Cellular localization of apelin and its receptor in the anterior pituitary: evidence for a direct stimulatory action of apelin on ACTH release

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Submitted 26 October 2005; accepted in final form 22 July 2006

Reaux-Le Goazigo A, Alvar-Perez R, Zizzari P, Epelbaum J, Bluett-Pajot MT, Llorens-Cortes C. Cellular localization of apelin and its receptor in the anterior pituitary: evidence for a direct stimulatory action of apelin on ACTH release. Am J Physiol Endocrinol Metab 292: E7-E15, 2007. First published August 8, 2006; doi:10.1152/ajpendo.00521.2005.—Apelin is a bioactive peptide recently isolated from bovine stomach extracts and identified as the endogenous ligand of the human orphan G protein-coupled receptor APJ. The presence of apelin-immunoreactive nerve fibers, together with the detection of apelin receptor mRNA in the parvocellular part of the paraventricular nucleus and the stimulatory action of apelin on corticotropin-releasing hormone release, indicate that apelin modulates adenocorticotropin (ACTH) release via an indirect action on the hypothalamus. However, a direct action of apelin in the anterior pituitary cannot be excluded. Here, we provided evidence for the existence of an apelinergic system within the adult male rat pituitary gland. Double immunofluorescence staining indicated that apelin is highly coexpressed in the anterior pituitary, mainly in corticotropes (96.5 ± 0.3%) and to a much lower extent in somatotropes (3.2 ± 0.2%). Using in situ hybridization combined with immunohistochemistry, a high expression of apelin receptor mRNA was also found in corticotropes, suggesting a local interaction between apelin and ACTH. In an ex vivo perfusion system of anterior pituitaries, apelin 17 (K17F, 10^-6 M) significantly increased basal ACTH release by 41%, whereas apelin 10 (R10F, 10^-6 M), an inactive apelin fragment, was ineffective. In addition, K17F but not R10F induced a dose-dependent increase in K+ -evoked ACTH release, with maximal increase being observed for a 10^-6 M concentration. Taken together, these data outline the potential role of apelin as an autocrine/paracrine-acting peptide on ACTH release and provide morphological and neuroendocrine basis for further studies that explore the physiological role of apelin in the regulation of anterior pituitary functions.

hypothalamo-pituitary-adrenal axis; apelin; apelin receptor; adenocorticotropin

Apelin is a bioactive peptide recently isolated from bovine stomach extracts and identified as the endogenous ligand of the human orphan G protein-coupled receptor APJ (26, 40). Apelin is a 36-amino acid peptide derived from 77-amino acid precurser proapelin, for which cDNAs have been cloned from humans, cattle, rats, and mice (12, 18, 40). This precursor exhibits a fully conserved COOH-terminal sequence between Trp-55 and Phe-77, including the COOH-terminal 17 (Lys-Phe-Arg-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe, apelin 17, or K17F) and 13 (Gln-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe, apelin 13, or Q13F) amino acid sequences. In vivo, in the rat hypothalamus and plasma, we have characterized the predominant molecular forms of endogenous apelin as corresponding to the pyroglutamyl form of Q13F (pE13F) and, to a lesser extent, to K17F (8). These apelin fragments display the highest affinity for the APJ receptor (1, 3, 6, 7) and exhibit the highest activities on extracellular acidification rate, cAMP production, or apelin receptor internalization (7, 9, 12, 21, 40, 43). In contrast, apelin 10 (Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe, R10F), corresponding to the last 10 COOH-terminal amino acids of proapelin, has a low affinity for the APJ receptor (>10^-7 M) and has no activity in vitro and in vivo (9, 30).

The production of a polyclonal antibody with high affinity and selectivity for K17F and pE13F (8) made it possible to visualize, for the first time, apelinergic nerves in the rat central nervous system (31). The topographical distribution of apelin immunoreactivity shows that apelin-immunoreactive (IR) neuronal cell bodies are abundant in the hypothalamic supraoptic nucleus and the magnocellular part of the paraventricular nucleus (PVN) (3, 31). The density of IR nerve fibers and apelinergic nerve endings is highest in the inner layer of the median eminence and in the posterior pituitary (3, 31), suggesting that the apelin neurons of the supraoptic nucleus and PVN, like arginine vasopressin (AVP)- and oxytocin-containing magnocellular neurons, project toward the neural lobe of the pituitary. Subsequently, apelin and apelin receptor mRNA were shown to colocalize with AVP in magnocellular neurons (8, 25, 30, 32), and we recently reported (8) that, in lactating rodents, K17F given intracerebroventricularly inhibits the phasic electrical activity of AVP neurons and reduces plasma AVP levels, resulting in aqueous diuresis.

Apelin-IR nerve fibers (31), as well as apelin receptor mRNA (7, 23), are also detected in the parvocellular part of the PVN, and both apelin (3) and apelin receptor mRNA (7, 23) are located in the anterior pituitary. Altogether, these data suggest that apelin is involved in not only the regulation of posterior but also anterior pituitary hormone release. In agreement with this hypothesis, intracerebroventricular administration of pE13F to adult rats significantly increases plasma ACTH and corticosterone release (15, 39). Moreover, in vitro pE13F stimulates corticotropin-releasing hormone (CRH) release from hypothalamic explants (39).

Together, these neuroanatomical and biological data suggest that apelin modulates ACTH release via an indirect action on the hypothalamus involving CRH release. Similarly to other...
Anterior pituitary hormones, ACTH secretion is regulated not only by hypothalamic neuropeptides and peripheral hormones that reach the pituitary gland via portal vessels and the systemic circulation, respectively, but also by autocrine/paracrine peptides produced within the gland (33, 34). In this context, apelin could have a direct action on ACTH release in the anterior pituitary.

Thus the aim of the present study was to determine the phenotype of anterior pituitary cells expressing apelin using dual immunofluorescence staining and confocal analysis. In situ hybridization in combination with immunohistochemistry was then used to assess whether apelin receptor mRNA was expressed by corticotrophs. In addition, the adenohypophysial effect of apelin on ACTH release was evaluated by measuring the effects of active (K17F) or inactive (R10F) apelin fragments on the basal and K+-evoked ACTH release from perfused rat anterior pituitaries in vitro.

Materials and Methods

Drugs and antibodies. Apelin fragments (Lys-Phe-Arg-Arg-Gln-Arg-Pro-Ago-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe, K17F; and Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe, R10F) were synthesized by NeoMPS (Strasbourg, France). Rabbit polyclonal antibodies directed against the apelin fragment K17F were produced and characterized in the laboratory as previously described (8).

Anti-rat prolactin (PRL; 1:2,500), anti-rat luteinizing hormone (LH; 1:1,250), anti-rat thyrotropin (TSH; 1:1,000), anti-human ACTH (1:2,500), and anti-human follicle-stimulating hormone (FSH; 1:5,000) antibodies were provided by the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). The specificities of these antisera were assessed by the NIDDK and have been described previously (19). The anti-rat growth hormone (GH) (9B5 1:3,000) was kindly given by Dr. D. Grouselle.

Tissue preparation for immunohistochemistry and in situ hybridization. All animal experiments were carried out in accordance with present institutional guidelines for the care and use of experimental animals. Adult (250–350 g) male Wistar Kyoto rats (Charles River Laboratories, L’Arbresle, France) were kept on a 12:12-h light-dark cycle with free access to food and water. Under pentobarbital anesthesia, rats were perfused transcardially after a brief saline rinse with modified eagle’s medium (Dulbecco’s modified eagle’s medium containing 0.1% NGS) in PB for 1 h. They then were incubated in a blocking solution consisting of 3% normal goat serum (NGS) in PB for 1 h. They then were incubated in blocking solution containing 1% NGS for 24 h at 4°C. After several washes, sections were then incubated for 1 h at room temperature with a biotinylated goat anti-rabbit secondary antibody (diluted 1:1,000 in PB; Vector Laboratories, Abcys, Paris, France) followed by 1:5,000-conjugated streptavidin (diluted 1:250; Vector Laboratories) diluted 1:100 in PB. After three washes in PB, sections were then washed and coveredslipped with Aquamount. Control sections were processed in parallel in the absence of either primary or secondary antibodies. The sections were observed under a confocal microscope described above.

Synthesis of cRNA probes of the rat apelin receptor. The apelin receptor antisense and sense cRNA probes were synthesized by in vitro transcription, as previously described in detail (7). Briefly, clone 16.3, which contained the full-length rat apelin receptor cDNA, was digested with XhoI and EcoRI, and the resulting fragment was inserted between the SacI and EcoRI sites of the Bluescript KS plasmid (Stratagene, La Jolla, CA). The Bluescript KS plasmid was used to synthesize the sense and antisense riboprobes. The cDNA was linearized by digestion with SacI or XhoI, and in vitro transcription was performed using T3 or T7 RNA polymerase (Roche Diagnostics, Meylan, France) in the presence of [35S]-UTP (Amersham Pharmacia Biotech, Orsay, France). The adequacy and the yield of the transcription were verified by agarose gel electrophoresis and scintillation count as previously described (11).

Tissue preparation and in situ hybridization. The in situ hybridization protocol of Sibony et al. (35) was used. Briefly, after treatment with proteinase K and acetylation, each section received 30 μl of the hybridization mixture containing 5 × 106 cpm of the sense or antisense riboprobes and was incubated for overnight in a humid chamber at 50°C.

Immunohistochemistry for ACTH after in situ hybridization. Immediately after in situ hybridization, the sections were rinsed twice in phosphate-buffered saline (PBS; pH 7.4) and incubated in 3% NGS for 30 min and then with ACTH antibody (diluted 1:1,000 in PB) overnight. After being rinsed twice in PBS, sections were incubated in a 1:200 dilution of biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) for 30 min and then rinsed again twice in PBS and incubated with Vectastain ABC Elite for 30 min. Color reaction was developed using a solution of hydrogen peroxide-activated, 3,3’-diaminobenzidine tetrahydrochloride (0.2 mg/ml). The sections were washed in Tris-HCl buffer (50 mM; pH 7.6) overnight, dehydrated, dipped in Kodak NTB2 liquid emulsion, and exposed for 1 mo. The sections were then counterstained with toluidine blue and cresyl violet.

Pituitary ACTH release. Adult male Sprague-Dawley rats (200–250 g; Charles River Laboratories) were killed by decapitation between 900 and 1000. Pituitaries were rapidly dissected and the posterior lobes discarded. The anterior pituitaries were transferred in perfusion chambers (1 pituitary/chamber in a volume of 0.5 ml). The superfusion chamber rested in a water bath at 37°C, and the superfusion medium (Dulbecco’s modified eagle’s medium containing 0.1% bovine serum albumin) constantly gassed with 95% O2-5% CO2 was pumped through the chambers at a rate of 100 μl/min by a constant-speed peristaltic pump. After an initial 120-min stabilization period, the overflow from the superfusion chamber was collected during successive 5-min intervals. Basal ACTH release was evaluated over a 30-min period. In a first set of experiments, apelin peptides K17F (10−6 M) and R10F (10−6 M) or potassium (25 mM) alone were then added to the medium for a 15-min period. The samples were collected during 115 min after the beginning of peptides or K+-infusion. For each condition, three to five different chambers were used. In a second set of experiments, after a 120-min equilibration period, basal ACTH release was measured in the medium for 35 min. Next, perfused anterior pituitaries were exposed for 15 min to K+ (25 mM) alone or in the presence of different concentrations of K17F (10−9 to 10−6 M) or R10F (10−6 M). Samples were collected for 115 min as described above. For each condition, three different chambers were used.

ACTH levels in each collected fraction were determined on 0.05 ml using a specific RIA Kit ([1,25I]-ACTH; ICN Biomedicals, Orangeburg, NY). Sensitivity was 10 pg/ml, with intra- and interassay coefficients of variation <10%.
ACTH release was evaluated by determining the area under the curve (AUC) using a commercially available software (Prism 4.0; GraphPad Software, San Diego, CA). AUC of ACTH release was determined using the linear trapezoidal rule. This was calculated by summing the incremental areas of each trapezoid below the effect-time curve. The mathematical representation for AUC is: \( \int_{t_1}^{t_2} \), where \( \Delta t \) is the time interval between the two sampling times and \( H_1 \) and \( H_2 \) are the relative ACTH concentration at times 1 and 2, respectively. The AUC was calculated from 35 to 70 min, and each sampling time interval was 5 min. Data from each condition and all series were pretreated by the difference between the value and the baseline. AUC for each treatment group is expressed as pg·min·ml⁻¹.

Statistical analysis. The statistical significance of the difference between mean values regarding the effects of apelin fragments on ACTH release was determined using one-way ANOVA followed by Dunnett’s multiple comparison test. Statistical significance was set at \( P < 0.05 \).

Image acquisition. Fluorescent images were acquired in frame mode with a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) consisting of a Leica DM IRBE inverted microscope equipped with an argon-krypton laser through a 63×1.2 NA oil immersion objective. Bright-field and dark-field images were acquired with an Olympus microscope and photographed with a CoolSnap camera.

Quantification of double-labeled apelin-ACTH and apelin-GH cells. Quantification of the percentage of apelin immunoreactive cells that also express ACTH or GH was carried out by confocal microscopy (Leica Microsystems, Heidelberg, Germany) with a ×40 oil objective. Confocal microscopic images from double-labeled sections

Fig. 1. Dual-labeling immunofluorescence experiments performed on pituitary sections for apelin and anterior pituitary hormones. A: at low magnification, apelin immunoreactivity (red) is detected in numerous anterior pituitary cells and in the intermediate lobe (IPit) of the pituitary. B: this distribution is similar to that of ACTH immunoreactivity (green). C: no labeling is obtained with the apelin preimmune serum. D: apelin (red) widely colocalizes (yellow) with ACTH (green) in corticotrophs (arrowheads), but some cells contain exclusively apelin (*) or ACTH (>). E: apelin also colocalizes to a lesser extent with growth hormone (GH; green) in somatotrophs (arrowheads). F: apelin colocalizes <1% with luteinizing hormone. In contrast, thyrotropin- (G), prolactin- (H), and follicle-stimulating hormone-immunoreactive anterior pituitary cells (I) are devoid of apelin. Scale bar: A and B, 100 μm; C–J, 25 μm. APit, anterior lobe; PPit, posterior lobe.
were acquired. One dozen images per pituitary that represent the total volume of the pituitary were captured. Each image was the average of four scans (in the xy plane). Confocal images obtained as TIFF files were then analyzed. For each image, the number of apelin-IR cells (detected in red) and then the number of apelin cells that also express ACTH or GH (detected in green) were counted. Results were expressed as means ± SE.

Data presentation. All photomicrographs were prepared using Adobe Photoshop 5.5 for Macintosh (Adobe Systems, Mountain View, CA) by adjusting brightness and/or contrast and were labeled (including scale bars). The data content of the photomicrographs was not altered in any way.

RESULTS

Colocalization of apelin and anterior pituitary hormones. Using a specific and selective polyclonal antibody that recognizes bioactive apelin fragments (K17F and pE13F), but not inactive apelin fragments (R10F and G5F) (7), numerous anterior pituitary cells were immunoreceptor (IR) for apelin (Fig. 1A). The distribution of apelin-immunostained cells was similar to that of corticotrophs, with numerous apelinergic cells being detected in the anterior and intermediate lobes of the pituitary (Fig. 1B). Furthermore, apelin immunoreactivity was homogenously distributed throughout the cytoplasm of anterior pituitary cells (Fig. 1B). No labeling was obtained with the preimmune serum (Fig. 1C).

The phenotype (ACTH, GH, FSH, LH, PRL, or TSH) of apelin-IR pituitary cells was determined in double immunofluorescence experiments. Confocal analysis showed that apelin mainly colocalized with ACTH in corticotrophs, as illustrated in Fig. 1D. A few apelin-IR cells also contained GH (Fig. 1E). Quantification at the confocal level indicated that apelin is predominantly coexpressed by corticotrophs (96.5 ± 0.3% of the total population), to a much lower degree by somatotrophs (3.2 ± 0.2%), and by LH-containing cells (<1%).

Cellular localization of apelin receptor mRNA in corticotrophs. The in situ hybridization protocol (19) including microwave and proteinase K treatments did not lead to successful ACTH detection. Consequently, to improve ACTH immunostaining, the omission of the proteinase K treatment with the maintenance of the microwave treatment was necessary to obtain positive cellular immunolabeling for ACTH after the in situ hybridization.

The sections hybridized with the apelin receptor antisense riboprobe (Fig. 2, A and B) showed a specific signal, whereas no hybridization signal was obtained with the sense riboprobe (Fig. 2, C and D). The observation under a light microscope showed that apelin receptor mRNA was highly expressed in the anterior pituitary (Fig. 2, A and B). A large number of anterior pituitary cells express apelin receptor mRNA (Fig. 2B), as shown by the presence of positive (black arrowheads) and negative (open arrowheads) cells. A labeling with the antisense but not with the sense riboprobes was also detected in the intermediate lobe, whereas the posterior lobe was weakly labeled (Fig. 2A). We (19) previously verified that the ACTH immunohistochemical labeling after in situ hybridization was identical to that obtained by immunohistochemistry alone (not shown).

Combination of in situ hybridization with the apelin receptor antisense (Fig. 3, A–C) and sense (Fig. 3, C and D) riboprobes and immunohistochemistry for ACTH showed that apelin receptor mRNA expression is present in corticotrophs (Fig. 3, B and C). The pattern of the staining was characterized by clusters of silver grains located in ACTH-IR cells (brown precipitate, black arrowheads). However, we observed that all ACTH-IR cells do not express apelin receptor mRNA (Fig. 3B, open arrowheads). Apelin receptor mRNA expression was also detected in non-ACTH-IR pituitary cells, the phenotype of which remains to be determined. In pituitary sections hybr-
ized with the apelin receptor sense riboprobe, silver grains do not decorate ACTH-immunostained cells (Fig. 3, C and D, open arrowheads).

Effect of apelin fragments on basal ACTH release of rat perifused anterior pituitary. To test the hypothesis that apelin could directly stimulate corticotrophs, we examined the effects of apelin on the in vitro basal and stimulated ACTH release from superfused male rat anterior pituitaries. In the first set of experiments, basal ACTH release measured over a 30-min period was 336 ± 37 pg/ml (Table 1). Addition of K17F (10⁻⁶ M) to the medium significantly increased basal ACTH release by 41% (474 ± 21 vs. 336 ± 37 pg/ml, \( P < 0.05 \)), whereas the inactive apelin fragment R10F (10⁻⁶ M) was ineffective (332 ± 47 vs. 336 ± 42 pg/ml; Table 1). In the same experiment, addition of K⁺ (25 mM, for a 15-min period) to the medium rapidly stimulated ACTH release by 78% (600 ± 60 vs. 336 ± 37 pg/ml, \( P < 0.05 \)) over a 30-min period starting at the K⁺ application.

In the second set of experiments, the basal level of ACTH and the effect of K⁺ alone at 25 mM, respectively, are statistically not different compared with that obtained in the first set of experiments. The dose dependency of K17F (10⁻⁹ to 10⁻⁶ M) effects on K⁺-induced ACTH release was evalu-

Table 1. Effects of K17F, R10F, K⁺, and K⁺ combined with K17F (10⁻⁹ to 10⁻⁶ M) on ACTH release from perifused anterior pituitaries

<table>
<thead>
<tr>
<th>Conditions</th>
<th>ACTH levels, pg/ml</th>
<th>ACTH, %relative to basal levels</th>
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<tbody>
<tr>
<td>Basal ACTH release</td>
<td>336 ± 37</td>
<td>100 ± 13</td>
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<tr>
<td>K17F, 10⁻⁶ M</td>
<td>475 ± 21</td>
<td>141 ± 8</td>
</tr>
<tr>
<td>R10F, 10⁻⁶ M</td>
<td>332 ± 47</td>
<td>99 ± 16</td>
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<tr>
<td>K⁺, 25mM</td>
<td>600 ± 60</td>
<td>178.6 ± 10.4</td>
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<tr>
<td>K⁺ + K17F, 10⁻⁹ M</td>
<td>608 ± 34</td>
<td>180.8 ± 6.5</td>
</tr>
<tr>
<td>K⁺ + K17F, 10⁻⁸ M</td>
<td>618 ± 18</td>
<td>183.8 ± 13.9</td>
</tr>
<tr>
<td>K⁺ + K17F, 10⁻⁷ M</td>
<td>740 ± 47</td>
<td>220.3 ± 14.7</td>
</tr>
<tr>
<td>K⁺ + K17F, 10⁻⁶ M</td>
<td>1,016 ± 50</td>
<td>302.5 ± 17.9</td>
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ACTH levels are means ± SE of 3–5 chambers and are expressed in pg/ml or as %relative to mean basal. After a 120-min equilibration period, ACTH release in the medium by perifused rat anterior pituitaries was measured for 30 min (basal release) or after exposure (15 min) to K17F (10⁻⁶ M), R10F (10⁻⁶ M), K⁺ (25 mM) alone, or K⁺ combined with K17F (10⁻⁹ to 10⁻⁶ M). Basal ACTH release (pg/ml) was calculated as the means of basal levels measured every 5 min over a 30-min period starting at the beginning of the peptide or K⁺ application. *\( P < 0.05 \); *non-significant vs. basal level; \( P < 0.05 \); \( P < 0.001 \); *non-significant vs. K⁺ alone.
A significant stimulatory effect of K17F on K⁺-evoked ACTH release was obtained only for concentrations of 10⁻⁷ and 10⁻⁶ M (AUC between 35 and 70 min compared with that of K⁺ 25 mM alone; Fig. 5). At 10⁻⁸ M, the slight increase in ACTH release was not significant. The same profiles of ACTH release were observed in presence of K⁺ alone, K⁺ + 10⁻⁹ M K17F, and K⁺ + 10⁻⁶ M R10F (Fig. 4).

**DISCUSSION**

In the present study, we provided evidence for the existence of an apelinergic system within the adult rat pituitary gland. Apelin immunoreactivity is mainly colocalized with ACTH and to a lesser extent with GH, and apelin receptor mRNA is expressed by corticotrophs. Using a paradigm of anterior pituitary perfusion, apelin 17, but not apelin 10, significantly increases basal ACTH release and dose dependently potentiates the K⁺-evoked ACTH release. Thus, using morphological and pharmacological approaches, apelin present in corticotrophs or somatotrophs exerts a stimulatory action on ACTH release in an autocrine/paracrine fashion.

Using a polyclonal antiserum with high affinity and selectivity for K17F and pE13F, we observed in the anterior pituitary the presence of highly stained apelin-IR cells. This localization is in agreement with the high expression of proapelin mRNA in the rat pituitary (18) and apelin immunoreactivity detected with an antiserum directed against human apelin 36 (3). In addition, the distribution of apelinergic cells within the entire pituitary is similar to that of corticotrophs. Next, using double fluorescence immunohistochemistry, we determined the phenotype of apelin-IR anterior pituitary cells. By confocal analysis, we visualized apelin-IR staining mostly in anterior pituitary endocrine cells containing ACTH and, to a lesser extent, GH. In contrast, no apelin immunoreactivity was found in LH-, PRL-, FSH-, and TSH-containing cells pointing out on a predominant colocalization of apelin with ACTH in the...
anterior pituitary. Apelin is not the only “hypothalamic” peptide that colocalizes with both hormones. Three other peptides have also been detected within ACTH- and GH-containing cells, such as galanin (6, 37), neuromedin U (6), and leptin (41). Interestingly, these peptides are involved in the regulation of food intake (13, 17, 28), and adipocyte apelin mRNA expression was recently reported to be increased in various mouse models of obesity associated with hyperinsulinemia (2). Moreover, a positive correlation between plasma apelin levels and the body mass index, as already known for leptin (see review in Ref. 4), and an involvement of apelin in the control of food intake have been recently described (39).

By in situ hybridization using radioactive riboprobes, we then studied the expression of apelin receptor mRNA within the anterior pituitary. In agreement with previous data (7, 23), we found a high expression of apelin receptor mRNA in the anterior pituitary. A labeling was also detected in the intermediate pituitary, whereas the posterior pituitary was poorly labeled. Since apelin immunoreactivity is primarily localized within corticotrophs, we evaluated whether apelin receptors are synthesized by these cells. For this purpose, using in situ hybridization for the detection of apelin receptor mRNA in combination with immunohistochemistry for ACTH, we observed that apelin receptor mRNA, like vasopressin type 1B (20), angiotensin type 1B (19), and CRH type 1 (29) receptors, are synthesized by corticotrophs. By analyzing the double-labeled pituitary coronal sections, we cannot exclude that another type of endocrine cells (gonadotrophs, thyrotrophs, lactotrophs, and/or somatotrophs) could also express apelin receptor mRNA, which remains to be defined.

The fact that apelin is detected primarily by corticotrophs, and to a lesser extent by somatotrophs, and that apelin receptor mRNA is expressed by corticotrophs suggests that apelin in the anterior pituitary may directly or indirectly stimulate ACTH secretion via an autocrine or paracrine fashion.

To test this hypothesis, we evaluated the effects of apelin fragments (active K17F and nonactive R10F) on basal or K+-evoked ACTH release from adult male rat-perifused anterior pituitaries. An advantage of the perfusion system is the conservation of the anterior pituitary integrity. Perfusion studies also have the advantage of allowing analysis of the mechanisms that regulate ACTH secretion in the absence of the complexities of feedback control. We first verified that the basal ACTH levels obtained in our experimental conditions were similar to those previously published in the literature (10, 22, 27). We then observed that K17F significantly stimulates basal ACTH release by 41%, whereas R10F at the same dose was inactive. Thus apelin, similarly to other vasoactive peptides (angiotensin II, vasopressin), and CRH directly stimulate basal ACTH release via their receptors expressed by corticotrophs. In addition, K17F, but not R10F, significantly potentiated the K+-evoked ACTH release. ACTH release in anterior pituitary has been shown to result from the opening of L-type Ca2+ channels and subsequently extracellular Ca2+ influx (16, 38). We can hypothesize that apelin, by acting through its receptors, increases intracellular Ca2+ concentrations, thereby inducing ACTH release, knowing that numerous studies (7, 9, 21, 30) have reported that the apelin receptor stably expressed in eukaryotic cells is negatively coupled to adenylate cyclase, whereas others (5, 21) performed on NTera 2 human teratocarcinoma cells and on RBL-2H3 cells have shown that apelin 36, K17F, and pE13F increase intracellular calcium mobilization. Further investigations are needed to determine the mechanism by which apelin potentiates K+-evoked ACTH release.

![Fig. 5. Effects of K17F on K+-evoked ACTH release from perifused rat pituitary. The results are expressed as means ± SE of area under the curve (AUC; expressed in pg·min·ml⁻¹). AUC is the integral of the ACTH release calculated between 35 and 70 min (as shown in Fig. 4). Statistical differences were assessed using 1-way ANOVA followed by Dunnett’s multiple comparison test, with a threshold of significance set at P < 0.05. *P < 0.05 vs. K+-evoked ACTH release; NS, nonsignificant.](http://ajpendo.physiology.org/Downloadedfrom)
ACTH secretion, which reflects the activity of the hypothalamo-pituitary-adrenal (HPA) axis, is regulated by hypothalamic, circulating, and adrenohypophysal peptides (34). Thus apelin, present in the hypothalamus, the blood circulation, and the anterior pituitary could stimulate ACTH release by reaching corticotrophs via portal vessels, systemic circulation, or acting locally.

Administration of apelin by the intracerebroventricular route was shown to significantly increase plasma ACTH (39) and corticosterone (15, 39) release at least in part via a stimulatory action on CRH release (39). In a latter study (40), pE13F (100 nM) significantly stimulated the release of CRF and AVP from hypothalamic explants in vitro. From these experiments and from our work it appears that apelin increases ACTH secretion by a direct action in the anterior pituitary and an indirect action via stimulation of CRH release in the hypothalamus (Fig. 6).

Pituitary endogenous apelin (principally secreted by corticotropic and to a lesser extent by somatotrophs) stimulates ACTH release by a direct autocrine/paracrine action on corticotrophs via apelin receptors synthesized by these cells. Thus apelin, like angiotensin II/III (36) and AVP (1, 14), originating from the hypothalamus or from the pituitary gland itself, could stimulate indirectly or directly anterior pituitary ACTH release.

In agreement with this model, it has been shown (24) that, in rats submitted to acute stress (restraint stress), known to increase the activity of the HPA axis, apelin receptor mRNA expression was increased in the paravascular division of the PVN. More recently, Wei et al. (42) reported that dexamethasone, a glucocorticoid agonist, drastically decreased apelin mRNA levels in 3T3-L1 mouse adipocytes, whereas in adrenalectomized rats apelin receptor mRNA expression was increased (24), suggesting that glucocorticoids downregulated the expression of apelin and its receptor.

In conclusion, our study provides definitive evidence for the existence of a pituitary apelinergic system in the adult male rat. We outlined the potential role of apelin as a modulator of ACTH secretion in an autocrine/paracrine fashion and provided morphological basis for further studies exploring the physiological role of apelin in the regulation of the HPA axis.

ACKNOWLEDGMENTS

The expert technical assistance of M.-T. Morin is greatly acknowledged. We are very grateful to Dr. J.-M. Gasc for advice and encouragement. We thank the National Hormone and Pituitary Program and the NIDDK. We are grateful to Dr. D. Grouselle for kindly providing the GH antibody.

GRANTS

This study was supported by the Institut National de la Santé et de la Recherche Médicale.

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