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ENERGETICS OF RELAXATION IN FROG MUSCLE

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SUMMARY

1. Changes in the levels of phosphocreatine (PCr) and ATP during relaxation after tetanic contraction have been observed and compared with the heat + work production during the same period.

2. The results were the same using 2 sec or 15 sec tetani and using 15 or 30 Hz stimulation.

3. During relaxation the mean chemical change (of ATP + PCr) was $-0{\cdot}105 \pm 0{\cdot}078$ μmol/g muscle ($n = 53$). This change is less than would be required to explain the heat + work produced.

4. The PCr split during both 2 sec and 15 sec tetani (up to the last stimulus) was less than would be required to explain the heat + work produced. This confirms previous experiments.

5. It is concluded that the discrepancy between chemical change and heat + work production does not diminish during relaxation but actually increases.

INTRODUCTION

Gilbert, Kretzschmar, Wilkie & Woledge (1971) showed that, at the start of a tetanic contraction, the splitting of phosphocreatine (PCr) is much less than would be necessary to account for the heat (h) and work (w) produced. Much of the heat + work must be derived from some other process. In contrast it seems from their results that during relaxation there might be *more* splitting of PCr than would be expected from the heat + work production. This would suggest that the unknown process which produces heat + work early in a contraction is partially reversed during relaxation, absorbing part of the heat + work produced by PCr splitting at that time. Unfortunately this conclusion is not secure; the estimates obtained by Gilbert *et al.* of PCr splitting in relaxation have rather large standard errors because they were obtained indirectly from experiments not designed to measure this quantity. Their results, in terms of the ratio of the change in PCr to the total creatine (Ct) in the muscle (ΔPCr/Ct) are $-0{\cdot}035 \pm 0{\cdot}018$ for relaxation after a 15 sec tetanus and $-0{\cdot}012 \pm 0{\cdot}007$

after a 2 sec tetanus. For comparison, the change in PCr/Ct which would account for the heat + work production would be about −0·006. These results, particularly that for the longer tetanus, greatly exceed the more accurate estimate given by Kushmerick & Davies (1969) for the inorganic phosphate liberated during relaxation after a 2 sec tetanus of a dinitrofluorobenzene (DNFB) treated muscle which, in the same units, is 0·0075 ± 0·0029. However, as relaxation is certainly affected by DNFB treatment (Aubert, 1964; Lebacq, 1972), and as there are no heat measurements directly comparable with the results of Kushmerick & Davies, a sure conclusion cannot be reached from these either. To try to resolve these difficulties of interpretation, we have made direct measurements of ΔPCr/Ct in relaxation after both 2 sec and 15 sec tetani, and comparable observations of the heat + work production.

One process certainly occurring in relaxation is reduction in the level of free calcium in the cytoplasm. This process is probably coupled to PCr splitting and it may be the major cause of PCr splitting at this time. The recent studies of Rüdel & Taylor (1973) suggest that the amount of free calcium ion is much greater at higher stimulation frequencies. It might be that at higher stimulation frequency more PCr splitting would be needed during relaxation to remove this extra calcium. To test this possibility we have used two different stimulation frequencies in our experiments.

METHODS

The experiments were done on sartorius muscles of frogs (*Rana temporaria*) at 0° C, unpoisoned and oxygenated. The Ringer solution contained: NaCl, 106 mM; KCl, 2·0 mM; $CaCl_2$, 1·8 mM and $NaHCO_3$, 10 mM. It was gassed with 5 % CO_2 in O_2.

Heat experiments. Heat was measured with a thermopile as described by Dickinson & Woledge (1973). In these experiments both muscles of a pair were stimulated simultaneously (capacitor discharges of alternating polarity, 24 V, 0·04 μF, frequency 15 or 30 Hz). Each pair was stimulated only once and heat and tension were recorded during and after stimulation. After the experiment the muscles were frozen, and the weight and total creatine content were determined. The heat production records were corrected in the usual way for heat loss and thermopile lag. For these experiments it was necessary to calculate the amounts of thermoelastic heat production and heat derived from the dissipation of work stored in the series elasticity. The thermoelastic heat calculation was made using a value of 0·01 for the ratio of thermoelastic heat to tension × muscle length. This is the average value found by Woledge (1961, 1963). The work stored in the series elasticity of the muscle itself was calculated from the data of Jewell & Wilkie (1958), and that stored in the connexions and the transducer was calculated from the observed stress-strain curve of the apparatus. The heat record was corrected for the heat produced by the stimuli.

Chemical experiments. In most of these experiments one muscle of each pair was tetanized for either 2 or 15 sec and was frozen after a delay of 3 sec; the other muscle was tetanized for the same period but was frozen at the last stimulus. The onset of stimulation and the moment of freezing were set so that the two muscles were frozen simultaneously. Stimulation parameters were the same as in the heat experiments

and the tensions were recorded. In a few experiments unstimulated muscles were frozen. All the muscles were frozen on the hammer apparatus (Kretzschmar & Wilkie, 1969; Krezschmar, 1970; Gilbert *et al.* 1971) then weighed and extracted as described by Dydyńska & Wilkie (1966). The amounts of free creatine and total creatine (Ct) were measured by the method of Ennor (1957), and ATP was measured by the enzymatic method described by Scopes (1972).

Rejection of data. Some experiments were rejected because one of the chemical measurements (ΔPCr/Ct, ΔATP/Ct) was more than 2 S.D. away from the mean for the group. The S.D. used in this test were calculated including the values thought to be aberrant. Of fifty-eight otherwise satisfactory experiments, five were rejected in this way.

RESULTS

Changes during relaxation

The first section of Table 1 gives the chemical changes observed during relaxation from 2 sec and 15 sec tetani with either 15 or 30 Hz stimulation. None of the four groups gives a result significantly different (at a 5% level) from any of the others for the change in ATP, PCr or ATP + PCr. It is therefore legitimate to combine the groups in pairs to give a more sensitive test for any influence of either frequency or tetanus duration. This is done in the next section of the Table. Again no significant differences appear. This procedure might conceal an effect due to an interaction of frequency and duration; in particular the important factor might be the total number of stimuli. The individual group means show no such trend however. It thus seems that our results reveal no effect of either tetanus duration or stimulus frequency on the chemical changes during relaxation. However, since all the chemical changes are small, it remains possible that these variables do have effects which, although too small to detect, are proportionally quite large.

Tension development. There might be some doubt as to whether the muscles conducted action potentials at the higher frequency of stimulation. A failure to detect a difference between the two frequencies might be due to the muscle responding to only every other stimulus at the higher frequency. However, a comparison of the tension records suggests that this is not so, because with the higher frequency, tension was developed more rapidly and the maximum tension (per unit cross-section) was greater. The extra tension measured at 2 sec after the start of stimulation was $16 \pm 5\%$ of the maximum tension. By 15 sec this had declined to $5 \pm 6\%$. No difference in the time course of relaxation could be detected after tetani at the different frequencies. As expected from the results of Abbott (1951), relaxation after the 15 sec tetanus was considerably slower than that after the 2 sec tetanus.

Since no effect of frequency or duration can be detected in the chemical results we have combined all of them together. The final value of $\Delta(\text{ATP} + \text{PCr})/\text{Ct}$ so obtained is surprisingly small ($-0{\cdot}0039 \pm 0{\cdot}0029$), and is not significantly different from zero ($P = 0{\cdot}18$). The average amount of total

TABLE 1. Chemical changes and heat + work during relaxation. The chemical changes were determined by comparison of paired muscles, one of which was frozen after the last shock of a tetanus, the other 3 sec later, by which time it had relaxed. A negative sign indicates a decrease of concentration during relaxation. We have tested the significance of the difference between zero and the mean values in the last line and give the P values in brackets

Tetanus duration (sec)	Stimulation frequency (Hz)	ΔPCr/Ct × 1000			ΔATP/Ct × 1000		Δ(ATP + PCr)/Ct × 1000		Heat + work (mJ/μmol Ct)		
		n	Mean	S.E.	Mean	S.E.	Mean	S.E.	n	Mean	S.E.
2	15	11	−1·9	3·3	+4·6	2·6	+2·7	3·6	—	—	—
2	30	15	−6·6	7·0	−2·0	1·9	−8·6	7·2	4	0·460	0·050
15	15	14	−7·7	4·4	−0·4	1·4	−8·1	4·5	6	0·528	0·064
15	30	13	−1·9	5·9	+2·6	2·1	+0·5	5·9	—	—	—
2, 15	15	25	−5·2	2·9	+1·8	1·5	−3·4	3·2	—	—	—
2, 15	30	28	−4·4	4·6	+0·1	1·5	−4·3	4·8	—	—	—
2	15, 30	26	−4·6	4·3	+0·8	1·7	−3·8	4·6	—	—	—
15	15, 30	27	−4·9	3·6	+1·0	1·3	−3·9	3·8	—	—	—
2, 15	15, 30	53	−4·8	2·8	+0·9	1·0	−3·9	2·9	10	0·501	0·044
			(P = 0·09)		(P = 0·37)		(P = 0·18)				

creatine was $27{\cdot}0 \pm 0{\cdot}25$ μmol/g. The value of Δ(ATP + PCr) in μmol/g is thus $-0{\cdot}105 \pm 0{\cdot}078$.

Heat production. Observations were made of the heat production during the 3 sec after the last shock of tetanic contractions. A part of the heat produced consisted of work dissipation in the muscle and the thermoelastic effect of the falling tension (Hill, 1961). The size of these components was calculated as described above and they were subtracted from the observed quantity. The remainder is the heat + work required for comparison with the chemical change. For this purpose the heat + work is best expressed per μmol of total creatine. The mean values obtained were $0{\cdot}46 \pm 0{\cdot}05$ mJ/μmol from four observations of 2 sec tetani at 30 Hz, and $0{\cdot}53 \pm 0{\cdot}06$ mJ/μmol from six observations of 15 sec tetani at 15 Hz. As these results do not differ significantly, they can be combined for comparison with our combined chemical results, giving a value of $0{\cdot}5 \pm 0{\cdot}04$ mJ/μmol. Expressed in a more familiar way this result is 11·7 mJ/g which, although rather higher than the value of 7·5 mJ/g given by Hill (1961), probably does not differ from it significantly.

TABLE 2. Chemical changes and heat + work during contraction. The chemical changes are calculated, as shown in this Table, from the average values of PCr/Ct in muscle given different treatments. Observed quantities in roman type, calculated quantities in italics

Tetanus duration	PCr/Ct			Heat + work (mJ/μmol Ct)		
(sec)	*n*	Mean	S.E.	*n*	Mean	S.E.
0 (rest)	8	0·807	0·014	—	—	—
2	17	0·783	0·006	—	—	—
15	28	0·670	0·005	—	—	—
2–rest	—	*−0·024*	*0·015*	10	2·09	0·06
15–rest	—	*−0·137*	*0·015*	6	7·81	0·45
15–2	—	*−0·113*	*0·008*	6	5·78	0·38

Changes during contraction

Since the chemical changes we have found during relaxation are so much smaller than those we expected from the work of Gilbert *et al.* it is important to examine whether, in other respects, our results resemble theirs. In particular we need to know whether the relation of heat + work to chemical change during contraction is the same. In order to answer this question we froze some muscles without stimulation. By comparison of the mean value of PCr/Ct in these resting muscles with the mean value for muscles frozen at the end of 2 sec and 15 sec tetani, we can estimate the PCr split during these tetani. The experiments for heat + work during relaxation also provide measurements of the heat + work at the end of the

tetani. All these results are given in Table 2. In Fig. 1 the observed chemical changes are compared with those predicted from the heat + work. Two such predictions are given for different values of ΔH, the enthalpy change per mol of PCr split. The value of 34 kJ/mol for $-\Delta H$ is found *in vitro* (Woledge, 1972). The value of 46 kJ/mol is that used by Gilbert *et al.* Even with the larger value of $-\Delta H$ it is evident that, as Gilbert *et al.* describe, not enough PCr is split to account for the heat + work produced in either a 2 sec or a 15 sec tetanus. If $-\Delta H$ is taken as 46 kJ/mol the amount of unexplained heat + work at 2 sec is about 30 mJ/g (half of the total) which is very similar to the quantity reported by Gilbert *et al.* (40 mJ/g).

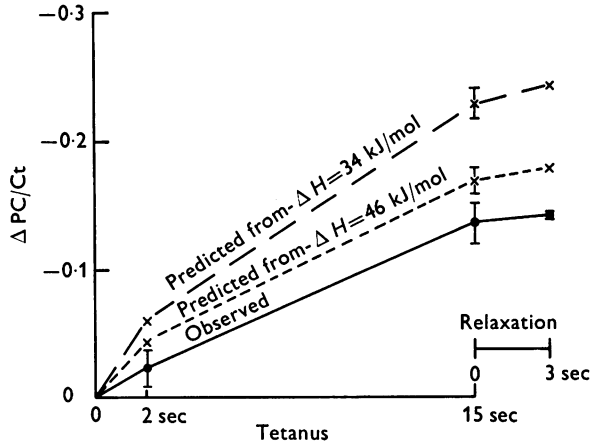

Fig. 1. Change of PCr level during and after a tetanus. The observed quantities (Tables 1 and 2) are plotted ●. The predicted values (×) are calculated from the measured heat + work and the values of ΔH indicated. The observed values for contraction were determined by comparing mean values of PCr/Ct for unpaired muscles, thus the standard errors are relatively large. The observed value for relaxation was found by comparing paired muscles: one contracted, the other contracted and relaxed. The mean ΔPCr/Ct found in this way was added to the mean value for a 15 sec tetanus to give the point plotted after relaxation.

DISCUSSION

Although the results of previous studies suggested that the amount of chemical change in relaxation might depend on the duration of the preceding tetanus, we have failed to find any such effect for a tetanus lasting 2 sec or longer. Following a 2 sec tetanus the chemical change was 'small', $-0{\cdot}102 \pm 0{\cdot}124$ μmol/g, [Δ(ATP + PCr)/Ct = $-0{\cdot}0038 \pm 0{\cdot}0046$]; this agrees with the finding by Kushmerick & Davies (1969) of a chemical change of $-0{\cdot}180 \pm 0{\cdot}072$ μmol/g in DNFB treated muscle. Following a

15 sec tetanus our value for $\Delta(ATP+PCr)/Ct$ was $-0{\cdot}0039 \pm 0{\cdot}0038$, the same as the change that occurred after the 2 sec tetanus. However, this result was much lower than the $\Delta(ATP+PCr)/Ct$, $-0{\cdot}041 \pm 0{\cdot}018$, found by Gilbert *et al.* (1971) with this tetanus duration. Although the difference between this value and our own is large, it is not statistically significant ($P \simeq 0{\cdot}1$). This is due to the large standard error of the result of Gilbert *et al.*; their experiments were not designed to determine the chemical break-down during relaxation and thus did not include any direct comparisons, using paired muscles, of a tetanus with a tetanus+relaxation. Furthermore, only four measurements of the break-down during a 15 sec tetanus were made.

The other study of relaxation to which we might compare our results is that by Mommaerts & Wallner (1967). In this experiment the DNFB treated muscles performed a very brief tetanus (0·2 sec); during relaxation (0·6 sec) the change in ATP found was $-0{\cdot}01 \pm 0{\cdot}02$ μmol/g. This result is not significantly different from ours, but we hesitate to make any other comment; it may be that the comparison of these results is inappropriate since the muscle is likely to be in a very different state after a few stimuli than it is after a 2 sec tetanus.

Changes in ATP level during relaxation

Gilbert *et al.* (1971) found a small but significant increase in ATP/Ct, $+0{\cdot}0039 \pm 0{\cdot}0015$, early in a tetanus. A possible hypothesis, based on this finding, is that all this newly synthesized ATP, equivalent to one ATP per myosin, is broken down during relaxation. In fact we did not find a break-down of ATP during relaxation; our value of $\Delta ATP/Ct$ was significantly different ($P = 0{\cdot}01$) from that postulated, $-0{\cdot}0039 \pm 0{\cdot}0015$. However, we have not measured the amount of ATP synthesis during stimulation. So it is possible that less ATP was synthesized by our batch of frogs than by the frogs used by Gilbert *et al.*; if this were so the hypothesis would require that less ATP be broken down during relaxation. Thus the hypothesis cannot be completely rejected on the available evidence, but if any break-down of ATP occurs during relaxation, the amount is less than one ATP per myosin.

Hypotheses about relaxation

Several hypotheses concerning the chemical change during relaxation can be evaluated on the basis of our results.

(1) It could be that there is no chemical change. This hypothesis cannot be ruled out by our results (see P values in Table 1) but it is unreasonable since it would require that all the reactions which are known to be occurring during stimulation should cease abruptly after the last stimulus. This

seems unlikely since in relaxation the muscle retains the properties of a tetanized muscle, albeit to a diminishing degree; heat production continues (Hill, 1961), tension persists, and the muscle can shorten and redevelop tension during relaxation (Jewell & Wilkie, 1960).

(2) A more attractive hypothesis is that the break-down of PCr and ATP in progress during the tetanus continues during relaxation: the actual amount depending on the degree to which the properties of a tetanized muscle are retained. On the basis of this hypothesis the chemical change during relaxation can be predicted from our results for contraction (Table 2) and can then be compared with the results for relaxation. The two properties of tetanized muscle which we have used for this purpose are (*a*) the tension expressed as $\int(Pl/m)dt$ (where P is the tension, l is the length of the muscle and m is the weight of the muscle) and (*b*) the heat + work produced by the muscle. Using the ratio of ΔPCr/Ct to $\int(Pl/m)dt$ during the plateau of the tetanus from 2 sec to 15 sec and the values for the $\int(Pl/m)dt$ for relaxation, the predicted ΔPCr/Ct is $-0{\cdot}008$ and $-0{\cdot}011$ for relaxation following a 2 and 15 sec tetanus, respectively. Using the ratio of ΔPCr/Ct to (heat + work)/Ct, the predicted ΔPCr/Ct for relaxation is $-0{\cdot}009$. The measured ΔPCr/Ct, $-0{\cdot}0048 \pm 0{\cdot}0028$, is smaller than these predicted values, but it is compatible with them. This suggests that the total chemical change during relaxation can be explained by the continuation of these processes which occur during the tetanus, and there is no need to suggest other processes to explain it. In particular there is no need to suppose that calcium pumping uses PCr faster during relaxation than during contraction.

(3) The fact that no special process is needed to account for the heat + work during relaxation suggests the hypothesis that the heat + work is completely derived from the enthalpy of PCr and ATP changes. Since the $-\Delta H$ measured *in vitro* for these reactions is 34 and 43 kJ/mol, respectively (Gellert & Sturtevant, 1960; Pin, 1965; Woledge, 1971, 1972), the hypothesis requires a chemical change of between $-0{\cdot}015 \pm 0{\cdot}001$, if only PCr/Ct changes, and $-0{\cdot}012 \pm 0{\cdot}001$, if only ATP/Ct changes. In fact it seems unlikely that there is any ATP break-down, so the first of these predicted values is the more relevant. The observed value of Δ(ATP + PCr)/Ct, $-0{\cdot}0039 \pm 0{\cdot}0029$, is significantly less than this ($P = 0{\cdot}02$). Thus unless the $-\Delta H$ for the reaction is greater than 34 kJ/mol, the hypothesis can be rejected. Some of the heat + work comes from some other process besides the splitting of ATP and PCr. This seems quite likely since Gilbert *et al.* (1971) have shown, and we have confirmed, that an unknown process or processes do occur during a tetanus.

(4) Finally we must test the hypothesis, originally suggested by Gilbert *et al.* that the discrepancy between chemical change and the output of

heat + work which is established during the tetanus may be resolved, in part or completely, during relaxation. Since there is an excess of heat + work production early in the tetanus, this hypothesis requires that during relaxation more ATP + PCr should be broken down than is expected from the heat + work produced and the ΔH values for the reactions. As we have just seen, the ATP + PCr broken down is in fact significantly less than the quantity predicted in this way.

The hypothesis which is in best accord with our results is (2). It seems that the chemical events in relaxation closely resemble those in contraction, not only in their relation to tension and heat + work, but also in that an unknown process continues to provide a considerable portion of the total heat + work produced.

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