Phosphoserine/threonine phosphatases in the rat adrenal cortex: a role in the control of steroidogenesis?

S B Sayed, B J Whitehouse and P M Jones

Cellular and Molecular Endocrinology Group, Biomedical Sciences Division, King’s College London, Campden Hill Road, London W6 7AH, UK

(Requests for offprints should be addressed to B J Whitehouse)

Abstract

The involvement of protein kinases in the signal transduction pathways controlling adrenal steroidogenesis is well established, and the phosphorylation of substrates by cAMP-dependent protein kinase is a major mechanism in ACTH action. However, the possibility that protein phosphatases (PPs) might also be involved in this process has not been investigated. The aim of this study was, therefore, to measure the function, expression and enzymic activity of PPs in zona glomerulosa (ZG) and zona fasciculata/reticularis (ZFR) tissue from the rat adrenal cortex. Immunoblot analysis using specific antisera demonstrated the presence in whole adrenals and capsules of PP type 1 (PP1) migrating with an apparent molecular mass of 37 kDa, and PP type 2A (PP2A) migrating with apparent molecular masses of 38 and 31 kDa. The PP inhibitors, okadaic acid (OA), calyculin A (CA), tautomycin and microcystin RR, caused a reduction in PP activity in vitro, at doses between 1 nM and 1 µM. In addition, treatment of ZG cells with the adenylate cyclase stimulator, forskolin (10 µM) resulted in a significant reduction in PP activity. The effects of CA and OA on steroid secretion by ZG and ZFR cells were also investigated. Neither CA nor OA had any effect on basal steroid secretion or on yields of steroid obtained from 22R-hydroxycholesterol at doses between 1 and 100 nM. However, both OA and CA (10 and 100 nM respectively) significantly reduced ACTH-stimulated aldosterone and corticosterone production by ZG and ZFR cells. CA and OA (10 and 100 nM respectively) also reduced steroid secretion by cells stimulated by forskolin (10 µM) or dibutyryl cAMP (200 µM). These results suggest that PPs may be involved in the intracellular mechanisms through which adrenocortical steroidogenesis is regulated, acting at a point after cAMP generation and action, but proximal to the side-chain cleavage of cholesterol.


Introduction

Adrenocorticotrophic hormone (ACTH) as the main hormonal regulator of adrenocortical function acts on the function of all zones of the gland, and stimulates the production of steroids by zona glomerulosa (ZG), fasciculata (ZF) and reticularis cells (see Vinson et al. 1992). The mechanisms involved in the response to ACTH begin with the binding of the peptide to its G-protein-coupled receptor on the surface of the adrenocortical cell and activation of adenylate cyclase leading to an increment in cyclic AMP concentration. This results in the activation of cAMP-dependent protein kinases and phosphorylation of proteins (Grahame-Smith et al. 1967, Gill & Garren 1971, see also Orme-Johnson 1990, Schimmer 1995). Phosphorylation of regulatory and structural proteins by protein kinases has long been known to be an important control mechanism within the cells of eukaryotes (Greengard 1979). However, it is not so widely appreciated that the phosphorylation state of a protein is also dependent on the activity of protein phosphatases (PPs). This group of enzymes is also highly regulated and is known to have important roles in several cellular processes, the best understood of which is their part in the control of glycogen metabolism (Cohen 1982, 1989, Cyert & Thorner 1989, Hubbard & Cohen 1993). The possibility that PPs might play a part in the control of secretory processes has also attracted attention recently, and PPLinked mechanisms have been invoked in several endocrine tissues including islets of Langerhans, corpora lutea, and chromaffin and pituitary cells (Gagliardino et al. 1991, Galindo et al. 1992, Iriuchijima et al. 1992, Murphy & Jones 1996, Abayasekara et al. 1996, Ford et al. 1996).

Serine/threonine PPs are divided into four main classes on the basis of their biochemical characteristics: type 1, type 2A, type 2B and type 2C (Cohen 1991). PP type 1 (PP1) dephosphorylates the β subunit of phosphorylase kinase and is inhibited by nanomolar concentrations of two endogenous heat-stable proteins, inhibitor 1 (I1) and inhibitor 2 (I2). PP type 2 (PP2) dephosphorylates the α subunit of phosphorylase kinase and can be further subdivided according to their requirements for different cations. PP2A dephosphorylates substrates in the absence of divalent cations whilst PP2B and PP2C are respectively...
Ca$^{2+}$- and Mg$^{2+}$-dependent (Ingebritsen & Cohen 1983a,b). PP2A activity can be modulated by reversible methylation or phosphorylation on tyrosine or threonine residues (Chen et al. 1992, Guo & Damuni 1993, Favre et al. 1994) and Li et al. (1995) have described two heat-stable inhibitors of the enzyme (I1PP2A and I2PP2A). In recent years, the study of the physiological aspects of PP function has been greatly facilitated by the identification of a number of potent, non-peptide inhibitors of phosphatase activity, of which the best known is okadaic acid, a fatty acid polyether first isolated from marine dinoflagellate sponges (Tachibana et al. 1981, Cohen et al. 1990).

There is some circumstantial evidence that PP may play a part in the mechanism of ACTH action. Thus, dephosphorylation of proteins in response to ACTH has been noted (Koroscil & Gallant 1980, 1981), and a study by Iyer and coworkers (1988) suggested that phospho-protein phosphatases might be involved in this process, but this possibility has not been systematically investigated. The aims of the present study were therefore: 1) to measure the expression of those PPs most commonly involved in intracellular control mechanisms, 2) to measure the enzyme activity using an in vitro assay and observe the effects of inhibition of this activity and 3) to assess the functional significance of these enzymes in ACTH-stimulated steroidogenesis by investigating the effects of PP inhibitors on basal and stimulated aldosterone and cortisol production by adrenocortical cells.

Materials and Methods

Materials

Synacthen (ACTH 1–24) was obtained from Ciba Geigy (Horsham, Sussex, UK). Protogel and Tris/glycine/SDS running buffer for PAGE were obtained from National Diagnostics (Hesse, Hull, UK). Polyclonal anti-human PP1 and PP2A antibodies (raised against the C-terminal peptides of their catalytic subunits) were obtained from TCS Biologicals Ltd (Botolph Claydon, Bucks, UK). Secondary antibodies linked to horse-radish peroxidase (HRP) were obtained from DAKO Ltd (High Wycombe, Bucks, UK). TCS Biologicals Ltd (Botolph Claydon, Bucks, UK). Secondary antibodies linked to horse-radish peroxidase were obtained from Amersham International (Little Chalfont, Bucks, UK). Okadaic acid, 1-norokadaone, tautomycin, calyculin A and microcystin RR were supplied by Calbiochem-Novabiochem Ltd (Nottingham, UK). Worthington type 1 collagenase was obtained from Lorne Labs (Twyford, Berks, UK). Bovine serum albumin, fraction V (BSA), forskolin (FSK), dibutyryl cyclic AMP (dbcAMP), 22R-hydroxycholesterol, aldosterone and corticosterone standards, leupeptin and phenylmethylsulphonyl fluoride (PMSF) were bought from Sigma Chemical Co. (Poole, Dorset UK).

Isolation of cells

Male Sprague–Dawley rats were bred and maintained by the Biological Services Unit of King’s College London. Cells were isolated using the protocol described by Purdy et al. (1991). Rats were killed by stunning and cervical dislocation, the adrenals were removed rapidly, and after removal of excess fat the glands were separated into capsules, which comprise mainly ZG tissue, and inner zones which consist of medulla and zona fasciculata/reticularis (ZFR) tissue. These fractions were incubated separately in 5 ml Krebs–Ringer bicarbonate buffer (3·6 mm K+, 11·1 mm glucose) containing 2% (w/v) bovine serum albumin (KRBGA) and 2 mg/ml collagenase (w/v) for 1 h at 37 °C in an atmosphere of 95% O2 and 5% CO2. The tissue was dispersed by pipetting and the resultant cell suspension was filtered through a nylon gauze (60 μm mesh) and pelleted by centrifugation (1000 g, 10 min).

Polyacrylamide gel electrophoresis, Western blotting and immunoprobing

Adrenal tissue was sonicated in sample buffer (2% w/v SDS, 5% v/v 2-mercaptoethanol, 0·1% w/v bromophenol blue, 10% v/v glycerol, 62·5 mm Tris, pH 6·8) and incubated at 100 °C for 4 min. Proteins were separated using a discontinuous buffering system (Laemmli 1970), and transferred to nitrocellulose membranes by Western blotting using a Multiphor II, Novablot, semi-dry electrophoretic transfer unit (Pharmacia–LKB, Milton Keynes, Bucks, UK). Non-specific binding sites were blocked by incubation (20 h) in a phosphate-buffered saline containing 0·05% v/v Tween-20 (PBST) supplemented with 5% w/v milk powder. Membranes were washed with PBST (4 × 15 min) and incubated with anti-PP1 or PP2A antibodies diluted 1:1000 in PBST for 24 h. After further washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h and HRP activity was detected by ECL according to the manufacturer’s instructions.

Measurement of PP1/PP2A activities in adrenocortical cells

PP1/PP2A activities in capsules and ZG cells were measured using a kit from GIBCO-Life Technologies (Paisley, Scotland, UK). A ~32P-labelled substrate for PP1/PP2A activity, glycogen phosphorylase a, was prepared by reacting purified phosphorylase b with [32P]ATP, and PP1/PP2A activities were measured as the extent of dephosphorylation of this substrate. Adrenal tissue (5000 ZG cells or one adrenal capsule) was sonicated in a buffer containing 20 mm Tris–HCl, 2·5 mm EDTA, 0·5 mm EGTA, 0·1% mercaptoethanol, 0·05 mg/ml leupeptin, 1 mm PMSF, pH 7·4. PP1/2A activities were measured according to the manufacturer’s instructions.
during incubations at 30 °C for 10 min. Reactions were terminated by the addition of 20% TCA and proteins were precipitated by incubation at 4 °C for 30 min and pelleted by centrifugation (12,000 g, 3 min). 32P released into the supernatant was measured by liquid scintillation counting and PP1/PP2A activities were calculated.

Secretion experiments

Cell were incubated at a density of 1 × 10^6 cells/ml (ZG cells) and 3 × 10^6 cells/ml (ZFR cells) in the presence or absence of ACTH (10 nm), dbcAMP (200 µm), FSK (10 µm), 22R-hydroxycholesterol (22ROHC; 2.5 µm) or PP inhibitors (1 nM - 1 µM) for 2 h at 37 °C. After incubation, samples were stored at −20 °C and aldosterone and corticosterone content were determined by RIA, as described previously (Purdy et al. 1991).

Statistics

Results were expressed as means ± s.e.m. and data from individual experiments were analysed by ANOVA and Bonferroni’s multiple comparison test. When results from several experiments were combined the data were expressed as a percentage of control values and analysed by Student’s paired t-tests. Differences between groups were considered significant when P<0.05.

Results

Protein phosphatases in rat adrenals and adrenal capsules

Western blots of rat adrenal and adrenal capsule extracts which have been immunoprobed with anti-PP1 antibody or anti-PP2A antibody are shown in Fig. 1a and b. The major immunoreactive form of the catalytic subunit of PP1 in rat adrenals and capsules was a protein with an approximate molecular mass of 37 kDa (Fig. 1a). The antibody raised against the catalytic unit of PP2A recognised two immunoreactive bands of approximate molecular mass 38 and 31 kDa (Fig. 1b). Whole adrenals (cortex and medulla) and adrenal capsules (mainly ZG tissue) were found to have similar levels of PP1 and PP2A immunoreactivity, when corrected for protein content.

Measurement of PP1/PP2A activities and the effects of inhibitors

Initially, optimum conditions for the measurement of PP1/PP2A activity were established as described by Ford et al. (1996), using serial dilutions of rat adrenal capsule extracts. Optimal measurements of PP1/2A activities are obtained using concentrations of enzymes which dephosphorylate approximately 0.02 nmol/min phosphorylase a during a 10 min incubation. Our preliminary experiments suggested that these levels of activity were obtained using tissue extract equivalent to 1/400 of an adrenal capsule per observation, and this dilution was used in all subsequent assays of PP1/2A activities.

In these experiments, the effects of four structurally dissimilar inhibitors of PP1/PP2A activity in adrenal capsules were investigated: okadaic acid (OA, from marine dinoflagellates), calyculin A (CA, from the marine sponge Discodermia calyx), tautomycin (TM, from soil bacteria), and microcystin RR (MC, from cyanobacteria). In addition, the effect of 1-norokadaone (N-Ok), a structural analogue of OA which has similar chemical properties but which lacks any effect on phosphoprotein phosphatase activity (Nishiwaki et al. 1990), was examined (Fig. 2). CA and MC, known to be equipotent inhibitors of PP1 and PP2A (Ishihara et al. 1989, MacKintosh et al. 1990), significantly reduced PP activity between 1 nM and 1 µM with 50% inhibition at approximately 5 nM for CA and 20 nM for MC. TM, which has a greater affinity for PP1 (MacKintosh & Klumpp 1990), exhibited a similar dose-response relationship to CA, with 50% inhibition at

Figure 1 Immunological detection of PP1 (a) and PP2A (b) in adrenal capsules. Proteins in extracts of adrenal capsules (lanes 1 and 2, 50 and 100 µg) and in whole adrenals (lanes 3 and 4, 50 and 100 µg) were separated by SDS-PAGE and transferred to nitrocellulose membranes and probed with antibodies raised against the catalytic subunit of PP1 and PP2A. Sites of antigen-antibody interaction were visualised by ECL. Arrows indicate molecular masses calculated from the migration positions of molecular mass standards.
The fatty acid polyether, OA, which shows a greater specificity for PP2A than for PP1 (Biolojan & Takai 1988) was effective between 100 nM and 1 µM, with 50% inhibition at approximately 300 nM. N-Ok was tested as a negative control for OA and had no effect on adrenal PP1/PP2A activities at concentrations up to 1 µM (Fig. 2). The effects of these inhibitors on PP1/2A activities in purified ZG cells were also investigated. OA (1 µM) reduced PP1/2A activities to 10% of control values, whilst N-Ok (1 µM) was without effect (control, 1250 ± 24(CA), 2410 ± 44(MC), 1860 ± 14 (TM) and 1790 ± 42 (N-Ok) (means ± S.E.M., n=4).

Effects of calyculin A and okadaic acid on aldosterone and corticosterone secretion

The effects of CA and OA (1–100 nM) on basal and ACTH-stimulated aldosterone and corticosterone secretion from ZG and ZFR cells preparations are shown in Fig. 3. CA (1–100 nM) had no effect on basal aldosterone production by ZG cells or basal corticosterone production by ZFR cells (Fig. 3a and b). In contrast, 10 and 100 nM CA caused a significant reduction in aldosterone and corticosterone secretion by ACTH-stimulated ZG and ZFR cells. Maximum inhibition was achieved at 100 nM CA, under which conditions secretion was not significantly different from basal (aldosterone, 105·5 ± 4% basal; corticosterone, 105·5 ± 4% basal, n=3 separate experiments). Similar effects were obtained using OA as an inhibitor of PP activities. As shown in Fig. 3c and d, OA had no effect on basal secretion of the steroids at any concentration tested but caused a significant reduction in ACTH-stimulated aldosterone and corticosterone secretion from ZG and ZFR cells respectively. The threshold for inhibition of steroidogenesis was 10 nM OA in ZG cells and maximum inhibition of aldosterone production was achieved at 100 nM OA (to 108 ± 2·9% of basal values; n=3). In ZFR cells the effects of OA were less striking, in the presence of 100 nM OA corticosterone production remained significantly greater than basal values (+ACTH, 827 ± 103% basal; +OA +ACTH, 427 ± 46% basal; n=3).

The PP inhibitors could also inhibit steroid production in response to elevations in endogenous cAMP induced by forskolin (FSK), and to direct activation of PKA by dibutyryl cyclic AMP (dbcAMP). Figure 4 shows that CA (10 and 100 nM) significantly inhibited the production of aldosterone and corticosterone from ZG and ZFR cells, respectively.
steroids by ZG and ZFR cells stimulated by FSK (10 µM) or dbcAMP (200 µM). Similar inhibitory effects of OA (100 nM) are shown in Fig. 5. In contrast, the PP inhibitors did not inhibit steroid production in response to exogenous 22ROHC. Table 1 shows that CA (1–100 nM) had no significant inhibitory effects on yields of aldosterone and corticosterone obtained from ZG and ZFR cells respectively, during a 2 h incubation at 37 °C in the presence of 22ROHC (2-5 µM). Similarly, OA (100 nM) had no significant inhibitory effect on 22ROHC-dependent steroid production by ZG and ZFR cells (+OA, aldosterone production 90 ± 3% stimulated values; corticosterone production, 99 ± 7%, n=4).

Discussion

Although phosphoprotein phosphatase activity has previously been detected in rat and beef adrenal tissue there was no attempt to characterise the proteins involved (Ullman & Perlman 1975, Li 1979, Iyer et al. 1988). In this study we provide the first evidence that the phosphoprotein phosphatases PP1 and PP2A are present in rat adrenal tissue and adrenal capsules. The catalytic subunit of PP1 (PP1C) was originally described as a monomer of 37 kDa molecular mass (Tung et al. 1984). The polyclonal anti-PP1 antibody used here recognises two forms of PP1C in rabbit skeletal muscle with 37-5 and 35-4 kDa molecular mass (Berndt et al. 1987, Cohen 1989). In rat adrenal tissue we were able to identify a single immunoreactive band corresponding to a protein of molecular mass 37 kDa, suggesting that the isoform of the catalytic subunit present in rat adrenal glands is PP1C. The antiserum directed against the catalytic subunit of PP2A recognised two immunoreactivities corresponding to molecular mass 38 and 31 kDa. The PP2A catalytic subunit is detected as a 36 kDa protein in rabbit skeletal muscle in the presence of protease inhibitors and as a 33-5 kDa protein in their absence (Paris et al. 1984, Silberman et al. 1984, Tung et al. 1984). Thus, it is probable that the 38 kDa protein in rat adrenal tissue is equivalent to the 36 kDa immunoreactivity seen in rabbit skeletal muscle, and that the 31 kDa immunoreactivity is a degradation product of the 38 kDa protein.

PP1 and PP2A were expressed to a similar level in rat whole adrenals (medulla plus cortex) and capsules...
(essentially the outer shell of the gland, consisting of ZG cells adhering to the fibrous capsule with a smaller number of ZF cells). Although the present study does not differentiate between the PP content of the ZFR tissue and medulla, it seems probable that the enzymes are present at similar levels throughout the gland. The assays of PP1/PP2A activity present in rat adrenal capsules and ZG cells suggested that the total enzyme level was of similar magnitude to that detected in rat islets of Langerhans (Murphy & Jones 1996) and at least one order of magnitude greater than that observed in rat luteal cells (Ford et al. 1996).

Inhibition of PP1/PP2A activity in vitro was observed with submicromolar concentrations of all four of the PP inhibitors used in the current study. OA has been shown to be more selective for PP2A than PP1 in vitro, although it may not distinguish between PP1 and PP2A in vivo (Cohen 1989, Cohen et al. 1989). On the other hand, TM is reported to be more selective for PP1 than for PP2A, whereas CA and MC have similar potencies for PP1 and PP2A (Carmichael 1989, Ishihara et al. 1989, MacKintosh & Klumpp 1990). However, it is difficult to draw any firm conclusions in the present experiments as to which PP is the predominant form in rat adrenocortical cells from the relative potencies of the various inhibitors. The higher inhibitory potency of TM over OA might suggest that PP1 is the dominant form in rat adrenal capsules. However, this conclusion is not consistent with the observations that CA was the most potent of all of the inhibitors tested and that CA and MC produced rather different patterns of

Figure 4 The effects of calyculin A (CA) on FSK- and dbcAMP-stimulated steroidogenesis. ZG and ZFR cells were incubated with (■) or without (□) FSK (10 μM) or dbcAMP (200 μM), in the absence or presence of calyculin A (10 and 100 nM), and aldosterone (upper panel) and corticosterone (lower panel) production were determined by RIA. Values are calculated as % stimulated values and shown as means ± SEM, n=5. **P<0.01 versus stimulated values.
Figure 5 The effects of okadaic acid (OA) on FSK- and dbcAMP-stimulated steroidogenesis. ZG and ZFR cells were incubated with (■) or without (□) FSK (10 μM) or dbcAMP (200 μM) in the absence or presence of okadaic acid (100 nM), and aldosterone (upper panel) and corticosterone (lower panel) production were determined by RIA. Values are calculated as % stimulated values and shown as means ± S.E.M., n=5. **P<0.01 versus stimulated values.

Table 1 The effects of calyculin A on basal and 22R-hydroxycholesterol-supported aldosterone secretion from ZG cells and corticosterone secretion from ZFR cells. Cells were incubated with or without 22R-hydroxycholesterol (2.5 μM) and calyculin A (1–100 nM) for 2 h at 37 °C. Values are means ± S.E.M., n=6

<table>
<thead>
<tr>
<th></th>
<th>Aldosterone secretion (fmol/10⁴ cells/2 h)</th>
<th>Corticosterone secretion (nmol/10⁴ cells/2 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>95 ± 3.5</td>
<td>0.42 ± 0.01</td>
</tr>
<tr>
<td>With 22R-hydroxycholesterol (2.5 μM)</td>
<td>972 ± 192</td>
<td>35.9 ± 0.46</td>
</tr>
<tr>
<td>With calyculin A (nM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>706 ± 145</td>
<td>40.6 ± 0.96</td>
</tr>
<tr>
<td>10</td>
<td>682 ± 142</td>
<td>34.9 ± 0.46</td>
</tr>
<tr>
<td>100</td>
<td>1022 ± 105</td>
<td>55.0 ± 10.6</td>
</tr>
</tbody>
</table>
inhibition. Nevertheless, the relatively high overall level of serine/threonine phosphatase activity favours the idea that these enzymes might play a physiologically significant role in rat adrenocortical tissue. This conclusion is further supported by our demonstration that the in situ activation of PKA in intact ZG cells by forskolin resulted in a significant inhibition of PP1/2A activities. We have not defined the mechanism responsible, but the most likely explanation is that protein kinase A (PKA) phosphorylates, and thus activates, the 11 protein within ZG cells, leading to diminished PP1 activity in subsequent extracts, as reported in other tissues (Purdy 1989).

The functional significance of PP1 in adrenal steroidogenesis was further supported by the results of our experiments using two structurally dissimilar PP inhibitors, CA and OA, which both significantly reduced the response to activators of the cAMP/PKA pathway. CA proved to be the more potent inhibitor of ACTH-stimulated steroidogenesis, giving a threshold for inhibition at a dose of 10 nM and reduction of the steroid output to basal levels with 100 nM CA. It inhibited the response to FSK and db-cAMP in a similar fashion in zona glomerulosa cells. However, FSK-stimulated ZFR cells proved to be particularly sensitive to CA with a reduction to basal levels at a 10 nM dose (Fig. 4), this may be related to the fact that these cells are known to respond atypically to this diterpen (Purdy et al. 1991). Generally, similar results were obtained with OA, although it was somewhat less effective at reducing steroid output, in line with its lower potency as a PP inhibitor. Importantly, however, neither compound had any effect on 22ROHC-supported steroidogenesis, nor did they reduce basal steroid output by ZG or ZFR cells suggesting that their effects cannot simply be ascribed to non-specific inhibition of biosynthesis. Taken together, these results are consistent with a site of inhibition of the ACTH signal-transduction pathway after cAMP generation and action but before the action of cytochrome P450 size chain cleavage (P450SCC).

Phosphorylation of protein substrates by PKA is part of the mechanism by which ACTH stimulates steroidogenesis, and intuitively, therefore, one might expect PP inhibitors to stimulate rather than inhibit secretion, by increasing phosphorylation of proteins. This would also be consistent with the reduction in PP1/2A activity found after forskolin pretreatment of ZG cells. One explanation for these seeming contradictions is that continued steroid production requires not only the phosphorylation of a protein but also its subsequent dephosphorylation and that PP inhibitors may interfere with this cycle of events by inhibiting the latter process. In support of this, other workers have also found that PP inhibitors such as OA and CA can block agonist-stimulated steroid production without interfering with the biosynthetic process. Abayasekara et al. (1996) showed that LH- and cAMP-stimulated progesterone production by rat luteal cells was inhibited by OA and CA. In addition, Azhar and coworkers (1991, 1994) reported that OA inhibited lipoprotein-stimulated corticosterone secretion from rat adrenocortical cells, although, in contrast to the present data, the drug was without effect on ACTH-stimulated steroidogenesis. The reason for this anomaly may well reside in the different experimental conditions used: in the former case the cells were cultured for periods of up to 24 h, and the earliest time interval reported was 3 h treatment with OA.

In the present studies, as well as those described above, none of the PP inhibitors tested had any effect on yields of steroid obtained from 22ROHC. This compound diffuses readily across the aqueous layer between the inner mitochondrial membranes and thus bypasses the rate-limiting step in steroid biosynthesis, cholesterol transport to the inner mitochondrial membrane where the cytochrome P450SCC enzyme complex resides (Simpson & Boyd 1967, Jefcoate et al. 1974, Toaff et al. 1982). This lack of inhibition of 22ROHC-supported steroidogenesis implies that PPs regulate steroidogenesis after PKA activation but before cholesterol delivery to the cytochrome P450SCC, thus raising the possibility that the unidentified substrate protein for PPs may be involved in cholesterol transport. Several proteins have been identified which are thought to be involved in cholesterol transport in steroidogenic cells, including the 13 kDa sterol carrier protein-2 (SCP-2, Chanderbahn et al. 1982, Vahouny et al. 1985), and the 30 kDa protein which is now known as StAR, the steroidogenic acute regulatory protein (Alberta et al. 1989, Epstein et al. 1989, Green & Orme-Johnson 1991, Stocco & Sodeman 1991, Clark et al. 1994). SCP-2 is phosphorylated by protein kinase C (PKC) (Steinschneider et al. 1989) and computer analysis of the protein sequence of StAR has identified three putative phosphorylation sites for PKA, calmodulin–dependent protein kinase II and PKC (Stocco & Clark 1996).

In conclusion, we propose that the protein phosphatases, PP1/2A, are involved in the regulation of a phosphoprotein that is important in ACTH action. It appears to act distal to the activation of PKA in the signal transduction pathway but proximal to transport of cholesterol to the inner mitochondrial membrane. The identity of this phosphoprotein has yet to be determined.

Acknowledgements

We are grateful to the Medical Research Council for financial support. S.B.S is supported by a Medical Research Council postgraduate studentship.

References

Alberta JA, Epstein LF, Pon LA & Orme-Johnson NR 1989 Mitochondrial localization of a phosphoprotein that rapidly accumulates in adrenal-cortex cells exposed to adrenocorticotropic hormone or to cAMP. *Journal of Biological Chemistry* 264 2368–2372.


Downloaded from Bioscientifica.com at 11/04/2018 01:54:11PM via free access


Stocco DM & Clark BJ 1996 Regulation of the acute production of steroids in steroidogenic cells. Endocrine Reviews 17 221–244.

Stocco DM & Sodeman TC 1991 The 30-kDa mitochondrial proteins induced by hormone stimulation in MA-10 mouse Leydig tumor cells are processed from larger precursors. Journal of Biological Chemistry 266 19731–19738.


Received 17 October 1996
 Accepted 4 April 1997