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Comparison of Protein Phosphorylation Patterns Produced in Adrenal Cells by Activation of cAMP-dependent Protein Kinase and Ca-dependent Protein Kinase

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Bovine adrenal fasciculata cells, exposed to either ACTH or AII, synthesize glucocorticoids at an enhanced rate. It is generally accepted that the signaling pathways triggered by these two peptides are not identical. ACTH presumably acts via a cAMP-dependent protein kinase (PKA) and AII, via a calcium-dependent protein kinase. We have found that either peptide hormone stimulates synthesis of a mitochondrial phosphoprotein pp37, leading to accumulation of its proteolytically processed products pp30 and pp29. On the basis of a number of criteria, this 37 kDa protein is the bovine homolog of the 37 kDa protein that we have characterized in rodent steroidogenic tissue (Epstein L. F. and Orme-Johnson N. R.: *J. Biol. Chem* 266 (1991) 19,739–19,745). Further, bovine pp37 is phosphorylated when PKA or protein kinase C (PKC) is activated directly by $(Bu)_2$cAMP or PMA, respectively. These studies indicate that either pp37 is a common substrate for PKA and PKC in these cells or there is a common downstream kinase, which is activated by exposure to either ACTH or AII. Rat adrenal glomerulosa cells, exposed to either ACTH or AII, show an enhanced rate of mineralocorticoid synthesis. As for bovine fasciculata cells, it is thought that the signaling pathway triggered by ACTH differs from that triggered by AII. As we found for bovine fasciculata, pp37 is phosphorylated when the rat cells are exposed to either peptide hormone. However, in contrast to the finding for bovine fasciculata, while exposure of the rat glomerulosa cells to $(Bu)_2$cAMP does cause the synthesis of pp37, exposure of the cells to PMA does not. Taken together, these findings provide further evidence that the subcellular signaling events, triggered by the action of AII on bovine adrenal fasciculata and rat adrenal glomerulosa cells, differ. Further, the fact, that pp37 is phosphorylated only when the rate of steroidogenesis is enhanced, reaffirms its potential involvement in the signaling pathway that causes stimulation of steroid hormone biosynthesis.

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INTRODUCTION

ACTH action on adrenal cells results in the synthesis of cAMP and the subsequent activation of cAMP-dependent protein kinase (PKA) [1, 2]. Several observations suggest that PKA activity is essential for acute ACTH-stimulation of steroid hormone synthesis: cAMP directly stimulates steroidogenesis [3–5]; PKA is thought to be the only cAMP-binding protein in mammals [6]; and ACTH-stimulated steroidogenesis is greatly diminished in mouse adrenal tumor cells containing a mutated PKA [7]. By contrast, AII stimulates steroid synthesis in adrenal cells by a cAMP-independent, calcium-dependent signaling mechanism [8, 9]. Although these ACTH- and AII-stimulated signal transduction pathways are distinct, both ACTH- and AII-stimulated steroidogenesis are inhibited by CHX [10, 11], indicating that the rate-limiting step in this metabolic process may be regulated by a shared mechanism. While AII-stimulated changes in intracellular calcium could affect a variety of metabolic processes without activating a kinase, by analogy to the action of

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ACTH, AII may also regulate steroidogenesis via activation of a cellular kinase.

Protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase (Ca^{2+}/Cam PK) are two types of widely-distributed calcium-dependent kinases [12, 13]. Of these two kinases, the role of PKC in steroidogenesis has been examined most extensively [11, 14–18]. In both bovine and in rat glomerulosa cells, AII stimulates an activating translocation of PKC from the cytosol to the membrane [16, 17]. However, direct activation of PKC with the phorbol ester PMA stimulates steroidogenesis in bovine glomerulosa [15] and fasciculata cells [11, 19] but not in rat glomerulosa cells [17]. It is difficult to reconcile the observations that while in AII-stimulated bovine glomerulosa and fasciculata cells, PKC is activated and can stimulate steroidogenesis, in rat glomerulosa cells, PKC is activated but does not affect steroidogenesis. Nonetheless, these studies of different species, suggest that PKC may not be universally involved in regulation of steroidogenesis in AII-stimulated cells.

In previous studies we [20], Stocco and co-workers [21] and Elliott *et al.* [22] have detected *ca* 30 kDa proteins that are synthesized in a number of steroidogenic tissues and transformed cell lines when PKA is activated. The mitochondrial phosphoprotein pp37, that we and Stocco and co-workers have found, is rapidly processed proteolytically to a 30 kDa product pp30, which accumulates in mitochondria of steroidogenic cells. In the mitochondria of unstimulated control cells an unphosphorylated form of the protein, p30, accumulates. In the studies presented in this paper we compare phosphorylation of this protein in bovine fasciculata to that in rat glomerulosa when distinct second messenger pathways are activated. Our initial studies confirmed that AII stimulates a subcellular translocation of PKC in bovine fasciculata. When we compared bovine fasciculata to rat glomerulosa cells, we found that direct activation of PKC with PMA stimulates phosphorylation of pp37 in the bovine but not in the rat cells. The calcium ionophore, A23187, stimulates phosphorylation of the protein in both. Thus, the physiological effects and second messenger agonists that stimulate steroidogenesis also stimulate phosphorylation of pp37. These results support the hypothesis that pp37 is involved in the regulation of steroidogenesis. Moreover, the observation that PMA stimulates phosphorylation of pp37 in bovine fasciculata cells but not in rat glomerulosa cells may explain the observation that PKC is involved in AII-stimulated steroid hormone synthesis in bovine fasciculata but not in rat glomerulosa cells.

METHODS

Primary bovine adrenal cells were prepared by a modification of the method of Gospodarowicz *et al.* [23]. Greater than 90% of the cell population appeared to be fasciculata/reticularis by visual inspection, with the contaminating 10% being red blood cells. AII-stimulated cortisol synthesis in these fasciculata cells was 3000×10^{-15} mol/10^6 cells/min, whereas AII-stimulated aldosterone synthesis in these cells was 8×10^{-15} mol/10^6 cells/min. AII-stimulated aldosterone synthesis in glomerulosa cells has been shown to be 555×10^{-15} mol/10^6 cells/min [15]. Before use, cells were washed into amino acid-free Krebs–Ringer–bicarbonate buffer supplemented with 0.5% BSA, 0.2% glucose and 25 mM HEPES (KRBAG) [24] and incubated at 37°C. All the experiments, except the ^{32}P-labeling experiments, were performed on cells suspended at 1–2 × 10^6 cells/ml in KRBAG.

Rat adrenal zona glomerulosa (ZG) cells were isolated by methods previously described [25]. In brief, ZG cells were preincubated for 50 min at 37°C with collagenase (3.7 mg/ml) and DNase (0.05 mg/ml) in a modified amino acid-free Krebs–Ringer–bicarbonate buffer containing 0.1% BSA, 200 mg/dl glucose, with the potassium concentration adjusted to 3.7 mEq/l (KRBAG). Cells were pelleted by centrifugation, washed twice with KRBAG, preincubated for another 30 min at 37°C and washed again. By microscopic examination, there was less than 5% contamination of these cell preparations by fasciculata cells.

For [^{35}S]methionine/cysteine incorporation, cells were suspended in KRBAG at 1–2 × 10^6 cells/ml and were stimulated with peptide hormone or second messenger analog for 5 min, then exposed to 1 mCi/ml [^{35}S]Met/Cys for 20–30 min at 37°C. Label incorporation was terminated by the addition of a 10-fold excess of cold phosphate buffered saline (PBS) composed of 10 mM KPi plus 9% NaCl (w/v), pH 7.4. The cells were pelleted at 150 ***g***, the supernatants were discarded and the cell pellets frozen at −20°C.

For [^{32}P]orthophosphate incorporation, cells were washed 5 × into PO_4-free Dulbecco's Modified Eagle's Medium (DMEM) and suspended in a final concentration of 2 × 10^6 cells/ml. The peptide hormones and other reagents were dissolved in KRBAG; a total of 3% KRBAG was added to each reaction tube, including control samples. This amount of KRBAG increased the unlabeled phosphate about 300-fold and was added so that the cells would not be limited for phosphate. The labeling reaction was quenched by the addition of a 10-fold excess of cold PBS. The cells were pelleted at 150 ***g*** as above and were washed again in 1 ml cold PBS. Then they were pelleted and frozen at −20°C.

Two-dimensional electrophoresis gels (2D-PAGE) of bovine and rat cell samples were run according to the procedures of O'Farrell *et al.* [26, 27]. Frozen cell pellets were solubilized directly with Garrels' buffer composed of 9.5 mM urea 4% NP-40, 100 mM dithiothreitol, and 2% ampholytes (from Pharmacia) [28] and loaded onto a first dimension tube gel. For bovine cell samples, the first dimension tube gels were 17 cm and were made with 2% ampholytes pH 6.0–8.0, 4%

acrylamide, 4% NP-40, and 8.5 M urea. The gels were loaded at the acidic end and were focused in reverse for 7500 Vh. Running the tubes in reverse yields a more basic gel than running them forward using the same ampholyte ratio in each case. For rat cell samples, the first dimension tubes were 15 cm and were made with 0.8% pH 5.0–7.0, 0.8% pH 6.0–8.0 and 0.4% pH 3.5–10.0 ampholytes, 4% acrylamide, 4% NP-40, and 8.5 M urea. The gels were loaded at the basic end and were focused forward for 10,000 Vh.

For both cell types the tube gels were equilibrated in 5 ml SDS-sample buffer for 10 min twice then frozen until used. 11% SDS–polyacrylamide slab gels were used to resolve protein in the second dimension. These gels were overlaid with 1% agarose stack containing 125 mM Tris, pH 6.8. The first-dimension tube gels were applied to the top of the stack along with agarose chips that contain dansylated molecular weight markers. These markers include, conalbumin (76 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (21 kDa). The second dimension gels were run overnight at 50 V, constant voltage. ^{35}S-labeled gels were washed twice with water for 10 min and incubated for 20 min in 1 M sodium salicylate with a pH between 5 and 7, then dried [29].

Quantification of pp37 and pp30

Proteins on the gels were quantified using a Millipore Bio Image Visage 110 computer-assisted image analysis system. To control for protein loading among these gels, an additional 3–14 well-resolved proteins that were common to each of the gels within an experiment were scanned. The values presented are ratios of the computer-generated integrated intensity of the proteins of interest over the sum of integrated intensities of the common proteins.

PKC blotting

These procedures are essentially those of Leach and co-workers [30]. 2×10^6 bovine fasciculata cells in the absence or presence of several stimulants for 15 min at 37°C. These samples were quenched in 10 vol of cold PBS containing 0.5 mM EDTA and 0.5 mM EGTA. The cells were pelleted, resuspended in 1 ml PBS + 0.5 mM EDTA + 0.5 mM EGTA and sonicated for 6 s. The sonicate was centrifuged for 2 min at 600 *g* and this supernatant was subsequently centrifuged at 100,000 *g* for 1 h. The resulting supernatant was considered to be the cytosol and the pellet was considered to be the membrane fraction.

Protein was measured in these samples by the method of [31]. 50–100 μg aliquots of protein were loaded in each lane of a one-dimension 7.5% SDS–PAGE gel. Pellet samples were loaded directly. Cytosol samples were precipitated with an equal volume of 20% trichloroacetic acid, on ice for 15 min. The precipitate was collected by centrifugation (15 min, microfuge). The supernatant was aspirated with a drawn-out 9 inch pipette and the pellet was gently washed with 100% ethanol. The pellet was dissolved in SDS-sample buffer and boiled. Adjustments to the pH of the SDS-sample buffer were done using Tris, pH 8.8. 50–100 μg aliquots of protein were loaded into each lane of a one-dimensional 7.5% SDS–PAGE gel. After electrophoresis the proteins were transferred to nitrocellulose by electrophoretic blotting and the blot was probed with a mixture of ^{125}I-labeled monoclonal antibodies directed against α-PKC and β-PKC [30]. The antibodies were the kind gift of Dr Karen Leach of the Upjohn Company, Kalamazoo, MI.

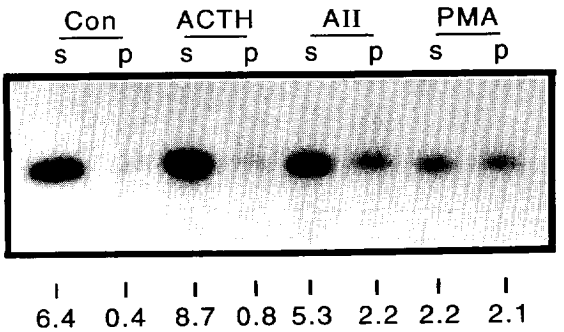

Fig. 1. AII-stimulated translocation of PKC in bovine fasciculata cells. 2×10^6 bovine fasciculata cells were incubated without stimulant or with 1 μg/ml ACTH or 1 μg/ml AII or 400 nM PMA for 15 min at 37°C. The cells were separated into soluble cytosolic (s) and membrane pellet (p) fractions as described in Methods. SDS–PAGE electrophoresis, blotting and probing were also carried out as described in Methods. Quantitation of the density of the PKC bands is indicated below the autoradiogram.

RESULTS

In bovine and rat glomerulosa cells, AII stimulates PKC to translocate from the cytosol to the plasma membrane [16, 17]. To determine if AII had a similar effect in bovine fasciculata cells, these cells were stimulated with ACTH, AII or PMA and membranes were separated from cytosol by differential centrifugation.. Equal amounts of cellular proteins were separated by 1D-PAGE, blotted and PKC was unidentified using a mixture of monoclonal antibodies directed against α-PKC and β-PKC and visualized by ^{125}I-labeled protein A. The resulting autoradiograph, shown in Fig. 1, was scanned and the integrated intensities of the PKC bands are presented in Table 1. These values

Table 1. Subcellular localization of PKC in bovine fasciculata cells

	Control	ACTH	AII	PMA
Supernatant*	6.4	8.7	5.3	2.2
Pellet†	0.4	0.8	2.2	2.1

These values represent the integrated intensities of bands.

*The 100,000 *g* supernatant.

†The 100,000 *g* pellet.

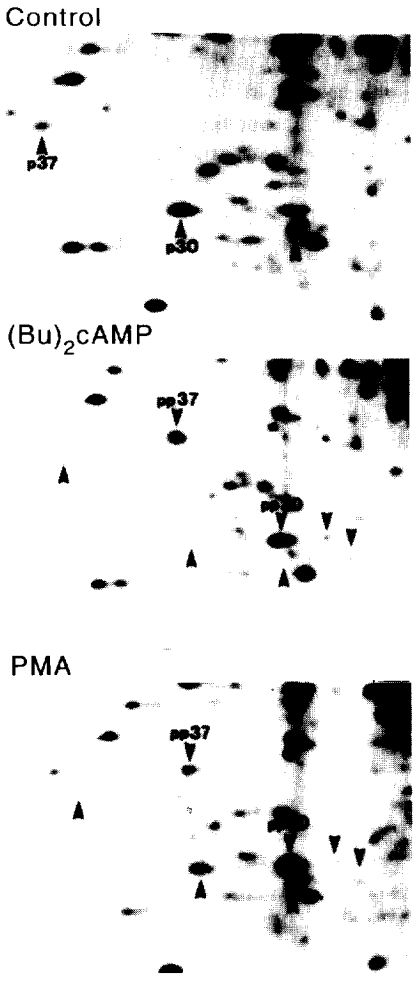

Fig. 2. p30 and pp30 in bovine fasciculata cells stimulated with $(Bu)_2$cAMP or PMA. Fluorograms of 2D-PAGE gels of bovine fasciculata cells labeled with radioactive amino acids and stimulated with $(Bu)_2$cAMP or PMA. Aliquots of 2×10^5 cells were incubated without stimulant or with 4.5 mM $(Bu)_2$cAMP or 400 nM PMA for 5 min at 37°C. Then these cells were labeled with 1.0 mCi/ml [^{35}S]Met/Cys for an additional 20 min at 37°C. After labeling, the cells were prepared for and subjected to 2D-PAGE as described in Methods. Up arrowheads indicate the proteins of interest in control cells, up arrowheads also indicate the relative positions of these proteins in stimulated cells. Down arrowheads indicate the proteins of interest in stimulated cells. These gels are representative of experiments that have been repeated three times for 8BrcAMP and more than ten times for $(Bu)_2$cAMP and PMA.

demonstrate that while ACTH has no effect on the subcellular location of the kinase, AII stimulates translocation of PKC from the cytosol to the membrane fraction. PMA also stimulates translocation of this enzyme. The sum of the PKC intensities (cytosol + membrane) in the PMA-stimulated lanes is about 50% of that in the control or peptide hormone-stimulated lanes suggesting that PKC is rapidly degraded in PMA-stimulated cells [12].

The involvement of PKC in the accumulation of the phosphoprotein, pp37, and its proteolytically processed product pp30, were examined using second messenger analogs to stimulate bovine fasciculata cells. Figure 2 shows 2D-PAGE of bovine fasciculata cells incubated in the absence or presence of second messenger analogs and labeled with [^{35}S]Met/Cys for 15 min. These cells were stimulated with PMA to activate PKC or, for comparison, with $(Bu)_2$cAMP to activate PKA. Figure 2 shows that the phosphoproteins, pp37 and pp30 were produced in cells stimulated with these second messenger agonists. Addition of the inactive phorbol ester (4α-phorbol 12,13-didecanoate) to the cells did not stimulate phosphorylation of these proteins (data not shown) suggesting that the effect of PMA is specific for the activation of PKC.

Figure 3 shows 2D-PAGE of bovine fasciculata cells labeled with [^{32}P]orthophosphate for 60 min. These

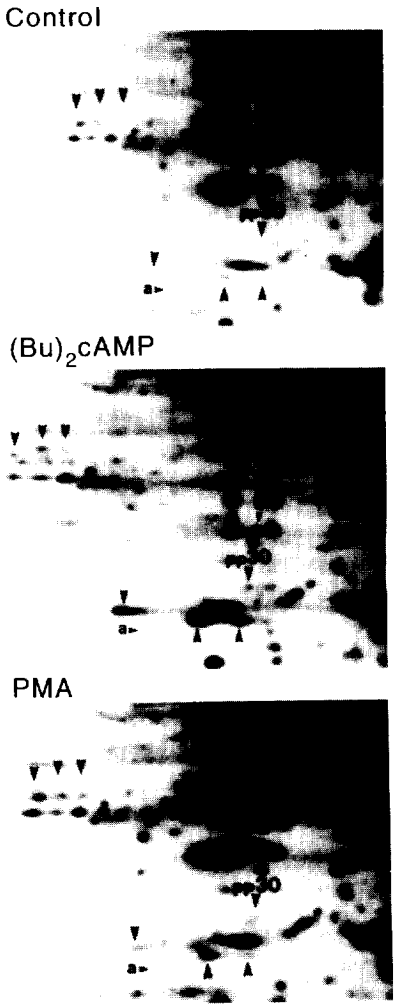

Fig. 3. Phosphate-labeling of $(Bu)_2$cAMP- and PMA-stimulated cells. Autoradiograms of 2D-PAGE gels of bovine fasciculata cells labeled with radioactive orthophosphate and stimulated with $(Bu)_2$cAMP or PMA. Bovine fasciculata cells were washed 5× in PO_4-free DMEM and resuspended in this medium at 2×10^6 cells/ml. 100 μl aliquots were labeled for 1 h with 1 mCi/ml [^{32}P]orthophosphate at 37°C. During this labeling period, these cells were incubated with no stimulant or with 4.5 mM $(Bu)_2$cAMP or 400 nM PMA. 2D-PAGE was performed as in Fig. 1. These gels are representative of five repetitions of this experiment.

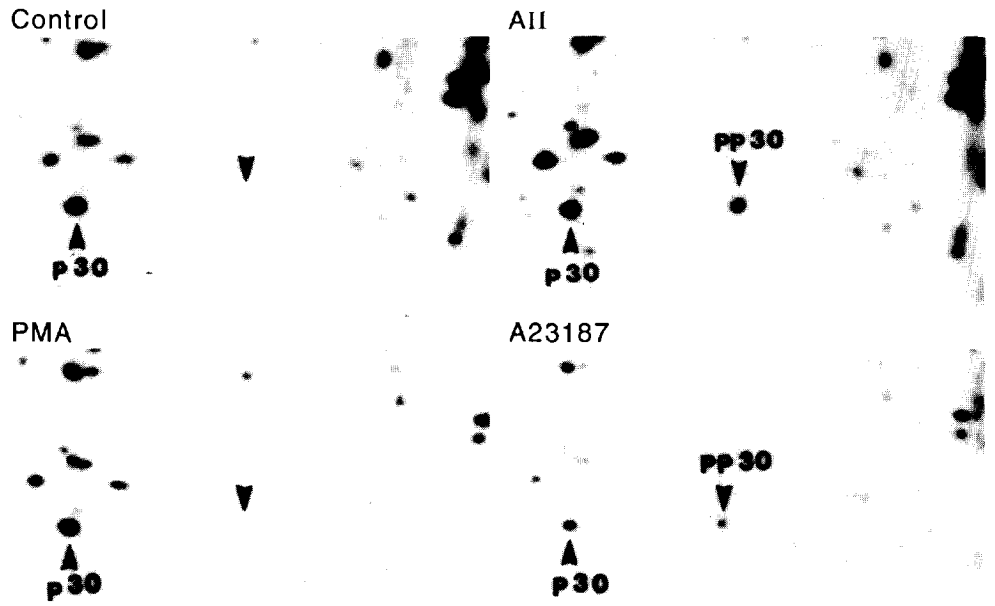

Fig. 4. p30 and pp30 in PMA- and A23187-stimulated rat glomerulosa cells. Fluorograms of 2D-PAGE of rat glomerulosa cells labeled with radioactive amino acids and stimulated with AII, PMA, or A23187. Aliquots of cells (100 μl containing 1×10^5 cells) were incubated for 5 min without stimulant or with 10 nM AII or 400 nM PMA or 10 μM A23187 at 37°C. The samples were then labeled for 30 min with 1.0 mCi/ml [^{35}S]Met/Cys. After labeling, the cells were prepared for and subjected to 2D-PAGE as described in Methods. Up arrowheads indicate p30 and p30′ in control cells, up arrowheads also indicate the relative positions of these proteins in gels of stimulated cells. Down arrowheads indicate pp30 and pp30′ in gels of stimulated cells. These gels are representative of gels obtained in 3 independent experiments.

cells were incubated without stimulant or with $(Bu)_2$cAMP or PMA during the labeling period and show that pp30 accumulates in response to either second messenger agonist. As we have shown for ACTH and AII (JAH and NROJ, in preparation), $(Bu)_2$cAMP and PMA simulate many changes in phosphorylation in these cells compared to control cells. Some changes are similar, indicated by large arrowheads, and some are specific, for the two agonists. Phosphoprotein "a" is specifically found in PMA-stimulated cells (and also in AII-stimulated cells) and phosphoprotein "b" is specific for $(Bu)_2$cAMP-stimulated cells (and ACTH-stimulated cells).

Published reports that PMA stimulates steroid synthesis in bovine fasciculata and glomerulosa cells but not in rat glomerulosa cells suggest that these species respond differently to direct activation of PKC. Thus it was of interest to evaluate the effect of PMA on phosphorylation of pp37 in rat glomerulosa cells. Glomerulosa cells were incubated without stimulant or with AII or PMA and also with A23187 for 5 min at 37°C. The cells were then labeled with [^{35}S]Met/Cys for 30 min. The long labeling time (30 min) and the rapidity of processing in rat adrenal cells [20] make it difficult to detect pp37 in the gels shown and only p30 and pp30 are indicated. The fluorograms in Fig. 4 show no detectable accumulation of pp30 in PMA-stimulated cells whereas A23187 stimulates the accumulation of this phosphoprotein. As was found in bovine fasciculata, A23187 stimulates only a small increase in phosphorylation of pp30 in rat glomerulosa. The results of scanning these fluorograms are shown in Table 2 and indicate that this small A23187-stimulated increase is similar to that observed in AII-stimulated rat glomerulosa cells.

As A23187 stimulates phosphorylation of pp37 in rat glomerulosa cells, it was also of interest to examine the effect of A23187 on phosphorylation of bovine pp37. Fasciculata cells were labeled with [^{35}S]Met/Cys and stimulated with A23187 or PMA. The cellular proteins were separated by 2D-PAGE and the resulting fluorograms were scanned. Table 3 shows the sum of the integrated intensities of the phosphoproteins pp30 and pp30′ for each condition and also these sums expressed as percentages of the sums of all 30 kDa forms. Both PMA and A23187 stimulated phosphorylation of these proteins in bovine fasciculata cells. These scanning

Table 2. PMA- and A23187-stimulated phosphorylation of rat proteins

	% unphosphorylated (p30)	% phosphorylated (pp30)
Control	100	0
AII	70	30
PMA	97	3
A23187	67	33

These values are calculated integrated intensities from scanned 2D-PAGE gels shown in Fig. 4.

Table 3. PMA- and A23187-stimulated phosphorylation of bovine proteins

	% unphosphorylated (p30)	% phosphorylated (pp37 + pp30)
Control	70	30
AII	10	90
PMA	33	67
A23187	52	48

results indicate that the background level of phosphorylation (control) is high, however, A23187 consistently stimulates a small increase in phosphorylation of the protein.

Several groups have published that steroid hormone synthesis is enhanced in bovine fasciculata and glomerulosa cells stimulated with PMA and A23187 [11, 15, 19]. These published experiments were repeated for bovine fasciculata cells. Cells were incubated without stimulus or with PMA or A23187 and aliquots of cells were quenched at 0 and 10 min. Cortisol was measured at each time point and steroidogenic rates were determined for each condition. The results of several experiments are shown in Table 4. PMA stimulates a mean 8.5-fold increase over control with a range between 2- and 17-fold and 10–30% of the response is stimulated by ACTH. The response to A23187 is more modest, with a mean 2.6-fold increase and a range of 0.2- and 6.7-fold increase above control.

DISCUSSION

To understand the role of PKC in the signal transduction pathway utilized by AII in bovine fasciculata and rat glomerulosa cells, it seemed reasonable to look for PMA-stimulated phosphorylation of pp37 in these cells. Our initial studies confirmed that AII activates PKC in bovine fasciculata cells. It has been shown previously, that AII stimulates translocation of PKC from the cytosol to the plasma membrane in bovine and rat glomerulosa cells [16, 17]. We repeated this experiment in bovine fasciculata cells and verified that AII stimulates an activating translocation of the kinase in these cells. Additionally, we labeled bovine fasciculata cells with [^{32}P]orthophosphate and found that PMA stimulated a similar phosphorylation pattern as AII (JAH and NROJ, in preparation). Specifically, PMA stimulates phosphorylation of protein "a" and did not stimulate phosphorylation of protein "b" found in ACTH- and $(Bu)_2$cAMP-stimulated cells (JAH and NROJ, in preparation). These results suggest that AII activates a similar kinase as PMA, that is PKC.

*Table 4. Cortisol synthesis in PMA- and A23187-stimulated bovine fasciculata cells**

	Exp. 1†	Exp. 2†	Exp. 3†	Exp. 4†
Control	1.48	5.99	3.55	1.42
PMA	10.45	12.98	28.66	23.94
A23187	1.95	7.61	4.12	9.51

*These values are ng cortisol/10^6 cells/10 min.

†The experiments presented here correspond to those shown in Table 3.

The results of our studies and those of others indicate that AII activates PKC [16, 17]. We compared the action of PKC in bovine fasciculata and rat glomerulosa cells and found that direct activation of PKC using PMA stimulated phosphorylation of pp37 in bovine fasciculata cells but not in rat glomerulosa cells. This result is of particular interest because AII also activates PKC in rat cells [17]. Moreover, this observation coincides with the apparent bovine-specific involvement of PKC in the regulation of steroidogenesis and thus increases the correlative evidence that pp37 contributes to this regulation. It is possible that bovine pp37 has PKC phosphorylation sites that are not present in the rat protein. Our results are consistent with such a mechanism and could explain the differential involvement of PKC in regulation of steroid hormone synthesis in bovine and rat adrenal cells.

The rat protein is not phosphorylated in cells stimulated with PMA, however, pp30 does accumulate in glomerulosa cells incubated with A23187. Both A23187 and AII stimulate accumulation of pp30 to a similar extent in rat glomerulosa cells consistent with the hypothesis that the ionophore stimulates the same calcium-dependent kinase utilized by AII in these cells. Additionally, pp37 is phosphorylated in bovine fasciculata cells stimulated with the ionophore. Because PKC phosphorylates bovine pp37, A23187 could be activating PKC in these cells. However, the effect of A23187 on phosphorylation of pp37 is modest in both bovine fasciculata and rat glomerulosa cells which could reflect activation of a similar mechanism by calcium influx in both species.

The second messenger agonists A23187 and PMA are reported to have similar and different effects, respectively, on steroidogenesis in bovine and rat adrenal cells. These similarities and differences are reflected in the ability of these effectors to stimulate phosphorylation of pp37. Although a quantitative relationship between steroid synthesis and protein phosphorylation was not found (determined for bovine only), the occurrence of phosphorylation of pp37 correlates with stimulated steroidogenesis. Thus, these results further support the hypothesis that pp37 is involved in regulation of steroid hormone synthesis.

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