Vol. 164, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1989 Pages 567-574

ISOLATION OF A NOVEL 38 RESIDUE-HYPOTHALAMIC POLYPEPTIDE WHICH STIMULATES ADENYLATE CYCLASE IN PITUITARY CELLS

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Received September 6, 1989

Summary: A novel neuropeptide which stimulates adenylate cyclase in rat anterior pituitary cell cultures was isolated from ovine hypothalamic tissues. Its amino acid sequence was revealed as: His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg- Lys- Gln- Met- Ala-Val-Lys-Lys-Tyr-Leu-Ala-Ala-Val-Leu-Gly-Lys-Arg-Tyr-Lys-Gln-Arg-Val-Lys-Asn-Lys-NH_2. The N-terminal sequence [1-28] shows 68% homology with vasoactive intestinal polypeptide (VIP) but its adenylate cyclase stimulating activity was at least 1000 times greater than that of VIP. It increased release of growth hormone (GH), prolactin (PRL), corticotropin (ACTH) and luteinizing hormone (LH) from superfused rat pituitary cells at as small a dose as 10^{-10}M (GH, PRL, ACTH) or 10^{-9}M (LH). Whether these hypophysiotropic effects are the primary actions of the peptide or what physiological action in the pituitary is linked with the stimulation of adenylate cyclase by this peptide remains to be determined.

The major hypothalamic hypophysiotropic stimulating hormones, *i.e.* luteinizing hormone releasing hormone (LHRH) (1), thyrotropin releasing hormone (TRH) (2), growth hormone releasing hormone (GHRH) (3) and corticotropin releasing hormone (CRH) (4), have been isolated and characterized, and using their synthetic preparations, their physiological roles were defined. However, whether these hypothalamic releasing hormones represent *all* the physiological releasing hormones remains to be determined. The possible presence of a follicle-stimulating

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Abbreviations: GH, growth hormone; PRL, prolactin; ACTH, corticotropin; LH, luteinizing hormone; VIP, vasoactive intestinal polypeptide; PHI, peptide histidine isoleucine amide; cAMP, cyclic adenosine 3', 5'monophosphate; LHRH, luteinizing hormone releasing hormone; TRH, thyrotropin releasing hormone; GHRH, growth hormone releasing hormone; CRH, corticotropin releasing hormone; SS14 or 28, somatostatin 14 or 28 residue; ACSA, adenylate cyclase stimulating activity; PACAP, pituitary adenylate cyclase activating polypeptide.

0006-291X/89 $1.50

hormone releasing hormone (FSHRH) distinct from LHRH is under debate (5). Presence of prolactin (PRL) releasing hormone has been described (6-8). The presence of pituitary hormones different from known anterior pituitary hormones has also been reported (9). Electronmicroscopic study with morphometry indicated that at least three types anterior pituitary cells remain unidentified (10). These pituitary cells would presumably also require their hypothalamic regulators. Such reports imply that additional hypothalamic hypophysiotropic hormones may be present.

Most of the hypothalamic releasing hormones so far isolated appear to stimulate pituitary adenylate cyclase and increase accumulation of cAMP, although the extent of stimulation varies. Increase of intracellular, as well as extracellular, cAMP in cultured pituitary cells is most strikingly demonstrated by GHRH (11), and to a lesser extent by CRH (12). LHRH and TRH also increase cAMP accumulation , although it may not be linked with secretion of these hormones (13,14). Along these lines, we considered it possible to discover novel hypothalamic releasing hormones by monitoring the pituitary adenylate cyclase stimulating activity (ACSA) in hypothalamic extracts. This report deals with the discovery of a novel hypothalamic polypeptide with a significant ACSA.

Materials and Methods

Tissue extraction: Fresh ovine hypothalami were collected at Colorado Lamb Co., Denver, CO, and immediately frozen on dry ice. Tissues were stored at -70° C until extracted. Ovine hypothalami (4,370 pieces; 2400 g) were heated in 10 vols of boiling water for 10 min to inactivate intrinsic proteases, and cooled on ice. After addition of glacial acetic acid (AcOH) and ß-mercaptoethanol (ß-ME) to a final concentration of 2 M and 0.02%, respectively, tissues were homogenized with a Polytron for 10 min at 4° C. Following centrifugation at 10,000 x *g* for 30 min, the supernatant was precipitated by addition of acetone (final: 66%). After removing precipitates by centrifugation, the supernatant was evaporated to remove acetone, and then diluted with 3 vols of water. One-sixth of the material was first pumped onto a C-18 silica column (750 ml, 218TPB2030, Vydac) and the column was then eluted step-wise with 10, 20, 30, 40, 50, and 60% acetonitrile (CH_3CN) in 0.1 % trifluoroacetic acid (TFA) to yield frs. A, B, C, D, E, and F, respectively. This step was repeated six times to process all tissue extracts. Aliquots of each of frs. A, B, C, D, E, and F were lyophilized, dissolved in the culture medium, and assayed for adenylate cyclase stimulating activity (ACSA). Frs. C which showed considerable activity was used as starting materials for this isolation. Lyophilized fr. C was dissolved in 1 M AcOH and applied on SP-Sephadex column (H^+ form, 4 x 48 cm, Pharmacia) pre-equilibrated with 1 M AcOH. Successive, step-wise elution with 1 M AcOH, 2 M pyridine, followed by 2 M pyridine-AcOH (pH 5.0) yielded frs. SP-1, SP-II, and SP-III, respectively. ACSA was demonstrated in SP-III which contained basic materials. Then, the SP-III was then subjected to Gel-filtration on a Sephadex G-50 column (fine, 5.5 x 97 cm, Pharmacia) using 2M AcOH / 0.02% ß-ME as the eluting buffer, individually. Column effluents were monitored for O.D. at 280 nm. An aliquot of each fraction was subjected to the bioassay. The fractions with dominant ACSA were pooled and lyophilized. The residue was reconstituted with 10mM ammonium formate (pH 6.5) containing 10% CH_3CN and adsorbed on a CM-52 cellulose column (1 x 38 cm, Whatman), which was preequilibrated with 10mM ammonium formate containing 10% CH_3CN. Then the chromatography was performed using a linear gradient of ammonium formate from 10 mM to 0.8 M as described in the legend of Fig.2a. The bioactive portion thus purified was then apllied on a column of TSK ODS 120T (4.6 x 250mm, ToyoSoda) and eluted with a linear gradient of acetonitrile as described in the legend of Fig.2b. Finally the active fraction was purified on a Vydac phenyl column (4.6 x 250 mm, Vydac), eluted with the same gradient described above. During HPLC, column effluents were monitored by measuring UV absorbance at 210 nm and 280 nm, simultaneously.

Structure analysis: Sequence analysis was performed by a pulse liquid phase sequencer (Model 477A/120A, Applied Biosystems). The resulting PTH-amino acids were analysed by reverse phase

HPLC in concert with the sequencer. PTH-amino acids were measurable as low as 1 pmol. Amino acid analysis was carried out with Pico Tag system (Waters) after hydrolysis of the highly purified peptide (ca. 200 pmol) in 6N HCl containing 0.1% phenol at 110 °C for 24 hrs. The carboxy terminal analysis of the peptide (100 picomole) was carried out by digestion with carboxypeptidase B or Y (Sigma Chemical Co., St. Louis, MO) in 10 µl of 0.1M ammonium bicarbonate (pH 8.0). Analysis of released amino acid was performed at the picomole level using a prelabeling method.
Synthesis: The 38 residue peptides with C-terminal amide and free form according to the sequencing result were synthesized by solid phase techniques as described previously (15), conducted on a 4-methyl benzhydrylamine resin and N-Boc-Lys(2-Cl-Z)-PAM resin, respectively. Purification was made by Sephadex G-50 gel-filtration and reverse phase HPLC. Correct synthesis was confirmed by amino acid analysis and sequencing.
Bioassay: 1) ACSA of test samples was determined *in vitro* using pituitary cell cultures as described previously (11,16). The accumulation of cAMP in culture media of rat pituitary cells during a 3-hr incubation with the test sample was used as the response parameter. The content of cAMP in the media was determined by radioimmunoassay (RIA) as described previously (17). 2) The effect on the release of anterior pituitary hormones [GH, ACTH, PRL, LH, FSH and TSH] was determined using a static *in vitro* method using rat pituitary cell cultures as described above and a superfusion method using dispersed rat pituitary cells as described elsewhere (18). All pituitary hormones in the culture media and superfusate were determined by the respective RIA supplied from National Hormone and Pituitary Program, NIDDK. 3) Vasodepressor activity of the test materials was determined in urethane anesthetized rats by monitoring arterial blood pressure after intravenous injection of the sample. The arterial blood pressure was determined through the canula inserted in the femoral artery by means of a pressure transducer (P-1000B) and Marcotrace 40 (Marco Biosystems, Houston, TX).

Results and Discussion

ACSA in ovine hypothalamic extract was observed in fr.C and D, which are eluates with 30% and 40% CH_3CN, respectively, from C-18 silica column. In our preliminary study, a small amount of frs. C and D was purified by gel-filtration, followed by ion exchange HPLC (IEX HPLC) and then reverse phase HPLC (RP HPLC). A two-dimensional map for individual ACSA based on hydrophobicity and electrical charge was prepared along with ACSA of known hypothalamic RHs (Fig. 1). Substances with ACSA derived from fr. D appeared to be GHRH- or

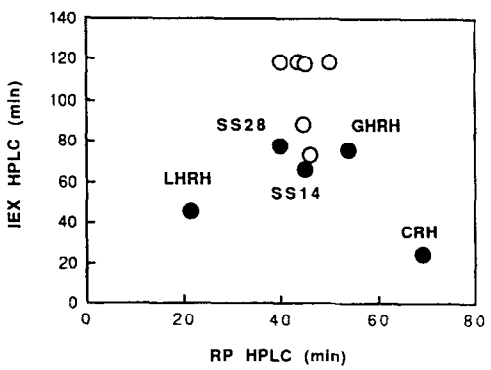

Fig. 1 Two dimension map of ACSA in fr. C. The retention times on RP HPLC which represent hydrophobicity were plotted on the abscissa, and those of IEX HPLC which represent electrical charge on the ordinate, thus creating a two-dimensional map for hydrophobicity and electrical charge of each active fraction. RP HPLC was performed on a TSK ODS 120T column under the same elution condition as described in Fig.2b legend. IEX HPLC was performed on a TSK CM 2SW column in a gradient of ammonium formate (pH 6.5) 10mM to 0.5M for 100 min, then increased to 1.0M for 20 min. Various known hypothalamic releasing hormones (●) were also mapped to show the presence of any novel hypothalamic substances (○) in locations different from those of known releasing hormones.

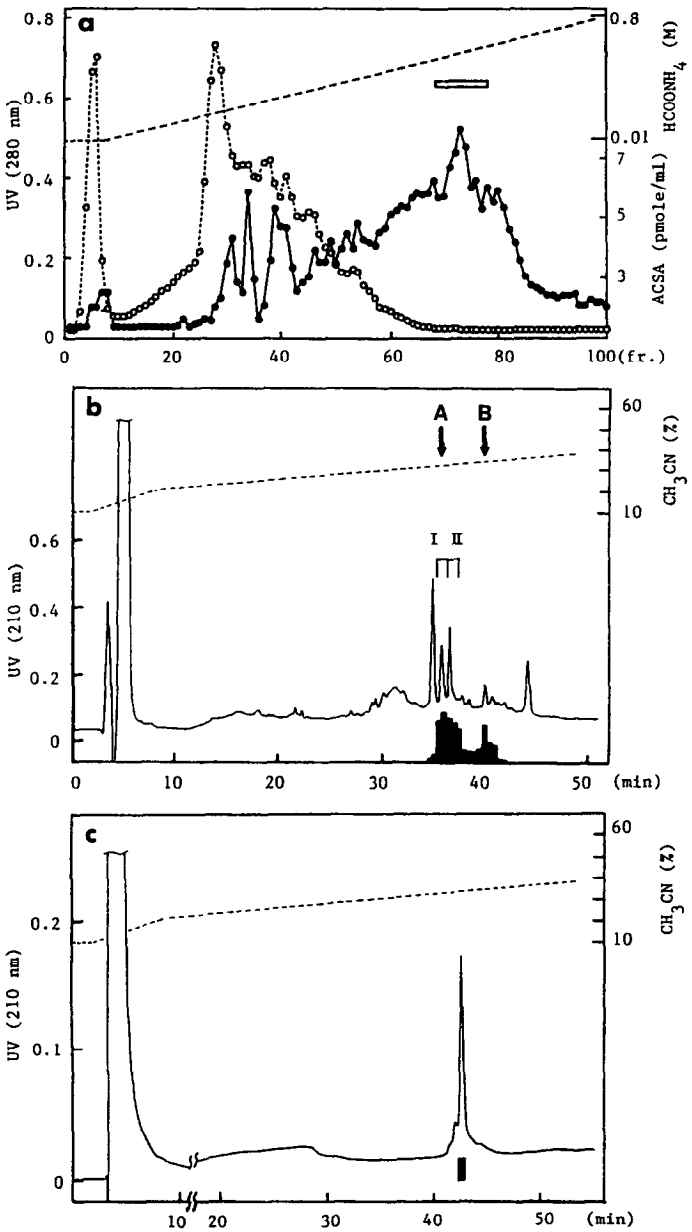

Fig. 2a Cation exchange chromatography of fr.74-80 with ACSA obtained by Sephadex G-50 gel-filtration. Sample: lyophilized fractions 74-80 of the gel filtration. Column: Whatman CM-52, 1 x 20 cm. Fraction size: 4 ml/tube. Flow rate: 8 ml/hr. Solvent system: linear gradient elution from (A) to (B); (A) 10 mM ammonium formate (pH 6.5) : CH_3CN = 90 : 10 (v/v) (B) 0.8M Ammonium formate (pH 6.5) : CH_3CN = 90 : 10. Cyclic AMP levels (●) accumulated in the rat pituitary cell culture media after 3-hr incubation were used as the response parameter of ACSA. An open box shows the most potent ACSA (fr. 70-77) which was processed further.

Fig. 2b Reverse phase HPLC of ion exchange chromatography fraction with ACSA. Sample: The ACSA active fraction eluted at fr.70-77. Flow rate: 1.0 ml/min. Column: TSK ODS 120T (4.6 x 250 mm, ToyoSoda). Solvent system: Linear gradient elution from (A) to (B) for 120 min; (A) H_2O : CH_3CN : 10%TFA = 90 : 10 : 1 (v/v) (B) H_2O : CH_3CN : 10%TFA = 40 : 60 : 1 (v/v). The black bar shows the ACSA (A and B). The major ACSA portions (A) were divided into I, 34-35min, and II, 35-36min, corresponding to the respective UV absorbance peaks.

Fig. 2c Final purification of PACAP by RP HPLC. Sample: The ACSA fraction [A-II] eluted at 35 - 36 min on previous RP HPLC. Flow rate: 1.0 ml/min. Column: Vydac phenyl (4.6 x 250 mm). Solvent system: Linear gradient elution from (A) to (B) for 120 min; (A) H_2O : CH_3CN : 10% TFA = 90 : 10 : 1 (v/v) (B) H_2O : CH_3CN : 10% TFA = 40 : 60 : 1 (v/v).

CRH-related materials because they were located in the positions overlapping those of GHRH or CRH on the map and had the respective immunoreactivity (data not shown). On the other hand, ACSA of fr. C contained at least six substances with ACSA located in areas different from those for any known hypothalamic releasing hormones (fig. 1). These substances did not have immunoreactivities for GHRH and CRH (data not shown). Especially four activities in the strong basic portion cannot be explained by known neuropeptides. Based on these findings, fr. C was used as a starting material to isolate novel hypophysiotropic factors. After step-wise ion exchange chromatography on SP-Sephadex column, the basic peptide fraction, SP-III was applied on a column of Sephadex G-50. ACSA eluted as a broad peak, corresponding to m.w. 1,000-7,000, suggesting the presence of varying molecular weight substances with ACSA (data not shown). The fractions corresponding to m.w. 3,000-4,000 which showed the greatest ACSA were pooled, lyophilized, and then subjected to cation exchange chromatography on a CM-52 cellulose column (Fig. 2a). As observed in the preliminary study, the major ACSA was found in the strong basic regions of frs.70-77, which were pooled and purified by RP HPLC on TSK ODS 120-T column. As shown in Fig.2b, a large (A) and a small (B) ACSA peak were observed. As two UV absorbance peaks emerged in the first large ACSA peak A, fractions (I = 34-35 min and II = 35-36 min) corresponding to each of these UV absorbance peaks were pooled and each was purified by another RP HPLC system on a Vydac phenyl column, which yielded, respectively, a pure material (Fig. 2c).

The primary stucture of the material isolated from the fraction II [35-36 min] was first determined by Edman degradation using a 477A Protein/Peptide Sequencer (Applied Biosystems). The phenylthiohydantoin (PTH) derivatives of the amino acids were identified by RP HPLC on a 120A PTH analyzer (Applied Biosystems). The peptide was sequenced from 1 to 38 residues using 100 to 200 picomole of the peptide. The structure of the peptide was revealed to be:
His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val- Lys-Lys-Tyr-Leu-Ala-Ala-Val-Leu-Gly-Lys-Arg-Tyr-Lys-Gln-Arg-Val-Lys-Asn-Lys-NH_2.
Its amidated C-terminus was indicated by the carboxypeptidase digestion. The C-terminal lysine was released by CPase Y , but not by CPase B after 1-hr incubation. Furthermore, two 38-residue peptides with free- and amidated-C terminus were respectively synthesized by a solid phase method (15), and their retention times were compared to that of the native peptide on RP HPLC. The native peptide co-eluted with the synthetic peptide with amidated C-terminus, and separated from the synthetic peptide with free C-terminus (data not shown). This peptide was named PACAP38 (Pituitary Adenylate Cyclase Activating Polypeptide with 38 residues). The synthetic PACAP38 showed the same ACSA potency as the native peptide (Fig. 3).

Based on the result of amino acid analysis of this peptide, the final yield of the peptide was about 1.2 nmole from 2,400 g of ovine hypothalami. The peptide which was purified from the fraction I [34 - 35 min] in the first RP HPLC on TSK ODS column (Fig. 2b) also showed the same sequence as PACAP38. It was identified as [$Met(O)^{17}$]-PACAP38, indicated by the same retention time of the synthetic PACAP38 oxidized with performic acid on RP HPLC (19).

A computer-assisted search for homology of sequence of PACAP38 revealed that the N-terminal portion [1-28] has 68% homology with ovine VIP, which has recently been revealed to

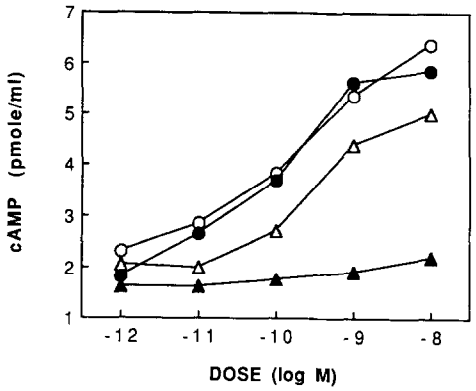

Fig. 3 Adenylate cyclase stimulation activity of native PACAP38 (●) and synthetic PACAP38 (○) as compared with VIP (▲) and CRH (△) determined using rat pituitary cell cultures as described in the text.

be identical with porcine VIP (20,21). Especially, the sequence common to that of VIP, Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu, may reflect a distant ancestral relatedness between VIP and PACAP38. On the other hand, the sequence of the C-terminal region [29-38] showed no homology with any other known peptides. The N-terminal structure of PACAP38 was very similar to that of an ovine hypothalamic peptide which we previously isolated (22). This was a VIP-like GH releasing factor (VLGRF) which stimulated GH release from rat pituitary fragments *in vitro* (22). Its N-terminal amino acid sequence was partially determined as shown in Fig.4. Due to the unavailability of a sufficient amount of ovine hypothalamic tissues at that time, we could neither complete nor confirm the primary structure of this peptide. It is possible that PACAP38 is the same, or is of a very closely-related family, as this peptide. Besides VIP, PACAP38 shows a

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                1                                   10                                  20
PACAP38:        His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys
VLGRF:          His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Lys-Arg-Tyr-Asn-Lys-Glu-Met-Ala-Lys----
VIP:            His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys
oGHRH:          Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Ile-Leu-Gly-Gln-Leu-Ser-Ala-Arg
PHI:            His-Ala-Asp-Gly-Val-Phe-Thr-Ser-Asp-Phe-Ser-Arg-Leu-leu-Gly-Gln-Leu-Ser-Ala-Lys
Secretin:       His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Asp-Ser-Ala-Arg-Leu-Gln
Glucagon:       His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln

                21                                  30
               -Lys-Tyr-Leu-Ala-Ala-Val-Leu-Gly-Lys-Arg-Tyr-Lys-Gln-Arg-Val-Lys-Asn-Lys-NH2

               -Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH2
               -Lys-Leu-Leu-Gln-Asp-Ile-Met-Asn-Arg-Gln-Gln-Gly-Glu-Arg-Asn-Gln-Glu-Gln----Leu44-NH2
               -Lys-Tyr-Leu-Glu-Ser-Leu-Ile-NH2
               -Arg-Leu-Leu-Gln-Gly-Leu-Val-NH2
               -Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr
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Fig. 4 Amino acid sequence of PACAP38 with related peptides: ovine VLGRF[22], VIP[20], ovine GHRH[23], PHI[24], Secretin[25], glucagon[26]. Residues which are underlined indicate amino acids identical to those for PACAP38. The number at the top refers to the position of the amino acid in the indicated peptide.

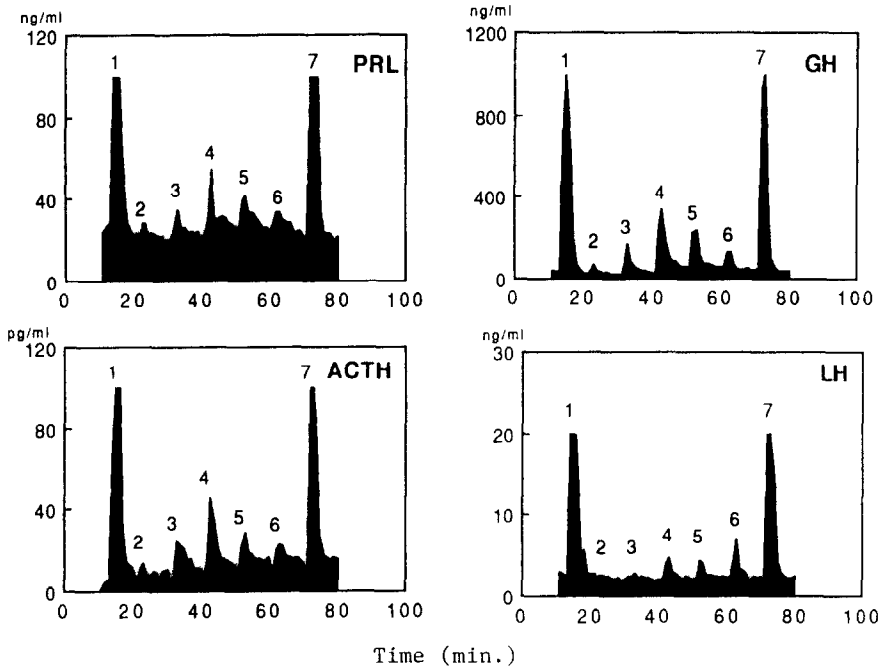

Fig.5 The effect of synthetic PACAP38 on the release of pituitary hormones from superfused pituitary cells. The peptide was applied as a 3-min pulse. 0.9ml fractions were collected every 3 min. Each fraction was determined for GH, Prl, ACTH, LH, FSH and TSH by the respective RIA. A depolarizing concentration (100mM) of KCl was used as an internal standard to confirm that the cells showed a normal large release of pituitary hormones. The numbers at the top indicate: 1,7: 100mM KCl, 2: 10^{-10}M PACAP38, 3: 10^{-9} M PACAP38, 4: 10^{-8} M PACAP38, 5: 10^{-7} M PACAP38, 6: 10^{-6} M PACAP38.

certain degree of homology also with GHRH (23), peptide histidine isoleucine amide (PHI) (24), secretin (25) and glucagon (26), and seems to be a chimera of these peptides (Fig. 4).

In view of the significant homology of PACAP38 with VIP, we examined them both to determine if they share common biological activities. In urethane-anesthetized rats, both peptides showed comparable vasodepressor activity in a dose range between 0.33 nmole and 1.0 nmole. On the other hand, PACAP38 showed at least 1000 times greater ACSA than that of VIP, a level comparable to that of CRH (Fig. 3). On the other hand, PACAP38 stimulated release of GH, PRL, and ACTH from superfused rat pituitary cells in a dose-dependent manner in a dose range between 10^{-10} M to 10^{-8} M. The responses declined as the doses increased and showed "bell-shaped" dose-response curves for these hormones (Fig. 5). On the other hand, LH response was roughly linear in the dose range of 10^{-9} M to 10^{-6} M (Fig. 5). The release of FSH and TSH was not altered under the same conditions (data not shown). Despite considerable cAMP accumulation, PACAP38 did not, however, induce a significant increase in the release of any known pituitary hormone in static rat pituitary cell cultures (data not shown). Whether the increase in GH, ACTH, PRL and LH release by PACAP38 from the superfused pituitary cells reflects the primary physiological action of the peptide, or which pituitary hormone's secretion is coupled with the adenylate cyclase stimulation by PACAP38, remains to be investigated. It is also possible that PACAP38 modulates the action of other hypophysiotropic hormones. This novel neuropeptide might also prove to have a broad range of biological activities in various tissues, as does VIP.

Physiological and immunohistochemical studies with PACAP38 are underway to reveal its functional roles.

Acknowledgments

This study was supported in part by NIH grant DK09094. We gratefully acknowledge Colorado Lamb Co., Denver, CO for providing us with ovine hypothalamic tissues, Dr. M. Tamura for his gift of cAMP antiserum, Yamasa Co. for the gift of Tyr-cAMP used for labelling, and Drs. M. Fujino, C. Kitada and M. Mori, Takeda Chemical Industries, Ltd., Tsukuba labs., for peptide synthesis as well as constructive discussion in this study. We also thank Mrs. P. Portilla and M. C. Noriega for their technical assistance and L. Powell for editorial assistance in preparation of this manuscript.

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