Oxidative Phosphorylation of Cardiac Mitochondria and Contraction of Glycerol-treated Fibers of Psoas Muscle

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In this paper are reported observations on a system in which adenosine diphosphate and orthophosphate are synthesized into adenosine triphosphate by a respiring mitochondrial suspension supplied with glutamate. The ATP thus synthesized is available to a glycercinated muscle fiber which, upon developing isometric tension, hydrolyzes the ATP back to ADP and P. The hydrolysis products are then available once more to the mitochondria (cf. (1)). The oxygen consumption of the mitochondria is followed polarographically, and the tension of the fiber is recorded by a strain gauge.

This compound system can be viewed in various ways. It is, for example, a crude reconstruction of the situation in the living muscle fiber, and its behavior reveals something about the availability of mitochondrial ATP to fibers. On the other hand, it is a new, self-regulating ATP “feeder” system, differing from earlier described feeder systems, such as creatine kinase (2) and pyruvate kinase (3), in that it is truly cyclic.

EXPERIMENTAL PROCEDURE

Glycerol-treated fibers of rabbit psoas were prepared according to Szent-Györgyi (4) and stored in 50% glycerol-water at about \(-10^\circ\) for 9 to 11 months. For an experiment, a fiber bundle of 100 \(\sim\) 150 \(\mu\) in diameter and about 1 cm in length was removed from the stock fiber bundle, fixed on a thin glass hook with acetone-diluted Duco cement, and washed with a solution of 0.1 \(M\) KCl buffered with 0.05 \(M\) Tris-maleic acid-KOH (pH 7.0) for 1 to 2 days at \(0^\circ\).

Rabbit heart mitochondria (sarcosomes) were prepared in a sucrose isolation medium containing 0.32 \(M\) sucrose and 0.001 \(M\) ethylenediamine-tetraacetate adjusted to pH 7.0 with NaOH according to the method of Cleland and Slater (5). However, ethylenediaminetetraacetate had to be removed from the preparation, since it is known to have a relaxing effect on glycercinated fibers (6). The preparation of mitochondria was washed once with 0.32 \(M\) sucrose alone and was finally suspended in a small volume of 0.32 \(M\) sucrose (10 to 20 mg of protein per ml). Mitochondria were used in experiments within 4 hours after preparation. During this time interval, the preparations retain their “tightly coupled” phosphorylative respiration (7, 8).

The general composition of the reaction medium used in experiments was 0.087 \(M\) KCl, 0.017 \(M\) phosphate buffer (pH 7.0), 0.088 \(M\) sucrose, 0.01 \(M\) sodium glutamate, various concentrations of ADP (pH 7.0), and mitochondria (1 to 6 mg per ml). Isometric tension was recorded by a system consisting of a Statham strain gauge transducer, a Millivac voltmeter, and a Rectiriter recorder. The dimensions of the fiber bundle were measured by means of an American Optical dissecting microscope (40 \(\times\)) micrometer. The cross-sectional area was calculated from the diameter, assuming a cylinder of circular cross section. Tension developed is thus expressed in kg per cm\(^2\) of the cross-sectional area. However, it has been noticed that differences in the cross-sectional shape of fiber bundles make the value in kg per cm\(^2\) quite variable. Therefore, in most cases, tension is expressed as the percentage of the “standard” tension, i.e. the tension developed upon addition of 5 \(mM\) ATP, 5 \(mM\) MgSO\(_4\), and 0.5 \(mM\) CaCl\(_2\) to the reaction medium described above. The rate of tension development is expressed by the reciprocal of the time in minutes which it would have taken to reach the standard ATP-Mg-Ca contraction level if the initial rate of tension development had continued. The rate is measured either from the slope of the tangent line drawn directly on the record or, in some cases, from the slope in the plot of the log of tension against time.

The platinum electrode technique (9) was employed for measurement of oxygen utilization and oxidative phosphorylation (10), by means of control circuits, a Millivac voltmeter, and a Rectiriter recorder. The oxygen consumption is expressed in micromoles of O\(_2\) per liter (\(\mu\)M O\(_2\)) calculated on the assumption that the air-saturated medium contains 240 \(\mu\)moles of O\(_2\) per liter at 25\(^\circ\), pH 7.0.

The temperature of the system was maintained at 25\(^\circ\) by circulation from a constant temperature water bath, and the reaction mixture was stirred by a magnetic stirring device.

RESULTS

1. ADP-induced Tension in Presence of Mitochondria—The fibers employed in the present work contained some myokinase, since the addition of 5 \(mM\) ADP plus 1 \(mM\) MgSO\(_4\) to the medium evoked tension in the absence of mitochondria or glutamate. Addition of the ADP without Mg\(^{++}\), however, was ineffective, since myokinase is Mg\(^{++}\)-activated. On the other hand, addition of ADP without Mg\(^{++}\) was effective when mitochondria were present.

The latter (from the logarithmic plot) usually gives a higher value than the former (directly from the record).
well as glutamate and oxygen were present. Therefore, it is clear that the ATP produced from the ADP (and the P of the medium) through oxidative phosphorylation was the actual agent effective in inducing tension (cf. Fig. 2).

2. Mitochondrial Respiration in Presence and Absence of Glycerol-treated Fibers—Less time lag was observed in respiratory acceleration when glutamate was added first and ADP was added last than when ADP was added first and glutamate last. Since the former is also the circumstance in which respiration and phosphorylation can best be separated, the former became the preferred order of addition in all subsequent experiments.

Respiration in the absence of a fiber bundle is illustrated in Fig. 1. The experiment was begun by mixing 2.6 ml of a medium (0.1 M KCl, 0.05 M sucrose, and 0.02 M phosphate buffer, pH 7.0) with 0.4 ml of a suspension of mitochondria (15 mg of protein per ml) in 0.33 M sucrose. At the first arrow, 30 μmoles of glutamate (in 0.03 ml; approximate final concentration, 10 mM) were added, and respiration began at the rate of 0.23 μM O₂ per second (0.7 × 10⁻³ μmoles of O₂ per second); we shall call this Phase I. At the second arrow, 1.92 μmoles of ADP (in 0.02 ml; final concentration, 630 μM) were added, and the respiratory rate was accelerated almost eightfold to 1.52 μM O₂ per second (5.55 × 10⁻³ μmoles of O₂ per second); we shall call this Phase II. Phase II rather abruptly ceases after 124 μM O₂ (0.372 μmoles of O₂) have been consumed. At the end of the phase, ample oxygen (74 μM or 0.271 μmole) and glutamate (approximately 8670 μM or 29.9 μmoles) remained, so presumably the limiting factor was exhaustion of the ADP. The minimal (neglecting any ATPase) P/O ratio during Phase II was 635 μM/124 × 2 μM (or 1.90/2 × 0.372) = 2.55. During the subsequent phase (Phase III), the respiratory rate fell to 0.19 to 0.10 μM O₂ per second (0.58 × 10⁻³ μmoles of O₂ per second to 0.3 × 10⁻³ μmoles of O₂ per second).

Respiration in the presence of a fiber is illustrated by Curve a of Fig. 2, in which the conditions of Fig. 1 are maintained, but there is also present in the system a glycerol-treated fiber bundle, 164 μ in diameter and 0.59 cm in length. As can be seen by comparing Fig. 1 with Curve a of Fig. 2, the presence of a fiber bundle developing isometric tension (Fig. 2, Curve b) has no perceptible effect on the respiration. This conclusion is reaffirmed by the result that the average P/O ratio of 2.4 is found in the presence of a fiber bundle (Fig. 8).

3. Kinetics of Respiration and Contraction—Curve b of Fig. 2 shows that after the tension reaches its maximal value (T₁) during the period when the respiration was also measured, the subsequent imposition of 5 mM ATP, 5 mM MgSO₄, and 0.5 mM CaCl₂ evoked a still higher tension, which is here designated the “standard” tension (Tₛ). Any particular tension, T, can therefore be expressed as T/T₁ or T/Tₛ; as already remarked, these variables are convenient in comparing fiber bundles the cross-sectional areas of which are difficult to measure with certainty.

The tension development shown by Curve b of Fig. 2 is reproduced in Fig. 3 with a logarithmic plot. Curve a (log (T/T₁), Fig. 3) suggest that tension development follows rapidly any change in availability of ATP, since the break point in Curve a or b of Fig. 3 coincides with the end point of Phase II of Fig. 2. Moreover, if ATP rather than ADP is added, the slower rate shown in Curves a and b of Fig. 3 is not observed; this indicates that in this system ATP production, not tension development, is the rate-limiting process. Further support for this is suggested by a third experiment (Fig. 4) in which stepwise addition of ADP evoked stepwise increases in tension.

*Ti is a function of ATP concentration. However, it can serve as a standard tension when comparing tensions developed in the presence of the same concentration of ATP or ADP with mitochondria and glutamate.
4. Dependence of Response upon Mitochondrial Concentration—With substrate and oxygen in excess, an initial ADP concentration of 320 μM was imposed on various concentrations of mitochondria in the presence of a fiber bundle. The eventual extents of tension development in the fiber and of oxygen consumption by the mitochondria in the phosphorylating phase (Phase II) were independent of mitochondrial concentration; \( T_i/T_{st} \approx 56 \) to 57% and approximately 0.4 μM O₂ were consumed. However, the initial rates of tension development and of oxygen consumption in Phase II increased roughly linearly with the increase in mitochondrial concentration (Fig. 5). These observations are consistent with supposing (a) that the extent of tension development at this concentration of ATP (viz. the initial concentration of ADP) is linear with the concentration of ATP (cf. 8), and (b) that the tension adjusts rapidly to any concentration of ATP, and (c) that all concentrations of mitochondria were saturated with ADP, in which case the concentration of ATP synthesized is a linear function of time.

When ATP is supplied to the fiber in the presence of mitochondria, the rate of tension development is slightly greater than in the absence of mitochondria (Fig. 6). Since the glutamate respiration of mitochondria is not at all accelerated by the addition of ATP under these conditions, it is unlikely that the ATP feeder system of mitochondria is responsible for this increased rate of tension development. It may be suggested that mitochondria contain the equivalent of an activator, e.g. the equivalent of 0.008 pmole of Mg++ per mg of mitochondrial protein (Fig. 6), but this effect still falls short of explaining the relations of Fig. 5 (e.g. the increased rate of tension development by mitochondria).

5. Dependence of Response on ADP Concentration—If the mitochondria are thought of as an enzyme saturated with respect to one of its substrates, namely P₃, and subjected to increasing concentrations of its second substrate, ADP, then one might expect the rate of production of its product, ATP, to be, on the one hand, proportional to the rate of O₂ consumption (“tightly coupled” oxidative phosphorylation), and on the other hand, related to the rate of tension development. The precise form of the latter relation is not obvious a priori. Bowen and Blum (11) have suggested that isotonic (actually zero tension) extent of contraction is a “Michaelis-Menten function” of ATP concentration, [ATP], so that for small values of [ATP] it is proportional to [ATP]. If we suppose that the same is true for isometric tension development, and that (see above) the tension adjusts to [ATP] much faster than [ATP] is increased, then one might expect the rate of tension development also to be proportional to the rate of ATP production or of ADP consumption. Thus either respiration versus [ADP] or tension development versus [ADP] should be a “Michaelis-Menten function” for the mitochondrial P-saturated enzyme with respect to ADP. This, in fact, seems to be the case (Figs. 7 and 8); either relation gives a Michaelis constant of \( 2.5 \times 10^{-4} \).
FIG. 7. Dependence of respiration rate on ADP concentration. Conditions were those of Fig. 2, but the concentration of ADP added was variable as indicated on abscissa. ○—○, preparation No. 9 (3.42 mg of mitochondrial protein per ml reaction mixture). △—△, Preparation No. 12 (2.13 mg per ml). The rate in Phase I was subtracted from the rate observed in Phase II. It was expressed in micromolar O₂ per second per mg of mitochondrial protein per ml of reaction mixture and therefore it is equivalent to μmoles of O₂ per second per gram of protein.

FIG. 8. Dependence of contraction rate on ADP concentration. Conditions were those of Fig. 7. ○—○, preparation No. 9 (3.417 mg of mitochondrial protein per ml of reaction mixture). △—△, Preparation No. 12 (2.13 mg per ml). The rate was expressed as velocity unit per minute per mg of mitochondrial protein per ml of reaction mixture.

When concentrations of ATP are imposed on the fiber by oxidative phosphorylation rather than by direct addition of ATP, the half-time of tension response to a given ATP generation rate within the mitochondria depends, among other things, on the rate at which the ATP can move from the mitochondrion to the fiber. Although magnetic stirring was used in these experiments, it was thought that perhaps the response time was being made short by virtue of tight specific adsorption of mitochondria on the fiber. That this is probably not so is suggested by the following experiment. A fiber immersed in a mitochondrial suspension was incubated at room temperature for about 20 minutes. It was then rinsed in medium for only a few minutes. Finally it was immersed in medium containing ADP and glutamate. Tension development did not occur unless mitochondria were added again.

6. Total Oxygen Consumption during Phosphorylation—Although the specific respiratory rate of mitochondria varies from preparation to preparation and with age, the total amount of oxygen consumed during the phosphorylation phase (Phase II) is proportional to the amount of ADP added (Fig. 9), and the relationship is not affected by the presence of a contracting fiber. Thus, despite the somewhat low value (e.g. 2.4) of the P/O ratios observed in this work, it seems certain that all the ADP added is converted to ATP, and that such ATPase activity as the fiber may have brought about no detectable cycling, through the reaction, ATP → ADP + P.

7. Degree of Tension Development and ADP Concentration—Curve a, Fig. 10, shows that the final extent of tension development “saturates” when plotted against ADP concentration, and that it reaches half its maximal value at 2.5 × 10⁻⁴ M ADP. Curve b, Fig. 10, is evidence that when ATP is added instead of ADP the half-maximal tension is reached at a lower concentration, viz. at about 1.2 × 10⁻⁴ M ATP.

DISCUSSION

As expected from current knowledge, ATP produced by the oxidative phosphorylation of ADP in mitochondria is available

FIG. 9. Amounts of oxygen consumption during phosphorylation and ADP concentration. Conditions were those of Fig. 2. The straight line is equivalent to the P/O ratio of 2.4. Mitochondrial preparations: ○, No. 12 (2.13 mg of mitochondrial protein per ml of reaction mixture); □, No. 10 (5.6 mg per ml); ●, No. 9 (3.4 mg per ml).

FIG. 10. Degree of tension development and ADP concentration. The final extent of tension developed on addition of ADP or ATP (Tf) was expressed as its ratio to that on addition of 5 mM ATP, 5 mM MgSO₄, and 0.5 mM CaCl₂ (Tst). Conditions were those of Fig. 2. Mitochondrial preparations: ○, No. 10 (5.6 mg of mitochondrial protein per ml of reaction mixture); △, No. 8 (3.0 mg per ml); □, No. 12 (2.13 mg per ml) with ADP; ●, preparation No. 10 with ATP.
to a model contraction system of the glycerol-treated fiber (Fig. 2). The availability of ATP newly synthesized by oxidative phosphorylation is estimated to be about $(1.2/2.5 ~\times~) 48\%$, since the tension developed by $2.5 \times 10^{-4}$ M ADP with mitochondria and glutamate is obtained upon addition of $1.2 \times 10^{-4}$ M ATP under the same conditions (in the presence of mitochondria and glutamate) (Fig. 10). One of the possible explanations for this limited availability of ATP is that all the ADP added was synthesized into ATP but that because of “compartmentation” in mitochondria, not all the newly synthesized ATP was available to the fiber bundle, the “compartmentation” effect having an “apparent” Michaelis constant of about $2.5 \times 10^{-4}$ M ATP. This possibility is now under investigation (cf. 12).

The mitochondrial system employed can feed back both products of ATP hydrolysis by ATPase, that is, orthophosphate as well as ADP, whereas earlier described feeder systems (creatine phosphate-enzyme and phosphoenolpyruvate-enzyme) return ADP only. Although no particular advantage of the mitochondrial system over the other systems has been shown in the present experiments, the method used for following the mitochondrial oxygen consumption shows an advantage in yielding a continuous recording of oxygen utilization and thus making easy a comparison of not only the extent but also of the rate of ATP production with those of tension development. Experiments presented in Figs. 2, 3, 4, 5, 7, and 8 indicate that tension development of the glycerol-treated fiber bundle follows rapidly any change in availability of ATP synthesized from ADP over a wide range of concentration of ADP (70 to 700 μM). Therefore, it is suggested that the rate-limiting step of this mitochondrial-fiber system is not the tension response to ATP but the oxidative phosphorylation of ADP; that is, tension development is faster than ATP production, so that the former can follow any change in the latter.

On the one hand, it is expected that the respiration rate after the oxidative phosphorylation period (viz. the respiration rate in P Phase III of Fig. 1) depends on the ATPase activity. In fact, in model experiments on mitochondria and myosin-ATPase, which will be reported elsewhere, the respiration rate after the phosphorylation period was observed to be dependent upon myosin concentration at the range of relatively higher concentrations of myosin (1 to 5 g per liter). Likewise, if the ATP synthesized by mitochondrial oxidative phosphorylation is hydrolyzed by ATPase of the fiber bundle, ADP is again available to mitochondria. Therefore, the respiration rate in Phase III is expected to be faster than that without the fiber bundle. However, in this fiber-mitochondria system, the amount of fiber is so little (myosin less than 8 mg per liter) that the ADP produced by a fiber bundle is apparently negligible when compared with the concentration of ADP added, and essentially no change is observed (Figs. 2 and 8). The rather abrupt fall in oxygen consumption from Phase II to Phase III also indicates that the rate of ATP hydrolysis by the fiber bundle is much slower than that of the ATP synthesis by mitochondria in all cases reported in this paper. If ATP hydrolysis by the fiber-ATPase is faster than ATP synthesis by the mitochondrial suspension, the accelerated oxygen consumption in Phase II should have continued. The situation is thus such that tension development by ATP is faster than ATP synthesis by mitochondria, whereas the latter is faster than ATP hydrolysis by a fiber bundle and, therefore, such that the cause for tension development in the fiber is unlikely to be ATP hydrolysis by the fiber.

However, it should also be mentioned that if the so-called "initial burst" of ATP hydrolysis by myosin (9, 10) is involved in the tension development, a role for ATP hydrolysis in tension development must be considered even in this particular system. In the burst, not all, but only a small part of available ATP is hydrolyzed, and the extent of the burst depends on the amount of fibers. Since the amount of fibers is very small in the reported experiments, the extent of ATP hydrolysis must be negligible, and thus this particular part of ATP hydrolysis by fibers could even be faster than the ATP synthesis by mitochondria, although the gross hydrolysis of ATP by fibers is still much slower than the ATP synthesis as observed. Therefore, the above tentative conclusion cannot be drawn directly. However, in order to explain the observed close relation between the rates of ATP synthesis and of tension development, this particular type of ATP hydrolysis must have at least one more special feature, i.e. that the extent of this hydrolysis depends proportionally also on the concentration of ATP synthesized over a wide range of ATP concentration. There are some other possibilities that ATP hydrolysis in the particular type can be the cause for the tension development in a glycerol-treated fiber bundle. Therefore, the study of the burst of ATP hydrolysis of a fiber system, if indeed it occurs, is very desirable.

**SUMMARY**

A new model for a cyclic ATP-generating system for muscular contraction has been studied in vitro. Glycerol-treated fiber bundles of rabbit psoas muscle develop tension in a medium containing rabbit heart mitochondria, glutamate, inorganic orthophosphate, and adenosine diphosphate. Simultaneous recordings of oxygen consumption in the phosphorylating mitochondrial system and of tension development in the fiber bundle indicate that the tension development can rapidly follow any change in the oxygen consumption during oxidative phosphorylation of the diphosphate to the triphosphate of adenosine.

Half the "standard" tension developed by 5 mM adenosine triphosphate, 5 mM MgSO$_4$, plus 0.5 mM CaCl$_2$ is obtained with $2.5 \times 10^{-4}$ M adenosine diphosphate or $1.2 \times 10^{-4}$ M adenosine triphosphate in the presence of mitochondria, glutamate, and orthophosphate. Therefore, the availability of adenosine triphosphate newly synthesized by oxidative phosphorylation in cardiac mitochondria for tension development of glycerol-treated fibers is suggested to be only $(1.2/2.5 =) 48\%$. The regulatory factors involved in the reconstructed system are discussed.

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