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Can the binding of Ca^{2+} to two regulatory sites on troponin C determine the steep pCa/tension relationship of skeletal muscle?

(skinned muscle fibers/cross-bridge cycle time)

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ABSTRACT **The relationship between tension and Ca^{2+} concentration in single skinned muscle fibers has been determined with a high density of experimental points and the data have been fitted by a least squares method to the Hill equation. We find that the mean Hill coefficient for the slope of the tension/Ca^{2+} relationship is between 5 and 6, and the pK_d is about 5.9. Because there are four Ca^{2+} binding sites on troponin C, and only two of these regulate hydrolysis of MgATP, we conclude that the regulation of tension by Ca^{2+} binding is greatly modified by other factors. One important factor is the time required for a cross-bridge to complete a cycle once initiated, relative to the time Ca^{2+} remains bound to troponin C. The pCa/tension relationship will shift to higher pCa values as the ratio of cross-bridge cycle time to the Ca^{2+} bound time increases. For example, the pCa/tension curve may progressively shift to the left with increase in tension because strain in the myofilament lattice progressively increases the cycle time. This left shift will produce a pCa/tension relationship that is steeper than the actual Ca^{2+} binding curve. The anticipated shift of the pCa/tension curve with cycle time also bears on interpretations of earlier experiments on the "active state" and on the effects of Ca^{2+} on the maximal velocity of shortening.**

It is widely accepted that the contractile activity of vertebrate skeletal muscle is initiated by the binding of Ca^{2+} to troponin C (TnC), one component of the complex of four regulatory proteins on the thin filaments (1). Binding of Ca^{2+} to two calcium-specific sites on TnC regulates the rate of MgATP hydrolysis by myofibrils (2, 3). Two other sites on TnC have a higher affinity for Ca^{2+} and also bind Mg^{2+}, but there is no biochemical evidence that these or any other sites participate in regulation (4). One might therefore expect tension to increase with Ca^{2+} concentration, following a slope similar to that for the hydrolysis rate. Yet the estimated slope of the pCa/tension relationship in skinned muscle fibers is usually much steeper (5–9). A model for tension regulation similar to that for the regulation of hydrolysis would require that more than two sites bind Ca^{2+} with maximal cooperativitiy (Eq. 1 below). Either these reported slopes are overestimated or the relationship between Ca^{2+} binding and tension is not direct. To define better the slope of the pCa/tension relationship in chemically skinned rabbit psoas fibers (10, 11), we developed a high-precision technique in which the interval between points on the pCa/tension curve averages 0.07 pCa unit.

MATERIALS AND METHODS

Segments of single fibers are mounted in our apparatus (Fig. 1) by wrapping one end ¾ turn around a fine wire extending from a tension transducer (12) and the other end around a wire extending from a length-adjustment device. Only the ends of the fiber are slightly compressed under small clips. During contraction the wrapped part of the fiber "grips" the wire. Thus, no part of the fiber in series with the force-generating segment is crushed. While the fiber is being mounted and the sarcomere length set to 2.6 μm, the chamber contains 0.5 ml of a saline at pCa = 8 (10 nM Ca). After an equilibration period, the Ca^{2+} concentration is increased automatically in small steps by microprocessor-controlled injection of a predetermined volume of saline at pCa 5.0 (10 μM Ca) into the saline in the chamber.

High-precision pumps (Fluid Metering, Oyster Bay, NY) inject 50 μl of pCa 5.0 saline with each full revolution. The pumps are driven by stepping motors controlled by the microprocessor. The volume in the chamber is kept constant by aspirating fluid off the top through a vibrating tube. The vibration prevents a meniscus from forming, and withdrawal of solution stops uniformly at a predetermined level. A small

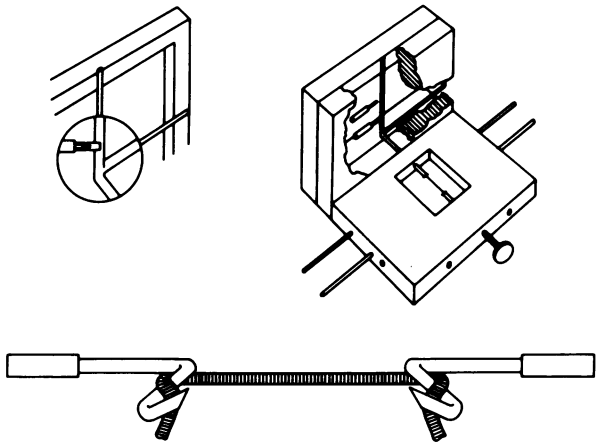

FIG. 1. Fiber is attached to a wire hook that extends from the tension gauge, fixed to the back of the chamber (top), and to a similar hook attached to the length-adjustment device. The fiber is wrapped ¾ turn around the wires and the ends are wedged into small tapered slots formed in a bend at the ends of the wires. This is shown diagramatically at the bottom of the figure. When the fiber contracts, it transmits force to the hooks through the wrapped segments. This avoids the use of clamps, which may crush part of the fiber at the point of attachment to the apparatus. The hooks are made from stainless steel wire 0.35 mm in diameter. At the left is an enlargement of the contact between a strain gauge element and the stainless steel tubing that holds the wire hook. Details of this tension transducer will be published elsewhere. It is a stable system, with drift limited to a few milligrams per hour. The chamber is made of an aluminum block and is kept at a constant temperature by circulating temperature-controlled fluid through channels milled in the block. Solution changes are made by pumping fluid into the chamber through stainless steel tubes forced through holes in the chamber walls.

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Abbreviations: TnC, troponin C; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

paddle on the end of the tube vigorously stirs the solution. The apparatus is programmed to wait after each addition until the tension change is less than 1 mg/10 sec before the next increase in calcium concentration.

With this automated serial method we avoid pulling the fiber through an interface for each change in pCa. Other advantages of the method are that the increment in the Ca^{2+} concentration can be made as small as needed, random pipetting errors are avoided, and the data are recorded in digital form on paper tape, which eliminates chart reading errors.

The accuracy of the automated technique was verified directly by injecting [^{3}H]inulin solution into the chamber by the procedure used to inject pCa 5.0 saline. Washout efficiency was determined by filling the chamber with [^{3}H]inulin solution and then washing out twice with unlabeled solution (2.4% of the cpm was left after the first wash; 0.4% left after the second). [^{3}H]Inulin solution was then serially injected and, after a predetermined total injected volume (V), four 25-μl samples were removed from the chamber and assayed for radioactivity. The entire sequence was repeated for several other Vs. The equation $C_v = C_s + (C_0 - C_s)e^{-V/V_0}$ is solved and C_v is compared to the sample cpm; C_v is cpm in the chamber (or concentration) at V, C_s is cpm in the stock inulin, C_0 is initial cpm, and V_0 = chamber volume (0.5 ml). All cpm are per 25-μl sample volume. The accuracy of the serial method was confirmed to be within 1% (or 0.004 pCa unit).

In our experiments the substrate [MgATP(13)] was at 5.0 mM [approximately the *in vivo* level (14–17)], a concentration sufficient to maximize the frequency for oscillatory work in skinned rabbit psoas (18). Free ATP was kept at 5.0 mM, a concentration sufficient to buffer the substrate without further "back-up" enzyme systems (19). The saline also contained 7.5 mM phosphate, 6.0 mM total ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), up to 6 mM total calcium (depending on the pCa), 10 mM morpholinopropanesulfonic acid to buffer the pH to 7.00, 40 mM Na propionate, and 21 mM Na_2SO_4 (ionic strength = 0.200). Temperature was maintained at 20 ± 0.1°C.

After the fiber was mounted in the apparatus and the length adjusted so that the sarcomeres were 2.6 μm long, a protocol was keyed into the microprocessor memory. Usually specified was an initial test of the fiber with pCa 5 solution and then, following the relaxation, serial decreases of the pCa from 8 to 5 to determine the pCa/tension relationship.

An experiment in which about 20 data points were collected took approximately 10 min. The data from the automated experiments were read into a Nova 800J minicomputer, a plot of the pCa/tension curve was printed, and further data analysis was carried out. The Hill coefficient (n) and the association constant (K) were estimated by fitting the data to the equation

$$P/P_0 = (C{\cdot}K)^n/[1 + (C{\cdot}K)^n] \qquad [1]$$

by using a nonlinear least-squares method; C is [Ca^{2+}] and P/P_0 is normalized tension. This equation assumes maximum cooperativity in calcium binding and therefore yields the steepest possible pCa/tension relationship for a given number of sites.

RESULTS

Fig. 2 is a reproduction of the tension record from a microprocessor-controlled experiment. In Fig. 3*A*, pCa/tension data from three different fibers are plotted; a continuous line calculated from the fitted parameters is superimposed. The results obtained with the automated apparatus should be obtainable by conventional methods suitably designed to maximize the accuracy of the curve.

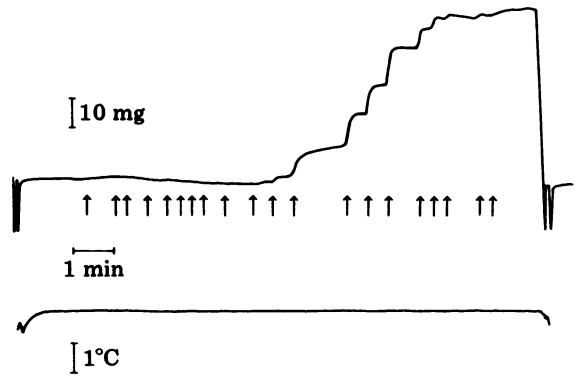

FIG. 2. Pen recorder tracing of a microprocessor-controlled pCa/tension experiment. The upper trace is tension and the lower trace is temperature. At the beginning of the record, pCa 8 saline was washed in as a complete solution change; at the end of the record the fiber was relaxed with a similar change. Between these washes, 50- or 100-μl volumes of pCa 5 saline were injected (arrows). The last arrow indicates a 1-ml injection.

By keeping the interval between pCa steps small, data similar to those of Fig. 3*A* were obtained by a conventional technique.

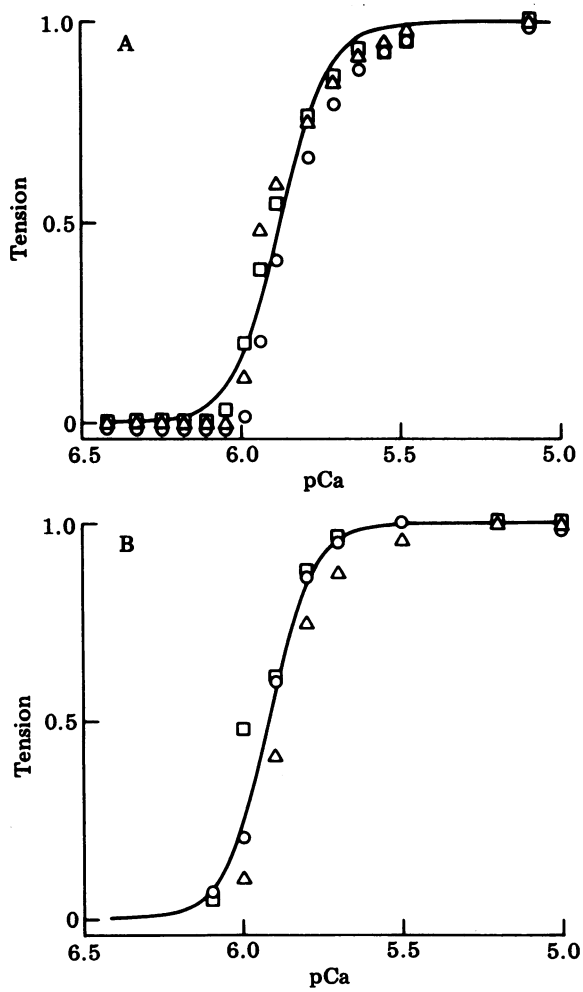

FIG. 3. (*A*) Normalized tension plotted against pCa for three microprocessor-controlled experiments. The line is drawn according to Eq. 1 with $n = 5.8$ and $\log K = 5.87$. (*B*) Plot of the data from three experiments done by the manual method. The line is from Eq. 1 with $n = 6.1$ and $\log K = 5.92$.

Table 1. Comparison of data collected automatically and manually

	n	SD	log K	SD	N
Microprocessor	5.7	±0.9	5.88	±0.06	13
Manual	5.2	±1.1	5.80	±0.12	7

n, Hill coefficient; N, number of curves.

The data in Fig. 3*B* were collected with a complete relaxation between each pCa point. For each data point the relaxing solution was washed out with a special solution that contained all the components for a pCa data point except the CaEGTA, which was added from a stock solution after the fiber was equilibrated in the special solution. This procedure avoided activating the fiber during the solution change and in this respect was like the automated procedure. The pCa solutions used in the manual experiments contained (in addition to those components in the solutions for the automated experiments) 16 mM creatine phosphate and 74 units of creatine phosphokinase (Sigma) per ml. Na_2SO_4 concentration was adjusted to keep the ionic strength at 0.200. These manual experiments demonstrate that substrate supply in the fiber is adequate, that ADP build up is not a factor, and that the quicker and more precise automated method is not biased in any obvious way.

The mean n (Hill coefficient) and association constant for curves collected automatically from 13 fibers and from 7 collected by the manual method are listed in Table 1. The tension observed at high pCa increased too steeply, and at low pCa too slowly, to fit well to Eq. 1. As a consequence, the confidence range for n is wide. The observed tension increased with a slope corresponding to $n \approx 10$ at high pCa; after the midpoint of the curve, it increased with a slope corresponding to $n = 3.5$. We interpret the large n for the best fit curve as evidence that tension is not a simple or exclusive function of bound calcium.

DISCUSSION

There are reports (5–7, 9, 20) in which the Hill coefficient of the pCa/tension curve is 3–4 and other reports (21, 22) of smaller n values. That our values are even larger than 4 is probably due to a number of factors. We have avoided averaging tension data from separate experiments to construct a single curve, a procedure that lowers n. The structural continuity of the fibers at the point of attachment to the apparatus, the small pCa interval between data points, and the high substrate concentration may also contribute to the steep slope we obtain.

The four potential calcium binding sites on TnC are divided into two classes (3): a high-affinity class ($K_a = 2 \times 10^7\ M^{-1}$) and a low-affinity class ($K_a = 3 \times 10^5\ M^{-1}$), each class having two sites. In high Mg^{2+}, the four sites have about equal affinity for Ca^{2+} due to competition between Mg^{2+} and Ca^{2+}. In our studies the Mg^{2+} was kept at ≈0.1 mM, a concentration in which the high-affinity sites should be saturated with calcium before threshold tension is reached. Because the affinities of all the TnC sites increase by 10 when TnC is combined with troponin I and T, the low-affinity sites have a $K_a \approx 5 \times 10^6\ M^{-1}$. To compare our results to those of Potter and Gergely (3), we have to take into account that we use an apparent Ca·EGTA affinity constant ($2 \times 10^6\ M^{-1}$ at pH 7.00) that is about half of what they used to analyze the binding and hydrolysis data. Recalculating the pK_d of the low-affinity sites on this basis we obtain $pK_d = 6.3$. If we assume that only these two sites regulate tension, that they bind Ca^{2+} independently, and that both must be occupied for an actin unit to become functional, then the midpoint of the pCa/tension curve should occur about pCa 5.92. In fact, our midpoint is about pCa 5.88 (Table 1). The close approximation of these midpoints is not particularly significant because our curve would be shifted about 0.5 unit higher if we had used an ionic strength as low as that used by Potter and Gergely (100 mM) or had omitted phosphate from the solution (unpublished observations). Fuchs and Bayuk (23) referenced their data to a Ca·EGTA affinity constant similar to our value and obtained a pK_d for Ca^{2+} binding to glycerinated psoas bundles of about 5.9.

It is apparent that the steep slope of the pCa/tension relationship observed in our experiments cannot be interpreted by conventional binding schemes involving two or even four regulatory sites. If four Ca^{2+} binding sites and maximum cooperativity are assumed, $n = 4$ has to be the theoretical upper limit if tension is controlled exclusively by Ca^{2+} binding. At present, there is little evidence for maximum cooperativity between Ca^{2+} binding to four regulatory sites on TnC, whether purified or on myofibrils (3, 24, 25), although there has been a suggestion that the sites interact negatively (26).

The suggestion (2) that attachment of myosin to actin relieves inhibition of actin and enhances Ca^{2+} binding to TnC (ref. 27; cf. ref. 24) could generate a wide range of ns. We cannot test these proposals against our data, however, until a more quantitative description is available.

Among mechanisms that could alter both the position and slope of the pCa/tension curve relative to the pCa/binding curve is the relationship between the time a cross-bridge takes to cycle back to the relaxed state once a cycle is initiated and the mean life-time of the TnC–Ca^{2+} complex. If Ca^{2+} remains bound to TnC in intact myofilaments for a mean lifetime that is shorter than the duration of a cross-bridge cycle, the concentration of Ca^{2+} required to reach half-maximal tension will be less than that required to half-saturate the regulatory sites on TnC. The half-time for the dissociation of Ca^{2+} from TnC has recently been estimated to be 2–3 msec (28), and presumably becomes 20–30 msec when TnC is combined with troponin I. In this discussion the cross-bridge cycle is assumed to begin when myosin heads become strongly bound to actin (29) and to end when the refractory period is over. It excludes, therefore, the time myosin heads spend in the postrefractory or relaxed state. If the mean cross-bridge cycle time does not vary as tension increases, the pCa/tension curve will parallel the Ca^{2+} binding curve and the position relative to the binding curve will depend on the (constant) ratio cycle time/Ca^{2+} bound time, in which mean Ca^{2+} bound time is estimated from the half-time for the dissociation of Ca^{2+} from TnC.

In intact myofilament systems, however, internal strain is believed to decrease the rate of cross-bridge cycling. This strain dependence of cycle rate is the basis for the observation that the rate of energy mobilization by a muscle is coupled to the rate at which it can shorten under load [Fenn effect (30–32)]. If tension increase in the skinned fiber introduces strain changes that affect the mean cycle time, the pCa/tension curve will no longer parallel the Ca^{2+} binding curve.

If the time it takes for a cross-bridge to complete a cycle increases progressively with increase in tension, the experimentally observed pCa/tension relationship will be a composite curve produced by shifting through a family of curves. This family consists of curves all parallel to the Ca^{2+} binding curve but with midpoints shifted to the left depending upon the cycle time. The progressive shift would result in a pCa/tension relationship that is steeper than the binding curve upon which it is based. This concept is illustrated in Fig. 4, which shows actual data points (circles) superimposed on a family of hypothetical curves (lines). Each curve represents a different apparent pK_d, corresponding to a given value of the ratio defined

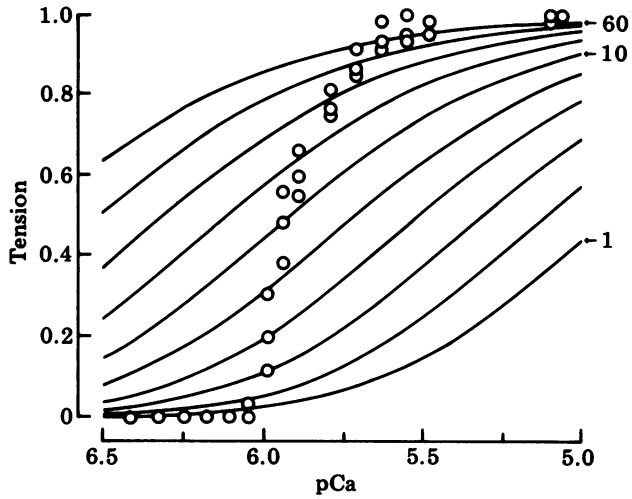

FIG. 4. The lines represent a family of hypothetical pCa/tension curves calculated according to the assumptions that Ca^{2+} binds independently to two sites on TnC and that both must be occupied to permit tension to develop. The curve labeled 1 is drawn assuming $pK_d = 5.3$; the other curves are drawn by assuming 0.2-unit increments in the pK_d. The observed pCa/tension data, shown here as circles, initially follow a hypothetical curve near that labeled 1. With increase in tension, the observed curve rises steeply through hypothetical curves centered progressively more to the left. The labels 10 and 60 refer to the factor by which the binding constant of TnC–Ca^{2+} must increase if tension were assumed to be strictly proportional to bound Ca^{2+}. The empirically observed steep slope of the pCa/tension curve could result, for example, from a tension-dependent increase in the ratio of the time myosin heads require to cycle through the attached states (see text) to the time Ca^{2+} is complexed to TnC.

above. The right-hand curve is drawn assuming a pK_d of 5.3. The other curves are shifted left by 0.2-unit increments to approximate the shift anticipated from a doubling of the ratio.

If change in strain leads to an increase in slope of the pCa/tension relationship, then elimination of change in strain should reduce the slope to that of the binding curve. Whenever the hydrolysis rate is measured in isolated protein systems or in myofibrillar preparations that cannot support tension, the strain in the myofilament lattice is minimal and probably negligible. The hydrolysis rate of myofibrillar preparations, measured as a function of pCa, parallels the Ca^{2+} binding curve (3)—i.e., has a slope much lower than slopes we and others find for the pCa/tension relationship. The pCa/hydrolysis rate relationship of skinned fibers held isometric and supporting tension is steeper (33) than that for myofibrils.

The dependence of the position of the pCa/tension curve on the mean cross-bridge cycle time may make it impossible to define uniquely the "active state" of muscle during the rising or falling phase of tension by imposing rapid length changes (34, 35). Changes in lattice strain produced by rapid length changes should alter the mean cross-bridge cycle time and thus change the number of myosin units that a given concentration of Ca^{2+} will keep in the cycle. A similar problem arises when the effect of pCa on the maximum velocity of muscle shortening is studied (36–38). As in the definition of the active state, the assumption is made that change in load does not alter the apparent Ca^{2+} sensitivity. But, according to the above argument, the decrease in cycle time that accompanies an increase in shortening velocity will lead to a decrease in apparent Ca^{2+} sensitivity. If so, the muscle may produce less power at each fractional load than is expected from the isometric force and the maximal velocity of shortening may be underestimated.

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