Biochemical Pharmacology, Vol. 44, No. 7, pp. 1357–1365, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

DE NOVO SYNTHESIS OF CALMODULIN BINDING PROTEIN IN SUBSTANCE P-INDUCED STEROIDOGENESIS IN BOVINE ADRENOCORTICAL CELLS

TOMOKAZU YOSHIDA, MITSUNOBU MIO and KENJI TASAKA*

Department of Pharmacology, Faculty of Pharmaceutical Science, Okayama University, Okayama 700, Japan

(*Received* 14 *April* 1992; *accepted* 2 *July* 1992)

Abstract—In order to clarify the mechanism of substance P (SP)-induced cortisol secretion from bovine adrenocortical (BAC) cells, protein synthesis at the early stage of SP-stimulation in BAC cells was investigated. Both SP and adrenocorticotropic hormone (ACTH) increased [^{3}H]leucine uptake into BAC cells in a dose-dependent fashion. Although the SP-induced [^{3}H]leucine uptake precedes the cortisol secretion, ACTH was slower in inducing [^{3}H]leucine uptake and cortisol secretion. Protein synthesis inhibitors, actinomycin D and cycloheximide, were potent in inhibiting the SP-induced cortisol secretion. SDS–PAGE analysis, revealed that a 240 kDa protein is newly synthesized in BAC cells in response to SP but not ACTH. It was also indicated that the production of this 240 kDa protein was elicited about 30 min after stimulation by SP. Moreover, A23187 and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) also caused a rapid [^{3}H]leucine uptake and production of 240 kDa protein. In contrast, dibutyryl cAMP did not induce the synthesis of this 240 kDa protein. Calmidazolium, a calmodulin inhibitor, effectively inhibited not only [^{3}H]leucine uptake but also 240 kDa protein production due to SP. On the other hand, KT-5720, an inhibitor of protein kinase A, had no effect on [^{3}H]leucine uptake or 240 kDa production. Using the [^{125}I]calmodulin-membrane overlay method, it was found that the 240 kDa protein was a newly synthesized calmodulin binding protein. From the present study, it was concluded that the *de novo* synthesis of this 240 kDa protein may be intimately related to the cortisol secretion in SP-stimulated BAC cells associated with an activation of the Ca–calmodulin pathway.

After the stimulation of adrenocortical cells with various steroidogenic peptides, such as adrenocorticotropic hormone (ACTH†) and substance P (SP), steroidogenesis takes place with several minutes of lag time [1, 2]. In the case of ACTH-stimulation, this lag time is ascribed to the synthesis of labile proteins, which may participate in the production and/or secretion of glucocorticoids [13]. Grower and Bransome [4] reported that the contents of cytosolic protein in mouse Y-1 adrenal tumor cells increased by 20% within 15 min after exposure to ACTH. In the case of ACTH stimulation, it has been suggested that the cAMP–protein kinase A system may play an important role not only in the steroidogenesis but also in rapid protein synthesis [5]. On the other hand, as we reported previously, Ca^{2+} and calmodulin may play predominant roles in the intracellular signal transduction of SP-induced cortisol secretion from bovine adrenocortical (BAC) cells [2]. However, there has been no report concerning Ca^{2+}- and calmodulin-mediated protein synthesis in adrenocortical cells. In order to clarify the mechanism of SP-induced steroidogenesis, protein synthesis taking place in the early stage of SP stimulation and Ca^{2+}-mediated cortisol secretion were investigated.

* Corresponding author. Tel. (81) 862-52-1111 ext. 7939; FAX (81) 862-55-7456.

† Abbreviations: ACTH, adrenocorticotropic hormone; SP, substance P; BAC cells, bovine adrenocortical cells; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; FCS, fetal calf serum; HS, horse serum; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; KRBH solution, Krebs–Ringer bicarbonate–HEPES solution; TPA, 12-*O*-tetradecanoyl-phorbol - 13 - acetate; db - cAMP, N^6,2′ - *O* - dibutyryladenosine 3′,5′-cyclic monophosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Preparation of BAC cell. BAC cells were prepared as described previously [2]. In brief, bovine adrenal cortex, freshly excised immediately after slaughter, was sliced (0.5–1.0 mm of thickness) in an ice-cold bath. The slices were minced and incubated for 30 min at 37° in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3 mM KCl, 1.1 mM $CaCl_2$, 0.2% collagenase Type 1 and 2% bovine serum albumin (BSA). Thereafter, DMEM containing 10% fetal calf serum (FCS) was added and the cells were dispersed by repeated pipettings. To obtain the cell suspension, both incubation and dispersion were repeated under the same condition. The dispersed cells were passed through stainless meshes (200 and 400 mesh) and washed three times with DMEM. The isolated cells were primarily cultured in DMEM supplemented with 10% FCS, 5% horse serum (HS), 100 U/mL penicillin and 100 μg/mL streptomycin for 48–96 hr at 37° in a CO_2 incubator with 5% CO_2 in humidified air. Approximately 5×10^7 adrenocortical cells per g tissue were collected and the cell viability, as assessed by 0.3% Trypan blue dye exclusion test, was higher than 90%. The viable cells were incubated for a further 24 hr in DMEM

containing 10% FCS and 5% HS before the experiments.

Measurement of cortisol output. Cultured BAC cells were washed in serum-free DMEM and preincubated for 120 min at 37° in a 24-well plastic plate. The cell density was adjusted to 10^5 cells/mL in each well. Thereafter, SP and ACTH dissolved in serum-free DMEM at various concentrations were added into each well and subsequent incubation was continued for 60 min at 37°. Protein synthesis inhibitors were added into the medium simultaneously with both stimulants. By adding ice-cold DMEM into the medium, the reaction was terminated and the cells were sedimented by centrifugation and the cortisol content in the supernatant was measured using a radioimmunoassay kit.

[^{3}H]*Leucine uptake into BAC cells.* Cultured BAC cells (10^6 cells in each well of a six-well culture plate) were washed three times with Krebs–Ringer bicarbonate–HEPES solution (in mM: NaCl 145, $NaHCO_3$ 5, $CaCl_2$ 2, KCl 5, $MgCl_2$ 1.3, NaH_2PO_4 1.2, HEPES 20, glucose 5.5, 0.2% BSA; pH 7.4: KRBH solution), and incubated in a leucine-free DMEM for 120 min in a CO_2 incubator. Thereafter, 6.7 pM (1 μCi/mL) [^{3}H]leucine was added and incubation was continued for another 60 min. After that, the cells were stimulated with 10 nM SP, 100 pM ACTH, 1 μM A23187, 10 nM 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) or 1 μM dibutyryl cyclic AMP (db-cAMP) for various incubation periods. The reaction was terminated by addition of ice-cold DMEM containing 1 mM leucine. After an aspiration of the medium, the cells were washed three times with ice-cold DMEM and solubilized by adding 0.5 N NaOH. The lysate was neutralized with 0.5 N HCl and the radioactivity was determined by means of a liquid scintillation counter (Aloka, LSC-700).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of proteins synthesized in BAC cells. Cultured BAC cells (10^6 cells in each well of a six-well culture plate) were preincubated in a serum-free DMEM for 120 min at 37° in a CO_2 incubator. Thereafter, various steroidogenic stimulants were added and incubation was continued for another 60 min. Inhibitors of protein kinase A or calmodulin were added to the incubation medium simultaneously with the stimulants. The reaction was terminated by addition of ice-cold KRBH solution and the cells were washed three times with ice-cold KRBH solution. SDS–PAGE sample solution (9% SDS, 150 mM Tris–HCl, 3% 2-mercaptoethanol, 15% glycerol; pH 6.8) was added into the medium, and the mixture was incubated in boiling water for 5 min to accomplish the protein denaturation. SDS–PAGE was carried out according to the method of Laemmli [6], using a slab gel (13 cm × 14.3 cm, 2 mm in thickness) containing 5% polyacrylamide. In each run, proteins obtained from 10^4 cells, which correspond to approximately 10 μg of protein, were applied to each lane. After electrophoresis, the gel was stained with Coomassie brilliant blue R250. In some cases, BAC cells were similarly stimulated by substance P or ACTH in the presence of 6.7 pM (1 μCi/mL) of [^{3}H]leucine and the proteins were analysed by SDS–PAGE. Thereafter, the protein band, which corresponds to newly synthesized protein, was cut out of the gel and homogenized and the radioactivity was determined.

Analysis of calmodulin binding proteins in SP-stimulated BAC cells by means of [^{125}I]*calmodulin-membrane overlay method.* After SDS–PAGE of BAC cell proteins, the gel was washed for 30 min with a transfer solution containing 20 mM Tris, 50 mM glycine and 20% methanol. The proteins in the gel were transferred to a nitrocellulose membrane using an electroblotting apparatus (Sartorius) at a constant current of 250 mA for 90 min. Thereafter, the membrane was washed with Tris–NaCl solution (20 mM Tris–HCl, 200 mM NaCl, 1 mg/mL BSA; pH 7.4) for 120 min, and incubated overnight with 2 μCi/mL of [^{125}I]calmodulin dissolved in Tris–NaCl solution supplemented with either 1 mM $CaCl_2$ or 5 mM EGTA. The membrane was rinsed with the same solution and washed for 180 min with Tris–NaCl solution supplemented with 0.1 mM $CaCl_2$. Subsequently, the membrane was air-dried and exposed to X-ray film at −80° [7].

Chemicals. The compounds used were as follows (sources are indicated in parentheses): DMEM (Nissui, Tokyo, Japan), collagenase Type 1, BSA, actinomycin D, cycloheximide, db-cAMP and W-7 (Sigma Chemical Co., St Louis, MO, U.S.A.), FCS and HS (Hezelton, Lenaxa, KS, U.S.A.), penicillin (Toyo Jozo, Shizuoka, Japan), streptomycin (Meiji, Tokyo, Japan), SP and ACTH (Peptide Institute Inc., Osaka, Japan), cortisol radioimmunoassay kit (NIPPON DPC Corp, Tokyo, Japan), L-[4,5-^{3}H]-leucine (149 μCi/nmol) and [^{125}I]calmodulin (58.9 μCi/mg) (Amersham International, Amersham, U.K.), A23187 and TPA (Calbiochem, San Diego, CA, U.S.A.), calmidazolium (Janssen, Beerse, Belgium), trifluoperazine (Wako Pure Chemicals, Osaka, Japan), KT-5720 (Kyowa Hakko, Tokyo, Japan). Other chemicals used were all reagent grade and were purchased from commercial sources.

Statistical analysis. A one-way analysis of variance with Dunnett's test was used to determine the statistical significance.

RESULTS

In order to study the relationship between the protein synthesis and cortisol secretion, the effects of SP and ACTH on both [^{3}H]leucine uptake and cortisol secretion were compared. SP and ACTH elicited [^{3}H]leucine uptake and cortisol secretion in a dose-dependent fashion, at concentrations higher than 10 pM (Fig. 1a). The maximal responses in cortisol secretion due to SP and ACTH were obtained at concentrations of 100 nM and 100 pM, respectively, whereas the [^{3}H]leucine uptake due to SP and ACTH reached the maximum at concentrations of 100 and 1 nM, respectively. Figure 1b indicates the time courses of [^{3}H]leucine uptake into BAC cells and cortisol secretion induced by submaximal concentrations of SP (10 nM) and ACTH (100 pM). In the case of SP stimulation, the onset of both [^{3}H]leucine uptake and cortisol

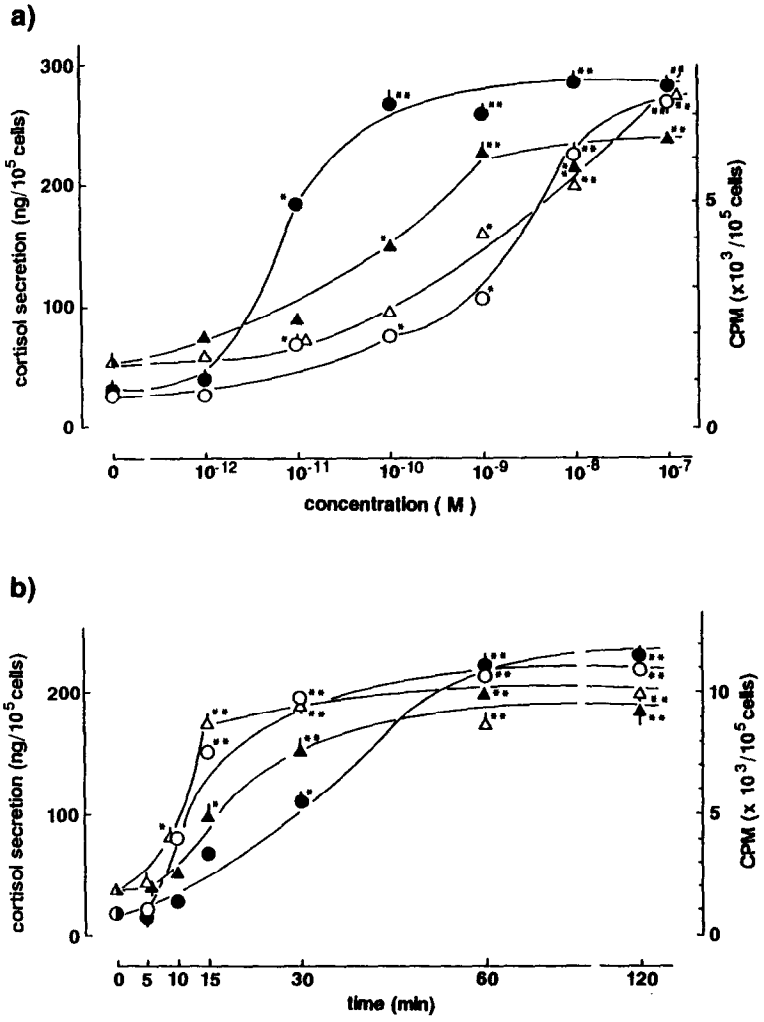

Fig. 1. Cortisol secretion and [^{3}H]leucine uptake into BAC cells induced by SP and ACTH. (a) Dose–response curves of cortisol secretion and [^{3}H]leucine uptake. The cells were stimulated with either compound for 60 min at 37°. (b) Time courses of cortisol secretion and [^{3}H]leucine uptake. The cells were incubated with SP (10 nM) and ACTH (100 pM) for various incubation periods. (○) and (●) represent cortisol secretion induced by SP and ACTH, respectively. (△) and (▲) represent [^{3}H]leucine uptake induced by SP and ACTH, respectively. In both figures, these symbols are commonly used. Each point represents the mean ± SEM of data obtained from five separate experiments. * and ** represent statistical significance compared to resting levels at $P < 0.05$ and $P < 0.01$, respectively.

secretion was observed approximately 10 min after stimulation. The [^{3}H]leucine uptake in BAC cells reached close to the maximum 15 min after stimulation and cortisol secretion reached the maximum 30 min after stimulation, indicating that protein synthesis precedes cortisol synthesis in SP-stimulated BAC cells. In the case of ACTH stimulation, [^{3}H]leucine uptake was initiated almost simultaneously with cortisol secretion and the onset was slower (about 15 min after stimulation) than SP stimulation. The slower elevation in cortisol output and [^{3}H]leucine uptake was also noticed.

The effects of protein synthesis inhibitors, actinomycin D and cycloheximide on SP-induced cortisol secretion from BAC cells are shown in Fig. 2. Both compounds dose-dependently inhibited the cortisol secretion induced by SP (10 nM) and ACTH (100 pM); significant inhibitions were observed at the same concentration of 10 nM in both cases. In accordance with this, both actinomycin D and cycloheximide inhibit [^{3}H]leucine uptake at the same concentration range which inhibits cortisol secretion, and a significant inhibition was observed at concentrations higher than 10 nM (data not shown).

The newly synthesized proteins in BAC cells in response to SP were analysed by means of SDS–PAGE (Fig. 3a). In the resting cells, several proteins were detected in SDS–PAGE as seven major bands; the molecular masses were 51, 65, 100, 120, 160, 200 and 420 kDa, respectively. In the present

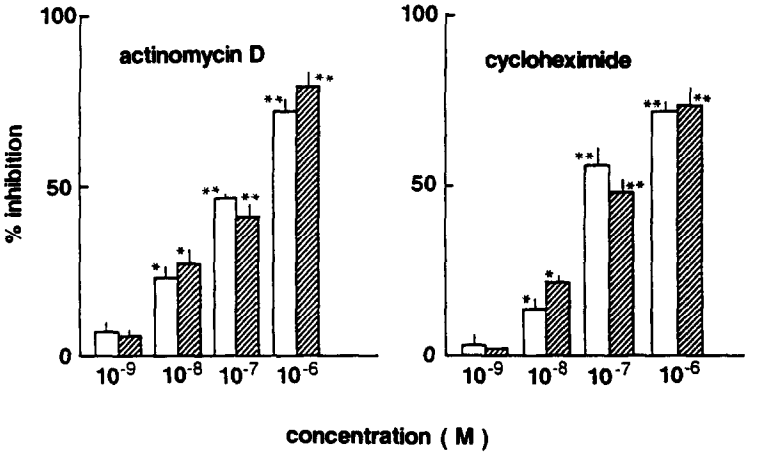

Fig. 2. Inhibitory effects of actinomycin D and cycloheximide on the cortisol secretion from BAC cells induced by SP and ACTH. Various concentrations of protein synthesis inhibitors were added to the medium simultaneously with SP (10 nM; open columns) and ACTH (100 pM; hatched columns). Each column represents the mean ± SEM of data obtained from five separate experiments. * and ** represent statistical significance compared to control groups at $P < 0.05$ and $P < 0.01$, respectively.

experiments, proteins having molecular masses smaller than 40 kDa migrated to the front. In the SP-stimulated cells, 240 kDa protein band was revealed, as well as the proteins which appeared in the control cells. This protein was not detected when ACTH-stimulated cells were analysed under the same experimental condition; the molecular weights of visible bands were the same as those seen in control cells. On the other hand, the 51 kDa protein, which corresponds to one of the labile proteins [3], increased after stimulation with ACTH but no such increase was detected after SP treatment. It was confirmed that this 240 kDa protein appeared 30 min after stimulation but this was not detected 6 hr after stimulation (Fig. 3b).

As indicated in Table 1, SP significantly increased [^{3}H]leucine uptake into the 240 kDa protein of BAC cells in a dose-dependent manner, though ACTH did not induce [^{3}H]leucine uptake in this protein at all. Table 2 represents the sequential changes in [^{3}H]-leucine uptake in the 240 kDa protein in SP-stimulated BAC cells. As indicated here, the incorporation of [^{3}H]leucine to 240 kDa protein was 10 times that detected in the control 30 min after addition of SP, and this high level of [^{3}H]leucine uptake was sustained for about 6 hr. These results indicate that *de novo* synthesis of the 240 kDa protein was elicited by SP.

When the BAC cells were stimulated with A23187 or TPA, both cortisol secretion and [^{3}H]leucine uptake occurred in a dose-dependent manner at concentrations higher than 1 and 0.1 nM, respectively (Fig. 4a). The [^{3}H]leucine uptake due to A23187 (100 nM) and TPA (10 nM) precedes the cortisol secretion as in the case of SP (Fig. 4b). The A23187-induced responses started at 5 min and reached a plateau at 15 min after stimulation. The time courses of [^{3}H]leucine uptake and cortisol secretion elicited by A23187 were similar to those seen in SP. On the other hand, the TPA-induced responses took place about 10 min after stimulation and continued even 120 min after stimulation. The 240 kDa protein was also detected in the BAC cells stimulated by either A23187 (100 nM) or TPA (10 nM), and the SDS–PAGE profiles were similar to that of SP-stimulated cells (Fig. 4c).

db-cAMP was also effective in inducing cortisol secretion and [^{3}H]leucine uptake (Fig. 5a). In contrast to SP, A23187 and TPA, db-cAMP induced simultaneous increases in [^{3}H]leucine uptake and cortisol secretion and the time courses were similar to those seen after ACTH-stimulation (Fig. 5b). The 240 kDa protein synthesis was not detected in the db-cAMP-stimulated cells (Fig. 5c). In the SDS–PAGE of db-cAMP-stimulated BAC cells, seven major protein bands with molecular masses of 51, 65, 100, 120, 160, 200 and 420 kDa were detected. This SDS–PAGE profile was similar to that of ACTH-stimulated cells but not to that seen after SP stimulation.

As indicated in Fig. 6a, the [^{3}H]leucine uptake induced by SP was potently inhibited by calmodulin inhibitors, such as calmidazolium, trifluoperazine and W-7, although KT-5720, an inhibitor of protein kinase A [8], had no effect. Similarly, calmidazolium, but not KT-5720, was effective in inhibiting the SP-induced production of the 240 kDa protein (Fig. 6b). This seems to suggest that calmodulin may be involved in the synthesis of the 240 kDa protein. In contrast, the [^{3}H]leucine uptake induced by ACTH was strongly inhibited not only by calmidazolium, trifluoperazine and W-7 but also by KT-5720.

Calmodulin-binding proteins in BAC cells were determined according to the [^{125}I]calmodulin-membrane overlay method. As shown in Fig. 7, when [^{125}I]calmodulin was applied onto the blotted membrane to which the proteins of BAC cells were transferred, several proteins were detected. In control cells, five protein bands with the molecular masses of 57, 68, 160, 200 and 220 kDa were

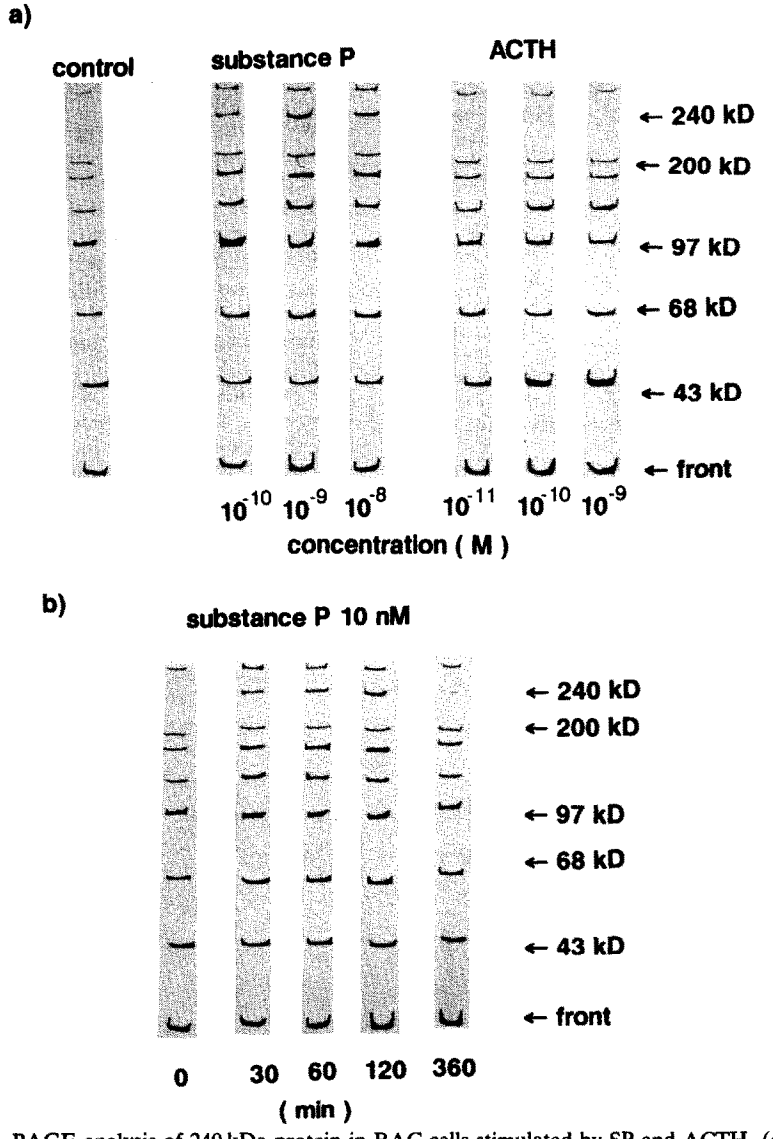

Fig. 3. SDS–PAGE analysis of 240 kDa protein in BAC cells stimulated by SP and ACTH. (a) SDS–PAGE profiles of BAC cells stimulated by various concentrations of SP and ACTH. (b) Sequential SDS–PAGE analysis of BAC cells stimulated by SP (10 nM).

Table 1. [^{3}H]Leucine uptake into the 240 kDa protein band in SDS–PAGE analysis of BAC cells stimulated by SP and ACTH

Stimulant	Concn (M)	[^{3}H]Leucine uptake (cpm)
Control		15.9 ± 0.5
SP	10^{-10}	162.2 ± 1.7*
	10^{-9}	254.4 ± 3.9*
	10^{-8}	406.2 ± 4.1*
ACTH	10^{-9}	15.5 ± 0.6

BAC cells were stimulated by various concentrations of SP or ACTH in the presence of [^{3}H]leucine for 60 min, and SDS–PAGE was carried out. Thereafter, the radioactivity in the 240 kDa protein band was determined.

Each value indicates the mean ± SEM of the data obtained from five separate experiments.

* $P < 0.01$.

detected. When the cells were stimulated with SP, the 240 kDa protein was also detected, as well as the proteins observed in the control cells. Since these proteins bound [^{125}I]calmodulin only in the presence of Ca^{2+}, these proteins appear to be the specific calmodulin-binding proteins. By comparing the SP-stimulated cells with the resting cells, it became evident that only the 240 kDa protein band is a newly synthesized calmodulin-binding protein.

DISCUSSION

It has been suggested that in the ACTH-induced cortisol secretion from adrenocortical cells, both cAMP and Ca^{2+} cooperatively regulate the secretion of corticosteroids [9, 10]. In a previous study, it was indicated that the cortisol secretion due to ACTH is much more dependent upon cAMP than Ca^{2+} [2].

Table 2. Sequential changes in [^{3}H]leucine uptake into the 240 kDa protein band in SDS–PAGE analysis of BAC cells stimulated by SP (10 nM)

Time after stimulation (min)	[^{3}H]Leucine uptake (cpm)
0	14.8 ± 0.7
3	27.4 ± 1.1
10	88.7 ± 1.6*
30	148.7 ± 3.6†
60	406.2 ± 4.1†
120	528.3 ± 5.0†
360	121.5 ± 2.2†

BAC cells were stimulated by 10 nM SP in the presence of [^{3}H]leucine for various periods, and SDS–PAGE was carried out. Thereafter, the radioactivity in the 240 kDa protein band was determined.

Each value indicates the mean ± SEM of the data obtained from five separate experiments.

* $P < 0.05$; † $P < 0.01$.

It is also suggested that the early synthesis of labile proteins, which is mainly regulated by the cAMP–protein kinase A system, may trigger the formation of corticosteroids in adrenocortical cells [11]. However, as shown in Fig. 1a, when BAC cells were stimulated with ACTH, cortisol secretion took place at a lower concentration than that necessary for [^{3}H]leucine uptake, and this seems to indicate that some portion of the cortisol secretion may occur independently of the protein synthesis. In contrast, it has been shown that SP-induced cortisol secretion is predominantly dependent upon the Ca^{2+}–calmodulin system; SP caused a rapid but sustained uptake of ^{45}Ca, and SP-induced cortisol secretion was potently inhibited by calmodulin inhibitors [2]. However, in adrenocortical cells, it was not clear whether or not the SP-induced and Ca^{2+}-mediated protein synthesis plays some important roles in the process leading to cortisol secretion. In the present study, it became apparent that [^{3}H]leucine uptake precedes the SP-induced cortisol secretion. In accordance with this, actinomycin D and cycloheximide inhibited cortisol secretion from BAC cells, indicating that protein synthesis may be

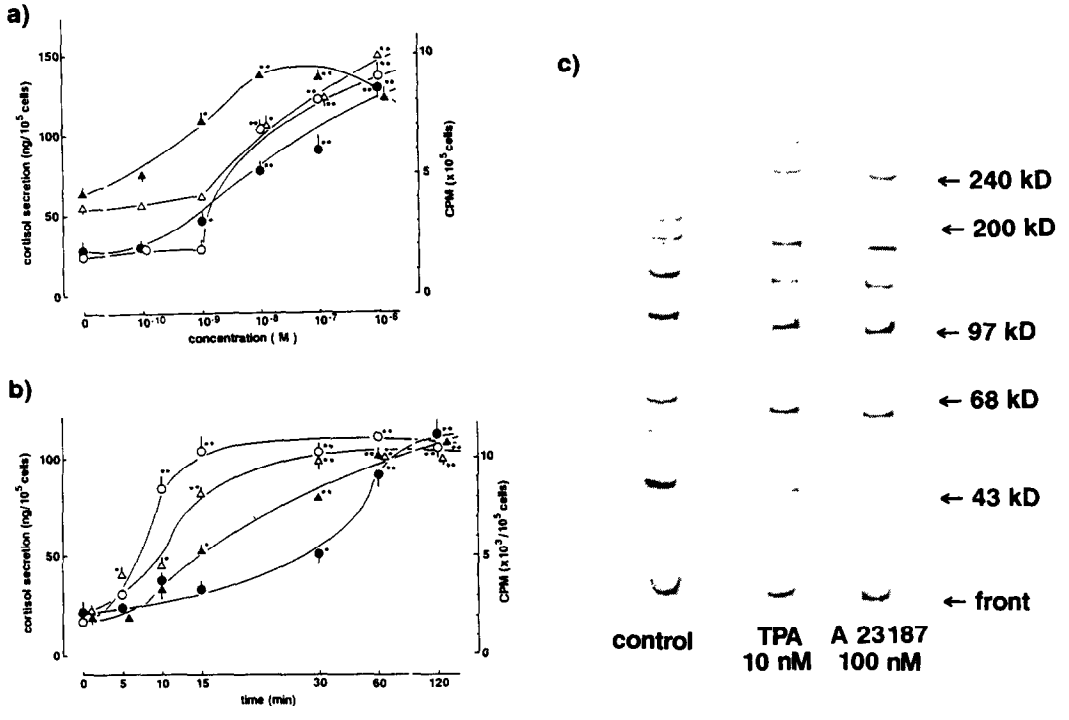

Fig. 4. Cortisol secretion, [^{3}H]leucine uptake and production of 240 kDa protein in BAC cells induced by A23187 and TPA. (a) Dose–response curves of cortisol secretion and [^{3}H]leucine uptake. The cells were stimulated with either compound for 60 min at 37°. (b) Time courses of cortisol secretion and [^{3}H]leucine uptake. The cells were incubated with A23187 (100 nM) and TPA (10 nM) for various incubation periods. (○) and (●) represent cortisol secretion induced by A23187 and TPA, respectively, (△) and (▲) represent [^{3}H]leucine uptake induced by A23187 and TPA, respectively. Each point represents the mean ± SEM of data obtained from five separate experiments. * and ** represent statistical significance compared to resting levels at $P < 0.05$ and $P < 0.01$, respectively. (c) SDS–PAGE profile.

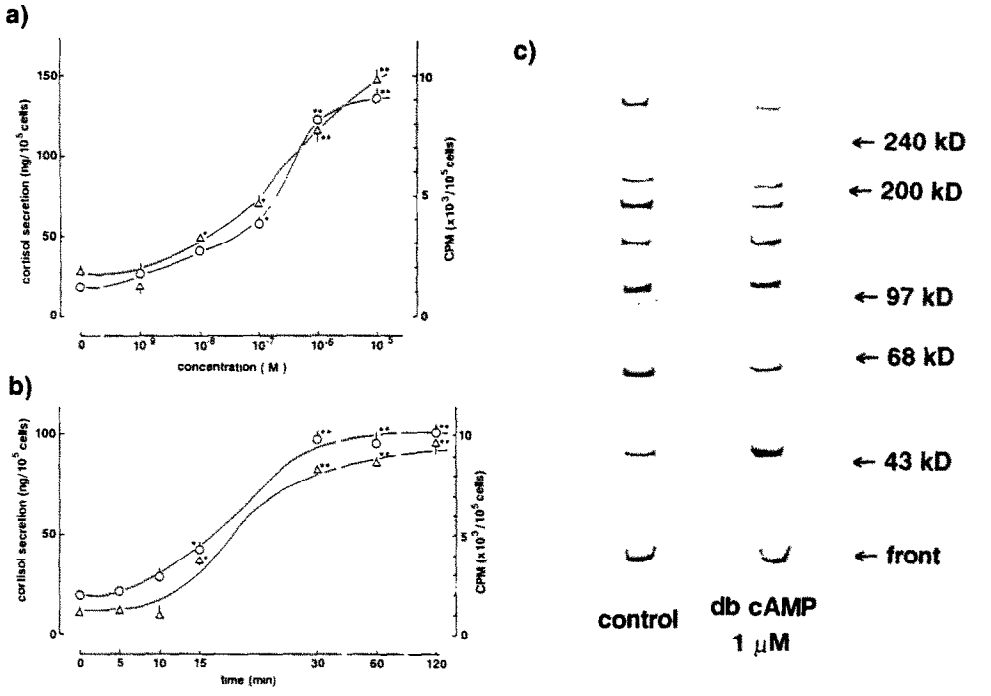

Fig. 5. Cortisol secretion, [^{3}H]leucine uptake and production of 240 kDa protein in BAC cells induced by db-cAMP. (a) Dose–response curves of cortisol secretion (○) and [^{3}H]leucine uptake (△). The cells were stimulated for 60 min at 37°. (b) Time courses of cortisol secretion (○) and [^{3}H]leucine uptake (△). The cells were incubated with db-cAMP (1 μM) for various incubation periods. Each point represents the mean ± SEM of data obtained from five separate experiments. * and ** represent statistical significance compared to resting levels at $P < 0.05$ and $P < 0.01$, respectively. (c) SDS–PAGE profile.

necessary for the induction of cortisol secretion from SP-stimulated BAC cells. These results may indicate that cortisol secretion is linked to a rapid protein synthesis in BAC cells. Comparing the time courses of SP- and ACTH-induced [^{3}H]leucine uptake, it became clear that SP-induced protein synthesis occurs much more rapidly than that seen with ACTH, as in the case of cortisol secretion [2]. A23187 also caused a rapid [^{3}H]leucine uptake similar to SP, though the time course of db-cAMP-induced [^{3}H]leucine uptake was slower in its onset and it was similar to ACTH. Although TPA was also effective in eliciting [^{3}H]leucine uptake and cortisol secretion, both sequences were slower than those seen after SP or A23187. Since such a slow activation by TPA was similar to that observed in TPA-induced histamine release from mast cells [12], it was supposed that slow activation by TPA can be ascribed to the slow penetration of TPA through the cell membrane. These results seem to be in agreement with the previous findings that ACTH-induced cortisol secretion is mainly mediated by an increase in intracellular cAMP level, while SP-induced cortisol secretion is predominantly influenced by an increase in intracellular Ca^{2+} levels. In addition, SP-induced [^{3}H]leucine uptake was inhibited in the presence of calmodulin inhibitors but not with an inhibitor of protein kinase A, though both inhibitors were effective in inhibiting ACTH-induced [^{3}H]leucine uptake. The result seems to indicate that both Ca^{2+}–calmodulin and protein kinase C play some important roles in the SP-induced rapid protein synthesis and the subsequent cortisol secretion. It is also suggested that the protein synthesis caused by ACTH in BAC cells is mediated not only by the cAMP–protein kinase A system but also by the Ca^{2+}–calmodulin system.

By comparing the SDS–PAGE profiles of SP- and ACTH-stimulated BAC cells, it was found that *de novo* synthesis of a 240 kDa protein was characteristic for SP-induced steroidogenesis. On the other hand, an increase in 51 kDa protein was also a distinctive feature of ACTH-stimulated BAC cells as reported previously [3]. It can be assumed that this 240 kDa protein may be intimately related to the cortisol secretion after SP stimulation. Moreover, the incorporation of [^{3}H]leucine into 240 kDa protein indicated that *de novo* synthesis of 240 kDa protein was elicited by SP but not by ACTH as shown in Table 1.

The *de novo* synthesis of 240 kDa protein was also induced by TPA and A23187. Since no 240 kDa protein was detected after ACTH- and db-cAMP stimulations, the synthesis of 240 kDa protein may represent a specific feature of Ca^{2+}-mediated cortisol secretion. Furthermore, since TPA also caused 240 kDa protein synthesis, it was supposed that protein kinase C may also participate in 240 kDa

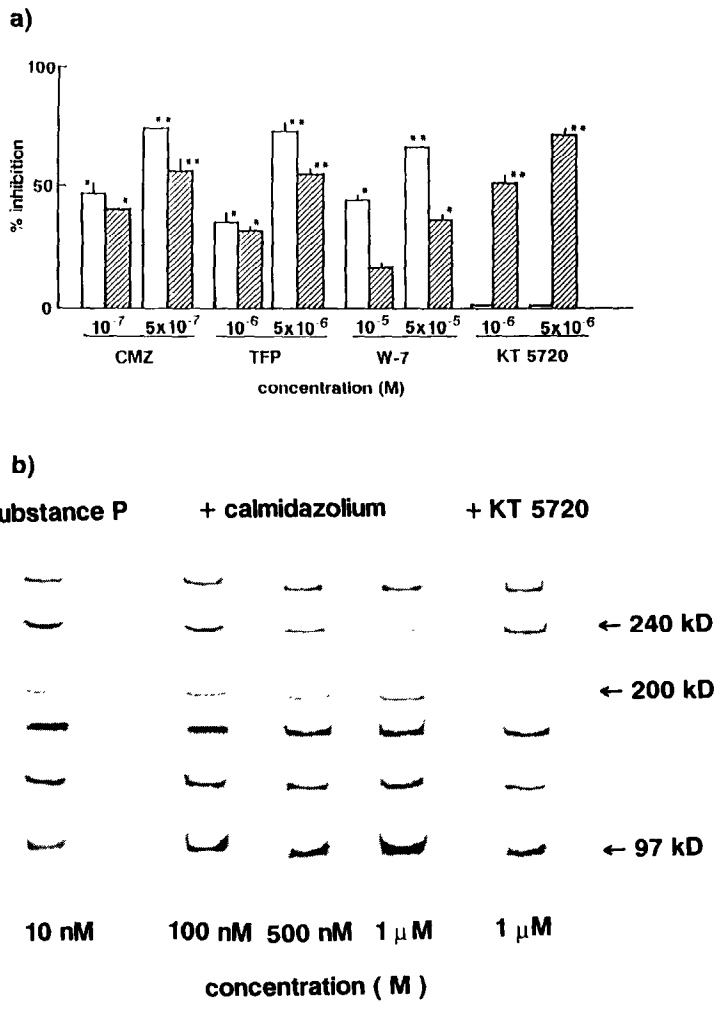

Fig. 6. Inhibitory effects of calmodulin inhibitors and protein kinase A inhibitor on [^{3}H]leucine uptake and 240 kDa protein synthesis in BAC cells elicited by SP and ACTH. (a) [^{3}H]Leucine uptake. The cells were incubated with calmodulin inhibitors and protein kinase A inhibitor simultaneously with SP (10 nM; open columns) or ACTH (100 pM; hatched columns). Each column represents the mean ± SEM of data obtained from five separate experiments. * and ** represent statistical significance compared to control groups at $P < 0.05$ and $P < 0.01$, respectively. CMZ, calmidazolium; TFP, trifluoperazine. (b) SDS–PAGE analysis.

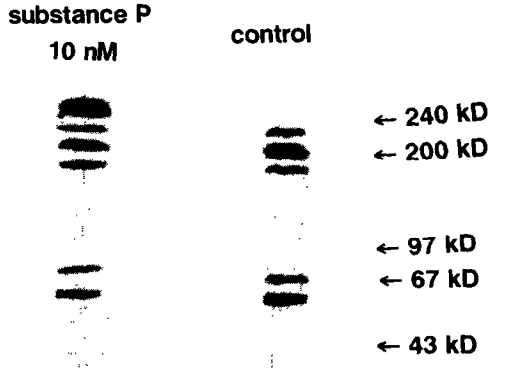

Fig. 7. Detection of calmodulin-binding proteins in BAC cells according to [^{125}I]calmodulin membrane overlay method.

protein synthesis. Since TPA is effective in inducing ^{45}Ca uptake in BAC cells (data not shown), it is also possible that activation of calmodulin may take place after TPA stimulation in association with an increase in the cytosolic Ca^{2+} level. Accordingly, calmidazolium, but not KT-5720, inhibited the synthesis of 240 kDa protein elicited by SP. From these results it was assumed that SP-induced 240 kDa protein synthesis is predominantly mediated by the Ca^{2+}–calmodulin system. At present, it is not known why TPA (or protein kinase C) induces the Ca^{2+} uptake, but cross-talk between Ca^{2+}–calmodulin and protein kinase C systems may take place inside the BAC cells through an increase in cytoplasmic Ca^{2+} levels.

As reported previously, calmodulin inhibitors are effective in inhibiting ACTH-induced cortisol secretion [13]. It has also been shown that ACTH induces Ca uptake into the adrenocortical cells,

though the ACTH-induced Ca uptake actually takes place after an increase of intracellular cAMP levels [14]. In accordance with these findings, calmodulin inhibitors were also effective in inhibiting ACTH-induced [^{3}H]leucine uptake, though ACTH did not induce 240 kDa protein synthesis. This suggests that a part of the newly synthesized proteins may be the same in ACTH- and SP-stimulated cells, though such proteins were not identified in the present SDS–PAGE analysis. It has been reported that the molecular masses of labile proteins synthesized at the early stage of ACTH-induced steroidogenesis are 2.3, 12, 28 and 51 kDa [3], but such proteins (with the exception of 51 kDa protein) were not detected in the present study.

It has been reported that a gel-overlay method of radiolabeled protein after SDS–PAGE is a useful technique for analysing the protein binding to calmodulin, actin and vinculin [7]. However, there have been some difficulties in reducing the SDS concentrations from the gels with low polyacrylamide concentration, so that the proteins in the SDS–polyacylamide gel were transferred to a nitrocellulose membrane, and [^{125}I]calmodulin was overlaid on the blotted membrane as in the case of usual gel-overlay method [7]. Using this method, several protein bands were detected in the resting BAC cells only in the presence of Ca^{2+} but not in the absence of Ca^{2+} (supplemented with EGTA); this may indicate that these proteins are actually calmodulin-binding proteins. After stimulation with SP, only the 240 kDa protein was a newly synthesized calmodulin-binding protein. The 240 kDa protein synthesis was also observed in A23187- and TPA-stimulated BAC cells, but not in ACTH- and db-cAMP-stimulated cells. In addition, 240 kDa protein synthesis was inhibited by calmidazolium, but not by KT-5720. From these findings it can be concluded that the *de novo* synthesis of 240 kDa protein may be intimately related to the cortisol secretion in SP-stimulated BAC cells via an activation of Ca–calmodulin pathway.

REFERENCES

1. Epstein LF and Orme-Johnson NR, Regulation of steroid hormone biosynthesis. Identification of precursors of a phosphoprotein targeted to the mitochondrion in stimulated rat adrenal cortex. *J Biol Chem* **266**: 19739–19745, 1991.
2. Yoshida T, Mio M and Tasaka K, Cortisol secretion induced by substance P from bovine adrenocortical cells and inhibition by calmodulin inhibitors. *Biochem Pharmacol* **43**: 513–517, 1992.
3. Krueger RJ and Orme-Johnson NR, Evidence for the involvement of labile protein in stimulation of adrenal steroidogenesis under conditions not inhibitory to protein synthesis. *Endocrinology* **122**: 1869–1875, 1988.
4. Grower MF and Bransome ED Jr, Adenosine 3′,5′-monophosphate, adrenocorticotropic hormone and adrenocortical cytosol protein synthesis. *Science* **168**: 483–485, 1970.
5. Alberta JA, Epstein LF, Pon LA and Orme-Johnson NR, Mitochondrial localization of phosphoprotein that rapidly accumulates in adrenal cortex cells exposed to adrenocorticotropic hormone or to cAMP. *J Biol Chem* **264**: 2368–2372, 1989.
6. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
7. Glenny JR Jr and Weber K, Detection of calmodulin binding polypeptides separated SDS-polyacrylamide gel by sensitive [^{125}I]calmodulin gel over assay. *Methods Enzymol* **102**: 204–210, 1983.
8. Kase H, Iwahashi S, Nakanishi S, Matsuda Y, Yamada K, Takahashi M, Murakata C, Sato A and Kaneno M, K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinase. *Biochem Biophys Res Commun* **121**: 499–506, 1984.
9. Rasmussen H and Barrett PQ, Calcium messenger system. *Physiol Rev* **64**: 938–984, 1984.
10. Podesta EJ, Milami A, Steffan H and Neher R, Steroidgenic action of calcium ions in isolated adrenocortical cells *Biochem J* **186**: 391–397, 1980.
11. Wong M, Rice DA, Paker KL and Schimmer BP, The role of cAMP and cAMP dependent protein kinase in the expression of cholesterol side chain cleavage and steroid 11β-hydroxylase genes in mouse adrenocortical tumor cells. *J Biol Chem* **264**: 12864–12871, 1989.
12. Cantwell ME and Foreman JC, Characteristics of the effect of phorbol ester on rat mast cells: interaction with anti-IgE. *Agents Actions* **18**: 77–80, 1986.
13. Papadopoulos V, Widmaier EP and Hall PF, The role of calmodulin in responses to adrenocorticotropin of plasma membranes from adrenal cells. *Endocrinology* **126**: 2465–2473, 1990.
14. Sala GB, Hayashi K, Catt KJ and Dufan ML, Adrenocorticotropin action in isolated adrenal cells. *J Biol Chem* **254**: 3861–3865, 1979.