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Loss of Sensitivity to ACTH of Adrenocortical Cells Isolated from Maturing Domestic Fowl[1] (42096)

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Abstract. Maturation of domestic fowl corticosteroidogenesis was evaluated using purified adrenocortical cells. Basal corticosterone production decreased steadily from 2 days to 26 weeks after hatching. However, maximally stimulated corticosterone production was not changed. In contrast, the half-maximal steroidogenic concentrations (ED_{50} values or effective doses for 50% maximal effect) of ACTH analogs increased approximately 40 times by 26 weeks, but the ED_{50} values of 8-bromo-cyclic AMP and pregnenolone were not changed. This suggests that adrenocortical cell sensitivity to ACTH decreases with maturation of the domestic fowl.

In vivo studies have shown that maturation of the domestic fowl is accompanied by a fall in plasma corticosterone in the absence or presence of exogenous ACTH (1, 2). However, another study suggests an increased sensitivity of the domestic fowl adrenal gland to ACTH from 1 day to 3 weeks after hatching (3). These studies appear contradictory. In addition, they were *in vivo* studies and thus, the presence of extraadrenal factors complicates the interpretation of the results.

In an effort to explain these results of *in vivo* studies and to evaluate adrenocortical cell function per se, we have used an *in vitro* system. We and other investigators have used isolated cells to study mammalian adrenocortical function during development (4, 5) and aging (6). The intrinsic properties of these cells can be defined because they are removed from influences that operate *in vivo*. However, this approach has not yet been used to investigate the effect of age on adrenocortical function of the domestic fowl.

Recently, we have characterized some of the functional and ultrastructural properties of isolated domestic fowl adrenocortical cells (7). In work reported here, we prepared adrenocortical cells from fowl of various posthatching ages, and tested the response of these cells to steroidogenic agents.

Materials and Methods. Male White Leghorn domestic fowl (*Gallus domesticus*) were housed at 20±1°C under a 16-hr light:8-hr dark photocycle. They were fed a standard commercial diet and water *ad libitum*. Birds (2 days, 2 weeks, 6 weeks, and 26 weeks after hatching) were killed by decapitation and exsanguinated. The number of birds used in each of three experiments for each age were respectively, 30, 20, 15, and 10. These numbers of birds reflect the amount of adrenal gland tissue required to yield a sufficient quantity of cells to test cell responses to steroidogenic agents and to achieve equal cell concentrations for each age studied. Adrenal glands were quickly removed, trimmed free of connective tissue, and then diced into small pieces (about 2 mm^3) for cell isolation.

The standard medium for cell isolation and incubation was Krebs-Ringer Hepes (*n*-2-hydroxyethylpiperazineethanesulfonic acid) buffer (24.2 m*M* Hepes, 118.5 m*M* NaCl, 4.75 m*M* KCl, 2.54 m*M* $CaCl_2$, 1.20 m*M* KH_2PO_4, 1.20 m*M* $MgSO_4 7H_2O$, 11.1 m*M* glucose), pH 7.5. Adrenocortical cells were isolated as follows: Diced adrenal glands were placed in 50-ml plastic conical centrifuge tubes with 5–10 ml of cell isolation medium containing 0.2% collagenase (Type I), 0.01% lima bean trypsin inhibitor, and 0.2% bovine serum albumin (fraction V) (all agents from

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Sigma Chemical Co.). The tubes were oscillated horizontally (80–90 oscillations/min) at 37°C for 20 min in a Dubnoff metabolic shaker. The supernatant fluids were decanted and stored on an ice bath. Fresh cell isolation medium was added to the tubes and the tissue fragments were triturated with plastic pipets. These mechanical steps were repeated until tissue fragments were completely dissociated. The pooled supernatant fluids were filtered through sterile gauze and then centrifuged at 100*g* for 15 min at 4°C. The cell pellets were dispersed in 1 ml of incubation medium (standard medium containing 0.5% bovine serum albumin), placed on a 40% Percoll continuous density gradient (kit provided by Pharmacia Fine Chemicals), and centrifuged at 400*g* for 15 min at 4°C to separate adrenocortical cells from cell fragments and other cell types. After centrifugation, the cell fractions found between 1.04 and 1.05 g/ml (as shown by density marker beads) were removed by pipet. These fractions were diluted to twice their volumes with incubation medium and then centrifuged at 100*g* for 15 min at 4°C to remove the cells from the Percoll. The cell pellets were resuspended in incubation medium. At each age studied, light microscopy was used to establish the paucity of medullary cells and to establish that 85–93% of the cell populations were comprised of adrenocortical cells, as determined by the presence of lipid droplets. Our recent work (7) using electron microscopy has confirmed the light microscopic identification of adrenocortical cells. A hemacytometer (Improved Neubauer, 0.1 mm deep; American Optical) was used to determine adrenocortical cell concentrations. Cell suspensions for each age studied were then adjusted to 200,000 cells/ml. Aliquots of the cell suspensions of equal cell concentrations were incubated with steroidogenic agents for 2 hr at 39.5–41.0°C in a Dubnoff metabolic shaker. The incubation volumes (90% cell suspension, 10%, a solution containing a steroidogenic agent) were 200–500 μl. Steroidogenic agents were purified ostrich ACTH-1-39 (gift from Ryno Naudé, Department of Biochemistry, University of Port Elizabeth, P.O. Box 1600, Port Elizabeth 6000, Republic of South Africa), ACTH-α-1-24 (Cortrosyn; Organon Inc.), 8-bromo-cyclic AMP (8Br-cAMP) and pregnenolone (Sigma Chemical Co.). Prior to the addition of these agents to the cell suspensions, ACTH analogs were dissolved in a solution of 0.9% NaCl, pH adjusted to 2.6 with 1.0 *N* HCl, containing 0.1% bovine serum albumin; however, 8Br-cAMP and pregnenolone were made up in the standard medium. In each experiment at least 85% of the cells were viable after incubation at indicated by trypan blue dye (4%) exclusion.

Corticosterone, the major glucocorticoid secreted by the domestic fowl adrenal gland (8), was measured by a modification of the radioimmunoassay procedure of Roy *et al.* (9) using specific antibody (Miles Research Products). Cross-reactivity with pregnenolone was low (≤0.83%). In our hands as little as 0.1 ng/ml corticosterone could be detected. Radioimmunoassay of aliquots of the same pooled cell suspensions (performed with each radioimmunoassay) showed intraassay and interassay coefficients of variation of 7.3 and 11.9%, respectively.

The effect of fowl age on the half-maximal steroidogenic concentration (effective dose for 50% maximal effect or ED_{50}) of steroidogenic agent was determined. Corticosterone values were plotted by an Eadie-Hofstee modification (10) of the method described by Sayers *et al.* (11). The data approached linearity as indicated by their high coefficients of correlation (≥0.91) as measured by linear regression. The absolute values of the calculated negative slopes of the lines were the ED_{50} values. These values were used as indices of the potencies of steroidogenic agents or of the sensitivities of cells to these agents. The ED_{50} value varies inversely with agent potency or cellular sensitivity.

Analysis of variance (12) was used to evaluate the data; data are expressed as means ± SEM, and were deemed significantly different when $P \leq 0.05$.

Results. Fig. 1 shows the acute effect of ACTH analogs on corticosterone production of adrenocortical cells isolated from fowl of various ages. In the absence of ACTH analogs, there was an age-related reduction in corticosterone production (a steady 85% decrease from 2 days to 26 weeks after hatching). The greatest decrease (55–64%) occurred between 2 weeks and 6 weeks after hatching. In

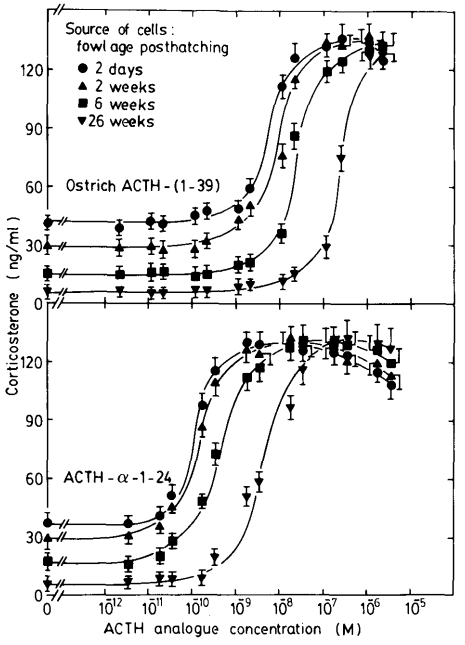

FIG. 1. ACTH analog-induced corticosterone production by adrenocortical cells (200,000 cells/ml) isolated from domestic fowl of different ages. Each symbol represents the mean of corticosterone values from nine cell suspensions (three suspensions from each of three experiments). SEM are represented by bars.

contrast, in the presence of ACTH analogs, maximal corticosterone production was the same for all ages.

ACTH analogs stimulated cellular corticosterone production in a dose-dependent manner over approximately two log orders of concentration, regardless of fowl age (Fig. 1). The synthetic mammalian ACTH, ACTH-α-1-24, was more potent than ostrich ACTH-1-39 (13) in stimulating corticosterone production (Table I). Regardless of fowl age, the ED_{50} of ostrich ACTH-1-39 was 54–60 times greater than the ED_{50} of ACTH-α-1-24.

Although age did not affect maximal ACTH-induced corticosterone production, it did decrease cell sensitivity as indicated by increased ED_{50} values. Decreases in cell sensitivity from 2 days to 2 weeks after hatching were slight. However, ED_{50} values of ACTH analogs at 6 weeks and 26 weeks after hatching were 3–4 times and 39–41 times greater than the ED_{50} values at 2 days after hatching, respectively.

Cells from fowl of all ages achieved the same level of maximal corticosterone production when stimulated with 8Br-cAMP (1 m*M*) or when supported by pregnenolone (10 μ*M*) (Table II). These results are similar to those obtained with maximal steroidogenic concentrations of ACTH. On the other hand, although cell sensitivity to ACTH decreased with age, it did not change for corticosterone production stimulated by 8Br-cAMP or supported by pregnenolone (Table I).

Discussion. There are reports that basal plasma corticosterone values decrease with domestic fowl age (1, 2). Our data on isolated adrenocortical cells are consistent with these reports and indicate that adrenocortical cells themselves are responsible for the decline. From 2 days to 26 weeks after hatching, there was an 85% reduction in basal corticosterone production by isolated adrenocortical cells.

There are also reports of an age-related decrease in maximal ACTH-induced plasma corticosterone values (2). However our data on isolated cells show equivalent levels of maximal ACTH-induced corticosterone production at all ages tested (Table II). Further-

TABLE I. HALF MAXIMAL STEROIDOGENIC CONCENTRATION (ED_{50})[a] OF A STEROIDOGENIC AGENT WITH ISOLATED DOMESTIC FOWL ADRENOCORTICAL CELLS

Source of cells: fowl age posthatching	ACTH-α-1-24 (*M*)	Ostrich ACTH-1-39 (*M*)	8 Br-cAMP (m*M*)	Pregnenolone (μ*M*)
2 days	$(1.21 \pm 0.43) \times 10^{-10}$	$(6.69 \pm 0.34) \times 10^{-9}$	0.25 ± 0.01	0.18 ± 0.01
2 weeks	$(1.57 \pm 0.39) \times 10^{-10}$	$(7.38 \pm 0.41) \times 10^{-9}$	0.26 ± 0.01	0.19 ± 0.02
6 weeks	$(3.80 \pm 0.27) \times 10^{-10}$[b]	$(2.28 \pm 0.23) \times 10^{-8}$[b]	0.28 ± 0.02	0.16 ± 0.02
26 weeks	$(4.93 \pm 0.38) \times 10^{-9}$[b]	$(2.66 \pm 0.40) \times 10^{-7}$[b]	0.27 ± 0.02	0.18 ± 0.01

[a] Each value is the mean ± SEM of ED_{50} values from three experiments.
[b] ED_{50} values that are significantly different ($P \leq 0.05$) from the values for 2 days fowl age posthatching.

TABLE II. MAXIMAL CORTICOSTERONE PRODUCTION IN RESPONSE TO STEROIDOGENIC AGENTS

Source of cells: fowl age posthatching	ACTH-α-1-24	Ostrich ACTH-1-39	8Br-cAMP	Pregnenolone
2 days	129.7 ± 11.1	135.7 ± 10.3	237.6 ± 8.7	283.5 ± 18.0
2 weeks	132.6 ± 10.0	138.3 ± 7.4	229.6 ± 11.3	261.9 ± 23.7
6 weeks	130.2 ± 9.6	130.6 ± 9.4	223.0 ± 10.1	277.1 ± 15.4
26 weeks	134.1 ± 11.2	129.1 ± 7.8	235.3 ± 8.2	265.2 ± 21.3

Note. Each value (ng/ml) is the mean ± SEM of nine cell incubations (three incubations from each of three experiments).

more, similar results were obtained with 8Br-cAMP-induced and pregnenolone-supported corticosterone production (Table II). These data, calculated on an equal cell concentration basis, suggest that aging results in decreased basal corticosterone production without a change in the amount of corticosterone that the cells can produce when maximally stimulated. The reported decrease in maximal ACTH-induced plasma corticosterone with age (2) may not be inconsistent with our *in vitro* results if the age-related decrease in the adrenal to body weight ratio is taken into account.

There are conflicting reports from *in vivo* studies regarding the effect of age on the sensitivity of the domestic fowl adrenal gland to ACTH (1–3). Our *in vitro* work was an attempt to resolve this problem by considering the intrinsic properties of isolated adrenocortical cells. Fig. 1 shows that cells from older fowl required greater concentrations of ACTH analogs to achieve maximal corticosterone production than cells from younger fowl. Thus, there was a striking loss in cellular sensitivity to ACTH. This conclusion is further indicated by the increase in ED_{50} values of ACTH analogs with age (Table II).

In contrast to the ED_{50} values for ACTH analogs, the ED_{50} values for 8Br-cAMP and pregnenolone were not changed with age. The data suggest that 8Br-cAMP and pregnenolone act at sites that are not altered with age and that the loss in ACTH sensitivity is the result of membrane changes in ACTH receptor function.

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