The Role of Extracellular Calcium in Corticotropin-stimulated Steroidogenesis*

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The role of extracellular Ca\(^{2+}\) in the binding of corticotropin (ACTH) to adrenocortical cell receptors as well as in the post-binding events involved in steroidogenesis were investigated. Binding studies using \[^{125}\text{I}^{-}\text{Tyr}^{3},\text{Phe}^{5},\text{Nle}^{7}\]ACTH (1–38) peptide showed that extracellular Ca\(^{2+}\) is essential not only for the interaction of ACTH with its receptor, but also for continued occupancy of the receptor. In view of the requirement of Ca\(^{2+}\) for binding the hormone to its receptor, the role of Ca\(^{2+}\) in post-receptor events was investigated by covalently attaching the hormone to its receptor by photoaffinity labeling in the presence of Ca\(^{2+}\). Persistent activation of steroidogenesis induced by photoaffinity labeling in the presence of Ca\(^{2+}\) was depressed when cells were incubated in medium containing EGTA but was unaffected when the cells were merely washed and incubated in Ca\(^{2+}\)-free medium. In the presence of EGTA, 8-Br-cAMP partially restored persistent activation of steroidogenesis. The concentration of extracellular Ca\(^{2+}\) required for restoring steroidogenesis was 10-fold lower than the concentration of Ca\(^{2+}\) needed for optimal binding of ACTH to its receptor. These results suggest that the primary role of extracellular Ca\(^{2+}\) in the action of ACTH is to facilitate the association of the hormone with its receptor.

The importance of calcium in the stimulation of steroidogenesis in the adrenal cortex by ACTH was first pointed out by Birmingham et al. (1). Several groups have shown, subsequently, that extracellular calcium is required for ACTH-induced steroidogenesis in isolated adrenocortical cells derived from several species (2–5). There have been numerous attempts to elucidate the precise role of the ion in the actions of ACTH. Lefkowitz et al. (6) investigated the binding of \[^{125}\text{I}\]labeled ACTH preparations to a subcellular fraction derived from a mouse adrenal tumor and proposed their results as evidence for the existence of two classes of ACTH receptors with vastly differing affinities and capacities. However, the existence of the very high affinity sites (apparent \(K_D\), \(1.1 \times 10^{-15}\) M; 60 sites/cell) were not confirmed, and other investigators concluded that calcium is not required for the interaction of ACTH with the adrenal receptor(s) (6, 7). This conclusion has been widely accepted, and most of the studies of the role of calcium have focused on events subsequent to the binding of the hormone to its receptor.

We have recently synthesized an analog of ACTH, \[^{125}\text{I}\]-[\(\text{Phe}^{5},\text{Nle}^{7}\)]ACTH (1–38) peptide, which retains the full biological potency of the hormone after radioiodination (8, 9). \[^{125}\text{I}\]-[\(\text{Tyr}^{3},\text{Phe}^{5},\text{Nle}^{7}\)]ACTH (1–38) peptide (referred to as \[^{125}\text{I}\]ACTH analog hereafter) was obtained in a homogeneous state by reverse phase HPLC and found to have a specific radioactivity of 1800 ± 75 Ci/mmol (9). \[^{125}\text{I}\]ACTH analog was found to be equipotent with ACTH in stimulating corticosterone production in isolated rat adrenocortical cells (8, 9). Binding studies with \[^{125}\text{I}\]ACTH analog showed that the adrenocortical cells contain a single class of receptors with an apparent \(K_D\) of 4.1 ± 0.21 nM and a capacity of 3840 ± 1045 sites/cell (10). Binding correlated very well with the ability to stimulate cAMP production, but maximal steroidogenesis was produced by the occupancy of only 3% of the receptors, indicating receptor reserve. The concentration-response curves for cAMP production for a series of ACTH analogs were found to be superimposable on the binding inhibition curves (10). In view of the large discrepancies between our results and those reported by Lefkowitz et al. (6), we have reinvestigated the role of calcium in the actions of ACTH including the primary step of interaction with its receptor. In this study, we have utilized specific photoaffinity labeling of ACTH receptors (11) to distinguish between the role of calcium in the binding of the hormone to its receptor and the role of the ion in post-binding events.

MATERIALS AND METHODS

Highly purified porcine ACTH and its \([2\text{-nitro-5-azidophenylsulfonyl-Tyr}^{3}]\) derivative were prepared as previously described (12). EGTA and CaCl\(_2\) were obtained from J. T. Baker Chemical Co. 8-Br-cAMP was obtained from Sigma. Medium 199 and custom RPMI medium were provided by the Cell Culture Facility, University of California, San Francisco.

\[^{125}\text{I}\]ACTH Analog.—[\(\text{Phe}^{5},\text{Nle}^{7}\)]ACTH (1–38) peptide was synthesized by the solid-phase method and purified by partition chromatography as described (8). \[^{125}\text{I}\]ACTH analog was prepared and separated from free iodide by gel filtration on Sephadex LH-20 as described (9). It was stored at 4 °C and freshly purified by reverse phase HPLC for each experiment (9). The HPLC solvents were removed by exchange on a Sephadex G-25 column (1 x 20 cm) equilibrated with medium.
199 containing 0.5% BSA and 0.01% bacitracin. The Sephadex column was treated with 0.1 mg of polylysine in the same solvent prior to exchanging the HPLC solvent. The specific radioactivity of the 

$^{125}$I-ACTH analog used in these studies was $1800 \pm 75$ Ci/mmol (mean $\pm$ S.E.). The steroidogenic potency of the $^{125}$I-ACTH analog was identical to that of ACTH (9).

Binding Studies—Adrenocortical cells were isolated from the degenerated adrenal glands of adult male Sprague-Dawley rats (350-400 g) by digestion with collagenase and DNase as previously described (13). Cells were suspended in medium containing 10% fetal bovine serum and 0.004% gentamicin (Schering Corp.) at a density of $7.6 \times 10^5$ cells/ml and incubated overnight (18 h) in sterile bacterial Petri dishes (Falcon, no. 1029) at 37 °C in 5% CO$_2$/95% air. Cells were harvested as described (10) and resuspended in medium 199 containing 0.5% BSA and 0.01% bacitracin (4 x $10^6$ cells/ml) and incubated at 24 °C with 200 pm $^{125}$I-ACTH analog in the presence or absence of excess unlabeled ACTH (440 nM) for 1 h in an atmosphere of 5% CO$_2$/96% O$_2$. Cells were then processed by centrifugation through a cushion of medium 199 containing 1.5% BSA as previously described for determining cell-associated radioactivity (10). Aliquots from each incubation were analyzed for corticosterone and cAMP by radioimmunoassay, and the DNA content of the cell pellet was measured by the method of Short et al. (14).

Photolysis of Adrenocortical Cells—Cells were plated at a density of $10^6$ cell/ml/well as described elsewhere (15) and used after 5 days in primary culture. Cells were washed (3 times) with medium 199/0.5% BSA/0.01% bacitracin and then incubated with or without 2.5-NA$^{3}$P-ACTH (100 nm) for 15 min at 24 °C in the dark. Photolysis was then performed using a Blak-Ray lamp emitting principal radiation at 366 nm as previously described (11). Cells were washed 2 times after photolysis with the incubation medium and incubated with ACTH antiserum 46-2 (9) at a 1:20 dilution for 30 min to remove noncovalently bound peptide. Cells were then washed 2 times with the incubation medium with 0.5 mM EGTA and finally incubated in 0.25 ml of the same medium containing 0.5% BSA and 0.01% bacitracin and then incubated with or without 2,5-NA$^{3}$P-ACTH (100 nm) for 15 min at 24 °C in the dark.

RESULTS

$^{125}$I-ACTH analog was used in direct binding studies with isolated rat adrenocortical cells to elucidate the role of calcium in the interaction of ACTH with its physiological receptor. The results presented in Fig. 1 show that there is an absolute requirement for extracellular calcium for both the binding of $^{125}$I-ACTH analog to adrenocortical cells and for stimulation of corticosterone production. Binding of the peptide and steroidogenesis which are maximal in medium 199 (bars in Fig. 1) are both abolished by the addition of 3 mM EGTA which is more than sufficient to chelate the calcium ions (1.8 mM) present in medium 199. Addition of 1 mM Ca$^{2+}$ does not restore binding or function since EGTA is still in excess; however, when the Ca$^{2+}$ concentration exceeded that of EGTA, both binding and steroidogenesis were restored in parallel. cAMP production in response to ACTH was also found to be dependent on extracellular Ca$^{2+}$ as reported earlier (2-4). cAMP production in the experiment shown in Fig. 1 was 11.85 ± 0.11 pmol/h in medium 199 alone, <0.1 pmol/h in the presence of 3 mM EGTA, and 11.9 ± 0.4 pmol/h in the presence of 3 mM EGTA and 3 mM Ca$^{2+}$. Studies using calcium-free RPMI medium confirmed the above findings. No significant binding of $^{125}$I-ACTH analog was observed in the absence of Ca$^{2+}$, and addition of Ca$^{2+}$ resulted in a parallel increase in binding as well as corticosterone production (Table 1).

![Effect of Ca$^{2+}$ on the binding of $^{125}$I-ACTH analog to adrenocortical cells](image1.png)

**FIG. 1.** Effect of Ca$^{2+}$ on the binding of $^{125}$I-ACTH analog and stimulation of steroid synthesis in adrenocortical cells. Adrenocortical cells were incubated in medium 199/0.5% BSA/0.01% bacitracin with 200 pm $^{125}$I-ACTH analog in the absence or presence of 3 mM EGTA containing various concentrations of Ca$^{2+}$ (0-6 mM), and cell-bound radioactivity and corticosterone production were assessed as described under "Materials and Methods." Nonspecific binding measured in the presence of 440 nM ACTH has been subtracted from the values which are means ± S.E. of duplicate analyses of duplicate incubations.

**TABLE 1**

| Ca$^{2+}$ concentration (mM) | $^{125}$I-ACTH analog bound (pmol/50 µg DNA) | Corticosterone (ng/h) |
|-----------------------------|--------------------------------------------|----------------------|
| 0                           | 0.01 ± 0.21                                | 26.2 ± 1.7           |
| 0.5                         | 2.37 ± 0.11                                | 193 ± 22             |
| 1.8                         | 4.04 ± 0.18                                | 175 ± 13.1           |
| 8.0                         | 3.95 ± 0.18                                | 178 ± 31.7           |

![Effect of Ca$^{2+}$ on the dissociation of $^{125}$I-ACTH analog from adrenocortical receptors](image2.png)

**FIG. 2.** Effect of Ca$^{2+}$ on the dissociation of $^{125}$I-ACTH analog from adrenocortical receptors. Cells were incubated with 200 pm $^{125}$I-ACTH analog for 1 h at 24 °C and then centrifuged at 700 x g for 7 min. The supernatant was removed, and the cells were resuspended in fresh medium, divided into two parts, and incubated in the absence and presence of 3 mM EGTA. Aliquots were removed in triplicate at various times and processed as described under "Materials and Methods." Nonspecific binding measured in the presence of 440 nM ACTH has been subtracted. Points are means from two separate experiments.
Role of Ca²⁺ in ACTH Action

In this experiment, Mg²⁺ up to a concentration of 8 mM could not replace Ca²⁺ (data not shown).

In order to ascertain whether extracellular Ca²⁺ is required for continued occupancy of the receptor, the dissociation of ¹²⁵I-ACTH analog from adrenocortical cells was investigated in the presence and absence of Ca²⁺. The results in Fig. 2 show that removal of calcium from the medium dramatically accelerates the dissociation of ACTH from the receptor. Half-maximal dissociation of ¹²⁵I-ACTH analog occurred in 32 min in the presence of Ca²⁺ and 3.5 min in the absence of the ion.

The absolute requirement of extracellular Ca²⁺ for the binding and continued occupancy of the receptor makes it difficult to study the effect of Ca²⁺ on events subsequent to the hormone-receptor interaction. We solved this problem by covalent attachment of ACTH to its receptor. Adrenocortical cells in primary culture were incubated with 100 nM 2,5-NAPS-ACTH in the presence or absence of 3 mM EGTA, photolyzed, and washed as described under “Materials and Methods.” Cells were then incubated in the presence (▲) or absence (○) of 3 mM EGTA. Aliquots were removed at the times indicated and assayed for corticosterone. After 90 min both groups of cells were washed with medium 199, 0.05% BSA, 0.01% bacitracin, and the incubation media were switched. Cells incubated in the presence of EGTA and cells previously kept in the presence of EGTA were now incubated in medium 199 alone. It is apparent that the rate of steroid production in the former is now attenuated and the rate of steroidogenesis in the latter is restored to normal (Fig. 4, left panel). After 90 min, the incubation media were reversed. Cells incubated in medium 199 alone were now incubated in the presence of EGTA and cells previously kept in the presence of EGTA were now incubated in medium 199 alone. It is apparent that the rate of steroid production in the former is now attenuated and the rate of steroidogenesis in the latter is restored to normal (Fig. 4, right panel).

The concentration of extracellular Ca²⁺ necessary to restore persistent activation of steroidogenesis was investigated. The results in Fig. 5 show that as little as 0.18 mM Ca²⁺ is sufficient to restore the steroidogenic response elicited by covalent attachment of ACTH to adrenocortical cells. This is 10-fold lower than the concentration of Ca²⁺ needed for maximal binding of the ¹²⁵I-ACTH analog. Since EGTA tends to deplete the medium as well as the cells of Ca²⁺, the effect of washing persistently activated cells in Ca²⁺-free medium alone was investigated. Cells washed with Ca²⁺-free RPMI medium after photoaffinity labeling with 2,5-NAPS-ACTH and incubated in Ca²⁺-free medium remained activated and continued steroid production in the absence of extracellular Ca²⁺ (Fig. 6).

Since cAMP is known to stimulate steroidogenesis in the adrenocortical cells in the absence of ACTH, we investigated whether cAMP could restore steroidogenesis in persistently activated cells in the absence of Ca²⁺. It is apparent from Fig. 7 that exogenous cAMP can at least partially restore the
steroidogenic response to ACTH which was abolished by EGTA.

**DISCUSSION**

The role of Ca$^{2+}$ in the actions of ACTH has been the subject of numerous investigations over the past 25 years (summarized in Refs. 16 and 17). Haksar and Pérone (3) found that the requirement for Ca$^{2+}$ in ACTH action on isolated rat adrenocortical cells was greater for events preceding the formation of CAMP than for those that follow. However, because of the studies of Lefkowitz et al. (7) it was generally accepted that Ca$^{2+}$ was not required for the interaction of ACTH with the adrenal receptors. All previous studies attempting to characterize corticotropin receptors on rat (10) and human (18) adrenocortical cells and rat adipocytes (19) as well as 3T3-L1 cells (20). The results obtained using this fully active radioligand clearly show that the primary role of extracellular Ca$^{2+}$ is at the first step, namely binding of the hormone to its receptor on the adrenocortical cell surface. Ca$^{2+}$ is required not only for the association of ACTH with its receptor but also for continued occupancy of the receptor.

Whereas the actions of ACTH on adrenocortical cells have generally been studied in media containing millimolar concentrations of Ca$^{2+}$, all studies with adrenal membrane preparations in the past have been performed in the absence of added Ca$^{2+}$. This was necessitated, in part, by the finding that Ca$^{2+}$ in millimolar concentrations inhibited adenylate cyclase activity (7, 21, 22). The concentration of ACTH required for half-maximal stimulation of adenylate cyclase activity in rat and bovine adrenal membrane preparations was 2-3 orders of magnitude higher than that required with intact cells. In the light of our results on the requirement of Ca$^{2+}$ for the binding of the hormone to the receptor, the inability of ACTH to activate adenylate cyclase at low concentrations in membrane preparations in the absence of Ca$^{2+}$ is understandable. We have found that Ca$^{2+}$ is required for specific binding of 125I-ACTH analog to bovine (23) and rat adrenal membranes. The conflicting requirements of the presence of physiological concentrations of Ca$^{2+}$ for binding of the hormone to the receptor and the absence of such concentrations of Ca$^{2+}$ for optimal adenylate cyclase activity make it virtually impossible to measure binding of ACTH and activation of adenylate cyclase activity in adrenal membrane preparations under the same conditions.2

In view of the stringent requirement of Ca$^{2+}$ for binding ACTH to its adrenal receptor, we resorted to covalent attachment of the hormone to its receptor in order to evaluate the role of extracellular Ca$^{2+}$ in post-binding events. No covalent attachment of ACTH to the receptor occurred when photolysis was conducted in the presence of EGTA, supporting the conclusion derived from the binding studies that Ca$^{2+}$ is necessary for the interaction of the hormone with the adrenocortical cell receptor. Once the hormone was covalently linked to the receptor in the presence of Ca$^{2+}$, the role of the ion in post-binding events could be studied. Although persistent activation of steroidogenesis induced by photoaffinity

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labeling was suppressed in the presence of EGTA, steroidogenesis continued unabated when the persistently activated cells were washed with Ca\textsuperscript{2+}-free medium and reincubated in Ca\textsuperscript{2+}-free medium. These results suggest that extracellular Ca\textsuperscript{2+} is not needed for steroidogenesis once the hormone is bound to the receptor and is able to maintain continued occupancy due to covalent attachment. Influx of Ca\textsuperscript{2+} from the extracellular medium under the influence of ACTH has been considered as a possible mechanism involved in steroidogenesis (24). Our results clearly show that such influx of Ca\textsuperscript{2+} from the extracellular fluid is not necessary for ACTH-induced steroidogenesis.

Minute quantities of Ca\textsuperscript{2+} bound to the membrane may, of course, play an important role in the events beyond the binding of the hormone to its receptor. Depletion of Ca\textsuperscript{2+} by incubation with excess EGTA is clearly deleterious and causes reversible cessation of steroidogenesis even when the hormone is covalently attached to the receptor. It is possible that Ca\textsuperscript{2+} is needed to maintain the hormone in a conformation favorable for productive interaction with the receptor even when covalently bound to its receptor. Photoaffinity labeling of Xenopus melanophores with a photoactive derivative of \( \alpha \)-melanocyte-stimulating hormone has shown (25) that Ca\textsuperscript{2+} is required both for binding of the hormone to its receptor and for one or more intracellular events involved in melanin dispersion. Since the amino acid sequence of \( \alpha \)-melanocyte-stimulating hormone is present in the first 13 residues of ACTH, it is tempting to speculate that Ca\textsuperscript{2+} may be involved in stabilizing a conformation of the peptide segment common to the two hormones.

Ca\textsuperscript{2+} may play a role at a site beyond the binding of the hormone to the receptor in addition to its effect on the binding step. One possible locus of Ca\textsuperscript{2+} action may be the membrane-bound adenylate cyclase. Several laboratories have found that steroidogenesis and cAMP formation in response to ACTH were impaired when Ca\textsuperscript{2+} was omitted from the extracellular medium (21, 26, 27). However, cAMP was able to at least partially overcome the consequences of the lack of Ca\textsuperscript{2+}. This is in agreement with our results and suggests an effect of Ca\textsuperscript{2+} on adenylate cyclase activity. Several groups have suggested that Ca\textsuperscript{2+} exerts its stimulatory effect at the level of the interaction of the guanine nucleotide-binding protein with the enzyme (28, 29). The concentration of free Ca\textsuperscript{2+} required for the stimulation of adenylate cyclase appears to be in the 0.1–1.0 \( \mu \)M range (30).

It is also conceivable that translocation of Ca\textsuperscript{2+} bound to the plasma membrane or other organelles of the adrenocortical cell to another compartment in response to the interaction of ACTH with its receptor may play a role in the steroidogenic action of the hormone. Studies using inhibitors of the actions of calmodulin have shown that Ca\textsuperscript{2+} plays an important regulatory role at intracellular sites (31). It is clear, however, from the results presented above that the primary and exclusive role of extracellular Ca\textsuperscript{2+} in the action of ACTH is to facilitate the binding of the hormone to its receptor.