Acute regulation of the bovine gene for the steroidogenic acute regulatory protein in ovarian theca and adrenocortical cells

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ABSTRACT

Upregulation of the steroidogenic acute regulatory protein (StAR) is implicated in the rapid synthesis and secretion of steroidogenic cells to produce steroids in response to stimulation by trophic hormones of the gonadal and stress axes. In the present study, we have assessed the kinetics of both StAR gene transcription and protein biosynthesis in primary cell cultures of bovine adrenocortical and ovarian theca cells, under conditions of acute stimulation by corticotrophin (ACTH) and luteinizing hormone (LH), respectively. In both cell systems, detectable upregulation of StAR gene transcription occurred within 1–2 h, reaching maxima at 4 h (theca cells) or 6 h (adrenocortical cells). mRNA levels returned rapidly to baseline, by 12 h or 24 h, respectively. Specific StAR protein levels were assessed by western blotting using a novel antibody raised against a bovine StAR peptide, and showed a similar fast upregulation, albeit delayed by 1–2 h compared with the mRNA. The response of the cultured theca cells was more acute than that of the adrenocortical cells, possibly reflecting the propensity of the LH receptor to desensitize rapidly, unlike the ACTH receptor. The primary bovine theca cell cultures were also used for fully homologous transfection studies using various deletion promoter–reporter constructs of the bovine StAR gene. Kinetic analysis of the results indicated that the acute transcriptional response resides within the proximal (−315 bp) promoter region, which includes two putative responsive elements for the steroidogenic factor-1. More distal promoter regions may be involved in modulating the specificity of expression by combining enhancer and inhibitory functions.

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INTRODUCTION

In steroidogenic tissues, the rate-limiting step in the acute hormone-dependent upregulation of steroid biosynthesis is considered to be the transport of the cholesterol substrate to the inner mitochondrial membrane, where it is converted to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage complex (reviewed in Thomson 1998). This rate-limiting mitochondrial transport is dependent upon the de novo biosynthesis of new protein (reviewed in Stocco & Clark 1996). An important candidate for this role is the recently cloned steroidogenic acute regulatory protein (StAR; Clark et al. 1994), which is rapidly and highly upregulated after a steroidogenic stimulus, and becomes associated with mitochondria (Stocco & Clark 1996). Furthermore, the apparently indispensible nature of StAR is revealed by the severe impairment of steroidogenesis after inactivation of the StAR gene in mice by homologous recombination (Caron et al. 1997b), or in the naturally occurring human deficiency disease, congenital lipoid adrenal hyperplasia (Miller 1997).
The cDNA and gene sequence encoding the StAR protein have been exploited, initially for the mouse (Clark et al. 1994), and subsequently for other species, including the bovine (Hartung et al. 1995). Initial experiments investigated the basal levels of StAR mRNA in different tissues in vivo, or in tumour cell lines, and the levels attained after long-term stimulation. Although several studies have looked at factors regulating the StAR gene promoter (reviewed in Reinhardt et al. 1999), most of these used a prolonged (>12 h) stimulation protocol, and so the molecular mechanisms responsible for the acute (<4 h) cAMP-dependent upregulation of StAR gene transcription remain unclear.

In order to investigate this in a homologous system, we have exploited the ability of bovine ovarian theca cells and bovine adrenocortical cells to produce large amounts of steroids in response to acute stimulation by the natural adenylate cyclase-linked secretagogues, luteinizing hormone (LH) and adrenocorticotrophic hormone (ACTH), respectively. Using northern and western blotting for ACTH, secretagogues, luteinizing hormone (LH) and stimulation by the natural adenylate cyclase-linked large amounts of steroids in response to acute cells and bovine adrenocortical cells to produce factors regulating the acute (<4 h) cAMP-dependent upregulation of StAR gene transcription remain unclear.

Materials and methods

Cell culture

Bovine theca interna cells were prepared from large antral follicles (10–25 mm diameter) of ovaries collected from mid- to late-cycle cows at the local abattoir. Cell preparation and primary culture conditions were exactly as described by Bathgate et al. (1999). After Percoll purification, cells were resuspended in 1:1 Dulbecco’s minimal essential medium and Ham’s F-12 medium, supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 0-1% BSA, 5 µg/ml transferrin, and 5 ng/ml sodium selenite. For mRNA and protein preparation, approximately 10^6 cells were seeded into vitrogen-coated six-well plates and cultured at 37 °C under 95% air–5% CO₂ for 6 days (with medium changed every 2 days), attaining in that time approximately 80% confluence. For measurements of endogenous mRNA and protein production, after the medium change on day 6, cells were supplemented in addition with 100 ng/ml bovine insulin with or without 10 ng/ml bovine luteinizing hormone (bLH; a kind gift of NIADDK and the National Hormone and Pituitary Program, NIH, Bethesda, MD, USA), and culture continued for the times indicated. Primary zona fasciculata/reticularis (ZFR) cells were isolated from sliced bovine adrenocortical tissue as previously described (Williams et al. 1989, Nicol et al. 1998) and were plated at a density of 1·5 · 10^6 per well of a six-well plate for both mRNA and protein studies. After plating, cells were maintained for 48 h in Ham’s F-10 medium containing 10% v/v controlled serum replacement-1 (Sigma, Poole, Dorset, UK), 100 IU penicillin, 100 µg/ml streptomycin and 5 µg/ml amphotericin B at 37 °C under 95% air–5% CO₂, with one medium change after 24 h, and then for a further 16 h in Ham’s F-10 medium containing 0-2% BSA and antibiotics as above, before hormone treatment using 10 nM Synacthen (synthetic ACTH(1–24) peptide, Novartis Pharmaceuticals, Camberley, Surrey, UK) in the same medium.

Northern hybridization

For the ovarian theca cells, total RNA was extracted using thepeqGOLD RNA-Pure (peqLab, Erlangen, Germany) reagent and pooled from two wells per experiment for each data point. Two micrograms total RNA per slot were subjected to northern hybridization using 1-3% agarose–2-2 M formaldehyde gels in morpholinopropanesulfonic acid running buffer (Sambrook et al. 1989). RNA was transferred to a nylon membrane (Nytren; Schleicher & Schüll, Dassell, Germany) by overnight capillary transfer and fixed by u.v. crosslinking. Hybridization with a 750 bp gene-specific probe from the 5’ region of the bovine StAR cDNA was exactly as previously described (Hartung et al. 1995). To control for even loading and transfer of the RNA, blots were rehybridized using a radiolabelled probe specific for the bovine S15 ribosomal protein (Bathgate et al. 1999). For the ZFR cells, total RNA was isolated using the RNAzol B reagent (Biogenesis, Poole, Dorset, UK), and 20 µg RNA per slot was denatured with glyoxal (Thomas 1983), resolved on a 1-2% agarose gel in 10 mM sodium phosphate pH 7-0 buffer, capillary-transferred to positively charged nylon membranes (Stratagene) and fixed by u.v. crosslinking. Hybridization for StAR mRNA was as above, but using a somewhat
shorter 5′ cDNA probe (accession number S79908, nucleotides 210–608). Even loading and transfer were confirmed by rehybridization using a β-actin probe. All northern and western (see below) blotting experiments were repeated at least twice for fully independent batches of cells, and gave fully reproducible results.

**Antibody production and western blotting**

Total cellular protein (ZFR cells 25 µg, theca cells 15 µg per sample), was prepared by homogenization in PBS containing 0.1% SDS and 1% sodium deoxycholate, resolved on a 12% acrylamide gel, and electroblotted onto Immobilon-P membrane (Sigma). Western analysis was carried out using, and electroblotted onto Immobilon-P membrane (Sigma). Western analysis was carried out using, as primary antibody, a polyclonal sheep antiserum (Sigma). Western analysis was carried out using, as primary antibody, a polyclonal sheep antiserum raised against a peptide fragment (AMQRALGIL – amino acids 82–107; Hartung et al. 1995) from the predicted bovine StAR protein sequence attached to a lysine-web-based eight-branched antigen scaffold (The Binding Site, Birmingham, UK), and a donkey anti-sheep horseradish peroxidase-conjugated second antibody. After preliminary optimization (data not shown), primary and secondary antibodies were used respectively at 1 : 10 000 and 1 : 25 000 dilutions in PBS containing 10% blocking buffer (Pierce, Rockford, IL, Staffs, USA) and 2% w/v non-fat milk (Marvel Original; Premier Beverages, Stafford, UK) for immunodetection of adrenocortical proteins, and at 1 : 7500 and 1 : 10 000 dilutions, respectively, in PBS containing 10% blocking buffer (Pierce) and 1% non-fat milk for theca cell proteins. The final signal was visualized by chemiluminescence (SuperSignal ULTRA kit; Pierce). The specificity of the antibody was demonstrated first by its ability to detect a protein in adrenocortical protein extracts at the anticipated size of approximately 30 kDa, the pattern of expression of which followed closely behind that of the StAR mRNA (see later), and, secondly, by the complete elimination of the specific 30 kDa band as a result of the addition of an excess (>3 ng/ml) of the peptide used to generate the antibody (not shown).

**Cell transfection and analysis**

All bovine StAR promoter–reporter DNA constructs, in addition to control vectors, were exactly as previously described (Rust et al. 1998). DNA was purified using the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany) as described by the manufacturer. For transfection experiments, 10⁶ bovine theca cells per well, prepared as above, were seeded into 12-well plates, and cultured as above in the presence of 100 ng/ml insulin for 2 days. Cells were then washed briefly in PBS before the addition of OPTI-MEM (Gibco-BRL, Deisenhofen, Germany) transfection medium. Transfection was carried out by adding 5 µg per well LipofectAMINE (Gibco-BRL) at a ratio of 2 : 1 with the DNA to be transected, and incubating for 5 h. Medium was then replaced by the standard culture medium and incubation continued for a further 3 days, before treatment or not with 10 ng/ml bLH, for the times as indicated. Cells were then washed rapidly in PBS and immediately extracted into 40 µl reporter lysis buffer (Luciferase Assay System; Promega, Madison, WI, USA) before luciferase activity was measured using the same kit. As cotransfected control, a β-galactosidase reporter gene driven from the cytomegalovirus (CMV) promoter was used, measuring the resulting activity using the Galacto-Light kit (Tropix, Bedford, MA, USA). All transfections were performed in triplicate for any batch of primary cells. All experiments were repeated at least twice using independent batches of cells, with fully reproducible results.

**RESULTS**

**Expression of endogenous StAR mRNA and protein in primary cultures of bovine ovarian theca and adrenocortical (ZFR) cells**

The endogenous StAR mRNA in bovine ovarian theca and adrenocortical (ZFR) cells was expressed as transcripts of three different sizes (Figs 1, 2), as shown previously also for bovine corpus luteum (Hartung et al. 1995, Pescador et al. 1996) and predicted from the positions of alternative polyadenylation sites within the 3′ UTR of the cloned bovine cDNA (Hartung et al. 1995). These migrated as two major bands at approximately 3·0 kb and 1·8 kb, and a minor band at 1·6 kb, evident only in the theca cells. Levels in untreated cells were very low for both cell types (Figs 1B, 2B). After treatment of theca cells with bLH or adrenocortical cells with ACTH, there was a similar rapid increase in the relative amounts of the two major StAR transcripts in both cell systems. This increase was already detectable after 1 h of stimulation, by comparison with the untreated controls, and reached a maximum at about 4 h (theca cells, Fig. 1A) and 6 h (adrenocortical cells, Fig. 2A) of treatment. At the time of maximal induction of StAR mRNA, the shorter 1·6 kb transcript became readily detectable in the theca cell cultures (Fig. 1A). Also of significance was the rapid decline in transcript levels at subsequent
Northern hybridization of StAR mRNA in extracts of bovine theca cells cultured for the time indicated in the presence (A) or absence (B) of 10 ng/ml LH. As control for even loading and transfer, blots were rehybridized against a probe for the bovine ribosomal protein, S15. The blots in (A) and (B) are from parallel experiments using the same batch of cells. Duplicate lanes represent RNA from independent parallel cell cultures from within the same experiment, to indicate the extent of within-batch variation.

**Figure 1.** Northern hybridization of StAR mRNA in extracts of bovine theca cells cultured for the time indicated in the presence (A) or absence (B) of 10 ng/ml LH. As control for even loading and transfer, blots were rehybridized against a probe for the bovine ribosomal protein, S15. The blots in (A) and (B) are from parallel experiments using the same batch of cells. Duplicate lanes represent RNA from independent parallel cell cultures from within the same experiment, to indicate the extent of within-batch variation.
time-points, such that, at 12 h (theca cells) or 24 h (adrenocortical cells), StAR mRNA had returned to basal (unstimulated) levels. The apparent and slight long-term increase in StAR mRNA in the absence of bLH (Fig. 1B) probably reflected the differentiation of these cells in culture under the influence of insulin only (Bathgate et al. 1999).

Total cellular protein was also prepared from similar cultures of stimulated and unstimulated cells and subjected to western blotting using a newly developed anti-StAR polyclonal antibody. This antibody was raised against a 26-mer sequence from the predicted external surface of the bovine StAR protein (Hartung et al. 1995) and specifically recognized a 30 kDa protein (Fig. 3). Protein bands of similar size were detected in adrenocortical and luteal protein extracts using another polyclonal antibody raised against mouse recombinant StAR protein (Pescador et al. 1996, Ronen-Fuhrmann et al. 1998, and data not shown). The time-course of changes in StAR protein levels in the two cell types was similar (Figs 3, 4), with a first detectable increase at 2 h and maximum levels being maintained through to 6 h (theca cells) or somewhat longer (adrenocortical cells), but with a return to near basal values by 12–24 h. Thus StAR protein production was marginally delayed, both in appearance and disappearance, with respect to the changes in levels of the specific mRNA. Absolute levels of StAR protein, both basal and stimulated, appear to be much greater in the adrenocortical cells than in the bovine theca cells, as indicated by the control lanes (1 and 2) in Fig. 3, in which the same amount of total protein as in the other lanes had been loaded.

Acute regulation of the bovine StAR gene promoter in homologous cell culture

In a previous study (Rust et al. 1998), we have been able to characterize the role of certain cis elements within the upstream promoter region of the bovine StAR gene responsible for expression in a heterologous cell system cotransfected or not with the transcription factor, steroidogenic factor-1 (SF-1; Ad4 BP; Fig. 5). In this system, deletion promoter–reporter constructs had been transfected into non-steroidogenic cells together with appropriate expression vectors. In order to determine whether the SF-1-responsive elements are also involved in endogenous StAR gene expression in primary cultures of bovine steroidogenic cells (i.e. in a homologous system as close to the in vivo situation as possible), and are sufficient to account for the acute up- and downregulation of the StAR gene, a time-course for activation of defined promoter–reporter constructs was determined under acute bLH stimulation after transfection into primary ovarian theca cells (Fig. 6). The results show that there was consistently detectable upregulation in response to bLH by 3 h of stimulation. The maximum level of luciferase activity accumulated in the cells was reached by 4–6 h, and was not further increased at later times. A comparable time-course was obtained irrespective of the use of a long promoter fragment (−1245 bp; Fig. 6A), or a shorter fragment (−315 bp; Fig. 6B) attached to the luciferase reporter gene. However, it should be noted that, in repeated experiments, the response to LH appeared generally to be more robust, consistent, and somewhat earlier in these primary cell
cultures, for the shorter –315 bp construct (cf. Fig. 7).

Experiments using different promoter–deletion constructs transfected into theca cells optimally stimulated by bLH for 4 h indicated that the acute stimulation of the bovine StAR gene requires only the minimal promoter up to –315 bp (Fig. 7), which includes the first two proximal SF-1 binding motifs (Rust et al. 1998). Indeed, even shorter constructs comprising only one (–203) or none (–101) of the proximal SF-1 sites, also appear to be responsive to LH. Longer promoter constructs, however, do not increase the level of luciferase activity obtained. As already indicated in Fig. 7, these appear to have reduced activity by comparison with the shorter constructs. This would suggest that the region upstream of the –315 construct may include a negative factor that could modulate the activity of the proximal promoter.

**DISCUSSION**

The acute response of steroidogenic cells to the anterior pituitary hormones, LH or ACTH, is an essential part of the regulatory mechanisms promoting steroid biosynthesis. Both peptide hormones are released in short pulses or trains of pulses, and elicit both immediate and sustained effects. Amongst the immediate effects are those influencing steroid secretion (reviewed in Thomson 1998). In addition to such very acute events, there is also an effect at the transcriptional level. This effect was quite rapid, newly transcribed StAR mRNA being detectable in both adrenocortical and ovarian thecal cells after about 1 h of stimulation. Equally important, however, is the observation, also for both cell types, that levels of mRNA peaked at about 4–6 h and then declined to basal values by, maximally, 12–24 h. This shows that the stimulatory effect upon transcription is short-lived, and that mechanisms are present in these steroidogenic cells for the rapid degradation of the newly synthesized StAR mRNA. It is important to note that the primary cell cultures were exposed continuously, through the treatment period, to the effectors LH or ACTH. It is known that the LH receptor is rapidly downregulated (desensitized, internalized) within the first few minutes of hormone exposure (e.g. for bovine luteal cells; Budnik & Mukhopadhyay 1987), thus all subsequent LH-dependent events will be receptor-independent consequences of this initial stimulation. For the effect of ACTH on adrenocortical cells, the situation appears to be different. Instead of being desensitized, the cognate receptor appears rather to be upregulated (Penhoat et al. 1989). This could explain the more sustained transcriptional response of the StAR gene to ACTH treatment evident in the adrenocortical cell cultures.

By transfecting different promoter–reporter constructs into ovarian theca cells, under a treatment paradigm similar to that above in which we have
shown acute upregulation of the endogenous StAR gene, we were also able to show that luciferase activity (i.e. expression of active protein) follows the same time-course of induction as the native StAR protein. Increased luciferase activity was evident first at 2–3 h, just like the endogenous StAR protein seen in the western blots, suggesting that both StAR and luciferase mRNAs are translated in a comparable manner. The induction of reporter activity was, as expected, slightly delayed by comparison with the induction of transcription. The disappearance of the luciferase activity will probably have a

**Figure 4.** Western blot of immunoreactive StAR protein in extracts of bovine theca cells cultured as indicated for increasing times in the absence or presence (+) of 10 ng/ml bovine LH. It should be noted that the absolute levels of the StAR protein are considerably less than in the adrenocortical cells (lanes 1 and 2), and there is thus an increased non-specific background. As for the adrenocortical cells, the StAR protein appears to be represented as a doublet. In all lanes, 15 µg protein were loaded.

**Figure 5.** Schematic representation of the bovine StAR gene and its promoter, indicating putative transcription factor binding elements.
time-course different from that for the endogenous protein, as this will depend on the function of substrate-specific proteases. Nevertheless, it is evident that there is a plateau in luciferase expression at 4–6 h, with no further increase after this, reflecting precisely the short phase of transcriptional induction. This experiment showed that the promoter information required for this acute upregulation of transcription resides within the first 1200 bp upstream of the transcription start site, and probably within the first 315 bp of this. Use of promoter–deletion constructs confirmed what we have previously shown for this gene using a heterologous transfection system (Rust et al. 1998), namely that LH-induced, cAMP-dependent gene activation is maximal where the first two SF-1-responsive elements, which reside within the –315 bp immediate upstream region of the promoter, are present. It should be noted, though, that a marked LH-dependent stimulation was also evident for the very short –101 construct (Fig. 7), which does not include an SF-1 binding element, suggesting that SF-1 may not be absolutely required to mediate the stimulatory effect of LH. Other genes have also been described in the same or related cell types, for example, P450SCC in luteal cells (Liu & Simpson 1997) or oxytocin in granulosa cells (Wehrenberg et al. 1994), which also depend upon SF-1 interaction, and respond to LH stimulation. These responses, however, have a quite different time kinetic and cell specificity. Thus other factors must act in conjunction with SF-1 to encode the cellular and temporal specificity observed for the StAR gene. One candidate in the case of the mouse StAR gene is the transcription factor, CCAATT/enhanced binding protein β (C/EBPβ) (Reinhardt et al. 1999a, Silverman

FIGURE 6. Primary cultures of bovine theca cells transfected with luciferase reporter constructs linked with either the –1245 (full-length, A) or the –315 (short, B) bovine StAR gene promoters. Three days after the transient transfection, cells were stimulated or not as indicated by addition of 10 ng/ml bovine LH. The amount of luciferase protein synthesized is estimated from its enzymatic activity expressed in relative light units (RLU; relative to the cotransfected CMV-β-galactosidase constitutive construct) as a percentage of the level attained with the strong constitutive promoter of the pGL3-C construct (C2) in the absence of LH. C1, luciferase activity of the empty reporter vector, pGL3-B. Data are expressed as means ± s.e.m from parallel triplicate experiments using the same batch of primary cells.

FIGURE 7. Primary cultures of bovine theca cells transfected with luciferase reporter constructs linked with different deletion fragments of the bovine StAR gene promoters. The promoter fragments correspond to DNA sequences extending upstream from the transcription start site to the restriction sites for BanI (–101), HpaII (–203), PstI (–315), KpnI (–1009) and BamHI (–1245), respectively. Results are expressed in relative light units (RLU) as a percentage of the luciferase activity obtained using the strong constitutive promoter construct, pGL3-C, in the absence of LH.
et al. 1999). Support for this view is provided by a comparison of the present transfection results, using cells endogenously expressing the StAR gene, with the heterologous system used previously. In the previous study, the transcriptional activity induced by SF-1 attained maximally only 2% of the level reached by the same constitutively active control plasmid (pGL3-C) as that used here; this compares with greater than 50% in the present homologous system. Similarly, a constitutively active protein kinase A subunit was able to increase the SF-1-dependent transcriptional activation by only about 50% in the previous study whereas, in the homologous system, LH induced up to a 10-fold increase in reporter gene activity. The molecular mechanisms by which SF-1 could be involved in cAMP-dependent signal transduction are not yet understood. In a recent study, a potential serine acceptor site for protein kinase A (PKA) phosphorylation, and the C-terminal activation domain were both implicated in the transcriptional upregulation of the high-density lipoprotein receptor gene in rat luteal cells via SF-1 (Lopez et al. 1999). Also, possible mitogen-activated protein kinase phosphorylation of SF-1 has been suggested in the context of cofactor recruitment (Hammer et al. 1999).

The requirement for other factors to act with SF-1 in mediating cAMP-regulated signal transduction would also offer an explanation for the anomalous findings regarding specific nuclear protein-binding to the bovine StAR promoter (Rust et al. 1998). Of the three putative SF-1 binding motifs evident in the bovine StAR promoter (Fig. 5), only the distal element bound purified SF-1 with high affinity. The second proximal element bound SF-1 only weakly, and the most proximal element failed to show any binding activity (Rust et al. 1998). Yet it is the proximal promoter containing the first SF-1 motifs which, in this and other studies, appeared to mediate SF-1-dependent activation of the StAR gene by adenylate cyclase in (for example) humans (Sugawara et al. 1996), mouse (Caron et al. 1997a), rat (Sandhoff et al. 1998) and pig (LaVoie et al. 1999). Findings of a recent study using the mouse StAR gene promoter even suggested that an interaction of SF-1 in the proximal promoter may not be essential, and that adenylate cyclase-induced activation can be mediated by a combination of the transcription factors C/EBPβ and GATA-4 only (Silverman et al. 1999). This opinion is supported by findings of the present study, in which the –101 construct of the bovine StAR gene was able to transduce LH-stimulation to the reporter gene. This fragment does include an inverse GATA motif at position –65 (Rust et al. 1998).

Other regions of the promoter may be involved in the definition of expression specificity. There is evidence, from the transfection studies, for a mildly inhibitory element in the region upstream of nucleotide –315. There is also, in this region, marked nuclear protein binding unrelated to SF-1 (Rust et al. 1998). Furthermore, although not apparently contributing to gene activation, a very good binding site for SF-1 exists in the upstream region of the promoter at –1100 (Rust et al. 1998). Concerning the rapid switching off of the StAR gene, this might involve the activation of the negative transcription factor, DAX-1, which has been shown to interact with the mouse StAR gene promoter, possibly interfering directly with SF-1 binding in the proximal promoter region (Zazopoulos et al. 1997, Reinhardt et al. 1999b, Sandhoff & McLean 1999).

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