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Research Article

A C-terminally elongated form of PHI from porcine intestine

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Abstract. A C-terminally elongated form of peptide histidine isoleucine amide (PHI) was isolated from porcine intestine based on its effect on cAMP production in IMR-32 cells. The structure was determined by amino acid sequence analysis of tryptic fragments and by mass spectrometry. The peptide has 42 amino acid residues like those described from human, rat and mouse, but the amino acid sequence of the C-terminal extension of pig PHI is unique. Unlike the other peptides, it has a C-terminal Ala and it differs at five positions from the human form and at six positions from the rat form, while the human and the rat forms differ by only two substitutions. To avoid confusion arising from different C-terminal residues, a unifying nomenclature is proposed: PHI-27 for the hormone and PHI-42 for the elongated product.

Key words. Prohormone processing; VIP/PHI precursor; cAMP production.

PHI, a 27-residue peptide hormone was first isolated and characterized from porcine intestinal extracts, and was named after its peptide (P) N-terminal histidine (H) and C-terminal isoleucine (I) amide [1–4]. To indicate its length, it is denoted as PHI-27. For the human form, PHM is used to indicate the C-terminal residue.

PHI-27 is produced from a precursor it shares with vasoactive intestinal peptide (VIP). These two active peptides have close structural similarities with each other [5], and also with secretin [6], glucagon [3], growth-hormone-releasing factor [7], gastric inhibitory polypeptide [8], and some other hormones, which all are known as members of the secretin-glucagon-VIP group [3, 4].

The amino acid sequences of the full-length precursor proteins of PHI/VIP, deduced from their corresponding nucleotide sequences, are known for human [9], rat and mouse [10], chicken [11], and turkey [12].

It has been demonstrated that in humans [13] and in rats [14], there is an elongated form of PHI, so-called PHV-42, consisting of PHI-27 combined with the flanking sequence between PHI-27 and VIP. In such a processing scheme, a cleavage has occurred at the N-terminal histidine residue of VIP followed by a carboxypeptidase action to remove the C-terminal Arg and Lys.

To date, entire PHI/VIP precursor sequence from pig has not been described. Thus, the connecting amino acid sequence in the precursor protein between PHI-27 and VIP is not known from this species. In the present report, we describe the isolation of the C-terminally elongated form of PHI from porcine intestine and demonstrate its unique sequence compared to those of the other active PHI peptides already known.

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Materials and methods

Materials. Porcine intestines were obtained from an abbatoir. IMR-32 neuroblastoma cells were obtained from ATCC (Bethesda, Md.). cAMP Flashplates were from NEN (Boston, Mass.). All reagents were purchased from Sigma (St. Louis, Mo.) or Merck.

Tissue extraction and fractionation. A concentrate of thermostable intestinal peptides, CTIP, was prepared from porcine intestines as described [15]. Briefly, the uppermost meter of the intestines was boiled for 10 min, frozen, minced and extracted with 0.5 M acetic acid (20 h at 4–8 °C) under constant stirring. The extract was filtered, peptides in the filtrate were adsorbed onto alginic acid at pH 2.7 ± 0.1, eluted with 0.2 M HCl, and precipitated at pH 3.5 ± 0.1 with NaCl to produce the CTIP. An aqueous solution of CTIP was fractionated with ethanol as described [16].

Soluble material was adsorbed to alginic acid at pH 2.7 ± 0.1, eluted with 0.2 M HCl, the pH was adjusted to 3.5 with Na acetate and the peptides were salted out with NaCl. The salted-out peptides were dissolved in 0.2 M acetic acid and chromatographed on a Sephadex G25f column (35×135 cm) with 0.2 M acetic acid. Fractions between 30 and 60 l were collected and the peptides precipitated with NaCl (320 g/l). The precipitate was extracted with methanol for 10 min with stirring. The solution was filtered and the pH of the filtrate was adjusted to 7.2 ± 0.1 with 0.1 M NaOH in methanol. The precipitate formed was collected and used for further purification.

Purification of PHI. All chromatographies were carried out using ÄKTAexplorer and ÄKTApurifier systems from Amersham Pharmacia Biotech.

The material (1.5 g), obtained from the methanol extraction and neutral-pH precipitation, was dissolved in the equilibration buffer and the pH adjusted to 5. The first cation exchange chromatography was carried out on a Resource S column (6 ml, Amersham Pharmacia Biotech). The column was equilibrated with eluent A (20 mM Na acetate, pH 5.2, containing 20% acetonitrile), the sample applied and the peptides eluted with eluent B (eluent A containing 2 M NaCl) using a gradient 0–15% B in 20 column volumes (CV). The active fractions eluting early in this chromatography and containing cAMP-producing activity were pooled and further purified. Peptides were detected by monitoring changes in the absorbance at 214 nm.

The next cation exchange chromatography involved the same column using 20 mM Na phosphate, pH 2.5, containing 20% acetonitrile as eluent A, and the same buffer containing 2 M NaCl as eluent B. A gradient from 6 to 19% B in 30 CV was employed. The active fractions were pooled and further processed using reverse-phase high-performance liquid chromatography (HPLC).

Reverse-phase HPLC was carried out on a C18 column (YMC ODS-AP, 10×100 mm, 5 μm, 300 Å). Eluent A consisted of 0.1% trifluoroacetic acid (TFA) in water and eluent B of 0.1% TFA in 95% acetonitrile/water. A gradient 20–40% B in 30 CV was used to elute the peptides. The active fractions were further purified by a second round of reverse-phase HPLC.

Final purification was performed with a μRPC C2/C18 column (4.6×100 mm, 3 μm, 100 Å, Amersham Pharmacia Biotech) with the same solvent system as described for the previous purification step, and with a gradient 30–45% B in 15 CV.

Mass spectrometry and sequence analysis. Mass spectrometry (MS) was carried out on a Finnigan Lasermat 2000 (Thermo Bioanalysis, UK) matrix-assisted laser desorption/ionization (MALDI) instrument using α-cyano-4-hydroxycinnamic acid as the matrix as described [17]. An aliquot (0.5 μl) of the sample and a saturated solution of matrix (0.5 μl, 70% acetonitrile/water) were mixed on a stainless steel target and allowed to dry on a heated plate. Mass spectra were acquired from the average of five laser shot recordings at 337 nm using positive ion mode.

Amino acid sequences were determined using a Procise HT sequencer (PE-Biosystems) for N-terminal and a Procise Ct sequencer (PE-Biosystems) for C-terminal degradations.

Tryptic digestion. The peptide was digested with trypsin at an enzyme-to-substrate ratio of 1:10 (w/w) in 0.2 M ammonium bicarbonate, pH 7.8, for 4 h at 37 °C. After incubation, the solution was heated at 100 °C for 10 min. The tryptic fragments were separated by HPLC on Vydac C18 (4.6×50 mm) using 0–35% B in a gradient in 30 CV in the solvent system described above for reverse-phase HPLC.

Cyclic AMP accumulation. Cells were plated on 96-well plates. Overnight confluent cultures were incubated with DMEM-F12 (GIBCO) media containing isobutylmethylxanthine (2 mM) for 20 min. Cells were treated with aliquots of crude lyophilized fractions (diluted in DMEM-F12 media) for 20 min. The reaction was stopped with 1/5 vol 0.5 N HCl. Cells were then either kept at 4 °C for a minimum of 1 h or frozen overnight (-20 °C). There was no difference between results obtained in either manner. Cell media were tested for cAMP concentration by radioimmunoassay using cAMP Flashplates.

Results and discussion

The elongated form of PHI was purified to homogeneity using four chromatographic steps (fig. 1). In the first cation exchange step, several groups of fractions that activated adenylate cyclase in IMR-32 cells were ob-

served. This activity was well separated from the other activities and was purified further by a second step of cation exchange chromatography, at a lower pH. After that step, three fractions with the highest activity were pooled and chromatographed using two rounds of reverse-phase HPLC. The pure peptide was subjected to structural analysis.

N-terminal sequence analysis for 12 steps revealed a sequence identical to that of PHI. However, molecular mass analysis by MALDI MS gave a mass value of 4493.5, which is considerably higher than that for PHI-27. From these data, a C-terminally extended form of PHI was suspected. The peptide was cleaved with trypsin, the fragments were separated by reverse-phase HPLC and subjected to sequence analysis. One fragment had the sequence VSNGISEDQGPA. Since this fragment did not end in Lys or Arg, it was deduced to be the C-terminal fragment. To obtain overlapping information, the peptide was degraded from the N terminus for 43 cycles. The mass value (4493.0) calculated from this sequence is in very good agreement with that obtained experimentally. The peptide was also degraded for three cycles using a C-terminal sequencer. In the first cycle, an alanine residue was observed. Combined, the results give the sequence as shown in figure 2.

Comparison of the novel amino acid sequence with those from other species shows differences at five positions towards the human form and at six positions towards the rat form, whereas the human and rat forms differ from each other by only two substitutions. The C-terminal parts of the peptides from chicken and turkey are identical, but four residues shorter than those from mammals.

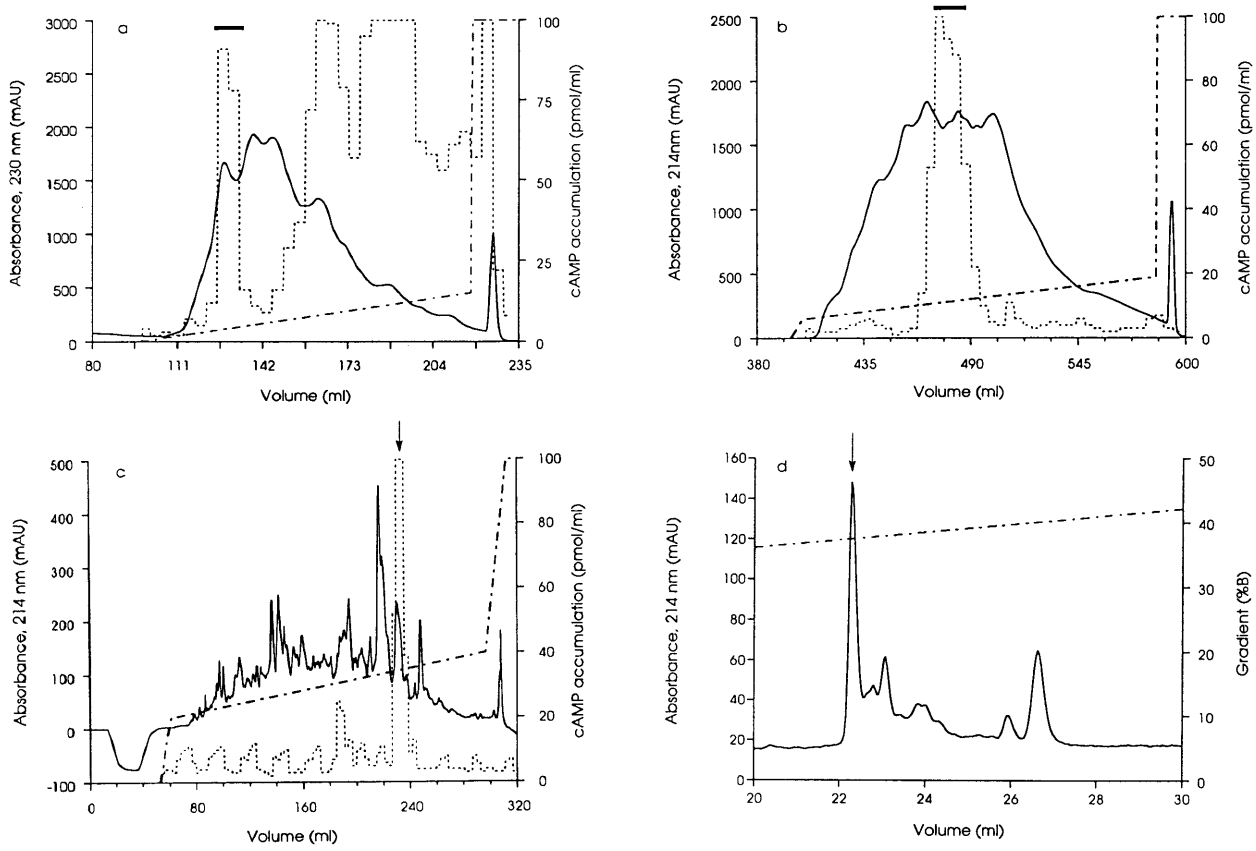

Figure 1. Purification of the elongated form of porcine PHI. (*a*) Cation exchange chromatography on a Resource S, 6-ml column. Eluent A, 20 mM Na acetate, pH 5.2, containing 20% acetonitrile. Eluent B, eluent A containing 2 M NaCl. Gradient: 0–15% B in 20 column volumes (CV). The fractions indicated by the bar were purified further. (*b*) Cation exchange chromatography on a Resource S, 6-ml column. Eluent A, 20 mM Na phosphate, pH 2.5, containing 20% acetonitrile. Eluent B, eluent A containing 2 M NaCl. Gradient: 6–19% B in 30 CV. The active fractions were pooled and processed further. (*c*) Reverse-phase HPLC on an YMC ODS-AP, 10 × 100 mm column (5 μm, 300 Å). Eluent A, 0.1% TFA/water. Eluent B, 0.1% TFA/95% acetonitrile/water. Gradient: 20–40% B in 30 CV. The fraction indicated by the arrow was purified further. (*d*) Final purification of the elongated form of PHI by reverse-phase HPLC on a μRPC C2/C18 column (4.6 × 100 mm). Eluent A, 0.1% TFA/water. Eluent B, 0.1% TFA/95% acetonitrile/water. Gradient: 30–45% B in 15 CV. The peak indicated by the arrow was analyzed.

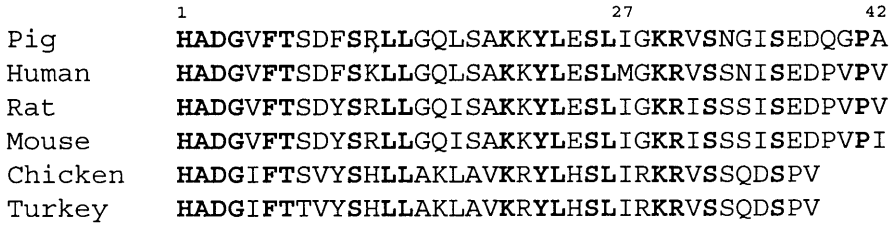

Figure 2. Alignment of the C-terminally elongated forms of PHI from different species.

These data indicate high variability in the C-terminal part of the molecule compared to the remaining 27 residues of PHI itself, which is highly conserved throughout all these species. It is therefore possible that the elongated form of PHI represents a processing intermediate rather than a true hormone in itself. However, biological effects involving different potency of the 27-residue peptide and its 42-residue relative have been reported in mice and rats. In several assay systems, the 42-residue peptide has shown higher potency than the 27-residue peptide [13, 18, 19]. In addition, human PHV-42 has a specific tissue distribution, is the most abundant circulating prepro-VIP-derived product in patients with VIP-producing tumors, and has been isolated and characterized from an adrenal pheochromocytoma [13, 20–22]. These results indicate that the C-terminally elongated PHI may have a distinct biological role, at least in humans.

Notably, both the 27- and 42-residue forms have different C-terminal residues in different species. There is a C-terminal Met in the human 27-residue hormone and an Ile in those from the other species, while the elongated forms have Ala in pig, Ile in mouse, and Val in the rest. We propose that a single name, PHI, will be used for all forms, together with a number indicating the length of the peptide. Thus, the 27-residue hormone would be named PHI-27 and its 42-residue elongated form PHI-42, independent of the actual C-terminal residue, which may be different.

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