CYTOCHALASIN B AND THE ADENOSINE TRIPHOSPHATE CONTENT OF TREATED FIBROBLASTS

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INTRODUCTION

Cytochalasin B, a mold metabolite from *Helminthosporium dematioideum*, was shown by Carter (1) to prevent motility and cytokinesis in cultured cells. This compound has since come into wide use, and has been shown to affect morphogenic development, endocytosis, and intracellular and extracellular motility (12). Studies with intact cells indicate that cytochalasin B produces its effect by depolymerization of cytoplasmic microfilaments. These microfilaments are considered to be actin-like. However, recent studies with isolated rabbit actin have provided conflicting views concerning the direct effect of the drug on the integrity and function of these structures (4, 6–8). Kletzien et al. (5) have observed that cytochalasin B rapidly and reversibly inhibits glucose and glucosamine transport. At a concentration of 0.07 μg/ml, cytochalasin B inhibits 2-deoxyglucose transport by 50%. This inhibition was detected within 2 min of the addition of the drug. Concentrations of about 3 μg/ml were required to observe altered cell morphology. The possibility was considered that depolymerization of microfilaments—with a resulting change in cell shape and function—resulted from a rapid depletion of intracellular energy levels brought about by the inhibition of sugar transport.

The effects of cytochalasin B and iodoacetate on the adenosine triphosphate (ATP) content of cultured fibroblasts were determined by the luciferase-luciferin method with a liquid scintillation counter (9). During a period of 30 min, under conditions in which the cells became round, cytochalasin B did not decrease the energy levels of the treated cells. However, pretreatment with cytochalasin B may reduce the effects of a 30-min exposure to the metabolic inhibitor, iodoacetate.

MATERIALS AND METHODS

Cytochalasin B was made to a 5 mg/ml solution in dimethyl sulfoxide (DMSO). A 1×10^{-3} M standard

solution of disodium adenosine 5-triphosphate (Sigma Chemical Co., St. Louis, Mo.) was prepared in Tris buffer (0.02 M, pH 7.4) and stored at −20°C in 2.0-ml vials. This solution was diluted to 1×10^{-7} M and contained the same concentration of perchloric acid as the diluted cell extracts in order to correct the perchloric anion quenching. Iodoacetate was purchased from Calbiochem (Los Angeles, Calif.) and made to 0.1 M in H_2O. All other chemicals were of reagent grade.

Luciferase Enzyme

Desiccated firefly lantern extract (Sigma Chemical Co.) was stored at −20°C. Luciferase enzyme was prepared for use by reconstituting one vial of extract with 5.0 ml of 0.05 M $MgSO_4$–0.1 M $NaAsO_4$, pH 7.4 buffer (10). The reconstituted extract was incubated at 24°C for 4 hr and then centrifuged to remove the cloudy precipitate. This incubation was necessary in order to degrade any endogenous ATP. The supernatant was diluted with 40 volumes of Mg^{2+}-arsenate buffer before its use in ATP determinations.

Cell Culturing and Treatment

Fertilized chicken eggs were supplied by the Sunnyside Hatchery of Oregon, Wisconsin. Modified minimal essential medium (Eagle's) was obtained from Schwarz/Mann (Orangeburg, N. Y.), and fetal calf serum from Grand Island Biological Co. (Grand Island, N. Y.) Primary cultures of 12-day-old chick embryo fibroblasts were prepared by a method described by Temin (11). The cells were grown at 37°C in a humidified CO_2 incubator in Eagle's modified essential medium containing 4% fetal calf serum. 5 days later, the fibroblasts were transferred to Falcon tissue culture dishes (60-mm diameter) (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) at a concentration of 5×10^5 cells per plate. The medium used to culture the secondaries contained 4% fetal calf serum.

Cells cultured for 1, 2, or 4 days after transfer were removed from the incubator in groups of three plates. Each group received one of the treatments outlined in Table I. A group of three plates, which received no treatment, served as controls. Three plates of untreated cells were trypsinized and the cells counted with a hemocytometer.

After a specified period of treatment, the plates were removed from the incubator, the medium was removed, the plates were rinsed with cold 0.16 M NaCl, and 2.0 ml of cold 0.4 M $HClO_4$ was added to extract the ATP (2). The extraction was carried out for 30 min at 4°C and the extract frozen at −20°C.

Cell Extract Preparation

Frozen perchloric acid cell extracts were thawed, and samples were diluted with cold distilled water to bring the ATP concentration within the optimal, linear range for scintillation counting (1×10^{-9}–1×10^{-8} M ATP). Scintillation vials containing 1.0 mg of Mg^{2+}-arsenate buffer were placed on ice, and to each was added, with mixing, 50 µl of diluted cell extract. The background level of the luciferase and the standard curves of ATP were determined for each experiment.

Liquid Scintillation Determination

A vial containing unknown, blank, or standard ATP concentration was removed from the ice. With mixing, 1.0 ml of diluted firefly luciferase was added, and the vial was lowered into the counting chamber. With experience, the length of time from the addition of the enzyme to the initiation of the machines' counting could be maintained at 16 sec. A Packard model 314 EX liquid scintillation counter was used to measure the emitted light quanta. The chamber was maintained at 4°C. Determinations were made with

TABLE I

The Per Cent of ATP Remaining in Cultured Fibroblasts Exposed to Cytochalasin B and Iodoacetate

Treatment at 37°C	ATP	Student's t	$t_{.01}$
2 µl/ml DMSO for 30 min	99.0 ± 1.67	0.289	3.065
10 µg/ml cytochalasin B for 30 min	101 ± 2.26	0.353	3.06
1.0 mM iodoacetate for 30 min	18.2 ± 2.21	45.4	2.98
10 µg/ml cytochalasin B for 60 min with 1.0 mM iodoacetate during final 30 min	77.2 ± 7.62	1.62 2.15*	2.92 2.98

The calculated means ± SE of the per cent ATP remaining after treatment are based on five to nine experiments (control = 100%). Student's t with one exception noted compares the treatment and the control.

* Student's t compares the treatment with 1.0 mM iodoacetate for 30 min.

the analyzer circuit and with the coincidence switch off. Voltage was set at 1225 v, and window divisions were set for a range of 50–1000 on the tritium channel. The gain setting was 500. The light emissions were counted for 0.3 min.

Treatment of Data

The mean number of counts for the blanks was subtracted from the values of the unknowns and the standards. The ATP content of the unknowns was computed from the standard regression curve. It was observed that these curves varied with the different enzyme preparations. The moles of ATP/cell and the per cent of the untreated cells wre computed for each treatment. The significance of the data was determined by Student's t test.

RESULTS AND DISCUSSION

Untreated cultured fibroblasts had a mean ATP level of $8.09 \pm 0.52 \times 10^{-15}$ moles/cell. This level is similar to that observed by Colby and Edlin, who, using $^{32}PO_4$ and chromatography, reported ATP levels of 1.59 to 2.68×10^{-15} moles/cell in cultured chick embryo fibroblasts (2). Chapman et al. (3), using the luciferase-luciferin system, reported ATP levels of $4.0–8.0 \times 10^{-15}$ moles/cell in synchronized cultures of Chinese hamster cells.

The levels of ATP in fibroblasts treated with DMSO (Student's t value 0.289) and cytochalasin B (Student's t value 0.353) were not significantly less than the level in untreated cells ($t_{.01}$ limit of 3.06); this indicates that neither treatment affects the cells' energy level during 30 min of incubation. By this time, however, the cells clearly are morphologically altered by cytochalasin B. By Student's $t_{.01}$ test, a 30-min exposure to iodoacetate significantly reduced the ATP level of the cells to $18.2 \pm 2.21\%$ of the untreated cells without altering cellular morphology. However, ATP levels of cells treated with cytochalasin B before exposure to iodoacetate maintained $77.2 \pm 7.62\%$ of the ATP concentration of untreated cells. Student's t test indicates that there is a significant difference between the latter treatment and the iodoacetate treatment at the $t_{.05}$ level but not at the $t_{.01}$ level. The loss of iodoacetate's effect after cytochalasin B treatment may be the result of either a decrease in cell membrane permeability to iodoacetate or a change by the cell to an ATP generating pathway (other than Embden-Meyerhoff) that is insensitive to this inhibitor.

These data indicate that the rapid and almost complete inhibition of glucose transport by cytochalasin B (5) does not result in a detectable decrease in the energy levels of the treated cells although they have become round. This rules out the possibility that depolymerization of cytoplasmic microfilaments resulted from a decrease in cellular ATP. These data support the suggestion that the drug has a multiplicity of effects. It inhibits transport, it alters cell shape, and it may alter the permeability barrier of the cell surface, as evidenced by the reduced effect of iodoacetate after cytochalasin B pretreatment, on intracellular ATP levels.

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REFERENCES

1. Carter, S. B. 1967. *Nature (Lond.).* **213:**261.
2. Colby, C., and G. Edlin. 1970. *Biochemistry.* **9:** 917.
3. Chapman, J. D., R. G. Webb, and J. Borsa. 1971. *J. Cell Biol.* **49:**229.
4. Forer, A., J. Emmersen, and O. Behnke. 1972. *Science (Wash. D. C.).* **175:**774.
5. Kletzien, R. F., J. F. Perdue, and A. Springer. 1972. *J. Biol. Chem.* In press.
6. Sanger, J. W., S. Holtzer, and H. Holtzer. 1971. *Nat. New Biol.* **229:**121.
7. Sanger, J. W., and H. Holtzer. 1972. *Proc. Natl. Acad. Sci. U.S.A.* **69:**253.
8. Spudich, J. A., and S. Lin. 1972. *Proc. Natl. Acad. Sci. U.S.A.* **69:**442.
9. Stanley, P. E., and S. G. Williams. 1970. *Anal. Biochem.* **29:**381.
10. Strehler, B. L. 1965. *In* Methods of Enzymatic Analysis. U. Bergmeyer, editor. Academic Press, Inc., New York. 559.
11. Temin, H. M. 1960. *Virology.* **10:**182.
12. Wessells, N. K., B. S. Spooner, J. F. Ash, M. D. Bradley, M. A. Laduena, E. L. Taylor, J. T. Wrenn, and K. M. Yamada. 1971. *Science (Wash. D. C.).* **171:**135.