Regulation of Tension in Skinned Muscle Fibers

Effect of Cross-Bridge Kinetics on Apparent Ca^{2+} Sensitivity

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ABSTRACT   Three different ways of shifting the pCa/tension curve on the pCa axis have been studied and related to changes in the rate constants of the cross-bridge cycle. The curve midpoint shifts to higher pCa's when the substrate (Mg-ATP) is reduced from 5 to 0.25 mM, when the phosphate concentration is reduced from 7.5 mM to 0, and when the ionic strength is reduced from 0.200 to 0.120. The Hill coefficients of the pCa/tension curve in our standard saline (5 mM substrate, 5 mM free ATP, 7.5 mM phosphate, ionic strength 0.200, 15°C) are between 5.1 and 5.6 and fall to 3.0 with the left shift of the curve brought about by reducing both substrate and phosphate. Left shifts of the curve produced by reduction in the ionic strength do not result in a lower Hill coefficient. Reducing either substrate or phosphate is associated with a reduction in the optimal frequency for oscillatory work, but reduction in ionic strength is not so associated. Maximum tension increases with the left shift of the curve brought about by reducing phosphate concentration or ionic strength, but tension decreases with the left shift of the curve accompanying substrate concentration reduction in a phosphate-free saline. We argue that one mechanism for the observed shift of the curve along the pCa axis is the relationship between the time a cross-bridge takes to complete a cycle and the time Ca^{2+} stays bound to troponin C (TnC). If the cycle rate is decreased, a smaller fraction of TnC sites must be occupied to keep a given fraction of cross-bridges active. To illustrate this concept, we present a simplified model of the cross-bridge cycle incorporating the kinetics of Ca binding to TnC.

INTRODUCTION

Evidence that the concentration of Ca^{2+} in the myoplasm regulates the state of activity of skeletal muscle comes from many sources (Ebashi et al., 1969; Weber and Murray, 1973; Fabiato and Fabiato, 1979; Gergely, 1980). Some of the most direct evidence has been obtained from experiments on skinned fibers, a simplified preparation of myofibrils that can be exposed to a range of

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buffered pCa's ($-\log[Ca^{2+}]$) in a solution containing substrate (Mg-ATP), a pH buffer, and salts. When the Ca^{2+} concentration is below 0.1 μM (pCa >7.0), the fibers are relaxed; then, as the Ca^{2+} concentration is increased, tension increases over an approximately 2- to 10-fold range. By 10 μM (pCa 5.0), maximum tension is usually obtained.

Although an appropriate increase in Ca^{2+} can induce a skinned fiber to go from essentially zero to full activity, there is evidence that the regulatory mechanism for tension does not depend exclusively on Ca^{2+} binding. Even in such a simple system as the skinned fiber preparation, the shape and position of the pCa/tension curve depend on a variety of solution parameters (Brandt et al., 1972; Donaldson and Kerrick, 1975; Fabiato and Fabiato, 1978). Hill coefficients of published pCa/tension curves range from 1 to 6 with values between 2 and 3 most common; the midpoints range from pCa 6.5 to 5.5 (cf. Brandt et al., 1980). Coefficients >4 are too large to be explained by a control mechanism governed exclusively by Ca^{2+} binding (Brandt et al., 1980), and many of the solution parameters that shift the curve on the pCa axis are not likely to modify the affinity of troponin C (TnC) for Ca^{2+}. It is of interest, therefore, to examine various conditions that modulate the position and slope of the pCa/tension curve.

Recently we developed a method for obtaining very precise and reproducible pCa/tension curves on skinned muscle fibers. In an initial report (Brandt et al., 1980), we established the accuracy of the method and demonstrated that the pCa/tension relation of fibers in 5 mM substrate has a Hill coefficient (n) of 5.2–5.8, where n is the assumed number of binding sites acting with maximum cooperativity. Because n is larger than the number of known binding sites, we concluded that other factors must be sought. Based on the observation (Johnson et al., 1979) that Ca^{2+} remains bound to TnC for a period shorter than the mean cross-bridge cycle time, we proposed that a change in the cycle time will affect the position and possibly the slope of the pCa/tension curve.

In the present report we describe a series of experiments that were designed to test this proposal. Changes in solution parameters were chosen that were expected to modify the kinetics of the actomyosin ATPase cycle in a predictable way or that were known to change the optimum frequency of oscillatory work (Kawai and Brandt, 1980). Specifically, we expected the pCa/tension curve to shift to the right (to lower pCa's) in conditions that favor more rapid cross-bridge movement through the attached states or higher rates of oscillatory work, and to the left in conditions that slow these transitions. We also studied the effect of change in ionic strength because earlier work on skinned crayfish fibers (Brandt et al., 1974) showed that change in this parameter shifts the pCa/tension curve.

METHODS

Muscle Preparation

We use single fibers of chemically skinned psoas from adult rabbit (Wood et al., 1975; Kawai and Orentlicher, 1976). Strips of muscle ~1–2 mm in diameter and 30 mm in

length are separated from psoas muscles of rabbit anesthetized by CO_2 inhalation and killed by decapitation. These strips are tied at two places to small wooden sticks, cut free from the muscle, then chemically skinned in a saline containing: 5 mM EGTA, 2 mM Mg-ATP, 180 mM K propionate, and 5 mM imidazole (pH 7.0) for 48 h at 2°C. Some of the fiber bundles are stored in this solution in the refrigerator and used in experiments for up to 2 wk; the rest are equilibrated for 24 h in iso-ionic glycerol saline (skinning saline plus 6 M glycerol), then stored in a deep freeze. By comparing the refrigerated preparation with that stored in the deep freeze, we eliminate the possibility that either glycerol or freezer storage further modifies or damages the preparation. For more details on the preparation and its ultrastructure, see Eastwood et al., 1979.

Muscle Chamber

The muscle chamber is machined from aluminum and painted with inert plastic. It has an inside capacity of 0.5 ml (see Fig. 1, Brandt et al., 1980). Stainless steel (SS) tubes from the length adjustment device and force transducer assembly, respectively, enter the chamber through air gaps in teflon collars; a small amount of vaseline is used to seal the outer ends of the gaps. The chamber is heated by a focused incandescent lamp and cooled by water circulating through channels in the chamber walls. A thermistor attached to the chamber block is connected to a feedback network to maintain the chamber block temperature at the desired level. A second thermistor in the chamber is used to monitor the solution temperature. The saline is constantly stirred by a vibrating paddle to minimize local heterogeneities in ionic concentrations ("unstirred layer;" Orentlicher et al., 1974) and in temperature. Other SS tubes forced through holes in the walls of the chamber carry solutions in or out. Because of good thermal contact between the chamber walls and the tubes, the temperature of the incoming fluid closely approaches that of the chamber. A small slit in the side of the hollow SS stirrer is used to aspirate excess solution. The constant agitation of this orifice breaks up the meniscus so that solution withdrawal stops at a constant level. This allows maintenance of a constant volume of fluid in the chamber.

Fiber-mounting Procedure

A segment of a single fiber 6–10 mm long is dissected from a stock bundle and transferred to the experimental chamber, which is initially filled with a relaxing saline appropriate to the experiment. The ends of the preparation are wrapped around bent SS wires and wedged in small slots formed by doubling over the ends of the wires (Brandt et al., 1980). A HeNe laser beam is directed onto the fiber and the sarcomere length is adjusted to 2.5–2.6 μm by measuring the separation of the first-order diffraction lines. These lines are projected onto a Reticon RL256G image sensor (EG Reticon, Sunnyvale, CA) ∼4 mm below the specimen. The array output is displayed on an oscilloscope where the diffraction peaks can be read to within one array element (25 μm). Muscle at this length, defined as L_0, is ∼10% above the slack length.

Force Transducer

The force transducer (Brandt et al., 1980, and manuscript in preparation) has low compliance (2 nm/dyn), high sensitivity (0.1 dyn change can be detected), and minimal drift (0.005%/°C). The resonant frequency is 2 kHz with a muscle holder attached.

Automated Apparatus

This is composed of a PLS 441 microprocessor (Pro-log Corp., Monterey, CA), a paper tape punch, pumps, valves, and a hexadecimal keyboard used to input the

experimental parameters, which in turn select the program modules. The tension preamplifier output goes to a digital meter that ranges ± 2,000 mV (1 dyn/mV). Four digits (binary-coded decimal) obtained from the meter are input separately to the processor as is time (hours, minutes, and seconds) from a clock chip. Digital outputs of the processor control valves and high-precision pumps driven by stepping motors. For a visual record, tension, temperature, and timing signals are also recorded on a two-channel Brush recorder (Gould Inc., Instruments Div., Cleveland, OH) equipped with two event markers.

The experimental control program is modular and includes eight separate general purpose routines. Usually these routines end in a subroutine in which the system waits until the tension becomes stable (P <0.1 dyn/s) and then punches the steady state data on paper tape. At the time of an experiment a series of codes (two hexadecimal numbers) are entered on the keyboard. These specify the program module and the control data (valve, pump, volume, timing, etc). It takes about 6 codes to specify a pCa/tension curve, but commonly 12 or more are entered—one set for a control curve and one for an experimental curve. Because 256 codes can be stored in the processor, a very complex experimental sequence can be automated. Once a fiber is mounted, the solutions are placed in the appropriate reservoirs, and the protocol is keyed in, the experiment proceeds without further intervention by the investigator. Automation eliminates timing errors, bias in estimating the steady tension, baseline adjustment and truncation errors on the pen recorder, and, most importantly, chart-reading errors.

Solutions

The standard "relaxing" solution contains: 10 mM EGTA (ethylene glycol bis(β-aminoethylether)$N,N,N'N'$-tetraacetic acid), 5 mM Mg-ATP, 5 mM ATP, 7.5 mM Pi (phosphate), 40.1 mM Na propionate, 17.2 mM Na_2SO_4, and 10 mM MOPS (morpholinopropane sulfonic acid) at pH 7.00. The "activating" solutions contain 10 mM total EGTA (the ratio of Ca-EGTA to EGTA according to the pCa and other components as above). In all modified solutions the ionic strength and monovalent cation (Na^+) concentrations are adjusted by changing the concentration of Na propionate. Variations in Na_2Mg-ATP and Na_2HPO_4 are compensated by adjustment of Na_2SO_4 to maintain both ionic strength and Na^+ concentration constant. The temperature for most experiments was 15 ± 0.1°C but a few were performed at 10 or 20°C.

We mix the pCa solutions directly in the experimental chamber by injecting a pCa 4.85 stock saline while keeping the solution volume (v_0) constant. Assuming instantaneous mixing, the following equation defines the concentration of a constituent (C_v) at the volume (v) of injected stock (C_s), from the initial concentration (C_0) of the constituent in the chamber:

$$C_v = C_s + (C_0 - C_s)e^{-v/v_0}. \tag{1}$$

This equation is used to find the concentrations of EGTA and Ca-EGTA, from which the pCa is then calculated. By starting with pCa 8 in the chamber we obtain a pCa series to pCa 5.0.

To verify that the mixing is essentially instantaneous and that the calculated pCa is correct, we injected a solution containing ^3H inulin into the chamber, which had been initially filled to 0.5 ml with a "cold" solution. After a given volume of inulin solution was injected, the procedure was stopped and aliquots of the fluid in the chamber were taken and counted in a liquid scintillation counter. We calculated how

many counts should be in each sample of the chamber solution from Eq. 1 and the activity of the stock inulin. Table I compares the expected counts and the experimentally determined counts. In all cases the predicted and observed counts agree within 1%. The last column is calculated by assuming that our usual starting solutions were used instead of the inulin solution.

Starting solutions (that in the chamber or at pump stations) are all calculated by a computer program that prints the volume of each stock solution required to obtain a solution as specified. Specifying an outcome is much simpler than calculating the outcome of a given mix of stocks (Brandt et al., 1972). The following apparent association constants (log values at pH 7.00) are used: Ca-EGTA, 6.28; Mg-EGTA, 1.6; Ca-ATP, 3.70; Mg-ATP, 4.05.

Data Reduction

In the tables, slopes of the curves are reported as the Hill coefficient and the midpoint position by the pK ($-\log K$) where K is a dissociation constant. These are obtained by fitting the data points for each curve to the equation

$$\frac{P}{P_0} = (C/K)^n/[1 + (C/K)^n] \tag{2}$$

by a nonlinear least-squares method (Brandt et al., 1980). C is the Ca^{2+} concentration and P/P_0 is the normalized tension. This equation assumes maximum cooperativity

TABLE I

ACCURACY OF SERIAL DILUTION METHOD

Volume	Measured cpm	Predicted cpm	Deviation	Equivalent pCa
0.5	8,502	8,593	1%	6.09
0.75	11,496	11,527	<1%	5.79
1.5	13,355	13,302	<1%	5.25

in calcium binding and therefore yields the lowest estimate for the number of assumed binding sites (n). Tables II–VI list the mean values for these fitting parameters, the standard errors of the mean (SEM), and the number of curves (N) in each experiment.

RESULTS

We expect that left shifts of the curve on the pCa axis will accompany an increase in the time cross-bridges spend in the attached states (see Appendix; Brandt et al., 1980). We have therefore collected pCa/tension curves for several experimental conditions that we anticipate from our sinusoidal analysis data (Kawai and Brandt, 1980) or from the biochemical literature will change the rate of cross-bridge cycling.

Kawai (1978) observed that the optimum frequency for oscillatory work increases as the substrate concentration is increased up to ~5 mM, where the effect saturates. We (Kawai and Brandt, 1978) have observed a similar increase in the optimal frequency of oscillatory work with increasing phosphate; this effect saturates by ~7.5 mM. The data in Table II show that the pCa/tension curve shifts to lower pCa's as the concentration of substrate or phosphate is increased; these effects are additive.

Effect of Phosphate

Fig. 1 is a reproduction of four records of the tension change with time following a series of increments (arrows) in the Ca^{2+} concentration in salines of different phosphate concentrations. In A and B, respectively, 7.5 and 0 mM phosphate are compared for one fiber, and in C and D, 7.5 and 15 mM
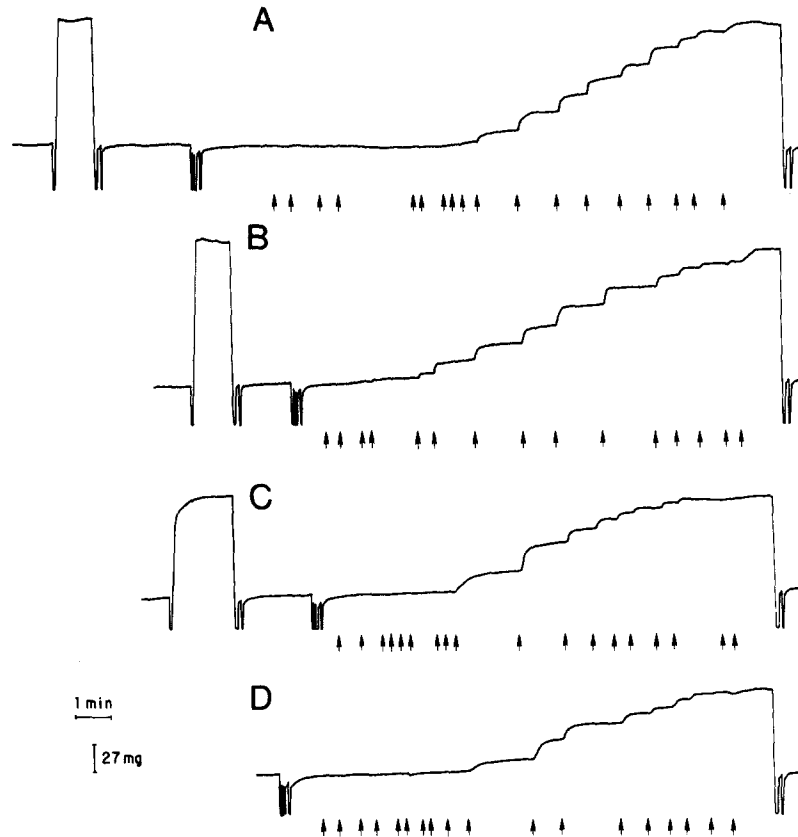

FIGURE 1. Reproduction of four pen recorder tracings showing the tension change with time after incremental additions of Ca^{2+} (arrows) to the bathing saline. At the beginning of curves A-C, a single test tension is generated by changing the relaxing saline for a pCa 4.85 saline. The large downward deflections are solution change artifacts. After the large test tension, the fiber is put into a pCa 8.0 solution, then a series of incremental decreases in pCa is generated by 100-μl injections of a pCa 4.85 stock. At the last arrow a 1-ml injection is made to ensure that a maximum tension is obtained. Because the solution is vigorously stirred, and the volume in the chamber is kept constant, the pCa can be calculated from Eq. 1 for any given injection volume. Curve A was recorded from a fiber bathed in a 7.5 mM phosphate saline, whereas curve B is from the same fiber bathed in a 0-phosphate saline. On a second fiber, the effect of 7.5 mM phosphate (C) is compared with the effect of 15.0 mM phosphate (D).

phosphate are compared for another. The substrate is fixed at 5 mM. After the tension change levels off (<1 dyn/10 s), the time and tension data are punched on paper tape. The data thus collected from each curve are fit to Eq. 2. The fitted parameters are then used to compute the mean parameters listed in the tables. The effects of 0, 7.5, and 15 mM phosphate (left to right) are presented graphically in Fig. 2A and B. In this figure the data points (symbols) and best-fit curves (lines) are shown. The mean parameters obtained by averaging all the best-fit parameters for individual phosphate experiments are listed in Table II and are plotted as computed pCa/tension curves in Fig. 3A.
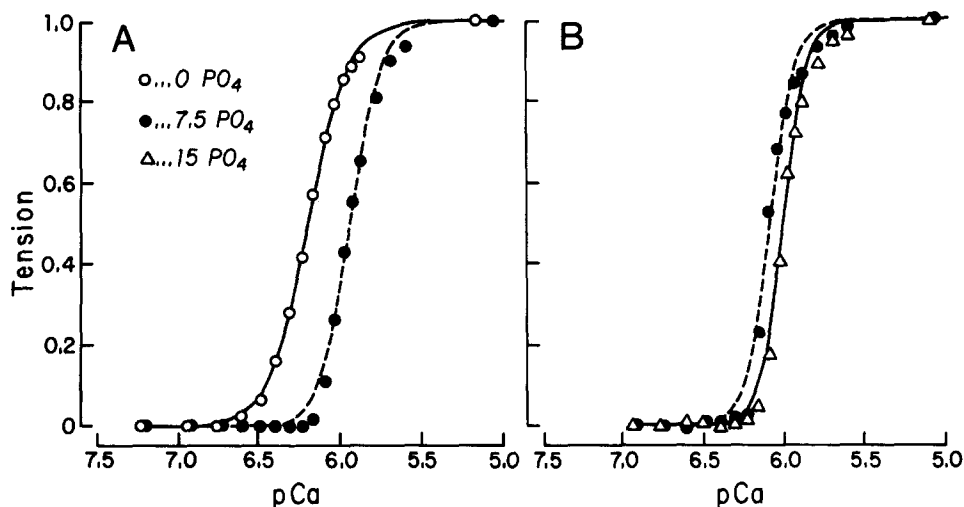

FIGURE 2. Data from the four curves shown in Fig. 1 are plotted in Fig. 2 as pCa/tension curves. Each data point represents the steady tension just before the next increment in [Ca^{2+}]. The lines are calculated from Eq. 2 and parameters that best fit the points. The curve on the left in A (—O—) is from the fiber bathed in 0-phosphate saline (Fig. 1B), whereas the curve on the right (--●--) is from the same fiber bathed in 7.5 mM phosphate saline (Fig. 1B). Record of 7/30/80, fiber 2. In B, data from a second fiber are used to compare 7.5 mM phosphate (--●--) with 15 mM phosphate (—△—). Record of 6/24/80, fiber 2.

From these results we conclude that removal of phosphate shifts the curve to the left.

To illustrate the significance of the standard errors associated with our data, mean parameters for three independent sets of standard curves (Table V) are used to construct the curves of Fig. 3D. The restricted range in curve positions and slopes in Fig. 3D can be compared with the much larger shifts of the pCa/tension curve that are observed with an increase in phosphate from 0 to 7.5 mM. As we expect from the observation that the optimal frequency of oscillatory work increases with phosphate up to 7.5 mM (at which it saturates),

the shift in the pCa/tension curve between 0 and 7.5 mM phosphate is large and significant compared with that for increasing phosphate from 7.5 to 15 mM. The confidence levels for assuming a difference between means are listed in Table VII.
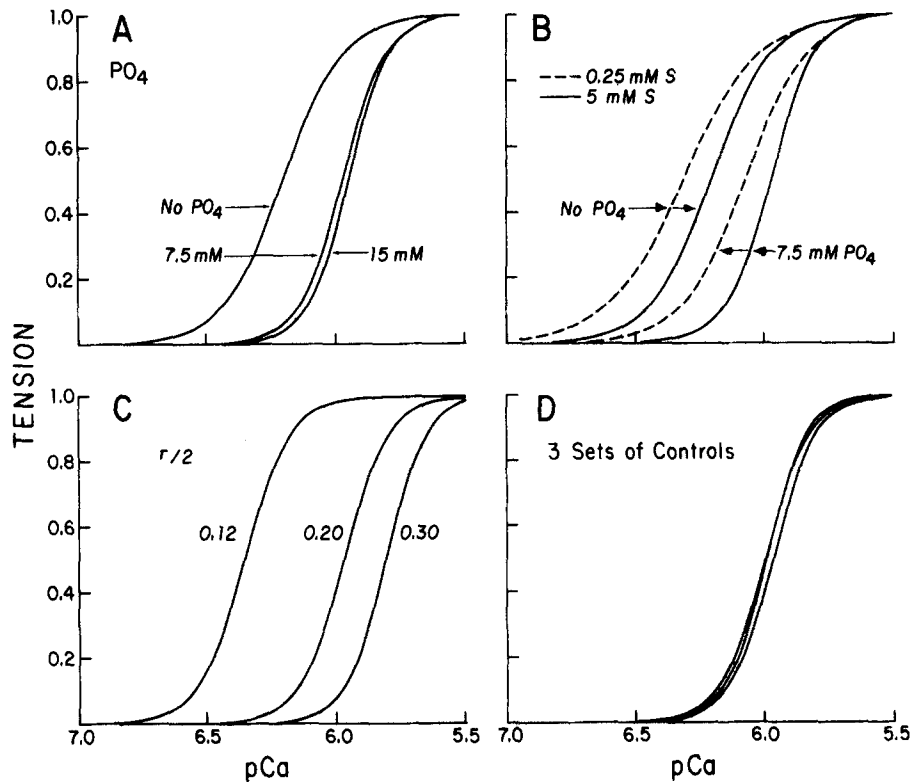

FIGURE 3.   pCa/tension curves computed according to Eq. 2 and the parameters of Tables II–V. In A, the three curves represent, from left to right, the effects of 0, 7.5, and 15 mM phosphate. In B, the pair of curves to the left are for fibers tested in 0 phosphate and the right-hand pair for fibers in 7.5 mM phosphate. Within each pair, the right-hand curve is for 5.0 mM substrate, the left-hand member is for 0.25 mM substrate. The curve shifts for the change in phosphate concentration are the same for the two substrates. Thus the shifts caused by substrate and phosphate concentration changes are additive, an indication that they act on different transitions in the cross-bridge cycle. In C, the ionic strength for the leftmost curve is 0.120, for the middle curve 0.200, and for the right-hand curve 0.300. Unlike the shifts that accompany changes in substrate or phosphate concentration, those that accompany reduction in ionic strength are not associated with a reduction in the slope. In D, there are three curves that represent three separate batches of fibers bathed in a standard saline (Table V; 49 fibers). These served as controls for the other experiments described above. The small shifts in these curves demonstrate the degree of reproducibility we obtain.

TABLE II
EFFECTS OF SUBSTRATE AND Pi CONCENTRATION
(FREE ATP 5.0, IONIC STRENGTH 0.200)

Substrate	Pi	Slope (n)	pK	N	P_o
0.25	0	3.0±0.1	6.31±0.02	25	84±4.5,
0.25	7.5	3.6±0.2	6.07±0.03	27	82±4.6
5.0	0	3.7±0.2	6.20±0.01	22	94±3.4
5.0	7.5	5.1±0.1	5.97±0.01	36	79±4.2
5.0	15.0	5.6±0.3	5.95±0.01	11	65±6.7

TABLE III
EFFECTS OF IONIC STRENGTH
(5.0 SUBSTRATE, 5.0 FREE ATP, 7.5 Pi)

Ionic strength	Slope (n)	pK	N	P_o
0.120	5.0±0.2	6.35±0.01	31	91±5.9
0.200	5.1±0.1	5.97±0.01	36	79±4.2
0.300	5.6±0.1	5.81±0.01	29	60±3.1

TABLE IV
EFFECTS OF FREE ATP
(5.0 SUBSTRATE, 7.5 Pi, IONIC STRENGTH 0.200)

Free ATP	Slope (n)	pK	N	P_o
1.0	6.9±0.3	5.92±0.02	8	88±9.5
5.0	5.1±0.3	6.00±0.02	13	75±5.7

TABLE V
THREE SEPARATE SETS OF STANDARD CURVES
(5.0 SUBSTRATE, 7.5 Pi, 5.0 FREE ATP, IONIC STRENGTH 0.200)

Control from	Slope (n)	pK	N	P_o
Table II, III	5.1±0.1	5.97±0.01	36	79±4.2
Table IV	5.1±0.3	6.00±0.02	13	75±5.7
15 PO_4 experiments	5.6±0.2	6.00±0.01	16	77±7.2

TABLE VI
EFFECTS OF TEMPERATURE
(0.25 SUBSTRATE, 5.0 FREE ATP, IONIC STRENGTH 0.200)

$[PO_4]$	Temperature	Slope (n)	pK	N	P_o
0	10	3.2±0.1	6.30±0.04	10	77
0	15	3.1±0.2	6.33±0.03	8	89
0	20	2.7±0.3	6.29±0.05	7	87
7.5	10	3.8±0.4	6.01±0.06	9	70
7.5	15	3.8±0.3	6.04±0.06	8	82
7.5	20	3.4±0.2	6.13±0.05	10	94

Concentrations are given in millimolar, P_o is in dynes (see text for mean diameters). The mean force for our standard curves (Table IV) is 1.43×10^6 dyn cm^{-2}. N is the number of fibers in each data set, and the slope and pK are the average of the parameters obtained by fitting the data for the individual fibers to Eq. 2. The ± sign precedes standard errors of the mean.

Effect of Substrate

Fig. 3B compares the effects of two substrate concentrations (0.25 and 5.0 mM) at two phosphate concentrations (0 mM, left pair; 7.5 mM, right pair). The right-hand member (solid lines) of each pair is the higher substrate. Decrease in substrate concentration from 5.0 to 0.25 mM shifts the pCa/tension curve to the left, both in 0 and in 7.5 mM phosphate. The amount of the shift associated with change in substrate is exactly the same in 0 and 7.5 mM phosphate (Table II); thus the shifts induced by phosphate and substrate are additive. As curves shift to the left with a decrease in substrate or phosphate concentration, the Hill coefficients decrease (Table II).

Effect of Ionic Strength

Large shifts in the pCa/tension curve accompany ionic strength changes produced by altering Na propionate concentration (Table III). Decreasing ionic strength from 0.200 to 0.120 shifts the pCa/tension curve midpoint 0.38 unit to the left, whereas increasing ionic strength from 0.200 to 0.300 shifts the curve 0.16 unit to the right. These data are used to construct the curves of Fig. 3C. The tendency for the Hill coefficient to decrease with left shift is minimal in contrast to the slope decrease accompanying the shifts induced by reducing phosphate or substrate concentration.

Tables II through VI list the force in dynes rather than force per unit cross-sectional area of fiber. This can be readily obtained from the mean diameters described below. Our reasons for not normalizing the force include the observation that the variance of the force increases slightly with normalization. This is probably because the variation in fiber diameters is small, and what variation there is is not caused by differences in the number of myofibrils acting in parallel. The mean fiber diameters of the control experiments were 82 ± 1 μm and for all other experiments, the mean diameters range from 79 to 84 μm with the exception of the low substrate experiments, where the mean diameters were 90 and 94 μm. Having no reason to believe that this latter difference is a sampling error, we suspect that the diameters can be affected by the experimental solutions. If so, normalizing the force against cross-sectional area could introduce an error.

The mean force at maximum activation for an ionic strength of 0.300 is 60 dyn; at an ionic strength of 0.120 it is 91 dyn—a 52% increase in tension with left shift of the curve. This increase can be compared with the maximum variation in the force of the standard curves at an ionic strength of 0.200 (75–79 dyn). The left shift with decrease in phosphate concentration from 15 to 0 mM (5 mM substrate, Table II) is also accompanied by an increase in force from 65 to 94 dyn.

The tension change accompanying the left shift brought about by a decrease in substrate follows a pattern opposite to that for shifts brought about by changes in phosphate concentration or ionic strength. There is a decrease in force in zero phosphate and no change in force in 7.5 mM phosphate as substrate is decreased from 5 to 0.25 mM (Table II).

Table IV compares data from experiments with 1.0 and 5 mM free ATP.

These data are included as evidence that 5 mM free ATP was sufficient to buffer the substrate, Mg-ATP, in the absence of an ATP-regenerating system. If the substrate concentration had been significantly depleted in the core of the fibers, reducing the free ATP from 5 to 1 mM should have produced a left shift of the curve (cf. Table II). However, the data in Table IV show that a reduction in the free ATP is accompanied by a small right shift and by an increase in the Hill coefficient. We have not studied this further, but we suspect that the changes are related to the increase in Mg^{2+} concentration (from ~0.1 to 0.5 mM; Donaldson and Kerrick, 1975; Solaro and Shiner, 1976) that must accompany a reduction in free ATP. In addition to these experiments on the adequacy of substrate buffering, we note that in an earlier report (Brandt et al., 1980) we collected pCa/tension curves in the presence and absence of an ATP-regenerating system. Both sets of solutions contained 5 mM free ATP. The pCa/tension curves from the two series were identical. Because the above argument makes it reasonable to assume that the 5 mM substrate experiments are well buffered, we argue that the experiments with 0.25 mM substrate are also well buffered. This is because the ratio of the optimal frequency for oscillatory work for fibers in 0.25 mM substrate to those in 5 mM substrate is 1/6. Thus if 5 mM substrate is buffered by 5 mM ATP, 0.25 mM substrate must also be adequately buffered by 5 mM ATP. This leaves the possibility that ADP buildup in the fiber core distorts the results. If so, then large-diameter fibers should have larger pK's than small fibers and we have found no such correlation.

Some of the curves used to obtain data for Table II (those for experiments with 0.25 mM substrate) were conducted at 10 and 20°C in addition to 15°C. We found, as had others (Ashley and Moisescu, 1977; Stevenson and Williams, 1980), that, with the exception of maximum tension, there is no effect of temperature change in this range on the normalized steady state parameters of fibers bathed in 0 phosphate saline and only a small effect on fibers bathed in 7.5 mM phosphate saline. Consequently, in Table II we pooled the results from these experiments with the data from experiments at 15°C. Table VI contains these experimental results separated according to temperature.

DISCUSSION

The data for the standard pCa/tension curves reported here were collected under conditions nearly identical to those of an earlier study (Brandt et al., 1980), in which we observed that the Hill coefficient of this curve is too steep ($n = 5.2-5.8$) to be accounted for simply by the binding of Ca^{2+} to TnC. In the present study n is between 5.1 and 5.6 for curves collected in comparable solutions (Table V). Again n is >4, the largest possible value if all four calcium binding sites bind with maximum cooperativity. It is beyond the scope of the present report to consider further the problem of why n is so large except to note that determining which factors, in addition to calcium binding to TnC, regulate the position of the pCa/tension curve may be a step towards understanding why n is so large.

The mean of the midpoints (pK) of all the standard curves in the present report is 5.99 ± 0.02, which is ~0.1 p units higher than the midpoint reported

earlier (Brandt et al., 1980). The only obvious difference in the two solutions used was the concentration of EGTA, which was 10 mM in the present study and 6 mM in the earlier one. Because the ionic strength was the same in the two studies, this difference in EGTA concentration does not seem sufficient to account for the small but significant difference in midpoints. One possible explanation is that we may have worked at a longer sarcomere length in the present study. This is because in the present study we introduced a test tension at pCa 4.85 before collection of data for a curve, and set the sarcomere length to 2.6 μm after the first such test. In the earlier experiments we set the sarcomere length to 2.6 μm before conducting the experiments. This procedure was modified because we observed that shortening of the sarcomeres often occurred during the first full tension. Left shifts of ~0.1 p units have been reported for fibers stretched above slack length (Fabiato and Fabiato, 1978; Moisescu and Thieleczek, 1979).

It may not be relevant to compare our value for the curve midpoint with values reported by other laboratories because the latter include a wide variety of species almost none of which are mammalian. Even confining the comparison to data on frog muscle, the most frequently used species, there is a wide range of reported midpoints, i.e., from pCa 6.7 (Hellam and Podolsky, 1969; Julian, 1971) to ≤5.4 (Donaldson and Kerrick, 1975). The bulk of the reported values, however, are clustered around pCa 6.0 (6.2, Fabiato and Fabiato, 1978; 6.05, Moisescu and Thieleczek, 1979; 6.25, Moisescu, 1976; 6.3, Julian and Moss, 1980; 5.9, Julian and Moss, 1981). The only value reported for rabbit psoas from another laboratory is pCa 6.6, in an ionic strength of 0.150 (Fuchs and Black, 1980). For this value (as for all those above), the differences in the assumed affinity of EGTA for Ca^{2+} must be taken into account. In this last case the midpoint value of pCa 6.6 will correct to pCa ~6.2, one very close to that expected from our results. The wide range of midpoints reported for the pCa/tension curve noted above cannot be caused exclusively by differences in the assumed affinity of EGTA for Ca^{2+}, and other factors must be sought.

The main objective of the work reported here is to examine the relation between the position of the pCa/tension curve and the rate of cycling of cross-bridges through the various states. We show in Results that reductions of substrate and phosphate concentration that decrease the optimum frequency for oscillatory work are accompanied by a left shift of the pCa/tension curve. Curve shifts such as these can come about by at least two mechanisms: alterations in the affinity of TnC for Ca^{2+}, and, as we suggest (see Appendix), by modulation of the rate constants of the cross-bridge cycle.

The pCa/tension curve is generally assumed to be directed by the Ca^{2+} binding properties of TnC, i.e., an increase in isometric tension in skinned fibers is presumed to parallel the binding of Ca^{2+} to regulatory sites on TnC (Kerrick et al., 1980). There are, however, several theoretical and factual difficulties with this assumption. The theoretical problems result from the large number of presumed and incompletely understood chemical and physical processes that link Ca^{2+} binding to tension generation. Free Ca^{2+} is in equilibrium with Ca-TnC and the probability of formation of Ca-TnC must
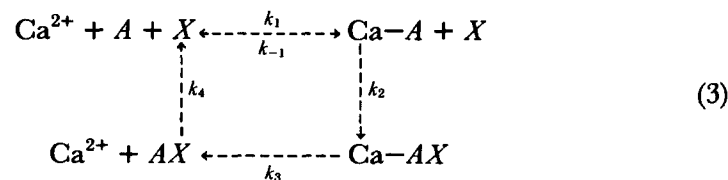
be related to the probability of cross-bridge formation. The function that describes this relation is not immediately obvious. The purpose of this report is, in fact, to explore one component of this linkage.

Among the factual difficulties are the following. The pCa/tension curve is too steep to be described by a binding equation (Brandt et al., 1980). It can be shifted along the pCa axis by solution changes not known or expected to affect Ca^{2+} binding to TnC. For example, studies on isolated Z-I band assemblies show no effect of change in ionic strength on Ca^{2+} binding (Solaro et al., 1976). In a study on the effect of ionic strength decrease on the pS/tension curve of skinned crayfish fibers (Brandt et al., 1974), Ca^{2+} sensitivity was shown to be little affected, although there was a marked reduction in the capacity of substrate to inhibit tension.

There are conditions in which the pCa/tension curve shifts may be caused by changes in the affinity of TnC for Ca^{2+}. For example, the pCa/tension curve shifts to the right when the Mg^{2+} concentration is raised from 0.03 to 5.0 mM (Donaldson and Kerrick, 1975), possibly because this ion competes with Ca^{2+} for the high-affinity binding sites on TnC (Potter and Gergely, 1975) or modifies TnC by binding to very-low-affinity sites (Andersson et al., 1981). (However, Potter and Gergely believe that only the calcium-specific sites are regulatory.) Bremel and Weber (1972) have reported that the affinity of TnC for Ca^{2+} increases with cross-bridge attachment (but see Fuchs, 1977). The possibility, therefore, that the time cross-bridges spend in the attached and refractory states contributes to shifts in the pCa/tension curve must be considered in conjunction with the possibility that binding itself can change.

Two limits to the relation between the lifetime of the Ca-TnC complex and the attached time of the cross-bridges can be considered. If the attached time is assumed to be very long compared with the lifetime of the Ca-TnC complex, the concentration of Ca^{2+} necessary to initiate all myosin heads into the cross-bridge cycle will be much lower than that needed to saturate the TnC sites. At the other limit (i.e., the attached time is assumed to be short compared with the time Ca^{2+} stays bound to TnC), each myosin head may complete several cycles before Ca^{2+} dissociates from TnC.

To illustrate our hypothesis that the kinetics of the cross-bridge cycle can affect the position of the pCa/tension curve, we introduce a model based on the assumption that attachment of myosin to actin requires that Ca^{2+} be bound to TnC only in the initial phase of attachment (see Appendix for a brief discussion of the assumptions behind the model).

$$Ca^{2+} + A + X \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} Ca-A + X$$
$$\Big\downarrow k_4 \qquad\qquad \Big\downarrow k_2$$
$$Ca^{2+} + AX \underset{k_3}{\longleftarrow} Ca-AX$$

(3)

A is regulated actin in the thin myofilaments, X is any one of a number of myosin product complexes, $1/k_4$ is the mean lifetime of the attached state,

and $1/k_2$ is the mean time myosin is detached (weakly attached; Stein et al., 1979) from actin. k_{-1} (and we assume $k_3 = k_{-1}$) is the dissociation rate constant for the Ca-TnC complex.

In the appendix, several equations are derived from the model. One equation (5) describes the relation between the maximum tension and the rate constants, whereas another (6) describes the relation between the midpoint of the pCa/tension curve and the rate constants. We note that Eq. 3 includes only one Ca^{2+} per TnC as a simplification even though more than one must be simultaneously bound to activate a cross-bridge set (Potter and Gergely, 1975). Other properties of the scheme in Eq. 3 are discussed briefly in the appendix. Although the scheme is limited (for example, it does not take into account the steep slope of the pCa/tension curve), it does allow for qualitative predictions about the direction of shift of the pCa/tension curve with change in the attached time, change in the lifetime of the Ca-TnC complex, and change in the time in the refractory state (detached time). In addition, useful predictions of changes in magnitude of tension can be made.

As shown in Eqs. 3 and 6 (Appendix), the ratio of the dissociation rate constant for Ca-TnC to the rate constant for cross-bridge movement through the attached states (k_{-1}/k_4) affects the predicted midpoint of the pCa/tension curve. The ratio has a large effect when it is greater than unity, i.e., in the condition when the attached time is longer than the lifetime of the Ca-TnC complex. Below we review the possible range of values for k_{-1} and k_4, but we note that the available values are, for the most part, from studies on isolated proteins. We do not know how directly they can be applied to skinned fibers.

Johnson et al. (1979) report that Ca^{2+} stays bound to TnC with a half-time of 2-3 ms, which corresponds to a value for k_{-1} of $\sim$300 s^{-1}. This may decrease to $\sim$30 s^{-1} if TnC is part of a regulated thin filament because the affinity of the Ca^{2+}-specific sites on TnC is reported to increase by a factor of 10 when TnC is combined with TnI (Potter and Gergely, 1975). The relaxation rate in skinned fibers (Moisescu, 1976) has been reported to be on the order of 2 s^{-1}, which may be a lower boundary for k_{-1}. The relaxation rate is probably limited by transitions in the attached states, however, and not by the rate of Ca^{2+} dissociation from TnC.

Assuming that k_4 is roughly equivalent to the hydrolysis rate constant, we estimate it to be $\sim$0.5-1 s^{-1} from studies on glycerinated muscle (Hayashi and Tonomura, 1968; Levy et al., 1976; Takashi and Putnam, 1979). Using these values and taking k_{-1} to equal 30 s^{-1}, we obtain a range for the ratio k_{-1}/k_4 of 30-60. From this (also see Eq. 6) we conclude that activation of a muscle to a given fraction of maximum tension requires a Ca^{2+} concentration such that a lower fraction of Ca^{2+} binding sites are occupied. In addition, each cycle of a cross-bridge will coincide with more than one cycle of association and dissociation of Ca^{2+} and TnC. We can calculate from the association constant of TnC for Ca^{2+} ($\sim$10^6, Potter and Gergely, 1975; and see Brandt et al., 1980) and the above value for k^{-1} that in our skinned fibers each TnC binding site combines about 2 times s^{-1} with Ca^{2+} at pCa 7, and 10 times s^{-1} at pCa 6, assuming that the sites are independent. Because it is probable that two sites

must be occupied simultaneously for activation, each occupancy is not a window for the activation of postrefractory myosin heads.

If we take the pCa/tension curve midpoint as 6.0 (standard curve of Table V), and assuming k_{-1} lies between 2 and 30 s^{-1} (see above), we estimate from Eq. 6 that the true pK_1 for Ca^{2+} binding to TnC is between 5.56 and 4.73. If we assume that a pK of 4.73 is possible, then right shifts of the pCa/tension curve by means that increase k_4 could in theory force the curve midpoint towards this value. In fact, we cannot shift the curve much below 5.8 by any means we have tried so far.

If left shifts of the pCa/tension curve accompany an increase in attached time, it follows from Eq. 5 that maximum Ca^{2+}-activated tension will also increase. An increase in maximum tension does accompany the left shifts of the curve induced by ionic strength reduction and reduction in phosphate concentration. Similar increases in tension with reduction in ionic strength have been reported by others for skinned fibers (Ashley and Moisescu, 1977; Julian and Moss, 1981; Gordon et al., 1973; Thames et al., 1974) and for intact fibers (April et al., 1968; Edman and Hwang, 1977; Gordon and Godt, 1970). We observe a large increase in tension with reduction of phosphate from 7.5 to 0 mM in 5 mM substrate but no such increase in 0.25 mM substrate. Thus reduction of substrate in zero phosphate is associated with a significant decrease in maximum tension (Table VII).

TABLE VII

SIGNIFICANCE OF THE DIFFERENCE BETWEEN MEAN
PARAMETERS OF TABLES II–IV

	Parameters compared		
Two conditions compared	P$_o$	Slope (n)	pK
7.5 Pi vs. 0 in 0.25 S	NS*	>99.5	>99.5
7.5 Pi vs. 0 in 5 S	99	>99.5	>99.5
7.5 Pi vs. 15 in 5 S	NS	NS	NS
5 S vs. 0.25 in 0 Pi	95	>99.5	>99.5
5 S vs. 0.25 in 7.5 Pi	NS	>99.5	>99.5
0.200 Γ/2 vs. 0.120	95	NS	>99.5
0.200 Γ/2 vs. 0.300	>99.5	>99.5	>99.5

* No significant difference between means.
The other values are percent confidence that the means are different. S = substrate, P = maximum isometric force, slope (n) = the Hill coefficient, and pK = the midpoint of the pCa/tension curve (see text and eq. 2). The number of fibers in each condition is listed in Tables II–IV.

Contrary to the effect of substrate concentration change (in zero phosphate) reported here, tension in skinned crayfish fibers increases when the substrate is lowered from ~15 to 1 mM (Orentlicher et al., 1977). At constant pCa, pS/tension curves are bell-shaped (Reuben et al., 1971). In pCa 8, tension is maximal at 3 μm or so, then the fibers relax with further increase in substrate. As the pCa is lowered, the point of maximum tension shifts to lower pS (Brandt et al., 1972; Orentlicher et al., 1977; Cooke and Bialek, 1979). To

cogently compare our present data on the effect of substrate concentration on the maximum tension with that in the literature, we will have to take into account normalization procedures (see Methods).

Although further work must be done to establish the reason for the difference in effect of substrate change on tension (in zero phosphate) between our present results and those cited above, one explanation for our results may be found in the assumption that cross-bridges in the rigor state do not actively generate tension. This assumption is not unreasonable because in the absence of substrate a muscle cannot generate tension, although it can support considerable force if stretched. The fraction of cross-bridges in rigor will increase with reduction in substrate concentration, and the other states, including those that generate tension, will be proportionately reduced. Rigor bridges may also, by opposing sarcomere shortening, reduce measurable tension further.

Substrate and phosphate concentration reduction have opposite effects on tension. Their effects on the curve position are additive; therefore they probably act on different cross-bridge states. Phosphate increase and substrate increase seem to shorten the time cross-bridges spend in the attached states because these shift the curve to the right and increase the optimal frequency for oscillatory work from ~7 to 20 Hz (Kawai and Brandt, 1978; also see Herzi and Ruegg, 1977; White and Thorson, 1972). One interpretation of the decrease in tension and the right shift that accompany an increase in phosphate concentration is that phosphate affects the lifetime and hence the steady state population of the tension-generating state. Thus an increase in substrate concentration shortens the lifetime of the rigor state and an increase in phosphate concentration shortens the lifetime of the tension state.

Two of the three conditions that we find are accompanied by left shift of the curve are also associated with reductions in the optimal frequency of oscillatory work. An ionic strength decrease at least to 0.128, however, is not associated with a change in this frequency (Kawai et al., 1981), although the magnitude of oscillatory work increases. V_{max} of shortening (Edman and Hwang, 1977) and maximum tension (April and Brandt, 1973) have been reported to increase in intact fibers bathed in hypotonic media. In skinned fibers bathed in media of reduced ionic strength, no increase in V_{max} has been observed, although the maximum tension does increase (Julian and Moss, 1981). Gulati and Podolsky (1978) argue from their skinned fiber data that some kinetic parameters of the cross-bridge cycle must change with reduction in ionic strength.

When ionic strength is reduced, the hydrolysis rate of myofibrils increases (Goodno et al., 1978). The fraction of myosin products (X in Eq. 3) in reversible association with actin also increases (Stein et al., 1979). Because k_2 includes the refractory state transition, which is believed to be rate limiting in isolated protein systems (Eisenberg and Greene, 1980), and because the rate of hydrolysis increases in myofibrils with a decrease in ionic strength (over the range 0.1–0.2; Goodno et al., 1978), we argue that reduction in ionic strength decreases the lifetime of the refractory state, i.e., increases k_2. This will produce

the effects we observe (Table II): a left shift in the pCa/tension curve (Eq. 6), an increase in the steady state population of the attached states (Eq. 5), and a concomitant decrease in the population of refractory myosin. Furthermore, increases of 50% in tension are recorded, which implies that the refractory state pool may contain as much as one-third of the myosin under our standard conditions. At present there is little direct biochemical evidence that ionic strength can affect the lifetime of the refractory state, except that relating to hydrolysis rate noted above. The skinned fiber may exibit biochemical properties that are not found in less structured systems.

APPENDIX

From the kinetic scheme given in Eq. 3 we can derive the steady state probability (P) that a cross-bridge is in a tension-generating state (AX) + (CaAX):

$$P = \frac{1/k_{-1} + 1/k_4}{\{K_1/k_2 + 1/k_1\}/C + 1/k_{-1} + 1/k_2 + 1/k_4}.$$ (4)

At infinite Ca^{2+} concentrations, this assumes a maximal value of

$$P_{oc} = \frac{1}{1 + \dfrac{k_{-1} \cdot k_4}{k_2\{k_4 + k_{-1}\}}}.$$ (5)

The [Ca^{2+}] that gives half P_o (half tension or half maximum rate of hydrolysis) is (where $K_1 = k_{-1}/k_1$):

$$[Ca^{2+}]^{P_{oc}/2} = K = \frac{K_1}{1 + \dfrac{k_{-1}}{k_4} \cdot \dfrac{k_2}{k_{-1} + k_2}}.$$ (6)

In Eq. 6 the ration k_{-1}/k_4 affects the position of the pCa/tension curve midpoint on the pCa axis. Because k_{-1} is assumed to be a constant, an increase in the time cross-bridges spend in the attached states (= $1/k_4$) may shift the curve to the left, whereas a decrease in attached time may shift the curve to the right until the limit $K = K_1$ is reached.

One concept, difficult to introduce into kinetic schemes in a simple way, is included in Eq. 3 by the transition k_3. This transition is meant to signify that Ca^{2+} is unable to initiate another cross-bridge attachment until it dissociates from the one it last initiated. A problem with this representation is that it also implies that a cross-bridge cannot complete its transition to the rigor state until Ca^{2+} dissociates. We do not believe this is necessary, and as long as k_3 is larger than k_4 it is reasonably safe to ignore this implication. Rate constant k_4 comprises transitions through the attached states of the cross-bridge cycle, including the tension-generating and rigor states. k_2 comprises transitions through all of the "detached states" or weakly associated states including the refractory state (Eisenberg et al., 1972). We assume that k_2 is determined by the transition through the refractory state because this limits the rate of hydrolysis in isolated protein systems (see Eisenberg and Greene, 1980). Recently the maximal hydrolysis rate of a system of minifilaments of myosin and F-actin, a preparation reasonably close to skinned fibers, has been reported to be ~12.5 s^{-1} (Reisler, 1980). This is still 10 times larger than any measured value for skinned or glycerinated fibers (see Discussion).

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