Hormonal regulation of focal adhesions in bovine adrenocortical cells: induction of paxillin dephosphorylation by adrenocorticotrophic hormone

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A study of bovine adrenocortical cell shape on adrenocorticotrophic hormone (ACTH) challenge showed that the cells round up and develop arborized processes. This effect was found to be (1) specific for ACTH because angiotensin II and basic fibroblast growth factor have no effect; (2) mediated by a cAMP-dependent pathway because forskolin reproduces the effect of the hormone; (3) inhibited by sodium orthovanadate, a phosphotyrosine phosphatase inhibitor, but unchanged by okadaic acid, a serine/threonine phosphatase inhibitor; and (4) correlated with a complete loss of focal adhesions. Biochemical studies of the focal-adhesion-associated proteins showed that pp125^{fa}, vinculin (110 kDa) and paxillin (70 kDa) were detected in the Triton X-100-insoluble fraction from adrenocortical cells. During

cell adhesion on fibronectin as substratum, two major phosphotyrosine-containing proteins of molecular masses 125 and 68 kDa were immunodetected in the same fraction. A dramatic decrease in the extent of tyrosine phosphorylation of these proteins was observed within 60 min after treatment with ACTH. No change in pp125^{fa} tyrosine phosphorylation nor in Src activity was detected. In contrast, paxillin was found to be tyrosine-dephosphorylated in a time-dependent manner in ACTH-treated cells. Sodium orthovanadate completely prevented the effect of ACTH. These observations suggest a possible role for phosphotyrosine phosphatases in hormone-dependent cellular regulatory processes.

INTRODUCTION

The organization of cell adhesion sites is driven by a family of heterodimeric transmembrane glycoproteins known as integrins [1] that bind to components of the extracellular matrix such as fibronectin, vitronectin and laminin. The binding of integrins to ligands catalyses the recruitment of multiple cytoskeleton-associated proteins into structures called focal adhesions and simultaneously induces the reorganization and polymerization of actin stress fibres [2–4]. In addition to providing a structural link between cells and the extracellular matrix, focal adhesions represent important sites for signal transduction [4]. Notably, several known signalling enzymes including protein kinase C [5], c-Src [6] and the non-receptor tyrosine kinase, focal adhesion kinase (pp125^{fa}) [7], are localized at these sites. Integrin activation induces a substantial increase in the tyrosine phosphorylation of pp125^{fa}, which is correlated with enhanced pp125^{fa} activity [8,9]. Thus pp125^{fa} is a likely candidate for mediating integrin-regulated signalling pathways. The focal adhesion protein paxillin is also phosphorylated on tyrosine in response to integrin ligand interaction, and several studies suggest that paxillin is a substrate for pp125^{fa} [10,11]. One of the primary functions of phosphotyrosine residues is to provide docking sites for proteins that contain Src homology 2 (SH2) domains [12]. It follows that the tyrosine phosphorylation of proteins such as paxillin might facilitate the formation of protein complexes that could be essential for both the assembly of focal adhesion structures and the efficient processing of extracellular stimuli. In support of this hypothesis, treatment of fibroblasts with tyrosine kinase inhibitors blocks integrin-stimulated focal adhesion and stress fibre formation [13], and the inhibition of

tyrosine phosphatases stimulates focal adhesion formation [14].

We have used primary cultures of bovine adrenocortical cells from the fasciculata-reticularis zone as a model of endocrine cell regulation. These cells are highly responsive to adrenocorticotrophic hormone (ACTH), which acts via a G_s-coupled receptor. The effects of ACTH include increased steroid production, inhibition of thymidine incorporation into DNA [15], modification of extracellular matrix production [16] and cytoskeletal alterations [17]. In addition, it has long been known that ACTH stimulation affects cultured cell morphology by inducing the retraction of cell margins and the rounding-up of cells [17]. This effect has been observed in both normal and tumoral adrenocortical cells from various species and can be reproduced by the addition of permeant analogues of cAMP, the second messenger of ACTH [18,19]. In several other cell types, such as Chinese hamster ovary cells [20] and Sertoli cells [21], cAMP has been shown to induce similar changes in cell morphology. This suggests that a common cAMP-dependent pathway might exist to regulate cytoskeletal structure and cell adhesion.

The mechanism of ACTH-induced changes in cell shape has not been characterized in bovine adrenocortical cells. In the present study we have characterized the focal adhesions in these cells and the profile of tyrosine phosphorylation of their constituent proteins on ACTH treatment. We find that focal adhesions are disassembled after ACTH addition. This effect of ACTH seems to result mostly from the activation of phosphotyrosine phosphatases, leading to the dephosphorylation of paxillin. These observations suggest a possible role for phosphotyrosine phosphatases in ACTH-dependent cellular regulatory processes.

Abbreviations used: ACTH, adrenocorticotrophic hormone; ECL, enhanced chemiluminescence; FGF-2, basic fibroblast growth factor; PTPase, phosphotyrosine phosphatase.

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MATERIALS AND METHODS

Reagents

ACTH was from Ciba-Geigy (Rueil Malmaison, France). [γ - 32 P]ATP (3000 Ci/mmol) and the enhanced chemiluminescence (ECL) detection reagents were purchased from Dupont-NEN (Les Ulis, France). The anti-phosphotyrosine monoclonal antibody (clone 4G10) was from Upstate Biotechnology (New York, NY, U.S.A.). Monoclonal antibodies against human vinculin and chick paxillin were from Sigma (St. Louis, MO, U.S.A.) and from Transduction Laboratories (Lexington, KY, U.S.A.) respectively. The anti-Src antibody (Ab-1) was obtained from Oncogene Science (Cambridge, MA, U.S.A.). Rhodamine-conjugated rabbit Ig fraction against mouse IgG was from Cappel (Durham, NC, U.S.A.). Horseradish peroxidase-conjugated affinity-purified rabbit anti-(mouse IgG) was from Bio-Rad (Hercules, CA, U.S.A.). The peptide AEEEIYGEFEAKKK was synthesized by Neosystem (Strasbourg, France). Nitrocellulose was obtained from Schleicher and Schuell (Ecquevilly, France). The micro-bicinchoninic acid protein assay reagent kit was from Pierce (Oud Beijerland, The Netherlands). Aquamount Mountant was from BDH (Poole, Dorset, U.K.).

Cell culture

Bovine adrenocortical fasciculata reticularis cells were prepared from adrenal glands and grown at 37 °C in Ham's F-12 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% (v/v) horse serum, 2.5% (v/v) fetal calf serum and 1 mM glutamine as described previously [22]. At the onset of the culture more than 99% of the cells were immunoreactive for *P*-450_{scc} and 80–95% were immunoreactive for *P*-450 17 α , indicating that the cell population consisted of pure adrenocytes essentially of fasciculata reticularis origin [23].

Immunofluorescence studies

Adrenocortical cells were plated on coverslips precoated with 20 μ g/ml fibronectin. Cells were then fixed with 3.5% (w/v) paraformaldehyde in PBS for 20 min at room temperature and washed three times with PBS+ (10 mM Na₂HPO₄/137 mM NaCl/1.5 mM KH₂PO₄/2.7 mM KCl/0.5 mM MgCl₂/1 mM CaCl₂). Permeabilization was performed with methanol at –20 °C for 10 min followed by incubation in Triton X-100 (0.1% in PBS) for 10 min. The cells were washed three times in PBS+ and three times with 1 mM NH₄Cl, twice with PBS+; non-specific binding sites were then saturated with PBS/1 mg/ml BSA for 30 min. Incubation with the primary antibody was performed in PBS for 1 h at room temperature. After three washes with PBS+, the cells were then incubated for 1 h with the rhodamine-labelled secondary antibody. After three washes in PBS+, the nuclei were labelled with 1 μ g/ml Hoechst for 5 min and then washed three times with PBS+. The coverslips were then rinsed, dried in ethanol and mounted on glass slides with Aquamount mountant.

Preparation of cell extracts and immunoblotting

Adrenocortical cells (5×10^6) were grown on fibronectin-coated plates for 4 days, then stimulated with various factors for the indicated periods. For analysis of focal adhesion-associated proteins, at the end of the incubation, the medium was quickly removed and the cells were rinsed in CSK100 buffer [10 mM Pipes (pH 6.8)/100 mM NaCl/300 mM sucrose/3 mM MgCl₂/

0.5 mM CaCl₂/1 mM PMSF/3 μ g/ml leupeptin/3 μ g/ml aprotinin/1% (v/v) Triton X-100] for 5 min on ice. Cytoskeleton-associated proteins were solubilized by scraping and flushing in lysis buffer [20 mM Tris/acetate (pH 7)/0.27 M sucrose] [24] containing 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM benzamide, 4 μ g/ml leupeptin and 1 μ g/ml pepstatin A. The homogenate was then centrifuged at 12000 *g* at 4 °C for 10 min and the supernatants were kept at –80 °C until use. The protein determination was performed with the micro-bicinchoninic acid method.

For immunoblots, 80 μ g of protein of adrenocortical cell extract were analysed by SDS/PAGE [10% (w/v) acrylamide/0.2% bisacrylamide]. Proteins were then transferred from the gel to nitrocellulose for 1 h; the residual binding sites were blocked by incubating the filters for 1 h in PBS containing 0.05% (v/v) Tween 20 and 5% (v/v) non-fat milk. The blots were subsequently incubated for 1 h with rabbit anti-phosphotyrosine antibody (1 μ g/ml) or anti-vinculin antibody (1:500 dilution). After being washed, the blots were incubated for 1 h with horseradish peroxidase-conjugated rabbit anti-(mouse IgG) diluted in PBS/0.05% (v/v) Tween 20 (1:3000 dilution). Immunoreactive proteins were detected by chemiluminescence.

Determination of c-Src activity

Src activity was assayed essentially as described in [25]. In brief, 5 mg of protein from the cytoskeletal fraction of control or ACTH-treated cells were loaded on a 0.2 ml DEAE-cellulose column. The column was washed with 1 ml of 10 mM Tris/HCl pH 7.5, containing 2 mM dithiothreitol and 2% (v/v) glycerol. The enzyme was eluted with 200 μ l of Tris buffer containing 0.4 M NaCl. The eluate was diluted 1:10 with Tris buffer and loaded on a 0.2 ml heparin-Sepharose column. The enzyme was eluted with Tris buffer containing 50–500 mM NaCl (stepwise, 0.4 ml elution). Enzymic activity was assayed in each fraction in a final volume of 18 μ l containing 3 μ l of peptide (AEEEIYGEFEAKKK) [26], 6 μ l of enzymic fraction, 3 μ l of kinase buffer [10 mM Tris/HCl (pH 7.5)/2 mM dithiothreitol/10 mM MgCl₂/10 mM MnCl₂] and 6 μ l of a reactive solution containing 1 μ M [γ - 32 P]ATP (2×10^6 c.p.m., specific radioactivity 4500 Ci/mmol). After a 15 min incubation at 30 °C, the reaction was stopped by the addition of 10% (w/v) trichloroacetic acid with BSA as a carrier protein. The samples were centrifuged and the supernatants were spotted on phosphocellulose papers (Whatman P81), washed three times in 5 mM H₃PO₄ and counted in a β -counter.

Reproducibility of data and data presentation

Each experiment was repeated at least three times in identical or similar configurations with similar results. Each data point represents the mean $\pm$ S.D. for measurements from three different wells or dishes in the same experiment. Statistical analyses were performed with Student's *t* test.

RESULTS

Tyrosine phosphatases are required for ACTH-induced changes in adrenocortical cell morphology

Phase-contrast micrographs of adrenocortical cells grown on fibronectin-coated plates and subsequently treated with 10 nM ACTH, 1 μ M forskolin, 10 ng/ml basic fibroblast growth factor (FGF-2), or 10 nM angiotensin II are presented in Figure 1.

Figure 1 ACTH-induced changes in adrenocortical cell morphology can be blocked by sodium orthovanadate

Adrenocortical cells grown on fibronectin-coated plates were treated for 45 min with F12 alone (A); forskolin (B); 10 nM ACTH (C); 10 nM ACTH plus 1 mM sodium orthovanadate (D); 10 nM ACTH plus okadaic acid, either 5 nM (E) or 50 nM (F); 10 ng/ml FGF-2 (G); or 10 nM angiotensin II (H). Magnification $\times 320$. This experiment is representative of five additional experiments.

Control adrenocortical cells exhibited a fibroblastic aspect (Figure 1A). ACTH, which acts through a G_s -coupled receptor, induced a rapid rounding of the cells, a pronounced thickening around the nucleus, and a decrease in cytoplasmic phase detail (Figure 1B). The same effect was obtained with forskolin (Figure 1C). In contrast, angiotensin II and FGF-2, two biological factors regulating adrenocortical cells, were also tested because they activate the inositol phosphate and tyrosine kinase pathways respectively. As shown in Figures 1(G) and 1(H) respectively, neither FGF-2 nor angiotensin II had an effect on adrenocortical cell shape. In a time-course experiment we observed that the effect of ACTH was detectable after 45 min of hormone addition and up to 48 h after a single addition of the hormone (results not shown).

The rapid kinetics of this effect suggested that it was triggered through post-translational modification rather than through transcriptional control. This prompted us to examine the role of protein phosphorylation in this process. As shown in Figure 1(D), pretreatment of cells with sodium orthovanadate, a potent inhibitor of phosphotyrosine phosphatases (PTPases), com-

Figure 2 Characterization of focal adhesions in adrenocortical cells

Control adrenocortical cells (A,C) and ACTH-treated cells (B,D) were labelled with monoclonal anti-vinculin (upper panels) and anti-phosphotyrosine (lower panels) antibodies, followed by rhodamine-conjugated rabbit anti-(mouse IgG) antibodies. Magnification $\times 1000$.

pletely blocked the ACTH-induced modification of cell shape. In contrast, cells pretreated with okadaic acid, an inhibitor of serine/threonine phosphatases (Figures 1E and 1F) retained the ability to respond to ACTH. This observation strongly favours the involvement of protein tyrosine phosphatases in ACTH-induced adrenocortical cell shape modifications.

ACTH induces disruption of focal adhesions

Focal adhesions can be visualized by immunofluorescence with antibodies against vinculin and phosphotyrosine. We thus attempted to determine whether adrenocortical cells attach to a substratum through focal adhesions and whether these could be modified by ACTH. As shown in Figure 2(A), immunostaining of vinculin in control cells was typical for focal adhesions, distributed mainly at the periphery of the cells. This staining was detected only when the cells were grown on fibronectin as substratum. On poly-(L-lysine)-coated plates, no focal adhesions were detected (results not shown). When the cells were stimulated by 10 nM ACTH, the majority of vinculin staining was redistributed throughout the cytoplasm and no focal adhesions were detected (Figure 2B). Anti-phosphotyrosine staining in control adrenocortical cells was similar to that observed with vinculin (Figure 2C), indicating that tyrosine-phosphorylated proteins were concentrated in focal contacts. As shown in Figure 2(D), phosphotyrosine staining was greatly decreased at focal contacts on exposure to ACTH.

To expand and to confirm the results obtained by immunofluorescence studies, we analysed the amounts of vinculin and phosphotyrosine-containing proteins in control and ACTH-treated adrenocortical cells by Western blotting. Figure 3(B) shows that vinculin was detected as a 110 kDa protein, present in equivalent amounts in control and in ACTH-treated adrenocortical cells. This suggests that the loss of vinculin staining

Figure 3 Detection of phosphotyrosine-containing proteins in the Triton X-100-insoluble fraction from adrenocortical cells

Protein (80 μ g) from the cytoskeletal fraction from control (CTL) or ACTH-treated cells were immunoblotted with the monoclonal anti-phosphotyrosine antibody (4G10) (A) or the anti-vinculin antibody (B). Bands were detected by ECL. The positions of molecular mass standards (in kDa) are shown at the extreme left and right. This experiment is representative of three additional experiments.

observed in immunofluorescence experiments resulted mostly from a redistribution of vinculin within the cell rather than from a degradation of the protein on treatment with ACTH. As shown in Figure 3(A) the anti-phosphotyrosine antibody detected two major bands in control cells (lane 1) with apparent molecular masses of 120–130 and 70 kDa. Minor bands were also present with molecular masses of approx. 210, 60 and 40 kDa.

The addition of phosphatase inhibitors paralleled the results of the immunofluorescence studies. Figure 3(A), lane 2, shows that the phosphotyrosine signal increased in the presence of sodium orthovanadate. When 50 nM okadaic acid was present, the pattern was similar to that of untreated cells (Figure 3A, lane 3). No signal was detected when the cells were grown on poly-(L-lysine)-coated plates (results not shown). In ACTH-treated cells (Figure 3A, lane 4), a marked decrease was observed in the phosphotyrosine signal, especially for the 130 and 70 kDa bands, whereas the minor bands remained unchanged. When the cells were pretreated with sodium orthovanadate before the addition of ACTH (Figure 3A, lane 5) the pattern of phosphotyrosine was similar to cells treated with sodium orthovanadate alone (Figure 3A, lane 2). Treatment of the cells with okadaic acid (50 nM) before ACTH gave a pattern similar to that with ACTH alone (Figure 3A, lane 4). As sodium orthovanadate is a potent inhibitor of PTPases, we concluded that ACTH might stimulate a PTPase activity.

Effect of ACTH on tyrosine phosphorylation of focal adhesion proteins

Three tyrosine-phosphorylated components of focal adhesions are pp125^{fa}, c-Src and paxillin. We therefore tested adrenocortical cell extracts for the presence of pp125^{fa} and paxillin via Western blot, and for c-Src via an activity assay. Protein (80 μ g) from 1% (v/v) Triton X-100-soluble and Triton X-100-insoluble fractions was analysed by SDS/PAGE and Western blot with a mixture of anti-pp125^{fa} and anti-paxillin monoclonal antibodies. As shown in Figure 4 (right panel), pp125^{fa} was detected as a

Figure 4 Detection of pp125^{fa} and paxillin in the detergent-insoluble fraction of adrenocortical cells

Adrenocortical cells grown on fibronectin-coated plates were treated with or without ACTH (10 nM) for 45 min, then rinsed in 1 ml of CSK-100 buffer for 5 min at 4 °C. The insoluble fraction was called the cytoskeletal fraction. An 80 μ g sample of each fraction from control cells (C) and ACTH-treated cells (A) was immunoblotted with the monoclonal anti-pp125^{fa} antibody and the monoclonal anti-paxillin antibody (1 μ g/ml) as described in the Materials and methods section. Bands were detected by ECL. The positions of molecular mass standards (in kDa) are shown at the left. This experiment is representative of two additional experiments. Abbreviations: FAK, pp125^{fa}; PAX, paxillin.

125 kDa protein and paxillin as a 68–70 kDa protein in the Triton X-100-insoluble (cytoskeletal) fraction. No signal was detected in the detergent-soluble (cytoplasmic) fraction (Figure 4, left panel). Thus we used the Triton X-100-insoluble fraction to perform further biochemical analysis of focal adhesion proteins.

Immunoprecipitation of pp125^{fa} was performed from adrenocortical cells treated with ACTH (10 nM) for 5–60 min. Figure 5 (upper panel) shows that one phosphotyrosine-labelled protein at 125 kDa was detected in the immunoprecipitates from control adrenocortical cells. On challenge with ACTH, the tyrosine phosphorylation of pp125^{fa} was not significantly modified compared with control cells at any time of stimulation. Figure 5 (lower panel) shows that pp125^{fa} was present in an equivalent amount in all immunoprecipitates. We conclude that neither pp125^{fa} concentration nor phosphorylation state is affected by the addition of ACTH.

Figure 5 Characterization of pp125^{fa} in adrenocortical cells

Bovine adrenocortical cells were treated with 10 nM ACTH for 0, 5, 15, 30 or 60 min and the cytoskeletal fractions were prepared as described in the Materials and methods section. Proteins (500 μ g) were immunoprecipitated (IP) with the monoclonal anti-pp125^{fa} antibody (FAK). The immunoprecipitates were then Western blotted (WB) with the anti-phosphotyrosine (α -P Tyr) antibody (upper panel) or the anti-pp125^{fa} (α -FAK) antibody (lower panel). Bands were detected by ECL. The positions of molecular mass standards (in kDa) are shown at the left. This experiment is representative of two additional experiments.

Figure 6 Characterization of c-Src activity in adrenocortical cells

Bovine adrenocortical cells were treated for 45 min with or without 10 nM ACTH; the cytoskeletal fractions were obtained and c-Src activity was isolated as described in the Materials and methods section. The heparin Sepharose column was eluted with increasing salt concentrations and the activity was assayed in each fraction with the peptide AEEIYGEFEAKKK as substrate. Activity is shown as a function of fraction number. Each fraction was examined for the presence of pp60^{c-Src} via Western blotting with anti-Src antibody. Symbols: ●, Src activity; □, NaCl concentration. This experiment is representative of two additional experiments.

Transformation of embryonic chicken cells by pp60^{v-src} causes an extensive reorganization of focal contacts [27], which leads to reduced adhesion and rounded morphology. We therefore examined the endogenous c-Src kinase activity in the detergent-insoluble fractions from adrenocortical cells treated with or without ACTH, by using the optimal and specific peptide substrate for c-Src, AEEIYGEFEAKKK [26]. Figure 6 (upper panel) illustrates the elution profile of kinase activity observed when the Triton X-100-insoluble fraction of control adrenocortical cells was analysed on a heparin-Sepharose column. A single peak of activity was detected, eluting from the column between 150 and 250 mM NaCl. Analysis of each fraction by Western blot with an anti-Src antibody showed the presence of the immunoreactive band that was eluted with activity. Thus we conclude that in adrenocortical cells, c-Src activity and focal adhesion proteins are found in the same fraction. Equivalent

Figure 7 ACTH stimulates tyrosine dephosphorylation of paxillin in adrenocortical cells

Bovine adrenocortical cells were treated with 10 nM ACTH for the indicated periods. The cytoskeletal fractions were prepared as described in the Materials and methods section. (A) Cytoskeletal proteins (80 μg) were immunoblotted with the anti-phosphotyrosine antibody (WB α-P-Tyr) or with the anti-paxillin antibody (WB α-PAX). Bands were detected by ECL. The position of a molecular mass standard (in kDa) is shown at the left. (B) Protein (500 μg) was immunoprecipitated with the monoclonal anti-paxillin antibody. The immunoprecipitates were then Western blotted with the anti-phosphotyrosine antibody. Bands were detected by ECL. The lower band (50 kDa) is the Ig heavy chain. The arrow indicates the paxillin (PAX) protein. The positions of molecular mass standards (in kDa) are shown at the left. This experiment is representative of three additional experiments. Abbreviation: CTL, control.

amounts of cell lysates from ACTH-treated cells showed the same activity profile as control cells (Figure 6, lower panel) and no change in the specific activity of the endogenous c-Src was found after the addition of hormone.

ACTH triggered a rapid tyrosine dephosphorylation of paxillin

Treatment of adrenocortical cells with ACTH resulted in the dephosphorylation of a 70 kDa protein. The kinetics of the effect was characterized. As shown in the upper panel of Figure 7(A), the phosphotyrosine content of the 70 kDa protein decreased rapidly within 5–15 min of treatment with ACTH. Its size and phosphotyrosine content suggested that it might be paxillin. When the same blot was incubated with the anti-paxillin antibody (Figure 7A, lower panel), one protein was detected in equal amounts in each lane. To confirm that the 70 kDa phosphoprotein was indeed paxillin, immunoprecipitation with an anti-paxillin antibody was performed on the detergent-insoluble fraction. Adrenocortical cells were treated for between 0 and 60 min with ACTH in the presence or absence of sodium

Figure 8 Forskolin stimulates tyrosine dephosphorylation of paxillin in adrenocortical cells

(A) Adrenocortical cells grown on fibronectin-coated plates were treated for 45 min with F12 alone (control), 10 μ M forskolin or 10 μ M forskolin plus 1 mM sodium orthovanadate. Magnification $\times 320$. This experiment is representative of five additional experiments. (B) Bovine adrenocortical cells were treated with forskolin (FSK; 10 μ M) for the indicated time. The cytoskeletal fractions were prepared as described in the Materials and methods section. Protein (500 μ g) was immunoprecipitated (ip) with the monoclonal anti-paxillin antibody and analysed as described in the legend to Figure 7(B). Bands were detected by ECL. The lower band (50 kDa) is the Ig heavy chain (IgG). The upper arrow indicates the paxillin protein. The positions of molecular mass standards (in kDa) are shown at the left. This experiment is representative of three additional experiments. Abbreviations: WB, Western blot; α P-Tyr, anti-phosphotyrosine.

orthovanadate; the immunoprecipitates were analysed by Western blotting with an anti-phosphotyrosine antibody. As shown in Figure 7(B), control cells (lane 1) and control cells treated with sodium orthovanadate (lane 2) presented a broad band of molecular masses in the range 68–74 kDa. This pattern was typical for paxillin. ACTH treatment for 5 min (Figure 7B, lane 3), 15 min (lane 4) or 60 min (lane 5) led to a time-dependent decrease in paxillin phosphorylation. No modification in tyrosine phosphorylation of paxillin was observed when a pretreatment with sodium orthovanadate was performed before stimulation with ACTH (60 min; Figure 7B, lane 6). These results strongly suggest that treatment with ACTH induces tyrosine dephosphorylation of paxillin, which can be prevented by sodium orthovanadate.

cAMP is a mediator for tyrosine dephosphorylation of paxillin

Most effects of ACTH are mediated by a G_s -coupled receptor that stimulates cAMP production. To determine whether ACTH acts on cell shape and paxillin dephosphorylation via a cAMP-dependent pathway, we used forskolin to activate adenylate cyclase. Treatment of adrenocortical cells with 10 mM forskolin mimicked the effect of ACTH: cells rounded up and developed arborized processes. As with the hormone, the effect of forskolin was completely blocked by pretreatment with orthovanadate, as shown in Figure 8(A) (see also Figure 1). Similarly, paxillin phosphorylation decreased in response to treatment with forskolin, with the same kinetics as for treatment with ACTH. As shown in Figure 8(B), paxillin was immunoprecipitated from cells treated from 0 to 60 min with forskolin in the presence or in the absence of vanadate, then analysed by Western blotting with an anti-phosphotyrosine antibody. This decrease in phosphorylation was blocked when cells were pretreated with orthovanadate (Figure 8B, right lane). These results show that cAMP elevation can reproduce the effects of ACTH on cell shape and paxillin phosphorylation.

DISCUSSION

In the present study we found that in adrenocortical cells ACTH or its second messenger cAMP triggered a characteristic modification of cell shape. Angiotensin II, which activates the phospho-

inositide pathway [28], and FGF-2, which acts through its tyrosine kinase receptor [29], had no effect on cell morphology. In several other cell types, such as Chinese hamster ovary cells [20] and Sertoli cells [21], cAMP has been shown to alter cell morphology. The rat PC12 pheochromocytoma cell line responds to forskolin, a direct activator of adenylate cyclase, by proliferation arrest and neurite outgrowth [30]. In fibroblasts, marked changes in the actin microfilament network have been observed in response to cAMP [31]. The diversity of cell types exhibiting similar morphological changes in response to cAMP suggests that they involve common cellular mechanisms to regulate cytoskeletal structure and cell adhesion.

The mechanism by which ACTH induces adrenocortical cell shape changes has not previously been characterized. At the cellular level, we found that ACTH treatment causes a gradual thinning of the stress fibres (results not shown) accompanied by a decrease in the size of vinculin- and phosphotyrosine-containing adhesions at their extremities, leading to a loss of focal adhesions within 1 h. Focal adhesion disassembly has also been observed in fibroblasts treated with agents such as thrombospondins or vascular endothelial growth factor [32,33]. In adrenocortical cells, strikingly, the effect of ACTH or forskolin on cell shape can be completely blocked by the phosphotyrosine phosphatase inhibitor sodium orthovanadate. In contrast, the serine/threonine phosphatase inhibitor okadaic acid had no effect. This suggests strongly that the effect of ACTH via cAMP is dependent on the activation of PTPase activities.

For many cells in culture, focal adhesions are the most concentrated sites of tyrosine phosphorylation, which has a role in focal adhesion assembly [13]. Several tyrosine kinases have been identified in focal adhesions, including members of the Src family and pp125^{fa}. In bovine adrenocortical cells, we have analysed these tyrosine kinases and the regulation of their phosphorylation on tyrosine by ACTH. Tyrosine-phosphorylated pp125^{fa} was detected in adrenocortical cells grown on fibronectin as substratum; surprisingly, ACTH had no effect on its tyrosine phosphorylation. It has been reported [34–36] that tyrosine phosphorylation of pp125^{fa} can be stimulated by peptides binding to G_i / G_q -coupled receptors (i.e. bombesin, angiotensin and bradykinin) independently of integrin ligation and precedes focal adhesion assembly. As ACTH, which acts

through a G_s -coupled receptor, induces the disassembly of focal adhesions, we expected to see tyrosine dephosphorylation of pp125^{fak} on the addition of hormone; however, no change in the amount of protein or state of phosphorylation could be correlated with focal adhesion disassembly. However, several reports [37,38] suggest that pp125^{fak} has little or no role in the assembly of focal adhesions.

We further characterized the other tyrosine kinase, c-Src, known to associate with focal adhesions. In adrenocortical cells, pp60^{c-src} was present in both control and ACTH-treated cells at a lower level than in other tissues [25], and no difference in enzyme activity was found after the addition of hormone. It has been reported that c-Src activity can be abolished completely by the phosphorylation of a single tyrosine residue, Tyr-527 [39]. In transformed cells, where v-Src is constantly active, the adhesion sites are much smaller than in normal cells and adhesion is weakened. Our results show that in adrenocortical cells the tyrosine kinases pp125^{fak} and c-Src are neither activated nor inhibited by treatment with ACTH; thus it seems unlikely that they might be implicated in the focal adhesion disassembly induced by the hormone.

Examination of cytoskeletal tyrosine-phosphorylated proteins in adrenocortical cells after adhesion to fibronectin showed that proteins of molecular masses 210, 60 and 40 kDa remained unchanged on challenge with ACTH. A 130 kDa protein whose identity is as yet unknown was found to be tyrosine-dephosphorylated. Among the other cytoskeletal proteins, a 68 kDa protein was identified as paxillin and was found to be dephosphorylated within 10 min after the addition of either hormone or forskolin. Paxillin is commonly phosphorylated on tyrosine by pp125^{fak} during cell adhesion to fibronectin, but also on serine residues by a serine kinase of unknown identity [40]. In turn, phosphorylated paxillin recruits other signalling proteins by way of interactions with SH2 and/or SH3. Indeed, paxillin has been described to bind to the C-terminus of vinculin *in vitro* and *in vivo* [41] as well as to the SH3 domain of pp60^{c-src} [42] and to the SH2 domain of v-Crk [44]. Paxillin phosphorylation has previously been correlated with focal adhesion assembly [34–36]. Thus the tyrosine dephosphorylation of paxillin observed in ACTH-treated adrenocortical cells could represent a mechanism for the dissociation of complexes with proteins containing SH2 domains.

Interestingly, our results show that the effect of ACTH or cAMP on cell rounding is blocked by a tyrosine phosphatase inhibitor and that ACTH and cAMP decrease the tyrosine phosphorylation of a number of proteins associated with cytoskeletal structures. Thus PTPases are probably the regulators of focal adhesion disassembly in bovine adrenocortical cells. This hypothesis is supported by results showing that inhibition of tyrosine phosphatases stimulates focal adhesion formation [14]. In contrast, overexpression of PTPases in cells leads not only to the dephosphorylation of focal adhesions but also results in the disassembly of both focal adhesions and stress fibres [45]. Preliminary results from our laboratory show that the cytoskeletal fraction of adrenocortical cells contains two major PTPases as measured by an in-gel assay (S. Rocchi, I. Gaillard, E. Van Obberghen, E. M. Chambaz and I. Vilgrain, unpublished work) and both activities are rapidly activated in ACTH-treated cells. Investigations are in progress to examine whether, in adrenocortical cells, cAMP/cAMP-dependent protein kinase directly phosphorylates and activates a PTPase. The direct activation of PTPases by tyrosine phosphorylation has been well documented [45,46], whereas their regulation by serine/threonine phosphorylation has been documented indirectly [47,48].

The mechanism for focal adhesion disassembly is poorly understood. Inhibition of tyrosine kinases, activation of protein

tyrosine phosphatases, inhibition of Rho activity, cell detachment and various inhibitors of actin–myosin interaction all lead to the rapid disassembly of focal adhesions [44]. In bovine adrenocortical cells, our results could suggest the following mechanism for ACTH-induced focal adhesion disassembly: increase in cAMP → cAMP-dependent protein kinase activation → PTPase(s) phosphorylation and activation → paxillin dephosphorylation → dissociation of pp125^{fak}, paxillin, vinculin complex → focal adhesion disassembly. This model is supported by our time-course experiments, in which paxillin dephosphorylation precedes the redistribution of vinculin and subsequent loss of focal adhesions. This model could be relevant *in vivo* during the embryonic development of the adrenal cortex, which is under the control of ACTH [49]. Indeed, the fetal adrenal cortex is a dynamic organ in which cells proliferate in the periphery, migrate centripetally, differentiate to form the specialized cortical compartments and then undergo senescence [49].

In summary, bovine adrenocortical cell primary cultures, which are highly responsive to ACTH, are an interesting model for the examination of the mechanism of action of the hormone and its second messenger on cell shape. Our results strongly suggest that the activation of PTPases by cAMP and the tyrosine dephosphorylation of paxillin are important steps leading to focal adhesion disassembly. These observations suggest a role for PTPases in hormone-dependent cellular regulatory processes.

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REFERENCES

- Hynes, R. O. (1992) *Cell* **69**, 11–25
- Maher, P. A., Pasquale, E. B., Wang, J. Y. and Singer, S. J. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6576–6580
- Guan, J. L., Trevithick, J. E. and Hynes, R. O. (1991) *Cell. Regul.* **2**, 951–964
- Bockholt, S. M. and Burridge, K. (1993) *J. Biol. Chem.* **268**, 4565
- Barry, S. T. and Critchley, D. R. (1994) *J. Cell. Sci.* **107**, 2033–2045
- Clark, E. A., Shattil, S. J. and Brugge, J. S. (1994) *Trends Biochem. Sci.* **19**, 464–469
- Schaller, M. D. and Parsons, J. T. (1993) *Trends Cell. Biol.* **3**, 258–262
- Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. R. and Parsons, J. J. (1994) *Mol. Cell. Biol.* **14**, 1680–1688
- Xing, Z., Chen, H.-C., Nowlen, J. K., Taylor, S. J., Shalloway, D. and Guan, J. L. (1994) *Mol. Cell. Biol.* **5**, 413–421
- Turner, C. E., Glenney, J. R. and Burridge, K. (1990) *J. Cell Biol.* **111**, 1059–1068
- Bellis, S. L., Miller, J. T. and Turner, C. E. (1995) *J. Biol. Chem.* **270**, 17437–17441
- Turner, C. E. and Miller, J. T. (1994) *J. Cell Sci.* **107**, 1583–1586
- Burridge, K., Turner, C. E. and Romer, L. H. (1992) *J. Cell Biol.* **119**, 893–903
- Defillipi, P., Bozzo, C., Volpe, G., Romano, G., Venturino, M., Silengo, L. and Tarone, G. (1994) *Cell. Adhes. Commun.* **2**, 75–86
- Ramachandran, J. and Suyama, A. T. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 113–117
- Feige, J. J., Savona, S., Pellerin, S., Lefeuvre, B., Keramidas, M., Shi, D. L., Jiang, Z. and Chambaz, E. M. (1992) *Colloque INSERM* **222**, 259–271
- Rainey, W. E., Hornsby, P. J. and Shay, J. (1983) *Endocrinology (Baltimore)* **113**, 48–54
- Buckley, D. I. and Ramachandran, J. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7431–7435
- Mountjoy, K. G., Robbins, L. S., Mortrud, M. T. and Cone, R. D. (1992) *Science* **257**, 1248–1251
- Hsie, A. W. and Puck, T. T. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 358–361
- Spruill, W. A., White, M. G., Steiner, A. L., Tres, L. L. and Kierszenbaum, A. L. (1981) *Exp. Cell Res.* **13**, 131–148

- 22 Duperray, A. and Chambaz, E. M. (1980) *J. Steroid Biochem.* **13**, 1359–1364
- 23 Negoescu, A., Labbat-Moleur, F., Brambilla, E., Chambaz, E. M. and Feige, J. J. (1995) *Mol. Cell. Endocrinol.* **105**, 155–163
- 24 Chabre, O., Cornillon, F., Bottari, S. P., Chambaz, E. M. and Vilgrain, I. (1995) *Endocrinology (Baltimore)* **136**, 956–964
- 25 Chedin, M., Filhol, O., Duminy, C., Bolla, M., Roche, S., Chambaz, E. M. and Cochet, C. (1997) *Carcinogenesis* **18**, 1463
- 26 Songyang, Z. and Cantley, L. C. (1995) *Trends. Biochem. Sci.* **20**, 470–475
- 27 Zheng, X. M., Wang, Y. and Pallen, C. J. (1992) *Nature (London)* **359**, 336–339
- 28 Clyne, C. D., Nicol, M. R., MacDonald, S., Williams, B. C. and Walker, S. W. (1993) *Endocrinology (Baltimore)* **132**, 2206–2212
- 29 Savona, C., Chambaz, E. M. and Feige, J. J. (1991) *Growth Factors* **5**, 273–282
- 30 Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T. and Hidaka, H. (1990) *J. Biol. Chem.* **265**, 5267–5272
- 31 Downey, G. P., Elson, E. L., Schwab, III, B., Erzurum, S. C., Young, S. K. and Worthen, G. S. (1991) *J. Cell Biol.* **114**, 1179–1190
- 32 Murphy-Ullrich, J. E. and Hook, M. (1989) *J. Cell Biol.* **109**, 1309–1319
- 33 Enomoto, T., Okamoto, T. and Sato, J. D. (1994) *Biochem. Biophys. Res. Commun.* **202**, 1716–1723
- 34 Sinnett-Smith, J., Zachary, I., Valverde, A. M. and Rozengurt, E. (1993) *J. Biol. Chem.* **268**, 14261–14268
- 35 Leduc, I. and Meloche, S. (1995) *J. Biol. Chem.* **270**, 4401–4404
- 36 Leeb-Lundberg, L. M. F., Song, X.-H. and Mathis, S. (1994) *J. Biol. Chem.* **269**, 24328–24334
- 37 Hildebrand, J. D., Schaller, M. D. and Parsons, J. T. (1993) *J. Cell Biol.* **123**, 993–1005
- 38 Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sabue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., Yamamoto, T. and Aizawa, S. (1995) *Nature (London)* **377**, 539–542
- 39 Kaplan, K. B., Bibbins, K. B., Swedlow, J. R., Arnaud, M., Morgan, D. O. and Varmus, H. E. (1994) *EMBO J.* **13**, 4745–4756
- 40 Bellis, S. L., Perrotta, J. A., Curtis, M. S. and Turner, C. E. (1997) *Biochem. J.* **325**, 375–381
- 41 Wood, C. K., Turner, C. E., Jackson, P. and Critchley, D. R. (1994) *J. Cell Sci.* **107**, 709–717
- 42 Turner, C. E. and Miller, J. T. (1994) *J. Cell Sci.* **107**, 1583–1586
- 43 Shaller, M. D. and Parsons, J. T. (1995) *Mol. Cell. Biol.* **15**, 2635–2645
- 44 Burrridge, K., Chrzanowska-Wodnicka, M. and Zhong, C. (1997) *Trends Cell Biol.* **7**, 342–347
- 45 Bouchard, P., Zhao, Z., Banville, D., Dumas, F., Fischer, E. H. and Shan, S. H. (1994) *J. Biol. Chem.* **269**, 19585–19589
- 46 Den-Hertog, J., Tracy, S. S. and Hunter, T. (1994) *EMBO J.* **13**, 3020–3032
- 47 Brautigan, D. L. and Pinault, F. M. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6696–6700
- 48 Gaits, F., Li, Y., Bigay, J., Ragab, A., Ragab-Thomas, J. M. F. and Chap, H. (1996) *J. Biol. Chem.* **271**, 20151–20155
- 49 Mesiano, S. and Jaffe, R. B. (1997) *Endocr. Rev.* **18**, 378–403