Studies on Adrenocorticotropic Hormone Receptor Using Isolated Rat Adrenocortical Cells

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Synopsis

To clarify the function of ACTH receptors, the actions of ACTH on cyclic AMP formation, Ca²⁺-influx across cell membrane, and corticoidogenesis were examined using dispersed adrenocortical cells prepared from the rat adrenal gland.

1) There are two types of ACTH receptors from Scatchard analysis of ¹²⁵I-ACTH₁₋₂₄ binding to the cell, the one receptor is of high affinity and low capacity (dissociation constant (Kd₁)=2.6×10⁻¹⁰M and 7,350 sites per cell), and the other one is of low affinity and high capacity (dissociation constant (Kd₂)=7.1×10⁻⁹M and 57,400 sites per cell). 2) Both apparent dissociation constants derived from the effects of ACTH on corticoidogenesis and Ca²⁺-influx well correspond with Kd₁ of the high affinity receptor. 3) Apparent dissociation constant obtained from the effect of ACTH on cyclic AMP formation is in good agreement with Kd₂ of the low affinity receptor.

Thus it could be deduced from these data that the high affinity receptor is concerned with an increased Ca²⁺-influx to regulate corticoidogenesis at physiological levels of ACTH, whereas the low affinity receptor is coupled to adenylate cyclase at supraphysiological concentrations of ACTH.

There is increasing evidence to support an assumption that there are two different ACTH receptors on the adrenocortical cell surface (Lefkowitz et al., 1971; Wolfsen et al., 1972; McLhinney and Schulster, 1975). Nevertheless, the functional roles of these two receptors have not yet been well elucidated.

The present study was performed to clarify the functional roles of two receptors in the corticoidogenesis regulation using dispersed adrenocortical cells prepared from rat adrenal.

Materials and Methods

Preparation of the cell suspension

The adrenal cells were dispersed by trypsin digestion of decapsulated rat adrenal gland (female Wistar-Imamichi rat, weighing 200 to 250 g). The trypsin digestions were performed with gentle stirring for 15 min at 37°C under 95% O₂-5% CO₂ mixture as gas phase. The digestion solution was 0.25% trypsin (Difco 1:250) in Ca²⁺-free Krebs Ringer bicarbonate glucose buffer (pH 7.4) containing 10 μM EGTA and 0.3% bovine serum albumin (Ca²⁺-free KRBGA). The adrenal cortical tissue obtained from 20 to 30 adrenals was digested in 25 ml of the digestion solution. The digestion procedure was repeated 5 times, and the fresh digestion solution was used for each incubation. The first crop was discarded by decantation, the following 4 crops were pooled by filtration through a 80-mesh platinum sieve. The pooled cell suspension was spun at 100×g for 10 min at 4°C, and the cellular sediment was resuspended in 30 ml of Ca²⁺-free KRBGA containing 0.05% trypsin.

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inhibitor for washing. The washed cells were suspended in Ca²⁺-free KRBGA containing 0.2% trypsin inhibitor to be about $5 \times 10^6$ cells per ml, and this cell suspension was used in this experiment.

Corticosterone was measured fluorometrically by the method described by Silber et al. (1958).

$^{125}$I-Labeled ACTH₁₋₂₄ was prepared by the slightly modified method of McLhinney and Schulster (1974).

Cyclic AMP was determined using the commercial assay kit from Radio Chemical Centre, Amersham.

Incorporation of Ca²⁺ into the cell was measured by the method described by Keppens et al. (1977).

**Chemicals**

Na$^{125}$I and $^{45}$CaCl₂ were purchased from New England Nuclear Corp. Synthetic ACTH₁₋₂₄ was kindly gifted from Daiichi Seiyaku Co., Ltd. Tokyo, Japan. Trypsin inhibitor and bovine serum albumin were purchased from Sigma Chemical Co., and all other chemicals were of reagent grade.

**Results**

Scatchard plotting analysis of $^{125}$I-ACTH₁₋₂₄ binding with the cell was presented in Fig. 1. From this analysis, it could be assumed that there are two distinct ACTH-receptors in rat adrenocortical cells. The one is of high affinity and low capacity (dissociation constant (Kd₁) $= 2.6 \times 10^{-10}$ M, and 7,350 sites per cell), and the other one is of low affinity and high capacity (dissociation constant (Kd₂) $= 7.1 \times 10^{-9}$ M, and 57,400 sites per cell). Table 1 shows the relation between ACTH concentrations and the occupied receptors according to the hypothetical method of Lefkowitz et al. (1971). The high-affinity receptor filled more rapidly at physiological concentrations of ACTH, whereas the low-affinity receptor filled further in response to sharp rises in ACTH concentration even up to $10^{-8}$-$10^{-7}$M. The presented result may reinforce the presence of two different types of ACTH receptors in the adrenocortical cell.

The experiment depicted in Fig. 2 was designed to contrast ACTH-induced corticoidogenesis in the presence or absence of Ca²⁺ in the incubation medium. As shown

![Fig. 1. Scatchard plot of binding data obtained by incubation of isolated cells with $^{125}$I-ACTH for 15 min at 37°C. From regression analysis by the method of least squares, the following equations were obtained: (1) $Y_1=0.07531-0.00392X_1$, from which Kd₁ = $2.6 \times 10^{-10}$ M with 7,350 sites per cell, and (2) $Y_2=0.02049-0.00014X_2$, from which Kd₂ = $7.1 \times 10^{-9}$ M with 57,400 sites per cell.](image)

**Table 1. Relation of ACTH concentration to the number of receptors occupied.**

<table>
<thead>
<tr>
<th>[ACTH]</th>
<th>Sites filled per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>High affinity R.</td>
</tr>
<tr>
<td>$10^{-15}$</td>
<td>2</td>
</tr>
<tr>
<td>$10^{-14}$</td>
<td>28</td>
</tr>
<tr>
<td>$10^{-13}$</td>
<td>272</td>
</tr>
<tr>
<td>$10^{-12}$</td>
<td>2,041</td>
</tr>
<tr>
<td>$10^{-11}$</td>
<td>5,833</td>
</tr>
<tr>
<td>$10^{-10}$</td>
<td>7,163</td>
</tr>
<tr>
<td>$10^{-9}$</td>
<td>7,330</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>7,348</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>7,350</td>
</tr>
</tbody>
</table>

The data were expressed as the number of receptors occupied by any given ACTH concentrations according to the hypothetical method of Lefkowitz et al. (1971). The dissociation constant and number of binding sites of each receptor were derived from known values in Fig. 1. R.; receptor.
in Fig. 2, addition of increasing concentrations of ACTH in the presence of Ca\(^{2+}\) (1.2 mM) resulted in an increased corticoidogenesis. The maximum stimulatory effects of ACTH were obtained at the level of 10\(^{-9}\) M of ACTH. On the other hand, in the absence of Ca\(^{2+}\) little corticoidogenesis was observed until ACTH concentration exceeded beyond 10\(^{-9}\) M, and the higher concentrations of ACTH were necessary to induce the detectable corticoidogenesis. From double reciprocal plotting analysis of corticoidogenic effect of ACTH, the apparent dissociation constants of ACTH binding to the cell were estimated to be as 2.6 × 10\(^{-10}\) M in the presence of Ca\(^{2+}\), and 9.1 × 10\(^{-9}\) M in the absence of Ca\(^{2+}\), respectively as shown in Fig. 2. The apparent dissociation constant derived from corticoidogenic effect of ACTH in the presence of Ca\(^{2+}\) well agreed with Kd\(_1\) of the high-affinity ACTH receptor, whereas the value obtained in the absence of Ca\(^{2+}\) was almost the same as Kd\(_2\) of the low-affinity ACTH receptor.

Ca\(^{2+}\) in the incubation medium may act to facilitate a binding affinity of ACTH to the one receptor, thus the effect of Ca\(^{2+}\) on ACTH binding to the receptor was examined and the obtained result was shown in Fig. 3. As shown in Fig. 3, Ca\(^{2+}\) could not facilitate the binding affinity of ACTH to the cell, but rather interfered the binding at the higher concentrations.

The effect of ACTH on Ca\(^{2+}\)-influx across the cell membrane was shown in Fig. 4. From double reciprocal plotting analysis of ACTH effect on Ca\(^{2+}\)-influx, the apparent dissociation constant was calculated as 2.1 × 10\(^{-10}\) M, and this value was very close to Kd\(_1\) (2.6 × 10\(^{-10}\) M) of the
high-affinity receptor. These results may suggest that an interaction of ACTH with the high-affinity receptor might result in an increased cell membrane permeability to Ca$^{2+}$, and Ca$^{2+}$ might act as the second messenger of ACTH to facilitate corticoidogenesis by unknown mechanisms.

The effect of ACTH on cyclic AMP formation was shown in Fig. 5. From double reciprocal plotting analysis of this effect, the apparent dissociation constant of ACTH was estimated as $5.4 \times 10^{-9}$ M, and this value was very close to $K_d$ ($7.1 \times 10^{-9}$ M) of the low-affinity receptor. Thus it could be deduced from these data and shown in Fig. 2 that ACTH might interact with the low-affinity receptor to activate adenylate cyclase to produce more cyclic AMP, and an increased cyclic AMP acted to facilitate corticoidogenesis.

**Discussion**

It is clear from Scatchard analysis of binding of $^{125}$I-ACTH$_{1-24}$ to adrenal cortical cell that there are at least two different types of ACTH receptors on the cell. Such a concept is in good agreement with recent observations in rat adrenocortical cells (McIlhinney and Schulster, 1975), in the cell membrane fractions prepared from rabbit adrenal (Wolfson et al., 1972) and mouse adrenal tumour (Lefkowitz et al., 1971).

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**Fig. 4.** ACTH-induced incorporation of Ca$^{2+}$ into the cell. Incubation was carried out for 30 min at 37°C in the presence of both 0.8μCi $^{45}$Ca and unlabeled 1.2 mM Ca$^{2+}$. The values shown are the means of duplicate determinations.

**Fig. 5.** ACTH-induced cyclic AMP formation in the presence of 1.2 mM Ca$^{2+}$. Incubation time was 1 hr at 37°C. The values shown are the means of duplicate determinations.
Cyclic AMP has been recognized to be second messenger of several polypeptide hormones (Robison et al., 1971). However, numerous observations for ACTH action on corticoidogenesis have been reported to be some contradictions in regard of sole “cyclic AMP theory” (Hudson and McMartin, 1975; Honn and Chavin, 1977). Namely, ACTH can act to increase corticoidogenesis submaximally even at the lower concentrations without any detectable increment of cyclic AMP formation (Mackie et al., 1972; Bowyer and Kitabchi, 1974). From the present experiment on the effects of ACTH in the presence or absence of Ca2+, it could be assumed that there are two different mechanisms of ACTH action on corticoidogenesis; the one is strictly depending upon extracellular presence of Ca2+ and the other is mediated by the other factors, one of which might be cyclic AMP.

The apparent dissociation constant of ACTH to the cell estimated from its corticoidogenic effect in the presence of Ca2+ was $2.6 \times 10^{-10} \text{M}$, and that estimated from its effect on Ca2+-influx was $2.1 \times 10^{-10} \text{M}$. These values were almost identical with $Kd_1 \left(2.6 \times 10^{-10} \text{M}\right)$ of the high-affinity ACTH receptor. The apparent dissociation constant of ACTH to the cell estimated from its corticoidogenic effect in the absence of Ca2+ was $9.1 \times 10^{-9} \text{M}$, and that estimated from its effect on cyclic AMP formation was $5.4 \times 10^{-9} \text{M}$. These values were very close to $Kd_2 \left(7.1 \times 10^{-9} \text{M}\right)$ of the low-affinity ACTH receptor.

It could be concluded from these facts that the corticoidogenic effect of ACTH in the presence of Ca2+ might be mediated by its interaction with the high-affinity receptor, whereas the corticoidogenic effect of ACTH in the absence of Ca2+ could be mediated by its interaction with the low-affinity receptor. An interaction of ACTH with the high-affinity receptor might result in an increased membrane permeability to Ca2+, which may act as the second messenger of ACTH to facilitate corticoidogenesis in the adrenal. Such an assumption might be reasonable from numerous observations that Ca2+ is essential for ACTH action on corticoidogenesis (Birmingham et al., 1953; Berridge, 1975). There is a good deal of evidence supporting a concept that Ca2+ may act as the second messenger of ACTH in corticoidogenesis regulation (Leier and Jungmann, 1973; Berridge, 1975). The precise role of Ca2+ as the second messenger of ACTH has not yet been elucidated except an interesting suggestion proposed by Farese (1971) and Farese and Prudente (1977). Our previous observation has shown that the addition of Ca2+ into incubation medium could stimulate corticoidogenesis in the dispersed adrenocortical cell obtained from bovine adrenocortical tissue even without ACTH addition, and this effect of Ca2+ was abolished by cycloheximide (Yanagibashi and Matsuba, 1976).

On the other hand, the dissociation constant $(7.1 \times 10^{-9} \text{M})$ of the low-affinity receptor was very close to the apparent dissociation constant $(5.4 \times 10^{-9} \text{M})$ calculated from the double reciprocal plotting analysis of ACTH effect on cyclic AMP formation described previously. McIlhinney and Schulster (1975) have also reported a similar observation and suggested that the low affinity receptor is functionally coupled to adenylate cyclase. These results would account for either the apparent discrepancy in ACTH effects on corticoidogenesis and cyclic AMP formation or the overproduction of cyclic AMP at the higher concentrations of ACTH than it enhances corticoidogenesis maximally. It was found in our unpublished experiment that removal of Mg2+ did not affect ACTH effect on corticoidogenesis in the presence of Ca2+, but removal of Mg2+ could abolish both ACTH effects on corticoidogenesis and cyclic AMP formation in the absence of Ca2+. Moyle et al. (1973) have found that o-nitrophenyl
sulfenyl derivative of ACTH$_{1-39}$ (NPS-ACTH) acts as competitive antagonist on ACTH action on cyclic AMP formation, but NPS-ACTH acts as agonist on corticoidogenesis, and they have suggested the presence of two distinct ACTH-receptors in adrenocortical cells. It would be deduced from their suggestion and our presented data that NPS-ACTH may interact with the high affinity receptor as an agonist, but it interacts with the low-affinity receptor as a competitive antagonist of ACTH.

The concentrations of ACTH in human plasma were about $5 \times 10^{-12}$ M in the resting state, and hard to exceed $1 \times 10^{-9}$ M even under severe stress such as surgical operation (Yanagibashi and Matsuba, 1977). Thus it could be suggested that only the high-affinity receptor in two ACTH receptors is functioning to regulate corticoidogenesis in adrenal under physiological conditions.

Possible functions of two distinct ACTH-receptors are depicted in Fig. 6. Namely, the high affinity receptor could mediate Ca$^{2+}$-influx to regulate corticoidogenesis at the lower concentrations of ACTH, and the low-affinity receptor might be coupled to adenylate cyclase to produce more cyclic AMP to support corticoidogenesis at the higher concentrations of ACTH.

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References