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High-energy phosphates in quiescent, beating and contracted cardiac cells

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Isolated myocytes from ventricles are quiescent in the presence of 0.9 mM calcium. However, it is possible to induce beating by adding 0.5 mM $BaCl_2$ to the media or to induce a contracture by elevating the external concentration of potassium (72.5 mM K). During the viable stage of contracture, which is up to 1 h, the sarcomere spacing is 1.7 ± 0.1 μm and no leakage of intracellular components is observed. The metabolic properties of the cells in quiescent, beating and contracted states were compared. The O_2 consumption (natom per mg cell protein per min) increased from 10–11 in quiescent cells to 60–66 in beating cells and 90–99 in contractured cells. In contrast no significant difference was found in the metabolite levels in the three cellular states: (nmol per mg cell protein ± S.E.M.) ATP, 20.9 ± 1.5; CrP, 22.3 ± 2.2; ADP, 6.03 ± 0.67; Cr, 10.8 ± 1. It is proposed that the combined action of myosine ATPase, ATP synthase and cytosolic and mitochondrial creatine kinases serves to buffer the metabolite levels during periods of enhanced oxygen consumption.

Introduction

The development of force in cardiac muscle involves a rise in internal calcium. Heart beat is controlled by movement of calcium through membranes (sarcolemma, sarcoplasmic reticulum, mitochondria) and the interaction of calcium with proteins (troponin C). A contracture may be induced by calcium overload in conditions where both the membrane is depolarized and the voltage-dependent Na/Ca exchange [1] is activated; these conditions are obtained in high external potassium and low external sodium [2–7]. The metabolic consequence of force development is increased ATP splitting by the contractile apparatus. Therefore, it is of the utmost importance to study the levels of the high-energy phosphates. ATP, ADP, creatine phosphate, as well as creatine, during beating or contractures.

Contractures induced by high external potassium ions have been shown to be either transient and reversible [6,8] or permanent and irreversible [4,7], on whole hearts, ventricular strips and papillary muscles. It is difficult in multicellular preparations to distinguish between direct cellular effects and indirect phenomena due to cell-to-cell interactions. To avoid the events due to sarcolem lesions and squeezing out of metabolites, linked to cell-to-cell interactions, experiments were conducted on isolated cells. Besides the absence of cell–cell interactions, it is also very easy to change and to control the external medium.

Abbreviations: CrP, creatine phosphate; Cr, creatine; MOPS, 4-morpholineprepanesulfonic acid.

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In the present work we used isolated ventricular cells from the frog heart. Previously we have demonstrated that the technique for cell isolation provides a high yield of cells that are similar to myocytes in situ, and are resistant to physiological concentrations of calcium [9]. The quiescent cells were exposed to different external media to induce either beating (Ba 0.5 mM) or a contracture (high K/low Na). The levels of ATP, ADP, creatine phosphate and creatine, as well as the O_2 consumption, were studied on these three states.

Materials and Methods

Cell isolation. Cells were isolated from hearts of frog *Rana esculenta,* as previously described, by enzymatic perfusion with collagenase and trypsin [9]. After isolation in medium nominally Ca^{2+}-free, cells were preincubated for 20 min in Ringer medium without calcium (88.4 mM NaCl, 2.5 mM KCl, 23.8 mM $NaHCO_3$, 0.6 mM NaH_2PO_4 and 3.8 mM $MgCl_2$, bubbled with 95% O_2/5% CO_2) in the presence of 5 mM pyruvate, as substrate, and 0.1% BSA free of fatty acids. After this preincubation step, 70–90% of the isolated cells were morphologically similar to frog heart cells in situ, i.e., spindle like (200–300 μm length, 5 μm diameter, Fig. 1A) and resistant to physiological concentrations of calcium for at least 10 h.

During experiments, cells were incubated at 22° C in Ringer medium (above) containing 0.9 mM $CaCl_2$. In this medium they are quiescent but it is possible to induce beating by the addition of 0.5 mM $BaCl_2$ [10]. Quiescent or beating cells are maintained alive for more than 5 h [9]. The viability was determined by the intactness of the sarcolemma, as shown by trypan blue exclusion and by retention of lactate dehydrogenase [11,12].

Contractures were induced by increasing of the external K^+ concentration to 72.5 mM and decreasing of the external Na^+ concentration to 42.8 mM. 0.6 ml of cell suspension were mixed with 1 ml of a medium where NaCl and $NaHCO_3$ were substituted for KCl and $KHCO_3$.

Metabolite and enzyme determination. For a comparison between cells quiescent, beating and contractured, Ba or K ions were added 15 min after incubation of the cells in the presence of calcium. Aliquots of the cell suspensions were then taken after further incubation periods of 10, 15, 20 and 60 min.

These aliquots were centrifuged through silicon oil (either 508V70 from Rhône Poulenc or a mixture of 2 vol. DC200 + 10 vol. AR200 from Serva) in 10% perchloric acid. The protein content of the pellet was determinated by the Lowry method. To determine metabolite levels, the perchloric acid extract was used after neutralization with KOH-MOPS and ATP, ADP and creatine phosphate were determined by bioluminescence and creatine by fluorescence as previously described [9]. Lactate dehydrogenase activity was studied according to Bergmeyer [13] using the supernatants; aliquots of the supernatants were also quenched by perchloric acid and neutralized to determine ATP and creatine phosphate leakage.

Respiration rates. Oxygen consumption was determined polarographically with a Clark electrode (Yellow Springs Instruments) in a 1 ml stirred cuvette, thermostated at 20° C (Gilson). Stirring was performed at the lowest speed allowing a stable electrode signal. The shape and the viability of the cells were checked in the end of the oxygen consumption determination. 0.5 mg cell protein per ml were used. To determine the maximum oxygen consumption, ADP was added and the cells were lysed by digitonin [11,14].

Results

Morphological changes induced by high K/low Na medium

When the external K^+ concentration is increased from 2.5 to 72.5 mM and the external Na^+ concentration is decreased from 112.8 to 42.8 mM, an increased influx of Ca^{2+} is observed. Observation of the cells under light microscopy after addition of KCl to the suspension shows three stages of morphological change.

During the first minutes, the cells progressively contracted. At this stage, it was still possible to separate the spindle-like cells and the contracted ones by centrifugation on Percoll gradient (unpublished work). After 5 min, all the cells initially spindle-like appeared contracted as in Fig. 1B. The contracted state was stable for 1 h and finally the cells became either hypercontractured or

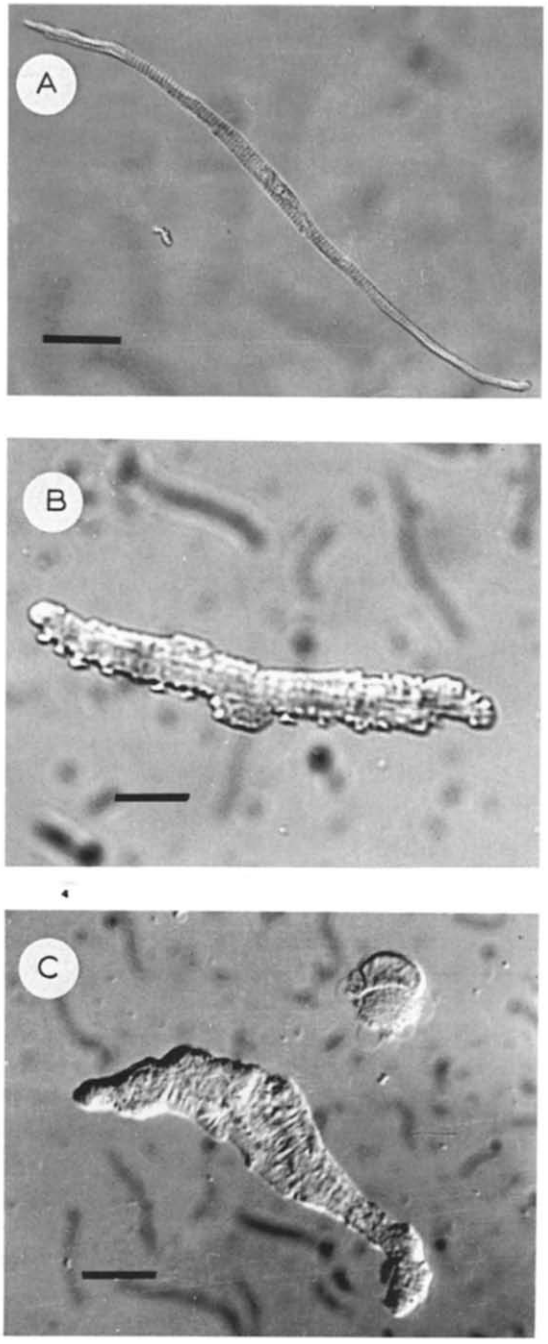

Fig. 1. Isolated myocytes from frog ventricles, observed by interference contrast optics (Nomarski). Effect of a high K/low Na medium. (A) A quiescent cell; the bar represents 35 μm. (B) A contractured viable cell, as observed between 5 and 60 min after incubation in a high K/low Na medium; the bar represent 10.5 μm. (C) An hypercontractured cell and a rounded one, as observed after incubation for 1 h in the high K/low Na medium; the bar represents 12 μm.

rounded as shown in Fig. 1C: during this last stage, the cells no longer excluded trypan blue.

In the contracted state, the sarcomere length was 1.7 ± 0.1 μm ± S.D. (20 determinations), instead of 2.2 ± 0.1 μm found in quiescent state [9]. These observations, obtained by observation with interference contrast optics, have been confirmed by electron microscopy (not shown). The stable contracted state was characterized by no loss of intracellular components, as shown by determination of lactate dehydrogenase activity and small metabolites in the supernates of silicone oil centrifugation [14]. After 1 h incubation in high K/low Na medium the apparition of hypercontracted cells is correlated with the increase of lactate dehydrogenase and small metabolites in the supernates of silicone oil centrifugation.

Metabolite concentrations and O_2 consumption

It had been previously shown [14] that the rate of O_2 consumption increased 6 times when beating was induced by either 5 mM KCl or by 0.5 mM $BaCl_2$. The O_2 consumption was compared in the present work for the quiescent, beating and contracted states from the same population of isolated cells (five determinations). The previous values for beating and resting cells was confirmed (Table I) and it was observed that the O_2 consumption was further increased to 90–99 natom per mg cell protein per min in the high K/low medium.

The concentration of ATP, ADP, creatine and creatine phosphate has been determined for aliquots of the cell suspension, after centrifugation through silicone oil of density 1.03 g/ml. The stability of the levels of the high energy phosphate for more than 5 h in quiescent and beating frog heart cells had already been observed [9]: during the time of the present experiments (within 1 h incubation) this stability was observed for the five cellular isolations and the values are indicated in Table I. For the cells incubated in high K/low Na medium, the aliquots taken after 60 min incubation were not taken into account. This was because there was a loss of ATP, ADP and creatine phosphate in the supernates of silicone oil centrifugation and furthermore because some rounded or hypercontracted cells with a decreased density, remained in the oil layer. The levels of the high-

TABLE I

METABOLIC CONTENTS AND O_2 CONSUMPTION OF ISOLATED FROG-HEART CELLS IN THE STATES: QUIESCENT, BEATING AND CONTRACTED

The values corresponded either to two or three determinations (metabolites) or to one determination (O_2 consumption), for five cellular isolations. They are expressed in nmol per mg cellular protein ± SEM for metabolic contents and in natom per mg protein and per min for the O_2 consumption. 1 mg cell protein corresponds to 2 mg dry wt, or 10 mg wet wt, and approx. to 6 µl cellular water (Refs. 16 and 9 and cited references therein). The maximum O_2 consumption determined after lysis by digitonin was 145 ± 8 natom · mg^{-1}. min^{-1} (three determinations).

Cells	ATP	CrP	ADP	Cr	ATP/ADP	CrP/C	O_2 consumption (natom/mg per min)
Quiescent	21.7 ± 1.1	23.1 ± 2.0	6.64 ± 0.67	11.2 ± 1.8	3.1 ± 0.4	2.5 ± 0.2	10–11
Beating	21.0 ± 2.1	20.7 ± 2.1	5.82 ± 0.67	9.8 ± 1.6	3.5 ± 0.4	2.1 ± 0.3	60–66
Contractured	19.9 ± 1.1	23.1 ± 2.5	5.64 ± 0.47	11.2 ± 1.3	3.2 ± 0.3	2.4 ± 1.0	90–99

energy phosphates obtained after 10, 20 and 30 min in high K/low Na medium were constant and their averaged values are indicated in Table I. The comparison of the values obtained for quiescent and beating or for quiescent and contracted cells (Student's *t*-test) indicated that the difference was not significant and mean values were (nmol per mg cell protein ± S.E.M.): ATP, 20.9 ± 1.5, CrP, 22.3 ± 2.2, ADP, 6.08 ± 0.67 and Cr 10.8 ± 1.8. The mean of the ratio ATP/ADP and creatine phosphate/creatine determined for each cellular preparation is also indicated in the table. This ratio provides an indication of the level of free ADP in the cytosolic compartment determined by the equilibrium of the creatine kinase reaction in the cytosol [14].

Discussion

Increased beating and contracture indicates an increased rate of ATP splitting which is correlated with an increased O_2 consumption. The results of the present study show that for isolated heart cells maintained in three different metabolic states the levels of the high-energy phosphates, ATP, ADP and creatine phosphate, are highly regulated. These results have been observed on isolated ventricular cells, an in vitro system where there are no cell-to-cell constraints and where the level of the work is very low. In contrast, in the working heart, as the level of work is increased from low to high, the level of ATP is constant but the creatine phosphate/creatine ratio is decreased [15,16]. This indicates an increase of free cytosolic ADP according to the creatine kinase equilibrium. During high K contractures of whole heart or papillary muscle and ventricular strips [4,7,8], there was a fall of creatine phosphate and a more or less important leakage of total adenine nucleotides. The mechanism of adenine nucleotide leakage is by formation of AMP by myokinase, deamination and dephosphorylation [16]. In isolated cells where the formation of ADP is not very high, this mechanism of leakage of adenosine nucleotides is not efficient.

Although the increase of free cytosolic ADP is low, as shown by the moderate decrease of the creatine phosphate/creatine ratio, the increase of O_2 consumption is high. The problem is to understand how oxidative phosphorylation is controlled in these cells.

Many studies of the control of oxidative phosphorylation have been performed on isolated heart mitochondria [17,18]. According to Doussière et al. [17], the membrane-bound ATPase and the respiratory chain are the major factors controlling the rate of oxidative phosphorylation; the relative importance of the adenine nucleotide carrier in this control depends on the mode of regeneration of ADP from ATP exported outside mitochondria. Bishop and Atkinson [18] demonstrated that in the regulation of oxidative phosphorylation both the ATP/ADP ratio and the concentration of phosphate are important. However, it is not very easy to extrapolate these observations to cellular behaviour. Most of the studies on isolated mitochondria have neglected an important component: the creatine phosphate-creatine kinase sys-

tem. This system is important in muscle cells, both to optimize the free-energy level of ATP hydrolysis and to facilitate the diffusion of ADP from myofilaments to mitochondria (see Ref. 19 and cited references). Furthermore, there is evidence of a limitation of diffusion of ADP toward adenine translocator which can be compensated for by the mitochondrial creatine kinase [20]. Unfortunately, the only study of control of oxidative phosphorylation by the extramitochondrial concentration of creatine and creatine phosphate [21] was performed in the presence of a very low adenylate concentration for which the control strength of the adenine carrier becomes noticeable [17].

In the present study, the buffering capacity of the cytosolic (and myofilament) creatine kinase is high enough to maintain almost stable cytosolic concentration of ATP, and mitochondrial creatine kinase is able to restore the levels of creatine phosphate and creatine. There was little increase in the level of free ADP as deduced from the small decrease in the level of creatine phosphate-to-creatine ratio. The flux of high-energy phosphate from mitochondria is increased, but the O_2 consumption in contracted cells is still below the maximum capacity of the cells (Table I and Ref. 16). In these conditions of low work, the level of the high-energy phosphates does not seem to be the signal controlling the rate of O_2 consumption.

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