Lecomte et al., 1986). Nevertheless, at the dose used (50 mg/kg, i.p. ,twice daily, 10 days), limited antinociceptive responses were observed in the hot plate test in mice after acute injection (Noble et al., 1990, 1992a). The naloxone-induced withdrawal syndrome in rats has also been studied after central infusion of comparable antinociceptive doses of the mixed inhibitor RB38A, the selective NEP inhibitor RB38B, the $\mu$-agonist DAMGO, and the $\delta$-agonist DSTBULET. DAMGO induced a severe withdrawal syndrome characterized by a large weight loss, hypothermia, jumping, mastication, teeth chattering, diarrhea, lacrimation, and salivation. DSTBULET and RB38A produced a moderate physical dependence with only the incidence of wet dog shakes and changes in body temperature being statistically significant. No physical dependence was observed after chronic selective inhibition of NEP by RB38B (Maldonado et al., 1990a).

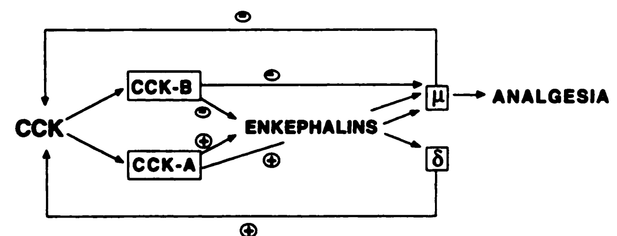


FIG. 7. Hypothetical model of the interactions between CCK, via CCK-A, CCK-B receptors, and the opioid system via $\delta$-opioid and $\mu$-opioid receptors.

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Antinociceptive responses observed after i.v. administration of acetorphan were significantly reduced in animals receiving chronic i.c.v. infusion of thiorphan for 14 days versus chronically saline-treated controls (Bousselmame et al., 1991a). In addition, chronic i.c.v. administration of DAMGO, DSTBULET, and RB38A produced a time-dependent reduction in their analgesic effects, and, after 120 h of continuous infusion, only RB38A was still able to induce a significant antinociceptive effect (Maldonado et al., 1990a). These data suggest that, even under the drastic conditions of chronic i.c.v. infusion of a mixed inhibitor, long-term complete inhibition of enkephalin catabolism induces only a partial tolerance and a moderate physical dependence similar to that produced by $\delta$-opioid agonists.

The promising results obtained with the mixed inhibitor RB38A were recently confirmed after systemic administration of RB101, which induces strong antinociceptive responses (Noble et al., 1992a). Thus, repeated administration of RB101 (twice daily for 5 days) at a dose that produces a potent analgesic effect (160 mg/kg, i.p.) did not induce a naloxone-precipitated abstinence syndrome (fig. 6B). Under the experimental conditions used, no signs of withdrawal that are accepted as being reliable indices for quantifying the degree of physical dependence in mice (jumps, paw shakes, tremor, body weight loss) were observed in animals treated with the mixed inhibitor prodrug (Noble et al., 1992c). Moreover, chronic administration of RB101 (80 mg/kg, i.p., twice daily for 8 days) did not induce tolerance or cross-tolerance with morphine (Noble et al., 1992b). A lack of cross-tolerance between morphine and acetorphan also was observed in another study (Bousselmame et al., 1991b).

Addiction, following chronic treatment with opiates, is probably due to multiple cellular events. The opioid receptors have been shown to be functionally coupled to their second-messenger systems through guanine nucleotide-binding proteins (Childers, 1991). One of the well-documented mechanisms implicated in these side effects is the desensitization of opioid receptors (uncoupling of the receptors from their associated G-proteins, protein phosphorylation, alteration in adenylate cyclase functioning) (Wüster et al., 1985; Christie et al., 1987; Nestler and Tallman, 1988; Terwilliger et al., 1991). These effects have also been explained by a regionally specific down-regulation of receptors (Rogers and El-Fakahany, 1986; Morris and Herz, 1989; Steece et al., 1989).

The moderate degree or the lack of tolerance or physical dependence observed after chronic treatment with the mixed inhibitors (RB38A or RB101) could be explained by a weaker, but more specific, stimulation of the opioid-binding sites by the tonically released endogenous opioids, thus minimizing receptor desensitization or down-regulation that usually occurs after the general stimulation of opioid receptors by exogenously administered agonists. This assumed limited opioid receptor occupation by the endogenous enkephalins is in agreement with in vivo binding studies (Meucci et al., 1989; Ruiz-Gayo et al., 1992a). The local concentration of the exogenous opioid substances probably largely exceeds the concentration of endogenous enkephalins in brain regions, such as the periaqueductal gray or the locus coeruleus of rats, which are rich in $\mu$-receptors and where local injection of morphine or enkephalin analogues has been shown to cause exceptionally pronounced pharmacological effects (Bodnar et al., 1988; Al-Rodhan et al., 1990; Bodnar et al., 1991) and where microinjections of methylnaloxonium precipitate a severe withdrawal syndrome in morphine-dependent rats (Maldonado et al., 1992). The locus coeruleus contains a densely packed group of noradrenaline-containing neurons whose firing is selectively inhibited by $\mu$-agonists (Williams et al., 1987). Chronic morphine treatment induces a hypersensitivity of these neurons, and this is considered to be one of the main causes of the withdrawal syndrome (Aghajanian, 1978).

This latter hypothesis appears to be supported by the reduction in the effects of morphine abstinence by clonidine, a presynaptic blocker of noradrenaline release. It is interesting to observe that in slices of rat pons kelatorphan was able to potentiate strongly the firing of the locus coeruleus that had been induced by exogenous Met-enkephalin but had no intrinsic effect, indicating that there is little or no tonic endogenous opioid action in this brain region (Williams et al., 1987). This is probably one of the major reasons why the withdrawal syndrome is significantly milder after chronic treatment by peptidase inhibitors as compared to exogenous opioids. Indeed, it has been clearly demonstrated that the locus coeruleus is the most critical structure implicated in the development of dependence (Duman et al., 1988; Nestler and Talman, 1988; Rasmussen et al., 1990; Maldonado et al., 1992).

Another possible reason for the lack of side effects following chronic RB101 treatment could be a more efficient release of antiopiate peptides induced by morphine than by the natural ligands of opioid receptors. Indeed, the concept of an "endogenous antiopioid system" has been suggested, involving several neuropeptides such as MIF-1, CCK, and FMRF amide, Phe-Met-Arg-Phe $NH_2$ (Faris et al., 1983; Tang et al., 1984; Galina and Kastin, 1986). This latter peptide is released from rat brain by morphine infusion (Tang et al., 1984). There are numerous reasons to suspect that peptides of this family may be endogenous antiopiate substances. Indeed, there is a good correspondence between localization of binding sites of these peptides and that of the endogenous opioid peptides (Allard et al., 1992). Moreover, several authors have demonstrated that FMRF amide-related peptides potently antagonize the analgesic effects of opiates thereby inducing withdrawal syndrome (Tang et al.,

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1984; Brussaard et al., 1989). In addition, it has been shown that the cerebrospinal fluid of morphine-dependent rats contains a peptide, the octapeptide F8Fa (FMRF amide-like octapeptide), which is also designated neuropeptide FF (Malin et al., 1990a), that precipitates the withdrawal syndrome when centrally injected into morphine-dependent rats. Neuropeptide F8Fa appears to be necessary for the full expression of naloxone-precipitated opioid withdrawal, because its inactivation by antibody or antagonist peptide largely prevents the appearance of the classical signs of naloxone-precipitated abstinence (Malin et al., 1990b, 1991). Tolerance to the analgesic effect of morphine is also prevented by simultaneous treatment with antagonists of CCK receptors (Watkins et al., 1984; Xu et al., 1992), and immunoneutralization of neuropeptide F8Fa appears to selectively restore morphine sensitivity in opiate-tolerant animals (Lake et al., 1991). These results support the hypothesis that endogenous CCK and neuropeptide F8Fa contribute to opiate tolerance.

The lack of cross-tolerance between morphine and acetorphan observed on the hot plate test (Bousselmame et al., 1991b) led the authors to suggest that the enkephalins protected by acetorphan act on the $\delta$-receptor site to produce antinociception. However, in the study with the mixed inhibitor RB101, where a lack of cross-tolerance also has been shown, the highly $\delta$-selective antagonist naltrindole was unable to block the antinociceptive effects of RB101 observed in morphine-tolerant mice. Moreover, several previous studies have shown the preferential involvement of $\mu$-receptors in supraspinal opioid analgesia using the hot plate test after central (Chaillet et al., 1984; Fang et al., 1986) or peripheral (Baamonde et al., 1991; Noble et al., 1992a) administration of opioid agonists or enkephalin-degrading enzyme inhibitors.

The binding characteristics of enkephalins and morphine to the same site, which have been reported to be different (Abdelhamid and Takemori, 1991), could lead to different cellular responses characterized by a rapid development of tolerance to the effects of morphine, with no major changes in the effects induced by the enkephalins. On the other hand, the lack of cross-tolerance between morphine and RB101 could be due to the higher intrinsic efficacy of enkephalins compared to morphine and analogues. In morphine-treated mice, a large fraction of the receptors appear to be uncoupled from the associated G-proteins, resulting in a loss of transduction of the agonist effect via second-messenger effectors. Thus, the lack of cross-tolerance of RB101 in morphine-tolerant mice could be explained if morphine requires a larger fraction of functional receptors to produce a given effect than do the endogenous enkephalins. The higher intrinsic efficacy of enkephalins has been demonstrated by the greater efficiency of modified enkephalins as compared to morphine and analogues in several studies (Christie et al., 1987; Gacel et al., 1988; Porreca et al., 1990b).

A major side effect of opiate analgesia is a central respiratory depression, which is mainly due to the inhibition of bulbar respiratory neurons. Activation of $\mu$- and $\delta$-receptors decreases the firing of these neurons with a subsequent diminution in respiratory rhythm and tidal volume (Morin-Surun et al., 1984). Kelatorphan, ionophoretically applied into the nucleus ambigus of cats, produces a low, partially naloxone-reversible, reduction of respiratory frequency, suggesting a weak tonic release of endogenous enkephalins in the neuronal network that generates the motor respiratory activity. A dysfunctioning in the opioidergic system involved in the control of breathing could, therefore, be one cause of sudden infant death syndrome (Morin-Surun et al., 1992; Boudinot et al., 1988). Moreover, after i.v. injection of kelatorphan in freely moving rats at concentrations that give analgesic responses in the vocalization threshold to paw pressure in arthritic rats (Kayser et al., 1989), there was no significant effect on respiratory frequency and tidal volume (Boudinot et al., 1988). The NEP inhibitors acetorphan (Lecomte et al., 1986), SCH 34826 (Chipkin et al., 1988), and SCH 32615 (Chipkin and Coffin, 1991) have also been reported to have no respiratory effects in rodents or squirrel monkeys.

It is well documented that chronic opiate treatment induces psychic dependence, a major side effect limiting their clinical utilization. Numerous studies with microinjections of selective agonists have shown that the brain areas most frequently implicated in opioid reward are the VTA and the nucleus accumbens. The motivational properties of endogenous enkephalins have been studied after microinjections of thiorphan into the VTA; reinforcing effects were observed (Glimcher et al., 1984). Moreover, kelatorphan appears to differentially affect intracranial self-stimulation behavior with respect to the neuroanatomical locus of administration. Microinjection of this inhibitor into the lateral ventricle increased intracranial self-stimulation behavior, whereas local administration into the nucleus accumbens reduced it (De Witte et al., 1989). Unlike morphine, repeated systemic administration of the mixed inhibitor RB101 failed to establish a conditioned place preference and did not induce psychic dependence (Noble et al., 1993a) (fig. 6C).

The biochemical mechanisms involved in the reward systems are unknown, but several studies have shown that dopaminergic neurons, particularly those that project from the VTA to the nucleus accumbens, may play a major role in the rewarding properties of opiates (Phillips and LePiane, 1982; Bozarth, 1986; Cador et al., 1991). Activation of the mesolimbic dopamine system could be necessary and possibly sufficient to produce a conditioned place preference. Thus, a reduction in the functional responsiveness of dopamine transmission in opiate dependence was proposed to contribute to the maintenance of opioid self-administration to maintain a tonically active basal dopamine release and subsequent re-

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warding effects (Acquas and Di Chiara, 1992). As discussed above, chronic opiate treatment desensitizes opioid receptors by decoupling them from their associated G-proteins leading to a loss of extracellular messages. This desensitization could also be due to an alteration of cyclic AMP-dependent protein kinase which phosphorylates and activates several intracellular proteins (Duman et al., 1988; Beitner et al., 1989; Cox, 1991). Accordingly, Beitner-Johnson and Nestler (1991) have recently shown that chronic treatment with morphine decreases the phosphorylation, and thus the activity, of tyrosine hydroxylase (Zigmond et al., 1989), the rate-limiting enzyme in dopamine biosynthesis, specifically in regions implicated in drug reinforcement such as the VTA and the nucleus accumbens. The reduced phosphorylation of this enzyme could explain the recently reported decrease in opioid-stimulated dopamine release (Ostrowski et al., 1982; Leone et al., 1991) following chronic morphine treatment (Acquas et al., 1991) and could lead to the "dysphoric" properties of opiate abstinence (Bozarth and Wise, 1984; Koob et al., 1989; Stinus et al., 1990).

The failure of RB101 to establish a conditioned place preference probably results from a lower recruitment of opioid receptors, as more fully discussed above, and the relatively poor capability of endogenous enkephalins to modify intracellular events such as protein phosphorylation with subsequent modulation of the dopaminergic transmission in the nucleus accumbens. This hypothesis is supported by the minimal changes in dopamine release in the nucleus accumbens after local administration of kelatorphan into the VTA (Daugé et al., 1992) and by the apparent absence of any effects on the levels of dopamine and its metabolites in the nucleus accumbens following i.v. administration of acetorphan (Dourmap et al., 1990a). Accordingly, when injected in the VTA of rats, DAMGO and to a lesser extent the $\delta$-agonist DSTBULET were shown to increase the rate of intracranial self-stimulation behavior which reflects the reinforcing value of abusive drugs such as morphine. In contrast, under the same conditions, kelatorphan had either no effect or slightly decreased the rate of intracranial self-stimulation (Heidbreder et al., 1992).

### H. Effect of Peptidase Inhibitors on Morphine Withdrawal

The peptidase-resistant enkephalin analogues, FK 33-824 and metkephamid, completely suppress the opiate withdrawal syndrome in monkeys (Gmerek et al., 1983). The nonspecific peptidase inhibitors, apotropin and bacitracin (Pinsky et al., 1982), or the NEP inhibitors, phosphoramidon (Dzoljic et al., 1986), thiorphan (Haffmans and Dzoljic, 1987), acetorphan (Livingston et al., 1988) and orally active compounds (Dzoljic et al., 1992), and the mixed inhibitor, phelorphan (Haffmans et al., 1987), also minimize the severity of the naloxone-precipitated morphine withdrawal syndrome in rats. Phelorphan was more effective than thiorphan, suggesting that attenuation of the narcotic withdrawal syndrome is related to the level of opioid receptor occupancy by exogenous or endogenous agonists. This was confirmed when the effects of i.c.v. thiorphan, kelatorphan, and RB38A were compared in the same model (Maldonado et al., 1989). Jumping, chewing, and tooth chattering were decreased by all three inhibitors with an order of potency: RB38A > kelatorphan > thiorphan. The increase in plasma corticosterone and hypothermia were reduced by kelatorphan and RB38A, whereas rhinorrhea was blocked by thiorphan, tremor by kelatorphan, and diarrhea by RB38A. Other signs remained unchanged.

The greater efficiency of the mixed inhibitors in attenuating the morphine abstinence syndrome is probably due to the resulting greater increase in enkephalins in certain brain regions, especially those enriched in opioid $\mu$-receptors, such as the caudal part of the periaqueductal gray matter, which also contains high levels of NEP in rats (Waksman et al., 1986a) and could be an important site of action for the development of physical morphine dependence (Laschka et al., 1976). In line with this, wet dog shakes and teeth chattering have been found to decrease after direct administration of thiorphan into the periaqueductal gray matter (Haffmans and Dzoljic, 1987). Furthermore, REM sleep deprivation decreases the grooming and shaking behavior induced by opiate withdrawal (Ukponmwan et al., 1985). As expected, acetorphan alleviated the withdrawal syndrome in humans (H. Loo, unpublished results). On the other hand, the putative nonspecific enkephalinase inhibition produced by D-Phe has been claimed to improve the treatment of alcohol and polydrug abusers (Blum et al., 1989).

### I. Behavioral Effects of Neutral Endopeptidase 24.11 and Neutral Endopeptidase 24.11/Aminopeptidase N Inhibitors

In addition to its strong analgesic effect, morphine induces euphoria and sensations of well-being in humans (reviewed by Wise and Bozarth, 1987). Enkephalins have also been shown to be self-administered by rats (Stein and Belluzi, 1978) and to produce either hyperlocomotor activity or sedative effects, depending on the dose administered. Some of these behavioral responses are not completely reversed by the $\mu$-selective antagonist naloxone (Havemann and Kuschinsky, 1985), suggesting that $\mu$- and $\delta$-receptors could be differentially involved.

High densities of both opioid receptors and NEP are found in the nucleus accumbens and the caudate nucleus (Waksman et al., 1986a), forebrain structures that are involved in emotional, cognitive, and motor functions and receive a rich innervation from dopaminergic neurons located in the VTA and substantia nigra. Various pharmacological and biochemical studies have also shown that morphine and enkephalins are involved in

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the control of behavior such as arousal, locomotion, self-administration, self-stimulation, learning, and memory functions through modulation of the motor (nigrostriatal) and limbic cortical (mesocorticolimbic) dopaminergic systems.

Chronic administration of antidepressant drugs increases Met-enkephalin-like immunoreactivity in the striatum and nucleus accumbens of rat brain (De Felipe et al., 1985). A similar increase has also been observed after electroconvulsive shock or following chronic administration of lithium (Hong et al., 1979; Holoday et al., 1986; Staunton et al., 1982). The physiological relationships between the enkephalinergic and dopaminergic systems have been demonstrated clearly by the strong enhancement of both proenkephalin mRNA and Met-enkephalin-like immunoreactivity in rat striatum and nucleus accumbens after chronic administration of dopamine antagonists or after production of 6-hydroxydopamine-induced lesions of dopamine neurons in the substantia nigra and VTA (Hong et al., 1979; Thal et al., 1983; Scott Young et al., 1986; Morris et al., 1988). Moreover, in situ hybridization experiments have shown that, in the rat forebrain, all detectable enkephalin neurons contain the $D_2$ receptor mRNA and that chronic haloperidol treatment increases the levels of both the $D_2$ receptor and preproenkephalin mRNA (Le Moine et al., 1991).

Several pharmacological studies have been carried out to clarify the role of opioids on dopaminergic systems. Local injection of thiorphan into the rat VTA produced a naloxone-reversible conditioned reinforcement in a place preference paradigm, suggesting activation of the dopaminergic mesolimbic pathway by the endogenous opioids (Glimcher et al., 1984). An increase in dopamine metabolism in the nucleus accumbens and a potentiation of the behavioral effect of dopamine injected into this region were induced by foot-shock stress or local infusion of thiorphan (Kalivas and Bronson, 1985; Kalivas et al., 1986), or kelatorphan into the VTA (Calenco-Choukroun et al., 1991b), suggesting a phasic control of the dopamine mesocorticolimbic pathway by endogenous enkephalins. This was recently confirmed using in vivo microdialysis with simultaneous measurement of motor behavior. Under these conditions, microinjection of kelatorphan into the VTA produced a dose-related increase in both motor activity and extracellular dopamine in the nucleus accumbens (fig. 8A) (Daugé et al., 1992). However, these effects are weaker than those induced by DAMGO.

Moreover, stimulation of $\delta$-opioid receptors in the VTA by local injection area of the selective agonists DSTBULET, DTLET, BUBU, or kelatorphan induced hyperactivity in familiar (home cage) or unfamiliar (open field and four-hole box) environments. These effects were suppressed by a $\delta$-selective antagonist. The $\mu$-agonist DAMGO also increased locomotion in the actimeter but decreased the activity in the open field and four-hole box tests, possibly reflecting an increase in emotion and fear (Calenco-Choukroun et al., 1991a). The differences in the responses induced by kelatorphan or $\delta$-agonists with those produced by DAMGO suggest that $\mu$- and $\delta$-receptors are involved in different neuronal pathways in the VTA. This is supported both by the association of only a part of enkephalinergic terminals with tyrosine hydroxylase-containing neurons in the rat VTA (Sesack and Pickel, 1992) and by a study in which 6-hydroxydopamine-induced lesions of the rat mesoaccumbens pathway were found to abolish the effects of kelatorphan or BUBU in the VTA but not those elicited by the $\mu$-agonist DAMGO (Calenco-Choukroun et al., 1991b). Taken together these results show that the endogenous enkephalins preferentially bind to $\delta$-receptors to induce hyperactivity. The conditions under which they could activate $\mu$-receptors remain an open question.

The nucleus accumbens is one of the major targets of the dopaminergic neurons originating from the VTA. Direct injection of modified enkephalins, such as DAMGO, DTLET, or kelatorphan, into this area led to opposite effects, i.e., hypoactivity in the case of the $\mu$-agonists versus hyperactivity in the case of the $\delta$-agonists and kelatorphan (Daugé et al., 1988). The responses obtained were antagonized by naloxone or ICI 174,864, but not by the nonselective but preferential $D_2$ dopamine antagonist thioproperazine (Daugé et al., 1989). The apparent lack of a direct relationship between dopaminergic and opioidergic systems in the nucleus accumbens seems to be supported by the absence of change in the in vitro release of newly synthesized dopamine that is observed following addition of $\mu$- and $\delta$-agonists or kelatorphan to superfused slices of rat nucleus accumbens (Petit et al., 1986) and by the absence of modification in dopamine levels in rat nucleus accumbens following i.v. injection of acetorphan (Dourmap et al., 1990a) (table 6). However, the locomotor stimulant effects produced by $\delta$-receptor stimulation could be dependent on the activation of $D_1$ receptors (Longoni et al., 1989).

The complexity of the interactions between dopaminergic and opioidergic systems in the nucleus accumbens is illustrated by the results of 6-hydroxydopamine-induced lesions of the dopaminergic neurons of the VTA and chronic neuroleptic treatment, both of which potentiate the behavioral effects of exogenous opioids (Stinus et al., 1985, 1986; Kalivas and Bronson, 1985) or kelatorphan (Maldonado et al., 1990b) infused into the nucleus accumbens. In agreement with the presence of $D_2$ receptors on the forebrain enkephalin neurons, $D_2$-dopamine receptor antagonists, such as sulpiride, or the mixed $D_1$-$D_2$ antagonist haloperidol, but not the $D_1$ antagonist SCH23390, have been found to facilitate the opioid behavioral effects induced by kelatorphan (Maldonado et al., 1990b). Because both the enkephalinergic and dopaminergic systems in the nucleus accumbens appear to function in parallel to increase locomotor ac-

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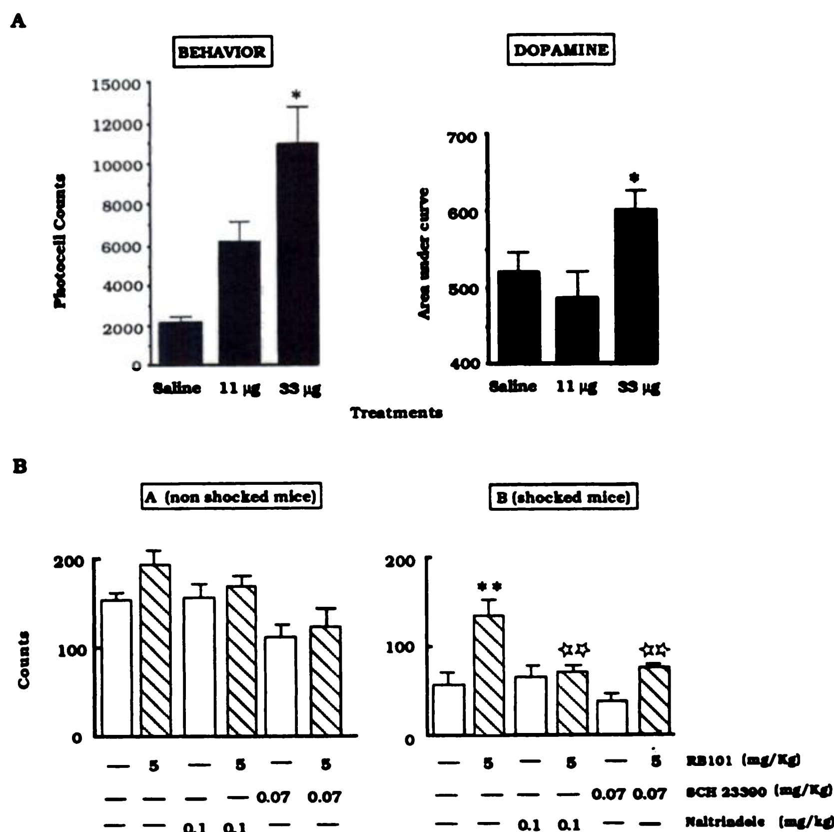


FIG. 8. A, Effects of kelatorphan injected in the VTA on motor activity and extracellular dopamine levels in the nucleus accumbens of rats. B, Effects of naltrindole (δ-antagonist) and SCH 23390 ($D_1$ antagonist) on the effect induced by i.v. injected RB101 in the conditioned suppression of motility test in mice.

tivity (Kalivas et al., 1983), the supersensitivity of the opioid system after long-term dopaminergic blockade could be interpreted as a homeostatic mechanism to maintain normal locomotor activity. Moreover, the behavioral supersensitivity to endogenous opioids protected by kelatorphan in the nucleus accumbens appears to be maximum after 2 to 3 weeks. This delay corresponds to the first appearance of the antipsychotic effects of neuroleptics, suggesting that alterations in the opioidergic system, very likely through its interrelations with the dopaminergic pathway, could be taking place in a neuronal system critically involved in the control of mood (Roques et al., 1985; McLennan and Maier, 1983).

In the nucleus accumbens, the enkephalinergic pathway projecting to the ventral pallidum also seems to control the functioning of SP and/or dynorphin-containing neurons which bear $D_1$ receptors and project to the substantia nigra. Kelatorphan also produces the same pattern of increase in brain stimulation or locomotor activity as i.c.v. administered amphetamine and δ-opioid agonists (Heidbreder et al., 1988; Michael-Titus et al., 1990).

A link between opioidergic and dopaminergic systems has also been demonstrated by the clear antidepressant-like effects observed in the forced swimming and suppression of motility test following i.v. administration of the systemically active mixed inhibitor RB101 (fig. 8B) in mice. These effects, which were shown to be related to δ-receptor and $D_1$ receptor activation, produced an increase in dopamine turnover in the striatum (Baamonde et al., 1992). Subcutaneous administration of SCH 32615 has also been reported to increase DOPAC, 3,4-dihydroxyphenylacetic acid in the nucleus accumbens of rats (Giorgi et al., 1991). Furthermore, the following results all support a relationship between the protection of the endogenous enkephalins and behavioral modifications: (*a*) catatonic or hypertonic immobility induced in mice by i.c.v. injection of µ-agonists or thiorphan plus bestatin (Chaillet et al., 1983a); (*b*) naloxone-reversible potentiation of the stereotypes induced by phencyclidine (Hiramatsu et al., 1986) after i.c.v. administration of bestatin and thiorphan in the cannulated rat; (*c*) potentiation by thiorphan or bestatin of the antidepressant actions of a subeffective dose of imipramine, measured using the forced swimming test (De Felipe et al., 1985, 1989); (*d*) the slight reduction in immobility time induced in the same test by either thiorphan or bestatin (Ben Natan et al., 1984); (*e*) the suppression of motility in a conditioned emotional response to an environment associated previously with foot shock following i.c.v. administration of either thiorphan or the combination of thiorphan and bestatin (Nabeshima et al., 1988); (*f*) the reduced behav-

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TABLE 6

*Relationships between dopaminergic and opioidergic systems determined using behavioral tests and dopamine turnover**

| Compounds | Administration | Behavioral effects | Effects on dopamine system | References |
|---|---|---|---|---|
| Thiorphan | VTA | ↑ behavioral effects | ↑ DA metabolism (N. Acc) | Kalivas and Bronson, 1985<br>Kalivas et al., 1986 |
| Kelatorphan | VTA | ↑ behavioral effects | ↑ DA metabolism (N. Acc.) | Calenco-Choukroun et al., 1991b |
| Kelatorphan | VTA | ↑ motor activity | ↑ Extracellular DA (N. Acc.) | Daugé et al., 1992 |
| Kelatorphan | N. Acc. | ↑ motor activity ($\delta$) | Behavioral effects not antagonized by thioproperazine | Daugé et al., 1989 |
| RB101 | i.v. | Antidepressant-like effects ($\delta$) | Effects antagonized by $D_1$-antagonist | Baamonde et al., 1992 |
| SCH 32615 | s.c. | | ↑ DA metabolism (N. Acc.) | Giorgi et al., 1991 |
| Acetorphan | i.v. | | ↑ DA levels in the olfactory tubercle (no effects in the striatum and N. Acc.) | Dourmap et al., 1990a |
| SCH 34826 | p.o. (chronic) | | ↓ $D_1$ receptors (striatum) (no effects on $D_2$ receptors) | Trampus et al., 1991 |
| Thiorphan | Striatum | | ↑ DA metabolites (striatum) | Wood, 1982 |
| Kelatorphan | Striatum | ↑ motor activity ($\delta$) | Behavioral effects antagonized by thioproperazine | Daugé et al., 1989 |
| Kelatorphan | Striatum (perfusion) | | ↑ Extracellular DA (striatum) | Dourmap et al., 1990b |

* DA, dopamine; N. Acc., nucleus accumbens.

ioral responses triggered by learned helplessness after i.c.v. treatment with RB38 (Tejedor-Real et al., 1993).

Injection of DTLET or kelatorphan into the rat striatum increased locomotor activity (Roques et al., 1985; Daugé et al., 1988). This effect was reversed by the dopamine antagonist thioproperazine and could be related to a specific $\delta$-induced increase in the spontaneous and $K^+$-induced release of newly synthesized striatal dopamine (Petit et al., 1986; Daugé et al., 1989). A modulation of the dopaminergic system in the striatum by the endogenous opioid system was also demonstrated using NEP inhibitors, such as phosphoryl-Phe-Leu (Algeri et al., 1981) and thiorphan (Wood, 1982), which increased striatal dopamine metabolites. Furthermore, kelatorphan that had been infused by microdialysis in the striatum of anesthetized rats increased extracellular dopamine, supporting a tonic enkephalinergic modulation of the dopaminergic nigrostriatal pathway (Dourmap et al., 1990b). Changes in monoamine turnover have also been reported following i.c.v. injection in mice of thiorphan plus bestatin or acetorphan alone (Llorens-Cortes and Schwartz, 1984; Lecomte et al., 1986). The inhibitors decreased noradrenaline and increased dopamine and serotonin levels in the cortex and striatum.

Chronic haloperidol administration, or 6-hydroxydopamine-induced lesions of dopamine neurons of the substantia nigra, has also been shown to enhance both proenkephalin mRNA and Met- and Leu-enkephalin levels in the striatum (Scott Young et al., 1986; Hong et al., 1979). However, $D_1$ antagonists as well as $D_1$ agonists were reported to be devoid of effects on in vitro and in vivo Met-enkephalin release from the rat striatum, whereas $D_2$ receptor stimulation enhanced the extracellular level of Tyr-Gly-Gly (Llorens-Cortes et al., 1991). Peripheral administration of the selective NEP inhibitor acetorphan increased dopamine levels in the olfactory tubercle, but not in the striatum and nucleus accumbens, of rats (Dourmap et al., 1990a) and chronic treatment with SCH 34826 reduced $D_1$ receptors in rat striatum without changing $D_2$- or opioid-binding site levels (Trampus et al., 1991).

The tonic inhibition of the striatal opioid neurons by the nigrostriatal dopaminergic input suggests that, under normal conditions, dopamine release is under the control of $\delta$-receptors, tonically stimulated by endogenous enkephalins (Petit et al., 1986). Because haloperidol was shown to increase the expression of the striatal $D_2$ receptors located on enkephalins neurons (Le Moine et al., 1991), the tardive dyskinesia syndrome induced by long-term treatment with neuroleptics might be, at least partially, due to excessive $\mu$ (akinesia) and $\delta$ (tremor) effects induced by disinhibition of the enkephalinergic neurons normally negatively controlled by the dopaminergic input. The molecular events that control the relationships between these interactions, however, are still largely unknown.

The observed euphorogenic and anxiolytic properties of opiates suggest that a defectively operating opioidergic system may be involved in the pathogenesis of various kinds of mental illness (Roques et al., 1985). This hypothesis is supported by (*a*) the well-known disinhibitory effects of laudanum tincture in humans, (*b*) the antipsychotic effects of opiates (methadone, buprenorphine, $\beta$-endorphin, and related peptides), and (*c*) the involvement of endogenous enkephalins in behavioral reinforcement (reward system). The interrelationships between the opioidergic and dopaminergic systems in the mesocorticolimbic and nigrostriatal pathways provide strong

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support for a crucial role of endogenous opioids in the control of mood. Amphetamine enhances the release of central dopamine, and chronic use of this drug results in psychotic symptoms resembling schizophrenia. Several recent studies have shown a cross-sensitization among amphetamine, stressful stimuli, and thiorphan or kelatorphan. This suggests that a hypersecretion of endogenous opioid peptides in the mesocorticolimbic pathway could induce an exaggerated behavioral response to stressful environmental stimuli, whose repetition could induce psychotic symptoms (McLennan and Maier, 1983, and references cited therein). Conversely, depression might result from a deficiency in enkephalin release, minimizing their rewarding and euphorogenic effects. Likewise, drug abuse could be caused by a deficiency in the internal opioid-controlled rewarding system.

### *J. Gastrointestinal Effects of Enkephalin-degrading Enzyme Inhibitors and Their Clinical Use as Antidiarrheal Agents*

The well-known antidiarrheal effect of opioids has been therapeutically exploited for many years. The clinically used loperamide, an opiate agonist unable to cross the blood-brain barrier, slows down intestinal transit (Guandalini et al., 1984; Kachel et al., 1986). Likewise, the enkephalin analogue, nifalatide (Tyr-D-Met(O)-Gly-(pNO$_2$)-Pro-NH$_2$, inhibits prostaglandin E$_2$ and castor oil-induced diarrhea in rats and humans. In this latter case, a double-blind study showed that orally administered nifalatide also decreased the overall stool frequency, the frequency of abdominal cramping, and the incidence of nausea and vomiting. Moreover, no clinical signs were observed related to a possible central action of this modified enkephalin, including respiratory depression and behavioral changes, in agreement with the inability of nifalatide to cross the blood-brain barrier (Ryan et al., 1986).

The mechanism of the antidiarrheal activity of opioids has been extensively studied and shown to occur by a reduction in gastrointestinal motility (Shang et al., 1986) and especially through changes in ion and fluid transport across the mucosal cell membrane (reviewed by Coupar, 1987). The latter effects are caused by activation of opioid receptors localized on neurons that innervate the enterocytes of the mucosa, with a subsequent enhancement of basal absorption of Na$^+$ and reduction in secretion of Cl$^-$. Pharmacological studies with selective agonists have shown that opioid control of intestinal electrolyte transport is predominantly mediated by δ-opioid receptors (Sheldon et al., 1990). Thus, when given peripherally at doses much lower than those needed to cause analgesia, the δ-agonist DPDPE was equipotent with morphine in inhibiting diarrhea but did not affect the rate of transit (Shook et al., 1989). This could be explained by the differences in the distribution of opioid receptors in the intestine. Indeed, whereas the myenteric plexus contains mainly μ-receptors (Surprenant and North, 1985), only δ-receptors seem to be present at the surface of submucous plexus neurons (Mihara and North, 1986; Binder et al., 1984). Stimulation of δ-receptors may result in a decrease in the release of a neurotransmitter, i.e., acetylcholine which stimulates mucosal secretion (Hautefeuille et al., 1985). Moreover, the inhibition of the electrogenic pump by opioids results in net salt and fluid absorption, counteracting the secretory actions of the mucosa.

Numerous studies have shown that NEP, APN, and ACE inhibitors potentiate the inhibitory effects of exogenously administered enkephalins on the electrically stimulated contractions of the guinea pig ileum (Aoki et al., 1984). NEP and APN are codistributed all along the gastrointestinal tract, not only at the surface of the brush border of mucosal cells but also, although in lower concentrations, in the myenteric plexus and submucosal layers (Matsas et al., 1986; Pollard et al., 1991) where enkephalin-like immunoreactivity and μ- and δ-opioid receptors are also localized (Nishimura et al., 1986). Accordingly, pretreatment of cells with phosphoramidon alone, amastatin alone, or phosphoramidon plus amastatin caused 20-, 2-, and 100-fold, respectively, increases in the ability of Met-enkephalin to stimulate the contraction of gastric smooth muscle cells. This indicates that opioid receptors, topologically located in the vicinity of NEP and aminopeptidases, contribute to the degradation of enkephalins in these cells (Menozzi et al., 1991).

The possible tonic participation of endogenous enkephalins at different levels of the gastrointestinal tract has been investigated, essentially through inhibition of NEP by thiorphan (reviewed by Checler, 1991). Owing to the reported involvement of central opioid receptors in gastrointestinal motility (Porreca et al., 1986), different routes have been used for administering the inhibitor and opioid antagonists. The antidiarrheal effects of thiorphan and acetorphan have been compared to those of loperamide in a model of castor oil-induced diarrhea in rats (Marçais-Collado et al., 1987). When administered i.v. (or orally, for acetorphan), but not when administered i.c.v., both compounds produced a delayed onset of diarrhea, with no reduction in the gastrointestinal transit as was observed with loperamide. The naloxone-antagonized antidiarrheal effect of thiorphan and its prodrug seem to result from an antisecretory effect, probably due to the stimulation of peripheral δ-opioid receptors by the endogenous enkephalins tonically released from the submucosal plexus neurons but sparsely from the myenteric plexus neurons (Kachur et al., 1980). Orally administered acetorphan was 6-fold more active than i.v. thiorphan, probably due to its easier access to the enzyme located in the peripheral tissues, either by direct diffusion across the epithelial cells or after a first passage into the circulation. Naloxone, administered i.c.v., failed to prevent the antidiarrheal effect of orally

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administered acetorphan, showing that the inhibitor acts peripherally. Given these results, it would be interesting to test the effects of orally administered mixed inhibitors, because both NEP and APN are present in the gastrointestinal tract. Moreover, the advantages of peptidase inhibitors over opioid agonists, especially δ-agonists that are unable to enter the brain, warrant investigation.

Nevertheless, the main advantage of the use of inhibitors is, as in the case of analgesia, a more physiological and selective antisecretory effect occurring probably through the stimulation of δ-opioid receptors by the protected endogenous enkephalins, thereby reducing the risk of tolerance and such rebound effects as a severe reduction in gastrointestinal propulsion induced by stimulation of μ-receptors (O'Brien et al., 1988; Larvol et al., 1992; Baumer et al., 1992). For this reason, acetorphan is now entering the market as an antidiarrheal agent under the registered trademark TIORFAN (Bergmann et al., 1992). It will be interesting to see the possible effects of repetitive treatments with TIORFAN on blood pressure homeostasis and the CNS.

The carboxyl-containing NEP inhibitor, SCH 34826, has been reported to have no gastrointestinal effects at doses 30 to 100 times higher than those affecting NEP or producing analgesia (Chipkin et al., 1988). One possible reason could be the less favorable pharmacokinetic properties of this inhibitor.

Central opioid receptors seem to play a major role in the facilitatory effect of morphine and thiorphan-protected opioids in the colonic motor response to feeding in the dog (Fioramonti et al., 1985). In addition, the localization of both enkephalins and NEP in the wall of the cat gall bladder could explain the decrease in fluid secretion that is elicited by 3 mg/kg, i.v., acetorphan (Jivegard et al., 1989) in the inflamed and distended gall bladder. This finding suggests that opioid peptidase inhibitors could also be of clinical interest in the treatment of acute cholecystitis.

The presence of enkephalins and opioid receptors in the myenteric plexus and smooth muscle of the lower esophagus could explain the weak (12% maximum) inhibition of the lower esophageal sphincter observed in humans after perfusion with acetorphan (2.5 mg/kg, i.v., in 20 min) (Chaussade et al., 1988).

In conscious rats equipped with chronic gastric fistulas, both i.c.v. thiorphan and i.v. acetorphan potently inhibited both the basal and gastrin-evoked gastric acid output, suggesting a centrally mediated inhibition of gastric secretion, very likely through a decrease of the vagal drive to the stomach (Chicau-Chovet et al., 1988). However, these effects were not reversed by naloxone, a finding at variance with the effects observed in cats (Bado et al., 1987) which were partially related to an opioid-controlled mechanism. A possible explanation could be that the distribution of NEP is different in the two species and/or that another peptidase is involved in the control of gastric acid secretion in the cat. This peptidase might be ACE because at the high dose used in these studies, thiorphan could inhibit this enzyme. This hypothesis seems to be confirmed because captopril can inhibit gastric acid secretion stimulated by pentagastrin in rats. The peptide responsible for this centrally mediated effect remains to be determined.

Food intake is facilitated by morphine and other opiates, and this effect seems to involve both central and peripheral stimulation of opioid receptors. Moreover, in rats, i.c.v. administration of thiorphan did not modify alimentary behavior, suggesting a lack of tonic control of food intake by endogenous brain enkephalins (Jackson and Sewell, 1985). In contrast, the critical role of peripheral opioid receptors that had been phasically stimulated by endogenous opioids is supported by the finding that acetorphan (5 mg/kg, i.v.) increases food intake in cats under sham feeding conditions (Bado et al., 1989).

*K. Role of Neutral Endopeptidase 24.11 in Airways*

Neuropeptide-containing nerves have been found in airway smooth muscle epithelia and blood vessels of several species, including humans (Lundberg et al., 1984). SP directly contracts airway smooth muscle and potentiates vagal motor pathways. Removal of the epithelium, which is enriched in NEP, potentiated SP-induced contractions of the airway smooth muscle. Thiorphan or phosphoramidon also facilitated SP action by inhibiting NEP located in the nerve plexus and/or submucosa layers (Djokic et al., 1989). NEP activity was found to be decreased by 40% in the tracheal layer of animals with a viral respiratory infection, suggesting that the resulting enhanced asthma and bronchoconstrictor responses (cough) could be due to increased levels of SP (Dusser et al., 1989; Nadel and Borson, 1988; Nadel, 1990). In agreement with this, human recombinant NEP, administered by aerosol to guinea pigs, reduced the drastic cough induced by SP inhalation (Kohrogi et al., 1989). If the loss of NEP activity in the viral infection is not entirely due to a loss of epithelial cells, then the mechanism by which viruses down-regulate the enzyme remains an interesting question (see section VIII).

Both NEP and ACE have been shown to be involved in inhibiting kinin-induced potentiation of ferret trachea contraction (Dusser et al., 1988). Although the lung contains both peptidase activities, the enzymes are differentially distributed between airway and vascular tissue. ACE is localized at the luminal surface of the vascular endothelium, whereas NEP is localized within epithelial cells of the alveolar septa and within tracheal smooth muscle and epithelium (Johnson et al., 1985). Nevertheless, it has been suggested that an NEP-like enzyme, but not ACE, regulates the response to SP released endogenously from sensory nerve endings in the airway epithelial layer (Lötvall et al., 1990). Given this apparently critical regulatory role of NEP in SP metab-

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olism in airways, coughing could be a possible side effect of NEP inhibitors. It would be important to test the effects of NEP inhibitors in diseases, such as adult respiratory distress syndrome and septicemia (Johnson et al., 1985), in which a drastic increase in NEP levels in blood has been observed, probably due to the release of the peptidase from lung fibroblasts or bronchial epithelial cells.

The effects of SP and capsaicin, which release tachykinins, on human bronchial smooth muscle contraction in the presence or absence of an NEP inhibitor in vitro have been studied to determine the role(s) of endogenously released tachykinins in human bronchial tissues and the role(s) of NEP in regulating the effects of the tachykinins. Phosphoramidon potentiated the SP-induced contraction in a dose-dependent fashion and increased and prolonged the bronchial smooth muscle contraction caused by capsaicin. Captopril did not alter the contractile response to SP, suggesting that ACE does not regulate the contractile response to exogenous SP in human bronchial smooth muscle in vitro (Honda et al., 1991). These results suggest that NEP could regulate the contractile effects of exogenous SP and endogenous substances, probably tachykinins, released by capsaicin in the human bronchi. Nevertheless, it has been shown that the magnitude of potentiation by NEP inhibitors was smaller for capsaicin (<10-fold leftward shift) than for the substrate agonists (about 100-fold leftward shift). This quantitative difference could be explained, at least in part, by the release of peptides that are not substrates for NEP (Warner et al., 1990). Recently, oral administration of acetorphan (200 mg) in six asthmatic subjects did not modify the bronchoconstriction produced by inhalation of metabisulfite, although this reflex is related to tachykinin release (Nichol et al., 1992). The effects of chronically administered NEP or NEP/ACE inhibitors have yet to be investigated.

### *L. Inhibition of Neutral Endopeptidase 24.11-induced Inactivation of Atrial Natriuretic Peptide: Pharmacological and Clinical Implications*

This section is not meant to be a comprehensive review of the abundant literature on ANP because recent articles have focused on its molecular biology (Genest and Cantin, 1987; Michel and Arnal, 1990), physiology and distribution (Inagami, 1989), degradation (Kenny and Stephenson, 1988), and pharmacological and clinical effects issuing from its protection by NEP inhibitors (Needelman et al., 1989; Roques and Beaumont, 1990). ANF was the term originally given to a family of peptides derived from a 126-residue precursor (Pro-ANF) that are mainly synthesized in atrial cardiocytes (De Bold et al., 1981; De Bold, 1985). The major circulating form in humans and rats appears to be the COOH-terminal fragment of Pro-ANF (99–126) or ANP, a peptide of 28 amino acids with a 17-residue disulfide-linked loop. Human α-ANP has the sequence $^{99}$SLRRSSCFGGRMDRIGAQSGLGCNSFRY$^{126}$, and in rat ANP, Met$^{110}$ is replaced by Ile. The integrity of the disulphide loop and the COOH-terminal tripeptide FRY have been shown to be important for biological activity, whereas neither the COOH-terminal tyrosine nor the $NH_2$-terminal tetrapeptide seem to be essential. Brain natriuretic peptide is a cyclic 26-residue peptide encoded by a different gene from ANP and which might be involved in the control of blood pressure by modifying vasopressin release. Despite its name, this peptide is not confined to the CNS (Tateyama et al., 1990; Aburaya et al., 1989).

ANP is released into the circulation from the atria in response to stimuli that increase the stress-strain relationship in auricular tissue (Arai et al., 1988; reviewed by Michel and Arnal, 1990). Its primary sites of action are the kidney, the vascular endothelium, and smooth muscle of the blood vessels where it acts on specific receptors to induce diuresis, natriuresis, vasodilation, fluid shifts from the intravascular to the interstitial compartments, interference with the basoreflex control of circulation, and a functional antagonism of many actions of angiotensin II (Koseki et al., 1986; Chabardes et al., 1987; Butlen et al., 1987; Hamet et al., 1989). ANP also inhibits renin release from the juxtaglomerular cells by increasing both the concentration of NaCl at the level of the macula densa cells of the distal tubule and aldosterone secretion from the zona glomerulosa cells of the adrenal gland. Elevated plasma levels of ANP are found in cases of cardiac insufficiency, especially congestive heart failure, pathological hypertension, and renal dysfunction (Burnett et al., 1986; Cody et al., 1986); the amounts of circulating ANP appear to be linked to the gravity of the conditions. In the kidney, ANP could act at three distinct levels: the glomeruli, the proximal tubule, and the collecting ducts. Binding sites have also been reported on brain microvessels in the area postrema and choroid plexus, i.e., structures outside the blood-brain barrier where the peptide could alter the production of cerebrospinal fluid, suggesting that ANP could play a role in cerebral edema (Steardo and Nathanson, 1987).

The physiological actions of ANP (fig. 9) seem to be associated with a large increase in extracellular levels of cyclic GMP that are released from smooth muscle cells (Hamet et al., 1984, 1989) following the binding of the cyclic peptide to two distinct (A and B) ANP receptors (Chinkers et al., 1989; Chang et al., 1989). Both have an extracellular ligand-binding domain as well as a large intracellular domain proposed to contain a guanylate cyclase and an ATP-binding domain. A third ANP receptor (C) has only a short intracellular domain (Fuller et al., 1988) and behaves as a clearance receptor, internalizing bound ANP for lysosomal degradation (Maack et al., 1987). Some preliminary studies have shown that B-receptors can be down-regulated and desensitized by

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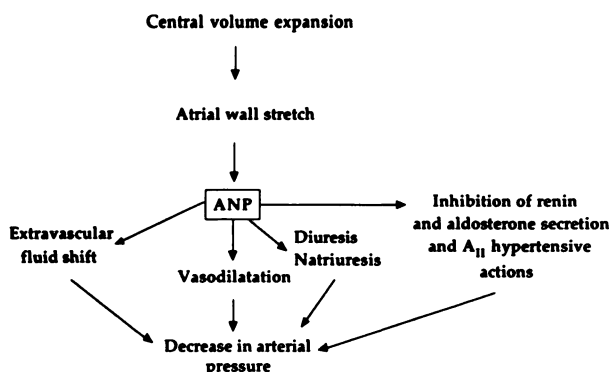


FIG. 9. Scheme of the functional actions of ANP in the regulation of blood pressure homeostasis. $A_{II}$, angiotensin II.

exogenous ANP or selective ligands and that the levels of C-receptors are slightly decreased (reviewed by Chabrier et al., 1990).

In addition to ANP clearance occurring through the C-receptors, enzymatic degradation, probably both in the kidney and in the vasculature, was proposed to be responsible for the short half-life of ANP in the circulation; this was reported as ranging from 20 to 40 s in the rat and 3 to 5 min in humans. Several groups independently showed that kidney membrane preparations degrade ANP and that the initial attack is through cleavage of the $Cys^7$-$Phe^8$ bond, with consequent opening of the disulphide ring (Koehn et al., 1987; Stephenson and Kenny, 1987a; Sonnenberg et al., 1988). These results explain the increased in vivo activity of ANP analogues having protected $Cys^7$-$Phe^8$ bonds, even though these peptides are intrinsically less active (Schiller et al., 1987). The inactivation of ANP was inhibited by thiorphan and phosphoramidon and NEP copurified with the ANP-inactivating activity from rat kidney membranes (reviewed by Kenny and Stephenson, 1988). Cleavage of ANP, hypothesized to be due to NEP in the kidney (Ura et al., 1987), was shown to modify renal secretion (Seymour et al., 1987). In addition, ANP was shown to be cleaved in vascular tissue by a thiorphan-sensitive metallopeptidase (Tamburini et al., 1989) which has since been identified as NEP by gel electrophoresis using the highly potent inhibitor [$^{125}$I]RB104 (Soleilhac et al., 1992). NEP mRNA is also present in cultured endothelial cells of arterial and venous origin (Llorens-Cortes et al., 1992).

Following these findings, numerous in vivo studies with NEP inhibitors were carried out. Thiorphan was shown to protect infused ANP, enhancing both its diuretic and natriuretic effects (Trapani et al., 1989; Olins et al., 1989; Webb et al., 1989). It was also shown at 30 mg/kg (i.v.) to induce a modest increase in diuresis and urinary sodium excretion in anesthetized or conscious normotensive rats without changing arterial pressure or the basal levels of ANP (Trapani et al., 1989). The *SS*-isomer of kelatorphan, which has a high affinity for NEP ($K_I$ = 1.8 nM), appears to be more potent than thiorphan in protecting exogenous ANP. Further in vivo studies with bidentate-containing inhibitors might prove interesting (Olins et al., 1989).

Potentiation of ANP activity has also been observed using other NEP inhibitors (Seymour et al., 1989a,b). Thus, the thiorphan-related inhibitor, SQ 29,072, increased the magnitudes, and especially the durations, of the depressor, natriuretic, and cyclic GMP responses induced by exogenous ANP in the conscious SHR in which the elevated blood pressure can be normalized by ACE inhibitors. Likewise, the carboxy-containing inhibitor, SCH 39,370, potentiated the effects of exogenous ANP and Pro-$ANF_{103-105}$ in the SHR (Sybertz et al., 1989).

Although having no effect on arterial pressure in the SHR, SCH 39,370 significantly decreased blood pressure in the desoxycorticosterone acetate-$Na^+$ rat, a model of hypertension characterized by increased levels of ANF and an insensitivity to ACE inhibitors and angiotensin II antagonists. SQ 28,603 (Seymour et al., 1991b) and recently developed orally active NEP inhibitors such as SCH 34,826 (Sybertz et al., 1990a) and the highly selective NEP inhibitor retrothiorphan (Pham et al., 1992) or sinorphan (Lecomte et al., 1990), whose affinity for NEP is only 40 times higher than for ACE, have been shown to induce a dose-dependent increase in diuresis, urinary cyclic GMP, and sodium excretion in desoxycorticosterone acetate-$Na^+$ rats. This natriuretic action is associated with the appearance of detectable ANP-like immunoreactivity in the urine. The NEP inhibitors also produced a significant decrease in mean arterial pressure in these animals. In the SHR, this effect was barely or not observed at all. The carboxyl inhibitor, UK 69,578, increased the diuretic and natriuretic effects of exogenous ANP in rats and dogs with a surgically induced atrioventricular block as models of chronic heart failure. The inhibitor doubled plasma ANP concentrations and increased $Na^+$ excretion 4-fold without changing plasma renin levels. Chronic administration of candoxatrilat to rats with aortocaval fistula produced a significant down-regulation of renal medullary and up-regulation of glomerular ANP-binding sites in experimental heart failure compared to shams, but no further change occurred during chronic dosing with candoxatrilat. Moreover, candoxatrilat significantly attenuated the development of cardiac hypertrophy following aortacaval fistula surgery (Wilkins et al., 1992). Interestingly, in cardomyopathic hamsters, which have a significantly lower basal mean arterial pressure compared to normal hamsters (90 versus 135 mm Hg), thiorphan (10 mg/kg, i.v.), although having no effect on blood pressure in either group, produced a 3-fold increase in urinary $Na^+$ excretion and a doubling of plasma ANP in cardiomyopathic but not normal animals (Smits et al., 1990a). This indicates that the renal effects of NEP inhibitors are not hampered by a decrease in arterial pressure.

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The role of both NEP and the C-receptors in controlling the biological activity of ANP has been clarified. At low plasma concentrations, ANP is probably internalized through an interaction with the C-receptors and degraded in the lysosomes (Maack et al., 1987), whereas at higher ANP concentrations NEP inactivation also intervenes (Krieter et al., 1989; Koepke et al., 1989; Olins et al., 1989).

As expected, the combination of the NEP inhibitor SCH 34,826 and c-ANP (4–23), a truncated ANP analogue capable of selectively inhibiting the binding of ANP to the C-receptors, produced a greater reduction in blood pressure and greater increases in plasma and urinary excretion of cyclic GMP than either agent alone in conscious desoxycorticosterone acetate-$Na^+$ rats but not in normotensive rats (Sybertz et al., 1990a; Vemulapalli et al., 1991). The additive effects of c-ANP (4–23) are probably due to blockade of both NEP and C-receptors but could also involve inhibition of c-ANP (4–23) degradation by NEP.

The mechanism by which NEP inhibitors exert their effects remains a matter of discussion. Thus, in anesthetized dogs with congestive heart failure, identical increases in plasma ANP levels were induced either by administration of the NEP inhibitor SQ 28,603 or by infusion of exogenous ANP. The former treatment resulted in diuresis, natriuresis, and increases in urinary ANP and cyclic GMP, whereas no significant changes, including natriuresis, were observed with infused ANP. Arterial pressure did not change in either group. These studies suggest that NEP inhibitors may potentiate the natriuretic action of endogenous ANP by a mechanism that is independent of systemic or renal hemodynamics and is unrelated to increases in plasma ANP but may be associated with the appearance of large amounts of ANP in the urine (Cavero et al., 1990). Thus, NEP inhibition has been found to produce greater natriuresis and diuresis than that produced by equivalent plasma levels of ANF obtained from exogenous administration of the peptide. Accordingly, the high concentrations of undegraded ANP was postulated to act directly on ANP receptors, although they are located on basolateral membranes on the contraluminal side (Cavero et al., 1990).

The possible role of bradykinin, another substrate of NEP, on the effects induced by NEP inhibitors is controversial. Bradykinin has potent vasodilator effects through interaction with vascular receptors. Administration of an NEP inhibitor is associated with a significant increase in urinary bradykinin. In desoxycorticosterone acetate-$Na^+$ rats, the antihypertensive response to the NEP inhibitors, SCH 34,826 and SCH 42,495, was abruptly reversed by an i.v. injection of a polyclonal antiserum to ANP but unaffected by administration of a bradykinin receptor antagonist (Sybertz et al., 1990b). Likewise, in the SHR, the thiorphan-induced natriuresis was substantially attenuated by antiserum against ANP but not by a bradykinin antagonist (Hirata et al., 1991). The natriuretic effect of retrothiorphan is also found in kininogen-deficient rats that do not synthesize bradykinin (Pham et al., 1992). In contrast, in anesthetized normotensive rats, the thiorphan-induced potentiation of the renal effects of exogenously infused ANP (diuresis and natriuresis) was completely abolished by the specific bradykinin antagonist [D-Arg$^0$, Hyp$^3$, Thi$^5$, D-Phe$^7$, Thi$^8$] bradykinin (Smits et al., 1990b). Similarly, in anesthetized volume-expanded rats, the renal responses elicited by thiorphan, including the increase in urinary cyclic GMP, were significantly reduced, not only by anti-ANP antibodies but also by antibradykinin antibodies (Bralet et al, 1991). These data suggest that NEP inhibitors can potentiate the renal activity of ANP by a mechanism that may involve accumulation of bradykinin as well as protection of ANP.

The depressor, natriuretic, and cyclic GMP responses induced by i.v. administered brain natriuretic peptide in conscious SHR and conscious cynomolgus monkeys are potentiated by the NEP inhibitor SQ 28,603 (Seymour et al., 1992). Brain natriuretic peptide, whose plasma concentration matches or surpasses that of ANP in disease states such as heart failure and hypertension, primary aldosteronism, and renal failure, could, therefore, contribute to the beneficial effects of NEP inhibitors (Seymour et al., 1992, and references cited therein). On the other hand, the very potent vasoconstrictor cyclic peptide endothelin has been shown to be degraded in vitro (Vijayaraghavan et al., 1990) and in vivo (Abassi et al., 1992) by NEP. SQ 29,072 produces a 55% increase in endothelin after i.v. administration in rats (60 mg/kg). Therefore, it is important to verify that prolonging the lifetime of endothelin by NEP inhibitors does not produce undesirable effects.

Following the promising results obtained with NEP inhibitors in various animal models of hypertension, several studies have been carried out in humans with systemically active compounds. In 16 normal volunteers, doses of 0.025 to 10 mg/kg, i.v., UK 69,578 were well tolerated and led to a significant increase in plasma levels of ANP and sodium excretion and a slight reduction in plasma renin (Northridge et al., 1990). A 2-fold increase in plasma ANP was reached within 2 h and declined to control values by 8 h after 10 mg/kg, i.v., UK 69,578 (Jardine et al., 1990). In six patients with mild heart failure in which the basal levels of ANP are already increased, UK 69,578 had beneficial effects by increasing diuresis and decreasing natriuresis and cardiac pressure (Northridge et al., 1989).

In healthy volunteers a recently developed orally active carboxyl NEP inhibitor, UK 79,300, also designated candoxatril, was administered alone (50 mg each 48 h) or during 5 days before an exogenous ANP infusion. In both cases, UK 79,300 was shown to enhance plasma ANP concentrations with a concomitant decrease in plasma

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renin activity and ANP aldosterone concentrations. Moreover, a significant increase in urinary excretion of sodium, phosphorus ANP, and cyclic GMP, without change in urinary potassium, was observed (Richards et al., 1990, 1991).

In patients with mild heart failure, acute administration of UK 69,578 and UK 79,300 elevated plasma ANP levels and urinary sodium excretion. No significant changes in heart rate, systemic arterial blood pressure, or echocardiographic left ventricular dimensions were observed (Northridge et al., 1990; Kromer et al., 1991); however, a small decrease in pulmonary artery wedge pressure was measured (Northridge et al., 1990). Repeated NEP inhibition over a 24-h period with candoxatril (150 mg, i.v.) in patients with severe chronic heart failure was shown to produce persistent elevation of plasma ANF levels, hemodynamic unloading, and suppression of neurohumoral activity. However, candaxotril did not elicit significant diuresis in these patients. These results suggest that NEP inhibitors would be appropriate as diuretic agents in the first stages of disease rather than in patients with advanced chronic heart failure (Münzel et al., 1992). In normal human volunteers, acetorphan was shown to be orally active at 100 to 300 mg, leading to a 2-fold increase of ANP levels in the plasma. Urinary volumes were increased by 35% and 75% following 100 and 300 mg of acetorphan, respectively (Gros et al., 1989). In patients with congestive heart failure, sinorphan, the *S*-isomer of acetorphan, was shown to produce an increase in plasma ANF, diuresis, and natriuresis. However, as was shown for the other NEP inhibitors, these changes were not accompanied by significant effects on left ventricular hydraulic load (Kahn et al., 1990).

Sinorphan, at a dose of 100 mg, inhibited 70% of plasma NEP activity and increased sodium urinary excretion in cirrhotic patients with ascites, suggesting that ANP could play a role in the control of sodium homeostasis in liver cirrhosis with ascites (Dussaule et al., 1991). In healthy volunteers who had maintained a high sodium intake for 5 days, the orally active NEP inhibitor, SCH 34,826, produced natriuresis and phosphate excretion without modifying blood pressure and heart rate (Burnier et al., 1991). In preclinical trials, the same results were observed with the thiol inhibitor SQ 28,603 tested in normotensive conscious female cynomolgus monkeys (Seymour et al., 1991a).

Taken together these results show that, especially under conditions of heart loading, inhibition of NEP potentiates the effects of endogenous ANP, thereby inducing a significant increase in natriuresis, diuresis, and cyclic GMP excretion without changing potassium elimination. However, the change in blood pressure is slight in the SHR and in patients with congestive heart failure in spite of a large increase in circulating ANP. The activation of the renin angiotensin-aldosterone system was suggested to antagonize the increase in renal hemodynamics and sodium excretion induced by ANP in humans and animals with congestive heart failure (reviewed by Margulies et al., 1991; Michel and Arnal, 1990). This suggestion is in harmony with the general properties of angiotensin II which promotes sodium retention and vasoconstriction, whereas ANP promotes sodium excretion and vasorelaxation. Accordingly, ACE inhibitors have been shown to potentiate hemodynamic responsiveness in rats (Raya et al., 1989) and dogs with congestive heart failure (Villarreal and Freeman, 1989).

Based on the above data, we proposed to extend the concept of mixed inhibitors that were initially developed to block both NEP and APN also to dual inhibition of NEP and ACE (Roques and Beaumont, 1990). The design of such compounds was facilitated by our knowledge of the structural requirements for inhibition of either enzyme. Thus, in 1982, two thiol inhibitors, ES34 and ES37 (table 1), were shown to inhibit both NEP and ACE with affinities in the nanomolar range (Roques, 1982; Fournié-Zaluski et al., 1984b). Based on these results, new mixed inhibitors, such as compounds PC57 and PC61 (table 2), with improved affinities and bioavailabilities have been recently synthesized (B. P. Roques and M.-C. Fournié-Zaluski, unpublished results).

As expected, in dogs with congestive heart failure chronic ACE inhibition potentiated both renal hemodynamic and excretory responses to NEP inhibitors (Margulies et al., 1991). Likewise, in the SHR, the maximal depressor responses (−44 ± 4 mm Hg) induced either by the combination of the selective NEP inhibitor SQ 29,072 and captopril or by the mixed inhibitor ES34, since designated SQ 28,133, were greater than the responses to any of the inhibitors given alone (Seymour et al., 1991c). Similar results have been obtained by using either a combination of retrothiorphan and captopril (Pham et al., 1993) (fig. 10) or a newly designed mixed NEP/ACE inhibitor (J. B. Michel, M. C. Fournié-Zaluski, and B. P. Roques, unpublished results). Inhibition of NEP by oral administration of candoxatril (100 mg every 12 h) in volunteers did not affect the level of endogenous angiotensin II but reduced clearance of infused angiotensin II. This could be due to inhibition of NEP-produced cleavage of the Tyr-Ile bond of angiotensin II when the plasma concentration of this peptide is acutely increased 3- to 5-fold (Richards et al., 1992). This suggests that patients with high renin hypertensive diseases may show enhancement of plasma angiotensin II and subsequent exacerbation of the hypertensive state after NEP inhibitor administration. Conversely, in patients with volume-expanded states, characterized by low renin activity and high ANP levels, the hypotensive effect of NEP inhibition may be potentiated. Clinical trials with NEP inhibitors, and probably in the near future with mixed NEP/ACE inhibitors, should indicate the advantages and the limits of these approaches in the treatment of essential

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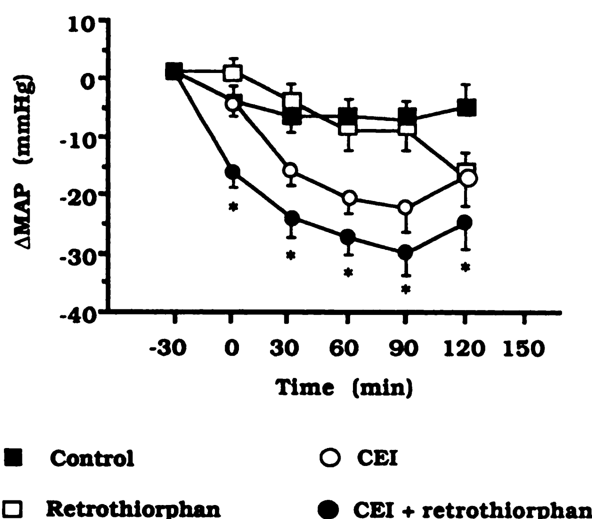


FIG. 10. Hypotensive effects of selective inhibitors of NEP (retrothiorphan) and ACE (CEI, captopril) in the SHR. The inhibitors were administered alone or together by i.v. perfusion (10 mg/kg) at time 0. Retrothiorphan alone had little effect but significantly potentiated the hypotensive effect of captopril. CEI, converting enzyme inhibitor.

hypertension, chronic cardiac failure, and sodium retention states. It is interesting to observe that, in all of the now large number of studies performed with NEP inhibitors no major side effects have been observed. This suggests that the cough, sometimes associated with chronic treatment with ACE inhibitors would not be potentiated by NEP inhibitors.

The main advantage of peptidase inhibitors is their physiological mechanism of action. Like the enkephalins in the CNS, use of the peptidase inhibitors could minimize the problem of receptor desensitization that occurs with agonists such as modified ANP or antagonists of angiotensin II receptors (Roques, 1988a). Moreover, the dual inhibition of NEP and ACE will eliminate the administration of diuretics that are required to potentiate the action of ACE inhibitors but that trigger increases of plasma renin and aldosterone and produce hypokaliema. In addition, the NEP inhibitor-induced enhancement of ANP is expected to normalize the decrease in glomerular hydrostatic pressure and filtration observed during treatment with ACE inhibitors in those pathological states characterized by very low plasma volume and renal artery stenosis (Corvol and Menard, 1988). Moreover, ANP and the inhibitor SCH 42,495 prevents myointimal proliferation after vascular injury in rats and could, therefore, have therapeutic value in inhibiting smooth muscle cell proliferation in vivo (Davis et al., 1992).

### *M. Possible Roles of the Common Acute Lymphoblastic Leukemia Antigen Identified as Neutral Endopeptidase 24.11 on Lymphoblastic Cells*

The discovery that the CALLA (CD10) is NEP provides an interesting example of how two, apparently unrelated areas of research can suddenly be brought together to the mutual benefit of both. In 1975, 1 year after the first purification of NEP, Greaves et al. injected rabbits with ALL cells that had been precoated with rabbit antibodies against normal lymphocyte antigens. The resulting antisera recognized an apparently ALL-specific protein which became known as CALLA. CALLA was subsequently identified in most cases of non-T-ALLs which have a B-precursor phenotype, as well as in some T-ALLs and lymphoblastic lymphomas. However, although originally thought to be a leukemia-specific protein, CALLA was also found to appear transiently during the development of normal B-cells (pre-B) and was also identified in a subset of lymphoid (Burkett's lymphoma, myeloma) and nonlymphoid (glioma) malignancies (reviewed by Le Bien and McCormack, 1989). In addition, CALLA was detected in other nonlymphoid tissues, and, at about the same time as the peripheral distribution of NEP was being determined, the antigen was found in the proximal tubes of the kidney and fetal intestine and on the cell surface of neutrophils, fibroblasts, and macrophages.

CALLA was also purified and shown to be a glycoprotein with a molecular weight varying between 95 and 110 kDa, depending on the tissue source. The sequencing of CALLA cDNA from human kidney cortex (Letarte et al., 1988) and a pre-B CALLA$^+$ cell line, Nalm 6 (Shipp et al., 1988), finally showed that this protein was NEP and CALLA+ cells, and cell lines have since been shown to have high levels of NEP activity (Arock et al., 1989; Beaumont et al., 1989; Tran-Paterson et al., 1990). It should be pointed out, however, that when a more sensitive assay was used (see section II) low NEP activity was also found in some cells previously classified as CALLA$^-$, including normal B- and T-cells, suggesting that the enzyme might be expressed all along the differentiation pathway in the B- and T-cell lineage (Beaumont et al., 1989). Interestingly, in agreement with the presence of CALLA at the surface of macrophages (Lai et al., 1985), a low but significant NEP activity was detected on these cells (Beaumont et al., 1989).

Anti-CALLA monoclonal antibodies are used to diagnose and follow the progression of a disease. Several studies have shown, for example, that children with CALLA$^+$ ALL have a more favorable prognosis than those with CALLA$^-$ ALL. The antibodies have also been used to try to eliminate leukemic cells ex vivo or in vivo. The latter treatment has met with limited success, probably because the antibody-protein complex is rapidly internalized (Le Bien and McCormack, 1989). Some attempts have been made to use this internalization process to target CALLA$^+$ cells, and a monoclonal antibody coupled to the ricin A chain has been reported to kill an ALL cell line in vitro (Pelham et al., 1987), although considering the very high toxicity of ricin A and the wide tissue distribution of NEP, it is difficult to see this having any in vivo therapeutic application. How-

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ever, given the high concentrations of NEP on a CALLA$^+$ lymphoblast, this approach might be feasible with material less toxic than ricin A or more specifically toxic to the leukemic cells. In addition, local destruction of NEP-bearing cells can be envisioned by vectorization of cytotoxic drugs. The coupling of a monoclonal antibody against NEP designated $ALb_1$, to the deglycosylated A chain of ricin which has a lower hepatotoxicity is more adapted to vectorization in brain tissue and has led to the development of an immunotoxin that keeps intact the cytotoxicity of the toxin (Milhiet, 1992). Very recently, a conjugate formed between mitomycin-C and a CD10 monoclonal antibody (NL-1) was shown to exert a selective in vivo cytotoxicity for a CD10$^+$ tumor with less myelosuppression than the mitomycin-C alone (Shida et al., 1992).

The transient, high expression of NEP on pre-B cells suggests a role for the enzyme in hematopoiesis. Because NEP generally seems to function as an inactivating enzyme, this role could be to temporarily protect the developing cell from a peptidergic signal, although an activation process cannot be ruled out. The identity of the substrate(s) involved, however, is, for the moment, a matter for conjecture. Numerous factors, such as the cytokines, have been shown to affect lymphocyte growth and development. NEP has been shown to cleave interleukin 1 in vitro (Pierart et al., 1988), although it is probably not involved in the regulation of interleukin-1-induced responses in vivo (Kimura et al., 1991) because the protein appears to be a poor substrate for the enzyme. As previously discussed, the addition of the NEP inhibitors, thiorphan or phosphoramidon, to cultured T-lymphocytes (Jurkat T-cells) led to inhibition of interleukin-2 release, suggesting a role of the ectopeptidase in the paracrine control of cell secretion by a still unknown regulatory peptide (Mari et al., 1992). NEP is also present on stromal cells where it has been shown to be capable of cleaving the thymic humoral factor $\gamma 2$ (Indig et al., 1989) which could play a role in differentiation and proliferation of hematopoietic cells.

Among the small peptides already known as NEP substrates, some, such as the enkephalins and SP, have been reported to influence immune cell function, although their roles here are far from clear. This is particularly true of the opioids which have been reported to activate natural killer cells through a binding to opioid receptors (Huckelbridge et al., 1990). Neutrophils were shown to have a relatively high density of NEP/CALLA at their surface, the role of which could be to cleave peptides such as Met-enkephalin, SP, and formyl-Met-Leu-Phe which are involved in inflammatory responses (Shipp et al., 1991a). Therefore, NEP inhibitors could potentiate the local effects of these peptides (Nichol et al., 1992).

Elucidating the role of NEP in lymphocyte development might help to clarify its role, if any, in leukemia and other CALLA$^+$ malignancies. There are various arguments that would suggest that the presence of the enzyme is merely a secondary consequence of the cell transformation. Loss of NEP from the cell surface by antibody-induced internalization appears to have no effect on lymphoid cell growth, either in vivo or in vitro. Similarly, inhibition of NEP activity by thiorphan had no effect on the growth of the CALLA$^+$ ALL cell line, although, if the in vivo substrate is exogenous and not present in the growth medium, this is hardly surprising (Arock et al., 1989). The expression of CD10/NEP on murine pre-B-cells and bone marrow stromal cells suggests a role for the enzyme in early B-cell ontogeny. Indeed, in cultures of progenitors, the addition of specific CD10/NEP inhibitors such as thiorphan or phosphoramidon increased the number of lymphoid colonies at days 5 to 7 by 34% (Salles et al., 1992). This suggests that CD10/NEP participates in the regulation of the earliest stages of stromal cell-dependent B-cell lymphopoiesis (Salles et al., 1992). It remains to be determined which peptide substrate is involved in this effect and whether NEP acts in this case as a processing or inactivating enzyme.

It is now apparent that other peptidases are transiently present in elevated levels on lymphocyte membranes. Significant ACE-like activity has been found in CALLA$^+$ (Reh), one CALLA$^-$ (JEA) cell line, and a CALLA$^+$ ALL membrane preparation, all of the pre-B phenotype (Beaumont et al., 1989). In addition, the human myeloid leukemia marker, CD13, has recently been shown to be APN (Look et al., 1989). Although there are clear differences in the expression of CD13/APN and CALLA/NEP in normal and leukemic hematopoietic cells, it is also probable that other as yet unidentified lymphoid markers will turn out to be proteolytic enzymes. Because many peptides can be cleaved by more than one enzyme, it is possible that modulation or inhibition of only one will not be sufficient to produce a measurable response. Interestingly, levels of both NEP and ACE have been shown to be susceptible to modulation by $\gamma$-interferon for NEP on chronic lymphocytic leukemia B-cells and by T-cell-conditioned medium for ACE on macrophages (Beaumont et al., 1989, and references cited therein).

The elevated levels of NEP, ACE, and APN in certain leukemias, even if not directly implicated in the disease itself, could affect the circulating levels of a number of peptides such as ANP, bradykinin, and angiotensin I. It is perhaps surprising, therefore, that no clinical symptoms, such as changes in blood pressure homeostasis, resulting from an enhanced degradation of these peptides seem to have been reported in CALLA$^+$ leukemias.

## XI. Conclusions and Perspectives

NEP became an interesting pharmacological "target" following the discovery that it acts to regulate the physiological functions of the enkephalins in the brain. Inter-

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**POSSIBLE CLINICAL USE OF SELECTIVE NEP INHIBITORS AND MIXED NEP/APN OR NEP/ACE INHIBITORS**

*Selective NEP inhibitors*

- antidiarrheal agents (without constipation effect)
- treatment of acute cholecystitis
- diuretic and natriuretic agents (protection of atrial natriuretic peptide)
- new antidepressants

*Mixed NEP/APN inhibitors*

- analgesics devoid of tolerance and dependence (protection of endogenous enkephalins)

*Mixed NEP/ACE inhibitors*

- new antihypertensive agents

FIG. 11. Possible clinical use of selective or mixed inhibitors.

est was further heightened when it was shown to degrade ANP in the periphery. The resulting research, carried out in many laboratories around the world, has led to the synthesis of numerous inhibitors, some of which have proved invaluable experimental and clinical tools. The main advantage of modifying the concentration of endogenous peptides by use of NEP inhibitors is that pharmacological effects are induced only at receptors tonically or phasically stimulated by the natural effectors. Moreover, the mechanisms involved in the synthesis of the clearing peptidase, and of its target peptide precursors as well as the secretion of the active peptides, have been found to be barely, or not at all, influenced by changes in the extracellular concentration of these effectors. The latter effect minimizes receptor overstimulation and the development of tolerance that occur after chronic treatment with exogenous drugs. The goal of discovering analgesics endowed with a potency similar to that of morphine, but devoid of major side effects, may now have been reached with the mixed NEP/APN inhibitors, although these compounds have yet to be evaluated in clinical trials. In addition to its antidiarrheic property, acetorphan was shown to induce natriuresis and diuresis in humans. The marketing of acetorphan will give important information concerning the possible extension of the clinical indications for NEP inhibitors, for instance, as new antidepressive agents. Likewise, it seems from initial studies that the use of NEP, and especially NEP/ACE inhibitors, as new antihypertensive agents has a promising future (fig. 11).

Although it might be thought that inhibiting a multifunctional enzyme would produce undesirable side effects, from the studies carried out so far this does not seem to be the case. A similar situation can be found with ACE, also a widely distributed enzyme capable of cleaving a number of biologically active peptides, because, after several years of wide utilization, the major in vivo effects of ACE inhibitors appear to be related to their actions in reducing arterial pressure. One possible explanation could be that in some tissues NEP is initially expressed to participate in tissue differentiation and thereafter remains at a certain level at the cell surface, although devoid of a major functional role.

The selective and mixed inhibitors of NEP, APN, and ACE have been, and will continue to be, of immense value in determining not only the role of these enzymes but also the role of their endogenous substrates, as is amply illustrated in this review for the enkephalins. These molecules can also be used, in association with molecular biology studies, to shed light on the intracellular traffic of NEP, the regulation of its expression at the cell surface, and its potential use as a target to vectorize compounds, and it will certainly aid studies aimed at elucidating the role of NEP/CD10 in virus infection and immunological and inflammatory processes. The concept of mixed inhibitors could also conceivably be extended in the future to molecules capable of selectively inhibiting more than two enzymes, e.g., the three Zn metallopeptidases involved in blood pressure regulation: NEP, ACE, and the putative big-endothelin-converting enzyme (Roques, 1988a).

There is no doubt that, in the near future, structural studies of NEP will lead to important progress in modern approaches to drug development. NEP was the first Zn ectopeptidase to be cloned, and the association of site-directed mutagenesis experiments and structural studies will be invaluable in the rational design of selective and mixed inhibitors through molecular modeling studies. ACE and APN have also now been cloned, which will allow this approach to be extended to these enzymes, enabling the proposed analogies and differences in the active sites of exo- and endo-Zn metallopeptidases to be more precisely determined. The experience gained in all of the studies reported in this review should permit the characterization of other still unknown biologically relevant Zn metallopeptidases and aid in the rapid design of efficient inhibitors endowed with appropriate bioavailabilities. One attractive target could be the light chain of tetanus and botulinum neurotoxin which contains the consensus sequence HExxH of Zn metallopeptidases. Indeed, the extraordinary neurotoxicity of these toxins seems to be related to their abilities to inactivate synaptobrevin, a protein that plays a key role in neurotransmitter release (Schiavo et al., 1992).

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