Specific Inhibition of Protein Kinase A in Granulosa Cells Abolishes Gonadotropin Regulation of the Proopiomelanocortin Promoter*

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Gonadotropins (follicle-stimulating hormone (FSH), luteinizing hormone, and human chorionic gonadotropin (POMC)) and β-adrenergic agonists have been shown to stimulate expression of the proopiomelanocortin (POMC) gene in ovarian granulosa cells. The current studies investigate the intracellular mechanisms by which gonadotropins regulate gene expression. Primary cultures of rat granulosa cells were transfected with the plasmid POMC-CAT-150, which expresses chloramphenicol acetyltransferase (CAT) reporter gene under the regulation of the rat POMC 5′-flanking region. CAT activity was stimulated by treatment of the cells with either 20 ng/ml FSH or 1 μM isoproterenol. To assess the role of protein kinase A (PKA:protein phosphotransferase; EC 2.7.1.37) in the gonadotropin and adrenergic response, an expression vector, MtR-AB, encoding a mutant RI regulatory subunit was cotransfected with POMC-CAT-150. The mutant protein kinase A regulatory subunit encoded by MtR-AB lacks functional CAMP-binding sites but effectively binds and specifically inhibits the catalytic activity of protein kinase A. The results of this analysis demonstrated that gonadotropin and adrenergic agonist stimulation of the POMC-CAT reporter construct in primary cultures of rat granulosa cells was abolished by cotransfection with MtR-AB; whereas a control SV40-promoter construct was unaffected by either gonadotropin treatment or cotransfection with MtR-AB. Basal expression directed by the POMC promoter was also decreased by cotransfection with the MtR-AB, implying that basal expression from the POMC promoter may also depend on protein kinase A. Deletion analysis of the POMC sequence indicated regions (−40 to −33 and +4 to +63) important for basal and FSH-stimulated expression. These studies suggest that both gonadotropin and adrenergic stimulation of the POMC promoter are mediated by protein kinase A and that regions proximal to the promoter are essential for gonadotropin-regulated expression from the promoter.

The gonadotropins FSH1 and luteinizing hormone regulate a number of ovarian functions, including follicular growth, ovulation, and corpus luteum function. Studies of the intracellular mechanisms by which gonadotropins regulate granulosa cell function have focused primarily upon the activation of adenylate cyclase with subsequent increases in cAMP and cAMP-dependent protein kinase activity (1–6). The ability to enhance or mimic gonadotropin action by compounds that stimulate adenyl cyclase, membrane-permeable CAMP analogues, or phosphodiesterase inhibitors has supported the concept that CAMP may be the principal intracellular mediator of gonadotropin activation. However, recent studies have raised the possibility of alternate intracellular mechanisms. Stimulation with luteinizing hormone, FSH, or human chorionic gonadotropin has been shown to stimulate increases in phosphoinositol turnover and calcium influx, although higher levels of gonadotropin are required to elicit these effects (7, 8). The relative roles of these different mechanisms are difficult to assess and are a strong rationale for the current studies.

Among the functions stimulated by gonadotropins are progesterone synthesis (9, 16) and proopiomelanocortin (POMC) gene transcription (15, 19, 20). Previous studies demonstrated that both steroidogenesis and POMC promoter activity were stimulated, whereas the activity of a control promoter, that of the SV40 virus, was unresponsive to gonadotropins (20). These studies also demonstrated stimulation of POMC promoter activity by a CAMP analog and a phosphodiesterase inhibitor, indicating that the gonadotropins were most likely exerting their actions on gene expression by their well characterized stimulation of cAMP levels. The stimulation of POMC promoter activity by cAMP is interesting since this promoter lacks a well conserved cAMP regulatory element (CRE) sequence (22, 45) or a well conserved AP-2 element that also confers CAMP regulation (23). The specific sequence elements involved in CAMP-mediated stimulation of POMC promoter activity are therefore of interest due to the potential of either divergence from the CRE consensus sequence or the mediation by novel control elements.

Initial studies concerned with delineating regulatory elements of the POMC promoter in multiple species have been conducted. Interpretation of the data have been made more difficult by the apparent proximity of sequences sufficient for CAMP response to the TATA box. For example, studies of a rat POMC promoter-reporter gene construct transfected into AtT-20 anterior pituitary tumor cells indicated that hormone and cAMP-regulated sequences were closely linked to basal promoter elements (33).
The cAMP-dependent protein kinases exist as multiple isoforms with closely related subunits. The holoenzyme consists of two regulatory (R) subunits and two catalytic (C) subunits (24). Upon the binding of two molecules of cAMP to each of the R subunits, the C subunits dissociate and catalyze the phosphorylation of specific proteins. The different isoforms are composed of unique R subunits (designated RIα, RIβ, RIIα, and RIIβ), as well as unique C subunits (Cα and Cβ) that have been identified as distinct gene products with tissue-specific patterns of expression (28). Granulosa cells and Sertoli cells are two of the limited number of sites of RIIβ isoform expression (25, 26).

The aim of these studies is to evaluate the role of protein kinase A (cAMP-dependent protein kinase) in the actions of gonadotropins and adrenergic agents on POMC gene expression. Previous studies that examined the role of protein kinase A in the regulation of gene expression have used peptide inhibitors containing the kinase pseudosubstrate site found in the regulatory subunit (10–13, 17). Recently, this issue has been examined using an expression plasmid that overexpresses a mutated regulatory subunit with a much lower affinity for cAMP than this mutant acts as an inhibitor of the kinase (14, 27). To investigate the mechanism of action of gonadotropins, primary cultures of granulosa cells were transfected with a plasmid containing the chloramphenicol acetyltransferase (CAT) gene (18) under the regulation of the rat POMC promoter and 5′-flanking region, either alone or cotransfected with an expression plasmid overexpressing a mutant RI subunit, to examine the role of cAMP and protein kinase A activity in gonadotropin-regulated gene expression.

MATERIALS AND METHODS

Hormones—Pregnant mare's serum gonadotropin and human follicle-stimulating hormone (hFSH, 4822B) were gifts of the National Hormone and Pituitary Program of the University of Maryland School of Medicine and the National Institute of Diabetes and Digestive and Kidney Diseases. In addition, pregnant mare's serum gonadotropin was also obtained from Sigma.

Granulosa Cell Preparation and Culture—Granulosa cells were isolated 48 h after stimulation with 20 IU of pregnant mare's serum gonadotropin by the method of Campbell et al. (129). Approximately 3 × 10⁶ viable cells were plated on 35-mm wells of six-well plates (GIBCO) coated with 35 µg of Cell-Tak (Collaborative Research, Inc., Bedford, MA). The cells were then cultured in 3 ml of serum-free medium 199 (GIBCO) supplemented with 18 ng/ml corticosterone (Steraloids, Wilton, NH), 0.1% bovine serum albumin (low endotoxin, Sigma), and ITS (insulin, transferrin, selenium premix, Collaborative Research, Inc.) at a final concentration of 1 µg/ml insulin and transferrin and 1 ng/ml selenium acid.

Granulosa Cell Transfection—Granulosa cells were transfected with a calcium phosphate/DNA coprecipitate as described (20). Briefly, newly isolated granulosa cells were cultured in serum-free medium for 4–6 h. The cells were then treated with 0.25 µl of precipitate containing 45 µg of DNA/well for 4 h, followed by 3.5 min of glycerol shock (15% glycerol in Dulbecco's phosphate-buffered saline). The cells were rinsed with 2 ml of phosphate-buffered saline, and fresh serum-free media was added with or without hormone supplementation. An important aspect of reproducible transfection was the use of Cell-Tak-coated plates, which allowed the granulosa cells to attach firmly and quickly to the plates.

CAT Assay and Quantitation—After 12 h of culture in the presence or absence of hormone, cells were scraped from each well and collected by centrifugation at 450 × g for 10 min. The cells were brought up in 100 ml of Tris saline buffer, pH 7.4, and lysed by three freeze-thaw cycles in a dry ice ethanol bath and a 37 °C water bath. Soluble extract was separated from insoluble cell components by centrifugation at 15,000 × g for 5 min. Cell extracts (85 µl) were incubated with a solution of 1 M Tris buffer, pH 7.4, containing 25 µl of [14C] chloramphenicol (Du Pont-New England Nuclear) and 55.4 mg/ml acetyl coenzyme A (Sigma) at 37 °C for 4 h. The chloramphenicol forms were then extracted from the solution with 1 ml of ethyl acetate. The extract was dried in a Speed-Vac, solubilized in 15 µl of ethyl acetate, and loaded onto the stacking zone of a TLC plate (type LK6, Whatman). These conditions were adapted from those used in Young et al. (21) and Gorman et al. (30). Quantitation was achieved by scintillation counting of the spots on TLC plates detected by autoradiography and was expressed as percent conversion of chloramphenicol to its acetylated products.

Plasmids—Plasmids were prepared by alkaline lysis followed by CsCl density gradient centrifugation according to Hardy (31), except that 500 ml of bacterial culture were used instead of 250 ml. The lack of protein contamination was verified using ultraviolet absorbance at 260 and 280 nm. The identities of the plasmids were confirmed by restriction analysis. The plasmid POMC-CAT-150 (−130 to +43) relative to the transcription start site) was constructed by exonuclease III/S1 nuclease deletion of POMC-CAT-704 (20) using a kit (Stratagene). The plasmids diagrammed in Fig. 3 were created either by restriction enzyme digestion of POMC-CAT-704 followed by filling with Klenow fragment of DNA polymerase and religation or by using fragments of the POMC promoter generated by oligonucleotide primers and the polymerase chain reaction (AmpliTaq, Perkin-Elmer Cetus Instruments) and ligation into a plasmid containing a promoterless CAT gene (XhoI/HindIII digest of POMC-CAT-704). The pMtr(A,B) plasmid was a gift from Dr. S. McKnight at the University of Washington, Seattle, and the pSV2-CAT plasmid was a gift from Dr. B. Howard at the National Cancer Institute (Bethesda, MD).

DNA Sequencing—All constructs were sequenced using dyeoxy chain termination (37) from a double-stranded template with Sequenase (U. S. Biochemical Corp.). A sequencing primer (GGTTA TAGCTGATCAGCTCCG) identical with bases 4867–4885 of vector pSV2CAT was prepared by Genosys. All sequencing reactions were run on a 6% acrylamide (acylamide:bis, 19:1), 8.3 µa urea gel and analyzed using the program MacVector (International Biotechnologies, Inc.).

Experimental Design and Data Analysis—Each figure represents data from a representative experiment of replicates. Each experiment utilized plasmid from one preparation, whereas replicate experiments utilized plasmid from a different preparation. Statistical significance was calculated using Student's t distribution.

RESULTS

The mechanisms of gonadotropin and adrenergic stimulation of the POMC promoter were investigated by transfection of primary cultures of rat granulosa cells with the POMC promoter-directed CAT expression vector, POMC-CAT-150. This plasmid contains a segment of the rat POMC gene from −150 to +43 fused to the CAT gene.

Granulosa cells transfected with POMC-CAT-150 demonstrated a high basal level of CAT activity that was significantly stimulated 3- and 2-fold above controls by either 20 ng/ml FSH or 1 µM isoproterenol, respectively (Fig. 1, A and B, open bars). Granulosa cells taken from the same original pool of cells were cotransfected with POMC-CAT-150 and Mtr-AB, an expression vector for a mutant form of the RI regulatory subunit of kinase A. Cotransfection with Mtr-AB, which specifically inhibits protein kinase A (14), not only reduced basal CAT expression to ∼10% of controls but also abolished gonadotropin and adrenergic stimulation of POMC-CAT-150 (Fig. 1B, solid bars). These data imply that protein kinase A is necessary for both high basal and hormone-stimulated activity of the POMC promoter fragment contained in POMC-CAT-150.

To control for specificity of both the gonadotropin and Mtr-AB effects, a control vector, pSV2-CAT, was used. This vector contains the CAT gene under control of the SV40 virus early gene promoter. Neither gonadotropin stimulation nor cotransfection with Mtr-AB caused any significant alteration in the level of CAT expression (Fig. 2, A and B). These control experiments argue against any generalized effect of either gonadotropin or Mtr-AB on the processes of transcription and translation in this system.

Initial deletion analysis of the POMC promoter was performed to begin characterizing the sequences involved in protein kinase A-dependent regulation. Deletions of the 5′
region from -704 to -480 (designated -480), from -704 to -160 (designated -160), and from -704 to -40 (designated -40) all preserved both basal and gonadotropin-stimulated expression of the CAT gene (Fig. 3). Interestingly, basal and gonadotropin-stimulated expression from the promoter increased with deletion of the segment from -480 to -160.

To further probe the sequences required for gonadotropin-stimulated expression, 5' deletion mutants were constructed that were closer to the basal promoter. Specifically, an 8-base pair palindromic (CCTGCAGG) immediately adjacent to the TATA box was partially deleted by utilizing a PstI restriction enzyme cleavage site in this region (Fig. 4). This deletion mutant from -40 to -33 (designated pSLY002) completely eliminated FSH-responsive expression and lowered basal expression from the promoter without interrupting the TATA box (Fig. 5). These data imply that the region from -40 to -33 is necessary for gonadotropin-stimulated expression of POMC in rat granulosa cells.

The role of the sequences downstream of the transcription start site in the gonadotropin-stimulated expression was addressed in a further series of experiments. A 3' deletion mutant was constructed deleting from +63 to +44 but containing the sequence to -150 (designated -150/no exon). (Fig. 4). This deletion mutant also abolished gonadotropin-stimulated expression and lowered basal expression from the promoter. These last two deletion mutants suggest that sequences from -40 to -33 and +4 to +63 are both necessary, but neither one is sufficient for FSH-stimulated expression.

**DISCUSSION**

These data argue that gonadotropin stimulation of the POMC promoter requires protein kinase A activity. Previous studies have shown stimulation of the POMC promoter in granulosa cells by cAMP analogues, phosphodiesterase inhibitor, and the gonadotropins hFSH and human luteinizing hormone (20). From those data, it was hypothesized that gonadotropins exert their effect on gene regulation through a cAMP-dependent pathway. These data confirm that hypothesis and suggest that the cAMP-mediated promoter stimulation acts via protein kinase A. Although a majority of the previous evidence suggested that cAMP is the principal intracellular mediator of gonadotropin action, other potential mediators have been implicated (7, 8). Hence, the relative roles of other intracellular signals such as Ca2+ and diacylglycerol are unknown. This evidence is also of interest due to the absence of definitive CRE sequences or AP-2 elements in the proximal promoter region of POMC. This suggests that there is divergence in the sequences recognized by these factors or the existence of some new, undefined elements that mediate cAMP action on transcription. The existence of diverse CRE sequences would not be surprising given the apparent diversity of CREB or CREB-like transcription factors (45).

Multiple gonadotropin-responsive cell functions appear to be mediated by cAMP. Wang and Ascoli (32) recently showed that the development of Leydig tumor cell lines stably transfected with a similar mutant RI subunit expression vector demonstrated reduced gonadotropin-responsive steroidogenesis and c-fos mRNA expression. The partial effects of the transfected expression plasmid obtained by these investigators may be related to the use of a mutant RI subunit with partial cAMP binding activity or to the selection of stable cell clones with potential compensatory survival mechanisms. In
addition to inhibiting gonadotropin stimulation, overexpression of the RI subunit also inhibited isoproterenol stimulation of the POMC promoter. These data are consistent with previous data on the mechanism of action of β-adrenergic agonists and isoproterenol in granulosa cells. The positions of the deletions are indicated above their locations. An 8-base pair palindrome adjacent to the TATA box and hypothesized to be important for gonadotropin-stimulated promoter activity is delineated by a box.

FIG. 3. Structures of POMC-CAT fusion genes and their corresponding responsiveness in transfected granulosa cells. The positions of the deletions are depicted by spaces and the resulting numbered nucleotides relative to the transcription start site. Restriction enzyme sites are depicted (e.g. H3, HindIII; BglII, BglII; and PstI, PstI), and the constructs obtained using polymerase chain reaction (PCR) primers are noted. The CAT activities are expressed as percent conversion of chloramphenicol to its acetylated products. The open bars depict CAT activity from cells under basal conditions, and the closed bars depict CAT activity from cells stimulated with hFSH (20 ng/ml). rPOMC, rat POMC.

POMC promoter in anterior pituitary tumor cells demonstrated that corticotropin-releasing hormone- and forskolin-stimulated promoter activity were not deleted by a series of 5' deletion mutants, suggesting that elements responsible for basal and cAMP responsiveness are closely linked (35). Although a potential cAMP-responsive region of the rat POMC 5'-flanking region was determined, it required the excision of the POMC promoter and fusion to a heterologous promoter, making the relevance of this site difficult to assess. Other studies have examined the regulation of the human POMC promoter by transfection into rat C6 glioma cells (34). In this system, cAMP responsiveness was localized to a region between -417 and +67 of the human gene, although C6 cells do not express POMC mRNA. Recent evidence suggests that the choice of the cell line for transfection studies can determine the responses obtained with cAMP-responsive gene promoters (35). Therefore, current data from multiple laboratories suggest that the human and rat POMC promoters are responsive to cAMP, but the exact identification of the sequence elements responsible for this stimulation is still unclear.

It is possible that a cAMP-responsive element interacts with another enhancer element to yield gonadotropin-responsive expression. In the adrenal cortex, ACTH-responsive expression of the 21-hydroxylase gene promoter requires both an upstream element between -330 to -150 and a proximal element between -68 to -62 (42). Initial deletion analysis suggests that an element important for high basal and cAMP-stimulated expression of the rat POMC gene is interrupted by deletion from -40 to -33. Although this region has no homologies to regulatory elements, it does contain a perfect 8 base pair palindrome of CCTGCAGG (Figure 4). The relative importance of this element in gonadotropin responsiveness is currently under examination. These data suggest that the cAMP-responsive region of the rat POMC gene lies between -33 and +63. Furthermore, it appears that two regions are necessary but neither is sufficient for FSH-stimulated expression. This suggests that an element in the +4 to +63 region may therefore interact with sequences in the region of -40 to -30 to elicit the full gonadotropin- and cAMP-responsive expression. It is interesting to note that the +4 to +63 region required for gonadotropin responsiveness
cholesterol side chain cleavage is regulated by gonadotropins and may be generally important to other gonadotropin-regulated genes. In Leydig cells, this effect is blocked by protein synthesis inhibitors such as cycloheximide (41). Therefore, the mechanism of action of gonadotropins on target cell genes may involve the induction of a trans-acting or autocrine/paracrine factor.

The data presented in this manuscript strongly suggest that the mechanism of action of gonadotropins on POMC promoter activity in granulosa cells requires protein kinase A. Furthermore, basal expression also requires protein kinase A. Initial deletion analysis of the sequence elements in the POMC promoter responsible for this regulation suggest that a palindrome in close proximity to the POMC promoter and sequences downstream of the transcription start site may be important for gonadotropin-stimulated activity of the promoter.

REFERENCES

Gonadotropin Stimulation of the POMC Promoter via Kinase A