Regulation of rat pituitary cocaine- and amphetamine-regulated transcript (CART) by CRH and glucocorticoids


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Stanley, S. A., K. G. Murphy, G. A. Bewick, W. M. Kong, J. Opacka-Juffry, J. V. Gardiner, M. Ghatel, C. J. Small, and S. R. Bloom. Regulation of rat pituitary cocaine- and amphetamine-regulated transcript (CART) by CRH and glucocorticoids. Am J Physiol Endocrinol Metab 287: E583–E590, 2004.—Cocaine- and amphetamine-regulated transcript (CART) cDNA was originally isolated from rat brain by polymerase chain reaction differential display as a transcript whose expression was upregulated by acute administration of cocaine or amphetamine (15). CART mRNA is highly conserved across species (14, 34), suggesting that CART plays an important physiological role. CART has been reported as a hypothalamic satiety factor (21, 23), although more recently, intranuclear injection of the active fragment CART-(55–102) has been demonstrated to increase food intake (1). However, CART immunoreactivity (CART-IR) and mRNA are widespread throughout the central nervous system and also the periphery (12, 19, 20, 30), including the pituitary gland. This suggests that CART may have physiological roles beyond appetite regulation.

COCAINE- AND AMPHETAMINE-REGULATED TRANSCRIPT (CART) cDNA was originally isolated from rat brain by polymerase chain reaction differential display as a transcript whose expression was upregulated by acute administration of cocaine or amphetamine (15). CART mRNA is highly conserved across species (14, 34), suggesting that CART plays an important physiological role. CART has been reported as a hypothalamic satiety factor (21, 23), although more recently, intranuclear injection of the active fragment CART-(55–102) has been demonstrated to increase food intake (1). However, CART immunoreactivity (CART-IR) and mRNA are widespread throughout the central nervous system and also the periphery (12, 19, 20, 30), including the pituitary gland. This suggests that CART may have physiological roles beyond appetite regulation.

There is both anatomic and functional evidence that hypothalamic CART peptide is involved in the regulation of the hypothalamo-pituitary function and, in particular, the hypothalamo-pituitary-adrenal (HPA) axis. CART mRNA and CART-IR have been colocalized with neurotransmitters known to modulate the HPA axis. Proopiomelanocortin (POMC) mRNA and CART mRNA (17) are highly coexpressed in the arcuate nucleus (ARC), and CART mRNA and melanin-concentrating hormone are colocalized in the lateral hypothalamus (7). CART peptide is also present in the paraventricular division of the paraventricular nucleus (PVN), an area containing corticotropin-releasing hormone (CRH)-expressing neurons (10). ARC CART expression is regulated by the glucocorticoid environment (4). In vitro, CART peptide stimulates the release of CRH from ex vivo hypothalamic explants (31), and intracerebroventricular injection of CART-(55–102) increases the expression of the early gene, c-fos, in CRH-containing PVN neurons (35) and raises circulating adrenocorticotropin hormone (ACTH) and corticosterone (CORT) (31, 35). CART-IR is released into the hypophysial-portal circulation (24) and may modulate pituitary hormone release.

Little is known of the localization or function of anterior pituitary CART. CART is both synthesized and stored in the anterior pituitary, as demonstrated by the presence of CART mRNA and high concentrations of CART-IR (26). Although Western blotting has demonstrated that there are at least five CART immunoreactive species in the whole pituitary (22), the predominant form in the anterior pituitary is CART-(55–102) (33). CART-IR cells in the rat anterior pituitary are found in discrete clusters (20), but the identity of these CART-IR-containing pituitary cells remains ill defined. Similarly, the factors regulating pituitary CART remain to be determined.

Because hypothalamic CART is modulated by the HPA axis, we hypothesized that pituitary CART is also influenced by the HPA axis. We have examined the effects of CRH and of the glucocorticoid corticosterone on pituitary CART expression, synthesis, and release in vitro and in vivo. The colocalization between pituitary CART mRNA and ACTH expression was partly localized to corticotrophs.

METHODS

Materials

Rat CART-(55–102) was purchased from the Peptide Institute (Osaka, Japan). Human CRH and CORT were supplied by Sigma

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Chemical. All tissue culture materials were provided by GIBCO (Life Science Technology, Paisley, Renfrewshire, UK).

Animals

Male Wistar rats (specific pathogen free; Imperial College School of Medicine, London, UK) weighing 250–300 g were maintained in groups of five for pituitary quarter studies and diurnal variation studies. Male Wistar rats (Charles River, Margate, Kent, UK) were individually housed for studies to examine the effect of intraperitoneal CRH. Male sham-operated control and adrenalectomized Wistar rats, weighing 200–300 g, were obtained from Charles River. Adrenalectomy was carried out by the supplier. Animals were maintained in individual cages for 14 days. Adrenalectomized rats were maintained on oral 0.9% sodium chloride. A nonoperated group received CORT (25 μg/ml dissolved in 1 ml of ethanol and then in water, orally) for 14 days.

All animals were maintained under controlled temperature (21–23 °C) and light (12:12-h light-dark cycle, and lights on at 0700) with ad libitum access to food (RM1 diet, SDS, Witham, UK) and water. Animal procedures were approved by the British Home Office Animals Scientific Procedures Act 1986 (Project licenses 90/1077 and 70/8888).

Clonal Cell Line Culture

The corticotroph cell line AtT-20/D16–16 (16) was grown in 175-ml culture flasks in DMEM (GIBCO) with 0.1 g/l sodium pyruvate, 4.5 g/l glucose, 100 IU/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), and 10% FCS (GIBCO). The cells were maintained in a humidified atmosphere at 37 °C with 95% O2-5% CO2. The culture medium was replaced with fresh DMEM on alternate days, and the cells were subcultured, at one in four, once a week, by use of 0.25% trypsin.

Study 1: Effect of CRH on CART-IR Release in Vitro

The effects of CRH on pituitary CART-IR release were determined using anterior pituitary segments. The method was a modification of that previously described (9). Rats were decapitated, and anterior pituitary glands were harvested immediately and then divided into four pieces of approximately equal size. The segments were randomly placed (1 segment/well) in the wells of a 48-well tissue culture plate (Nunc International, Roskilde, Denmark) and incubated in 500 μl of artificial cerebrospinal fluid [αCSF: 20 mM NaHCO3, 126 mM NaCl, 0.09 mM Na2HPO4, 6 mM KCl, 1.4 mM CaCl2, 0.09 mM MgSO4, 5 mM glucose, 0.18 mg/ml ascorbic acid, and 100 μg/ml aprotinin (BDH, Poole, Dorset, UK)]. The anterior pituitary segments were maintained at 37 °C in a humidified environment saturated with 95% O2-5% CO2 for 2 h, with the medium changed after 1 and 2 h. The segments were then incubated in αCSF alone or CRH (100 nM) for 4 h. At the end of this period, the αCSF was collected and stored at −20 °C until radioimmunoassay (RIA) for CART. The study was repeated on three occasions.

Study 2: Effect of CRH on Plasma CART-IR in Vivo

The acute action of CRH on plasma CART-IR was assessed in vivo in male Wistar rats. All studies were carried out during the light phase (0930–1100). Rats were intraperitoneally injected with 1 ml of saline for 3 days to acclimatize them to the injection procedure; then they also underwent sham decapitation for 2 days before the study day. On the following day, rats (n = 10/group) were intraperitoneally injected with either saline or 10 μg of CRH (32). At 10, 20, or 60 min after injection, animals were killed by decapitation, and trunk blood was collected into plastic tubes containing potassium EDTA (final concentration of 1.2–2 mg EDTA/ml blood; Sarstedt, Leicester, UK). Plasma was separated by centrifugation, frozen on dry ice, and stored at −70 °C until measurement of CART-IR and CORT byRIA and ACTH by immunoradiometric assay (IRMA).

Study 3: Effect of Endocrine Manipulation on CART Expression and Content

Adrenal manipulation results in long-term modulation of hypothalamic CRH expression and release. The effects of long-term alterations in CRH on pituitary CART expression, content, and release were assessed in sham-operated control, adrenalectomized, and CORT-treated rats (n = 15 per group).

Fourteen days after surgery, animals were killed by decapitation. Pituitary glands were harvested, snap-frozen, and then used for RNA extraction and Northern blot analysis of CART expression (n = 7) or individual frozen anterior pituitary glands using TRI-Reagent (Helena Biosciences, Sunderland, UK) according to the manufacturer’s protocol. The RNA concentration was determined by absorbance at 260 nm and the RNA reprecipitated and resuspended in glass-distilled water to give a final concentration of 5 mg/ml.

Fifty micrograms of total RNA from each tissue were size-separated on a denaturing MOPS [3-(N-morpholino)propanesulfonic acid]/formaldehyde gel (1% agarose) and transferred to a Hybond-N membrane (Amersham International, Buckinghamshire, UK). The RNA was fixed by baking at 80 °C for 2 h before a riboprobe corresponding to nucleotides 101 to 411 of CART cDNA was used as probe. The riboprobe was synthesized using [α-32P]CTP (Amersham International) and T7 RNA polymerase (Promega, Southampton, UK). Hybridization was carried out overnight at 55 °C in 5X standard saline citrate (SSC; 1X SSC contains 0.15 M sodium chloride and 15 mM sodium citrate), 5X Denhardt’s solution, 50% deionized formamide, 100 μg/ml of denatured sonicated herring sperm DNA, and 100 μg/ml of yeast tRNA with 1.5 MBq radiolabeled probe. Nonspecific hybridization was removed by increasingly stringent washes, the final one being in 0.1X SSC/0.1% SDS at 70 °C for 30 min. The filter was then exposed to a phosphorimager screen, and radiolabeled bands were quantified by image densitometry with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The data were normalized by oligo(DT) probing, as previously described (29). Results are expressed in arbitrary units calculated as a ratio of the CART mRNA signal to the oligo(DT) signal.

Study 4: Diurnal Variation in Plasma CART and ACTH

The diurnal variation in plasma CART-IR and correlation with plasma ACTH and CORT were determined. For 2 days before the study, animals were acclimatized to the guillotine apparatus to minimize the hormonal consequences of stress on the study day. On the study day, animals were decapitated, and trunk blood was collected at 8 time points: 0700, 1100, 1500, 1900, 2200, 0100, 0400, and 0700 (n = 8–9 per group). Plasma was separated and stored as described for study 3 until assay for CART-IR, ACTH, and CORT.

Study 5: CART Release From AtT-20 Cells

To determine whether CART-IR might be released from pituitary corticotrophs, the CART-IR content of medium was determined from...
The cells had reached confluence. When the cells had reached confluence, the medium was aspirated and separated from loose cells and debris by low-speed centrifugation. Conditioned medium (4 ml) and control medium (4 ml) were decanted and resuspended in 250 μl of 0.06 M phosphate buffer for determination of CART-IR content by RIA and ACTH content by IRMA. The study was repeated on four occasions.

**Study 6: Dual Immunocytochemistry and in Situ Hybridization**

Dual in situ hybridization (ISH) and immunocytochemistry (ICC) were performed to determine whether CART mRNA and ACTH-IR were colocalized in native pituitary glands.

**ICC.** Rats were decapitated and the pituitary glands quickly removed. Whole pituitary glands were maintained in Bouin’s fixative for 24 h before being transferred to 70% ethanol. The pituitary glands were paraffin-embedded, and sections were cut using an AS500 microtome (Anglia Scientific, Cambridge, UK) and mounted on polylysine-coated slides (VWR International, Poole, UK). Paraffin sections were dewaxed in xylene and rehydrated through graduated ethanol. Endogenous peroxidase was blocked by incubation for 15 min with a solution of 1% hydrogen peroxide. Antigen retrieval was performed by microwaving sections in 0.01 M citrate buffer, pH 6.0, for 10 min at 800 W. Sections were incubated for 10 min at room temperature with normal donkey serum diluted 1:20 in 0.01 M PBS, 0.1% sodium azide, and 0.1% BSA (Jackson Immuno Research, West Grove, PA). The rabbit anti-ACTH antibody (Peninsula Labs, Belmont, CA) was diluted 1:1,000 in 0.01 M PBS, 0.1% sodium azide, and 0.1% BSA, and sections were incubated overnight at 4°C. For negative controls, the primary antibody was replaced with PBS (data not shown). Sections were then incubated in biotinylated donkey anti-rabbit (Jackson Immuno Research) diluted 1:50 for 30 min at room temperature before being rinsed in Tris-buffered saline and incubated with an avidin-biotin complex-horseradish peroxidase kit (Dako, High Wycombe, UK) at room temperature for 30 min. The chromagen was developed with 3-amino-9-ethylcarbazole (Sigma Chemical).

**ISH.** A rat CART riboprobe was used for the detection of preproCART (ppCART) mRNA in rat pituitary. The CART probe was directed to nucleotides 1065 to 1374 (accession no. NM031569) by primers 5’-TGA GAA CTT CAA GGC CTG CT and 3’-TCT TCC AGT GAC CTG CT and 3’-TCT TCC AGT GAC CTG CT and 3’-TCT TCC AGT GAC CTG CT and 3’-TCT TCC AGT GAC CTG CT. Linearized DNA was transcribed using T7 polymerase and T3 polymerase (Promega, Madison, WI) to produce antisense and sense riboprobes, respectively, labeled with 35S CTP (Amersham Biosciences UK, Little Chalfont, Bucks, UK). Immediately after chromagen development in the ICC protocol, the slides were washed in 0.1 M PBS, incubated in 1 μg/ml of proteinase K for 30 min at 37°C, acetylated in 0.25% acetic anhydride for 10 min at room temperature, and dried under vacuum. Sections were hybridized at 60°C with 35S CART riboprobes (7 × 106 counts/ml) overnight, washed, and then exposed to BioMax MR scientific imaging film (Eastman Kodak, Rochester, NY). Film was developed 3 days later to determine the hybridization signal, and slides were dipped in Ilford Scientific G5 emulsion (Ilford Imaging UK, Mobberley, Cheshire, UK) and exposed for 10 days at 4°C before development. Slides were overlapped and observed for colocalization using Nikon Eclipse E800 microscope linked to a Xillix Micromager PMI and Image Pro-Plus 4.0 software. Percentage colocalization was determined by counting cells in eight matched sections from the rat pituitary (n = 2). Cells were considered colocalized if they exhibited >10% of the background level of silver grains overlaying them.

**RIAs**

An RIA directed against CART-(55–102) was developed. Briefly, the peptide was purchased from Peptide Institute. The antiserum was raised in rabbit immunized with human CART-(55–102) peptide conjugated to BSA by glutaraldehyde (28). A New Zealand white rabbit was initially inoculated with 200 μg of peptide conjugate [200 μl of 0.25% glutaraldehyde plus 20 nmol of CART-(55–102)] mixed overnight with 5 nmol BSA] mixed with an equal volume of complete Freund’s adjuvant. Inoculations were administered at two sites subcutaneously. Boosts consisted of 100 μg of peptide conjugate in an equal volume of incomplete Freund’s adjuvant and were administered subcutaneously along the flanks. Boosts were given 70 and 180 days after initial inoculation. Test bleeds were performed 60, 80, 170, and 210 days after initial inoculation, production bleeds 220 days after initial inoculation, and exsanguination bleeds 250 days after initial inoculation. The antiserum showed full cross-reactivity with synthetic rat and mouse CART-(55–102) (Peptide Institute). The 125I-labeled synthetic CART-(55–102) was prepared by the direct iodogen method and purified by reverse-phase HPLC on a C18 (Waters) column over a 15–45% 90-min gradient of acetonitrile (ACN)-water-0.1% TFA.

The assay was performed in a total volume of 350 μl of 0.06 M phosphate buffer, pH 7.2, at 4°C for 3 days. The antibody-bound label was then separated from free label by addition of 250 μl of a charcoal suspension containing 4 mg of charcoal and 0.4 mg of dextran (average mol wt, 70,000; Sigma). The tubes were centrifuged at 2,500 g for 20 min at 4°C followed by immediate separation of the supernatant.

The assay had a sensitivity of 1.25 ± 0.5 (SE) fmol/tube, n = 4, with 95% confidence. The midrange was 16.2 ± 0.9 fmol/tube, n = 8. Inter- and intra-assay variations were established to be 15 ± 1 and 7.8 ± 0.7%, respectively, n = 5. Commercially available CART fragments were tested for cross-reactivity. The assay showed 20% cross-reaction with CART-(61–102) and CART-(62–102) and <0.1% cross-reaction with CART-(1–39) (Phoenix Pharmaceuticals, Belmont, CA). The assay showed <0.1% cross-reaction with ≤1 nmol/tube of agouti-related peptide, arginine vasopressin, bombesin, brain natriuretic peptide, calcitonin gene-related peptide, follicle-stimulating hormone, galanin, glucagon, growth hormone-releasing hormone, neuropeptide Y, oxytocin, prolactin, prolactin-releasing peptide, somatostatin, and substance P. Recovery of CART-(55–102) from liver homogenate was >85%. The specificity of freshly prepared CART-(55–102) peptide label, as estimated by self-displacement in the assay, was 40.8 Bq/fmol. A standard curve of 10–50 fmol/tube of CART-(55–102) was set up in conjunction with dilution curves of hypothalamus, stomach, anterior pituitary, and posterior pituitary extracts to assess their parallelism (n = 5). The resulting binding curves were almost parallel (Fig. 1).

Anterior pituitary tissue extracts (n = 3) were fractionated as previously described (26) by high-resolution reverse-phase FPLC with a (Pep RPC HR 5/5) C18 column (Pharmacia, Uppsala, Sweden). The elution profile showed a single peak of CART-(55–102) immunoactivity (Fig. 1).
Plasma CART-IR was significantly increased compared with measured in response to intraperitoneal administration of CRH.

Study 2: Effect of CRH on CART-IR Release in Vivo

CART-IR was detected in aCSF after short-term incubation of anterior pituitary segments. Under basal conditions, CART-IR release was 0.52 ± 0.18 fmol·explant⁻¹·h⁻¹. However, incubation with CRH for 4 h significantly increased pituitary CART-IR release 15-fold (100 nM CRH, 7.81 ± 1.02 fmol·explant⁻¹·h⁻¹, P < 0.001 vs. basal, n = 23; Fig. 2).

Study 2: Effect of CRH on CART-IR Release in Vivo

To determine whether CRH released pituitary CART-IR acutely in vivo, plasma ACTH, CORT, and CART-IR were measured in response to intraperitoneal administration of CRH. Plasma CART-IR was significantly increased compared with saline controls at both 20 min (plasma CART-IR at 20 min: saline 45.1 ± 3.0 pmol/l, CRH 64.8 ± 4.0 pmol/l, P < 0.05, n = 8–10) and 60 min after CRH injection (plasma CART-IR at 60 min: saline 49.5 ± 3.0 pmol/l, CRH 90.7 ± 4.4 pmol/l, P < 0.01, n = 8–10; Fig. 2A). As expected, ACTH was significantly increased compared with saline controls at all time points (plasma ACTH at 10 min: saline 39.2 ± 4.2 ng/ml, CRH 59.7 ± 6.6 ng/ml, P < 0.05; plasma ACTH at 20 min: saline 40.6 ± 5.0 ng/ml, CRH 67.3 ± 6.7 ng/ml, P < 0.01; plasma ACTH at 60 min: saline 35.5 ± 1.9 ng/ml, CRH 74.2 ± 7.7 ng/ml, P < 0.001; Fig. 2B). CORT was also significantly increased in CRH-treated animals compared with control at all time points (plasma CORT at 10 min: saline 181 ± 31 ng/ml, CRH 348 ± 37 ng/ml, P < 0.001; plasma CORT at 20 min: saline 64 ± 10 ng/ml, CRH 466 ± 17 ng/ml, P < 0.001; plasma CORT at 60 min: saline 16 ± 5 ng/ml, CRH 610 ± 26 ng/ml, P < 0.001). There is a significant positive correlation between plasma CART-IR and ACTH (r = 0.49, P < 0.001) and between plasma CART-IR and CORT (r = 0.72, P < 0.001).

Study 3: Effect of Endocrine Manipulation on CART Expression and Content

The influence of the glucocorticoid environment on pituitary CART was examined in adrenal-manipulated animals. Pituitary CART mRNA, CART-IR content, and circulating CART-IR were measured in control, adrenalectomized, and CORT-treated animals. Plasma CORT levels were measured to ensure appropriate manipulation (plasma CORT in control 14.6 ± 5 ng/ml, in CORT-treated 196 ± 21 ng/ml, and in adrenalectomized not detected).

CART expression. CART expression in the anterior pituitary gland was significantly decreased by supraphysiological replacement with CORT (pituitary CART mRNA: control 6.76 ± 0.9, CORT-treated 4.59 ± 0.3 relative units, P < 0.05 vs. control, n = 7). CART expression was not significantly increased in the anterior pituitary glands from adrenalecto-
mized animals (pituitary CART mRNA: control 6.76 ± 0.9, adrenalectomized 7.89 ± 0.56 relative units, P = not significant, n = 7; Fig. 4A).

**CART-IR Content.** The pituitary gland content of CART peptide was determined in anterior pituitary lobes harvested from endocrine-manipulated animals. CART-IR was significantly increased in the anterior pituitary lobes from adrenalectomized rats compared with sham-operated animals (pituitary CART-IR; control 5.0 ± 1.4 pmol/gland, adrenalectomized 11.5 ± 1.7 pmol/gland, P < 0.05 vs. control, n = 8). Pituitary CART-IR content was significantly suppressed in those animals treated with high levels of CORT (pituitary CART-IR; control 5.0 ± 1.4 pmol/gland, CORT-treated 1.1 ± 0.4 pmol/gland, P < 0.05 vs. control, n = 8; Fig. 4B).

**Plasma CART-IR.** Circulating concentrations of CART-IR reflected the changes in pituitary CART-IR. Plasma CART-IR was significantly increased in adrenalectomized animals compared with sham-operated controls (plasma CART-IR: control 48.8 ± 3.0 pmol/l, adrenalectomized 69.2 ± 8.1 pmol/l, P < 0.05; n = 13–15). Conversely, chronic supraphysiological CORT treatment significantly reduced circulating CART-IR (plasma CART-IR: control 48.8 ± 3.0 pmol/l, CORT-treated 30.7 ± 3.1 pmol/l, P < 0.05; n = 13–15; Fig. 4C).

**Study 4: Diurnal Variation in Plasma CART**

Plasma CART-IR varied significantly throughout the day, with circulating levels significantly greater at 1500, 2200, and 0100 compared with plasma concentrations at 0700 (plasma CART-IR: 0700 94.7 ± 4.9 pmol/l, 1500 124.5 ± 6.5 pmol/l, 2200 146.6 ± 8.4 pmol/l, and 0100 128.7 ± 12.1 pmol/l, P < 0.05; Fig. 5). Plasma CORT was significantly elevated at 1900 and 2200 compared with the nadir at 0700 (plasma CORT: 0700 39.3 ± 15.3 ng/ml, 1900 258.4 ± 31.6 ng/ml, 2200 262.2 ± 40.8 ng/ml, P < 0.05). There was significant correlation between plasma CART-IR and CORT (plasma CART-IR and CORT: r = 0.329, P < 0.01). Although there was a significant correlation between plasma CART-IR and ACTH (r = 0.569, P < 0.001), correlation between plasma CART-IR and ACTH failed to reach significance.
Study 5: CART Release from AtT-20 Cells

CART-IR was detectable in the medium of the corticotroph cell line AtT-20 but not in nonconditioned medium (culture medium CART-IR: AtT-20 1.73 ± 0.54 fmol·ml⁻¹·24 h⁻¹). ACTH was detectable in the conditioned medium (culture medium ACTH: 24.3 ng·ml⁻¹·24 h⁻¹).

Study 6: Dual ICC and ISH

Sections of the anterior and neurointermediate lobes of the pituitary were inspected for evidence of ppCART mRNA expression. Silver grains indicative of ppCART mRNA expression were found in clusters throughout the anterior pituitary. ppCART mRNA was prevalent in distinct areas of the anterior pituitary, and an oval-shaped nucleus was apparent in each wing of the anterior pituitary and also around its periphery (Fig. 6A). Dual ICC for ACTH and ISH for ppCART demonstrated a subpopulation of ACTH-IR cells that expressed pp-CART. Of these ppCART-expressing cells, 28% of cells were found to colocalize with ACTH-IR (Fig. 6, B, C, and D).

DISCUSSION

The regulation and role of CART in the hypothalamus have been extensively studied. There is strong evidence that CART peptide acts as a neurotransmitter in the control of hypothalamo-pituitary function. CART-IR is found in the dense core vesicles of dendrites (30), and its release from ex vivo hypothalami is calcium dependent (26). Intracerebroventricular CART administration increases plasma ACTH and corticosterone (31, 35) and also induces anxiety-like behavior (11). Thus hypothalamic CART and the HPA axis appear to be closely linked. However, little is known of the distribution or regulation of CART in the pituitary and whether either is also influenced by the HPA axis.

Previous studies have shown CART mRNA and CART peptide to be located within the same anterior pituitary cells.

Fig. 6. Dual immunocytochemistry for ACTH and in situ hybridization for prepro-CART (ppCART) on sections of anterior lobe of the pituitary. Silver grains indicate ppCART mRNA expression, and red staining demonstrates ACTH immunoreactivity. Magnification: ×40 (A), ×200 (B), ×400 (C and D).
CART-(79–102) developed a sensitive assay directed to CART-(55–102) and, using this, determined the effects of CRH and glucocorticoids on pituitary CART synthesis and release. This assay does show some cross-reactivity with CART-(62–102). However, Thim et al. (33) extracted only one form of CART from the anterior pituitary, CART-(55–102), and Murphy et al. (26) previously demonstrated one major CART-IR peak from anterior pituitary gland extract by use of fast performance liquid chromatography. CART-(55–102) is therefore likely to be the major endogenous form of CART peptide in the anterior pituitary. The CART-IR detected by our assay from the anterior pituitary is therefore likely to be CART-(55–102).

The effects of CRH, the primary hypothalamic regulator of the HPA axis, on pituitary CART-IR release were examined. In vitro, anterior pituitary fragments released CART-IR into the bathing aCSF, and this release was significantly increased by CRH. The effects of CRH on CART-IR release were also examined in vivo: acutely, in response to intraperitoneal CRH, and chronically by examination of CART expression, content, and circulating levels in adrenal-manipulated animals. Acute administration of CRH increased plasma ACTH and CORT after 10 min, whereas plasma CART-IR was significantly increased after 20 min and this effect was maintained at 60 min. Pituitary CART mRNA, pituitary CART peptide content, and circulating CART levels were examined in adrenalectomized and glucocorticoid-treated rats. Adrenalectomy significantly increased pituitary CART-IR peptide content and circulating levels, and exogenous corticosterone significantly reduced pituitary CART expression, synthesis, and release. To determine whether CART-IR and ACTH were coregulated under physiological conditions, the diurnal variation in circulating CART-IR and its relationship to ACTH and CORT were examined. Circulating CART-IR varied over the course of a day, with low levels at 7 AM rising to significantly higher levels at 7 and 10 PM. This profile was comparable to that of plasma CORT. Moreover, there was a significant correlation between plasma CART-IR and plasma CORT.

Adrenalectomy increased hypothalamic CRH immunoreactivity sevenfold (25). Previous studies have demonstrated that adrenalectomy also results in a two- to threefold increase in corticotroph number, a doubling of corticotroph size, and an increase in pituitary ACTH content (18). Conversely, supra- physiological concentrations of exogenous corticosterone significantly suppress hypothalamic CRH and reduce pituitary corticotroph number and ACTH content (2, 3), CRH mediates its actions via CRH R1 receptors in the anterior pituitary by using cAMP as a second-messenger system. Barrett et al. (5) have previously shown pituitary CART mRNA expression to be upregulated in response to cAMP. However, CART mRNA may also be responsive to circulating glucocorticoids, independent of CRH. Modulation of hypothalamic CART expression in adrenalectomized animals (36) suggests that the CART gene may contain a glucocorticoid response element.

Given the regulation of pituitary CART by CRH and glucocorticoids, we examined the expression of CART in pituitary corticotrophs. Using dual ICC and ISH, we have shown that ppCART mRNA is colocalized to a subpopulation of ACTH-immunoreactive cells. Furthermore, the corticotroph AtT-20 cell line releases CART-IR. These findings suggest that at least some pituitary CART is localized and released from pituitary corticotrophs under the control of CRH and glucocorticoids.

There has been little work examining the identity of pituitary cells containing CART. Barrett et al. (6) demonstrated CART mRNA in AtT-20 cells by PCR but also in the mammosomatotroph cell line, GH3. In addition, Broberger et al. (8) demonstrated CART-IR in prolactin-immunoreactive pituitary cells. Our study examined only the colocalization between pituitary ppCART mRNA and ACTH immunoreactivity. However, the evidence would suggest that CART is localized to more than one anterior pituitary cell population (6, 8). Our dual ICC and ISH results suggest that CART is also found in pituitary cell populations that do not express ACTH. Interestingly, ppCART-expressing cells are closely apposed to a further subpopulation of pituitary corticotrophs, suggesting that there may be interactions between other CART-containing cells and corticotrophs.

The changes in pituitary CART expression and content after adrenal manipulation are very similar to the alterations in galanin under the same conditions. Pituitary galanin is upregulated by adrenalectomy and suppressed by dexamethasone treatment in rats (27). However, in females, galanin is localized primarily to the lactotrophs, and in males, galanin is found in the thyrotrophs and somatotrophs (27) rather than in corticotrophs. Thus, in addition to the corticotroph populations that express CART, the steroid environment may also regulate the CART-expressing lactotroph and somatotroph populations.

The origin of circulating CART peptide has yet to be identified. CART peptide is present in the adrenal medulla (20), but the elevation rather than reduction of CART peptide observed in adrenalectomized animals would suggest that adrenal CART does not contribute greatly to circulating levels. CART-IR has been demonstrated in the pituitary portal circulation that is released from the hypothalamus. However, because paraventricular, arcuate, and supraoptic expressions of CART are reduced in adrenalectomized animals (4, 36), it seems unlikely that hypothalamic CART contributes significantly to circulating CART-IR. The pituitary intermediate lobe also secretes ACTH and is responsive to CRH, and it is possible that this might be the origin of CART-IR in vivo and in vitro. However, CART-IR has not been identified in either rodent or primate pituitary intermediate lobes (13, 20). Because alterations in plasma CART-IR mirror the changes in anterior pituitary CART expression and content in adrenal-manipulated animals, anterior pituitary CART may be an important source of circulating CART-IR.

The data presented here suggest that the steroid environment regulates pituitary CART. Pituitary CART mRNA and peptide are influenced by CRH and by glucocorticoids. CART mRNA is colocalized to or closely associated with a subpopulation of pituitary ACTH-immunoreactive cells. Further work is needed to determine whether this modulation of pituitary CART peptide is involved in regulation of the HPA axis itself or plays another role.

GRANTS

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REFERENCES


