ROUNDING AND STEROIDOGENESIS OF ENZYME AND ACTH TREATED Y-1 MOUSE ADRENAL TUMOR CELLS

THESIS

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Cultured Y-1 mouse adrenal tumor cells exhibited varying degrees of rounding when treated with ACTH, proteolytic or glycolytic enzymes. ACTH (0.5 U/ml) caused rounding, formation of filopodia and numerous thin microvilli, and stimulated steroidogenesis. Rounding, filopodia and bleb formation occurred in trypsin (0.01%) and hyaluronidase (0.1%) treated cells; the enzymes failed to stimulate steroid production or prevent ACTH stimulation in subsequent incubations. Treatment with 20 mU/ml neuraminidase resulted in rounding, formation of thin microvilli and blebs. Steroid production was slightly increased, although subsequent effects of ACTH were prevented. It is suggested that rounding may not always be associated with steroidogenesis. In affecting the binding of ACTH while stimulating steroid production, neuraminidase appeared to be altering a carbohydrate-containing ACTH receptor.

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CHAPTER I

INTRODUCTION

In the adrenal cortex, the initial event in the stimulation of steroid production by adrenocorticotropin (ACTH) is the binding of ACTH to the plasma membrane (Taunton et al., 1969). This process results in the activation of adenyl cyclase and an increase in the intracellular levels of cyclic 3',5'-adenosine monophosphate (cAMP) (Sutherland et al., 1965; Grahame-Smith et al., 1967; Lefkowitz et al., 1970). Exogenous cAMP mimics the effects of ACTH in stimulating steroidogenesis, but the intracellular sites of action of endogenous and exogenous cAMP have yet to be fully characterized (Garren et al., 1971). The uptake of cholesterol by mitochondria is the rate-limiting step in steroidogenesis and appears to require an intact microfilament network (Mrotek et al., 1982A).

The binding of ACTH to adrenocortical membranes is thought to occur at a specific cell surface receptor (Schulster and Schwyzer, 1980; Buckley and Ramachandran, 1981); however, the receptor for ACTH has not been isolated nor has its exact composition and structure been determined (Buckley and Ramachandran, 1981). Insulin, and other polypeptide hormones, exert their effects through glycoprotein

receptors (Schulster and Schwyzer, 1980; Jacobs et al., 1980); whether the ACTH receptor is also a glycoprotein is uncertain (Buckley and Ramachandran, 1981), although several investigations present evidence consistent with this possibility. Haksar and coworkers (1973; 1974A) showed that neuraminidase diminished the steroid response of isolated rat adrenal cells to ACTH stimulation. Since neuraminidase cleaves terminal sialic acid residues, this indicates that a carbohydrate moiety may be involved in the binding or activating sequence of ACTH (Haksar et al., 1973; 1974A). Because the carbohydrate binding lectin, Concanavalin A, interacts with adrenal membranes, protecting the ACTH binding site from destruction (Schlegel, 1976), this also suggests that the ACTH receptor contains carbohydrates. As exemplified by the techniques of Haksar and coworkers (1973; 1974A) proteolytic and glycolytic enzymes may, therefore, provide a means for evaluating the involvement of proteins and glycoproteins in the binding of ACTH by adrenal cells.

The cultured Y-1 mouse adrenal tumor cell is a model system used by many investigators to study the mechanism of action of ACTH in stimulating steroid synthesis (Kowal, 1970; Mrotek and Hall, 1975; 1977; Rae et al., 1979; Mrotek et al., 1982A). The Y-1 responds to ACTH, exogenous cAMP and cholera toxin in a manner identical to non-tumor adrenal cells (Grahame-Smith et al., 1967; Kowal, 1970; Donta et

al., 1973; Wishnow, 1973); however the major steroid secreted by this adrenal cell is 20 α -hydroxypregn-4-en-3-one (20 α -dihydroprogesterone, 20-DHP) instead of corticosterone (Kowal, 1970). In addition to their effects in stimulating steroid synthesis, ACTH and cAMP also cause morphological changes in the cultured mouse adrenal tumor cell (Yasumura et al., 1966; Masui and Garren, 1971; Cuprak et al., 1977; Mattson and Kowal, 1980; Mrotek et al., 1982A). This effect of ACTH and cAMP produces a morphology described as rounding; rounding is the retraction of the peripheral margins of the cell toward the nucleus, elevation of the nuclear region from the growth surface and extensive development of long filopodia (Kawaoi et al., 1977; Cuprak et al., 1977; Mattson and Kowal, 1980; Mrotek et al., 1982A). Rounding results from a rearrangement of microfilaments and a loss of stress fibers within the cell, while filopodia formation involves intermediate filaments and microtubules (Mrotek et al., 1982A). Thus ACTH, and presumably cAMP, act through microfilaments to both increase steroid production and transform the Y-1 adrenal cell from a flattened to a rounded morphology (Mrotek and Hall, 1975; 1977; Mrotek et al., 1982A). Whether ACTH-stimulated steroid production is dependent on cell rounding is presently unknown.

In non-adrenal cell lines, proteolytic enzymes induce rounding by rearranging cytoskeletal components and altering membrane-associated proteins or glycoproteins (Hartwig

and Stossel, 1975; Huggins et al., 1976; Furcht and Wendelschafer-Crabb, 1978). Cell surface glycoproteins, such as fibronectin, provide cell-substrate adhesion and are sensitive to neuraminidase or proteolytic digestion (Zetter et al., 1976; Yamada and Olden, 1978). Evidence suggests that trypsin digests intramembrane connecting proteins, such as the actin binding protein which maintains cell shape (Hartwig and Stossel, 1975; Moore and Carraway, 1978) by anchoring intracellular actin filaments to the plasma membrane (Carraway, 1978). Other proteolytic and glycolytic enzymes, such as pronase and hyaluronidase, used in cell culture and isolation of cells from tissues produce cell suspensions which contain rounded cells (Prop and Wiepjes, 1973). Thus, enzymes appear to be agents causing rounding by altering cell attachments and intracellular cytoskeletal fibers.

Although the ACTH response of Y-1 cells depends on surface receptors and microfilaments, it has not been determined whether the enzymes capable of affecting receptors and microfilaments also affect rounding, steroid production and the action of ACTH.

The purpose of this study is to determine whether rounding and steroid production are associated. Using various proteolytic and glycolytic enzymes, enzymatic effects on rounding, steroidogenesis and subsequent response to ACTH will be examined. From these experiments it is

expected that information will be obtained to provide clues for the characterization of the ACTH receptor.

CHAPTER II

MATERIALS AND METHODS

Tissue Culture

Y-1 mouse adrenal tumor cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and were maintained as previously described (Mrotek and Hall, 1975; 1977; 1978). All cells used in the experiments were from the fifty-first to the sixty-first population doubling.

Experimental Procedure

Y-1 cells were subcultured into culture plates (10 cm^2, Costar, Cambridge, MA, USA) two to four days prior to experimentation. Serum containing medium (SCM) was removed and at half-hourly intervals, non-serum containing medium (NSM) was added. The first two intervals served as a preincubation to remove serum residues (Mrotek et al., 1982B, in press), the third provided a measurement of basal steroid production using a specific radioimmunoassay (Maroulis and Abraham, 1975; with modifications by Mrotek and Hall, 1977). To estimate the duration of incubation with trypsin, pronase, and hyaluronidase, a series of 15 min consecutive incubations were conducted to determine the time required for cells to round and detach from the culture plate; in subsequent experiments, enzymes were

removed from the cells 10-15 min prior to their detachment. Total incubation times determined by this method were: pronase, 5 min; trypsin, 30 min; hyaluronidase, 60 min. Alternatively, the 15 min incubation time for neuraminidase was previously described by Haksar et al. (1973; 1974A). After enzyme treatment, cells were then incubated with ACTH for 30 min. The medium from both the enzyme and subsequent ACTH incubations was collected in vials containing 0.2 M sodium azide and 0.155 N sodium cyanide and saved for determination of steroid content.

Experimental Solutions

All experiments were conducted using NSM consisting of Eagle's Minimum Essential Medium supplemented with Earle's salts, 2.0 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, 2.0 gm/l $NaHCO_3$, 2.5% (v/v) fetal calf serum (Lot # 007443) and 12.5% (v/v) horse serum (Lot# 010680) (pH 7.2-7.4). ACTH was dissolved in 1.0% (w/v) bovine serum albumin/saline (pH 4.0); 50 µl of this solution in NSM gave a final ACTH concentration of 0.5 U/ml. Enzymes were dissolved in NSM in the following concentrations: 0.01% (w/v) trypsin, 0.1% (w/v) hyaluronidase, 0.001% (w/v) pronase, and 20 mU/ml neuraminidase.

Radioimmunoassay

Following treatment, 20-DHP in the incubation medium was measured by radioimmunoassay using tritium labeled

20-DHP (55.0 Ci/mmole, New England Nuclear, Boston, MA, USA) (Maroulis and Abraham, 1975; with modifications by Mrotek and Hall, 1977).

Scanning Electron Microscopy

Y-1 cells grown on glow discharged carbon-coated glass coverslips two days prior to experimental incubations were washed three times with Hank's buffer (pH 7.4), fixed in 2.9% (v/v) glutaraldehyde for 20 min and after three more washings with Hank's buffer, were stained with 1.0% (w/v) osmium tetroxide in Hank's buffer for 10 min. Following three deionized water washes, cells were dehydrated in an ethanol/deionized water series (20,30,50,70,90,100,100, 100% v/v) and then critically point dried using ethanol/ liquid carbon dioxide as the transition fluids. Before the cells were examined in an ETEC Autoscanning Electron Microscope, they were sputter-coated with gold/palladium discharged at a rate of 20 mA/60 sec with a Model E5100 series II Cool Sputter-Coater (Polaron Instruments, Inc., Line Lexington, PA, USA).

Chemicals

ACTH (Porcine, Grade II, 93 U/mg) and trypsin inhibitor (Type II Soybean) were obtained from Sigma Chemical Co. (St. Louis, MO., USA). The enzymes used in this study were: 0.25% (w/v) trypsin from GIBCO (Grand Island, NY, USA), hyaluronidase (Type III ovine testis, 610 U/mg) and

neuraminidase (Type V from C. perfringes, 0.31 U/mg) both from Sigma Chemical Co., and pronase (B Grade, 45,000 P.U.K./gm) from CalBiochem (San Diego, CA, USA). Tritium labelled 20-DHP (55.0 Ci/mmole) was purchased from New England Nuclear (Boston, MA, USA). Antibody against 20-DHP was a gift from Dr. Guy Abraham (Harbor General Hospital, Torrance, CA, USA). Eagle's Minimum Essential Medium with Earle's salts and glutamine were obtained from KC Biological Inc. (Lenexa, KS, USA). All serums were purchased from Irvine Scientific (Santa Ana, CA, USA).

CHAPTER III

RESULTS

Morphological Features of Control, ACTH- and Enzyme-Treated Y-1 Cells

Control- -Cultured Y-1 mouse adrenal tumor cells observed with phase contrast light microscopy (PCLM) or scanning electron microscopy (SEM) were spread on the growth surface in flattened polymorphic shapes (Figure 1A and B). Although an occasional rounded mitotic cell was

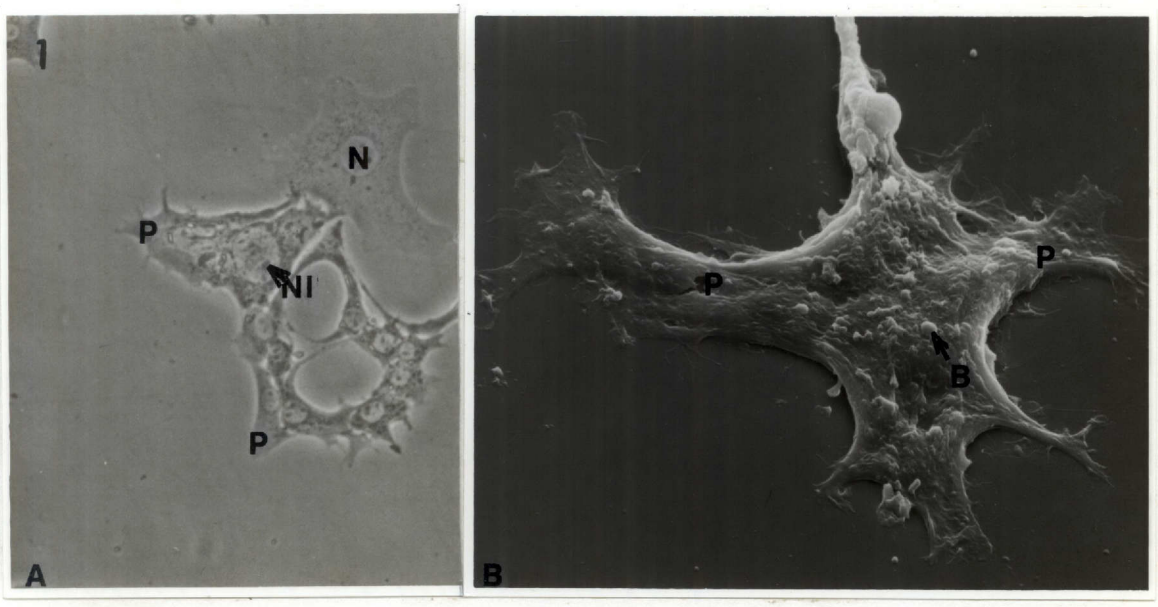

Figure 1. Control Y-1 adrenal cells. A. Phase contrast light micrograph. X400. B. Scanning electron micrograph. X1000. (B: surface bleb; N: nucleus; NI: nucleolus; P: pseudopodia). Note the blebs, pseudopodia and the absence of thin microvilli.

seen, most cells were tightly associated in colonies and there was little space between the adjacent flattened cells. As seen with PCLM, individual cells had numerous pseudopodia and cell nuclei with distinct nucleoli were apparent. The surface of the cells observed with SEM were covered with many blebs but there were few thin microvilli.

ACTH- -Morphological changes occurred in Y-1 cells incubated for 30 min with ACTH (0.5 U/ml) (Figure 2A and B).

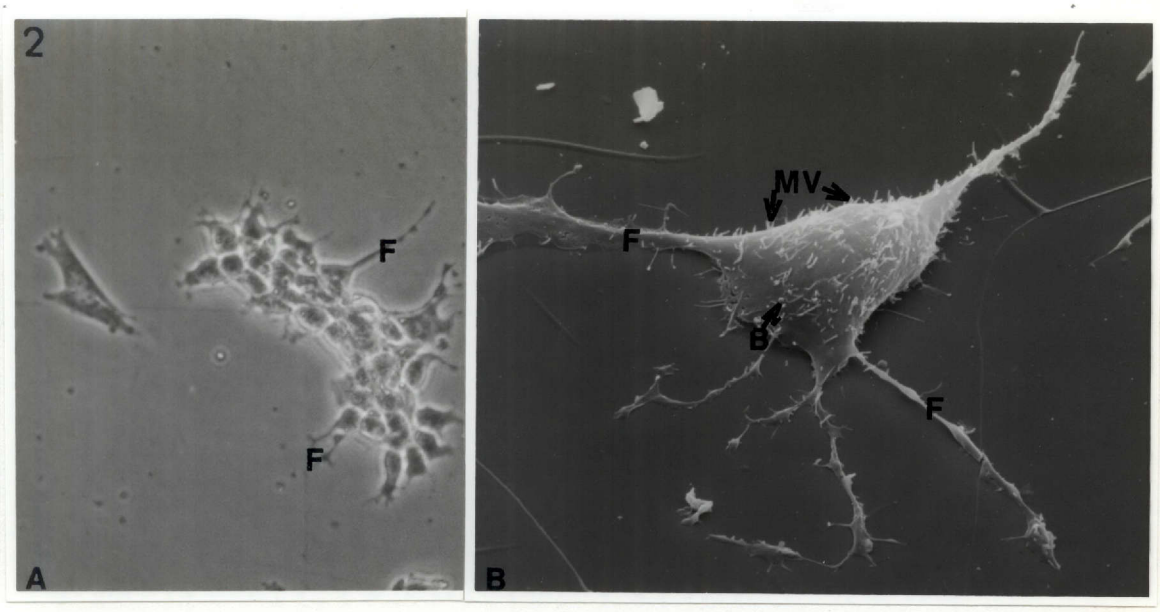

Figure 2. Y-1 adrenal cells treated with 0.5 U/ml ACTH. A. Phase contrast light micrograph. X400. B. Scanning electron micrograph. X2000. (B: surface bleb; F: filopodia; MV: thin microvilli). Note the rounded stellate morphology with prominent filopodia and numerous thin microvilli.

Both PCLM and SEM revealed that the peripheral margins of the cell retracted toward the nucleus and filopodia of

varying lengths extended outward from the cell body. Time-lapse video microscopy of ACTH-treated cells revealed that filopodia actively grew outward from various points on the cell periphery while other areas of the peripheral margin retracted toward the nucleus. As the intercellular space between adjacent cells increased, the cell body thickened making it difficult to discern the nucleus using PCLM and videomicroscopy. Occasionally a flattened cell was present in the groups of cells examined with PCLM. In SEM micrographs, the cell surface contained numerous thin microvilli and a reduced number of blebs.

Trypsin- -Phase contrast microscopy revealed that about 50% of the cells treated 30 min with 0.01% trypsin exhibited

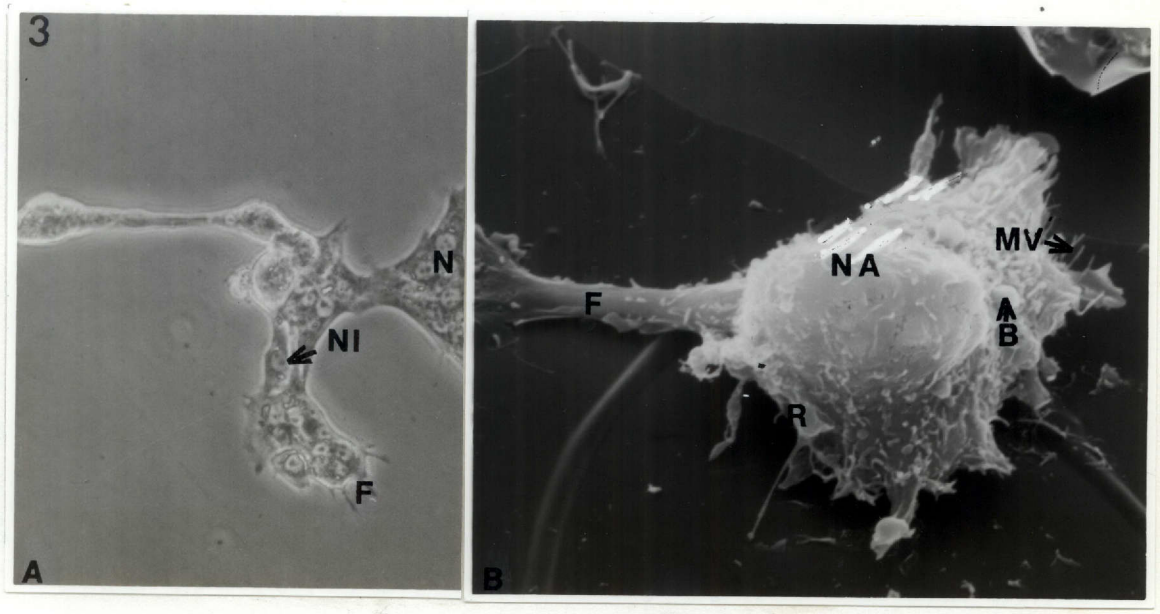

Figure 3. Y-1 adrenal cells treated with 0.01% trypsin. A. Phase contrast light micrograph. X400. B. Scanning electron micrograph. X4000. (B: surface bleb; F: filopodia; MV: thin microvilli). Note the rounded cell with the large nuclear area, filopodia, blebs and thin microvilli.

rounding, although only 30% of the cells seen in Figure 3A were rounded. Using SEM, a trypsin-treated, rounded cell was found to develop prominent filopodia and a large nuclear area (Figure 3B). Blebs, ruffles and a few microvilli were seen on the surface of the cell.

Pronase- -Y-1 cells treated 5 min with 0.001% pronase rounded and were removed from the culture plate so radidly, that PCLM and SEM examination was not possible.

Hyaluronidase- -A light micrograph of Y-1 cells incubated for 15 min with 0.1% hyaluronidase is shown in Figure 4A. Although it appears that these cells rounded slightly

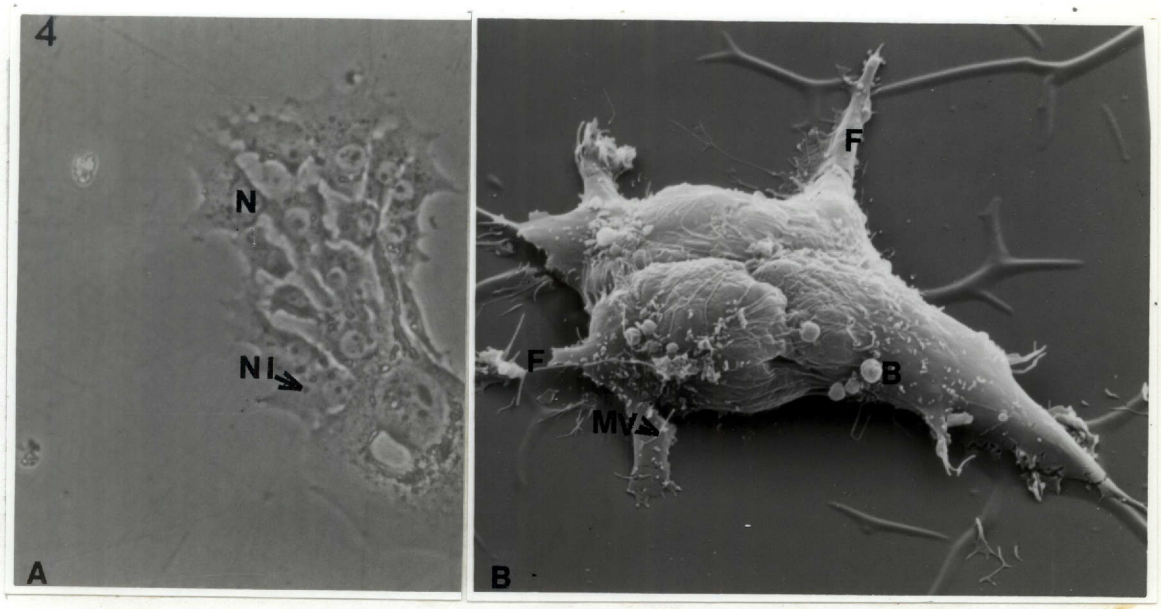

Figure 4. Y-1 adrenal cells treated with 0.1% hyaluronidase. A. Phase contrast light micrograph. X400. B. Scanning electron micrograph. X2000. (B: surface bleb; F: filopodia; N: nucleus; NI: nucleolus; MV: thin microvilli). Note the slightly rounded cells in 4A. 4B shows a more extensively rounded cell with blebs, filopodia and few thin microvilli.

leaving increased space between cells, all nuclei were visible. In the SEM micrograph a similarly treated cell appeared to be more extensively rounded (Figure 4B). Broad filopidia, blebs and only a few thin microvilli were seen on the surface of the cell.

Neuraminidase- -The effect of incubating Y-1 cells with 20 mU/ml neuraminidase for 15 min can be seen in the micrograph in Figure 5A. The cells were rounded slightly;

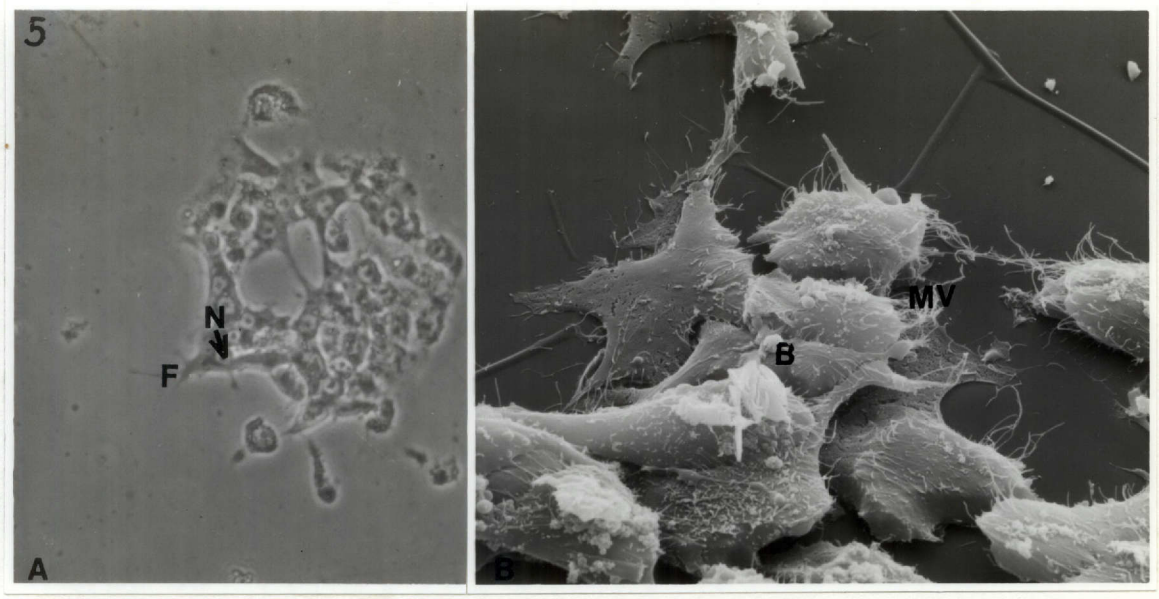

Figure 5. Y-1 adrenal cells treated with 20 mU/ml neuraminidase. A. Phase contrast light micrograph. X400. B. Scanning electron micrograph. X1000. (B: surface bleb; F: filopodia; N: nucleus; MV: thin microvilli). Note the slightly rounded appearance with filopodia, blebs and numerous thin microvilli with overlapping margins of the lifted cell edges.

however, the increased intercellular space and difficulty in obtaining completely focused nuclei suggested a morphological change had occurred. Examination of the neuraminidase treated cells by SEM revealed that cell peripheries

began to lift from the substrate, cell edges overlapped and few filopodia were present (Figure 5B). Occasional blebs and numerous microvilli were also present on the cell surface.

Steroidogenesis of ACTH- and Enzyme-Treated Y-1 Cells

Trypsin- -Data for steroid production by Y-1 mouse adrenal tumor cells incubated with ACTH (0.5 U/ml) or 0.01% trypsin is compared in Figure 6. During the first

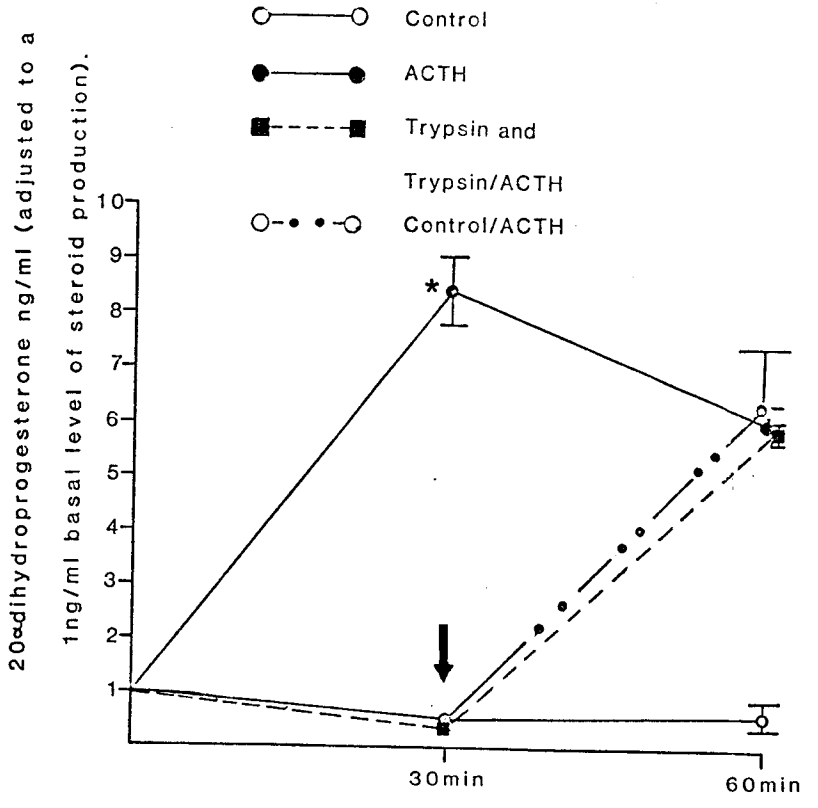

Figure 6. Y-1 cells were incubated with 0.01% trypsin and ACTH (0.5 U/ml) for 30 min. Trypsin inhibitor (0.5 mg/ml) was added to trypsin-treated cells prior to addition of ACTH to these and control cells (arrow). ACTH treatment was allowed to continue for 30 min.

*Values are the mean ± range for two determinations.

30 min treatment, incubation with trypsin alone did not stimulate steroid production. After the addition of trypsin inhibitor and a second incubation of the previously trypsin-treated cells with ACTH, steroid production increased in a manner similar to control cells initially treated with ACTH (Figure 6).

Pronase- -Since pronase removed cells from the culture plate, steroid content of the incubation medium could only be determined in medium centrifuged to remove cells. There was no change from control cell steroid production. (Table I).

TABLE I

EFFECT OF A FIVE MIN INCUBATION WITH 0.001% PRONASE ON STEROID PRODUCTION BY Y-1 CELLS

Treatment	20-DHP (ng/plate)*
Control	0.37 ± 0.11
Pronase	0.20 ± 0.10

*Values are the mean ± range of two determinations.

Hyaluronidase- -Y-1 cells treated with 0.1% hyaluronidase during four 15 min periods produced the same amount of steroids as control cells (Figure 7). In subsequent incubation, the addition of 0.5 U/ml ACTH to washed preparations of control or 0.1% hyaluronidase-treated cells stimulated production of similar amounts of 20-DHP.

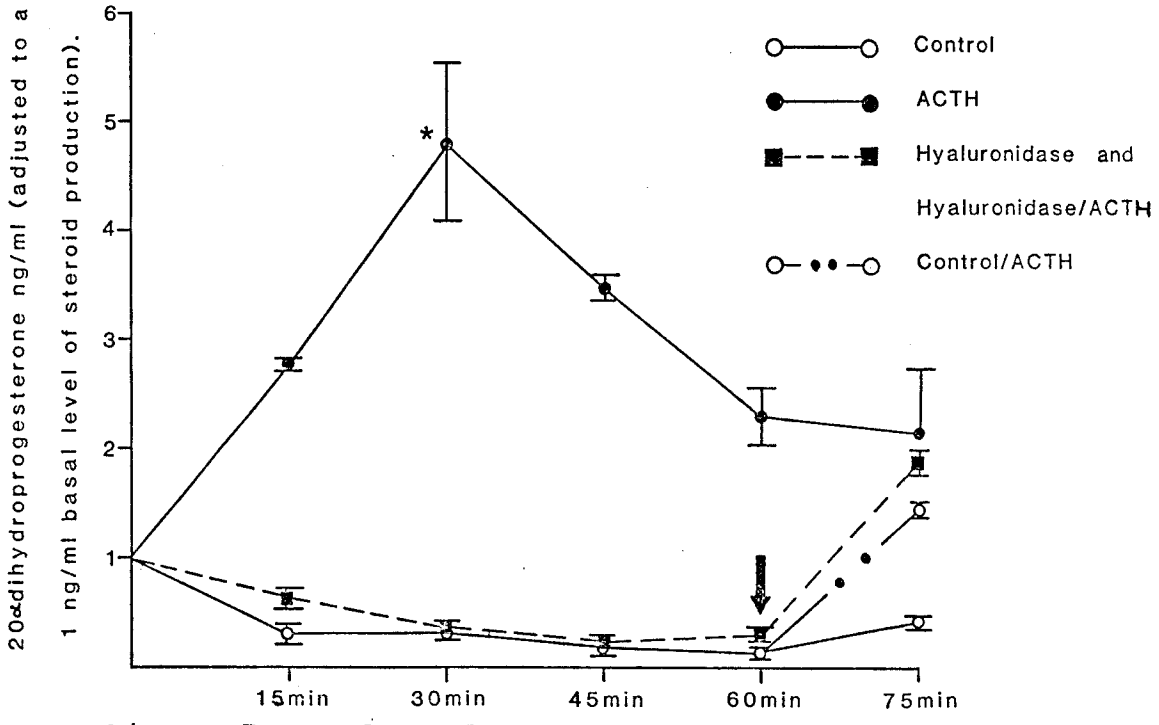

Figure 7. Y-1 cells treated with 0.1% hyaluronidase and 0.5 U/ml ACTH every 15 min for one hour, and then cells were washed with NSM. Control and hyaluronidase-treated cells were incubated with ACTH for 30 min (arrow).

*Values are the mean ± range for two determinations.

Neuraminidase- -To determine what effects neuraminidase treatment would have on steroid production, Y-1 cells were incubated for 15 min with 20 mU/ml neuraminidase. As seen in Figure 8, neuraminidase alone stimulated steroid production. After washing these and similarly treated control cells, they were incubated for 30 min with 0.5 U/ml ACTH. Cells previously treated with neuraminidase did not respond to ACTH to the same degree as the washed control cells (Figure 8).

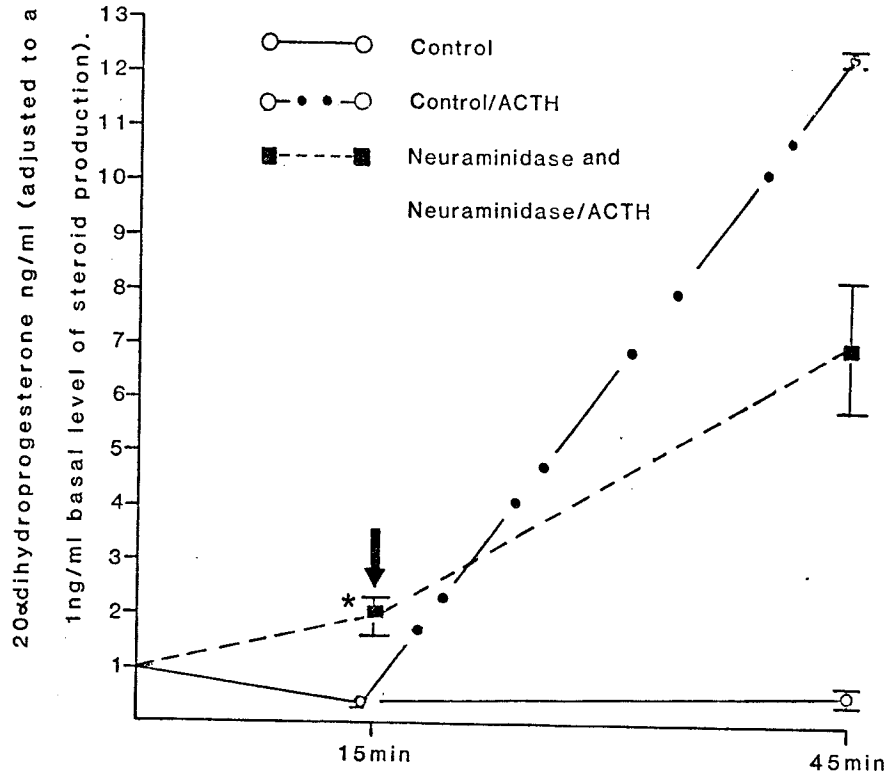

Figure 8. Y-1 cells were incubated for 15 min with 20 mU/ml neuraminidase, and washed with NSM prior to addition of ACTH (0.5 U/ml) to control and neuraminidase-treated cells; this incubation was allowed to proceed 30 min.

*Values are the mean ± range for two determinations.

CHAPTER IV

DISCUSSION

Previous studies showed that cultured Y-1 adrenal tumor cells both rounded and increased steroid synthesis in response to ACTH (Yasumura et al., 1966; Donta et al., 1973; Kawaoi et al., 1977). However, few investigators attempted to directly associate rounding with the steroidogenic response of adrenal cells to stimulation. Since proteolytic or glycolytic enzymes caused cells to round and detach from culture plates (Prop and Wiepjes, 1973; Huggins et al., 1976; Harrison and Allen, 1979), these enzymes were used with cultured adrenal cells to produce rounding in order to examine the associations between rounding and steroid production. In addition, the ACTH response was determined after enzymatic treatment to determine the effect of enzymes on the ACTH receptor. In these studies the association between rounding and steroidogenesis was examined. Phase contrast and scanning electron microscopy was used to evaluate rounding and steroidogenesis was examined with a radioimmunoassay methodology.

Although the enzymes trypsin and hyaluronidase caused extensive rounding of adrenal cells, steroid output was

not increased above control levels. Pronase rapidly rounded the cells and removed them from the culture plate; steroid levels could not be measured with confidence because cells were present. When neuraminidase was used, space between adjacent cells increased and the nuclear outlines became less distinct, suggesting that these cells had rounded slightly. Neuraminidase alone also caused a small increase in steroid production. Although previous reports indicated that trypsin caused rounding of Y-1 adrenal cells during subculturing (Yasumura et al., 1966), the present study is the first to examine its effects on steroidogenesis. Similarly, the effects of pronase, hyaluronidase, and neuraminidase on Y-1 cell rounding and steroid production were not reported previously. The preceding observations suggested that rounded cells do not necessarily produce steroids and that fully rounded cells may not be required for steroid production.

In preliminary studies, treatment of the Y-1 cell with neuraminidase alone elevated intracellular cAMP production (results not shown). Examination of data from previous studies indicated that intracellular cAMP levels were increased during neuraminidase treatment of isolated rat adrenal cells (Haksar et al., 1974B). Any treatment, such as exogenous cAMP initiated rounding and steroidogenesis in Y-1 cells (Masui and Garren, 1971; Donta et al., 1973; Wishnow, 1973; Cuprak et al., 1977). However, the precise

explanation for the increased intracellular cAMP in neuraminidase-treated Y-1 and isolated rat adrenal cells is unknown. If terminal sialic acids were removed from integral glycoproteins of the plasma membrane surface by neuraminidase, it perhaps allowed intramembrane molecules responsible for activating adenyl cyclase to aggregate. Alternatively, neuraminidase bound to the membrane in a manner similar to its reported binding to erythrocyte membranes (LaMont and Isselbacher, 1977) might also result in activation of adenyl cyclase. Regardless of the mechanism involved, the present evidence for increased intracellular cAMP, taken together with the findings of Haksar and coworkers (1973; 1974A), suggested that neuraminidase could affect rounding and steroid production, perhaps independently, through its stimulation of intracellular cAMP synthesis.

On the other hand, trypsin, hyaluronidase, and pronase seemed to affect Y-1 cell rounding in a manner different from neuraminidase, since steroid production was not increased. Several investigators reported possible mechanisms for these enzymes to cause cell rounding. The extracellular glycoprotein fibronectin, appears to be responsible for maintaining adhesion between a cell and its growth substrate (Yamada and Olden, 1978); because trypsin, hyaluronidase, and pronase may use fibronectin as a substrate, rounding will occur if it is digested. It should also be

noted that some fibronectins contain sialic acid residues (Yamada and Olden, 1978), the possibility cannot be excluded that neuraminidase may also cause rounding by removal of sialic acid from fibronectin. Alternatively, the proteolytic enzymes might also digest a transmembrane actin-binding protein as suggested by Carraway (1978); in this event actin-containing microfilaments would dissociate from the plasma membrane producing rounding.

SEM examination of Y-1 mouse adrenal tumor cells treated with ACTH or enzymes revealed some similarities in the morphological responses to treatment. The prominent topographical feature of ACTH-treated cells was numerous thin microvilli. Similar features were observed by Cuprak and coworkers (1977); they suggested that microvilli were a characteristic ACTH-dependent response in the Y-1 cell. However, in the present study thin microvilli were observed on both trypsin- and neuraminidase-treated Y-1 cells. This observation indicates, at least for trypsin-treated cells, that microvilli are not a characteristic response to stimulation, but may serve as a reservoir for excess membrane during the rounding process (Knutton et al., 1975).

The effects of the enzymes in altering steroid production was also examined during a subsequent incubation with ACTH. The concentrations of trypsin and hyaluronidase used in the present experiment had no effect on the response of Y-1 cells to ACTH, pretreatment with neuraminidase

inhibited ACTH-stimulated steroid production. Similar findings were obtained using neuraminidase-treated isolated rat adrenal cells (Haksar et al., 1973; 1974A). Sialic acid removal from the adrenal cell surface may, therefore, interfere either with the binding of ACTH to the plasma membrane receptor or with the activation of adenyl cyclase by ACTH. The ACTH molecule consists of a sequence of amino acid residues which activate adenyl cyclase while a second lysine- and arginine-rich region of the ACTH molecule binds to the receptor (Schulster and Schwyzer, 1980). At physiological pH it seems possible that a net negative charge exists on terminal sialic acids found in carbohydrate-containing membrane molecules. The present observations using neuraminidase are, therefore, more consistent with the view that neuraminidase abolished the binding of ACTH to its receptor.

The present results offer the first information regarding the composition of the ACTH receptor in the Y-1 adrenal cell. Together with the observations of Haksar and coworkers (1973; 1974A) and the sensitivity of the adrenal cell to Concanavalin A (Schlegel, 1976), this study provided a clue that the ACTH receptor contained carbohydrates and may be glycoprotein or glycolipid in character. Finally, it appeared that rounding was not always associated with increased steroid production.

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