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Gap Junctions and ACTH Sensitivity in Y-1 Adrenal Tumor Cells

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Initial studies of adrenocorticotropin-sensitive (ACTH-sensitive) and ACTH-insensitive Y-1 adrenal cortical tumor cell lines suggest a relationship between responsiveness to ACTH and the presence of gap junctions. An ACTH-sensitive clone of Y-1 cells possesses gap junctions and these junctions appear to enlarge with ACTH treatment. Gap junctions have not been observed, however, in an ACTH-insensitive clone of Y-1 tumor cells even when stimulated to produce cyclic adenosine monophosphate and steroids with cholera toxin.

Key words: adrenal cortical tumor cells, ACTH, gap junctions, steroidogenesis

Adrenocorticotropin (ACTH) interacts with receptors in the plasma membrane of the adrenal cortical cell to initiate a sequence of events resulting in the formation of specific steroid hormones [1]. In addition, ACTH stimulates cultured ACTH-responsive mouse Y-1 adrenal cortical tumor cells to transform from a flattened to a rounded shape [2, 3, present study]. These spherical cells remain attached to each other and to the surface of the culture flask by long, slender processes. The production of steroids and the cell rounding of cultured adrenal cortical cells appear to be dependent on cyclic AMP production [4, 5]. Cholera toxin, however, induces adenylate cyclase activity, steroid production, and cell rounding in ACTH-insensitive cells as well as in ACTH-sensitive clones [6–8, present study].

Preliminary studies [9] have provided evidence that gap junctions may enlarge in response to ACTH in cultured ACTH-sensitive Y-1 tumor cells. Gap junction growth is also apparent in the adrenal cortex of a patient with an ectopic ACTH-secreting tumor [10, 11]. Several other investigators have recently reported effects of several hormones and vitamin A on the gap junctions of their respective target tissues [12–17].

These observations have led us to investigate the nature of the apparent relationship between ACTH sensitivity and responses of gap junctions in Y-1 adrenal cortical cells.

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MATERIALS AND METHODS

Cell Lines

Y-1 (BS-1) ACTH-sensitive mouse adrenal tumor cells were obtained from Dr Bernard Schimmer of the University of Toronto and ACTH-sensitive Y-1 (D) (originally studied by Decker [9] were obtained from Dr Gordon Sato of the University of California at San Diego. These cells were derived originally from the same clone. Y-1 ACTH-insensitive adrenal tumor cells were also originally obtained from Dr Sato as ACTH-sensitive cells. The cells were maintained in plastic tissue culture flasks (25 cm^2, Falcon Plastics) in Ham's nutrient mixture F-10 medium supplemented with 15% horse serum, 2.5% fetal calf serum, penicillin (60 μg/ml). The cells were incubated at 37°C in an atmosphere containing 5% CO_2. Experiments were performed one to two weeks after subculturing, when cells were confluent. ACTH-sensitive cells responded to ACTH stimulation with increased steroidogenesis and by rounding, indicating that these cells were functional. Y-1 ACTH-insensitive adrenal tumor cells do not respond to ACTH stimulation but do respond to cholera toxin.

Chemicals

Purified cholera toxin was provided by Dr R. A. Finkelstein, University of Texas, and was stored frozen in a 0.05 M Tris–0.001 ethylenediaminetetraacetic acid (EDTA) buffer at pH 7.5. Concentrations of 3–50 ng/ml medium were used in these experiments. ACTH was purchased from Armour Pharmaceutical Company (Chicago) or Sigma (St Louis) and diluted in distilled water for use at concentrations of 10–100 munits/ml medium.

Assays of Cyclic AMP and Steroids

A commercially available radioimmunoassay (Schwartz-Mann) was used for assays of cyclic AMP levels. Steroids elaborated by Y-1 tumor cells were measured using a modification of the procedure of Vernikos-Danellis et al [18].

Freeze Fracture

Cells were prepared for freeze fracture by fixing in their culture flasks for 10 min in 2.5% glutaraldehyde in 0.05 M cacodylate, and 2% sucrose or 3% glutaraldehyde in 0.1 M cacodylate (pH 7.4) at room temperature; then they were infiltrated with 30% glycerol on ice. The cells were then collected from the bottom of the flasks with a rubber policeman and placed in specimen holders designed for use in the Balzers BAE 121 or BAF 301 freeze fracture device (Balzers, Santa Ana, California). The tissue was rapidly frozen in freon cooled by a bath of liquid nitrogen, then was fractured and replicated. Replicas were viewed in a Philips 200 or 300 electron microscope. The freeze fracture nomenclature of Branton et al [19] is used.

Scanning Electron Microscopy

Cells were prepared for scanning electron microscopy by fixation in their flasks in 2.5% glutaraldehyde in 0.05 M cacodylate and 2% sucrose at 37°C for 1 h. The cultures were washed in cacodylate buffer (pH 7.2) for 30 min at room temperature, dehydrated in alcohol, critical point-dried, and sputter-coated with gold-platinum alloy. The intact cells were then scanned in a Cambridge S4 scanning electron microscope.

RESULTS

Cell Rounding

ACTH-sensitive Y-1 (BS-1) adrenal tumor cells began to round within 15 min after addition of ACTH (10 munits/ml). By 60 min, 85% of the population was spherical as revealed by phase contrast microscopy (Fig. 1a,b). These cells became flattened again after 24–48 h. Cholera toxin (10 ng/ml) also induced rounding in this cell line but the kinetics of the rounding response were different. Cells began to round after 1–2 h and 90% of the population had rounded after 7 h. Approximately 85% remained rounded after 72 h (Fig. 1c,d). Scanning electron microscopy revealed that dense areas of ACTH-sensitive Y-1 tumor cells did not round in response to ACTH, whereas nearly all cells rounded in response to cholera toxin (Fig. 1e, f). These parameters of rounding were dependent to some degree on the concentrations of ACTH and cholera toxin.

ACTH-insensitive Y-1 adrenal cortical cells rounded only in response to cholera toxin. Cells began to round within 1–2 h and 75% of the population was spherical by 7 h and remained rounded for as long as 72 h (Fig. 2a,b).

Cyclic AMP Production

One ACTH-sensitive (BS-1) and the ACTH-insensitive Y-1 tumor cells elaborated enhanced amounts of cyclic AMP into the medium in response to cholera toxin, while increased production of cyclic AMP also resulted when ACTH was added to the medium of the ACTH-sensitive (BS-1) cells (Table I). We have not tested the ability of the ACTH-insensitive cells to respond to ACTH by increased cyclic AMP production. Another ACTH-insensitive Y-1 tumor cell line (OS_3), however, has been reported to be insensitive to ACTH with respect to adenylate cyclase activation and steroidogenesis even though it possesses the capacity to produce steroids in response to cyclic AMP [20].

Steroidogenesis

ACTH-sensitive Y-1 tumor cells (BS-1) liberated enhanced amounts of fluorogenic steroids into the medium in response to both ACTH (10–100 munits/ml) and cholera toxin (3–50 ng/ml) whereas ACTH-insensitive cells increased steroid production only after cholera toxin treatment (Fig. 3). ACTH-sensitive Y-1 (D) cells also produced fluorogenic steroids in response to ACTH (Fig. 4). In this line of Y-1 cells steroid secretion is significantly enhanced within 8 h and steroidogenesis increases linearally during the next 24 h.

Membrane Structure With Particular Reference to Gap Junctions

Small particle clusters resembling gap junctions have been observed in one line of untreated ACTH-sensitive Y-1 (D) tumor cells (Fig. 5a). These aggregates consist generally of 5–10 8.5-nm particles. However typical gap junctional E fracture face depressions have yet to be observed in this cell line. Furthermore, no typical gap junctional particle aggregates on the P fracture face or pits on the E fracture face were observed in untreated cultures of another line of ACTH-sensitive Y-1 cells (BS-1). When Y-1 (BS-1) and Y-1 (D) ACTH-sensitive cells were treated with ACTH, many circular or lentiform particle aggregates resembling gap junctions were observed in the P fracture face. Complementary E fracture face pits were also abundant (Fig. 5b–j). When Y-1 (D) cells were treated with

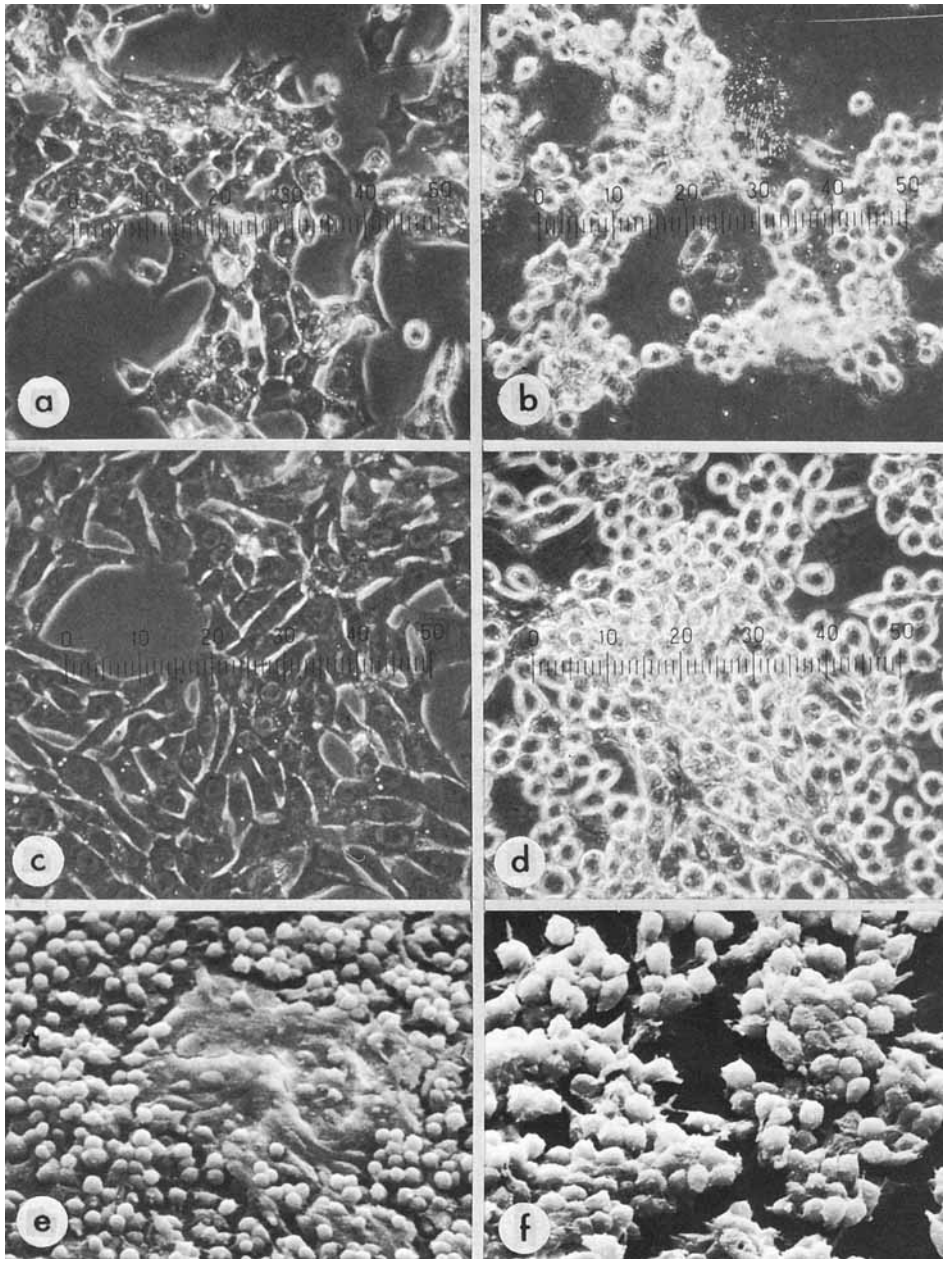


Fig. 1. Rounding responses of ACTH-sensitive tumor cells (BS-1) to ACTH and cholera toxin. a) control (0.2 ml of distilled water added to medium); b) rounded cells 8 h after the addition of 20 munits/ml ACTH in 0.2 ml distilled water; c) control; d) rounded cells 8h after the addition of 20 ng/ml cholera toxin (a–d, 200 × phase contrast); e) scanning electron micrograph of BS-1 cells 2 h after the addition of 20 munits/ml ACTH; note flattened cells (250 ×); f) scanning electron micrograph of BS-1 cells, 8 h after the addition of 20 ng/ml cholera toxin (302 ×).

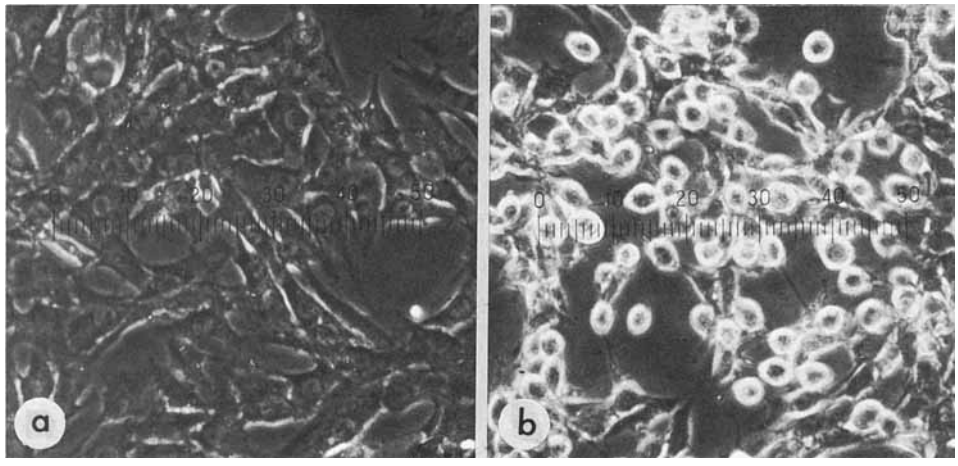


Fig. 2. Rounding response of ACTH-insensitive Y-1 tumor cells to cholera toxin. a) control; b) rounded cells 8 h after the addition of 3 ng/ml cholera toxin (a,b, 200 × phase contrast).

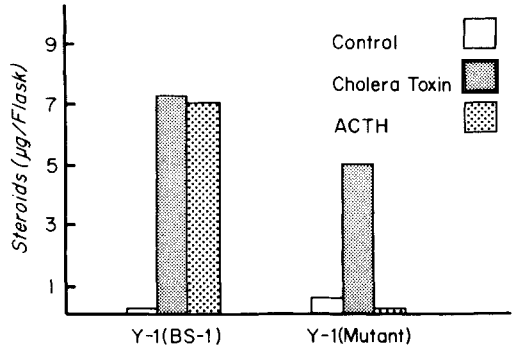


Fig. 3. Production of steroids by ACTH-sensitive (BS-1) and -insensitive Y-1 tumor cells in response to ACTH and cholera toxin. In these experiments, cultures were incubated in duplicate at 37°C in 3 ml growth medium, containing 100 munits/ml ACTH, or 10 ng/ml cholera toxin. Steroids were measured after 24 h of culture.

TABLE I. Cyclic AMP Production by ACTH-Sensitive (BS-1) and ACTH-Insensitive Y-1 Tumor Cells

	1 h	2 h	3 h	24 h
BS-1 cells				
Control	0.67 ± 0.2		1.2 ± 0.1	14.1 ± 3.8
CT[a] (5 ng/ml)	0.79 ± 0.1		20.8 ± 3.0	47.2 ± 16.1
ACTH (20 munits/ml)	14.5 ± 3.3		52.0 ± 0.1	7.4 ± 2.8
ACTH-insensitive cells				
Control	0.8 ± 0.1	1.8 ± 0.1	1.2 ± 0.1	
CT (10 ng/ml)	15.4 ± 1.7	44.4 ± 5.6	71.2 ± 3.4	

[a]cholera toxin.

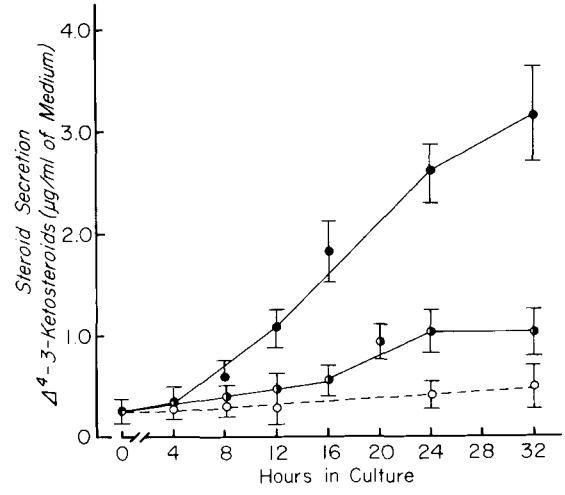
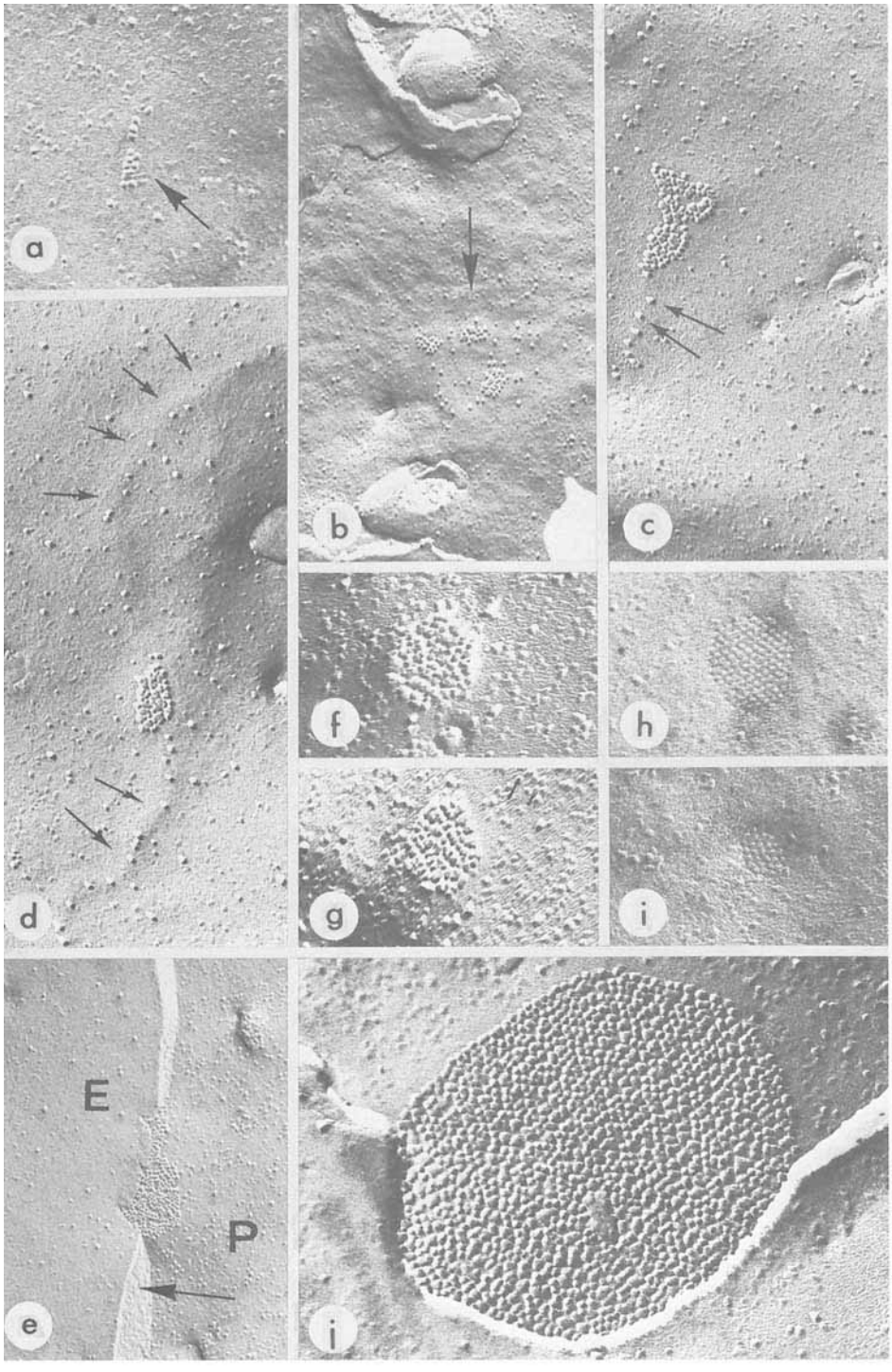


Fig. 4. Production of steroids by ACTH-sensitive Y-1 (D) tumor cells. ACTH (20 munits/ml) was added to the medium of Y-1 (D) cells removed 1 month (•) or 6 months (◑) earlier from liquid nitrogen storage. These cells were seeded at the same density. Controls had been removed from liquid nitrogen 1 month earlier (○). Each point is the mean steroid concentration from four flasks.

ACTH (10 munits/ml) for 4–8 h, particle aggregates failed to exceed 0.2 μm in diameter (Fig. 5b), whereas cultures treated for longer periods (8–16 h) exhibited gap junctional particle aggregates as large as 0.4 μm in diameter (Fig. 5e–h). When Y-1 (BS-1) cells were treated with ACTH (20 munits/ml for 7 h many P face particle aggregates and E face pits were observed (5f–j). One junctional aggregate was 0.5 μm in diameter (Fig. 5j). In addition, freeze cleaving further disclosed images that were indicative of gap junction development 4–8 h after treatment of ACTH. Occasionally, small formation plaques revealing particle aggregates and neighboring 10-nm particles were observed on an elevated particle-free region of the P face membrane. These plaques are reminiscent of those reported by Johnson et al [21] in reaggregating Novikoff hepatoma cells (Fig. 5b).

Untreated or cholera toxin-treated (20 min to 3 h) ACTH-insensitive Y-1 (mutant) cells exhibited no P fracture face gap junctional particle aggregates or E face pits. Areas of cell-cell apposition, revealed in freeze fracture replicas as areas containing both P and E fracture faces separated by a narrow cleft, contained no specialized intramembranous structures. Occasionally, small areas of the P face appeared devoid of particles.

Fig. 5. Gap junctions in ACTH-sensitive Y-1 tumor cells. a) small aggregate of 8.5-nm particles on the P fracture face of untreated Y-1 (D) cells (95,000 ×). b) Apparent formation plaque with clusters of 8.5-nm particles and single 10– to 11-nm particles 4 h after the addition of 20 munits/ml ACTH to a culture of Y-1 (D) tumor cells (55,000 ×). c) Particle aggregates 8 h after addition of ACTH to culture of Y-1 (D) cells; note single particles at arrows (76,000 ×). d) From same membrane as in c; note single particles on raised ridge at arrows (76,000 ×). e) Gap junction in membrane of Y-1 (D) cells 12 h after the addition of ACTH; note apparent intercellular space (arrow) and P face particles and E face pits typical of gap junctions (50,000 ×). f,g) P face particle aggregates in Y-1 (BS-1), 7 h after the addition of ACTH (20 munits/ml). h,i) E face pits 7 h after the addition of ACTH (f–i, 105,000 ×). j) Aggregate of P face particles measuring 0.5–0.6 μm in diameter (100,000 ×).



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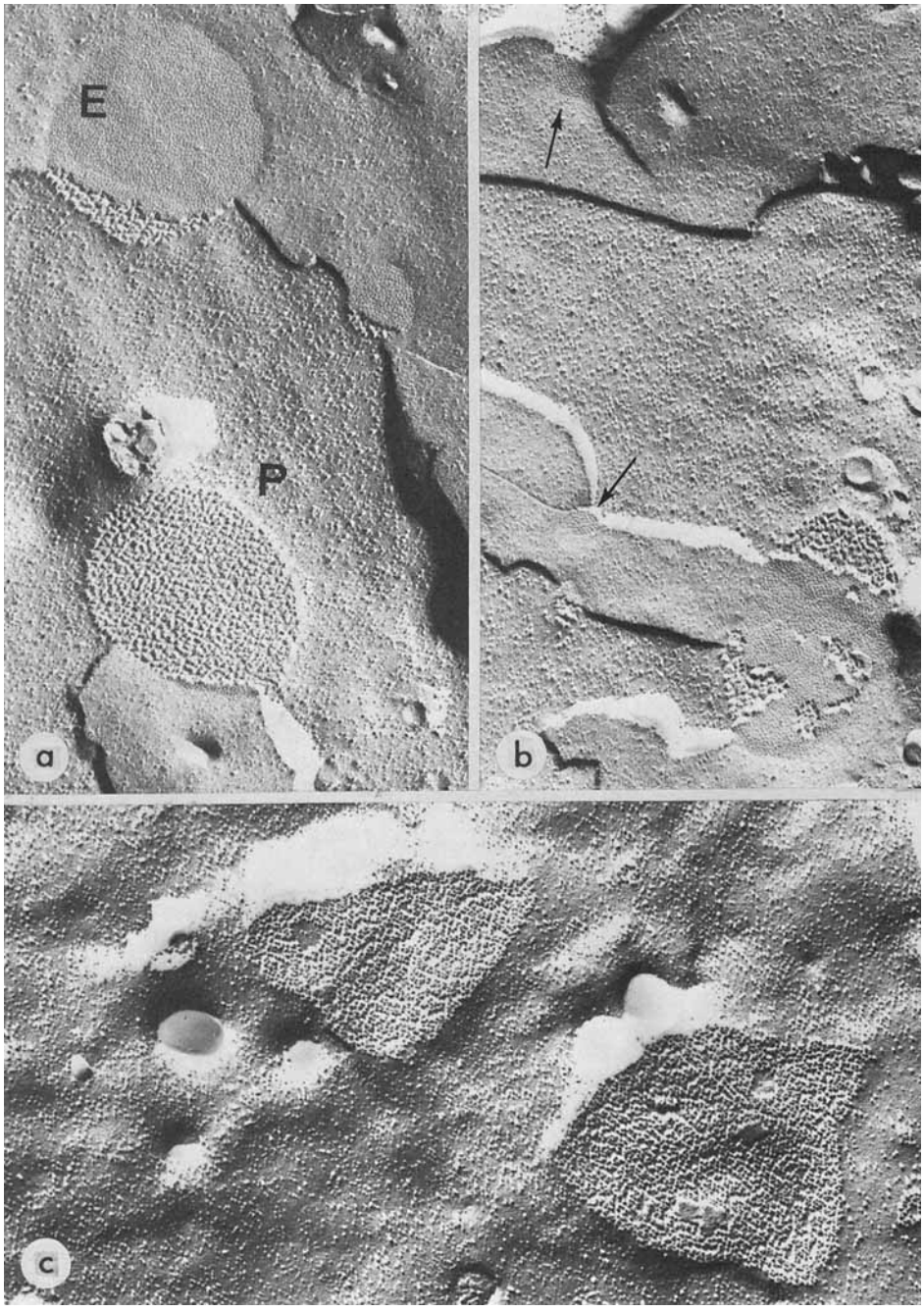

Fig. 6a, b). Gap junctions are numerous and relatively large in adrenal cortex of 5-week-old mouse (A^y); note E face pits (arrows) (52,000 ×). c) Gap junctions are relatively large and numerous in adrenal cortex of female New Zealand white rabbit (50,000 ×).

Loss of ACTH-Sensitivity and Gap Junctions During Culture

Both clones of ACTH-sensitive Y-1 cells slowly lost some of their sensitivity to ACTH during several months of culture. This loss was revealed by a reduced production of fluorogenic steroids after ACTH treatment in clone Y-1 (D) after 4–6 months in culture (Fig. 4), whereas a reduced rate and frequency of cell rounding was observed in clone BS-1 after 8 months in culture. These cells did not exhibit rounding until 40 min after the addition of ACTH (10 munits/ml). Freeze fracture studies of one clone, Y-1 (D), with reduced ACTH sensitivity failed to reveal the presence of gap junction arrays; only the small aggregates characteristic of the unstimulated clones were apparent.

Membrane Structure in Normal Adrenal Cortical Cells

Freeze fracture studies of normal, intact mouse and rabbit adrenal cortical cells revealed a large number of extensive gap junctions (Fig. 6a–c). Particle shape and packing differed in the mouse and rabbit. Our studies in the rabbit reveal that the majority of the gap junctions are found in the lower portion of the zona fasiculata and in the zona reticularis. Moreover, in the fetal rabbit cortex gap junctions appear in these regions as the developing adrenal cortical cells acquire a steroidogenic potential [22].

DISCUSSION

Friend and Gilula [23], utilizing both thin-section and freeze fracture electron microscopy, noted that the gap junctions of some glandular tissues were relatively extensive when compared to those in certain nonglandular epithelia. We have also demonstrated the presence of numerous, relatively large gap junctions in adrenal cortex of mouse and rabbit. Several other studies have also documented the presence of extensive gap junctions in several other secretory cells and these studies are discussed in a recent review [24]. These investigations have demonstrated the presence of extensive gap junctions and apparent cytoplasmic gap junctional vesicles in other steroidogenic cells including granulosa [25–28], luteal [29], and Leydig [30], in a variety of species.

A number of recent studies have also documented rapid and dramatic responses of gap junctions in several tissues to a variety of hormones and vitamin A [24]. Decker [9] has demonstrated the formation and growth of gap junctions in ependymoglial cells of the hypophysectomized frog larva in response to thyroxine. Other studies have disclosed that vitamin A may induce the growth of gap junctions in normal chick shank skin in culture [12], while extensive junctions and apparent interiorized gap junctional vesicles appear in a dry keratoacanthoma of the rabbit ear in response to vitamin A [13]. In both cases, vitamin A also induces mucous secretion. Extensive gap junctions and interiorized gap junctional vesicles are also frequent in an estrogen-dependent tumor of proximal convoluted tubule in the hamster [31].

The data presented in a preliminary report [9] and in this study suggest that the presence and behavior of gap junctions in Y-1 tumor cells may be related to ACTH sensitivity. We have documented the presence of gap junctions only in ACTH-sensitive clones of Y-1 tumor cells, while none were observed in untreated or cholera toxin-treated ACTH-insensitive cells even though cyclic AMP levels and steroid production were dramatically elevated. In addition, these studies also provided evidence that gap junctions in ACTH-

sensitive cells respond to ACTH by enlarging within the first few hours of treatment. Finally, we have observed an apparent correlation between the loss of ACTH sensitivity and gap junctions in clones originally sensitive to ACTH over several months in tissue culture.

If the apparent relationship between ACTH sensitivity and the presence of gap junctions is valid, we are still left with the question of the intimacy of this relationship. Does the presence of gap junctions depend on the presence of receptors to ACTH or are ACTH receptors and gap junctions simply coincidental differentiations of the membrane? Is the growth of gap junctions in ACTH-sensitive Y-1 tumor cells dependent only on the interaction of ACTH with its membrane receptor, or do subsequent steps in the chain of events elicited by this interaction regulate the behavior of gap junctions in these cells?

Ongoing studies are presently directed toward the validation of the apparent relationship between ACTH sensitivity and the behavior of gap junctions in adrenal cortical cells discussed in this paper. Other experiments in progress are directed to the question of the intimacy of this postulated relationship.

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