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Research Article

Identification of the bioactive peptide PEC-60 in brain

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Abstract. PEC-60 is a 60-residue peptide originally isolated from pig intestine. It inhibits glucose-induced insulin secretion from perfused pancreas in a hormonal manner and also has biological activity in the immune system. PEC-60-like immunoreactive material has been reported in catecholamine neurons of the central and peripheral nervous systems, but the peptide has not been identified from that material. We have now isolated PEC-60 from pig and rat brains with a method that combines column purification procedures with the specificity of a radioimmunoassay and the sensitivity of mass spectrometry to directly identify the peptide. The results show that PEC-60, like many other peptides, is expressed in the gastrointestinal tract and the central nervous system. The specific regional brain distribution and interaction with classical neurotransmitters raise the possibility that PEC-60 may play a role in the central nervous system disorders involving dopamine dysregulation.

Key words. PEC-60; radioimmunoassay; brain; pig; rat.

PEC-60, a peptide (P) with N-terminal glutamic acid (E), C-terminal cysteine (C), and a total of 60 residues (PEC-60), is a peptide originally isolated from pig intestines and shown to affect insulin release from the endocrine pancreas. It is structurally related to the pancreatic secretory trypsin inhibitor (41% residue identity). The disulfide arrangement of these two peptides is identical, showing related overall conformations [1], but PEC-60 does not inhibit trypsin. PEC-60-like immunoreactive material (PEC-60-LI) has been detected in rat brain cells staining for tyrosine hydroxylase [2], in particular in dopaminergic cell areas A9 and A10 (substania nigra pars compacta and ventral tegmental area) and in noradrenergic cells (locus ceruleus). However, attempts to identify mRNA for PEC-60 in the same cells have not been successful and the immunoreactive material has not been identified as PEC-60

itself. Initial attempts to isolate the peptide were not successful because of the very low peptide concentrations. However, as now reported, isolation was made possible by a combination of column purification methods, a sensitive radioimmunoassay (RIA), and screening with an LC-MS technique.

Materials and Methods

Purification

Purification was carried out from 50 kg pig brain raw material. Frozen brains were minced, boiled for 10 min, and cooled to 10–15 °C. Peptides were extracted with 3% acetic acid (150 l) for 16 h under constant stirring, after which solids were filtered off. The pH was adjusted to 2.5 ± 0.2 with 2 M HCl, 5 kg alginic acid was added, and the material was stirred for 30 min and allowed to sedi-

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ment for 1 h. After filtration, 1 kg of Amberlite XAD 16 HP (Supelco, Bellefonte, P.) for each 40 l of liquid was added. The preparation was stirred for 45 min and allowed to sediment for 30 min. The liquid was decanted and Amberlite collected on a filter. Amberlite was washed with water (10 l) and the material adsorbed was eluted with 2 l 45% isopropanol/water. After removal of the solvent, 4.5 g peptide remained.

Four chromatographies were carried out. (i) The material was fractionated on a Sephadex G-25 (Fine) column, 10×100 cm (Amersham Biosciences, Uppsala, Sweden) in 0.2 M acetic acid. Fractions 21–42, corresponding to 1050–2100 ml, contained most of the PEC-60-LI and were used for further purification. The subsequent three HPLC steps were carried out on an ÄKTAexplorer HPLC system (Amersham Biosciences). (ii) The material obtained from the Sephadex G-25 (Fine) column (1.25 g) was dissolved in water (350 ml). After pH adjustment to 4.1 with 20 mM NaOH, it was adsorbed onto a Resource S 6-ml column (Amersham Biosciences) equilibrated with 20 mM sodium acetate, pH 4.1, containing 20% acetonitrile (buffer A), and eluted between 2.7–3.8% B (buffer A containing 2 M NaCl) in a linear gradient 0–15% B in 35 column volumes (CV). (iii) This chromatography step was carried out using the same column, but at a lower pH. The column was equilibrated with 20 mM sodium phosphate, pH 2.5, containing 20% acetonitrile. The sample was diluted three times with water, the pH was adjusted to 2.5, and the material was pumped onto the column. The peptides were eluted in a linear gradient from 0 to 15% B in 35 CV at a flow rate of 30 ml/min. The fractions were analyzed by RIA. (iv) The last step was a reverse-phase HPLC on a C18 column (ODS-AP, 10×100 mm, 5 µm; YMC, Germany). Eluent A was 0.1% TFA/water and eluent B 0.1% TFA/95% acetonitrile/water; the gradient was 20–40% B in 30 CV.

LC-MS screening
The fraction showing PEC-60-LI in RIA was analyzed on an Ettan ESI-ToF mass spectrometer (Amersham Biosciences) connected to an Ettan LC, using a reverse-phase column (Xterra MS C18, 3.5 µm, 3×50 mm) eluted with buffers A (0.1% acetic acid/water) and B (0.1% acetic acid/95% acetonitrile/water) in a gradient of 15–35% B in 12 CV at a flow rate of 0.6 ml/min.

Rat brain
Sprague-Dawley rats, approximately 3 months old, were sacrificed with focused high-energy microwave irradiation, as this method results in high peptide recoveries [3]. Following brain dissection into frontal cortex (FC), striatum (STR), occipital cortex (OCC), hippocampus (HIPP), and hypothalamus (HYP) according to [4], the tissues were freeze-dried and the PEC-60 extracted using identical extraction methods as for other neuropeptides [5, 6].

After lyophilization, the samples were resuspended in assay buffer and used for RIA.

PEC-60 RIA
Lyophilized samples were reconstituted in the assay buffer. PEC-60-LI was quantified by RIA using PEC-60 standard, antiserum and ^{125}I-labeled PEC-60. The assay was run in duplicates as follows: 100-µl standards (prepared in the same buffers as samples) and samples were incubated at 4 °C for 48 h with 100 µl antibody to give approximately 30% 'zero binding'. Following this incubation, 100 µl labeled PEC-60 was added and the solution was incubated for an additional 24 h. Free and antibody-bound PEC-60 were separated by 50 µl Sac-Cel (anti-rabbit solid-phase second antibody-coated cellulose suspension; IDS, Bolton, UK). Samples were left for 30 min at room temperature, the reaction was then blocked with 1 ml distilled H_2O. After centrifugation at 3000 g for 20 min at 4 °C, the supernatants were decanted and the pellets were counted in a gamma counter. The detection limit of the assay was 0.15 ng/ml and the intra-assay coefficient of variation was 3%. PEC-60 standard, antiserum and ^{125}I-labeled PEC-60 were prepared as described previously [7]. PEC-60 standard was purified from pig intestine as described previously [1].

Results and discussion

The results show the presence of PEC-60 in both pig and rat brain. PEC-60-LI was purified by four chromatography steps, involving a size exclusion, a cation exchange at pH 4.1 (fig.1), a cation exchange at pH 2.5 (fig. 2), and a
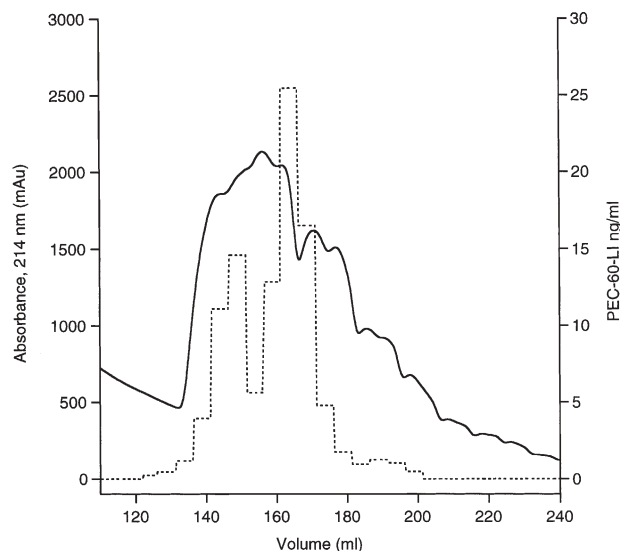

Figure 1. Ion exchange on a Resource S column (6 ml). Gradient: 0–15% B in 35 CV. Solvent system: A = 20 mM sodium acetate (pH 4.1) + 20% acetonitrile, B = A + 2 M NaCl. PEC-60-LI is indicated by the histogram.
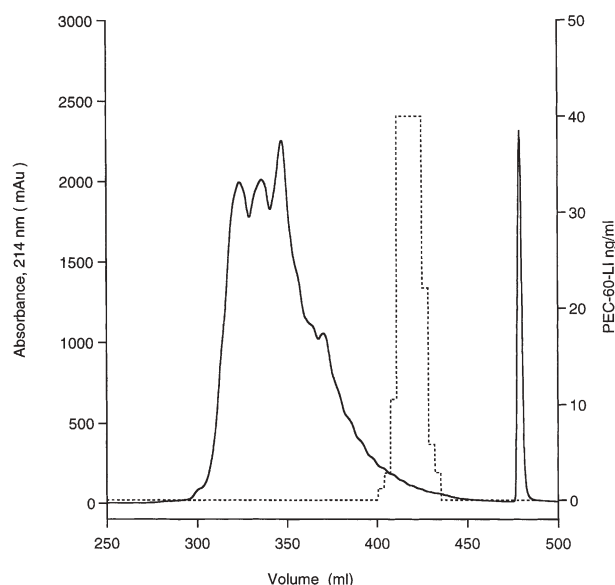
Figure 2. Ion exchange on a Resource S column (6 ml). Gradient: 0–15% B in 35 CV. Solvent system: A=20 mM sodium acetate (pH 2.5)+20% acetonitrile, B=A+2 M NaCl. PEC-60-LI is indicated by the histogram.
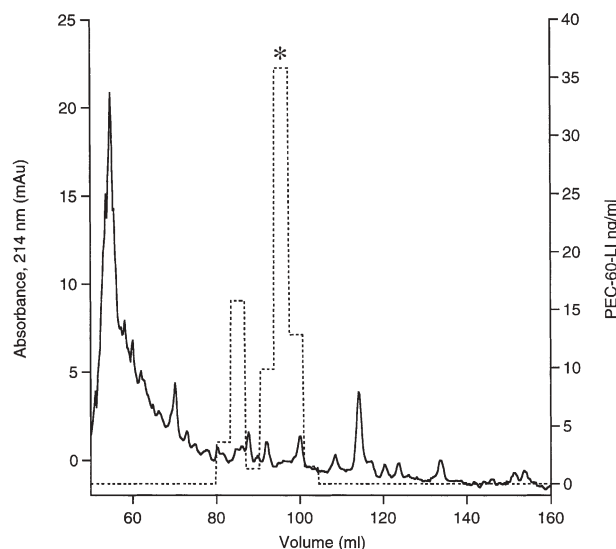
Figure 3. Reverse-phase chromatography on an YMC ODS-AP column (10×100 mm, 5-µm particles). Gradient: 20–40% B in 30 CV. Solvent system: A=0.1% TFA/water, B=0.1% TFA /95% acetonitrile/water. PEC-60-LI is indicated by the histogram.
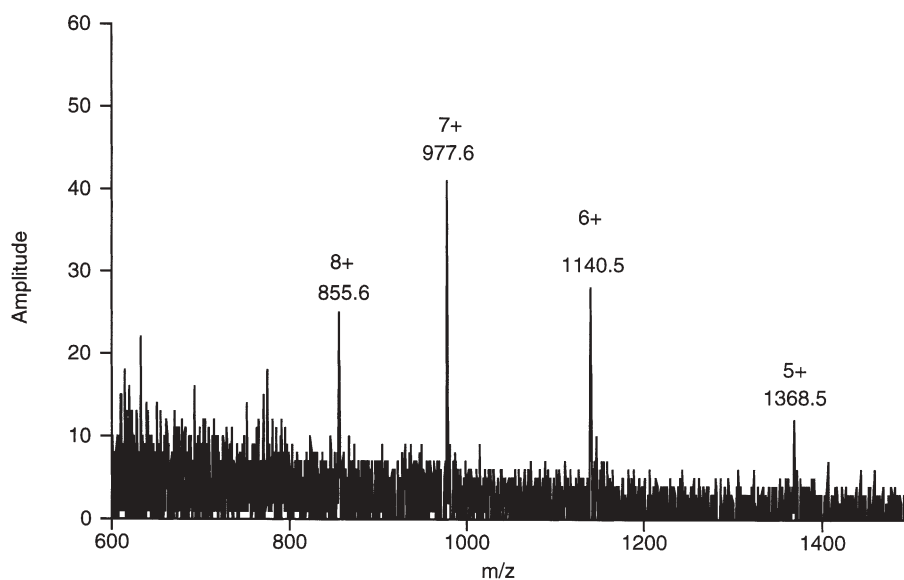

Figure 4. Mass spectrum of the immunoreactive fraction indicated by an asterisk in figure 3. The sample was run by Ettan LC-ESI-TOF mass spectrometry (Amersham Biosciences, Uppsala).

reverse-phase step (fig. 3). All fractions were analyzed by RIA and the fractions with the highest immunoreactivity were subjected to analysis by LC-MS (fig. 4). In the LC-MS, extracted ion chromatograms were created for 5+ (m/z 1368.36), 6+ (m/z 1140.47), 7+ (m/z 977.69) and 8+ (m/z 855.60) ions of PEC-60. The peak positions in all the extracted ion chromatograms overlapped perfectly. The ion species corresponding to the four different charge states were present in the same spectrum and gave a strong

evidence for the existence of full-length PEC-60 in the tissue.

As shown in table 1 sequential dilution of samples resulted in a proportional decrease of the immunoreactivity. Moreover, in a separate RIA, addition of an internal standard resulted in proportional increase of the measured immunoreactivity (table 1). These data, together with those obtained from the pig brain samples, show that PEC-60 is present in the central nervous system (CNS). Interestingly,

Table 1. PEC-60 in rat brain.

	Frontal cortex	Occipital cortex	Hippocampus	Striatum	Hypothalamus
(a) PEC-60-LI in RIA samples (expressed as ng/ml)					
Dilution*		8.5	1.5	1.4	
1:2	6.6	5.1	ND	ND	
1:4	3.9	3.1	ND	ND	
1:8	2.3	ND	ND	ND	
1:16	ND				
Sample*	1.3	2.5			
Sample + standard (5 ng/ml)*	6.1	6.9			
(b) PEC-60-LI in brain regions (n = 12 region) (expressed as ng/ml wet weight tissue)					
Mean (± SE)	2.40 ± 0.27	2.19 ± 0.16	0.74 ± 0.14	0.56 ± 0.07	ND

ND, not detectable.   * RIA samples from different brain regions were run in duplicate. Results are expressed in ng/ml.

PEC-60-LI was detectable in all major rat brain regions, albeit at low concentrations. The highest concentration was measured in the frontal cortex and the occipital cortex (table 1). The experiment was rerun 6 months later and the same results were obtained for both absolute levels and relative distribution in the brain regions (results not shown).

In previous work [3, 5, 6, 8], we showed that other neuropeptides, i.e., neurotensin, CGRP, galanin, neurokinin A, substance P, somatostatin, and neuropeptide Y, show a different relative distribution in the brain than PEC-60. Thus, their highest concentrations are found in the hypothalamus, followed by several times lower concentrations in the striatum and hippocampus, with lowest levels in the frontal cortex. In contrast, the PEC-60 concentration was highest in the cortex, both frontal and occipital, and only very low concentrations were measured in the subcortical areas such as striatum. Interestingly, immunohistochemistry showed the presence of PEC-60 in the limbic areas but no mRNA could be identified in the same regions [2]. The reason for such a different relative distribution is not clear, but would seem to imply a specific function for PEC-60 in the CNS. In view of its specific distribution in rat brain and interaction with dopamine and GABA systems [9, 10], PEC-60 may play a role in the pathophysiology of Parkinsonism and schizophrenia and is also altered in Alzheimer's disease [11, 12]. Independent of its role, however, we have conclusively demonstrated the occurrence of PEC-60 in pig and rat brain.

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