Culture of the terminally differentiated ventricular cardiac muscle cell

Characterization of exogenous substrate oxidation and the adenylate cyclase system

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Received 14 February 1984; revised version received 6 March 1984

Oxidation of several exogenous substrates by cultured adult rat ventricular cardiac muscle cells has been assessed. Unlike freshly isolated cardiac muscle cells which oxidize glucose preferentially, the cultured cells more closely resemble metabolically the in situ heart and the isolated perfused heart, in that their preference for exogenous substrates is in the order of fatty acid > glucose. This switch in metabolic preference from glucose to fatty acid is complete within 12 h after placing freshly isolated cells in culture. Glucose oxidation is stimulated by insulin and isoproterenol and inhibited by β-hydroxybutyrate and octanoate. The adenylate cyclase system has also been examined in these cultured cells. Isoproterenol, norepinephrine and epinephrine stimulate the accumulation of cyclic adenosine 3′:5′-monophosphate (cyclic AMP) in a concentration-dependent manner. The order of potency is isoproterenol > norepinephrine ≅ epinephrine. This stimulation is potentiated by 1-isobutyl-3-methylxanthine and inhibited by 1-propranolol.

Substrate oxidation *Cardiac muscle* *Adult rat* *Cell culture* *Metabolism* *Adenylate cyclase*

1. INTRODUCTION

Methodology to culture successfully the adult rat ventricular cardiac muscle cell has only recently been perfected [1–8]. Freshly isolated cells are striated and cylindrical in shape, typical of the intact heart muscle. When placed in culture, these cells round up, lose their highly organized morphology, attach to the surface of the culture flask, and extend pseudopod-like processes. With increased time in culture the cells spread out and become entirely flattened. We have characterized these cells during both the spreading-out process and after they have become entirely flattened using the light, scanning electron, and transmission electron microscopes [2,5,6]. We have concluded that once they become established in culture they are similar morphologically to the in vivo adult rat ventricular cardiac muscle cell except that they are spread out and flattened. Compared to cultured neonatal myocytes [9], culture myocytes derived from adult rats more closely resemble in vivo adult ventricular muscle cells; in particular, the cultured adult cells contain a very well developed transverse tubular system, whereas cultured neonatal myocytes have at most only a rudimentary transverse tubular system [5].

The adult ventricular cardiac muscle cell grown for weeks or months in culture offers a new and unique system which can be used to study the adult mammalian heart muscle cell. This system provides the following: (1) a pure homogeneous population of cardiac muscle cells since growth of other cell types in the culture has virtually been elimi-

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00145793/84/$3.00

nated; (2) heart cells that are free of neural or hormonal influences; (3) a system in which multiple samples (40 to 50 T-25 culture flasks) can be obtained from the same preparation; (4) a system in which the immediate environment of the cells can easily be controlled and manipulated. We present here the results of studies that we have carried out to characterize selected aspects of the metabolism and the denylate cyclase system of these cells.

2. MATERIALS AND METHODS

2.1. Materials

[U-^{14}C]Glucose (spec. act. 5 mCi/mmol), [U-^{14}C]-lactate (spec. act. 100 mCi/mmol), [U-^{14}C]octanoate (spec. act. 20 mCi/mmol), [^{3}H]cyclic AMP (spec. act. 9 μCi/mmol) and omnifluor scintillation fluid were purchased from New England Nuclear; isoproterenol, epinephrine, norepinephrine, and IBMX were from Sigma and l-propranolol was a gift from Ayerst Laboratories. The source of all other chemicals and materials was as in [2,8].

2.2. Cell isolation and culture

Ventricular cardiac muscle cells were isolated from adult female Holtzman rats (200–250 g) and cultured as in [2,8]. The cells were cultured for the first 7 days in T-25 flasks precoated with rat tail collagen in a conditioned medium prepared from a rabbit corneal cell line (ATCC CCL 60 SIRC, American Type Culture Collection, Rockville, MD) [8]. To prepare this conditioned medium the corneal cells are cultured until they become confluent in T-75 flasks in Eagle's MEM containing 2× vitamins, non-essential amino acids, penicillin, streptomycin and 10% preselected fetal calf serum. They are then trypsinized, resuspended in the above medium, divided into 20 aliquots and replated in 12 ml of medium in T-75 flasks. After 2 days the medium is removed and frozen. Medium is removed and frozen every 2 days thereafter until the cells have again become confluent. When needed this conditioned medium is thawed and used without filtering. For the initial 7 days the cardiac muscle cells are cultured in medium which contains cytosine 1-β-D-arabinofuranoside (14 μg is added to the medium on day 1 and day 3 of culture) to eliminate (>99%) growth of fibroblasts and other nonmuscle cells. The medium is not changed during this initial 7 day period; after this time period the medium is changed every other day and the cells are cultured in Eagle's MEM, 10% fetal calf serum, vitamins, non-essential amino acids, penicillin and streptomycin. Experiments reported here were carried out on cells that had been in culture between 12 and 18 days.

2.3. Exogenous substrate oxidation

The production of $^{14}CO_2$ during a 2 h incubation period was used to estimate the oxidation of exogenous substrates [10]. Tissue culture medium was removed from the T-25 flasks and the cells were washed several times with and then incubated in Krebs-Ringer bicarbonate buffer (5.0 ml) equilibrated with 95% O_2–5% CO_2 (pH 7.4) at 37°C in a shaking water bath. The buffer also contained 5 μCi of either [U-^{14}C]glucose, [U-^{14}C]lactate or [U-^{14}C]octanoate. The final concentration of radioactive plus non-radioactive substrate in the incubation medium was: glucose (5.5 mM), lactate (1.0 mM) or octanoate (0.1 mM). The plastic caps on the T-25 flasks were replaced with rubber stoppers fitted with a center well containing 0.4 ml hyamine hydroxide. Following the incubation period perchloric acid was injected into the flasks and the $^{14}CO_2$ was trapped [11]. The cups containing the hyamine hydroxide were placed in scintillation vials containing 10 ml omnifluor scintillation cocktail and the radioactivity measured in a liquid scintillation spectrometer. The cells were scraped from the flasks with a rubber policeman, mixed with the perchloric acid solution and transferred to a test tube. Protein was pelleted by centrifugation and estimated as in [12].

2.4. Cyclic AMP accumulation

Cultured cells were washed 3 times with 5 ml Krebs-Ringer phosphate buffer for the following composition: 128 mM NaCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, 5.2 mM KCl and 10 mM Na_2HPO_4. The cells were incubated in a total volume of 2 ml Krebs-Ringer phosphate buffer for 15 min at 37°C and then were scraped from the surface of the flask with a rubber policeman and transferred in the buffer to a centrifuge tube. The reaction was terminated by placing the tube in a boiling water bath for 1 min after addition of 0.2 ml of 2 N HCl. When the contents of the tube had cooled to room temperature, 0.2 ml of 2 N NaOH was added, the tube vortex-mixed, and centrifuged at 3000 × g for

10 min. A 50 μl aliquot of the supernatant fluid was assayed for cyclic AMP content by a modification of the protein kinase binding procedure in [13] using the 10000 × g supernatant fraction of homogenized bovine adrenal glands [14] to eliminate possible interference by ATP. The free cyclic AMP was separated from the bound cyclic AMP by charcoal adsorption [14]. Cyclic AMP standards were prepared using incubation medium treated in the same manner as the unknown samples.

2.5. *ATP analysis*

The cellular concentration of ATP was determined fluorometrically as in [15].

3. RESULTS AND DISCUSSION

The oxidation of exogenous glucose, lactate and octanoate by cultured adult rat cardiac muscle cells is linear for at least 4 h of incubation (fig.1). In all subsequent experiments a 2 h incubation period was used. It was observed that the preference for these 3 substrates was in the order of octanoate > glucose > lactate (fig.1). These data are expressed in terms of oxygen equivalents so that the oxidation of the different substrates can be compared with each other and with data obtained by others in studies utilizing the in situ and isolated perfused heart and freshly isolated myocytes [18–22]. Similar to data reported by others [19,21,22] we (table 1) also find that freshly isolated heart myocytes prefer carbohydrate over fatty acid as a substrate. To determine when during the culture period this change in preferring glucose (freshly isolated cells) to preferring octanoate (cultured cells) occurs, we measured the oxidation of these substrates at various times after initially placing the cells in culture. As shown in table 1 this change in metabolic pattern has occurred by the twelfth hour of culture.

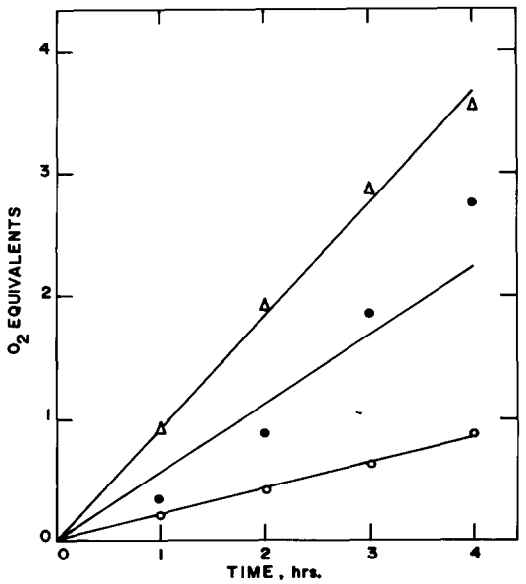

Fig.1. Oxidation of exogenous substrates by cultured adult rat cardiac muscle cells. Cells that had been in culture for 14 days were incubated in Krebs-Ringer bicarbonate buffer with [U-^{14}C]glucose (●—●), [U-^{14}C]-lactate (○—○), [U-^{14}C]octanoate (Δ—Δ). $^{14}CO_2$ production was used to estimate substrate oxidation. The values for the oxidation of glucose, lactate and octanoate were calculated as μmol of substrate oxidized/mg protein and were converted to oxygen equivalents by multiplying by 6, 3 or 11, respectively. Values are the mean of 6 determinations.

To characterize further the metabolism of these cells we tested the effect of several other agents which are known to influence the oxidation of glucose in other types of heart preparations. Octanoate and β-hydroxybutyrate were observed to inhibit, and insulin and isoproterenol to stimulate glucose oxidation (table 2). The concentration of ATP in these cells was measured and was found to be similar (25 nmol/mg protein) to that reported for intact heart muscle [16,17,19].

We next carried out a series of experiments to characterize the adenylate cyclase system in these

Table 1

Effect of time in culture on substrate preference by adult rat cardiac muscle cells

Time in culture	O_2 equivalents (glucose) / O_2 equivalents (octanoate)
O (freshly isolated)	1.46
6 h	0.56
12 h	0.10
36 h	0.12
12 days	0.18

Freshly isolated or cultured cells were incubated with either [U-^{14}C]glucose or [U-^{14}C]octonoate for 2 h. Data are expressed as a ratio of glucose oxidation to octonoate oxidation. Values are the mean of 4 individual determinations

Table 2

Effect of several metabolites and hormones on the oxidation of glucose by cultured adult rat cardiac muscle cells

Addition	Glucose oxidation (% of control)
None	100
β-Hydroxybutyrate (0.01 mM)	43
Octanoate (0.1 mM)	88
Insulin (30 μunits/ml)	121
Insulin (300 μunits/ml)	182
Isoproterenol (1.0 μM)	142

Cells that had been in culture for 18 days were incubated for 2 h in Krebs-Ringer bicarbonate buffer with [U-^{14}C]-glucose. Values are the mean of 3–6 individual determinations

cultured adult cardiac muscle cells. Isoproterenol was observed to stimulate the accumulation of cellular cyclic AMP in a concentration-dependent manner and this stimulated accumulation could be potentiated by the cyclic AMP-dependent phosphodiesterase inhibitor, IBMX (fig.2). Norepinephrine and epinephrine were also observed to stimulate the accumulation of cyclic AMP in a concentration-dependent manner (fig.3). The order of potency of these 3 catecholamines was isoproterenol > norepinephrine ≅ epinephrine (fig.4). This catecholamine-stimulated accumulation of cyclic AMP can be inhibited by the β-blocker, propranolol (fig.4). The polypeptide hormone glucagon, which has been shown to stimulate the accumulation of cyclic AMP in other types of heart preparations [23], was also tested and found to increase the concentration of cellular cyclic AMP in a dose-dependent manner.

These studies demonstrate that the cultured adult rat ventricular cardiac muscle cell retains the metabolic pattern of the intact heart in that it has a similar substrate preference and responds in an appropriate manner to several metabolic effectors. This is in agreement with our ultrastructure studies

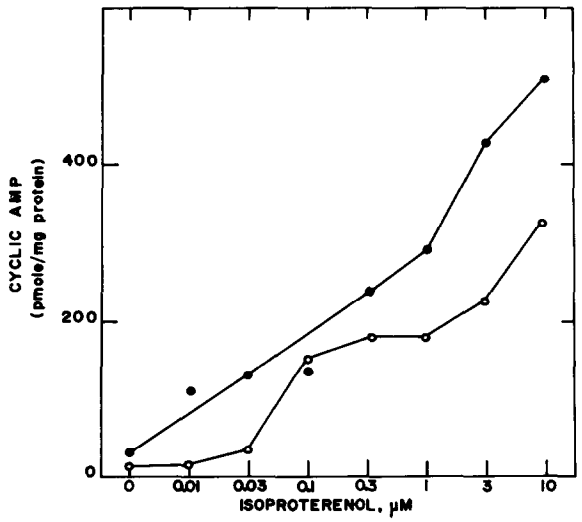

Fig.2. Dose-dependent stimulation of cyclic AMP accumulation in cultured adult rat cardiac muscle cells by isoproterenol and potentiation by 1-isobutyl-3-methylxanthine (IBMX). Cells that had been in culture for 12 days were incubated for 15 min with the indicated amount of isoproterenol without (○—○) or with (●—●) IBMX (100 μM) and the accumulation of cyclic AMP measured. Values are the mean of determinations made on 3 individual culture flasks.

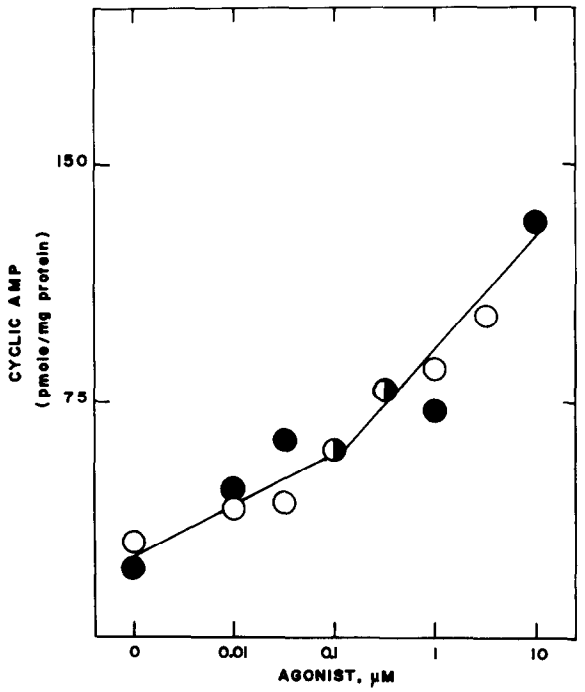

Fig.3. Dose-dependent stimulation of cyclic AMP accumulation in cultured adult rat cardiac muscle cells. Cells that had been in culture for 15 days were incubated with the indicated amount of epinephrine (○) or norepinephrine (●) and 100 μM IBMX. Values are the mean of determinations made on 3 individual culture flasks.

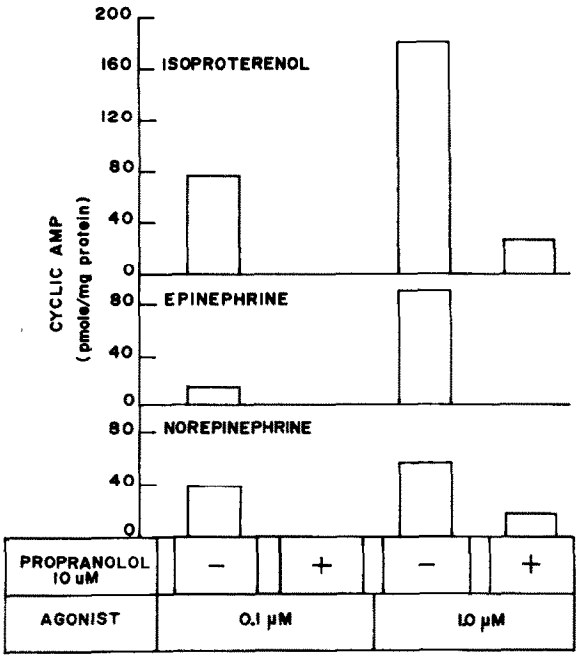

Fig.4. Inhibition of catecholamine-stimulated accumulation of cyclic AMP in cultured adult rat cardiac muscle cells by propranolol. Cells that had been in culture for 16 days were incubated with propranolol (10 μM) for 5 min and then the catecholamine at the specified concentration was added and the incubation continued for an additional 15 min. Values are the mean of determinations made on 3 individual culture flasks.

[5,6] which conclude that these cultured cells are similar morphologically to the in vivo cardiac muscle cell except that they are flattened and spread out.

The preferred substrate of the intact heart is known to be fatty acids [18–20]. For some reason freshly isolated cardiac muscle cells prefer carbohydrate (glucose plus lactate) over fatty acid as a substrate ([19,21,22] and table 1). Perhaps this is due to changes in membrane permeability or other damage done to the cells during the isolation procedure. In earlier studies it was observed that the damaged area of the membrane at the intercalated disc of freshly isolated myocytes appears to be repaired or sealed off after only a few hours of culture [2,6]; this and other membrane repair processes may thus reestablish normal permeability to these cells. Another possibility for this change in metabolic preference may be that mitochondrial damage causes a switch from oxidative metabolism to anoxic or partially anoxic metabolism. Our morphological studies [2,5,6] have shown that freshly isolated cells are damaged and during the early culture period their surfaces are composed of blebs filled with disrupted and bloated mitochondria. In any case, whatever causes this alteration in metabolic pattern is apparently repaired or returned to the pattern of the intact heart by the twelfth hour of culture (table 1).

In conclusion, the cultured adult rat ventricular cardiac muscle cell appears to have a metabolic pattern similar to the intact adult heart and provides a new and unique system in which to investigate biochemical and physiological control mechanisms operating in the heart muscle cell.

ACKNOWLEDGEMENTS

We thank Nicholas Lanson, jr and Mary Giamo for excellent technical assistance. This investigation was supported by Grants HL 25873 and AM 28521 from the National Institutes of Health and a Grant from the American Heart Association.

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