Pituitary Adenylate Cyclase-activating Polypeptide and Cyclic Adenosine 3′,5′-Monophosphate Stimulate the Promoter Activity of the Rat Gonadotropin-releasing Hormone Receptor Gene via a Bipartite Response Element in Gonadotrope-derived Cells

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Specific type I receptors for pituitary adenylate cyclase-activating polypeptide (PACAP) are present in gonadotrope cells of the anterior pituitary gland. By transient transfection of mouse gonadotrope-derived αT3-1 cells, which are direct targets for PACAP and express gonadotropin-releasing hormone receptor (GnRH-R), a marker of the gonadotrope lineage, we provide the first evidence that PACAP stimulates rat GnRH-R gene promoter activity. The EC_{50} of this stimulation is compatible with a mediation via activation of the cyclic AMP-dependent signaling pathway and, consistently, co-transfection of an expression vector expressing the protein kinase A inhibitor causes reduction in PACAP as well as cholera toxin-stimulated promoter activity. Deletion and mutational analyses indicate that PACAP activation necessitates a bipartite response element that consists of a first region (−272/−237) termed PACAP response element (PARE) I that includes a steroidogenic factor-1 (SF-1)-binding site and a second region (−136/−101) referred to as PARE II that contains an imperfect cyclic AMP response element. Gel shift experiments indicate the specific binding of the SF-1 and a potential SF-1-interacting factor to PARE I while a protein immunologically related to the cyclic AMP response element-binding protein interacts with PARE II. These findings suggest that PACAP might regulate the GnRH-R gene at the transcriptional level, providing novel insights into the regulation of pituitary-specific genes by hypothalamic hypophysiotropic signals.

The hypothalamic neuropeptide gonadotropin-releasing hormone stimulates the synthesis and release of gonadotropins, luteinizing hormone, and follicle-stimulating hormone, acting through a specific membrane receptor belonging to the family of heptahelical G protein-coupled receptors. The pituitary gonadotropins then enter the systemic circulation to regulate gonadal function, including steroid hormone synthesis and gametogenesis. The responsiveness of gonadotrope cells to GnRH is dependent on the number of cell surface GnRH-R, and changes in the number of these receptors often correlate with changes in the level of receptor mRNA (1–3). To investigate this issue at the transcriptional level, namely the tissue-specific and regulated expression of the GnRH-R gene, the promoter regions of the mouse, rat, human, and ovine genes have been isolated and characterized (4–8).

Transient transfection assays in the mouse gonadotrope-derived αT3-1 cell line have provided evidence that gonadotrope-specific activity of the mouse promoter is mediated by a bipartite basal enhancer that includes an SF-1-binding site, a consensus AP-1 element, and a novel element termed GnRH-R-activating sequence (GRAS) (9). Similarly, the interaction of SF-1 with a gonadotrope-specific element motif in the human gene has been shown to mediate gonadotrope-specific expression (10). Regarding the rat promoter, we and others have reported that full gonadotrope specific activity required a distal regulatory domain in addition to the SF-1 and AP-1 elements present in the proximal domain (7, 8, 11). We have found that the basal expression of the GnRH-R gene in the gonadotrope-derived αT3-1 cell line is highly dependent on a distal enhancer that is active in the context of the GnRH-R gene promoter only and therefore is termed GnRH receptor-specific enhancer. We have shown that GnRH receptor-specific enhancer activity (−1135/−753) was mediated through a functional interaction with a proximal region (−275/−226) that included the SF-1-binding site (11).

In addition, the availability of these promoters has allowed the study of the hormonal regulation of the GnRH-R gene and led to the conclusion that some of the elements involved in constitutive expression were also implicated in hormonal regulation. The GRAS element was indeed demonstrated to be involved in the autocrine/paracrine stimulation of the mouse GnRH-R promoter by activin (12). Likewise, two reports based on deletion and/or mutational analysis and functional transfection studies, as well as electrophoretic mobility shift assays, have revealed the involvement of the AP-1 element in the mechanism of the homologous regulation of the mouse GnRH-R

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1 The abbreviations used are: GnRH, gonadotropin-releasing hormone; GnRH-R, gonadotropin-releasing hormone receptor; SF-1, steroidogenic factor-1; AP-1, activating protein 1; GRAS, GnRH receptor-activating sequence; PKC, protein kinase C; PKA, protein kinase A; PACAP, pituitary adenylate cyclase-activating polypeptide; TPA, 12-O-tetradecanoylphorbol-13-acetate; PRL, prolactin; bp, base pair(s); PKI, protein kinase A inhibitor; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase; CRE, cAMP response element; CREB, CRE-binding protein; ATF, activating transcription factor; CBP, CREB-binding protein; PARE, PACAP response element; PCR, polymerase chain reaction.
gene by GnRH in αT3-1 cells (13, 14). An auxiliary element localized 10 bp upstream was found to be necessary for optimal activation by GnRH (13). These elements interact with members of the Fos/Jun heterodimer superfamily in agreement with PKC dependence of GnRH-R response to GnRH (13, 14) with the probable implication of a mitogen-activated protein kinase pathway in this regulation (14). In contrast, the homologous, PKC-mediated desensitization of the human GnRH-R promoter activity in αT3-1 cells has been shown to involve a different AP-1 motif located in the distal part of the promoter (15). Much less is known regarding the regulation of the GnRH-R promoter activity by the PKA-dependent pathway. Using transient transfection in the somatotactropotrope GH3 cell line stably expressing the GnRH-R gene, the mouse GnRH-R gene was found to be responsive to cAMP (16). Similarly, treatment of αT3-1 cells with forskolin or a cAMP analog significantly increased luciferase activity of the transfected rat GnRH-R promoter (7). These data led us to examine the regulation of the rat GnRH-R gene transcription by cAMP as well as by a physiological activator of the cAMP-dependent signaling pathway, the putary adenylate cyclase-activating polypeptide (PACAP).

PACAP, a member of the vasoactive intestinal polypeptide/secretin/glucagon family of peptides, was isolated from ovine hypothalamic extracts based upon its adenylate cyclase stimulating activity in rat pituitary cells (17). The major form of PACAP is a C-terminal amidated 38-amino acid polypeptide, but a shorter form, PACAP27, corresponding to the N-terminal 27 residues of PACAP38, is also found in the hypothalamus. Two major PACAP receptors have been identified: 1) PAC1 receptors highly specific for PACAP that activate not only adenylate cyclase but also phospholipase C. These receptors have been shown to be expressed in the anterior pituitary, adrenal medulla, hypothalamus, testis, and αT3-1 pituitary cell line (18). 2) VPAC1 and VPAC2 receptors, which bind both vasoactive intestinal polypeptide and PACAP but activate almost exclusively adenylate cyclase (19–21) and have been found in lung, liver, prostate gland, and seminal vesicles. At the pituitary level, PACAP stimulated the release of luteinizing hormone in vivo (22), whereas in vitro in cultured pituitary cells, it weakly stimulated luteinizing hormone and follicle-stimulating hormone release. Interestingly, it notably enhanced GnRH-induced gonadotropin secretion, thus suggesting intriguing possibilities for this peptide in regulating gonadotropin secretion and reproductive function (23). Consistently, PACAP has been shown not only to stimulate glycoprotein hormone α-subunit synthesis and release (24, 25) but also to increase α-subunit mRNA concentrations in primary rat pituitary cells and αT3-1 cells (26). Recently, Burrin and collaborators (27) have demonstrated using transfection assays in αT3-1 cells that PACAP regulated the expression of the human α-subunit gene at the transcriptional level.

In the present study, we examined the effects of PACAP and cAMP on GnRH-R promoter activity using αT3-1 cells. We employed deletion, mutation, and heterologous constructs of the rat GnRH-R promoter to delineate DNA sequences responsive to PACAP and cAMP. Gel shift assays were performed to assess the binding capacity of the putative elements with nuclear factors. We show that both PACAP and cAMP responsiveness of the GnRH-R gene promoter in αT3-1 cells is supported by two distinct proximal regions, one of which includes the SF-1-binding site localized at −245/−237 and is described previously as crucial for tissue-specific expression and the other, an imperfect cAMP response element present at position −110/−103.

EXPERIMENTAL PROCEDURES

Materials—PACAP, 12-O-tetradecanoylphorbol-13-acetate (TPA), and cholera toxin were obtained from Biovallay (Merseyside, UK), Calbiochem (San Diego, CA), and Sigma, respectively. All oligonucleotides were provided by Eurobio (Les Ulis, France) or Genaxis (Montigny-le Bretonneux, France).

Vector Construction—The series of 5′-deletion mutants termed pCAT1.2, pCAT0.5, and pCAT0.18 GnRH-R, spanning regions −1257/−32, −515/−32, and −180/−32 (all numbering is relative to the translational start site), respectively, have been described previously (8). A further series of 5′-deletion mutants spanning regions −433/−32, −381/−32, −316/−32, −297/−32, −272/−32, −247/−32, and −222/−32 were generated according to the same protocol using Deep Vent DNA polymerase (New England Biolabs, Montigny-le Bretonneux, France) with selected sense primers that included an HindIII restriction site to facilitate cloning and the antisense primer −32 SaI, which was complementary to bases −20 to −39.

A series of block replacement mutations at 8-bp intervals in the region from −260 to −221 within the pCAT0.27 GnRH-R construct (−272/−32) was generated by PCR amplification using a series of sense/antisense primers (overlapping over 14 bp) designed to place a PstI restriction site with T and C flanking bases at the 5′ and 3′ ends, respectively (TCTGCAGC). Overlapping fragments were generated from pCAT0.43 GnRH-R (from −433 to −32) as a template in separate PCR reactions using the mutated sense primer and antisense primer −32 SaI or the mutated antisense primer and sense primer −433 Hind. The amplified products were digested with HindIII and SaI, gel-purified, and inserted into the pCAT Basic vector (Promega, Lyon, France) digested with the same enzymes. Because of a superstimulated basal activity of construct −272 Mut D, the PstI site was replaced by a KpnI site (TGCTA/CC).

To subclone the artificial promoter constructs upstream of the luciferase reporter gene (Promega), the multiple cloning site of the pGL3-Basic vector was altered to provide compatible restriction sites in the appropriate orientation (11). A minimal proline (PRL) promoter and a single 50-bp module containing the SF-1 element were synthesized as described previously (11). The 50-bp module was introduced into the modified pGL3-Basic containing upstream either the minimal PRL promoter or a rat GnRH-R promoter fragment subcloned in place of the minimal PRL promoter (see below). The −136/−32 and the −101/−32 proximal regions of the GnRH-R promoter were generated using selected sense primers −136 Bst and −101 Bst, respectively, and antisense primer −32 SaI. The −56/−32 region was directly obtained by self annealing two oligonucleotides with an overlap of 27 bp and thus reconstituting a double-stranded DNA, which included the −32 SaI and SaI half-sites at each end for cloning into the BstEII/SaI sites in the modified pGL3-Basic vector. By serially replacing 8-bp segments from position −260 to −237 in the 50-bp module with the PstI restriction site flanked by T and C at the 5′ and 3′ ends, respectively (TCTGCAGC), three separate mutants were created and ligated upstream of the −136/−32 promoter region in place of the 50-bp module in the modified pGL3-Basic. The series of block replacement mutations eco1 to eco4 scanning the sequence from −136 to −101 in the −136/−32 promoter region were also generated by PCR amplification using a series of sense/antisense overlapping primers designed to place an EcoRI restriction site flanked by a GC at the 3′ end (GAATTCGC). All mutant plasmids were identified by restriction digest of midi-prep DNA and ultimately verified by nucleotide sequencing.

The PKI expression vector was prepared by subcloning the rat cDNA encoding PKI into the XbaI/EcoRI site of the pcDNA3 (Invitrogen, Leek, The Netherlands) upstream of the CMV promoter. This CDNA was initially cloned from rat pituitary by reverse transcription-PCR and ligated into pUC18 (Amersham Pharmacia Biotech) in our laboratory, whereas an identical rat cDNA sequence was simultaneously isolated from the brain by another group (28).

Cell Culture and Transient Transfection—The mouse gonadotrope αT3-1 cells were maintained in monolayer cultures in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate (Sigma) at 37°C in humidified 5% CO2, 95% air (29). Transfection was achieved with CAT or luciferase reporter constructs. The amplified plasmids were digested with a restriction enzyme site flanked by T and C at the 5′ and 3′ ends. The amplified products were combined and submitted to a second round of dIII restriction site digestion with T and C flanking bases at the 5′ and 3′ ends. The amplified products were combined and submitted to a second round of digestion with HpaI (32) was generated for transient transfection in T3-1 cells with forskolin or a cAMP analog significantly increased luciferase activity of the transfected rat GnRH-R promoter (7). These data led us to examine the regulation of the rat GnRH-R gene transcription by cAMP as well as by a physiological activator of the cAMP-dependent signaling pathway, the putary adenylate cyclase-activating polypeptide (PACAP).

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PACAP Regulation of the Rat GnRH Receptor Gene Promoter

RESULTS

PACAP Stimulates GnRH-R Promoter Activity through the PKA-signaling Pathway—To assess the potential implication of PACAP in the regulation of the rat GnRH-R gene, immortalized pituitary gonadotrope cells (aT3-1 cells) were transiently transfected with GnRH-R promoter CAT fusion constructs then treated with 20 nM PACAP for 16 h (Fig. 1A). The effect of PACAP was compared with that exerted by activators of the PKA- and PKC-dependent pathways, cholera toxin (1 nM) and TPA (25 nM), respectively (Fig. 1B). Both PACAP and cholera toxin stimulated CAT expression of the −1257/+32 construct equivalently, with 2.5 ± 0.5-fold and 2.3 ± 0.1-fold increases over untreated cells, respectively. As a consequence of the presence of the enhancer in the distal part of the promoter, deletion of the sequence extending from −1257 to −515 caused a decrease in basal CAT activity (see the Introduction and Ref. 11). Nevertheless, the resulting construct −515/+32 still displayed an increased response to both PACAP and cholera toxin. A further deletion from −515 to −180 abrogated both PACAP and cholera toxin response, suggesting that response elements for PACAP and cholera toxin were localized within a proximal

Fig. 1. Characterization of the PACAP response of the rat GnRH-R gene promoter. A, structure of the full-length and of 5′-deleted GnRH-R promoter CAT constructs used in transient transfection assays in B. The GnRH receptor-specific enhancer (GnRH-R specific enhancer), as well as the GRAS, AP-1, and SF-1 elements are indicated by black boxes at their corresponding locations within the rat promoter sequence. The major transcription start sites are located at positions −110/ −107 and −99/ −97 (relative to the ATG codon) are indicated by bent arrows. GnSE, GnRH receptor-specific enhancer. B, aT3-1 cells were transfected for 6 h with various GnRH-R promoter CAT constructs and then treated for 16 h with either 20 nM PACAP, 1 nM cholera toxin (Ctx), 25 nM TPA, or a combination of cholera toxin and TPA. CAT activity was calculated as CAT activity/β-galactosidase activity and then normalized as fold induction over that of the promoterless pCAT Basic vector. The values represent the means ± S.D. *, p < 0.001 compared with untreated cells. C, cells were transfected with the 500-bp 5′-flanking region of the rat GnRH-R gene (−515/−32) or the promoterless construct as control, followed by treatment with increasing concentrations of PACAP (2 × 10⁻⁶, 2 × 10⁻⁵, 2 × 10⁻⁴, 2 × 10⁻³, 2 × 10⁻², 2 × 10⁻¹, and 2 × 10⁻⁰ M). Measurements, which were normalized to the CMV promoter-containing vector pCDNA3, were expressed as CAT/β-galactosidase. D, cells were co-transfected with the 500-bp 5′-flanking region and increasing amounts of pCDNA3, used as a control, followed by treatment with 20 nM PACAP or 1 nM cholera toxin (Ctx). The measurements are expressed as CAT/β-galactosidase. All results are the means ± S.D. of duplicate samples in at least three independent transfection experiments.

Preparation of Nuclear Extracts and Gel Mobility Shift Assays—The cells were seeded at 3 × 10⁵ cells/100-mm tissue culture dish in triplicate and cultured for 24 h in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate. The culture medium was then replaced by OptiMEM medium, and the cells were cultured for an additional 6 h. The serum-free medium was finally replaced by Dulbecco’s modified Eagle’s medium supplemented with 2% fetal bovine serum, 10 units/ml penicillin, and 10 μg/ml streptomycin sulfate, and cells were incubated in the presence or the absence of 20 nM PACAP for 16 h. Thereafter, cells were harvested, and nuclear extracts were prepared by the method of Andrews and Faller (30).

Synthetic double-stranded oligonucleotides, designed to contain 5′ protruding ends, were labeled (5 pmol) by filling in the recessed 3′ termini with Klenow fragment from Escherichia coli DNA polymerase I and 50 μCi of [α-³²P]dUTP (3000 Ci/mmol; Amersham Pharmacia Biotech). The CRE/PARE II and CREB consensus probes were labeled with 50 μCi of [γ-³²P]ATP (3000 Ci/mmol; Amersham Pharmacia Biotech) using a T4 polynucleotide kinase. All probes were then purified on a poly(dI-dC) (1 mg/ml) column.

DNA-protein interactions were analyzed by gel retardation assays with nuclear extracts from T3-1 cells transfected with the 500-bp 5′-flanking region of the rat GnRH-R gene (−515/−32) or the promoterless construct as control. The DNA-protein complexes were resolved on 5% nondenaturing polyacrylamide gel electrophoresis. Gels were then transferred to Hybond-N + membrane, and bound radioactivity was detected by autoradiography.

Antibody Abrogation Gel Shift Assays—Antibody abrogation gel mobility shift assays were used to identify DNA-binding factors that bind to the GnRH-R promoter. The procedure for antibody abrogation gel shift assays was conducted as described above with nuclear extracts from T3-1 cells transfected with the 500-bp 5′-flanking region of the rat GnRH-R gene (−515/−32) or the promoterless construct as control. Nuclear extracts were incubated with an excess of unlabeled competitor, and the incubation was continued for 30 min at 20 °C. In antibody abrogation gel shift assays, nuclear extracts were incubated with either a rabbit polyclonal antibody directed against the DNA-binding domain of murine SF-1 (Upstate Biotechnology), a rabbit polyclonal anti-CREB antibody (Santa Cruz Biotechnology, Inc.), or an equal concentration of mouse IgG for 1 h at 4 °C prior to the addition of radiolabeled probe. Free probe was separated from bound probe by electrophoresis in 5% nondenaturing polyacrylamide gels that were run at 120 V for 2 h in 1 × Tris-borate-EDTA buffer. Gels were transferred to blotting paper and probed for CAT activity.

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Statistical Analysis—The data were analyzed by one-way analysis of variance. If the F-test was significant, then the means were compared using Tukey-Kramer’s method of multiple comparisons. For Figs. 1C, 5, and 6, the data were logarithmically transformed before proceeding to analysis of variance.
PACAP Regulation of the Rat GnRH Receptor Gene Promoter

GRAS AP-1 SF-1

-515
-433
-381
-316
-297
-272
-247
-222
-180

Promoter region extending from –515 to –180. Additional experiments using similar GnRH-R promoter sequences but fused to the luciferase gene reporter gave equivalent results, even following treatments with the adenylate cyclase activator, forskolin, or the permeant cAMP analog, 8-bromo-cAMP. In response to 10 μM forskolin, the luciferase constructs containing either the full-length (–1135/-32) or the enhancerless (–433/-32) promoter were stimulated 2.8 ± 0.3- and 3.5 ± 0.3-fold, respectively, whereas the construct containing the shortest GnRH-R promoter (–180/-32) remained unaffected (data not illustrated). Likewise, 2 mM 8-bromo-cAMP significantly stimulated the luciferase activity of both the full-length and the enhancerless constructs, by 2.0 ± 0.2- and 1.9 ± 0.2-fold, respectively. In contrast, unlike PACAP and activators of the PKA-dependent signaling pathway, TPA had no effect on the expression of any construct (Fig. 1B). These data are in accordance with a previous report showing that forskolin and cAMP analogs, but not TPA, increased the activity of the rat promoter transfected into aT3-1 (7). In addition, these results suggested the involvement of a PKA-mediated pathway for the activation of the GnRH-R promoter by PACAP.

To evaluate the specificity of the response of the rat GnRH-R promoter, increasing doses of PACAP ranging from 2 × 10^{-16} to 2 × 10^{-7} M were tested in aT3-1 cell cultures transfected with the –515 GnRH-R CAT construct (pCAT0.5 GnRH-R) or with the promoterless pCAT Basic vector. As shown in Fig. 1C, PACAP induced a concentration-dependent increase in GnRH-R CAT activity, with a maximal response observed at 2 nM. As expected, the activity of the promoterless vector was not affected by PACAP. More importantly, the EC_{50} value for stimulation of GnRH-R promoter activity by PACAP (0.2 ± 0.06 nM) was in closer agreement with signaling through the cAMP/PKA cascade (3 nM) than through stimulation of the inositol-phosphate turnover (20 nM) in aT3-1 cells (18). Taken together these findings implied that the stimulatory effect of PACAP on GnRH-R promoter was most probably mediated through coupling of PAC1 receptors to the PKA-dependent signaling pathway.

To test this hypothesis, aT3-1 cells were transfected with pCAT0.5 GnRH-R and co-transfected with variable amounts of a vector expressing the rat PKI cDNA under the control of the CMV promoter (pcDNA3PKI). A vector containing only the CMV promoter (pcDNA3) was used as a control (Fig. 1D). Co-transfected pcDNA3PKI significantly decreased (p < 0.01) basal GnRH-R CAT activity by about 50%. Furthermore, 0.5 μg of pcDNA3PKI markedly reduced (p < 0.001) both cholera toxin- and PACAP-activated GnRH-R CAT activity by 76.5 and 61.4%, respectively, whereas 1 μg led to a maximal inhibition of 84 and 70.5%, respectively. Again, these findings were consistent with the implication of the cAMP-dependent signaling pathway in PACAP-stimulated GnRH-R promoter activity.

**Effect of 5'-Deletions of the GnRH-R Gene Promoter on PACAP- and Cholera Toxin-stimulated CAT Activity**—To localize the putative response elements for both PACAP and cAMP, serial 5'-deletion mutants with 5'-termini located between –515 and –180 within the GnRH-R gene promoter were designed. Transient transfection experiments were performed with aT3-1 cells, which were then treated with either PACAP, cholera toxin, or vehicle as above. As shown in Fig. 2 (left panel), the –515 GnRH-R CAT construct elicited a 5.3 ± 0.9-fold increase in the basal activity over the promoterless vector, and deletions from –515 to either –433 or –381 did not significantly affect the basal activity (6.5 ± 1.1 and 6.7 ± 1.1-fold over promoterless vector, respectively). However, the basal GnRH-R promoter activity was significantly decreased after 5'-deletion from –381 to –316, which eliminated the AP-1-binding site, in agreement with a previous report from our laboratory (11). Additional deletions from –297 to –247 similarly decreased constitutive expression, whereas a further deletion from –247 to –222 that included the SF-1 element was inefficient. The latter observation was surprising because the SF-1 element is crucial for constitutive activity of the rat GnRH-R promoter. Block replacement mutagenesis of the SF-1 element in the context of the full-length (–1135/-32) or proximal (–450/-32) promoter strongly decreased basal expression (11). Because deletion of the SF-1 element in the present experiment was inefficient, this suggested that sequences upstream of –247 could be necessary for SF-1 activity.

Consistent with mediation of PACAP action through the PKA-dependent pathway, an equivalent pattern of expression was obtained in PACAP- as well as cholera toxin-treated cells (Fig. 2, right panel). Transfections using the –515 GnRH-R CAT construct resulted in a 2.6 ± 0.4- or 2.8 ± 0.3-fold increase in CAT activity in response to PACAP or cholera toxin stimu-
The mutation extending from 270 to 221 is shown. The putative SF-1 site is boxed. The location and nucleotide sequence of the introduced block replacement mutations were indicated and labeled as MutA to MutE. B, the series of block replacement mutations were generated in the context of the −272/32 GnRH-R CAT construct using PCR amplification as described under "Experimental Procedures." The major transcription start sites located at positions −110/−107 and −99/−97 (relative to the ATG codon) are indicated by bent arrows. T3-1 cells were transfected for 6 h with these vectors and then treated for 16 h with 20 nM PACAP.

The CAT activity was adjusted for β-galactosidase activity. The values are expressed as fold increases over untreated cells. The results shown are the means ± S.D. from three separate experiments, each performed in duplicate.

In addition to SF-1, other protein factor(s) interact(s) specifically with the PARE I region—To analyze the binding capacity of the PARE I region (−260/−237), nuclear extracts from αT3-1 cells were prepared for use in gel retardation assays. Radiolabeled synthetic oligonucleotides corresponding to sequence −264 to −231, either intact (wild probe), or mutated at position −260/−253 (MutA probe), −252/−245 (MutB probe), or −244/−237 (MutC probe or SF-1 mutant) were incubated with αT3-1 nuclear extracts and tested for protein-DNA interactions. PACAP stimulation (20 nM for 16 h) of αT3-1 cells prior to preparation of nuclear extract gave results equivalent to those obtained with nonstimulated cells. As shown in Fig. 4, three DNA-protein complexes were formed when wild probe was used as a labeled oligonucleotide (lane 2, complexes I, II, and III), and all complexes were competed by the addition of an excess of homologous competitor (lanes 3–5). However, complex III appeared to be of relatively weak affinity because a marked amount of this complex was still detected in the presence of a 1000-fold molar excess of unlabeled wild probe (lane 5). In addition, neither mutation A, B, or C seemed to affect the formation of complex III (lanes 17, 12, and 7, respectively). Taken together these data suggested that complex III was DNA sequence-independent and therefore nonspecific. More impor-
tantly, radiolabeled MutC probe failed to form complex II (lane 7), indicating that the sequence corresponding to the SF-1-binding site (−245/−237) was involved in the formation of this complex, which was consistent with our previous study (11). Moreover, the abundance of complex I formed with the MutC probe was significantly diminished as compared with that formed with the wild probe (lane 7), suggesting that complex I was also affected by mutation C, yet to a lesser extent than complex II. In contrast, MutA and MutB probes could form complex II but were incapable of binding protein(s) of complex I (lanes 17 and 12, respectively), which actually correlated with their inability to compete for binding with the radiolabeled MutC probe (data not shown). Thus, these data suggested that the sequences covered by mutations A and B were necessary for complex I formation, whereas the sequence covered by mutation C was required for both complex II formation and high affinity binding of the factor(s) involved in complex I. To validate further the identity of the protein(s) that interacted with the PARE I region, antibody abrogation gel shift experiments were conducted using a rabbit polyclonal antibody directed against the DNA-binding domain of the murine SF-1 protein (Fig. 4, lanes 26–28). As is apparent from the figure, addition of the anti-SF-1 antibody to the binding reaction abrogated complex II formation in a dose-dependent manner, indicating that a factor immunologically related to SF-1 interacted with the SF-1 response element. Interestingly, the intensity of complex I was attenuated in the presence of increasing concentrations of anti-SF-1 antibody, which suggested that SF-1 also favored complex I formation. Collectively, these data point out the ability of nuclear protein(s) to bind the 16-bp sequence adjacent to the SF-1 element, in addition to SF-1 binding to its own site, which is in concordance with the results obtained with the transfection assays.

**The Association of the PARE I Region with the −136/−32 Region Is Necessary to Confer Full Responsiveness to PACAP**—To determine whether PARE I was not only necessary but also sufficient to mediate full PACAP response, a PCR-generated 50-bp module (−275/−226) encompassing the PARE I region was placed under the control of the minimal PRL promoter, and the response to PACAP stimulation was measured. Transfection with this construction demonstrated only a 2.3 ± 0.2-fold increase (p < 0.001) in luciferase activity in response to PACAP stimulation (Fig. 5A), suggesting that other elements most probably localized downstream of the PARE I region in the GnRH-R promoter were required for full stimulation. The minimal PRL promoter was then replaced by the GnRH-R promoter region extending from −136 to −32. Although this downstream region alone was insensitive to PACAP stimulation, it was capable of cooperating with the PARE I-containing module. Indeed, when both elements were linked together, full response to PACAP was recovered because the fusion promoter showed an optimal 5 ± 0.9-fold response under PACAP-stimulation (p < 0.001). To investigate the importance of the −136/−32 proximal region in PACAP regulation of the GnRH-R gene, two additional PCR-generated fragments, −101/−32 and −56/−32, were fused to the 50-bp module encompassing PARE I. As shown in Fig. 5A, the −101 and −56 GnRH-R LUC constructs, either alone or fused to the 50-bp module, were unresponsive to PACAP (p > 0.05). These findings suggested that element(s) located in the −136/−101 region, hereafter referred to as PARE II, participated in PACAP responsiveness and that cooperation of PARE I and PARE II was necessary for an optimal response to PACAP.

To further establish the requirement of PARE I for the PACAP response, the block replacement mutations A, B, and C were generated in the context of the 50-bp module placed upstream of the −136/−32 region, and the resulting constructs were tested for PACAP-stimulated expression (Fig. 5B). In accordance with the data in Fig. 5, mutations A, B, and C (SF-1 mutant) led to significant decreases of 82, 95, and 79%, respectively, in PACAP-activated transcription (p < 0.001). As expected, the cooperative action of PARE I and PARE II in mediating PACAP response was abrogated by targeted mutagenesis across the PARE I region.

**Mutation of the cAMP Response Element within the PARE II Region Strongly Reduced PACAP-induced Stimulation**—Analysis of the PARE II region revealed the presence of an imperfect CRE (5'-TGACGTTT-3') at position −110/−103. To determine whether this CRE was critical for PACAP responsiveness of the GnRH-R gene, four block replacement mutations (eco1 to eco4) were synthesized across the PARE II region in the context of the −136/−32 region linked to the PARE I-containing module. These mutations consisted of replacing the wild-type sequence with a 8-bp sequence that included an EcoRI restriction site. As shown on Fig. 6, mutation of the imperfect CRE (eco4) elicited a weak and insignificant decrease (p > 0.05) in the basal activity of the fusion construct, whereas it resulted in a major 83% loss of PACAP-stimulated promoter activity as compared with the wild-type promoter (1.4 ± 0.1-fold versus 3.6 ± 0.3-
Fig. 6. The CRE-like sequence located within the PARE II region is involved in PACAP responsiveness of the rat GnRH-R promoter.

A. The region of the GnRH-R promoter spanning from −142 to −90 is shown. The CRE-like sequence is boxed. The location and nucleotide sequence of the introduced block replacement mutations are indicated and labeled as eco1 to eco4. B. The series of block replacement mutations were generated by PCR amplification into the context of the chimeric 50-bp module/proximal GnRH-R promoter/Luc construct. The major transcription start sites located at positions −110/−107 and −99/−97 (relative to the ATG codon) are indicated by bent arrows. The constructs were transfected into αT3-1 cells as described above. The luciferase activity was corrected for transfection efficiency by normalizing to TK-Renilla luciferase expression vector and expressed as fold stimulation over pLuc/PRL construct. The results shown are the means ± S.D. of duplicate samples of at least three independent experiments. (fold, p < 0.001). Mutations eco1 and eco3 also led to a significant but moderated decrease in PACAP stimulation (2.3 ± 0.1-fold and 2.4 ± 0.2-fold), and an unaltered stimulated activity was observed for mutation eco2 as compared with the wild-type construct (3.6 ± 0.3-fold, p > 0.05). Therefore, the imperfect CRE located at −110/−103 appeared as the most active element that could cooperate with PARE I in mediating PACAP responsiveness. Altogether these data demonstrated that two distinct sequences were involved in the mediation of PACAP-stimulated GnRH-R gene expression: PARE I, extending from −260 to −237 and including an SF-1 site, and PARE II, which contained a functional although imperfect CRE.

DISCUSSION

In the present study, we have examined the regulation of the activity of the GnRH-R gene promoter by PACAP using transient transfection in αT3-1 cells, a well characterized mouse pituitary gonadotrope cell line that expresses a functional GnRH-R (31) and a PACAP-selective receptor (PAC1-R). It is established that in these cells, PAC1-R are coupled to both cAMP and inositol phosphate production as well as to increases in intracellular Ca²⁺ concentration (18). By transient transfection in αT3-1 cells, we demonstrate for the first time that PACAP stimulates the activity of the rat GnRH-R gene promoter through the cAMP pathway, providing a mechanism by which this hypophysiotropic peptide may operate as a modulator of GnRH action in the anterior pituitary. This is reminiscent of previous findings showing that cAMP and PACAP enhance GnRH-induced hormone secretion in perfused rat pituitary cells (25, 32).

It is noteworthy that PACAP action on the GnRH-R gene promoter activity is mediated through the sole activation of the PKA-dependent signaling pathway, whereas PACAP may act in αT3-1 cells via both the PKA- and PKC-dependent pathways (18). Indeed, cholera toxin-induced production of endogenous cAMP, unlike TPA, stimulates promoter activity. Moreover, PACAP effects on promoter activity are compatible with cAMP mediation based on the similarity between the dose dependence relationship determined in this study and those previously established for cAMP generation in these cells (18). Furthermore, co-transfection using a vector expressing the PKI dramatically decreased the stimulatory effect of PACAP, providing additional evidence that PACAP acts primarily through the PKA-dependent pathway. Finally, and most importantly, the cis-acting sequences that promote PACAP action are localized with those of cholera toxin and involve, in addition to the SF-1 element containing domain (PARE I region), a CRE that binds a protein most likely belonging to the CREB/ATF family (PARE II region).

The sensitivity of the rat GnRH-R gene to PACAP is also the property of the gonadotropin α-subunit gene, another important marker gene of the gonadotrope lineage (26). Interestingly, the action of PACAP on the α-subunit gene is similarly mediated by the PKA-dependent pathway. This conclusion was
based notably on results obtained in transient transfection experiments of αT3-1 cells stimulated with PACAP, cAMP, or PMA, in the presence or in the absence of selective inhibitors of the PKA- or PKC-dependent pathways (33). Likewise, Burrin et al. (27), using deletion and mutational analysis combined with transfection studies in αT3-1 cells, have localized PACAP response elements of the human α-subunit gene to a 50-bp sequence in the proximal promoter, which includes an SF-1-binding site. In addition, full PACAP activation was shown to require the two intact CREs, located further downstream to this 50-bp region. Altogether these data suggest that the rat GnRH-R and the α-subunit genes can be coordinately regulated by PACAP via similar intracellular mechanisms in gonadotrope cells of the pituitary gland.

Our results showing that luciferase expression driven by the 1.2-kilobase rat GnRH-R promoter is stimulated by forskolin and cAMP analogs are consistent with the data obtained by Reinhart et al. (7). Together, these and our data contrast with those obtained with the mouse promoter, which is unaffected by forskolin treatment in transiently transfected αT3-1 cells (13, 14). This suggests that the differential sensitivity of the rat and murine genes regarding the activation of the PKA-dependent pathways may be an intrinsic property of their respective promoters.

This is somewhat intriguing because the mouse promoter contains sequences highly homologous to the PARE regions and located at positions similar to those identified within the rat promoter. Consequently, it would be potentially able to respond to cAMP stimulation. The main difference identified to date between the two promoters is that regarding the efficiency of the GRAS element. This element was shown to be crucial for cell-specific expression of the mouse promoter (9), whereas it was much less efficient in the rat promoter context (11). This may provide a possible explanation for the differential sensitivity of the rat and mouse promoter with respect to the activation of the cAMP/PKA signaling pathway. The αT3-1 cells are known to produce activin, and its autocrine/paracrine stimulatory action on the mouse GnRH-R promoter is mediated through the GRAS element (see the Introduction and Ref. 12). Also, in αT3-1 cells, it was previously established that PACAP could activate the follistatin promoter via the cAMP-dependent PKA pathway (34). Because follistatin is a powerful inhibitor of activin action, PACAP could neutralize the activin-induced stimulation of the mouse GnRH-R promoter activity by stimulating follistatin production. The direct positive action of PACAP on the mouse promoter would therefore be masked by the indirect and opposite action of follistatin. Regarding the rat promoter, because the GRAS element is very poorly active (11), the direct effect of PACAP on promoter activity would be predominant. This hypothesis is consistent with data obtained in the GGH3 cell line, a somatolactotrope cell line stably expressing the mouse GnRH-R (16). These cells do not produce activin; the mouse GnRH-R promoter can thus be stimulated by activation of the PKA-dependent signaling pathway (see the introduction and Refs. 16 and 35) and deletion of the CRE (TGACGTTT) within the mouse promoter prevented cAMP-dependent stimulation (36).

As an initial step toward the identification and localization of PACAP- and cAMP-responsive elements within the GnRH-R promoter, serial 5′-deletion mutants were tested and exhibited molar excess. R, nuclear proteins (9 μg) were incubated with an affinity-purified rabbit polyclonal anti-CRE antibody or an equal concentration of mouse IgG prior to the addition of radiolabeled probes consisting of the CRE/PARE II or the CRE consensus probe. All binding reactions were subjected to electrophoresis through nondenaturing 5% polyacrylamide gels as described under "Experimental Procedures."
It is noteworthy that SF-1 is involved in both constitutive and cAMP-regulated expression of various genes, viz. an SF-1 motif is required for both basal and cAMP-induced regulation of the rat HDL receptor promoter (37). In the human ACTH receptor promoter, two SF-1-binding sites, SF-35 and SF-98, must be present to elicit a response to cAMP, whereas full constitutive activity necessitates both sites plus a third, SF-209 (38). Furthermore, SF-1 that mediates basal and cAMP-regulated transcription of the rat steroid cytochrome P450c17 gene can be phosphorylated in vitro by PKA, which diminishes its binding and hence may play a regulatory role in transcriptional activation (39).

Alternatively, the imperfect CRE located at $-110/-103$ within the PARE II region appears crucial for PACAP responsiveness, because the CRE mutant (eco4) elicits a 83% decrease in PACAP stimulation. This response element binds a protein immunologically related to the CREB family, suggesting that such a factor participates together with SF-1 and the AB factor in PACAP regulation of the GnRH-R gene. As targeted mutagenesis of either the SF-1 site, the neighboring region AB or the CRE sequence significantly impairs PACAP-stimulated activity of the GnRH-R fusion construct, the cooperative action of PARE I and PARE II seems to occur in a synergistic manner. Similarly, by using selected mutants of the CREB and SF-1-binding sites within the context of the rat aromatase promoter, Carlone and Richards (40) showed that CREB and SF-1 interact synergistically to confer high constitutive activity in R2C Leydig cells. Likewise, mutation in either the CRE or the SF-1 regulatory element completely eliminates synergistic stimulation of the rat inhibit $\alpha$-promoter activity by SF-1 and the cAMP pathway in cells co-transfected with PKA and SF-1 expression vectors (41). In the same report, it was stated that SF-1 interacts directly with CREB through the likely recruitment of CBP/p300, because this co-activator further enhances transcription by these pathways. SF-1 was shown to interact with two domains of CBP/p300 that were distinct from the CREB-binding domain (42), which raised the possibility that CBP/p300 may serve as a signal integrator for both SF-1 and CREB factors. Based on these studies and our findings, we propose a model for the activation of the GnRH-R gene promoter by PACAP, which involves the contribution of CBP/p300 (Fig. 8). Under basal conditions, the AB factors together with SF-1 bind to the PARE I region within the GnRH-R gene promoter. The co-activator CBP/p300 is subsequently recruited through SF-1 interaction and promotes constitutive promoter activity. Under PACAP stimulation, CREB-related factors are phosphorylated and can then bind to CBP/p300 with high affinity. SF-1 and CREB-related factors, through their interaction with CBP/p300, are then in sufficiently close proximity to establish protein-protein interactions with a resulting increase in transcriptional activity. In contrast, the absence of one of these factors would preclude the formation of the multi-component complex, leading to nearly complete suppression of PACAP response. Such a situation occurs when the action of either AB, SF-1, or CREB-related factors is abrogated experimentally by targeted mutagenesis of their cognate elements. This model does not exclude the interaction of SF-1 with other cofactors besides CBP such as steroid receptor co-activator-1 (43, 44). Further experiments will be necessary to refine the model and elucidate the precise mechanisms involved in this transcriptional regulation, which represents an important and novel aspect in the neurohormonal control of gonadotrope function.

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