THE MECHANISM OF CORTISOL ACTION IN CULTURED RAT HEART CELLS
Effects on RNA and Protein Synthesis

ROBERT C. SHALER and RICHARD L. MccCARL. From the Department of Medicinal Chemistry, University of Pittsburgh School of Pharmacy, Pittsburgh, Pennsylvania 15213, and the Department of Biochemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

INTRODUCTION

Heart cells growing as monolayer cells in tissue culture have the ability to beat (1). After several days in culture these same cells cease functioning and lose the morphology characteristic of beating cells. This process by which heart cells lose their ability to beat in culture has been described as dedifferentiation (2). While studying the effect of steroids on beating, McCarl et al. (3) demonstrated that cortisol not only will enhance the per cent of beating in heart cell cultures, but also will re-initiate beating in those cells which have ceased to function. Thus, cortisol restores and maintains the functionally differentiated state of the heart cell in culture.

Since cortisol is instrumental in maintaining the differentiated state of heart cells in culture, studies were performed to determine how the hormone controls the beating phenomenon in heart cells. This report is concerned with the effects of cortisol on RNA and protein synthesis in heart cells and their relationship to beating.

MATERIALS AND METHODS

Method of Heart Cell Culture

The animals used for the culture of heart cells were mixed strains and sexes obtained from the rat laboratory in the Department of Biochemistry at The Pennsylvania State University. All animals used were 2-4 days old.

The method of heart cell culture was that described by McCarl, et al. (3), with one modification. The trypsinization flask described by Harary and Farley (1) was substituted for the automatic trypsinizing flask used by McCarl et al. (3).

The Study of Protein Synthesis in Heart Cells

Cells to be studied under the influence of cortisol acetate (purified by thin-layer chromatography) were given 25 µl of a stock solution dissolved in absolute ethanol. The final concentration of steroid was 30 µg/ml. The control cells received the same amount of absolute ethanol as the cortisol-treated cells.

Phenylalanine-14C (300 mCi/mmmole, New England Nuclear Corp., Boston, Mass.), or amino acid-14C mixture (1 mCi/mg, New England Nuclear Corp.) was added to 3 ml of growth medium of the heart cells, giving a final concentration of 20 µCi/3 ml of culture medium. At 1-, 2-, and 3-hr periods after incorporation of label and exposure to the cortisol, the cells were harvested by decanting the medium and rinsing the attached cells three times with cold isotonic saline (0.9% NaCl). They were scraped off the plate with a stirring rod equipped with a rubber policeman, were pooled with the contents of two other Petri dishes treated in the same way, and were placed in a 12 ml conical centrifuge tube. The cells were centrifuged at the middle setting in an International clinical centrifuge (International Equipment Co., Needham Heights, Mass.) for 2 min. The cell pellet was resuspended in cold isotonic saline (1 ml) and transferred to a 7 ml capacity Dounce Homogenizer with a tight-fitting pestle (Kontes Glass Co., Vineland, N.J.) and homogenized with 20 strokes. A portion of the homogenate was then placed onto a millipore (0.22 µ) filter (Millipore Corp., Bedford, Mass.). To this was added 2 ml of cold 15% trichloroacetic acid (TCA), and then the material was filtered by using a vacuum. The TCA-soluble portion was also collected. The TCA-insoluble material was heated to 95°C for 15 min and washed three times with 5% TCA and three times with cold diethyl ether. The filter was air-dried overnight and placed into a scintillation vial.
containing 10 ml of toluene-based scintillation fluid (Liquifluor, Beckman Instruments, Inc., Fullerton, Calif.). The TCA-soluble portion was analyzed for radioactivity in Bray's solution (6). The activity was measured in a Beckman LS-200B Liquid Scintillation instrument. Quenching was determined by the channels ratio method and the efficiency of counting was 85% (7). A second portion of the homogenate was assayed for protein content according to the method of Oyama and Eagle (8).

**Incorporation of Amino Acids-14C into Heart Cell Polysomes**

Heart cells (grown in 100 mm Petri dishes) were incubated for 3 hr with an equivalent amount of absolute ethanol. At the end of the cortisol incubation period, the medium was decanted and replaced with 7 ml of amino acid incorporation medium containing amino acids-14C (1 mCi/mg, New England Nuclear Corp.) at a concentration of 5 μCi/3 ml of culture medium. The cells were exposed to the label for 5 min at 37°C and then cooled to 0°C with ice, washed, and harvested as described in the section on protein synthesis in heart cells. The solutions used for the washings, homogenization, and sucrose gradients contained: 10 mm Tris-HCl, 10 mm MgCl₂, 250 mm KCl (pH 7.4). The homogenization consisted of only 6–10 strokes in the 7 ml capacity Dounce Homogenizer with the tight-fitting pestle. The homogenate was then centrifuged for 10 min and the supernatant (1 ml) was layered over a 27 ml linear sucrose gradient (15–40%). The sucrose gradient was then centrifuged for 2 hr at 25,000 rpm in the SW 25.1 rotor in a Spinco Model L Ultracentrifuge. After centrifugation, the gradients were assayed at 254 mµ with an ISCO Density Gradient Fractionator connected to a UV flow-through cell (Instrumentation Specialties Co., Lincoln, Neb.). Fractions (1 ml) were collected into test tubes containing 2 ml of cold 15% TCA. The contents of the TCA-treated fractions were collected onto millipore filters and the radioactivity was measured as previously described. The hot TCA-insoluble material will be referred to as nascent peptide chains. All of the above operations were carried out at 0–4°C except for the initial incubation which was at 37°C.

**In Vitro Binding of Cortisol to Low Ionic Strength 105,000 x g Heart Cell Supernatant**

Heart cells (grown in 100 mm Petri dishes) were washed and homogenized as described for the polysome isolation procedure. The homogenate was centrifuged at 105,000 g for 60 min and the resulting supernatant was dialyzed for 12 hr with three changes of buffer against a solution containing 10 mm Tris-HCl (pH 7.4). The low ionic strength supernatant (1 ml) was incubated with cortisol-14C at a final concentration of 0.33 μCi/ml for 2 hr at 0°C and then layered onto a Sephadex G-100 column (1.5 X 18 cm) overlaid with 4 g of Sephadex G-25. The eluting buffer contained 10 mm Tris-HCl at pH 7.4. The fractions were collected (5 ml at 0.5 ml/min) and assayed for radioactivity by taking 1 ml samples and placing them into Bray's solution (6). The protein content was measured by the method described in the section on protein synthesis in heart cells.

**RESULTS**

The effect of cortisol on protein synthesis was examined in two ways. First, the incorporation of either phenylalanine-14C or amino acids-14C into TCA-soluble and -insoluble fractions of heart cell homogenates was measured. Analysis of the TCA-soluble fraction showed that there is no effect of cortisol on the incorporation of the amino acid-14C mixture into the amino acid pools of the cells up to 3 hr of incubation (Table I). The same results were also seen when labeled phenylalanine was used, suggesting that cortisol does not affect the permeability of heart cells to protein precursors. Fig. 1 is a typical experiment showing that there is a significant decrease in the incorporation of labeled precursor into the TCA-insoluble

| Minutes after treatment with cortisol | 5   | 60  | 120 | 180 |
|-------------------------------------|-----|-----|-----|-----|
| Control                             | 2506 ± 102 | 2790 ± 324 | 1968 ± 105 | 1431 ± 207 |
| Cortisol                            | 2703 ± 227 | 2506 ± 352 | 2299 ± 313 | 1766 ± 362 |

* Values are expressed as cpm/A660 mµ.
† Standard deviation.
Figure 1 Incorporation of phenylalanine-14C into TCA-insoluble portion of rat heart cell homogenate under the influence of cortisol. Label and cortisol were added at 0 time. These results are from a typical experiment on a single culture of cells. The bars on the graph indicate the standard deviation of each point. X, cells treated with cortisol; O, control cells.

Fraction of the heart cells after 3 hr, suggesting that there is an inhibitory effect of cortisol on protein synthesis in cultured heart cells. These effects are very small, and therefore another method was used to substantiate these effects on protein synthesis.

Protein synthesis was also measured by examining the effect of cortisol on the incorporation of labeled protein precursors into nascent peptides (see Methods) growing on free polysomes (Fig. 2). Since the above studies suggest that there is a significant effect of cortisol on protein synthesis without an effect on intracellular amino acid pools in 3 hr, the incorporation of labeled protein precursors into free polysomes was examined after the cells had been exposed to cortisol for 3 hr. Fig. 2 shows that cortisol causes a decrease in the incorporation of precursor into the free polysomes. Fraction 7 contains monomers as evidenced by electron microscopic observation. These results lend support to the above data that cortisol causes a decrease in at least some proteins syn-

Figure 2 Effect of cortisol on amino acid-14C incorporation into heart cell polysomes. Heart cells were exposed to cortisol acetate for 3 hr. After a 5 min pulse with amino acids-14C in 7 ml per Petri dish in incorporation medium, the cells were homogenized in polysome buffer (10 mm Tris-HCl, 10 mm MgCl₂, 250 mm KCl pH 7.4). They were separated on a 15–40% linear sucrose gradient by centrifugation in a SW 25.1 rotor at 25,000 rpm for 3 hr. Fractions (1 ml) were collected into test tubes containing 2 ml of cold TCA by fractionating the gradient with an ISCO density gradient fractionator and flow-through cell set at 254 mg. The results from the control and cortisol experiments are normalized for both absorbance at 254 mg and position in the gradient. O, 14C-incorporation into control cells; Δ, 14C-incorporation into cortisol-treated cells; and X, absorbance at 254 mg.

Brief Notes
thesized in heart cells, namely those synthesized on free polysomes.

In line with current thought on the mechanism of steroid action in target tissues (4), it was decided to determine whether cortisol binds to some material in the cytoplasm. Fig. 3 shows that cortisol does bind to some macromolecular material in the 105,000 g, low ionic strength (0.3 µ) cultured heart cell supernatant. The low ionic strength was used to precipitate and eliminate myosin as a binding protein. Fraction number 6 of Fig. 3 is the fraction in which the Folin-positive material, which binds cortisol, is eluted from the gel filtration column. A cortisol-binding substance also can be demonstrated in whole heart supernatant. These studies are not designed to determine quantitatively the amount of cortisol which is bound by this substance in the heart supernatant but only to demonstrate that a substance exists which can bind cortisol. As a control, we also have shown that cortisol does not bind to myosin (the contractile protein in heart cells).

Studies on the effect of cortisol on RNA synthesis showed that cortisol has no effect on RNA synthesis in cultured rat heart cells as measured by the incorporation of labeled RNA precursor into TCA-insoluble material or in intact rat hearts as measured by RNA polymerase activities.

**DISCUSSION**

The effects of cortisol on RNA synthesis are in contrast to those reported in the literature concerning the effects of cortisol on RNA synthesis in the rat liver and the rat thymus systems. In the former, cortisol is known to increase RNA synthesis, whereas in the latter it causes a decrease in RNA synthesis. Thus, the rat heart system (whether in the cultured or intact animal system) is different than either of these systems in that there is no response to cortisol in terms of RNA synthesis. It is difficult to explain this discrepancy since all three tissues are highly differentiated ones. It is interesting that all three responses are seen in the same animal, thus indicating the complexity of an organism's response to steroid hormones.

It is difficult to speculate concerning the
specificity of cortisol action on generalized protein synthesis in heart cells, since these studies are concerned with the synthesis of proteins on free polysomes and not with nuclear, mitochondrial or membrane-bound polysomes. In the heart system, the measurement of protein synthesis on membrane-bound polysomes is difficult since the use of such high ionic strengths (to keep myosin-synthesizing particles in solution) makes the use of the detergent sodium deoxycholate impossible. However, the effect of cortisol on protein synthesis on free polysomes is clear.

We have shown in our laboratory that actinomycin D has no effect on the re-initiation effect of cortisol on the beating in heart cells (5). That is, the antibiotic does not block the action of the steroid. These findings along with the current findings suggest that the re-initiating effect of cortisol as well as its effect on protein synthesis is not a genetic mechanism but may well be acting at a cytoplasmic site in the cells.

Current dogma suggests that steroids bind to some receptor molecule in or on the cell prior to evoking their action in appropriate target organs or tissues (4). The cultured heart cells and the intact heart both have a macromolecular substance which will bind cortisol. It is not known at present whether or not this material is involved in the steroid’s action on the beating phenomena, on protein synthesis in the cells, or both. This material may only bind the cortisol and have no function, or may act as a transporting agent carrying the steroid to its site of action.

The cultured heart cell system is supposed to be representative of the intact heart system. This is one culture system which is unique in terms of function. The beating in the cells can be recorded visually and is interpreted as being representative of the whole heart system. Until recently, there had been no biochemical evidence (direct or indirect) that related these two systems. Harary and Slater (10) have shown that the cultured heart cells will react to cardiac-active agents in a similar manner as the intact system. This study presents further evidence that the two systems are not only functionally but also biochemically similar. This similarity is seen in the response of the two systems to cortisol in terms of RNA synthesis. Cortisol does not affect RNA synthesis in either system. Also, both systems have a macromolecular substance located in their respective soluble supernatant fractions which binds cortisol as demonstrated by gel filtration. This does not necessarily mean that these substances are the same.

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