Biochimica et Biophysica Acta, 347 (1974) 483–490

BBA 46748

APPARENT P/O RATIO AND CHEMICAL ENERGY BALANCE IN FROG SARTORIUS MUSCLE IN VITRO

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(Received October 8th, 1973)
(Revised manuscript received February 4th, 1974)

SUMMARY

We tested the hypothesis that there is a constant relation between the amount of chemical energy used during a single tetanus and the extent of subsequent aerobic recovery metabolism. Breakdown of high energy compounds ($\Delta \sim P$) during contraction was measured by rapid freezing techniques using isometric contractions of unpoisoned frog sartorius muscles at 0 °C. A stable and sensitive method was designed to study recovery O_2 consumption of similar contractions. In some cases, initial chemical changes and recovery O_2 consumption were measured in the same muscle. The ratio of these chemical changes yields a value for the P/O ratio of whole muscle. This ratio was found to be 2 and was constant for the range of contraction durations studied (5–20 s). Splitting of phosphorylcreatine or ATP after mechanical relaxation and glycolytic resynthesis of ATP could not be detected. Thus, the tested hypothesis is valid; but there is no ready explanation why this estimate of the P/O ratio of whole muscle differs from the ratio measured in isolated mitochondria. Because of this constancy and the observed stoichiometry unknown energy yielding reactions, postulated to occur either early or late in a maintained tetanus, are excluded by these experiments.

The identification of ATP hydrolysis as the driving chemical reaction during muscular contraction and its later resynthesis by recovery metabolic pathways is an axiom of skeletal muscle physiology. While the temporal sequence [1] of these reactions is generally accepted, there is little direct evidence on the quantitative relationships between the extent of the driving chemical reaction during contraction and oxidative recovery metabolism [2]. In addition, a missing reaction during contraction has been postulated on the basis of discrepancies between measured heat and chemical change during isometric tetani [3, 4]. Objections to this type of interpretation on experimental [5, 6] as well as theoretical [7] bases remain. We have, therefore, reinvestigated the questions concerning the chemical balance in muscle using an alternative method which relies only on chemical measurements. Our procedure was to measure the chemical energy used during a contraction and compare it to the extent of aerobic recovery metabolism. Comparison of the two measurements yields a value

for an apparent P/O ratio which, to our knowledge, has not been directly measured in whole tissue preparations.

Single isometric tetani in frog sartorius muscles (*Rana pipiens*) in a physiological saline solution without added substrate at rest length at 0 °C were studied. The recovery O_2 consumption was measured by a polarographic technique using a Clark-type electrode. Previously reported technical problems [8] of adequate sensitivity at 0 °C and stimulation in solution have been overcome by the use of a glass and stainless steel muscle chamber of small volume with alternating condenser discharge stimulation (RC $<$ 1 ms) through two fine platinum wires very near the muscles. The time constant of the oxygen measuring system was approx. 30 s. All chemical measurements are expressed herein per gram blotted weight (g) and normalized to the mean force per cross-section area, estimated by blotted weight/standard rest length ($g \cdot L_0^{-1}$). This normalization procedure reduced the scatter for muscles of varying sizes and for various batches of frogs.

Fig. 1 shows a typical experimental record of O_2 consumption. Stable basal rates of O_2 consumption were observed; the average value, 7 ± 1 nmoles $\cdot$ min$^{-1} \cdot$ g^{-1} (11 observations of at least 100 min), is in reasonable agreement with the generally used, though extrapolated value of Hill [9]. Following a 10-s isometric tetanus suprabasal O_2 consumption began in 2–3 min and generally 40 min were required before the rate returned to baseline. Tetani of longer duration were associated with a somewhat earlier onset of this increased rate of O_2 utilization and a longer duration before return to baseline. The recovery O_2 consumption, ΔO_2, was measured from baseline to baseline and used as a measure of the recovery metabolism. ΔO_2 for a 10-s tetanus was 0.323 ± 0.0065 μmoles $\cdot$ kg$^{-1} \cdot$ cm^{-1} ($N = 20$). For standard sar-

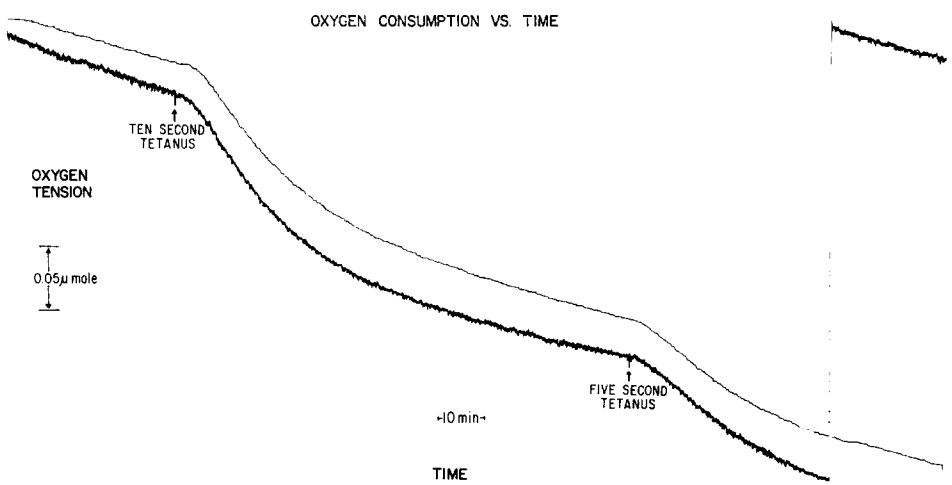

Fig. 1. Photograph of an experimental record of O_2 consumption following a single tetanus. Upper and lower traces are from one member of a pair of sartorii, each in a separate chamber. The abscissa is time and runs from left to right. The ordinate is the output of the O_2 electrode scaled to the amount of O_2 in the closed chambers (1 μmole = 14.6 mV). A downward deflection indicates O_2 uptake by the muscle. The rapid vertical rise in the lower trace is an artefact due to a shift in bias voltage. The O_2 tension during the experiment was kept at that of air ± 20 %. The muscle of the upper trace was stimulated 1 min after the lower.

TABLE I

CHEMICAL CHANGES AND TENSION–TIME INTEGRALS MEASURED IN SINGLE ISOMETRIC TETANI OF FROG SARTORIUS MUSCLES AT 0 °C

Measurements of O_2 consumption were made as described in the text and Fig. 1; n is the number of observations in 15 frogs. The tension–time integral was measured from the area of the isometric force–time record. Values given are the means ± standard error of the content of an experimental muscle minus the content of its paired control; N is the number of such paired measurements. Muscles not poisoned were equilibrated with air; poisoned muscles were treated for 45 min at 0 °C in a phosphate-buffered physiological saline solution containing 0.5 mM iodoacetate and gassed with 100 % N_2. For 10-s tetani $\Delta P_i \cdot g^{-1}$ in the unpoisoned muscle is significantly ($P < 0.05$) greater than that in the poisoned experiments. The reason for this is not known, but this difference is opposite to what would be expected if there were rapid oxidative phosphorylation during the tetanus in the unpoisoned muscles. For the 20-s tetani the statistically significant difference between $\Delta P_i \cdot g^{-1}$ and Δphosphorylcreatine $\cdot g^{-1}$ or Δcreatine $\cdot g^{-1}$ ($P < 0.05$) is consistent with hexose phosphate formation known to occur during prolonged tetani and recovery periods [3, 13]. PCr, phosphorylcreatine, Cr, creatine.

Experimental conditions	N	ΔPCr (μmoles·g^{-1})	ΔCr (μmoles·g^{-1})	ΔP_i (μmoles·g^{-1})	$\Delta \sim P$ (μmoles·g^{-1})	$\int P dt$ (kg·cm·s^{-1}·g)		ΔO_2 (μmoles·g^{-1})	n
1. Unpoisoned muscles									
5-s tetanus*	10	−2.05±0.11	1.98±0.12	1.83±0.13	−1.95±0.08	11.3±0.6	13.9±0.6***	0.557±0.017	6
10-s tetanus*	16	−2.81±0.18	2.97±0.20	3.22±0.16	−3.00±0.15	24.4±0.9	26.0±1.0	0.840±0.017	20
20-s tetanus*	10	−6.18±0.19	6.08±0.25	5.07±0.26	−5.78±0.22	46.8±2.3	46.0±2.0	1.29 ±0.09	8
2. Poisoned muscles									
10-s tetanus*	5	−3.24±0.52	3.36±0.46	2.48±0.18	−3.03±0.37	23 ±1	—	—	—
3. 10-s tetanus, frozen 60 s after last stimulus; poisoned*	6	−3.61±0.27	3.65±0.20	2.87±0.22	−3.38±0.20	26 ±1	—	—	—
10-s tetanus, frozen 200 s after last stimulus; poisoned**	9	−0.20±0.36	−0.18±0.32	0.16±0.16	0.06±0.24	0.3±0.9†	—	—	—
10-s tetanus, frozen 200 s after the last stimulus; unpoisoned**	10	0.07±0.34	−0.12±0.42	−0.23±0.25	−0.14±0.25	2.3±0.8†	—	—	—

* Control muscle was not stimulated.

** Control muscle frozen 10 s after onset of an isometric tetanus.

*** This column refers to the tension–time integral in oxygen consumption experiments.

† Differences, experimental minus control.

torius, that is one with a force per cross-section area ($P_0 \cdot L_0 \cdot g^{-1}$) of 2 $kg \cdot cm^{-2}$, this ΔO_2 is 0.65 $\mu mole \cdot g^{-1}$.

Because of the observed delay in the onset of recovery O_2 consumption, measurements of chemical utilization during contraction could be made in unpoisoned muscles without concurrent oxidative phosphorylation. Pairs of sartorii were frozen using a rapid-freezing hammer apparatus [3] while a tetanic stimulation was applied to one muscle. The extent of chemical energy utilization during the tetanus was estimated from the observed breakdown of phosphorylcreatine and increases in free creatine and inorganic phosphate (P_i) in an experimental muscle compared to its paired control. The mean of these three measurements, defined as $\Delta \sim P$, was used as the most precise measure. $\Delta \sim P$ for a 10-s tetanus was 1.23 ± 0.06 $\mu moles \cdot kg^{-1} \cdot cm^{-1}$ ($N = 16$) or 2.5 $\mu moles \cdot g^{-1}$ for a standard sartorius. The chemical data are presented in Table I. The equivalence of $-\Delta$phosphorylcreatine $= \Delta$creatine $= \Delta P_i$ and the absence of a change in the ATP content (-0.03 ± 0.11 $\mu mole \cdot g^{-1}$ in 15 independent experiments of 5-s tetani) is consistent with the operation of the Lohmann reaction without side reactions.

The comparison of recovery metabolism and initial chemical energy utilization is most easily expressed by taking the ratio of $\Delta \sim P$ and ΔO_2, forming an apparent P/O ratio. Based on mean values of the independent measurements of $\Delta \sim P$ ($N = 16$) and ΔO_2 ($N = 20$), for 10-s tetani, the apparent P/O is 1.89. To avoid possible errors associated with variation amongst animals, ΔO_2 and $\Delta \sim P$ were measured on the same frog. Recovery O_2 consumption was first measured, then in a following tetanus, the muscle and its paired control were frozen and assayed for chemical changes. The mean P/O for five such paired experiments was 1.70 ± 0.07.

Lactate production above that in an unstimulated control muscle was not detectable during the 10-s tetanus itself ($+0.05 \pm 0.07$ $\mu mole \cdot g^{-1}$; $N = 6$) nor after 3 min of recovery ($+0.08 \pm 0.11$; $N = 6$) nor at the end of the aerobic recovery period ($+0.02 \pm 0.07$; $N = 5$). Hence the possible contribution of aerobic glycolysis to recovery metabolism is small and was not considered.

It is possible that our method systematically underestimates $\Delta \sim P$: (1) there may be a breakdown of ATP and phosphorylcreatine after the tetanus which would not be measured by our protocol for rapid freezing; (2) there may be a resynthesis of ATP or phosphorylcreatine during the tetanus. A breakdown of ATP and phosphorylcreatine after contraction was experimentally tested in a straightforward manner. The results of two series of experiments using poisoned as well as unpoisoned muscles are given in Table I. No statistically significant additional chemical breakdown was found 60 s after the end of stimulation in poisoned muscles, or at 200 s after the end of a 10-s stimulation in the more restrictive pair-wise comparisons. Our present results agree with the finding that no detectable ATP and phosphorylcreatine splitting occurs for periods of up to 1000 s after contraction [10, 11].

Rapid resynthesis of ATP and phosphorylcreatine by glycolytic reactions is excluded, but our experiments with unpoisoned muscles cannot exclude rapid oxidative resynthesis during a tetanus, using stored O_2 in the muscle or oxidizing equivalents in the mitochondria*. To test this possibility, measurements of chemical

* Non-mitochondrial O_2 utilization in frogs muscle is excluded because 0.001 M CN^- completely blocks basal and contraction induced O_2 consumption.

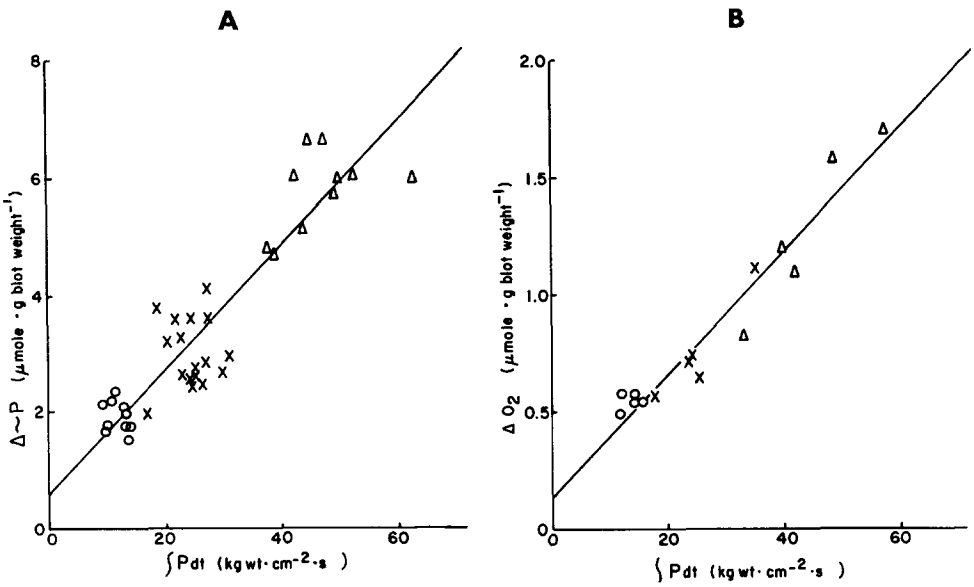

Fig. 2. Chemical change as a function of the tension–time integral for isometric contraction in frog sartorius at L_0 at 0 °C. Ordinates are: (A) $\Delta \sim$ P, chemical breakdown during contraction measured from independent determinations of P_i, phosphorylcreatine and creatine on each muscle. (B) ΔO_2, recovery O_2 consumption. Abscissa is tension–time integral normalized to muscle cross-section area: $L_0 \cdot g^{-1} \cdot \int P dt$. Each point represents measurements on one frog; (○) 0.5-s tetanus, (×) 10-s tetanus, (△) 20-s tetanus. Solid line is the least-square linear regression for each: $\Delta O_2 = 0.0263$ ($\pm$ 0.0019) $\cdot \int P dt + 0.138$ (± 0.058) and $\Delta \sim P = 0.104$ (± 0.0075) $\cdot \int P dt + 0.685$ (± 0.229). The mean of ΔP_i is less than Δphosphorylcreatine and Δcreatine for the 20-s tetani (Table I). Thus, the slope of the regression of $\Delta \sim$ P vs the tension–time integral is less by 7 % than that of Δphosphorylcreatine and Δcreatine; however, the effect is not significant.

changes were made on muscles poisoned with IAA to block glycolysis and N_2 to stop respiration and reduce mitochondrial electron carriers. As is clear from the data given in Table I, $\Delta \sim$ P for a 10-s tetanus in unpoisoned muscles was not different from that found in poisoned preparations.

Under certain conditions, mitochondrial suspensions from frog muscle can yield higher P/O values [12]. This raises the logical possibility that our measured value of P/O indicates the existence of a missing reaction ($\Delta \sim$ Y) such that the complete relation between initial chemical utilization and recovery metabolism is given by $\Delta \sim P + \Delta \sim Y / \Delta O_2 \cong 3$. To investigate this hypothesis ΔO_2 and $\Delta \sim$ P were independently measured in similar 5-, 10- and 20-s tetani (Table I). The regressions of $\Delta \sim$ P and ΔO_2 against the tension–time integral (which may be viewed as tetanus duration, corrected for any fall in tension) are shown in Fig. 2. Both relations were linear with statistically significant intercepts. The slope from the regression shown in Fig. 2A divided by that of Fig. 2B provides a third estimate of the P/O ratio in the muscle of 1.98. These three estimates are not statistically different. Using this value to scale ΔO_2 to $\Delta \sim$ P the average intercepts of these regressions are also not statistically different; however, variability precludes detection of small differences.

This variability plagues all such studies on intact muscles and is in a large

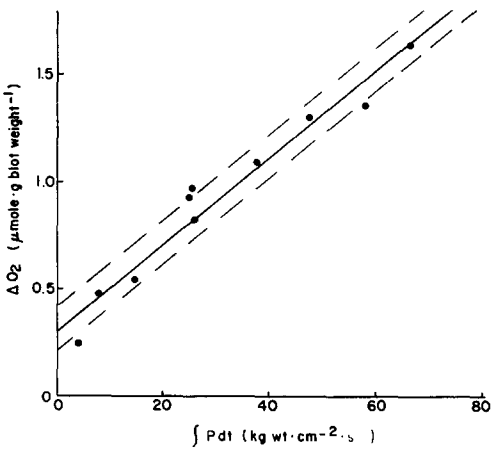

Fig. 3. ΔO_2 as a function of the tension–time integral; repeated measurements on the same muscle. Abscissa and ordinate are as in Fig. 2B. Data for single isometric tetani were obtained in the following order: 10-, 10-, 20-, 15-, 5-, 25-, 2.5-, 1-, 10-, 30-s duration. Solid line is the least-square fit linear regression. The cluster of three points (10-s tetani) and the standard error of estimate, $\pm s_{y \cdot x}$ (broken lines), indicate the error associated with repeated measurements.

part due to destructive chemical methods which permit only one measurement per animal. Often the real relationship between two variables masquerades as linear when grouped data are analyzed. The O_2 measurements allow multiple measurements on the same muscle and were exploited to clarify the fine structure of the relation. Fig. 3 shows the relationship between ΔO_2 and tension–time integral measured on one muscle. The linearity observed in the range from 5 to 20 s was quite typical; additional experiments ($N = 4$), extend the linear range to 2.5–30 s. The intercepts for 11 experiments showed a considerable range, whereas the slopes were found to be less variable. Thus the linearity as well as the variability in the intercepts noted in the grouped regressions appears to be a property of individual muscles, and not an artifact due to sampling from a population of frogs.

The agreement of the point estimate of the P/O ratio (10-s tetanus) with that estimated from the slopes means ΔO_2 vs the tension–time integral is superimposable on $\Delta \sim P$ using P/O ratio of about 2. Thus, the P/O ratio is found to be constant at least for tetani of durations of 5–20 s, within the statistical limitations imposed by sampling from a population of frogs. It remains possible that a hypothetical reaction $\Delta \sim Y$, independent of the duration, exists; however, this superposition constrains it to have the same dependence on the tension–time integral as does $\Delta \sim P$. Therefore, the possibility that a missing reaction only occurs during a part of the tetanus is excluded. Furthermore, the stoichiometry of our results requires either that any unidentified reaction during the tetanus be coupled to oxidative metabolism in some manner not involving net changes in ATP, phosphorylcreatine or P_i, an unlikely possibility, or that the supposed missing reaction be compartmentalized such that coupling with the "high energy" phosphate pools in the cell be delayed at least until 200 s into the recovery period. Finally it is also a logical possibility that significant chemical energy utilization occurs during the aerobic recovery period, i.e. recovery O_2 consumption measures both contractile and post-contractile energy-

requiring processes. Examples of such post-contractile processes are active ion transport and glycogen resynthesis from hexose phosphates. Our experiments on this point consider the first 200 s of the recovery period following a 10-s tetanus. No $\Delta \sim P$ was measurable (S.E. of the mean $\cong 0.25\ \mu mole \cdot g^{-1}$) whereas the amount required to raise the apparent P/O from the observed value of 2 to a P/O ratio of 3 would be equivalent to about $2\ \mu moles \cdot g^{-1}$ of ATP ([ΔO_2 for a 10-s tetanus] $\times 6$ minus observed $\Delta \sim P$). We cannot exclude the possibility that this extent of high energy phosphate utilization occurs during the recovery period; however, this is contrary to other published work [10, 11]. Thus it is not clear why the observed P/O ratio differs from that found in suspensions of tightly coupled mitochondria, however, this may be a previously unrecognized aspect of chemical energy balance in muscle.

EXPERIMENTAL

Rana pipiens were stored at 10 °C and remained in good condition for at least two months. Sartorius muscles were dissected from decapitated animals. The pelvic bone was split in the midline to preserve the in vivo attachments. The dissected muscles were kept for 1 h at room temperature aerobically in a physiological saline solution before use. The composition of this solution was NaCl (115 mM), KCl (2.5 mM), Na_2HPO_4/NaH_2PO_4, pH 7.1 (3 mM), $CaCl_2$ (1.8 mM) and $MgSO_4$ (1 mM); this solution was equilibrated with air or 100 % N_2 as required.

O_2 consumption was measured using a Clark-type electrode in a glass and stainless steel chamber containing 4 ml of the saline solution. The baseline for the O_2 measuring system includes the O_2 consumption of the electrode. At these extremely low levels of O_2 consumption rates (< 1 nmole/min), the baseline of the system becomes important. The system baseline was measured prior to mounting the muscle in the chamber and in general, finite values were measured. Initially these rates could be as large as the basal O_2 consumption of the muscle. The control rates were found to decrease continuously over the course of the experiment (approx. 12 h) to values about 40 % of the basal $\dot{O}_2$. Thus, for a typical measurement period (approx. 2 h), baseline changes were on the order of 10 %. ΔO_2 was calculated using the arithmetic mean of the initial and final rates. Repeated measurements of ΔO_2 (approx. ± 10 %) (cf. Fig. 3 for example) provide experimental justification for this procedure. These slowly decreasing baselines further indicate that bacterial oxidation and tissue damage, both associated with increasing baselines, were not present. We calibrated the electrode output using the value 0.437 μmole O_2/ml saline solution at 0 °C equilibrated with air at 1 atm. ATP, phosphorylcreatine, creatine, inorganic phosphate and lactate were extracted from the thin frozen muscles by simple diffusion into a mixture of equal volumes of absolute methanol and 10 mM sodium EDTA, pH 8, at -35 °C for four days. Standard chemical assays were used [3, 13, 14].

The electrode configuration chosen and the use of alternating condenser discharges (10 Hz, 9 V) completely avoided the problem of electrolysis of water while continuously recording the O_2 tension (cf. Fig. 1).

ACKNOWLEDGEMENTS

The authors thank Ms Margaret Coppe for technical assistance. The work was supported by a post-doctoral fellowship from the Southeast Chapter of the Massachusetts Heart Association to R.J.P., by grants from The Massachusetts Heart Association and The Medical Foundation, Inc. and in part by a P.H.S. Research Grant from the National Institute of Arthritis and Digestive Diseases.

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