

RAPID COMMUNICATION

Possible Involvement of Calcium/Calmodulin-Dependent Protein Kinase in ACTH-Induced Expression of the Steroidogenic Acute Regulatory (StAR) Protein in Bovine Adrenal Fasciculata Cells

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Abstracts. Steroidogenic acute regulatory (StAR) protein plays a crucial role in the regulation of cholesterol transport from the outer mitochondrial membrane to the inner membrane, where P450_{scc} participates in a rate-limiting step of adrenal steroidogenesis. We have already reported that both of cAMP- and protein kinase C-dependent processes may play a crucial role in the regulation of expression of StAR protein when bovine fasciculata cells are stimulated with ACTH. In the present study, ACTH increased cytosolic calcium movement and activated expression of StAR protein, resulting in enhancing cortisol production by bovine adrenal fasciculata cells. The role of the calcium/calmodulin-dependent protein kinase process in the regulation of expression of the StAR protein by ACTH was studied. The activating effects of ACTH on the StAR protein and cortisol production were inhibited by pretreatment with KN-93, a specific inhibitor of calcium/calmodulin-dependent protein kinase II. These findings suggest that ACTH can enhance expression of the StAR protein as well as cortisol synthesis in bovine adrenal fasciculata cells, in part via a calcium/calmodulin-dependent protein kinase process.

Key words: ACTH, Calcium/calmodulin-dependent protein kinase, StAR protein

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ACTH affects the conversion of cholesterol to pregnenolone by cholesterol side-chain cleavage complex, resulting in enhancing cortisol biosynthesis [1]. It has recently been reported that a 30 kDa protein was purified as the steroidogenic acute regulatory (StAR) protein [2] which facilitates the translocation of cholesterol from the outer to the inner mitochondria membrane, where P450_{scc} is located [3–5]. Several observations suggest that cAMP-dependent protein kinase activity is essential for acute ACTH-stimulation of steroid hormone synthesis [1], while calcium messenger systems also

play roles in the regulation of steroidogenesis activated by ACTH. We recently reported that ACTH stimulates expression of StAR protein via both a cAMP-dependent process and a protein kinase C-dependent system regulated by calcium [6], but there are no reported studies of the role of the calcium/calmodulin-dependent kinase system in ACTH-stimulated expression of StAR protein in bovine adrenal fasciculata cells.

We therefore examined the effect of ACTH on expression of StAR protein and cortisol production by bovine adrenocortical fasciculata cells. Our findings suggested that the calcium/calmodulin-dependent protein kinase system may be involved in ACTH-induced expression of StAR protein in bovine adrenals.

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Materials and Methods

Chemicals

2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-93), and 2-N-(4-methoxybenzenesulfonyl)-amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-92) were purchased from Seikagaku-Kogyo Co. (Tokyo, Japan). ACTH was purchased from Daiichi Pharmaceutical Co. (Tokyo, Japan). Bovine serum albumin (BSA, fraction V), collagenase type I, deoxyribonuclease type I, and N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) were obtained from Sigma chemical Co. (St. Louis, Mo, USA). Anti-rabbit [125 I]-goat IgG was purchased from Du Pont-New England Nuclear (Boston, MA, USA). Eagle's minimum essential medium (MEM) was obtained from GIBCO. Anti-StAR antibody was generously supplied by Dr. Stocco.

Preparation of bovine adrenal fasciculata cells

Bovine adrenal glands were obtained from a local slaughterhouse. Isolated bovine adrenal fasciculata cells were prepared by a previously reported method [6,7]. The cells isolated from bovine adrenal fasciculata tissues were resuspended with Krebs-Ringer bicarbonate buffer, pH 7.4, containing 20 mM HEPES, 1.3 mM CaCl_2 , 0.2% glucose, 0.1% BSA (KRBGA-HEPES).

Cytosolic calcium movement and cyclic AMP formation

Isolated bovine adrenal fasciculata cells were loaded with fura II/AM, and cytosolic calcium movement ($[\text{Ca}^{2+}]_i$) was monitored, as previously reported [7]. The cells were incubated with and without ACTH in the presence of 0.5 mM isobuthylmethylxanthine for 30 min after 15 min-pretreatment with KN-92 and KN-93. Then trichloroacetic acid was added to the incubation mixture containing the cells and the media to stop the reaction. Cyclic AMP content was determined as previously reported [7].

StAR expression in fasciculata cells

Expression of StAR protein in bovine adrenal fasciculata cells was examined by methods previously reported [6]. Bovine adrenal fasciculata cells were preincubated with or without various inhibitors for 15 min in KRBGA-HEPES. After preincubation, ACTH was added to the incubation mixture, and incubation was performed for 2 h. Samples were then centrifuged, and the pelleted cells were obtained. The cells were soon solubilized by Seprazol and subjected to SDS-PAGE containing linear gradient of 10–20% acrylamide gel which was purchased from Daiichi-Kagaku-Yakuhin (Tokyo). All proteins were electrophoretically transferred to nitrocellulose membrane (Amersham, England) and analyzed by standard procedures for immunoblotting. The immuno-specific StAR protein was detected with a rabbit anti-peptide antibody as the primary label, a goat anti-rabbit immunoglobulin G labelled by [125 I] for the secondary label, and the signal detected by autoradiography.

Steroidogenesis

Adrenal fasciculata cells were resuspended to a final concentration of 10^4 cells/0.5 ml in KRBGA-HEPES and incubated in the presence of various additives for 2 h under constant agitation at 37 °C after 15-min preincubation with or without various inhibitors. The amount of cortisol in the media was then measured directly by specific radioimmunoassay [6].

Results

Effect of various inhibitors on ACTH-induced increase in $[\text{Ca}^{2+}]_i$ concentration, cyclic AMP formation and cortisol production

ACTH significantly increased cortisol production, cyclic AMP formation and the $[\text{Ca}^{2+}]_i$ concentration (Table 1). Pretreatment with KN-93, a specific inhibitor of calcium/calmodulin-dependent protein kinase II, significantly inhibited ACTH-stimulated cortisol production, but KN-92, an analogue of KN-92 which does not inhibit calcium/calmodulin

kinase, did not affect this production (Table 1). Cyclic AMP formation and the increase in the $[Ca^{2+}]_i$ concentration induced by ACTH were not affected by pretreatment with KN-93, as shown in Table 1.

Effect of inhibitor of calcium/calmodulin-dependent protein kinase on ACTH-induced expression of StAR protein

As shown in Fig.1, enhancement of expression

of StAR protein by 10^{-7} M ACTH was inhibited by pretreatment with KN-93. KN-92 had no effect on ACTH-induced expression of StAR protein.

Discussion

Our findings clearly demonstrated that ACTH stimulated expression of StAR protein in bovine adrenal fasciculata cells, which was detected as 30 kDa and 28 kDa proteins by immunoblotting

Table 1. Effect of pretreatment with KN-92 and KN-93 on cortisol production, changes in $[Ca^{2+}]_i$ and cyclic AMP formation induced by ACTH in bovine adrenal fasciculata cells

| Pretreatment | Cortisol production (ng/ 10^4 cells/120 min) | $[Ca^{2+}]_i$ (nM) | Cyclic AMP formation (pmol/ 10^4 cells/30 min) |
|--|---|-----------------------|---|
| Control | 0.16 ± 0.02 | 268 ± 11 | 0.16 ± 0.01 |
| + 10^{-7} M ACTH | $3.0 \pm 0.1^a)$ | $321 \pm 15^b)$ | $3.07 \pm 0.16^a)$ |
| + 10^{-7} M ACTH after 15 min pretreatment with 10μ M KN-92 | $2.8 \pm 0.3^a)$ | $311 \pm 9^b)$ | $3.44 \pm 0.11^a)$ |
| + 10^{-7} M ACTH after 15 min pretreatment with 10μ M KN-93 | $0.8 \pm 0.1^c)$ | $318 \pm 11^b)$ | $3.10 \pm 0.09^a)$ |

Cells were pretreated with KN-92, KN-93 and buffer for 15 min. Then 10^{-7} M ACTH was added, and incubation was performed. Pretreatment with KN-92 and KN-93 did not affect basal production of cortisol, $[Ca^{2+}]_i$ and cyclic AMP formation (data not shown). Details of methods are given in the text. These experiments were performed with three different preparations of cells isolated from 6 different bovine adrenals. Results are expressed as means \pm SEM. a) $P < 0.001$ vs. control, b) $P < 0.05$ vs. control, c) $P < 0.001$ vs. 10^{-7} M ACTH.

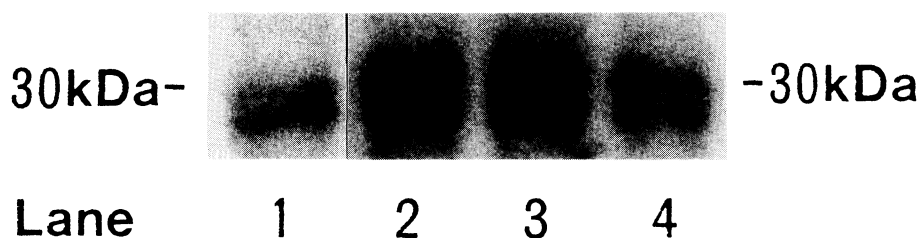


Fig. 1. Effect of ACTH on StAR expression with and without pretreatment with KN-92 and KN-93. Bovine adrenal fasciculata cells were pretreated with 10μ M KN-92 and KN-93 for 15 min, and then incubated with buffer or 10^{-7} M ACTH for 2 h. After incubation, proteins from the pelleted cells which were solubilized with Seprazol were analyzed by SDS-PAGE. Immunoblotting analysis was performed as described in Materials and Methods. Lane 1: Cells incubated with buffer for 2 h without pretreatment, Lane 2: Cells incubated with 10^{-7} M ACTH for 2 h without pretreatment., Lane 3: Cells incubated with 10^{-7} M ACTH after pretreatment with 10μ M KN-92., Lane 4: Cells incubated with 10^{-7} M ACTH for 2 h after pretreatment with 10μ M KN-93.

analysis. Our previous findings also demonstrated that ACTH could enhance expression of StAR protein in these cells [6]. Thus the present findings are therefore consistent with these previous findings [6].

The present findings also demonstrated that activation of both cortisol production and expression of StAR protein by ACTH was decreased by pretreatment with KN-93, a specific antagonist for calcium/calmodulin-dependent protein kinase II [8]. We also found that ACTH increased $[Ca^{2+}]_i$, which activates calcium/calmodulin kinase. It has already been reported that ACTH-evoked cortisol secretion is also regulated by calmodulin kinase, suggesting that the calcium/calmodulin system may play a crucial role in the regulation of cortisol production in fasciculate cells [9]. The present study provides new insights into the role of the calcium/calmodulin-dependent protein kinase system in the regulation of expression of StAR protein and cortisol biosynthesis by ACTH in fasciculata cells. We have already reported that both cAMP- and protein kinase C-dependent processes may play a crucial role in the regulation of expression of StAR protein when bovine fasciculata cells are stimulated by ACTH [6].

Computer analysis of the StAR protein sequence has identified three putative cAMP-dependent protein kinase/calmodulin-dependent protein kinase II phosphorylation sites and one protein kinase C phosphorylation site [10]. These findings suggest that ACTH-induced StAR expression is regulated in part by a calcium/calmodulin-dependent protein kinase system as well as by cAMP- and protein kinase C-dependent processes, and that phosphorylation is a key step in the mechanism of action of StAR.

In conclusion, our findings suggest that the effect of ACTH on expression of StAR protein is regulated in part by calcium/calmodulin-dependent protein kinase processes, resulting in activation of cholesterol side-chain cleavage and cortisol production in bovine adrenal fasciculata cells.

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