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Cloning and Characterization of *Aplysia* Neutral Endopeptidase, a Metallo-Endopeptidase Involved in the Extracellular Metabolism of Neuropeptides in *Aplysia californica*

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Cell surface metallo-endopeptidases play important roles in cell communication by controlling the levels of bioactive peptides around peptide receptors. To understand the relative relevance of these enzymes in the CNS, we characterized a metallo-endopeptidase in the CNS of *Aplysia californica*, whose peptidergic pathways are well described at the molecular, cellular, and physiological levels. The membrane-bound activity cleaved Leu-enkephalin at the Gly^3–Phe^4 bond with an inhibitor profile similar to that of the mammalian neutral endopeptidase (NEP). This functional homology was supported by the molecular cloning of cDNAs from the CNS, which demonstrated that the *Aplysia* and mammalian NEPs share all the same amino acids that are essential for the enzymatic activity. The protein is recognized both by specific anti-*Aplysia* NEP (apNEP) antibodies and by the [^{125}I]-labeled NEP-specific inhibitor RB104, demonstrating that the *apNEP* gene codes for the RB104-binding protein. *In situ* hybridization experiments on sections of the ganglia of the CNS revealed that apNEP is expressed in neurons and that the mRNA is present both in the cell bodies and in neurites that travel along the neuropil and peripheral nerves. When incubated in the presence of a specific NEP inhibitor, many neurons of the buccal ganglion showed a greatly prolonged physiological response to stimulation, suggesting that NEP-like metallo-endopeptidases may play a critical role in the regulation of the feeding behavior in *Aplysia*. One of the putative targets of apNEP in this behavior is the small cardioactive peptide, as suggested by RP-HPLC experiments. More generally, the presence of apNEP in the CNS and periphery may indicate that it could play a major role in the modulation of synaptic transmission in *Aplysia* and in the metabolism of neuropeptides close to their point of release.

Key words: Aplysia*; neutral endopeptidase; CNS; neuropeptide degradation; buccal ganglion; SCP*

Specific behaviors and various physiological functions from yeast to mammals are controlled by a wide range of bioactive peptide hormones. The use of peptides as messengers usually involves the following steps: production and release of the peptide by a specific cell, interaction of the peptide with a receptor on the surface of the target cell, and degradation of the peptide to terminate its action. The first and last steps of this scheme require the participation of proteases/peptidases. It is now acknowledged that a small number of membrane peptidases, with a broad range of specificity, act together to put an end to the biological actions of neuropeptides (McKelvy and Blumberg, 1986; Maroux, 1987; Turner, 1986; Turner et al., 1987). Most of these neuropeptidases are zinc integral membrane proteins with their active site facing the exoplasmic side of the cell (Maroux, 1987). One of the best known of these peptidases is probably neutral endopeptidase-24.11 (NEP, enkephalinase, neprilysin, CALLA), which has been implicated in the physiological degradation of several bioactive peptides (for review, see Kenny, 1993; Roques et al., 1993).

To study the importance of neuropeptide-degrading enzymes in the CNS, we used the marine snail *Aplysia californica*. This animal has been used extensively to study a wide range of behaviors and physiological functions. The simplicity and accessibility of its neuronal components contributed to link cellular, biochemical, molecular, and physiological studies and to finely characterize peptidergic pathways (Miller et al., 1993a,b; Brezina et al., 1995; Byrne and Kandel, 1996).

So far, three peptidase activities have been characterized and linked to the extracellular metabolism of peptides in *Aplysia*. A leucine aminopeptidase activity (Squire et al., 1991), an aminopeptidase N activity (Bawab et al., 1992), and a neutral endopeptidase activity (Bawab et al., 1993). In mammals, NEP is a cell surface metallo-endopeptidase ubiquitously distributed in the CNS and the peripheral organs (Roques et al., 1993). Previous studies demonstrated that NEP plays a critical role in atrial natriuretic factor-mediated hypotension and diuresis (Gros et al., 1989, 1990; Seymour et al., 1995; Thompson and Morice, 1996), enkephalin-mediated analgesia (Roques et al., 1980), tachykinin-mediated modulation of synaptic transmission (Barnes et al., 1993; Saleh et al., 1996), endothelin-mediated vasoconstriction

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(Vijayaraghavan et al., 1990), and peptide-mediated inflammatory responses (for review, see Connelly et al., 1985; Martins et al., 1990; Shipp et al., 1990, 1991; for review, see Kenny, 1993; Roques et al., 1993).

In a previous study, we identified and characterized a neutral endopeptidase activity in the kidney membranes of *A. californica* (Bawab et al., 1993). As a means to better define the physiological role of apNEP in *Aplysia*, we have characterized a NEP-like activity in the CNS and cloned the corresponding cDNA. We have characterized apNEP by Western blotting and apNEP mRNA in the CNS by *in situ* hybridization. We have also demonstrated that inhibitors of the NEP-like activity potentiate the action of endogenous neuropeptides in the buccal ganglion, and in particular of small cardioactive peptide (SCP). All together these results support the importance of peptidases in the modulation of synaptic transmission and will further our investigation into the role of the extracellular regulation of neuropeptides in behavior.

MATERIALS AND METHODS

Peptides, chemicals, and solutions. Peptides Tyr-Gly-Gly and [Leu]enkephalin were purchased from IAF Biochem International (Montréal, Quebec, Canada), L-tyrosine was from Life Technologies-BRL (Burlington, Ontario, Canada), and amastatin, 1–10 phenanthroline, phosphoramidon, phenylmethyl-sulfonyl fluoride (PMSF), and 1-O-*n*-octyl-B-D-glucopyranoside (octylglucoside) were from Sigma (St. Louis, MO). Captopril was obtained from Squibb (Princeton, NJ). Thiorphan, (3-hydroxyamino-carbonyl-2-benzyl-1-oxopropyl)-glycine (HACBO-Gly) and 2[(3-iodo-hydroxy)phenylmethyl]-4-*N*-[3-(hydroxyamino-3-oxo-1-phenylmethyl)propyl]amino-4-oxobutanoic acid (RB104) were obtained from Bernard P. Roques (Université René Descartes, Paris, France). The labeled substrate (tyrosyl-3,5-^{3}H)[Leu]enkephalin was obtained from New England Nuclear (Boston, MA). [^{125}I]Na was purchased from Amersham (Ontario, Canada). Phosphoramidon (Sigma) was added directly to a static bath (2 ml volume) to obtain a final desired concentration. Artificial seawater (ASW) contained (in mM): NaCl 460, KCl 10, $CaCl_2$ 11, $MgCl_2$ 55, and HEPES buffer 10, pH 7.6.

Animals and preparations. A. californica (200–250 gm) were purchased from Marine Specimen Unlimited (Pacific Palisades, CA) or from the *Aplysia* Resource Facility (Miami, FL). They were maintained in a large 900 l tank at 15°C. All physiological experiments were performed at room temperature (22°C) on isolated buccal ganglia. Before dissection, the mollusks were anesthetized with an injection of an isotonic $MgCl_2$ solution (385 mM) corresponding to approximately one-third of their volume. Dissection of the buccal ganglion was performed in an extracellular medium made from equal volumes of isotonic $MgCl_2$ and ASW. The ganglia were pinned to the bottom of a Sylgard-coated chamber (3 ml volume) filled with 2 ml of ASW. Both branches of the radula nerve were aspirated into a suction electrode for electrical stimulation. All preparations were rested under constant superfusion of ASW for at least 90 min before the start of an experiment.

Enzyme assays and metabolite analysis. A. californica plasma membranes were prepared as described (Bawab et al., 1992). For the enkephalin-degrading activity, 5–8 μg membrane proteins were preincubated for 15 min at 25°C in 100 μl of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5, in the presence of amastatin, at a concentration of 10 μM, alone or combined with different peptidase inhibitors. The labeled substrate [^{3}H][Leu]enkephalin [(tyrosyl-3,5-^{3}H)leu-enkephalin] (30–40 Ci/mmol) was added, and the metabolites were separated from the substrate by RP-HPLC as described previously (Bawab et al., 1993). For the SCP_B-degrading activity, 50 μg membrane proteins were preincubated for 15 min at 25°C in 100 μl of 50 mM MES, pH 6.5, in the presence of 10 μM amastatin and 1 μM captopril. The substrate SCP_B (10 μg) was added and incubated for 1 hr at 25°C, and the metabolites were separated from the substrate by RP-HPLC on a μBondapak C-18 column (Waters). A linear gradient from 95% solvent A [0.1% trifluoroacetic acid (TFA) in water]/5% solvent B (80% acetonitrile/0.1% TFA) to 100% solvent B was developed for 50 min at a flow rate of 1 ml/min.

Molecular identification of [^{125}I]RB104 binding proteins in Aplysia *tissues.* RB104 was iodinated by the chloramine T method and purified as described previously (Bawab et al., 1993). Membrane preparations from *Aplysia* CNS were solubilized for 1 hr at 4°C in Tris-buffered saline, pH 7.5, containing 1% (w/v) octylglucoside. The solubilized proteins were separated by electrophoresis, electroblotted to a nitrocellulose membrane, and labeled with [^{125}I]RB104 as described previously (Bawab et al., 1993).

Molecular cloning of the apNEP cDNA. Filter replicates of a λJ1 genomic library were hybridized at low stringency with a 760 bp *Hin*dIII–*Apa*I fragment (nucleotides 1616–2376) isolated from the rabbit cDNA, in 6× SSC, 5× Denhardt's solution, 20% formamide, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA, at 42°C for 16 hr. Filters were washed in 2× SSC, 0.1% SDS at 42°C for 1 hr. Restriction fragments of the genomic DNA were subcloned into pUC19 and sequenced. To clone the corresponding cDNA, a [^{32}P]-labeled 68 bp genomic exon was used to screen random-primed λGT10 CNS and ovotestis cDNA libraries. Filters were hybridized at 42°C for 16 hr in 6× SSC, 5× Denhardt's solution, 0.5% SDS, 50% formamide, and 100 μg/ml denatured salmon sperm DNA. After hybridization, filters were washed in 0.1× SSC, 0.1% SDS at 55°C for 1 hr and exposed to Kodak x-ray film at −80°C. Positive clones were identified, purified, and subcloned into pBluescript (Stratagene, La Jolla, CA). Double-stranded DNA was sequenced by the dideoxynucleotide method (Sanger et al., 1977) according to Sequenase protocols (United States Biochemical Corp.). The 5′ end of the cDNA was cloned by 5′-RACE (rapid amplification of 5′ cDNA extremities) using *Aplysia* CNS poly(A^+) RNA as described by Chen (1996). The first-strand cDNA was synthesized with SuperScript reverse transcriptase (Life Technologies, Burlington, ON) using a specific primer CTTGACGATCCACTTTTTCCCC (nucleotides 639–660). An oligo (dA) anchor was added to the 3′ end of the first strand cDNA with terminal deoxynucleotidyltransferase. A short 12-cycle round of PCR was performed as described by Chen (1996) with the same specific 3′ primer and the 5′ anchor primer TGAGGTGGTTGCCACAGGAGG$(T)_{20}$.The product of this PCR reaction was subjected to a second amplification using a nested, specific 3′ primer TCAAGGCTGCTGAGTCTTTGGG (nucleotides 601–622) and the 5′ anchor primer TGAGGTGGTTGCCACAGGAGG. The product was subcloned into the pCR II plasmid (Invitrogen, Carlsbad, CA) and sequenced.

cRNA probes. cRNA probes of 930 bp were obtained by *in vitro* transcription of the *Hin*dIII–*Eco*RI fragment of the apNEP cDNA, subcloned in pBluescript. Probes were labeled with digoxigenin-UTP (Boehringer Mannheim, Laval, Quebec, Canada) using T7 or T3 RNA polymerase (Pharmacia Biotechnology, Baie d'Urfé, Quebec, Canada) according to the manufacturer's instructions. The size and amounts of labeled RNAs were evaluated by Northern blotting after separation on a formaldehyde-agarose gel. Probes were aliquoted and stored at −80°C until use.

In situ *hybridization. In situ* hybridization was performed essentially as described in Panoskaltsis-Mortari and Bucy (1995) on either frozen or paraffin tissue sections. Sections were hybridized with 3 ng of heat-denatured cRNA probe in 100 μl of 50% deionized formamide, 2× SSC, 500 μg/ml heat-denatured herring sperm DNA, 250 μg/ml yeast tRNA, 10% dextran sulfate, for 16 hr at 50°C. After hybridization, slides were successively washed in 2× SSC for 5 min at room temperature, treated with RNase A (40 mg/ml in 500 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA) at 37°C for 30 min, washed in 2× SSC, 50% formamide at 50°C for 15 min, and in 1× and 0.5× SSC at room temperature for 5 min each. Positive signals were detected using anti-digoxigenin antibodies (Boehringer Mannheim). Tissues were equilibrated for 1 min in antibody dilution buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl), blocked for 30 min in the same buffer containing 2% normal goat serum, and incubated at room temperature for at least 1 hr with sheep anti-digoxigenin antibodies diluted 1:500. Sections were then washed in the antibody dilution buffer for 5 min, transferred to the detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 10 min, and incubated in 340 μg/ml nitroblue tetrazolium/175 μg/ml 5-bromo-4-chloro-3-indolylphosphate/4 toluidine salt (Boehringer Mannheim) in detection buffer. Staining was allowed to proceed overnight in the dark at 4°C. The coloring reaction was stopped in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. Sections were mounted in 33% glycerol, 1× PBS, and stored at 4°C.

Antibodies and immunoblotting. Antibodies directed against apNEP were produced by injecting rabbits with a pool of bacterially expressed C-terminal (amino acid 288–453) and N-terminal (amino acids 454–761) apNEP protein fragments fused to a 6-His tag (Qiagen, Mississauga, ON). Immunoblot analysis was performed using horseradish peroxidase-conjugated anti-rabbit IgG antibodies (Dako, Mississauga, ON) and the

SuperSignal substrate (Pierce, Rockford, IL) as recommended by the manufacturer.

Electrophysiology. Intracellular microelectrodes were pulled from omega-dot borosilicate glass (WPI, Sarasota, FL) and filled with 2 M KAc. Their resistances were between 10 and 20 MΩ. The experiments were performed in current-clamp mode, and the voltage signals were amplified using Axoclamp 2B amplifiers (Axon Instruments). Neurons in the buccal ganglion were identified on the basis of the classification suggested by Fiore and Meunier (1979): these were A neurons corresponding to cells B4 and B5 of Gardner's classification (Gardner, 1971), and B neurons. A and B neurons and one or two other large silent neurons located near the B neurons were impaled in each experiment. The radula nerve was stimulated with a suction electrode with 3 msec pulses; at the beginning of the experiment, the stimulus intensity was adjusted to evoke several spikes in A neurons (usually 2–3 V). Then the radula nerve was stimulated with trains of 30–50 stimuli (20 Hz) to evoke several (two to three) waves of synaptic and electrotonic potentials in A and B neurons (see Fig. 9). The intertrain interval was 10 min. The evoked responses as well as the spontaneous background activity were continuously monitored during the experiment using a DASH iV (Astro-Med) chart recorder (25 mm/min chart speed).

RESULTS

Evidence for a neutral endopeptidase-like activity in the *Aplysia* CNS

To reveal the presence of a membrane-associated neutral endopeptidase activity in the *Aplysia* CNS, we incubated plasma membranes from pooled ganglia with [^{3}H][Leu]enkephalin. Amastatin was added at a concentration of 10 μM to reduce as much as possible the strong aminopeptidase N activity present in this tissue (Bawab et al., 1992). The resulting metabolites were analyzed by RP-HPLC (Fig. 1). As expected for a NEP-like activity, a peak that comigrated with the Tyr-Gly-Gly peptide is visible (Fig. 1*A*). It corresponds to the degradation of 1.6 pmol of substrate per milligram of protein per minute. The tyrosine peak is probably generated by the residual aminopeptidase N activity (Bawab et al., 1992).

To characterize the nature of the [Leu]enkephalin-degrading activity, we used various peptidase inhibitors. The cation chelating agent 1-10-phenanthroline completely inhibited the hydrolysis of [^{3}H][Leu]enkephalin (Table 1), suggesting that the activity is produced by a metallopeptidase. NEP inhibitors such as RB104 (Fig. 1*B*), HACBO-Gly, thiorphan, and phosphoramidon (Table 1) were shown to abolish the Tyr-Gly-Gly peak. In contrast, captopril, an inhibitor of the dipeptidylcarboxypeptidase (Fig. 1*C*), and PMSF, an inhibitor of serine proteases (Table 1), had no effect on the activity of our enzyme preparation. All of these results suggest that a metallopeptidase with an inhibitor profile similar to that of the NEPs found in *Aplysia* kidney and in mammals is present in the CNS of *Aplysia*.

Binding of the highly specific NEP inhibitor [^{125}I]RB104 to NEP-like proteins in the *Aplysia* CNS

RB104 is a highly specific NEP inhibitor that was shown to detect as little as 2 ng of rat NEP on a Western blot (Fournié-Zaluski et al., 1992). We first tested the affinity of the enkephalin-degrading enzyme in CNS plasma membranes for [^{125}I]RB104 and found that the K_D is similar to that of the rat NEP and the *Aplysia* kidney enzyme (Table 2) (Fournié-Zaluski et al., 1992; Bawab et al., 1993). [^{125}I]RB104 was then used in inhibitor gel electrophoresis experiments. Solubilized CNS membrane proteins or purified rabbit NEP, which was used as a control, were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with [^{125}I]RB104. As shown in Figure 2, [^{125}I]RB104 binds to proteins of 100 and 200 kDa in the *Aplysia* CNS membranes and to the 94 kDa rabbit protein; this binding was completely abolished by NEP inhibitors such as HACBO-Gly (10 μM) and phosphoramidon (10 μM). In contrast, the labeling was not affected by specific inhibitors of other peptidases such as captopril (10 μM) or amastatin (10 μM). These results demonstrate that

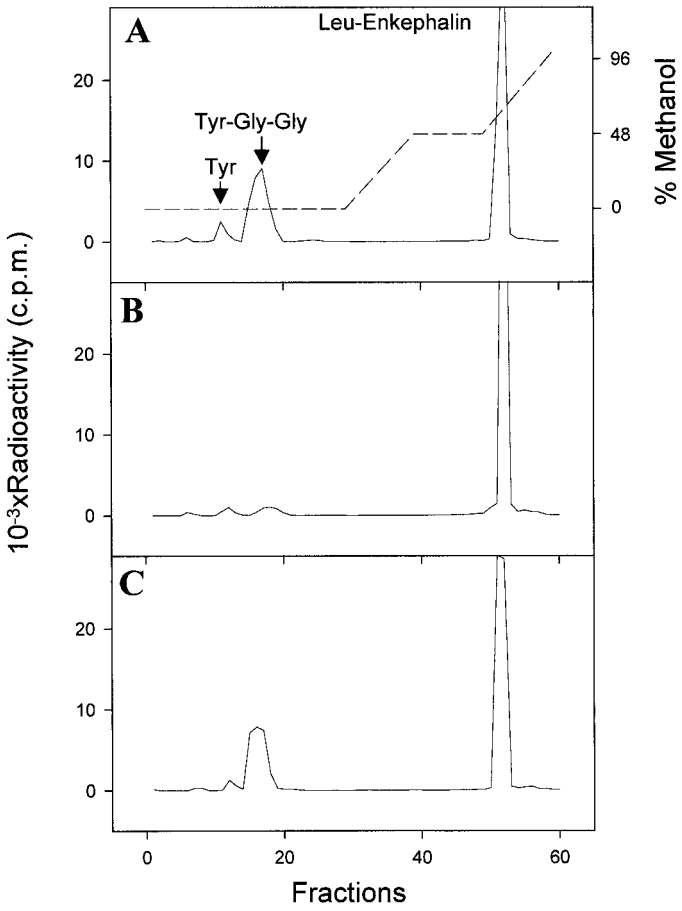

Figure 1. RP-HPLC analysis of degradation metabolites of [^{3}H][Leu]enkephalin in the *Aplysia* CNS. The substrate was incubated with CNS plasma membranes, in the absence (*A*) or presence of 1 μM RB104 (*B*) or 1 μM captopril (*C*). *Arrows* indicate the elution position of standard peptides. The *dashed line* represents the methanol gradient used in the HPLC.

Table 1. Comparison of the action of peptidase inhibitors on apNEP activity present in *Aplysia californica* CNS and kidney membranes

Inhibitors	Concentration (μM)	% Inhibition (head ganglia membranes)	% Inhibition (kidney membranes)[a]
RB104	1	87	91
HACBO-Gly	10	91	74
Thiorphan	10	41	33
Phosphoramidon	10	41	45
1-10 Phenantroline	5	100	100
Captopril	10	11	0
PMSF	100	0	0

Plasma membranes from head ganglia or kidney [results from Bawab et al. (1993)] were incubated, before the addition of the [^{3}H]Leu-enkephalin, in the absence or presence of various peptidase inhibitors. The enzymatic assays were performed as indicated in Materials and Methods. Results are expressed as a percentage of inhibition of substrate degradation. The percentage inhibition was calculated by comparing the radioactivity under the Tyr-Gly-Gly peak in the presence and absence of inhibitors.

[a]Bawab et al. (1993).

Table 2. Comparison of the NEP-like enzymes in the CNS and kidney of *Aplysia californica*

Characteristics	CNS	Kidney[a]
Activity[b]	1.6 pmol/mg protein per minute	3.5 pmol/mg protein per minute
RB104-K_D[c]	0.16×10^{-10}	0.26×10^{-10}
RB104-B_{max}[e]	12 fmol/mg protein	20 fmol/mg protein
RB104-inhibitor gel electrophoresis (M.W.)	100 and 200 kDa	140 kDa
Western blot (M.W.)[d]	100 and 200 kDa	140 kDa
Northern blot[e]	+[f]	++

[a]Bawab et al. (1993).

[b]Membrane preperations were incubated with [^{3}H][Leu]enkephalin, in the presence of amastatin at a concentration of 10 μM. The metabolites were separated by RP-HPLC, and the number of counts per minute under the Tyr-Gly-Gly peak were measured as described in Bawab et al. (1993).

[c]Kidney plasma membranes were incubated in the presence of [^{125}I]RB104 and different dilutions of cold I-RB104. The K_D and B_{max} values were calculated from Scatchard analysis as reported previously (Bawab et al., 1993).

[d]Western blot realized with specific apNEP antibodies.

[e]Northern blot realized with an apNEP cDNA probe.

[f]Intensity of the hybridization signal.

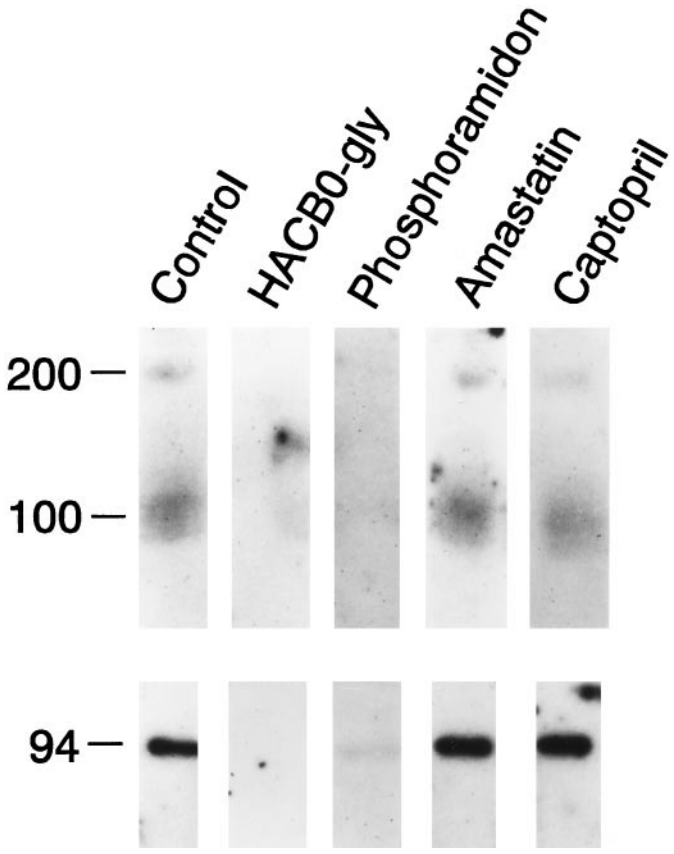

Figure 2. Inhibitor gel electrophoresis with [^{125}I]RB104 and different peptidase inhibitors. Solubilized *Aplysia* CNS membrane proteins (*top panel*) and purified rabbit NEP (*bottom panel*) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. NEP-like proteins were labeled with 100 pM [^{125}I]RB104 in the presence or absence of peptidase inhibitors: absence of inhibitor (*Control*); *HACBO-gly* at 10 μM; *Phosphoramidon* at 10 μM; *Amastatin* at 10 μM; *Captopril* at 10 μM.

NEP-like proteins are expressed in the CNS and that their molecular sizes are different from that of the 140 kDa NEP-like enzyme already observed in the *Aplysia* kidney membranes. However, their active site is likely to be structurally and functionally similar, because they all bind [^{125}I]RB104 with high affinity. These results raise the question of whether the NEP-like proteins in the kidney and CNS are differentially glycosylated isoforms of the same protein or whether they are expressed from two closely related NEP-like genes.

Isolation of cDNA clones encoding an apNEP

To answer this question, we cloned cDNAs coding for the NEP-like activity. We first screened an *Aplysia* genomic library at low stringency, using a 760 bp rabbit NEP cDNA fragment as a probe (Devault et al., 1987). One of the 13 clones (λNEPg1) found was further characterized, and a 400 bp fragment was subcloned and sequenced. A short segment of 68 bp, flanked by splicing consensus sequences, showed high sequence similarity to the rabbit NEP sequence (Fig. 3). Interestingly, the 3′ splicing site is identical to the one described for all of the NEP-like family members, and the 5′ splicing site is common to endothelin-converting enzyme (ECE), a human phosphate-regulating gene with homologies to endopeptidases on the X-chromosome (PHEX) and kell blood group protein (KELL) but not to NEP (Fig. 3). Considering the high level of conservation of exon/intron boundaries, these results not only suggest that apNEP is a member of the NEP family, but also indicate that the apNEP and mammalian NEP-like enzymes are likely to be derived from a common ancestor (see also below).

This 68 bp segment was PCR-amplified, subcloned, and used as a probe to screen *Aplysia* CNS and ovotestis cDNA libraries by plaque hybridization. Of eight positive recombinant phages, the inserts of the λ5.1, λNEPc, λNEPe, and λNEPf clones were sequenced. Their sequences indicated that they represented overlapping cDNAs derived from the same apNEP mRNA but that the 5′ region of the coding region was missing. Because we did not succeed in cloning the 5′ part of the cDNA by rescreening the libraries, we performed a 5′-RACE protocol using a set of nested specific internal primers and mRNA isolated from the CNS. This yielded one overlapping PCR product that covered the missing coding sequence and part of the 5′ UTR. The first ATG is found at position 164 and is followed by an open reading frame of 2361 nucleotides that codes for a putative apNEP protein of 787 amino acids (Fig. 4). This protein is ~35% identical to human NEP.

Southern blot analysis of the *apNEP* gene

The cloning of one small exon of the *apNEP* gene suggests that it could be fragmented into many exons as observed for the mammalian homologs. To assess this point, a Southern blot of *A.*

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                                                                                   Tyr Leu
apNEP                                                                   tttcag A TAC CTG
hKELL                                                                   ccctag A GCC GTG
hPHEX                                                                   ccatag A TCT CTG
hECE1                                                                   ctccag G GCC TTA
hNEP    tctccttgtag TC TTC CCA GCC GGC ATT CTG CAG CCC CCC TTC AGT GCC CAG CAG TCC AAC TCA TTG

        Asn Tyr Gly Ser Ile Gly Val Ile Ile Gly|His Glu Ile Thr His|Gly Phe Asp Asp Lys
apNEP   AAC TAT GGA TCT ATA GGA GTT ATC ATA GGT|CAC GAA ATC ACT CAC|GGT TTT GAT GAT AAA G gtaa
hKELL   AAC TTT GGC GCT GCT GGC AGC ATC ATG GCC|CAC GAG CTG TTG CAC|ATC TTC TAC CAG CTC T gtgg
hPHEX   AGT TAT GGT GCT ATA GGA GTA ATT GTC GGA|CAT GAA TTT ACA CAT|GGA TTT GAT AAT AAT G gtag
hECE1   AAC TTT GGT GGC ATA GGT GTC GTC GTG GGC|CAT GAG CTC ACT CAT|GGC TTC GAT GAC AAT G gtac
hNEP    AAC TAT GGG GGC ATC GGC ATG GTC ATA GGA|CAC GAA ATC ACC CAT|GGC TTC GAT GAC AAT G gtaa
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Figure 3. Comparison of the apNEP (λNEPg1 clone), hNEP, hECE1, hKELL, and hPHEX exons that code for the zinc-binding domain. Nucleotide sequences of exons and flanking introns are shown in *capital* and *small letters*, respectively. Splicing consensus sequences are *underlined*. The deduced amino acid sequence of the *apNEP* exon is shown above the nucleotide sequence. The codons for identical amino acids are in *bold type*, and the pentapeptide consensus sequences (His-Glu-Xaa-Xaa-His) that are part of the metalloprotease zinc-binding domain are *boxed*.

californica genomic DNA was digested with *Bgl*II, *Eco*RI, *Hin*dIII, *Sac*I, and *Xba*I and hybridized with a short probe. Considering the fact that no *Sac*I or *Bgl*II site and only one *Xba*I restriction site exists in this probe, the multiple bands that hybridized in each lane indicate that this small cDNA region corresponds to at least three exons in the genomic DNA (Fig. 5*A*). Consistent with the cloning of a small exon (see above), this result suggests that the genomic organization of the *apNEP* gene may be similar to that of the members of the NEP-like family, which are all fragmented into several exons (D'Adamio et al., 1989).

Tissue expression of the apNEP mRNA, and cellular localization in the *Aplysia* CNS

Northern blots of poly(A^+) RNA extracted from various tissues were probed with a 316 bp apNEP cDNA fragment and used to determine the size of the apNEP transcript and its specificity of expression (Fig. 5*B*). A single transcript of ~3.8 kb was abundantly present in ovotestis and kidney and very little was expressed in the CNS, gill, and heart where the signal could only be detected after a long period of exposure. By comparison with the size of the cDNA, it is likely that additional 5′ and/or 3′ untranslated sequences are present in the transcript. The presence of apNEP in these tissues was confirmed by Western blot experiments (see below). These results confirm that apNEP is expressed in both the CNS and kidney as well as in many other organs.

To determine the type(s) of cells that express apNEP in the *Aplysia* CNS, we performed preliminary *in situ* hybridization experiments on paraffined sections of the ganglia. A positive signal can be observed with a cRNA probe in many neurons of all the ganglia (Fig. 6*A*), demonstrating that neurons are the source of apNEP in the CNS. The signal is not restricted to the cell bodies and can also be observed in the neuropil and ganglion peripheral nerves in structures that look like neurites (Fig. 6*C*). The specificity of the signal was confirmed by the absence of any signal when the same experiments were performed on adjacent sections using a sense probe (Fig. 6*B*). At this point we did not try to identify individual neurons.

Primary structure of apNEP

The apNEP cDNA sequence encodes a putative protein of 88 kDa, which shares important structural features with mammalian NEP. (1) As predicted by the Kyte and Doolittle (1982) hydrophobicity plot (Fig. 7*A*), apNEP is a type II integral membrane protein with a short N-terminal cytoplasmic tail of 31 amino acids, a hydrophobic region of 23 residues, which represents a putative transmembrane helix, and a large extracellular C-terminal domain of 686 amino acids. (2) The extracellular portion of apNEP contains the highly conserved zinc-binding motif (residues 622–626) (Fig. 6*B*) and thus probably constitutes the catalytic domain. (3) apNEP contains 10 putative sites for N-glycosylation (Asn-Xxx-Ser/Thr), suggesting that apNEP is highly glycosylated. (4) The 10 cysteine residues found in the extracellular domain of apNEP coalign with those of the mammalian NEP (Fig. 7*B*). (5) Nearly all of the amino acids that are essential for the enzymatic activity of the mammalian NEP (for review, see Roques et al., 1993) are found in the same position on the cDNA encoding apNEP (Table 3). All together, these results suggest that the apNEP cDNA codes for an *Aplysia* neutral endopeptidase homolog.

The CNS 100 kDa and the kidney 140 kDa [^{125}I]RB104-binding proteins are likely to be coded by the *apNEP* gene

To determine whether the NEP-like molecules in the CNS and kidney membranes are both expressed from the *apNEP* gene, immunoblots of membrane extracts from the kidney and CNS were performed, using anti-apNEP antibodies. As expected from the inhibitor gel electrophoresis experiment, a band of 140 kDa was detected in the kidney plasma membranes, whereas a single band of 100 kDa was detected in the membranes of the CNS (Fig. 8*A*). Under nonreducing electrophoresis conditions, an additional band of ~200 kDa was detected in the membranes of the CNS (Fig. 8*C*), suggesting strongly that the 200 kDa protein is a dimer of the 100 kDa protein, as observed in mammals (Kenny and Maroux, 1982). These results demonstrate the presence of apNEP in both tissues and clearly link the RB104-binding proteins in the membranes of both the CNS and kidney to the product of the *apNEP* gene.

The discrepancy in the apparent molecular mass of the CNS and kidney NEP-like enzymes in *Aplysia* membranes may be the result of post-translational modifications, such as glycosylation. To examine this point, membrane extracts from these tissues were deglycosylated with PNGase F; the resulting proteins were sepa-

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5′  GACAGTTAGGCCTTGAGAGCGCGGAGAGCTACACCTTTCACTCCTTGCGAGTTCTGAGACGGGGCGCCTCAGGAGAAGATTTCAAACCTTGGGCTGGAGTCCCTCATCAGTTCATCTGT  119

                                                Met Ala Pro Glu Ile Pro Leu Asp Ile Gln Arg Leu Gln Phe Gln Arg Thr Thr Ser His   20
GAAACTTGCGTGAAGAGCTAGAAGGACATCAGCCACGCCAGTG ATG GCC CCG GAG ATC CCA CTC GAC ATA CAA CGT TTG CAG TTT CAG AGA ACG ACA TCA CAC  222

Trp Pro Glu Glu Gln Lys Ser Lys Ala Val Trp Arg Leu Ile Ala Leu Leu Val Val Leu Val Leu Ala Leu Val Ala Ile Val Val Leu Ser   51
TGG CCA GAA GAG CAA AAG AGC AAA GCT GTC TGG CGG CTG ATT GCA CTG CTG GTG GTG CTA GTC CTT GCC CTG GTA GCT ATT GTG GTG CTT TCA  315

Ile Val Ile Val Arg Ile Arg Glu Asp Asn Lys Thr Thr Val Lys Pro Glu Pro Ser Ser Gly Ser Asp Gly Thr Thr Ser Ala Ala Ser Gly   82
ATA GTC ATC GTG AGA ATC AGA GAA GAC AAC AAA ACC ACC GTC AAA CCT GAA CCT TCT TCA GGC TCA GAT GGC ACA ACA TCA GCA GCT TCA GGC  408
                  *
Ser Gly Gly Thr Thr Ser Ala Ala Ser Gly Ser Gly Gly Thr Thr Ser Ala Asp Ser Asp Gly Lys Ile Cys Val His Glu Gly Cys Val Thr  113
TCA GGC GGC ACA ACA TCA GCA GCT TCA GGC TCA GGA GGC ACA ACA TCA GCA GAC TCA GAC GGT AAA ATT TGC GTG CAC GAG GGT TGT GTT ACG  501

Ala Ala Ala Arg Ile Met Ser Asn Leu Asp Lys Ser Val His Pro Cys Asp Asn Phe Tyr Asn Phe Ala Cys Ala Asn Trp Glu Tyr Asp Arg  144
GCA GCC GCC CGC ATC ATG AGT AAC CTC GAC AAA TCA GTT CAT CCC TGT GAC AAC TTC TAC AAT TTT GCT TGT GCG AAT TGG GAA TAT GAC CGC  594

Asp Ile Pro Lys Asp Ser Ala Ala Leu Ser Val Leu Ser Glu Leu Gly Lys Lys Val Asp Arg Gln Val Lys Leu Ile Ile Glu Ala Pro Ser  175
GAC ATT CCC AAA GAC TCA GCA GCC TTG AGT GTT TTA TCA GAG CTG GGG AAA AAA GTG GAT CGT CAA GTA AAA CTT ATC ATC GAA GCG CCG TCA  687

Pro Pro Asp Glu Leu Pro Val Val Thr Lys Ala Lys Asn Leu Tyr Lys Ser Cys Leu Asp Leu Glu Thr Met Asp Ala Arg Gly Ile Asp Pro  206
CCA CCA GAC GAG CTG CCA GTA GTG ACG AAG GCC AAG AAT TTG TAC AAA TCC TGT CTC GAT CTA GAA ACA ATG GAT GCA CGT GGA ATC GAC CCA  780

Tyr Lys Gln Trp Leu Ser Ser Thr Ile Gly Glu Trp Pro Leu Ile Ser Ser Ala Phe Asn Glu Ser Glu Phe Asp Ile Thr Asp Ala Ile Arg  237
TAC AAG CAA TGG CTC AGT TCT ACT ATC GGA GAG TGG CCT TTG ATC TCC AGT GCT TTC AAC GAG TCG GAA TTT GAC ATC ACT GAC GCC ATC AGA  873
                                                              *
Lys Ala Asn Ile Tyr Gly Pro Gly Pro Val Val Ala Phe Tyr Val Gly Thr Asp Asp Lys Asn Ser Ser Lys Lys Ile Leu Lys Ile Asp Gln  268
AAA GCC AAT ATC TAC GGT CCC GGC CCC GTT GTC GCT TTC TAT GTT GGT ACT GAC GAC AAA AAC TCT TCT AAA AAG ATA CTT AAG ATA GAC CAG  966
                                                                  *
Pro Ala Phe Gly Leu Pro Gly Gln Arg Tyr Tyr Arg Val Pro Arg Asn Asp Thr Tyr Ile Lys Ala Tyr Glu Thr Tyr Leu Tyr Arg Val Ala  299
CCT GCC TTT GGT CTT CCA GGC CAG AGA TAC TAC CGA GTA CCC AGG AAC GAC ACG TAT ATC AAG GCC TAT GAG ACG TAC CTC TAC AGA GTG GCA 1059
                                                        *
Glu Ala Leu Gly Phe Ala Asp Pro Ala Thr Ala Glu Lys Asp Val Ala Asp Val Val Asp Phe Glu Met Gln Ile Ala Gln Ile Ser Val Arg  330
GAG GCG CTC GGA TTT GCT GAC CCC GCT ACG GCA GAG AAG GAT GTC GCG GAC GTT GTA GAT TTT GAA ATG CAG ATC GCC CAG ATT TCC GTG AGA 1152

Pro Ala Ile Arg Arg Asn Ala Asn Ala Val Phe Asn Pro Met Thr Leu Ala Glu Leu Asp Gln Asp Phe Ser Ser Pro Glu Leu Asn Phe Ser  361
CCT GCG ATC AGA CGT AAC GCC AAT GCA GTA TTT AAC CCA ATG ACC CTG GCT GAG CTC GAC CAA GAC TTC AGT TCG CCA GAG CTC AAC TTC TCG 1245
                                                                                                                  *
Arg Leu Ile Thr Thr Val Met Ser Ala Pro Glu Val Ala Val Ser Val Gly Asp Asp Glu Ile Ile Met Asn Arg Ser Pro Pro Tyr Phe Arg  392
AGG TTA ATC ACA ACA GTG ATG TCA GCA CCA GAA GTA GCA GTC AGT GTC GGA GAC GAT GAA ATC ATT ATG AAC AGA TCA CCC CCT TAC TTT CGG 1338
                                                                                    *
Asn Leu Thr Asp Leu Leu Arg Asn Thr Pro Lys Lys Thr Ile Ala Asn Tyr Ile Ile Trp Arg Ile Thr Ile Ser Tyr Leu Gly Thr Leu Thr  423
AAC CTT ACA GAC CTT CTT AGA AAC ACA CCA AAA AAA ACA ATA GCT AAC TAC ATC ATA TGG CGG ATT ACT ATC TCT TAC CTC GGT ACT CTG ACA 1431
 *
Gln Val Phe Lys Asp Ile Arg Phe Glu Phe Thr Lys Ala Ile Tyr Gly Ile Glu Thr Val Gln Pro Arg Glu Leu Phe Cys Thr Ser Phe Val  454
CAG GTT TTT AAA GAC ATC CGA TTT GAG TTC ACA AAG GCC ATA TAT GGT ATA GAA ACT GTT CAA CCA CGA GAG TTG TTC TGT ACA AGC TTT GTG 1524

Arg Arg Asn Val Gly Phe Ile Ile Ser Lys Pro Phe Val Asp Lys Phe Phe Ser Pro Glu Ala Lys Asp Val Ala Leu Glu Met Ile Ser Gly  485
AGA AGA AAT GTT GGA TTC ATC ATC AGC AAA CCG TTT GTG GAC AAG TTC TTC TCC CCA GAA GCT AAA GAT GTG GCT CTA GAA ATG ATC TCC GGT 1617

Leu Gln Ser Ala Phe Asn Glu Ile Val Asp Glu Val Glu Trp Met Asp Glu Glu Thr Lys Val Val Ala Arg Glu Lys Asn Asp Ala Ile Val  516
CTT CAG TCA GCT TTT AAT GAG ATT GTA GAT GAA GTA GAA TGG ATG GAC GAA GAA ACA AAA GTA GTA GCT CGA GAA AAG AAT GAT GCA ATT GTC 1710

Ser Lys Ile Gly Tyr Pro Glu Phe Val Ile Asn Ser Thr Arg Leu Thr Glu Leu Tyr Glu Asn Tyr Thr Tyr Gly Asn Asp Thr Tyr Phe Glu  547
TCC AAG ATT GGA TAC CCC GAA TTT GTG ATC AAT TCC ACA CGA CTG ACA GAA TTG TAC GAA AAT TAC ACA TAT GGA AAT GAT ACG TAC TTC GAA 1803
                                  *                 *                 *
Asn Ile Leu Ser Lys Asn Lys Val Asn Val Asp Ser Ser Phe Arg Ser Leu Arg Glu Leu Val Asp Lys Glu Gln Trp Phe Arg Ser Pro Pro  578
AAC ATA TTG TCC AAG AAC AAA GTG AAT GTA GAC AGT TCT TTC CGT AGT CTG AGG GAG CTG GTG GAC AAA GAG CAA TGG TTC AGA AGT CCG CCC 1896

Thr Val Asn Ala Tyr Tyr Asn Lys Ala Gly Asn Glu Ile Val Phe Pro Ala Gly Ile Leu Gln Ser Pro Val Phe His Val Asp Phe Pro Lys  609
ACA GTT AAT GCC TAC TAT AAC AAA GCT GGC AAC GAA ATA GTG TTT CCA GCG GGA ATC TTG CAG TCA CCA GTT TTC CAT GTG GAC TTC CCG AAA 1989

Tyr Leu Asn Tyr Gly Ser Ile Gly Val Ile Ile Gly His Glu Ile Thr His Gly Phe Asp Asp Lys Gly Arg Leu Tyr Asp Lys Asn Gly Asn  640
TAC CTG AAC TAT GGA TCT ATA GGA GTT ATC ATA GGT CAC GAA ATC ACT CAC GGT TTT GAT GAT AAA GGC AGG CTG TAT GAC AAG AAC GGA AAC 2082

Leu Asn Gln Trp Trp Ser Asn Ser Ala Ile Glu Lys Phe Asn Thr Gln Lys Gln Cys Ile Ile Asp Gln Tyr Gly Ala Tyr Val Met Asp Gln  671
CTG AAT CAA TGG TGG AGT AAC TCT GCT ATC GAG AAG TTT AAC ACC CAG AAG CAA TGT ATC ATT GAC CAG TAC GGA GCT TAT GTC ATG GAC CAA 2175

Val Glu Leu Asn Leu Asn Gly Ile Arg Thr Gln Gly Glu Asn Ile Ala Asp Asn Gly Val Ser Lys Ser Leu Ser Gly Arg Thr Lys Leu Gly  702
GTG GAG CTC AAT CTG AAT GGA ATA CGT ACG CAG GGA GAA AAT ATT GCT GAC AAT GGC GTC TCA AAG AGT CTT TCA GGG CGT ACA AAA CTG GGT 2268

Gln Ser Thr Arg Lys Glu Glu Pro Thr Leu Pro Gly Leu Gln Tyr Thr Asn Asp Gln Leu Phe Phe Ile Ser Phe Gly Gln Thr Trp Cys Asn  733
CAA AGC ACA AGA AAA GAA GAG CCT ACC TTG CCA GGA CTT CAG TAC ACG AAC GAT CAA CTC TTT TTC ATC AGT TTT GGG CAG ACC TGG TGT AAC 2361

Met Tyr Arg Asn Asp Ala Leu Ile Ser Ser Ile Arg Ser Gly Val His Ser Pro Gly Gln Tyr Arg Val Ile Gly Ser Leu Gln Asn Ser Glu  764
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Asp Phe Ala Arg Val Phe Asn Cys Pro Ser Thr Ser Tyr Met Asn Ala Ala Asn Lys Cys Gln Val Trp ***                              787
GAT TTT GCC AGA GTG TTC AAC TGT CCT TCA ACA TCG TAC ATG AAC GCG GCC AAC AAA TGT CAA GTC TGG TGA ACAGGCCAATGAACTGGTCATTATTCC 2553

AGATCCGTTGATTATCGATCTGCTGTTCTTGTTTTTGTTGGTTCGTGCTTATTTTATGATGAAAGTGGAGAAAGAAAACGTTTCGTTTCAAAAACAATTCGACATTCCTTTGACCAGTTGTAG 2676

ATAAATACACACTTCAAACTG 3′                                                                                                    2697
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Figure 4. Nucleotide and deduced amino acid sequence of the *Aplysia* neutral endopeptidase. Amino acids are numbered starting at the first ATG of the open reading frame. The putative transmembrane region is *underlined*. Potential sites of *N*-glycosylation are indicated by an *asterisk*, and the cysteine residues are *bold*. The zinc-binding signature HEXXH is *boxed*. The nucleotide sequence has been submitted to the GenBank Data Bank with accession number AF104361.

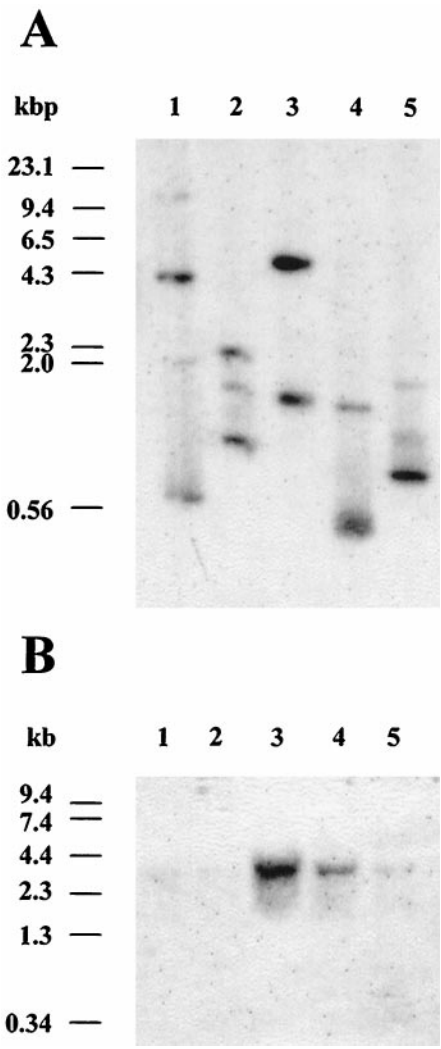

Figure 5. Southern and Northern blot analysis of the *apNEP* gene. *A*, Genomic DNA was isolated from ovotestis and digested with either *Bgl*II (*lane 1*), *Eco*RI (*lane 2*), *Hin*dIII (*lane 3*), *Sac*I (*lane 4*), or *Xba*I (*lane 5*). Digested DNA (10 μg/lane) was run on a 0.8% agarose gel, transferred to a nitrocellulose membrane, and hybridized at high stringency with the [^{32}P]-labeled *Hin*dIII–*Acc*I apNEP fragment (nucleotides 1142–1458) as described previously (Wickham and DesGroseillers, 1991). DNA molecular weight markers are indicated in kilobase pairs (*kbp*) on the *left*. *B*, Northern blot analysis of the apNEP transcript. Total RNA was extracted from different tissues, and poly(A$^+$) RNA (5 μg) isolated from gill (*lane 1*), heart (*lane 2*), ovotestis (*lane 3*), kidney (*lane 4*), and CNS (*lane 5*) was fractionated on a 1% formaldehyde/agarose gel, blotted to a nitrocellulose membrane, and hybridized at high stringency with the [^{32}P]-(*Hin*dIII–*Acc*I) apNEP fragment, as performed previously (Auclair et al., 1994). RNA molecular weight markers are indicated in kilobases (*kb*) on the *left*. To control the amounts of RNA in each lane, filters were stripped and rehybridized with an *Aplysia* actin probe (data not shown).

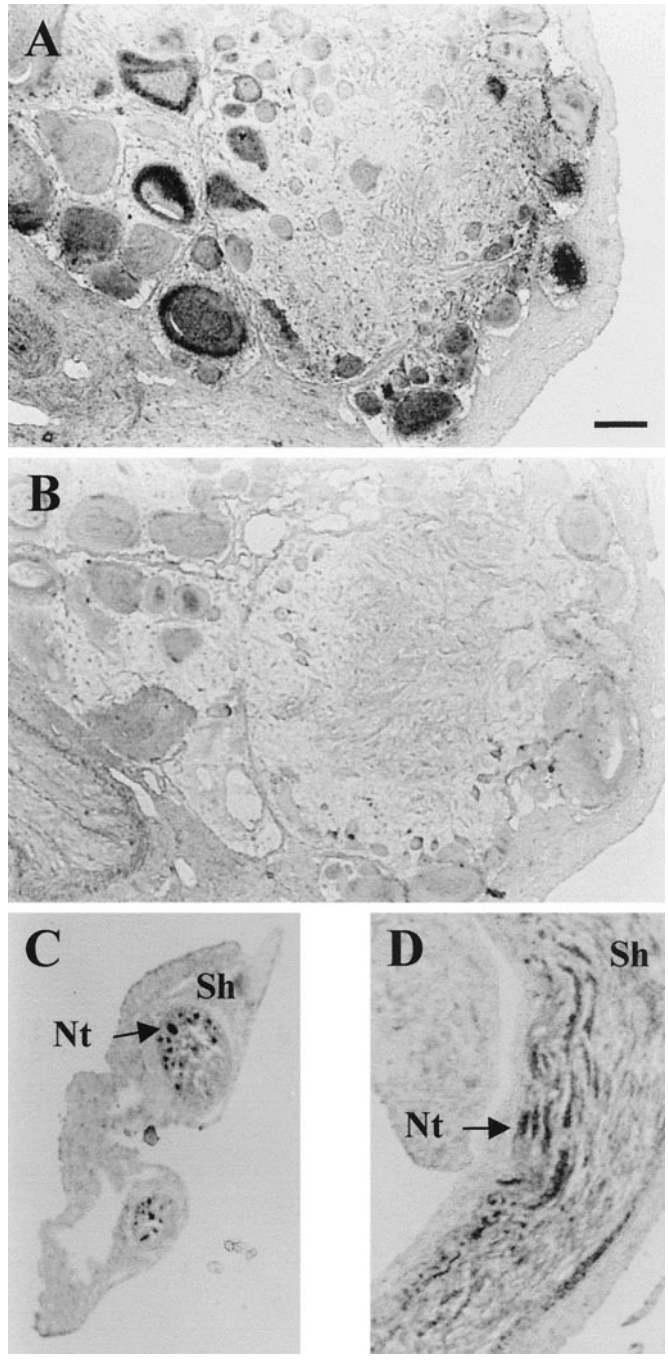

Figure 6. In situ hybridization of apNEP on paraffined sections of *Aplysia* ganglia. Sections of the abdominal ganglion (*A, B*) and of a buccal ganglion nerve (*C, D*) were hybridized with either an apNEP cRNA antisense (*A, C, D*) or sense (*B*) probe. Positive signal is seen in neurons (*A*) and neurites (*Nt*) extending into the nerve. No signal is detected in the sheath (*Sh*). The same results were obtained with sections from all the major ganglia. Scale bar, 100 μm.

rated by SDS-PAGE and detected by Western blotting. After PNGase F treatment, the molecular size of apNEP in the kidney was reduced to ~88 kDa (Fig. 8*B*), which is the predicted size from the cDNA sequence. This demonstrates that the 140 kDa protein is highly glycosylated and confirms that it is probably the product of the *apNEP* gene. On the other hand, the size of the 100 kDa protein in the CNS (Fig. 8*B*), heart, and gill was unchanged. To determine whether PNGase F can remove sugars from glycoproteins expressed in the CNS membranes of *Aplysia*, we probed the blot with antibodies directed against 5-HT$_{ap1}$, another highly glycosylated protein (Angers et al., 1998). This protein was not deglycosylated either (data not shown), indicating that several glycosylated proteins in the CNS are PNGase F resistant.

We cloned the apNEP cDNA from the CNS in pCDNA3/RSV, and the recombinant plasmid was introduced into mammalian HEK 293 cells, as reported previously (Angers et al., 1998). Plasma membranes were purified and the protein was detected by Western blotting after PNGase F treatment. As seen in Figure

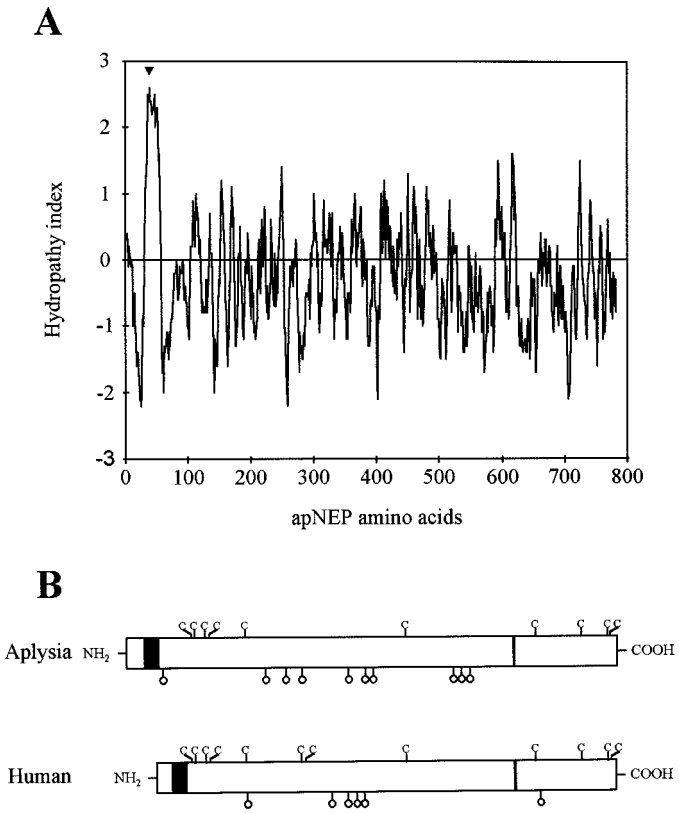

Figure 7. Molecular structure of apNEP. *A*, Hydropathy analysis of apNEP. The 787 amino acid-long apNEP sequence was scanned using the computer program of Kyte and Doolittle (1982). *Numbers* on the horizontal axis refer to the amino acid sequence. Negative values correspond to hydrophilic regions and positive values to hydrophobic regions. The *arrowhead* indicates the only potential membrane-spanning segment of apNEP. *B*, Schematic representation of the primary sequences of the human and *Aplysia* NEP proteins. The cysteine residues in the two proteins are indicated by the one-letter code *C*. The *black rectangle* represents the transmembrane region, and the *thin rectangle* represents the HEXXH gluzincin domain. The position of the possible *N*-glycosylation sites is indicated by *open lollipops*.

8*B*, the results suggest that the enzyme found in *Aplysia* kidneys is likely to be coded by the same gene as the cDNA we isolated from the CNS because they are of the same size.

The application of a NEP-specific inhibitor potentiates the action of endogenous neuropeptides on the buccal ganglion and prevents the *in vitro* degradation of SCP_B by *Aplysia* CNS membranes

In situ hybridization and Western blotting experiments (Fig. 8*A*) showed that apNEP is present in the buccal ganglion of *Aplysia*. To determine whether apNEP could be responsible for the inactivation of neuropeptides *in vivo*, we studied a well understood behavior in invertebrates, which is feeding. In *Aplysia*, feeding consists of a number of different rhythmic motor patterns, including biting, swallowing, and rejection (Kupfermann, 1974; Weiss et al., 1986). Different reports have characterized the critical roles of several neuropeptides, including SCP_B, FMRFamide, egg-laying hormone (ELH), buccalin, and myomodulin, as well as serotonin and acetylcholine, in the modulation of various aspects of the feeding behavior (Kreiner et al., 1987; Lloyd et al., 1987; Sossin et al., 1987; Lloyd, 1988; Miller et al., 1993a,b). Inhibition of apNEP by an apNEP-specific inhibitor should potentiate the action of secreted peptides that are normally substrates for this enzyme. Therefore, to recruit at least some of the peptidergic neurons in the ganglion, we decided to stimulate the radula nerve, because this nerve contains processes of SCP-containing neurons (Miller et al., 1994). Trains of stimuli to the radula nerve were delivered every 10 min (see Materials and Methods for more details); the evoked responses were recorded in A and B neurons and one or two other large cells located near the B cells. After three to four control responses, phosphoramidon (10–100 μM) was added to the bath, and three to four responses were monitored in the presence of the drug; 5–10 more responses were recorded after the inhibitor was washed out. The results of an experiment, in which 10 μM phosphoramidon was added, are shown in Figure 9. In the control period during the stimulation itself, there was in general a burst of action potentials and a burst of PSPs with oscillations of membrane potentials in the monitored neurons (Fig. 9*A*). The later parts of the evoked responses were greatly prolonged in the presence of phosphoramidon. In the example in Figure 9*A*, one can notice that the delayed firing is increased in three of the neurons. These effects were reversible after washout. The summary of five experiments (18 neurons) is shown in Figure 9*B*. These results suggest that the action of several endogenous peptides in the buccal ganglia can be enhanced because of the decrease of their degradation by a NEP-like enzyme present in this ganglion.

Because exogenous applications of SCP_B induce the same physiological responses on these neurons as those obtained after radula nerve stimulation (data not shown), we tested whether SCP_B is a substrate for apNEP *in vitro*. Using RP-HPLC, we showed that SCP_B is cleaved by *Aplysia* CNS membrane extracts and that this cleavage is inhibited by the NEP inhibitor phosphoramidon (10 μM). As seen in Figure 10, the peak corresponding to the uncleaved SCP peptide is clearly preserved in the presence of phosphoramidon. In the absence of the inhibitor, this peak is strongly reduced, and other peaks appeared, probably corresponding to the metabolites resulting from the degradation of SCP_B by a NEP-like enzyme present in the membrane protein extract.

DISCUSSION

Endopeptidase activity in the CNS

Previous studies showed that an endopeptidase with catalytic properties similar to those of neutral endopeptidase-24.11 is present in the kidney of *A. californica* (Bawab et al., 1993). In this paper, we demonstrate that this activity also exists in the CNS of this mollusk. The HPLC profiles of [Leu]enkephalin degradation, the sensitivity of this activity to specific NEP inhibitors, and the binding of [^{125}I]RB104 to the protein all strongly suggest that the CNS and kidney endopeptidases are similar. However, the CNS endopeptidase migrates as a 100 kDa protein band on a Western blot. Although consistent with the size of the mammalian (Kenny et al., 1987; Fournié-Zaluski et al., 1992) and the mollusk *Mytilus edulis* (Shipp et al., 1990) NEPs, the CNS protease is much smaller than the one found in the *Aplysia* kidney (Bawab et al., 1993). Our results demonstrate that not only is the pattern of glycosylation of apNEP different in the kidney and CNS, as observed in mammals (Roques et al., 1993), but the nature of the sugars that are added to the glycoproteins is likely to be different in these tissues. This could be attributable to the presence of a fucose residue on the first *N*-acetylglucosamine of the oligosaccharide chain in the CNS; this addition is known to inhibit the cleavage of sugar chains by PNGase F, and fucose residues have been reported in different glycoproteins isolated from the CNS of *Aplysia* (Thompson et al., 1976; Ambron et al., 1985; Goldberg

Table 3. Comparison of the essential amino acids of thermolysin (TLN), mammalian neutral endopeptidase (mamNEP), and *Aplysia californica* neutral endopeptidase (apNEP)

Action	TLN[a]	mamNEP	apNEP	References
Coordination of the zinc atom	His 142	His 583[b]	His 622	Colman et al., 1972; Hangauer et al., 1984; Devault et al., 1988; Le Moual et al., 1993
	His 146	His 587[b]	His 626	Colman et al., 1972; Hangauer et al., 1984; Devault et al., 1988; Le Moual et al., 1993
	Glu 166	Glu 646[b]	Glu 684	Colman et al., 1972; Le Moual et al., 1991, 1993
Catalysis	Glu 143	Glu 584[b]	Glu 623	Colman et al., 1972; Weaver et al., 1977; Devault et al., 1988
Substrate binding		Arg 102[b]	Ala 152	Bateman et al., 1989; Beaumont et al., 1991; Kim et al., 1992
	Arg 203	Arg 717[b]	Arg 755	Colman et al., 1972; Holmes and Matthews, 1982; Marie-Claire et al., 1997
	Asn 112	Asn 542[b]	Asn 581	Roderick et al., 1989; Dion et al., 1995
	Ala 113	Ala 543	Ala 582	Weaver et al., 1977
Stabilization of the transition state	His 231	His 711[b]	His 749	Colman et al., 1972; Dion et al., 1993
	Asp 170	Asp 650[b]	Asp 688	Colman et al., 1972; Christianson and Alexander, 1990; Le Moual et al., 1994

[a]Determined by crystallographic studies.

[b]Determined by site-directed mutagenesis experiments.

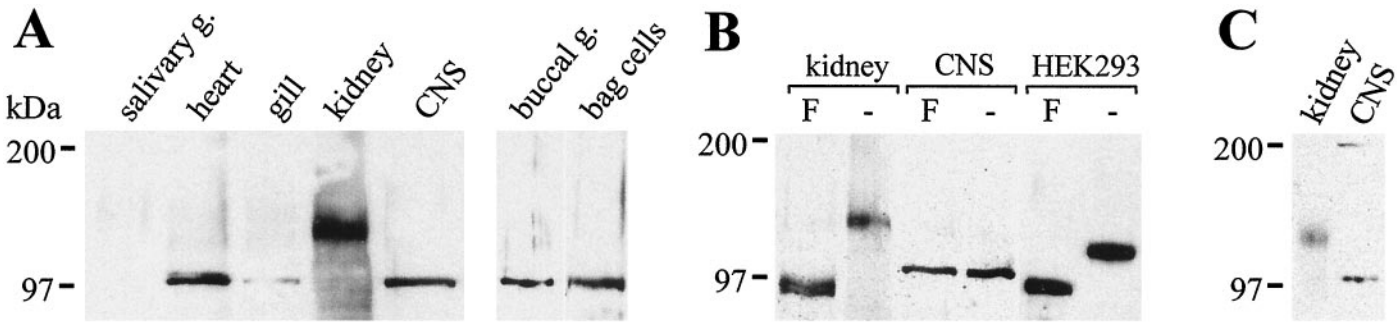

Figure 8. Immunoblot analysis of the expression and glycosylation of apNEP in different *A. californica* tissues. *A*, Twenty micrograms of solubilized membrane proteins (salivary gland, heart, gill, kidney, and CNS) and 30 μg of total protein extracts (buccal ganglion and bag cells) were separated on a 6% SDS-polyacrylamide gel under reducing conditions, blotted, and detected with an anti-apNEP antisera. *B*, Plasma membrane protein extracts isolated from *Aplysia* tissues (kidney, CNS) or from transiently transfected mammalian HEK293 cells (*HEK293*) were incubated in the absence (−) or presence (*F*) of PNGase F, before loading on the gel. *C*, SDS-PAGE under nonreducing conditions. The *arrow* indicates the position of the 200 kDa band.

and Ambron, 1986; Cleary and Schwartz, 1987). The meaning of this differential glycosylation is unknown because both proteins seem to exhibit similar [Leu]enkephalin-degradation activities, affinities for RB104 (Table 2), and responses to different NEP inhibitors (Table 1).

More significantly, our results with [^{125}I]RB104 and the anti-apNEP antibodies clearly link the RB104-binding protein in the plasma membranes of both the kidney and CNS to the product of the *apNEP* gene. We do not yet know whether the enkephalin-degrading activity in these membranes is generated by apNEP, although the binding of [^{125}I]RB104 to a single protein in both the CNS and kidney is a strong indication for the expression of a single *NEP-like* gene in these tissues.

Structure/function of apNEP

As described previously for the kidney endopeptidase, the activity of the CNS enzyme is low and does not allow us to fully characterize it. The molecular cloning of the apNEP cDNA represents a first step toward achieving this goal. The predicted molecular size, topological localization in the membranes, and peptidic sequence of the protein places apNEP in the large family of NEP-like enzymes (Turner and Tanzawa, 1997). Indeed, a phylogenetic analysis localizes apNEP at the branching point of mammalian NEP-like enzymes, suggesting that *apNEP* may be considered as the ancestor of these genes (Fig. 11). After the separation of vertebrates from invertebrates, it is likely that the ancestor *NEP* gene duplicated and diverged to generate peptidases involved in several physiological processes (NEP) (Roques et al., 1993), in bone and tooth mineralization processes (PHEX) (The HYP Consortium, 1995; Ruchon et al., 1998), in the control of blood pressure (ECEs) (Turner and Murphy, 1996; Webb et al., 1997), or in a still uncharacterized function in erythrocytes (KELL) (Lee et al., 1991). Consistent with this hypothesis, most of the residues that have been shown to be essential for the activity and/or conformation of human NEP are conserved at the same position in apNEP (Table 3). Such high conservation in the nature and position of all these residues is very significant when we consider that the two proteins originated from organisms whose ancestors diverged 600 million years ago, and it suggests that these residues were subjected to severe evolutionary constraints to keep the proper folding of its active site. In particular, the 10 cysteine residues in the ectodomain of apNEP and PHEX, which contribute to the stabilization of the active enzyme's conformation (Tam et al., 1985), not only align perfectly in each protein but are also conserved in NEP, ECEs, and KELL. This again suggests that the structure of apNEP is close to that of the ancestor protein and that some of the mammalian NEP-like enzymes may have evolved by acquiring extra pairs of cysteine residues. Interestingly, the cluster of four cysteines (C-X_4-C-X_{17}-C-X_7-C), which is located a few amino acids downstream from the transmembrane domain in all members of the NEP-like

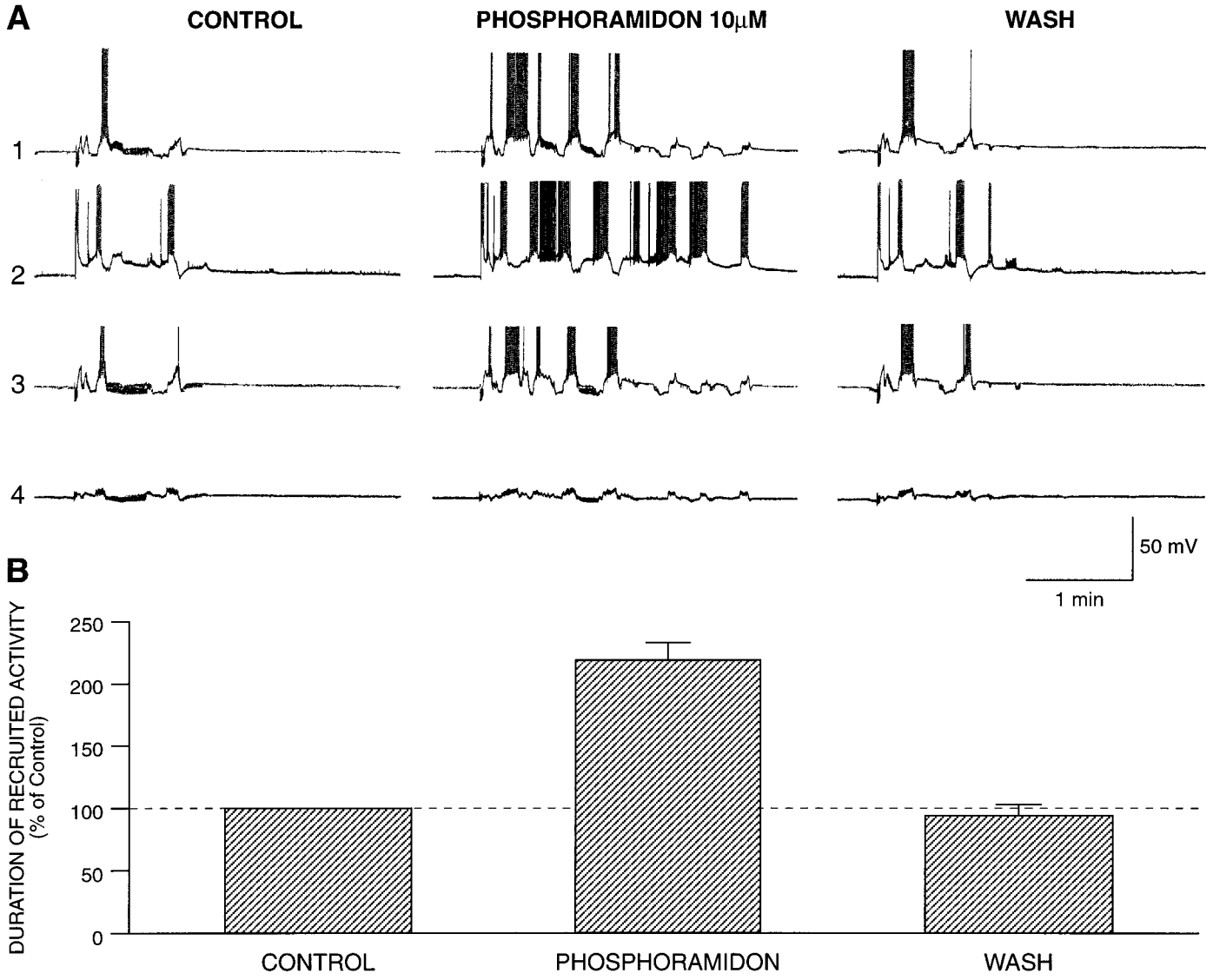

Figure 9. Phosphoramidon prolongs the responses of the buccal neurons to radula nerve stimulation. *A*, Simultaneous recordings from four neurons before, during, and after exposure to phosphoramidon (10 μM). In all cases the activity evoked is prolonged: trace *1*, B neuron; trace *2*, A neuron; trace *3*, B neuron; trace *4*, unidentified cell (see Results for details). *B*, Summary of five experiments (18 neurons) with phosphoramidon (10 or 100 μM). Prolongation of the responses evoked by radula nerve stimulation was observed in all the monitored neurons. The duration of the recruited activity was normalized to each respective control. The percentage average of every neuron in one experiment contributed to the average score of that experiment.

family, is separated from the transmembrane domain by a spacer of 50 amino acids in apNEP. This spacer contains many serine and threonine residues, suggesting that it may allow O-glycosylation of the protein and/or a better exposure of the active site at the cell surface. Alternatively, it may promote the cleavage of apNEP by a specific protease. Such a feature, which would either modulate the activity of apNEP at the membrane or liberate the protein into the extracellular fluid, has been described for human NEP (hNEP) (Almenoff et al., 1984; Johnson et al., 1985; Deschodt-Lanckman et al., 1989; Soleilhac et al., 1996). This may be particularly useful in *Aplysia*, which has an open circulatory system with arteries leading directly to open tissue spaces (Kandel, 1979). The presence of soluble peptidases in the hemocel may be a more efficient way to degrade peptides, a possibility supported by the description of metallopeptidase activities in *Aplysia* hemolymph (Squire et al., 1991; Bawab et al., 1992; Owens et al., 1992; Rothman et al., 1992).

Arg^{102} is the only functional residue that is not shared by apNEP and mammalian NEP. It is known to play a role in substrate binding and to interact with the free carboxy group of the P′2 residue of some substrates (e.g., enkephalins), allowing a dipeptidyl-carboxypeptidase-like activity (Beaumont et al., 1991). The absence of this arginine in the active site of apNEP could explain the weak enzymatic activity of apNEP toward enkephalins. In addition, we demonstrated previously that the enkephalin-degrading enzyme in kidney plasma membranes is a real endopeptidase because it degrades [Leu]enkephalinamide, a peptide that is protected from degradation by carboxypeptidases (Bawab et al., 1993).

Physiological role of apNEP

As observed in mammals (Roques et al., 1993), apNEP is found in many tissues, suggesting that it could be involved in the regulation of different peptidergic pathways. Indeed, neuropep-

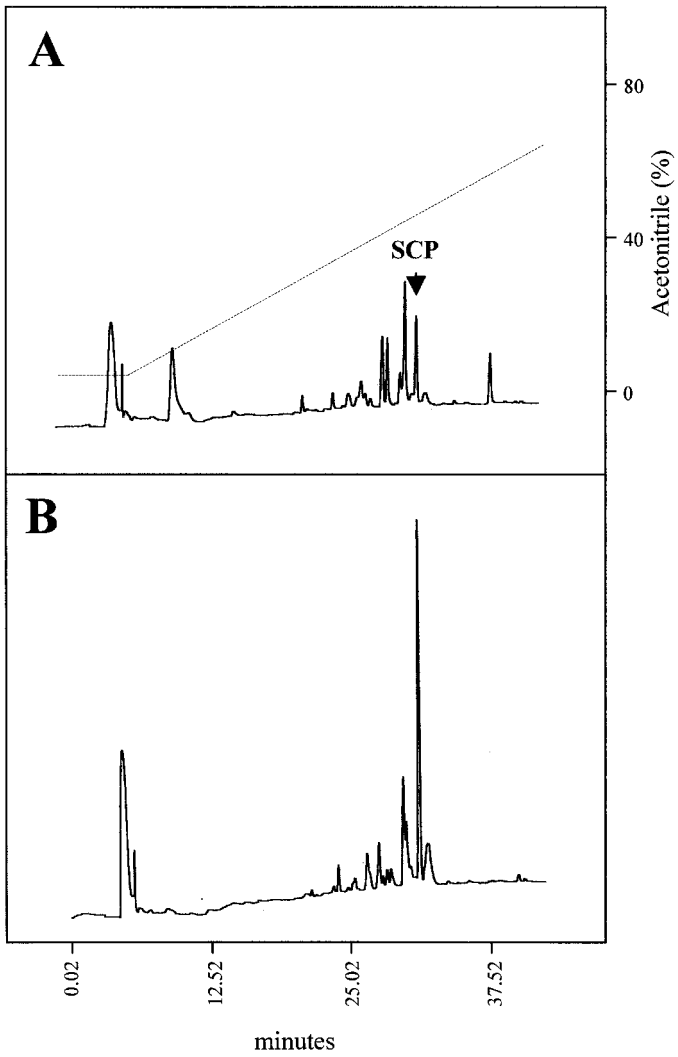

Figure 10. SCP_B is degraded by an *Aplysia* CNS NEP-like enzyme. SCP_B was incubated with CNS plasma membranes, in the absence (*A*) or presence of 10 μM phosphoramidon (*B*). The *arrow* indicates the elution position of the uncleaved SCP_B. The *dashed line* represents the acetonitrile gradient used in the HPLC.

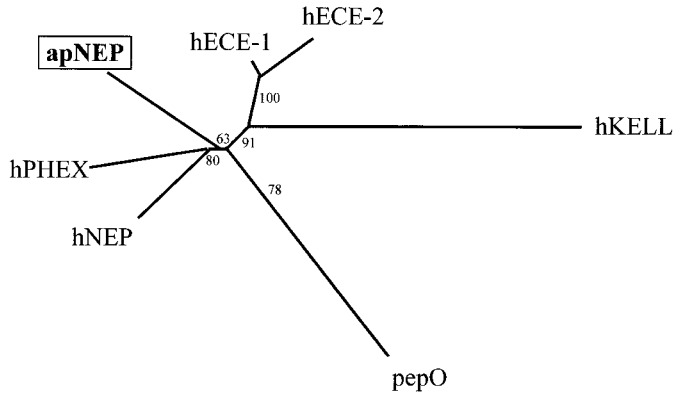

Figure 11. Phylogenetic analysis of the members of the NEP-like family. Sequences were aligned using the Clustal V program (Thompson et al., 1994). The phylogenetic tree was constructed using the Neighbor Joining method (Saitou and Nei, 1987) with a bootstrap analysis that calculates the probability of occurrence of the presented branching for 100 possible trees (Felsenstein, 1993). *hNEP*, Human neutral endopeptidase (accession number M26605); *hECE-1*, human endothelin-converting enzyme 1 (accession number Z35307); *hECE-2*, human endothelin-converting enzyme 2 (accession number AB011179); *apNEP*, *A. californica* neutral endopeptidase (accession number AF104361); *hPHEX*, human phosphate-regulating gene with homologies to endopeptidases on the X-chromosome (accession number Y10196); *hKELL*, human kell blood group protein (accession number M64934); *pepO*, lactococcus lactis *PepO* gene (accession number L04938). Sequences were aligned, and only the peptide regions that could be aligned with the PepO sequence were retained for the analysis; this roughly corresponds to the extracellular parts of the human and mollusk enzymes.

tides are ubiquitously present in *Aplysia*, and many of them are potential substrates for apNEP. Localization of apNEP by *in situ* hybridization and/or immunohistochemistry can provide important clues concerning its physiological roles and may guide the search for its physiologically relevant substrates. Colocalization of apNEP and specific peptides, and potentialization of the action of the peptides by specific enzyme inhibitors *in vivo*, are the two most important criteria to establish the physiological relevance of a peptidase in the regulation of a peptidergic pathway.

Our results suggest that apNEP-like peptidases in the buccal ganglion may be involved in the regulation of the feeding behavior. apNEP is expressed in this ganglion, and NEP-inhibitors potentiate the action of the peptides, most likely by controlling their rate of degradation. In this pathway, SCPs, myomodulin, and buccalin are potential substrates (Kreiner et al., 1987; Lloyd et al., 1987; Sossin et al., 1987; Miller et al., 1992). We have shown that one of these peptides, SCP_B, is effectively degraded by a CNS NEP-like enzyme. Similarly, in the abdominal ganglion, α-bag cell peptide (α-BCP) (Owens et al., 1992), which is a neuropeptide that mediates the bag cell-induced inhibition of left upper quadrant cells (LUQ) and acts together with ELH to coordinate long- and short-lasting events in the egg-laying program (Rothman et al., 1985), was reported to be rapidly degraded by endogenous peptidases when applied to the abdominal ganglion in the absence of peptidase inhibitors (Rothman et al., 1985). Analysis of the metabolites revealed that among other peptidases, a NEP-like activity is involved in α-BCP degradation. The expression of apNEP by the LUQ cells (data not shown) and the presence of apNEP in the bag cell extracts (Fig. 7*A*) is consistent with the possibility that it could be involved in this α-BCP-degrading activity.

As observed in mammals (Barnes et al., 1988; Roques et al., 1993), the *apNEP* gene is expressed in neurons. This suggests that the protein may be present in proximity to peptide receptors where it can play a major role in the modulation of synaptic transmission by controlling the metabolism of neuropeptides close to their site of action. The presence of *apNEP* mRNA in neurites that come from the ganglia via peripheral nerves suggests that a finer regulation in the level of apNEP may be exerted by local translation of the transcript in neurites. Transport and local translation of mRNAs is now well documented (Wilhelm and Vale, 1993; Steward, 1997), although the significance of this phenomenon is not completely understood. There is building evidence that local translation of mRNA in neurites serves to locally modulate the action of the translated product in response to changing physiological conditions (Van Minnen, 1994; Martin et al., 1997).

The *Aplysia* nervous system uses a wide variety of neuropeptides to modulate its behavior and physiological functions, and several peptidases are responsible for the regulation of the actions of these peptides. A global understanding of the function of any

neuropeptide requires knowledge of its synthesis, release, target tissues, and regulation. The present study provides insight into the nature and distribution of the *Aplysia* neuropeptidase apNEP and provides the necessary tools to further investigate the role that the extracellular regulation of neuropeptides plays in behavior.

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