Effects of Some Metabolic Influences on the Spontaneous Activity of Cultured Rat Heart Cells (37087)

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There is a rather extensive literature on the electrophysiological and biochemical properties of cultured heart cells (1–12). However, the contribution of various metabolic fuels to the functional activity of cardiac cells has not yet been fully characterized. Results obtained with isolated heart preparations (13–20) have indicated that the glycolytic pathway is of importance for the maintenance of certain aspects of cardiac function.

In view of these findings, it was of interest to determine if similar mechanisms were operative in isolated rat heart cells. Therefore, 2-deoxy-D-glucose (2-DG), a glucose analogue which is known to inhibit glycolysis in various animal tissues, was selected for this study in order to investigate the role of glucose metabolism in the initiation and maintenance of the beating of cultured heart cells. 2-DG reduces glucose utilization by cells (21, 22), and is phosphorylated by hexokinase to 2-deoxy-D-glucose-6-phosphate (2-DG-6-P), which appears not to be further metabolized to any great extent (23–25). 2-DG also produces ATP depletion (14, 26, 27).

Material and Methods. Isolated cells from hearts of postnatal rats (2–7 days old) were prepared by a method essentially similar to that described by Harary and Farley (28). The cells were dispensed in T-15 flasks, and incubated at 37°, with air as the gas phase. The growth fluid used was Eagle's basal medium in Hanks' balanced salt solution (HBSS), supplemented with 10% fetal bovine serum, 10% human serum, and Eagle's nonessential amino acids.

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Experiments were carried out with cells from 3 to 6 days in culture. The experiments were done in HBSS, composed as follows (m*M*): Na^+ 141, K^+ 5.8, Ca^{2+} 1.22, Mg^{2+} 0.8, Cl^- 143, HCO_3^- 4.16, SO_4^{2-} 0.8, PO_4^{2-} 0.77, glucose 5.5. Cultures were equilibrated for 60 min prior to use. Changes in rates of beating were followed visually in a constant temperature enclosure, at 37°, by means of a phase contrast inverted microscope. Beating rates were measured for 15 min before starting the experiment. Control cultures were included in each experiment. The pH was maintained at 7.2 during the experimental period. All drugs were of reagent grade, and dissolved in HBSS at the time of use. 2-DG was used at 2.5 m*M*, Na pyruvate at 5 m*M*, adenosinetriphosphate (ATP) at 0.04 m*M*, glucose at 5.5 m*M* and Na fluoroacetate at 10 m*M*.

For ATP measurements, the adherent cell layer was washed three times with HBSS and drained; then, 1 ml hot distilled water was added to the flask, and the cells quickly removed with a rubber policeman. The cells were extracted for 10 min in a hot water bath, cooled and centrifuged. ATP determinations were performed in 0.5 ml aliquots of the supernatant by means of the firefly-luciferase method, in a Farrand fluorometer (29).

The precipitate was used for protein determinations, by the biuret reaction (30). For the electrical stimulation of the cells, 2 platinum electrodes connected to a Grass S4 stimulator were used. The cells were stimulated with suprathreshold stimuli with a duration of 1.0 msec, at frequencies in the range of 60 to 200 per min.

Results. Effects of 2-DG, ATP or pyruvate

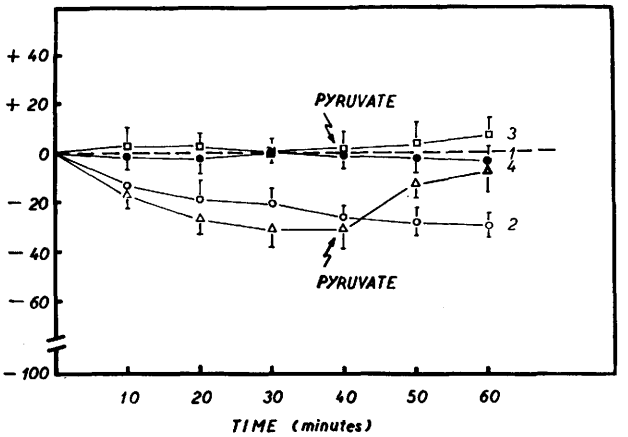

FIG. 1. Effects of 2-DG and/or pyruvate on the rate of beating of cultured heart cells incubated in glucose medium. After the equilibration period the initial control rate of beating was recorded. The values indicated on the ordinate represent the % changes from the initial control rate of beating. Vertical lines indicate the SEM. The drugs concentrations are expressed as mmole/liter. 1 (●): Untreated control cells beating in medium with 5.5 m*M* glucose (28 experiments). 2 (○): 2-DG (2.5 m*M*) added at 0 time (28 experiments). 3. (□): Pyruvate (5.0 m*M*) added at arrow to untreated control cells (31 experiments). 4 (△): 2-DG m*M*) added at 0 time and pyruvate (5.0 m*M*) added at arrow (17 experiments).

on the rate of beating of cultured heart cells incubated in HBSS with glucose as the substrate. In Hanks' medium, the behavior of untreated cells did not differ from that observed in nutrient medium. The initial spontaneous rate (Mean ± SEM) of 76 cultures was 84.98 ± 3.59 beats/min, and this frequency of beating remained regular throughout the experiment (Fig. 1, curve 1). Furthermore, the heart cells maintained rhythmic activity for many hours in this medium. 2-DG produced a progressive decline of the rate of beating, a maximal depression of 30% being reached approximately at 40 min after addition (Fig. 1, curve 2). The ability of pyruvate and ATP to modify the depression

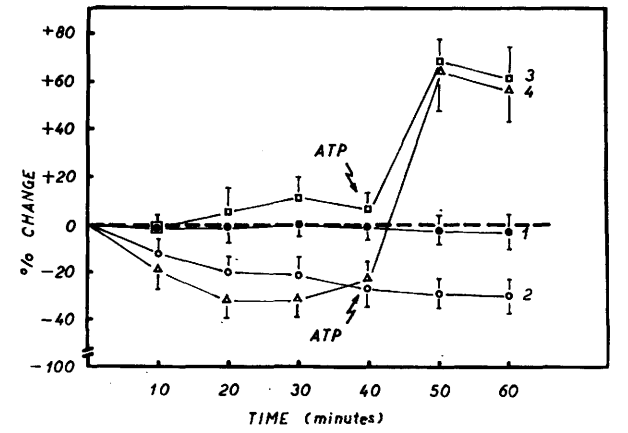

FIG. 2. Effect of ATP on the rate of beating of cultured heart cells incubated in glucose medium. Conditions and details as in Fig. 1. 1 (●): Untreated control cells beating in medium with 5.5 m*M* glucose (28 experiments). 2 (○): 2-DG (2.5 m*M*) added at 0 time (28 experiments). 3 (□): ATP (0.04 m*M*) added at arrow to untreated control cells (14 experiments). 4 (△): 2-DG (2.5 m*M*) added at 0 time. ATP (0.04 m*M*) added at arrow (10 experiments).

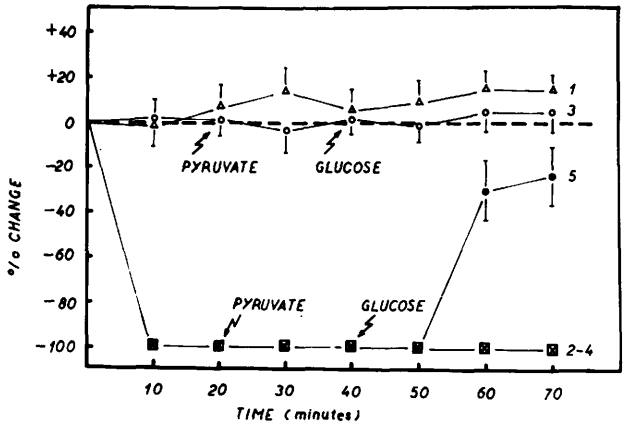

FIG. 3. Effects of 2-DG, pyruvate or glucose on the rate of beating of cultured heart cells incubated in the absence of glucose. After the equilibration period the cells are washed three times with glucose-free medium, 15 min before 0 time, and the initial control rate of beating was recorded. Other details as in Fig. 1. 1 (△): Control cells beating in glucose-free medium (12 experiments). 2 (X): 2-DG (2.5 m*M*) added at 0 time (15 experiments). 3 (○): Pyruvate (5 m*M*) and glucose (5.5 m*M*) added at arrows to control cells in glucose-free medium (12 experiments). 4 (□): 2-DG (2.5 m*M*) added at 0 time and pyruvate (5 m*M*) added at arrow (12 experiments). 5 (●): 2-DG (2.5 m*M*) added at 0 time; pyruvate (5 m*M*) and glucose (5.5 m*M*) added at arrows (14 experiments).

produced by 2-DG was investigated. Addition of pyruvate 40 min after 2-DG, restored the rate toward the control level (Fig. 1, curve 4), but it had no significant effect on the heart rate of untreated cultures (Fig. 1, curve 3). The inhibition produced by 2-DG was also reversed by ATP. Furthermore, added ATP produced striking enhancement of the rate both in untreated and in treated cultures (Fig. 2, curves 3 and 4).

The beating frequency of the heart cells incubated in a glucose medium, was not modified by one hour exposure to fluoroacetate, an inhibitor of the Krebs cycle.

Effects of 2-DG, pyruvate or glucose on the rate of beating of cultured heart cells incubated in Hanks' medium without glucose. In the absence of exogenous substrate, the average initial rate (Mean ± SEM) of 25 cultures was 76.56 ± 4.51 beats/min. The beating frequency remained for 70 min without decrement (Fig. 3, curve 1). Cultures observed for a period of 6 hr exhibited no appreciable changes in rates of beating. The addition of 2-DG to cells incubated in substrate-free medium produced in 10 min a complete inhibition of the automatism (Fig. 3, curve 2). Pyruvate was ineffective in reversing the influence of 2-DG (Fig. 3, curve 4), but the addition of glucose alone led to a partial recovery of the beating (Fig. 4, curve 4). Neither pyruvate nor glucose had a significant effect on the rate in untreated cultures. When both pyruvate and glucose were added, whatever the order of addition, reversal of the 2-DG depression was obtained (Figs. 3 and 4, curve 5), and this reversal was greater than that observed with glucose alone. On the other hand, addition of glucose and pyruvate to control cultures did not modify their rate of beating (Figs. 3 and 4, curve 3). In glucose-free medium, added ATP did not restore the beating in 2-DG treated cells. Furthermore, it was surprising to find that ATP elicited only a weak transient stimulation of the rate in untreated control cultures. In the absence of glucose, the beating frequency of the heart cells was not modified by 1-hr exposure to Na fluoroacetate.

Effects of 2-DG on the ATP concentration of cultured heart cells. The cellular levels of ATP were also studied (Table I). The ATP concentration at the end of the experimental

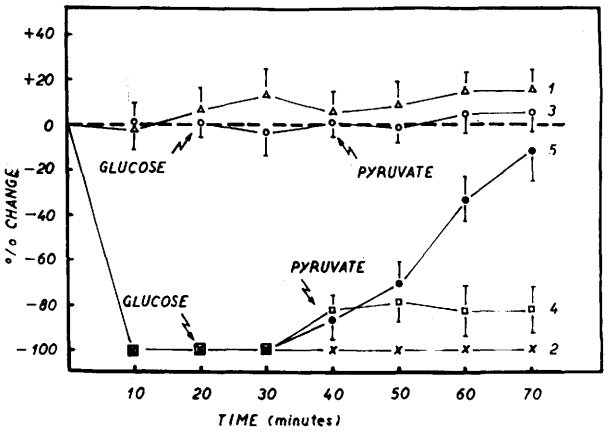

FIG. 4. Effects of 2-DG, glucose or pyruvate on the rate of beating of cultured heart cells incubated in the absence of glucose. Conditions and details as in Fig. 3. 1 (△): Control cells beating in glucose-free medium (12 experiments). 2 (X): 2-DG (2.5 m*M*) added at 0 time (15 experiments). 3 (○): Glucose (5.5 m*M*) and pyruvate (5 m*M*) added at arrows to control cells in glucose-free medium (12 experiments). 4 (□): 2-DG (2.5 m*M*) added at 0 time and glucose (5.5 m*M*) added at arrow (21 experiments). 5 (●): 2-DG (2.5 m*M*) added at 0 time; glucose (5.5 m*M*) and pyruvate (5 m*M*) added at arrows (14 experiments).

period was 3.21 μmole/g protein in control cultures, and 1.03 μmole in 2-DG treated cells. Pyruvate restored the levels of ATP to 2.11 μmole in 2-DG depressed cells and also increased the control values to 4.95 μmole. Addition of ATP (0.040 m*M*) also increased the ATP concentration in both 2-DG treated and untreated cells.

After 60 min incubation in substrate-free medium, the ATP concentration in control cultures was 2.75 μmole/g protein, and was markedly reduced by 2-DG to 0.43 μmole.

Response of cultured heart cells incubated in glucose-free medium to electrical stimulation. These experiments were carried out in order to investigate whether the lack of activity in the cultures under some experimental conditions (glucose removal; 2-DG addition) was due to a failure in the generation and discharge of impulses from pacemaker cells, or to deficiencies in the contractile elements. The possibility should be considered that conduction failures could also result in cessation of beating.

TABLE I. Effect of 2-DG on ATP Concentration of Cultured Heart Cells.

Conditions[a]	ATP concentration μmole/g protein[b]
Control (60′)	3.20 ± 0.284[c]
2-Dg (60′)	1.03 ± 0.096[d]
Control 40′ + Pyruvate (20′)	4.95 ± 0.393[e]
2-DG 40′ + Pyruvate (20′)	2.11 ± 0.197[f]
Control 40′ + ATP (20′)	4.10 ± 0.310[g]
2-DG 40′ + ATP (20′)	2.72 ± 0.263[h]

[a] The ATP determinations were performed after the time (min) indicated by the parentheses.

[b] Mean ± SEM ($n = 6$)

c vs d, $p < 0.001$; c vs e, $p < 0.005$; d vs f, $p < 0.001$; c vs g, $p < 0.05$; d vs h, $p < 0.001$.

After one hour in HBSS without glucose, 8 control cultures responded well to the electrical stimulus and followed the various frequencies applied. In the case of those cultures stopped by 2-DG, 6 out of 8 cultures did not respond to the stimulus, and in 2 cases, the application of the stimulus elicited only a slight beating in few cells.

The effect of electrical stimulation in heart cells treated for 20 min with 2-DG in glucose-free medium, followed by a 20-min exposure to pyruvate, glucose or ATP was investigated. 7 out of 9 2-DG and pyruvate-treated cells responded and followed the stimulus similar to control cells. Cells exposed to 2-DG and glucose responded to the stimulus in all the 6 cases studied, and followed the frequencies well, but the contrac-

tions were weak and incomplete, especially at high rates. In cultures treated with 2-DG and ATP, 3 out of 4 cultures responded and followed the applied electrical stimulation.

Discussion. In the present study, highly specialized cells cultured *in vitro* can maintain their functional activity without appreciable changes in a simple buffered salt medium. It has been found that isolated chick heart cells, incubated in a similar environment, suffer no appreciable membrane injury for at least two hr (31).

The moderate depression in rate brought about by 2-DG addition to cultured heart cells in glucose medium might be attributed to an impairment of the utilization of glucose. The restoration of the rate of beating in 2-DG depressed cells by pyruvate might be explained on the basis of its ability to enter the tricarboxylic acid cycle and to elevate the intracellular ATP content, insofar as 2-DG leads to a reduced level of ATP.

Similar to pyruvate, exogenous ATP contributes to the energy requirements of the cultured heart cells. However, in glucose medium, added ATP, in contrast to pyruvate, produces a marked increase of the rate in both 2-DG treated and untreated cultures. We cannot offer any explanation to account for the interesting effects of ATP from the data obtained in the present study. In any event, it appears that the ATP levels cannot be strictly related to the rate of beating of these cells. A dissociation between ATP concentration and heart function has been shown to occur by other workers (20, 32).

The stability of the rhythm in the absence of a carbon source in the salt medium, indicates that endogenous sources are capable of sustaining function in these cells. The finding that under these conditions, 2-DG leads to rapid cessation of beating, would indicate that glycogen utilization is important for spontaneous activity.

The incomplete recovery obtained with glucose in cardiac cells depressed by 2-DG and substrate-free medium may be attributed to an inhibition of glucose penetration of 2-DG-6-phosphate accumulation, and to a reduced availability of ATP. Pyruvate could provide energy necessary for glucose uptake and phosphorylation. However, in the absence of glucose, pyruvate failed to restore spontaneous activity in cells depressed by 2-DG.

As reflected in the experiments with electrical stimulation, 2-DG treated cells incubated in glucose-free medium appear to have an impairment of their automatism and contractile mechanism. These results also indicate that, in the absence of glucose, pyruvate, although capable of reestablishing contraction, fails to meet the demands required for spontaneous beating. Furthermore, the beating frequency of the heart cells was not depressed by high concentrations of fluoroacetate.

Thus, it would appear that an adequate utilization of glucose is required for the initiation of automatism in cultured heart cells.

Summary. The effects of 2-deoxy-D-glucose (2-DG) were studied on postnatal rat heart cultures in Hanks' balanced salt solution. In this medium, the cardiac cells maintained a stable rhythm for a prolonged period of time. When glucose was the substrate, 2-DG depressed the rate of beating; this depression was reversed by ATP or pyruvate. ATP also stimulated the rate in cells without 2-DG, whereas pyruvate had no effect. Intracellular ATP levels were decreased by 2-DG, and incubation with pyruvate increased the ATP levels both in treated and untreated cultures. In the absence of glucose, the rate of beating remained unchanged, but addition of 2-DG stopped the beating, whereas glucose was only partially effective in counteracting the 2-DG depression. However, pyruvate and glucose, whatever the order of addition, restored the rate. Electrical stimulation induced beating in cultures treated with 2-DG and pyruvate or ATP in glucose-free medium. These findings indicate that, although the energy derived from the Krebs cycle is of importance for supporting contractility, adequate glucose utilization is needed for automatism in cardiac cells.

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