Influence of Temperature upon Contractile Activation and Isometric Force Production in Mechanically Skinned Muscle Fibers of the Frog

ROBERT E. GODT and BARRY D. LINDLEY

From the Departments of Physiology, Medical College of Georgia, Augusta, Georgia 30912, and Case Western Reserve University, Cleveland, Ohio 44106

ABSTRACT Increasing temperature (4–22°C) increases the Ca²⁺ concentration required for activation of mechanically skinned frog muscle fibers. The pCa required for 50% maximal force (pCa₀) was inversely proportional to absolute temperature. Assuming that relative force is directly related to fractional occupancy of the Ca²⁺-binding sites on troponin that regulate force, the shift was consistent with a Gibbs free energy change of binding (ΔG) of about −7.8 kcal/mol. This is close to the ΔG for Ca²⁺ binding to the calcium-specific sites on troponin C reported by others. Decreasing Mg²⁺ from 1 mM to 60 μM shifts the force-pCa curves at either 4 or 22°C to higher pCa, but the shift of pCa₀ with temperature over this range (0.4 log units) was the same at low and high Mg²⁺. Maximal force increased with temperature for the entire range 4–22°C with a Q₁₀ of 1.41, and over the restricted range 4–15°C with a Q₁₀ of 1.20. From the dual effects of temperature on Ca²⁺ activation and maximal force, one would expect that force would respond differently to temperature change at high or low Ca²⁺. At high Ca²⁺, a temperature increase will lead to an increased force. However, at low to intermediate Ca²⁺ levels (below the intersection of the force-pCa curves for the initial and final temperatures), steady state force should decrease with increasing temperature. The inverse responses should occur with a decrease in temperature. These responses are observed when temperature is changed by rapid solution exchange.

INTRODUCTION Temperature may affect a number of steps in muscle contraction, e.g., membrane excitation, excitation-contraction coupling (which includes release and re-uptake of calcium by the sarcoplasmic reticulum), activation of contraction by calcium, and cross-bridge mechanics. Using the simplified, mechanically skinned fiber preparation, one can control the free calcium concentration, and hence the level of activation, and study the activation and cross-
bridge properties directly. However, previous work with temperature effects on activation of skinned fibers is meager and contradictory. Ashley and Moisescu (1977) report that in mechanically skinned fibers of the barnacle the relation between isometric force and pCa (−log Ca\(^{2+}\)) is similar at 4 and 20°C. Orentlicher et al. (1977), however, find that increasing the temperature from 5 to 20°C causes the force-pCa relation of mechanically skinned crayfish fibers to shift to higher pCa (i.e., less Ca\(^{2+}\) is required for activation at higher temperatures). Similarly, Brandt and Hibberd (1976) find in chemically skinned cat ventricular muscles that increasing temperature from 20 to 25°C causes the force-pCa curve to shift to higher pCa, although a further increase to 29°C causes no additional shift. Recently, Stephenson and Williams (1981) have reported that increasing temperature in the range 3–25°C shifts the force-pCa relation of skinned fast-twitch fibers of the rat to lower pCa. Under the same conditions, however, the force-pCa curve of rat slow-twitch fibers was unaffected by temperature.

A priori, one might expect, however, that increasing temperature would cause a shift of the force-pCa curve to lower pCa because the enthalpy of Ca\(^{2+}\) binding to troponin C is negative (Potter et al., 1977) and, hence, the affinity of troponin for Ca\(^{2+}\) should decrease with increasing temperature. Inasmuch as most of these previous studies had other aims than precise investigation of temperature effects, we felt a re-examination in frog muscle fibers with close attention to temperature influences on equilibrium constants of bathing solution constituents might be helpful in resolving these contradictions.

Our experiments demonstrate that, as expected from calcium binding to troponin, increasing temperature shifts the force-pCa curve of skinned frog muscle fibers toward lower pCa. Furthermore, the shift with temperature of the pCa needed for half-maximal activation is quantitatively similar to that seen for Ca\(^{2+}\) binding to the calcium-specific sites on troponin C (Potter et al., 1977). Maximal force increases with temperature as well. These findings would indicate that the force changes seen with rapid microwave-induced heating of single intact frog muscle fibers (Lindley and Kuyel, 1978) may well be caused by changes in contractile system properties (i.e., activation and cross-bridge properties), in addition to influences upon excitation and excitation-contraction coupling.

**METHODS**

Single fiber segments from the semitendinosus muscle of the frog (*Rana pipiens*) were mechanically skinned at room temperature in a low-calcium skinning solution (Ca\(^{2+}\) \(\leq 10^{-9}\) M) whose constituents are given below. In experiments on steady state temperature effects, one end of the skinned segment was placed in a clamp attached to a micromanipulator, the other in a clamp attached to a photoelectric force transducer based on a design by Hellam and Podolsky (1969). Further details of skinning and mounting are given in Godt (1974). In experiments on the effects of rapid temperature changes, the skinned segment was attached with small silk threads between a static arm and a force transducer using a semiconductor strain gauge (Akers AE-803, Horten, Norway). To obviate length effects on the activation curves
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The bathing solutions contained: 1 mM Mg\textsuperscript{2+}, 3.12 mM MgATP (pMgATP = \(-\log MgATP = 2.5\)), 5 mM EGTA, 20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffer, 15 mM phosphocreatine, 16-30 mM KCl so that ionic strength was 0.150 M, 0.5 mg/ml creatine phosphokinase (100-150 U/mg), pH 7.00, and various amounts of CaCl\textsubscript{2}. Some experiments were conducted in solutions identical to these but with 60 μM Mg\textsuperscript{2+} and 10 mM PIPES. The skinning solution used had similar composition but contained 70 mM KCl and no CaCl\textsubscript{2}, phosphocreatine, or creatine phosphokinase. Calcium chloride in a 1-M solution was obtained from BDH Chemicals Ltd., Poole, England; KCl was from Fisher Scientific Co., Fair Lawn, NJ; and MgCl\textsubscript{2} and EGTA were obtained from Baker Chemical Co., Phillipsburg, NJ; the other chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Calculation of the total concentration of ingredients needed to produce the desired free concentrations of constituents in the bathing solution was accomplished using a computer program similar in principle to that recently published by Fabiato and Fabiato (1979), but written in HPL for an HP9825 (Hewlett-Packard Co., Palo Alto, CA) and in Basic for a PET 2001 microcomputer (Commodore Business Machines, Santa Clara, CA). The stability constants used at the four temperatures investigated are given in Table I. Stability constants appropriate to our temperatures were interpolated by fitting stability constants from the literature to a Van't Hoff isochore, \( K = a \exp (b/T) \), where \( T \) is absolute temperature and \( a \) and \( b \) are constants.

All stability constants, except that for HPIPES, are expressed as concentration constants involving only concentration terms. The constants for HEGTA and \( \text{H}_2\text{EGTA} \) in Boyd et al. (1965), are, however, mixed constants calculated using H\textsuperscript{+} activity. As suggested by Martell and Smith (1974) (cf. Tsien and Rink, 1980), these constants were converted to concentration constants by multiplying by the hydrogen ion activity coefficient determined in KCl solution. For 0.15 M ionic strength, we used an activity coefficient of 0.765, a value midway between the values given in Harned and Owen (1958; their Table 14-2-1A) for HCl in 0.1 and 0.2 M KCl solutions.

The true stability constants for CaEGTA given in Table I were calculated from apparent stability constants determined in solutions containing 150 mM KCl and 5 mM PIPES at pH 7.0 by Allen and Blinks (unpublished observations) using an aequorin technique (Allen et al., 1977). Their apparent constants, expressed as logs, were 6.33, 6.40, 6.41, 6.45, and 6.46 for temperatures of 0, 10, 21, 30, and 40°C, respectively. Note that the log of the true stability constant at 22°C, calculated from these data and the correlated stability constants for HEGTA and \( \text{H}_2\text{EGTA} \), is 10.95, which compares favorably with the widely used value of 10.97 for 20°C given in Schwartzenbach et al. (1957).

Before an experiment, the pH of each solution was adjusted to 7.00 (±0.01) at the desired temperature. For experiments on steady state temperature effects, solutions were placed in 6-ml troughs in a temperature-controlled solution changer. The temperature of the solutions was maintained at ±0.25°C by flowing coolant from a constant temperature bath and was continuously monitored with a thermistor temperature probe. The solution changer was mounted on springs, in a design similar to that of Hellam and Podolsky (1969). To minimize surface tension and functionally to compromise the sarcoplasmic reticulum in the fibers (Solaro et al., 1971) a drop of Triton X-100, a non-ionic detergent, was added to each solution trough. Between experiments, solution troughs were kept covered with glass slides to prevent evapora-
TABLE I

STABILITY CONSTANTS

| Description of ionic species | 4°C  | 10°C  | 15°C  | 22°C  | r        | Source                      |
|------------------------------|------|-------|-------|-------|----------|-----------------------------|
| $K_1$ = KATP/K-ATP           | 8    | 8     | 8     | 8     | 8 (25°C) | Botts et al. (1965)          |
| $K_2$ = MgCP/Mg-ATP          | 40   | 40    | 40    | 40    | 40 (30°C) | O'Sullivan and Perrin (1964) |
| $K_3$ = CaCP/Ca-CP           | 14   | 14    | 14    | 14    | 14 (25°C) | Smith and Alberty (1956)     |
| $K_4$ = CaATP/Ca-ATP         | $1.14 \times 10^4$ | $1.08 \times 10^4$ | $1.03 \times 10^4$ | $9.71 \times 10^2$ | 0.92 | Taquikhan and Martell (1966) |
| $K_5$ = CaHATP/Ca-HATP       | $1.93 \times 10^2$ | $1.78 \times 10^2$ | $1.67 \times 10^2$ | $1.33 \times 10^2$ | 0.92 | Taquikhan and Martell (1966) |
| $K_6$ = CaEGTA/Ca-EGTA       | $3.19 \times 10^{11}$ | $2.04 \times 10^{11}$ | $1.43 \times 10^{11}$ | $8.04 \times 10^{10}$ | 0.99 | Allen and Blinks (unpublished observations). $K_6$ calculated from apparent constant using $K_{12}$ and $K_{13}$ |
| $K_7$ = CaHEGTA/Ca-HEGTA     | $2.0 \times 10^8$ | $2.0 \times 10^8$ | $2.0 \times 10^8$ | $2.0 \times 10^8$ | (20°C) | Anderegg (1964)              |
| $K_8$ = MgATP/Mg-ATP         | $2.35 \times 10^4$ | $2.68 \times 10^4$ | $2.97 \times 10^4$ | $3.41 \times 10^4$ | 0.99 | Phillips et al. (1966)       |
| $K_9$ = MgHATP/Mg-HATP       | $1.09 \times 10^2$ | $1.24 \times 10^2$ | $1.37 \times 10^2$ | $1.57 \times 10^2$ | 0.94 | Taquikhan and Martell (1966) |
| $K_{10}$ = MgEGTA/Mg-EGTA    | $1.62 \times 10^8$ | $1.62 \times 10^8$ | $1.62 \times 10^8$ | $1.62 \times 10^8$ | (20°C) | Schwartzbenach et al. (1957) |
| $K_{11}$ = MgHEGTA/Mg-HEGTA  | $2.51 \times 10^8$ | $2.51 \times 10^8$ | $2.51 \times 10^8$ | $2.51 \times 10^8$ | (20°C) | Anderegg (1964)              |
| $K_{12}$ = HEGTA/H-EGTA      | $6.81 \times 10^8$ | $5.15 \times 10^8$ | $4.12 \times 10^8$ | $3.05 \times 10^8$ | 0.99 | Boyd et al. (1965)           |
| $K_{13}$ = H2EGTA/H-HEGTA    | $1.21 \times 10^9$ | $9.71 \times 10^8$ | $8.17 \times 10^8$ | $6.47 \times 10^8$ | 0.99 | Boyd et al. (1965)           |
| $K_{14}$ = H2EGTA/H-H2EGTA   | $4.47 \times 10^8$ | $4.47 \times 10^8$ | $4.47 \times 10^8$ | $4.47 \times 10^8$ | (20°C) | Schwartzbenach et al. (1957) |
| $K_{15}$ = H2EGTA/H-HEGTA    | $1.0 \times 10^8$ | $1.0 \times 10^8$ | $1.0 \times 10^8$ | $1.0 \times 10^8$ | (20°C) | Schwartzbenach et al. (1957) |
| $K_{16}$ = HATP/H-ATP        | $3.57 \times 10^8$ | $3.51 \times 10^8$ | $3.47 \times 10^8$ | $3.42 \times 10^8$ | 0.98 | Taquikhan and Martell (1966) |
| $K_{17}$ = H2ATP/H-HATP      | $1.77 \times 10^8$ | $1.52 \times 10^8$ | $1.34 \times 10^8$ | $1.14 \times 10^8$ | 0.99 | Taquikhan and Martell (1966) |
| $K_{18}$ = HPipes/H-PIPES    | $8.63 \times 10^8$ | $7.67 \times 10^8$ | $6.96 \times 10^8$ | $6.07 \times 10^8$ | 0.99 | Good et al. (1966)           |

Table I is a list of stability constants with (M⁻¹) values for 4, 10, 15, and 22°C obtained in solutions with physiological ionic strength (0.1-0.2 M). A value appropriate to these particular temperatures was interpolated by fitting the stability constants given in the reference to a Van't Hoff isotherm: $K = a \exp (b/T)$, where $T$ is absolute temperature, using an exponential curve-fitting program on an HP-65 hand calculator (Hewlett-Packard Co., Palo Alto, CA) to obtain the constants $a$ and $b$. The linear correlation coefficient, $r$, is given to indicate "goodness of fit" of the Van't Hoff relation. In some cases a stability constant is available at only one temperature. This is shown in the table by listing the temperature of measurement in brackets in the row for this constant. The constants for PIPES buffer were calculated given that the $p\text{Ka}$ at 20°C is 6.8 and that $\Delta p\text{Ka}/^\circ C$ is −0.0085 (Good et al., 1966).
tion. For experiments on rapid temperature changes, the fibers were mounted in a small groove (9-mm² cross-sectional area). Temperature-equilibrated solutions could be flushed rapidly through the groove such that a complete change took <0.5 s.

Three series of experiments were conducted to establish the influence of temperature on calcium-activated force production. In the first, the relation between force and pCa was determined at a fixed temperature. Because force tends to decrease somewhat when fibers are repeatedly activated, each fiber was subjected to only one submaximal calcium concentration, followed immediately by a supramaximal calcium concentration. Fibers generating maximal force <10 N/cm² (referred to the diameter of the skinned fiber, assuming a circular cross section) were rejected.

In the second series, force-pCa curves at different temperatures were scaled relative to one another by comparing maximal force at different temperatures. At one temperature, a fiber was activated maximally by transfer to a supramaximal calcium solution appropriate to that temperature, and was then relaxed by transfer back to relaxing medium. The temperature of the solution baths was changed to a second temperature and the fiber maximally activated a second time in a supramaximal calcium solution appropriate to the second temperature. Each solution was adjusted to pH 7.00 at its appropriate temperature before the experiments. Just before a fiber was activated, the pH was checked again with a miniature pH electrode to ensure that it had not changed more than ±0.01.

To compensate for the more severe force decreases (5–10%) seen with repeated activation at supramaximal calcium levels, the following procedure was adopted. Assume provisionally that force declines by some factor α with repeated activation. (That is, if a fiber is activated twice with the same solution, the force seen with the second activation, T₂, is equal to α T₁, where T₁ is the force observed in the initial activation.) Thus, when a fiber is maximally activated first at temperature a and then at temperature b, the force observed at temperature b (Tᵇ) is equal to αβ Tᵃ, where Tᵃ is the tension at temperature a and β is the ratio of force in the absence of deterioration. If the sequence of temperatures is reversed, i.e., b precedes a, then the force at temperature a, Tᵃ, is equal to αβ/β, where Tᵇ is the force at temperature b. Thus the deterioration factor α can be eliminated and the ratio β of force at temperature b over that at temperature a is given by

\[ \beta = \frac{T_b}{T_a} = \left( \frac{T_b}{T_a} \frac{T_a}{T_a} \right)^{1/2} \]

In practice for any temperatures a and b, we obtained β from the average force ratio of three fibers taken from a to b \((Tᵇ/Tᵃ)\) and three other fibers taken from b to a \((Tᵇ/Tᵃ)\).

In the last series of experiments, we tested the scaled force-pCa curves by observing the change in force with temperature at a fixed submaximal calcium concentration. This was done in two ways. In one case the fiber was mounted in a small groove and temperature-equilibrated solutions were flushed rapidly through the groove. The other experiments were similar to those for scaling the maximal force at two temperatures. The fiber was activated first at one temperature and allowed to relax. The temperature of the solutions was then changed and the fiber was reactivated in a solution of similar pCa at the second temperature. The force ratio was scaled in the manner described above to control for deterioration.

The force-free calcium data were fitted to a Hill equation of the form

\[ \text{percent force} = 100 \frac{[Ca^{2+}]^N}{(K^N + [Ca^{2+}]^N)} \]
using a nonlinear least-squares fitting program. This relation was used primarily as a convenient description of the general pattern of the relation to aid in comparison of data at different temperatures, and to relate our data to that of others. We impute no physical significance to the parameters $N$ and $K$.

RESULTS

Force vs. $pCa$

The influence of temperature upon the activation of contractile force by $Ca^{2+}$ and upon maximal force is shown in Figs. 1 and 2 for two levels of free Mg$^{2+}$ (1 mM) ($n = 4\text{–}6$ for all points). Smooth curves represent a nonlinear least-squares fit of Hill equation to data, given parameters $N$ and $K$ in Table II. A. Normalized data illustrating effect on $Ca^{2+}$ sensitivity of contractile apparatus. B. Data normalized relative to maximal force at 4°C, illustrating the effect upon maximal force. Standard error bars are omitted, since in most cases they fell at the edges of the symbols. □, 4°C; ×, 10°C; ●, 15°C; +, 22°C.
magnesium. Increasing temperature decreases the calcium sensitivity of the contractile apparatus (seen best in Figs. 1A and 2A) while increasing the maximal force that can be generated at saturating levels of Ca^{2+} (Figs. 1B and 2B). These force-pCa data were fitted by the Hill equation using the parameters given in Table II. The shift of the curves with temperature was similar at high and low Mg^{2+}. That is, compared with solutions at 22°C, the pCa required for 50% force (pCa_{50}) in 1-mM Mg^{2+} solutions was 0.4 log units higher at 4°C, whereas in 60-μM Mg^{2+} solutions it was 0.42 log units higher at 4°C. The maximal force ratios were unaffected by Mg^{2+}. At high Mg^{2+} maximal force at 22°C was 1.88 times that at 4°C, whereas at low Mg^{2+} maximal force at 22°C was 1.85 times that at 4°C. Furthermore, maximal

![Graph](image-url)
force at 4°C was unaffected by Mg\textsuperscript{2+} in this range, in that maximal force at low Mg\textsuperscript{2+} was 0.98 (±0.02 SEM) times that at high Mg\textsuperscript{2+}.

The adequacy of scaling of the force-pCa relationships shown in Fig. 1 was tested by activating fibers submaximally at two temperatures. Three fibers were activated at 10°C in a solution with pCa 5.94, allowed to relax, and then activated at 15°C in a solution with pCa 5.87. Three other fibers were activated in identical solutions but with the temperature sequence reversed to control for deterioration. With correction for deterioration, the force at 15°C was 84% of that at 10°C, in good accord with the value of 85% taken from the data of Fig. 1.

Shift of Ca\textsuperscript{2+} Sensitivity with Temperature

The shift of the force-pCa curves with temperature might reflect the influence of temperature upon the binding of Ca\textsuperscript{2+} to the regulating sites on troponin. If one assumes that the level of normalized force is directly related to the occupancy of the troponin regulatory sites by Ca\textsuperscript{2+}, then, as demonstrated in the Appendix, one can obtain the total Gibbs free energy change of binding per mole of ligand (ΔG) from the calcium concentration required for half-maximal force activation (Ca\textsubscript{50}). The ΔG calculated from the data in Fig. 1A was (in kcal/mol) −7.85, −7.80, −7.73, and −7.75 at 4, 10, 15, and 22°C, respectively. Similarly, the slope of a plot of the natural logarithm of Ca\textsubscript{50} vs. reciprocal temperature (Fig. 3) reflects the enthalpy change of binding (ΔH), which, for the data in Fig. 3, was −8.6 kcal/mol.

Increase of Maximal Force with Temperature

Table III shows that maximal calcium-activated force increases with temperature. The Q\textsubscript{10} for all four temperatures, calculated from a least-squares fit, is 1.41 (r = 0.95), whereas that for the lower three temperatures alone is 1.20 (r = 0.99). The natural logarithm of maximal force is plotted against the reciprocal temperature in Fig. 4. From a least-squares fit of the data, the activation energy (negative of the slope) over all four temperatures is 5.50 kcal/mol (r = 0.94), whereas that for the lower three temperatures is 2.94 kcal/mol (r = 0.99).
FIGURE 3. Effect of temperature on calcium concentration required for half-maximal force (Ca₅₀) at high Mg²⁺. Bars indicate standard errors.

Response to Rapid Temperature Change

Although the previous experiments were performed at fixed temperatures, it is interesting to observe the behavior of fiber force when temperature is rapidly altered. This was accomplished by flushing the experimental chamber with a solution at a different temperature. Fig. 5 shows the response of a fiber to rapid temperature changes imposed by flushing. At high levels of Ca²⁺ (pCa 5), where fiber force is maximal, decreasing the temperature from 22 to 10°C causes force to fall, whereas increasing the temperature from 10 to 22°C causes force to rise. At low to intermediate Ca²⁺ concentrations, on the other hand, force responds to a temperature change in a reverse manner. From Fig.

**Table 111**

| Temperature | Maximal force relative to 4°C (SE) |
|-------------|----------------------------------|
| 4°C         | 1.00                             |
| 10          | 1.13 (0.03)                      |
| 15          | 1.23 (0.05)                      |
| 22          | 1.88 (0.08)                      |
1B one would expect that, at pCa 5.8, steady state force would fall if temperature were changed from 10 to 22°C. As can be seen in the lower panel of Fig. 5, changing the temperature from 10 to 22°C at pCa 5.8 leads to a fall in force to a lower level, as expected. Inversely, changing the temperature from 22 to 10°C causes a rise in force to a higher level.

**DISCUSSION**

Our data on the relation between force and pCa for *R. pipiens* muscle fibers is similar to that seen at 22°C by Robertson and Kerrick (1979). In their solutions with 1 mM Mg\(^{2+}\) and 2 mM MgATP at pH 7, they found \(N\) to be 3.27 and \(K\) to be \(3.89 \times 10^{-6}\) M (10^{-5.41}). This can be compared with our values of 3.81 for \(N\) and of \(1.63 \times 10^{-6}\) M for \(K\). On the other hand, these data do not agree with those reported for frog by Kerrick and Donaldson (1975), who fitted their data with a slightly different form of the Hill equation. In solutions at 20°C with 1 mM Mg\(^{2+}\) and 2 mM MgATP at pH 7, they found an \(N\) of 1.5 and a \(K\) (calculated from their data) of \(1.03 \times 10^{-5}\) M. We have no explanation for this discrepancy.

Increasing temperature has at least two effects upon the contractile apparatus of frog muscle: the calcium sensitivity decreases (Fig. 1A) and the maximum tension increases (Fig. 1B). The latter finding has been observed by other workers. In intact muscle, tetanic force increases with increasing temperature (Casella, 1950; Blinks et al., 1978; Lindley and Kuyel, 1978; Bressler, 1981), as does maximum K\(^+\) contracture force (Caputo, 1972). Similarly,
Ashley and Moisescu (1977) found that maximal calcium-activated force in skinned barnacle fibers at 4°C was only 50% (or lower) of that at room temperature. This agrees favorably with our data for frog skinned fibers, where maximal force at 4°C is but 55% of that at 22°C. In addition, Kuhn et al. (1979) found that maximal force of glycerinated single fibers from the giant water bug (*Lethocerus maximus*) increases with increasing temperature. Their data indicate that the increase is quantitatively similar to the increase we report for frog skinned fibers, being ~1.8–1.9-fold higher at 22 than at 5°C. Stephenson and Williams (1981), on the other hand, observed a much
greater effect of temperature in skinned muscle fibers of the rat, where an increase in temperature from 5 to 22°C produced a roughly threefold increase in maximal Ca²⁺-activated force.

In the conventional cross-bridge model, force depends upon the number of attached cross-bridges and the force per bridge. In glycerinated fibers from the dorsal longitudinal muscle of Lethocerus maximus, the number of attached cross-bridges in fully activated fibers, as determined from rapid force transient experiments, does not change with temperature in the range 5–35°C (Kuhn et al., 1979). Thus, the increase of maximal force with increasing temperature is probably caused by an increase of force per attached cross-bridge, as is expected in the Huxley-Simmons (1971) model. This notion is bolstered by observations of Ford et al. (1977) on instantaneous stiffness of intact single frog muscle fibers. As temperature increases in the range of 0–8°C, instantaneous stiffness during tetanus (a function of the number of attached bridges and stiffness per bridge) increases somewhat but not as much as does tetanic force, which indicates that force per attached bridge probably increases with increasing temperature.

Our data on calcium sensitivity changes with temperature are, however, discrepant with those of a number of others. In barnacle fibers, Ashley and Moisescu (1977) observed similar force-pCa curves at 4 and 20°C. In contrast, Orentlicher et al. (1977) found that the calcium sensitivity of skinned crayfish fibers increases by about an order of magnitude, i.e., the force-pCa curve shifts to higher pCa by ~1.0 log unit, when temperature is increased from 5 to 20°C. Similarly, Brandt and Hibberd (1976) found, in chemically skinned cat ventricular muscle, that increasing the temperature from 20 to 25°C shifts the force-pCa curve to higher pCa by ~0.3 log units, whereas a further increase from 25 to 29°C has no further effect. Stephenson and Williams (1981) reported that skinned fibers from rat slow-twitch (soleus) and fast-twitch (extensor digitorium longus) muscle respond differently to temperature changes. They found that the mean pCa₂⁰₀ of typical slow-twitch fibers is similar at low (3–5°C), intermediate (22–25°C), and high (35°C) temperatures. In contrast, the mean pCa₂⁰₀ for fast-twitch fibers is decreased by 0.33 log units when the temperature is raised from 3–5°C to 22–25°C; an increase of temperature from 25 to 35°C, however, had no further effect on pCa₂⁰₀. As with rat fast-twitch fibers, we find that in frog phasic fibers an increase of temperature from 4 to 22°C shifts the tension-pCa curves to lower pCa, with the pCa₂⁰₀ decreasing by 0.4 log units.

The experiments in Fig. 1 were conducted using solutions with a free Mg²⁺ concentration somewhat higher than those used by some others (1 mM as compared with 0.034–0.04 mM for Ashley and Moisescu, 1977, 0.01–0.9 mM for Orentlicher et al., 1977, and ~0.1 mM for Brandt and Hibberd, 1976). Differences in free magnesium concentration, however, do not appear to explain the discrepancies with these workers because experiments with frog muscle conducted at a low Mg²⁺ concentration (0.06 mM, Fig. 2) show that, although the activation curves are shifted to lower pCa values, the curve at 4°C still lies well to the left of the curve at 22°C. In fact the shift of the pCa₂⁰₀
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between 4 and 22°C at low Mg\(^{2+}\) (0.42 log units) is similar to that seen in high-Mg\(^{2+}\) solutions (0.4 log units).

Some of the discrepancy between our experiments and those of Orentlicher et al. (1977) and Brandt and Hibberd (1976) may be ascribed to species differences. Another possibility is that we, unlike these workers, used data on the temperature sensitivity of many of the stability constants in an attempt to keep ionic conditions as similar as possible at all temperatures. Clearly, both species and fiber-type differences do play a role in the response to temperature, as shown by Ashley and Moisescu (1977) and Stephenson and Williams (1981), who also were aware of the effect of temperature on solution binding constants.

Our study can be contrasted best with that of Stephenson and Williams (1981) on rat skinned fibers. For comparison it is important to note that in their study the most important constants, those for CaEGTA and MgATP, were similar to those we used. Over the temperature range 3–25°C, they used an apparent constant for CaEGTA of \(5 \times 10^6\) M\(^{-1}\) at pH 7.1, derived from measurements at pH 5.8–6 and extrapolated to pH 7.1 (Ashley and Moisescu, 1977; Moisescu and Thieleczek, 1978, 1979). At pH 7 this apparent constant would be \(3.2 \times 10^6\) M\(^{-1}\), which is close to those we used after Allen and Blinks (unpublished observations), i.e., an apparent stability constant of \(2.14 \times 10^6\) M\(^{-1}\) at 0°C rising to \(2.57 \times 10^6\) M\(^{-1}\) at 21°C. The apparent stability constant they used for MgATP corresponds to a true constant of \(2.2–2.6 \times 10^4\) M\(^{-1}\) over the temperature range of 0–20°C, whereas ours varied from 2.35 to 3.41 \(\times 10^4\) M\(^{-1}\) over the range 4–22°C.

It is significant that Stephenson and Williams (1981) found that the force-pCa relation of skinned fast-twitch fibers of the rat was affected by temperature, whereas that of slow-twitch fibers was not. Over the range 3–5°C to 22–25°C, the pCa\(_{50}\) of rat fast-twitch fibers decreased by 0.33 log units as compared with the shift of 0.4 log units over the range 4–22°C we observed with frog phasic muscle fibers. This similarity is not unexpected insofar as mammalian fast-twitch fibers are similar in other ways to amphibian phasic fibers. For example, the contractile apparatus of both is more sensitive to Ca\(^{2+}\) than Sr\(^{2+}\), whereas that of slow-twitch fibers shows similar sensitivities to these ions (Ebashi et al., 1969; Donaldson and Kerrick, 1975; Kerrick et al., 1976; Takagi and Endo, 1977; Hoar and Kerrick, 1979). The maximum velocity of shortening (at the same temperature) is similar in frog phasic and mammalian fast-twitch fibers, whereas that of slow-twitch fibers is lower (Close, 1972). Post-tetanic potentiation is observed in frog phasic and mammalian fast-twitch fibers, but not in slow-twitch fibers (Close, 1972; Kirby and Lindley, 1981). In addition, twitch speed is similar and relative tetanic force is higher in frog phasic and mammalian fast-twitch fibers than are those of slow-twitch fibers. This similarity is strengthened by the quantitative agreement between temperature effects on the force-pCa relation we observed with frog phasic fibers with that expected from effects on calcium binding to rabbit troponin derived from white (i.e., fast-twitch) muscle (see below).

Based upon studies of divalent cation binding to troponin, Potter and co-
workers (Potter and Gergely, 1975; Johnson et al., 1979) have identified the low-affinity, Ca\(^{2+}\)-specific binding sites on troponin C (TnC) as the putative sites that regulate contraction. Potter and Gergely (1975) obtained a good fit of their model with the relation between myofibrillar ATPase and Ca\(^{2+}\) by assuming that ATPase is directly proportional to the occupancy of the Ca\(^{2+}\)-specific sites on troponin by Ca\(^{2+}\). If force production is also assumed proportional to occupancy of the Ca\(^{2+}\)-specific sites, one can calculate the Gibbs free energy change of binding (ΔG) from the force-pCa curves (see Appendix). The ΔG obtained from the data of Fig. 1 at 4, 10, 15, and 22°C (−7.7 to −7.8 kcal/mol) compares favorably with the value of −7.7 kcal/mol for binding of Ca\(^{2+}\) to the Ca\(^{2+}\)-specific sites of TnC (Potter et al., 1977).

A model relating force production to fractional occupancy of the Ca\(^{2+}\)-specific sites, however, may be too simple. In the first place, although the data on ΔG for activation of force and for occupancy of Ca\(^{2+}\)-specific sites on purified TnC are gratifying similar, the Ca\(^{2+}\) binding constant of these sites increases by about an order of magnitude when TnC is complexed with troponin I and troponin T in the complete troponin complex. Preliminary evidence, however, indicates that attachment of Tn to the thin filament decreases the affinity of Ca\(^{2+}\) for the Ca\(^{2+}\)-specific binding sites to 5 × 10\(^{5}\) M\(^{-1}\) in the presence of Mg\(^{2+}\) (Potter and Zot, 1982), so that ΔG for the attached Tn, as for TnC, is −7.7 kcal/mol. In the second place, simultaneous measurements of bound Ca\(^{2+}\) and isometric force in glycerinated rabbit psoas fibers indicate that fractional saturation of myofibrillar Ca\(^{2+}\) binding sites is, in fact, greater than normalized force (Fuchs and Fox, 1981). Furthermore, if myofibrillar ATPase is directly proportional to fractional occupancy of these sites, contractile ATPase activity will be activated at lower Ca\(^{2+}\) levels than isometric force. This has been observed in a number of different muscles (Schädler, 1967; Levy et al., 1976; Godt, unpublished data).

It is clear, however, that the regulatory reaction governing the activation of force by Ca\(^{2+}\) does follow a simple binding isochore since the shift of the pCa\(_{50}\) with temperature can be fitted nicely by a single value of ΔG, i.e., the shift of pCa\(_{50}\) is directly proportional to the reciprocal temperature (cf. Fig. 3), as in a simple binding reaction. Furthermore, because the regulatory reaction involves troponin, one would expect increasing temperature to shift the force-pCa curve to lower pCa, since binding affinity of Ca\(^{2+}\) to TnC (and thus presumably to thin filament Tn as well) decreases with temperature (see Appendix).

When temperature is changed rapidly by flushing through a solution with different temperature, the force of activated skinned fibers changes rapidly as the contractile apparatus shifts to a force-pCa curve appropriate to the new conditions. The new steady level should be higher than the previous level for pCa values below (i.e., Ca\(^{2+}\) levels above) the intersection point of the tension-pCa curves for the initial and final temperatures. Inversely, the new steady level will be lower for pCa values above the intersection of the two curves. Note that the force changes shown in Fig. 5 bear out these predictions.

The influence of temperature on both Ca\(^{2+}\) activation and maximal force
might provide an explanation for the effects of rapid microwave heating on potassium contracture force of intact frog fibers. In fibers activated at high K+, Lindley and Kuyel (1978) found that heating caused a rapid monotonic increase of force. In fibers activated submaximally at lower K+, on the other hand, heating caused a rapid rise of force followed by a slower fall to a lower steady force level. Given our observations with skinned fibers, these changes in steady force might be explained by effects of temperature upon the contractile system itself, without considering any influence of temperature on the excitation-contraction (EC) coupling mechanism.

Other effects of temperature upon intact fibers can be reinterpreted in view of the marked influence of temperature upon the force-pCa relation. For instance, Caputo (1972) showed that, in frog muscle fibers, the relation between peak force production and membrane potential in potassium contractures shifted to the right (i.e., toward lower potentials) with temperature. (Similarly, in voltage-clamped snake muscle fibers, Washio [1974] observed that the relation between force and potential shifts to the right with temperature.) Lacking data on temperature effects on troponin, Caputo chose to interpret this shift solely as an effect of temperature upon the EC coupling mechanism, being the result of a shift of the relation between Ca\(^{2+}\) release and membrane potential to the right. It is not clear what effect temperature will have on EC coupling, however. For example, nonlinear intramembranous charge movements are thought to reflect key steps in the EC coupling process (Schneider and Chandler, 1973). Hollingworth and Marshall (1981) have shown that, although increasing temperature (2–15°C) increases the kinetics of charge movement, it has no effect on the amount or steady state distribution of charge in mammalian fast and slow fibers or in frog phasic muscle fibers. Thus, they conclude that temperature effects on force production in these muscles is likely to be the result of direct effects upon the contractile machinery, as we have shown. In addition, force production could also be influenced by the temperature dependency of the Ca\(^{2+}\) pumping rate of the sarcoplasmic reticulum (de Meis, 1981). In any event, although changes in temperature may well affect some facets of EC coupling, it is clear from our data that the Ca\(^{2+}\) sensitivity of the contractile apparatus can change with temperature as well. Thus, the shift of the K+ contraction force vs. potential curve with temperature in the frog is more likely caused by the synergistic effects of an increased Ca\(^{2+}\) requirement for force production coupled with any decrease in Ca\(^{2+}\) released. In this regard, Sakai and Kurihara (1974) have shown that intact fibers, bathed in low concentrations of caffeine that are subthreshold for producing force, will undergo a contracture when bath temperature is quickly reduced. These “rapid cooling contractures” may be caused in part by the shift of the force-pCa relation to higher pCa when temperature is decreased.

**APPENDIX**

As a first approximation, it is plausible to assume that the activation of isometric force with calcium is directly related to the binding of Ca\(^{2+}\) to sites on troponin.
Under the assumption that the normalized force level at any level of \( \text{Ca}^{2+} \) is directly proportional to the fractional occupancy of the calcium-binding sites on troponin responsible for contractile regulation, one can calculate the free energy change of \( \text{Ca}^{2+} \) binding from force-pCa data, such as presented in Fig. 1A. For a general class of binding sites, one plots the ligand equilibrium curve, that is, the relation between fractional occupancy of the sites (on the abscissa) vs. the natural logarithm of the ligand activity, \( x \) (on the ordinate). From this curve one can define the mean ligand activity, \( x_m \), as that ligand activity such that the area under the curve to the left of \( \ln x_m \) is equal to the area above the curve to the right of \( \ln x_m \). If the ligand equilibrium curve is symmetrical, \( x_m \) is equal to that ligand activity where half the sites are occupied by ligand. Wyman (1964) has demonstrated that, in general, the total Gibbs free energy change of binding per mole of ligand \( (\Delta G) \) is equal to \( RT \ln x_m \). This result holds regardless of the degree of cooperativity of binding of ligand to the sites. The value of the concept of mean ligand activity is to permit an estimate of free energy changes without making assumptions about the molecular details of the binding reaction.

As has been shown, the Hill equation provides a good fit of our data relating force and free calcium. Since the Hill equation is symmetrical, if the fractional activation level of isometric force is assumed to be directly proportional to the fractional occupancy by \( \text{Ca}^{2+} \) of the regulatory sites on troponin, the mean ligand activity is that concentration of \( \text{Ca}^{2+} \) at which normalized force is half-maximal \( (C_{a0}) \). From the data in Fig. 1A, the free energy change of binding calculated in this manner was \(-7.85\) kcal/mol at \( 4^\circ C \), \(-7.80\) kcal/mol at \( 10^\circ C \), \(-7.73\) kcal/mol at \( 15^\circ C \), and \(-7.75\) kcal/mol at \( 22^\circ C \). This compares favorably with the value of \(-7.7\) kcal/mol obtained for binding of calcium to the \( \text{Ca}^{2+} \)-specific sites on TnC at \( 25^\circ C \) (Potter et al., 1977). Since \( \Delta G = \Delta H - T\Delta S \), where \( \Delta H \) is the enthalpy change, \( T \) is absolute temperature, and \( \Delta S \) is the entropy change, one can obtain an estimate of \( \Delta H \) for binding from the slope of a plot of \( \ln C_{a0} \) vs. \( 1/T \) (Fig. 3). The \( \Delta H \) obtained in this manner from the data of Fig. 1A was \(-8.6\) kcal/mol. This can be compared with a \( \Delta H \) of \(-7.7\) kcal/mol obtained from calorimetry for both the \( \text{Ca}^{2+} \)-Mg\(^{2+} \) and the \( \text{Ca}^{2+} \)-specific sites on TnC at \( 25^\circ C \) (Potter et al., 1977). From these data one can estimate that \( \Delta S \) is between \(-2.7\) and \(-3.0\) cal/mol °K as compared with a value of zero for \( \Delta S \) of the \( \text{Ca}^{2+} \)-specific sites on TnC (Potter et al., 1977).

Furthermore, since

\[
\Delta G = -RT \ln K_{\text{Ca}}
\]

then

\[
K_{\text{Ca}} = \exp \left( \frac{\Delta S}{R} - \frac{\Delta H}{RT} \right)
\]

and, thus,

\[
\frac{\partial K_{\text{Ca}}}{\partial T} = \frac{K_{\text{Ca}} \Delta H}{RT^2}.
\]

Since \( \Delta H \) for \( \text{Ca}^{2+} \) binding to either class of sites on TnC is negative, one would expect \( \text{Ca}^{2+} \) affinity to troponin to decrease with increasing temperature.

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