The inducible isoform of CREM (inducible cAMP early repressor, ICER) is a repressor of *CYP19* rat ovarian promoter

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Abstract

The synthesis of estradiol by the granulosa cells is a prominent event in ovarian physiology and depends on the expression of $P450_{AROM}$. FSH induces the expression of $P450_{AROM}$ in granulosa cells as a result of the presence in the ovarian promoter of a CRE (cAMP response element)-like sequence (CLS). In rodents, LH downregulates aromatase expression during luteinization by an as yet undescribed mechanism. In granulosa cells, LH increases the expression of the inducible cAMP early repressor (ICER), an isoform of CREM (cAMP-responsive element modulator) that represses cAMP-induced transcription. The possibility that ICER represses the activity of the aromatase ovarian promoter, thus being part of the mechanism underlying the effects of LH was investigated. We have found that: (1) nuclear proteins from forskolin-stimulated granulosa cells were specifically bound to an oligonucleotide containing the CLS sequence of the *CYP19* ovarian promoter and one out of the two protein–DNA complexes formed was supershifted by an anti-CREM antibody; (2) in granulosa cells, forskolin-induced increases in $P450_{AROM}$ promoter luciferase reporter gene activity were prevented by the transient overexpression of ICER; (3) similar results were obtained in 8-Br-cAMP-stimulated R2C cells, a Leydig tumor cell line routinely used for the study of $P450_{AROM}$ promoter activity; (4) both ICER mRNA levels and $P450_{AROM}$ promoter-driven luciferase activity were elevated 6 and 12 h after stimulation of R2C cells with 8-Br-cAMP and were decreased 24 and 48 h later; (5) in an R2C polyclonal line overexpressing ICER, the promoter activity at early stages of stimulation was completely attenuated, while 24 and 48 h downregulation was prevented in another R2C line stably transfected with an antisense ICER construct. These results suggest that ICER represses *CYP19* ovarian promoter and that LH-induced expression of ICER may serve to downregulate $P450_{AROM}$ transcription in granulosa cells during luteinization.

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Introduction

Aromatase cytochrome P450 ($P450_{AROM}$) is the rate-limiting enzyme required for the conversion of androstenedione and testosterone into 17β-estradiol (Kellis & Vickery 1987, Simpson *et al.* 1994). In the female gonad the expression of $P450_{AROM}$ constitutes one of the hallmarks of granulosa cell differentiation, because the production of large amounts of estradiol determines the outcome of follicular development (McNatty *et al.* 1979, Hsueh *et al.* 1984).

$P450_{AROM}$ is the product of the *CYP19* gene, a member of the P450 superfamily of genes which contains over 300 members in some 36 gene families (Nelson *et al.* 1993). $P450_{AROM}$ expression can be detected in several tissues including human placenta, brain, adipose tissue and the gonads (Simpson *et al.* 1997*b*). The coding region of *CYP19*, and hence the protein, is identical for every tissue where the enzyme is expressed. However the 5′ end of the transcripts changes and the use of alternative tissue-specific promoters allows differential regulation in each tissue (Means *et al.* 1991, Simpson *et al.* 1997*a*). Thus follicle-stimulating hormone (FSH) activates *CYP19* expression in the ovary because a CRE-like sequence bestows cAMP-dependent inductibility on the ovarian promoter (Fitzpatrick & Richards 1994, Michael *et al.* 1997).

The CRE 8 bp palindromic consensus sequence binds homodimers of serine 133-phosphorylated CREB (Gonzalez *et al.* 1989, Dwarki *et al.* 1990, Yun *et al.* 1990, Shaywitz & Greenberg 1999) or CREB heterodimers with ATF-1 (activating transcription factor 1) or CREM (cAMP-responsive element modulator), two other bZIP factors that share with CREB a high degree of identity within the bZIP region (Hai *et al.* 1989, Foulkes *et al.* 1991).

CREM gene is composed of several exons and encodes a set of proteins generated by alternative splicing that

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function as activators or repressors of cAMP-induced transcription (Foulkes *et al.* 1991, 1992, Laoide *et al.* 1993). An alternative intronic promoter in the CREM gene directs the rapid, cAMP-dependent transcription of a repressor isoform, therefore named inducible cAMP early repressor (ICER) (Molina *et al.* 1993). Alternative RNA processing of the CREM binding and dimerization domain (DBD) and γ exon leads to the generation of four types of ICER transcripts: ICER-I and ICER-II (composed of exon γ plus DBD-I or DBD-II), and the γ exon-deficient isoforms: ICER-Iγ and ICER-IIγ. The four proteins of the ICER family are the smallest CRE-binding nuclear factors described to date. They act as repressors of cAMP-induced transcription, because while sharing with other CREM isoforms the DNA binding and dimerization domains, they are devoid of the kinase-inducible and transactivation domains. This unique feature endows ICER with a key role as a repressor that mediates the attenuation of cAMP-dependent transcription.

Upon binding to their membrane-bound G-protein-coupled receptor (Tsai-Morris *et al.* 1991, Heckert *et al.* 1992) both FSH and luteinizing hormone/human chorionic gonadotropin (LH/hCG) induce CREB phosphorylation in rat ovarian granulosa cells (Mukherjee *et al.* 1996). However, while FSH induces aromatase expression, LH promotes the enzyme downregulation during the luteinization process (Hickey *et al.* 1990, Fitzpatrick *et al.* 1997) by mechanisms that remain unknown. In granulosa cells, ICER expression in response to LH/hCG has been proposed to mediate the suppression of α-inhibin subunit gene that occurs during the preovulatory surge of LH (Mukherjee *et al.* 1996). Similar to $P450_{AROM}$ the expression of inhibin α-subunit in the ovary is induced by FSH through a cAMP-dependent mechanism and is downregulated by LH after ovulation (Turner *et al.* 1989, Pei *et al.* 1991). Here, we have investigated whether ICER may also attenuate *CYP19* transcription, thus being part of the mechanism underlying the effects of LH.

To test this hypothesis, we have first measured the effects of transient overexpression of ICER on the activity of $P450_{AROM}$ promoter-luciferase reporter in granulosa and R2C cells, a rat Leydig tumor cell line routinely used for the study of the aromatase promoter, and thereafter we have measured the activity of the promoter in R2C cells stably transfected with ICER sense and antisense constructs.

Materials and Methods

Reagents and hormones

All culture media were from Invitrogen Co. (San Diego, CA, USA). Diethylstilbestrol (DES), forskolin, 8-Br-cAMP, protease and phosphatase inhibitors and all other reagents were from Sigma Co. (Madrid, Spain). Anti-CREM and anti-CREB antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and UBI (Lake Placid, NY, USA). Poly [dI-C] and T4 polynucleotide kinase were from Amersham Pharmacia Biotech AB (Uppsala, Sweden). AMV reverse transcriptase (M519), RNAsin ribonuclease inhibitor (N211) and *Taq* DNA polymerase were from Promega (Madison WI, USA). The pcDNA 3·1/Zeo(+) plasmid and Trizol were purchased from Invitrogen Co.

Animals and cell culture

Immature 19- to 21-day-old female Sprague–Dawley rats were purchased from Charles Rivers Laboratories (Barcelona, Spain). Housing conditions and experimental procedures were approved by the University of Las Palmas Committee on Animal Care that enforces European Union rule 86/609. Granulosa cells were obtained by follicle puncture from the ovaries of rats implanted with DES for 3 days and cultured in McCoy's 5a medium (modified, without serum). R2C Leydig cells obtained from ATCC (Marassas, VA, USA) were grown and cultured in Waymouth MB752/1 medium supplemented with 20 mM HEPES, 1·12 g/l $NaHCO_3$, 15% horse serum and 50 μg/ml gentamycin.

PCR and constructs

Total RNA was extracted from duplicated cultures of granulosa and R2C cells, using a modified guanidinium isothiocyanate method. The samples were stored frozen (−70 °C) in diethylpyrocarbamate-treated water until use. After drying and optical density determination, reverse transcription was performed by standard protocols. Briefly, equal amounts of RNA (1 μg) were incubated for 75 min at 42 °C in 20 μl (final volume) of 1 × PCR buffer (10 mM Tris–HCl, 50 mM KCl, 5 mM $MgCl_2$, and 0·1% Triton X-100, pH 9), 500 ng of polydeoxythymidine primers, 1 mM dNTP, 5 U AMV reverse transcriptase and 20 U RNAsin ribonuclease inhibitor. Each cDNA was amplified using specific oligonucleotides designed to prime the different ICER isoforms in 25 μl of 1 × PCR buffer containing (final concentrations) 2·5 μM DIG-dUTP, 0·625 U *Taq* DNA polymerase and 125 ng (10–15 pmol each) of the appropriate gene-specific synthetic primers. ICER primers were: ICER-5′ ACTTA GGATCCACTGTGTACGGCCAAC; ICER-3′ GTT AAATAGAATTCACTAATC TGTTTTGGG, the underlined sequences being restriction sites for BamHI in the 5′ primer and EcoRI in the 3′ primer that were introduced to facilitate the posterior subcloning. PCR products were detected by chemiluminescence and due to the high sensitivity of the method the thermal profile had to be adjusted as follows: 94 °C for 1 min for denaturation, and 2 min at 62 °C and 72 °C for annealing and elongation

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respectively. Under these conditions unspecific background signals were reduced to undetectable levels and linearity was ensured for up to 29 cycles for ICER, and 17 cycles for G6PDH that was used as an internal amplification control. The amplified products were resolved by 1·8% agarose gel electrophoresis and transferred to positively charged nylon membranes. Detection was performed with a commercially available DIG luminescent detection kit (Boehringer Mannheim, Barcelona, Spain) following the instructions of the manufacturer. The membranes were recorded and visualized in an imaging system with a cooled CCD capture system for chemiluminescence (Diana III from Raytest, Straubenhardt, Germany) using the software provided by the manufacturer (AIDA, 1D, 2D).

AROM-luc is − 161 bp of the 5′ flanking sequence of the rat *CYP19* promoter (Fitzpatrick & Richards 1994) ligated to pGL2-Luc (Promega) and was donated by Dr J S Richards (Baylor College, Houston, TX, USA).

ICER sense (ICERs) and antisense (ICERas) expression vectors were constructed with cDNA obtained from forskolin-stimulated granulosa cells amplified with the Expand High Fidelity PCR System kit (Promega). In addition to the already described oligonucleotides, another set of primers was used where EcoRI in the forward and BamHI in the reverse primer were introduced to subclone ICER in the antisense orientation. A band corresponding to ICER-I was dissected out from the agarose gel and the DNA was isolated (using Quantum Prep Freeze and Squeeze DNA Gel Extraction Spin columns, Bio-Rad Laboratories, Richmond, CA, USA) and sequenced before the purified fragments were cloned into the pcDNA 3·1/Zeo(+) plasmid (Invitrogen Co.) polylinker region to obtain pcDNA-ICER-Is and pcDNA-ICER-Ias constructs.

Transfections, establishment of ICER-expressing R2C clones and luciferase assays

Transient transfections were performed in 5×10^5 granulosa cells or 40% confluent R2C cells plated into 35 mm dishes. Plasmid DNA (2·5 μg) in 100 μl serum-free medium was mixed with 5 μl lipofection reagent (Superfect, Qiagen Inc., Valencia, CA, USA), incubated for 10 min to form the transfection complex and added to PBS rinsed cells. After 4 h the DNA–lipid complexes were removed with PBS. Granulosa cells or R2C cells were thereafter reincubated in McCoy's 5a modified medium or 15% horse serum-supplemented Waymouth medium containing the appropriate treatments for the times indicated in each experiment.

To prepare stable R2C cell lines, 10 μg plasmid DNA in 300 μl serum-free Waymouth medium were transfected to R2C cells (10^5). After thorough washing, reincubation for 3 weeks in Waymouth medium supplemented with a previously determined optimal dose (400 μg/ml) of Zeocin was performed to select the stably transfected clones. Media were changed every 3 days to eliminate cellular debris. The transfection produced similar numbers of clones, suggesting that ectopic expression of ICER was not toxic for the cells. About 100 clones of Zeocin-resistent cells were pooled to obtain the stably transfected cell lines for each construct.

The luciferase activity in 20 μl cellular extracts was assayed in an analytical luminescence detector (Luminova 1254–001, Bio-Orbit OY, Turku, Finland) for 10 s using the Luciferase Assay System kit (Promega) according to the manufacturer's protocol, and levels of luciferase activity were expressed as light units per μg protein.

Electrophoretic mobility shift assays

Nuclear cell extracts were prepared from forskolin-stimulated granulosa cells (Andrews & Faller 1991). Briefly, granulosa cells were scraped off the culture dishes into 1·5 ml cold PBS, pelleted and resuspended in cold buffer A (10 mM HEPES-KOH pH 7·9, 1·5 mM $MgCl_2$, 10 mM KCl, 0·5 mM dithiothreitol, 0·2 mM phenylmethylsulfonyl fluoride (PMSF)). The cells were kept on ice for 10 min, vortexed and centrifuged before the supernatant fraction was discarded. The pellet was resuspended in cold buffer B (20 mM HEPES-KOH pH 7·9, 25% glycerol, 420 mM NaCl, 1·5 mM $MgCl_2$, 0·2 mM EDTA, 0·5 mM dithiothreitol, 0·2 mM PMSF) and incubated on ice for 20 min. Cellular debris was removed by centrifugation. The supernatants were stored at − 70 °C until use.

Nuclear proteins (15–20 μg), were incubated for 20 min on ice in binding buffer (10 mM HEPES pH 7·6, 0·2 M NaCl, 5 mM $MgCl_2$, 0·5 mM EDTA, 0·5 mM EGTA, 5 μg/ml poly [dI-C] acid) and a 24-bp double-stranded oligonucleotide containing the CRE-like sequence of the rat $P450_{AROM}$ promoter (− 161/ − 138 sequence) previously labeled with [^{32}P-γ]ATP by T4 polynucleotide kinase. Where indicated, increasing concentrations of the unlabeled probe, an AP-1 probe or a specific antibody to CREM-1 or CREB were added to the incubation mixture 30 min before the addition of the labeled DNA probe. After non-denaturing electrophoresis (0·5 × TBE, Tris–borate-EDTA) at 150 V, the gels were dried and recorded as autoradiograms in a Molecular Dynamics 400A PhosphorImager.

Statistical analysis

Results are expressed as means ± S.E.M., and significance was determined by analysis with Student's *t*-test for two group comparison or ANOVA for multiple group comparison.

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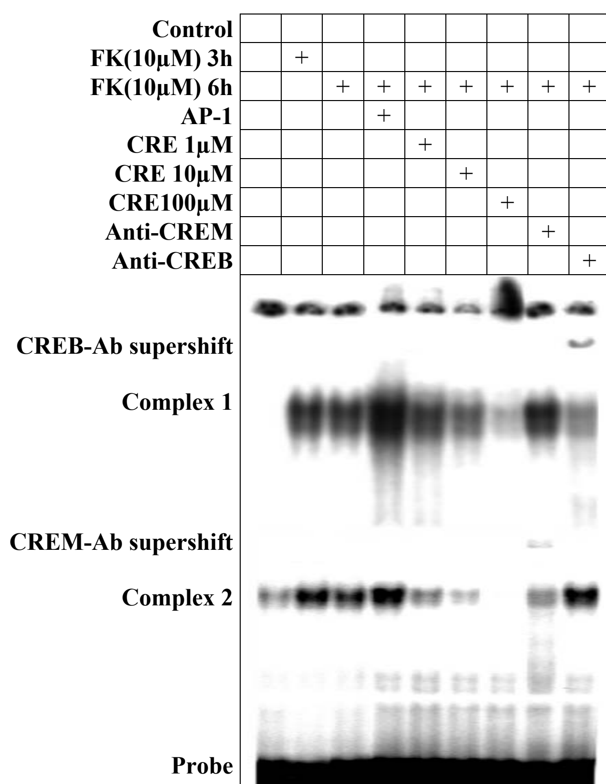

Figure 1 Mobility shift assay of protein extracts from forskolin-stimulated granulosa cells. Nuclear extracts obtained from granulosa cells that were stimulated for 3 or 6 h with forskolin (FK; 10 μM) were incubated with a labeled oligonucleotide spanning the region of the CRE sequence of aromatase promoter. Pre-incubation with increasing concentrations of the unlabeled oligonucleotide, a consensus AP-1 oligonucleotide, CREM or CREB antibodies was performed where indicated. DNA–protein complexes were electrophoresed in non-denaturing conditions and visualized after recording as autoradiographs by photostimulable-storage imaging.

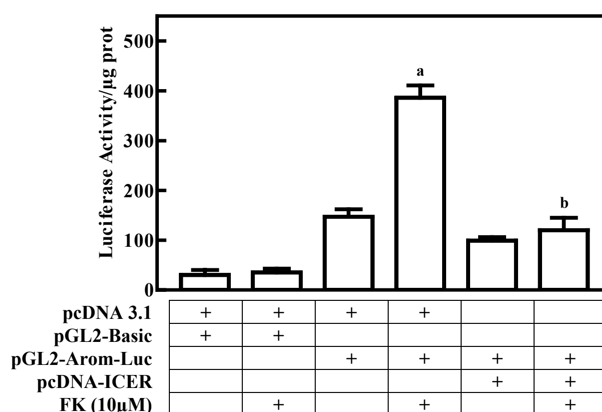

Figure 2 Activity of the $P450_{AROM}$ promoter in rat granulosa cells overexpressing ICER. pcDNA-ICERs was transiently cotransfected along with the pGL2-Arom-Luc plasmid into granulosa cells that were thereafter stimulated with forskolin (FK; 10 μM) for 6 h, and luciferase activity was determined as described in the Materials and Methods section and represented as activity per μg cellular protein. Data from triplicate measures of three different experiments were pooled and results are the means ± S.E.M. a and b, $P<0{\cdot}01$ compared with control unstimulated cells and with forskolin-treated ICER untransfected cells respectively.

Results

Mobility shift assay and transient overexpression of ICER in granulosa cells

To determine if proteins synthesized in granulosa cells in response to cAMP analogs interact with the CRE in the ovarian aromatase promoter, a radiolabeled double-stranded oligonucleotide probe containing the CRE-like sequence (CLS) of $P450_{AROM}$ promoter was incubated with protein extracts from granulosa cells treated for 3 and 6 h with forskolin. Figure 1 shows that proteins from forskolin-stimulated granulosa cells specifically bind the CLS sequence from *CYP19* ovarian promoter as demonstrated by the fact that competition was achieved when incubations were performed with an excess of unlabeled $P450_{AROM}$ promoter CRE, but not with an oligonucleotide containing the AP-1 response sequence. In agreement with previously reported data (Fitzpatrick & Richards 1994, Michael *et al.* 1997) the slower mobility complex was supershifted after pre-incubation with an anti-CREB antibody, thus indicating that CREB is a component of the complex. A second, faster migrating protein–CLS complex was also detected. This complex most likely contains ICER since: (1) it was supershifted by an anti-CREM antibody; (2) consistent with the notion that proteins encoded by ICER are the smallest among the CREM isoforms as well as any other CRE-binding proteins, the complex has a very low molecular mass; (3) except for CREM-τ expressed in a specific and hormone-regulated fashion in the germ cells of the testis (Foulkes *et al.* 1992, 1993), no CREM isoforms other than ICER have been described to date as inducible.

The expression of $P450_{AROM}$ promoter-driven reporter genes transiently transfected into granulosa cells can be induced by forskolin (Fitzpatrick & Richards 1994). Therefore, to assess the effect of ICER on $P450_{AROM}$ promoter activity the active −161 bp 5′ flanking sequence of the rat *CYP19* promoter ligated to pGL2-Luc was transiently transfected into granulosa cells and the promoter activity in response to forskolin was measured when overexpression of ICER was simultaneously obtained by cotransfection with the pcDNA-ICER plasmid (Fig. 2). Luciferase activity was raised 2·5-fold in forskolin-stimulated cells ($P<0{\cdot}01$). Cotransfection of the ICER construct completely prevented the forskolin-increased luciferase activity, thus providing support to the hypothesis that ICER can repress $P450_{AROM}$ expression in rat granulosa cells.

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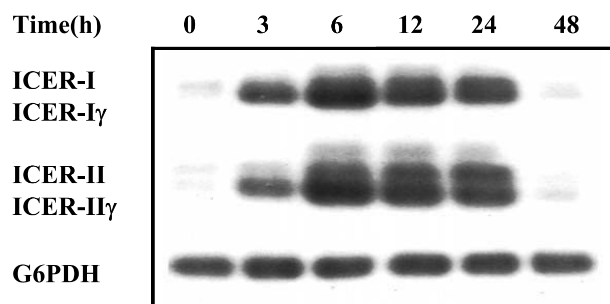
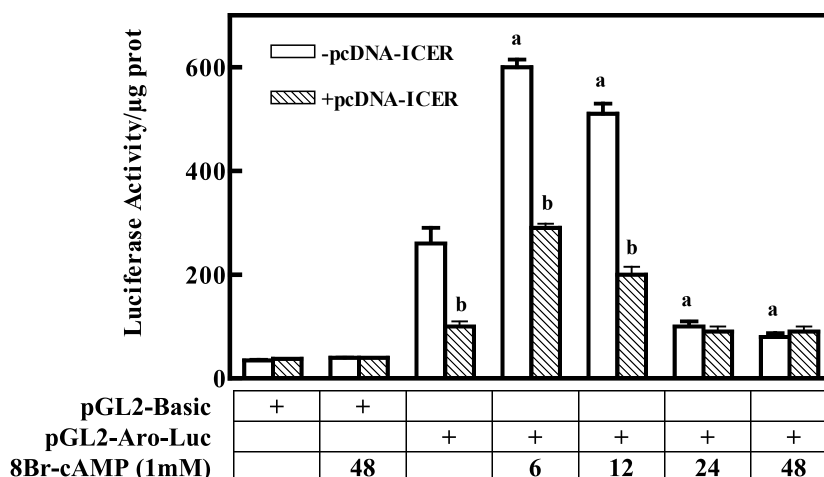


Figure 3 Effects of 8-Br-cAMP on ICER mRNA levels in R2C Leydig tumor cells. R2C cells were stimulated with 8-Br-cAMP (1 mM) for the times indicated, and ICER mRNA levels were determined by RT-PCR as described in the Materials and Methods section. G6PDH was used as an internal amplification control.

ICER mRNA levels in response to cAMP and effects of the transient overexpression of ICER on $P450_{AROM}$ promoter activity in R2C tumor Leydig cells

Early studies have shown that R2C tumor Leydig cells express high basal levels of aromatase mRNA but do not respond to the same levels of forskolin used in granulosa cells (Fitzpatrick & Richards 1994). In these studies the transcriptional activation of a $P450_{AROM}$ promoter reporter gene was assessed after 48 h stimulation with cAMP analogs, due to the fact that the full activity of the reporter gene is reached only 48 h after transfection. It seemed a reasonable possibility that a parallel cAMP-induced expression of ICER could account for the apparent unresponsiveness of $P450_{AROM}$. To test this possibility, ICER mRNA levels were assessed by RT-PCR in unstimulated and 8-Br-cAMP-activated cells. As shown in Fig. 3, basal levels of ICER were undetectable whereas the exposure to 8-Br-cAMP (1 mM) resulted in a rapid induction of ICER mRNA with maximal induction of the four isoforms occurring after 6 h of 8-Br-cAMP stimulation. ICER mRNA levels remained high for up to 24 h and returned to the levels of unstimulated cells 48 h after 8-Br-cAMP treatment.

Thus it seemed likely that the length of stimulation routinely used would allow for ICER synthesis and $P450_{AROM}$ downregulation, resulting in the apparent lack of response to cAMP. To explore this hypothesis, the activity of luciferase as reporter gene of the $P450_{AROM}$ promoter was measured in R2C cells transiently transfected with the pGL2-Arom-Luc construct. R2C cells were stimulated with 8-Br-cAMP (1 mM) for the usual 48 h and also 6, 12 and 24 h before the end of the 48-h experimental period. Figure 4 (open bars) shows that the stimulation of R2C Leydig tumor cells with 8-Br-cAMP for 6 and 12 h resulted in a twofold ($P<0{\cdot}01$) increase in the luciferase activity. As in earlier reported data (Fitzpatrick & Richards 1994), treatment with the cAMP analog for 24 and 48 h significantly ($P<0{\cdot}05$) decreased the activity of the reporter gene below the levels of unstimulated cells. The use of this slightly different experimental approach has permitted the observation that in R2C cells $P450_{AROM}$ activity can be stimulated with cAMP, the previously reported unresponsiveness being more likely attributable to ICER repression.



pGL2-Basic	+	+					
pGL2-Aro-Luc			+	+	+	+	+
8Br-cAMP (1mM)		48		6	12	24	48

Figure 4 Activity of the $P450_{AROM}$ promoter in 8-Br-cAMP-stimulated R2C Leydig tumor cells and effects of the transient overexpression of ICER. R2C cells were transiently transfected with the pGL2-Arom-Luc construct (open bars) alone or with the pcDNA-ICERs (hatched bars) and treated with 8-Br-cAMP (1 mM) for 6, 12, 24 and 48 h before the end of the experiment. Luciferase activity in the cellular extracts was assessed as described in the Materials and Methods section. Data from triplicate measures of three different experiments were pooled and the results are the means ± S.E.M. a and b, $P<0{\cdot}01$ compared with control unstimulated cells and with ICER untransfected cells respectively.

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To support this latter assumption, we have also studied the effects of transiently cotransfecting the ICER expression vector on the basal and 8-Br-cAMP-stimulated activity of the AROM-luc construct. Figure 4 (hatched bars) shows that both basal and cAMP-stimulated activity of the luciferase reporter gene are significantly attenuated ($P < 0{\cdot}01$) when ICER is overexpressed, indicating that ICER can also repress aromatase expression in R2C cells.

Effects of the stable expression of ICER sense and antisense constructs on $P450_{AROM}$ promoter-luciferase reporter expression in R2C tumor Leydig cells

To strengthen the hypothesis of ICER being able to repress *CYP19* transcription, we next compared the activity of the $P450_{AROM}$ promoter luciferase construct in three polyclonal lines of R2C tumor cells stably transfected with the pcDNA 3·1/Zeo(+) empty vector or the pcDNA-ICER-Is or pcDNA-ICER-Ias. The results obtained with the pcDNA 3·1/Zeo cell line mimic those in wild-type cells. Figure 5 shows that the activity of the reporter gene increases 6 and 12 h after 8-Br-cAMP and decreases after 24 and 48 h stimulation. ICER-Is over-expressing cells behaved as wild-type R2C cells transiently transfected with the ICER expression vector, and did not respond to 8-Br-cAMP stimulation. Stimulation of ICER-Ias R2C cells with the cAMP analog resulted in greater activity of the reporter gene as compared with wild-type cells. Also, downregulation of the promoter activity in the ICER-Ias R2C cells after 24 and 48 h stimulation with 8-Br-cAMP was less pronounced than in pcDNA 3·1/Zeo transfected cells, thus further supporting the possibility that ICER is a repressor of *CYP19* ovarian promoter.

Discussion

The transcription of ICER, the best characterized among the CREM repressor isoforms is induced by cAMP, and four CRE-like elements termed CAREs (cAMP autoregulatory elements) convey cAMP-responsiveness to the ICER promoter thus making it possible for ICER to autoregulate its own expression in a phosphorylation/dephosphorylation independent fashion (Molina *et al.* 1993). These distinctive features provide the grounds for ICER to function as a regulator of the cyclic expression of cAMP-dependent genes (Lamas & Sassone-Corsi 1997).

Cyclicity is a key functional property of many cells in the neuroendocrine, endocrine and reproductive systems. ICER has been reported to be involved in the repression of melatonin synthesis during the course of the normal circadian rhythm in the pineal gland (Stehle *et al.* 1993), to block adrenocorticotropin secretion from the corticotropes (Lamas *et al.* 1997), to function as a repressor of para-thyroid hormone up-regulation of the vitamin D receptor in osteoblastic cells (Huening *et al.* 2002), and also participates in the long-term desensitization of the FSH and thyrotropin receptors (Lalli & Sassone-Corsi 1995, Monaco *et al.* 1995).

Estrogen production by the ovary is also a cyclic process and despite the presence during the periovulatory surges of high levels of cAMP mobilizing hormones, the granulosa cells in the dominant preovulatory follicles are deprived of aromatase expression during luteinization (Hickey *et al.* 1990, Fitzpatrick *et al.* 1997).

The existence of a cycloheximide-sensitive factor involved in LH-dependent downregulation of *CYP19* transcription was described in early studies on the regulation of aromatase expression (Fitzpatrick & Richards 1991). Because LH/hCG-dependent increases in all the four isoforms of ICER mRNA have been reported (Mukherjee *et al.* 1998), we propose that proteins encoded by ICER may be this formally described cycloheximide-sensitive factor and that induction of ICER expression by LH may be part of the molecular mechanism whereby aromatase is downregulated by this hormone. To support this role of ICER as a repressor of $P450_{AROM}$ expression in the rat female gonad, we provide the following experimental observations: (1) the lower molecular mass complex of proteins from forskolin-stimulated granulosa cells specifically bound to the CRE of the ovarian aromatase promoter was supershifted by anti-CREM but not by anti-CREB antibody; (2) forskolin-induced luciferase activity was suppressed when the $P450_{AROM}$ promoter-driven luciferase gene was transiently cotransfected with an ICER expression vector into granulosa cells or wild-type R2C cells, a Leydig tumor cell line routinely used for the study of $P450_{AROM}$ promoter activity; (3) $P450_{AROM}$ promoter activity in wild-type R2C cells was elevated 6 and 12 h after stimulation with 8-Br-cAMP and downregulated to levels below basal activity 24 and 48 h after treatment. However, the promoter activity in response to the cAMP analog was completely attenuated in an R2C polyclonal cell line overexpressing ICER, while another R2C line stably transfected with a construct of ICER in the antisense direction exhibited increases in the cAMP-inducible $P450_{AROM}$ promoter activity that were not downregulated to the same extent as in the wild-type cells.

Other, not necessarily mutually exclusive mechanisms, may help to explain LH downregulation of aromatase expression. In addition to the CRE-like sequence (Fitzpatrick & Richards 1994, Michael *et al.* 1997) the ovarian promoter of *CYP19* contains an hexameric steroidogenic factor-1 (SF-1) site downstream of the CRE-like sequence (Fitzpatrick & Richards 1993, Young & McPhaul 1998). The synthesis and phosphorylation of SF-1 and the increased levels of phosphorylated CREB appear to be equally necessary in order to obtain the optimal activation of hormone-dependent *CYP19* transcription (Carlone & Richards 1997).

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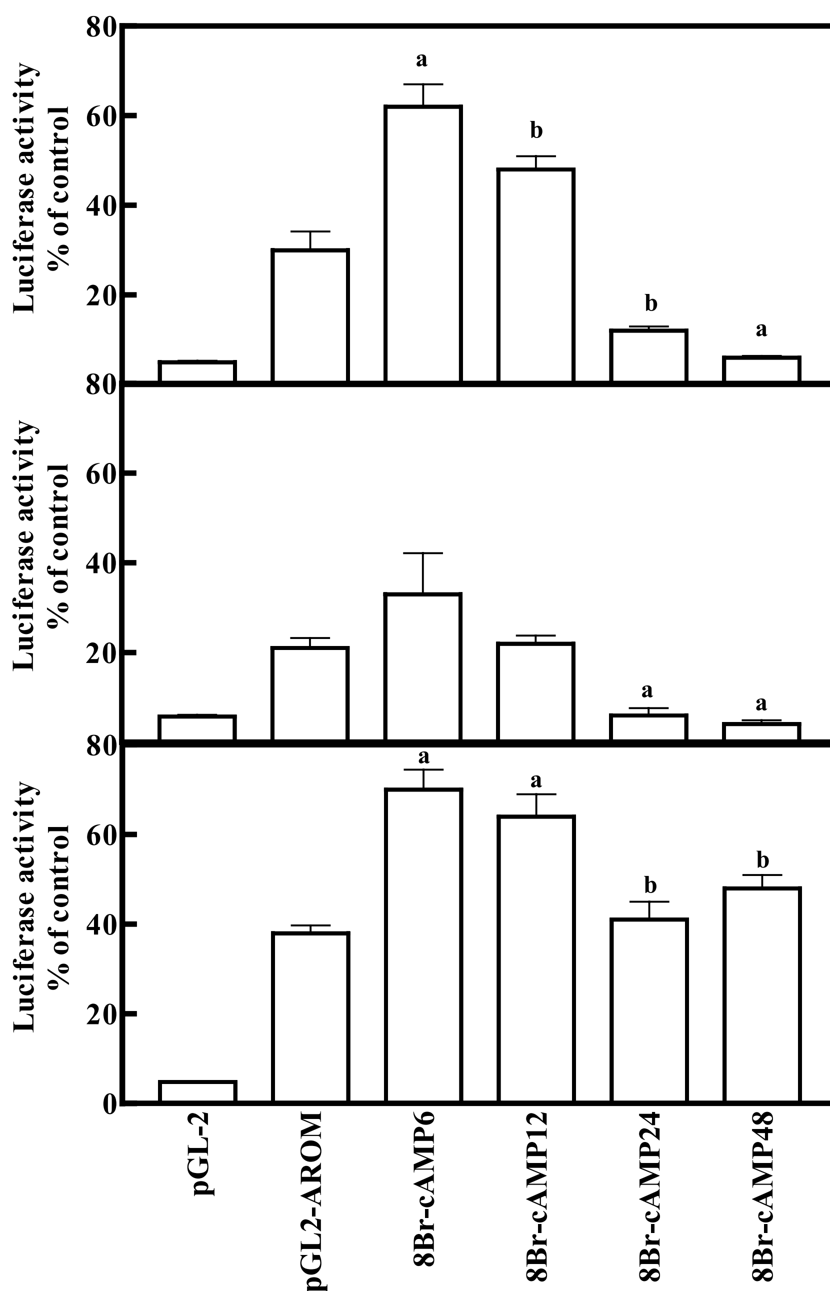

Figure 5 Functional activity of the $P450_{AROM}$ promoter in wild-type and polyclonal lines of R2C Leydig tumor cells overexpressing ICER sense (s) or antisense (as). Wild-type (upper panel), ICERs (middle panel) or ICERas (lower panel) stably transfected R2C cells were transiently transfected with the pGL2-Arom-Luc construct. Luciferase activity was measured 48 h after the transfection in control or 8-Br-cAMP (1 mM)-stimulated cells that were treated for 6, 12, 24 or 48 h (8Br-cAMP6, -12, -24, -48) before the end of the experiment as described in the Materials and Methods section. Data from triplicate measures of three different experiments were pooled and results are the means ± S.E.M. a, $P<0{\cdot}01$ and b, $P<0{\cdot}05$ compared with control unstimulated cells.

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The preovulatory surge of LH almost completely abolishes SF-1 expression (Carlone & Richards 1997). Therefore, inadequate levels of phosphorylated SF-1 together with ICER competition for CRE sites in the $P450_{AROM}$ promoter may cooperate to repress aromatase expression in granulosa cells.

Finally, although extensively used to study $P450_{AROM}$ promoter activity, the usefulness of R2C cells has been greatly reduced due to their apparent unresponsiveness to hormonal stimulation. The interference of ICER in the assessment of CRE-dependent expression using CRE-mediated reporter genes has recently been documented in several cell lines (Kemp *et al.* 2002). We have shown here the existence of this experimental pitfall in R2C Leydig tumor cells, thus contributing to enlarge the possibilities of these cells as a potential model system to study not only basal but also hormone-induced expression of $P450_{AROM}$.

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