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Regulation of Expression of the Steroidogenic Acute Regulatory (StAR) Protein by ACTH in Bovine Adrenal Fasciculata Cells

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Immunocytochemical studies and immunoblotting analysis demonstrated that there exists the StAR protein in bovine adrenal fasciculata cells, and ACTH activated expression of the StAR protein. Then roles of intracellular signal transduction systems in the regulation of expression of the StAR protein were studied. The addition of Bt_2cAMP and forskolin, or phorbol ester plus calcium ionophore 23187 activated expression of the StAR protein as well as cortisol production, suggesting that cyclic AMP- or protein kinase C-dependent process plays a crucial role in the regulation of expression of the StAR protein. Activating effects of ACTH which activates cyclic AMP formation on the StAR protein and cortisol production were inhibited by pretreatment with calphostin C which is a protein kinase C inhibitor, suggesting that ACTH enhances expression of the StAR protein possibly via both of two signal transduction systems such as cyclic AMP- and protein kinase C-dependent processes.

The conversion of cholesterol to pregnenolone by cholesterol side-chain cleavage complex, is the first enzymatic step in adrenal steroidogenesis and occurs in the inner mitochondrial membrane (1). A crucial event in the acute regulation of adrenal steroidogenesis by ACTH is the delivery of cholesterol into the mitochondria where it is converted to pregnenolone by the cholesterol side-chain cleavage enzyme (2–5). These data suggested that a newly synthesized regulatory protein fascilitated the translocation of cholesterol from the outer to the inner mitochondrial membrane where P450scc resides (6–8). It had recently been reported that the 30-kDa protein was purified as the steroidogenic acute regulatory (StAR) protein (9). In general, the action of ACTH regulating cortisol production by adrenocortical fasciculata cells has been considered to be mediated via the cyclic AMP (cAMP)-dependent systems (10). Moreover, it was reported that Ca^{2+} is essential for ACTH action on corticoidogenesis (11). Thus it is suggested that cAMP-dependent process as well as Ca^{2+} messenger system play a crucial role in the regulation of adrenal steroidogenesis. However, there was no report describing the effect of ACTH on expression of the StAR protein, and also the role of signal transduction systems such as cAMP-dependent system and protein kinase C-dependent process on the regulation of the StAR protein in the adrenocortical tissues.

Thus we investigated the effect of ACTH and also the effect of various agonists for activating protein kinase A and C on expression of the StAR protein by bovine adrenocortical fasciculata cells. The present experiments demonstrated that both systems of cAMP-dependent protein kinase and protein kinase C may be involved in ACTH-induced expression of the StAR protein in bovine adrenals.

MATERIALS AND METHODS

Chemicals. Calphostin C was purchased from Kyowa Medex, Co., Ltd. (Tokyo, Japan). Bovine serum albumin (BSA, fraction V), collagenase type I, deoxyribonuclease type I, N^6, 2′-O-dibuthyryladenosine 3′:5′-cyclic monophosphate (Bt_2cAMP), 12-0-tetradecanoylphorbol-13-acetate (TPA), 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

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Du Pont-New England Nuclear (Boston, MA, USA). ACTH was purchased from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). 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 according to the method previously reported (12) with minor modifications. The pooled slices mainly consisting of fasciculata tissues were minced and incubated in MEM containing 0.1% BSA, 0.25% collagenase type I and 0.05 mg/ml deoxyribonuclease type I at 37°C for 10 min. After each incubation period, cells were dispersed mechanically by pipetting them 20 times through a 5-ml pipette. The dissociated cells were filtered through cotton gauze and washed three times 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).

Immunocytochemistry. The procedure was described in detail by Sasano et al. (13,14). The smear slide containing isolated bovine adrenal fasciculata cells were washed five times with cold PBS for 5 min prior to the immunostaining. The slides were incubated with the anti-StAR antibody for 18 hrs at 4°C in a moisture chamber after blocking endogenous peroxidase by hydrogen peroxide. Then the slides were incubated for 30 min at room temperature with biotinylated anti-rabbit immunoglobulin and peroxidase-conjugated streptavidin. Specific staining was identified by the presence of a brown reaction product.

Steroidogenesis. Adrenal fasciculata cells were resuspended to a final concentration of 20×10^4 cells/ml in KRBGA-HEPES and incubated in the presence of various additives for 2 hrs under constant agitation at 37°C after 15-min preincubation with or without various inhibitors. The content of cortisol in the media was measured directly by its specific radioimmunoassay.

StAR expression in fasciculata cells. Bovine adrenal fasciculata cells were preincubated with or without various inhibitors for 15 min in KRBGA-HEPES. After the preincubation, various concentrations of ACTH were added to the incubation mixture and incubated for 2 hrs. The samples were 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, Japan. All proteins were electrophoretically transferred to nitrocellulose membrane (Amersham) and analyzed using standard procedures for immunoblotting. The immuno-specific StAR protein was detected using a rabbit antipeptide antibody as the primary label, a goat anti-rabbit immunoglobulin G labelled by [^{125}I] for the secondary label, and the signal detected by autoradiogram.

RESULTS

Immunocytochemistry

Immunoreactivity was observed in the cytoplasm of the fasciculata cells (Fig. 1). No immunoreactivity was observed in the control slide containing isolated cells which were stained only by biotinylated second antibody after not incubating with the anti-StAR antibody.

Effects of Incubation Time and Various Concentrations of ACTH on Expression of the StAR Protein and Cortisol Production

Fig. 2 demonstrates the effect of incubation time with and without 10^{-7} M ACTH on expression of the StAR protein. Two bands of the StAR-immunoreactive protein were detected by immunoblotting, and 30 kDa and 28 kDa proteins were visible by autoradiogram. The expression of two bands gradually increased for 1–6 hrs (Fig. 2A,B) and reached maximally at 6 hrs when incubated with 10^{-7} M ACTH (Fig. 2B). The effect of various concentrations of ACTH on expression of the StAR protein was shown in Fig. 3A. Expression of the StAR protein and cortisol production were enhanced in dose-dependent manner by ACTH (Fig. 3A,B). Each concentration of ACTH showed a significant increase in cAMP concentrations in these cells (data not shown).

Effects of Calphostin C on ACTH-Induced Expression of the StAR Protein and Cortisol Production

Pretreatment with calphostin C, which is an antagonist against protein kinase C, suppressed ACTH-induced expression of the StAR protein and also ACTH-induced cortisol production (Fig. 4A,B). Calphostin C did not affect cAMP formation induced by ACTH (data not shown).

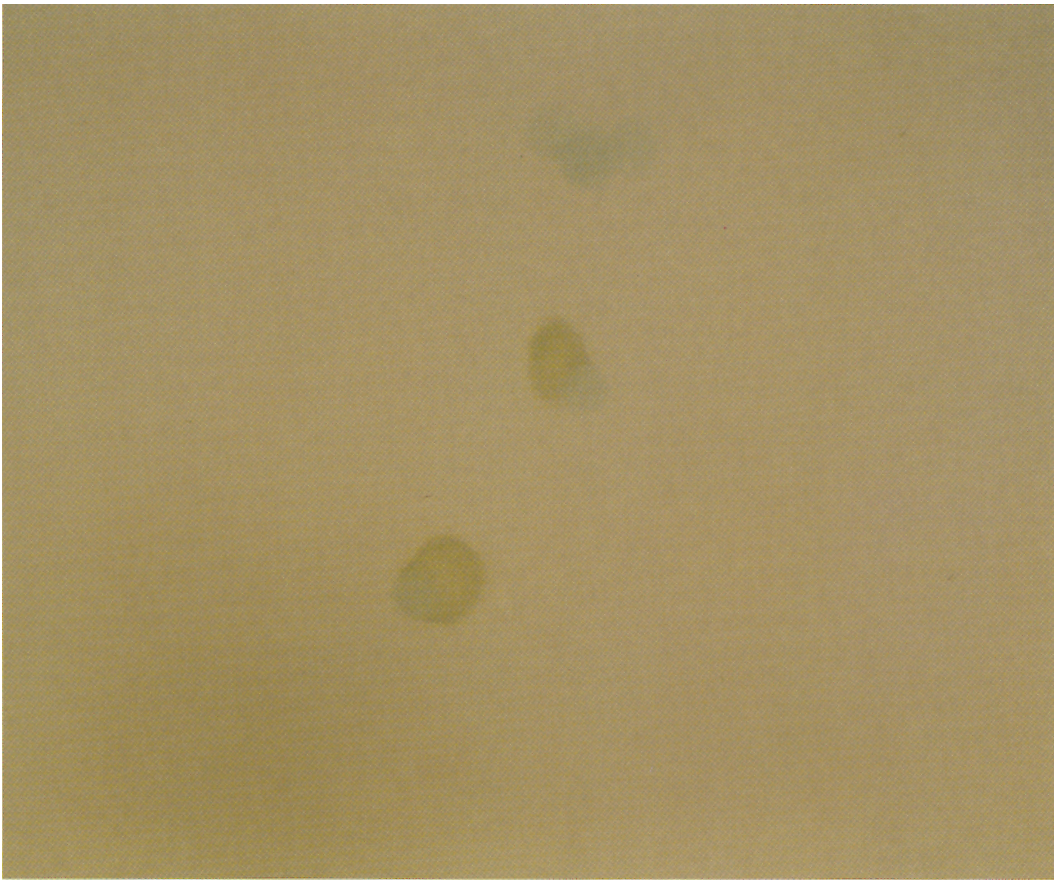

FIG. 1. Immunocytochemistry of the StAR protein in isolated bovine adrenal fasciculata cells. The fasciculata cells were stained as described in Materials and Methods. Immunoreactivity is shown as brown colour.

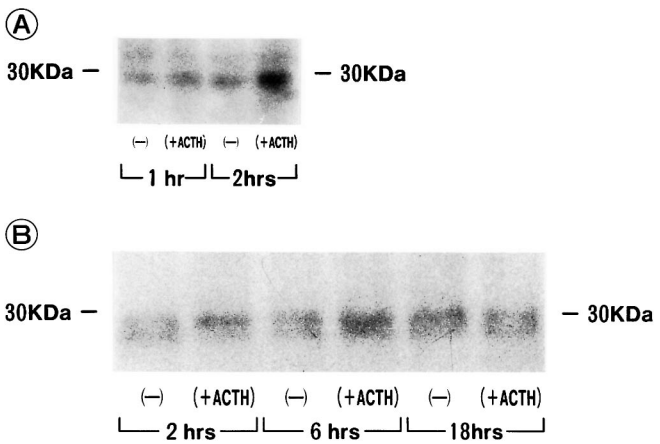

FIG. 2. Effect of incubation time with and without 10^{-7} M ACTH on expression of the StAR protein by bovine fasciculata cells. The cells isolated from bovine adrenal fasciculata tissues were incubated with ACTH for 1, 2, 6 and 18 hrs. Then proteins from the pelleted cells which were solubilized by Seprazol was analyzed by SDS-PAGE. Immunoblotting analysis was performed as described in Materials and Methods. The cells were prepared at different day for A and B.

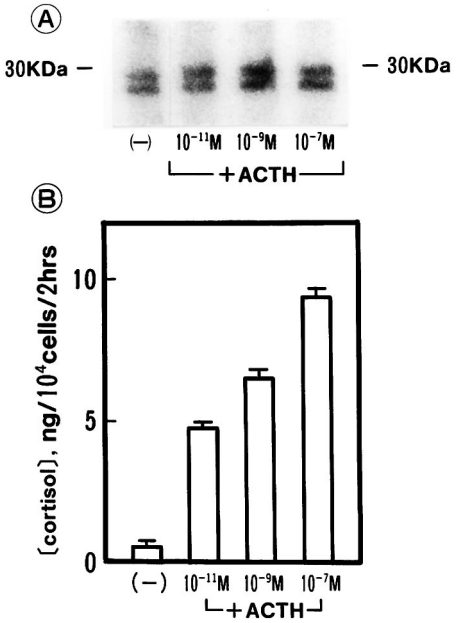

FIG. 3. Effect of various concentrations of ACTH on expression of the StAR protein (A) and cortisol production (B). The cells were incubated with and without ACTH for 2 hrs for determination of the StAR protein and cortisol production. The results of cortisol production described in B which were estimated from the samples of 3–5 tubes incubated are expressed as Mean ± S.E. (n = 3–5 tubes).

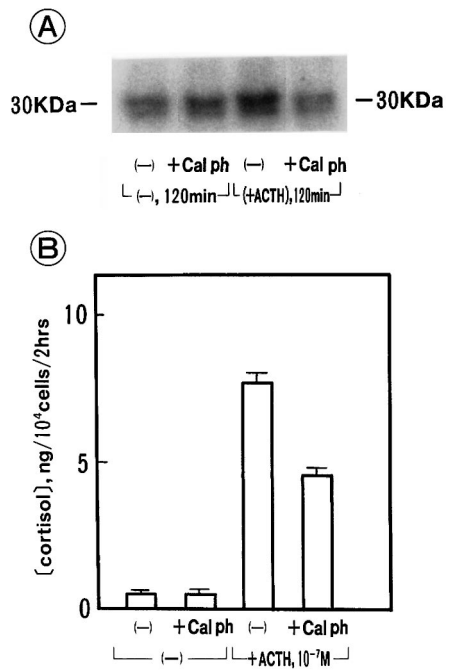

FIG. 4. Effect of pretreatment with calphostin C (Calph) on ACTH-induced expression of the StAR protein (A) and cortisol production (B). The cells were pretreated with 1 μM calphostin C for 15 min. Then 10^{-7} M ACTH or buffer was added to the incubation mixture and incubated for 2 hrs. Expression of the StAR protein and cortisol production were analyzed as described in the legends of Figs. 2 and Fig. 3. The results of cortisol production are expressed as Mean ± S.E. Calph: calphostin C

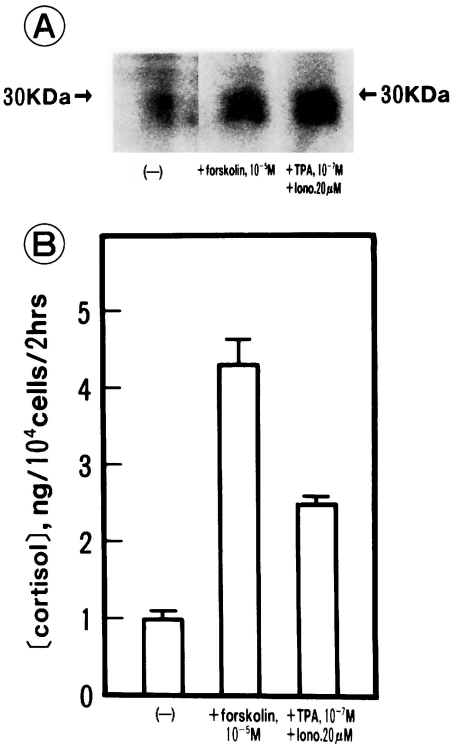

FIG. 5. Effect of forskolin and phorbol ester (TPA) plus calcium ionophore A23187 (Iono) on expression of the StAR protein (A) and cortisol production (B). The cells were incubated with 10^{-5} M forskolin and 10^{-7} M TPA plus 20 μM calcium ionophore A23187 for 2 hrs. Expression of the StAR protein and cortisol production were analyzed as described in the legends of Figs. 2 and Fig. 3. The results of cortisol production are expressed as Mean ± S.E. Iono: calcium ionophore A23187

Effects of Bt_2cAMP, Forskolin and TPA plus Calcium Ionophore A23187 on Expression of the StAR Protein and Cortisol Production

Fig. 5 shows the effect of forskolin or TPA with A23187 on expression of the StAR protein and cortisol production. Both of them showed an enhancement in expression of the StAR protein and also cortisol production. The activating effects of Bt_2cAMP on expression of the StAR protein and cortisol production was abolished by pretreatment with H-89 and H-88, both of which are an inhibitor for cAMP-dependent protein kinase (Fig. 6A,B).

DISCUSSION

The present experiments clearly demonstrated that ACTH can stimulate expression of the StAR protein which was detected as 30 kDa and 28 kDa proteins by immunoblotting analysis in bovine adrenal fasciculata cells and it was increased maximally at 6 hrs by 10^{-7} M ACTH. The present data also showed that both proteins in bovine adrenal fasciculata cells which were detected by anti-StAR antibody were decreased when pretreated with cycloheximide (data not shown). The previous experiments reported that 30 kDa protein was detected by immunoblotting analysis of isolated mitochondrial fraction of mouse Leydig tumor cells (9), while whole homogenates of the cells isolated from bovine adrenal tissues were used for analysis of the StAR protein in the present experiments. The present experiments demonstrated that two bands in bovine adrenal whole homogenate were detectable by anti-StAR antibody although species differences such as mouse and bovine may explain the difference in molecular weight of the proteins detected by anti-StAR

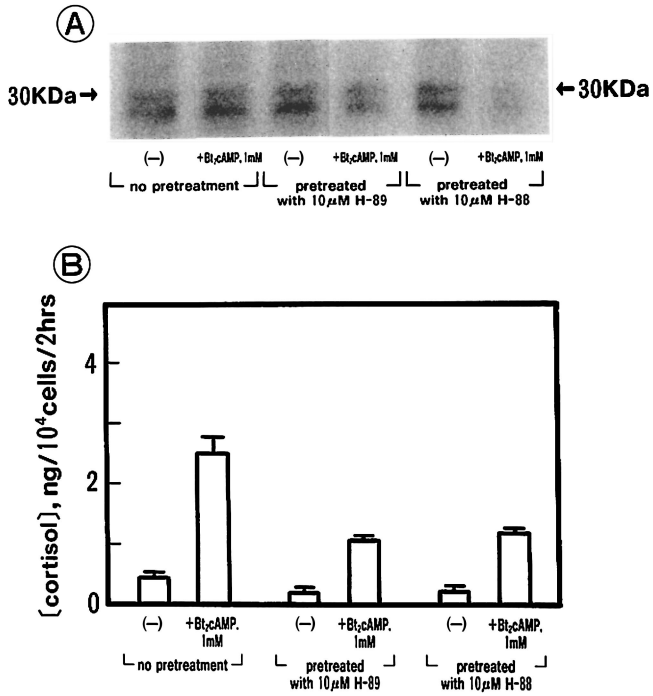

FIG. 6. Effect of pretreatment with H-89 and H-88 on Bt_2cAMP-induced expression of the StAR protein (A) and cortisol production (B). The cells were pretreated with 10 μM H-89 and 10 μM H-88 for 15 min. Then 1 mM Bt_2cAMP was added to the incubation mixture and incubated for 2 hrs. Expression of the StAR protein and cortisol production were analyzed as described in the legends of Figs. 2 and 3. The results of cortisol production are expressed as Mean ± S.E.

antibody. It is also speculated that the 30 kDa protein seems to act in mitochondria as the StAR protein and the 28 kDa protein may be a degradated metabolite of the StAR protein inside the cells, although further experiments will be needed.

The present study provides new insights into the roles of cAMP-dependent protein kinase system and protein kinase C-dependent processes in the regulation of expression of the StAR protein. Those results indicated that ACTH enhanced expression of the StAR protein simultaneously with increasing cortisol production. Expression of the StAR protein activated by ACTH was diminished by pretreatment with calphostin C which is a specific antagonist for protein kinase C (15). TPA plus calcium ionophore A23187 that can stimulate protein kinase C (16) also enhanced the expression of the StAR protein as well as cortisol production. Thus it is suggested that ACTH-induced expression of the StAR protein is regulated possibly via protein kinase C-dependent processes. The present data also demonstrated that Bt_2cAMP and forskolin which stimulates cAMP accumulation in adrenocortical tissues (17) increased expression of the StAR protein with enhancing cortisol production, suggesting that expression of the StAR protein is also regulated by the cAMP-dependent systems which are well known to be activated by ACTH. Thus cAMP- and protein kinase C-dependent processes may play a crucial role in the regulation of the expression of the StAR protein in bovine fasciculata cells. It had already been reported that expression of the StAR protein was significantly enhanced in mouse MA-10 Leydig tumor cells incubated with cAMP (18), and reporter gene expression of StAR in the Y-1 adrenocortical cells was increased more than 2-fold by 8-Br-cAMP (19). These previous findings suggest that cAMP-dependent regulation exists in expression of the StAR protein and also in its expression at DNA level in the steroidogenic tissues. Thus our data are consistent with those previous findings (18,19), and both cAMP- and protein kinase C-dependent processes are closely related to the action of ACTH for expression of the StAR protein in bovine fasciculata cells.

In conclusion, ACTH can activate expression of the StAR protein via both of cAMP- and protein kinase C-dependent processes, resulting in activation of cholesterol side-chain cleavage and cortisol production.

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