Cyclic AMP-induced expression of steroidogenic acute regulatory protein is dependent upon phosphoprotein phosphatase activities

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ABSTRACT

In addition to the well-documented role of protein kinases in the regulation of steroid production, phosphoprotein phosphatase (PP) activity is required for steroidogenesis. In the present study, we have used the mouse Y1 adrenocortical cell line to identify the site of action of PPs on steroid production by measuring the effects of PP inhibition on the expression of the steroidogenic acute regulatory (StAR) protein and on steroid production. Forskolin-induced activation of cyclic AMP-dependent protein kinase (PKA) enhanced steroidogenesis and this was accompanied by an increased

expression of StAR protein. Both steroidogenesis and StAR protein expression were inhibited by two structurally dissimilar inhibitors of PP1 and PP2A activities, okadaic acid and calyculin A. These results suggest that inhibition of PP1 and PP2A inhibits steroid production by preventing the expression of the StAR protein, implicating PP1/2A dephosphorylation reactions as important regulators of stimulus-dependent StAR protein expression, and thus of steroidogenesis.

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INTRODUCTION

Agonist-dependent activation of the cyclic AMP-dependent protein kinase (PKA), and the consequent phosphorylation of cellular proteins is a critical stage in the stimulation of steroid production (see Orme-Johnson 1990, Schimmer 1995), although the identities and functions of the PKA substrates are still uncertain (Stocco & Clark 1996, Kim *et al.* 1997, Papadopoulos *et al.* 1997). PKA-mediated events are rapidly reversible through the actions of phosphoprotein phosphatases (PPs; Cohen 1989), and we have demonstrated that at least three of these enzymes, PP1, PP2A and PP2B, are expressed in primary luteal and adrenal cells (Ford *et al.* 1996, Sayed *et al.* 1997). PP1 and PP2A activities appear to be obligatory for stimulated steroid production since inhibitors of these enzymes have profound inhibitory effects on PKA-dependent steroidogenesis (Azhar *et al.* 1992, 1994, Abayasekara *et al.* 1996, Ford *et al.* 1996, Sayed *et al.* 1997). This action of PP inhibitors is

before the delivery of cholesterol to the inner mitochondrial membrane since there was no inhibition of steroid production from 22R-hydroxy cholesterol (22ROHC), which freely crosses the aqueous space between outer and inner mitochondrial membranes (Abayasekara *et al.* 1996, Sayed *et al.* 1997).

Delivery of cholesterol to the inner mitochondrial membrane is the rate-limiting step in steroidogenesis and evidence is accumulating that mitochondrial cholesterol transport is dependent on the expression of the steroidogenic acute regulatory (StAR) protein (Clark *et al.* 1994, Stocco & Clark 1996, Stocco 1997, 1998). For example, expression is restricted to steroidogenic tissues (Clark *et al.* 1995a) and correlates well with changes in steroid production (Nishikawa *et al.* 1996, Liu & Stocco 1997, Ramnath *et al.* 1997, Stocco 1997), experimental over-expression of StAR protein increases steroid production (Clark *et al.* 1994) and the reduced steroid production in congenital lipoid adrenal hyperplasia has been attributed to mutations in the

StAR protein gene (Lin *et al.* 1995). Furthermore, a number of studies have shown that pharmacological or receptor-operated activation of PKA stimulates steroid production by increased expression of the StAR protein (Clark *et al.* 1994, Liu *et al.* 1996, Balasubramanian *et al.* 1997, Pescador *et al.* 1996). In the present study, we have investigated whether StAR protein expression is also the site of regulation of steroidogenesis by PP1 and PP2A.

MATERIALS AND METHODS

Materials

Tissue culture reagents and plastics were from Gibco BRL (Paisley, Strathclyde, UK). Calyculin A (Cal A) and okadaic acid (OA) were from Calbiochem (Nottingham, Notts, UK). Polyclonal antisera against PP1 and PP2A, and a monoclonal antibody against PP2B were from TCS Biologicals (Botolph-Claydon, Bucks, UK). A polyclonal anti-serum against StAR protein was a kind gift from Professor Douglas Stocco (Texas-Tech University, TX, USA). Horseradish peroxidase-coupled goat anti-rabbit immunoglobulin G (IgG) and goat anti-mouse IgG were from Dako A/S (Glostrup, Denmark). Enhanced chemiluminescence (ECL) reagents, Hyperfilm and 1,2,6,7-[³H]progesterone for use in radioimmunoassay (RIA) were from Amersham International plc (Amersham, Bucks, UK). All other biochemicals were from the Sigma Chemical Company Ltd (Poole, Dorset, UK).

Cells

Mouse adrenocortical Y1 cells were obtained from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK), and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin and 10% (v/v) foetal bovine serum in a humidified atmosphere of 5% CO₂. Cell viability was assessed by microscopic assessment of Trypan blue exclusion after incubation in 0.1% (w/v) Trypan blue in DMEM for 15 min at 37 °C. Mitochondrial function was assessed using a tetrazolium assay to measure dehydrogenase activities, in which cells were incubated in the presence of 3-(4,5,-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml). The reaction was terminated by the addition of acidified propanol, and the conversion of MTT to formazan was quantified by measuring the absorbance at 570 nm.

Steroid production

Steroid production by monolayers of Y1 cells seeded in 96-well microculture plates at a density of 2×10^5 cells per well was assessed as described previously for primary adrenal cells (Sayed *et al.* 1997). Y1 cells secrete predominantly 20 α -hydroxyprogesterone (Schimmer 1979, 1981), which showed parallel displacement of 1,2,6,7-[³H]progesterone when introduced into an RIA for progesterone over the range 0.25–16 nmol/ml. Steroid production by Y1 cells was therefore measured using a progesterone RIA (Sauer *et al.* 1986), but using 20 α -hydroxyprogesterone to construct standard curves.

Immunodetection of proteins

PP immunoreactivities were detected in Y1 cell extracts by polyacrylamide gel electrophoresis and immunoblot analysis as described for primary luteal and adrenal cells (Ford *et al.* 1996, Sayed *et al.* 1997). For detection of StAR immunoreactivities, mitochondria-enriched fractions were prepared by differential centrifugation. Y1 cells were homogenised (4 °C) in a buffer containing 250 mM sucrose, 10 mM Tris, 0.1 mM EDTA, pH 7.4. The homogenate was centrifuged (4 °C, 600 g, 30 min), the pellet was discarded, and the supernatant was re-centrifuged (4 °C, 10 000 g, 30 min) to produce a mitochondria-enriched pellet, which was resuspended in a buffer containing 20 mM Tris, 2 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 50 µg/ml leupeptin, 0.1% (v/v) β -mercaptoethanol, pH 7.4, and dispersed by sonication. Total protein in extracts was measured using the Bradford assay (Bradford 1976), and mitochondrial fractions contained $9 \pm 1\%$ ($n=6$) of total homogenate protein. In some experiments, immunoreactivity was quantified using densitometric scanning (UVP Easy system) of the ECL signal on the film, as described by Parker *et al.* (1996).

Data analysis

Differences between means were assessed using one way analysis of variance and Bonferroni's multiple comparisons test, as appropriate, and were considered significant when $P < 0.05$.

RESULTS

Stimulation of StAR protein accumulation and steroidogenesis

Under basal conditions, little StAR immunoreactivity was detected in mitochondria-enriched fractions prepared from Y1 cells, confirming that there is

FIGURE 1. Forskolin-induced StAR protein expression and steroidogenesis in Y1 cells. (Left panel) Immunoreactive StAR protein in extracts of Y1 cells after exposure to forskolin (1 μ M) for various times was quantified by densitometric scanning and expressed as a percentage of the immunoreactivity in control extracts (no forskolin) from the same experiments. Bars show means $\pm$ S.E.M., $n=3-6$ separate experiments, except 3-h exposure to forskolin where $n=1$. The inset panel shows a representative immunoblot of StAR expression after 20-h exposure to 1 μ M forskolin (lane 2), compared with untreated Y1 cells (lane 1). (Right panel) Steroid production by Y1 cells incubated in the presence of forskolin is expressed as a percentage of the production rate in the absence of forskolin (13.5 ± 0.5 pmoles/ 10^6 cells/h). Bars show means $\pm$ S.E.M., $n=8$.

minimal expression of the StAR protein under unstimulated conditions (Clark *et al.* 1995a). However, a 30 kDa protein with StAR immunoreactivity was detected in extracts of Y1 cells which had been exposed to the adenylate cyclase activator, forskolin (1 μ M), as shown in the immunoblot inset in Fig. 1. Figure 1 (left panel) shows the cumulative results from a number of experiments demonstrating that forskolin-induced increases in StAR immunoreactivity were time-dependent, with enhanced accumulation of the 30 kDa StAR being seen after 3 h and increasing further after 6 and 20 h exposure to forskolin (Fig. 1, left panel). In parallel experiments, steroid production by Y1 cells was also stimulated by exposure to 1 μ M forskolin (Fig. 1, right panel). Enhanced steroidogenesis was detected after 3 h, and enhanced steroid production was maintained for up to 20 h incubation in the presence of forskolin (Fig. 1).

Expression of phosphoprotein phosphatases in Y1 cells

Figure 2 shows immunoblots of PP1, PP2A and PP2B immunoreactivities in Y1 cell extracts. Y1 cell

PP1 migrated on electrophoresis on 15% polyacrylamide gels with an apparent molecular mass of 37 kDa, and PP2A and PP2B migrated with apparent molecular masses of 36 kDa and 16 kDa respectively, in good agreement with the reported sizes of these enzymes in primary steroidogenic tissue (Ford *et al.* 1996, Sayed *et al.* 1997).

Effects of inhibitors of PP1/2A on steroidogenesis and StAR protein accumulation in Y1 cells

The PP1 and PP2A inhibitors Cal A (1–100 nM) or OA (10 nM–1 μ M) produced a concentration-dependent inhibition of forskolin-induced steroid production by Y1 cells during a 6-h incubation, as shown in Fig. 3. These concentrations of the inhibitors had no effect on unstimulated steroid production by Y1 cells over a 6-h incubation (Cal A 100 nM, $101 \pm 3\%$ control; OA 1 μ M, $103 \pm 3\%$, $n=6$, $P>0.2$). The StAR immunoblot inset into Fig. 4 demonstrates that inhibition of steroidogenesis was accompanied by an inhibition of forskolin-induced expression of the 30 kDa StAR protein. This was a reproducible observation, as shown in

FIGURE 2. PP expression in Y1 cells. The figure shows immunoblots for PP1, PP2A and PP2B in Y1 cell extracts. Molecular masses were calculated from the migration positions of chromophore-tagged proteins of known molecular masses.

Fig. 4, in which 10 nM Cal A markedly reduced forskolin-stimulated accumulation of the StAR protein in three separate experiments, although Cal A alone was without effect on StAR accumulation in Y1 cells (Fig. 4). In a similar experiment, OA (0.1 μ M) completely blocked the forskolin-induced (1 μ M) increase in StAR expression.

FIGURE 3. Effects of PP1 and PP2A inhibitors on forskolin-induced steroidogenesis. Steroid production by Y1 cells is expressed as a percentage of the basal production in the absence of forskolin, Cal A and OA (213 pmoles/10⁶ cells/h, open bar). Forskolin (1 μ M; cross-hatched bar) significantly stimulated steroid production over basal levels ($P < 0.01$). The presence of 1–100 nM Cal A (forward hatching) or 100 nM–1 μ M OA (backward hatching) significantly inhibited forskolin-induced steroid production ($P < 0.01$). Bars show means $\pm$ S.E.M., $n = 6$.

Effects of inhibitors of PP1/2A on Y1 cell function

The inhibition of steroidogenesis and of StAR protein expression by PP1/2A inhibitors was not due to cytotoxic effects of these compounds on the Y1 cells in incubations of up to 6-h duration. Estimation of plasma membrane integrity by Trypan blue exclusion showed >95% of cells excluding the dye in controls and no detectable differences in cells incubated for up to 6 h in the presence of forskolin (1 μ M), Cal A (up to 100 nM), or OA (up to 1 μ M). Similarly, the inhibitors did not affect Y1 cell dehydrogenase activities, even when used at 10-fold higher concentrations than those required to suppress StAR protein expression (Cal A 100 nM, 6 h, $110 \pm 14\%$ control; OA 1 μ M, 6 h, $110 \pm 7\%$, $n = 3$, $P > 0.2$). The reduction in StAR immunoreactivity in response to inhibition of PP1/2A activities (see Fig. 4) was selective for the StAR protein since re-probing the immunoblots with an anti-actin antibody showed no significant changes in actin immunoreactivity after a 6-h incubation in the presence of Cal A (+10 μ M forskolin, $113 \pm 18\%$ control; +10 μ M forskolin + 10 nM Cal A, $107 \pm 24\%$, $n = 5$, $P > 0.2$ vs control for both treatments). The effects of PP1/2A inhibitors on forskolin-induced steroidogenesis were not due to direct inhibition of the steroidogenic enzymes in Y1 cells, since Cal A did not inhibit steroid production from Y1 cells supplied with 22ROHC or pregnenolone, both of which enter the steroidogenic pathway after cholesterol transport to the inner mitochondrial membrane, as shown in Table 1. In these experiments forskolin (1 μ M), 22ROHC (5 μ M) and pregnenolone (5 μ M) all produced significant increases in steroid production by

FIGURE 4. Inhibition of StAR protein accumulation in Y1 cells. StAR protein immunoreactivity was quantified by densitometry and expressed as a percentage of expression in unstimulated controls. Incubation in the presence of forskolin (1 μ M) for 6 h caused a marked increase in StAR immunoreactivity in mitochondria-enriched fractions, and the presence of Cal A (10 nM) significantly reduced forskolin-induced StAR accumulation ($P < 0.05$). Exposure of Y1 cells to Cal A alone did not affect StAR expression. Data are means $\pm$ S.E.M. from three separate experiments. The inset panel shows a representative immunoblot from an experiment in which StAR immunoreactivity was measured after 6-h incubation in normal medium (lane 1), or in medium supplemented with 1 μ M forskolin (lane 2), 1 μ M forskolin plus 10 nM Cal A (lane 3), or 10 nM Cal A alone (lane 4). The molecular mass of StAR immunoreactivity was calculated from the migration positions of chromophore-tagged proteins of known molecular masses.

Y1 cells during a 6-h incubation. Cal A (3–30 nM) completely inhibited forskolin-stimulated steroid production, but had no significant effect on steroid production induced by 22ROHC or pregnenolone (Table 1).

DISCUSSION

The Y1 mouse adrenal cell line offered a useful experimental model for the present studies. StAR protein is not abundant in steroidogenic tissues, so an *in vitro* cell line provides large amounts of tissue from which to prepare mitochondria-enriched fractions, whilst avoiding the use of large numbers of experimental animals. Although Y1 cells have a reduced capacity for 21-hydroxylation and 11-hydroxylation reactions, the earlier parts of the biosynthetic pathway from cholesterol to pregnenolone are intact, and the PKA-dependent signalling pathways are thought to reflect those in primary

adrenal cells (Schimmer 1979, 1981). We have now shown that Y1 cells express immunoreactive PP1, PP2A and PP2B of molecular masses similar to those reported in primary adrenal cells (Sayed *et al.* 1997), and that two structurally dissimilar inhibitors of PP1 and PP2A cause a concentration-dependent inhibition of steroid production in Y1 cells, in accordance with their effects in primary tissues (Abayasekara *et al.* 1996, Sayed *et al.* 1997).

In the present study, forskolin stimulated the accumulation of the 30 kDa StAR protein in Y1 cell mitochondria and increased steroid synthesis over time-courses similar to those reported for other steroid-producing cells (Clark *et al.* 1995a,b), in accordance with the StAR protein being required for cholesterol transport and steroid synthesis, suggesting that Y1 cells are similar to primary adrenal tissue and to other steroidogenic cells in this respect (Stocco 1997, 1998). The enhanced rate of steroid production by Y1 cells was maintained for up to 20 h, and the increased accumulation of the

TABLE 1. Effects of calyculin A on 22R-hydroxycholesterol- and pregnenolone-induced steroid production in Y1 cells. Y1 cells were incubated for 6 h in the absence or presence of 1 µM forskolin, 5 µM 22R-hydroxy cholesterol (22ROHC) or 5 µM pregnenolone and in the absence or presence of 3–30 nM Cal A as shown, and steroid production was determined as described in the Methods section. Data are means ± s.e.m.

Treatment	Steroid production (pmol/10 ⁵ cells/h)			
	0 Cal A	3 nM Cal A	10 nM Cal A	30 nM Cal A
Control	17.4 ± 6.3	17.3 ± 3.3	20.3 ± 3.2	22.7 ± 2.8
Forskolin (1 µM)	75.9 ± 6.6	24.3 ± 2.3*	14.1 ± 1.9*	11.7 ± 2.4*
22ROHC (5 µM)	62.9 ± 4.9	69.9 ± 2.2	57.2 ± 5.6	71.9 ± 7.8
Pregnenolone (5 µM)	68.5 ± 17.1	55.3 ± 9.9	68.4 ± 5.4	90.1 ± 9.1

*All concentrations of Cal A significantly inhibited forskolin-stimulated steroidogenesis (*P*<0.01), but Cal A was without effect on basal steroid secretion, and on steroidogenesis in response to 22ROHC or pregnenolone.

30 kDa StAR protein in mitochondria over this time is consistent with the accumulation of a 30 kDa product from a cytosolic active 37 kDa precursor involved in mitochondrial cholesterol transport (see Stocco & Clark 1996, Stocco 1997, 1998). Our measurements of immunoreactive StAR protein also demonstrated unequivocally that inhibitors of PP1 and PP2A inhibited both steroid production and the accumulation of the 30 kDa StAR protein in Y1 cells. Since the inhibitors had no generalized cytotoxic effects on Y1 cells when used over the time-course and concentration range of the present studies, the simplest explanation for these observations is that inhibiting PP1 and PP2A activities blocks PKA-induced steroid production primarily through preventing the increased expression of StAR protein.

These novel results have several implications for the regulation of steroid production. First, our observations imply that PP1 and/or PP2A activities are essential for the PKA-dependent increases in the expression of StAR protein which precede enhanced steroid production in many steroidogenic tissues. This absolute requirement for a dephosphorylation process in addition to PKA-dependent phosphorylation events suggests the involvement of a cyclic rather than a linear transduction process, the precise nature of which is unknown at present. Secondly, the coupling of an inhibition of StAR protein expression to an inhibition of steroid production lends further support to the notion that StAR protein is the rate-limiting factor in steroidogenesis, and complements previous studies in which expression of StAR protein has been linked to increased steroid production (see Stocco & Clark 1996 for review). Thirdly, StAR protein is itself a potential substrate for protein kinases and PPs as it contains

consensus sites for PKA-dependent phosphorylation, and its phosphorylation state may influence its action in promoting steroid production (Stocco 1998). The realization that manipulating the phosphorylation/dephosphorylation cycle in steroidogenic cells may influence the expression of StAR protein, as well as its function once expressed, will be important for the design of future studies into the function of StAR protein *in situ*. Finally, the identification of PP1/2A activities as being crucial for the maintenance of stimulated steroidogenesis through StAR protein expression may offer potential for therapeutic manipulation of steroid production by pharmacological modification of PP activities.

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