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SUBSTRATE DEPENDENCE OF ENERGY METABOLISM IN ISOLATED GUINEA-PIG CARDIAC MUSCLE: A MICROCALORIMETRIC STUDY

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SUMMARY

1. The effects of glucose, pyruvate and lactate on basal metabolism and on contraction-related energy expenditure of thin trabeculae isolated from guinea-pig heart were studied using a microcalorimetric technique.

2. Resting heat rates of cardiac ventricular muscle measured in the presence of substrate-free solution (56 ± 20 mW (g dry weight)$^{-1}$), 10 mM-lactate (54 ± 12 mW (g dry weight)$^{-1}$) and 10 mM-glucose (63 ± 24 mW (g dry weight)$^{-1}$) did not differ significantly. Increasing the external glucose concentration (up to 100 mM) and/or adding insulin (up to 80 units l^{-1}) had virtually no effect on the measured resting heat rate.

3. With 10 mM-pyruvate as substrate resting heat rate was substantially larger (106 ± 40 mW (g dry weight)$^{-1}$) than with glucose, lactate or substrate-free solution. The concentrations of pyruvate producing a half-maximal increase in resting heat rate as compared to substrate-free solution ranged between 0·4 and 1·2 mM.

4. In order to test whether the development of an anoxic core contributed to the substrate dependence of resting heat production the critical P_{O_2} (i.e. the P_{O_2} that produced a just-noticeable decrease in heat rate) was determined in cylindrical preparations of various diameters. It was found that none of the preparations had an anoxic core at rest in a solution equilibrated with 100% oxygen.

5. From the dependence of the critical P_{O_2} on the diameter of the preparation the diffusion coefficient of oxygen through cardiac muscle was calculated using a modification of Hill's equation (Hill, 1928). The O_2 diffusion coefficient was found to be $1{\cdot}09 \times 10^{-5}$ cm^2 s^{-1}.

6. Contraction-related heat production was also found to be dependent on the substrate used. In the presence of 10 mM-pyruvate it was about 60% larger than in the presence of 10 mM-glucose, 10 mM-lactate or with substrate-free solution.

7. Isometric force of contraction showed the same substrate dependence as contraction-related heat production and increased with a similar time course during repetitive stimulation.

8. The possible mechanisms underlying the substrate dependence of myocardial energy metabolism are discussed. It is suggested that the increased energy expenditure observed in the presence of pyruvate may be related to a decrease in intracellular phosphate and/or to an increase in intracellular pH.

INTRODUCTION

It has been known for a long time that basal metabolism of cardiac muscle depends on the nature of the metabolic substrate supplied (Krebs, 1950). For example, in isolated cardiac muscle in the presence of pyruvate the rate of heat production was found to be increased by 60–80% when pyruvate was supplied as substrate instead of glucose (Chapman & Gibbs, 1974; Daut & Elzinga, 1988*b*). In isolated cardiomyocytes the rate of oxygen consumption was found to be increased by about 50% when glucose was replaced by lactate or palmitate (Montini, Bagby & Spitzer, 1981). In working isolated heart preparations it was found that in the presence of pyruvate the viablity and metabolic stability was improved (Bünger, Haddy, Querengässer & Gerlach, 1975; Bardenheuer & Schrader, 1983) and maximal developed left ventricular pressure and oxygen consumption were increased (Zweier & Jacobus, 1987).

The mechanisms underlying the pronounced effects of metabolic substrate on myocardial energy expenditure are still obscure. Two types of mechanisms have been invoked to explain the beneficial effect of pyruvate on contractility of various cardiac preparations. (i) The energy expenditure of contracting cardiac muscle may be substrate-limited. This is implied by the hypothesis that glucose alone may not be sufficient to supply the energy requirements of the heart (Bünger *et al.* 1975; Montini *et al.* 1981; Bardenheuer & Schrader, 1983; Gibbs & Kotsanas, 1986), or by the hypothesis that the regulatory mechanisms acting on glycolysis may be bypassed when pyruvate is supplied externally (Chance, Leigh, Kent, McCully, Nioka, Clark, Maris & Graham, 1986). (ii) On the other hand, one would expect the control of mitochondrial oxidative phosphorylation by the cytosolic phosphorylation potential, and/or by free cytosolic ADP, to keep the rate of respiration constant as long as the rate of ATP hydrolysis is unchanged (for review, see Hassinen, 1986). This means that the energy expenditure should be limited by the degree of activation of the various cytosolic ATPases. An increased metabolic rate in the presence of pyruvate must therefore be related to a (direct or indirect) stimulation of the rate of cytosolic ATP hydrolysis.

In order to get a better understanding of the factors contributing to the regulation of energy metabolism in the intact cardiac cell we studied the effects of glucose, pyruvate and lactate on resting heat production and contraction-related heat production of isolated guinea-pig ventricular muscle. Some of the results have been reported in preliminary form (Daut & Elzinga, 1987, 1988*d*, *e*).

METHODS

In the earlier experiments the dissecting procedure and the solutions were exactly as described in Daut & Elzinga (1988*b*). In the more recent experiments the following simplified dissection procedure was used. Guinea-pigs weighing 200–250 g were killed by a sharp blow on the head; the heart was quickly removed and rinsed in Tyrode solution (composition see below). Then the heart was submerged in oxygenated dissecting solution containing (mM): 60 NaCl; 60 K_2SO_4; 1 $CaCl_2$; 3 $MgSO_4$; 10 sodium pyruvate; 10 HEPES; the pH was adjusted to 6·8 with Tris. Several thin trabeculae were cut out quickly in this dissecting solution at room temperature and transferred to a small 'recovery chamber' (see below) containing modified Tyrode solution of the following composition (mM): 144 NaCl; 6 KCl; 2 $CaCl_2$; 1 $MgSO_4$; 1 NaH_2PO_4; 10 glucose; 10 HEPES. The

pH was adjusted to 7·4 by addition of Tris, the solution was equilibrated with 100% O_2 and the temperature was 37 °C. The results obtained with the two different dissection procedures were the same. In some experiments the external P_{O_2} was varied by equilibrating the superfusing solution with a known mixture of oxygen and nitrogen using a gas mixer (Gould). The corresponding P_{O_2} in the recording chamber was determined with an automatic acid–base laboratory (ABL 330, Radiometer).

The recording chamber consisted of a Perspex tube of 0·8 mm i.d. and 1·0 mm o.d. The tube had a length of 12 mm and was glued into a stainless-steel block. The stainless-steel block contained a 'recovery chamber' of 8 mm diameter which was used for introducing the preparations into the recording chamber. In the recovery chamber the contractility of the preparations was checked visually under the microscope by electrical stimulation.

The ends of a suitable trabecula were tied to two small platinum loops using a nylon thread of 10 μm thickness. The platinum loops were connected to two micrometer screws; they also served as stimulating electrodes. The dimensions of the preparation were measured carefully under the microscope at 50× magnification. By turning the two micrometer screws simultaneously the preparation was then pulled into the actual recording chamber. During the experiment the recovery chamber was sealed with a tightly fitting Teflon plug.

The Perspex tube was perfused at a rate of 1 μl s^{-1} by means of a suction pump. This corresponds to a velocity of the solution in the recording chamber of about 2 mm s^{-1}. The temperature difference between the upstream side and the downstream side of the preparation was measured with 2 × 6 constantan–chromel thermocouples. The rate of heat production of the preparation, $\dot{H}$ (W), was calculated from the equation

$$\dot{H} = \Delta T f C_h / y, \tag{1}$$

where ΔT is the temperature difference between the upstream and the downstream thermocouples (°C), f is the flow rate of the perfusing solution (cm^3 s^{-1}), C_h is the heat capacity of water (J $°C^{-1}$ cm^{-3}), and y is the yield of the system (dimensionless), i.e. the fraction of the applied heat that is actually recorded by the thermocouples. With improved thermal isolation of the system the yield of the recording system was 0·77, i.e. somewhat higher than in previous experiments (Daut & Elzinga, 1988*b*).

The distance between the stimulating electrodes and the ends of the preparation was about 6 mm so that the stimulating electrodes were always outside the actual temperature recording chamber. The magnitude of the stimulus artifact was tested by varying the duration of the stimulating current while keeping applied voltage constant. It was found that 3 Hz stimulation at just-subthreshold level did not produce any detectable heat signal. The amplified temperature signal was filtered at 1 Hz, unless otherwise indicated, before feeding it into the pen-recorder. Further details of the recording apparatus can be found in Daut & Elzinga (1988*b*).

In some experiments isometric force of contraction of thin ventricular trabeculae was measured. These measurements were carried out in a different chamber, which was also perfused with Tyrode solution pre-warmed to 37 °C. Stimulation was applied through platinum plate electrodes. The force transducer was built using a piezoresistive element (AE 801, Aksjeselskapet Mikroelektronikk, Horten, Norway).

All results are expressed as mean ± standard deviation. The number of preparations (n) from which the data are obtained is indicated in parentheses.

RESULTS

Substrate dependence of resting heat production

As a first step in investigating the nature of the substrate dependence of myocardial metabolism we studied the effects of glucose, pyruvate and lactate on resting heat production. The preparation was superfused with physiological salt solution containing 10 mM-pyruvate at a rate of 1 μl s^{-1} and the resting heat rate at 37 °C was determined as described previously (Daut & Elzinga, 1988*b*). Figure 1*A* shows that replacing 10 mM-pyruvate by 2 mM-glucose resulted in a decrease in heat rate by more than 50% within 3 min.

In order to test whether glucose uptake across the cell membrane was rate-limiting we increased glucose concentration and added insulin. Raising the glucose concentration to 100 mM or adding 80 units ml^{-1} insulin had virtually no effect on the heat rate measured in the steady state, as can be seen from Fig. 1*A* and *B*. Note,

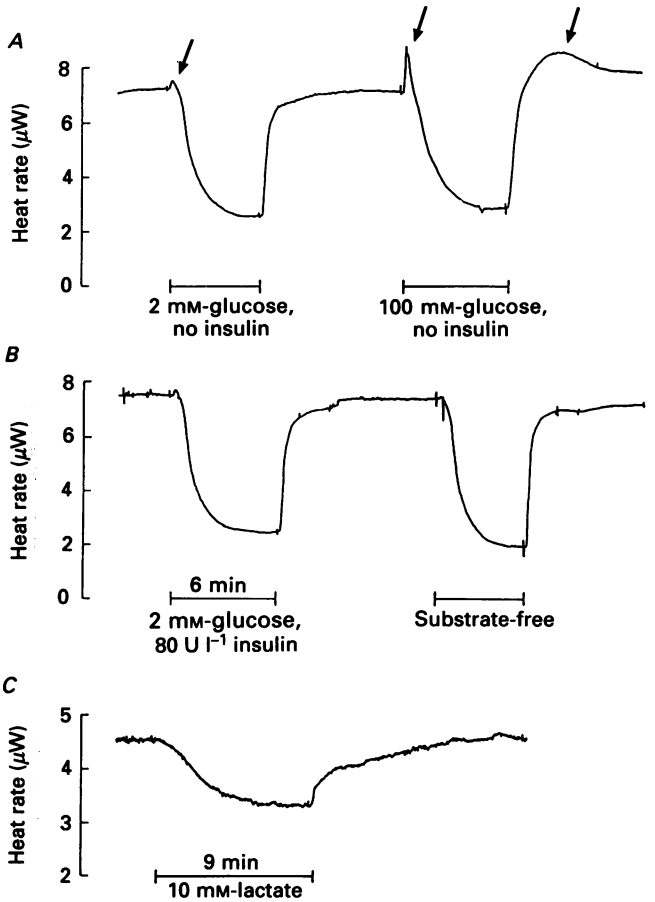

Fig. 1. *A*, effects of pyruvate, glucose, insulin and lactate on resting heat rate. *A*, change of substrate from 10 mM-pyruvate to 2 mM-glucose and to 100 mM-glucose. The arrows indicate the overshoot in heat rate recorded during the transition period when both substrates are expected to be present in the cells. *B*, same preparation as in *A*; change of substrate from 10 mM-pyruvate to 2 mM-glucose plus insulin, and to substrate-free solution. *C*, different preparation; change of substrate from 10 mM-pyruvate to 10 mM-lactate.

however, that during the transition period after switching the solution, when both substrates are expected to be present in the intracellular space (arrows), there was a transient increase in the rate of heat production. Increasing glucose in the presence of insulin was also ineffective (not shown). When pyruvate was replaced by substrate-free solution the measured resting heat rate was only a little smaller than with glucose as sole substrate (Fig. 1*B*).

The resting heat rate in the presence of lactate was also much lower than that

observed with pyruvate. Figure 1*C* shows a typical experiment in which a trabecula was exposed to 10 mM-lactate for 9 min. In this preparation resting heat rate decreased from 4·6 to 3·3 μW after switching from 10 mM-pyruvate to 10 mM-lactate. The average resting heat rate in this series of experiments was 106 ± 40 mW

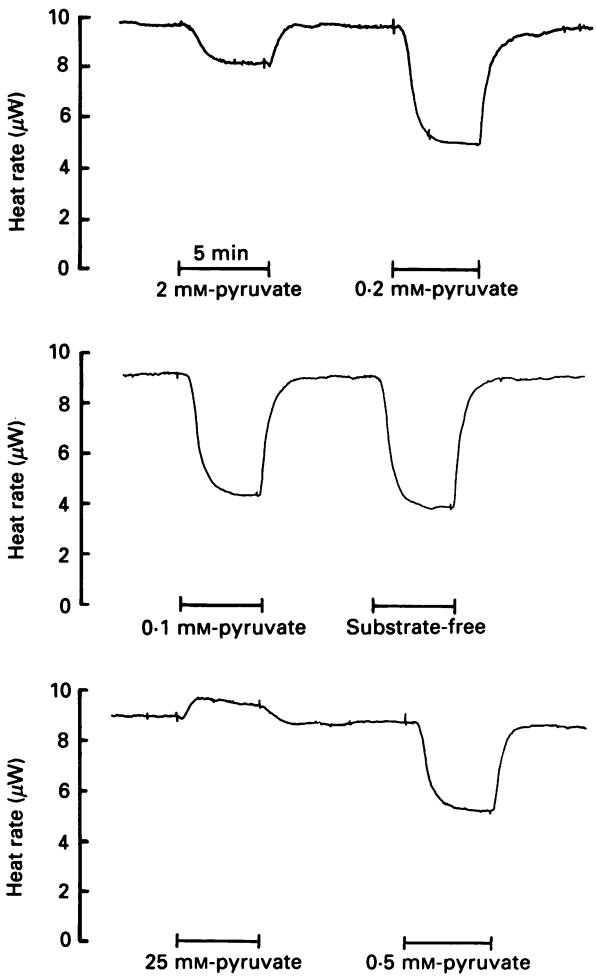

Fig. 2. Effects of changes in pyruvate concentration on resting heat rate. The control solution between the interventions contained 10 mM-pyruvate.

(g dry weight)$^{-1}$ with 10 mM-pyruvate (n = 14); 63 ± 24 mW (g dry weight)$^{-1}$ with 10 mM-glucose (n = 8); 54 ± 12 mW (g dry weight)$^{-1}$ with 10 mM-lactate (n = 4); and 56 ± 20 mW (g dry weight)$^{-1}$ with substrate-free solution (n = 8). To convert the measured resting heat rates to specific resting heat rates (mW cm^{-3}) we multiplied these figures with the fresh dry weight-to-volume ratio, which was 0·22 ± 0·02 g dry weight cm^{-3} (n = 10). The average specific resting heat rates with pyruvate, glucose, lactate and substrate-free solution were 23·3, 13·9, 11·9 and 12·3 mW cm^{-3}, respectively.

These data show that amongst the substrates tested only pyruvate caused a large rise in basal metabolism compared to substrate-free solution. The concentration dependence of the effects of pyruvate was determined as illustrated in Fig. 2. It can be seen that reduction of pyruvate from 10 mM to concentrations between 2 and 0·1 mM resulted in large decreases in the rate of heat production of the preparation.

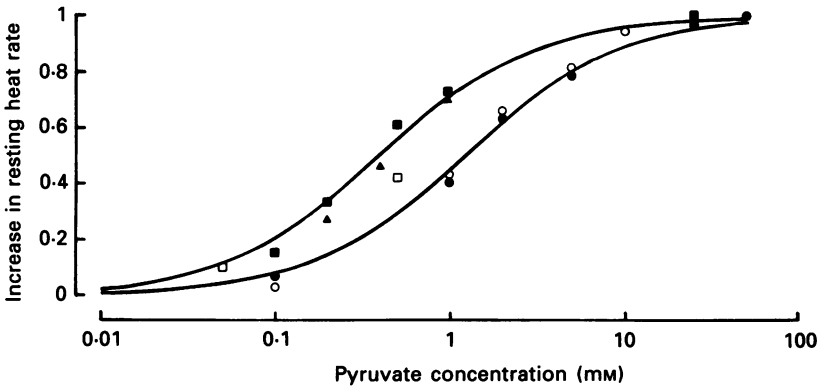

Fig. 3. Normalized dose–response curves of the effect of pyruvate on resting heat rate. The continuous lines were calculated from the equation $y = [\text{pyruvate}]/([\text{pyruvate}]+K_{0.5})$, where $K_{0.5}$, the pyruvate concentration giving a half-maximal effect, was 0·4 mM (left) and 1·2 mM (right).

Usually a new steady state was reached within 5 min. Even in the presence of 0·1 mM-pyruvate the resting heat rate recorded in the steady state was a little higher than in the presence of substrate-free solution (middle trace).

Figure 3 shows two typical dose–response curves of the effects of pyruvate on resting heat rate. The results were normalized, the heat rate measured in substrate-free solution was taken as zero and the maximal heat rate (measured with 25 or 50 mM-pyruvate) was taken as 1. It can be seen that the curves are sigmoid with a slope of about 1. The position of the curve on the abscissa was somewhat variable from one preparation to the next and seemed to depend on the diameter. In the two largest preparations (○ and ●, 300 μm diameter) the half-maximal increase in resting heat rate was observed with 1·2 mM-pyruvate (continuous line); in the smallest preparation (■, 140 μm diameter) the half-maximal effect was found with 0·4 mM-pyruvate (left-hand curve). The curves from the other preparations were in between these two extremes. Dose–response curves from very small preparations were difficult to obtain because of the relatively low signal-to-noise ratio.

Effects of external P_{O_2} on resting heat production

In cylindrical multicellular cardiac preparations there are always radial gradients of oxygen. In order to test whether the development of an anoxic core might affect the measured resting heat rate and whether this may influence the differences in heat rate seen with various substrates we studied the effect of external P_{O_2} on resting heat rate. The critical P_{O_2} which just produced an anoxic core was determined by varying external P_{O_2} stepwise between 0·03 and 0·95 atm. This was achieved by switching to

different solutions equilibrated with various mixtures of O_2 and N_2. The upper traces in Fig. 4 show such a determination of the critical P_{O_2} in the presence of 20 mM-glucose plus 2 mM-pyruvate. It can be seen that with 40% external P_{O_2} there was a just-noticeable decrease in resting heat rate and that the response became progressively larger when the preparation was exposed to 30, 20 and 0% O_2.

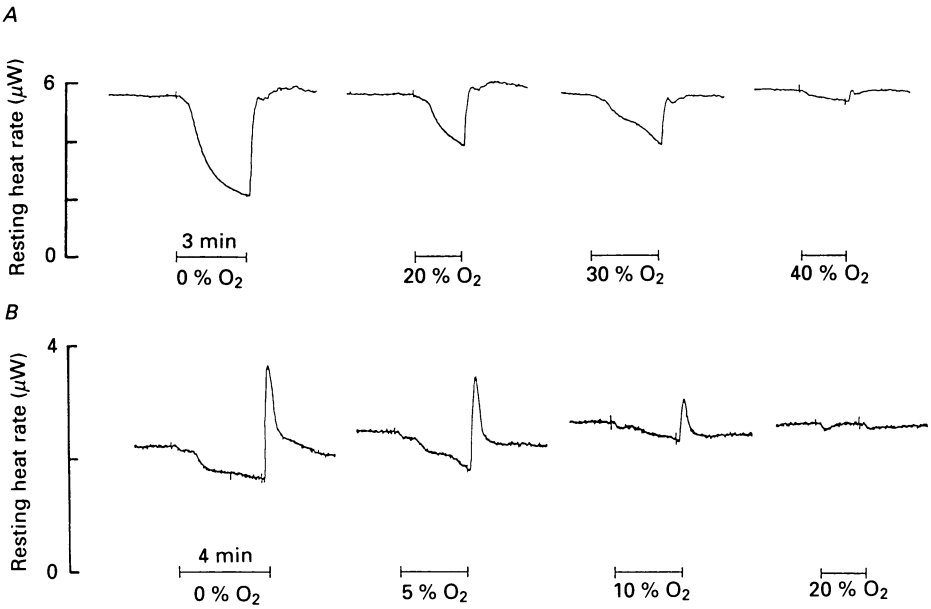

Fig. 4. Effects of different oxygen concentrations on resting heat rate. Normally the solution was equilibrated with 100% oxygen at 37 °C, which resulted in a P_{O_2} of 0·95 atm in the recording chamber (see Daut & Elzinga, 1988*b*). At the times indicated the superfusing solution was changed to solutions equilibrated with various mixtures of oxygen and nitrogen. In *A* the superfusate contained 20 mM-glucose plus 2 mM-pyruvate as substrate; in *B* it contained 20 mM-glucose, no pyruvate. *A* and *B* are from the same preparation.

The lower traces in Fig. 4 show a similar experiment carried out in a solution containing 20 mM-glucose as sole substrate. In contrast to the experiments in solutions containing pyruvate, there was a distinct overshoot after recovery from exposure to hypoxic solution. A similar overshoot was usually observed following recovery from hypoxia in substrate-free solution (not shown). In the thin preparation of Fig. 4 (diameter 220 μm) the external P_{O_2} had to be reduced to relatively low values to produce a response. With glucose as sole substrate there was no significant decrease in resting heat rate at a P_{O_2} of 20% (lower trace). In experiments of this kind we took the value in between the trace just showing a response (10% P_{O_2}) and the response showing no response (20% P_{O_2}), in this example 15%.

Hill's equation for radial diffusion of oxygen in a cylindrical preparation (Hill, 1928, 1965) can be written in the form:

$$\text{critical}\, P_{O_2} = (1/4K)r^2 \text{MVO}_2, \tag{2}$$

where the critical P_{O_2} (atm) is defined as the external P_{O_2} that would just lead to the development of an anoxic core in a cylindrical preparation consuming oxygen at a constant and homogeneous rate in all cells. K is the Krogh permeation constant for oxygen (ml O_2 cm^{-1} min^{-1} atm^{-1}), r is the radius of the preparation, and MVO_2 is the

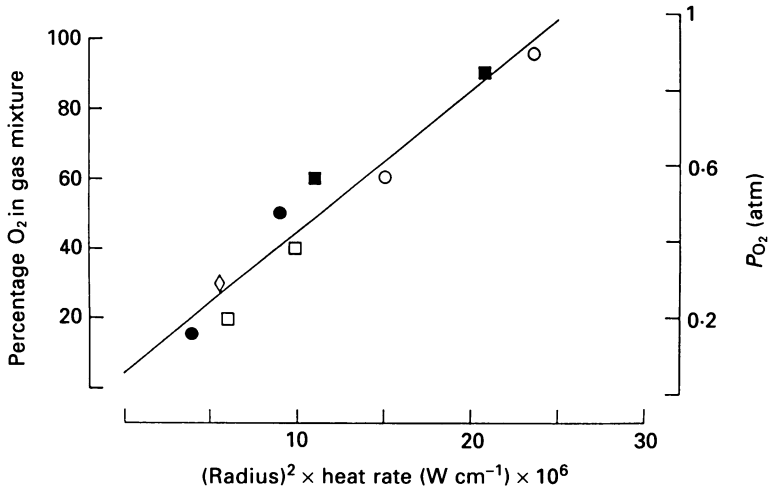

Fig. 5. Plot of nine determinations of critical P_{O_2} in five different preparations. In some preparations the critical P_{O_2} was determined in two different solutions as shown in Fig. 4. In these cases open and filled symbols of the same shape have been used. The straight line was calculated by linear regression through all the data points. It had a slope of 0·038 atm cm W^{-1}.

myocardial oxygen consumption (ml O_2 min^{-1} cm^{-3}). Using the energy equivalent of oxygen consumption (β = 20·3 J (ml O_2)$^{-1}$) and the solubility of oxygen in muscle at 37 °C (α = 0·0296 ml O_2 cm^{-3} atm^{-1}, see Mahler, Louy, Homsher & Peskoff, 1985) eqn (2) can be rewritten as:

$$\text{critical}\, P_{O_2} = \frac{1}{4\alpha\beta D} r^2 \dot{H}_s, \tag{3}$$

where $\dot{H}_s$ is the specific heat rate of the preparation (W cm^{-3}) and D is the diffusion constant of oxygen in cardiac muscle (cm^2 s^{-1}). Inspection of eqn (3) shows that a plot of critical P_{O_2} against $r^2\dot{H}_s$ should give a straight line with a slope of $1/(4\alpha\beta D)$, from which the diffusion constant of oxygen can be calculated.

Such a plot is shown in Fig. 5, where our determinations of the critical P_{O_2} in preparations with different specific heat rates and different radius have been compiled. We found all critical P_{O_2} values to be below 100% which suggests that the substrate effect was not influenced by the occurrence of an anoxic core. From the slope of the line fitted to the data of Fig. 5 a diffusion constant of $1{\cdot}09 \times 10^{-5}$ cm^2 s^{-1} was calculated. This agrees well with the diffusion constant of O_2 in muscle determined by others (see Discussion). The simplest interpretation of the results shown in Fig. 5 is that all of the heat produced by quiescent cardiac muscle preparations is indeed due to oxidation of substrates.

Substrate dependence of contraction-related heat production

In trying to measure contraction-related heat production it is important to know the speed of the response of the recording system. Figure 6*A* shows an experiment

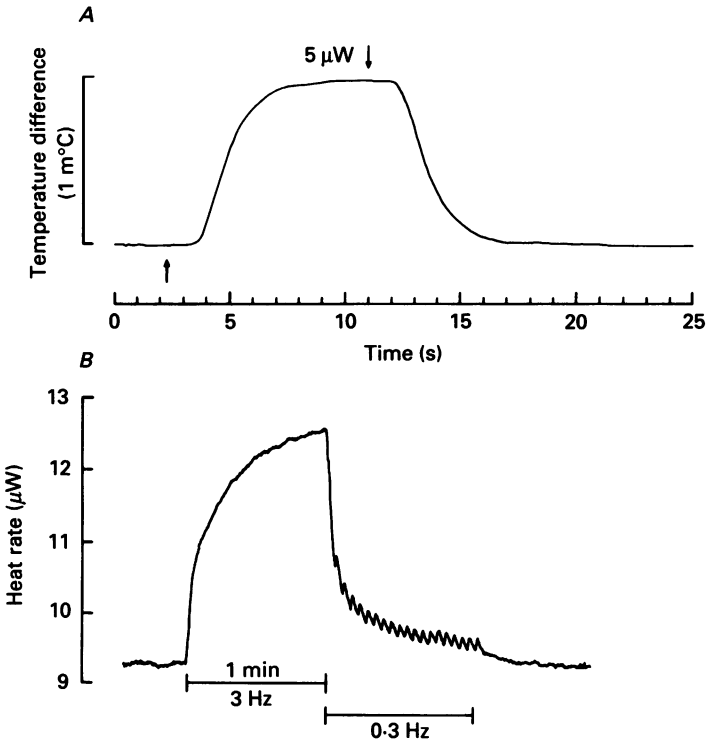

Fig. 6. The time resolution of the recording system. *A*, the temperature difference between the upstream and the downstream thermocouples produced by application of a 5 μW calibration pulse. The arrows indicate the points in time when the heating pulse was switched on and off. *B*, the change in heat rate produced by stimulation of the preparation through two platinum electrodes. Filter, −3 dB at 2 Hz. The substrate was 10 mM-pyruvate. After stimulation at a rate of 3 Hz for 3 min the stimulation rate was reduced to 0·3 Hz.

in which the time resolution of the system was analysed by applying a rectangular heating pulse through a small thermistor bead mounted in the middle of the recording chamber (see Daut & Elzinga, 1988*b*). After applying a heating pulse of 5 μW there was a delay of about 1 s before the voltage output at the thermocouples started to rise. This delay represents the time needed for the solution to move from the centre of the chamber to the downstream thermocouples. The heat signal reached 90% of its steady-state value within 1·5 s.

Figure 6*B* shows a measurement of the heat production induced by stimulation of a ventricular trabecula isolated from guinea-pig heart. Care was taken to ensure that the stimulating current did not make any contribution to the measured heat signal (see Methods). When the trabecula was stimulated at a rate of 3 Hz for 1 min, the

measured heat rate rose from 9·3 to 12·8 μW. When the contraction-related heat production had reached a steady state the rate of stimulation was reduced to 0·3 Hz. The heat rate dropped rapidly and the heat production of individual contractions, superimposed on a slow decay of heat rate, could be discriminated. After termination

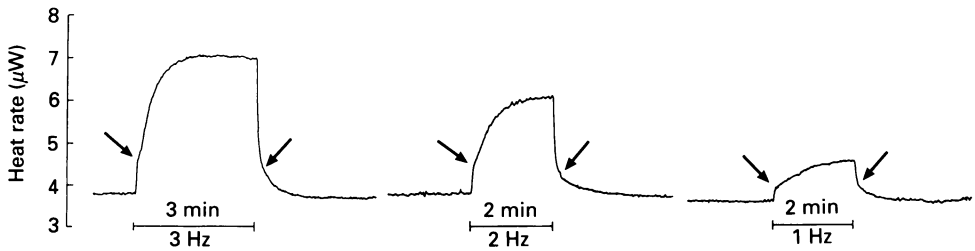

Fig. 7. The dependence of contraction-related heat production on stimulation rate. The substrate was 10 mM-pyruvate. The point of transition between the fast and the slow phase of the rise and fall in heat rate (which could be more clearly seen on a semilogarithmic plot) is indicated by arrows.

of stimulation the slow decay of heat rate continued. It is obvious from Fig. 6 that the time resolution of the recording system is much faster than this slow decay in heat rate.

Figure 7 shows the frequency dependence of contraction-related heat production. Four phases of contraction-related heat production could be discriminated (arrows): (i) A rapid rise of the rate of heat production at the beginning of stimulation. The speed of this phase is limited by the time resolution of the recording system. (ii) A slow rise of heat rate which continued for up to 3 min during stimulation. This phase became even slower when the stimulation rate was reduced to 2 or 1 Hz. The slow rise of heat rate is related to the slow phase of the positive staircase (see Fig. 9) and seems to be associated with a rise in intracellular Na^+ and Ca^{2+} (Cohen, Fozzard & Sheu, 1982; Lado, Sheu & Fozzard, 1982; Seibel, 1986). (iii) A rapid fall of heat rate at the end of stimulation. The speed of this phase is also limited by the frequency response of the recording system. (iv) A slow decay of heat rate after termination of stimulation. This phase represents the time course of recovery metabolism. It seemed to be independent of stimulation rate. The time constant of the final exponential phase was 20 ± 2 s ($n = 8$).

The magnitude of contraction-related heat production changed only little with time in the course of an experiment. This is illustrated in Fig. 8. On the left, the contraction-related heat production at the beginning of the experiment is shown. In the middle, the same stimulation of 3 Hz was repeated after 11 h of experimentation, during which the preparation was challenged with many different solutions. It can be seen that the amplitude of contraction-related heat production was only about 30% smaller and that the change of heat rate during and after stimulation had become somewhat slower. The experiments reported below were all carried out in the first 5 h after removal of the heart.

Figure 8 also illustrates the large difference between the heat production associated with high-K^+ contractures and the heat production associated with repetitive

contraction. The amplitude of the heat production during a contracture was markedly dependent on the size of the preparation. With the type of preparation normally used (250–350 μm diameter) the rate of heat production induced by a high-K^+ contracture (150 mM-K^+) was typically at least 4 times larger than the rate of

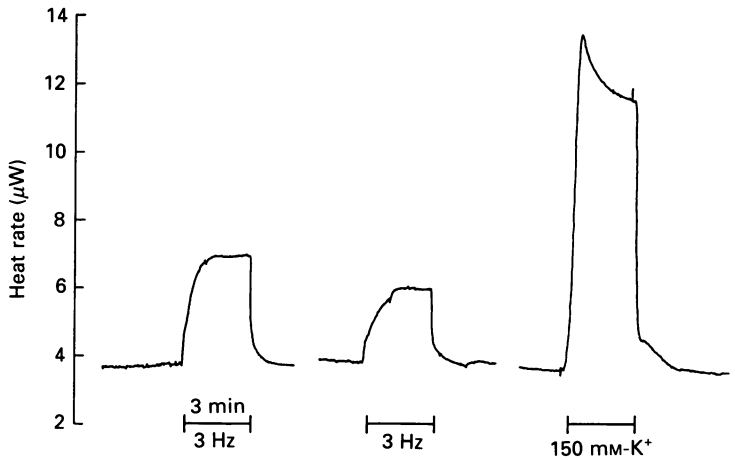

Fig. 8. The change of contraction-related heat production during the course of an experiment. The first record (left) was taken 1 h, and the second record (middle) was taken 12 h after removal of the heart. On the right the heat production due to a high-K^+ contracture in the same preparation (also 12 h after removal of the heart) is shown for comparison.

heat production induced by 3 Hz stimulation. It has been shown previously that the heat rate measured during a high-K^+ contracture is limited by the development of an anoxic core (Daut & Elzinga, 1988*b*).

The amplitude of contraction-related heat production was also dependent on the metabolic substrate used. Figure 9*A* shows an experiment in which the contraction-related heat production measured in the presence of 10 mM-glucose was compared to that in the presence of 10 mM-pyruvate. First, the preparation was stimulated at 3 Hz with 10 mM-glucose as substrate. Then the preparation was equilibrated with 10 mM-pyruvate for 15 min and the same stimulation was repeated. It can be seen that resting heat rate increased from 2·8 to 4·8 μW and that contraction-related heat production was almost doubled. Subsequently, the preparation was again equilibrated with 10 mM-glucose for 15 min and the 3 Hz stimulation was repeated. The re-control (right) was very similar to the original control run with 10 mM-glucose (left). On average, contraction-related heat production with 10 mM-pyruvate was increased by a factor of 1·6 compared to that observed with 10 mM-glucose ($n = 5$).

Figure 9*B* shows an experiment in which the isometric force of contraction was measured in a different preparation using the same protocol. When equilibrated with 10 mM-pyruvate (middle) the very thin trabecula produced 48% more tension than with 10 mM-glucose (left, right). The fast and the slow phase of the positive staircase were very similar to the two corresponding phases of contraction-related heat production depicted in Fig. 9*A*.

With 10 mM-lactate as substrate, contraction-related heat production and isometric tension had nearly the same magnitude as with 10 mM-glucose ($n = 3$). Surprisingly, contraction-related heat production in substrate-free solution was always found to be somewhat larger than that in the presence of 10 mM-glucose.

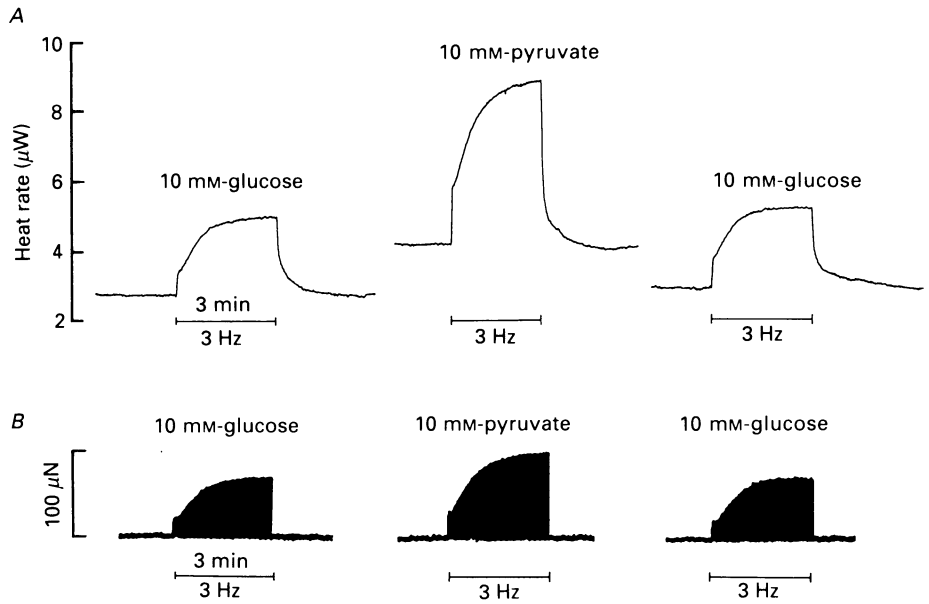

Fig. 9. Effects of pyruvate on contraction-related heat production and tension. *A*, contraction-related heat production with 10 mM-glucose (left, control), 10 mM-pyruvate (middle) and 10 mM-glucose (right, re-control); stimulation frequency 3 Hz. *B*, isometric force of contraction of a different trabecula superfused with 10 mM-glucose (left, control), 10 mM-pyruvate and 10 mM-glucose (right, re-control); stimulation frequency 3 Hz. Between measurements the preparation was allowed to equilibrate in the new solution for 15 min.

despite the fact that the resting heat rate was usually lower with substrate-free solution than with 10 mM-glucose in the same preparation (Fig. 1*B*). On average, contraction-related heat production was increased by 10% with substrate-free solution compared to that observed with 10 mM-glucose ($n = 4$).

DISCUSSION

The specific heat rate of resting guinea-pig ventricular muscle

With glucose as substrate, the rate of heat production of quiescent guinea-pig ventricular muscle was found to be 63 ± 24 mW (g dry weight)$^{-1}$. This is in reasonable agreement with our previous results (Daut & Elzinga, 1988*b*). However, the specific resting heat rate of 13·9 mW cm^{-3} of tissue in the presence of glucose reported here is substantially less than the value published in our previous study. The main reason for this is that our present value for the dry weight-to-volume ratio is 0·22, whereas previously a value of 0·28 was used.

This discrepancy is due to the fact that in the HEPES-buffered physiological salt solution the preparations shrink considerably during experiments lasting 6 h or longer. This loss of cellular water apparently does not affect the rate of heat production of the preparation (Fig. 4 of Daut & Elzinga, 1988*b*). In our previous study the volume of the preparation measured at the end of the experiment was used to calculate the dry weight-to-volume ratio. This means that our previous values for specific heat rate (Daut & Elzinga, 1988*b*) have to be multiplied by a factor of $0{\cdot}22/0{\cdot}28 = 0{\cdot}79$ to give the specific heat rate per cm^3 of freshly dissected cardiac muscle.

Even with maximal glucose and insulin concentration resting heat rate could not be significantly increased above the value recorded with 2 mM-glucose. This finding is in line with recent NMR measurements in isolated guinea-pig heart (Zweier & Jacobus, 1987) showing that insulin had no effect on intracellular high-energy phosphates or left ventricular developed pressure in the presence of 16·7 mM-glucose. The saturation of the effects of glucose might be due to the fact that one of the glycolytic reactions is rate-limiting at glucose concentrations above 2 mM.

The average resting heat rates measured with 10 mM-lactate and with substrate-free solution were not significantly different from the heat rate measured with 10 mM-glucose. However, all of these heat rates were significantly lower than the heat rate recorded with 10 mM-pyruvate as substrate (106 ± 40 mW (g dry weight)$^{-1}$). The increase of resting heat rate by a factor of 1·68 when switching from glucose to pyruvate is in good agreement with earlier calorimetric measurements by Chapman & Gibbs (1974).

The apparent dependence of the half-maximally effective pyruvate concentration on the diameter of the preparation may be due to the fact that radial gradients of pyruvate exist in larger preparations. In the isolated perfused heart and *in vivo* the half-maximal effect is probably reached with less than 0·5 mM-pyruvate, because diffusion distances between capillaries and cardiac muscle cells are small.

The diffusion constant of oxygen in cardiac muscle

To test whether all of the heat measured with glucose and pyruvate was due to substrate oxidation we calculated the diffusion constant of oxygen from the dependence of critical P_{O_2} on the radius of cylindrical trabeculae. The calculation was based on a solubility of oxygen (α) in muscle of 0·0296 ml O_2 cm^{-3} atm^{-1} at 37 °C (Mahler *et al.* 1985), which is larger than the estimate made by A. V. Hill (1965). Our value for the diffusion constant D ($1{\cdot}09 \times 10^{-5}$ cm^2 s^{-1}) corresponds to a permeation constant K (Krogh, 1919) of $1{\cdot}94 \times 10^{-5}$ ml O_2 cm^{-1} min^{-1} atm^{-1} ($K = D\alpha$). Our results are consistent with the values determined by different methods in hamster skeletal muscle ($D = 1{\cdot}2$–$2{\cdot}6 \times 10^{-5}$, Ellsworth & Pittman, 1984), frog skeletal muscle ($D = 1{\cdot}45 \times 10^{-5}$, Mahler *et al.* 1985), rat skeletal muscle ($K = 2{\cdot}2 \times 10^{-5}$, Kawashiro, Nüsse & Scheid, 1975) and chicken smooth muscle ($K = 2{\cdot}3 \times 10^{-5}$, DeKoning, Hoofd & Kreuzer, 1981).

The agreement of the diffusion constant we obtained with the values reported by others suggests that myoglobin contributes very little to oxygen transport in isolated trabeculae. This may be due to the fact that diffusion distances are much greater than for instance in the intact heart. Our findings that a plot of r^2 times specific heat

rate against critical P_{O_2} (Fig. 5) gives a straight line and that the calculated value of D is consistent with previous determinations suggest that in the presence of glucose and/or pyruvate all of the heat production is due to oxidation of substrates. Therefore we consider it unlikely that the development of an anoxic core affected the substrate dependence of resting heat production.

Using Hill's equation the critical radius (which would just lead to the development of an anoxic core in a cylindrical preparation) is calculated to be 330 μm in the presence of 10 mM-extracellular pyruvate at an external P_{O_2} of 720 mmHg. This confirms the conclusion that none of the preparations used in the present investigation had an anoxic core at rest (see also Fig. 5 of Daut & Elzinga, 1988*b*). Using our value for D a critical heat rate of 110 mW cm^{-3} is calculated for a typical trabecula of 300 μm diameter. This suggests that our measurements of contraction-related heat production are not distorted by anoxia at the core of the preparation.

The cellular mechanisms underlying the substrate dependence of energy metabolism

We have shown that both resting metabolism and contraction-related metabolism are stimulated when pyruvate is used as substrate. The increase in energy expenditure must ultimately be related to an increase in the activity of the major intracellular ATPases and to the concomitant increase in the rate of ATP synthesis. A large contribution of the Na^+,K^+-ATPase is unlikely, since we have recently found that blockage of the Na^+–K^+ pump reduces resting heat rate only by 5–10% (Daut & Elzinga, 1988*c*). In the resting preparation the transmembrane Ca^{2+} fluxes are also likely to be small. Therefore, the main candidate for the enzyme responsible for the substrate dependence of energy metabolism is the actomyosin ATPase.

Intracellular pH

One possible explanation for our results is that pyruvate may cause a rise in intracellular pH. Intracellular alkalinization leads to a shift of the pCa–tension relation towards lower Ca^{2+} concentrations (Fabiato & Fabiato, 1978), probably due to increased binding of Ca^{2+} to troponin (Blanchard & Solaro, 1984). This could give rise to an increased rate of ATP hydrolysis by the actomyosin ATPase.

As far as resting heat production is concerned such a mechanism is unlikely, since application of pyruvate would be expected to produce a transient intracellular acidosis due to pyruvic acid entering the cell in undissociated form. Indeed, recent experiments by Blatter & McGuigan (1988) using pH-sensitive microelectrodes have shown that switching from glucose to pyruvate produces intracellular acidification in ferret cardiac muscle. In our experiments no transient decrease in heat rate upon switching from pyruvate to glucose was observed. Furthermore, we have recently studied the effects of intracellular pH on heat production in guinea-pig ventricular muscle (Daut & Elzinga, 1988*a*). Application of 10 mM-NH_4Cl, which would be expected to change intracellular pH by at least 0·1 unit, produced only a 10% change in resting heat rate. Thus basal metabolism seems to be relatively insensitive to changes in intracellular pH.

In the case of contraction-related heat production a contribution of intracellular pH to the effects of pyruvate cannot be ruled out. Bountra, Kaila & Vaughan-Jones (1988) have shown that in cardiac Purkinje fibres trains of impulses lead to an

intracellular acidification. A similar frequency-dependent intracellular acidification has been found in a ^{31}P-NMR study on Langendorff-perfused ferret hearts (Allen, Elliot & Smith, 1986). This acidification was abolished when glycolysis was prevented by glycogen depletion and removal of glucose. If the decrease in intracellular pH was caused by lactate production through anaerobic glycolysis, it might be argued that the frequency-dependent acidification is absent with pyruvate as substrate and that this explains the larger force and heat production.

It is possible that in isolated guinea-pig ventricular muscle there is also a frequency-dependent intracellular acidification. However, there are several findings which argue against a major contribution of changes in pH_i to the effects of glucose, pyruvate and lactate on energy metabolism reported here. (i) The heat measurements with varying oxygen concentrations suggest that our preparations had no anoxic core at low rates of stimulation. Whereas in isolated whole hearts perfused with physiological salt solution hypoxic regions producing lactate may exist, we consider it unlikely that lactate production caused by hypoxia could have caused a significant change in pH_i in our experiments. (ii) When substrate was changed from glucose to pyruvate during *continuous* stimulation the same total change in heat rate was observed as in the experiments reported above, where the solution change was usually carried out in between stimulation periods. No transient decrease in heat rate was found after switching to pyruvate during stimulation at 2 or 3 Hz. (iii) External application of lactate would also be expected to bypass glycolysis and thus to prevent intracellular acidification in the steady state. No major change in heat rate was observed when switching from 10 mM-glucose to 10 mM-lactate in quiescent or in continuously stimulated preparations.

Although we cannot exclude that a stimulation-dependent change in intracellular pH may be influenced by different substrates we consider it unlikely that this is the principal mechanism underlying the 60% increase in contraction-related heat production in the presence of pyruvate.

Intracellular phosphate

Another possible explanation for our results is that the effects of pyruvate may be mediated by a change in inorganic phosphate (P_i). Inorganic phosphate is one of the main factors determining the force produced by the actomyosin ATPase, probably by a direct action on the contractile machinery. As P_i increases maximal force is depressed and the pCa–tension relation is shifted to higher Ca^{2+} concentrations (Reiermann, Herzig & Rüegg, 1977; Kentish, 1986). The combination of these effects may cause a relatively large change in twitch tension and resting tension.

The possible link between substrate supply and P_i can best be explained by referring to a simplified scheme of the regulation of energy metabolism in myocardial cells (Fig. 10). We subdivide the control of oxidative phosphorylation in three stages: (i) Control by modulation of substrate supply ('substrate control'). This includes all processes contributing to the supply of NADH to the mitochondrial matrix, e.g. substrate transport across the sarcolemma and across the inner mitochondrial membrane, glycolysis, the pyruvate dehydrogenase complex and the citric acid cycle. (ii) Control of ATP synthesis at the inner mitochondrial membrane ('phosphorylation

control') by the thermodynamic driving force, i.e. by the difference between the redox potential available for synthesizing one molecule of ATP and the free energy of cytosolic ATP hydrolysis (see Gyulai, Roth, Leigh & Chance, 1985; Daut, 1987).

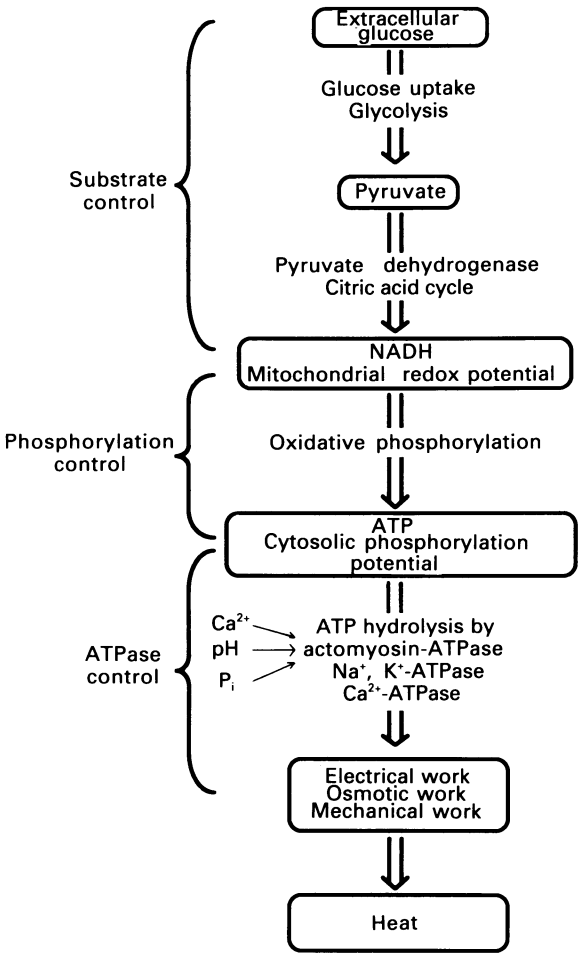

Fig. 10. Schematic diagram of some of the factors contributing to the regulation of energy metabolism in cardiac muscle.

This includes the respiratory chain, the F_0F_1-ATPase, the adenine nucleotide translocase and the phosphate translocase. (iii) Control of cytosolic ATP hydrolysis ('ATPase control'). This includes all factors modulating the rate of ATP hydrolysis in the cytoplasm, in particular the concentrations of free cytosolic Ca^{2+}, P_i and H^+.

The hypothetical sequence of events leading to an increase in heat rate with externally supplied pyruvate could then be envisaged as follows. (i) The rate-limiting steps contributing to substrate control (e.g. phosphofructokinase) are bypassed when pyruvate is supplied externally (Bishop & Atkinson, 1984). (ii) The NADH/NAD^+ redox potential in the mitochondria is increased by excess pyruvate (Kobayashi & Neely, 1983). (iii) Hence, the cytosolic phosphorylation potential is increased and

inorganic phosphate (P_i) is reduced (see e.g. Zweier & Jacobus, 1987; Koretsky & Balaban, 1987; Katz, Koretsky & Balaban, 1988). (iv) As a consequence, the tension (and the heat rate) at a given Ca^{2+} concentration are increased (Kentish, 1986). This means that ATPase control and substrate control may be linked through the change in P_i.

As far as substrate-dependent changes of P_i in resting cardiac muscle and their effect on metabolism are concerned there is little direct experimental evidence available, apart from the finding of Kentish (1986) that in the presence of 200 nM-Ca^{2+} the resting force of skinned rat cardiac trabeculae was usually decreased after increasing P_i from 0 to 2 mM (his Fig. 1). There are also some measurements of the effects of P_i on the ATPase activity of skinned cardiac and skeletal muscle fibres (Herzig, Peterson, Rüegg & Solaro, 1981; Altringham & Johnston, 1985; Kawai, Güth, Winnikes, Haist & Rüegg, 1987). These studies have shown that force and cardiac actomyosin ATPase activity do not necessarily change in parallel. Unfortunately, all of these measurements of ATPase activity have been carried out with saturating Ca^{2+} concentrations and therefore cannot be easily applied to our experimental conditions.

The substrate-dependent changes in P_i measured in isolated Langendorff-perfused hearts are consistent with the hypothesis outlined above. Recent ^{31}P-NMR measurements in both rat (Flaim, Kochel, Kira, Kobayashi, Fossel, Jefferson & Morgan, 1983; Katz *et al.* 1988) and guinea-pig heart (Zweier & Jacobus, 1987) show that, at least in the beating myocardium, switching from glucose to pyruvate produces a pronounced decrease in P_i.

Substrate control could also be present with 10 mM-lactate as sole substrate, due to the fact that the cardiac type of lactate dehydrogenase is subject to pronounced product inhibition, i.e. cardiac lactate dehydrogenase is strongly inhibited by low concentrations of pyruvate. The presence of substrate control through phosphofructokinase and lactate dehydrogenase would have the advantage of preventing intracellular accumulation of metabolic intermediates (e.g. pyruvate) under low-work conditions.

In conclusion, it is not yet clear to what extent the hypothetical mechanisms described above may be operative in intact cardiac muscle. It cannot be ruled out that mediators other than H^+ or P_i are involved in the modulation of myocardial energy metabolism by different substrates.

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REFERENCES

Allen, D. G., Elliott, A. C. & Smith, G. L. (1986). Intracellular acidosis on increasing the frequency of stimulation in isolated ferret hearts is partly attributable to increased lactic acid production. *Journal of Physiology* **377**, 113*P*.

Altringham, J. D. & Johnston, I. A. (1985). Effects of phosphate on the contractile properties of fast and slow muscle fibres from an antarctic fish. *Journal of Physiology* **368**, 491–500.

BARDENHEUER, H. & SCHRADER, J. (1983). Relationship between myocardial oxygen consumption, coronary flow, and adenosine release in an improved isolated working heart preparation of guinea pigs. *Circulation Research* **51**, 263–271.

BISHOP, P. D. & ATKINSON, D. E. (1984). Adenine nucleotide control of the rate of oxygen uptake by heart mitochondria over a 15 to 20-fold range. *Archives of Biochemistry and Biophysics* **230**, 335–344.

BLANCHARD, E. M. & SOLARO, R. J. (1984). Inhibition of the activation and troponin calcium binding of dog cardiac myofibrils by acidic pH. *Circulation Research* **55**, 382–391.

BLATTER, L. A. & MCGUIGAN, J. A. S. (1988). Effects of metabolic substrates on intracellular pH in ferret ventricle. *Biophysical Journal* **53**, 165a (abstract).

BOUNTRA, C., KAILA, K. & VAUGHAN-JONES, R. D. (1988). Effect of repetitive activity upon intracellular pH, sodium and contraction in sheep cardiac Purkinje fibres. *Journal of Physiology* **398**, 341–360.

BÜNGER, R., HADDY, F. J., QUERENGÄSSER, A. & GERLACH, E. (1975). An isolated guinea pig heart preparation with in vivo like features. *Pflügers Archiv* **353**, 317–326.

CHANCE, B., LEIGH, J. S., KENT, J., MCCULLY, K., NIOKA, S., CLARK, B. J., MARIS, J. M. & GRAHAM, T. (1986). Multiple controls of oxidative metabolism in living tissues as studied by phosphorus magnetic resonance. *Proceedings of the National Academy of Sciences of the USA* **83**, 9458–9462.

CHAPMAN, J. B. & GIBBS, C. L. (1974). The effect of metabolic substrate on mechanical activity and heat production in papillary muscle. *Cardiovascular Research* **8**, 656–667.

COHEN, C. J., FOZZARD, H. A. & SHEU, S.-S. (1982). Increase in intracellular sodium ion activity during stimulation in mammaliam cardiac muscle. *Circulation Research* **50**, 651–662.

DAUT, J. (1987). The living cell as an energy-transducing machine. A minimal model of myocardial metabolism. *Biochimica et biophysica acta* **895**, 41–62.

DAUT, J. & ELZINGA, G. (1987). Oxygen dependence of heat produced by quiescent guinea-pig ventricular muscle. *Journal of Physiology* **392**, 39*P*.

DAUT, J. & ELZINGA, G. (1988*a*). Effects of intracellular pH on energy expenditure of isolated cardiac ventricular muscle. *Pflügers Archiv* **412**, suppl. 1, R29.

DAUT, J. & ELZINGA, G. (1988*b*). Heat production of quiescent ventricular trabeculae isolated from guinea-pig heart. *Journal of Physiology* **398**, 259–275.

DAUT, J. & ELZINGA, G. (1988*c*). Heat production of the sodium pump in guinea-pig cardiac muscle. *Pflügers Archiv* **411**, suppl. 1, R12.

DAUT, J. & ELZINGA, G. (1988*d*). Substrate dependence of contraction-related heat production in isolated guinea-pig ventricular muscle. *Journal of Physiology* **407**, 125*P*.

DAUT, J. & ELZINGA, G. (1988*e*). Substrate-induced alterations of heat production in guinea-pig ventricular muscle. *Journal of Molecular and Cellular Cardiology* **20**, suppl. IV, S54.

DEKONING, J., HOOFD, L. J. C. & KREUZER, F. (1981). Oxygen transport and the function of myoglobin. Theoretical model and experiments in chicken gizzard smooth muscle. *Pflügers Archiv* **389**, 211–217.

ELLSWORTH, M. L. & PITTMAN, R. N. (1984). Heterogeneity of oxygen diffusion through hamster striated muscles. *American Journal of Physiology* **246**, H161–167.

FABIATO, A. & FABIATO, F. (1978). Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. *Journal of Physiology* **276**, 233–255.

FLAIM, K. E., KOCHEL, P. J., KIRA, Y., KOBAYASHI, K., FOSSEL, E. T., JEFFERSON, L. S. & MORGAN, H. E. (1983). Insulin effects on protein synthesis are independent of glucose and energy metabolism. *American Journal of Physiology* **245**, C133–143.

GIBBS, C. L. & KOTSANAS, G. (1986). Factors regulating basal metabolism of the isolated perfused rabbit heart. *American Journal of Physiology* **250**, H998–1007.

GYULAI, L., ROTH, Z., LEIGH JR, J. S. & CHANCE, B. (1985). Bioenergetic studies of mitochondrial oxidative phosphorylation using ^{31}phosphorus NMR. *Journal of Biological Chemistry* **260**, 3947–3954.

HASSINEN, I. E. (1986). Mitochondrial respiratory control in the myocardium. *Biochimica et biophysica acta* **853**, 135–151.

HERZIG, J. W., PETERSON, J. W., RÜEGG, J. C. & SOLARO, R. J. (1981). Vanadate and phosphate ions reduce tension and increase cross-bridge kinetics in chemically skinned heart muscle. *Biochimica et biophysica acta* **672**, 191–196.

HILL, A. V. (1928). The diffusion of oxygen and lactic acid through tissues. *Proceedings of the Royal Society* B **104**, 35–96.

HILL, A. V. (1965). *Trails and Trials in Physiology*. London: Arnold.

KATZ, L. A., KORETSKY, A. P. & BALABAN, R. S. (1988). Activation of dehydrogenase activity and cardiac respiration: a ^{31}P-NMR study. *American Journal of Physiology* **255**, H185–188.

KAWAI, M., GÜTH, K., WINNIKES, K., HAIST, C. & RÜEGG, J. C. (1987). The effects of inorganic phosphate on the ATP hydrolysis rate and the tension transient in chemically skinned rabbit psoas fibres. *Pflügers Archiv* **408**, 1–9.

KAWASHIRO, T., NÜSSE, W. & SCHEID, P. (1975). Determination of diffusivity of oxygen and carbon dioxide in respiring tissue: results in rat skeletal muscle. *Pflügers Archiv* **359**, 231–251.

KENTISH, J. C. (1986). The effects of inorganic phosphate and creatine phosphate on force production in skinned fibres from rat ventricle. *Journal of Physiology* **370**, 585–604.

KOBAYASHI, K. & NEELY, J. R. (1983). Mechanism of pyruvate dehydrogenase activation by increased cardiac work. *Journal of Molecular and Cellular Cardiology* **15**, 369–382.

KORETSKY, A. P. & BALABAN, R. S. (1987). Changes in pyridine nucleotide levels alter oxygen consumption and extra-mitochondrial phosphates in isolated mitochondria: a ^{31}P-NMR and NAD(P)H fluorescence study. *Biochimica et biophysica acta* **893**, 398–408.

KREBS, H. A. (1950). Body size and tissue respiration. *Biochimica et biophysica acta* **4**, 249–269.

KROGH, A. (1919). The rate of diffusion of gases through animal tissues, with some remarks on the coefficient of invasion. *Journal of Physiology* **52**, 391–408.

LADO, M. G., SHEU, S.-S. & FOZZARD, H. A. (1982). Changes in intracellular Ca^{2+} activity with stimulation in sheep cardiac Purkinje strands. *American Journal of Physiology* **243**, H133–137.

MAHLER, M., LOUY, C., HOMSHER, E. & PESKOFF, A. (1985). Reappraisal of diffusion, solubility, and consumption of oxygen in frog skeletal muscle, with applications to muscle energy balance. *Journal of General Physiology* **86**, 105–134.

MONTINI, J., BAGBY, G. J. & SPITZER, J. J. (1981). Importance of exogeneous substrates for the energy production of adult rat heart myocytes. *Journal of Molecular and Cellular Cardiology* **13**, 903–911.

REIERMANN, H. J., HERZIG, J. W. & RÜEGG, J. C. (1977). Ca^{2+} activation of ATPase activity, ATP–P_i exchange and tension in briefly glycerinated heart muscle. *Basic Research in Cardiology* **72**, 133–139.

SEIBEL, K. (1986). The slow phase of the staircase in guinea-pig papillary muscle, influence of agents acting on transmembrane sodium flux. *Naunyn-Schmiedeberg's Archives of Pharmacology* **334**, 92–99.

ZWEIER, J. L. & JACOBUS, W. E. (1987). Substrate-induced alterations of high energy phosphate metabolism and contractile function in the perfused heart. *Journal of Biological Chemistry* **262**, 8015–8021.