Primary Structure of a New Neuropeptide, Cerebral Peptide 2, Purified from Cerebral Ganglia of Aplysia †

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Primary Structure of a New Neuropeptide, Cerebral Peptide 2, Purified from Cerebral Ganglia of *Aplysia*†

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ABSTRACT: We report the purification and characterization of a novel neuropeptide from *Aplysia* nervous tissue. The peptide was termed cerebral peptide 2 (CP2) because it was the larger of two peptides predominantly synthesized in the cerebral ganglia and transported to other regions of the central nervous system. The purification of CP2 from extracts of cerebral ganglia using three sequential modes of high-pressure liquid chromatography (HPLC) was followed using the [^{35}S]methionine-labeled peptide obtained from transport experiments. The primary structure of CP2 was determined by automated Edman degradation of native CP2 and its proteolytic fragments in conjunction with mass spectrometry. CP2 is a 41 amino acid peptide with an amidated carboxyl terminal. A peptide with the proposed sequence of CP2 was synthesized and compared by HPLC with the native peptide. Chromatographic properties of the synthetic and native peptide labeled *in vivo* were found to be identical. CP2 does not appear to be a member of any previously identified peptide family.

We are interested in peptidergic neurotransmission in the marine snail. The central nervous system (CNS)[1] of *Aplysia* has several advantages for biochemical and electrophysiological studies. It consists of four paired (cerebral, buccal, pleural, and pedal ganglia) and one unpaired ganglion (abdominal ganglion). Each ganglion contains only several thousand neurons, and the neurons are relatively large (Kandel, 1979). The *Aplysia* CNS has been used extensively to study the structure, function, and processing of neuropeptides (Kaldany et al., 1985; Sossin et al., 1989).

Neuropeptides are an important class of neurotransmitters and hormones (Krieger, 1983). The sequences of a number of neuropeptides from *Aplysia* have been determined (Scheller et al., 1984). These peptides were generally chosen for study because of their biological activity or because they were expressed either by very large individual neurons or in homogeneous clusters of neurons. Such peptides are amenable to sequencing either through molecular genetic approaches or through conventional protein sequencing procedures (Kaldany et al., 1985; O'Shea & Schaffer, 1985; Shyamala & Scheller, 1987). Different approaches to peptide identification may reveal physiologically important neuropeptides that do not fall into three categories. One such approach is to identify neuropeptides that are synthesized preferentially in a particular ganglion. This approach has been used to identify and sequence pedal peptide (Pep) which is localized predominantly to neurons in the pedal ganglia (Lloyd & Connolly, 1989; Pearson & Lloyd, 1989). Another approach is to identify peptides that are synthesized in one ganglion and selectively transported to other central ganglia by fast axonal transport. Using this procedure, several uncharacterized peptides have been found to be synthesized in the cerebral ganglion and transported to other central ganglia, predominantly the pedal and abdominal ganglia (Lloyd, 1988, 1989).

We have taken advantage of this interganglionic peptide transport to generate radiolabeled peptide probes that have been used to follow the purification of neuropeptides from the extracts of cerebral ganglia. We have reported the sequence of one of these peptides, designated cerebral peptide 1 (CP1; Phares & Lloyd, 1994), an unamidated, 13 residue peptide. In this paper, we report the purification and sequence analysis of a second novel peptide from *Aplysia* cerebral ganglia termed cerebral peptide 2[2] (CP2). The primary structure of CP2 has been determined by automated Edman degradation of native CP2 and its proteolytic fragments in conjunction with mass spectrometry. CP2 has no sequence homology to any previously characterized peptide or to CP1.

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[1] Abbreviations: Asn-C, jack bean asparaginyl endopeptidase; ASW, artificial seawater; CNS, central nervous system; CP1, cerebral peptide 1; CP2, cerebral peptide 2 (fragments are followed by the inclusive residue numbers in brackets); CX-HPLC, cation-exchange high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; ESI-MS, electrospray ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; HFBA, heptafluorobutyric acid; LC/MS, liquid chromatography/mass spectrometry; Lys-C, *Achromobacter* endoprotease I; MALDI-TOF-MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; 2-ME, 2-mercaptoethanol; M_r, relative mass; RP-HPLC, reverse-phase high-pressure liquid chromatography; SCP_B, small cardioactive peptide B, ARPGYLAFRMamide; TEA Ac, triethylamine acetate; TFA, trifluoroacetic acid; TPCK, L-(tosylamino)-2-phenylethyl chloromethyl ketone; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

[2] The sequence of CP2 has been deposited in the Protein Identification Resource databank and given Accession Number A57272.

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EXPERIMENTAL PROCEDURES

Animals. *Aplysia californica* (50−500 g) were obtained from Marinus, Inc. (Long Beach, CA), and maintained in circulating artificial seawater (ASW) tanks at 16 °C. Prior to dissection, animals were immobilized by an injection of isotonic $MgCl_2$ equal to 50% of their body weight.

Transport. Transport experiments were carried out as previously described (Lloyd, 1989). Briefly, cerebral, pedal-pleural, and abdominal ganglia were removed with their connective nerves intact, and transferred to a transport chamber. The cerebral ganglia were isolated from the other ganglia by running the intact cerebral connectives through a polypropylene and petroleum jelly diffusion barrier. The cerebral ganglia were incubated for 24 h with 0.5 mCi of [^{35}S]methionine (>1000 Ci/mmol; Amersham) in 0.5 mL of 50% ASW (460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, and 5 mM $NaHCO_3$, pH 7.8), 50% hemolymph, 0.01% glucose, 25 units/mL penicillin, 25 μg/mL streptomycin, and 0.01% 2-mercaptoethanol (2-ME, Sigma). The remaining ganglia were incubated in 150 mL of ASW with 10% L15 culture medium (Sigma) modified to have the same salt composition as ASW, 0.01% glucose, 25 units/mL penicillin, 25 μg/mL streptomycin, and 0.01% 2-ME. The labeling period was followed by a 24 h chase with 1 mM unlabeled methionine in the ASW, L15 medium. The abdominal ganglion was extracted in 0.5 mL of 20 mM trifluoroacetic acid (TFA, Pierce) and 28 mM 2-ME, heated to 100 °C for 10 min, homogenized, and centrifuged at 10000*g* for 10 min. The supernatant was filtered (0.45 μm, LC13, Gelman, or 0.2 μm, Microspin) and run on reverse-phase (RP)-HPLC on a Pierce Aquapore RP-300 C8 column (4.6 × 220 mm).

Peptide Extraction and Purification of CP2. The cerebral ganglia used for the purification of CP2 were dissected from animals used for physiology experiments. Individual ganglia were extracted in 500 μL containing 20 mM TFA and 28 mM 2-ME, heated to 100 °C for 10 min, homogenized, and centrifuged at 10000*g* for 10 min. The supernatants from cerebral ganglia extracts were pooled and applied to a C18 cartridge (Sep-Pak, Waters Associates). The peptides were eluted with 3 mL of 75% acetonitrile (CH_3CN, Baker, VWR Scientific), 10 mM TFA. The eluate was lyophilized to 50 μL and diluted with 600 μL of 10 mM triethylamine acetate (TEA Ac, Pierce) with 20% CH_3CN and subjected to cation-exchange (CX) HPLC on a Pierce Aquapore CX-300 column (4.6 × 100 mm) using a gradient from 10 mM TEA Ac to 300 mM TEA Ac in 15 min at a flow rate of 2 mL/min. All CX-HPLC solutions contained 10% CH_3CN and were adjusted to pH 6. Each sample contained the equivalent of 25−35 ganglia and 10 nmol of Tyr^8−Substance P (American Peptides) as a standard. [^{35}S]Methionine-labeled CP2 purified from transport experiments was added to the sample as a probe. Fractions were collected, and small aliquots were counted in a liquid scintillation counter. 2-ME was added (50 mM final concentration) to the remainder of the fractions to minimize oxidation of methionine residues. CX-HPLC fractions containing the radioactive CP2 were injected onto the RP-HPLC with an Aquapore RP-300 C8 column (4.6 × 220 mm), and the peptide was eluted using a gradient from 18% CH_3CN to 30% CH_3CN in 5 min, to 38% CH_3CN in 40 min, and to 50% CH_3CN in 5 min with 10 mM HFBA as a counterion. Fractions containing the radiolabeled CP2 probe were pooled and partially dried before running them on a second step of RP-HPLC. The final purification step used 10 mM TFA as the counterion and a gradient from 15.5% CH_3CN to 23.5% CH_3CN in 32 min, and to 65% CH_3CN in 9 min.

Sequencing and Mass Spectrometry. Automated Edman degradation was carried out on Applied Biosystems Models 475A and 477A automated protein sequencer at the University of Wisconsin Biotechnology Center, or on an Applied Biosystems Model 470A automated protein sequencer at the Department of Biochemistry, University of Washington, using sequencing protocols specified by and reagents from the instrument manufacturer. Data analyses were conducted by direct inspection of on-line analog chart recording to 5 pmol full scale with 1 pmol of PTH-amino acid standards. Amino acid composition analysis was done at the Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI. Fast atom bombardment mass spectrometry (FAB-MS) was conducted at the Massachusetts Institute of Technology Mass Spectrometry Facility, Cambridge, MA.

Electrospray Ionization Mass Spectrometry (ESI-MS). Electrospray mass spectra were acquired on a Sciex API III triple quadrupole spectrometer fitted with a nebulization-assisted electrospray source (PE/Sciex, Thornhill, Ontario). Native CP2 was dissolved in 0.1% formic acid in 1:1 methanol/H_2O and infused at 5 μL/min through a 50 μm i.d. fused silica transfer line using a Harvard apparatus Model 11 syringe pump. Masses were scanned repetitively with a mass step of 0.1 Da and 1 ms/step. Resolution was adjusted to a 20% valley between adjacent isotope peaks in a singly charged cluster.

Liquid Chromatography/Mass Spectrometry (LC/MS). The masses of peptides from proteolytic digests were determined in a LC/MS mode either using a splitter for larger columns and larger flow rates or directly using capillary HPLC columns. Alternatively, fractions from the HPLC were infused directly using the Harvard pump. Capillary columns were 548 μm i.d. × 200 mm long with C18 packing and constructed following the procedure of Davis and Lee (1992).

Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS). MALDI-TOF mass spectra were acquired on a PerSeptive Voyager Elite mass spectrometer utilizing a reflectron to record post-source decay mass spectra (PerSeptive, Framingham, MA). The CP2[38−41]tetrapeptide from the Lys-C digest of CP2 (see below) was examined by MALDI-TOF-MS in an α-cyano-4-hydroxycinnamic acid matrix.

Digestion of CP2 by Achromobacter Endoprotease I (Lys-C). Two different Lys-C digests were conducted. For the first Lys-C digest, 100 pmol of purified CP2 was dissolved in 50 mM Tris-HCl (pH 9) and digested in 3 M urea/50 mM ammonium bicarbonate (NH_4HCO_3, pH 8−9) at 37 °C for 6 h with enzyme provided by Dr. T. Masaki, Ibaragi University, Japan. The digest was analyzed by LC/MS with 90/10 split stream for collection of fractions. For the second Lys-C digest, purified CP2 (250 pmol) was dissolved in 20 μL of 4 M urea/50 mM Tris-HCl buffer (pH 8) and heated to 30 °C for 30 min. Lys-C (approximately 1:250 molar ratio, Wako) was added in 5 μL of 50 mM Tris buffer and incubated at 36 °C for 8 h. The reaction was stopped by transferring the reaction tube to an ice bath. The resulting peptide fragments were purified by RP-HPLC using a Pierce

Aquapore-300 C18 column (2.1 × 220 mm) at a flow rate of 100 μL/min (counterion: 10 mM TFA) run at 0% CH_3CN for 15 min, then to 70% CH_3CN in 70 min, and remained at 70% CH_3CN for another 10 min. Absorbance peaks were collected manually.

Digestion of CP2 by Trypsin. A CP2 cleavage product from the first Lys-C digest with a relative mass (M_r) of 4039 Da was dried and digested with TPCK-trypsin (Worthington) in 3 M urea/50 mM NH_4HCO_3 (pH 8−9). Peptides were purified on an Applied Biosystems C18 column (2.1 × 30 mm) in an LC/MS mode with a 90/10 split stream for collection of fractions. Fractions containing peptide were sequenced by automated Edman degradation.

Digestion of CP2 by Endopeptidase Asn-C. CP2 was digested with asparaginyl endopeptidase (Takara, Japan), a proteolytic enzyme specific for the carboxyl terminus of asparaginyl residues (Abe et al., 1993; Kanda et al., 1995). The peptide was digested in sodium acetate buffer (pH 5) containing EDTA and dithiothreitol. Peptides were purified on an Applied Biosystems C18 column (2.1 × 30 mm) in the LC/MS mode with a 90/10 split stream for the collection of fractions.

Preparation of [^{3}H]Histidine-Labeled CP2 Fragments. A pair of cerebral ganglia were incubated for 20 h with [^{3}H]-histidine (0.2 mCi dried to 20 μL; 40 Ci/mmol; Amersham) added to 0.4 mL of ASW containing glucose and antibiotics (as described above) and 1 mM colchicine. This was followed by a 4 h chase period in the same medium with 1 mM cold histidine (Sigma) substituted for the radiolabel. Preliminary studies indicated that neurons in the ventral B cluster and in the H cluster synthesize CP2 (Phares and Lloyd, unpublished results), so neurons from these clusters were removed, extracted, filtered (0.2 μm, Microspin), and run on two modes of RP-HPLC using a Pierce Aquapore RP-300 C8 column (4.6 × 220 mm) to purify [^{3}H]histidine-labeled CP2 (^{3}H-His CP2). The first gradient (counterion: 10 mM HFBA) was from 20% CH_3CN to 40% CH_3CN in 20 min, and then to 60% CH_3CN in 3 min. The predominant radiolabeled peak was then run on the final gradient used for the purification of CP2 (see above). Small cardioactive peptide B (10 nmol of SCP_B; American Peptides) was added as a positive control (Lloyd et al., 1987), the sample was dried to 20 μL, and then, after the addition of 20 μL of 0.2 M NH_4HCO_3, peptide digestion was initiated by the addition of 5 μL of 40 μM TPCK-trypsin (Worthington) in 0.1 M HCl (1:50 molar ratio of trypsin to SCP_B) for 7 h at 37 °C. Half of the digest was run on the HFBA gradient described above. After the addition of 50 nmol each of unlabeled histidine and histidine amide (Sigma), the second half of the sample was run on a Rainin C18 column (4.6 × 220 mm) with 10 mM HFBA as a counterion with a flow rate of 1 mL/min. The gradient was held isocratic at 2% CH_3CN for 12 min, and then increased to 9.5% CH_3CN in 26 min.

Chromatographic Comparison of the Native and Synthetic Peptides. A synthetic peptide with the sequence of CP2 was prepared by a commerical source (AnaSpec Inc., San Jose, CA). Synthetic CP2 (5 nmol) was combined with [^{35}S]-methionine-labeled CP2 and run on the final TFA gradient used for the original purification. Fractions were collected, and aliquots of each were counted. The fractions containing the coeluting absorbance and radiolabeled peaks were pooled and combined with an additional 5 nmol of synthetic CP2, and the sample was split and dried. Both samples were dissolved in 45 μL of 4 M urea/50 mM Tris buffer (pH 9). Lys-C (12 pmol in 5 μL of buffer; final molar ratio was 1:400) was added to one sample while 5 μL of buffer without the protease was added to the other sample. Both samples were incubated for 7 h at 35 °C and analyzed by RP-HPLC (counterioin: 10 mM HFBA) using the gradient described above (see Preparation of [^{3}H]Histidine-Labeled CP2 Fragments).

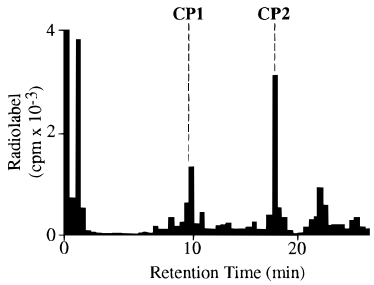

FIGURE 1: RP-HPLC profile (counterion: TFA) of peptides labeled in the cerebral ganglion and transported to the abdominal ganglion. Profile is from an extract of one abdominal ganglion. Labeled peaks with short retention times (<2 min) include unincorporated [^{35}S]-methionine, [^{35}S]cysteine, and [^{35}S]glutathione (Lloyd, 1988). Label in peaks marked "CP1" and "CP2" is associated primarily with a single peptide in each peak. Radiolabeled CP2 was used to follow the purification of this peptide.

Database Searches. The CP2 sequence was searched using the FASTA protocol (Pearson & Lipman, 1988) against the SwissProt database (Bairoch & Böckmann, 1991) compiled by the European Molecular Biology Laboratory and the Protein Identification Resource database (PIR; Barker et al., 1991) compiled by the National Biomedical Research Foundation.

RESULTS

Transport experiments in the *Aplysia* CNS have revealed the presence of several unidentified [^{35}S]methionine-labeled peptides (Lloyd, 1988, 1989). These transported peptides may be previously unknown peptide transmitters. We have begun to purify some of these neuropeptides using the *in vivo* labeled peptides from the transport experiments as probes to monitor the purification of the peptides from unlabeled ganglia. Figure 1 shows an example of RP-HPLC of an abdominal ganglion extract after labeling the cerebral ganglia with [^{35}S]methionine and allowing time for synthesis, processing, and fast axonal transport of radiolabeled peptides along the nerves connecting the ganglia. Two of the radiolabeled peaks were targeted for purification: CP1 and CP2. We have previously reported the purification and sequence analysis of CP1 (Phares & Lloyd, 1994). In this paper, we concentrated on CP2.

Purification of CP2. CP2 was purified from extracts of approximately 1500 cerebral ganglia in batches of 300−600 ganglia. Each purification consisted of three sequential modes of HPLC: CX-HPLC, RP-HPLC with HFBA as the counterion, followed by RP-HPLC with TFA as the counterion. A portion of the absorbance trace and the corresponding histogram of radiolabel in fractions collected during the final RP-HPLC step from one of the purifications are shown in Figure 2. The major absorbance peak corresponds to the radiolabeled peak of CP2. Approximately 3−4 pmol of CP2 was obtained per ganglion, making a total of approximately 4 nmol available for structural analysis.

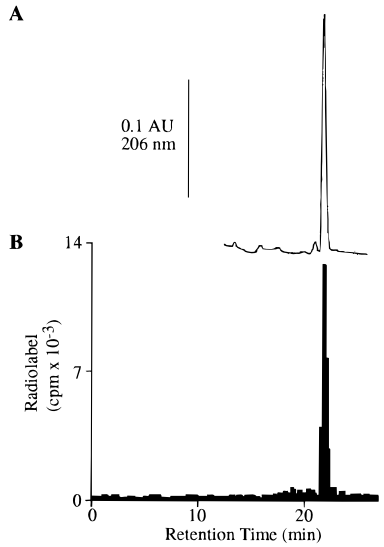

FIGURE 2: (A) Portion of the absorbance profile measured at 206 nm of CP2 from the final RP-HPLC step (counterion: TFA) of a purification of the peptide from 600 cerebral ganglia extracts. The absorbance profile suggested that the peptide had been purified to homogeneity which was confirmed by subsequent microsequencing and MS analyses. A small absorbance peak which elutes just prior to CP2 may be an oxidized form of the peptide which is also found in mass spectra. (B) Purification was monitored using radiolabeled CP2 transported from the cerebral ganglia to the abdominal ganglia (see Figure 1). Histogram represents elution of the radiolabeled CP2 probe measured in aliquots from collected fractions.

Table 1: CP2 Amino Acid Composition Analysis

residue[a]	pmol	calcd[b]	no. of residues estimated	actual[c]
D/N	1178.7	4.2	4	5
T	465.3	1.7	2	2
S	900.7	3.2	3	3
E/Q	959.1	3.4	3	3
P	276.4	1.0	1	1
G	1670.7	6.0	6	5
A	982.1	3.5	4	4
V	73.8	0.3	0	0
M	573.8	2.1	2	3
I	270.5	1.0	1	1
L	748.7	2.7	3	3
Y	419.3	1.5	2	2
F	632.8	2.3	2	3
H	469.8	1.7	2	2
K	289.3	1.0	1	1
R	682.6	2.4	2	2

[a] C and W were not determined. [b] Based on 1 residue = 279 pmol (the average of P, I, and K). [c] Based on sequence data.

Sequence Analysis. The amino acid composition of CP2 is shown in Table 1 while the results of sequence analyses and mass spectrometry of CP2 are summarized in Table 2. Several attempts to sequence intact CP2 by automated Edman degradation yielded only 27 amino acid residues. Amino acid composition analysis (Table 1) and the mass spectrometry data indicated that this sequence was incomplete by 13−15 residues. The calculated mass average of the 27 residues was 2970.2 Da, but the M_r of purified CP2 was 4590.8 (FAB-MS) or 4591.7 ± 0.5 Da (ESI-MS).

Amino acid composition analysis indicated that the sequence of CP2 should include two arginines and a single lysine (Table 1). The 27-residue sequence contained 2 arginines, but no lysine residues (Table 2); thus, the predicted lysine must be present in the region not yet sequenced. In order to obtain more sequence, CP2 (100 pmol) was digested with Lys-C and analyzed by LC/MS. A peptide from the amino terminus with a M_r of 4039.4 Da, CP2[1−37], was observed. Digestion of this peptide with trypsin yielded four fragments, a portion of each of which was sequenced. These analyses yielded the sequences of three trypsin fragments (CP2[1−16], CP2[17−23], CP2[24−37]; Table 2). The sequence of residues 17−23 was confirmed in a separate tryptic peptide, CP2[17−37], in which the bond following Arg−23 was not cleaved. The masses detected were in accord with the sequences.

To obtain the sequence of the remaining carboxyl terminus, CP2 was again digested with Lys-C which produced two fragments, CP2[1−37] and CP2[38−41], that were purified by RP-HPLC. An absorbance peak eluting just after the breakthrough peak yielded the sequence MQRH by Edman degradation. MALDI-TOF-MS analysis of this peak gave an M_r of 569.4 Da, which is 1 Da lighter than the mass predicted from the sequence. Addition of this M_r of CP2-[38−41] to the mass of CP2[1−37], less the mass of H_2O, gives a mass of 4590.8 Da, which agrees well with M_r measured by FAB-MS (4590.8 Da) and ESI-MS (4591.7 ± 0.5 Da), indicating that the sequence of CP2 was complete. Digestion of CP2 with Asn-C produced the carboxyl-terminal fragment CP2[34−41], and its sequence provided an overlap between the two Lys-C fragments and confirmed the carboxyl-terminal sequence (Table 2).

Carboxy-Terminal Amide Determination. Many neuropeptides are amidated at their carboxyl terminus, and this feature is usually important for the biological activity of the molecule (Eipper et al., 1992). Amidation reduces the mass by 1 Da, and the carboxyl-terminal peptide CP2[38−41] was observed to be 1 Da lighter than calculated from the sequence. The calculated average mass of the free acid form of intact CP2 is 4592.2 Da, and that of the amidated form is 4591.2 Da whereas the M_r was in the range 4590.8−4592.2 Da. Hence, mass measurements of CP2 did not provide an unequivocal assessment of whether the peptide was amidated at its carboxyl terminus. We demonstrated amidation of the CP2 carboxyl terminus by two independent techniques.

We took advantage of the carboxyl-terminal Arg-His sequence which can be cleaved by trypsin. Cerebral ganglia were incubated in [^{3}H]histidine, and radiolabeled CP2 was purified from the extracts of identified neuron clusters that appear to contain CP2 based on preliminary immunocytological evidence (Phares and Lloyd, unpublished observations). Tryptic digestion of ^{3}H-His CP2 should yield two labeled products. CP2[1−17] and either labeled histidine or histidine amide from the carboxyl terminus of CP2. We analyzed the digest on two different RP-HPLC gradients. Half of the digest was analyzed using a gradient designed to separate intact CP2 from the larger tryptic fragments. All the intact labeled CP2 had disappeared, indicating digestion was complete. Two radiolabeled products were generated: one in the breakthrough, and one later in the gradient, presumably CP2[1−17] (data not shown). The other half of the digest was analyzed with a RP-HPLC procedure that separates histidine and histidine amide. Only labeled histidine amide was observed, indicating that the carboxyl terminus of CP2 is indeed amidated (Figure 3).

The carboxyl-terminal amidation of CP2[38−41] tetrapeptide was confirmed by accurate mass measurement (M_r 569.4 Da) by MALDI-TOF-MS, which was consistent with the

Table 2: Edman Degradation and Mass Spectrometry of CP2 and Its Digestion Products

Treatment	Fragment	Sequence determined by Edman Degradation	Mass[a] Calc'd	Mass[a] M_r	MS Procedure(s)
None					
	CP2[1-41]	FDFGFAGLDTYDAIHRALEQPARGTSN...		4590.8	FAB-MS
LYS-C					
	CP2[1-37]	FDFGFA[b]...	4039.5	4039.4	ESI-MS, MALDI-TOF
	CP2[38-41]	[c,d]MQRHamide	569.4	569.4	MALDI-TOF
Trypsin[e]					
	CP2[1-16]	FDFGFAGLDTYDAIHR	1843.9	1844.5	ESI-MS
	CP2[17-23]	ALEQPAR	783.9	783.9	ESI-MS
	CP2[17-37]	ALEQPARGTSNSGSGYNMLMK	2212.5	2212.0	ESI-MS
	CP2[24-37]	GTSNSG_-_YNMLMK	1446.6	1447.2	ESI-MS
ASN-C					
	CP2[1-27]	FDFGFA[b]...		N.D.[f]	
	CP2[34-41]	MLMKMQRH		N.D.	
		5 10 15 20 25 30 35 40			
Complete	Sequence:	FDFGFAGLDTYDAIHRALEQPARGTSNSGSGYNMLMKMQRHamide	4591.2	4591.7 ± 0.5	ESI-MS

[a] Masses are reported as calculated average masses or observed relative masses (M_r). [b] Sequence was intentionally terminated. [c] Carboxyl-terminal amidation at His-41 cannot be detected by Edman degradation but was suggested by the mass of this fragment and was confirmed by data in Figures 3 and 4. [d] Sequence was confirmed by postsource decay analysis. [e] Trypsin digest of the 4039 Da Lys-C fragment. [f] Mass not determined.

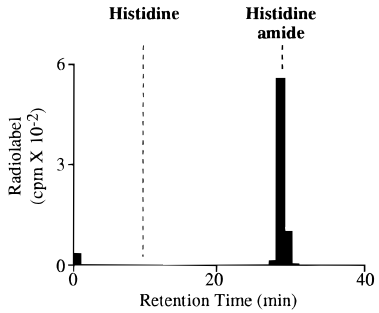

FIGURE 3: [^{3}H]Histidine amide was released by tryptic digestion of [^{3}H]histidine-labeled CP2. Histogram represents the elution of ^{3}H collected in fractions from a gradient that separates histidine and histidine amide. Elution of unlabeled histidine and histidine amide standards is represented by dashed lines. A second histidine residue occurs in the CP2[1−17] tryptic fragment. This fragment did not elute under these conditions, so a portion of the sample was analyzed using a different column and gradient. No intact CP2 remained in the sample, and two radiolabeled peaks were present: CP2[41] in the breakthrough volume and CP2[1−17] which eluted much later (not shown).

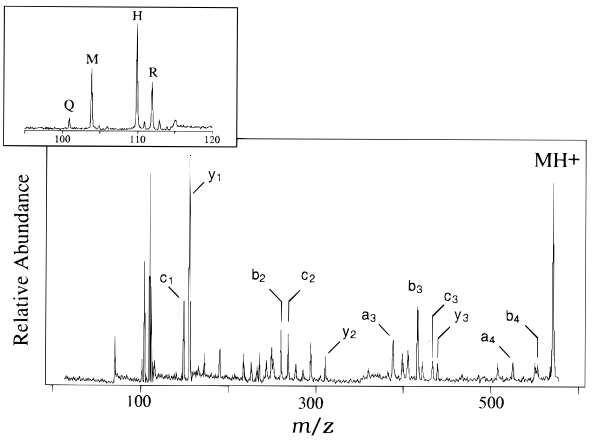

FIGURE 4: Determination of sequence and carboxyl-terminal amide by MALDI-TOF-MS postsource decay of CP2[38−41]. RP-HPLC of a Lys-C digestion of CP2 was used to obtain CP2[38−41]. The molecular ion MH$^+$ 570.4 Da and fragments thereof (produced by postsource decay) were analyzed by MALDI-TOF-MS in the reflectron configuration. Ions b_2 through b_3 and y_2 through y_3 were identified, as well as the immonium ions of Gln, Met, His, and Arg, accounting for the total sequence, MQRH-amide, including the carboxyl-terminal amide.

calculated mass of the amide not the acid form of the peptide. In addition, the sequence of the CP2[38−41] tetrapeptide was confirmed by mass analysis of product ions that were produced from postsource decay of the molecular ion MH$^+$ 570.4 Da (Figure 4 and Table 3). Product ions b_2 through b_4 and y_2 through y_3 were observed, as well as the immonium ions of Gln, Met, His, and Arg, in agreement with the sequence MQRH-amide.

Comparison of in Vivo Labeled CP2 with Synthetic CP2 by RP-HPLC. The sequence of CP2 was further confirmed by comparison of *in vivo* radiolabeled peptide with a commercially prepared synthetic peptide with the sequence shown in Table 2. Native [^{35}S]methionine-labeled CP2 was combined with synthetic CP2 and run on the final RP-HPLC gradient used in the purifications. Comparison of the elution of the synthetic peptide monitored by absorbance with the native radiolabeled peptide monitored by counting small aliquots from the collected fractions demonstrates that the two peptides coelute on this gradient (Figure 5A). The fractions containing the absorbance and radiolabel peaks were pooled and combined with additional synthetic peptide. Half of the sample was digested with Lys-C while the other served as a control. These samples were run on a second gradient using a different counterion, and the native and synthetic peptides continued to coelute (Figure 5B). In addition, both the native and the synthetic CP2 were digested by Lys-C, producing two radiolabeled peaks and two absorbance peaks which corresponded to CP2[1−37] and CP2[38−41]. The native radiolabeled fragments coeluted with the synthetic peptide fragments (Figure 5B). As predicted for the sequence, CP2[1−37] which contains two methionine residues had twice the label as CP2[38−41] which contains a single methionine. These procedures both confirm our sequence analysis and demonstrate that the synthesis was correct.

DISCUSSION

We have used a new approach to identify potential peptide transmitters: selective axonal transport of *in vivo* radiolabeled peptides from one region of the CNS to another. Using this approach, we have identified several previously unknown peptides in the CNS of *Aplysia* (Lloyd, 1988, 1989). The technique also provides probes to follow the purification of these neuropeptides. In this study, we detail the purification and sequence analysis of CP2, a newly identified peptide that is selectively transported from the cerebral ganglia to other ganglia in the *Aplysia* CNS. This approach may be useful to identify other neuropeptides in *Aplysia* as well as in other species.

Table 3: PSD Analysis of CP2[38−41]

M_r measured	M_r calcd	% error	assignment
569.4	569.3	0.02	MQRH-amide
552.4	552.3	0.02	b_4
549.3			ND[a]
524.6	524.3	0.06	a_4
507.5			a_4 - 17
438.6	438.3	0.07	y_3
432.6	432.2	0.09	c_3
421.6			y_3 - 17
415.6	415.2	0.10	b_3
404.6			ND[a]
398.7			b_3 - 17
387.7	387.2	0.13	a_3
310.2	310.2	0.00	y_2
293.2			y_2 - 17
276.3	276.1	0.07	c_2
267.3			ND[a]
259.3	259.1	0.08	b_2
248.4			ND[a]
242.4			b_2 - 17
234.1			ND[a]
216.1			ND[a]
189.0			ND[a]
171.0			ND[a]
153.9	154.1	0.13	y_1
147.9	148.1	0.13	c_1
110.9			R[b]
108.9			H[b]
102.9			M[b]
99.9			Q[b]

[a] Not determined. [b] Denotes $H_2N{=}CHX$.

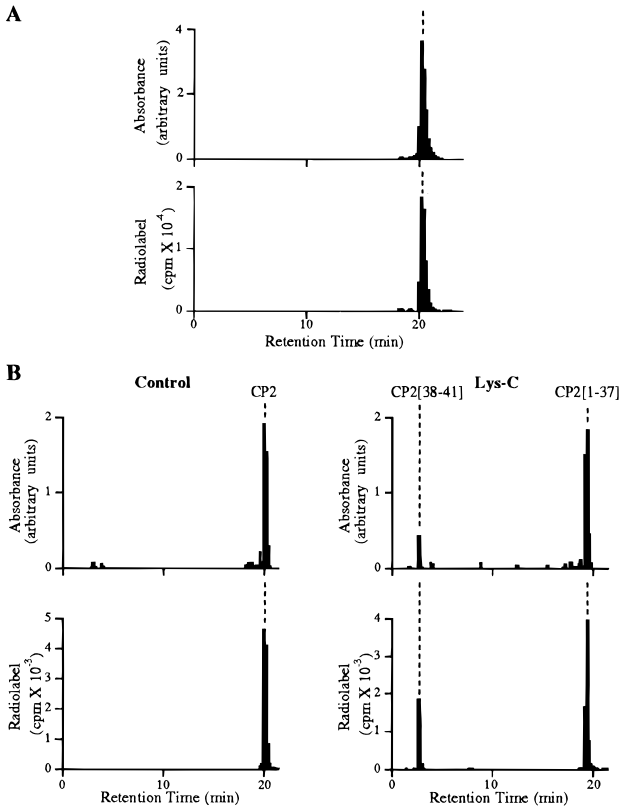

FIGURE 5: Absorbance of synthetic CP2 measured at 206 nm coelutes with CP2 labeled *in vivo* with [^{35}S]methionine. (A) Unlabeled synthetic CP2 was combined with native [^{35}S]methionine-labeled CP2, and the sample was analyzed using the conditions of the final RP-HPLC purification step (see Figure 2; counterion: TFA). The main absorbance and radiolabel peaks coeluted. There were also small peaks of label and absorbance that precede CP2 peak which are likely to be oxidation products. (B) After the addition of more synthetic CP2, the peak from (A) was divided, and half was digested with Lys-C while the other half was incubated in buffer without the protease as a control. The samples were analyzed by RP-HPLC (counterion: HFBA). The sample without the protease (Control) shows that synthetic and radiolabeled CP2 continued to coelute under these conditions and that no other peaks were generated. When the Lys-C-treated sample was analyzed on the same gradient (+Lys-C), no intact CP2 remained, but two peaks were generated from both the synthetic and radiolabeled CP2 which correspond to CP2[38−41] and CP2[1−37]. The fragments of synthetic and radiolabeled CP2 precisely coeluted. The absorbance in the breakthrough volume has been removed from absorbance histograms in both (A) and (B).

Using a combination of protease digestions, automated Edman degradation, and mass spectrometry, we were able to determine the full amino acid sequence of CP2. CP2 is 41 amino acids in length and is amidated at the carboxyl terminal. Searches of PIR and SwissProt protein sequence databases indicate that CP2 is apparently not related to any previously characterized peptide or protein.

Peptide transmitters are processed from larger precursor proteins (Eipper et al., 1986). It is interesting to note that the −RXamide (where X is any amino acid) is a common carboxyl-terminal sequence in invertebrate peptide transmitters (Walker, 1992). On the putative precursor protein, the carboxyl terminal of CP2 would appear as the sequence RHG. This sequence would conform to a consensus sequence, RXG, for precursor processing at a monobasic site if followed by a single R (Benoit et al., 1987; Devi, 1991).

Many neuropeptide precursors include more than one biologically active peptide (Eipper et al., 1986; Sossin et al., 1989), including those in *Aplysia* (Scheller et al., 1984). So, other biologically active peptides may be found on the CP2 precursor protein. The CP2 precursor protein may also encode CP1 which is also transported to the abdominal ganglia (Figure 1), or other peptides that do not contain methionine and thus were not detected in the transport experiments.

Interganglionic Communication. One goal of this study was to purify and characterize a new peptide transmitter candidate that may play important roles in interganglionic communication. Peptides transported from the cerebral ganglia may be particularly important in behavioral regulation since they receive inputs from a number of sensory structures and appear to be involved in the higher-order control of many behaviors (Kandel, 1979), including locomotion (Fredman & Jahan-Parwar, 1983), appetitive and consummatory aspects of feeding (Teyke et al., 1990; Rosen et al., 1991), and reproductive behaviors (Ferguson et al., 1989; Painter et al., 1988). Several neuronal groups within the cerebral ganglia are thought to contain neurons that project to the abdominal ganglia (Hawkins, 1989; Jahan-Parwar & Fredman, 1976; Mackey et al., 1989; McPhearson et al., 1991). Immunocytological studies and analyses of peptides synthesized by single neurons indicate that neurons within some of these groups synthesize CP2 (Phares and Lloyd, unpublished data), but the identity of the neurons that transport CP2 to the abdominal ganglia is still unclear.

Transmitter Role for CP2. CP2 is a potential peptide transmitter. In previous studies, many of the peptides that were transported from one region of the *Aplysia* CNS to another were found to be previously identified and characterized neuropeptides with transmitter-like actions (Lloyd, 1988, 1989). Also, CP2 is amidated as its carboxyl terminal, a posttranslational modification that when present is usually

an important structural feature for biological activity (Eipper et al., 1992). Finally, preliminary physiological studies indicate that synthetic CP2 potently depolarizes identified neurons in the abdominal ganglion of *Aplysia*. The availability of the synthetic peptide will allow further study of the biological role of CP2.

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