JBC Papers in Press. Published on June 15, 2001 as Manuscript M102063200

ERKs regulate cyclic AMP-induced steroid synthesis through transcription of the steroidogenic acute regulatory (StAR) gene.

Shân L. Gyles[1], Chris J. Burns[1], Barbara J. Whitehouse[1], David Sugden[1], Phil J. Marsh[2], Shanta J. Persaud[1] and Peter M. Jones[1]*

(SLG and CJB share first authorship of this study)

Endocrinology and Reproduction Research Group (1) and The Randall Centre (2), GKT School of Biomedical Sciences, King's College London, London SE1 1UL, UK

* - To whom correspondence should be addressed:

Dr. Peter Jones

Endocrinology and Reproduction Research Group

New Hunt's House (3rd floor, North)

GKT School of Biomedical Sciences

King's College London

London

SE1 1UL

UK

Telephone 0207 848 6273

Fax 0207 848 6280

E-mail peter.jones@kcl.ac.uk

Running title: StAR gene transcription is regulated by the ERK cascade

Summary

Cyclic AMP-dependent expression of the steroidogenic acute regulatory (StAR) protein is thought to be the controlling step for steroid production but the mechanisms through which external signals are translated into increased transcription of the StAR gene are unknown. We here demonstrate that cyclic AMP-induced steroid synthesis is dependent upon the phosphorylation and activation of ERKs, and that ERK activation results in enhanced phosphorylation of SF-1, and increased steroid production through increased transcription of the StAR gene. Adenylate cyclase activation with forskolin (FSK) caused a time-dependent increase in ERK activity and translocation from cytoplasm to nucleus which correlated with an increase in StAR mRNA levels, StAR protein accumulation and steroidogenesis. Similarly, ERK inhibition led to a reduction in the levels of FSK-stimulated StAR mRNA, StAR protein and steroid secretion. These effects were attributed to the finding that ERK activity is required for SF-1 phosphorylation, a transcription factor required for the regulation of StAR gene transcription. This conclusion was supported by our demonstration of an ERK-dependent increase in the binding of SF-1 from FSK-treated Y1 nuclei to three consensus double stranded DNA sequences from the StAR promoter region. These observations suggest that the activation of ERK2/1 by increasing cAMP is an obligatory and regulated stage in the stimulation of steroid synthesis by cyclic AMP-generating stimuli.

Introduction

Stimulus recognition in steroidogenic cells is dependent initially on the receptor-mediated activation of adenylate cyclase and subsequent increases in intracellular cyclic AMP that activate the cyclic AMP-dependent protein kinase (PKA) [1,2]. PKA activation stimulates the rapid mobilisation of intracellular stores of cholesterol to the outer mitochondrial membrane (OMM), and promotes cholesterol transport to the inner mitochondrial membrane (IMM) where it is converted to pregnenolone by the cytochrome P450 side chain cleavage complex ($P450_{SCC}$) [3]. The delivery of cholesterol to the IMM is the rate-limiting step in steroid synthesis, and so regulates the rate of secretion of steroid hormones [3]. It is now generally accepted that a mitochondrial phosphoprotein, known as steroidogenic acute regulatory (StAR) protein, is essential for cholesterol transport to the IMM, and that the expression of StAR protein is the key regulatory event in steroid synthesis [4, 5]. Thus, transcription of the StAR gene is closely coupled to steroidogenesis in a variety of tissues [6-8]; the experimental expression of StAR cDNA enables and enhances steroid production [9, 10]; and mutations in the StAR gene produce endocrine pathologies with a phenotype of reduced or absent steroidogenesis [11].

The regulation of StAR gene transcription is not fully understood, although the StAR gene contains binding sites for transcriptional regulators such as SF-1 (steroidogenic factor-1, at least 3 separate), DAX-1 (dosage-sensitive sex reversal- adrenal hyperplasia congenital critical region on the X-chromosome) and AP-1 (activator protein–1), whose transcriptional activities are phosphorylation-dependent [12, 13]. Although PKA-mediated protein phosphorylation is undoubtedly important in regulating steroid synthesis, other signalling systems have also been implicated in

StAR gene expression [*e.g.* 14-17]. The mitogen activated protein kinase (MAPK) family is a point of convergence for diverse signalling pathways, in which extracellular signal-regulated kinases (ERK2/1 or p42/44 MAPKs), c-jun N-terminal kinases (JNKs) and p38 kinases are the terminal kinases in three distinct yet interacting signal transduction cascades [18]. These enzymes commonly regulate target gene expression by the activation of downstream transcription factors, and ERK2/1 have been implicated in the regulation of SF-1 and AP-1 activity in human breast cancer MCF-7 cells and in human kidney COS cells [19]. We have now demonstrated that cyclic AMP-induced steroid synthesis is dependent upon the activation of the ERK cascade, and that ERK2/1 activation leads to increased phosphorylation of SF-1, enhanced SF-1 binding to regions of the StAR promoter and to enhanced steroid production by increasing the availability of StAR protein through increased transcription of the StAR gene.

Experimental Procedures

Materials: Tissue culture reagents and plastics were from Gibco BRL (Paisley, UK). Trilostane was a kind gift from Dr. George Margetts (Stegram Pharmaceuticals, Sussex, UK). PD098059 (PD) and UO126 (UO) were obtained from Calbiochem-Novabiochem (UK) Ltd. (Notts, UK). A polyclonal antibody to pregnenolone was obtained from Biogenesis (Poole, UK). [7-^{3}H(N)]-pregnenolone (specific activity 22.5 Ci/mmol) for use in radioimmunoassay was obtained from NEN Life Science Products, Inc (Boston, MA, USA). The anti-active p42/44 MAPK antibody was from Promega (Southampton, UK) and the anti-p42/44 MAPK antibody was obtained from Transduction Laboratories (Lexington, Kentucky). The anti-SF-1 antibody was

obtained from Upstate biotechnology (Lake Placid, New York). A polyclonal anti-serum against StAR protein was a kind gift from Professor Ian Mason (Edinburgh, UK). Horseradish peroxidase-coupled goat anti-mouse immunoglobulin G (IgG), goat anti-rabbit IgG and goat anti-sheep IgG were from Pierce (Rockford, UK). The monoclonal anti-phosphoserine antibody was from Calbiochem-Novabiochem (San diego, California). Alexa fluor 488 -conjugated goat anti-rabbit IgG was from Molecular probes (PoortGebouw, The Netherlands). Enhanced chemiluminescence (ECL) reagents, Hyperfilm and X-ray film were from Amersham International plc (Bucks, UK). T4 DNA kinase and buffer were obtained from Promega (Southampton, UK). [γ^{32}P]-ATP was from Amersham International plc (Bucks, UK). Complete protease inhibitor cocktail was from Roche Diagnostics (Sandhofer Strasse, Germany). The pCMV6 plasmid containing the full-length StAR cDNA sequence was a kind gift from Professor Douglas Stocco (Texas-Tech University, TX, USA). PCR primers and EMSA probes were prepared in house (King's College London, Molecular Biology Unit). The QIAquick gel extraction kit was obtained from Qiagen (Crawley, UK). The Dynabeads Oligo (dT) $_{25}$ kit was obtained from Dynal (Oslo, Norway). Moloney murine leukemia virus-reverse transcriptase (MMLV-RT, Superscript II) was from Gibco BRL (Paisley, UK). PCR was performed using a LightCycler rapid thermal cycler system from Roche Dignostics Ltd (Lewes, UK). All other biochemicals were from the Sigma Chemical Company Ltd. (Dorset, UK).

Cells: Mouse adrenocortical Y1 cells were obtained from the European Collection of Animal Cell Cultures (Wiltshire, UK) and maintained in DMEM supplemented with 100μg/ml streptomycin, 100U/ml penicillin and 10% (v/v) foetal bovine serum. MA-10 cells were a kind gift from Professor Mario Ascoli [20] and were maintained in Waymouth MB752/1 supplemented with 20mM Hepes, 15% horse serum and

50μg/ml gentamycin. Both cell lines were cultured at 37°C in an atmosphere of 5% CO_2.

Steroid Production: Y1 cells and MA-10 cells were seeded in 96-well microculture plates at a density of $1x10^6$ cells per well, and incubated overnight at 37°C, 5% CO_2 to allow the cells to adhere to the plates. The culture medium was replaced with medium alone (control), or medium supplemented with 1μM forskolin (FSK) with or without PD (50μM) or UO (10μM) and the incubation was continued for a further 3h. Steroid production was measured using a radioimmunoassay for pregnenolone over the range 0.8-100pmol/ml. The conversion of pregnenolone to other steroids was prevented by the addition of 2μM trilostane [21, 22], an inhibitor of 3β-hydroxysteroid dehydrogenase, to the incubation medium.

Immunoprecipitation of SF-1: Y1 cells were seeded in 6-well microculture plates at a density of $5x10^6$ cells per well. The medium was replaced with fresh growth medium (control) or with medium supplemented with 1μM FSK, with or without PD (50μM) and UO (10μM) and the incubation was continued for a further 3h after which the cells were washed with fresh growth medium. Ice cold lysis buffer (20mM Tris, 2mM EDTA, 0.5mM EGTA, 1mM PMSF, 50μg/ml leupeptin, 250μM NaF, 10μM E64 and 10μM TLCK) was added to each well and the cell extracts were transferred to tubes on ice. Anti-SF-1 antibody (5μg/tube) was added to the tubes which were incubated at 4°C on a rotating mixer. Protein A agarose (10%) was added to the tubes and the incubation was continued for a further 3 h at 4°C. After this time, the tubes were centrifuged at 9000g for 3 min and the pellet was washed three times with lysis buffer. Sample buffer (12.5% Tris, 10% glycerol, 5% β-mercaptoethanol, 2% (w/v) SDS and 0.1% (w/v) bromophenol blue) was added to each pellet and the

tubes were boiled briefly. Immunoreactivity was detected by polyacrylamide gel electrophoresis and immunoblot analysis using a mouse anti-phosphoserine primary antibody and a goat anti-mouse secondary antibody or an anti-SF-1 primary antibody and a goat anti-rabbit secondary antibody. Immunoreactivity was quantified using densitometric scanning (UVP Easy system) of the ECL signal on the film.

Electrophoretic mobility shift assay (EMSA): Double stranded oligonucleotides were generated and end labeled with [γ^{32}P]-ATP according to previously described protocols [12]. Y1 nuclear extracts (5µg) were incubated with 50 fmol radiolabeled oliognucleotide for 30 min on ice in a binding buffer described in Wooton-Kee *et al* [12]. Antibody supershift assays were performed with 5µg Y1 nuclear extract proteins and 10-25µg polyclonal SF-1 antibodies. Antibody and nuclear extract were pre-incubated with all components of the binding reaction, except for radiolabeled probe, for 1 h. Probe was then added and the incubation was continued for a further 30 min. Binding reactions were resolved on a 6% non-denaturing polyacrylamide gel. The gels were dried and the radioactive bands were visualised by autoradiography.

Sequences of oligonucleotides used for EMSA probes: The following sequences of the mouse StAR promoter region were used [12]:

SF1-1 (-135/-83), 5'-CTCCCTCC**CACCTT**GGCCAGCACT-3';

SF1-2 (-51/-29), 5'-ATGATGCA**CAGCCTT**CCACGGGA-3';

SF1-3 (-105/-83), 5'-CATTC**CATCCTT**GACCCTCTGCA-3'

In each case SF1 consensus binding sequences are indicated in bold and positions refer to the number of nucleotides upstream of the transcription start site.

Nuclear extract preparation: A confluent T75 flask of Y1 cells per treatment was incubated for 3 h with growth media alone, or media supplemented with 1µM FSK, with or without 50µM PD. The cells were homogenised in 5 volumes of buffer

containing 10mM Hepes, 1.5 mM $MgCl_2$, 10mM KCl, 0.25M sucrose and 0.5mM dithiothreitol, pH 7.4 and the nuclei collected by centrifugation (2400g, 5min, 4°C). 4 volumes of buffer containing 25% glycerol, 0.2mM EDTA, 0.5mM dithiothreitol, 0.64M KCl and 1 tablet of Complete protease inhibitor cocktail were added to each pellet and centrifuged at 18000g, 4°C for 2 min. The supernatants containing nuclear proteins were stored at –80°C.

Immunodetection of proteins*:* Y1 cells were incubated in the presence or absence of FSK (1μM) for 15 min-6 h at 37°C in a humidified atmosphere of 5% CO_2. Protein samples were prepared as described in Jones *et al*. [23]. Protein extracts (15μg) were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with an antibody that recognises the 42 and 44 kDa non-phosphorylated and phosphorylated (total) isoforms of ERK and with an antibody that recognises only the phosphorylated (active) isoforms. StAR immunoreactivity was detected in mitochondrial-enriched Y1 cells fractions using an antibody to the mature 30kDa form of StAR [23]. Antibody binding was detected by enhanced chemiluminescence. Total protein in extracts was measured using the Bradford assay [24].

Confocal Immunohistochemistry: Y1 cells were seeded onto coverslips in 6 well plates at a density of 5x 10^4 cells per coverslip. Cells were incubated overnight to adhere and then incubated with DMEM alone (control) or DMEM supplemented with 1μM FSK for 3 h at 37°C, before paraformaldehyde fixation (30 min, 4% paraformaldehyde), followed by incubation (1 h) with 2% goat serum in PBS/0.01% triton-X. Fixed cells were incubated with an antibody to the active (phosphorylated) form of ERK2/1 (1/100, 20 h, 4°C), washed with PBS/0.01% triton and were further

incubated (2 h, 20°C) with an Alexa fluor-488-conjugated second antibody. Antibody binding was visualised using a Biorad 1024 confocal microscope.

Isolation of Y1 cell messenger RNA and reverse transcription: Y1 cells were seeded in 24 well plates at a density of $1x10^5$ cells/well. After 24 h to ensure adherence, the cells were incubated for 3 h in the presence of DMEM alone (control) with or without 50μM PD, or in the presence of 1μM FSK with or without 50μM PD. Cells which were incubated with PD were pre-incubated for 30 min with the inhibitor. mRNA was isolated from the cells using the Dynabeads $Oligo(dT)_{25}$ kit. Briefly, cells were lysed by adding 300μl lysis buffer (supplied with kit). 20μl of Dynabeads ($6.6x10^7$ beads) was then added to the cell lysate and the mRNA was allowed to anneal to the beads. After repeated washes, the mRNA was eluted from the beads with 12μl 10mM Tris (pH7.5). cDNA was synthesised simultaneously from all mRNA samples using MMLV-RT, Superscript II. $Oligo(dT)_{18}$ (1μg) and random 10-mers (1μg) were added to the mRNA (10μl) and the mixture heated (70°C, 5 min) to remove secondary RNA structure, then cooled on ice. DTT (10mM), dATP, dCTP, dTTP and dGTP (all 0.5mM), recombinant ribonuclease inhibitor (80u, RNAsin), MMLV-RT (200u) and diethyl pyrocarbonate-treated water were added to make the final volume 20μl, and the mixture was incubated at 42°C for 50min. MMLV-RT was inactivated by heating at 70°C for 15 min. The cDNA was diluted 20-fold with tRNA (10μg/ml) and used immediately in PCR reactions or stored at -20°C for future use. An aliqout of mRNA was not reverse transcribed and was diluted with tRNA and stored at -85°C.

***PCR primers*:** 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 860bp. Forward and reverse GAPDH PCR primers were: sense

primer 5'-CCC ATC ACC ATC TTC CAG GAG C-3'; antisense primer 5'-CCA GTG AGC TTC CCG TTC AGC-3'. The predicted size of the GAPDH PCR product was 473bp. Forward and reverse cytochrome P450scc primers were: sense primer 5'-AGT GGC AGT CGT CGG GAC AGT-3'; antisense primer 5'-TAA TAC TGG TGA TAG GCC ACC-3'. The predicted size of the P450scc PCR product was 411bp.

Standard curves The product amplified by the GAPDH or P450scc primers was separated by agarose gel electrophoresis (1.8% v/v) and visualised by staining with ethidium bromide (EtBr, 0.5μg/ml). This product was then cut from the gel, spin column purified using a Qiaquick gel extraction kit and ten-fold serial dilutions were prepared as standards. StAR standards were prepared by ten-fold serial dilutions of the pCMV6 plasmid containing the 1.4kb mouse StAR cDNA sequence (standards were used over the range 3.21ng/μl-3.21fg/μl).

Quantitative PCR*:* In initial experiments StAR mRNA levels in Y1 extracts were quantified by competitive PCR, as described previously in Burns *et al* [25]. In subsequent experiments, real-time PCR was performed using a LightCycler rapid thermal cycler system. Reactions were performed in a 10μl volume containing nucleotides, Taq DNA polymerase and buffer (all included in the LightCycler-DNA Master SYBR Green I mix), 3mM $MgCl_2$ and 0.5μM primers. Reactions also included either Y1 cDNA standard or a mRNA/tRNA blank. All PCR protocols included a 10s denaturation step and then continued for 45 cycles consisting of a 95^{o}C denaturation for 0s, annealing for 10s at 55^{o}C (GAPDH), 58^{o}C (P450scc), 62^{o}C (StAR), and a 72^{o}C extension phase for 19s (GAPDH), 16s ($P450_{SCC}$), 34s (StAR). Fluorescence measurements were taken at the end of the 72^{o}C extension phase. The amplification product of each primer pair was subjected to melting point analysis and subsequent gel electrophoresis to ensure specificity of amplification.

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

Results

Cyclic AMP-induced ERK2/1 activation, StAR gene transcription, StAR protein accumulation and steroid synthesis: The mouse adrenocortical Y1 cell line has a reduced capacity for 21-hydroxylation and 11-hydroxylation reactions compared to primary adrenal cells, but retains intact the cyclic AMP-responsive early rate-limiting stages of steroid synthesis, including the transport of cholesterol to the IMM and its $P450_{scc}$ –mediated conversion to pregnenolone [26, 27]. The adenylate cyclase activator forskolin (FSK) stimulated steroid production by Y1 cells (Fig. 1[A](iii)), and increased steroid production was accompanied by increases in StAR mRNA and protein expression (Fig. 1[A](i) and (ii)). FSK also caused a time-dependent increase in ERK2/1 activities (Fig. 1[B] (i)) without causing any consistent changes in total ERK2/1 immunoreactivity. Results are expressed as a ratio of active ERK2/1 to total ERK2/1 in cell extracts (Fig. 1[B] (ii)). The immunoblot measurements of ERK2/1 activation were confirmed by confocal immunohistochemistry of active ERK2/1 (Fig. 2). Control cells contained low amounts of active ERK2/1, and this was localised to discrete areas outside the nucleus but associated with the nuclear membrane. On activation with forskolin there was a marked increase in the overall levels of active ERK2/1, and the immunofluorescence had translocated from the extranuclear accumulations to within the nucleus, giving areas of intense intranuclear fluorescence.

Inhibition of ERK2/1 activation blocks cyclic AMP-induced StAR gene transcription, StAR protein accumulation and steroid synthesis: ERK2/1 are activated by phosphorylation by a dual specificity tyrosine/threonine kinase, MAPK/ERK kinase (MEK), and so ERK2/1 activation can be selectively blocked by

compounds that inhibit MEK activity. Figure 3[A] demonstrates that cyclic AMP-induced steroid production was fully inhibited by two structurally dissimilar inhibitors of MEK, PD098059 (PD) [28] and UO126 (UO) [29], when used at concentrations which have been shown to inhibit MEK activity. The inhibition of steroid production by MEK inhibitors was not confined to Y1 cells (Fig 3[A](i)), since both MEK inhibitors totally inhibited FSK-stimulated steroid production by the mouse testicular MA-10 cell line [20], as shown in Figure 3[A](ii). Neither MEK inhibitor had any significant effect on unstimulated steroid production by Y1 cells nor MA-10 cells (Y1 cells, basal: 30 ± 4 pmoles/10^5 cells, PD: 21 ± 3 pmoles/10^5 cells, UO: 46 ± 7 pmoles/10^5 cells; MA-10 cells, basal: 16 ± 5.7 pmoles/10^5 cells, PD: 9 ± 3 pmoles/10^5 cells, UO: 15 ± 4 pmoles/10^5 cells). In contrast to their effects on cyclic AMP-dependent steroid synthesis, neither MEK inhibitor had any effect on steroid production from cells supplied with the water-soluble cholesterol analogue 22R-hydroxy-cholesterol (22-ROHC) (Fig. 3[A](iii)). 22-ROHC passes unassisted from the OMM to the IMM where it is converted to pregnenolone by $P450_{SCC}$, and thus supports steroid synthesis independently of StAR. These observations demonstrate that MEK inhibitors do not inhibit cyclic AMP-dependent steroid production by interfering with $P450_{SCC}$ function, and identify the site of action of ERK2/1 as proximal to cholesterol delivery to the IMM. FSK-induced accumulation of StAR protein in Y1 cells was assessed by immunoblotting extracts of Y1 cells incubated for 3 h with FSK alone or with FSK in the presence of the MEK inhibitors PD or UO (Fig. 3[B] (i-iv). MEK inhibition reduced FSK-induced accumulation of StAR protein to near basal levels. StAR mRNA levels in Y1 cell extracts were measured by real-time quantitative RT-PCR using a standard curve generated with a pCMV6 plasmid containing the 1.4Kb mouse StAR cDNA sequence (r = -0.99). A standard curve for

the amplification of $P450_{SCC}$ was constructed from product amplified by the primers and recovered and purified from an agarose gel (r = -1.00). In each case the expression of mRNA was normalised against the content of GAPDH mRNA in the same extracts. Agarose gel electrophoresis and melting point analyses of products revealed the presence of a single species in each sample (Fig. 4[A] (i) – (iv)). FSK caused a marked (~15-fold) increase in the levels of StAR mRNA (Fig. 4[B] (i)), of a comparable magnitude to that measured by competitive PCR in similar experiments (Fig. 1[A] (i)). Inhibiting ERK2/1 activation using the MEK inhibitor PD produced a significant reduction in the effects of FSK on StAR mRNA levels (Fig. 4[B] (i)). This reduction is consistent with the effects of MEK inhibition on StAR protein expression (Fig. 3[B]) and on PKA-dependent steroid production (Fig. 3[A] (i) and (ii)). FSK also enhanced levels of $P450_{SCC}$ mRNA in Y1 cells (~3-fold) but MEK inhibition had no effect on the levels of $P450_{SCC}$ mRNA (Fig 4[B] (ii)), suggesting a selective effect of MEK inhibition on the regulation of StAR expression rather than a general reduction in gene transcription.

Effects of ERK2/1 inhibition on SF-1 phosphorylation: The orphan nuclear transcription factor, SF-1, is known to play a major role in regulating the transcription of the StAR gene [15]. FSK was seen to cause an increase in the phosphorylation of SF-1 as assessed by SF-1 immunoprecipitation and phospho-serine immunoblotting (Fig 5). However, MEK inhibition with PD caused a reduction in FSK-stimulated SF-1 phosphorylation levels (Fig 5). Results were quantified by densitometric scanning and normalised by stripping the membrane and re-probing it for total SF-1 (Fig 5 [B]). The graph (Fig 5 [B]) represents the level of phosphorylated SF-1 expressed as a ratio to the level of total SF-1 in each sample. In the absence of FSK stimulation MEK inhibition had no effect on basal SF-1 phosphorylation (data not shown). These results

are consistent with the observed reduction in StAR protein accumulation upon MEK inhibition.

Effects of ERK2/1 inhibition on SF-1 binding: The DNA binding site of SF-1 recognises the CAXCCTT motif (where X represents any nucleotide) in genomic DNA in the StAR promoter region. To date, three separate regions encoding this consensus sequence and having high affinity for SF-1 have been identified in the region upstream of the StAR gene transcription start site, and have been called SF-1-1, SF-1-2 and SF-1-3 [12, 30]. Our results demonstrate that FSK treatment of Y1 cells increases the binding of Y1 cell nuclear proteins to synthetic double-stranded oligonucleotides corresponding to SF-1-1, SF-1-2 and SF-1-3 as shown in figure 6. At least part of this nuclear protein binding to SF-1 consensus DNA sequences could be attributed to SF-1 as determined by antibody supershift assays (fig 6 [A]). Furthermore, the FSK-induced binding of SF-1 to sequences in the StAR gene promoter region was dependent upon the activation of ERK2/1, since the effects were fully reversed by the presence of the MEK inhibitor, PD (fig 6 [B, C, D]).

Discussion

Mammalian steroid-producing tissues share a number of common general features. Steroid production is normally regulated by extracellular trophic signals (e.g. LH, FSH, ACTH) which act through specific cell-surface receptors to activate intracellular effector systems, and thus increase cholesterol metabolism [1, 2]. The enzymic events involved in the conversion of cholesterol to the various steroid hormones share common elements that are now fairly well understood [3]. Similarly, evidence is accumulating that the control of mitochondrial cholesterol transport in most steroid-

producing cells involves the expression of StAR protein and its import into the mitochondria [3].

A number of recent studies have suggested that the MAPK cascade is involved in cellular regulation in steroidogenic tissues, although many of the observations reported to date appear to be contradictory. MAPKs are known to be involved in proliferative responses in many tissues [31], and there is evidence that this is an important mechanism in adrenal tissue [32]. Thus, the activation of ERK2/1 has been implicated in the mitogenic responses of human H295R or mouse Y1 adrenocortical cells to angiotensin ll or ACTH, respectively [33-35], although inhibition of other MAPK activities has also been reported during ACTH-induced cell-cycle progression in Y1 cells [36]. A similar confusion surrounds the potential role(s) of ERK2/1 as transduction elements in the regulation of steroidogenesis. Thus, for example, the anterior pituitary trophic hormones LH and FSH are reported to activate ERK2/1 and enhance steroid production in ovarian cells [37,38], but ERK activation has also been associated with the inhibition of agonist-induced steroidogenesis in granulosa-derived cell lines [39]. Similarly, the activation of ERK2/1 has been linked to enhanced steroid production in human granulosa-luteal cells, and LH-induced steroidogenesis was inhibited by inhibitors of ERK2/1 [40], however the inhibitory effects of prostaglandin $F_{2\alpha}$ [41] or gonadotrophin-releasing hormone agonists [42] on steroidogenesis in this tissue have also been attributed to the activation of ERK2/1. There are several likely explanations for these apparently contradictory reports. A prime reason may be the existence of multiple transduction pathways coupled to cell surface receptors, with differences in receptor-effector coupling between tissues, cell-lines and species. One of the major intracellular effector systems in the regulation of

steroidogenesis is the adenylate cyclase/cyclic AMP system, although this may not be the sole mechanism through which trophic hormones regulate steroid production in their target tissues [43]. In the present study we have circumvented receptor expression, agonist binding, and receptor-effector coupling by using forskolin, an adenylate cyclase activator, to directly elevate intracellular cyclic AMP and thus allow us to focus on the downstream events in the signalling cascade. We chose mouse adrenocortical Y1 cells line for these studies since an *in vitro* cell line can provide the large amount of starting material required for the immunodetection of StAR and for the immunoprecipitation and analysis of the phosphorylation state of transcription factors. In addition, Y1 cells offer an excellent adrenocortical model for the present experiments since they retain intact the cyclic AMP-responsive early rate-limiting stages of steroid synthesis, including the transport of cholesterol to the inner mitochondrial membrane and its $P450_{SCC}$ –mediated conversion to pregnenolone [26, 27].

An additional reason for disparate conclusions between studies may result from differences in the experimental protocols, particularly with respect to different time courses of the events being measured. Thus, in other tissues, ERK2/1 activation tends to be a rapid and often transient event [44] while it normally takes longer for increased steroid production to be detectable, partly because of the requirements for increased expression of the StAR protein [1-6]. In the present study we measured cyclic AMP-dependent ERK2/1 activation over the same time-course as enhanced steroid production, to demonstrate that the timing of both events is consistent with ERK2/1 activation being involved in initiating the steroidogenic response. Our results clearly demonstrate that cyclic AMP-induced steroidogenesis in Y1 cells is dependent

on activation of the ERK2/1 signalling cascade and that steroid production is accompanied by a prolonged activation of ERK2/1. Thus activation of adenylate cyclase induced the activation of ERK2/1 in Y1 cells and the translocation of active ERK2/1 into the nucleus, and we were able to link this activation to steroid synthesis, since ERK2/1 inhibition reduced cyclic-AMP-dependent steroid production. Furthermore, our demonstration of similar effects in the adrenocortical Y1 cell line and the testicular MA10 cell line suggests that the involvement of ERK2/1 in steroidogenic responses to cyclic AMP may be a general effect, rather than a cell-type specific event.

The importance of ERK2/1 activation in steroid production was confirmed by our quantitative measurements of the expression of StAR mRNA and protein in Y1 cells. It is now well established that StAR protein plays a major role in regulating steroid synthesis [1-5], and that the stimulation of steroid production by agonists or by pharmacological elevations in cyclic AMP is dependent upon enhanced transcription of the StAR gene, elevations in StAR mRNA and the intracellular accumulation of StAR protein [3, 5, 25]. Our results confirm the importance of StAR in steroid production since forskolin caused a rapid and prolonged increase in the levels of StAR mRNA and protein in Y1 cells, associated with increased production of steroid. More importantly, our data show that ERK2/1 activation is required for the effects of cyclic AMP on StAR expression, since preventing the activation of ERK2/1 by inhibiting their upstream activator, MEK-1, was alone sufficient to reduce the cyclic AMP-dependent increases in StAR mRNA and protein. This effect was selective for StAR mRNA, since inhibition of MEK-1, and thus of ERK2/1, did not affect the accumulation of $P450_{SCC}$ mRNA induced by forskolin. These observations also imply

multiple effects of cyclic AMP on the regulation of gene expression in Y1 cells, some of which (*e.g.* StAR) are mediated through ERK2/1 while others (*e.g.* $P450_{SCC}$) do not require the activation of ERK2/1.

Our current results therefore explain the inhibitory effects of two structurally-distinct MEK-1 inhibitors on cyclic AMP-induced steroid production in two different cell lines, and are consistent with a transduction sequence in which increased intracellular cyclic AMP leads to the phosphorylation and activation of MEK-1, presumably through activation of PKA [45]. Activated MEK-1 in turn phosphorylates and activates ERK2/1, which accumulates in the nucleus and increases the transcription of the StAR gene and hence the accumulation of StAR mRNA and protein. Since StAR protein is rate-limiting for cholesterol transport into the mitochondria [1-6], this sequence of events inevitably results in enhanced delivery of cholesterol to the $P450_{SCC}$ complexes inside the mitochondria, and to increased production of steroids. This sequence of events also explains the lack of effect of inhibitors of MEK-1 on steroid production from cells supplied with 22ROHC. This cholesterol analogue is water-soluble and is therefore not dependent on the ERK2/1-driven expression of StAR to enable it to reach the inner mitochondrial membrane and act as a substrate for the $P450_{SCC}$.

In other tissues activated ERK2/1 modify cellular function primarily by regulating the expression of numerous genes through phosphorylating a variety of transcriptional regulators [18,46]. Our measurements of the cellular localisation of active ERK2/1 by confocal immunohistochemistry demonstrated that the low level of active ERK2/1 found in unstimulated cells were localised to small extranuclear ‘caps’ associated with

specific areas of the nuclear membrane. Activation of Y1 cells by forskolin caused a large increase in active ERK2/1 in the intranuclear compartment, consistent with an intranuclear location for the protein substrates of ERK2/1 in Y1 cells. The complex mechanisms regulating basal and stimulated transcription of the StAR gene are not fully understood, although the cyclic AMP–responsive region is thought to be coded within the first 254 base pairs of the StAR promoter [47], and several activating factors have been implicated in the regulation of StAR gene transcription [48-50]. Among these is the 53 kDa steroidogenic factor 1 (SF-1) whose presence appears to be an absolute requirement for StAR gene expression, since SF-1 knockout mice fail to express StAR mRNA [51]. SF-1 contains a serine residue (Ser-203) residing within the ERK2/1 consensus phosphorylation sequence (PX_nS/TP) [19] suggesting that the functional status of SF-1 can be modified by ERK2/1 and our direct measurements of ERK2/1-dependent SF-1 phosphorylation support this as an important regulatory mechanism in cyclic AMP-induced steroid production.

The promoter region of the StAR gene contains at least three sites encoding potential binding sites for activated SF-1 [12], and our results suggest that SF-1 binding to these sites may be the mechanism through which ERK2/1 activation leads to enhanced steroid production. Thus, we have demonstrated that the ERK2/1-dependent phosphorylation of SF-1 enhances its ability to bind to all three sites in the StAR promoter, supporting a role for ERK2/1-dependent phosphorylation of SF-1 as an important regulator of activational activity. These observations do not rule out an additional regulatory role for direct phosphorylation of SF-1 by PKA [52-54] in the regulation of StAR gene expression, nor are they inconsistent with the loss of SF-1 trans-activational activity in PKA-deficient cell lines [55,56], but they do suggest that

the ERK2/1-dependent event is obligatory for cAMP-induced StAR expression and steroid synthesis.

In summary, the current study provides a direct link from PKA activation, through the MEK/ERK cascade to the phosphorylation of SF-1, enhanced binding to the StAR promoter region, increased expression of StAR protein and enhanced steroid production in adrenal cells.

Footnotes

Acknowledgements: This work was supported by the Welcome Trust (Grant 054789/Z/98/Z) and a Research and Development Grant from the Guy's and St. Thomas's Charitable Foundation. S.G. is a BBSRC postgraduate student. We are grateful to Ron Senkus for his assistance with the confocal microscopy.

Abbreviations: StAR, steroidogenic acute regulatory protein; ERK, extracellular signal-regulated kinase; PKA, protein kinase A; SF-1, steroidogenic acute regulatory protein; $P450_{SCC}$, cytochrome P450 cholesterol side chain cleavage system; FSK, forskolin; MAPK, mitogen-activated protein kinase;

References

[1] Stocco, D. M., and Clark, B. J. (1996) *Endocrine Rev*. **17**, 221-244.

[2] Clark, B. J., Soo, S. C., Caron, K. M., Ikeda, Y., Parker, K. L. and Stocco, D. M. (1995). *Mol.Endocrinol*. **9**, 1346-1355

[3] Christenson, L. K. and Strauss III, J.F. (2000) *Biochem. et Biophys. Acta.* **1529**, 175-187.

[4] Stocco, D.M. (1998) *Rev. Reprod*. **3**, 82-85.

[5] Stocco, D.M. (2000) *Nat. Struct. Biol.* **7**(6), 445-447.

[6] Liu, J., Hekkila, P., Kahri, A., and Voutilainen, R. (1996) *J. Endocrinol.* **150**, 43-50.

[7] Clark, B. J., Combs, R., Hales, K. H., Hales, D. B., and Stocco, D. M. (1997) *Endocrinology* **138**, 4893-4901.

[8] Caron, K. M., Ikeda, Y., Soo, S. C., Stocco, D. M., Parker, K. L. and Clark, B. J. (1997) *Mol. Endocrinol*. **11**, 136-147.

[9] Clark, B. J., Wells, J., King, S. R., and Stocco, D. M. (1994) *J. Biol. Chem*. **269**, 1346-1355.

[10] Sugawara, T., Holt, J. A., Driscoll, D., Strauss, J. F. 3rd, Lin, D., Miller, W. L., Patterson, D., Clancy, K. P., Hart, I. M., Clark, B. J., and Stocco, D. M. (1995) *Proc. Natl. Acad. Sci. USA.* **92**, 4778-4782.

[11] Bose, H. S., Sato, S., Aisenberg, J., Shalev, S. A., Matsuo, N., and Miller, W.L. (2000) *J. Clin. Endocrinol. Metab*. **85**(10) 3636-3639.

[12] Wooton-Kee, C.R. and Clark, B.J. (2000) *Endocrinology* **141** (4) 1345-1355.

[13] Stocco, D. M. (2000) *Biochim. Biophys. Acta.* **1486** (1), 184-197.

[14] Reyland, M. E., Evans, R. M., and Elizabeth, K. W. (2000) *J. Biol. Che*m. **275** (47), 36637-36644.

[15] Sugawara, T., Saito, M., and Fujimoto, S. (2000) **141** (8), 2895-2903.

[16] Wang, X., Walsh, L. P., Reinhart, A. J., and Stocco, D.M. (2000) *J. Biol. Chem.* **275** (26), 20204-20209.

[17] Zenkert, S., Schubert, B., Fassnacht, M., Beuschlein, F., Allolio, B., and Reincke, M. (2000) *Eur. J. Endocrinol*. **142**(3), 294-299.

[18] Seger, R., and Krebs, E. G. (1995) *FASEB J*. **9**(9), 726-735.

[19] Hammer, G. D., Krylova, I., Zhang, Y., Darimont, B. D., Simpson, K., Weigel, N. L. and Ingraham, H. A. (1999) *Mol. Cell*. **3**, 521-526.

[20] Ascoli, M. (1981) *J. Biol. Chem.* **256** (1), 179-183.

[21] Potts, G. O., Creange, J. E., Harding, H. R., and Schane, H. P. (1978) *Steroids* **32**, 257-267.

[22] Aguilera, G., Menard, R. H., and Catt, K. J. (1980) *Endocrinology* **107**, 55-60.

[23] Jones, P. M., Sayed, S. B., Persaud, S. J., Burns, C. J., Gyles, S. L. and Whitehouse, B.J. (2000) *J. Mol. Endocrinol*. **24**, 233-239.

[24] Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254.

[25] Burns, C. J., Gyles, S. L., Persaud, S.J., Sugden, D., Whitehouse, B.J., and Jones, P.M. (2000) *Biochem. Biophys. Res. Commun*. **273**, 35-39.

[26] Schimmer, B. P. (1979) *Meth. Enzymol*. **58**, 570-574.

[27] Schimmer, B. P. (1981) *Functionally differentiated cell lines* (Sato, G., Ed.), pp. 61-92, A.R. Liss, New York.

[28] Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) *J. Biol. Chem.* **270**, 27489-27494.

[29] Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F., Copeland, R. A., Magolda, R. L., Scherle, P. A., Trzaskos, J. M. (1998) *J. Biol. Chem*. **273**, 18623-18632.

[30] Sandhoff, T.W., Hales, D.B., Hales, K.H. and Mclean, M.P. (1998) *Endocrinology* **139** (12) 4820-4831.

[31] Bonventre, J. V., Force, T. (1998) *Hypertension* **31** (1 Pt 2), 152-161.

[32] Lotfi, C. F., Lepique, A. P., Forti, F. L., Schwindt, T. T., Eichler, C. B., Santos, M. O., Rebustini, I. T., Hajj, G. N., Juliano, L., Armelin, H. A. *Braz. J. Med. Biol. Res*. (2000) **33** (10), 1133-1140.

[33] Watanabe, G., Lee R. J., Albanese, C., Rainey, W. E., Batle, D., Pestell, R. G. (1996) *J. Biol. Chem*. **271** (37), 22570-22577.

[34] Lotfi, C. F., Todorovic, Z., Armelin, H. A., Schimmer, B. P. (1997) *J. Biol. Chem*. **272** (47), 29886-29891.

[35] Lotfi, C. F., Costa, E. T., Schwindt, T. T., Armelin, H. A. (2000) *Endocr. Res*. **26** (4), 873-877.

[36] Watanabe, G., Pena, P., Albanese, C., Wilsbacher, L. D., Young, J. B., Pestell, R. G. (1997) *J. Biol. Chem*. **272** (32), 20063-20069.

[37] Cameron, M. R., Foster, J. S., Bukovsky, A., Wimalasena, J. (1996) *Biol. Reprod*. **55** (1), 111-119.

[38] Das, S., Maizels, E. T., DeManno, D., St Clair, E., Adam, S. A., Hunzicker-Dunn, M. (1996) *Endocrinology* **137** (3) 967-974.

[39] Seger, R., Hanoch, T., Rosenberg, R., Dantes, A., Merz, W. E., Strauss III, J. F., Amsterdam, A. (2001) *J. Biol. Chem*. In Press.

[40] Dewi, D. A., Abayasekara, D. R. E., Wheeler-Jones, C. P. D. (2000) *J. Endocrinol*. **167**, OC17.

[41] Tai, C., Kang, S. K., Choi, K., Tzeng, C., Leung, P. C. K. (2001) *J. Clin. Endocrinol. Metab*. **86** (1), 375-380.

[42] Kang, S. K., Tai, C., Nathwani, P. S., Choi, K., Leung, P. C. K. (2001) *Endocrinology* **142**, 671-679.

[43] Cooke, B.A. (1990) *Mol. Cell Endcorinol*. **69** (1), C11-15.

[44] Lopez-Ilasaca, M. (1998) *Biochem. Pharmacol*. **56** (3), 269-277.

[45] Yarwood, S. J., Kilgour, E., Anderson, N. G. (1996) *Biochem. Biophys. Res. Commun.* **224** (3), 734-739.

[46] Lewis, T. S., Shapiro, P. S., Ahn, N. G. (1998) *Adv. Cancer Res*. **74**, 49-139.

[47] Reinhart, A. J., Williams, S. C., Clark, B. J., and Stocco D. M. (1999) *Mol. Endocrinol*. **13,**729-741.

[48] Sirois, J., and Richards, J. S. (1993) *J. Biol. Chem*. **268,** 21931-21938.

[49] Nalbant, D., Williams, S. C., Stocco, D. M., and Khan S. A. (1998) *Endocrinology* **139,** 272-279.

[50] Christenson, L. K., McAlliste, J. M., Martin, K. O., Javitt, N. B., Osborne, T. F., and Strauss J. F. III (1998) *J. Biol. Chem*. **273,** 30729-30735.

[51] Caron, K. M., Ikeda, Y., Soo, S. C., Stocco, D. M., Parker, K. L., and Clark, B. J. (1997) *Mol. Endocrinol*. 11, 136-147.

[52] Zhang, P. and Mellon, S. H. (1996) *Mol. Endocrinol*. **10**, 147-158.

[53] Carlone, D. L. and Richards, J. S. (1997) *J. Steroid Biochem. Mol. Biol*. **61**, 223-231.

[54] Jacob, A. L. and Lund J. (1998) *J. Biol. Chem*. **273**, 13391-13394.

[55] Wong, M., Rice, D. A., Parker, K. L. and Schimmer, B. P. (1989) *J. Biol. Chem*. **264**, 12867-12871.

[56] Parissenti, A. M., Parker, K. L., Schimmer, B. P. (1993) *Mol. Endocrinol*. **7**, 283-290.

Figure 1 - Effects of FSK on Y1 cells. Y1 cells were exposed to FSK (1μM) for various times (0-6h), as shown. Cell extracts were prepared for PAGE and immunoblotting and mRNA extracts were prepared for quantitative PCR. Steroid synthesis during the incubation period was measured by radioimmunoassay.

[A] StAR mRNA, StAR protein and steroid production. (i) Changes in StAR mRNA in Y1 cell extracts were measured by competitive PCR amplification using primers to mouse StAR cDNA and a competitor sequence generated from *E.coli* DNA. Exposure to FSK (1μM, 3h) caused an approximately 15-fold increase in StAR mRNA. Data are expressed as ag of mRNA/10,000 Y1 cells. (ii) Changes in StAR protein expression were assessed by immunoprobing mitochondria-enriched fractions of Y1 cells. Exposure to FSK (1μM, 3h) caused an approximately 12-fold increase (control: 100%, FSK: 1152%) in the accumulation of a 30kDa immunoreactive StAR protein, as assessed by scanning densitometry. The blot shown is representative of 3 similar experiments. (iii) Pregnenolone production by Y1 cells was stimulated by exposure to FSK (1μM, 3h, mean+SEM, n=8).

[B] ERK2/1 immunoreactivity. PVDF membranes were probed with an antibody that recognises the phosphorylated (active) forms of ERK1 and ERK2, migrating with apparent molecular masses of 44kDa and 42kDa respectively (i). The same membrane was then stripped and re-probed with an antibody that recognises total (phosphorylated and non-phosphorylated) ERK2/1 immunoreactivities. The graph represents active ERK2/1 expressed as a ratio to the total ERK2/1 in each sample (ii). Exposure to FSK caused the activation of ERK2/1 in these cell extracts.

Figure 2 – Effects of FSK on the localisation of ERK2/1 in Y1 cells. Y1 cells were seeded onto coverslips and incubated with normal media or media supplemented with

1μM FSK for 3 h. Localisation of active ERK2/1 in the cells was assessed using a primary antibody to the active forms of ERK2/1, and a fluorescently labeled second antibody that could be visualised by confocal microscopy. Images were taken under a x 40 oil objective lens. The figure clearly shows that FSK causes an increase in the activation of ERK2/1, with the movement of the kinases from extra-nuclear regions to within the nucleus.

Figure 3 - Effects of ERK2/1 inhibition on steroid synthesis and StAR protein. *[A] Effect of ERK2/1 inhibition on steroid synthesis.* (i) – (iii) Steroid production by (i) and (iii) adrenocortical Y1 cells, or (ii) testicular MA-10 cells was measured after incubation for 3 h at 37°C. Steroid production was stimulated by the presence of (i) and (ii) FSK (1μM), or (iii) 22ROHC in the presence or absence of the MEK inhibitors PD (50μM) or UO (10μM). Results are expressed as % basal secretion in the absence of any treatment (30±4 pmoles pregnenolone/10^5 Y1 cells; 16.7±5.7 pmoles pregnenolone/10^5 MA-10 cells). Bars show mean + SEM, n=8, ***p< 0.01 versus stimulated steroid production. *[B] Effect of MEK inhibition on StAR protein levels.* MEK inhibition reduced FSK-induced accumulation of StAR protein in Y1 cells as assessed by immunoblotting extracts of Y1 cells incubated (3 h, 37°C) (i) under unstimulated conditions; (ii) in the presence of 1μM FSK; (iii) in the presence of 1μM FSK and 50μM PD; (iv) in the presence of 1μM FSK and 10μM UO (control: 100%, FSK: 1385%, FSK+PD: 333%, FSK+UO: 428%).

Figure 4 - Effects of ERK2/1 inhibition on mRNA expression in Y1 cells. Real time PCR using SYBR green 1 fluorescence to measure product accumulation. *[A] Product analysis.* Each of the primer pairs amplified a single product of the

appropriate predicted lengths as assessed by agarose gel electrophoresis and ethidium bromide staining (i). Melting point analysis revealed a single and specific product for (ii) GAPDH and (iv) $P450_{SCC}$. However, melting point analysis of StAR product generated with cell cDNA or plasmid gave 2 peaks (iii) although a single band was observed by gel electrophoresis (i). Sequencing of the full-length StAR plasmid, which generates 2 peaks, revealed a single sequence, which showed 100% identity to mouse StAR. Double peaks may be caused by a high guanine (G) and cytosine (C) content in an area of the product sequence, however this was not obvious on analysis of this sequence. Another possibility is the formation of secondary structures that may give rise to more than 1 melting peak. Mouse Y1 cDNA and mouse GAPDH cDNA were compared (ii) to show that there are no melting point differences between these standards. *[B] mRNA quantification.* (i) FSK (3 h, 1μM) caused an approximately 15-fold increase in the accumulation of StAR mRNA and the effects of FSK were significantly inhibited by the presence of PD (*P<0.05, n=3 separate experiments). (ii) In similar experiments, FSK caused an approximately 3-fold increase in $P450_{SCC}$ mRNA but this effect was not inhibited by the presence of PD.

Figure 5 - Effect of ERK2/1 inhibition on SF-1 phosphorylation levels

Y1 cells were incubated in (i) growth medium (ii), 1μM FSK and (iii) 1μM FSK+50μM PD for 3 h and protein fractions were prepared. The 53kDa SF-1 protein was isolated from these fractions by immunoprecipitation, transferred to a nitroceullose membrane and probed with an anti-phosphoserine antibody (Fig. 5[A]). The same membrane was stripped and re-probed with an anti-SF-1 antibody to normalise the results. Immunoreactive bands from both the total SF-1 and from the phosphorylated SF-1 only blots were quantified by densitometric scanning and

expressed as a percentage of basal SF-1 phosphorylation levels. Figure 5 [B] clearly demonstrates that FSK stimulates SF-1 phosphorylation and that this increase is reduced upon MEK inhibition (control: 100%, FSK: 732%, FSK+PD: 293%).

Figure 6 – Effects of ERK2/1 inhibition on SF-1 binding to the StAR gene promoter

Y1 cells were incubated in growth medium with or without 1μM FSK and 50μM PD for 3 h. EMSAs were performed to measure binding of SF-1 in nuclear extracts to each of three (SF-1-1, SF-1-2 and SF-1-3) radiolabeled probes of regions of the StAR promoter that contain consensus SF-1 binding sequences. In each case antibody supershift assays were performed in order to locate the position of the SF-1/DNA complex. Figure 6[A] shows an example of an antibody supershift assay using a probe to the SF-1-2 region of the StAR promoter. Figures 6 [A, B and C] show that FSK-stimulation caused an increase in binding of SF-1 to each of the three regions of the StAR promoter sequence. Furthermore, this FSK-stimulated increase was ERK-dependent since treatment with PD caused a depletion in binding. Results shown are representative of 2 separate experiments.

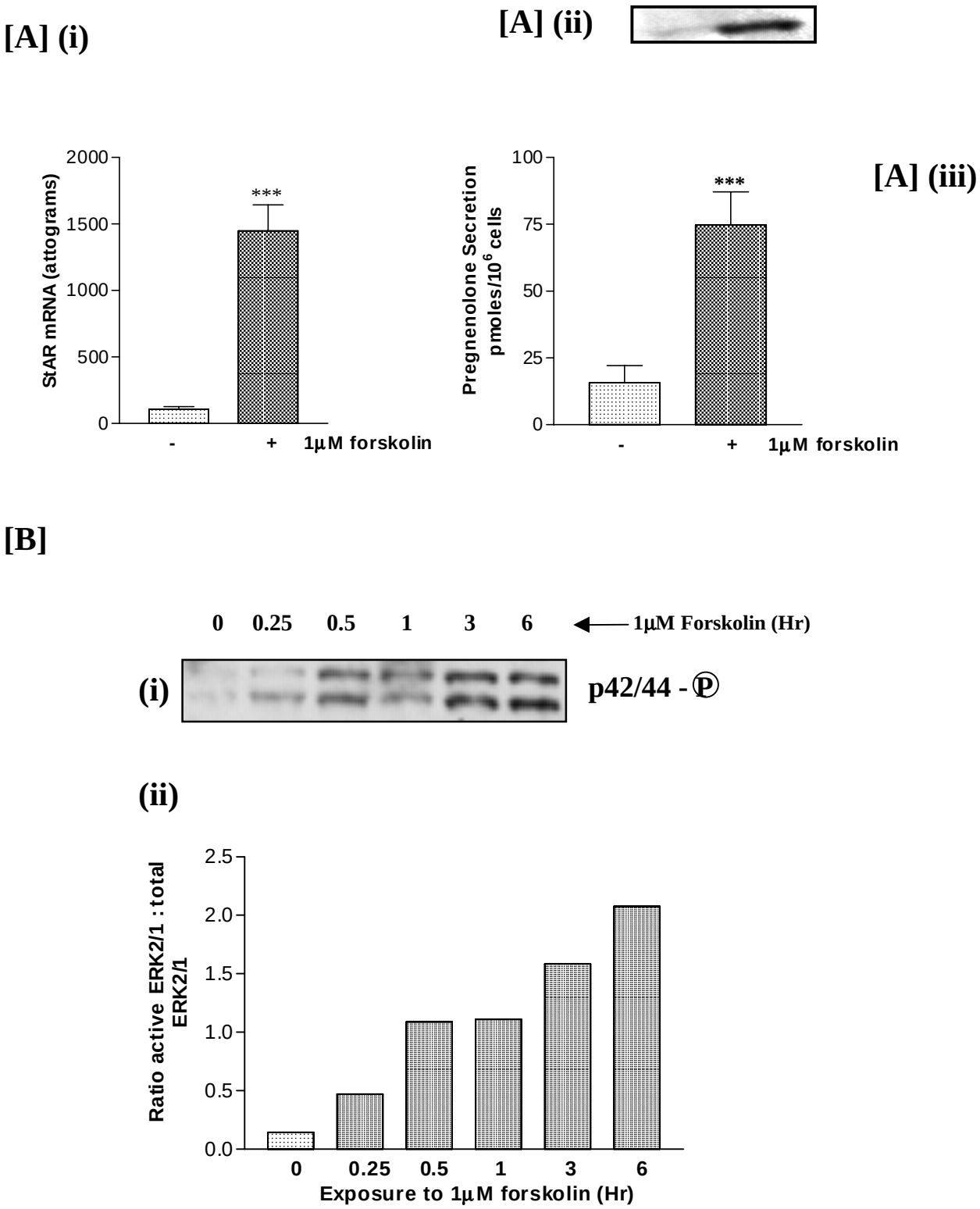


Figure 1: Effects of forskolin on Y1 cells.

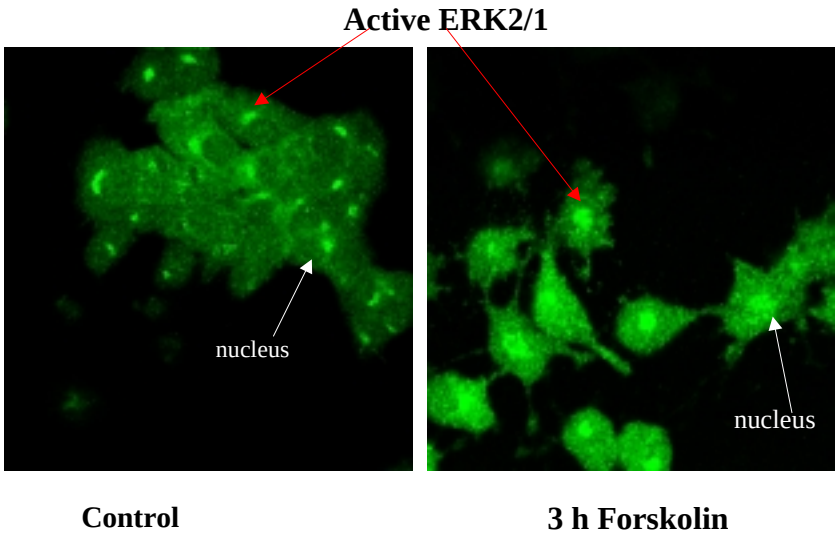


Figure 2. Effect of FSK on active ERK2/1 localisation in Y1 cells

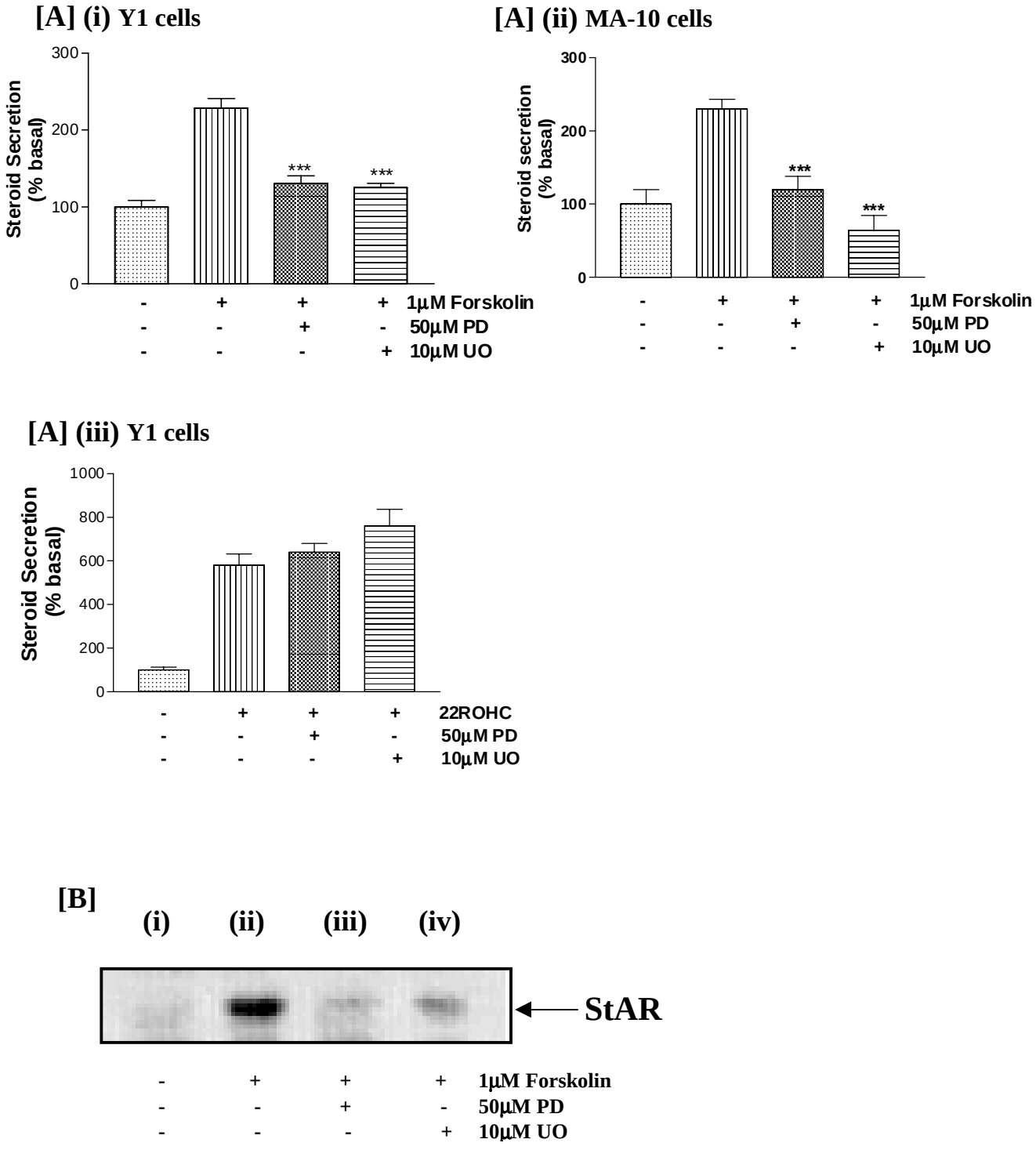
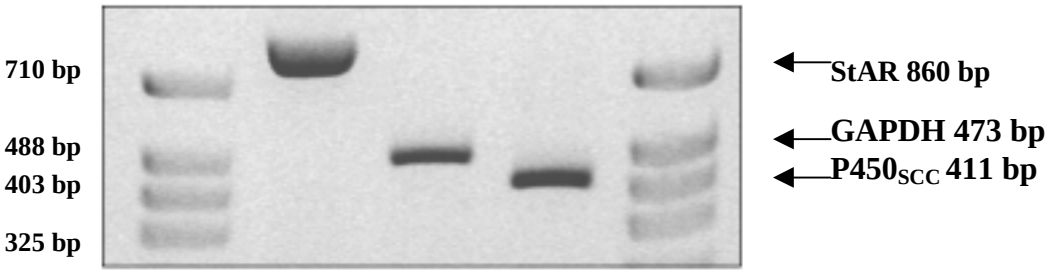
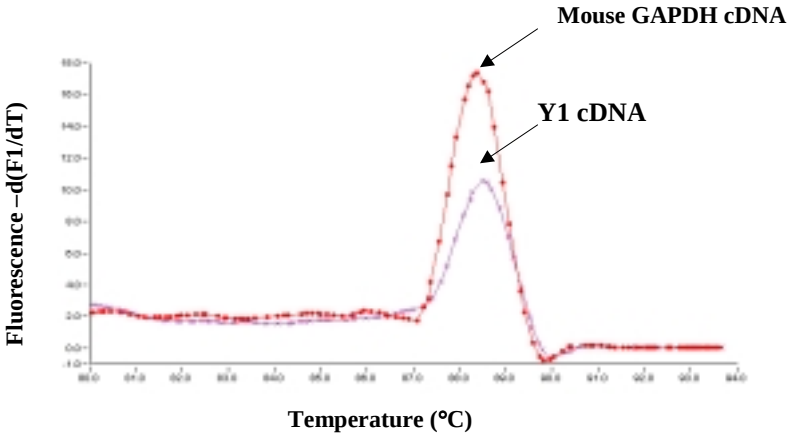


Figure 3: Effects of ERK2/1 inhibition on steroid synthesis and StAR protein.

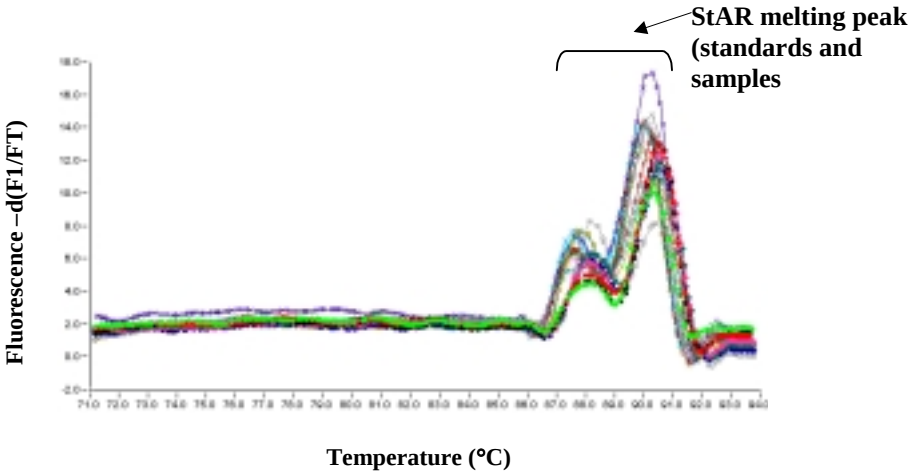
[A] (i)



(ii)



(iii)



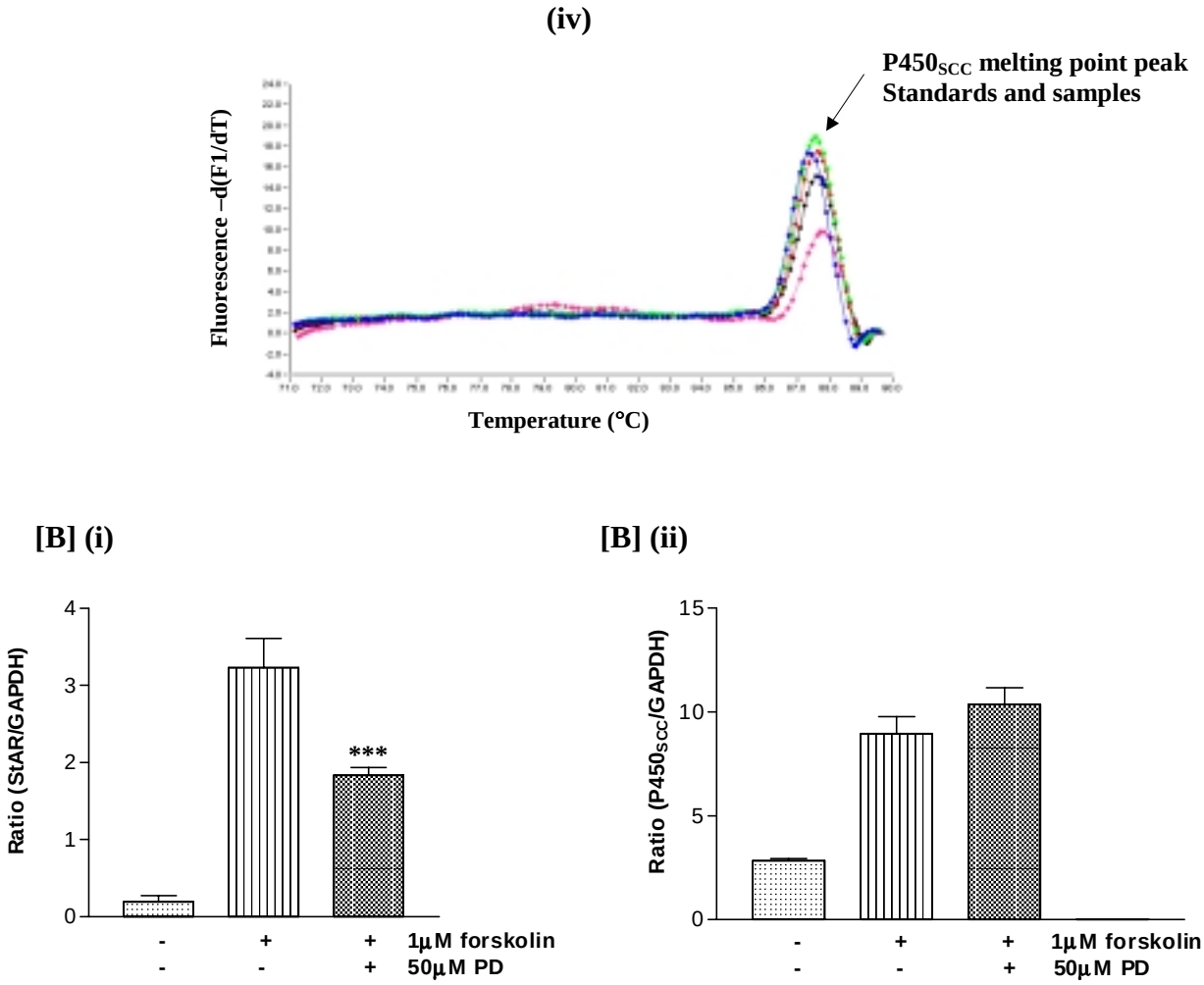


Figure 4. Effects of ERK2/1 inhibition on mRNA expression in Y1 cells.

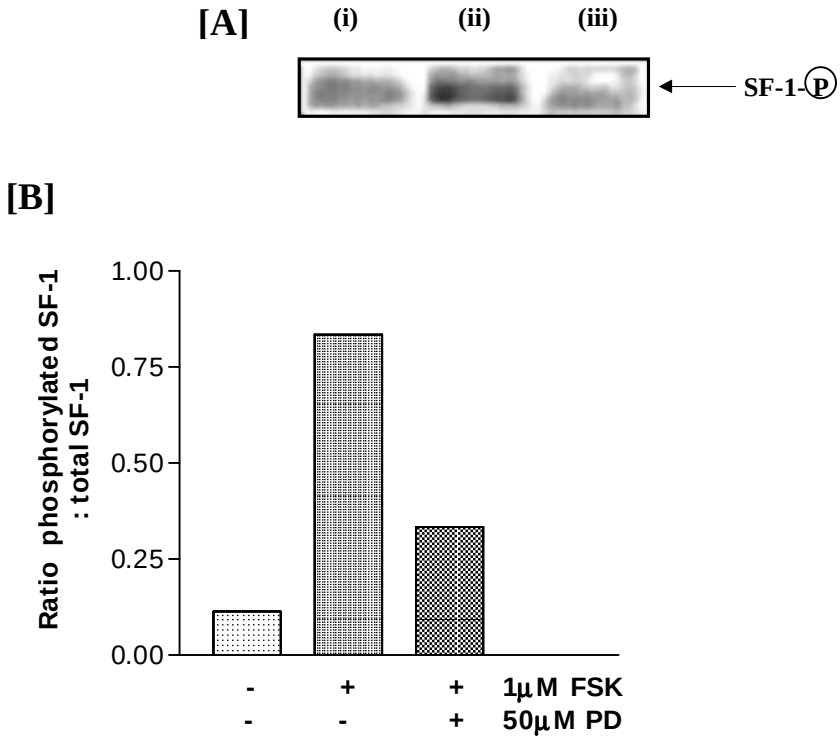


Figure 5. Effect of ERK2/1 inhibition on FSK-stimulated SF-1 phosphorylation

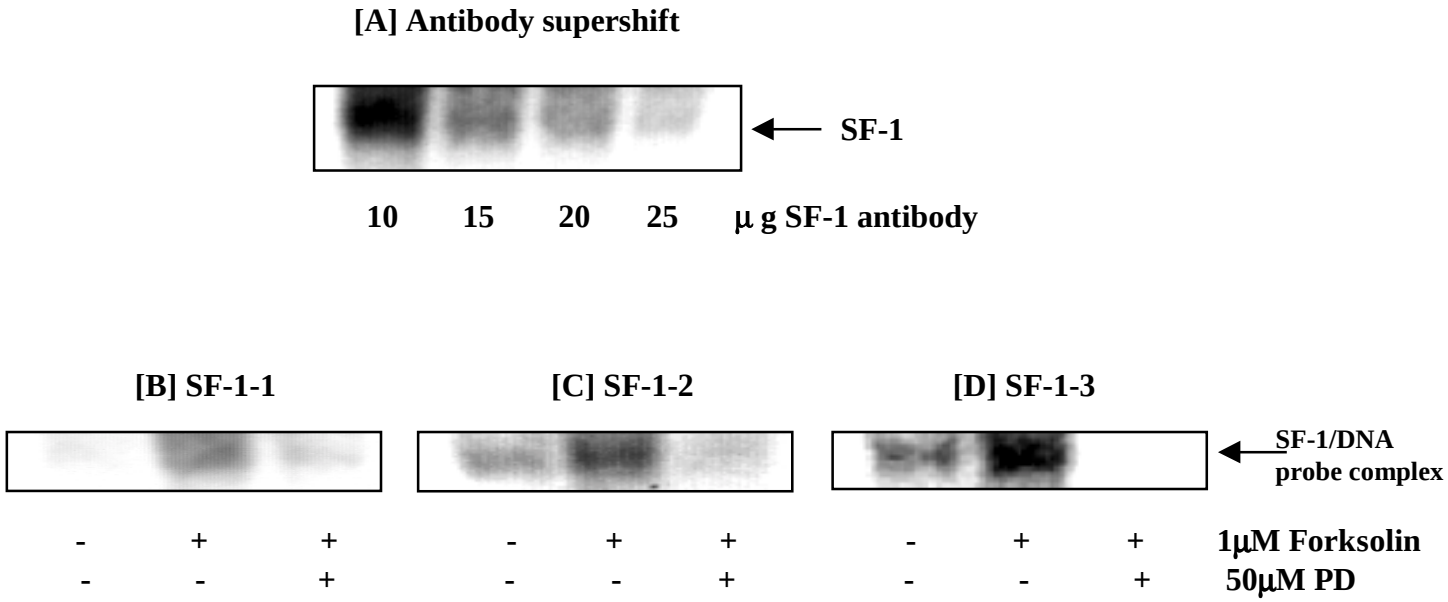


Figure 6 - Effect of ERK2/1 inhibition on SF-1 binding to the StAR promoter

ERKs regulate cyclic AMP-induced steroid synthesis through transcription of the steroidogenic acute regulatory (StAR) gene

Shân L. Gyles, Chris J. Burns, Barbara J. Whitehouse, David Sugden, Phil J. Marsh, Shanta J. Persaud and Peter M. Jones

J. Biol. Chem. published online June 15, 2001
