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Calcium Ion as a Second Messenger for *o*-Nitrophenylsulfenyl-Adrenocorticotropin (NPS-ACTH) and ACTH in Bovine Adrenal Steroidogenesis*

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ABSTRACT

o-Nitrophenyl sulfenyl-modified ACTH (NPS-ACTH) stimulated steroidogenesis acutely in bovine fasciculata-reticularis cells without increase in cellular cAMP synthesis. Application of NPS-ACTH to the cultured cells induced Ca^{2+} signals in individual cells as detected by video-enhanced microscopic fluorescence measurements. The percentage of Ca^{2+} signaling cells corresponded well with the increase of steroidogenesis induced by NPS-ACTH below 1 nM. Treatment of the cells with nicardipine, a Ca^{2+} channel blocker, suppressed the Ca^{2+} signals except for the transient increase just after the addition of NPS-ACTH and also blocked completely the stimulative effect on the steroidogenesis of NPS-ACTH below 1 nM. At a dosage of NPS-ACTH higher than 10 nM, the stimulative effect of steroidogenesis was partly suppressed by nicardipine and also by AA-861, a lipoxygenase inhibitor. The action of NPS-ACTH might be mediated by both Ca^{2+} and lipoxygenase metabolite(s) of arachidonic acid as dual second messengers. The effect of ACTH in pM range on the steroidogenesis was suppressed completely by the treatment with nicardipine and AA-861 at the same time, indicating that the action was mediated by both Ca^{2+} and the lipoxygenase metabolite(s) but not by cAMP. cAMP plays a significant role as a second messenger for ACTH action only at ACTH concentrations greater than 10 pM. (*Endocrinology* **139:** 4765–4771, 1998)

GLUCOCORTICOID synthesis in adrenal glands is mainly regulated by ACTH. ACTH acutely stimulates intracellular transport of cholesterol to mitochondrial inner membrane within a few minutes, which is the rate-determining step in overall steroidogenesis of the cells (1, 2). cAMP is one of the second messengers for the action of ACTH because cAMP synthesis in the adrenal cells was stimulated by pharmacological concentrations of ACTH (>100 pM) and the application of cAMP analogs activates adrenal steroidogenesis (3). ACTH modified at a tryptophan residue with *o*-nitrophenyl sulfenyl chloride, NPS-ACTH, stimulates rat, mouse, and bovine adrenal steroidogenesis without detectable increase of cellular cAMP level, although the dose required for the maximal stimulation was several hundred-times higher than that for ACTH (3, 4). It has been speculated, however, that cAMP is an essential mediator of NPS-ACTH action in Y-1 mouse adrenal tumor cell line because steroidogenic activity in A-kinase- or adenylate cyclase-deficient mutants of the Y-1 cell lines was very weak even on stimulation by the NPS-ACTH (5). In contrast to the normal adrenal cells, cellular cAMP synthesis in the Y-1 cells was significantly stimulated by the application of NPS-ACTH (5). The second messenger system of NPS-ACTH in normal adrenal cells might be different from Y-1 cells and requires more detailed investigation.

Calcium ion, Ca^{2+}, has been suggested to be a second messenger for ACTH action in adrenal fasciculata-reticularis cells because steroidogenesis of the cultured cells was stimulated by addition of Ca^{2+} in the medium both in the presence and absence of ACTH (6). Extracellular Ca^{2+} is required for binding of ACTH to its receptor and also for maximum stimulation of steroidogenesis (7). At low ACTH concentrations, Ca^{2+} influx via T-type Ca^{2+} channels might participate in the stimulation of steroidogenesis in bovine fasciculata cells (8, 9). Increase of cellular Ca^{2+}, however, is barely detectable in ACTH-stimulated cells with conventional methods (10, 11). Recently, we have observed Ca^{2+} signals induced by 0.1–100 pM ACTH in individual cultured bovine adrenal fasciculata cells using a microscopic fluorescence measurement technique (12). This observation is strong evidence for a role for Ca^{2+} as a second messenger of ACTH action.

Physiological second messenger(s) for ACTH action had been discussed for a long time. Application in the pM range of ACTH stimulated steroidogenesis in fasciculata-reticularis cells without significant increase in cellular cAMP synthesis (3, 13, 14). We have reported that 15-lipoxygenase metabolite(s) of arachidonic acid is a second messenger for ACTH at the physiological condition because pM concentrations of ACTH-stimulated bovine adrenal 15-lipoxygenase activity, which corresponded well with the stimulation of the steroi-

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dogenesis and addition of 15-hydroperoxyeicosatetraenoic acid (15-HPETE), a 15-lipoxygenase metabolite of arachidonic acid, stimulated steroidogenic activity of the cells (14). The action of ACTH in bovine fasciculata-reticularis cells may be mediated by at least three messengers: cAMP, Ca^{2+}, and the lipoxygenase metabolite(s). On the other hand, the second messenger system of NPS-ACTH is expected to be simpler because cAMP likely does not have a significant role in the action of the modified peptide.

In this report, we clarified the roles of Ca^{2+} and lipoxygenase metabolite(s) as second messengers for the acute action of NPS-ACTH on the steroidogenesis in cultured bovine adrenal fasciculata-reticularis cells. The relative importance of second messengers, *i.e.* cAMP, Ca^{2+} and lipoxygenase metabolite(s), in the acute stimulation by physiological concentrations of ACTH was also characterized.

Materials and Methods

Preparation of NPS-ACTH

ACTH (1–24, Cortrosyn, Daiichi Seiyaku, Tokyo, Japan) was modified by *o*-nitrophenylsulfenyl chloride at tryptophan-9 in the acidic condition, as reported previously (15). The synthesized NPS-ACTH was purified by reverse phase HPLC on a LiChrospher RP-18 column (5 μm, 250 × 4 mm, Merck), with a linear gradient of 20 min from 100% of solution A; 10% CH_3CN, 0.3% CF_3COOH, 89.7% H_2O, to 69% dilution by solution B; 89% CH_3CN, 2% CF_3COOH, 9% H_2O, at the flow rate, 1 ml/min. The NPS-ACTH and ACTH were eluted at 20 and 18 min after the injection, respectively, which were monitored by UV absorption at 280 nm. The eluted NPS-ACTH was lyophilized and dissolved in sterile water. The purity of the NPS-ACTH was confirmed by HPLC analysis and measurement of the absorption spectrum. NPS-ACTH concentration was estimated using an absorption coefficient of 4.1 mM^{-1} at 365 nm (16).

Cell culture, steroidogenic activity, and cAMP analysis

Fresh adrenal glands of Holstein-Friesian cows were obtained from a local slaughterhouse, and fasciculata-reticularis cells were isolated as described (17). Contamination of glomerulosa cells was less than 2% for each preparation because no aldosterone synthesis was detected in culture medium by specific RIA after incubation of the cells without addition of any inhibitors (18). Cells grown to confluence on a 24-well culture plate (Sumitomo Bakelite, Tokyo, Japan) were incubated with a fresh medium containing the pregnenolone metabolism inhibitors, 2 μM trilostane and 20 μM SU-10603 (kindly supplied by C. R. Jefcoate), and steroidogenic stimulators, *i.e.* ACTH, NPS-ACTH, or dibutyryl cAMP ($(Bu)_2$cAMP, Boehringer Mannheim, Mannheim, Germany). Various inhibitors, *i.e.* 40 μM nicardipine (Sigma Chemical Co., St. Louis, MO), and 50 μM AA-861 (Wako, Osaka, Japan) were added 15 min before beginning the incubation for metabolism of cholesterol to pregnenolone. After 2 h incubation of the cells under 5% CO_2 at 38.5 C, accumulated pregnenolone in the medium was extracted with hexane and measured by specific RIA, as described previously (19). Cell morphology did not change during the 2 h incubation with the inhibitors.

For measurement of cAMP accumulation, the cells were incubated for 2 h with fresh medium containing 0.2 mM 3-isobutyl-1-methylxanthine (Sigma Chemical Co.). The cAMP accumulated in the cells was extracted with ethanol and analyzed with a Biotrak cAMP EIA system (Amersham International, Amersham, UK) as described (14).

Microscopic imaging of Ca^{2+} fluorescence and its analysis

For Ca^{2+} imaging, cells were grown in glass-bottom dishes coated with collagen. The cells were loaded for 1 min at 37 C with 5 μM Fura-2/AM (Dojin, Kumamoto, Japan) in the presence of 0.01% Triton X-100 in 1 ml of physiological salt solution (PSS) containing 120 mM NaCl, 4 mM KCl, 1.25 mM $CaCl_2$, 1 mM $NaHPO_4$, 0.5 mM $MgSO_4$, 5 mM $NaHCO_3$, 0.1% BSA, 0.1% glucose, and 10 mM HEPES (pH 7.4). After three washes with PSS, the cells were preincubated for 20 min with or without 40 μM nicardipine at 37 C. The glass-bottom dish was mounted on a video-enhanced fluorescence microscope as previously described (12). The cells were excited by 340 and 380 nm in the presence of various concentrations of NPS-ACTH, and fluorescence intensity was recorded on video tape. After digitizing the intensity of the recorded fluorescence, Ca^{2+} concentrations in individual cells were estimated from image analysis of fluorescence intensity ratio between excitation at 340 nm and 380 nm.

Other methods

Cellular protein content was determined with a BCA protein assay kit (Pierce, Rockford, IL). Data were statistically analyzed by Student's *t* test, using the Microsoft Corp. Excel computer program.

Results

Effects of NPS-ACTH on the steroidogenesis and cAMP synthesis

Steroidogenic activity of cultured bovine adrenal fasciculata-reticularis cells can be estimated from the accumulation of pregnenolone in the presence of trilostane and SU-10603, which are blockers of further metabolism by 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase and cytochrome $P450_{17\alpha,\ lyase}$, respectively, because the pregnenolone formation is the rate-determining step in overall steroidogenesis (1, 2, 14). The acute stimulation of steroidogenesis was estimated from the accumulation of pregnenolone during a 2-h incubation after the application of NPS-ACTH or ACTH. The amounts of accumulated pregnenolone increased with the dose of NPS-ACTH as well as ACTH, and leveled off at a similar level (Fig. 1a). The concentration required for half-maximum stimulation (EC_{50}) is about 1 nM for NPS-ACTH, which is a hundred times higher than that for ACTH (10 pM).

Stimulation of cellular cAMP synthesis by ACTH in the adrenal cells was estimated from the accumulation of cAMP

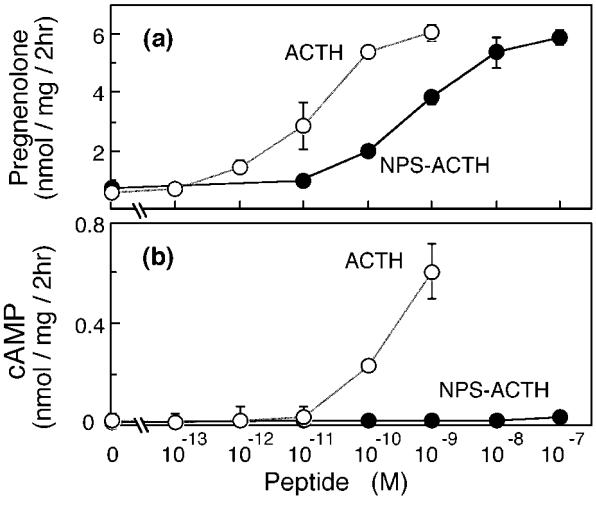

FIG. 1. Dose dependences of the activation of steroidogenesis and cAMP synthesis on the concentrations of NPS-ACTH and ACTH in bovine fasciculata-reticularis cells. The cultured cells were incubated for 2 h with various concentrations of NPS-ACTH (*closed circles*) or ACTH (*open circles*). Accumulated pregnenolone (a) and cAMP (b) were extracted and measured by RIA and enzyme immune assay, respectively, as described in *Materials and Methods*. Values are means of three separate experiments, performed in duplicate. *Error bars* indicate SD.

during the 2-h incubation in the presence of 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor. The ACTH concentration required for the stimulation of cAMP synthesis was more than 10 times higher than that for steroidogenesis as previously reported (3, 13, 14) (Fig. 1b). On the other hand, application of NPS-ACTH affected the cellular cAMP synthesis only slightly. No stimulation of cAMP synthesis was observed on addition of 1 nM NPS-ACTH, upon which steroidogenesis was stimulated to half maximal levels (Fig. 1b). Even on maximal stimulation of the steroidogenesis by 100 nM NPS-ACTH, cAMP accumulation was increased to only 5% of that in the presence of 1 nM ACTH. Similar dose-responses on cAMP accumulation and steroidogenesis induced by NPS-ACTH have been reported in rat and mouse adrenal cultured cells (3). The results indicate that increase of cellular cAMP is not essential for the stimulation of steroidogenesis by NPS-ACTH.

NPS-ACTH induced Ca^{2+} signaling

The effect of NPS-ACTH on the Ca^{2+} concentration in individual cells was analyzed by video enhanced microscopic fluorescence imaging. Application of NPS-ACTH induced typically three types of Ca^{2+} signals in individual cells, *i.e.* (a) series of Ca^{2+} spikes with a step-like Ca^{2+} elevation; (b) a step-like elevation of Ca^{2+} concentration; (c) series of Ca^{2+} spikes without Ca^{2+} elevation (Fig. 2, a–c, respectively). When the Ca^{2+} signals of 100–200 cells induced by 1 nM NPS-ACTH were analyzed in each experiment, signal types in the cells were found to constitute 63 ± 5%, 28 ± 4%, and 9 ± 2% of the relative population for (a), (b), and (c), respectively (mean of four separate experiments). The frequency of Ca^{2+} spikes was variable in individual cells (Fig. 2, a and c), but it did not show apparent correlation with NPS-ACTH concentration. The increase of the dosage of NPS-ACTH increased total population of Ca^{2+} signaling cells without affecting the signal height in individual cells. Similar tendency of Ca^{2+} signals was observed in the same cell system stimulated by 0.1–100 pM ACTH whose frequency and intensity were also independent on the dosage of ACTH (12). The dose-dependence of percentage of total number of Ca^{2+} signaling cells, (a) + (b) + (c), per number of monitored cells on NPS-ACTH concentration corresponded well to the curve for the stimulation of steroidogenesis up to 1 nM NPS-ACTH (Fig. 3). In the presence of 10 nM of NPS-ACTH, 95 ± 4% of cells show Ca^{2+} signals, but only 13 ± 1% in the absence of NPS-ACTH (mean of four

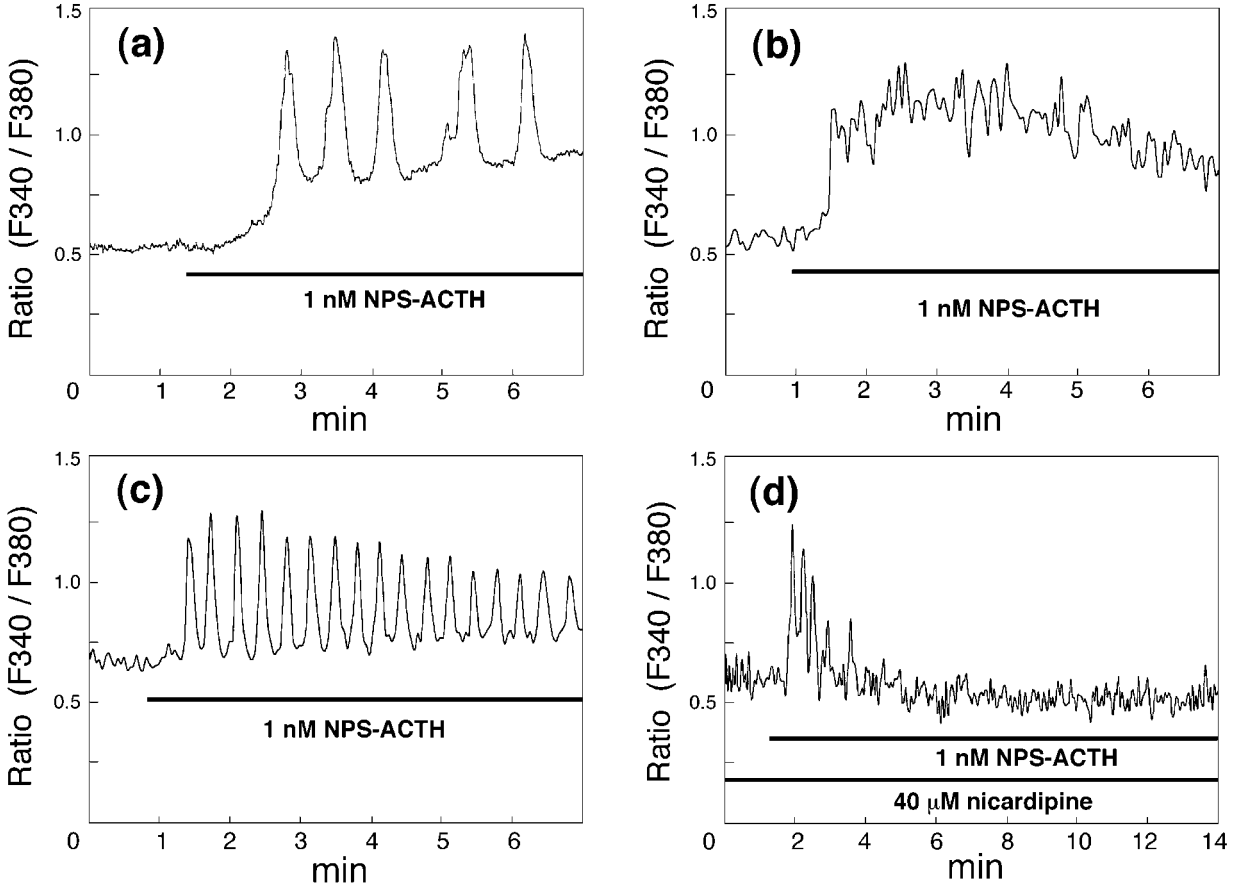

FIG. 2. NPS-ACTH induced Ca^{2+} signals in the individual cells. Ca^{2+} contents in individual bovine fasciculata-reticularis cells were measured by a video enhanced microscopic fluorescence technique as described in *Materials and Methods*. The vertical scale shows the ratio of fluorescence intensities of Fura-2 excited at 340 and 380 nm. The presence of 1 nM NPS-ACTH, indicated by lines in the figures, induced three types of Ca^{2+} signals in individual cells as described in the text (a–c). d, A typical Ca^{2+} signal in the cells pretreated with 40 μM nicardipine 20 min before the application of 1 nM NPS-ACTH.

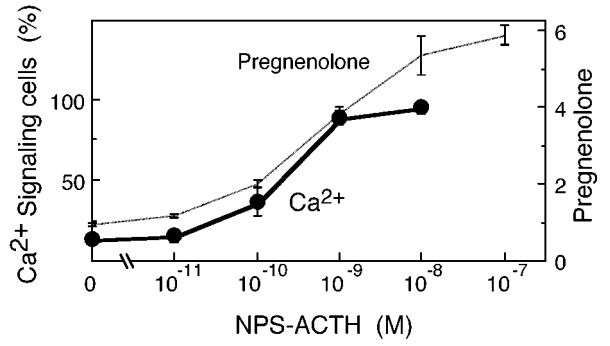

FIG. 3. Dose dependences of the percentage of total number of Ca^{2+} signaling cells and activation of steroidogenesis in the cultured cells on NPS-ACTH concentration. The cells were incubated for 2 h with various concentrations of NPS-ACTH. Ca^{2+} contents of individual cells were measured as for Fig. 2. At each NPS-ACTH concentration, cellular Ca^{2+} contents of 138–208 cells were individually monitored and the percentage of total number of Ca^{2+} signaling cells per number of monitored cells was plotted (*thick line*). Pregnenolone accumulation was determined as Fig. 1 (*thin line*). Values are means of three separate experiments. *Error bars* indicate SD.

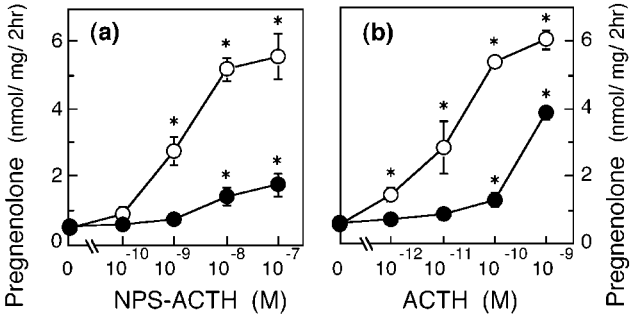

FIG. 4. Effects of nicardipine on NPS-ACTH and ACTH-stimulated cellular steroidogenesis. Incubation of the cells for 2 h with various concentrations of NPS-ACTH (a) or ACTH (b) and the determination of pregnenolone production were carried out as described in *Materials and Methods*. Some cells were pretreated with 40 μM of nicardipine 15 min before the beginning of the 2 h incubation (*closed circles*). Values are means of three separate experiments, performed in duplicate. *Error bars* indicate SD. *, Significantly different from activity of nonstimulated cells ($P < 0.01$).

separate experiments). It must be noted that the percentage of total Ca^{2+} signaling cells was almost saturated around 1 nM NPS-ACTH, while steroidogenesis was increased further with the increase of the peptide to 100 nM (Fig. 3). The action of NPS-ACTH above 1 nM might be mediated by other second messenger(s) beside Ca^{2+}.

Suppression of the action of NPS-ACTH and ACTH by Ca^{2+} channel blocker

When the cells were pretreated with nicardipine, a potent inhibitor of T- and L-type Ca^{2+} channels, the stimulative action of 0.1 and 1 nM NPS-ACTH on the steroidogenesis was completely suppressed (Fig. 4a), indicating the voltage-dependent Ca^{2+} influx might be essential for stimulative action of NPS-ACTH at these concentrations. The concentration of nicardipine required for the complete suppression of the stimulative effect of 1 nM NPS-ACTH was 40 μM in the culture medium containing 10% FCS. In the serum-free medium, the stimulation of steroidogenic activity was suppressed by 1 μM of nicardipine. The steroidogenic activity of the cells in the serum-free medium, however, was fluctuating in each experiment and a mean of the activities was about a half of that in the serum containing medium. Therefore, we used serum containing medium and 40 μM nicardipine to obtain clear results in this experiment. Because nicardipine affected neither nonstimulated nor 0.1 mM $(Bu)_2$cAMP-stimulated steroidogenesis (data not shown), the voltage dependent Ca^{2+} influx might not be essential for the basal steroidogenesis and cAMP-induced stimulation. The NPS-ACTH-induced Ca^{2+} signals were also affected by the inhibitor. In the cells pretreated with nicardipine, the Ca^{2+} elevation of NPS-ACTH-induced (a) type Ca^{2+} signals disappeared within a few minutes although the inhibitor has only a slight effect on the quick rise in Ca^{2+} (Fig. 2d). Most of the Ca^{2+} signaling cells ($90 \pm 5\%$, mean of three separate experiments) showed Ca^{2+} increase only transiently upon the addition of 1 nM NPS-ACTH to the nicardipine pretreated cells. It can be concluded that the voltage-dependent Ca^{2+} channels are responsible for the slow phase (>3 min) of Ca^{2+} signaling which is essential for the stimulatory action of NPS-ACTH in the steroidogenesis. When the cells were stimulated by 10 and 100 nM NPS-ACTH, nicardipine did not suppress the stimulative effect on steroidogenesis completely (Fig. 4a). The results indicate again that a second messenger(s) beside Ca^{2+} affects the steroidogenesis in the presence of NPS-ACTH above 10 nM.

ACTH-induced stimulation of steroidogenesis was also inhibited by the nicardipine pretreatment (Fig. 4b). The inhibitor completely suppressed the stimulation in steroidogenesis induced by 1 and 10 pM of ACTH, indicating that the Ca^{2+} influx is essential for the action of ACTH at these concentrations. In the presence of concentrations of ACTH higher than 100 pM, the inhibitory action of nicardipine on the stimulated steroidogenesis was not complete.

Suppression of the action of NPS-ACTH and ACTH by lipoxygenase inhibitor

ACTH action on the steroidogenesis in bovine fasciculata-reticularis cells is mediated by 15-lipoxygenase metabolite(s) of arachidonic acid as a second messenger (14). The role of the metabolite(s) in the action of NPS-ACTH could be elucidated by treatment of the cells with AA-861, a lipoxygenase inhibitor. Addition of AA-861 had no effect on the steroidogenesis stimulated by 0.1 and 1 nM of NPS-ACTH but suppressed to half the stimulative effects by 10 and 100 nM NPS-ACTH (Fig. 5a). The role of 15-lipoxygenase pathway on the stimulation of steroidogenesis is significant only at higher NPS-ACTH concentrations, at which the percentage of total number of Ca^{2+} signaling cells has already leveled off (Fig. 3). The action of NPS-ACTH at 10 and 100 nM might be mediated by lipoxygenase metabolite(s) together with Ca^{2+}. By contrast, the stimulative effect of ACTH on steroidogenesis was suppressed by AA-861 at any ACTH concentration (Fig. 5b). Stimulation by 1 pM ACTH was completely suppressed by the inhibitor. These results were consistent with a previous observation; 15-lipoxygenase activity was stimulated by pM concentrations of ACTH (14).

The inhibitory effect of nicardipine or AA-861 was not complete when the steroidogenesis was stimulated by high

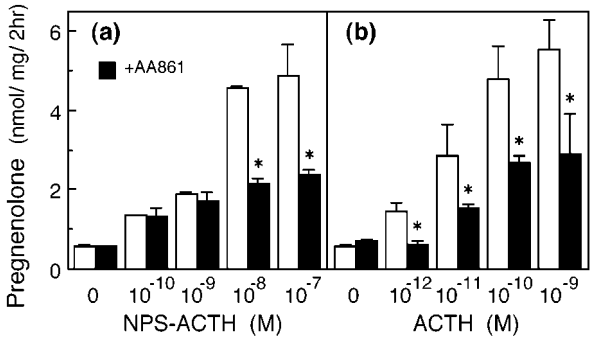

FIG. 5. Effects of AA-861 on NPS-ACTH and ACTH stimulated cellular steroidogenesis. Incubation of the cells for 2 h with various concentrations of NPS-ACTH (a) or ACTH (b), and the determination of pregnenolone production were carried out as described in *Materials and Methods*. Some cells were pretreated with 50 μM of AA-861 15 min before the beginning of the 2 h incubation (*filled columns*). Values are means of three separate experiments, performed in duplicate. *Error bars* indicate SD. *, Significantly different from activity without AA-861 ($P < 0.05$).

concentrations of NPS-ACTH or ACTH (Figs. 4 and 5). The maximally stimulated steroidogenesis by 100 nM NPS-ACTH was completely suppressed by the coaddition of the inhibitors (Table 1). Because AA-861 did not affect 0.1 mM $(Bu)_2$cAMP-stimulated steroidogenesis (14) as like as nicardipine, the data indicate that the second messengers for 100 nM NPS-ACTH were Ca^{2+} and lipoxygenase metabolite(s) but not cAMP. In the case of ACTH-stimulated steroidogenesis, the suppression induced by the coaddition of the inhibitors was not complete upon the addition of 1 nM ACTH. Because the ACTH at concentrations higher than 0.1 nM stimulated cellular cAMP synthesis significantly (Fig. 1b), the action of 1 nM of ACTH might be mediated by cAMP beside Ca^{2+} and lipoxygenase metabolite(s).

Discussion

Application of NPS-ACTH hardly stimulated cellular cAMP synthesis in bovine fasciculata-reticularis cells (Fig. 1b). Stimulation of steroidogenesis by lower concentrations (<1 nM) of NPS-ACTH might be mediated by increase of cellular Ca^{2+} as a sole, or major, second messenger, because dose responses of the percentage of total number of Ca^{2+} signaling cells and the stimulation of steroidogenesis induced by NPS-ACTH were quite similar (Fig. 3). Under the above conditions, nicardipine completely blocked the stimulative action of NPS-ACTH on the steroidogenesis (Fig. 4a). Upon the addition of higher doses (>10 nM) of NPS-ACTH, the metabolite(s) of lipoxygenase activity might be important in stimulation of the steroidogenesis together with Ca^{2+}, because the stimulation was partly suppressed by AA-861 (Fig. 5a). Although a slight increase of cAMP synthesis was observed upon the addition of 100 nM NPS-ACTH (Fig. 1b), the stimulation of steroidogenesis was completely suppressed by pretreatment of nicardipine and AA-861 at the same time (Table 1). Cellular cAMP can be concluded not to be essential in the acute action of NPS-ACTH at any concentration.

ACTH-induced acute stimulation of steroidogenesis is mediated by at least three second messengers, cAMP, Ca^{2+}, and

TABLE 1. Effects of nicardipine and AA-861 on the steroidogenesis of bovine fasciculata-reticularis cells stimulated by NPS-ACTH or ACTH

	NPS-ACTH (100 nM)	ACTH (1 nM)	Nonstimulated
	nmol pregnenolone/mg protein/2 h		
Without inhibitor	6.1 ± 0.8	5.0 ± 0.04	0.47 ± 0.03
Nicardipine (40 μM)	1.7 ± 0.3	3.9 ± 0.2	0.44 ± 0.1
AA-861 (50 μM)	2.4 ± 0.1	2.9 ± 1.0	0.50 ± 0.09
Nicardipine + AA-861	0.44 ± 0.11	1.2 ± 0.2	0.46 ± 0.05

Treatments of the cells and measurements of pregnenolone production were carried out as described in *Materials and Methods*. Values are means ± SD of three separate experiments.

lipoxygenase metabolite(s), in bovine fasciculata-reticularis cells. Application in the pM range of ACTH stimulated both Ca^{2+} signals and 15-lipoxygenase activity, while increase of total cellular cAMP was too small to be detected by conventional methods (12–14). Essential roles of Ca^{2+} and the lipoxygenase metabolite(s) in the stimulative effect of ACTH on the steroidogenesis were confirmed by the experiments using nicardipine and AA-861, both of which inhibited the ACTH-induced stimulation without affecting basal steroidogenesis. It must be noted that 0.1 mM $(Bu)_2$cAMP-stimulated steroidogenesis was not affected by either of the inhibitors, indicating that the stimulative action of ACTH at pM concentrations is not attributable to the increase in cellular cAMP. Although no increase of cellular cAMP was observed at 1 pM ACTH-stimulated condition, basal A-kinase activity might be essential for steroidogenesis, which has been indicated in A-kinase deficient adrenal tumor cells and in normal adrenal cells where an A-kinase inhibitor, H-89, suppressed the basal A-kinase activity (14, 20, 21). cAMP might be a second messenger in the action of ACTH at concentrations above 10 pM, at which a slight increase of cellular cAMP was observed (Fig. 1b). Upon the addition of pharmacological concentrations of ACTH (>100 pM), cellular cAMP synthesis was significantly increased, where the inhibitory effects of nicardipine and AA-861 on the steroidogenesis were incomplete (Table 1). cAMP might be a significant second messenger under these conditions, although Ca^{2+} and lipoxygenase metabolite(s) still have roles in the action. It has been reported for the chronic effect of ACTH in the same cell system that cAMP does not function as a sole intracellular mediator of induction of steroidogenic enzymes, even upon the application of very high concentrations of ACTH, 1 μM (22).

In the bovine fasciculata cells, voltage-dependent Ca^{2+} channels have been predicted to function in stimulation of steroidogenesis (13). Enyeart *et al.* (9) reported that T-type rather than L-type Ca^{2+} channels are required for ACTH-induced stimulation of steroidogenesis. They found with patch clamp experiments that T-type Ca^{2+} channels operate in ACTH action with an EC_{50} of 10.4 pM (8). The concentration of the EC_{50} is consistent with our previous observation that showed the percentage of Ca^{2+} signaling cells was increased by 0.1–100 pM of ACTH (12). Ca^{2+} is one of the major second messengers in angiotensin II or potassium-induced stimulation of aldosterone synthesis in adrenal glomerulosa cells (23). The T- and L-type Ca^{2+} channels in the bovine glomerulosa cells were also completely blocked by nicardi-

pine, which abolished potassium-induced stimulation of aldosterone synthesis (24). The frequency of series of Ca^{2+} spikes in the glomerulosa cells, however, was significantly increased by the increase in concentration of angiotensin II, although that in fasciculata-reticularis cells was not correlated with the concentration of NPS-ACTH or ACTH (25). The Ca^{2+} signaling mechanism in fasciculata-reticularis cells, which was stimulated by ACTH or NPS-ACTH might not be the same as in the glomerulosa cells induced by angiotensin II and potassium.

The Ca^{2+} and 15-lipoxygenase metabolite(s) are second messenger for ACTH and NPS-ACTH induced stimulation in the bovine adrenal cells. Interaction of those two second messenger systems has been reported in various cells. Cellular Ca^{2+} signals were reported to activate phospholipase A_2, which stimulated release of arachidonic acid in adrenal cells, and also to activate cytosolic 15-lipoxygenase activity in human polymorphonuclear leukocyte (26, 27). Addition of lipoxygenase metabolites of arachidonic acid has been reported to stimulate Ca^{2+} releases from mitochondria that were isolated from the bovine zonae fasciculata-reticularis and also from rat liver cells (28, 29). In the bovine fasciculata-reticularis cells, there is a possibility of cross-talk between Ca^{2+} signals and the lipoxygenase pathway in the ACTH-induced stimulation because the stimulation was inhibited by nicardipine and AA-861 at the same ACTH concentrations, indicating the Ca^{2+} and 15-lipoxygenase metabolite(s) function at the same time (Figs. 4b and 5b). On the other hand, NPS-ACTH-stimulated steroidogenesis was inhibited by nicardipine and AA-861 at different NPS-ACTH concentrations (Figs. 4a and 5a). These data might indicate Ca^{2+} and 15-lipoxygenase metabolite(s) stimulate steroidogenesis independently. The interaction between Ca^{2+} signals and the lipoxygenase pathway in the bovine fasciculata-reticularis steroidogenesis might not be simple and remain to be clarify.

Only one class of ACTH-receptor has been identified in adrenal fasciculata-reticularis cells (30, 31). NPS-ACTH might bind to the ACTH receptor because ACTH-induced cAMP synthesis was antagonized by NPS-ACTH (32, 33). Truncated ACTH, $ACTH^{11-24}$, antagonized ACTH-induced cAMP synthesis in mouse ACTH receptor expressed in HeLa cells (34). The IC_{50} of the truncated ACTH was about 1 nM, similar to EC_{50} of NPS-ACTH for the stimulation of the steroidogenesis in this study. The $ACTH^{11-24}$ could stimulate steroidogenic activity in rat adrenal cells (35). Because the NPS-ACTH is modified at Trp^9 of $ACTH^{1-24}$, the nitrophenyl sulfenyl residue might prevent binding of the amino-terminal region of ACTH to the specific site in the ACTH receptor. The remaining region of $ACTH^{11-24}$ might stimulate steroidogenesis without increase of cAMP.

For investigation of the mechanism for Ca^{2+}-mediated stimulation of steroidogenesis in the fasciculata-reticularis cells, ACTH-induced stimulation might not be suitable because the stimulative action of ACTH is mediated by not only Ca^{2+} but also other second messengers at any ACTH concentration. On the other hand, Ca^{2+} is a solo, or major, second messenger for the action of 1 nM NPS-ACTH at which steroidogenesis was stimulated to a half maximum level. The NPS-ACTH-induced Ca^{2+} signals were indistinguishable from those induced by ACTH in terms of both signal shape and intensity. Therefore, NPS-ACTH-stimulated cultured cells might be a suitable experimental system for investigating the mechanism of stimulation of steroidogenesis by Ca^{2+} signals. Further experiments on Ca^{2+}-induced cellular responses are underway in our laboratory.

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References

1. **Orme-Johnson NR** 1990 Distinctive properties of adrenal cortex mitochondria. Biochim Biophys Acta 1020:213–231
2. **Jefcoate CR, McNamara BC, Artemenko I, Yamazaki T** 1992 Regulation of cholesterol movement to mitochondrial cytochrome $P450_{scc}$ in steroid hormone synthesis. J Steroid Biochem Mol Biol 43:751–767
3. **Schimmer BP** 1980 Cyclic nucleotides in hormonal regulation of adrenocortical function. Adv Cyclic Nucleotide Res 13:181–214
4. **Rubin RP, Laychock SG, End DW** 1977 On the role of cyclic AMP and cyclic GMP in steroid production by bovine cortical cells. Biochim Biophys Acta 496:329–338
5. **Rae PA, Zinman HZ, Ramachandran J, Schimmer BP** 1980 Responses of Y1 adrenocortical tumor cells to *o*-nitrophenyl sulfenyl ACTH. Mol Cell Endocrinol 17:171–179
6. **Yanagibashi K** 1979 Calcium ion as "second messenger" in corticoidogenic action of ACTH. Endocrinol Jpn 26:227–232
7. **Cheitlin R, Buckley DI, Ramachandran J** 1985 The role of extracellular calcium in corticotropin-stimulated steroidogenesis. J Biol Chem 260:5323–5327
8. **Mlinar B, Biagi BA, Enyeart JJ** 1993 A novel K^+ current inhibited by adrenocorticotropic hormone and angiotensin II in adrenal cortical cells. J Biol Chem 268:8640–8644
9. **Enyeart JJ, Mlinar B, Enyeart JA** 1993 T-type Ca^{2+} channels are required for adrenocorticotropin-stimulated cortisol production by bovine adrenal zona fasciculata cells. Mol Endocrinol 7:1031–1040
10. **Braley LM, Menachery AI, Brown EM, Williams GH** 1986 Comparative effect of angiotensin II, potassium, adrenocorticotropin, and cyclic adenosine 3′,5′-monophosphate on cytosolic calcium in rat adrenal cells. Endocrinology 119:1010–1029
11. **Iida S, Widmaier EP, Hall PF** 1986 The phosphatidylinositide-Ca^{2+} hypothesis does not apply to the steroidogenic action of corticotropin. Biochem J 236:53–59
12. **Kimoto T, Ohta Y, Kawato S** 1996 Adrenocorticotropin induces calcium oscillation in adrenal fasciculata cells: single cell imaging. Biochem Biophys Res Commun 221:25–30
13. **Yanagibashi K, Papadopoulos V, Masaki E, Iwaki T, Kawamura M, Hall PF** 1989 Forskolin activates voltage-dependent Ca^{2+} channels in bovine but not in rat fasciculata cells. Endocrinology 124:2383–2391
14. **Yamazaki T, Higuchi K, Kominami S, Takemori S** 1996 15-Lipoxygenase metabolite(s) of arachidonic acid mediates adrenocorticotropin action in bovine adrenal steroidogenesis. Endocrinology 137:2670–2675
15. **Ramachandran J, Lee V** 1970 Preparation and properties of the *o*-nitrophenyl sulfenyl derivative of ACTH: an inhibitor of the lipolytic action of the hormone. Biochem Biophys Res Commun 38:507–512
16. **Scoffone E, Fontana A, Rocchi R** 1968 Sulfenyl halides as modifying reagents for polypeptides and proteins. Biochemistry 7:971–979
17. **Yamazaki T, Nawa K, Kominami S, Takemori S** 1992 Cytochrome $P\text{-}450_{17\alpha,\,lyase}$-mediating pathway of androgen synthesis in bovine adrenocortical cultured cells. Biochim Biophys Acta 1134:143–148
18. **Chu FW, Hyatt PJ** 1986 Purification of dispersed rat adrenal zona glomerulosa cells by Percoll density gradient centrifugation and the isolation of a population of cells highly responsive to adrenocorticotropin. J Endocrinol 109:351–358
19. **Yamazaki T, McNamara BC, Jefcoate CR** 1993 Competition for electron transfer between cytochrome $P450_{scc}$ and $P450_{11\beta}$ in rat adrenal mitochondria. Mol Cell Endocrinol 95:1–11
20. **Clegg CH, Abrahamsen MS, Degen JL, Morris DR, McKnight GS** 1992 Cyclic AMP-dependent protein kinase controls basal gene activity and steroidogenesis in Y1 adrenal tumor cells. Biochemistry 31:3720–3726
21. **Miyamoto N, Seo H, Kanda K, Hidaka H, Matsui N** 1992 A 3′,5′-cyclic adenosine monophosphate-dependent pathway is responsible for a rapid increase in c-*fos* messenger ribonucleic acid by adrenocorticotropin. Endocrinology 130:3231–3236
22. **Hanukoglu I, Feuchtwanger R, Hanukoglu A** 1990 Mechanism of corticotropin and cAMP induction of mitochondrial cytochrome P450 system enzymes in adrenal cortex cells. J Biol Chem 265:20602–20608
23. **Barrett PQ, Bollag WB, Rasmussen H** 1988 Mechanism of action of angiotensin II. In: Cooke BA, King RJB, van der Molen HJ (eds) Hormones and Their Actions, Part II. Elsevier, New York, pp 211–229
24. **Burnay MM, Python CP, Vallotton MB, Capponi AM, Rossier MF** 1994 Role

of the capacitative calcium influx in the activation of steroidogenesis by angiotensin-II in adrenal glomerulosa cells. Endocrinology 135:751–758
25. **Rössig R, Z-lyomi A, Catt KJ, Balla T** 1996 Regulation of angiotensin II-stimulated Ca^{2+} oscillations by Ca^{2+} influx mechanisms in adrenal glomerulosa cells. J Biol Chem 271:22063–22069
26. **Schrey MP, Rubin RP** 1979 Characterization of a calcium-mediated activation of arachidonic acid turnover in adrenal phospholipids by corticotropin. J Biol Chem 254:11234–11241
27. **Nichols RC, Vanderhoek JY** 1991 Calcium regulation of the human PMN cytosolic 15-lipoxygenase. Biochim Biophys Acta 1085:77–81
28. **Nishikawa T, Omura M, Noda M, Yoshida S** 1994 Possible involvement of lipoxygenase metabolites of arachidonic acid in the regulation of pregnenolone synthesis in bovine adrenocortical mitochondria. J Biochem 116:833–837
29. **Richter C, Frei B, Cerutti PS** 1987 Mobilization of mitochondrial Ca^{2+} by hydroperoxy-eicosatetraenoic acid. Biochem Biophys Res Commun 143: 609–616
30. **Buckley DI, Ramachandran J** 1981 Characterization of corticotropin receptors on adrenocortical cells. Proc Natl Acad Sci USA 78:7431–7435
31. **Zhang SH, Hennessy DP, McCauley I, Cranwell PD** 1993 Adrenocortical ACTH receptors in pigs of differing *in vivo* response to adrenocorticotropin. Comp Biochem Physiol 104A:43–49
32. **Seelig S, Sayers G** 1973 Isolated adrenal cortex cells: ACTH agonists, partial agonists, antagonis: cyclic AMP and corticosterone production. Arch Biochem Biophys 154:230–239
33. **Moyle WR, Kong YC, Ramachandran J** 1973 Steroidogenesis and cyclic adenosine 3′,5′-monophosphate accumulation in rat adrenal cells. Divergent effects of adrenocorticotropin and its *o*-nitrophenyl sulfenyl derivative. J Biol Chem 218:2409–2417
34. **Kapas S, Cammas FM, Hinson JP, Clark AJL** 1996 Agonist and receptor binding properties of adrenocorticotropin peptides using the cloned mouse adrenocorticotropin receptor expressed in a stably transfected HeLa cell line. Endocrinology 137:3291–3294
35. **Szalay KS, Wied DD, Stark E** 1989 Effects of ACTH-(11–24) on the corticosteroid production of isolated adrenocortical cells. J Steroid Biochem 32:259–262