BBA 45719

IN VITRO STUDIES OF BEATING-HEART CELLS IN CULTURE

XI. THE ATP LEVEL AND CONTRACTIONS OF THE HEART CELLS

MARIA W. SERAYDARIAN, ISAAC HARARY AND ED SATO

Department of Biophysics, Laboratory of Nuclear Medicine and Radiation Biology, and the Department of Biological Chemistry, UCLA School of Medicine, and the Institute of Molecular Biology, University of California, Los Angeles, Calif. 90024 (U.S.A.)

(Received April 11th, 1968)

SUMMARY

1. The beating of heart cells in tissue culture is not affected by the inhibition of either glycolysis or oxidative phosphorylation alone. When both pathways are inhibited simultaneously, beating stops.

2. The ATP concentration does not change significantly when either glycolysis or oxidative phosphorylation is inhibited alone. When both glycolytic and oxidative phosphorylation inhibitors are present, the ATP level drops significantly. The combined effect of both inhibitors exceeds the sum of the effects of the individual inhibitors.

3. Dinitrophenol inhibits the beating of heart cells with a concomitant small decrease in ATP level. The addition of oligomycin restores the ATP level to control values and beating resumes.

4. Heart cells in which beating is inhibited, respond to electrical stimulation, even though the ability to beat spontaneously has been lost. Contractions in response to stimulation can be observed until the decrease of ATP has reached 85–90 %.

5. It is concluded that heart cells must have a mechanism for maintaining a high steady-state level of ATP, since such a high level is required for spontaneous beating. The possible mechanisms for the maintenance of high steady-state level of ATP are discussed.

INTRODUCTION

Evidence has accumulated that fatty acids are the main fuel for the heart[1,2]. The dependence on lipids of the long-term functioning of cultured heart cells has also been demonstrated[3,4]. However, it was subsequently demonstrated that either oxidative phosphorylation or glycolysis could presumably supply enough ATP needed for spontaneous contractions[5]. Heart cells continued beating in the presence of either oligomycin or iodoacetate alone but ceased to beat when both inhibitors were present. Thus, either source of energy could supply enough ATP for beating. However, from these results it was not clear, whether the spontaneous beating of the heart cells could continue with a greatly diminished ATP level which could be supplied either by glycolysis or oxidative phosphorylation alone or whether the beating required an

undiminished high level of ATP. The latter alternative also implies that either of these processes, working alone, could maintain the necessary high ATP level. These alternatives were investigated by an examination of the ATP level under various experimental conditions.

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[6]. Each plastic plate had a layer of cells derived from either ½ rat or 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 20 min at 22–24° prior to the experiment. All inhibitors used were prepared 100-fold the final concentration and neutralized so that upon addition to the medium the change of concentration of the latter was negligible. Plates without the inhibitors, but otherwise treated identically served as controls. The control and experimental plates were always from the same batch, cultured for the same length of time. The rate of beating was determined visually under the microscope.

Either 2-deoxyglucose was added to the medium 10 min prior to oligomycin, or oligomycin was added prior to 2-deoxyglucose, and the extraction followed immediately after the cessation of beating, usually 10–20 min after the last addition.

For the dinitrophenol experiments the extraction either followed immediately the cessation of beating, usually after 5 min in $1 \cdot 10^{-3}$ M dinitrophenol or immediately after the beating was restored, usually 1–2 min after the addition of oligomycin.

The extraction procedure was carried out at 0°. The medium was decanted and the plates rinsed immediately with salt solution and drained, without disrupting the cells. This was necessary because the presence of serum in the medium would introduce errors in the protein determination. 1 ml of cold 0.3 M perchlorate was added, the cells were scraped and the suspension centrifuged in the cold, the precipitate was washed with cold perchlorate, the extract and washing combined, neutralized with KOH and diluted to a final volume of 2 ml. The potassium perchlorate was centrifuged off and aliquots of the extract taken for ATP analysis. The perchlorate precipitate was used for protein determination. This procedure was followed in all experiments, except when dinitrophenol was used. Because of easy reversibility of dinitrophenol effect, the extraction procedure followed the decantation of medium, with no washing of the plate, and the value for protein was taken from an average of 3 control plates.

Protein was determined according to the method of LOWRY *et al.*[7]. ATP according to the method of ESTABROOK AND MAITRA[8], using the ATP kit from Calbiochem. Glycogen was determined by a modification of the method of JOHNSON AND FUSARO[9]. Oligomycin B was dissolved in 96 % ethanol, and dinitrophenol, iodoacetate and 2-deoxyglucose in water. All reagents were of analytical grade.

RESULTS

Metabolic inhibitors and beating

It was considered possible that the effect of dinitrophenol or oligomycin and iodoacetate in causing cessation of beating was not related to the effect of these

compounds on energy metabolism. To investigate this, other specific metabolic inhibitors were tried. Monofluoroacetate, which inhibits the tricarboxylic acid cycle and effectively diminishes respiratory phosphorylation did not inhibit the beating of the heart cells. Up to 10^{-3} M, monofluoroacetate did not affect the rate. Monofluoroacetate, at only 10^{-6} M, coupled with 10^{-4} M iodoacetate inhibited beating (Fig. 1).

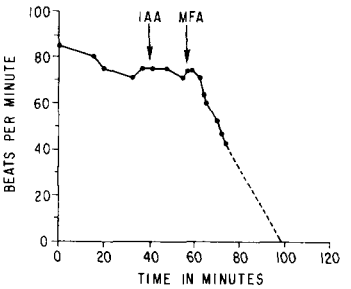

Fig. 1. The effect of monofluoroacetate (MFA) on iodoacetate (IAA)-pretreated cells.

Similarly, 10^{-3} M malonate alone was ineffective, but 10^{-3} M malonate and 10^{-4} M iodoacetate effectively stopped the spontaneous beating. The addition of $5 \cdot 10^{-6}$ M ATP restored the beating. Experiments with 2-deoxyglucose, an inhibitor of glycolysis, were consistent with the above observations and supported the conclusion that the action of these inhibitors was a result of their specificity as inhibitors of metabolic reactions and not due to some unknown side-action.

The effect of inhibitors on the ATP level and on the beating rate

The values for ATP and the rate of beating resulting from the addition of oligomycin and 2-deoxyglucose were determined (Table I).

Neither 2-deoxyglucose, nor oligomycin when present alone altered appreciably the ATP levels, 2-deoxyglucose did not alter the beating rate either, while oligomycin tended to slow it down. When both inhibitors were present simultaneously, the beating ceased in all plates and the ATP decreased between 27 % and 56 % below the control level. Furthermore, in each case the drop of ATP in the presence of both inhibitors exceeded the sum of the 2-deoxyglucose and oligomycin effects, when added separately.

Similarly, the inhibition of glycolysis by iodoacetate, did not change the level of ATP nor the rate of beating. Oligomycin alone tended to lower the level of ATP very slightly, but when both inhibitors were present, the level of ATP dropped considerably, to between 51 % and 66 % of control, exceeding the sum of the effects of either inhibitor alone and the beating ceased (Table II).

It was previously shown[5] that $5 \cdot 10^{-4}$ M dinitrophenol stopped the beating of heart cells in culture, and ATP, added to the medium of dinitrophenol-inhibited cells, restored the beating. Also oligomycin when added to dinitrophenol-inhibited cells, restored the beating of the heart cells, while oligomycin alone had either no effect on the beating or sometimes decreased its rate. Table III shows the actual values of ATP in the cultured cells under conditions of dinitrophenol inhibition of beating and the restoration of function upon the subsequent addition of oligomycin. As expected,

TABLE I

EFFECT OF OLIGOMYCIN AND 2-DEOXYGLUCOSE ON ATP LEVEL

After the plates were equilibrated for 20 min at room temperature, the inhibitors were added directly to the incubation medium. Either 2-deoxyglucose was added to the medium 10 min prior to oligomycin, or oligomycin was added 10 min prior to 2-deoxyglucose. The experiment was terminated by the addition of ice-cold 0.3 M perchloric acid at the time when beating stopped in the presence of both inhibitors between 10 and 20 min.

Expt. No.	*Additions*		*ATP (mμmoles/mg protein)*	*Beating (beats/min)*
	2-Deoxyglucose (10^{-2} M)	*Oligomycin (μg)*		
1	—	—	63	63
	3	—	53	70
	—	25	58	52
	3	25	36	0
2	—	—	60	56
	3	—	53	76
	—	1	53	27
	3	1	40	0
3	—	—	72	19
	3	—	58	18
	—	1	60	17
	3	1	33	0

TABLE II

EFFECT OF OLIGOMYCIN AND IODOACETATE ON ATP LEVEL

Conditions as in Table I. Iodoacetate was added to the medium 30 min prior to oligomycin. The experiment was terminated when beating stopped in the presence of both inhibitors, usually within 15 min.

Expt. No.	*Iodoacetate (10^{-4} M)*	*Oligomycin (μg)*	*ATP (mμmoles/mg protein)*	*Beating (beats/min)*
1	—	—	67	67
	2	—	67	24
	—	25	55	*
	2	25	49	0
2	—	—	71	63
	1	—	71	71
	—	25	55	22
	1	25	47	11
3	—	—	52	75
	2.5	—	58	80
	—	1	46	78
	2.5	1	30	0
4	—	—	41	77
	2.5	—	42	79
	—	1	39	67
	2.5	1	21	0

* Rate irregular.

TABLE III

EFFECT OF DINITROPHENOL AND OLIGOMYCIN ON ATP LEVEL

Cells were held in dinitrophenol until beating stopped, usually 5 min. Oligomycin was added and the beating resumed in 1–2 min, the experiment was terminated at this time. Controls using dinitrophenol alone were incubated for the same length of time and the beating did not resume. Conditions similar to those reported in Table I.

Expt. No.	*Additions*		*ATP* (*mμmoles/mg protein*)	*Beating* (*beats/min*)
	Dinitrophenol (10^{-4} *M*)	*Oligomycin* (*μg*)		
1	—	—	46	52
	2	—	43	38
	5	—	35	0
	10	—	31	0
	10	50	43	52
2	—	—	41	67
	2	—	37	43
	10	—	26	0
	10	50	34	30
3	—	—	42	15
	10	—	25	0
	10	4	36	35
4	—	—	48	54
	10	—	43	0
	10	50	51	52

dinitrophenol caused a drop in ATP levels, when beating was inhibited. The range of decrease was from 40 % to 90 % of the control, and it is of interest to note that even a drop of ATP to 90 % of the control resulted in the cessation of beating. The addition of oligomycin to the dinitrophenol-inhibited cells resulted in a restoration of beating and an increase in the ATP level back to the control level. It seemed that a small drop in the ATP seriously affected the spontaneous beating.

As a summary, data from many experiments were collected and statistically analyzed. The results are shown in Table IV. The concentration of ATP of cells treated with 2-deoxyglucose *plus* oligomycin was significantly different from the control and also from the cells treated with oligomycin alone. A decrease of ATP of about 40 % resulted in a complete cessation of beating. Iodoacetate *plus* oligomycin caused a significant decrease in the ATP, and a complete cessation of beating. Iodoacetate alone had no significant effect on either ATP or beating rate. Dinitrophenol alone showed a significant decrease of about 26 % in the ATP level, and a cessation of beating, while dinitrophenol *plus* oligomycin showed no significant change in the ATP and restoration of beating.

The response to electrical stimulation

It was of interest to investigate whether the decrease in ATP affected only the ability of the cells to beat spontaneously and whether at that lower ATP level the cells might still respond to electrical stimulation. The inhibited cells were therefore

TABLE IV

SUMMARY OF THE EFFECTS OF INHIBITORS ON ATP AND BEATING

Experimental conditions	*Number of experiments*		*ATP (mμmoles/mg protein)*		P**	*Beating$_{average}$ (beats/min)*	
	*Control**	*Expt.*	*Control**	*Expt.*		*Control**	*Expt.*
$1 \cdot 10^{-4}$–$5 \cdot 10^{-4}$ M iodoacetate	8	8	52 ± 4.3***	52 ± 5.1		68	54
$1.5 \cdot 10^{-2}$–$3 \cdot 10^{-2}$ M 2-deoxyglucose	14	13	76 ± 5.6	69 ± 7.3	$0.4 < P < 0.5$	49	47
1–25 μg oligomycin	27	35	65.6 ± 3.7	56.5 ± 2.6	$0.05 < P < 0.1$	55	27
$1 \cdot 10^{-4}$–$2 \cdot 10^{-4}$ M iodoacetate *plus* 12.5–25 μg oligomycin	5	7	57 ± 6.1	40 ± 2.9	$0.02 < P < 0.05$	55	5
1 μg oligomycin *plus* $2.5 \cdot 10^{-4}$–$5 \cdot 10^{-4}$ M iodoacetate	5	3	49 ± 2.3	26 ± 2.2	$P < 0.001$	74	0
$1.5 \cdot 10^{-2}$–10^{-2} M 2-deoxyglucose *plus* 1–25 μg oligomycin	14	14	76 ± 5.6	45 ± 5.5	$P < 0.001$	49	0
$5 \cdot 10^{-4}$–$1 \cdot 10^{3}$ M dinitrophenol	10	18	44.3 ± 4.4	29 ± 1.9	$0.001 < P < 0.01$	55	0
$1 \cdot 10^{-13}$ M dinitrophenol *plus* 25–50 μg oligomycin	8	5	43 ± 14.8	43 ± 11.8			§
$1 \cdot 10^{-3}$ M dinitrophenol *plus* 4 μg oligomycin	4	16	54 ± 7.6	45 ± 8.5	$0.4 < P < 0.5$		§

* Cells in complete growth medium, but without inhibitors, served as controls.

** Calculated from Student's t test.

*** Means ± S.E.

§ Rate often irregular.

TABLE V

BEATING IN RESPONSE TO ELECTRICAL STIMULATION

Electrical stimulation: frequency, 2 pulses/sec; pulse duration, 2 msec; voltage through the medium, 30 V. Stimulation was applied, at the time when beating stopped in the presence of dinitrophenol and oligomycin *plus* 2-deoxyglucose.

Additions			*Beating (beats/min)*	
Dinitrophenol (10^{-3} *M*)	*Oligomycin* (μg)	*2-Deoxyglucose* (10^{-2} *M*)	*Before stimulation*	*In response to stimulation*
—	—	—	21	86
—	—	—	28	86
—	—	—	40	86
2	—	—	0	100
2	—	—	0	100
—	1	—	28	86
—	1	—	40	86
—	1	3	0	100

tested for their response to electrical stimulation (Table V). The beating of a preparation of heart cells was inhibited by dinitrophenol or by 2-deoxyglucose and oligomycin. The cells were then stimulated electrically, with a frequency of 2 pulses/sec, and a pulse duration of 2 msec. The voltage delivered was approx. 30 V, but it was delivered through the medium and no attempt was made to estimate the amount of current delivered to the layer of cells in the plate.

In all cases tested the non-beating, inhibited cells responded to electrical stimulation and continued to beat as the stimulus was applied between 5 and 30 min. The results demonstrate that although the spontaneous beating was inhibited by a decrease in the ATP level, the contraction process was not.

ATP level and the response to electrical stimulation

An attempt was made to establish the response to stimulation at the lowest possible level of ATP. With the use of the inhibitors, discussed so far, the decrease of ATP never exceeded 43 % and in all such cases the cells still responded to stimulation. By increasing the concentration of dinitrophenol the ATP concentration decreased further, but no response to stimulation was observed. It seemed likely that at higher concentrations dinitrophenol might have a secondary effect.

It was observed, in the course of this investigation, that when the medium was substituted with salt solution, the addition of 1–2 μg of oligomycin resulted in a decrease of ATP concentration to very low values in the course of 60 min. It seems that this amount of oligomycin in a protein-free medium is a massive dose. The effect of oligomycin is related to the concentration of protein in the system. The addition of 1–2 μg to a serum-free, and therefore protein-free medium, results in an increased oligomycin to protein ratio thus increasing the effective concentration of oligomycin with respect to the heart cells. The addition of 0.03 μg of oligomycin to cells in a protein-free medium was enough to inhibit oxidative phosphorylation. This was measured by the ability of this amount of oligomycin to potentiate a decrease in ATP with 2-deoxyglucose (Table VI). The addition of 0.03 μg oligomycin to cells in a complete growth medium (with serum) did not potentiate 2-deoxyglucose effect.

TABLE VI

EFFECT OF OLIGOMYCIN IN PROTEIN-FREE MEDIUM

The complete growth medium was changed to the medium indicated and incubated at 37° for 30 min, then equilibrated at room temperature for 20 min prior to the addition of inhibitors. Oligomycin and 2-deoxyglucose additions and termination of experiment as in Table I.

Medium	*Additions*		*ATP (mμmoles/mg protein)*
	Oligomycin (μg)	*2-Deoxyglucose (10^{-3} M)*	
Complete medium *minus* serum	—	—	55
	0.03	—	60
	—	30	58
	0.03	30	45
Balanced salt solution *plus* $1 \cdot 10^{-4}$ M glucose	—	—	53
	0.03	—	56
	—	2.5	56
	0.03	2.5	40
Balanced salt solution *plus* $1.4 \cdot 10^{-3}$ M glucose	—	—	49
	0.03	—	51
	—	30	61
	0.03	30	41

TABLE VII

ATP LEVEL AND RESPONSE TO ELECTRICAL STIMULATION OF INHIBITED CELLS

The complete growth medium was changed to balanced salt solution containing $1 \cdot 10^{-4}$ M glucose, and incubated at 37° for 30 min, then equilibrated at room temperature for 20 min prior to the addition of oligomycin. At the time indicated the cells were stimulated, rate of beating recorded and experiment terminated as in Table I.

Expt. No.	*Oligomycin addition (μg)*	*Beating (beats/min)*		*Time (min after addition of oligomycin)*	*ATP (mμmoles/mg protein)*
		Before stimulation	*In response to stimulation*		
1	—	13	120	—	45
	—	0	120	—	40
	—	*	120	—	38
	—	30	120	—	44
2	2	0	120	5	25
	2	0	60	60	11
	2	0	0	90	5
3	2	0	60	5	28
	2	0	60	30	14
	2	0	0	60	4

* Rate irregular.

The effect of 2 μg of oligomycin in a protein-free medium was utilized to decrease the concentration of ATP to low levels and test the response to stimulation, under these conditions. The results are demonstrated in Table VII. The complete growth medium was replaced by balanced salt solution, which sometimes results in

cessation of beating but does not alter the ATP concentration, nor the ability of these cells to contract when stimulated. Oligomycin was added and the ATP level and response to stimulation was measured. A decrease in the ATP level was sufficient to abolish spontaneous contraction but the cells could contract when stimulated. It was not till the decrease in ATP reached 85–90 % that the level was insufficient to support electrically-induced contractions.

The control of the ATP level

In view of the observation that partially-inhibited cells cease to contract spontaneously with a concomitant small drop in ATP level, it seems plausible that the cell must have a mechanism for maintaining a high steady-state concentration of ATP. The control of glycolysis may be one of the pertinent mechanisms.

The heart exhibits a Pasteur effect, and similar observations have been made in the cultured heart cells. The utilization of glycogen in the heart cells is much greater under anaerobic conditions than under aerobic (Table VIII). Glucose uptake is somewhat greater under anaerobic conditions, but ATP level is unchanged. These observations indicate an increased role of glycolysis in ATP synthesis under anaerobic conditions. It has also been observed that when oxidative phosphorylation is inhibited by oligomycin the utilization of glycogen is increased.

TABLE VIII

AEROBIC AND ANAEROBIC GLYCOGEN UTILIZATION

The complete growth medium was changed to balanced salt solution containing $1 \cdot 10^{-4}$ M glucose and incubated at 37° for 30 min. Half of the plates were then placed in 95 % N_2 *plus* 5 % CO_2 at 37°, and another half in the usual gas mixture of 95 % O_2 *plus* 5 % CO_2 at 37°. Duplicate plates were taken for glycogen analysis at the time indicated.

Time (*min*)	*Glycogen, as glucose* (*mμmoles/mg protein*)	
	Air	N_2
0	183	187
60	140	88
120	98	38

DISCUSSION

Small changes in ATP affect the spontaneous contraction of cultured heart cells. It is reasonable to expect therefore that the heart must have control mechanisms that carefully maintain the steady-state level of ATP to maintain this crucial function.

The fact that inhibition of one source of ATP, by metabolic inhibitors or by anaerobiosis, does not alter the concentration of ATP in the cell indicates that the uninhibited source of ATP, either glycolysis, oxidative phosphorylation, or creatine phosphate has a reserve capacity which can be tapped for use, when needed.

Supporting evidence for this hypothesis is derived from observations, in perfused hearts, that fatty acid oxidation inhibits glycolysis[10,11] and that anaerobiosis also in perfused hearts, does not change the ATP level[12]. BICKIS AND QUASTEL[13] found,

for example, that in the Ehrlich ascites carcinoma cells the ATP level is not affected by the presence of various metabolic inhibitors.

The creatine phosphate level in the cultured heart cells is approximately the same as ATP. This reserve source of ATP resynthesis undoubtedly plays a role in maintaining the ATP level. Its quantitative contribution has not yet been determined and is presently under study.

It is possible that the small drop in ATP caused by inhibition may represent a large drop in a compartmentalized area of the cell. The results obtained indicate that whether the drop represents a depletion of an important reservoir or a small change in totally available ATP, it can be seen that this change stops the spontaneous beating. Therefore if the ATP is compartmentalized for spontaneous beating it would seem that this ATP is the first to be depleted under the inhibitory conditions and it does not change the argument for the necessity of very careful control of the ATP level.

The experiments reported here indicate that mitochondrial ATP is not restricted. The observation that the dinitrophenol-activated ATPase located in the mitochondria causes a depletion of the glycolytic ATP indicates that extramitochondrial ATP can diffuse into the mitochondrial space containing the ATPase.

The mechanism of how ATP is involved in the spontaneous beating is unknown. The effect of the ATP drop may only be indirect. The ATP may be needed for the calcium pump, for correct membrane function, or for active transport of K^+ or Na^+. Whatever the mechanism, it depends upon ATP and a small change in the ATP level affects it.

ACKNOWLEDGEMENTS

This investigation was supported in part by Contract AT(04-1)GEN-12 between the Atomic Energy Commission and the University of California; and by Research Grant HE 11216 from the National Institutes of Health, U.S. Public Health Service.

REFERENCES

1 R. J. BING, *Physiol. Rev.*, 45 (1965) 171.
2 J. R. EVANS, *Circulation Res.*, 14 (1964) 96.
3 I. HARARY, R. MCCARL AND B. FARLEY, *Biochim. Biophys. Acta*, 114 (1966) 15.
4 A. FUJIMOTO AND I. HARARY, *Biochem. Biophys. Res. Commun.*, 29 (1965) 456.
5 I. HARARY AND E. C. SLATER, *Biochim. Biophys. Acta*, 99 (1965) 227.
6 I. HARARY AND B. FARLEY, *Exptl. Cell Res.*, 29 (1963) 451.
7 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
8 R. W. ESTABROOK AND P. K. MAITRA, *Anal. Biochem.*, 3 (1962) 369.
9 J. A. JOHNSON AND R. M. FUSARO, *Anal. Biochem.*, 15 (1966) 140.
10 E. A. NEWSHOLME AND P. J. RANDLE, *Biochem. J.*, 80 (1961) 655.
11 E. A. NEWSHOLME AND P. J. RANDLE, *Biochem. J.*, 83 (1962) 387.
12 J. R. WILLIAMSON, *J. Biol. Chem.*, 240 (1965) 2308.
13 I. J. BICKIS AND J. S. QUASTEL, *Nature*, 205 (1965) 44.