CALCIUM TRANSIENTS AND CALCIUM BINDING TO TROPONIN AT THE CONTRACTION THRESHOLD IN SKELETAL MUSCLE

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ABSTRACT Antipyrylazo III calcium transients from voltage-clamped, cut skeletal muscle fibers of the frog were recorded, and the calcium binding to the regulatory sites of troponin C was calculated. The strength–duration curve for the contraction threshold was determined. It was found that the increase in myoplasmic calcium concentration necessary to produce the same level of contractile activation, i.e., the just visible movement, was ~60% higher at more positive membrane potentials resulting from short depolarizing pulses than at rheobase. However, using biochemical data for the k_{ON} and k_{OFF} rate coefficients of the binding sites, the calculated maximums of the calcium binding curves were about the same at different voltages, and the time to maximum saturation was roughly equal to the latency of the contractions. To characterize the calcium binding in intact fibers more accurately, those values of the k_{ON} and k_{OFF} rate coefficients that gave equal peak saturations during threshold movement at different membrane potentials were determined.

INTRODUCTION

It is generally accepted that the contractile activation of intact skeletal muscle fibers is a membrane potential-dependent process (Hodgkin and Horowicz, 1960). This voltage dependency is based on the movement of intramembrane charged particles (Schneider and Chandler, 1973). Both the amount of charge moved and the rate of this movement depend on the membrane potential (Chandler et al., 1976). The voltage-dependent characteristics of the charge movement are reflected in the size and kinetics of the changes in myoplasmic Ca^{2+} concentration (Kovács et al., 1979). In the regulation of contractile activity, the essential event is the binding of calcium to the calcium-specific sites of troponin C (e.g., Ebashi and Endo, 1968); therefore, the contraction evolving in this way evidently must bear voltage-dependent features.

In this complex system investigation of the phenomena at the contraction threshold has specific advantages. The kinetics of contractile activation is voltage dependent in this case, too, but the degree of mechanical response (i.e., the just visible movement) is the same at different membrane potentials though occurring with varying latencies. Earlier work has revealed that similar extents of activation can be explained by the movement of the same amount of intramembrane charges (Horowicz and Schneider, 1981). It was also described that along the strength–duration curve for the contraction threshold, similar increases in myoplasmic calcium concentration resulted from various short (5–20 ms) depolarizing pulses (Kovács and Szücs, 1983).

In the experiments reported here the measurement of myoplasmic Ca transients was extended to the rheobase. Moreover, calculations were performed to study the quantitative and kinetic aspects of calcium saturation taking place on the regulatory sites of the troponin C molecules.

METHODS

These experiments employed the methods developed by Kovács and Schneider (1978), with some modifications (Kovács and Szücs, 1983). Fibers were isolated from the semitendinosus muscle of frogs (*Rana esculenta*), cut in a relaxing solution (120 mM K-glutamate, 2 mM $MgCl_2$, 0.1 mM EGTA, 5 mM Tris-Na-maleate buffer), and mounted in a single vaseline gap chamber. The length of the terminated segment was set to ~200–300 μm. After the completion of the vaseline isolation, the solution in the closed end pool was exchanged for the external solution (75 mM [tetraethylammonium]$_2SO_4$, 10 mM Cs_2SO_4, 8 mM $CaSO_4$, 3.1×10^{-7} M tetrodotoxin, 5 mM Tris-Na-maleate buffer). The internal solution at the open end (108 mM Cs-glutamate, 5.5 mM $MgCl_2$, 0.1 mM EGTA, 0.0082 mM $CaCl_2$, 4.5 mM Tris-Na-maleate buffer, 13.2 mM Tris-Cs-maleate buffer, 5 mM ATP, 5.6 mM glucose) contained 1 mM antipyrylazo III dye, which entered the myoplasmic space of the fiber. The membrane potential of the closed end was voltage clamped at −100 mV. To determine the contraction threshold, the movements of the terminated segment were observed visually through a compound microscope at a magnification of 400. To record the contractions, we attached a piece of aluminum foil to the fiber close to the tendon and monitored its movements at a wavelength of 850 nm. The timing of the pulses, data taking, and processing were carried out using a microcomputer system. All experiments were performed at a low temperature (4–6°C).

The calcium transients accompanying the depolarizing pulses were derived from the absorbance changes measured at 720 nm. The dye absorbance signals were previously corrected for changes in intrinsic absorbance, which were recorded at 850 nm (Melzer et al., 1986). For the calculation of the free myoplasmic calcium concentrations, the K_d value and the extinction coefficient taken from the literature (Kovács et al., 1983) were applied.

Calculation of Calcium Binding on Troponin

The time course of saturation on the calcium-specific binding sites of troponin C was calculated by numeric integration of the differential equation

$$d\,[CaTn]/dt = [Ca^{2+}]\,[Tn]\,k_{ON} - [CaTn]\,k_{OFF} \quad (1)$$

(Robertson et al., 1981; Baylor et al., 1983). [CaTn] denotes the concentration of the calcium–troponin complex, whereas the concentration of the free troponin-binding sites ([Tn]) was given by

$$[Tn] = [Tn]_{total} - [CaTn], \quad (2)$$

where $[Tn]_{total}$ is the concentration of the calcium-specific binding sites of troponin in the myoplasm, its value was taken as 240 μM (Baylor et al., 1983).

The resting free-calcium level was taken to be 0 μM.

RESULTS

Calcium Transients at the Contraction Thresholds

The strength–duration relation for the contraction threshold was determined in the experiment presented in Fig. 1. At different membrane potentials, different pulse durations were necessary to evoke the just visible movement. The calcium transients recorded at the contraction threshold are shown in Fig. 1 *A*. The latency and the rate of rise of the calcium signals exhibit voltage dependency as reported earlier (Kovács and Szücs, 1983). Fig. 1 *B* shows that the maximum Ca^{2+} concentration increase during depolarizing pulses of 5–20 ms in duration is the same, whereas the peak amplitude of the signal obtained with a 100-ms-long pulse corresponding to the rheobase is considerably smaller.

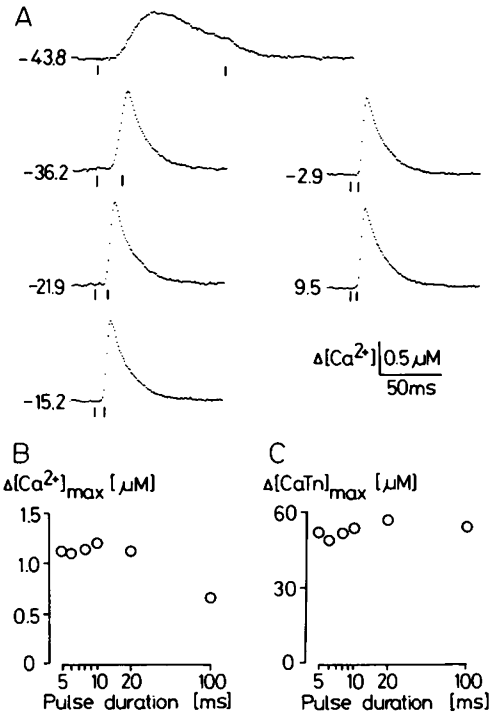

FIGURE 1 (*A*) Myoplasmic calcium transients belonging to the strength-duration relation for contraction threshold. In the experiment depolarization resulting in just visible contraction was determined at pulse durations of 5, 6, 8, 10, 20, and 100 ms. Calcium transients were recorded as an average of eight sweeps each. Duration of pulses is indicated by vertical bars; the membrane potential during the pulse is shown at the individual traces. (*B*) Peak values of the calcium concentration changes are plotted as a function of pulse duration. (*C*) Maximum values of troponin saturation calculated from the corresponding calcium transients using the parameters of model 2. Fiber 30223, *s* (sarcomere length) = 3.0 μm, *d* (horizontal diameter) = 100.0 μm, D_T (intracellular dye concentration) = 627–703 μM.

Quantitative Aspects of Troponin Saturation

The above observation is apparently in contrast with the fact that the applied pulses evoked the same contractile response, i.e., just perceptible movement. We had to suppose that the same degree of contractile activation is accompanied by the same occupancy of the calcium-binding sites on the troponin molecules, i.e., the calcium transients at the contraction threshold that have different amplitudes and kinetic properties can bring about the same percentage saturation at the regulatory sites. To test this assumption, the time course of calcium binding to troponin was calculated from the individual calcium transients using Eq. 1.

Inasmuch as there is no available data concerning the properties of binding sites in frog muscle fibers, three possible models were examined using the procedure described by Baylor et al. (1983). The parameter values of the models (Table I) were taken from previously reported biochemical data obtained in experiments on mammalian muscles using a purified troponin preparation (Potter and Gergely, 1975; Johnson et al., 1981) and a reconstructed thin filament (Potter and Zot, 1982) after making correction for the difference in temperature ($Q_{10} \approx 2$).

Fig. 2, *C* and *D* shows the saturation of troponin calculated from two calcium transients (pulse duration of 100 and 5 ms for Fig. 2, *A* and *B*, respectively) with the parameters of the three models. The maximum CaTn concentrations are different depending on the model chosen. It is obvious that, in models 1 and 3, the maximum saturation is higher at the rheobase than with the short pulse, whereas in model 2 the peaks are about the same. It

TABLE I
PARAMETER VALUES OF MODELS FOR CALCIUM-TROPONIN REACTION

Model	K_d	k_{OFF}	k_{ON}
	μM	s^{-1}	$M^{-1}\,s^{-1}$
1	0.2	5.8	2.9×10^7
2	2	57.5	2.9×10^7
3	2	5.8	2.9×10^6

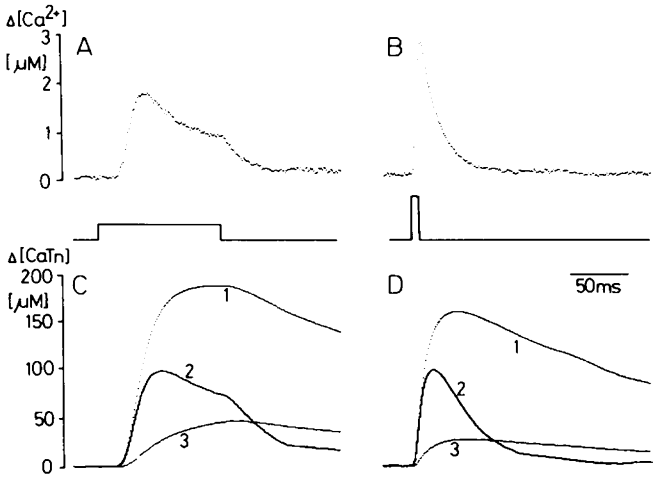

FIGURE 2 Calcium binding of troponin. The upper half shows calcium transients (*A*) belonging to the rheobase (100 ms, −33.3 mV) and (*B*) evoked by a short pulse, respectively (5 ms, 22.9 mV). The depolarizations are indicated by schematic pulses. Each of the curves was obtained by averaging eight sweeps. Fiber 30310, s = 2.6 μm, d = 71.4 μm, D_T = 448–507 μM. On the lower half the changes in concentration of the calcium–troponin complex are displayed (*C* and *D*). The individual curves were derived from the corresponding calcium transients (*A–C*, *B–D*) using Eq. 1. The numbers at the traces indicate which model was used in the calculation.

is also shown in Fig. 1 *C* that the troponin saturation calculated with model 2 is the same for all pulse durations tested, although the maximum amplitude of the calcium transient at the rheobase is considerably smaller than that of the other signals (Fig. 1, *A* and *B*).

The results obtained using model 2 are summarized in Table II. The maximum calcium concentration change measured using short pulses is 60% higher than that obtained at the rheobase. The peak values of the calcium binding to troponin are the same in both cases, in agreement with our supposition that the same extent of contractile activation is based on the same percentage of occupation of the regulatory sites.

Time Course of Troponin Saturation

Fig. 2 illustrates that, in the case of models 1 and 3, calcium binding approaches its maximum rather slowly (i.e., by the end of the depolarizing pulse at the rheobase), and the falling phase is also very slow. The time course of the troponin saturation calculated by model 2 seemed acceptable and was verified further by experimental observations.

In our experiments the rheobase was determined with 100-ms-long depolarizing pulses. It was, however, found that, although maintaining the pulse amplitude value obtained in this way, the just detectable movement could also be elicited after shortening the depolarizing pulses. The critical duration, which was still long enough to evoke the threshold movement at the rheobase voltage, was 55.6 ± 8.8 ms (mean ± SD) as an average of seven measurements. This value is comparable to the time course of troponin saturation at rheobase. In 25 cases when the calculations were carried out in a way similar to that shown in Fig. 2 *C*, the time necessary to reach maximum troponin saturation was 51.8 ± 8.3 ms (mean ± SD). We can conclude, therefore, that to elicit threshold movement, the rheobase voltage has to be maintained for a time long enough to reach sufficient saturation of troponin.

To support further the validity of our calculations, experiments were designed to study the relation between the onset of movement and the percentage of saturation of troponin. The results of measurements at two membrane potentials are shown in Fig. 3. The pulse durations evoking just detectable movement were determined (8 ms in Fig. 3 *A* and 30 ms in Fig. 3 *B*), and the corresponding calcium transients were then recorded. Thereafter, the pulse duration was increased to 15 ms (Fig. 3 *A*) and 40 ms (Fig. 3 *B*), respectively, and the movement of the terminated segment was observed. With this procedure the binding of Ca^{2+} to troponin at the contraction threshold was compared directly to the latency of contraction at the same membrane potential.

TABLE II
PEAK VALUES OF CALCIUM TRANSIENTS AND TROPONIN SATURATION AT THE RHEOBASE AND IN THE CASE OF SHORT PULSES

Fiber	1 $[Ca^{2+}]_{max}$ 100 ms	2 $[Ca^{2+}]_{max}$ 5–20 ms	3 2/1	4 $[CaTn]_{max}$ 100 ms	5 $[CaTn]_{max}$ 5–20 ms	6 5/4
	μM	*μM*		*μM*	*μM*	
50530	1.35	2.33	1.73	79.8	85.7	1.07
50429	2.71	3.30	1.22	126.5	118.3	0.94
50502A	1.17	2.31	1.97	81.3	99.6	1.23
50502B	1.55	1.86	1.20	95.9	80.0	0.83
30217	0.85	1.47	1.73	60.9	63.5	1.04
30310	1.78	2.92	1.64	103.2	109.0	1.06
30223	0.66	1.13	1.71	53.4	52.3	0.98
Mean ± SD	1.44 ± 0.68	2.19 ± 0.77	1.60 ± 0.29	85.9 ± 25.1	86.9 ± 23.9	1.02 ± 0.12

The displayed data are individual values for the rheobase (100 ms) and average of four to five values in the case of short pulses (5–20 ms). Ca^{2+} binding of troponin was calculated with the parameters of model 2.

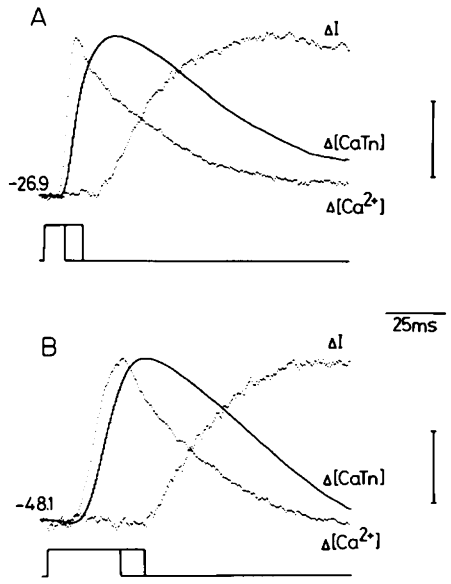

FIGURE 3 Comparison of the time course of myoplasmic calcium transient ($\Delta[Ca^{2+}]$), troponin–calcium binding (Δ[CaTn]) and the movement (ΔI). Calcium transients were evoked by (*A*) 8- and (*B*) 30-ms-long depolarizing pulses; the membrane potentials are given by numbers shown at the curves. To record movement the depolarizing pulses were increased to (*A*) 15 and (*B*) 40 ms, respectively. The depolarizations are indicated by schematic pulses below the traces. In the calculation of troponin saturation, the parameters of model 2 were used. The different signals were scaled to get the same peak amplitudes. Vertical calibration 1 μM $[Ca^{2+}]$, 33 μM [CaTn], 0.5 mV ΔI for both *A* and *B*. Fiber 50912, s = 2.3 μm, d = 100.0 μm, D_T = 284–304 μM.

To make the comparison easier, the traces representing the myoplasmic calcium concentration changes, troponin saturation, and contractile activity were adjusted to have the same peak values (Fig. 3). The voltage-dependent features are evident: at a more positive membrane potential (Fig. 3 *A*), both the latency of the calcium transient and the time to peak are shorter; therefore, the maximum of troponin saturation also develops earlier. In both cases the movement appears at the time when the calcium binding to troponin is close to the peak value corresponding to the threshold for contraction. Taking all results of the similar experiments into account, we conclude that the movement has started when the calcium saturation of troponin is above 90% of this value. A more accurate comparison does not seem to be reasonable because the events subsequent to the calcium binding on troponin (cross-bridge attachment, force generation, etc.) can cause some delay in the appearance of movement.

In using this type of movement recording, it was a disadvantage that the calcium transients and the corresponding movement traces were determined at different locations along the fiber, even though we tried to shorten the length of the terminated segment. To minimize the errors originating in the possible inhomogeneities of the different locations on the fiber, in a few cases contraction was recorded as a movement artifact at 850 nm at the same place where the calcium transients were measured. Although these movement records may have been contaminated by intrinsic absorbance changes, we obtained the same results as in the experiment described above (Fig. 3).

Best Fit of the k_{ON} and k_{OFF} Rate Coefficients

The above results show that the rate coefficients used in model 2 are probably close to those belonging to the regulatory sites of troponin C in intact fibers. The limits of these rate coefficients, valid for our circumstances, were determined below.

As an essential condition, it was supposed that the calcium transients belonging to both rheobase voltage and short depolarizing pulses at the contraction threshold brought about equal maximal troponin saturations. The values of the rate coefficients that fulfilled this condition were calculated. As expected, the maximums were equal in numerous combinations of k_{ON} and k_{OFF} rate coefficients, with each combination resulting in the equality of maximums at different troponin saturations. Taking either the peak values of the saturation curves or one of the rate coefficients as the given value, the others could be determined.

Table III shows the mean of those k_{ON} and k_{OFF} values that resulted in equal maximums of calcium-binding curves at given occupancy levels (20%, 50%, and 80%). Because we had no data as to how great this saturation of the regulatory sites at the contraction threshold is, we supposed a value between 20% and 80%. According to our results (Table III) the rate coefficients for intact fibers must be in the following ranges: $k_{ON} = 1.69 - 10.72 \times 10^7$ M^{-1} s^{-1} and $k_{OFF} = 37.6 - 98.1$ s^{-1}. If the limits for the percentage of occupancy of the binding sites at the contraction threshold could be further restricted, the limiting values for the rate coefficients would be in a narrower range, too.

Table III also shows how the time-to-peak values of calcium-binding curves belonging to rheobase vary with the percentage of troponin occupancy. It is obvious that, although the time course of the curves is different because of the different $k_{ON} - k_{OFF}$ values, the time necessary to

TABLE III
BEST FIT OF THE RATE COEFFICIENTS AND THE TIME TO PEAK OF CALCIUM BINDING TO TROPONIN C

	Maximum of troponin occupancy (%)		
	20	50	80
k_{ON} ($\times 10^7 M^{-1}s^{-1}$)	1.69 ± 0.78	5.08 ± 2.34	10.72 ± 4.91
k_{OFF} (s^{-1})	98.1 ± 61.7	72.7 ± 43.7	37.6 ± 21.3
Time to peak (*ms*)	57.3 ± 11.2	56.4 ± 10.2	54.7 ± 9.9

The values (mean ± SD, n = 10) were derived assuming equal maximums, with different pulse durations belonging to the contraction threshold, at given levels of troponin saturation. For further details see the text.

reach the maximum saturation was hardly changed. The time-to-peak values obtained on 10 fibers (between 57.3 ± 11.2 and 54.7 ± 9.9 ms, mean ± SD) agreed quite well with the critical duration (55.6 ± 8.8 ms) demonstrated above.

For further characterization of calcium binding to troponin C in intact fibers, the best fit of k_{OFF} and the maximal rate of occupancy were also determined using k_{ON} ($2.9 \times 10^7\ M^{-1}\ s^{-1}$ from model 2) as a constant. Carrying out the calculations on the same 10 fibers mentioned in Table III, the following rates were obtained (mean ± SD): $k_{OFF} = 90.1 \pm 55.8\ s^{-1}$; troponin occupancy at contraction threshold 36.4 ± 11.9%. The time-to-peak values for maximum occupancy at rheobase were found in the range shown in Table III.

DISCUSSION

In the experiments presented here, the calcium binding to the regulatory sites of troponin C was characterized in intact fibers. The measurements were made at the contraction threshold and the data were analyzed by supposing that, in the same fiber, the calcium transients having different time courses and amplitudes bring about an equal maximal level of calcium binding on troponin C. The agreement of the calculated time-to-peak calcium saturation with the critical duration at the rheobase and with the latency of contractions confirmed that our analysis is acceptable. However, on discussing the results, one also has to take into account those uncertainties that may decrease the reliability of the calculations.

One of the critical steps is the calculation of the free myoplasmic calcium concentration from the measured absorbance changes. The applied constants (K_d and the difference extinction coefficient of the dye) and the stoichiometry of the calcium–dye reaction are generally accepted in the literature. Recent data indicate, however, that antipyrylazo III binds to different intracellular sites (Irving et al., 1985). There is a possibility that either the bound dye molecules do not monitor calcium, or the characteristics of the calcium–dye reaction may differ from those determined in vitro. Although similar analyses with different dyes and different methods could improve the reliability of the measurements, we suppose that the ratio between the amplitudes and the time course of the transients recorded at the rheobase and with short depolarizing pulses is not modified significantly at the dye concentrations used in our experiments.

The determination of the k_{ON} rate coefficient is especially difficult because it appears in Eq. 1 in the term of k_{ON} [Ca]. Thus, k_{ON} may compensate for the uncertainties in the calibration of the calcium transients, and therefore the numerical value of the k_{ON} may be less accurate than that of k_{OFF}, the estimate of which appears to be independent of the free-calcium level.

The concentration of the regulatory sites on troponin C was taken to be 240 μM (Baylor et al., 1983). It follows from Eq. 1 that the value of $[Tn]_{total}$ only influences the absolute value of [CaTn] but has no effect on the relative occupancy ($[CaTn]/[Tn]_{total}$). Hence, the concentration of binding sites, in the first approximation, did not seem to be critical, thus, in determining the best fit of k_{ON} and k_{off} (Table III), the calcium binding was given in terms of relative saturation.

Inasmuch as we did not measure the resting calcium concentration, its value was taken to be zero in the calculations. Among the kinetic parameters defining the calcium binding to the troponin C, only the k_{OFF} would be influenced by an occasional change in the resting calcium concentration. The value of k_{OFF}*, valid for an elevated resting calcium concentration ([Ca]*) is given by Eq. 3,

$$k_{OFF}^* = k_{OFF} + k_{ON}[Ca]^*, \quad (3)$$

where k_{ON} and k_{OFF} are the rate coefficients at a 0 μM resting calcium concentration. Assuming a resting calcium level of 0.1 μM and using the parameters in model 2, this correction results in an ~5% increase in the k_{OFF} value.

The measurements were carried out using fibers with normal sarcomere length, varying from 2.3 to 3 μm. In the literature there are well-known data showing that stretching significantly increases the myofibrillar calcium sensitivity of different muscle preparations (Endo, 1972; Moss et al., 1983). Consistent with this effect it was previously established that the length of the muscle influences the apparent affinity of the calcium-binding sites on troponin (Fuchs, 1978). Hence, it looks very probable, that the rate coefficients determined here are not valid for stretched fibers, i.e., where sarcomere length is >3.6 μm. Inasmuch as there is no way to determine the contraction threshold under such conditions, the calculation of rate coefficients cannot be done in a way similar to the one described above.

In spite of all the difficulties, we believe that although further investigations may be necessary to characterize the calcium binding to the regulatory sites of troponin C in intact fibers, our results give reasonable limits for the k_{ON} and k_{OFF} rate coefficients.

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