EFFECT OF Ca ION CONCENTRATION ON CROSS-BRIDGE KINETICS IN RABBIT PSOAS FIBERS

EVIDENCE FOR THE PRESENCE OF TWO Ca-ACTIVATED STATES OF THIN FILAMENT

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ABSTRACT The effect of Ca ion concentration on cross-bridge kinetics in a small bundle (one to three fibers) of chemically skinned rabbit psoas muscle is studied. The length of the muscle is oscillated in small amplitude sine waves (0.2% L_0 peak-to-peak) at varying frequencies (0.125 – 167 Hz), and the resulting amplitude and phase shift in tension are measured. The frequency response function (complex stiffness) thus obtained can be divided into three parts, which we name process (A) (centered at 1 Hz), process (B) (3–17 Hz), and process (C) (50 Hz). Process (B), which represents oscillatory work, further splits into two processes (B′ and B) at partial Ca activation (<50% P_0), where the phase-frequency plot appears W-shaped. The slower of the two processes (B′) disappears by full activation, at which time the plot appears V-shaped. The characteristic frequencies associated with the minima of the plot do not shift in a graded way with Ca concentration, indicating that there is no change in apparent rate constants. Apparent rate constants of processes (A) and (C) are minimally affected by Ca. The above results are not altered when ionic strength is changed between 128 and 265 mM. We propose that activated thin filaments can have two "on" states and that Ca concentration controls the distribution of these two states. This mechanism generally supports the "switch" hypothesis of Ca regulation.

INTRODUCTION

Two apparently different mechanisms for the regulation of skeletal muscle function by Ca^{++} have been proposed, and evidence has been adduced in support of both. The difference between the two lies in the proposed effect of Ca binding on the attachment rate constant of myosin to actin. In one mechanism, Ca binding results in the rate constant going from zero to a maximum; corresponding actin segments are described as going from the "off" to the "on" state (Podolsky and Teichholz, 1970). In the other mechanism, the attachment rate constant is treated as increasing in a graded way, i.e., in proportion to the fraction of regulatory sites occupied by Ca (Julian, 1969; Julian and Moss, 1976).

Podolsky and Teichholz (1970) found identical maximum velocities of shortening (V_{max}) in fully and partially activated skinned frog fibers (see also Gulati and Podolsky, 1978; Edman, 1979; Brenner, 1980). Because V_{max} is believed to reflect attachment and/or detachment rate constants of myosin cross-bridges, their result supported the argument that the binding of Ca^{++} to the regulatory sites does not modify cross-bridge kinetics in a graded way, but rather

opens active sites to myosin in an all-or-none manner. In contrast, Julian (1971) reported that V_{max} varied with the degree of activation (Wise et al., 1971; DeClerck et al., 1977; Julian and Moss, 1981). A partial resolution of the above difference in experimental results seemed to be provided by a later report that either graded or all-or-none responses can be produced by changing the ionic strength (Thames et al., 1974).

That the rate constant(s) of the cross-bridge cycle may be graded in proportion to the amount of Ca bound to the regulatory sites on troponin-C (TnC) raises an important and challenging question about the mechanism of muscle activation by Ca^{++}. It is essential therefore to determine the action of Ca^{++} by an independent method. We have approached this problem by determining the effect of Ca^{++} concentration on the kinetics of actomyosin interaction using a method that we call sinusoidal analysis. With this method we detect three or more apparent rate constants, each of which is assumed to be a composite of many intrinsic rate constants of the cross-bridge cycle. An important advantage of this technique is that the results are not masked by internal loads, which often complicate the interpretation of physiological experiments. A preliminary account of these results has been presented (Kawai and Cox, 1980).

MATERIALS AND METHODS

All experiments were performed on adult rabbit psoas, for ease of comparison of our results with those of biochemical studies. EGTA, ATP, CP (creatine phosphate), CPK (CP kinase), and MOPS (morpholinopropane sulfonic acid) were purchased from Sigma Chemical Co. (St. Louis, Mo.); $CaCO_3$, MgO, NaOH, Na_2SO_4, NaH_2PO_4, Na_2HPO_4, and propionic acid from Fisher Scientific Co. (Pittsburgh, Pa.). Individual concentrations of multivalent ionic species are calculated by using our computer program, which assumes multiple equilibria with the following apparent association constants (log values at pH 7.00): CaEGTA 6.28, MgATP 4.00, CaATP 3.70.

A small bundle (one to three fibers) of chemically skinned psoas is dissected from a stock bundle (stored for 2–60 d) and placed in the experimental apparatus. Details of the chemical skinning and storage procedures and the resultant morphology have been published elsewhere (Eastwood et al., 1979). The preparation is first soaked in a relaxing saline containing (Na salts in mM): 5 EGTA, 2 MgATP, 8 phosphate, 41 propionate, 39 sulfate, 10 MOPS. The muscle is stretched to ~10% above its slack length (this stretched length is termed L_0), which generally yields sarcomere lengths of 2.5–2.6 μm.

The preparation is then washed with a saline containing (mM): 2 MgATP, 8 phosphate, 41 propionate, 44 sulfate, 10 MOPS to remove EGTA. This solution is twice replaced with an experimental saline containing all ingredients necessary for activation except for Ca and EGTA. After collecting a base-line record, a concentrated mixture of CaEGTA/EGTA at the desired pCa ($-\log[Ca^{++}]$) is injected into the experimental chamber (1:10 in volume). pH of the injecting solution is adjusted so that pH 7.00 is attained after mixing. Final concentrations of the species in the activating saline at standard ionic strength (201 mM) are (Na salts in mM): 5 MgATP, 5 free ATP, 7.5 phosphate, 4 sulfate, 42 propionate, 10 MOPS, 16 CP, 6 total EGTA, and Ca to achieve the desired pCa value. The solution also contains 80 U/ml CPK. Because we maintain total EGTA concentration constant, both ionic strength and monovalent cation (Na) concentration are kept constant as we change pCa (EGTA and CaEGTA are divalent anions at pH 7.0). For the lower ionic strength (128 mM) experiments, sulfate and proprionate are deleted from the saline and the CP concentration is reduced to 9 mM; for the higher ionic strength (265 mM) experiments, the sulfate concentration is elevated to 25 mM. The pH is adjusted to 7.00 ± 0.01, temperature is controlled to 20.0 ± 0.1°C, and the saline (0.5 ml in volume) bathing the muscle preparation is constantly stirred to avoid local heterogeneities in concentration and temperature.

A brief description of the sinusoidal analysis technique was published earlier (Kawai et al., 1977) and a detailed description recently (Kawai and Brandt, 1980). Data are collected at 17 frequencies

(0.125–167 Hz) for experiments at the standard ionic strength and at 16 frequencies (0.25–167 Hz) for experiments at the low ionic strength. Peak-to-peak amplitude of the length oscillations is kept to $\leq 0.2\% L_0$, which corresponds to ± 1.3 nm per half sarcomere.

RESULTS

At standard ionic strength (201 mM) with 5 mM MgATP, 5 mM free ATP, and 7.5 mM phosphate, tension threshold occurs at pCa 6.0 and maximum tension is observed by pCa 5–5.5 (Fig. 2 *B*). The pCa-tension relation is very steep, with a Hill coefficient of 4 or larger (Brandt et al., 1980); the plot is steepest at the foot of the curve and less so at the top. As soon as the steady isometric tension develops at each pCa condition, the computer is triggered and an experimental complex stiffness record is collected. Before each activation a record from the relaxed preparation is collected, and this is vectorially subtracted from the complex stiffness data obtained from the activated preparation. This procedure eliminates contributions from Ca-insensitive, in-parallel elements. However, the effect of the latter on the present experiments is already minimal, because the stiffness of the relaxed muscle at these sarcomere lengths is very small (Fig. 3 of Kawai and Brandt, 1980). The muscle is subsequently relaxed, and this sequence is repeated for each pCa value.

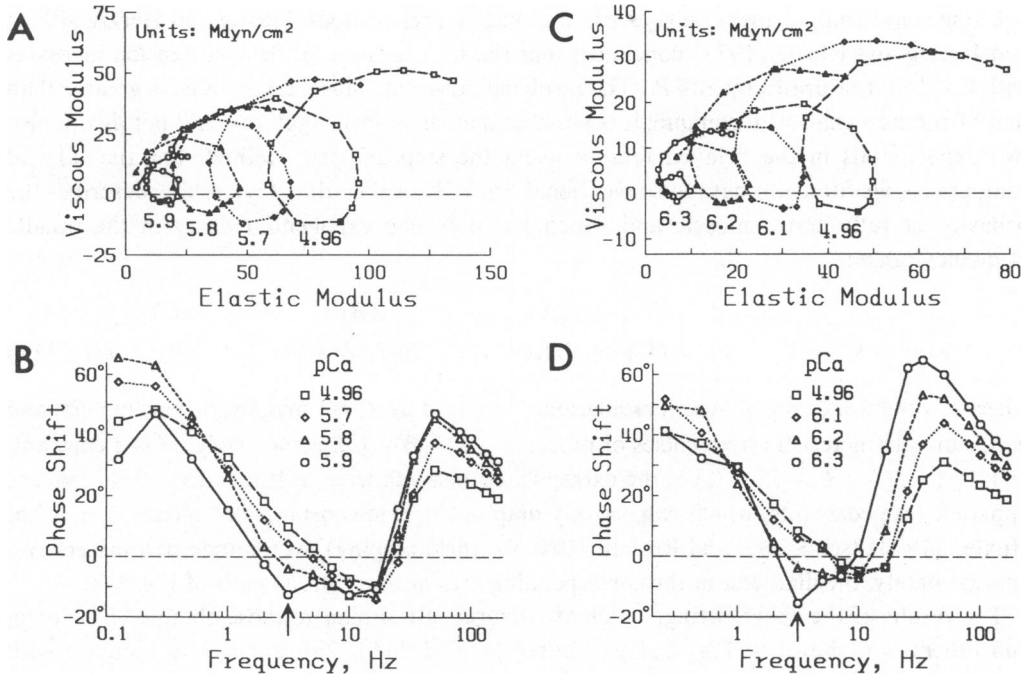

FIGURE 1 Nyquist plots (*A*, *C*) and phase-frequency plots (*B*, *D*) for various pCa (indicated in the figure). *A*, *B*: Obtained at the standard ionic strength (201 mM) on a bundle of three fibers; P_0 = 1.91 Mdyn/cm². Experiment of 4/6/79(1), Record Nos. 3–13. Peak-to-peak length change: 0.17% L_0. Frequencies used are: 0.125, 0.25, 0.5, 1, 2, 3.1, 5, 7.1, 10, 17, 25, 33, 50, 80, 100, 133, 167 Hz. (*C*, *D*) Obtained at a low ionic strength (128 mM) on a bundle of three fibers; P_0 = 1.36 Mdyn/cm². Experiment of 5/12/80(2), Record Nos. 10–23. Peak-to-peak length change: 0.20% L_0. Frequencies used are as above, except for 0.125 Hz. In *A* and *C*, filled symbols mark 10 Hz frequency points; in *B* and *D*, arrows indicate extra valleys evident at partial activation.

Complex stiffness data are displayed as Nyquist plots in Fig. 1 *A* and as phase-frequency plots in Fig. 1 *B*. The Nyquist plot has a typical bifoliate shape with three characteristic arcs. We name these process (A) (approximate frequency range <2 Hz), process (B) (2–33 Hz), and process (C) (>33 Hz). The phase-frequency plot from fully activated preparations is V-shaped (Fig. 1 *B*, pCa 4.96), i.e., there is only one region of negative phase (a phase delay). It is in this frequency range that the muscle generates "oscillatory work" (Pringle, 1967). The characteristic frequency defined by the minimum is designated by *b*. As Ca concentration is lowered the position of this minimum does not shift, indicating that there is little change in *b*. In contrast, in the plots from partially activated fibers there is an additional minimum in the lower frequency range (arrow in Fig. 1 *B*). The main minimum centers at ~14 Hz, the extra minimum at 3 Hz. Hence for partially activated fibers process (B) splits into two processes, which causes the phase-frequency plot to assume a W-shape. We designate these two processes as (B′) and (B), respectively. The slower process (B′) vanishes as the level of activation increases. A W-shaped plot at threshold activation and transition to a V-shape with increase in Ca concentration have been consistently observed in every preparation we have tested (~30). This is the first experimental evidence for the existence of two discrete rate constants in the oscillatory work component. (This component is equivalent to "phase 3" or "delayed tension" in step analysis experiments.)

Using step-length changes on glycerinated cardiac preparations, Herzig and Herzig (1974) and Herzig and Rüegg (1977) concluded that the rate constant of delayed tension increases with Ca^{++} and saturates by $50\%P_0$. The resolving power of sinusoidal analysis is greater than that of the step analysis technique; it is possible that these investigators could not distinguish two exponentials in the delayed tension using the step analysis method. For the sake of comparison, we fitted out data to conventional Eq. 1 (Kawai et al., 1977), which describes the behavior of fully active muscle and which has only one exponential delay in the middle frequency range:

$$\begin{matrix} & & & (A) & & (B) & & (C) & \\ Y(\nu) = Y_A + Y_B + Y_C = H + & A\nu\, i/(a + \nu i) & - & B\nu\, i/(b + \nu i) & + & C\nu\, i/(c + \nu i) & & (1) \end{matrix}$$

where ν is the frequency of length oscillation, $i\ \sqrt{-1}$; A, B, C are magnitude parameters, and a, b, c are characteristic frequencies of processes (A), (B), (C), respectively. H is a constant, and Y_∞ $(= H + A - B + C)$ is the extrapolated instantaneous stiffness. $2\pi a$, $2\pi b$, $2\pi c$ are apparent rate constants, which respectively map to the rate constants of "phases 4, 3, 2" of Huxley (1974) (see Kawai and Brandt, 1980, for this mapping). Magnitude parameters are, approximately, the diameter of the corresponding arcs in the Nyquist plots of Fig. 1 *A*.

The results of the above fitting, which are surprisingly similar to those obtained by Herzig and others, are shown in Fig. 2 *A* (see curve labeled $2\pi b$). $2\pi b$ appears to increase with increase in tension and saturates by $70\%P_0$. (It is apparent that this rate constant changes most rapidly at Ca concentrations where the pCa-tension curve is steepest.) This result is also similar to the V_{max} measurements of Wise et al. (1971) on glycerinated psoas.

It can also be seen from Fig. 2 *A* that rate constant $2\pi a$ increases slightly and saturates with Ca^{++} concentration. In the same figure rate constant $2\pi c$ appears to decrease to some extent, but this decrease is probably not significant because the maximum corresponding to characteristic frequency c (~50 Hz) does not shift with Ca concentration (Fig. 1 *B*). It is

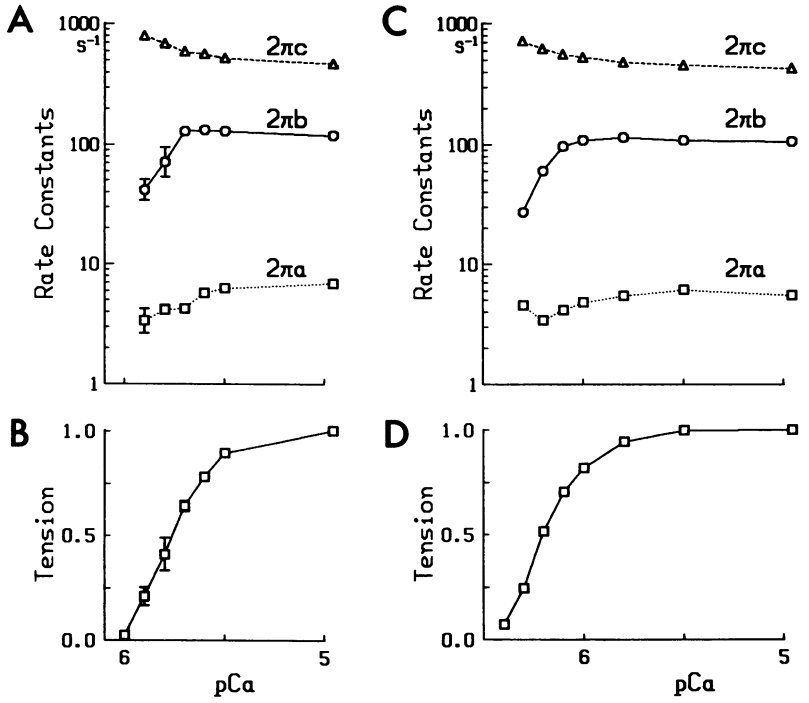

FIGURE 2 Apparent rate constants $2\pi a$, $2\pi b$, $2\pi c$ (*A*, *C*) and tension (*B*, *D*) as functions of pCa. (*A*, *B*) Obtained at the standard ionic strength (201 mM). Average of 10 experiments obtained between 3/30/79 and 4/11/79. SEM bars are shown when these are larger than the symbol size. P_0 = 1.97 ± 0.13 Mdyn/cm^2. (*C*, *D*) Obtained at a low ionic strength (128 mM). Single experiment. Data are deduced from a pCa series, parts of which are shown in Fig. 1 *C*, *D*.

evident from Fig. 2 *A*,*B* that there is no further change in the rate constants after tension reaches 70% P_0. Magnitude parameters (*A*,*B*,*C*) and instantaneous stiffness Y_∞ remain roughly proportional to tension (P) over the full range of Ca concentrations (compare Figs. 1 *A* and 2 *B*). We typically obtain P/Y_∞ = 1.2% L_0, which is the amount of length change required to release the full tension. This value is higher than that observed in intact preparations of frog or crayfish mostly because the fiber compliance increases on skinning (our unpublished observation). The proportionality between P and Y_∞ when Ca concentration is changed is consistent with the data observed by Herzig and Rüegg (1977) on cardiac preparations.

From experiments on skinned frog semitendinosus fibers, Thames et al. (1974) suggested that at lower ionic strengths the presence of "abnormal" (slowly cycling) cross-bridges reduces shortening velocity. Based on V_{max} data they concluded that these bridges cycle at a speed 1/10 normal or less. We would expect the presence of such bridges to change the low frequency component and possibly to decrease magnitude parameters *A*, *B*, *C* and to increase *H*. To investigate this possibility, we conducted experiments at an ionic strength below (128 mM) and above (265 mM) our control value.

We observed qualitatively similar results at these two ionic strengths. The data are analyzed similarly to those at the control ionic strength, and the results obtained at the lower

ionic strength are plotted in Fig. 1 *C, D* and in Fig. 2 *C, D*. As seen in Nyquist plots (Fig. 1 *C*), we observed no change in the complex stiffness data that might correlate with "abnormal" cross-bridges when the ionic strength was lowered. The existence of process (B′) is again evident at 3 Hz (Fig. 1 *D*, arrow) at partial activation. This is clearly different, however, from the "abnormal" cross-bridges Thames et al. (1974) observed in frog muscle because in the present report the ionic strength has no effect on the appearance of process (B′) (Fig. 1 *B, D*) and because tension and stiffness disappear on relaxation.

When the ionic strength was lowered we made the following additional observations: P_0 is larger than for the same preparation at the control ionic strength (April et al., 1968; Gordon et al., 1973); threshold tension develops at pCa 6.4; tension saturation occurs at pCa 5.8–5.5 (Fig. 2 *D*). The extra phase-delay (process B′) at 3 Hz is steeper, and there is a subtle graded shift with Ca in the position of the major phase delay (process B) at 10–14 Hz (Fig. 1 *D*). This latter observation may be comparable to the graded change of V_{max} with Ca at low ionic strength observed by Thames et al. (1974), although the effect is minor. Magnitude parameters are approximately scaled with tension except that *C* is larger at the low ionic strength. The three rate constants ($2\pi a$, $2\pi b$, $2\pi c$) at saturation do not differ in the ionic strength range 128–265 mM. These results are generally consistent with those of Gulati and Podolsky (1978) and of Julian and Moss (1981).

DISCUSSION

It is believed that myosin cross-bridges cyclically interact with actin to convert chemical energy stored in ATP into mechanical work. In physiological experiments we identify several cross-bridge states that are involved in cycling. These are (*a*) dissociated state, (*b*) tension state, and (*c*) rigor state. Each of these states may be divided into many substates as indicated by biochemical studies (Lymn and Taylor, 1971; Trentham et al., 1976; Stein et al., 1979). From one state to an adjacent state there is a finite transition probability, and this is represented by an intrinsic rate constant. The apparent rate constants (or characteristic frequencies) we observe in our experiments are, we must presume, complicated functions of the intrinsic rate constants. Our goal is to establish a correspondence between the apparent (experimentally observed) rate constants and the underlying biochemical transitions.

In vertebrate skeletal muscles, actomyosin ATPase is regulated via a control mechanism on the thin filaments in which Ca^{++} plays a central role (Ebashi and Endo, 1968; Weber and Murray, 1973; Fuchs, 1974; Szent-Györgyi, 1975). Ca binding to a TnC molecule is believed to turn on a segment of thin filament, consisting of seven monomeric actins, via the troponin–tropomyosin-mediated control system; increasing Ca^{++} concentration presumably results in an increased number of "on-state" actin units. Dissociated myosin heads charged with the hydrolysis product (Lymn and Taylor, 1971) readily react with actin units in the on-state to form cross-bridges.

A new approach to the Ca regulatory mechanism can be constructed from the recent report of Stein et al. (1979). They concluded that the "dissociated" actin and myosin species are in fact in rapid equilibrium with a "weakly associated" species. If we accept this proposal, then Ca binding to the regulatory proteins most likely affects a reaction subsequent to cross-bridge attachment, rather than the attachment itself. In other words, the attachment transition is

fully reversible and does not require Ca, whereas the subsequent reaction is not reversible but requires Ca-activated actin. After this second reaction myosin heads are strongly bound to actin and the back dissociation becomes a very low probability event. If this view is taken, the classical attachment transition must include these two sequential reactions. Henceforth we will refer to these as the "attachment" transition.

For the present report we must consider how our data relate to the mechanism of muscle activation by Ca^{++} and to previous observations on the effect of Ca^{++}. Our most significant observation is that the phase-frequency plot of partially activated fibers is W-shaped, whereas it is V-shaped for fully activated fibers (Fig. 1 *B, D*). It is also important to note that the characteristic frequencies of the corresponding minima do not change with Ca^{++} concentrations and the lower frequency component becomes undetectable by 70% P_0. This indicates that in the middle frequency range there are two rate constants, both of which are exponential delays. Therefore our (B) term in Eq. 1 should be rewritten to include this extra phase-delay for partial activation:

$$Y_B(\nu) = - B'\nu\, i/(b' + \nu i) - B\nu\, i/(b + \nu i) \tag{2}$$

where $b' = 3$ Hz, $b = 14$ Hz approximately, and these values appear to remain constant as Ca concentration changes. The effect of Ca is therefore very different from that of MgATP, which in low mM concentrations causes a graded shift in both b and c frequencies (Kawai, 1978, 1979; Cox and Kawai, 1981).

It might be suspected that the observed effect of Ca could result from ATP depletion or ADP accumulation in the fiber, especially at higher tensions. We exclude these possibilities for the following reasons: (*a*) oscillatory power output increases in direct proportion to the tension increase (B is scaled with tension); (*b*) there is no shift in the maximum or minimum positions in the phase-frequency plot, which is a very different result from that observed when we purposely limit MgATP (Kawai, 1978); (*c*) variation in CP/CPK and free ATP concentrations does not modify the result, which confirms our belief that the ATP supply is adequate under our experimental conditions (Kawai and Brandt, 1979).

Cross-bridge "attachment" apparently affects process (A), although to a lesser degree. We detect only a very small and possibly insignificant effect of Ca on rate constant $2\pi c$. We infer from this latter observation that the "attachment" transition does not influence process (C) under the present experimental conditions, an inference consistent with our earlier conclusion that, in the physiological MgATP range, process (C) reflects substrate (MgATP) binding to the myosin head and its subsequent dissociation from actin (Kawai, 1978, 1979). Since both MgATP and Ca^{++} concentration affect the rate constant of process (B) we have to conclude that both "attachment" and detachment transitions contribute significantly to process (B).

This conclusion is in accord with a model for glycerinated insect muscle (Thorson and White, 1969; Abbott, 1973*a*) in which attachment (f) and detachment (g) rate constants are related to the optimum frequency of oscillatory work by the following equation:

$$b = (f + g)/2\pi \tag{3}$$

Eq. 3 assumes a two state model in which f and/or g are strain sensitive; the principle is the same for models that incorporate many more states although Eq. 3 then becomes a more complicated expression.

Previous studies of the effect of Ca on the kinetics of vertebrate skeletal muscle were mainly based on force-velocity measurements, and the sensitivity of V_{max} to Ca^{++} was examined. At V_{max} unloaded muscle shortens at a constant speed, while in the present study the apparent rate constants are determined when the muscle is held in a near isometric condition. We assume that the distortion on cross-bridges is different in the unloaded vs. the near isometric condition, and strain-sensitive intrinsic rate constants may also assume different values. Because the apparent rate constants of sinusoidal analysis and V_{max} measure different aspects of cross-bridge behavior, correlation between them is not immediately obvious and will depend on the assumptions of a given model.

Because the apparent rate constants ($2\pi b$, $2\pi b'$) do not change with Ca concentration, our result is in accord with the "switch" hypothesis, which postulates that cross-bridges are simply recruited from inactive to active pools as the Ca concentration is raised. The switch hypothesis is consistent with the model developed by Huxley (1957), and was put forward as the Ca regulatory mechanism by Podolsky and Teichholtz (1970). The difference between their mechanism and ours is that Podolsky and Teichholtz proposed the presence of a single switch, while we propose two qualitatively different switches. In our mechanism the two switches correspond to two different activated states of the thin filament, each having a discrete rate constant for attachment to the myosin-hydrolysis product complex. A switch mechanism with two "on" states was suggested by Abbott (1973*b*) for glycerinated insect muscles. In his experiments, however, the rate constant did not saturate as he increased Ca^{++} concentration, nor did he identify an extra rate constant in the delay process.

We can propose two possible mechanisms to explain the presence of two activated states which differ in Ca sensitivity. One mechanism is that the binding of Ca to one of the two Ca specific sites (Potter and Gergely, 1975) on TnC permits a low "attachment" rate, while both sites have to be occupied for a maximum "attachment" rate. Alternatively, the two adjacent segments of actin in the helix might interact, so that 14 monomeric actins function together as a unit with 2 troponins and 2 tropomyosins. In this mechanism a partial on-state would occur when Ca^{++} is bound only to one of the two adjacent TnC molecules, while the maximum on-state would occur when both segments contain bound Ca on TnC.

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