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TETANUS RELAXATION

TEMPERATURE EFFECTS AND ARRHENIUS ANALYSIS

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Summary

Time constants (τ) have been accurately measured for the exponentially falling, latter 60—70% of the relaxation phase of maximal isometric tetani of the mouse extensor digitorum longus muscle over the range 15—35°C. Corresponding to the τ values, the rate constants (k = 25.0—189 s^{-1}) are assumed to describe a temperature-sensitive, first-order, rate-limiting reaction underlying, and determining the kinetics of, muscle relaxation. The mean Arrhenius plot for the k values of 6 muscles consists of 2 linear segments with a 25°C transition temperature. The activation energies at the relatively lower and higher temperatures are 22.9 and 12.6 kcal/mol, respectively. These values are qualitatively, and to some extent quantitatively, similar to corresponding known Arrhenius results of the Ca^{2+} active transport mechanism and the physical properties of the membrane of isolated sarcoplasmic reticulum. Thus, the present findings strongly indicate that relaxation of living muscle critically involves the 'relaxing factor' activity of Ca^{2+} uptake, as previously inferred from research on isolated sarcoplasmic reticulum. Using transition-state theory, the Arrhenius results indicate that $\Delta G^{\ddagger}$ of the assumed rate-limiting reaction is 14.8—15.0 kcal/mol at all temperatures studied, and $\Delta S^{\ddagger}$ is about 25 and —10 cal/degree per mol at temperatures below and above the transition temperature, respectively. These also correspond, at least qualitatively, to the values of the activation thermodynamic parameters of isolated sarcoplasmic reticulum. The negative $\Delta S^{\ddagger}$ at the higher temperature range, denoting an increase in order associated with the assumed activation process of the Ca^{2+} transport system, requires clarification.

† Deceased April 16, 1978.

There is clear evidence [1,2] that the contraction-relaxation cycle is regulated by the flux of activator Ca^{2+} between its storage sites in the sarcoplasmic reticulum and the sliding filament complex that comprises the contractile machinery. During excitation-contraction coupling, Ca^{2+} is released from the sarcoplasmic reticulum and combines with the filaments to activate contraction [3—5]. Relaxation then occurs when the sarcoplasmic reticulum performs its 'relaxing factor' function by pumping the Ca^{2+} back into itself, thus eluting the activator Ca^{2+} from the filaments and deactivating contraction [6,7]. Much evidence obtained from experiments on microsomes derived from the sarcoplasmic reticulum of skeletal muscle indicates that the relaxing function involves an active transport mechanism in which the energy to drive the pump is obtained by hydrolysis of ATP under action of a membrane-bound Ca^{2+}-ATPase [6,7]. Recently, interesting features of the mechanism of the pump have been revealed by temperature studies and their Arrhenius analyses of the Ca^{2+}-ATPase and associated Ca^{2+} uptake of such isolated microsomes [8—11].

We report effects of temperature on the kinetics of tetanus relaxation of a skeletal muscle. These effects are analyzable by the Arrhenius theory and the results bear certain resemblances to similar analyses of microsomal Ca^{2+}-ATPase and Ca^{2+}-uptake activities. We thus demonstrate more concretely than heretofore that relaxation of physiologically intact muscle involves the sarcoplasmic reticulum as inferred from studies of the uptake of Ca^{2+} by microsomal preparations.

Most previous determinations of the kinetics of muscle relaxation have been based on measurements of half-relaxation times, these mainly regarding the twitch. And only one of them indicated the possibility that an Arrhenius analysis was of interest in studying relaxation [13]. Though measures of half-relaxation time have some use, especially in comparing relaxation times of different muscles, or of the same muscle differently treated, they do not provide data that can be subjected to rigorous theoretical analysis, at least of the Arrhenius type. Furthermore, merely physiologically, the half-relaxation time of the twitch has limited significance since it relates to just a relatively quite small latter portion of the decay of the active state of the muscle, and thus reflects only a rather small segment of the full uptake of activator Ca^{2+} by the sarcoplasmic reticulum that is supposed to be basically involved in decay of the muscle's fully active state.

We have used the tetanus relaxation in the present work, because, first, it reflects the entire decay of the full, i.e., P_0, active state intensity [14]; and second, much of its latter part develops exponentially with a single, temperature-dependent time constant, τ, thus providing a set of temperature affected rate constants, k, which form the basis for an Arrhenius analysis (for a preliminary report on this feature, see Ref. 15).

Methods

We used excised extensor digitorum longus muscles of 3—6-month-old normal mice of the Jackson laboratory strain 129. The muscles were mounted in oxygenated mammalian Ringer's medium, initially at 15°C, in a massive

stimulation chamber, with square wave shocks, and connected to a type 5734 transducer tube isometric recorder, essentially as described previously [28].

The Ringer's medium contained $2 \cdot 10^{-5}$ g/ml *d*-tubocurarine (HCl) to ensure only direct stimulation of the muscles. Stimulating the muscles massively ensured that all response elements were synchronously activated, so that our records of whole muscle isometric contraction-relaxation cycles accurately reflect the course of the elementary cycles, especially here the relaxation events, occuring at the sarcomeric level. Each muscle was equilibrated to the Ringer's solution of the chamber at 15°C for 1 h before any testing. It was then tested for optimal length and strength of shock (always 0.3 ms in duration) to consistently produce maximal twitch output (i.e., the actual shocks were usually about 10% greater than just maximal). These stimulating conditions were used throughout our range of temperature since control tests showed they were adequate at each temperature studied. For the tetani, we determined at each temperature the frequency evoking a true maximal tension output, i.e. the P_0 for that temperature; at this frequency, fusion, moreover, was always present. Uniformly, such frequencies varied with temperature as follows: 15°C, 50 Hz; 20°C, 100 Hz; 25°C, 150 Hz; and both 30 and 35°C, 200 Hz.

Any muscle was tested for twitch and tetanus behavior sequentially at 15, 20, 25, 30 and 35°C. Each temperature was thermostatically controlled and varied by no more than about 0.1°C. In general, the entire series of tests involved a total time of about 1.5 h (i.e., exclusive of the initial equilibration period of 1 h). Thus, after the initial tests at 15°C (about 8—10 min) there were 4 periods, each averaging about 22 min, during which 12—15 min were used to heat the preparation to the next higher temperature and the remaining time to record several twitches at 2-min intervals and then the tetanus. Thus, each tested muscle spent about 45 min in Ringer's solution at 15—25°C, and a roughly equal duration at the higher range, 25—35°C. Hence, all muscle was exposed for only a quite short time to the higher temperatures and we observed nothing suggesting that such treatment caused any harmful effects to the muscle.

Results and Discussion

Fig. 1 presents semilogarithmic plots of the typical tetanus relaxations of a single muscle, obtained at our standard succession of temperatures. Clearly, the actual experimental points for the major, latter part (60—70%) of each plot quite exactly comprise a linear course and thus delineate an exponentially falling relaxation for which a time constant, τ, can be accurately measured. We have similarly tested six different muscles and they all yield tetanus relaxation plots like those of Fig. 1 at each temperature. Table I gives the means of the τ values for the six identically tested muscles. For each muscle the various plots of the latter part of the tetanus relaxations were as exactly linear as those of Fig. 1. However, as shown by the standard errors the corresponding τ values varied rather considerably among the muscles, the mean coefficient of variation being about ±8%. Nevertheless, the P values of Table I, show that the difference between the τ values for any pair of neighboring temperatures is highly signifi-

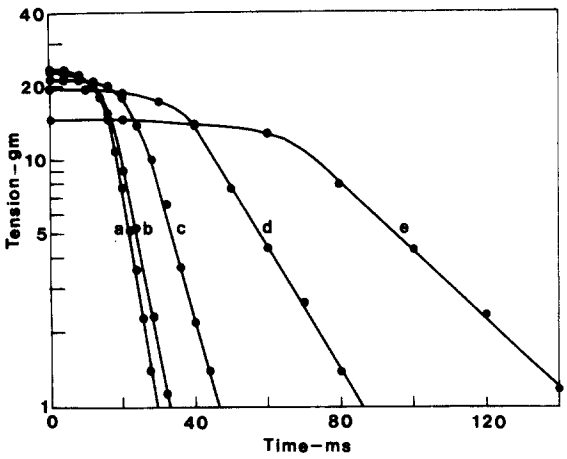

Fig. 1. Semilogarithmic plots of the relaxation phase of typical maximal isometric tetani of a single extensor digitorum longus muscle of the mouse at the standard series of temperatures in °C: a, 35; b, 30; c, 25; d, 20; e, 15. The zero of times marks the instant of the last shock of each tetanus. The dots represent measured values, and the curves have been drawn by eye through their respective points.

cant even for the higher temperature range involving relatively small differences in such paired values.

The presence of the definite τ values signifies that the reciprocal of each gives a rate constant, k (in s^{-1}), of some first order, rate-limiting reaction critically underlying the development of at least the large, latter segment of the tetanus relaxation (for a similar assumption, see Refs. 13, 16). The mean values of the k values are also given in Table I, and their Arrhenius plot is shown in Fig. 2. (It should be noted that in order to evaluate the logarithmic ordinate values of this graph statistically, we have calculated these values as indicated in the legend of this figure and not by taking the log of the mean k values listed in Table I). The plot evidently consists of two linear segments of different slopes whose transition temperature is 25°C. From the slopes of the two segments the

TABLE I

EFFECTS OF TEMPERATURE ON THE MEASURED TIME CONSTANT OF THE EXPONENTIALLY FALLING PORTION OF THE MAXIMAL TETANUS RELAXATIONS AND ON THE CORRESPONDING RATE CONSTANT OF THE ASSUMED RATE-LIMITING FIRST-ORDER REACTION DETERMINING RELAXATION

Note that the temperature coefficient (Q_{10}) for the 15—25°C range is 3.57 and for the 25—35°C range, 2.11. The difference between the means of τ for any pair of successive temperatures is highly significant with $P < 0.01$ or <0.005.

Temperature (°C)	t (ms)	k (s^{-1})
15	40.0 ± 3.5	25.0
20	23.4 ± 2.3	42.7
25	11.2 ± 1.2	89.3
30	7.2 ± 0.5	139
35	5.3 ± 0.3	189

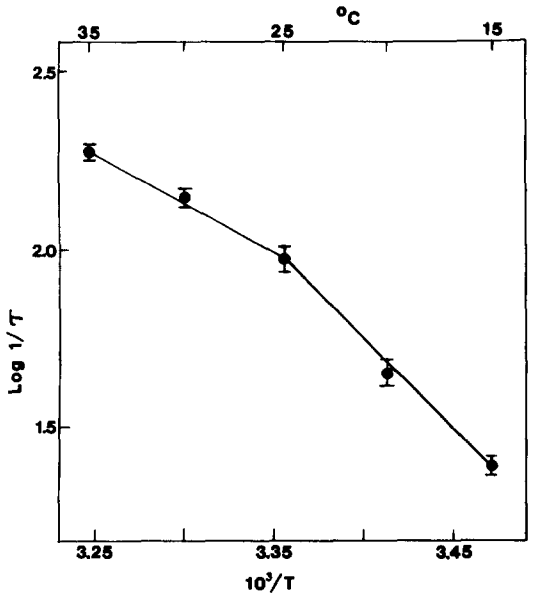

Fig. 2. Arrhenius plot of the means (±S.E.) of the values of log $1/\tau$ [= log k (s^{-1})] obtained from the exponentially falling tetanus relaxations of separate experiments on 6 different extensor digitorum longus muscles tested at the standard series of temperatures indicated by the reciprocals of the absolute temperature of the abscissa. Each τ was obtained from the linear portion of graphs similar to those of Fig. 1.

activation energies of the apparent reaction underlying the tetanus relaxation are 22.9 kcal/mol for the 15—25°C range, and 12.6 kcal/mol for the 25—35°C range, this lower value clearly reflecting the smaller Q_{10} for k at the higher temperature range (Table I).

We now infer that the rate-limiting reaction in question is concerned with some feature of the general mechanism by which the sarcoplasmic reticulum functions as a relaxing factor; i.e., by which it resequesters the activator Ca^{2+} it had released during the series of excitation-contraction couplings of the tetanus. Much experimentation on isolated preparations indicates that the essential feature of the sarcoplasmic reticulum's action as a relaxing factor is its powerful ability to accumulate Ca^{2+} by active transport [6,7]. But more specifically interesting here are the various findings, also regarding isolated microsomes, of the effects of temperature on its rates of Ca^{2+}-ATPase activity and uptake of the Ca^{2+} [8—12], and of the different physical features of the sarcoplasmic reticulum membrane as revealed by electron spin and nuclear magnetic resonance, and X-ray techniques [9,10,12]. It has been found generally that the Arrhenius plots of these temperature effects involve two linear segments of different slope whose transition temperature, depending on the particular determinations, varies from 11.5 to 20°C, and whose activation energies vary from 9—22 kcal/mol for the higher temperature range, and from 19.5 to 29 kcal/mol for the lower range. With the exception of our higher transition temperature of 25°C these properties of microsomal preparations resemble our comparable findings for the tetanus relaxations. But the Arrhenius values of the microsomal preparations are so variable that it is difficult to make

precise correlations between them and our tetanus relaxation values. Especially noteworthy is that our k values (Table I) are very different from those found for either microsomal Ca^{2+}-ATPase or Ca^{2+} uptake. Such values (i.e., in units of s^{-1}) are not explicitly given in the reports indicated above, but they can be derived from other mentioned data, e.g., by use of the relation between k (s^{-1}) and the free energy of activation (Table II). Thus, to mention only the Ca^{2+}-ATPase, the values of k calculated from the results of Hidalgo et al. [10], are 1.1 and 7.0 s^{-1} for 15 and 30°C, respectively, but our corresponding k values are 25 and 139 s^{-1}, i.e., about 20X higher. It is interesting, however, that Tonomura [17], suggests that the reaction rate of the Ca^{2+}-ATPase could be about 10 and 120 s^{-1} at 15 and 30°C, respectively and, thus, not too different from our values of k at these temperatures (Table I). It should be noted that his estimates may be high since he also mentions that Inesi measured a value of k at 25°C of 10 s^{-1} for the initial phase of Ca^{2+} uptake. However, the general differences between our various tetanus values and any of the others may stem from the obvious differences in method of studying relaxation: our tests concern actual tension relaxations of living mouse muscles (in which, e.g., the in situ sarcoplasmic reticulum ATPase and the coupled uptake of Ca^{2+} would be expected to perform much more expeditiously than in extracted preparations, which should account for our much greater values of k), while the others deal with the basic molecular, relaxing factor reactions of isolated rabbit sarcoplasmic reticulum. We must emphasize that our tetanus results resemble those for microsomal preparations in that both involve: (a) Arrhenius plots composed of two linear segments with a definite transition temperature and with a smaller slope for the higher temperature range; and (b) comparable activation energies which, in view of the temperature-dependent slopes, are less for the higher temperature range than for the lower. Hence, we infer that the tetanus relaxation of the living muscle depends essentially on sarcoplasmic reticulum mechanisms concerned with Ca^{2+} accumulation which are known to occur in microsomal preparations. That is, we propose that the course of tetanus relaxation, as at

TABLE II

THERMODYNAMIC VALUES FOR THE APPARENT ACTIVATION REACTION SUGGESTED BY THE KINETICS OF THE EXPONENTIALLY FALLING PHASE OF TETANUS RELAXATION OF MOUSE EXTENSOR DIGITORUM LONGUS MUSCLE

$\Delta G^{\ddagger} = -RT \ln(hk)/(k_B T)$ where R = gas constant, h = Planck's constant, k_B = Boltzmann's constant, T = degrees K, and k as given above. $\Delta S^{\ddagger} = (E_a^{\ddagger} - RT - \Delta G^{\ddagger})/T$, with $E_a^{\ddagger}$ = activation energy. Note that $H^{\ddagger} = E_a^{\ddagger} - RT$ and thus about 22.3 and 12.0 kcal/mol at 15—25 and 25—35°C, respectively.

Temperature (°C)	k (s^{-1})	Activation energy (kcal/mol)	$\Delta G^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (cal/degree per mol)
15	25.0		15.0	25.4
20	42.7	22.9 **	15.0	24.9
25 *	89.3		14.8	—
30	139	12.6 ***	14.8	−9.2
35	189		14.9	−10.4

* Transition temperature.
** For the 15—25°C range.
*** For the 25—35°C range.

least indicated by the exponentially falling part closely approximates the kinetics of the decrease in concentration of the myoplasmic free Ca^{2+} and thus of the Ca^{2+} bound to the contractile protein, which occurs as the sarcoplasmic reticulum, now free of excitation-contraction coupling effects, is able to perform its relaxing factor function of pumping the activator Ca^{2+} back into its vesicles.

Edwards et al. [16] have also studied the exponentially falling phase of tetanus, but in highly fatigued, slow rat muscles which have abnormally low rates of relaxation and parallel reduced ATP levels. They infer that the rate-limiting reaction of relaxation depends on the rate of utilization of ATP in the dissociation of the cross-bridges involved in myosin-ATPase cycling. However, they cannot rule out the possibility that an altered rate of Ca^{2+} uptake by the sarcoplasmic reticulum of their muscles could account for the changes in relaxation rate which they observe. Moreover, our experiments are on a fast, unfatigued mouse muscle, and our Arrhenius analyses, together with all the background evidence mentioned above strongly indicate that the sarcoplasmic reticulum's relaxing activity is the basic determinant of the rate of relaxation. Further support of this view is found in various results showing in isolated systems a direct dependence of reduction of contractile activity of myofibrils on uptake of Ca^{2+} by muscle microsomes [18], and in intact systems a positive correlation between relaxation speed of rat skeletal muscles and the Ca^{2+}-uptake rate of their isolated sarcoplasmic reticulum [19—21].

As already emphasized, our conception of tetanus relaxation is based directly on the exponential latter part of the relaxation. But we assume that this conception applies to the whole relaxation, i.e., to the slowly developing shoulder portion, as well as to the subsequent exponential phase. Support for this assumption is that, even though it would require the pumping of Ca^{2+} into the sarcoplasmic reticulum to follow the supposed first-order kinetics from the start, this would not be revealed in the parallel exponential fall of tetanus tension during the time of the shoulder because of the curved nature of the tension versus ordinary concentration curve of Ca^{2+} (rather than in terms of pCa) which is especially pronounced for high Ca^{2+} concentrations [14,18,22]. Further support for this assumption is that the Arrhenius plot (although not rigorously justifiable) of the reciprocals of the tetanus half-relaxation times also yields a bilinear relation, with a transition at 25°C, and gives the comparable activation energies of 17.9 and 10.5 kcal/mol at the lower and higher temperatures, respectively (cf. Table II).

The operation of the relaxing factor evidently involves a very complex series of possibly five reactions by which the membrane-bound Ca^{2+}-ATPase couples the energy it releases by splitting the terminal phosphate bond of ATP to the uptake of Ca^{2+} into the sarcoplasmic reticulum (e.g., Refs. 10, 17). Our findings do not reveal which unit of this reaction chain is the rate-limiting first-order reaction that seems to underlie our tetanus relaxations. However, whatever this reaction is, we can use our data according to transition-state theory [9,10] to determine changes in the values of the thermodynamic parameters of the already given activation energies. Table II shows that our values for $\Delta G^{\ddagger}$, and for $\Delta S^{\ddagger}$ at relatively low temperatures, are comparable with those found by others [9,10,23]. But our $\Delta S^{\ddagger}$ values at high temperatures are exceptional

since they are of negative sign. However, such negative values are consistent with the corresponding values at high temperatures given in the foregoing reports, since in these cases, as in ours, the shift in entropy with increase in temperature is at least toward smaller positive increments. For example, Inesi et al. [9] report that the $\Delta S^{\ddagger}$ for both Ca^{2+} uptake and ATPase activity is from +34 to +40 cal/degree per mol at 5—20°C, but only 0 to +4 cal/degree per mol at 20—35°C. These possible concordances with our findings add further support for our conclusion that the course of tetanus relaxation is essentially determined by the sarcoplasmic reticulum acting as a relaxing factor.

A most interesting feature of the theoretical development of the current research in muscle microsomes is concerned especially with the physical structure of the lipid-protein configuration of the sarcoplasmic reticulum membrane and the possible effects that certain thermotropic changes in it might have on the Ca^{2+}-ATPase pumping system [9,10,12,24]. Our present results and those of others suggest the possibility of a negative (or a lesser) entropy change during activation at temperatures above the transition temperature. If this is actually the case, there must be a relative increase in order of the physical structure (e.g. of the lipids?) [9,10] or of possibly the nature of the ATPase [25] of the sarcoplasmic reticulum membrane that conditions the activation of the Ca^{2+}-ATPase system. Certain workers [9,10] have stressed other results involving positive entropy values and therefore indicating decrease in physical order conditioning the activation reaction. The temperature dependent contrast between the negative and positive $\Delta S^{\ddagger}$ values raises certain problems that need clarification. Further work is also needed to relate the mechanism of relaxation we propose to different views of this process expressed by others [16,26,27]. It is hoped that projected studies on relaxation, which will deal with the effects of agents that are known to alter the sarcoplasmic reticulum membrane, will help elucidate these and other features of the general problem of muscular relaxation.

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