BBA 45806

IN VITRO STUDIES OF BEATING HEART CELLS IN CULTURE

XII. THE UTILIZATION OF ATP AND PHOSPHOCREATINE IN OLIGOMYCIN AND 2-DEOXYGLUCOSE INHIBITED CELLS

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(Received November 19th, 1968)
(Revised manuscript received February 5th, 1969)

SUMMARY

1. The ATP concentration in the cultured rat heart cells did not change significantly when either glycolysis or oxidative phosphorylation was inhibited with 2-deoxyglucose and oligomycin, respectively. When both glycolysis and oxidative phosphorylation inhibitors were present simultaneously, the ATP level dropped significantly. The combined effect of both inhibitors exceeded the sum of the effects of the individual inhibitors.

2. The utilization of ATP in the presence of either oligomycin or 2-deoxyglucose and in the presence of both inhibitors simultaneously was reflected in a decrease in phosphocreatine concentration.

3. The rate of high energy phosphate utilization in quiescent cells was calculated to be 0.289 $\pm$ 0.040 nmole/mg protein per min. The utilization of energy for contraction was calculated to be 0.055 $\pm$ 0.010 nmole/mg protein per contraction.

4. Taking into consideration the energy requirements of beating cells and the change in ATP + phosphocreatine when the inhibitors were present singly and simultaneously, it was concluded that a control of metabolism involving glycolysis and oxydative phosphorylation is operating under normal oxidative conditions. The sum to the rates of the two pathways, when operating alone, is greater than the sum of their rates when operating simultaneously.

INTRODUCTION

It has been demonstrated that heart cells in tissue culture continue to beat in the presence of either oligomycin, an inhibitor of oxidative phosphorylation[1], or 2-deoxyglucose, an inhibitor of glycolysis. When both inhibitors are present simultaneously, the cells cease to beat spontaneously but remain contractile when stimulated electrically[2].

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The ATP concentration in the cells was practically unchanged when the inhibitors were used singly, but dropped considerably when both inhibitors were present simultaneously. It was of interest to establish the mechanism for the maintenance of normal concentrations of ATP in the presence of individual inhibitors. From the many studies on the chemical energetics of muscle[3-6] and from the studies of the kinetics of phosphocreatine kinase[7], it was expected that phosphocreatine is used for ATP resynthesis. This has been demonstrated in the present study.

It has been suggested that normally glycolysis and oxidative phosphorylation operate below their maximum potential[2]. If this were so, oligomycin inhibition of oxidative phosphorylation would increase the rate of glycolysis and 2-deoxyglucose inhibition of glycolysis would increase the rate of oxidative phosphorylation. To investigate this, the utilization of total high energy phosphate, *i.e.* ATP and phosphocreatine, was determined in the presence of both inhibitors and compared to that when the inhibitors were used singly. Such comparison would indicate whether the sum of glycolysis and oxidative phosphorylation operating alone is greater than the sum of both operating together. The energy requirements under different conditions of inhibition had to be considered; therefore, an evaluation of resting metabolism and energy utilization per beat of the heart cells in tissue culture was made.

MATERIALS AND METHODS

The heart cells were derived from newborn rats and cultured in plastic plates in complete medium into a network of synchronously beating cells, by methods previously described[8]. Each plastic plate had a layer of cells derived from ½–1 rat. Cells cultured from 3 to 6 days were used in all experiments.

The volume of the medium in each plate was 4 ml and the plates were equilibrated for 20 min at 22–24° prior to the experiment. 2 μg oligomycin in 95 % ethanol (20 μl), and 2-deoxyglucose (0.12 ml), in a final concentration of 10 × the glucose in the medium, were added to the 4 ml medium in the plate either singly or simultaneously. Plates without the inhibitors, but otherwise identically treated, served as controls. The control and experimental plates were always from the same batch of cells, cultured for the same length of time. The rate of beating was determined visually under the microscope.

The extraction procedure was carried out at 0°. The medium was decanted and the plates were rinsed immediately with ice-cold salt solution and drained, without disrupting the cells. 1 ml of ice-cold 0.3 M $HClO_4$ was added, the cells were scraped and the suspension centrifuged in the cold. The precipitate was washed with cold $HClO_4$, the extract and washings combined, neutralized with KOH and diluted to a final volume of 2 ml. The potassium perchlorate was centrifuged, and aliquots of the supernatant extract taken for the determination of ATP, and total and free creatine. The $HClO_4$ precipitate was used for protein determination.

In the experiments in which hearts excised from newborn rats were used for analysis, six hearts were frozen immediately in isopentane, precooled with liquid nitrogen, and two groups of three hearts each extracted with ice-cold $HClO_4$ (ref. 9).

Protein was determined according to the method of LOWRY[10], ATP was determined with the fluorimetric enzyme assay technique[11], and phosphocreatine by the difference of total and free creatine determined according to a modification of

the method of ENNOR AND ROSENBERG[12]. All the enzymes and cofactors used in the enzymatic methods were obtained from Boehringer and Sons; all reagents were of analytical grade.

RESULTS

In two groups of 3 hearts each from newborn rats, the concentration of ATP was 62.2 and 47 nmoles/mg protein and phosphocreatine was 54.5 and 46.6 nmoles/mg protein, respectively. The cells in tissue culture have a range of 25–45 nmoles ATP/mg protein and 20–47 nmoles phosphocreatine/mg protein with only occasional values of less than 25 nmoles, and less than 20 nmoles, respectively. Thus the highest values obtained with cells in tissue culture are in the range of those of the intact hearts, while the lower values probably reflect the presence of cell types other than heart cells in the culture of those batches.

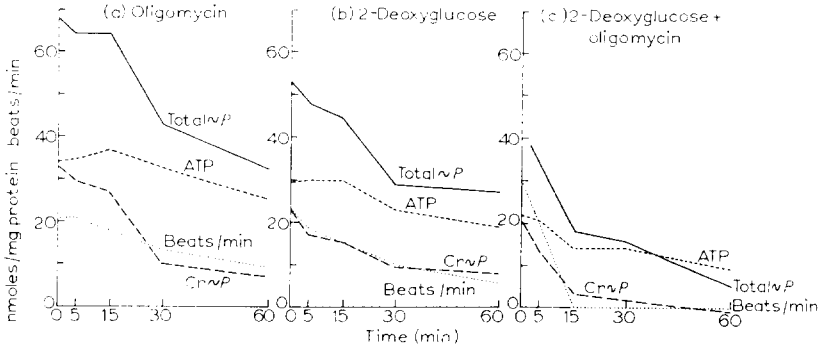

Fig. 1. The effect of addition of 2-deoxyglucose and oligomycin. The experimental points are the average values from 2 plates of the same batch of cells analyzed at the same time. ———, total ~ *P*; - - - -, ATP; – – –, phosphocreatine (Cr ~ *P*); ·····, beats/min.

Fig. 1 shows the effect of addition of oligomycin (Fig. 1a) and 2-deoxyglucose (Fig. 1b) singly, and simultaneously (Fig. 1c) on the beating rate and the concentration of ATP and phosphocreatine. A curve representing the concentration of the sum of ATP and phosphocreatine (total high energy phosphate) is also shown.

In all experiments the rate of decrease of phosphocreatine was much faster than the rate of change of ATP during the first 15–30 min after the addition of the inhibitors, while in the time interval between 15–30 and 60 min the rates of the decrease of ATP and phosphocreatine were roughly parallel. A new steady state seems to be established during the second period.

The change in ATP concentration was much greater when both inhibitors were present simultaneously than when either one was present alone. In most experiments ATP did not change during 15–30 min with either inhibitor alone. The change in phosphocreatine concentration was greater in the presence of both inhibitors, but either inhibitor alone invariably causes a drop in phosphocreatine. The beating ceased in the presence of both inhibitors and sometimes the rate decreased in the presence of either inhibitor alone, while control plates maintained the same rate of beating throughout the experiment. A decrease in phosphocreatine level was seen at each

time interval. The rate of decrease was greater in the first 30 min than in the 30–60 min interval. This change in the rate of decrease of phosphocreatine coincided with the change in the rate of beating. In this experiment when beating ceased phosphocreatine was reduced to about 1/6, while ATP was still maintained at about 75 % of the control.

The curve representing the change in the total high energy phosphate is similar to that of phosphocreatine. It is clear from Fig. 1 that the total high energy phosphate was not maintained when either inhibitor was present alone, and the decrease in the concentration was continuous. A statistical evaluation of the decrease in total high energy phosphate at random time intervals was made for 28 plates with oligomycin alone, 23 plates with 2-deoxyglucose alone and 31 plates with oligomycin and 2-deoxyglucose combined and a sign. test gave P values of 0.0192, 0.0008 and 0.0001 respectively[13], supporting the observation of continuous decrease in the concentration of high energy phosphate. It therefore appears that a control mechanism at the metabolic level does not compensate fully for an inhibited pathway, even though the energy requirements are diminished in more slowly beating cells.

Table I shows the results of one experiment of a series of 20. Duplicate plates were taken for control with no inhibitor added, for either inhibitor alone and for both inhibitors present simultaneously.

In considering the question of metabolic control, we are concerned with the changes in ATP and phosphocreatine obtained when both inhibitors are present simultaneously, (Δ[oligomycin + 2-deoxyglucose]) and with the sum of the changes

TABLE I

INHIBITOR EFFECTS ON HIGH ENERGY PHOSPHATES

The plates were equilibrated for 20 min at room temperature. The inhibitors were added directly to the incubation medium. The experiment was terminated by the addition of ice-cold 0.3 M $HClO_4$ at the time indicated. The beating rate was recorded just prior to the termination of experiment. D = (Δ[oligomycin + 2-deoxyglucose]) − (Δ[oligomycin] + Δ[2-deoxyglucose]).

Experimental conditions	*Time (min)*	*Beats per min*	*ATP (nmoles/mg protein)*	*ΔATP (nmoles/mg protein)*	*Phospho-creatine*	*Total high energy phosphate (nmoles/mg protein)*	*ΔTotal high energy phosphate (nmoles/mg protein)*
(a) Control		25	33.3		26.2	59.5	
Control		25	37.2		28	65.2	
(b) Oligomycin	30	0	18.3	a − b	7.7	26.0	a − b
+2-deoxyglucose	30	0	19.6	16.2			36.4
(c) Oligomycin	60	0	9.2	b − c	7.4	16.6	b − c
+2-deoxyglucose	60	0	12.7	8.0	8.3	21.0	7.1
(d) Oligomycin	30	25	26.8	a − d	9.2	35.0	a − d
	30	21	31.2	6.2	14.2	45.4	22.2
(e) Oligomycin	60	21	29.2	d − e	11.5	35.7	d − e
	60	23	26.7	1.1	9.9	31.5	6.6
(f) 2-Deoxyglucose	30	21	31.3	a − f	21.7	53.0	a − f
	30	21	32.7	3.2	22.8	55.5	8.1
(g) 2-Deoxyglucose	60	19	25.3	f − g	26.4	53.7	f − g
	60	20	29.4	4.6	33.9	53.3	0.8
D in 0–30 min				6.6			6.7
30–60 min				2.3			0.2

obtained when one inhibitor is present at a time (Δ[oligomycin] + Δ[2-deoxyglucose]). The difference, D = (Δ[oligomycin + 2-deoxyglucose]) − (Δ[oligomycin] + Δ[2-deoxyglucose]), if significantly greater than zero indicates an increased synthesis of high energy phosphate from the uninhibited source. The results of 20 experiments (the protocol of which was outlined above) were subjected to statistical tests of significance by the paired variates test, in which the difference D is obtained by choosing each change in the expression of D at random from experiments done with the same batch of cells[13]. This procedure eliminates the extraneous differences that exist between different batches of cells.

TABLE II

STATISTICAL TEST OF SIGNIFICANCE

The results of statistical test of significance of 20 experiments, conditions as in Table I. In all experiments, in the presence of both inhibitors the beating stopped between 20 and 30 min. D = (Δ[oligomycin + 2-deoxyglucose]) − (Δ[oligomycin] + Δ[2-deoxyglucose]).

Time interval	*D ATP ± S.E. (nmoles/mg protein)*	*Significance*	*D Total high energy phosphate ± S.E. (nmoles/mg protein)*	*Significance*
0 and 20–30 min	5.41 ± 1.79	$0.0005 < P < 0.005$	4.71 ± 3.34	$P > 0.1$
20–30 and 60 min	0.85 ± 1.48	$P > 0.1$	−3.00 ± 3.93	$P > 0.1$

Table II shows the results of the statistical evaluation of the experiments. The value, D, for ATP, *i.e.* 5.41 ± 1.19 nmoles/mg, is highly significant and demonstrates the ability of the cells to maintain the steady-state level of ATP when either glycolysis or oxidative phosphorylation are inhibited. The value, D, for the total high energy phosphate, *i.e.* 4.71 ± 3.34 is not significantly different from 0, however, the cells were beating in the presence of individual inhibitors, but ceased beating when both inhibitors were present. Actually, even when D = 0, a control of metabolism has to be assumed in view of the much greater energy requirement for beating than for quiescent cells. As calculated in the following section on resting metabolism and energy utilization, the beating cells utilize approx. 19.8 nmoles/mg protein of high energy phosphate more than the quiescent cells and thus even though the D value is not significantly different from 0 a control mechanism, supplying the 19.8 nmoles of high energy phosphate per mg protein, must be evoked.

The evaluation of the control mechanism during the 30–60 min interval is much more ambiguous. The cells which stopped beating in the presence of both inhibitors utilize the high energy phosphate for resting metabolism only, while the cells with single inhibitors continue to beat, very often at a slower rate. It is also probable that other secondary effects occur at the later time.

From the data of 20 experiments, the rate of utilization of energy for the resting metabolism, was calculated in cells inhibited with oligomycin and 2-deoxyglucose simultaneously, where no net resynthesis of high energy phosphate is presumed to occur. The difference in total high energy phosphate between cells which just stopped beating (usually at 20–30 min) and the level at 60 min was divided by the time in min. This gave the utilization of high energy phosphate per min for resting metabolism.

Plates from the same batch were paired at random. The value for the resting metabolism was 0.289 ± 0.040 nmole high energy phosphate/mg protein per min.

The average energy for contraction was calculated from the difference in high energy phosphate between control cells and cells inhibited with oligomycin and 2-deoxyglucose simultaneously, at the time when the latter stopped beating. In each batch a control was paired at random with cells that have received both inhibitors. This difference, divided by the time it took to stop beating represents the energy utilized for both contraction and resting metabolism per min. The resting metabolism (as calculated above) was subtracted from the total energy difference to give the energy for contractions per min. If we assume that the beating rate drops linearly from the initial value of the control plate to the final value of zero in the inhibited plates, then the average contraction rate can be taken as ½ of initial rate. The average energy for contraction was calculated by dividing the energy for contractions per min by the average number of beats per min, and the value for high energy phosphate obtained was 0.055 ± 0.010 nmole/mg protein per contraction.

Comparing the utilization of energy of the cells when both oligomycin and 2-deoxyglucose are present, with cells treated with one inhibitor only, one must take into consideration that the latter have an average rate of beating which is approximately twice that of the former. During the 30 min this represents approx. 360 beats more for the singly inhibited cells, thus utilizing at least 360 × 0.055 = 19.80 nmoles/mg protein. Thus even when the value D, as calculated in the above section, is not significantly different from 0, one has to account for an energy requirement of 19.8 nmoles high energy phosphate/mg protein, and that amount must have been supplied by the uninhibited source of metabolism.

DISCUSSION

The present study supports previous findings[2] that the inhibition of either glycolysis or oxidative phosphorylation does not alter significantly the concentration of ATP in cultured heart cells. The concentration of ATP decreased only when the phosphocreatine level was greatly diminished as a result of inhibition of both glycolysis and oxidative phosphorylation. The latter observation agrees with results of studies dealing with the kinetics of phosphocreatine kinase[7], where it was demonstrated that this enzyme is adapted for rapid phosphorylation of ADP.

When glycolysis and oxidative phosphorylation are inhibited the resynthesis of ATP is available only from the finite store of phosphocreatine. However, when only glycolysis or oxidative phosphorylation is inhibited the resynthesis of ATP is accomplished also by the uninhibited metabolic source. The problem then arises whether the rate of synthesis by the uninhibited source of energy is the same as its rate when both glycolysis and oxidative phosphorylation are functioning or if there is an increased rate of resynthesis by the uninhibited source. In order to answer this question the total high energy phosphate compounds *i.e.* ATP and phosphocreatine as well as the energy requirement of the cells have to be considered.

By comparing the change in ATP + phosphocreatine when the inhibitors were present singly and simultaneously and by taking into consideration the increased energy requirements for beating, it has been demonstrated that a control of metabolism involving glycolysis and oxidative phosphorylation operates under normal con-

ditions. The mechanism of this control is of particular interest and it seems plausible that either free creatine or phosphocreatine exerts such a control, possibly at the enzyme level. An allosteric effect of phosphocreatine on phosphofructokinase has recently been demonstrated (T. MANSOUR, personal communication).

The quantitation of the utilization of chemical energy for resting metabolism and for the contraction of the beating heart cells might however be underestimated if the inhibition of metabolism is not complete and some resynthesis occurs. This could account partially for the values reported in this study being considerably lower than those of POOL AND SONNENBLICK[6], a direct comparison, however, is not possible because of species difference and the difference between tissue culture and fresh tissue. The possibility must also be considered that the quantitation of energy utilization for contraction might have been overestimated. If the lower ATP concentration and/or phosphocreatine concentration causes a lower rate of resting metabolism, then the energy utilization of the beating cells would give a higher calculated value for the energy utilization for contraction.

ACKNOWLEDGEMENTS

The valuable help of Mr. Alfred Wallner in preparing the extracts of hearts of newborn rats is gratefully acknowledged.

These studies were supported in part by Contract AT(04-1) GEN-12 between the Atomic Energy Commission and the University of California, by Postdoctoral Fellowship 1-F2-HE-29, 840-01 from the National Heart Institute, and by Research Grant 9R01HE-11 216-09 from the National Institutes of Health, U.S. Public Health Service.

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