# Influence of Temperature on the Calcium Sensitivity of the Myofilaments of Skinned Ventricular Muscle from the Rabbit

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ABSTRACT The steady-state myofilament Ca sensitivity was determined in skinned cardiac trabeculae from the rabbit right ventricle (diameter, 0.13–0.34 mm) at 36, 29, 22, 15, 8, and 1°C. Muscles were stimulated to 0.5 Hz and stretched to a length at which maximum twitch tension was generated. The preparation was then skinned with 1% vol/vol Triton X-100 in a relaxing medium (10 mM EGTA, pCa 9.0). Each preparation was exposed to a series of Ca-containing solutions (pCa 6.3–4.0) at two of the six temperatures studied (temperature was regulated to ± 0.1°C). The pCa values (mean ± SD, $n = 6$) corresponding to half maximal tension at 36, 29, 22, 15, 8, and 1°C were 5.47 ± 0.07, 5.49 ± 0.07, 5.34 ± 0.05, 5.26 ± 0.09, 4.93 ± 0.06, and 4.73 ± 0.04, respectively. Mean (± SD) maximum tension ($C_{max}$) developed by the preparation as a percentage of that at 22°C was 118 ± 10, 108 ± 5, 74 ± 6, 57 ± 7, and 29 ± 5% at 36, 29, 15, 8, and 1°C, respectively. As cooling led to a shift of Ca sensitivity towards higher [$Ca^{2+}$] and a reduction of $C_{max}$, the Ca sensitivity curves over this range of temperatures do not cross over as has been described for canine Purkinje fibers (Fabiato 1985). Since tension is decreased by cooling at all levels of [$Ca^{2+}$] it is unlikely that changes in myofilament Ca sensitivity play a role in the large hypothermic inotropy seen in rabbit ventricular muscle. The increase in sensitivity of the myofilaments to Ca on warming from 1 to 29°C might be related to the increase in force seen on rewarming from a rapid cooling contracture in intact rabbit ventricular muscle.

## INTRODUCTION

Many isolated muscle experiments are performed between 20 and 37°C and over this temperature range there is little definitive information concerning the influence of temperature on myofilament Ca sensitivity, assessed as the pCa ($-\log_{10}$ [$Ca^{2+}$]) vs. tension relationship in cardiac muscle. Literature values for the temperature dependence of myofilament Ca sensitivity differ both in magnitude and direction, and have been ascribed to differences between species. Brandt and Hibberd (1976) reported that the Ca sensitivity of EGTA-treated cat ventricular muscle was unchanged between 29 and 25°C but decreased on cooling from 25 to 20°C. Recent evidence (Miller and Smith, 1985) has cast doubt on the efficacy of the EGTA "skin-

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ning" treatment and therefore these results must be interpreted with caution. Fabiato (1985) described an increase in Ca sensitivity of skinned canine Purkinje fibers on cooling from 22 to 12°C. Previous results from other striated muscle types are also, to some extent, mixed. Stephenson and Williams (1981, 1985) reported that the Ca sensitivity of the myofilaments of skinned skeletal muscle from the rat and toad was increased by cooling. Similarly, Godt and Lindley (1982) reported that the Ca sensitivity of skinned frog skeletal muscle was increased by cooling. However, Ashley and Moisescu (1977) found the Ca sensitivity of mechanically skinned barnacle muscle was unchanged between 22 and 4°C, and Orentlicher et al. (1977) showed that the myofilament Ca sensitivity of detergent-treated crayfish muscle was decreased by cooling.

Rapid cooling of mammalian cardiac muscle to below 4°C induces contractures that are thought to be due to a release of Ca from the sarcoplasmic reticulum (SR; Kurihara and Sakai, 1985; Bridge, 1986). These contractures can be used as a qualitative index of the amount of Ca available for release from the SR. Knowledge of how myofilament Ca sensitivity is affected at the temperature reached during rapid cooling contractures (RCCs) should allow a clearer and more quantitative interpretation of the results from studies in which this technique is used. For example, it has been observed that rewarming during an RCC in rabbit ventricular muscle results in a large transient increase in force (Bridge, 1986; Bers, 1989), which might be a reflection of a change in myofilament Ca sensitivity upon rewarming.

In most mammalian heart muscle preparations, cooling from 37 to 25°C leads to a large increase in twitch force (e.g., a more than fivefold increase for rabbit and rat ventricle; Shattock and Bers, 1987). Therefore, if cooling leads to an increase in the Ca sensitivity of the myofilaments (e.g., Fabiato, 1985), this could contribute to this hypothermia-induced inotropy.

The aim of the present study is to describe the effect of a wide range of temperatures (1–36°C) on the Ca sensitivity of skinned rabbit ventricular muscle. This will allow comparison with results from other studies in which the temperature dependence of Ca sensitivity was measured in other species or muscle types. This temperature range was chosen as it encompasses temperatures at which many physiological studies are carried out (20–37°C) down to the temperature reached during RCCs (1°C). Preliminary results of this study have been reported (Harrison and Bers, 1988).

## METHODS

Male New Zealand white rabbits were given 50–75 mg/kg of pentobarbital sodium administered intravenously. The heart was rapidly excised and flushed with warmed Tyrode of the following composition (in millimolar): 140 NaCl, 6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 5 HEPES, 10 glucose, pH 7.4 and equilibrated with 100% oxygen. Small loops of fine suture were tied around the ends of free-running trabeculae (mean diameter, 225 $\mu$m; range, 130–340 $\mu$m; $n = 23$) that were dissected from the free wall of the right ventricle and mounted on a tension transducer constructed from a piezoresistive element (AE801; SensoNor, Horten, Norway). The muscle was stimulated at 0.5 Hz at 29°C (pH 7.4) in the above solution and gradually stretched to a length at which maximum twitch tension was generated (mean twitch tension ± SD under these conditions was 22.2 ± 8 mN/mm$^2$, $n = 23$). Muscles generating < 10 mN/mm$^2$ were considered to be damaged and were discarded. With the muscle at optimum length the transducer assembly was moved to a multichamber bath assembly similar to that described

by Moisescu and Thieleczek (1978). The bath consisted of 17 oblong chambers (volume, 2.5 ml) milled from a plexiglass block. The areas underneath and between the chambers were removed so that the block could be perfused with a 25% propyleneglycol-water mixture to control the temperature of the solutions in the chambers. To effect a solution change the bath assembly was lowered away from the preparation and the plexiglass block was moved along until the desired chamber was underneath the preparation and the bath assembly was raised. Solution changes were completed within 1–2 s. As the bath is lowered and the muscle passes through the meniscus of the bathing solution, surface tension drags any superficial fluid from the preparation, thereby reducing carry-over and thus keeping contamination of the new bathing medium to a minimum. The solutions were stirred continuously with a small paddle driven by an electric motor and the temperature of the solutions was continuously monitored with a thermocouple (BAT-12; Sensortek, Clifton, NJ).

On transfer of the transducer-muscle assembly to the multichamber bath, the preparation was treated for 30 min with 1% vol/vol Triton X-100 (e.g., Kentish, 1982) in relaxing solution (R1, nominally Ca-free, 10 mM EGTA, pCa 9.0, see *Solutions* for composition). This treatment renders the muscle bundle freely permeable to the bathing solution by destroying the integrity of the sarcolemma, SR, and mitochondria.

In the initial studies, the steady-state Ca sensitivity of each preparation was measured at two temperatures (either at 36 and 29, 22 and 15, or 8 and 1°C). The muscle was initially equilibrated in a preactivating solution (R2, 50 $\mu$M EGTA, pCa 7.5) before being moved to an activating solution (10 mM EGTA, pCa 6.3–4.0). Once tension had reached a steady level, the muscle was moved to a solution (R1) to induce relaxation. Once tension had returned to baseline level the muscle was moved back to the preactivating solution (R2) in preparation for the next activation. This "Ca-jump" method of activation (Moisescu, 1976; Miller, 1975; Ashley and Moisescu, 1977) was used as it leads to a relatively synchronous activation of the muscle bundle, which helps to maintain the homogeneity of sarcomere length throughout the preparation. Maximal activating contractions (pCa 4.0) were interspersed amongst submaximal activations to enable accurate normalization of submaximal contractions.

A series of temperature-change experiments were then carried out to assess the Ca sensitivity of individual muscles over the whole range of temperatures. To achieve this the jacketing system of the bath assembly was modified to give three sets of wells, each jacketed separately. This allowed experimental solutions in the three sets of wells to be maintained at different temperatures. One group of seven wells was dedicated to 22°C so that all tension levels generated at other temperatures could be compared with that achieved at 22°C. The solutions in the other two sets of wells were replaced as necessary and the temperature of the water jacket around the wells altered accordingly.

The temperature-change studies were carried out to assess the effect of the whole range of temperatures on $C_{max}$ and the tension generated by the same submaximal $[Ca^{2+}]$ (pCa 5.2, solution A1). The total concentration of Ca had to be increased from 9.103 mM at 1°C to 9.463 mM at 36°C to achieve the same pCa over this temperature range as the affinity of EGTA for Ca is increased at higher temperatures (Harrison and Bers, 1987). At each temperature, the muscles were also exposed to a solution (A2) with $[Ca^{2+}]$ equal to that which produced half-maximal tension ($K_{1/2}$) in the steady-state experiments at that temperature. This provided an independent check on the $K_{1/2}$ values generated from the steady-state determinations. These experiments were designed to directly compare the effect of temperature on myofilament Ca sensitivity in individual preparations.

### *Solutions*

The composition of the majority of the solutions used to determine the Ca sensitivity of the contractile proteins is as follows (in millimolar): 10 EGTA + CaEGTA, 5 $Na_2ATP$, 15 $Na_2CrP$, 100 KCl, 7 $MgCl_2$, (free $[Mg^{2+}]$ 2.2–2.4 mM), 25 HEPES, titrated to pH 7.0 with

1 M KOH. 15 U/ml of creatine phosphokinase was added to the solutions though there is evidence (e.g., Saks et al., 1976; Kentish, 1982, 1984) that there is sufficient endogenous enzyme, bound to the myofilaments, to catalyze the Lohmann reaction following Triton-treatment. All chemicals were from Sigma Chemical Co. (St. Louis, MO) except for $MgCl_2$ (Fisher Scientific) and KOH (Fluka, Buchs, Switzerland). Activating solutions with $[Ca^{2+}]$ ranging from pCa 6.3 to 4.0 were made by varying the CaEGTA:EGTA ratio. Two nominally Ca-free relaxing solutions were prepared: R1 with 10 mM EGTA (pCa 9.0) and R2 with 0.05 mM EGTA (pCa 7.5), the EGTA was replaced with 9.95 mM HDTA (Fluka). Solution constituents were delivered from stocks with Gilson "pipetman" adjustable pipettes (Gilson Co., Inc., Worthington, OH). Volumes of water were weighed before each addition to ensure the accuracy of each delivery. After all the constituents were added, the solution was placed in a jacketed chamber that was perfused with a 25% propyleneglycol-water mixture to control the temperature of the solution to ±0.1°C. The pH electrode (Orion Research, Inc., Cambridge, MA) was calibrated as described by Harrison and Bers (1987). The solution was titrated to pH 7.00 with analytical grade 1 M KOH and brought to volume. 2-ml aliquots of each solution were transferred to polystyrene tubes and stored at −70°C to reduce the rate of spontaneous breakdown of high energy phosphate compounds. Great care was taken to control solution temperature to within ±0.1°C as the $pK_a$ of HEPES is temperature sensitive (Good et al., 1966) and the affinity of EGTA for Ca is both pH and temperature dependent (e.g., Boyd et al., 1965; Marini et al., 1986; Harrison and Bers, 1987).

The $[Ca^{2+}]$ of each solution at each temperature was calculated using a computer program (Smith, 1983) that was modified to include the effect of temperature on the binding constants of the solution constituents. Care was taken in the estimation of ionic strength (Smith and Miller, 1985), determination of EGTA purity (Bers, 1982; Miller and Smith, 1984; Harrison and Bers, 1987), measurement of Ca contamination (see Miller and Smith, 1984 for method), measurement of pH (Illingworth, 1981; Harrison and Bers, 1987), and choice of binding constants (see Harrison and Bers, 1987; Harrison, S. M., and D. M. Bers, manuscript submitted for publication; and below). The computer program adjusts the individual binding constants of all the ligands and metals present in the solutions for the ionic strength and temperature of the test solution (Miller and Smith, 1984; Smith and Miller, 1985; Harrison and Bers, 1987) and computes the free and bound concentrations of all metals and ligands. Ca contamination in a 140 mM KCl solution was found to be 3 μM using the pH metric method described by Miller and Smith (1984) and was included in the calculation of $[Ca^{2+}]$. This method of calculating the $[Ca^{2+}]$ of complex experimental solutions seems to be valid as Kentish (1986) reported that calculated $[Ca^{2+}]$ was within a few percent of the value determined from aequorin luminescence. In this study, the $[Ca^{2+}]$ of solutions was checked with an Orion Ca macroelectrode using the method described by Bers (1982). However, due to ionic interference, direct measurement is restricted to $[Ca^{2+}]$ above the effective detection limit of the electrode. This restriction meant that only a few solutions could be accurately checked. Solutions with $[Ca^{2+}]$ calculated to be 1.33, 2.02, and 3.64 μM were measured to be 1.4, 2.07 and 3.47 μM. The calculated and measured values compare favorably which supports the validity of the computational approach for assessing the $[Ca^{2+}]$ of experimental solutions.

The binding constants used in this study were taken from Martell and Smith (1974). These values were used as they proved to be the best fit to the experimental data on the temperature and ionic strength dependence of the Ca affinity of EGTA (S. M. Harrison and D. M. Bers, submitted) reported by Harrison and Bers (1987) and Harafuji and Ogawa (1980), respectively. The ΔH values used to correct binding constants for different temperatures were taken from various sources: EGTA and HDTA: Martell and Smith (1974) and Christensen and Izatt (1983), ATP: Christensen and Izatt (1983), Philips et al. (1965), Taqui Khan and Martell (1966), HEPES: Good et al. (1966). Results are presented as mean ± SD.

### *Statistical Analysis of Results*

Paired Student's $t$ tests were carried out to determine probabilities of significant difference between the paired (36 and 29, 22 and 15, and 8 and 1°C) $K_{1/2}$ and Hill coefficient values generated from the steady-state determinations of myofilament Ca sensitivity. Unpaired Student's $t$ tests were carried out to compare the values of $K_{1/2}$ from the steady-state and temperature-change determinations of Ca sensitivity. An analysis of variance (ANOVA) test was carried out on the $K_{1/2}$ values from the steady-state determinations of myofilament Ca sensitivity and on the values of the temperature dependence of $C_{max}$ from the temperature-change

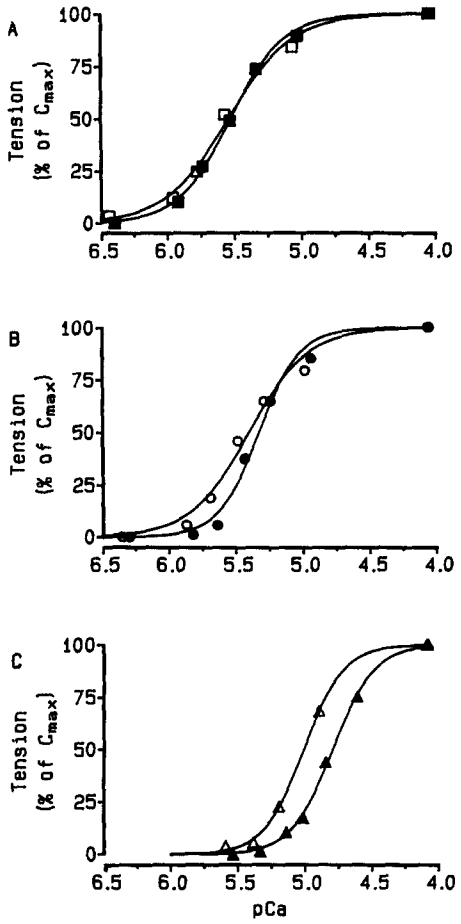


FIGURE 1. The effect of temperature on Ca sensitivity. Tension is expressed as a percentage of that generated by a solution with pCa 4.0 (pCa = $-\log[Ca^{2+}]$). (*A*) Data was generated as described in the text. □ represents data at 36°C. The $K_{1/2}$ was 5.546 and Hill coefficient was 1.82. ■ shows data at 29°C for the same muscle. $K_{1/2}$ = 5.529, Hill coefficient = 2.2. The solid lines represent the result of nonlinear least-squares curve-fitting to the Hill equation. Preparation 5-1-87, diameter-234 μm. (*B*) ○ shows data at 22°C. $K_{1/2}$ = 5.405, Hill coefficient = 1.95. ● represents data from the same preparation at 15°C. $K_{1/2}$ = 5.331, Hill coefficient = 2.8. Preparation 4-22-87, diameter = 130 μm. (*C*) △ represents data at 8°C. $K_{1/2}$ = 5.010, Hill coefficient = 2.9. ▲ represents data from same preparation at 1°C. $K_{1/2}$ = 4.794, Hill coefficient = 2.78. Preparation 7-28-87, diameter = 156 μm. Regression coefficients for the curve fits for all the data are >0.99.

experiments. Multiple comparisons were performed with Duncan's multiple range test if the data sets were found to have homogeneous variance.

## RESULTS

### *Steady-State Determination of Myofilament Ca Sensitivity*

Fig. 1 *A* shows an individual determination of the steady-state pCa vs. tension relationship at 29 and 36°C. These data were fitted to the Hill equation (e.g., Miller and

Smith, 1985; Harrison et al., 1988) through 0 and 100% using a nonlinear least-squares curve-fitting procedure. The fit returned values of the pCa at which 50% of maximum tension was generated ($K_{1/2}$) as well as the slope or Hill coefficient of the relationship. The Hill equation is a useful tool for the characterization and comparison of Ca sensitivity curves though we shall impute no stoichiometric significance to the Hill coefficient values given by the fitting procedure as it has been suggested that the Hill equation is not appropriate for analysis of the mechanism of Ca activation (Shiner and Solaro, 1984). The value of $K_{1/2}$ and the Hill coefficient were derived from each experiment and mean values were calculated (see Table I). The mean $K_{1/2}$ and Hill coefficient values generated as described above were no different ($P > 0.995$, Student's $t$ test) when the curve-fitting program was given complete freedom to change the upper and lower limits of the individual fits.

The mean $K_{1/2}$ at 36°C was 5.473 which was not significantly different from the

TABLE I

*Influence of Temperature on $K_{1/2}$ and Hill Coefficient*

| Temperature (°C) | $K_{1/2}$ (pCa) | | Hill coefficient | |
|---|---|---|---|---|
| 36 | 5.473 ± 0.067 | NS | 1.75 ± 0.1 | ‡ |
| 29 | 5.494 ± 0.070 | | 2.06 ± 0.1 | |
| 22 | 5.340 ± 0.052 | * | 2.15 ± 0.6 | NS |
| 15 | 5.259 ± 0.092 | | 2.49 ± 0.2 | |
| 8 | 4.932 ± 0.064 | ‡ | 3.06 ± 0.5 | NS |
| 1 | 4.732 ± 0.044 | | 2.94 ± 0.6 | |

Values are expressed as mean ± SD. These three sets of paired data (36 and 29, 22 and 15, and 8 and 1°C) were analyzed using a paired $t$ test. Probabilities of significance are shown to the right of each set of paired data. *NS* indicates no significant difference between the paired values, $^*P < 0.01$, and $^‡P < 0.001$. Duncan's multiple range test indicated that the values of $K_{1/2}$ were all significantly different from each other ($P < 0.01$) except for between 36 and 29°C (NS) and between 22 and 15°C ($P < 0.05$). Multiple comparisons could not be carried out on the Hill coefficient data as their variances were found to be nonhomogeneous.

mean $K_{1/2}$ at 29°C for the same group of muscles (5.494, $P > 0.95$). Fig. 1 *B* shows the Ca sensitivity of another muscle at 22 and 15°C. The mean $K_{1/2}$ at 22°C was shifted relative to that at 29°C by 0.15 pCa units towards higher $[Ca^{2+}]$ giving a $K_{1/2}$ of 5.340. The pCa vs. tension relationship was moved significantly ($P < 0.01$) towards higher $[Ca^{2+}]$ on cooling to 15°C, giving a $K_{1/2}$ of 5.259. Fig. 1 *C* shows a representative example of the pCa-tension relationship from a third group of muscles at 8 and 1°C. The mean $K_{1/2}$ at 8°C was 4.932 which was shifted significantly ($P < 0.001$) to 4.732 on cooling to 1°C. Table I shows the mean values for $K_{1/2}$ and Hill coefficient at each temperature and tests of significant differences are described in the legend.

The maximum tension that a skinned muscle can generate at any one temperature decreases with time (e.g., Jewell and Kentish, 1981; Miller and Smith, 1985; Kentish et al., 1986; Harrison et al., 1988). In the present study, the mean decline (±SD) of

$C_{max}$ at 36°C was 1.45 ± 0.6%/min ($n$ = 6), which fell to 0.26 ± 0.18 and 0.26 ± 0.17%/min ($n$ = 6) at 29 and 22°C, respectively, and to 0.18 ± 0.1%/min at 15°C. The high rate of tension decline at 36°C meant that preparations had deteriorated considerably (range, 17–68% of initial tension) by the end of the experimental protocol (but see Fig. 2). At 22°C the decline of $C_{max}$ reported here is similar to that observed by Harrison et al. (1988; 0.18%/min) and Kentish et al. (1986; 0.24%/min in the presence of dithiothreitol) though the rate reported here is much slower than that observed by Jewell and Kentish (1981) in the absence of S-H bond protecting agents (1.2%/min). At 8 and 1°C there was no noticeable reduction of $C_{max}$ during the course of an experiment. Pagani et al. (1986), using sodium dodecyl sulfate polyacrylamide gel electrophoresis of myofibrillar proteins, showed that saponin-treated muscles that exhibited a rapid decline of $C_{max}$ had suffered a significant loss of actin,

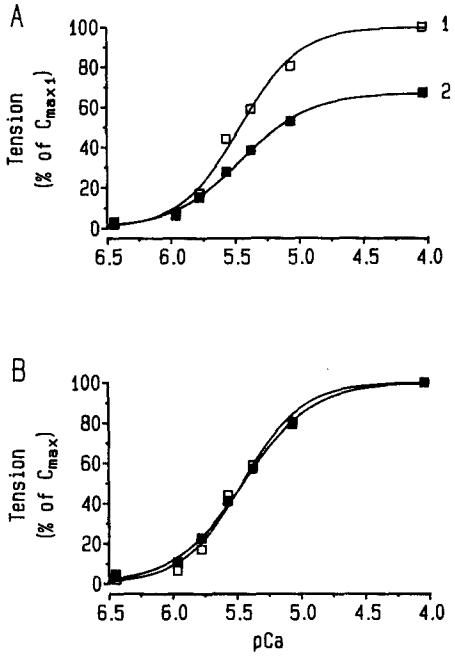


FIGURE 2. Reduction of $C_{max}$ does not affect Ca sensitivity. (*A*) (□) Determination of Ca sensitivity at 36°C. Tension is shown as a percentage of that generated by a solution having a pCa = 4.0, (preparation 6-21-87, diameter = 312 μm). ■ represents the Ca sensitivity (scaled as a percentage of $C_{max}$ from the initial determination) determined 15 min after the initial determination (*1*). (*B*) The same data as shown in *A* scaled to the $C_{max}$ of each determination.

tropomyosin, troponin, and myosin light chains. This raises the possibility that the Ca sensitivity of a preparation changes as muscle performance declines.

Fig. 2 *A* shows two successive determinations (separated by 15 min) of the pCa vs. tension relationship in the same muscle at 36°C where the rate of tension decline was 2.4%/min. This shows that peak force declined to 67% of its value 15 min previously, but when each data set is internally normalized (Fig. 2 *B*), the two curves can effectively be superimposed which indicates that there is not a selective wash out of regulatory proteins and that the muscle, though not generating as much force, has the same Ca sensitivity. Furthermore, there was no correlation ($r$ = 0.08) between the extent of deterioration of force production at 36°C and the Ca sensitivity exhibited by the preparations.

*Temperature-Change Experiments*

These experiments were carried out to determine the effect of the whole range of temperatures on submaximal and maximal force production ($C_{max}$) in individual muscles to eliminate differences between preparations. This protocol allowed a rapid comparison of force levels at different temperatures, thus obviating the necessity for normalization of tension responses at different temperatures. However, fewer [$Ca^{2+}$] were tested so Ca sensitivity curves determined from these data were less well characterized than those from the steady-state determinations.

Fig. 3 shows tension traces that demonstrate the effect of rapidly increasing temperature at constant [$Ca^{2+}$]. In Fig 3 *A* the muscle was first equilibrated in the preactivating solution (R2, pCa 7.5) before being exposed to a maximally activating solution (pCa 4.0) at 8°C. After tension had reached a steady level the temperature

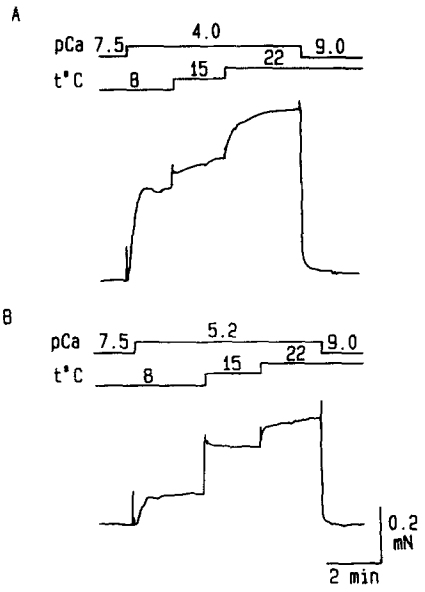


FIGURE 3. Effect of temperature changes on peak and submaximal force. (*A*) The preparation (9-10-87, diameter = 190 μm) was equilibrated in the preactivating solution (R2) at 8°C and then exposed to a solution with a pCa of 4.0 (8°C) that maximally activates the contractile proteins. With [$Ca^{2+}$] constant the temperature was rapidly (1 s) increased to 15 and 22°C. This led to a 32 and 85% increase in force, respectively, compared with that generated at 8°C, with little change in baseline force. (*B*) A similar experiment carried out at pCa 5.2 in the same fiber just after the contraction protocol shown in *A* was completed. At 8°C a solution of pCa 5.2 (A1) generated 33% of $C_{max}$ at 8°C. Similarly, at 15 and 22°C this pCa generated 65 and 64%, respectively, of $C_{max}$ at each temperature.

was raised to 15°C keeping the [$Ca^{2+}$] constant. This led to a 32% increase in force. Warming to 22°C led to a greater increase in force with little change in baseline tension. Fig. 3 *B* shows a similar experiment carried out at pCa 5.2 (solution A1) in the same preparation. At 8°C, this [$Ca^{2+}$] generated 33% of $C_{max}$ at 8°C. As the temperature was increased to 15°C the same [$Ca^{2+}$] generated 65% of $C_{max}$ at 15°C and further warming to 22°C led to the development of 64% of $C_{max}$ at 22°C.

Table II summarizes the temperature-dependence of $C_{max}$ (normalized to that at 22°C, see the legend of Table II for statistical analysis) and the tension generated by pCa 5.2 (solution A1) as a percentage of $C_{max}$ at each temperature from experiments in the temperature-change series. Table II also shows the percent of $C_{max}$ (for each

TABLE II

*Summary Table of Influence of Temperature Change on Maximal and Submaximal Force*

| Temperature (°C) | $C_{max}$ (% of $C_{max}^{22}$) | Tension in pCa 5.2 (% of $C_{max}$) | Tension in $pCa_{50}$ (A2) (% of $C_{max}$) | Mean $K_{1/2}$ from temperature change (pCa) |
|---|---|---|---|---|
| 36 | 118.5 ± 10.0 | 65.3 ± 3.8 | 54.4 ± 0.8 | 5.491 ± 0.01 |
| 29 | 108 ± 4.6 | 64.9 ± 7.7 | 40.5 ± 4.0 | 5.398 ± 0.07 |
| 22 | 100 | 65.4 ± 1.0 | 46.9 ± 4.4 | 5.312 ± 0.02 |
| 15 | 74.3 ± 6.0 | 65.0 ± 2.8 | 56.5 ± 3.0 | 5.305 ± 0.03 |
| 8 | 57.2 ± 7.0 | 38.8 ± 7.0 | 71.1 ± 2.5 | 5.113 ± 0.04 |
| 1 | 29.3 ± 5.4 | 15.4 ± 4.3 | 65.5 ± 7.0 | 4.897 ± 0.03 |

Column 2 shows the influence of temperature on $C_{max}$ (mean ± SD, $n = 4$), normalized to the degree of tension generated at 22°C. Duncan's multiple range test (excluding the data at 22°C, which had zero variance) indicated that all the mean values of $C_{max}$ differ significantly ($P < 0.01$) except for between 36 and 29°C ($P < 0.05$). Column 3 represents the mean (± SD, $n = 4$) tension generated after exposure to a Ca-activating solution with pCa 5.2 expressed as a percentage of the $C_{max}$ at each temperature. Column 4 shows mean (±SD, $n = 4$) percentages of $C_{max}$ generated by solutions (A2, referred to as $pCa_{50}$ in the table) having a $[Ca^{2+}]$ equal to the $K_{1/2}$ from the steady-state determinations of Ca sensitivity at each temperature. These $K_{1/2}$ values from the temperature-change series of experiments were also compared with the $K_{1/2}$ values from the steady-state determinations of Ca sensitivity (see Table I) using an unpaired $t$ test. The differences between the $K_{1/2}$ values using the two experimental approaches were significant ($P < 0.001$) only at 8 and 1°C.

temperature) generated by a solution (A2) having a $[Ca^{2+}]$ equal to that which generated half $C_{max}$ from the steady-state determinations of Ca sensitivity. At temperatures down to and including 15°C the values lie fairly close to 50% given that the $K_{1/2}$ lies on the steepest part of the Ca sensitivity curve. However, at 8 and 1°C >50% of $C_{max}$ was generated by these solutions. Each temperature-change experiment was fitted to the Hill equation as previously described using the two experimental points (tension achieved in solutions A1 and A2) and assuming that zero tension was generated by a pCa of 6.5 and maximum tension generated by pCa 4.0 to give the $K_{1/2}$ values shown in the last column of Table II.

Fig. 4 shows a comparison of the $K_{1/2}$ values determined from the steady-state Ca sensitivity determinations with those from the temperature-change experiments. This shows that these data compare favorably above 15°C (no significant difference,

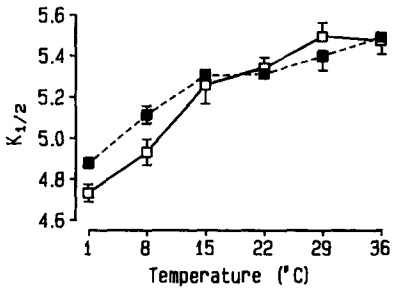


FIGURE 4. Comparison of the effect of temperature on $K_{1/2}$ from steady state and temperature-change experiments. □ shows the mean $K_{1/2}$ values (±SD) from the steady-state determinations of Ca sensitivity and ■ shows the $K_{1/2}$ values from the temperature-change series documented in Table I.

see Table II, legend) though it is apparent that at 8 and 1°C the difference between the steady-state and temperature-change determinations of $K_{1/2}$ is significantly different ($P < 0.001$) and indicates that the Ca sensitivity curves under these conditions are not shifted to as high a $[Ca^{2+}]$ as in the steady-state determinations. While this may represent a real difference in myofilament Ca sensivity between brief (as experienced by muscles during RCCs) and extended exposures to low temperatures, it should be noted that the temperature-change experiments were fitted to only two points over the critical region of the curve.

Using the $C_{max}$ values from Table II, the mean steady-state Ca sensitivity curves were scaled using the $C_{max}$ at each temperature, expressed as a percentage of that at 22°C. Fig. 5 illustrates that cooling leads to a shift of myofilament Ca sensitivity towards higher $[Ca^{2+}]$ in conjunction with a depression of peak force such that the curves do not cross over as has been reported for canine Purkinje fibers (Fabiato, 1985), frog skeletal muscle (Godt and Lindley, 1982), and for rat and toad skeletal muscle (Stephenson and Williams, 1981, 1985). The dashed curves at 8 and 1°C

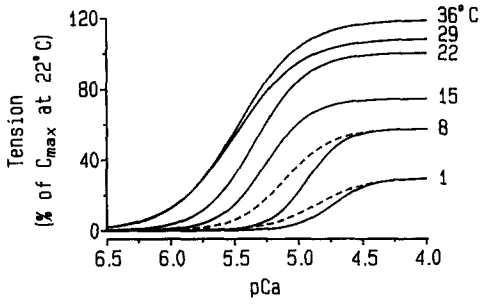


FIGURE 5. The influence of temperature on Ca sensitivity and $C_{max}$. The mean steady-state Ca sensitivity curves were scaled to take into account the effect of temperature on $C_{max}$ (see Table II). The two dashed curves at 8 and 1°C represent the result of curve fitting of data generated from the temperature-jump experiments (see text). The $K_{1/2}$ values for the dashed curves at 8 and 1°C are 5.113 and 4.879, respectively. Hill coefficient values for the dashed curves at 8 and 1°C are 2.35 and 2.25, respectively.

show the Ca sensitivity curves from the temperature-change experiments. This shows that the difference in Ca sensitivity between steady-state and temperature-change determinations does not alter the fact that at no point do the curves cross over.

The method used in this report by which the muscle is brought to optimum length in the intact state before treatment with Triton X-100, makes it possible to compare twitch force with maximum Ca-activated force at the same temperature. The size of the steady-state twitch at 29°C (muscle at optimum length before Triton treatment) was 40.7 ± 8.0% ($n$ = 10) of $C_{max}$ at 29°C. Fabiato (1981) reported that single pulse stimulation of single rabbit ventricular cells (at 22°C) led to twitch force equal to 19% of $C_{max}$ generated after mechanical skinning. It is possible that the preparations used in the present study (which generated only 2.5 times as much force upon skinning) had deteriorated during the skinning process. While our value for twitch force/$C_{Max}$ of 40% is higher than that reported by Fabiato (1985), this value is in agreement with the results of studies in which twitch force at 29°C in rabbit ventric-

ular muscle could only be augmented by 2.5–3 times by such interventions as elevated external Ca (Bers et al., 1981), low [Na] contractures or acetylstrophanthidin (Bers, 1987), BAY K 8644 (Bers, unpublished observation), or high frequency stimulation (Bers, 1989).

## DISCUSSION

### *Temperature-induced Shifts in Myofilament Ca Sensitivity*

The results presented here show that there was no significant difference in myofilament Ca sensitivity between 36 and 29°C though cooling from 29 to 1°C leads to a progressive shift in Ca sensitivity towards higher [$Ca^{2+}$] by 0.76 pCa units (see Table I). Brandt and Hibberd (1976) reported that the Ca sensitivity of cat papillary muscles was decreased by cooling from a $K_{1/2}$ of 5.80 at 25°C to 5.53 at 20°C with little change in Ca sensitivity between 25 and 29°C. These results are similar to those of this study found between 29 and 36°C. As indicated in the Introduction, these results must be interpreted with caution due to the possibility that the muscles were not completely "skinned" by the EGTA treatment (Miller and Smith, 1985). Fabiato (1985) reported that at 22°C the $K_{1/2}$ of canine Purkinje fibers was 5.354. This value is very close to the $K_{1/2}$ determined from the steady-state determinations of Ca sensitivity in the present study at 22°C (5.340). However, Fabiato (1985) found that the Ca sensitivity of canine Purkinje fibers was increased by cooling ($K_{1/2}$ shifted to 5.551 at 12°C), a shift in Ca sensitivity opposite in direction to that found here for rabbit cardiac muscle.

In skinned skeletal muscle, most determinations of the temperature dependence of myofilament Ca sensitivity (with the exception of Ashley and Moisescu, 1977; Orentlicher et al., 1977) show that cooling leads to an increase in Ca sensitivity. For example, Stephenson and Williams (1985), upon cooling the muscle from 22 to 5°C, showed a shift in $K_{1/2}$ from 5.74 to 6.1 in toad twitch muscle and from 5.94 to 6.25 in rat fast twitch muscle. Godt and Lindley (1982) reported a similar shift in $K_{1/2}$ from 5.788 at 22°C to 6.187 at 4°C in frog skeletal muscle. Pilot experiments (not shown) carried out as an extension of the present study on Triton-treated frog skeletal muscle fibers using the same solutions and protocol as described above yield results consistent with those of Stephenson and Williams (1985) and Godt and Lindley (1982). In contrast, Orentlicher et al. (1977) reported a decrease in Ca sensitivity in crayfish skeletal muscle upon cooling as the $K_{1/2}$ shifted from 6.194 to 5.214 between 20 and 5°C, and Ashley and Moisescu (1977) found no effect of temperature on the Ca sensitivity of barnacle muscle.

It is evident that there is a difference in both the magnitude and direction of the temperature-induced shift in Ca sensitivity between species and muscle types, which indicates that there are fundamental thermodynamic differences in the Ca binding and regulation of contraction in these tissues.

Comparison of results from the two protocols (see Figs. 4, and 5, and Table II) indicate that the Ca sensitivity of skinned ventricular muscle differs between short and long exposures to low temperatures (8 and 1°C). This might have implications for the interpretation of RCCs in that the Ca sensitivity is not moved to as high a [$Ca^{2+}$] during short exposures to cold temperatures. This phenomenon presumably

reflects some time- and temperature-dependent state of the muscle that cannot be readily identified by experiments of this nature.

As sarcomere length was not directly measured in these experiments there is a possibility that some of the variation in Ca sensitivity seen at each temperature is a reflection of the length dependence of Ca sensitivity of the myofilaments (e.g., Hibberd and Jewell, 1982). The results of studies in which cat papillary muscles were stretched until maximum twitch force was generated and sarcomere length was analyzed (e.g., Sonnenblick and Skelton, 1974) showed that mean sarcomere length was 2.2 μm. Gordon and Pollack (1980) also showed that active force generated by rat cardiac muscle peaked between a sarcomere length of 2.1–2.2 μm. These observations support the validity of this method to estimate the optimum working length of the preparation.

### *Does the Ca Sensitivity of Skinned Fibers Reflect the True Ca Sensitivity?*

Recent evidence (Harrison et al., 1985; Yue et al., 1986) suggests that the Ca sensitivity of intact cardiac muscle may be considerably higher than that exhibited by the same fiber after being subjected to chemical skinning. Harrison et al. (1985) reported that the Ca sensitivity of skinned cardiac muscle could be shifted to lower $[Ca^{2+}]$ by exposing skinned muscles to solutions containing imidazole or imidazole group–bearing compounds like carnosine, which are abundant in skeletal muscle (15–60 mM; Crush, 1970). Experiments using HPLC to assay for such compounds in cardiac muscle suggest that intact muscle contains many such compounds that would act to increase the Ca sensitivity of the myofilaments to Ca (Crichton et al., 1988). Therefore, whereas the skinned fiber is a useful preparation for the investigation of interventions that change the Ca sensitivity of the myofilaments, such as temperature, the $K_{1/2}$ values generated by this experimental approach may not reflect the Ca sensitivity in the intact state. Also, one cannot exclude the possibility that the temperature dependence of mechanisms controlling tension development differ between the skinned preparation and the intact state.

### *Do Changes in Ca Sensitivity Contribute to Hypothermia-induced Inotropy in Rabbit Cardiac Muscle?*

Our results indicate that between 36 and 29°C, where the majority of the hypothermic inotropy is induced (cooling from 37 to 29°C led to a fourfold increase in twitch force, Shattock and Bers, 1987), there is little or no change in myofilament Ca sensitivity or $C_{max}$ in these skinned fibers. However, these experiments were carried out at constant pH (7.00) and there is evidence that cooling intact muscle leads to an intracellular alkalosis (Saborowski et al., 1973; Aickin and Thomas 1977; but cf. Reeves and Wilson, 1969; Ellis and Thomas, 1976). If cooling (e.g., 36–29°C) leads to an alkalosis, the increase in $pH_i$ would increase myofilament Ca sensitivity (Fabiato and Fiabato, 1978*b*; Fabiato, 1985) over the temperature range where the majority of the hypothermic inotropy occurs. Such a $pH_i$-induced shift in myofilament Ca sensitivity could contribute to this inotropy.

On the other hand, this intropy may be more likely explained by changes in cellular Ca regulation. For example, cooling leads to an increase in intracellular sodium activity ($a^i_{Na}$, Chapman, 1986) presumably due to Na-pump inhibition (Eisner and

Lederer, 1980) and this may increase cellular Ca via Na/Ca exchange. Such a Ca gain could increase twitch force either by increasing diastolic $[Ca^{2+}]$ or by increasing SR Ca loading (and subsequent release). However, Shattock and Bers (1987) showed that a functional SR is not required for the hypothermic inotropy to occur, as inhibition of the SR by ryanodine leaves the inotropy unchanged. The increase in $a^i_{Na}$ with cooling will shift the Na/Ca exchange system such that more Ca may enter the cells during the action potential that is prolonged by cooling. These two factors would tend to favor Ca entry via the Na/Ca exchange (Bers, 1987*a*). Also, while peak Ca entry via Ca channels is depressed by cooling (Cavalie et al., 1985), the long action potential would again favor Ca entry via noninactivated Ca current (i.e., Ca window current).

### *Influence of Temperature on $C_{max}$*

Table II shows that $C_{max}$ is significantly reduced (see Table II legend) by cooling to below 36°C as has been previously reported (e.g., Ashley and Moisescu, 1977; Stephenson and Williams, 1981, 1985; Godt and Lindley, 1982; Ranatunga, 1982; Johnston and Sidell, 1984; Fabiato, 1985). In cases where cooling leads to an increase in Ca sensitivity (e.g., frog, rat, and toad skeletal muscle and dog Purkinje fibers) in conjunction with a reduction of $C_{max}$, then the Ca sensitivity curves at high and low temperature will cross over (e.g., Figs. 1 and 2 in Godt and Lindley, 1982) unlike those described for rabbit ventricle in this report (see Fig. 5).

The reason for the decrease in $C_{max}$ at low temperature is not clear. Stephenson and Williams (1981) reported that there was a reduction in the number of rigor complexes formed at 5°C (rigor tension was 15–20 times lower) compared with 22°C, and interpreted the fall in $C_{max}$ as a reduction in the number of crossbridges formed between myosin and actin at lower temperatures. However, Kuhn et al. (1979) showed that the number of attached cross bridges in fully activated fibers of the giant water bug (*Lethocerus maximus*) was unchanged between 35 and 5°C and therefore suggested that a decrease in force on cooling reflected a reduction in force per cross bridge. This result is supported by the observations of Ford et al. (1977) who showed that an increase in temperature from −0.1 to 8.1°C led to an increase in tetanic tension in frog muscle fibers, while a stretch of 1.5 nm per half sarcomere during the tetanus at each temperature did not cause a significantly different response. This was interpreted as an increase in force generated per cross bridge at higher temperatures.

### *Changes in Myofilament Ca Sensitivity during RCCs in Intact Cardiac Muscle*

Fig. 6 *A* shows an RCC in an intact cardiac ventricular trabecula from the rabbit. Cooling the muscle from 29 to 1°C leads to a large maintained contracture. RCCs are thought to be an index of the amount of Ca available for release from the SR (e.g., Kurihara and Sakai, 1985; Bridge, 1986) as they can be modified by the relatively specific agents ryanodine and caffeine, which interfere with SR Ca transport (Bers et al., 1987). On rewarming there is a transient increase in force, or rewarming spike, followed by relaxation as the processes capable of removing Ca from the sarcoplasm (e.g., SR Ca-ATPase, sarcolemmal Ca-ATPase, Na-Ca exchange) are reactivated. These rewarming spikes are not due to an increase of $[Ca^{2+}]_i$, as mea-

sured by indo-1 fluorescence (Bers et al., 1988). Furthermore, rewarming spikes are still observed in solutions containing nifedipine (Bers, 1987*b*), caffeine (Bers et al., 1987), and in the absence of Na and Ca ions (Bers, 1987*a*) which should block Ca channels, SR Ca release, and Na-Ca exchange.

In Fig. 6 *B* a Triton-treated rabbit cardiac trabecula was activated by increasing the $[Ca^{2+}]$ from pCa 7.5 to 5.2 at 1°C and then further to pCa 4.0. When the muscle is moved to a solution of pCa 9.0 at a higher temperature (22°C), a large transient increase in tension occurs before relaxation, which is absent if the temperature is constant throughout. This tension spike (Fig. 6 *B*) is qualitatively similar to the tension transient seen upon rewarming during an RCC in intact tissue (Fig. 6 *A*).

In rabbit ventricular muscle, these spikes are evident upon rewarming from RCCs at all levels of force (and therefore intracellular $[Ca^{2+}]$) though they are reduced in magnitude at lower levels of activation (Bers, 1989). As the Ca sensitivity curves do

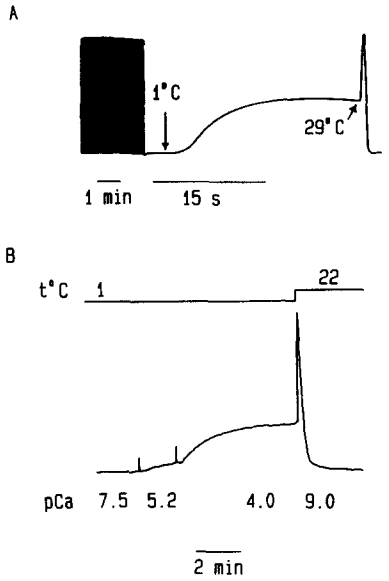


FIGURE 6. Rewarming spikes in intact and Triton-treated rabbit ventricular tissue. (*A*) Initial part of trace shows an intact rabbit cardiac trabecula paced at 0.5 Hz (see Bers et al., 1987). Stimulation is stopped and this is followed by rapid cooling to 1°C (downward arrow) in <0.5 s. At the second arrow, the superfusate is changed to one at 29°C. *B* shows a tracing from Triton-treated rabbit ventricular trabecula that is kept relaxed in a solution of pCa 7.5 (R2, 50 μM EGTA). $[Ca^{2+}]$ is increased from pCa 7.5 to 5.2, and then to 4.0 at 1°C. When steady tension is achieved, the preparation was moved to a solution of pCa 9.0 (10 mM EGTA) at 22°C to induce relaxation.

not cross over (Fig. 5) in skinned rabbit ventricular fibers, rapidly rewarming an intact preparation from 1 to 29°C while intracellular $[Ca^{2+}]$ is still above the threshold for tension development, would be expected to increase the Ca sensitivity and force at all $[Ca^{2+}]$. Rewarming would also reactivate the mechanisms capable of removing Ca from the sarcoplasm and thus induce relaxation. At lower force levels, where the rewarming spike is smaller, the temperature-induced shift in Ca sensitivity is less (see Fig. 6) and would therefore cause a smaller tension transient.

Extrapolation from the skinned fiber to intact muscle is limited by differences in the media bathing the contractile proteins in the two preparations. It is interesting, however, that tension changes resembling rewarming spikes can be simulated with temperature changes in Triton-skinned fibers. If the rewarming spike is purely a reflection of changes in myofilament Ca sensitivity and $C_{max}$ on cooling, one might expect to see differences in the magnitude of rewarming spikes from RCCs in spe-

cies in which the Ca sensitivity curves do cross over (e.g., dog Purkinje tissue, Fabiato 1985; frog skeletal muscle, Godt and Lindley, 1982).

### *Conclusions*

The results show that cooling leads to a reduction in the Ca sensitivity of the myofilaments of rabbit ventricular muscle such that a contribution to the hypothermia-induced inotropy observed in intact cardiac muscle is not possible. These data differ from some previous determinations of the temperature-dependence of the Ca sensitivity of the myofilaments in other species and muscle types which indicates that there is a variation between species and tissues both in the magnitude and direction of the shift in Ca sensitivity associated with cooling, and as to whether the Ca sensitivity curves cross over. The observed shifts in myofilament Ca sensitivity and $C_{max}$ as a function of temperature suggest that this effect could contribute to rewarming spikes in rabbit ventricular tissue.

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