Role of Actin in the Responses of Adrenal Cells to ACTH and Cyclic AMP: Inhibition by DNase I

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ABSTRACT Erythrocyte ghosts were loaded with pancreatic DNase I and fused with Y-1 adrenal tumor cells to test the possibility that this enzyme might inhibit the steroidogenic responses of the cells to ACTH and cyclic AMP. Fusion of erythrocyte ghosts loaded with DNase I, but not those containing albumin, ovalbumin, boiled DNase I, or DNase I with excess G-actin, inhibited the increase in production of 20α-dihydroprogesterone produced by ACTH and dibutyryl cyclic AMP; inhibition was concentration-dependent with 50% inhibition by 3×10^7 molecules of DNase I per cell. It was found that inhibition by DNase I was exerted at the step in the steroidogenic pathway at which cholesterol is transported to mitochondria where steroidogenesis begins. This was shown by measuring transport of cholesterol into the inner mitochondrial membrane, by measuring the production of pregnenolone by isolated mitochondria and by demonstrating that DNase I was without effect on the conversion of pregnenolone to 20α-dihydroprogesterone (an end-product of steroid synthesis). The actin content of Y-1 cells was measured by two methods based upon inhibition of DNase I and by SDS gels following centrifugation. The cells were found to contain $2\text{–}3 \times 10^7$ molecules of actin per cell of which two-thirds is present as G-actin. Since DNase I is known to bind to G-actin to give a one to one complex, these and other findings suggest that at least some of the G-actin in the cells may be necessary for the steroidogenic responses to ACTH and cyclic AMP.

The process of steroid biosynthesis involves the mobilization of stored cholesterol that is transported from the cytoplasm to the inner mitochondrial membrane, where the steroid binds to a cytochrome P-450 that converts it to pregnenolone (9, 24); this conversion is referred to as side-chain cleavage. Pregnenolone is converted to the final secreted steroid hormones by a series of enzymatic reactions most of which are extramitochondrial (9, 24).

For many investigations of steroid synthesis, an adrenal tumor cell called Y-1 (American Type Culture, Rockville, MD) has proved extremely useful (10–12, 15). These cells produce a number of steroids of which 20α-dihydroprogesterone can be taken as representative. It is possible to measure the transport of cholesterol to mitochondria (17), or into the inner mitochondrial membrane (10, 11) in adrenal cells, by inhibiting side-chain cleavage with aminoglutethimide—any cholesterol transported to the inner membrane accumulates there because it cannot enter the steroidogenic pathway in the presence of the inhibitor. Moreover, when the aminoglutethimide is removed, the production of pregnenolone can be measured by incubating isolated mitochondria; the conversion of the cholesterol to pregnenolone demonstrates that the accumulated cholesterol is used for steroid synthesis in the mitochondrial membrane (10, 11).

Considerable interest has been shown in the mechanism of intracellular transport of cholesterol because it is stimulated by ACTH and cyclic AMP (4, 5, 10, 22). Moreover, since this stimulation was shown to be inhibited by cytochalasin B (4, 5, 10, 21), it was proposed that microfilaments may be involved. This idea received strong support when it was reported that monospecific antibodies to actin, when injected into Y-1 cells via liposomes, inhibited the stimulation of cholesterol transport by ACTH and dibutyryl cyclic AMP (10). The major limitation of the liposome procedure lies in the fact that adherent liposomes containing the substance to be injected, remain attached to the cells so that it is difficult to determine how much of the substance in question has actually been delivered to the interior of the cell. The use of erythrocyte ghosts provides a possible solution to this problem since injection is efficient (e.g., references 3, 14), and the erythro-

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cytes can be destroyed by lysis after injecting their contents, with the result that uninjected material is removed (14). It was decided to determine whether DNase I, injected via erythrocyte ghosts into Y-1 cells, is capable of inhibiting the steroidogenic responses of these cells to ACTH and dibutyryl cyclic AMP. Since pancreatic DNase I is known to bind to G-actin in a quantitative manner (34) and thereby to prevent polymerization of actin, this approach has enabled us to study the role of actin in the responses to the two stimulating agents in greater detail.

MATERIALS AND METHODS

Cell Culture

Y-1 adrenal tumor cells were cultured in medium containing horse serum and fetal calf serum in plastic dishes as described elsewhere (10, 11). Each experiment was performed with cells from the same subculture, that is, all plates used in any one experiment were prepared from one batch of cells by subculture at one time. In preparation for an experiment medium was removed and cells were washed twice with phosphate-buffered saline. Experiments were performed in minimal Eagle's medium. In some studies, Y-1 cells were removed from plastic dishes by incubation with EDTA (0.5 mM) in phosphate-buffered saline at 37°C for 5 min. The cells were readily removed by scraping with a rubber policeman. The plates were washed with serum-containing medium and the cells were collected from the pooled washings by centrifugation.

Erythrocyte Loading and Fusion

Blood was taken from rabbits via the ear vein into a heparinized syringe. The procedure for loading and fusion was that described by Schlegel and Reichsteiner (25). Briefly, the erythrocytes were washed, swollen in hypotonic buffer containing the substance to be loaded, and then restored to their previous shape (sealed) by addition of one-tenth volume of 10-fold concentrated buffer. The erythrocyte suspension was added to Y-1 cells in minimal Eagle's medium containing bacterial phytohemagglutinin (50 μg/ml) to promote adhesion. Fusion was achieved by addition of polyethylene glycol 6000 (44% wt/vol). The polyethylene glycol was prepared as described in reference 25. After 1 min, the polyethylene glycol was diluted with a threefold excess of Hank's solution. After 30 min at room temperature, the medium was removed, cells were washed with phosphate-buffered saline, which was followed by addition of minimal Eagle's medium. The cells were then ready for use in an experiment. The loaded erythrocytes were fused with Y-1 cells either immediately after preparation or 12 h later. In a few studies, the loaded erythrocytes were kept for as long as 3 d before fusion without any discernible change in efficiency of loading. Unless otherwise stated, Y-1 cells were examined for steroidogenic responses and for efficiency of loading 12 h after fusion. Cells were examined at other times in studies not reported here. Our conclusions were not changed by these additional studies. If cells fused with erythrocytes containing buffer or control proteins, such as serum albumin, were incubated in the usual medium containing serum and incubated under our usual conditions, instead of being used in these experiments, the cells continued to divide and to secrete steroids at a normal rate during a period of at least 7 d. Such cells could be subcultured to produce viable cells that appeared normal by light microscopy.

When Y-1 cells were fused with erythrocytes loaded with fluorescent albumin or DNase I, 300 cells were counted per plate by phase-contrast microscopy and the percentage showing fluorescence was determined by fluorescence microscopy. In some studies, DNase I labeled with fluorescein isothiocyanate (FITC), was injected into Y-1 cells via erythrocyte ghosts. The cells were also incubated in medium containing the specific mitochondrial marker 3,3'-diethyloxacarbocyanine iodide, commonly abbreviated $DiOC_2(3)$. The dye was dissolved in 10 μl of ethanol and added to the medium at a final concentration of 20 μg/ml. The cells were examined by means of a Leitz diavert microscope using two filters in succession to determine the distribution of the injected fluorescent DNase I in relation to the fluorescently labeled mitochondria. The FITC label is detected by a Leitz N-2.1 filter, while $DiOC_2(3)$ is observed with a Leitz I-2 filter. The properties of $DiOC_2(3)$ as a specific mitochondrial marker are described in reference 27.

Steroid Synthesis

Three methods were used in these studies to measure cellular functions related to steroid production by Y-1 cells:

PRODUCTION OF 20α-DIHYDROPROGESTERONE: Samples of the incubation medium were subjected to radioimmunoassay by an established method (18). Measurement of this substance provides a reliable index of total steroid production by Y-1 cells. Duplicate determinations on one sample of medium show differences of less than 5% of the mean.

TRANSPORT OF CHOLESTEROL TO MITOCHONDRIA: To measure transport of endogenous cholesterol from stores in the cytoplasm to the inner mitochondrial membrane, we inhibited the conversion of cholesterol to pregnenolone in that membrane by incubating Y-1 cells in a medium containing aminoglutethimide (0.76 mM). Cholesterol transported to the membrane accumulates because it cannot enter the steroidogenic pathway. Subtraction of the cholesterol content of the membrane at zero time gives an accurate measure of cholesterol transport. The method has been described elsewhere (12). The inner mitochondrial membrane was prepared by the method of Yago et al. (37).

PRODUCTION OF PREGNENOLONE BY ISOLATED MITOCHONDRIA: Not all the cholesterol in adrenal mitochondria is available for conversion to pregnenolone. The steroidogenic pool of cholesterol can be studied by incubating the cells with aminoglutethimide (0.76 mM) and after incubation, cells are homogenized and mitochondria are prepared. The mitochondria are washed to remove aminoglutethimide and incubated at 30°C. The production of pregnenolone is measured. The method has been described elsewhere (12).

Miscellaneous

Cells were counted in a Coulter counter (Coulter Electronics Inc., Hialeah, FL) or in a hemocytometer. Iodination of DNase I was performed by the method of Bolton and Hunter (2). The specific activity of each preparation used was determined by adding a known amount of unlabeled DNase I to the iodinated preparation followed by determination of ^{125}I in the mixture. The mass of the [^{125}I]DNase I before dilution was negligible in relation to the amount of added DNase I. Cholesterol was measured by a fluorometric procedure using cholesterol oxidase (7). Methods for measuring pregnenolone and 20α-dihydroprogesterone by radioimmunoassay have been published (4, 20). The methods involved in measuring the conversion of [7α-^{3}H]pregnenolone to 20α-[^{3}H]dihydroprogesterone have been given elsewhere (10, 11). Rhodamine and FITC derivatives of proteins were prepared by a standard procedure (8). To measure the amount of injected [^{125}I]-labeled protein, one must remove bound erythrocytes after fusion with Y-1 cells. Lysis of erythrocytes after fusion was performed by adding a solution of 0.83% ammonium chloride in water (wt/vol) for 10 min. The solution was removed and the procedure was repeated twice more. The cells were then washed twice with phosphate-buffered saline and then examined (14). The procedure removes virtually all erythrocytes from the preparation without removing or detectably altering the Y-1 cells. In some studies, Y-1 cells, treated with NH_4Cl in this manner, were incubated in complete (serum-containing) medium. Basal steroid synthesis and the response to ACTH were not significantly different from values observed with cells similarly treated with normal saline instead of ammonium chloride. The Y-1 cells are then dissolved in 1.0 ml of 2 N sodium hydroxide to measure ^{125}I. Because commercial pancreatic DNase I shows a number of trace contaminants on SDS gels, highly purified DNase I was prepared by subjecting the commercial preparation to column chromatography as described by Wang and Moore (28). The purified enzyme shows a single band on SDS gels correspnding to a molecular weight of ~32,000. This DNase I was also used for iodination.

Protein biosynthesis was measured by incubating Y-1 cells with ^{3}H-leucine (0.5 μC; 0.5 μmol/ml) followed by precipitation with trichloroacetic acid (29). Synthesis of DNA was measured by incubating cells with ^{3}H-thymidine (1.0 μC; 0.5 nmol/ml) followed by measurement of [^{3}H]DNA (30). Oxygen consumption of mitochondria was measured by means of a Clark electrode (31). Adrenal cell actin was prepared as described previously (13).

Materials

DNase I from pancreas, bovine serum albumin, ovalbumin, and the reagents necessary for preparing fluorescent derivatives of proteins were obtained from Sigma Chemical Co. (St. Louis, MO). Radioactively labeled steroids and the Bolton Hunter reagents were obtained from New England Nuclear (Boston, MA). Aminoglutethimide was a generous gift from Ciba-Geigy Corp., (Pharmaceuticals Div., Summit, NJ). $DiOC_2(3)$ was purchased from Molecular Probes (Junction City, OR). Other materials were obtained as previously described (10, 11, 20, 21). Polyethylene glycol was obtained from Sigma Chemical Co.

RESULTS

Injection of Fluorescent Proteins

When erythrocyte ghosts were loaded with albumin labeled with rhodamine or FITC, >95% of the cells showed fluores-

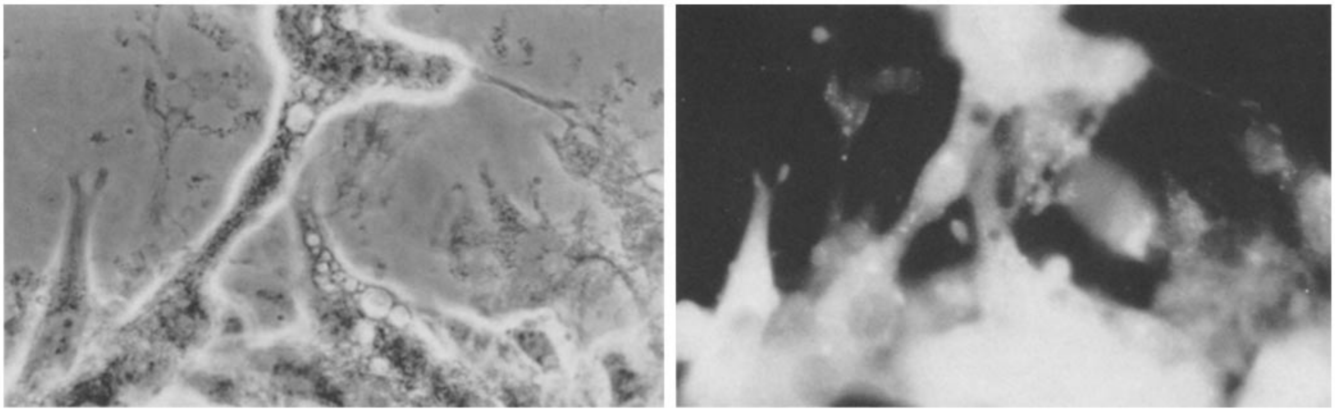

FIGURE 1 Loading of Y-1 cells with fluorescent albumin by erythrocyte ghosts. Y-1 cells were grown in plastic culture dishes. Cells were washed and fused with erythrocyte ghosts loaded with fluorescent albumin (rhodamine labeled). After loading, erythrocytes were subjected to lysis with ammonium chloride (see Materials and Methods) and then examined with an Olympus microscope. Phase-content (*left*) and fluorescence microscopy (*right*) are shown with the same field. ×210.

cence. When these erythrocytes were fused with Y-1 cells, 73–89% of the cells (81 ± 8% means and ranges for four determinations), showed intracellular fluorescence (Fig. 1). Attached fluorescent erythrocytes were clearly visible. Entry of fluorescent protein began within a few minutes of fusion and reached maximal intensity within 5 min as judged by fluorescence microscopy. On careful focusing, no fluorescence was seen in the nucleus. Moreover when $DiCO_2(3)$ was used to label mitochondria, double fluorescence labeling showed no FITC-labeled DNase I associated with the fluorescence mitochondria.

Injection of [^{125}I]DNase I

The amount of [^{125}I]DNase I injected into Y-1 cells from erythrocyte ghosts was measured by subjecting the erythrocytes to lysis after fusion with Y-1 cells. The Y-1 cells were then dissolved in NaOH and ^{125}I was determined by gamma counting. The amount of DNase I injected increased linearly with the number of loaded erythrocytes added until saturation was approached (Fig. 2*A*). Values shown on the ordinate represent the number of molecules of DNase I per cell; many of the cells added do not fuse with the Y-1 cells. The number of Y-1 cells present per plate was $1.48 \times 10^6 \pm 0.2 \times 10^6$ (mean and ranges for the 10 plates used in the experiment shown in Fig. 2*A*). At 2.5×10^7 erythrocytes the ratio of erythrocytes added to Y-1 cells was approximately 17. The values for DNase I per cell on the ordinate of Fig. 2 were calculated from the specific radioactivity of DNase I (10^6 cpm/mg) and the molecular weight of DNase I (31,500). The values for injected DNase I shown in Fig. 2, *A* and *B*, were determined 12 h after injection.

It should be pointed out that if only 80% of the cells were loaded, the loaded cells would contain more DNase I than that calculated on the basis of uniform distribution of the injected protein. However, the cells that did not appear to be loaded with fluorescence may have received some of the fluorescent protein that could escape detection.

When the concentration of [^{125}I]DNase I in the loading solution was varied and the number of erythrocytes and Y-1 cells remained constant, the amount of DNase I loaded per cell showed a linear relationship to the concentration of DNase I (Fig. 2*B*).

To determine whether DNase I released by erythrocyte lysis

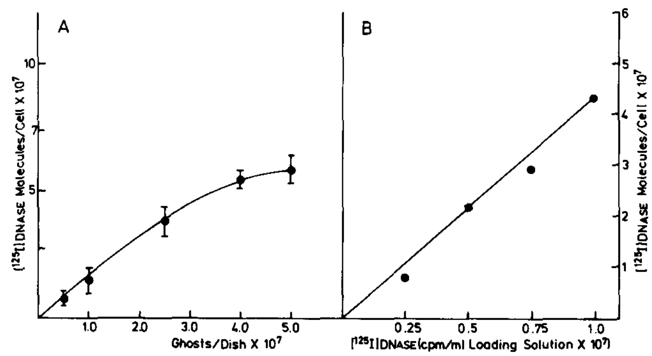

FIGURE 2 Loading of Y-1 cells with DNase I from erythrocyte ghosts. (*A*) erythrocyte ghosts were loaded with [^{125}I]DNase I (10^6 cpm/mg protein). Erythrocytes, in the numbers shown on the abscissa, were fused with Y-1 cells at ~80% confluence (i.e., 80% of the number of cells per dish at confluence: 2×10^6 for a 35-mm dish). After fusion, erythrocytes were subjected to lysis by ammonium chloride. Y-1 cells were washed and removed from the plates by incubation with EDTA. An aliquot of the cells was counted in a hemocytometer. The remaining cells were collected by centrifugation and dissolved in NaOH. The content of ^{125}I was determined in the NaOH solution. The values shown are means and ranges for duplicate determinations. (*B*) Studies performed in which concentration of [^{125}I]DNase I in the solution used to load the ghosts was varied. The number of ghosts added to the Y-1 cells was constant (2.5×10^7 per plate). The ordinate shows the number of molecules of DNase I per cell as before. Specific activity of [^{125}I]DNase I was 10^6 cpm/mg. Molecular weight of DNase I is 31,500.

bound to the surface of Y-1 cells, we added erythrocytes loaded with [^{125}I]DNase I to Y-1 cells and subjected the ghosts to lysis with ammonium chloride without fusion to the cells. The cells were washed as described in Materials and Methods, dissolved in NaOH and the solution was subjected to gamma counting to determine the amount of bound ^{125}I. Values were <1% of the radioactivity injected into the cells. Such low surface adsorption of [^{125}I]DNase I would not significantly alter the above calculation.

Influence of DNase I on the Steroidogenic Response to ACTH and Cyclic AMP

PRODUCTION OF 20α-DIHYDROPROGESTERONE: Fig. 3 shows that ACTH and cyclic AMP stimulate the

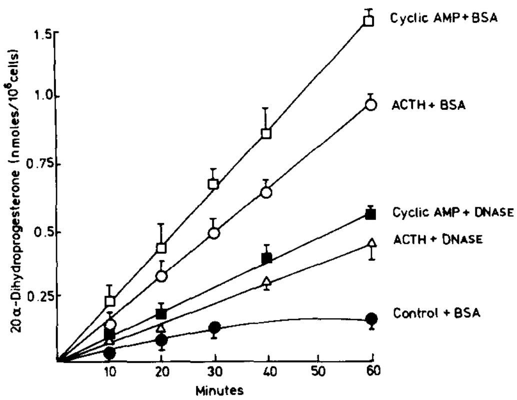

FIGURE 3 Influence of DNase I on steroidogenesis by Y-1 cells. Y-1 cells were fused with erythrocyte ghosts containing bovine serum albumin (*BSA*) or DNase I. 12 h after fusion, cells were washed, fresh medium was added, and the production of 20α-dihydroprogesterone was measured at the times shown. Values are means and ranges of duplicate determinations. In each such case, the range was <8% of the mean value shown. The concentrations of ACTH and dibutyryl cyclic AMP used were 86 mU/ml and 1 mM (final concentration), respectively. The loading solutions were prepared with albumin or DNase I at a concentration of 2 mg/ml. The cyclic AMP used was the dibutyryl ester.

production of 20α-dihydroprogesterone by Y-1 cells after fusion with erythrocyte ghosts containing albumin. In these cells the response to dibutyryl cyclic AMP is usually somewhat greater than that to ACTH and sodium butyrate is without effect on steroid synthesis by these cells (unpublished). Variation in the duration of linear production of 20α-dihydroprogesterone is seen from one subculture of Y-1 cells to another, but within one subculture little variation is seen from one dish to another. It can also be seen from Fig. 3 that the responses to ACTH and cyclic AMP are inhibited by fusion with erythrocyte ghosts containing pancreatic DNase I. For example, at 60 min the response to ACTH is inhibited by ~60% and that to dibutyryl cyclic AMP by >70%. Since not all cells are loaded with detectable amounts of the injected material (in this case DNase I), greater inhibition could have resulted with higher efficiency of injection. Numerous other studies have shown that fusion of erythrocytes containing phosphate-buffered saline is without effect on steroid production by the cells, e.g., control 0.11 $\pm$ 0.03; ACTH 0.82 $\pm$ 0.03; ACTH plus erythrocyte fusion 0.84 $\pm$ 0.06 nmol/10^6 cells incubated for 60 min (means and ranges for duplicate determinations). The effect of DNase I on the response to ACTH, when the number of added erythrocyte ghosts was varied, is shown in Fig. 4. The response is dose-dependent; 50% inhibition of the response corresponds to fusion with 2 $\times$ 10^7 erythrocytes per plate. From Fig. 2, it can be seen that this represents ~3.0 $\times$ 10^7 molecules of DNase I per cell.

TRANSPORT OF CHOLESTEROL TO INNER MITOCHONDRIAL MEMBRANE: Numerous studies already reported (10, 11) show that, under the conditions described in Fig. 5, there is no net transport of cholesterol to mitochondria within 30-min incubation of Y-1 cells in the absence of ACTH and cyclic AMP. Fig. 5 shows that fusion of Y-1 cells with erythrocyte ghosts containing pancreatic DNase I inhibits the stimulation of cholesterol transport produced by ACTH. Inhibition at 30 min is ~60%.

PRODUCTION OF PREGNENOLONE BY ISOLATED MITOCHONDRIA: Using the method described in Materials and Methods, it is possible to show that the stimulation of Y-1 cells by ACTH results in increased production of pregnenolone by isolated mitochondria (10, 11). It can be seen from Fig. 6, that this stimulation is inhibited by fusion of Y-1 cells with erythrocyte ghosts containing DNase I before addition of ACTH. In the experiment shown in Fig. 6, inhibition was ~75% at 30 min.

Numerous studies have shown that fusion of Y-1 cells with erythrocyte ghosts containing buffer or bovine serum albumin is without demonstrable effect on either of these last two responses to ACTH, i.e., transport of cholesterol to the inner mitochondrial membrane or production of pregnenolone by isolated mitochondria (data not shown).

DNase I and the Conversion of Pregnenolone to 20α-Dihydroprogesterone

The later steps in the steroidogenic pathway can be studied by incubating Y-1 cells with [^{3}H]pregnenolone and measuring the production of [^{3}H]-20α-dihydroprogesterone (12). When Y-1 cells are incubated with [7α-^{3}H]pregnenolone (10^6 cpm;

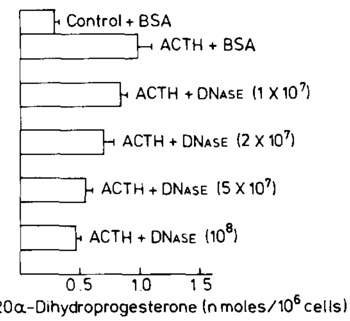

FIGURE 4 Influence of concentration of DNase I on the steroidogenic response to ACTH. Y-1 cells were fused with erythrocyte ghosts containing bovine serum albumin (*BSA*) or DNase I. Numbers in parentheses refer to the number of erythrocytes added to one plate of Y-1 cells. 12 h later cells were washed, fresh medium was added, and steroid production was measured after a 60-min incubation. Bars represent means and ranges for duplicate determinations. The concentration of ACTH used was 86 mU/ml and the loading solution contained DNase I at a concentration of 2 mg/ml.

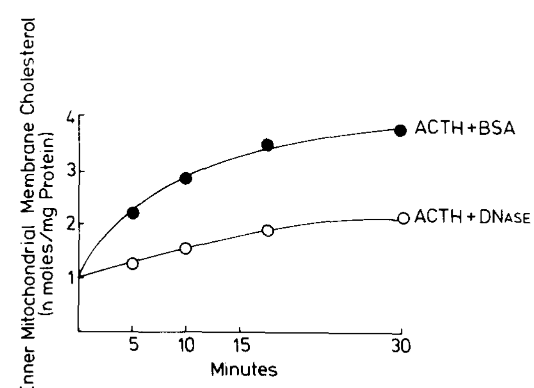

FIGURE 5 The effect of DNase I on the stimulation of cholesterol transport in Y-1 cells by ACTH. Large dishes of Y-1 cells (4 $\times$ 10^8 cells per dish) were fused with erythrocytes containing bovine serum albumin (*BSA*) or DNase I. 12 h later, cells were washed and incubated with medium containing aminoglutethimide (final concentration 0.76 mM) for the times shown. Thereafter inner mitochondrial membrane was prepared and the cholesterol content was determined.

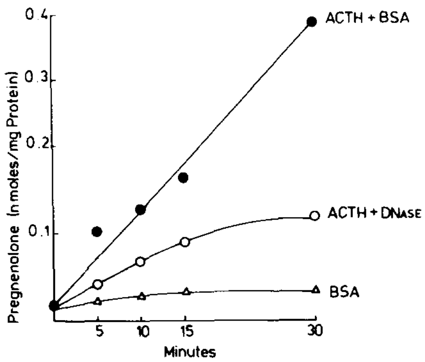

FIGURE 6 The effect of DNase I on production of pregnenolone by adrenal cell mitochondria. Y-1 cells were either fused with erythrocytes ghosts containing BSA (●) of DNase I (○) or were not fused with erythrocytes at all (△). 12 h later, cells were washed and incubated with medium containing aminoglutethimide (final concentration 0.76 mM) with (○, ●) and without (△) ACTH (86 mU/ml) for 30 min. Thereafter mitochondria were prepared and incubated for the times shown. Pregnenolone production was measured by extracting the medium and mitochondria (Materials and Methods).

150 nmol) for 30 min after fusion with ghosts containing albumin or DNase I, the [^{3}H]20α-dihydroprogesterone can be isolated by thin layer chromatography (10, 11), and measured by liquid scintillation spectrometry. In one experiment, the following values were found: erythrocyte ghosts containing albumin, 34,000 ± 4,000 cpm; erythrocyte ghosts containing DNase I, 35,000 ± 3,200 cpm (means and ranges for triplicate determinations).

Content of Actin in Y-1 Cells

The content of G- and F-actin in Y-1 cells was determined by three methods, namely, (*a*) an assay based on inhibition of DNase I (1), (*b*) an assay based upon immunoprecipitation of the complex formed between G-actin and DNase I (26), and (*c*) an assay based upon SDS gels following homogenization and centrifugation of the cells (23). The table shows that values from the three methods are in reasonable agreement. The lower values seen with the third assay may reflect limitations inherent in the method, e.g., limited proteolysis of actin and difficulties associated with densitometric determination of actin bands on SDS gels. It appears that two-thirds of the total actin is present in the monomeric form.

Examination of Mitochondria

After injection of [^{125}I]DNase I from erythrocyte ghosts, subcellular fractionation showed that <5% of the injected DNase I was associated with mitochondria. In other studies, oxygen consumption of isolated mitochondria was measured and found not to be altered significantly by injection of DNase I. For example, values (nanomoles O_2 per minute per milligram mitochondrial protein) were as follows with saturating levels of succinate: 94 ± 8 and 96 ± 4 (means and ranges for four determinations), for mitochondria injected with albumin and DNase I, respectively. Similarly values with malate and isocitrate were unaltered by DNase I (not shown).

Cellular Functions after Injection of DNase I

Fig. 7 shows that incorporation of [^{3}H]thymidine into DNA by Y-1 cells was not altered after injection of DNase I when compared with cells injected with albumin. When cell numbers were measured 2 d after injection of DNase I or albumin no difference was observed, e.g., values for four plates were as follows: 1.81 ± 0.2 × 10^6 for DNase I and 1.75 ± 0.3 × 10^6 for albumin (means and ranges for triplicate determinations). Incorporation of [^{3}H]leucine into protein was not affected by injection of DNase I, e.g., after 15-min incubation, incorporation was 61 ± 4 pmol of leucine/mg protein and 60 ± 5 pmol for DNase I and albumin, respectively (means and ranges of triplicate determinations).

It can be seen from Fig. 8 that DNase I treated with excess G-actin before injection into Y-1 cells was without significant effect on the response to ACTH when compared with injection of albumin, whereas injection of DNase I without actin produced the usual inhibition for this response. In previous studies we observed, by viscometry, that the loading solution does not cause polymerization of G-actin (13). In some studies, the time of onset of inhibition by injected DNase I was studied by interrupting the process of injection at various times after fusion to examine the response to ACTH. Significant inhibition was apparent within 10 min, e.g., control 0.13 ± 0.06; ACTH (albumin injection) 0.41 ± 0.08; ACTH (DNase injection) 0.23 ± 0.07 nmol 20α-dihydroprogesterone per 10^6 cells. In these studies steroid production was measured immediately after the process of injection was interrupted by treatment with ammonium chloride. Two dishes of Y-1 cells injected with [^{125}I]DNase I were used to demonstrate that ~2 × 10^7 molecules of DNase I were injected per cell at the end of 10 min.

DISCUSSION

The studies reported here demonstrate that injection of pancreatic DNase I into Y-1 adrenal tumor cells via erythrocyte ghosts, inhibits the increase in production of 20α-dihydroprogesterone that is produced by addition of either ACTH or dibutyryl cyclic AMP to these cells. Since various other proteins, including boiled DNase I, did not cause such inhibition, this effect appears to be specific for DNase I. Moreover, a highly purified DNase I prepared by the method of Wang and Moore (28) was also effective in producing inhibition (Results). This preparation shows a single band on SDS gels and has been used by these investigators for detailed structural studies of the enzyme. Evidently inhibition of DNase I cannot be attributed to the presence of trace contaminants seen in

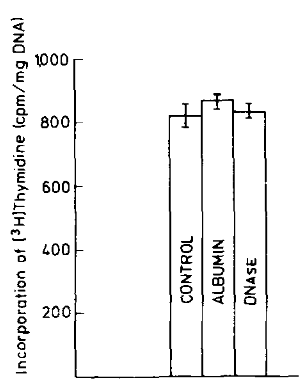

FIGURE 7 Incorporation of [^{3}H]thymidine in DNA by Y-1 cells injected with DNase I or bovine serum albumin. Incorporation of [^{3}H]thymidine was measured as described elsewhere (29). Y-1 cells were incubated with [^{3}H]thymidine (1.5 nmol; 3.0 μCi) per dish in 2 ml of minimal Eagle's medium. Incubation was continued for 2 h. After incubation the specific activity of DNA was measured. Values represent means and ranges of duplicate determinations. The Y-1 cells were injected with DNase I 12 h before incubation with [^{3}H]thymidine.

commercial preparations. The procedure of fusing erythrocyte ghosts with Y-1 cells does not itself affect steroid production or the response to ACTH (Results), and in any case the present studies were accompanied by control dishes in which the cells were fused with ghosts containing albumin or some other suitable protein, e.g., ovalbumin, boiled DNase I, or buffer.

In view of the well-known effect of DNase I in binding monomeric or G-actin (1), it would be reasonable to suggest that this property may be responsible for the inhibitory effects of this enzyme observed in our experiments, especially since the inhibitory effect of DNase I was overcome by addition of G-actin to the enzyme before injection (Fig. 8). This possibility is made more likely by previous reports from this laboratory showing that the actions of ACTH and dibutyryl cyclic AMP, on steroid production by Y-1 cells, are inhibited by antiactin antibodies (10). These findings are also consistent with evidence from studies in which cytochalasin B was found to inhibit the responses of Y-1 cells to the two stimulating agents (20, 21). In addition, inhibition by various members of the cytochalasin family was found to correlate closely with the binding affinity of these different cytochalasins to Y-1 cell actin (13). Other investigators have confirmed these findings in normal adrenal cells (5). Furthermore, antiactin antibodies have been shown to inhibit the steroidogenic responses of ovarian (32) and testicular cells (11) to luteinizing hormone.

The probability that these three substances (DNase I, antiactin, and cytochalasin) all act by inhibiting the normal functions of actin in these cells, is greatly increased by the fact that all three agents inhibit the same step in the steroidogenic pathway, namely the transport of cholesterol into mitochondria (Results and references 4, 10). It is now clear that increased transport of cholesterol from cytoplasm to mitochondria, is at least one cellular activity involved in steroid synthesis that is stimulated by ACTH and cyclic AMP and is likely to be important in increasing the synthesis of steroids (4, 10, 11, 20). Unfortunately little is known concerning the molecular basis of the transport process. The cholesterol must be moved from depots in the cytoplasm to the mitochondria and must then move to the inner mitochondrial membrane so that at least two steps are involved in this process (to the mitochondria and within the mitochondria). Since FITC-labeled DNase I was not seen in structures that showed fluorescence with $DiOC_2(3)$ and since mitochondria showed little ^{125}I after injection of $[^{125}I]$DNase I (Results), it would seem most likely that the enzyme acts outside these

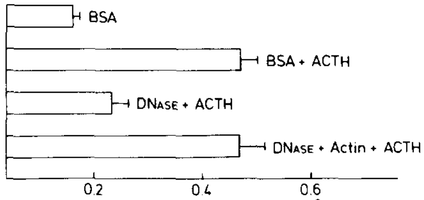

FIGURE 8 The influence of actin on the inhibitory action of DNase I. Y-1 cells were injected from erythrocyte ghosts, with the proteins shown (DNase I and bovine serum albumin), before incubation for 20 min with and without ACTH to determine production of 20α-dihydroprogesterone. The loading solution of DNase I contained 2 mg of this protein per milliliter. In one pair of flasks, DNase I was mixed with excess G-actin before loading into erythrocytes. Values are means and ranges of duplicate determinations.

organelles and hence that inhibition by DNase I takes place at the step(s) in which the cholesterol is moved through the cytoplasm. Moreover, there is at present no clear evidence that mitochondria contain actin (33). In the outer mitochondrial membrane, there is too much cholesterol, most of which is presumably not concerned with steroidogenesis, to make accurate measurements of the small amount of additional cholesterol transported to the membrane for steroid synthesis. The cholesterol content of the inner membrane is lower and the difference due to the steroidogenic cholesterol can be accurately measured (10, 11). Therefore, our earlier studies with cytochalasin (13, 21) and antiactin (10, 11) failed to distinguish between transport to, as opposed to transport *within*, the mitochondrion. The present experiments, however, provide some evidence in favor of involvement of actin (and hence ACTH) in transport of cholesterol through the cytoplasm as opposed to that within the mitochondrion although they do not exclude an additional effect produced by ACTH by some other mechanism.

In this connection, the specificity of the action of DNase I requires consideration to exclude possible effects on cellular components other than actin—especially DNA. In the first place, fluorescence microscopy suggested that the enzyme is excluded from the two principal sites of location of cellular DNA, namely, the nucleus and mitochondria (Results). Secondly, a variety of cell functions, including incorporation of $[^3H]$thymidine into DNA (nuclear function) and oxygen consumption by mitochondria, were unaffected by injection of DNase I. It seems clear that the action of DNase I reported here is not the result of a nonspecific effect on other cellular activities.

One advantage of erythrocyte ghosts as opposed to liposomes, as agents for the delivery of various compounds into cells, lies in the ease with which erythrocytes can be removed by lysis after they have delivered the entrapped material to the fused cells. This allows the investigator to measure the amount of a radioactive compound injected into the cells without the complication of bound but uninjected material. It was found that $>5 \times 10^7$ molecules of DNase I can be injected per cell. Unfortunately, we have not been able to measure the amount injected in the same cells as those used to measure the steroidogenic responses to ACTH and cyclic AMP. However, the accompanying data show that the procedure is reproducible, so that values for amounts injected determined on separate dishes provide a reasonable approximation for other dishes treated in the same way. It was found that 3×10^7 molecules of DNase I per cell caused a 50% inhibition of the steroidogenic response to ACTH (Fig. 4). Fluorescence studies show that ~80% of cells are loaded, so that as much as 3.6×10^7 molecules per cell may be present in the loaded cells. This should be considered an upper limit because some cells may have received some fluorescent protein without this being detectable under the microscope. In that event, the cells containing greater concentrations of DNase I may have been more severely inhibited than those containing smaller amounts. It is interesting to notice that the number of molecules of DNase I at 50% inhibition (3.0×10^7 per cell) is of the same order of magnitude as the total content of actin in these cells ($2–3 \times 10^7$ molecules per cell). Of this actin about two-thirds is monomeric of G-actin (Table I). DNase I acts by binding G-actin in a 1:1 complex (1). Moreover, Y-1 cells show large numbers of stress fibers, presumably

TABLE I
Content of Actin (G and F) in Y-1 Adrenal Tumor Cells

Method (reference)	Actin molecules/cell × 10^7* Total	G	F
DNase I (1)	2.0 ± 0.5	1.5 ± 0.3	0.5 ± 0.1
Anti-DNase I (26)	1.9 ± 0.4	1.3 ± 0.2	0.6 ± 0.1
SDS gels (26)	1.2	0.8	0.4

* Means and ranges for duplicate determinations.

composed largely of F-actin which does not bind readily to DNase I (1). These considerations suggest that DNase I inhibits the response to ACTH by inhibiting the normal function of a limited pool of Y-1 cell actin. Presumably the injected DNase I does not distribute at a uniform concentration throughout the cell. We (unpublished) and other workers (19) have noticed that stress fibers disperse under the influence of ACTH as seen on thin section electron microscopy. One possibility might be that DNase I prevents G-actin, newly released from the dispersing stress fibers, from discharging some function related to the intracellular transport of cholesterol, although there is, at present, no direct evidence for such a suggestion.

The best-known action of DNase I on actin involves the formation of a 1:1 complex between G-actin and the enzyme. This association is of high affinity (34–36) and would be expected to divert those molecules of G-actin that bind to DNase I from their normal functional activities. It is also known that DNase I binds F-actin and that this leads to depolymerization (34, 35). The binding to F-actin is characterized by two important differences from binding to G-actin. Firstly, binding to F-actin occurs with an affinity that is four orders of magnitude lower than that to G-actin (34–36), and secondly, binding is slower—1 h as opposed to 10 min to go to completion (35). If we assume uniform distribution of DNase I injected into Y-1 cells (approximate volume 2×10^{-6} ml), the calculated concentration of the enzyme in the cell would be $\sim 2 \times 10^{-7}$ M. Since the dissociation constants for G- and F-actin are, respectively, 10^{-8} and 10^{-4} M (34, 36), unless conditions within the cell (e.g., the influence of actin-binding proteins) greatly alter these affinities, it is clear that G-actin would be the major target for the injected DNase I. It was pointed out in Results that the onset of inhibition by the injected DNase is rapid, which also argues for an effect on G- as opposed to F-actin. In either case, however, the net effect of DNase I would be to shift the equilibrium between G- and F- in favor of G-actin either by binding G-actin or by depolymerization of F-actin. Moreover, at least some of the G-actin released from depolymerization would presumably be bound by DNase I so that the overall effect would be that of immobilizing G-actin and perhaps some loss of F-actin.

The three agents that inhibit the response to ACTH affect intracellular actin in different ways, so that a common factor capable of explaining inhibition of the response to ACTH, is not immediately obvious. Cytochalasin inhibits polymerization of G-actin (6) and inhibits association of F-actin into complex bundles (16). Antiactin antibodies could presumably affect actin and microfilaments in a variety of ways. The only available clue is that DNase I appears to act by binding to G-actin. This would presumably promote depolymerization of microfilaments by turnover. Since a major effect of cytochalasin is to prevent polymerization of G-actin and, since a similar effect could reasonably result from combination of antiactin with G-actin, the evidence favors inhibition of some function requiring G-actin as the basis of inhibition by DNase I and perhaps by cytochalasins. This, in turn, would suggest that at least some of the G actin in Y-1 cells must be free to polymerize if ACTH and cyclic AMP are to stimulate steroid synthesis. However, other possibilities cannot be excluded. Studies are planned to examine the changes in microfilaments seen after injection of DNase I using ultrastructural approaches.

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