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Phosphoprotein Phosphatases Regulate Steroidogenesis by Influencing StAR Gene Transcription

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The rate-limiting step in steroidogenesis is the transport of cholesterol into the mitochondria, and this is controlled by the steroidogenic acute regulatory (StAR) protein. We have previously shown that inhibition of phosphoprotein phosphatase 1 and 2A (PP1/2A) activities with the PP1/2A inhibitor calyculin A selectively reduces StAR protein expression and thus inhibits the synthesis of steroid hormones. The aim of this study was to determine whether this inhibition of StAR protein expression occurs at the level of transcription of StAR mRNA. We have used a competitive reverse transcription-polymerase chain reaction (RT-PCR) technique to determine whether inhibition of PP1/2A activities has any effect on the levels of StAR mRNA. Exposure of Y1 cells to forskolin significantly increased the expression of StAR mRNA and this forskolin-induced increase was reduced after exposure to Cal A at levels similar to those seen in the controls. These results suggest that cyclic AMP-induced increases in StAR mRNA levels are dependent upon phosphoprotein phosphatase activities. © 2000 Academic Press

Key Words: **steroidogenesis; steroidogenic acute regulatory protein; phosphoprotein phosphatase; Y1 cells; competitive reverse transcription-polymerase chain reaction.**

The acute regulation of steroid hormone biosynthesis is dependent on the expression of the steroidogenic acute regulatory (StAR) protein and its role in mediating the transfer of cholesterol to the inner mitochondrial membrane and the site of cholesterol side chain cleavage (1–5). Indeed, overexpression of StAR protein leads to increases in steroid production (6), and a number of studies have demonstrated that the increases in steroid production observed after PKA activation are dependent on increased expression of StAR protein (7–10). Furthermore, there is now strong evidence demonstrating that elevations in intracellular cAMP lead to elevations in StAR mRNA (7, 11, 12), suggesting that transcription of the StAR gene is important in the regulation of steroid production. This is supported by the finding that continued StAR synthesis and steroid production are dependent on transcriptional activity (13).

In our previous studies we have shown that cAMP-induced increases in steroid production in Y1 mouse adrenocortical cells (14) and primary tissues (15, 16) are dependent on the activity of phosphoprotein phosphatases 1 and 2A (PP1 and PP2A), since inhibition of these enzymes with the PP1/2A inhibitor calyculin A (Cal A) caused a dose-dependent inhibition of steroidogenesis. Furthermore, this inhibition of steroidogenesis correlated well with an inhibition in the expression of StAR protein (14). These results demonstrated that inhibiting the activities of PP1 and PP2A blocks cAMP-induced increases in steroid production by preventing the expression of StAR protein, although it was not determined whether this effect was at the level of transcription of new mRNA, or translation of existing mRNA. We have now addressed this question by using a quantitative, competitive RT-PCR procedure to determine whether or not the inhibition of StAR protein expression by inhibitors of PP1/2A occurs at the level of transcription of the StAR gene.

MATERIALS AND METHODS

Materials. Tissue culture reagents and plastics were from Gibco BRL (Paisley, UK). Calyculin A (Cal A) was from Calbiochem (Nottingham, UK). Trilostane was a kind gift from Dr. George Margetts (Stegram Pharmaceuticals, Sussex, UK). RQ1 RNase-free DNase, *Taq* DNA polymerase, RNAsin and avian Moloney murine leukemia virus-reverse transcriptase were from Promega (Southampton, UK). Qiaquick gel extraction kit was from Qiagen (Crawley, Sussex, UK). Oligo$(dt)_{18}$ and random 10-mers were prepared in house (King's College London, Molecular Biology Unit). All other biochemicals were from the Sigma Chemical Company Ltd. (Dorset, UK).

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Cells. Mouse adrenocortical Y1 cells were obtained from the European Collection of Animal Cell Cultures (Wiltshire, UK), and maintained in DMEM supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin and 10% (v/v) foetal bovine serum in a humidified atmosphere of 5% CO_2.

Steroid production. Y1 cells were seeded in 96-well microculture plates at a density of 1×10^5 cells per well, and incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 to allow the cells to adhere to the plates. The culture medium was replaced with DMEM alone (control) or DMEM supplemented with 1 μM forskolin with or without Cal A (1–100 nM), and the incubation was continued for a further 3 h. Steroid production by Y1 cells was measured using a radioimmunoassay for pregnenolone over the range 0.8–100 pmol/ml. The conversion of pregnenolone to other steroids was prevented by the addition of 2 μM trilostane (17, 18), an inhibitor of 3β-hydroxysteroid dehydrogenase, to the incubation medium.

Isolation of Y1 cell total RNA and reverse transcription. Y1 cells were incubated for 3 h in the presence DMEM alone (control) or in the presence of 1 μM forskolin with or without 10 nM Cal A and total RNA was extracted using the RNAzol B method. Briefly, approximately 15×10^6 Y1 cells were sonicated on ice in RNAzol B solution. The separation of RNA from DNA and proteins was initiated by the addition of chloroform (0.1 ml/ml cell lysate) followed by vigorous shaking and incubation on ice for 5 min. After centrifugation (12,000*g*, 4°C, 15 min), the aqueous RNA-containing upper phase was removed and an equal volume of isopropanol was added. The RNA was precipitated by incubation at 4°C for 15 min, followed by centrifugation (12,000*g*, 4°C, 15 min). The RNA pellet was then washed with 75% ethanol and air dried for 10–15 min. Finally, the RNA was dissolved in 1 mM EDTA and concentration was assessed by UV spectrophotometry at 260 nm. 5 μg of total RNA from each sample was treated with RQ1 RNase-free DNase (1 unit/μg RNA) to remove any remaining DNA contamination. cDNA was synthesised simultaneously from all purified RNA samples using avian Moloney murine leukemia virus-reverse transcriptase (MMLV-RT). Oligo$(dt)_{18}$ (1 μg) and random 10-mers (1 μg) were added to the RNA (1 μg) and the mixture heated (70°C, 3 min) to remove secondary RNA structure, then cooled on ice. DTT (10 mM), dATP, dCTP, dTTP, and dGTP (all 0.5 mM), recombinant ribonuclease inhibitor (80 u, RNAsin), MMLV-RT (200 u) and diethyl pyrocarbonate-treated water were added to make the final volume 20 μl, and the mixture was incubated at 37°C for 1 h. MMLV-RT was inactivated by heating at 98°C for 3 min. The cDNA was used immediately or stored at −20°C for future use.

Polymerase chain reaction. Forward and reverse PCR primers designed from the mouse StAR sequence were: sense primer 5′-CAG CAT GTT CCT CGC TAC GT-3′; antisense primer 5′-CCT TAA CAC TGG GCC TCA GA-3′. The predicted size of the StAR PCR product was 860 bp.

Competitive PCR. A competitor StAR DNA (310 bp) was prepared by amplification of *E. coli* DNA with the StAR primer pair under low stringency conditions, as described by Uberla *et al.* (19). A competitor was cut from the gel, spin column purified using a Qiaquick gel extraction kit, and quantified by comparing its density with known amounts of molecular weight standards (*Hpa*II digest of pBluescript SK+) which had been separated by agarose gel electrophoresis and stained with ethidium bromide (EtBr, 0.5 μg/ml). The efficiency of PCR amplification of the competitor and target was determined by removing a sample (2 μl) from a single reaction tube during successive cycles of the exponential phase of amplification and measuring the accumulation of target and competitor. This accumulation was measured by separating target and competitor by acrylamide gel electrophoresis (5% v/v), staining with EtBr and quantitating bands by densitometry. Competitive PCR was performed using a thermal cycler (Hybaid Omn-E) in a reaction (20 μl) containing 100 μM of each deoxynucleoside 5′-triphosphate, 0.5 μM of each primer, 1.5 mM $MgCl_2$, 1 μl of Y1 cell cDNA and one of seven threefold dilutions of StAR competitor DNA. Reactions were initiated by addition of 1 u of *Taq* DNA polymerase to each tube in a "hot start" procedure (20). Thermal cycling conditions were 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min for 40 cycles, followed by a final extension phase of 10 min at 72°C. Each competitive PCR assay contained the same amount of cDNA from each treatment group, and the same master mix of PCR reagents and range of competitor dilutions were used in all PCR reactions. PCR reaction products were resolved by agarose gel electrophoresis (1.8% w/v) and stained with EtBr.

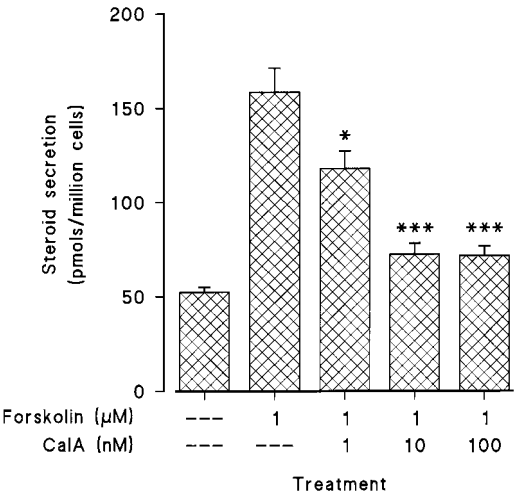

FIG. 1. The effects of inhibition of PP1/2A on forskolin-induced steroidogenesis in Y1 cells. Steroid production by Y1 cells is expressed as total steroid produced over 3 h. Forskolin (1 μM) significantly stimulated steroid production over basal levels ($P < 0.001$). The presence of 1–100 nM Cal A significantly inhibited forskolin-induced steroid production (1 nM Cal A: $*P < 0.05$; 10–100 nM Cal A: $***P < 0.001$). Bars show means + SEM, $n = 8$.

Data analysis. Differences between means were assessed using Student's *t*-test and considered significant when $P < 0.05$.

RESULTS

Effects of inhibitors of PP1/2A on steroidogenesis in Y1 cells. Figure 1 demonstrates that incubation of Y1 cells with the PP1 and PP2A inhibitor Cal A (1–100 nM) caused a concentration-dependent inhibition of forskolin-induced pregnenolone secretion during a 3 h incubation, with complete inhibition of stimulated steroidogenesis in the presence of 10 nM Cal A.

Quantitation of StAR mRNA expression in Y1 cells. PCR using StAR primers amplified a single product of the expected size (860 bp) for StAR from the Y1 cDNA samples. No PCR product was amplified from Y1-cell samples when the reverse transcription step was omitted. Low stringency PCR was used to generate a competitor DNA (310 bp) which had the same primer annealing sequences as StAR cDNA. This competitor DNA was shown to amplify with very similar efficiency to authentic StAR cDNA allowing it to be used for quantitative, competitive PCR. Competitive PCR with a fixed, small amount of target cDNA and between four and seven threefold dilutions of the competitor DNA, yielded two PCR products corresponding to StAR and competitor DNA (Fig. 2A). Density measurements of these products allowed the ratio of competitor/StAR to

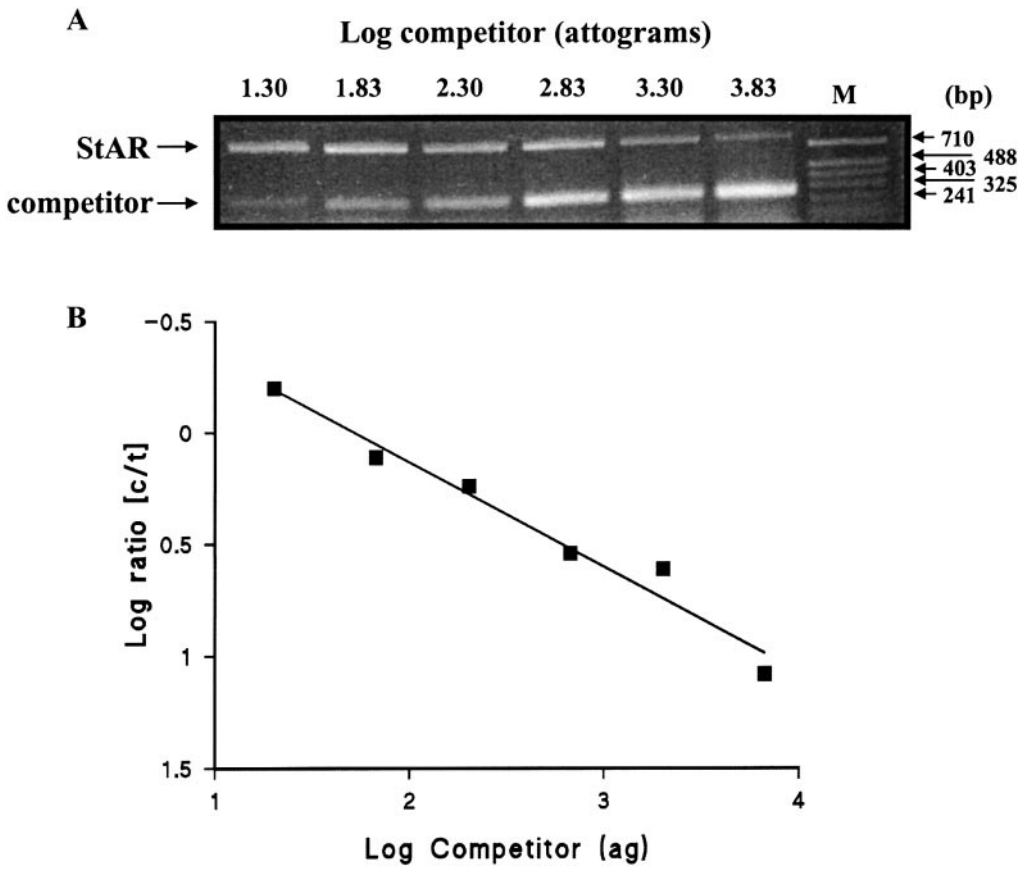

FIG. 2. Competitive RT-PCR for StAR mRNA in Y1 cells. (A) Representative gel image showing StAR product (860 bp) and competitor (310 bp). The amount of competitor (ag) included at the start of the PCR reaction is indicated above each lane. The gel image also shows the molecular weight markers (*Hpa*II digest of pBluescript SK+). (B) The densities of the competitor and StAR bands were measured by densitometry, corrected for the difference in size (× 310/860) and the ratio calculated and plotted against the amount of competitor in attograms (ag) added to the PCR reaction. From the regression line shown ($r^2 = 0.97$, $P = 0.0004$) the amount of StAR cDNA present at the start of the PCR can be calculated (i.e., when log ratio StAR/competitor = 0).

be calculated. Graphs of log ratio competitor/StAR versus log concentration of competitor initially added to the reaction demonstrated a linear relationship (Fig. 2B) and were highly significant when analysed by linear regression (P range = 0.029–0.0002; range r^2 = 0.90–0.98, n = 10). From these graphs the amount of StAR cDNA present at the start of the PCR was calculated (i.e., when log ratio competitor/StAR = 0). Figure 3 demonstrates the results of competitive PCR performed on all RNA samples, which show that exposure of Y1 cells to 1 μM forskolin caused a marked increase in the expression of StAR mRNA compared to basal, and this forskolin-induced increase was reduced after exposure to 10 nM Cal A to levels similar to those seen in the controls.

DISCUSSION

Y1 cells are a good model with which to study the biosynthetic pathway from cholesterol to pregnenolone, since the intracellular signalling components in this pathway reflect those in primary adrenal cells (21, 22). In this study we have confirmed that inhibition of PP1 and 2A activities with Cal A results in a concentration-dependent reduction in cAMP-induced steroid production from Y1 mouse adrenocortical cells. In addition, we have now demonstrated that the Cal A-induced reductions in StAR protein expression, which we have previously reported to be causal in the inhibition of steroidogenesis (14), are a consequence of a marked reduction in the levels of StAR mRNA expression. The highly sensitive competitive RT-PCR method we have used in this study demonstrated that under unstimulated conditions we were able to detect low level expression of StAR mRNA. This low level of StAR mRNA, which is present under unstimulated conditions, may explain earlier findings in which blocking transcription by treatment with actinomycin D failed to inhibit hormonal stimulation of corticosterone production in rats *in vivo,* or adrenal slices *in vitro* (23–25), which suggested that transcription may not be required for the acute steroidogenic response.

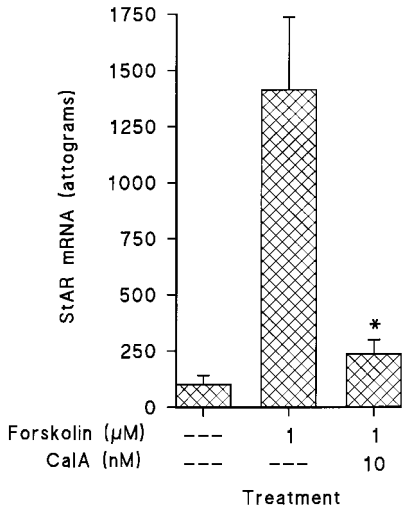

FIG. 3. The effects of inhibition of PP1/2A on forskolin-induced mRNA expression in Y1 cells. Summary of the results from competitive RT-PCR with Y1 samples treated with forskolin (1 μM) in the presence or absence of Cal A (10 nM). 3 h exposure of Y1 cells to forskolin significantly increased StAR mRNA levels compared to basal conditions ($P < 0.01$) and exposure of Y1 cells to Cal A significantly inhibited this forskolin-induced increase in StAR mRNA expression ($*P < 0.05$). Data are mean StAR mRNA levels + SEM and are expressed as ag of mRNA/10,000 Y1 cells, $n = 3–4$ experiments.

Previous studies have suggested that cAMP-mobilising stimuli such as forskolin stimulate increases in StAR mRNA levels (7, 11–13). However, the techniques used in these studies, namely northern hybridisation and RT-PCR, were qualitative rather than quantitative. Here we demonstrate quantitatively that, under conditions which we have previously shown result in increases in the expression of StAR protein (14), forskolin caused approximately a 14-fold increase in StAR mRNA levels compared to basal conditions, suggesting that cAMP-mediated stimuli increase StAR protein levels through an increase in the transcription of the StAR gene. The magnitude of this forskolin-induced increase in StAR mRNA levels was markedly greater than that found in previous studies (13, 26), presumably reflecting the increased sensitivity of competitive RT-PCR in comparison to alternative techniques of mRNA quantification. Importantly, we have also shown that this forskolin-induced increase in StAR mRNA levels was almost completely abolished after exposure of cells to the PP1/2A inhibitor Cal A. These novel data imply that cAMP-mediated increases in StAR mRNA levels are dependent on PP activities and the successful transcription of the StAR gene is absolutely dependent on a dephosphorylation event.

One possible target for the action of PPs in the regulation of StAR gene transcription is the 53 kDa transcription factor steroidogenic factor 1 (SF-1), whose activity is regulated by phosphorylation. It has previously been demonstrated that the StAR gene contains consensus binding sites for SF-1, and that SF-1 knockout mice do not express StAR mRNA, implicating SF-1 as a requirement for StAR gene expression (12). It is possible that a cycling transduction process involving both phosphorylation and dephosphorylation reactions may be involved in the regulation of SF-1, and perhaps other activating factors or co-regulators that have been implicated in the regulation of StAR gene expression, such as the CCAAT/enhancer binding proteins (C/EBPs; 27–29) and the sterol regulatory element binding protein (SREBP; 30). The therapeutic implications of regulating the production of steroid hormones are obvious, and since steroid production is implicitly dependent on the successful transcription of the StAR gene, the finding that PP1/2A activities are important in the regulation of this gene provides a possible site for the therapeutic manipulation of steroid production.

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