Regulation of Leydig cell steroidogenesis by extracellular signal-regulated kinase 1/2: role of protein kinase A and protein kinase C signaling

Pulak R Manna*, Youngah Jo* and Douglas M Stocco

Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, Texas 79430, USA

(Requests for offprints should be addressed to D M Stocco; Email: doug.stocco@ttuhsc.edu)

*(P R Manna and Y Jo contributed equally to this work)

Abstract

The steroidogenic acute regulatory (StAR) protein plays a central role in the regulation of steroid biosynthesis. While steroidogenesis is influenced by many processes, their modes of actions, in a few cases, remain obscure. In this study, we explored the mechanism of action of one such signaling pathway, the extracellular signal-regulated kinase 1/2 (ERK1/2), in regulating StAR expression and steroidogenesis in conjunction with the protein kinase A (PKA) and protein kinase C (PKC) pathways. Using MA-10 mouse Leydig tumor cells, we demonstrate that the activation of PKC and PKA signaling, by phorbol-12-myristate-13-acetate (PMA) and dibutyryl cAMP (dbcAMP)/human chorionic gonadotropin (hCG) respectively, was able to phosphorylate ERK1/2, an event markedly decreased by an upstream kinase inhibitor, U0126. Treatment with PMA enhanced StAR protein expression (associated with a slight increase in progesterone synthesis) but not its phosphorylation (P-StAR), which, in contrast, coordinately increased in response to dbcAMP/hCG. Inhibition of ERK1/2 activity by U0126

decreased PMA-treated StAR expression but increased dbcAMP/hCG-mediated StAR and P-StAR; however, progesterone levels were attenuated. U0126 was found to affect StAR expression and steroidogenesis both at the transcriptional and translational levels. Further studies demonstrated that the effect of U0126 on PMA- and dbcAMP/hCG-mediated StAR expression and steroid synthesis was tightly correlated with the expression of dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene 1 (*DAX-1*) and scavenger receptor class B type 1 (SR-B1). In fact, both *DAX-1* and SR-B1 appear to play important roles in hormone-regulated steroidogenesis. These findings clearly demonstrate that the ERK1/2 signaling cascade involved in regulating StAR expression and steroid synthesis is mediated by multiple factors and pathways and is stimulus specific in mouse Leydig cells.

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Introduction

Steroidogenesis in Leydig cells is predominantly mediated through the interaction of luteinizing hormone (LH)/human chorionic gonadotropin (hCG) with its specific receptor, which results in several intracellular modifications including the activation of cAMP-dependent protein kinase A (PKA) and phosphorylation of proteins (Cooke *et al.* 1992, Saez 1994, Ascoli *et al.* 2002, Stocco *et al.* 2005). The steroidogenic acute regulatory (StAR) protein regulates a key step in steroid biosynthesis, i.e. the delivery of cholesterol from the outer to the inner mitochondrial membrane (Clark *et al.* 1994, Lin *et al.* 1995, Stocco & Clark 1996). Compelling evidence regarding the crucial role of StAR in the regulation of steroid synthesis has been documented with both basic and clinical studies (Clark *et al.* 1994, Lin *et al.* 1995, Caron *et al.* 1997,

Hasegawa *et al.* 2000). Transcriptional or translational inhibition of StAR expression results in a dramatic decrease in steroid biosynthesis, whereas ≈ 10 –15% of steroid synthesis appears to be mediated through StAR-independent mechanisms (Lin *et al.* 1995, Stocco & Clark 1996, Clark *et al.* 1997, Manna *et al.* 2001). Two putative PKA phosphorylation sites have been identified in the mouse StAR protein at Ser56 and Ser194, and mutations (Ser $\rightarrow$ Ala) in these sites demonstrated the importance of Ser194 in the biological activity of StAR in steroid synthesis (Arakane *et al.* 1997).

Whereas the cAMP/PKA pathway is unquestionably the major signaling cascade for tropic hormone-induced StAR expression and steroidogenesis, PKC has also been shown to be involved in these processes (Jo *et al.* 2005, Stocco *et al.* 2005, Manna *et al.* 2006a, Renlund *et al.* 2006). We have recently reported that the PKC signaling pathway plays an

integral role in regulating the steroidogenic response in Leydig cells. While PKC-dependent induction of steroid synthesis is low when compared with that observed with PKA signaling, it is capable of potentiating gonadotropin and/or cAMP-stimulated steroidogenic responsiveness (Jo *et al.* 2005, Stocco *et al.* 2005, Manna *et al.* 2006a).

DAX-1 (also called Nr0b1 or Ahch), an orphan member of the nuclear hormone receptor superfamily of transcription factors, is expressed in the hypothalamic–pituitary–adrenal/gonadal axis and has been demonstrated to have roles in the regulation of steroidogenesis (Zazopoulos *et al.* 1997, Lalli *et al.* 1998, Osman *et al.* 2002, Tajima *et al.* 2003, Jo & Stocco 2004, Manna *et al.* 2006a) as well as in the gonadal development and function (Yu *et al.* 1998, Meeks *et al.* 2003a,b, Niakan & McCabe 2005). Targeted disruption of DAX-1 in mice results in testicular dysgenesis, which is manifested by decreased testicular size and decreased spermatogenesis, impaired Sertoli and Leydig cell function, and infertility (Yu *et al.* 1998, Jeffs *et al.* 2001, Meeks *et al.* 2003a,b). Another protein considered in the present studies was SR-B1, a high-density lipoprotein (HDL) receptor that binds various lipoprotein particles, facilitates HDL-derived cellular cholesterol influx, and thus replenishes cholesterol stores for steroid hormone biosynthesis (Ji *et al.* 1997, Azhar *et al.* 1998, Fidge 1999). In previous studies, both *in vivo* and *in vitro* approaches have demonstrated a striking correlation between the hormonal induction of SR-B1 expression (accompanied by enhanced lipid uptake from HDL) and steroid biosynthesis in steroidogenic cells (Landschulz *et al.* 1996, Rigotti *et al.* 1996, Azhar *et al.* 1998, Sun *et al.* 1999, Manna *et al.* 2006b).

The mitogen activated protein kinase (MAPK) signaling cascade has been implicated in the regulation of cell cycle progression, is used as a biochemical marker in evaluating mitogenic effects of a variety of stimuli, and is a point of convergence for diverse signaling pathways (Seger & Krebs 1995, Lewis *et al.* 1998, Le & Schimmer 2001, Manna *et al.* 2006b). Extracellular signal-regulated kinase 1/2 (ERK1/2), a member of the family of MAPKs, has been shown to be involved in StAR expression and steroid biosynthesis. However, its mode of action is poorly understood as a consequence of conflicting reports demonstrating stimulation, inhibition, or no effect in different steroidogenic cells (Gyles *et al.* 2001, Seger *et al.* 2001, Martinelle *et al.* 2004, Martinat *et al.* 2005, Tajima *et al.* 2005, Manna *et al.* 2006b). Despite the importance of the PKA and PKC signaling pathways in steroidogenesis, their precise roles in the regulation of MAPK/ERK-mediated steroidogenic responsiveness remain elusive. Utilizing MA-10 mouse Leydig tumor cells (that possess many characteristics of normal Leydig cells including low levels of LH receptors) as an experimental model, the present studies were aimed at elucidating these roles and demonstrated that ERK1/2 signaling has a variety of effects on the regulation of StAR expression and steroidogenesis in Leydig cells that are dependent on the specific experimental conditions and stimuli utilized.

Materials and Methods

Reagents

Phorbol-12-myristate-13-acetate (PMA), dibutyryl cyclic AMP (dbcAMP), hCG, and H89 were purchased from Sigma. GF-10923X (GFX) was obtained from AG Scientific (San Diego, CA, USA). U0126 and ERK1/2 antibody were purchased from Cell Signaling (Beverly, MA, USA). Phosphorylated ERK1/2 monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and treatments

MA-10 mouse Leydig tumor cells (Ascoli 1981) were grown in Waymouth MB 752/1 medium supplemented with 15% horse serum (Invitrogen) and antibiotics, as described previously (Manna *et al.* 2004a, Jo *et al.* 2005). Prior to experiments, cells were washed with 0.01 M PBS and incubated with or without additives (stimulators, inhibitors, etc.), which were freshly prepared and applied to the cells. Media were collected from different treatment groups and the accumulation of progesterone (increased several hundredfold over basal in response to tropic hormone or cAMP analog in 4–6 h) was determined (Manna *et al.* 2002b, Rao *et al.* 2003, Jo *et al.* 2005, Manna *et al.* 2006b). Some caution should be taken in measuring progesterone as the sole product of steroidogenesis in MA-10 cells, as a previous study has demonstrated that additional steroid metabolites are also present in these cells (Rommerts *et al.* 2001). However, the levels of such metabolites are low when compared with progesterone production in MA-10 cells.

Western blot analysis

Following the experiments, cells were washed, homogenized in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, and 1% NP-40), centrifuged at 12 000 g for 10 min, and the supernatant was assayed for total protein (Jo & Stocco 2004, Manna *et al.* 2006b). Equal amounts of protein were loaded on 10% SDS-PAGE gels and following electrophoresis were transferred to methanol-activated polyvinylidene difluoride membranes. Membranes were probed with specific antibodies that recognize phosphorylated (P)-ERK1/2, total ERK1/2, DAX-1 (Tamai *et al.* 1996; a gift from Dr Paolo Sassone-Corsi, University Louis Pasteur, Strasbourg, France), P-StAR (Manna *et al.* 2006b; a gift from Dr Steven R King, Baylor College of Medicine, Houston, TX, USA), and total StAR (Bose *et al.* 1999; a gift from Dr Walter L Miller, University of California, San Francisco, CA, USA). The antibody detecting P-StAR was generated against amino acids 190–199 of mouse StAR with phosphorylated Ser194. Horseradish peroxidase-conjugated secondary antibodies against rabbit, mouse, or goat IgG were used to recognize relevant primary antibodies.

Immunodetection of different proteins was determined by chemiluminescence following exposure to X-ray film (Marsh Bio Products, Inc., Rochester, NY, USA), and the immunoblots were quantified using a computer-assisted image analyzer (Visage 2000, BioImage Corp., Ann Arbor, MI, USA). Expression of phosphorylated and total proteins was assessed using identically processed membranes; however, where appropriate, the same membranes were also analyzed by stripping and reprobing.

Quantitative reverse transcription and PCR (RT-PCR)

Total RNA was isolated from different treatment groups using Trizol reagent (Invitrogen). RT-PCR was run sequentially in the same assay tube using 2 µg of total RNA, as described previously (Manna *et al.* 1999a, 2002b, 2004b). Isolation and amplification of StAR and SR-B1 cDNAs were carried out utilizing the following primer pairs: StAR sense, 5'-GACCTTGAAAGGCTCAGGAAGAAC-3' and StAR antisense, 5'-TAGCTGAAGATGGACAGACTTGC-3'; SR-B1 sense, 5'-CTCATCAAGCAGCAGGTGCTCAAGAA-3' and SR-B1 antisense, 5'-GGTGGGTAGACG-GACCCGTTGGCAAACA-3'. The variation in RT-PCR efficiency was assessed using L19 ribosomal protein gene using the sense primer 5'-GAAATCGCCAATGCCAACTC-3' and the antisense primer 5'-TCTTAGACCTGCGAGCCTCA-3'. The cDNAs generated were further amplified by PCR using the primer pairs listed above. The parameters used were optimized to be in the exponential phase of the PCR. The

molecular sizes of StAR, SR-B1, and L19 were determined on 1.2% agarose gels. Gels were vacuum dried, exposed to X-ray film (Marsh Bio Products) for 1–3 h, and the resulting signals were quantified (Visage 2000). Integrated optical density (IOD) values of each StAR band were quantified and normalized with the corresponding L19 bands.

Statistical analysis

All experiments were repeated at least three times. Data were analyzed by either Student's *t*-test or ANOVA followed by Fisher's least significant difference tests using the StatView program (Abacus Concepts, Inc., Berkeley, CA, USA). Data were expressed as the mean $\pm$ S.E.M. and the $P < 0.05$ values were considered significant.

Results

Roles of the PKC and PKA pathways in ERK1/2 signaling and their relevance to StAR expression and progesterone synthesis

The mechanism of the ERK1/2 signaling cascade in the regulation of StAR expression and steroidogenesis was studied in MA-10 cells. Cells were treated with PMA and dbcAMP in order to activate the PKC and PKA pathways respectively, and phosphorylation and expression of ERK1/2 were determined. Phosphorylation of ERK1/2 (P-ERK1/2), following PMA treatment increased by 3 min, was maximal between 10 and 120 min (~ 4.6 -fold) and decreased by

Figure 1 Effects of PMA and dbcAMP on phosphorylation and expression of ERK1/2 in MA-10 cells. Cells were treated with 10 nM PMA (A) and 0.5 mM dbcAMP (B) for different time periods (0–180 min). P-ERK1/2 and ERK1/2 in total cell lysates (22–25 µg protein) were determined by western blot analysis. Cells were also pretreated with the ERK1/2 inhibitor, U0126 (10 µM), for 30 min treated with PMA and dbcAMP for an additional 30 min as indicated, and P-ERK1/2 and ERK1/2 were determined. Immunoblots are representative of three independent experiments.

180 min, the longest time tested (Fig. 1A). Also, dbcAMP (0.5 mM) resulted in an increase in P-ERK1/2 by 3 min ($P<0.05$) and a 5.8 ± 0.9 -fold increase by 30 min, an induction that was sustained for 180 min (Fig. 1B). The total amount of ERK1/2 protein was unaltered in these groups. Pretreatment with a MAPK/ERK inhibitor, U0126 (10 μ M), for 30 min with either PMA or dbcAMP markedly inhibited P-ERK1/2 levels at a concentration shown to inhibit MAPK/ERK activity in mouse Leydig and adrenal cells (Gyles *et al.* 2001, Manna *et al.* 2002a, 2006a). These results demonstrate that activation of ERK1/2 by PMA and dbcAMP involves protein phosphorylation rather than protein synthesis.

The relevance of PMA- and dbcAMP-stimulated ERK1/2 signaling on StAR expression and steroid synthesis was determined. As illustrated in Fig. 2, U0126 was capable of increasing ($P<0.05$) StAR protein expression by 6 h but not its phosphorylation (P-StAR). PMA (10 nM) resulted in a 5.2 ± 1.4 -fold elevation in StAR expression but not P-StAR,

and a slight increase (3.2 ± 0.8 -fold) in progesterone synthesis when compared with controls. Addition of 0.05 mM dbcAMP (a dose incapable of increasing StAR expression and steroid synthesis by itself (Jo *et al.* 2005)) to PMA-treated cells dramatically elevated StAR, P-StAR, and progesterone to levels that are quantitatively similar to those seen with 0.5 mM dbcAMP (Fig. 2A and B). Notably, while U0126 decreased PMA and PMA plus dbcAMP-stimulated StAR and P-StAR, it increased ($P<0.05$) dbcAMP-mediated responses (Fig. 2A). However, progesterone levels in response to these stimuli were significantly decreased ($P<0.01$) by U0126. This inhibitor also decreased basal secretion of progesterone by 44% (control, 1.58 ± 0.21 vs U0126, 0.87 ± 0.14 ng/mg protein). U0126 had no apparent effect on 22R-hydroxycholesterol (22R-OHC, 25 μ M)-mediated progesterone synthesis, indicating that the cytochrome P450 side-chain cleavage (P450_{scc}) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) enzyme activities were not affected (Fig. 2B). These findings suggest that activation of ERK1/2 via PKC and PKA signaling influences StAR expression and steroid synthesis differently without affecting the activity of P450_{scc} and 3 β -HSD.

The specificity of PMA and dbcAMP effects on ERK1/2 signaling involved in StAR expression and steroid synthesis was assessed using inhibitors of the PKC (GFX) and PKA (H89) pathways. MA-10 cells treated with PMA (10 nM) and dbcAMP (0.5 mM) for 30 min activated ($P<0.01$) P-ERK1/2 levels, which were strongly decreased by GFX (20 μ M) and H89 (20 μ M) respectively (Fig. 3A). The concentrations of GFX and H89 used were based on previous studies (Jo *et al.* 2005, Manna *et al.* 2006b). Total ERK1/2 protein levels were unaltered by any of these inhibitors. The results summarized in Fig. 3B demonstrate that treatment with PMA and dbcAMP for 6 h resulted in 5.4 ± 1.2 - and 8.7 ± 1.9 -fold increases in StAR mRNA levels over untreated cells respectively. Inclusion of GFX and H89 in these treatments decreased PMA- and dbcAMP-induced StAR mRNA expression by 68 and 74% respectively. Treatment with PMA and dbcAMP increased progesterone synthesis over basal levels by 3.6- and 337 ± 29 -fold respectively. GFX and H89 decreased PMA- and dbcAMP-treated progesterone synthesis by 70 and 85% respectively.

Inhibition of ERK1/2 by U0126 was found to induce StAR expression but P-StAR was undetectable. Detection of P-StAR can be seen only in the presence of 0.1 mM dbcAMP. Both StAR and P-StAR were increased in a dose-dependent manner when U0126 and 0.1 mM dbcAMP were used in combination (Fig. 4A). Notably, while U0126 increased dbcAMP (0.1–0.5 mM)-stimulated StAR and P-StAR, it decreased progesterone synthesis approximately by 50% (Fig. 4B), an observation in agreement with previous studies (Renlund *et al.* 2006). By contrast, U0126 markedly inhibited PMA and PMA plus 0.05 mM dbcAMP-stimulated StAR/P-StAR and progesterone levels, demonstrating U0126 had effects on StAR expression and steroid synthesis in stimulus-specific manners.

Figure 2 Role of U0126 on PMA, PMA plus dbcAMP, and dbcAMP-stimulated StAR, P-StAR, and progesterone levels in MA-10 cells. Cells were pretreated with U0126 (10 μ M) for 30 min and then stimulated with PMA (10 nM), PMA plus 0.05 mM dbcAMP or 0.5 mM dbcAMP alone for 6 h. Cell lysates (22 μ g protein) were analyzed for StAR expression and P-StAR by western blot analysis. (A) Representative immunoblot illustrates the levels of StAR and P-StAR in different treatment groups. (B) Cells were also treated with 22R-OHC (25 μ M) in the presence or absence of U0126, and progesterone levels in the media were determined and expressed as ng/mg protein. Data represent the mean $\pm$ S.E.M. of four independent experiments, * $P<0.05$; $^{\dagger}P<0.01$; $^{\ddagger}P<0.001$.

Figure 3 Role of pathway-specific inhibitors on ERK1/2 signaling and its relevance to StAR expression and progesterone synthesis. (A) MA-10 cells were pretreated with inhibitors of PKC (GFX, 20 μ M) and PKA (H89, 20 μ M) for 30 min, and then treated with 10 nM PMA and 0.5 mM dbcAMP for an additional 30 min. Then P-ERK1/2 and ERK1/2 in cell lysates were determined using 20 μ g protein. Results shown are representative of three independent experiments. (B) Cells were pretreated for 30 min with the inhibitors as indicated, incubated for an additional 6 h with PMA and dbcAMP, and subjected to RNA extraction for RT-PCR analysis of StAR and L19 mRNA expression. A representative autoradiogram shows expression of StAR mRNA by RT-PCR analysis using 2 μ g total RNA. Integrated optical density (IOD) values of each StAR band were quantified and normalized with the corresponding L19 bands. Progesterone levels in the media of the same treatment groups were determined ($\pm$ S.E.M., $n = 3-4$). Note the different scales on both sides.

Since MA-10 cells possess functional LH/hCG receptors, the role of hCG on ERK1/2 signaling and their relevance to StAR expression and progesterone synthesis were investigated. Cells treated with hCG (100 ng/ml) for 0–180 min

demonstrated increases in P-ERK1/2 by 3 min ($P < 0.01$), reached maximal levels between 5 and 10 min, and slightly decreased by 180 min (Fig. 5A). U0126 abolished hCG-stimulated P-ERK1/2 when compared with the value at 10 min. The amount of ERK1/2 was unchanged during this time course. Also, hCG was capable of inducing StAR expression (4.8 ± 1.5 -fold) and P-StAR (3.5 ± 1.2 -fold) over their respective controls. Both GFX and H89 decreased hCG-stimulated StAR and P-StAR by 80–95%, and as a result progesterone levels were markedly attenuated (Fig. 5B). On the other hand, U0126 resulted in moderate but consistent increases (1.6- to 2.2-fold) in hCG-stimulated StAR and P-StAR, but significantly decreased ($P < 0.01$) progesterone synthesis.

Role of U0126 in StAR transcription and translation

To determine whether U0126 mediates the hormone-stimulated steroidogenic response at the level of transcription and/or translation, the effects of actinomycin D (Act, 4 μ M) and cycloheximide (Chx, 5 μ M) on dbcAMP-stimulated MA-10 cells were determined (Fig. 6). The increase in U0126-mediated StAR expression was repressed ($P < 0.05$) by both Act and Chx, indicating U0126 induced StAR expression both at the transcriptional and translational levels (Fig. 6A). Act and Chx also diminished basal progesterone synthesis between 37 and 45%. Furthermore, Act and Chx decreased ($P < 0.01$) dbcAMP (0.3 mM)-stimulated StAR, P-StAR, and progesterone synthesis (Fig. 6B). The effect of U0126 on dbcAMP-stimulated elevations in StAR and P-StAR was decreased by 86 and 65% with Act and 50 and 27% with Chx respectively. Furthermore, U0126 suppressed ($P < 0.05$) dbcAMP-stimulated progesterone production, a response further attenuated in the presence of both Act and Chx.

ERK1/2 signaling and its relevance to PMA- and dbcAMP/hCG-mediated expression of DAX-1, SR-B1, StAR, and progesterone synthesis

To obtain more insight into these mechanisms, the role of ERK1/2 signaling on PMA-, dbcAMP-, and hCG-mediated expression of DAX-1 and SR-B1 was studied and their relationship to StAR expression and progesterone synthesis was assessed. In MA-10 cells, the constitutive expression of DAX-1 was decreased to 42% by U0126 (10 μ M), an event that increased expression of the StAR protein. Treatment with PMA, dbcAMP, and hCG demonstrated ≈ 68 , 45, and 47% decreases in DAX-1 protein levels respectively, observations inversely correlated with StAR expression (Fig. 7A and B). Progesterone levels in response to these stimuli were inhibited by U0126 (Fig. 7C). While U0126 reversed the inhibition of PMA-treated DAX-1 expression, it further attenuated dbcAMP ($\sim 70\%$)- and hCG ($\sim 52\%$)-mediated DAX-1 protein levels.

Figure 4 Effect of U0126 on PMA- and dbcAMP-stimulated StAR, P-StAR, and progesterone synthesis in MA-10 cells. (A) Cells were pretreated with increasing doses of U0126 (0–50 μM) for 30 min, incubated with or without 0.1 mM dbcAMP for an additional 6 h and StAR and P-StAR were determined by western blot analyses. (B) Cells were also treated with 10 nM PMA, PMA plus 0.05 mM dbcAMP, or 0.1–0.5 mM dbcAMP in the presence or absence of U0126 (10 μM) and StAR and P-StAR were determined. Expression of StAR protein is illustrated following both short and long exposures. Representative immunoblots illustrate StAR and P-StAR in different treatment groups. The accumulation of progesterone in the same treatment groups was determined. Data represent the mean $\pm$ s.e.m. of three independent experiments using triplicate samples.

The differential effects of U0126 on StAR expression and steroid synthesis mediated by PMA and dbcAMP/hCG prompted us to study the role of SR-B1, an HDL receptor involved in providing cholesterol for steroidogenesis (Manna *et al.* 2006a,b). MA-10 cells treated with PMA, dbcAMP, and hCG resulted in 5.8 ± 1.6 -, 18.2 ± 3.6 -, and 11 ± 2.3 -fold increases in SR-B1 mRNA expression over basal levels

respectively (Fig. 8). Addition of U0126 to these treatments repressed SR-B1 mRNA expression between 40 and 55%, while at the same time progesterone levels were also significantly decreased ($P < 0.05$). Thus, the suppression of progesterone synthesis by U0126 could be, at least in part, due to inhibition of SR-B1 expression resulting in an insufficient uptake of cholesterol into these cells.

Figure 5 Effect of hCG on phosphorylation and expression of ERK1/2 and its relevance to PKC- and PKA-mediated StAR expression and steroid synthesis. (A) MA-10 cells were stimulated with 100 ng/ml hCG for different time periods (0–180 min), and cell lysates (24 μg protein) were analyzed for P-ERK1/2 and ERK1/2 by western blot analyses. (B) Cells were pretreated with two different doses (10 and 20 μM) of GFX, H89, and U0126 for 30 min and then incubated with 100 ng/ml hCG for an additional 6 h. Cell lysates were analyzed for StAR and P-StAR. Immunoblots are representative of three independent experiments having similar results. Progesterone levels in the media of the same treatment groups were determined (B). Data represent the mean $\pm$ S.E.M. of three independent experiments. * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$ vs hCG response.

Discussion

The MAPK/ERK signaling cascade has been demonstrated to regulate StAR expression and steroid biosynthesis. However, several contradictions to this observation have been reported in different steroidogenic tissues (Gyles *et al.* 2001, Seger *et al.* 2001, Dewi *et al.* 2002, Seto-Young *et al.* 2003, Martinelle *et al.* 2004, Martinat *et al.* 2005, Tajima *et al.* 2005, Manna *et al.* 2006b). For example, activation of ERK1/2 by hCG has been shown to increase StAR transcription and testosterone production in immature rat Leydig cells (Martinelle *et al.* 2004). Conversely, inhibition of ERK1/2 enhances LH- and FSH-mediated StAR expression and steroid synthesis in rat granulosa cells (Seger *et al.* 2001, Amsterdam *et al.* 2003).

In rat primary and mLTC-1 mouse Leydig tumor cells, ERK1/2 inhibition was shown to increase LH-, insulin-like growth factor-I-, and transforming growth factor- α -mediated StAR expression but decreased steroid synthesis (Martinat *et al.* 2005, Manna *et al.* 2006a,b). Also, ERK1/2 signaling resulted in differential effects on LH-induced StAR and CYP17 levels, producing an increase in progesterone but a decrease in androsterone synthesis in bovine theca cells (Tajima *et al.* 2005). In granulosa lutein cells, while inhibition of ERK1/2 had no effect on LH/hCG-induced steroidogenesis, it decreased (Bu)₂cAMP-, cholera toxin-, and forskolin-stimulated progesterone levels (Dewi *et al.* 2002). The present studies extend these observations by elucidating the molecular events in which the ERK1/2 cascade (in correlation with PKA and PKC signaling) acts to drive StAR expression and, thus, steroid biosynthesis in mouse Leydig cells.

Our findings demonstrate that both the PKC (activated by PMA) and PKA (activated by dbcAMP/hCG) signaling pathways are capable of rapidly phosphorylating ERK1/2. Selective inhibition of these pathways using specific inhibitors markedly repressed ERK1/2 phosphorylation and had a variety of effects on StAR expression and steroidogenesis. In previous studies, we examined the effect of several doses of these inhibitors and found them to have no adverse effects on cell viability or cellular DNA synthesis when compared with control Leydig cells (Manna *et al.* 2001, 2002a, Rao *et al.* 2003, Jo *et al.* 2005, Manna *et al.* 2006a). In addition, these inhibitors have also been widely used for blocking relevant signaling pathways in other steroidogenic cells (Gyles *et al.* 2001, Seger *et al.* 2001, Martinelle *et al.* 2004). Interestingly, inhibition of ERK1/2 by U0126 decreased PMA-stimulated StAR expression but increased dbcAMP/hCG responsiveness. However, progesterone levels were decreased by these treatments. The decrease in progesterone synthesis was not associated with inhibition of P450_{scc} and/or 3 β -HSD, as U0126 had no effect on 22R-OHC-mediated progesterone production. Consistent with this, ERK1/2 inhibition has been demonstrated to decrease forskolin-stimulated pregnenolone synthesis in MA-10 cells, but in those studies the expression of the StAR protein was not determined (Gyles *et al.* 2001). In human LH receptor expressing MA-10 cells, hCG was able to increase the PKA-mediated phosphorylation of Ras and ERK1/2 (Hirakawa & Ascoli 2003). Also, the effect of hCG on P-ERK1/2 was diminished by the inhibition of PKC and PKA responsiveness in immature rat Leydig cells (Martinelle *et al.* 2004). These observations indicate the complexity of the role of the ERK1/2 signaling cascade, which could be dependent on receptor-effector coupling and might involve signaling crosstalk and/or the involvement of additional factor(s) in the regulation of the steroidogenic response.

The regulation of StAR expression and steroidogenesis involves multiple second messenger systems including the PKA (Balasubramanian *et al.* 1997, Manna *et al.* 1999b, Sekar *et al.* 2000, Gyles *et al.* 2001, Martinat *et al.* 2005, Manna *et al.* 2006a) and PKC (Fiedler *et al.* 1999, Jo *et al.* 2005, Manna

Figure 6 Role of U0126 on StAR transcription/translation and progesterone production in MA-10 cells. (A) Cells were pretreated with either actinomycin D (Act, 4 μ M) or cycloheximide (Chx, 5 μ M) for 30 min, incubated with or without U0126 (10 μ M) for a further 30 min, and then stimulated for an additional 6 h in the presence or absence of 0.3 mM dbcAMP. Cell lysates (22 μ g protein) were analyzed for StAR and P-StAR by western blot analyses. Similar results were obtained from three independent experiments, and integrated optical density (IOD) values from representative immunoblots are given. (B) Accumulation of progesterone in the media was determined. Data represent the mean $\pm$ S.E.M. of three independent experiments.

et al. 2006a,b, Renlund *et al.* 2006) pathways. It is noted, however, that the effect of PKC signaling on steroid synthesis is far smaller than that of PKA signaling, usually being 1% of that seen with PKA activation, and the inability of PKC to phosphorylate StAR has been demonstrated to be a critical event for this dramatic difference (Jo *et al.* 2005, Manna *et al.* 2006a). Our present data corroborate previous findings which indicated that both the PKA and PKC pathways are involved in regulating StAR expression and steroidogenesis (Jo *et al.* 2005, Martinat *et al.* 2005, Manna *et al.* 2006a,b), and demonstrated that inhibition of ERK1/2 by U0126 elevated StAR protein expression but not its phosphorylation. Activation of PKC by PMA markedly increased the expression of the StAR protein but not P-StAR, thus producing inactive StAR and resulting in only a modest increase in progesterone synthesis. Increasing the PKA activity with a low dose of dbcAMP (one that is ineffective in increasing the steroidogenic response) in PMA-treated cells dramatically enhanced P-StAR and progesterone levels. While ERK1/2 inhibition resulted in decreases in PMA

and PMA plus dbcAMP-stimulated StAR/P-StAR and progesterone levels, it produced increases in dbcAMP/hCG-stimulated StAR expression and P-StAR, but diminished progesterone synthesis. These results are in support of the previous findings demonstrating that ERK1/2 inhibition increased interleukin-1 α (IL-1 α)-stimulated StAR and P-StAR but decreased androgen synthesis in rat Leydig cells (Renlund *et al.* 2006). Thus, it appears that ERK1/2 signaling plays a discrete role in StAR expression and steroid synthesis in pathway- and/or stimulus-specific manners in mouse Leydig cells.

In view of our present findings, it appears that the transcription factor DAX-1, which plays an important role in regulating StAR expression through the PKA pathway, is also regulated by PKC signaling. In addition to its role in the development of the adrenal and gonads, DAX-1 has been demonstrated to negatively regulate StAR expression and steroid synthesis both in Leydig and adrenal cells (Zazopoulos *et al.* 1997, Lalli *et al.* 1998, Manna *et al.* 1999a,b, Jo & Stocco 2004). In this study, we observed that inhibition of ERK1/2

Figure 7 Role of U0126 on PMA-, dbcAMP-, and hCG-stimulated DAX-1 expression and its relevance to StAR expression and steroid synthesis. MA-10 cells were pretreated with U0126 (10 μM) for 30 min and then incubated with 10 nM PMA, 0.5 mM dbcAMP, and 100 ng/ml hCG for an additional 6 h. Cell lysates (23–25 μg protein) were analyzed for DAX-1 and StAR protein expression by western blot analyses. (A) Representative immunoblots illustrate expression of DAX-1 and StAR proteins in different treatment groups. Expression of actin in different groups was determined as a loading control (lower panel). (B) IOD values of DAX-1 and StAR bands were quantified. (C) Accumulation of progesterone in the media of the same treatment groups was determined. Data represent the mean ± S.E.M. of three independent experiments.

was associated with a decrease in DAX-1 expression. While PMA, dbcAMP, and hCG increased StAR expression and progesterone synthesis, they inhibited expression of the DAX-1 levels. In support of this mechanism, an inverse relationship between DAX-1 and StAR expression has been reported in different steroidogenic cells (Tamai *et al.* 1996, Osman *et al.* 2002, Manna *et al.* 2006a,b). Furthermore, the inhibitory effect of PMA on DAX-1 expression could be reversed by U0126, which, in contrast, further diminished dbcAMP/hCG-mediated DAX-1 levels. Therefore, the

Figure 8 Effect of U0126 on PMA-, dbcAMP-, and hCG-stimulated SR-B1 mRNA expression and progesterone synthesis in MA-10 cells. (A) Cells were pretreated with U0126 for 30 min and then incubated with 10 nM PMA, 0.5 mM dbcAMP, and 100 ng/ml hCG for an additional 6 h, and the steady-state levels of SR-B1 mRNA were determined by RT-PCR analysis using 2 μg total RNA. A representative autoradiogram illustrates SR-B1 mRNA expression in different treatment groups. (B) Integrated optical density (IOD) values for each SR-B1 band were determined and normalized with the corresponding L19 bands. The accumulation of progesterone in the media following the same treatments was determined. Data represent the mean ± S.E.M. of four independent experiments. Note the different scales on both sides.

differential effects of U0126 on PKC- and PKA-mediated StAR expression might be explained, at least in part, by alterations in DAX-1 expression.

The present results also demonstrate the importance of SR-B1 in regulating steroid biosynthesis. SR-B1 is involved in providing cholesterol substrate for steroidogenesis in steroidogenic tissues. In MA-10 cells, we observed relatively low levels of SR-B1 mRNA by RT-PCR analysis. PMA and dbcAMP/hCG treatments were found to markedly increase SR-B1 mRNA expression, an event decreased by ERK1/2 inhibition, and are in agreement with our recent findings in mLTC-1 cells (Manna *et al.* 2006a,b). Previous studies have also documented that the expression of SR-B1 increases with hCG/ACTH and decreases with dexamethasone and is coordinately regulated with steroid synthesis in Leydig and adrenocortical cells (Landschulz *et al.* 1996, Rigotti

et al. 1996, Sun *et al.* 1999). In addition, the direct involvement of ACTH on the HDL-mediated cholesterol influx, the utilization of this cholesterol for steroid synthesis, and the induction of SR-B1 expression have been demonstrated in the adrenal gland (Rigotti *et al.* 1996). However, it has been shown that HDL binding to SR-B1 stimulates the ERK1/2 pathway following Ras activation (Grewal *et al.* 2003). Overall, it is conceivable that the decrease in progesterone synthesis in MA-10 cells caused by U0126 (even with elevated StAR) could be a result of a decreased uptake of cholesterol into cells due to the repression of SR-B1. Previously, U0126 has been shown to suppress IL-1 α -stimulated androgen production by dissipating the mitochondrial electrochemical potential in immature rat Leydig cells (Renlund *et al.* 2006). Hence, we cannot exclude the possibility that U0126 might inhibit PMA- and dbcAMP/hCG-stimulated progesterone production by affecting the integrity of the mitochondria.

Taken together, these findings provide insight into the mechanisms of ERK1/2 signaling in regulating PKC- and PKA-mediated StAR expression and steroidogenesis in mouse Leydig cells and illustrate the involvement of DAX-1 and SR-B1 in these processes. Thus, multiple and complex intracellular events appear to be involved in crosstalk among the different pathways and may function in regulating the steroidogenic response in Leydig cells. The exploration of the MAPK/ERK1/2 signaling pathway may help in understanding the mitogenic potential of a variety of hormones and growth factors in other steroidogenic cells.

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