Interdependence of steroidogenesis and shape changes in Y1 adrenocortical cells: studies with inhibitors of phosphoprotein phosphatases

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Abstract

Y1 adrenocortical cells respond to activators of the cyclic AMP-dependent protein kinase (PKA) signalling pathway not only with increases in steroid secretion but also with a characteristic change in cell morphology from flat and adherent to round and loosely attached. This change of shape, which may facilitate cholesterol transport to the mitochondrion, requires tyrosine dephosphorylation of the focal adhesion protein, paxillin, and can be blocked by inhibitors of phosphotyrosine phosphatase (PTP) activity. In a previous study we demonstrated that inhibition of phosphoserine/threonine phosphatase 1 and 2A (PP1/2A) activities caused a similar morphological response to PKA activation whilst opposing the effects on steroid production. We have now investigated the responses to PKA activation and inhibition of PP1/2A and used PTP inhibitors to examine the relationship between the morphological changes and enhanced steroid production.

Both forskolin (FSK) and the PP1/2A inhibitor, calyculin A (CA), caused rapid and extensive rounding of Y1 cells. FSK-induced cell rounding was reversible and accompanied by a reduction in the tyrosine phosphorylation of paxillin. Rounding was prevented by the PTP inhibitors pervanadate (PV) and calpeptin (CP) and was associated with the maintained tyrosine phosphorylation of paxillin. In contrast, CA-induced cell rounding was not reversible over a 2-h period and was not affected by the presence of PTP inhibitors, and CA had no effect on the tyrosine phosphorylation of paxillin. Although neither CA nor FSK produced any gross changes in cell viability as judged by Trypan Blue exclusion or mitochondrial activity, CA-treated cells showed a marked reduction in total protein synthesis assessed by ^{35}S-incorporation. The effects of FSK and the PTP inhibitors on cell rounding were reflected in their effects on steroid production since PV and CP also inhibited FSK-stimulated steroid production. These results suggest that the mechanism through which inhibition of PP1/2A activities induces morphological changes in Y1 cells is fundamentally different from that seen in response to activation of PKA. They are consistent with PKA-induced shape changes in adrenocortical cells being mediated through increased PTP activity and the dephosphorylation of paxillin, and support the view that the morphological and functional responses to PKA activation in steroidogenic cells are intimately linked.

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Introduction

It has long been known that activation of the widely used Y1 adrenocortical tumour cell line leads not only to increased steroidogenesis but also to a pronounced morphological transformation (Yasamura *et al.* 1966, Yasamura 1968; see also Schimmer 1980 & 1995, Hall & Almahbobi 1997). On application of a stimulant of the cyclic AMP-dependent protein kinase (PKA) signalling pathway, such as forskolin (FSK), cyclic AMP or adrenocorticotrophin (ACTH), the cell membrane retracts and the cell shape changes rapidly from flat and adherent to round and loosely attached (Kowal 1970, Mrotek & Hall 1975, Betz & Hall 1987). Similar morphological responses to stimulation with cyclic AMP have been found in other steroidogenic cell types, including bovine, rat and human adrenal cells (Rainey *et al.* 1983, Vilgrain *et al.* 1998), testicular Leydig and Sertoli cells (Hall *et al.* 1979, Spruill *et al.* 1981, Bilinska *et al.* 1997) and ovarian granulosa cells (Ben-Ze'ev *et al.* 1987, Aharoni *et al.* 1993).

One of the first steps in the cell rounding process in adrenal cells is the disassembly of focal adhesions at the cell margin and the loss of stress fibres from the cytoplasm (Rainey *et al.* 1983). This change precedes the

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steroidogenic response and it has been suggested that the reorganisation of the actin skeleton that occurs facilitates delivery of cholesterol to the outer mitochondrial membrane thus leading to increased steroid synthesis (Betz & Hall 1987, Shiver *et al.* 1992, Feuilloley & Vaudry 1996, Hall 1997). Recently, it has been demonstrated that an early effect of PKA activation is targeted to the protein paxillin, a component of focal adhesions which is dephosphorylated on tyrosine residues within minutes of exposure of Y1 cells and bovine adrenocortical cells to cyclic AMP (Han & Rubin 1996, Vilgrain *et al.* 1998).

In our recent studies of the role of phosphoserine/threonine phosphatases (PPs) in the control of adrenocortical cell function, we noted that inhibitors of PP1/2A activity, such as calyculin A (CA) and okadaic acid, also provoked a marked rounding response in Y1 cells (Sayed *et al.* 1997*b*). However, rather than promote steroid production, we found that the PP1/2A inhibitors blocked FSK-stimulated steroidogenesis and that this effect was secondary to reduced steroidogenic acute regulatory protein (StAR) mRNA and protein expression (Sayed *et al.* 1997*a*, Burns *et al.* 2000*a*, Jones *et al.* 2000). This apparent dissociation between cell rounding and steroid production seems paradoxical and raises the possibility that the morphological responses produced by PP inhibitors are separate and unrelated to activation of the PKA signalling pathway. In the present study we have, therefore, investigated the mechanisms underlying FSK- and CA-induced rounding of Y1 cells in the context of the divergent effects of these agents on steroidogenesis. A preliminary account of some of these findings was presented at the Ninth Conference on the Adrenal Cortex (Whitehouse *et al.* 2000).

Materials and Methods

Materials

Mouse adrenocortical Y1 cells were obtained from the European Collection of Cell Cultures (Salisbury, Wilts, UK). Tissue culture reagents and plastics were from Gibco BRL (Paisley, Strathclyde, UK). CA and calpeptin (CP) were from Calbiochem (Nottingham, Notts, UK). Anti-paxillin antiserum was from Zymed Laboratories (San Francisco, CA, USA) and mouse anti-phosphotyrosine antiserum was from Upstate Biotechnology (Lake Placid, NY, USA). Horseradish peroxidase-coupled goat anti-rabbit IgG and goat anti-mouse IgG were from Pierce (Rockford, IL, USA). Nitrocellulose membrane (0·2 μm) for Western blotting was from Scheicher and Schull (Dassel, Germany). Enhanced chemiluminescence (ECL) reagents, Hyperfilm and ^{35}S-cysteine were from Amersham International plc (Amersham, Bucks, UK). Polyclonal antibody to pregnenolone was obtained from Biogenesis (Poole, Dorset, UK) and [7-^{3}H(N)]-pregnenolone (specific activity 22·5 Ci/mmol) for use in radioimmunoassay (RIA) was obtained from NEN Life Science Products, Inc (Boston, MA, USA). All other biochemicals were from the Sigma Chemical Company Ltd (Poole, Dorset, UK).

Methods

Maintenance and viability of Y1 cells Y1 cells were maintained in DMEM supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin and 10% (v/v) foetal bovine serum in a humidified atmosphere of 5% CO_2. Cells were trypsinised and passaged (1:5) on achieving confluence. Cell viability after experimental treatments was assessed by Trypan Blue exclusion and by measurement of mitochondrial dehydrogenase activity, as described previously (Jones *et al.* 2000).

Real time estimate of shape changes The Ca^{2+}-fluorophore, Fura-2, was used as a marker of cell shape changes. Y1 cells were seeded onto 3-aminopropyltriethoxysilane (APES)-coated coverglass (Sigma) at a density of 30 000 cells per coverslip, which was sufficient to allow adherent cells to form two-dimensional clusters. Cells were allowed to adhere overnight in DMEM under standard tissue culture conditions. Cells were loaded for 30 min at 37 °C with 2·5 μM Fura-2/AM (Sigma). The coverslips were washed and transferred to a steel chamber, the volume of which was maintained under static conditions at 2 ml. A single 22-mm coverslip formed the base of the chamber, which was mounted into a heating platform on the stage of an Axiovert 135 Research Inverted microscope (Carl Zeiss Ltd, Welwyn Garden City, Herts, UK). All experiments were performed at 37 °C using a Na^{+}-rich balanced salt solution as the standard extracellular medium (Squires *et al.* 1994). Cells were illuminated at 380 nm using an Axon Imaging Workbench (Axon Instruments Inc, Foster City, CA, USA). Emitted light was filtered using a 510 nm long-pass barrier filter and detected using a Photonics Science ISIS camera (Robertsbridge, E. Sussex, UK). To reduce unnecessary bleaching of the fluorophore, data acquisition was recorded at a rate of 1 image/150 s. All records have been corrected for background fluorescence (determined from cell-free coverslip).

Cell rounding Photomicrographs were taken of Y1 cell monolayers before and at various times after treatments and the degree of cell rounding was assessed by counting all cells in a given field and expressing the number of rounded cells as a percentage of the total cells in view. Each field contained a minimum of 30 cells, and observations for each treatment were taken on three separate, randomly chosen fields of view. Assessment of cell rounding was performed blind by two separate operators.

Steroid secretion Y1 cells were seeded in 96-well microculture plates at an approximate density of

2×10^5 cells per well, and allowed to adhere during an overnight incubation at 37 °C in a humidified atmosphere of 5% CO_2. The culture medium was replaced with DMEM supplemented with test substances of interest, and the incubation was continued for 3 h, after which cells and medium were stored at − 20 °C to await assay for steroid content. Steroid production was assessed by measuring pregnenolone production in the presence of the 3-hydroxysteroid dehydrogenase inhibitor, trilostane, which prevents the further metabolism of the steroid (Potts *et al.* 1978, Aguilera *et al.* 1980).

Assessment of changes in phosphotyrosine content and immunoprecipitation of paxillin Y1 cell extracts were prepared as described previously (Sayed *et al.* 1987*a*) and total protein was measured using the Bradford (1976) assay. Proteins were separated on polyacrylamide gels and tyrosine phosphorylated products detected using an anti-phosphotyrosine antibody as described previously (Persaud *et al.* 1999). In some experiments paxillin was immunoprecipitated from the Y1 cell extracts using the procedure described by Burns *et al.* 2000*b*: briefly, cell lysates were treated with mouse anti-paxillin antibody (16 h, 4 °C) followed by protein A sepharose (4 h). After centrifugation the pellets were solubilised and the phosphorylated products detected after separation on polyacrylamide gels.

Measurement of total protein synthesis Y1 cells were seeded in 6-well microculture plates at a density of 2×10^6 cells per well. The cells were incubated in 2 ml growth medium containing 10 μCi L-^{35}S-cysteine per well (specific activity >1000 Ci/mmol) for 6 h. After washing in fresh medium containing 5 mM cysteine, the cells were lysed and protein content estimated. Proteins in the lysate were precipitated with trichloracetic acid (TCA; 10% final concentration, overnight at 4 °C) and trapped by vacuum filtration using GF/C glass microfibre filters (Whatman, Maidstone, Kent, UK). The filters were washed twice with 5 ml 5% TCA containing 1 mM cysteine, then transferred to counting vials for measurement of the ^{35}S content of the precipitates by β-counting. The rate of protein synthesis was calculated as ^{35}S c.p.m. incorporated/μg protein.

Data analysis

Unless otherwise stated, data are expressed as means ± S.E.M. Differences between means were assessed using one-way analysis of variance and Bonferroni's multiple comparisons test, as appropriate, and considered significant when $P<0{\cdot}05$.

Results

Time course and reversibility of cell rounding

The activation of PKA by treatment of Y1 cells with 1 μM FSK for 30 min caused the characteristic change in the

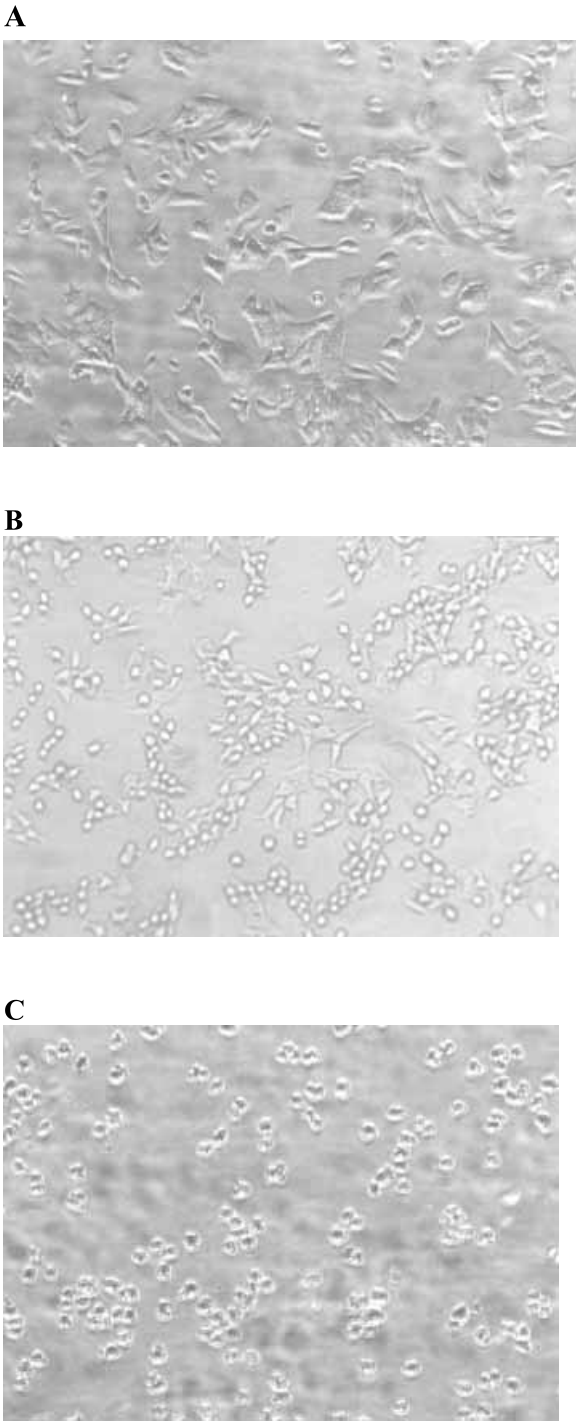

Figure 1 PKA-induced cell rounding in the Y1 cell line. The figure shows the mouse adrenocortical Y1 cell line growing as an adherent monolayer on tissue culture plastic. Under control conditions (A) the majority of the cells show a flattened epitheloid morphology. After exposure to forskolin (FSK) (1 μM, 30 min) many of the cells assume a characteristic rounded morphology, as shown in (B). After exposure to calyculin A (CA) (10 nM, 30 min) almost all the cells are rounded and loosely attached to the substratum (C).

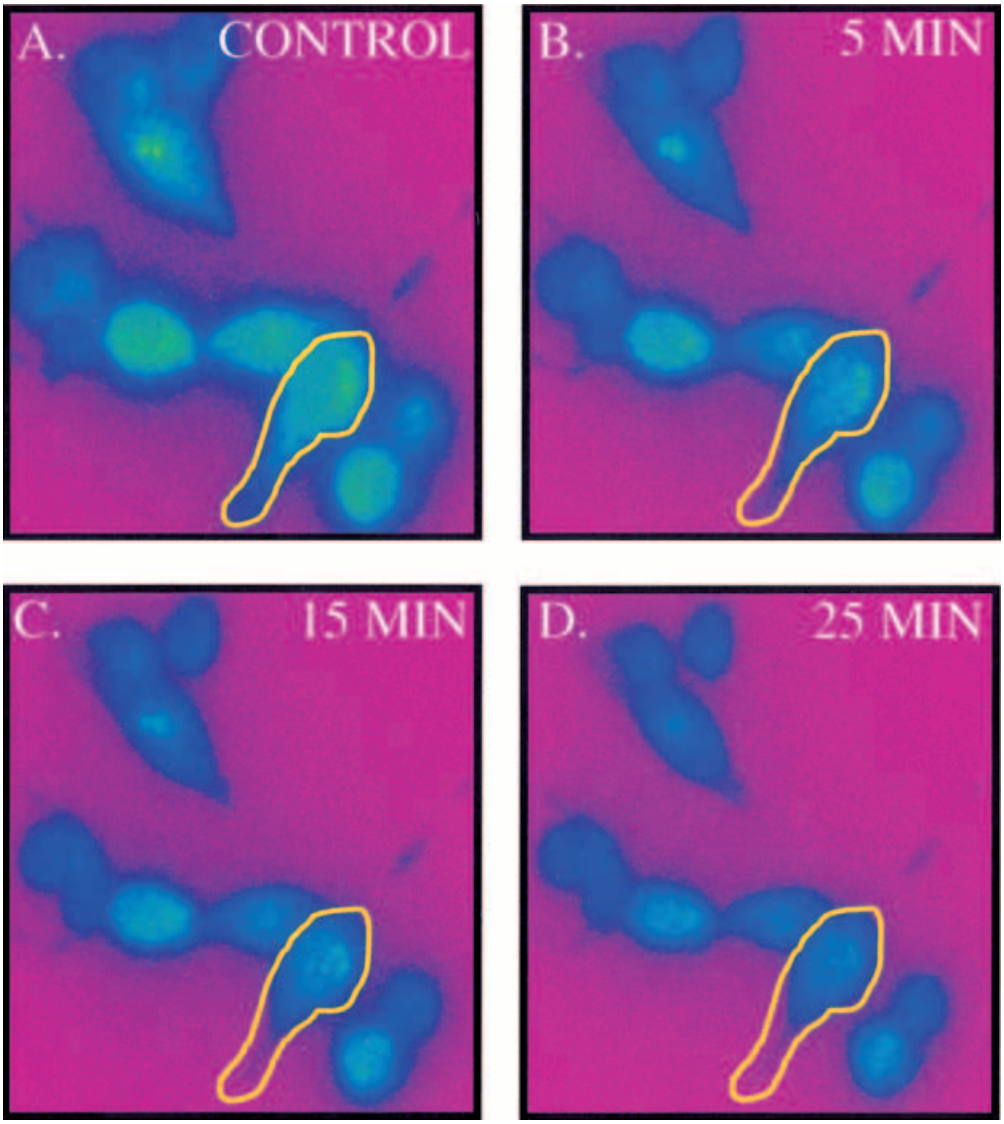

Figure 2(i)

morphology shown by the micrographs of Y1 cell populations (Fig. 1A,B), while exposure to 10 nM CA caused a more pronounced rounding of the cells as can be seen in Fig. 1C.

Figure 2(ii) shows the time course of the rounding in individual Y1 cells loaded with the Ca^{+}-fluorophore, Fura-2. Application of a single bolus of FSK (Fig. 2(i) final concentration 1 μM) produced a distinct change in cell shape, despite the strong adherence to the APES-coated coverslip. Shape changes were noticeable within 5 min (panel B), with clear rounding visible 15 min post application. Figure 2(ii) shows the effect of CA (final concentration 1 nM) with dramatic changes in cell shape visible as early as 4 min after exposure to the drug with the apparent formation of protruberances at one pole of the cell which appeared to retract over the next 12 min of observation.

Figure 3 shows the time course of changes in the extent of rounding of populations of Y1 cells in the presence of 1 μM FSK (upper panel) or 10 nM CA (lower panel). After 30 min, more than 70% of the cells exposed to FSK were rounded, while more than 90% were rounded after a similar exposure to CA. These morphological changes were maintained for up to 2 h in the presence of both agents. FSK-induced cell rounding was readily reversible, as shown in Fig. 3 (upper panel). Thus, when FSK was removed after 30-min exposure, increasing numbers of the rounded Y1 cells reverted to the normal flat, epitheloid appearance over a 90-min period (27·4 ± 0·5% versus

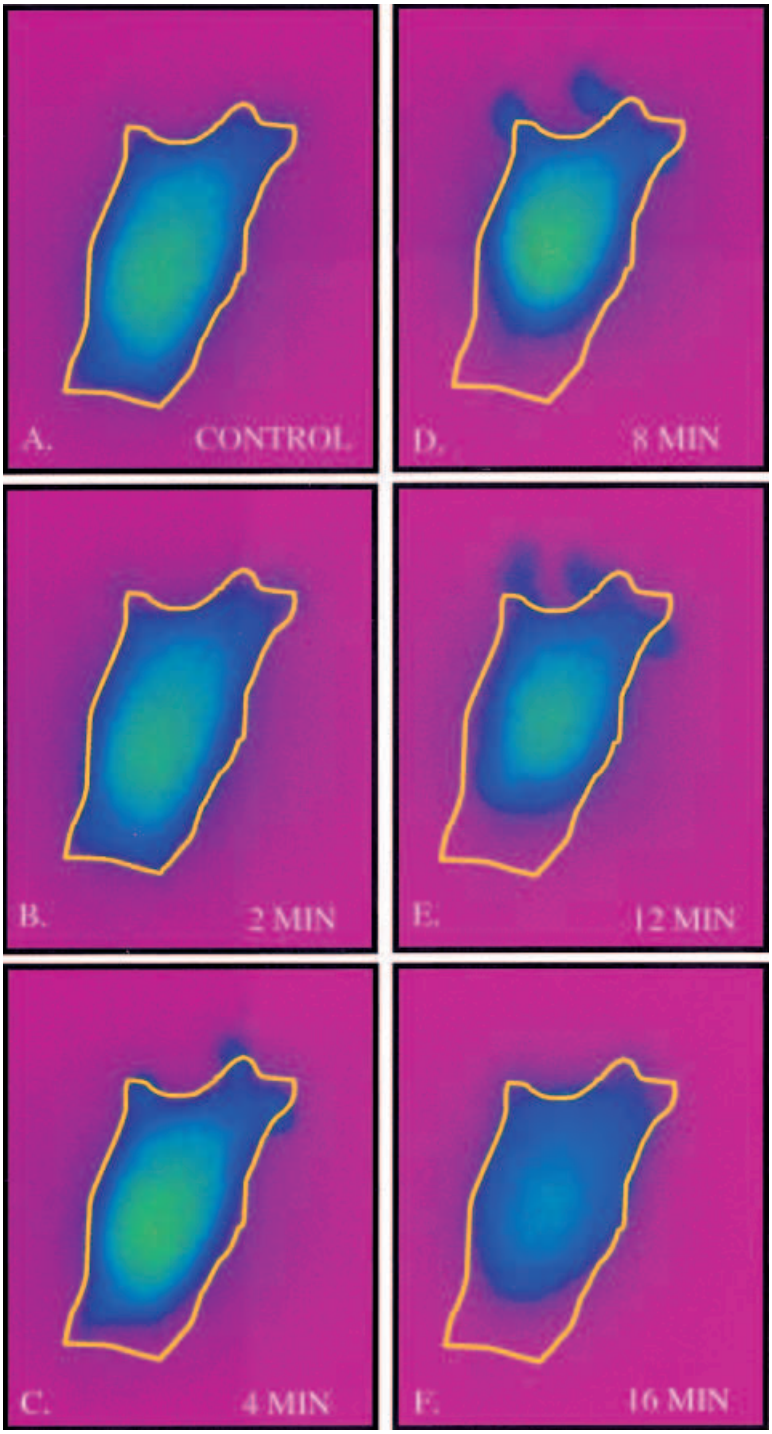

Figure 2(ii)

Figure 2 FSK- and CA-mediated shape changes in single Y1 cells. The figure shows pseudo-colour representations of Y1 cells loaded with the Ca^{2+}-fluorophore Fura-2. (i) After application of a single bolus of FSK (1 μM final concentration) shape changes were noticeable within 5 min (B), with clear rounding visible 15 min post application (C and D), as seen from the cell identified by the yellow perimeter. (ii) After application of CA (1 nM final concentration) extensive changes in shape were visible at 4 min (C), with apparent protruberances formed and retracted over the next 12 min (D–F).

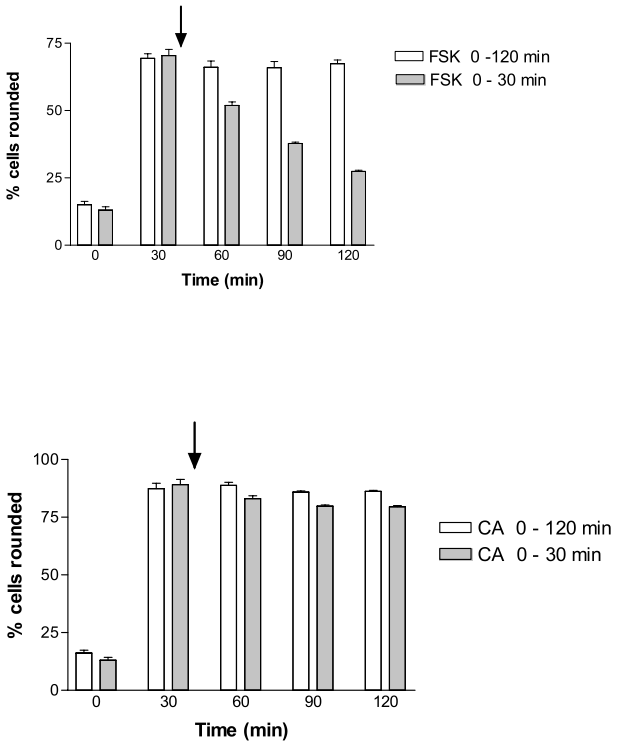

Figure 3 Time course and reversibility of cell rounding. Exposure to FSK (1 μM, upper panel) or CA (10 nM, lower panel) caused marked cell rounding in adherent Y1 monolayers within 30 min. FSK-induced cell rounding showed a time-dependent reversibility in experiments in which FSK was removed after 30 min (upper panel, solid bars), whereas CA-induced rounding was not reversible within the time-course of these experiments (lower panel, solid bars). Bars show means ± S.E.M. of 4 observations in an experiment typical of three separate experiments.

67·4 ± 1·4% cells rounded). In contrast, there was no significant reversal of the CA-induced rounding within the same period (Fig. 3, lower panel), although a significant number of CA-treated cells had reverted to the normal flattened morphology when the cells were examined 16 h after the removal of CA (37·9 ± 1·9% remained rounded versus 79·5 ± 0·5% rounded at 2 h). The cell rounding induced by either treatment was not accompanied by a detectable change in cell viability as assessed by the ability of the cells to exclude Trypan Blue (results not shown), or by changes in mitochondrial dehydrogenase activity (Table 1).

Effect of tyrosine phosphatase inhibitors on cell rounding

Y1 cell rounding induced by PKA activation was inhibited by the presence of phosphotyrosine phosphatase (PTP) inhibitors, as shown in Fig. 4. Thus, pretreatment of Y1 cells for 15 min with pervanadate (PV; 100 μM) or calpeptin (CP; 100 μg/ml) reduced the rounding response to a 60-min exposure to FSK (1 μM), while having little or

Table 1 Effects of PP1/2A inhibitors on NADH-dependent dehydrogenase activity in Y1 cells. Y1 cells were incubated for 3 or 6 hours in the presence of I μM forskolin (FSK) or calyculin A (CA, 0·1–10 nM) and NADH-dependent dehydrogenase activity was assessed by the formation of formazan from MTT during the incubation period. Values are expressed as % activity in control cells incubated in DMEM in the absence of FSK or CA and are shown as means ± S.E.M. (n=3)

	NADH-dependent dehydrogenase activity (% control)	
	3 hours	6 hours
Addition		
FSK 1 μM	109 ± 6	110 ± 4
CA 0·1 nM	108 ± 4	108 ± 3
CA 1·0 nM	105 ± 2	103 ± 4
CA 10 nM	106 ± 6	110 ± 7

Neither FSK nor CA had any significant effect on NADH-dependent dehydrogenase activity in Y1 cells (P>0·2).
MMT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

no effect on cell rounding in the absence of FSK. In contrast, neither inhibitor prevented the CA-induced changes in Y1 cell morphology. Pretreatment with PV had no effect on the rounding response to CA, while cells pretreated with CP became detached from the tissue culture plastic substrate on subsequent exposure to CA and could not be counted.

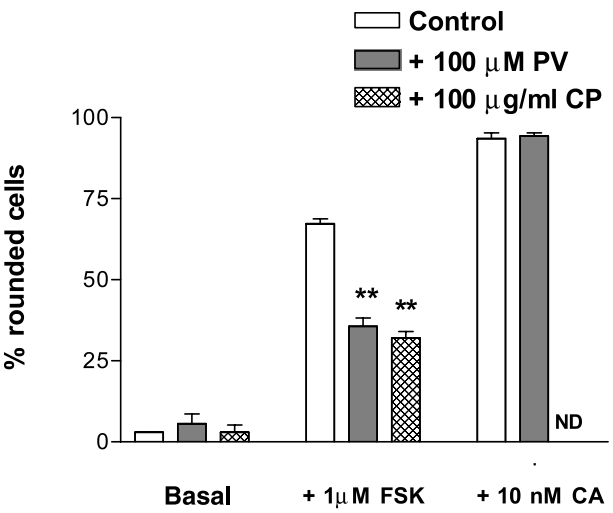

Figure 4 Effects of phosphotyrosine phosphatase (PTP) inhibitors on cell rounding. Exposure to FSK (1 μM) or CA (10 nM) for 60 min induced cell rounding in the majority of Y1 cells. FSK-induced rounding was significantly inhibited by pretreatment with the PTP inhibitors pervanadate (PV) (100 μM, solid bars) or calpeptin (CP) (100 μg/ml, hatched bars). In contrast, neither agent prevented CA-induced cell rounding. A numerical value for the effects of CP on CA-induced cell rounding could not be determined (ND) as this combination caused the cells to become non-adherent. Bars show means ± S.E.M., n=4. **P<0·01, significantly different from FSK-stimulated control.

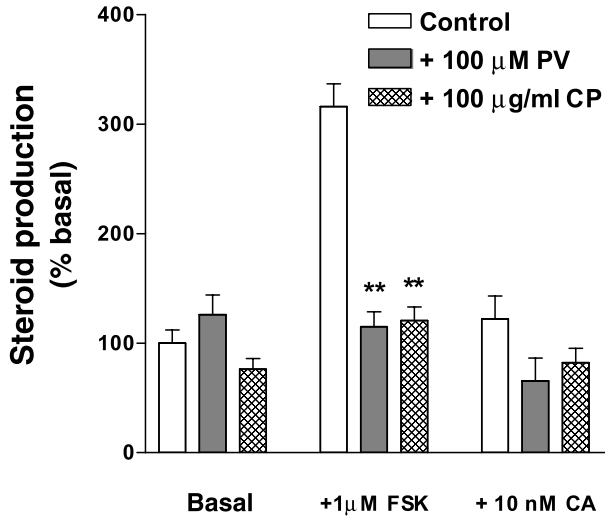

Figure 5 Effects of PTP inhibitors on steroid production. Exposure to FSK (1 μM, 3 h) caused a marked increase in steroid production by Y1 cells, which was eliminated in the presence of the PTP inhibitors PV (100 μM, solid bars) or CP (100 μg/ml, hatched bars). Neither PV nor CP significantly affected steroidogenesis in the presence of CA. Bars show means ± S.E.M., $n=8$. $^{**}P<0{\cdot}01$, significantly different from FSK-stimulated control.

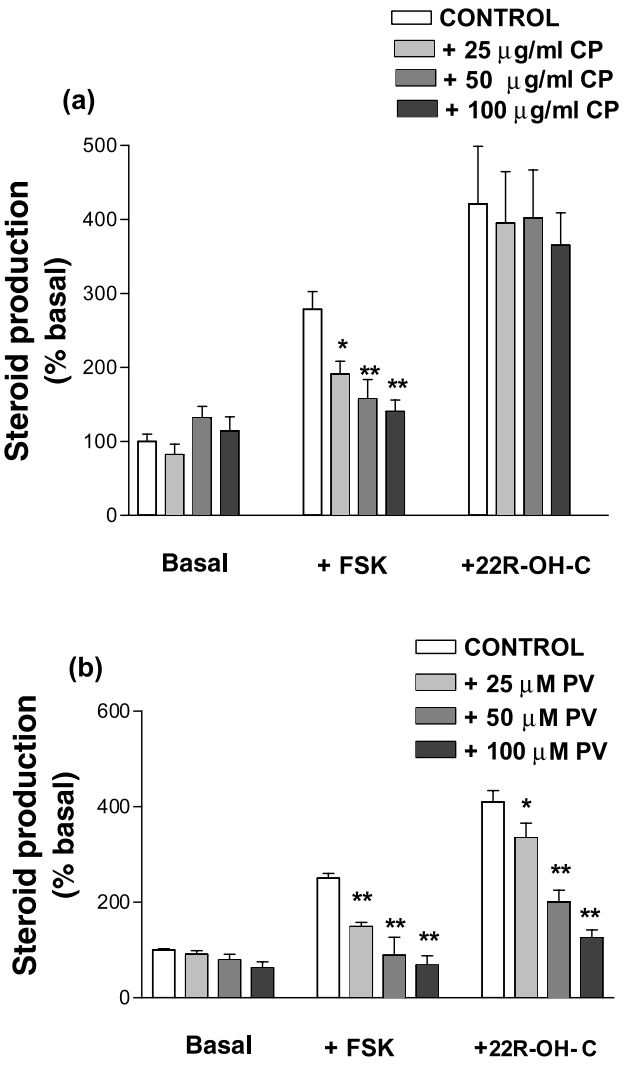

Figure 6 Effects of calpeptin and pervanadate on FSK- and 22R-hydroxycholesterol-stimulated steroidogenesis. (a) CP (25–100 μg/ml) reduced steroidogenesis stimulated by 1 μM FSK in a dose-related fashion but had no significant effect on basal production or the conversion of 22R-hydroxycholesterol (22R-OH-C) to pregnenolone by Y1 cells. (b) PV (25–100 μM) reduced both FSK-stimulated steroidogenesis and the conversion of 22R-OH-C to pregnenolone in a dose-related fashion. Bars show means ± S.E.M. $^{*}P<0{\cdot}01$, $^{**}P<0{\cdot}001$, significantly different from FSK or 22R-OH-C-stimulated control.

Steroid production

FSK-induced steroid production by Y1 cells was reduced to unstimulated levels by the presence of both PV and CP as shown in Fig. 5, while neither PTP inhibitor significantly affected basal steroid production. CA alone had no significant effect on steroid production when used at a concentration which induced cell rounding in >90% of Y1 cells (see Fig. 4). In accordance with this, combinations of CA plus PV or of CA plus CP had no marked effects on steroid production, despite the maintained cell rounding induced by these treatments. The effects of CP and PV on steroid production are examined in more detail in Fig. 6A and B, which shows a dose-related reduction of FSK-stimulated steroid production over the range 25 to 100 μg CP/ml with no significant effect on basal steroid production. It can also be seen that inhibition of PTP activity with CP did not affect the conversion of 22R-hydroxycholesterol to pregnenolone, even when CP was present at a concentration which greatly reduced FSK-induced cell rounding and steroid production. In contrast, while PV treatment (25, 50 and 100 μM) also significantly reduced FSK-stimulated steroid production, each of these doses also significantly reduced the yield of pregnenolone from 22R-hydroxycholesterol. (Fig. 6B).

Protein phosphorylation and protein synthesis

Figure 7A compares the effect of exposure to CP (100 μg/ml) and PV (100 μM) on tyrosine phosphorylation of proteins and shows a very marked increase in phosphorylation in the presence of PV which contrasts with the more selective changes obtained with CP. It is also noticeable that increased phosphorylation is visible in CP-treated tissue in the 76 kDa region, corresponding to the mobility of phosphorylated paxillin (Han & Rubin 1996).

Exposure to FSK reduced the tyrosine phosphorylation of paxillin in Y1 cell extracts, as shown in the blot obtained after paxillin immunoprecipitation (Fig. 7B, upper panel, lanes 1 and 2). The PKA-mediated reduction in paxillin

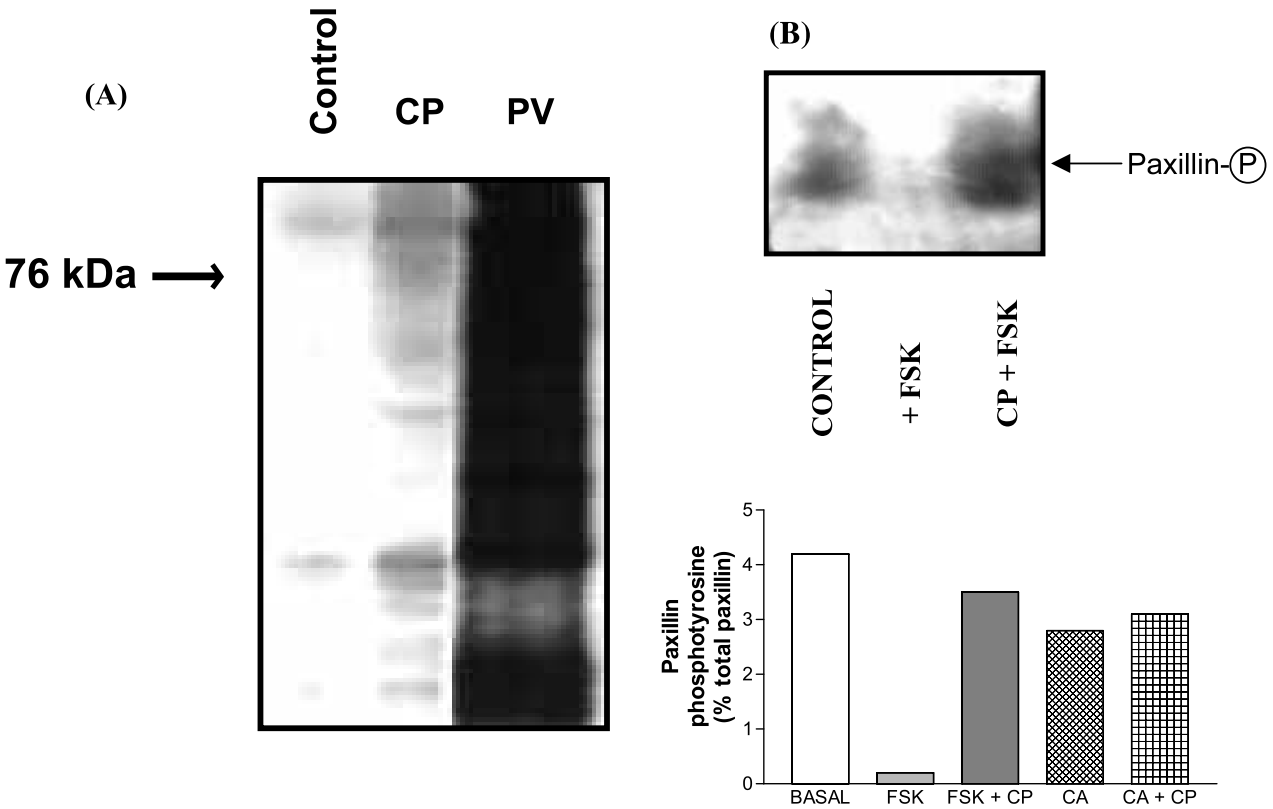

Figure 7 Tyrosine phosphorylation of proteins in Y1 cells. (A) The immunoblot shows tyrosine phosphorylation of Y1 cell proteins detected by probing with an anti-phosphotyrosine antibody following exposure to CP (100 μg/ml) and PV (100 μM) for 1 h. (B) The immunoblot (upper panel) shows tyrosine phosphorylation of paxillin after immunoprecipitation from Y1 cell extracts, detected by probing with an anti-phosphotyrosine antibody. The density of immunoblots was quantitated by scanning densitometry and expressed as a percentage of total paxillin as determined by stripping and reprobing the membrane with an anti-paxillin (phosphorylated and non-phosphorylated) antibody (lower panel). Exposure of Y1 cells to FSK (1 μM for 1 h) greatly reduced the tyrosine phosphorylation of paxillin in subsequent extracts compared with control cell extracts (Basal), and this FSK-induced reduction was blocked in the presence of CP (100 μg/ml). There was no difference in the tyrosine phosphorylation of paxillin between controls and extracts prepared after CA treatment (10 nM, 1 h) or following CA treatment in the presence of CP (100 μg/ml).

phosphorylation was prevented by the presence of CP (Fig. 7B, upper panel, lane 3), at a concentration which also inhibited FSK-induced cell rounding (Fig. 3). In contrast, exposure to CA alone, or to a combination of CA plus CP, had no marked effect on the extent of phosphorylation of paxillin (Fig 7B, lower panel).

Exposure to FSK for three hours had no detectable effect on the total synthesis of new proteins by Y1 cells, as assessed by ^{35}S-cysteine incorporation (+1 μM FSK, 90·1 ± 6·9% of control, mean ± S.E.M., n=5, P>0·2), whereas the additional presence of CA caused a marked reduction in total protein synthesis (1 μM FSK + 10 nM CA, 14·9 ± 7·1% of control, mean ± S.E.M., n=5, P<0·001).

Discusssion

The activation of the PKA-signalling pathway has been shown to exert dramatic effects on cytoskeletal architecture in a variety of secretory and non-secretory cell types; the responses include loss of actin stress fibres and focal adhesions, rounding of cells and in some cases detachment from the substratum (Spruill *et al.* 1981, Lampugnani *et al.* 1990, Glass & Kreisberg 1993, Han & Rubin 1996, Torgerson & McNiven 2000). In steroidogenic cells, these morphological changes often seem to be associated with enhanced steroid production, although details of the connection between the two processes remain uncertain (Cortese & Wolff 1978, Rainey *et al.* 1983, Betz & Hall 1987, Hall 1997). In the present study, we have investigated the relationship between changes in steroidogenesis and cell rounding in Y1 adrenocortical cells using inhibitors of phosphoprotein phosphatase activity to probe the system. The unstimulated Y1 cell has a flat epitheloid shape with structural support provided by a network of actin-containing filaments (stress fibres). These are anchored at the cell cortex by focal adhesions, multiprotein complexes which mediate signalling between the actin cytoskeleton and the extracellular matrix. The protein,

paxillin, has been identified as a component of focal adhesions and its tyrosine phosphorylation is known to be coupled to their assembly (Burridge *et al.* 1988, 1992). Cell rounding is associated with the dephosphorylation of paxillin and involves the disassembly of focal adhesions, dissolution of stress fibres and rearrangement of the cytoskeleton. In recent studies using Y1 and bovine adrenocortical cells, activation of PKA was found to cause the selective dephosphorylation of paxillin on tyrosine residues before any detectable change in cell shape occurred, and both events were blocked by inhibitors of protein tyrosine phosphatase activity, such as sodium orthovanadate and pervanadate (Han & Rubin 1996, Vilgrain *et al.* 1998). Recent evidence has identified one of the PTPs activated by ACTH in bovine adrenocortical cells as the cytosolic phosphatase, SHP2, suggesting that this enzyme plays a part in mediating the effects of stimulation (Rocchi *et al.* 2000).

Our results support and extend these observations by demonstrating that the PKA-mediated rounding of Y1 cells is prevented not only by pervanadate but also by the more specific PTP inhibitor, calpeptin. This compound, commonly used as an inhibitor of calpain, has recently been found also to inhibit tyrosine phosphatases with a preferential action on membrane-associated activities (Schoenwaelder & Burridge 1999). The inhibition of FSK-induced dephosphorylation of paxillin in Y1 cells by CP was accompanied by a reduction in FSK-induced cell rounding. This is consistent with a model of adrenocortical cell rounding in which the activation of PKA results in increased PTP activity leading to dephosphorylation of paxillin on tyrosine residues followed by disassembly of focal adhesions and stress fibres (Vilgrain *et al.* 1998).

The fact that PTP inhibition also inhibited steroid production further suggests that PKA-mediated cell rounding is inextricably linked with steroidogenesis. Both PV and CP reduced FSK-stimulated steroidogenesis to basal levels at the same time as reversing the effects of FSK on cell rounding to control levels. Furthermore, PTP inhibition by CP had no effect on steroid production from 22R-hydroxycholesterol, consistent with an action prior to cholesterol delivery to the inner mitochondrial membrane rather than any interference with mitochondrial steroidogenic enzyme activity. This conclusion is in accordance with recent reports that blocking PTP activity abolishes ACTH- and human choriogonadotrophin-stimulated steroidogenesis in rat adrenal and testicular interstitial cells. These observations were coupled with evidence demonstrating that activation of the PKA-signalling pathway leads to enhanced PTP activity (Paz *et al.* 1999, 2000, Cornejo Maciel *et al.* 2001). Our results suggest that one role for these enzymes may be in inducing the PKA-dependent morphological changes that precede increased steroid production, thereby enhancing the access of cholesterol to the mitochondrion.

PKA-dependent serine/threonine phosphorylation events are rapidly reversible by the actions of phosphoserine/threonine phosphatases, including PP1/2A, so it is perhaps not surprising at first sight that the inhibition of these activities in Y1 cells with CA produced morphological changes that appeared similar to those caused by the activation of PKA. However, our results suggest that the morphological changes induced by CA act through mechanisms different from those induced by FSK. First, there was a pronounced difference in the extent and reversibility of the cell rounding induced by FSK and CA, although it is possible that the slower reversibility of the CA-induced rounding may reflect a slower wash-out of CA from the Y1 cells. More importantly, and in contrast to FSK-induced cell rounding, CA-induced rounding was not blocked by inhibition of PTP activities and CA treatment did not lead to a reduction in the phosphotyrosine content of paxillin. These observations imply that PKA activation and inhibition of PP1/2A activity induce morphological changes in Y1 cells through fundamentally different mechanisms and also that CA-induced cell rounding is not linked to effects on steroid production. Since it is known that rounding of Y1 cells can occur as a non-specific response to cell injury (Betz & Hall 1987), our experiments might suggest that CA-induced rounding is related to the marked inhibition of total protein synthesis induced by this agent. However, the effects of CA did not appear to be cytotoxic in the short term as assessed by markers of cell viability; they may, instead, be related to the inactivation of elongation factors essential to protein synthesis when serine/threonine phosphatase activity is reduced (e.g. Jacobson & Peltz 1996). In addition, PP1/2A inhibitors have been found to cause shape changes in non-steroidogenic cell types and it has been suggested that microtubule-associated PP2A may play a role in controlling cytoskeletal organization (e.g. Usui *et al.* 1999). Nevertheless, it is clear from our results that inhibitors of PP1/2A have multiple effects on steroidogenic cell function and should, therefore, be used with caution in functional studies.

In conclusion, our results are consistent with PKA-induced shape changes in adrenocortical cells being mediated through the activation of tyrosine phosphatase activity and the dephosphorylation of paxillin, and support the view that the morphological and functional responses to activation of the PKA-signalling pathway in steroidogenic cells are intimately linked. In contrast, the mechanism through which inhibition of PP1/2A activities causes Y1 cells to round is fundamentally different from that seen in response to activation of PKA and is not associated with any facilitation of steroidogenesis.

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